Development of Combination Therapy with Anti-Cancer Drugs

ISBN: 978-94-6108-399-9

Cover design: **Ink dissolving in water** by unknown artist, representing the continuous evolvement in cancer research and the sometimes rather transient aspect of anti-cancer drug development; only a fraction of the preclinically discovered and/or developed drugs will 'make it' into the clinic, and very few of all drugs undergoing clinical evaluation can and will eventually be approved for general use in cancer treatment thereby becoming available for a large population.

Printed by: Gildeprint Drukkerijen

Development of Combination Therapy with Anti-Cancer Drugs

Ontwikkeling van combinatietherapie
met antikankermiddelen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op vrijdag 15 maart 2013 des ochtends te 10.30 uur

door

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geboren op 11 juni 1974 te Amsterdam

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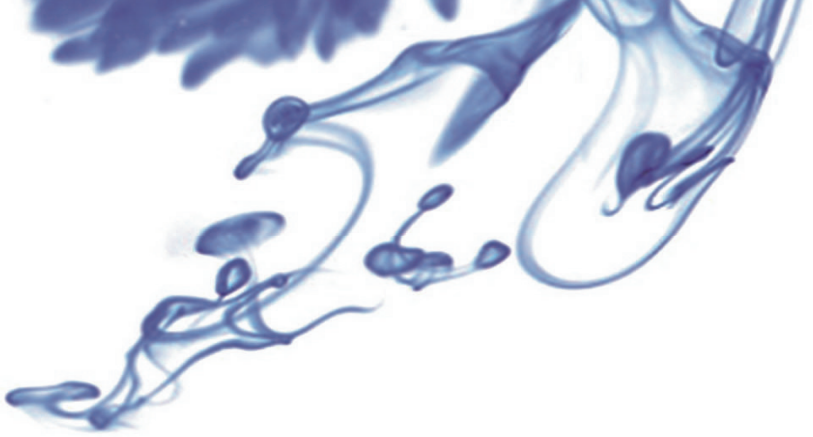
The research described in this thesis was performed at the Department of Clinical Pharmacology, The Netherlands Cancer Institute, Amsterdam, the Netherlands and at the Department of Pharmacy & Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands

Publication of this thesis was financially supported by:
MSD B.V., Haarlem, the Netherlands
Roche Nederland B.V., Woerden, the Netherlands
Boehringer Ingelheim B.V., Alkmaar, the Netherlands

"τὰ ὄντα ἰέναι τε πάντα καὶ μένειν οὐδέν"
(Ta onta ienai te panta kai menein ouden)
"All entities move and nothing remains still"
There is nothing permanent except change.
- *Heraclitus (Ἡράκλειτος)*
c. 535 – c. 475 BCE

Voor alle patiënten en hun dierbaren

Contents



CHAPTER 1

Introduction

1.1

The development of combination therapy with anti-cancer drugs, a general introduction

Preface

1.1
Preface

General Introduction

The way anti-cancer drugs are being developed is changing rapidly. The studies described in this thesis illustrate some of the ongoing changes. Traditional anti-cancer drugs have proven their value and their limitations are known. New targeted drugs have shown promising results, but further investigations are often needed. Combinations of anti-cancer drugs are not new (e.g. carboplatin and gemcitabine for the treatment of lung cancer), and combinations of traditional and/or targeted anti-cancer drugs can potentiate the activity of single agents and limit the development of resistance. Changes in drug development also influence the ideas about the best way to conduct early clinical trials, a subject that is further investigated in **Chapter 1.2**.

Traditional chemotherapy

Cisplatin and carboplatin are platinum based anti-neoplastic agents and one of the cornerstones of treatment for over a quarter of a century in many types of cancers like lung and ovarian cancer and germ cell tumors. Cisplatin inhibits cell growth through the formation of DNA cross links. Most notably are intrastrand DNA cross links, but interstrand cross link formation can also occur. The damaged DNA elicits DNA repair, but will induce apoptosis in case of excessive DNA damage and if DNA repair is impossible. Although first described in 1845 by M. Peyrone, cisplatin was approved for testicular and ovarian cancer by the USA Food and Drug Administration (FDA) in the late seventies of the 20th century. Nephrotoxicity is a known adverse effect of cisplatin. Nausea and vomiting are common side effects, but can be managed with anti-emetic treatment. Other adverse events consist of myelosuppression, neurotoxicity and ototoxicity. Carboplatin was developed after cisplatin and use in the clinic was approved in the late 1980s. The main advantages over cisplatin are the notably reduced side effects, particularly the elimination of nephrotoxic effects. Despite high initial response rates, a significant group of patients will develop platinum resistance.

Gemcitabine, administered as an infusion, is a nucleoside analogue that was first synthesized in the 1980s. Although it was initially intended as an

antiviral drug, during preclinical development inhibition of leukemic cells was observed. The triphosphate analogue replaces cytadine, one of the building blocks of nucleic acids during DNA replication, eventually resulting in apoptosis. Gemcitabine is used in the treatment of a variety of cancers like lung, pancreatic, bladder and breast cancer.

Cisplatin, carboplatin and gemcitabine are drugs that have been investigated in some of the studies described in this thesis, in combination with new anti-cancer drugs or in a different application form.

New concepts with existing drug combinations

Gemcitabine is a prodrug that first needs to be metabolized in the body in order to exert its action. This process is catalyzed by different enzymes. The enzyme deoxycytidine kinase (dCK) is the rate limiting step in this process. Fixed dose rate infusion, an infusion at a lower speed, is a concept that takes into account this rate limiting step. This new application of an existing drug is investigated in the study described in **Chapter 4** in combination with carboplatin in ovarian cancer patients after first line therapy. The pharmacokinetic results collected in this study have been further exploited by the use of population pharmacokinetics (PopPK), thereby increasing the information obtained from the study, without expanding the number of patients.

New concepts and combinations

Ruthenium is a heavy metal like platinum and ruthenium derivatives have been developed with the idea to create drugs as powerful as cisplatin, but with limited adverse events. NAMI-A was the first ruthenium derivative that has been evaluated in a clinical setting, as a single agent. Promising preclinical results of the combination of NAMI-A and gemcitabine resulted in a clinical study with these two agents in non small cell lung cancer (NSCLC) patients after first line therapy and has been described in **Chapter 5** of this thesis.

MK-1775 is a selective inhibitor of protein Wee1, and is thought to be especially active in cancer cells with p53 pathway mutations. Two checkpoints, named G1 and G2 are important in the repair of DNA damage.

For a well operating G1 checkpoint normal functioning of p53 is important. Since many tumors harbor p53 mutations, cancer cells are more dependent on the G2 checkpoint for DNA repair. Wee 1 plays a key role in the G2 checkpoint. By inducing DNA damage with chemotherapy and pharmacological inhibition of Wee1 by MK-1775, apoptosis can be induced especially in tumor cells. An preliminary analysis of the first in human phase I study with MK-1775 in combination with either gemcitabine, cisplatin or carboplatin has been described in **Chapter 3.2**. **Chapter 3.3** discusses the results of the preliminary analysis of the ongoing proof of concept phase II study in p53 mutated ovarian cancer patients after failure (during or within 3 months of treatment) of first line therapy and treated with carboplatin plus MK-1775.

MEK inhibitors are an example of targeted anti-cancer drugs. MEK is part of the mitogen-activated protein kinase (MAPK) pathway. MAPKs are initiated by ligand binding at the cell surface and ultimately lead to gene transcription in the nucleus. Genetic alterations can lead to overexpression and aberrant activation downstream of the mutation. **Chapter 2.1** describes the results a food effect study with MEK inhibitor selumetinib, while **Chapter 2.2** describes the results of the phase I part of the phase I and expansion study with MEK inhibitor RO4987655. Although in both these studies the MEK inhibitors are administered as single agents, it is not very likely that MEK inhibitors will eventually be used as single agents for the treatment of cancer. Combination with traditional chemotherapeutic agents or other targeted agents are likely to potentiate activity and reduce resistance. Although these two clinical studies described in this thesis are part of the development, they should also be considered as agents (eventually to be) used in combination therapy.

1.2

21st century early clinical trials in oncology

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Jan H.M. Schellens

Introduction

Abstract

Advances in tumor biology have resulted in different kinds of anti-cancer drugs, often referred to as molecularly targeted agents (MTAs). Assessment of MTAs in early clinical trials urges us to reevaluate traditional trial design. This overview discusses the challenges that are encountered and attempts to give a state of the art update of solutions and suggestions provided in available literature of how to deal with these challenges. Questions that are addressed include: what are the advantages and limitations of traditional trial designs? Are new trial designs able to offer adequate solutions? There is growing evidence that support rational combination of MTAs or with cytotoxic agents, but how to identify the best combinations and how to investigate these combinations in the clinic? The need for good biomarkers is explained in the light of personalized medicine and the basis that can be found in extensive analysis of genetic aberrations in tumors of patients.

Introduction

The primary goal of cancer research is to understand the underlying pathophysiological mechanisms that induce uncontrolled cell growth on a molecular level and to develop drugs that intervene with these processes, preferably without damaging healthy cells, and thus ideally resulting in adequate therapies that can cure patients from cancer without side effects. Despite the progress that has been made the past decades, major challenges are still to be conquered. With the increased understanding of tumor biology and that gene mutations can result in aberrant activation of signaling transduction pathways, cancer drug development, cancer prevention, diagnosis, and treatment are undergoing drastic changes. The shift away from the use of traditional cytotoxic chemotherapeutic agents to more specific molecularly targeted agents (MTAs), also affect the investigational phase of drug development in humans. This is related to the different pharmacological properties. Cytotoxic drugs tend to have a small therapeutic window, a close correlation between dose and observed toxicity, but also -although often less clearly established- a correlation between dose and efficacy (also see **Figure 1**).[1] Finally, efficacy is often measured as a decrease in tumor load. MTAs, on the contrary, tend to display less toxicity or very different toxicity profiles (e.g. long term toxicities). In regard to efficacy, inhibition of tumor growth is often more pronounced than the decrease in tumor size, which in general can be explained based on the mechanism of action (MOA) of MTAs. [2] Related to both the complexity of signaling pathways and cross-talk between different pathways, which is sometimes stimulated by pharmacological inhibition of special targets on one hand, and the high degree of specificity of cellular targets of MTAs on the other hand, more and more MTAs proof to be increasingly efficacious in combination with either other MTAs or traditional cytotoxic drugs.[3] Traditional trial designs that have been used for cytotoxic drugs might therefore not be optimal for MTAs and the main question is whether a different approach or different designs for first-in-human studies might be required for the investigation of new anti-cancer drugs. And what considerations should be taken into account when using

new designs? What are the traps and pitfalls? Additional reasons that justify thinking out-of the box include: 1) the fact that it takes 10-15 years to develop a new drug; 2) the extremely high costs that are involved in the development of new molecules (in general around $800 million for a new drug [4;5]), of which 70-90% of costs have been estimated to account for clinical drug failures.[6] Moreover, almost half of all clinical drug failures occur in late-stage Phase III of development[6]; and 3) the fact that in the past decade, despite ravishing and groundbreaking new scientific insights, a slowdown in the rate of approvals was observed instead of an acceleration.[2;7] Although the number of studies in clinical development has increased significantly to over 900 drugs, which is an impressive 2.5 fold increase compared to the year 2000,[8;9] only 1 in 20 drugs that enter clinical evaluation will eventually be approved for registration.[10]

The set-up of this overview is that the pros and cons of traditional Phase I designs and alternative trial designs are being discussed in the light of cytotoxic drugs and MTAs. Special attention is also given to combination of anti-cancer drugs. There is an increasing rationale for combining different drugs in early phases of development, but the proper way how to do this is an area of much debate. Since biomarkers play an increasingly important role in the evaluation of potential new MTAs, the requirements of good BMs and the limitations of BMs is being addressed. Finally, an attempt has been made to provide brief guidelines or points of attentions for future design of early clinical trials.

Traditional Phase I trials, and why they might not be good enough anymore

The primary objective of traditional Phase I studies is to investigate the safety, and to determine the dose and schedule of a drug. The maximal tolerable dose (MTD) is hereby used as an endpoint and is reached through predefined dose escalation steps, for which different schemes have been developed. The most often used modified Fibonacci sequence dictates dose increments that become smaller when the dose increases (e.g. first

100% dose increase, followed by 67%, 50%, 40% and 30% increases). [11;12] Most commonly, a 33% toxicity rate is used to define the MTD (also see **Figure 1**).[13;14]

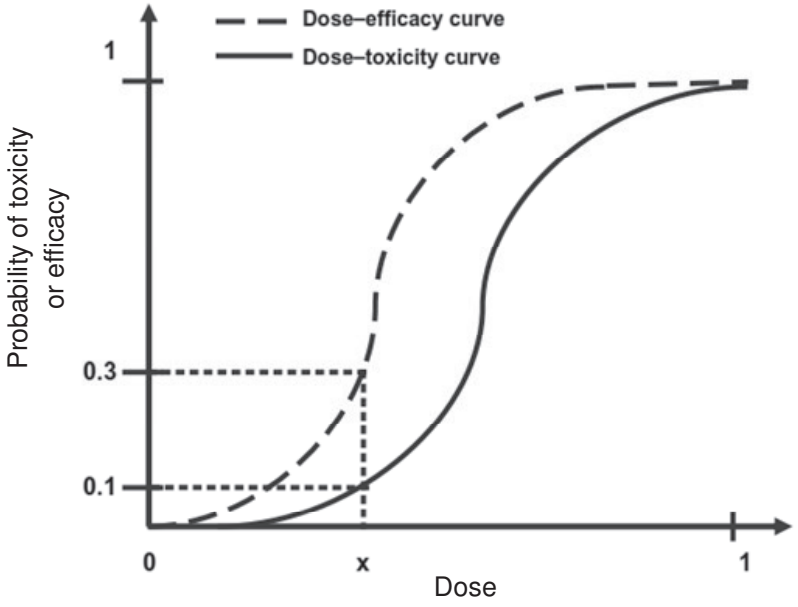

Figure 1. Relationship between toxicity and efficacy with dose for cytotoxic agents. Figure derived without adaptation (including figure legend) from Le Tourneau *et.al.* [11] "Typical dose-toxicity and dose-efficacy curves for cytotoxic agents. This example illustrates that at dose *x*, the probability of the efficacy is 30% and the probability of toxicity is 10%; hence the therapeutic index of the drug at dose x is 10% divided by 30% = 1/3."

Efficacy is evaluated in Phase II studies by applying the recommended phase 2 dose (RP2D) established in Phase I studies in a somewhat larger group of patients.[7] Phase II studies, in turn, are followed by large randomized Phase III trials to compare the new drug with the standard therapeutic drug. The information obtained from Phase I studies about safety, toxicity, PK and PD directly affect decisions about further development, thereby often dictating the fate of a novel compound.

In general Phase I trials are conducted in patients with (advanced) solid tumors for whom no standard treatment options are available anymore, but who do have a good performance status. The set-up most commonly used for traditional Phase I trials is referred to as the '3+3' design and allows dose escalation and de-escalation (an 'up-and-down' design). Initially three patients are included in one DL and if they tolerate the treatment without DLTs the dose is escalated. In case one of three patients experiences a DLT, the DL is expanded with three more patients. In case of two or more DLTs out of three to six patients, the DL is considered too toxic and the MTD is defined a DL lower. The design has proven its value in cytotoxic drug development because the rules for 'playing the game' of dose escalation are pragmatic and straightforward, and the minimal number of 3 patients per dose level (DL) is able to provide some information on interpatient pharmacokinetic variability. Because of the multiple dose escalation steps, it is a very safe design, but the downside of it is that a relatively large proportion of patients are potentially undertreated with subtherapeutic doses.[14]

Weaknesses of the traditional design in regard to MTAs include that the set-up is not optimal for drugs with a toxicity profile that does not correlate well to efficacy, or that does not comply with the common MTD definitions (e.g. chronic toxicities) and for exploring combinations of drugs. Furthermore, with the onset of rational drug design, in which the traditional methods of trial and error testing of chemical substances on cultured cells or animals is replaced by the inventive process of finding new drugs based on the knowledge of a biological target (e.g. ligand-based and three dimensional structure based drug design), decisions regarding superiority between a group of compounds or drug analogues based solid information about pharmacology, MOA, pharmacokinetics (PK) and pharmacodynamics (PD) are more relevant than ever before. Traditional Phase I trials tend to be too costly and too time consuming to be able to make fast decisions about superiority. Decisions however that pharmaceutical companies in the first place, but also doctors and everyone else involved in first-in-human testing need to make recurrently.

Are there better alternatives?

As explained previously, traditional trial designs have used toxicity as a primary endpoint. Although toxicity will always remain important, biologically based effects might serve as a more appropriate primary end point of first in human trials for MTAs, because the MTD of MTAs may differ from the dose resulting in substantial target-modulation.[15]

Phase 0 trials

Phase 0 trials attempt to meet the needs that the development of new anti-cancer drugs requires. These studies are named Phase 'zero' trials, which refers to the timeframe in which they are being conducted; prior to the traditional Phase I studies. The goal of Phase 0 studies is to enable go/no go decisions in an early stage of development in a human setting instead of only with the help of animal models.[9] One of the major strengths of Phase 0 trials is their emphasis on agent characterization and target effect or MOA through extensive target-assay development, and their increased flexibility regarding trial design: e.g. allowing intra-patient dose escalation, and exploring different drugs and schedules.[7] Phase 0 trials can be characterized by the following properties: 1) the studies are conducted in a limited number of patients (≤10 patients versus ± 20 patients in Phase I trials); 2) patients are exposed to the investigational drug for a limited period of time (≤ 7 days); 3) patients are exposed to a much lower dose (about a 100^{th} of the dose which is, based on animal data, expected to induce a pharmacological effect [16]). Consistent with this, is that the required toxicology testing needed before initiating a Phase 0 trial is often reduced compared to a Phase I trial (because the lower applied doses are generally associated with a decreased risk of toxicity).[7] However, another important ethical implication is that Phase 0 trials have no therapeutic intent. Instead, Phase 0 trials are considered to simplify drug selection, to help to bridge the gap of poor relation between preclinical and clinical PK and PD by gathering information about e.g. bioavailability, metabolism, and tissue distribution,[2] as well as to contribute to optimizing patient selection, response evaluation, and

selection of the starting dose. Altogether, Phase 0 trials might be able to compress the time lines for drug development.[7]

As Takimoto and colleagues discuss, three different types of Phase 0 trials are recognized by the Food and Drug Administration (FDA) investigational new drug (IND) guidance: microdose studies (with PK and imaging endpoints: radiopharmaceuticals can evaluate distribution and effect on a target by whole body imaging using techniques like PET [positron emission tomography], AMS [accelerated mass-spectrometry] and [dynamic contrast-enhanced magnetic resonance] DCE-MRI), pharmacologically relevant dose studies (with PD endpoints), and MOA related to efficacy studies (with PD biomarker endpoints, and for which more extensive safety testing is required).[17]

Despite the attention that Phase 0 trial designs received in literature, only very few Phase 0 trials have been conducted so far. This might be explained by the practical limitations involved. A biomarker, as well as a validated assay must be available in order to enable measurement of target effect, which is not always the case. Non-linear PK will also reduce the value of a Phase 0 study. From an ethical point of view, recruitment might be challenging since in contrast to Phase I studies, where -all be it limited- some therapeutic benefit could be expected, in Phase 0 studies there is none.[2] Participation might even negatively affect the chances for enrollment in future clinical studies.[7] Invasive procedures, like tumor biopsies, on the other hand, are mandatory for target characterization. Although opinions are ambiguous, Phase 0 studies expect a lot from patients in a very delicate stage of their lives, while personal benefit for patients is absent, and therefore Phase 0 studies demand a rather philanthropic mind set from patients and excellent information distribution from clinicians and other health care givers involved.

Clinical trials with accelerated titration designs

Different accelerated trial designs have been developed, e.g. by Simon and colleagues.[13] The designs were based on statistical models applied to twenty clinical trials already performed. All the designs from Simon *et al*.

with accelerated titration still make use of the traditional 3+3 design, after the accelerated phase of the trial. Main characteristics consist of using one-patient-cohorts and large dose increments between cohorts varying from 40-100%. Benefits include reducing the time of the trial, and the reduction of the number of patients treated at subtherapeutic doses (and thus potentially increasing the number of patients treating at an effective dose). But because of the intrapatient dose escalation long term toxicity will be more difficult to identify, as well as interpretation of the results of different dose levels might be hampered.[11]

Pharmacokinetically guided dose escalation (PGDE)

In PDGE trials preclinical and animal data are used to determine the starting dose in humans, and monitoring the pharmacokinetics of patients in a clinical Phase I trial is used to guide dose escalation and to reach the target area under the curve (AUC).[18] Samples need to be measured promptly during the trial at multiple time points. The use of different treatment schedules might complicate the use of this design. This design is also not recommended for compounds with high interpatient variability.[18;19]

Model based trials

Trials that use simulations are capable of determining the RP2D and might reduce the number of patients included in the trial, as well as reducing the number of patients, but need biostatistical expertise and appropriate software to be available.[11] Since most of these models are used to guide dose escalation all the data from previous patients is used during the trial and requires a data management system in which there is no delay in data entry.

Many simulation-based designs have been developed, only two will be mentioned briefly here (an extensive overview, although interesting is beyond the scope of this overview). The so called Bayesian model was the first continual reassessment method to be developed and uses a statistical approach of the shape of the dose toxicity curve as a basis for dose escalation.[20] A model based design that uses time to event as an endpoint is named time to event continual reassessment method,

abbreviated TITE-CRM, which is especially suited for toxicities that occur after the first cycle.[21]
For a more extended but very comprehensive overview of different designs for early phase clinical trials we kindly refer to Le Tourneau and colleagues.[11]

Trial designs for combination therapies

First-in-human clinical exploration of 1 novel drug is one thing, investigating a combination of drugs is a completely different story, because it adds more complexity than might initially be expected, for instance in determining the optimal dose and schedule of the combination. In addition, there is a difference of combining agents for which the MTD in a monotherapy schedule is already known (most common at present) and combining different agents of which for one or more drugs this information is lacking. However, although demonstrated single agent activity is required by the FDA for registration, it is actually debatable if for every novel compound a single agent Phase I or II trial demonstrating single agent activity should be required, especially if it is already very likely based on preclinical data that combination will be more effective, or if single agent activity is not at all to be expected. Besides considerations about costs and time lines, reservations are specifically in place with regard to the ethical aspect of exposing patients to a treatment which is expected to be inactive. Contributing to the complexity of this discussion, is that preclinical models can define anti-tumor effect relatively well, but that there are no standard preclinical models able to determine synergism and that include accurate determination of toxicity for a combination of drugs.[11]
Combining drugs most often implies that concessions need to be made regarding the applied doses in order to avoid excessive toxicity, although the ultimate goal is to assess what combination is most active and at the same time displays an acceptable safety profile. Despite a variety of strategies, there are 4 basic set-ups for dose escalation of combination therapies in Phase I trials, as Le Tourneau and colleagues[11] already nicely pointed out: 1) alternate dose escalation in multiple dose escalation steps; 2) simultaneous dose escalation in which both agents are increased at each

dose level; 3) single dose escalation (with one agent at a dose close to the MTD, and the dose of the other drug slowly increasing); 4) comprised dose escalation, in which both agents are escalated, but only one (almost) to RP2D. A way to obtain all necessary PK, especially if one novel compound is added, might be implementing a run-in period for the novel agent.[11] PK could be obtained with one agent and with the combination, in one patient. If for a drug the RP2D is known, simultaneous studies with different agents or schedules can be performed.[22] This might be especially suited for combinations of chemotherapeutic agents and new MTA's or drugs with nonoverlapping or little toxicities.

Novel study designs and Investigational Drug Steering Committee recommendations

Although many alternative trial designs have been developed, novel Phase I designs are scarcely used.[9;16] LoRusso and colleagues have published in 2010 a very practical overview which summarizes key aspects and formal recommendations made by the Clinical Trial Design Taskforce of the Investigational Drug Steering Committee, which include the following: 1) New designs have not yet succeeded in reducing the number of patients, nor the time needed for study completion; 2) Although the toxicity remains an important endpoint, in some cases it seems more appropriate to define the recommended dose and/or biologically active dose (BAD) than to define the MTD. In case of agents associated with minimal toxicity, or e.g. a limited absorption, the maximal potential dose (MPD) could be considered as a toxicity endpoint; 3) Intrapatient dose escalation can minimize the number of patients exposed to subtherapeutic doses and should therefore be promoted, but the rules for intrapatient dose escalation should be clearly described in the protocol (i.e. prior to study start). Decisions about dose escalation and RP2D should be made regardless of data from patients treated with intrapatient dose escalations; 4) The relevance of BMs is acknowledged, but the context, cost and feasibility of measuring a BM should determine decision making about implementation of a BM in a study. For trials that involve combinations of drugs, a rationale based on MOA or other preclinical research in which enhanced activity of the combination is demonstrated, preferably in

multiple models, should be promoted. The relevance of combination for future studies should be defined prior to the study. With determining the start dose, overlapping toxicity should be taken into account and anticipated. Finally, schedule and sequence should be explored, and novel designs which allow randomization are strongly recommended to enable analysis of the PK and PD effect of the combination versus the individual agents.

Drug combinations; how to combine sensibly?

We have addressed dose escalation and trial design for drug combinations, but another issue is: what agents to combine? Is there a way of choosing the 'right' agents to make the ultimate combinations? Which pharmacological effects can turn a combination into a success? Outside the field of oncology drugs have been successfully combined for many years and examples include tuberculosis, AIDS, and hypertension. In oncology, cytotoxic drugs have also often been combined and resulted in combination regimens, mostly referred to by acronyms; e.g. CHOP (cyclophosphamide, hydroxydaunorubicin [doxorubicin], vincristine [oncovin], prednisone) and FOLFOX (fluorouracil, leucoforin [folinic acid], oxaliplatin). But combinations of cytotoxic drugs and MTAs, or a combination of different MTAs, can also very well result in increased response rates.[3]

The main reasons for combining anti-cancer drugs include the following and are derived from the article written by Rodon and colleagues.[3] The first reason is cytotoxic enhancement; the effect can be additive (the effect of two agents is expected based on the concentration-response curve for each drug independently [23]) or synergistic (the effect of two agents is greater than expected [23]). These effects can be obtained by different strategies. Different pathways could be targeted by combining drugs that bind to different receptors (e.g. estrogen receptor and HER-2). A single receptor could also be targeted by combining agents with different MOAs (e.g. a monoclonal antibody [mAb] and a tyrosine kinase inhibitor [TKI], like trastuzumab and lapatinib).[24] Combination of two MTAs that inhibit

parallel signaling pathways can be an another option (e.g. combination of a MEK and a mTOR inhibitor [25]). The rationale for this approach is that secondary mutations can contribute to resistance and this strategy attempts to prevent this. Because feedback loops that mediate escape or resistance become especially activated after inhibition of a signaling pathway, this could theoretically be prevented by anticipating on this effect, and therefore adding another agent that is involved in the feedback loop (e.g. combining a MEK inhibitor with a PI3K inhibitor[26]).This strategy is closely related to the one of inhibiting both upstream and downstream targets of signaling pathways (e.g. EGFR and mTOR inhibitors [27]). As already explained, combination of different agents also attempts to avoid or at least slow down the development of resistance. Somewhat different is the idea of selective sensitization by combining a cytotoxic agent that induces DNA damage and adding another agent that prevents the repair of the induced DNA damage, specifically in tumor cells (e.g. cisplatin and a PARP inhibitor[28], or still in an early stage but with promising results carboplatin/cisplatin/gemcitabine in combination with a Wee1 inhibitor [29]).

These strategies are all theoretical. What should be done or what information should be available before a clinical trial with a certain combination of anti-cancer drugs can indeed be initiated? First of all, preclinical data with information about the PK, metabolism (including the effect on metabolic enzymes and transporters), PD and toxicology of each drug alone should be available. But what kind of information about preclinical research in regard to the hypothesized combination should be available is less well specified, and different approaches for preclinical investigation of drug combinations are actually characterized by different drawbacks. Chemosensitivity or cell viability assays (potential drug combinations added to different *in vitro* cultured cancer cell lines) are practical to perform, but commonly provide insufficient information about the MOA, compared to an animal model. Tumor models like xenografts and orhtotopic models, or transgenic mice models with specific mutations or deletions, are better suited for describing the MOA, but nevertheless often display a discrepancy with the clinical setting in patients with real,

heterogeneous tumors.[3] Combining agents based on efficacy results observed in a clinical setting in a single agent regimen for specific diseases, provides valuable information regarding toxicity, but even in such a scenario, providing a preclinical motivation before initiating a clinical trial should still be encouraged in an attempt to prevent unnecessary disappointments with expensive clinical trials performed in patients.

Another question is how many drugs should be combined? Based on the number of genes that in case of mutation can lead to e.g. tumor invasion and metastasis, the number of drugs that could (or should) be combined almost seem innumerous.[3] The shift to using high-through-put screens to define the genes that play the most pivotal role in a patient's tumor seems at present one of the most promising steps forward in tackling this huge problem.

The need for biomarkers in personalized medicine

Treatment with cytotoxic drugs could be described as 'one size fits all'. And although cytotoxic drugs have proven their value, there is an increased understanding that tumors are heterogeneous and 'evolve' over time and during treatment. Even primary tumors and metastases can display significant variations regarding e.g. mutational status, degree of vascularization, and tendency to grow or metastasize. Personalized medicine is a term that is commonly used nowadays, has raised increased interest with the introduction of MTAs, and refers to customized anti-cancer therapy that meets the needs of each patient or tumor based on individual tumor characteristics.[30] The principal thought behind the concept of personalized medicine is to divide cancer subtypes in smaller subtypes based on e.g. genetic alterations that are known to respond in a certain way to certain treatments or interventions. Validated markers that are able to identify and predict the outcome of certain interventions are mandatory to effectuate the idea of personalized medicine.[31]

One of the general definitions used to describe BMs, is worded as follows: 'characteristics that can be objectively measured as indicators of a biological or pathological process or pharmacological response to a therapeutic intervention' (Biomarkers Consortium – Foundation of National Institutes of Health).[32]

The use of BMs covers a broad range, and as a result BMs can be classified in multiple ways. An overview of the main types of BMs used in oncology is summarized in **Table 1**. BMs can help in early stages of development to estimate target effect and contribute in go/no go decisions, and assist in patient selection. BMs can facilitate diagnosis of cancer or help to monitor response to treatment, or to detect early recurrence. Finally, BMs can estimate the probability of response to treatment.

Pharmacodynamic BMs help to evaluate the (target) effect of a drug during preclinical investigation, but especially during clinical trials and therefore BMs are more important than ever in the era of targeted drugs, where the MTD does not necessarily corresponds with the biologically most effective dose. Requirements for a PD BM assay are based on several parameters: accuracy or sensitivity (ratio of BM positive results identified by the assay compared to the actual positive results), specificity (the proportion of negatives which are correctly identified), dynamic range (e.g. the concentration range in which the assay can measure a certain analyte), precision (variability of results compared to the true value), reproducibility (results of the assay on identical test material under different conditions, e.g. time of analysis or influence of freezing and thawing), robustness (stability over time, capability of transferring the assay to other laboratories).[7]

Other practical considerations consist of the type of biological material to perform the assay with and the presence of potential ethical limits in obtaining different samples at different time points (e.g. blood, serum or PBMCs which are less invasive than tumor biopsies) or the question if the BM assay can or should be performed on different kinds of material?

Table 1. Classification of cancer biomarkers modified from Dancey *et al.*[37] and Alymani *et al.* [33] BMs can be classified in different ways. For instance by modality of application, modality of assessment or based on their biological properties. A selection of most commonly used specifications has been included in the table. Sometimes BMs fit more than one category.

Type of Biomarker	Characterization	Example
Biochemical (or molecular)	Found in body fluids or tissue and often consist of genes, gene products or proteins	PSA (prostate cancer), CA-125 (ovarian cancer)
Diagnostic	Assists in identifying (the type of) disease, used to monitor treatment	PSA (prostate cancer), CA-125 (ovarian cancer)
Genomic	e.g. gene expression profiles	Mamma Print™ 70 gene (breast cancer)
Imaging	Evaluate pharmacologic or metabolic response	FDG-PET metabolic response
Pathological	e.g. immunohistochemistry (IHC), polymerase chain reaction (PCR)/sequencing	HER-2 (breast cancer) K-RAS (e.g. lung cancer)
Pharmacokinetic and pharmacodynamic	Demonstrate a direct pharmacologic effect of a drug, helps to predict the most effective dose	DPD (capecitabine)
Predictive	Provides information about the probability of benefit from a specific intervention, helps patient selection	HER-2 (breast cancer), K-RAS (e.g. lung cancer)
Prognostic	Provides information about the overall disease outcome independent of any specific intervention, assists in identifying patients that will respond to a drug	TIMP-1 multiple myeloma
Other	Miscellaneous	Circulating tumor cells (CTCs)

The development of a BM is characterized by different essential stages: discovery, validation, qualification, and implementation.[33] Starting with the discovery, it is important to know how the BM will be used and what is desired to be measured. It needs profound understanding of the pathogenesis on a molecular level. For BM validation similar principles can be applied as are used in bio-analytical method validation according to Good Laboratory Practice (GLP).[34] In the qualification process, sensitivity and specificity is defined, and clinical utility. However, for acceptance of BM qualification cost-effectiveness is also mandatory. For the final step, implementation of biomarkers a BM needs to be approved by the regulatory authorities, physicians need to be encouraged to use the BM and there should be a positive cost-effectiveness. The field of BM development is a field in progress. Development of methodology for BM is expected to improve BM development.[33] For more detailed information is referred to the existing literature.

Considerations for future early clinical trials

Teamwork, flexibility and creativity

One of the most obvious changes in cancer drug development is the increased complexity. Complexity in the sense of the necessity to understand the pathophysiology of different types and forms of cancer on a molecular level, but also complexity in all different areas and disciplines involved in both the preclinical and clinical phase of development of anti-cancer drugs. Cancer research and drug development is teamwork, now more than ever before. The extend to how the 'team' is functioning as a whole, is reflected in the outcome of clinical trials. The preclinical team constitutes molecular biologists, biochemists, pharmacists, bio-statisticians and many more. Mainly involved in the clinic are oncologists, surgeons, radiologists, nurse practitioners, nurses, data managers, to name few. But the line between pre-clinic and clinic is slowly fading out.

Cooperation, communication back and forth and the flexibility to respond and react to observed changes is of utmost importance for success in new early phase clinical trials. For instance, unexpected toxicities observed in

the clinic, can be investigated in the laboratory during a trial. The BM results and information about PK can result in adjustment of dose and even schedule. The use of simulation models could be used to assess the RP2D with fewer patients actually treated, or -equally important- can make optimal use of data collected in patients that have participated. Continuous reassessment and creative out of the box thinking, but with a profound rationale, should be encouraged.

Because of this complexity it is recommended to limit the number of participating sites of a trial to 3 different centers. First of all, because although it sounds as if a higher number of sites will speed up a trial, in reality it turns out that it will not.[14;35] Brutal competition for slots (mostly to avoid the need to disappoint a patient) is completely undesirable. But also can it hamper the experience of doctors to identify toxicity patterns of drugs. Within a participating hospital it is therefore recommended to assign doctors or nurse practitioners to clinical trials in order to maximize the experience with a drug from a clinical point of view.

Teamwork also involves close collaboration of 'pharma' versus health care givers and scientists. Pharmaceutical companies, academia and hospitals are encouraged to collaborate more closely. For instance in providing little amounts of pipeline drugs for e.g. testing in transgenic mouse models.

Finally, collaboration between pharmaceutical companies -although easily hampered by financial interests- is important. Too many companies focus independently on the development of a drug that has a similar drug target. Moreover, in trials with a combination of drugs, companies too often will give preference to combining their own novel compound of interest with another second compound they have in development, instead of choosing that compound (from another pharmaceutical company) that displays the most favorable pharmacological properties, or with which the most experience has been obtained.

Databases and the need for sharing information

Where would we be without internet and PubMed database? Many great initiatives to share information, to meet experts, and exchange ideas already exist, varying from annual cancer conventions like American Society of Clinical Oncology (ASCO), and American Association for Cancer Research (AACR), the International Agency for Research on Cancer (IARC) database for mutations and their clinical relevance, and the website clinicaltrials.gov where information about ongoing clinical trials can be obtained. Other initiatives are committees in which experts come together and develop guidelines, e.g. for BM development. Sharing information is necessary to prevent reinventing the wheel and to accelerate breakthroughs in cancer research.

Applying high tech technologies

Research has lead to new insights and new technologies, and thus is not limited to the development of new agents alone. Validated techniques can contribute to the development of better drugs. The use of BMs should indeed be promoted, despite the complexity and challenges involved. For early clinical trials a clinical trial track is included in **Figure 2**, which is adapted from Yap and colleagues.[36] and clearly visualizes the backbone of modern early clinical trials and a step forward to the actualization of personalized medicine. Essential in the scheme is that tumor analysis will be performed and screened for genetic alterations prior to study start and at progression. Based on the outcome of this genetic screen, the most appropriate trial or therapy will be selected. During treatment the patient will be monitored and besides PK sampling, pharmacologic BMs will be used to assess target effect, and response to treatment. At disease progression, tumor tissue (and other relevant tissue like blood or skin) is obtained for analysis of resistance mechanisms.

Initiatives like the Center for Personalized Cancer Treatment (CPCT http://www.cpct.nl) in the Netherlands are excellent examples of putting the scheme of **Figure 2** in practice. Three different cancer centers have joint their forces and CPCT aims to optimize patient selection for clinical trials by Next Generation Sequencing (NGS) on material obtained from tumor biopsies. Insight in the genetic changes in cancer cells can hereby be

obtained, and actually form a very solid and essential basis for progress that is expected to be made.

In conclusion, the shift from broadly acting cytotoxic drugs towards the use of more specific agents that target genetic alterations in tumors has drastic effects on the way clinical trials are conducted, but also on how different disciplines in academia, 'pharma' and hospitals (need to) work together.

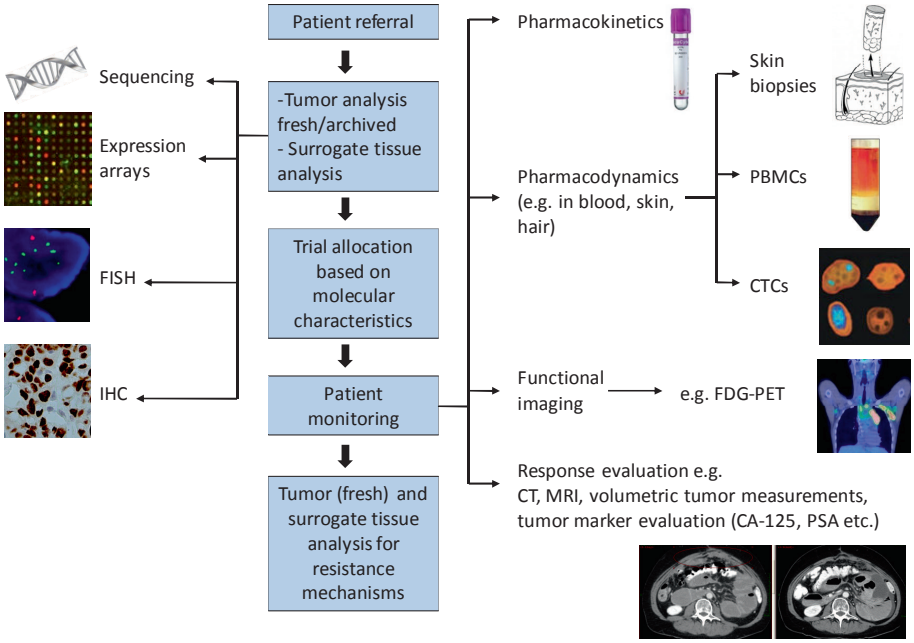

Figure 2. Clinical trial track for early-phase clinical trials modified from Yap *et al.*[36].

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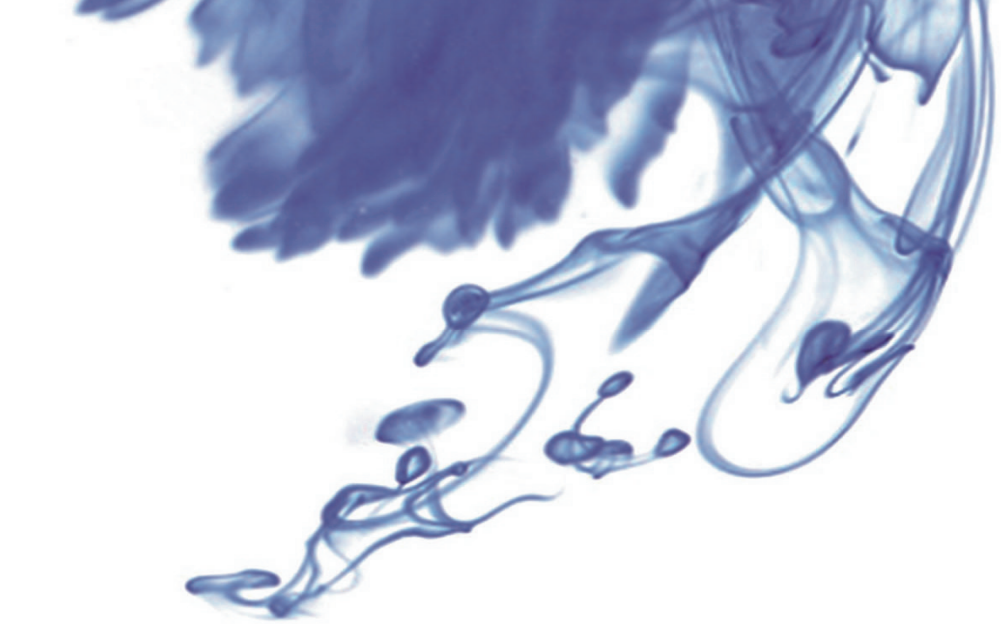

CHAPTER 2

Clinical pharmacological studies with MEK-Inhibitors

2.1

A phase I, open-label, randomized crossover study to assess the effect of dosing of the MEK 1/2 inhibitor selumetinib (AZD6244; ARRY-142866) in the presence and absence of food in patients with advanced solid tumors

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Cancer Chemother Pharmacol 2011 Dec;68(6):1619-28

Abstract

Purpose

This Phase I study assessed whether food influences the rate and extent of selumetinib absorption in patients with advanced solid malignancies, and determined the safety, tolerability and pharmacokinetic (PK) profile of selumetinib and its active metabolite N-desmethyl-selumetinib in fed and fasted states.

Methods

A single dose of 75 mg selumetinib was to be taken with food on Day 1 followed by a single dose of 75 mg after fasting for at least 10h on Day 8, or vice versa, followed by twice daily dosing of 75 mg selumetinib from Day 10. Plasma concentrations and PK parameters were determined on Days 1 and 8. Patients could continue to receive selumetinib for as long as they benefitted from treatment.

Results

In total, 31 patients were randomized to receive selumetinib; 15 to fed/fasted sequence and 16 to fasted/fed sequence. Comprehensive PK sampling was performed on 11 and 10 patients, respectively. The geometric least-squares means of C_{max} and AUC for selumetinib were reduced by 62% (ratio 0.38 90% CI 0.29, 0.50) and 19% (ratio 0.81 90% CI 0.74, 0.88) respectively under fed compared with fasting conditions. The rate of absorption (t_{max}) of selumetinib (fed) was delayed by approximately 2.5 hours (median). The food-effect was also observed for the active metabolite N-desmethyl-selumetinib. Selumetinib was well tolerated.

Conclusions

The presence of food decreased the extent of absorption of selumetinib. It is recommended that for further clinical studies selumetinib be taken on an empty stomach. Selumetinib demonstrated an acceptable safety profile in the advanced cancer population.

Methods

Aims

The primary objective of this study was to assess whether food influenced the rate and extent of selumetinib absorption. Secondary objectives were to determine the PK of both selumetinib and N-desmethyl-selumetinib in the presence and absence of food and to assess the safety and tolerability of selumetinib in patients with advanced solid malignancies.

An exploratory objective was included to assess the efficacy of selumetinib as measured by Objective Response Rate (ORR) based on Response Evaluation Criteria in Solid Tumors (RECIST 1.0) assessment in patients with measurable disease.

Patient selection

Male and female patients aged 18 years and over (World Health Organization (WHO)/Eastern Cooperative Oncology Group (ECOG) performance status 0-2) with advanced cancer, refractory to standard therapies or for whom no standard therapies existed, were enrolled in two investigational centers in The Netherlands and three in the United Kingdom. Patients had to be able to eat a high fat breakfast within a 30-minute time period. Required laboratory values consisted of absolute neutrophil count (ANC) ≥1500 per mm^3, platelets ≥100,000 per mm^3, hemoglobin >9.0 g/dL, serum bilirubin <1.5 x upper limit of normal (ULN), aspartate aminotransferase (AST) <2.5 x ULN, alanine aminotransferase (ALT) <2.5 x ULN and calculated serum creatinine clearance >50 mL/min (using Cockcroft-Gault formula or by 24h urine collection).

Exclusion criteria included: patients with refractory nausea and vomiting, chronic gastrointestinal diseases or significant bowel resection that would preclude adequate absorption; pregnant or lactating females; patients that received any radiotherapy or chemotherapy within 21 days prior to starting the study, or any investigational drug within the previous 28 days; patients with mean QTc (using Fridericia's correction) >450 ms at screening or with factors that increased the risk of QT prolongation or arrhythmic events or

patients using concomitant medication known to prolong QT interval; and patients with brain metastases or spinal cord suppression unless treated and stable for at least 1 month.

Informed written consent was obtained from all patients in accordance with federal and national guidelines and the study was conducted in compliance with GCP guidelines and the Declaration of Helsinki. An additional informed written consent was needed for optional genetic blood sampling.

Study design

This was a Phase I, multi-center, open-label, randomized crossover study (NCT00710515). The dose of selumetinib was selected based upon the PK, safety and tolerability data from the phase I clinical study of the Hyd-Sulfate capsule formulation [15]. Eligible patients were randomized to one of two sequences. The first sequence received a single dose of 75 mg selumetinib with food on Day 1, followed by a single dose of 75 mg selumetinib in the fasted state on Day 8. The second sequence received 75 mg selumetinib in the fasted state (Day 1) and then 75 mg selumetinib with food (Day 8) following a 7 day wash out period. Both groups continued on twice-daily 75 mg selumetinib from Day 10 onwards (extension period). Patients could continue to receive selumetinib until disease progression, unacceptable toxicity, or for as long as they continued to derive benefit from treatment. Selumetinib capsules were taken with approximately 240 mL of water. On Days 1 and 8 water could be taken freely up to 1h prior to, and from 1h after, dosing. In the fasted state, patients received no food or drink other than water for 10h prior to dosing. In the fed state patients were fed an FDA high fat breakfast [19]. Patients were required to completely ingest this breakfast within 30 minutes and were dosed with a single oral 75 mg dose of selumetinib 30 minutes after starting to eat breakfast. A standard meal was given at 4h post-dose on both days, and until that time no food or drink (other than water) was received. For the BD dosing from Day 10 onwards, the extension period, the doses were taken 12h apart. Both doses were taken in the fasted state. Breakfast could be

taken from 1h following dosing. Evening doses were not to be taken in the 1h preceding a meal or in the 2h after having finished a meal.

Treatment with selumetinib during the BD treatment phase was withheld if patients experienced an intolerable Adverse Event (AE) or any AEs ≥ Common Terminology Criteria (CTC) Grade 3. Selumetinib treatment could be restarted after the toxicity improved to a level considered by the Investigator to be manageable (i.e., CTC Grade 1, except for dermatological AEs where CTC Grade 2 was acceptable). Treatment could be resumed at the original dose or at a permanently reduced dose (50 mg selumetinib BD).

Plasma sampling and assay methods

Blood samples were collected on Days 1 and 8, at pre-dose (within 30 minutes of dosing), 15 and 30 minutes and 1, 1.5, 2, 4, 8, 12, 24, 36 and 48h post-dose for analysis of plasma selumetinib and N-desmethyl-selumetinib metabolite concentrations. For the analysis of selumetinib and N-desmethyl selumetinib in human plasma, solid phase extraction (SPE) was followed by high performance liquid chromatography (HPLC) with tandem mass spectrometric detection (MS/MS) with a calibration range that extended from 2-2000 ng/mL for selumetinib and 2-500 ng/mL for N-desmethyl selumetinib. $^{13}C_6$-selumetinib and $^{13}C_6$-N-desmethyl selumetinib were used as internal standards in the assay. The analytes and internal standards were extracted from human plasma by SPE using Phenomenex Strata-X plates Polymeric Reversed Phase 96-well plates and injected onto a Ultra Performance Liquid Chromatography (UPLC) column (Acquity(tm) UPLC® BEH Phenyl, 1.7 m, 2.1 x 50 mm) or an HPLC column (Phenomenex Synergi, 4µ Polar-RP, 2.0 x 50 mm). The chromatographic separation and detection was achieved by Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) using a Sciex API5000, respectively. Selumetinib and N-desmethyl selumetinib concentrations were determined by reference to calibration curves created by adding known concentrations of selumetinib and N-desmethyl selumetinib to control human plasma.

Pharmacokinetic analysis

Actual sample times were used for the PK analysis. All PK computations were performed at the Department of Clinical Pharmacology, Quintiles Overland Park, using WinNonlin Professional 5.2 and SAS® Version 8.2. To evaluate the PK characteristics of selumetinib and N-desmethyl-selumetinib in the presence and absence of food the following parameters were determined by non-compartmental analysis: 1) For selumetinib: C_{max}, t_{max}, AUC, area under the plasma-concentration-time curve from zero to the time of the last quantifiable plasma concentration (AUC_{0-t}), terminal rate constant (λ_z), $t_{1/2}$, volume of distribution (apparent) during terminal (λ_z) phase (V_z/F) and CL/F. Though not a true indicator of rate, t_{max} was used as an indicator of the effect of food on the rate of absorption of selumetinib. 2) For N-desmethyl selumetinib: C_{max}, t_{max}, AUC, AUC_{0-t}, λ_z and $t_{1/2}$.

Assessments

AEs were evaluated throughout the study and graded according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. In addition, the following safety assessments were performed: ophthalmological examination, ECGs, MUGA scan or echocardiogram and safety laboratory evaluations. Tumor assessments, performed as exploratory analysis, were done every 6-8 weeks and Response Evaluation Criteria in Solid Tumors (RECIST 1.0) was used to assess response to treatment.

Statistical evaluation

At least 24 patients were to be randomized to the two sequences to assess whether the 90% Confidence Interval for the ratio of the food effect on AUC and C_{max} lie entirely within the range of (0.8, 1.25). AUC and C_{max} were log-transformed and analyzed using an Analysis of Variance model allowing for the effect of food (fed or fasted), period, sequence and patient within sequence. The adjusted geometric means (gls means) were estimated for each treatment arm (fed or fasted). An estimate of the food effect (ratio of the gls means of selumetinib in fed state: fasted state) was calculated together with its 90% confidence interval (CI). A formal test of carry-over was not performed. The potential for any carry-over was

assessed by examination of pre-dose plasma concentrations on Day 8. An analysis of selumetinib t_{max} data was performed on untransformed data using a non-parametric analysis. The food effect, as measured by patient differences in t_{max} (fed minus fasted states), was analyzed using a Wilcoxon signed rank test. The Hodges-Lehmann estimator of median food effect was calculated and corresponding 90% CIs constructed.

The study included 3 main analysis populations: 1) the Per Protocol (PP) analysis set, 2) the Safety analysis set and 3) the Evaluable for PK analysis set. The PP analysis was to include all patients who were evaluable for PK analysis, had complied with the protocol requirements, and had no protocol violations. The Safety analysis set was to include all randomized patients who received at least 1 dose of study medication. Finally, the Evaluable for PK analysis set was to include all randomized patients who had completed at least 1 period of the study and had sufficient PK data available to evaluate the primary outcome variables AUC and Cmax. The analysis of primary PK outcome variables was presented for both the group 'Evaluable for PK' and the group 'Per Protocol (PP) analysis set'. The PP analysis was considered primary for interpretation of data, with supportive interpretation from Evaluable for PK analysis.

Results

Analysis sets and protocol deviations

Of 31 randomized patients, 30 were included into the PK and safety analysis set. One patient was excluded from the safety and PK set as she did not receive treatment. Nine patients were excluded from the PP analysis set due to protocol deviations. The following protocol deviations were observed: four patients had the drug administered more than 30 minutes after the breakfast; two patients did not complete both the fed and fasted periods of the crossover study; and five patients had insufficient breakfast consumption prior to dosing in the fed period.

Table 1. Demographic characteristics.

Demographic characteristics		Fed/Fasted (N=15)	Fasted/Fed (N=15)	Total (N=30)
Gender (n and % of patients)	Male	9 (60)	12 (80)	21 (70)
	Female	6 (40)	3 (20)	9 (30)
Age (years)	Mean (SD)	59.7 (9.01)	56.6 (10.25)	58.1 (9.61)
	Range	44/77	32/70	32/77
Age group (years) (n and % of patients)	≥ 18 -≤ 65	12 (80.0)	13 (86.7)	25 (83.3)
	> 65	3 (20)	2 (13.3)	5 (16.7)
WHO performance status (n and % of patients)	0	6 (40.0)	7 (46.7)	13 (43.3)
	1	2 (13.3)	7 (46.6)	9 (30.0)
	2	6 (40.0)	0	6 (20.0)
	3-4	0	0	0
Tumor site (n and % of patients)	Skin/Soft Tissue	3	5	8 (26.7)
	Colon/Colorectal/Rectal	3	3	6 (20)
	Head & Neck	1	1	2 (6.7)
	Esophagus	1	1	2 (6.7)
	Biliary Tract	2	0	2 (6.7)
	Liver	1	1	2 (6.7)
	Renal	2	0	2 (6.7)
	Bladder	1	1	2 (6.7)
	Other	1	3	4 (13.3)
Number of prior regimens (n and % of patients)	0	2 (13.3)	3 (20.0)	5 (16.7)
	1	5 (33.3)	2 (13.3)	7 (23.3)
	2	5 (33.3)	6 (40.0)	11 (36.7)
	>2	3 (20.0)	4 (26.7)	7 (23.3)

Race (n and % of patients)	Caucasian	12 (80)	15 (100)	27 (90)
	Black	1 (6.7)	0	1 (3.3)
	Asian – Non Japanese	1 (6.7)	0	1 (3.3)
	Other	1 (6.7)	0	1 (3.3)

Patient characteristics

Patient demographics and baseline disease characteristics are shown in **Table 1**. The most commonly reported sites of primary tumor were skin/soft tissue tumors [melanoma (8 patients)], and colorectal (3 patients). Overall, metastatic disease was more commonly reported than locally advanced tumors with the majority of metastatic disease occurring at the following sites: lymph nodes, hepatic (including gall bladder) and respiratory. The largest proportion of patients (36.7%) had received two prior chemotherapy regimens. This population was representative of typical Phase I population with pretreated patients.

Pharmacokinetics and bioavailability

Measurable concentrations of selumetinib were observed in both fed and fasted periods during the complete sampling period; up to 48 hours (**Figure 1A**).

Administration of selumetinib on an empty stomach resulted in significantly higher selumetinib exposure: geometric least-squares means Cmax and AUC were reduced by 62% (ratio 0.38 90% CI 0.29, 0.50) and 19% (ratio 0.81 90% CI 0.74, 0.88), respectively, under fed conditions, compared with exposure following 75 mg of selumetinib taken fasted (**Table** 2 and **Figure 2**).

The median t_{max} of selumetinib (75mg) was delayed by approximately 2.5 hours (90% CI 1.77, 3.03) in the presence of food. Results from a further t_{max}, AUC and C_{max} analysis conducted on data from all patients where PK data were evaluable were very similar to those observed in the PP population The geometric mean clearance of selumetinib was lower (19%) in the fasted state compared with the fed state reflecting the observed difference in AUC. The small difference in the volume of distribution (Vz/F)

observed between the fasted and fed states, did not translate into an appreciable difference in half-life (**Table 2**). Similar results were observed in the PK population.

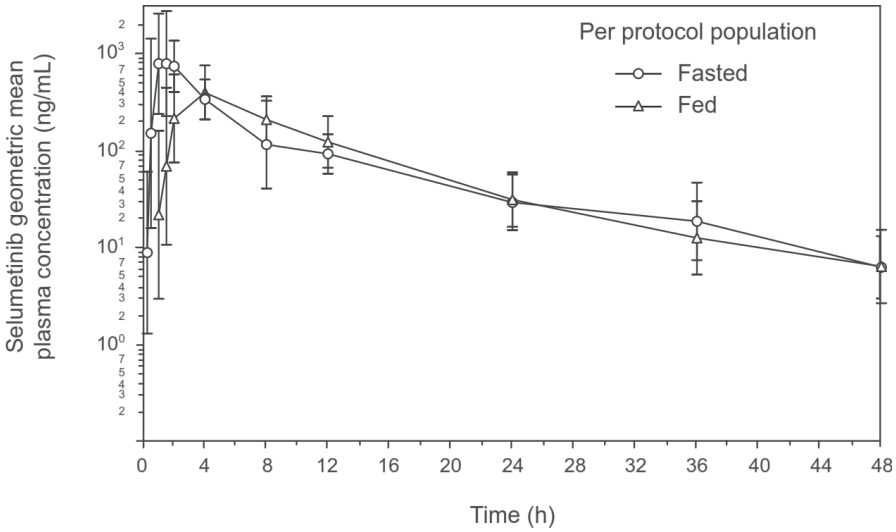

Figure 1A. Geometric mean (± SD) concentration profiles of selumetinib in fed and fasted state (PP population).

Table 2. Influence of food on the rate and extent of selumetinib absorption (**A**) and pharmacokinetic parameters for (**B**) selumetinib and (**C**) N-desmethyl-selumetinib in fed and fasted state [all PP population].

(A)				**Pairwise Comparison**	
Selumetinib PK parameters	**Treatment**	**n**	**Geometric LS mean**	**Ratio of fed/Fasted**	**90% CI**
AUC (ng*h/mL)	Fed	21	4664		
	Fasted	19	5782	0.81	0.74, 0.88
AUC_{0-t} (ng*h/mL)	Fed	21	4504		
	Fasted	21	5639	0.80	0.73, 0.88
C_{max} (ng/mL)	Fed	21	557		
	Fasted	21	1450	0.38	0.29, 0.50

(B)				
Selumetinib PK parameters	**Treatment**	**n**	**Geometric mean**	**CV (%)**
t_{max} (h)	Fed	21	4.0[a]	1.0, 11.6[a]
	Fasted	21	1.08[a]	0.5, 4.0[a]
$t_{1/2}$ (h)	Fed	21	8.6	44.5
	Fasted	19	9.4	33.0
CL/F (L/h)	Fed	21	16.0	35.8
	Fasted	19	12.9	29.9
V_z/F(L)	Fed	21	199.4	56.2
	Fasted	19	175.2	46.8

(C)				
N-desmethyl-selumetinib PK parameters	**Treatment**	**n**	**Geometric mean**	**CV (%)**
Cmax (ng/mL)	Fed	21	33.5	63.8
	Fasted	21	73.9	45.7
t_{max} (h)[a]	Fed	21	4.0[a]	1.0, 11.6[a]
	Fasted	21	1.50[a]	0.5, 4.0[a]
AUC (ng*h/mL)	Fed	13	379.1	49.0
	Fasted	15	418.4	36.5
AUC0-t (ng*/h/mL)	Fed	21	282.4	68.2
	Fasted	21	376.4	41.0
$t_{1/2}$ (h)	Fed	13	6.8	56.6
	Fasted	15	7.7	45.5

a Data are presented as median (min, max).

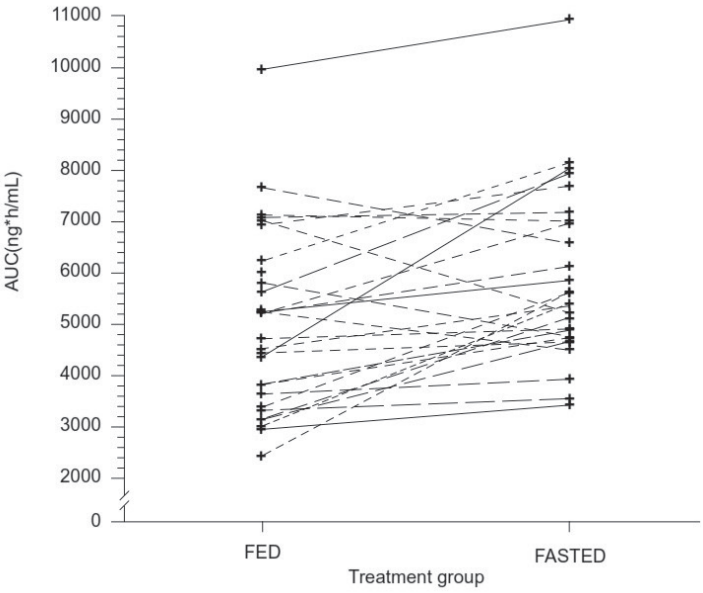

Figure 2A. AUC of selumetinib in fed and fasted state (PP population).

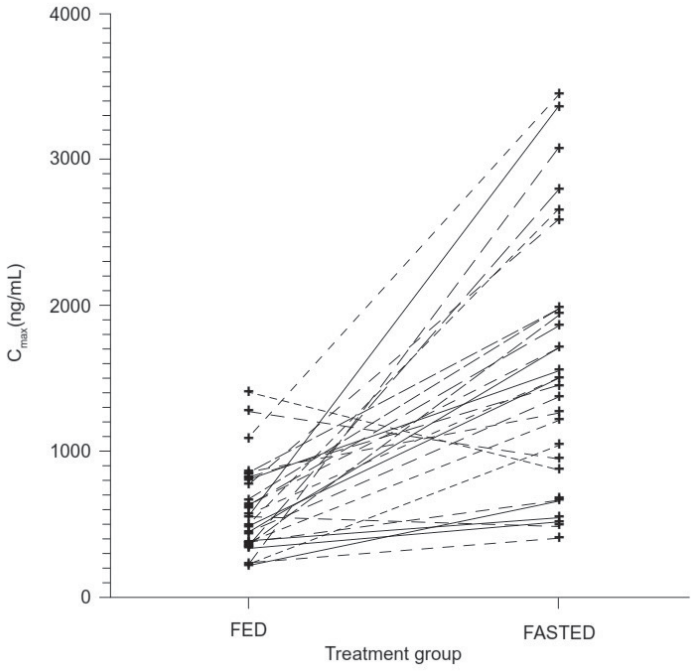

Figure 2B. C_{max} (B) of selumetinib in fed and fasted state (PP population).

Measurable concentrations of N-desmethyl-selumetinib were observed in both the fed and fasted periods for up to 24 hours (**Figure 1B**). Similar to selumetinib, the mean concentrations of N-desmethyl-selumetinib in the fed state were lower in comparison to the fasted state and the t_{max} for N-desmethyl-selumetinib was delayed when selumetinib was administered in the presence of food. A decrease of approximately 55% in the geometric mean Cmax and 9% in the geometric mean AUC of N-desmethyl-selumetinib were observed when selumetinib was given in the presence of food compared to the fasted state (**Table 2**). Whilst the presence of food reduced the exposure to selumetinib compared with the fasted state, the ratio of N-desmethyl-selumetinib to selumetinib (metabolite to parent) remained similar (0.06 and 0.07 ng*h/mL for fed and fasted state respectively). Hence, both the median plasma concentration of the parent compound and of the N-desmethyl-metabolite were decreased in a similar extent when selumetinib was given in the presence of food. The median tmax for N-desmethyl-selumetinib was delayed by approximately 3 hours in the fed condition, also comparable to the delayed tmax of selumetinib.

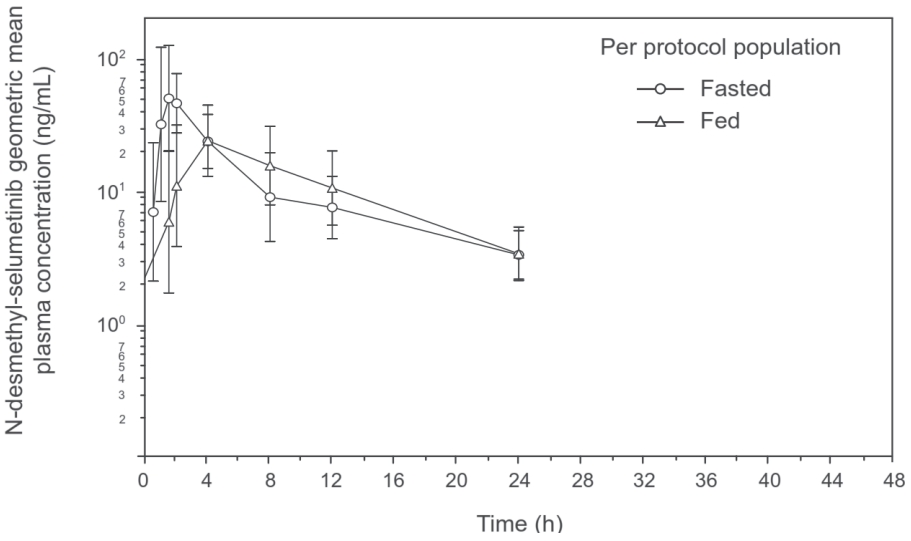

Figure 1B. Geometric mean (± SD) concentration profiles of N-desmethyl-selumetinib in fed and fasted state (PP population).

Safety and tolerability

Of the 31 randomized patients, 28 completed the food effect period which involved 2 single doses of selumetinib (on Day 1 and Day 8) and continued on BD dosing from Day 10 for a mean duration of 58.7 days (median 52.0 days; range 7 to 229 days). Two patients did not enter the extension period following voluntary discontinuation from the study. In the extension part 10 patients had one or more dose reductions largely due to adverse events (n=6) (AE).

Table 3. Most frequent AEs (≥10% of patients) in safety population.

	Number (%) of patients Total (n=30)
Nausea	18 (60.0)
Rash	16 (53.3)
Diarrhea	14 (46.7)
Fatigue	13 (43.3)
Constipation	11 (36.7)
Dyspnea	10 (33.3)
Vomiting	9 (30.0)
Abdominal pain	8 (26.7)
Dizziness	8 (26.7)
Edema peripheral	7 (23.3)
Anemia	7 (23.3)
Headache	7 (23.3)

A patient can have one or more preferred term reported under a given system organ class. Patients with multiple events in the same PT are counted only once for that PT, within each period. Adverse events with an onset date during the 30 day follow-up are assigned to the period in which their last dose was administered.

Adverse events

The most frequently reported AEs overall (regardless of severity, causality or seriousness) were nausea, rash, diarrhea, fatigue, constipation, dyspnea and vomiting (**Table 3**). Eighteen out of 30 (60.0%) patients (overall safety population) reported an AE of grade 3 or higher during the study, but only 4 (13.3%) of whom had grade 3 AEs (**Table 4**) which were assessed as potentially treatment-related. The 2 (6.7%) grade 4 AEs were not considered to be causally related to study treatment (one patient experienced worsening of dyspnea and one patient had hypercalcaemia). Serious AEs and AEs leading to discontinuation were reported by 40% and 20% of patients, respectively. One patient (3.3%) died whilst on study as a result of an intestinal perforation due to disease progression. Although the case was reported as an AE leading to death, it was in fact due to disease progression. AEs which were only reported following chronic exposure to selumetinib in the extension phase were peripheral edema, hypertension, dry mouth, vision blurred, skin fissures, joint swelling, acne (acne/dermatitis acneiform) and dyspepsia. The most common AEs reported following single exposure to selumetinib were diarrhea, nausea and abdominal pain.

Laboratory and vital sign findings in this study were generally consistent with those seen previously in selumetinib monotherapy studies. No notable changes in vital signs were observed.

Table 4. SAE of ≥CTC grade 3 reported in ≥2 patients (safety population).

	Number (%) of patients
	Total (N=11)
Dyspnea	3 (10.0)
Abdominal pain	2 (6.7)
Constipation	2 (6.7)
Vomiting	2 (6.7)
Anemia	2 (6.7)

Anti-tumor activity/efficacy

Although response evaluation was not the primary end point of this study, 28 patients were evaluable for objective response by RECIST. Two partial responses (7.1%) were observed (Day 71 and on Day 189). Both patients had cutaneous melanoma and were still alive and continuing study treatment at data cut-off. In addition, 5 (18%) patients had the best overall response of stable disease, 2 for >100 days (1 colorectal cancer and 1 melanoma patient), 2 patients for >50 days (1 liver cancer and 1 prostate cancer).

Discussion

In this Phase I, multi-center, open-label, randomized crossover study the influence of food on the rate and extent of selumetinib oral capsule absorption in male and female patients with solid malignancies was determined. In addition to selumetinib, concentrations of its 3-5-fold more active metabolite, N-desmethyl-selumetinib, were also determined [15], as any changes in the metabolite concentrations may influence the treatment efficacy. The present study showed that the extent (Cmax and AUC) and rate of absorption of selumetinib was significantly reduced in the presence of food. It is therefore recommended that for future clinical studies selumetinib should continue to be administered on an empty stomach (no food or drink other than water for 2 hours prior to dosing and 1 hour after dosing).

The exact mechanism for decreased selumetinib exposure in the presence of a high fat meal is unknown, and may be a result of saturation of drug dissolution in the intestinal fluids with an increased pH. In the average person, the basal gastric secretion in the fasted stomach is estimated to be 300 mL and in the fasted small intestine about 500 mL, and both can increase up to 5-fold after a high fat meal [16, 18, 20]. On the other hand, fatty foods also stimulate bile secretion which can cause a significant rise in the pH of the proximal small bowel, the major site for selumetinib absorption [16, 21]. The presence of more fluid in the gastrointestinal tract may actually improve the dissolution rate and can result in increased

solubility due to increased wetting of the drug and increased micellar solubilization [16, 22]. However, since the food effect consisted of a decrease in selumetinib exposure, the effect of the raised pH in the proximal bowel might be of greater importance and help explain the observed decrease in absorption of selumetinib. Additionally, the selumetinib capsule has a hydroxypropylmethylcellulose coating and will tend to disintegrate slowly thus preventing early activation by gastric acid and probably resulting in a controlled release.

Delayed gastric emptying is believed to be the major mechanism for delayed absorption and could be the underlying mechanism for the delayed t_{max} of selumetinib given in the presence of food. The presence of food in the stomach can decrease the rate of presentation of the drug to the small intestine and delay the onset of absorption, especially for rapidly absorbed drugs (C_{max} achieved within 2 hours in fasted state). For selumetinib in fasted state, C_{max} was determined in a previous study at1.5 h and therefore could fit in this category of drugs [15].

The study population was representative of the broader Phase I clinical population of patients with advanced solid malignancies in terms of baseline and demographic characteristics. As observed in previous selumetinib monotherapy studies [14, 15, 19], the exploratory efficacy analysis of this study suggests that selumetinib is biologically active. During the study, two patients with melanoma had partial responses and four patients had stable disease; two patients had stable disease for >100 days (1 patient with colorectal cancer and 1 patient with skin/soft tissue cancer) and two patients had stable disease for >50 days (1 patient with prostate cancer and 1 patient with liver cancer). However, it should be noted that this study was not designed to investigate the efficacy of selumetinib and tumor mutation status (e.g. BRAF V600E or KRAS) was not determined. Limited responses were to be expected from this advanced patient population who had progressed on multiple prior anti-cancer treatments, consistent with that seen in the Phase I trials.

The AE profile in this study was similar to that seen in previous selumetinib

monotherapy studies [14, 15, 19]. The most common AEs included gastrointestinal AEs, dermatological AEs, and fatigue. The majority of these events occurred during BD dosing.

2.1 Selumetinib food effect

Food effect data are available with other MEK inhibitors that have been used clinically including CI-1040 (PD-184352) and PD-0325901. CI-1040 was the first MEK inhibitor to enter the clinic and in the Phase I study dosing was initially performed in the morning on an empty stomach (after an overnight fast) [23]. It has been proposed that low aqueous solubility is responsible for the poor oral absorption of CI-1040 [23-25]; solubility of < 1µg/mL in pH 6.5 phosphate buffer. Administration of CI-1040 at 800 mg and 1,600 mg with a high fat meal resulted in a 4.5-fold increase in drug exposure of CI-1040 and a 5-fold increase of the metabolite PD-0184264. The MTD and recommended Phase II dose (RP2D) was determined at 800 mg BID administered with food. The mechanism postulated for the observed increase in CI-1040 exposure in the presence of food was facilitated dissolution either by the fat in the food or because of increased presence of bile salts. This would be in line with preclinical dissolution data in which the addition of detergents to the dissolution media increased dissolution. Development of CI-1040 was discontinued in Phase II due to lack of efficacy. PD-0325901, another non-ATP competitive MEK inhibitor [26], is a derivative of CI-1040 with improved aqueous solubility of 190 µg/mL in pH 6.5 phosphate buffer and is, like selumetinib, referred to as a second generation MEK-inhibitor. PD-0325901 had a 50-fold higher potency than CI-1040, improved bioavailability, increased metabolic stability and increased duration of target suppression (inhibition of ERK phosphorylation). In fasted conditions, PD-0325901 was absorbed rapidly with a tmax of 1 h, and C_{max} and AUC showed proportional increase at the tested doses, however, the effect of food on pharmacokinetics was not reported in the Phase I study. We would expect the food effect to be less than that for CI-1040 because of the improved solubility of PD-0325901. Due to lack of response and observed toxicity, development of PD-0325901 in Phase II was discontinued [27].

CH497655 (RO4987655) is a new MEK inhibitor and is currently being evaluated in a Phase I trial. Although a study has been performed in healthy volunteers, no information has been provided about the effect of food on the absorption. PK in healthy volunteers with doses up to 4 mg showed rapid absorption (median tmax of 1 h) and low variability [28].

In conclusion, PK parameters of orally administered drugs may be significantly altered in the presence of food due to changes in solubility, absorption and metabolism. The current study demonstrated that intake of a high fat meal decreases the extent and rate of selumetinib absorption and suggests that a 75 mg dose of selumetinib capsule formulation given twice a day has an acceptable safety profile in this advanced cancer population. It is recommended that for further clinical studies selumetinib should be taken on an empty stomach.

Acknowledgements

This study was sponsored by AstraZeneca.

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2.2

Phase I dose escalation study of the safety, pharmacokinetics and pharmacodynamics of the MEK inhibitor RO4987655 (CH4987655) in patients with advanced solid tumors

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Clin Cancer Res 2012 Sep 1;18(17):4794-805

Abstract

Purpose

This Phase I study of the MEK inhibitor RO4987655 (CH4987655) assessed its maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), safety, pharmacokinetics (PK)/pharmacodynamics (PD) profile and anti-tumor activity in patients with advanced solid tumors.

Patients and methods

An initial dose-escalation was performed using a once-daily (QD) dosing schedule, with oral RO4987655 administered at doses of 1.0–2.5 mg QD over 28 consecutive days in 4-week cycles. Doses were then escalated to 3.0–21.0 mg (total daily dose [TDD]) using a twice-daily (BID) dosing schedule.

Results

Forty-nine patients were enrolled. DLTs were blurred vision (n=1) and elevated creatine phosphokinase (n=3). The MTD was 8.5 mg BID (TDD, 17.0 mg). Rash-related toxicity (91.8%) and gastrointestinal disorders (69.4%) were the most frequent adverse events. The pharmacokinetic profile of RO4987655 demonstrated dose-linearity and a half-life of ~4 hours. At the MTD, target inhibition, assessed by suppression of ERK phosphorylation in peripheral blood mononuclear cells, was high (mean 75%) and sustained (90% of time $>IC_{50}$). Of the patients evaluable for response, clinical benefit was seen in 21.1%, including two partial responses (one confirmed and one unconfirmed). 79.4% of patients demonstrated a reduction in fluorodeoxyglucose uptake by positron emission tomography between baseline and Day 15.

Conclusion

In this population of heavily pre-treated patients, oral RO4987655 demonstrated manageable toxicity, a favorable PK/PD profile and promising preliminary anti-tumor activity, which has been further investigated in specific population of patients with RAS and/or RAF mutation driven tumors.

Introduction

Constitutive activation of the Ras-regulated mitogen-activated protein kinase (MAPK) signaling cascade has been identified in various human cancers. The MAPK cascade comprises three enzymes (RAF/MEK/ERK) involved in regulation of cell proliferation, differentiation, survival and migration. [1;2] Mutations of the Ras proto-oncogenes (*KRAS*, *HRAS* and *NRAS*) have been found in ~30% of cancers[3], while *BRAF* gene mutations have been identified in up to 66% of malignant melanomas.[4]
MAP kinase kinase (MEK) is the only known kinase capable of phosphorylating ERK; therefore, inhibition of MEK can potentially block the activation of multiple downstream pathways. Several small-molecule inhibitors of MEK are currently being investigated.[5-14] RO4987655 is a highly selective adenosine triphosphate non-competitive oral MEK inhibitor that has shown promising anti-tumor activity in a series of human cancer xenograft models (non-small cell lung cancer [NSCLC], pancreatic cancer and hepatocellular carcinoma).[15] RO4987655 has a unique ring structure with a high metabolic stability and slow dissociation from MEK which may confer better clinical efficacy compared with other MEK inhibitors (15). In a study of healthy volunteers, once-only single doses of oral RO4987655 (0.5–4 mg) were found to be safe and well tolerated. [16] Toxicity was typically mild/moderate, the most common adverse events (AEs) being skin-related or gastrointestinal [16]. Target effect was assessed by measuring the level of phosphorylated ERK (pERK) in peripheral blood mononuclear cells (PBMCs), which demonstrated pERK inhibition of >80% at higher RO4987655 doses. [16]

The objectives of this study were to determine the maximum tolerated dose (MTD), dose limiting toxicities (DLTs), the pharmacokinetics (PK)/pharmacodynamic (PD) profile, safety and tolerability, and preliminary anti-tumor activity of RO4987655 in a population of patients with advanced cancers. [17]

Methods

Patient selection

Patients were aged ≥18 years with advanced or metastatic solid tumors for which no standard therapy was available. All patients had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of ≤1, evaluable and/or measurable disease according to Response Evaluation Criteria In Solid Tumors (RECIST, v1.0) [18], a life expectancy of ≥12 weeks, and adequate organ functions (see Supplementary Information for full inclusion/exclusion criteria). Patients with a history of ocular disorders or other known risk factors were excluded, as were patients who had received recent corticosteroids or hormone therapy (within 2 weeks of first planned RO4987655 dose) or recent major surgery, chemotherapy, radiotherapy, immunotherapy or investigational agent (within 4 weeks). Unlimited prior systemic therapy for metastatic disease was permitted.

Study design and dose escalation

This Phase I, open-label, dose-escalation study (NCT00817518) was conducted at four European centers, was approved by an Independent Ethics Committee and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients prior to performing any study-related procedure. RO4987655 was administered as oral capsules at least two hours after a light meal, followed by at least one hour before the next meal.

Initial escalation was performed using a once-daily (QD) dosing schedule with oral RO4987655 administered over 28 consecutive days in 4-week cycles. Based on data from a toxicity study in cynomolgus monkeys [15] and clinical data from healthy volunteers [16], a starting dose of 1.0 mg/day was chosen. A regimen of twice-daily (BID) dosing was also investigated, with starting dose based on interim PK data from the QD regimen. A classical 3+3 dose-escalation design was used, with the dose escalated according to the grade/severity of toxicity during Cycle 1. Dose escalation was performed in 100% increments (according to nearest capsule strength) until the occurrence of Grade 2 toxicity, after which subsequent escalation

took place in increments of 50%. Following the occurrence of a Grade 3 toxicity that was not a DLT, dose-escalation was performed in increments of 33% until the first DLT was observed. This cohort was expanded to six patients and if further DLTs were not observed in these six patients, dose escalation continued by 25% increments. Escalation was stopped if two or more patients in a given cohort developed a DLT and the preceding dose level expanded to six patients to confirm the MTD (defined as the dose level below the lowest dose at which ≥2 DLTs were seen).

No dose reductions were permitted during the first 28 days of the study (DLT evaluation period). For any given patient, a maximum of one dose reduction or interruption was allowed after Day 28 of Cycle 1. Re-escalation was permitted for Grade ≥3 skin toxicity which improved to Grade ≤2 and for diarrhea or any other toxicity which improved to Grade ≤1 within 14 days. Patients were treated at their assigned dose until disease progression, unacceptable toxicity or patient withdrawal, whichever occurred first.

Assessments

Demographics and medical history were collected during screening. Physical examination, vital signs and safety assessments (ECOG-PS, 12-lead electrocardiogram [ECG], hematology/biochemistry, echocardiography/multigated acquisition [MUGA] scan and ophthalmological examination [fundoscopy]) were performed at baseline/screening and throughout treatment: ECG on Day 8 of Cycle 1 (pre-dose and 2 and 4 hours after drug administration); echocardiography/MUGA on Day 1 of Cycle 3; and all other assessments were done pre-dose on Days 1, 8, 15 and 22 of Cycle 1, Days 1 and 15 of Cycles 2 and 3, and thereafter on Day 1 of each cycle and at final visit. Following observation of creatine phosphokinase (CPK) elevation in one patient (17.0 mg, total daily dose), CPK was measured in all subsequent patients and retrospectively in blood collected from patients receiving lower doses.

Safety

AEs were graded according to the National Cancer Institute Common Toxicity Criteria (v3.0).[19] DLTs were defined as: Grade ≥3 non-hematological toxicity; Grade ≥3 nausea/vomiting, skin rash and/or diarrhea (despite adequate supportive care); Grade ≥3 skin toxicity that does not revert to Grade ≤2 within 14 days of the scheduled start date; febrile neutropenia (absolute neutrophil count [ANC] <1.0 x 10^9/L and fever ≥38.5°C), and/or documented infection (ANC <1.0 x 10^9/L); Grade 4 thrombocytopenia or bleeding requiring a platelet transfusion.

Pharmacokinetics and pharmacodynamics

Blood samples (4 mL in potassium EDTA vacutainers) were collected prior to dosing on Days 1 and 15 of Cycle 1 for PK analysis and at 1, 3, 7 and 12 hours following drug administration. Trough PK sampling was performed pre-dose on Days 8 and 22 of Cycle 1. The plasma concentration of RO4987655 was determined by a validated liquid chromatography tandem mass spectrometry (LC/MS/MS) method.[16] PK parameters were calculated via standard non-compartmental methods using WinNonlin V6.1 (Pharsight Corporation, Mountain View, CA) and PK measurements were fitted to a PK/PD model of pERK inhibition in PBMCs using NONMEM vVI (ICON, Maryland, US).

pERK inhibition

Target inhibition of 4 beta-phorbol 12-myristate 13-acetate (PMA)-induced pERK was measured in PBMCs (collected Days 1 and 15, Cycle 1from all patients) using flow cytometry as described previously.[16] NONMEM was used to fit PK/PD data to a model of serum and effect compartment RO4987655 concentration versus pERK inhibition in PBMCs (Supplementary Information). pERK inhibition was calculated as the percentage decrease in mean fluorescent intensity between pre- and post-dose samples, with adjustment for non-PMA stimulated pre-dose values. The antibody phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; clone D13.14.4E, Cell Signaling Technology, Beverly, MA) was used to detect endogenous levels of p44 and p42 MAPK (ERK1 and ERK2) when

phosphorylated either individually or dually at Thr202 and Tyr204 of ERK1 (Thr185 and Tyr187 of ERK2).

The effect of RO4987655 on cellular proliferation (Ki67 labeling) and target inhibition (pERK expression) was investigated by immunohistochemistry (IHC) in optional skin and tumor biopsies (collected at baseline/screening and on Day 15 of Cycle 1). Apoptosis was analyzed in tumor biopsies by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. A ≥20% change in PD biomarkers between baseline and Day 15 was considered significant. Mutational analyses for *KRAS, NRAS, BRAF* (V600), *HRAS, PI3KCA and PTEN* loss were performed if archival tumor samples were available.

Immunohistochemistry

Skin and tumor biopsies were formalin-fixed and paraffin-embedded according to standard procedures. Immunohistochemistry for Ki67 was performed using the ultraView detection kit (Ventana Medical Systems Inc, Tuscon, AZ) on a Ventana Benchmark XT platform according to the manufacturer's instructions. Slides were dewaxed, pretreated with mild Cell Conditioning 1 buffer (CC1, Ventana) and incubated for 12 minutes with a primary antibody against Ki67 (clone 30.9, Ventana). Slides were then counterstained with hematoxylin and bluing reagent, dehydrated and mounted. For pERK immunohistochemistry, the iView detection kit (Ventana) was used on a Ventana Benchmark XT platform. Slides were dewaxed and pretreated as before, and incubated for 1 hour with a phospho-p44/42 MAPK primary antibody (ERK1/2; Thr202/Tyr204; clone D13.14.4E, Cell Signaling Technology). To reduce non-specific staining by endogenous biotin present in cells and tissues, the Endogenous Biotin Blocking Kit (Ventana) was also used. Slides were counterstained and mounted as before.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Formalin-fixed tissue sections were dewaxed and washed in phosphate buffered saline. Sections were incubated in 3% citric acid for 1 hour to

decalcify the tissue and, after three washes with water, epitope retrieval was performed using proteinase K (Roche). Slides were again washed in water, and incubated with 100 µl of TUNEL reaction mixture (containing FITC-dUTP) at 37°C for 1 hour. Following another wash, slides were treated with 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity before incubating with a secondary antibody (anti-FITC-HRP, Roche) for 45 minutes. Finally, slides were washed and 3-Amino-9-Ethylcarbazole substrate was added for 10 minutes. Counterstaining was performed using hematoxylin for 30 seconds and slides were mounted using a gelatin-glycerin mounting medium.

Mutation analysis

Mutational analysis was performed centrally using formalin-fixed tissue. Biopsies were first assessed to ensure at least 50% tumor cell content and manually microdissected if required. Real-time polymerase chain reaction with fluorescently-labeled, sequence-specific probes was used to distinguish the wild-type *BRAF* (V600) sequence (GTG) from the mutant sequence (GAG). *KRAS* mutations were identified using an investigational assay based upon PCR and melting temperature analysis, with fluorescently-labeled, sequence specific probes designed to distinguish the wild-type sequence from mutation bearing sequences in exon 2 (specifically at codons 12 and 13) and in exon 3 (specifically at codon 61). All assays were performed on the cobas 4800 system (Roche Molecular Systems, Inc.) according to manufacturer's instructions. *NRAS* (in melanoma), *HRAS,* and *PI3K* mutations were screened for by standard sequencing methods and *PTEN* loss was determined by IHC (antibody clone 138G6, Cell Signaling Technology).

FDG-PET

Metabolic activity of tumors was investigated by fluorodeoxyglucose positron emission tomography (FDG-PET; at baseline; Day 15, Cycle 1; Day 1, Cycle 3). Baseline and follow-up PET scans were performed using a single scanner and under the same conditions (administered ^{18}F-FDG activity for all scans was maintained within 10% of the calculated activity administered at baseline and the same acquisition time per bed position was used for all

scans for each individual patient). Low-dose CT scans were performed for all PET scans for attenuation correction. Independent analysis of PET images was performed centrally based on European Organization for Research and Treatment of Cancer guidelines.[20] Lesions with the highest degree of FDG uptake were selected for quantitative analysis (up to five) and a 10 mm circular/spherical region of interest drawn. A standardized uptake value (SUV) was measured for each selected lesion and the delta change in SUV between baseline and Day 15 of Cycle 1 was calculated for each patient. FDG-PET scanning took place before tumor biopsies to avoid interference on FDG uptake. Patients with a recent history of diabetes were excluded from FDG-PET analysis.

Tumor response

Tumor assessments according to RECIST criteria (version 1) were performed at screening, every 2 cycles and on suspicion of disease progression.

Statistical analyses

PK/PD, safety, and tumor response data were analyzed by descriptive statistics. Correlations between specific AEs and anti-tumor activity or PK were assessed by logistic regression and Analysis of Variance (ANOVA).

Results

Forty-nine patients were enrolled between January 2009 and June 2010 (**Table 1**), all received at least one dose of RO4987655. The most common tumor types were melanoma (n=27, 55.1%; including choroidal melanoma, n=9) and colorectal cancer (CRC, n=11, 22.4%). None of the patients had previously received treatment with a MEK inhibitor. Thirteen patients received RO4987655 QD (1.0–2.5 mg) and 36 received RO4987655 BID (3.0–21.0 mg total daily dose [TDD]; **Supplementary Figure 1**). Patients received a median of two treatment cycles (range 0–12; 93.8% of patients completed at least one cycle), with a median duration of treatment of 57 days (range 2–337).

Table 1. Patient demographics and clinical characteristics.

	Total patients n=49
Sex	
Male	30 (61%)
Female	19 (39%)
Age, years	
Median	53
Range	22–79
Race	
White	48 (98%)
Asian	1 (2%)
Baseline ECOG performance status	
0	23 (47%)
1	25 (51%)
Unknown	1 (2%)
Prior anti-cancer therapies, median (range)	3 (1–9)
Primary cancer site and mutational status	
Melanoma	27
BRAF (V600)	2
No mutation	7
Unknown	18
Colon/large intestine and rectum	11
KRAS	6[a]
PI3KCA	1[a]
No mutation	1
Unknown	4
Lung	3[b]
Ovarian	2[b]
Pancreas	1[b]
Synovial sarcoma	1[b]
Thymoma	1[b]
Unknown	1[b]
Cervix	1[b]
Clear-cell sarcoma	1[b]

[a]One tumor contained both a *KRAS* and *PI3KCA* mutation; [b]Mutational status unknown. ECOG, Eastern Cooperative Oncology Group

Four DLTs were observed during the first 28 days of treatment, all with BID dosing (**Table 2**). At 8.5 mg BID (TDD, 17 mg) one patient experienced Grade 3 elevated CPK. No further DLTs were observed when this cohort was expanded to nine patients in total. After escalation to 10.5 mg BID (TDD, 21 mg), three patients experienced DLTs (Grade 3 blurred vision, Grade 3 elevated CPK and Grade 4 elevated CPK). Accordingly, 8.5 mg BID (TDD 17.0 mg) was defined as the MTD. All DLTs were reversible.

Safety

Patients experienced 189 treatment-related AEs, including 20 Grade 3 AEs (in 17 patients) and two Grade 4 AEs (in two patients). The most common AEs (≥10% of patients, **Table 2**) were skin toxicity (rash-related, n=45 [92% of patients]; dry skin, n=7 [14%]; skin fissures, n=6 [12%]) and gastrointestinal (diarrhea, n=16 [33%]; nausea, n=7 [14%]; vomiting, n=6 [12%]; stomatitis, n=5 [10%]). Grade 3/4 AEs were primarily limited to BID dosing of ≥5.0 mg. Among the rare (<10% patients) Grade ≥3 toxicities, isolated and reversible Grade 3 neutropenia occurred in two patients in the 1.5 mg and 10 mg dose cohorts and one case of reversible Grade 3 anemia occurred in the 13 mg dose cohort treated by transfusion on Day 63. One patient was reported with a Grade 3 left ventricle ejection fraction decrease in the 13 mg dose cohort which occurred on Day 56 when drug was stopped due to progression of the disease. The other Grade 3 toxicities included general disorders (asthenia, depression, decreased appetite) and skin disorders (**Table 2**). Most rash-related toxicities were Grade 1/2, with six patients experiencing Grade 3 events and no Grade 4 events reported. Median time to development of Grade 3 rash was 49.5 days (range 9–146). Skin toxicity developed primarily in the face, upper trunk and back; comprising red papulopustules and crusts, occasionally accompanied by swelling (mainly the nose). Severe psychological impacts were reported in patients experiencing substantial alterations in appearance.

Sixteen eye-related AEs occurred in 13 [27%] patients (including blurred vision, photopsia, corneal erosion, dry eyes, periorbital edema, chorioretinopathy, punctate keratitis and retinal vein occlusion [RVO]).

Table 2. Treatment-related toxicity.

		Total daily dose (mg)								
	Number of patients	QD dosing[a]	BID dosing[b]							
	(n)	1.5 (n=4)	3 (n=3)	4 (n=3)	5.5 (n=4)	7.5 (n=4)	10 (n=4)	13 (n=4)	17 (n=9)	21 (n=4)
DLTs										
Grade 3 blurred vision (n=1)										1
Grade 3 elevated CPK (n=2)									1[c]	1
Grade 4 elevated CPK (n=1)										1
Treatment-related AEs	**AEs (all grades)**		**AEs (Grade 3–4 only)**							
	n	**• %**								
Rash-related[d]	45	91.8		1				1	2		2
Diarrhea	16	32.7								
Eye-related[e]	13	26.5								1	1
Elevated CPK	9	18.4			1					3	2
Fatigue	8	16.3									
Dry skin	7	14.3									
Nausea	7	14.3									
Skin fissures	6	12.2									
Vomiting	6	12.2									
Stomatitis	5	10.2									

Pruritus	3	6.1					1	
Asthenia	3	6.1					1	
Neutropenia	3	6.1	1		1			
Anemia	2	4.1				1		
Decreased appetite	2	4.1					1	
Depression	1	2.0		1				
Decreased Ejection Fraction	1	2.0				1		
Treatment-related SAEs (all grades)	**n**	**%**		**SAEs (all grades)**				
Depression	1	2.0		1				
Anemia[f]	1	2.0				1		
Retinal vein occlusion	1	2.0					1	
Asthenia	1	2.0					1	
Decreased appetite	1	2.0					1	
Chorioretinopathy	1	2.0					1	
Elevated CPK	1	2.0					1	
Blurred vision	1	2.0						1

Data cut-off: January 28th 2011. Only the most severe intensity was counted for multiple occurrences of the same AE in one individual. Adverse events occurring in >10% of patients are shown above the dotted line. AEs, adverse events; BID, twice daily; CPK, creatine phosphokinase; DLT, dose-limiting-toxicity; QD, once daily; SAEs, serious adverse events.

[a]Only one Grade 3 toxicity (neutropenia) was reported in only one dosing-cohort in the QD regimen (1.5 mg QD);

[b]The most common AEs were reported at Grade 3 or 4 severity in the BID dosing cohorts. Two Grade 4 AEs occurred, both elevated CPK (8.5 mg and 10.5 mg, BID). No Grade 5 AEs occurred. Nausea/vomiting, skin rash and/or diarrhea were only considered a DLT if they reached Grade ≥3 severity despite adequate supportive care measures. SAEs were defined as any AE which was fatal; life-threatening; required in-patient hospitalization or prolongation of existing hospitalization; resulted in persistent or significant

disability/incapacity; was a congenital anomaly/birth defect; was medically significant or required intervention to prevent one or other of the outcomes listed above. No SAEs occurred in once-daily dosing cohorts;

[c]No further DLTs were observed when the 17.0 mg (BID) cohort was expanded to nine patients;

[d]Includes dermatitis acneiform, rash, dermatitis, papular rash, folliculitis, and genital rash;

[e]Includes blurred vision, photopsia, corneal erosion, dry eyes, periorbital edema, chorioretinopathy, punctate keratitis and retinal vein occlusion;

[f]No signs of chronic or acute gastrointestinal bleeding were observed and no hemoglobinuria was recorded

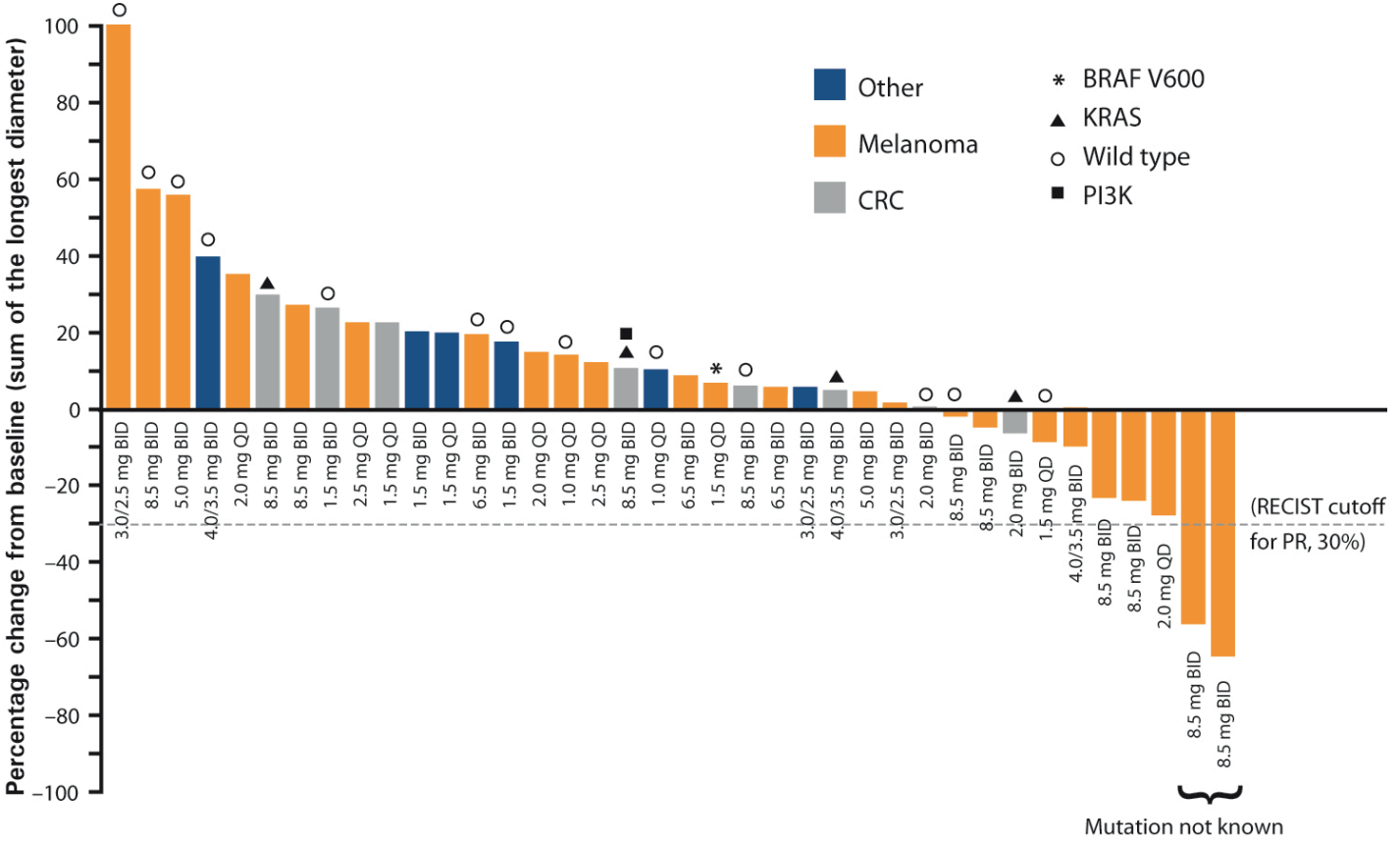


Figure 1. Relative change (%) in sum of lesion size from baseline at best overall response.

Thirty-eight patients were evaluable for tumor response. Target lesions were not measured in seven patients, three patients were not evaluable at Day 1 of Cycle 3 and tumor assessment was not completed for one patient. By the end of the study, 44 patients had progressed. All patients were eventually withdrawn from treatment: 43 due to disease progression, five due to AEs and one due to refusal of treatment. Tumor response assessments were performed according to RECIST criteria (version 1). Dotted line indicates the RECIST cut-off for partial response (–30%). The figure also depicts mutation status for patients with response data: BRAF (*), KRAS (▲); wild type (o), PI3K (■); one melanoma patient with *BRAF* mutation and two CRC patients with *KRAS* mutation were not evaluable for response and are not shown. BID, twice daily; CRC, colorectal cancer; QD, once daily; RECIST, Response Evaluation Criteria In Solid Tumors

Blurred vision was associated with fluid accumulation in the sub-retinal space, identified by optical coherence tomography (OCT), resulting in serous retinal detachment (SRD) in one patient. Two patients experienced Grade 3 ocular toxicity (RVO [8.5 mg BID] and blurred vision [10.5 mg BID]). Median time-to-onset of ocular toxicity was 12 days (range 1–175 days), and median duration of toxicity was 14 days (range 2–104 days). Ocular toxicity resolved either spontaneously or with drug interruption (two Grade 2 and two Grade 3) except for one patient with Grade 3 visual disturbances associated with RVO who improved to Grade 1 at study completion, and one with Grade 1 blurred vision whose condition remained unresolved at study completion.

Nine [18%] patients experienced elevated CPK including four Grade 3 (one at 2.0 mg BID, two at 8.5 mg BID and one at 10.5 mg BID) and two Grade 4 events (at 8.5 mg BID and 10.5 mg BID). CPK elevation was reversible with drug interruption and was asymptomatic in most patients and not associated with either clear rhabdomyolysis symptoms or cardiac dysfunction. Three cases of Grade 1 myalgia (in three patients), one Grade 1 joint swelling, one Grade 2 joint stiffness, one Grade 2 neck pain, one Grade 1 pain in extremity, and one Grade 2 muscular weakness were reported in association with CPK elevation.

Eight patients experienced dose reductions due to treatment-related AEs (one patient at 1.5 mg QD and 4.0/3.5 mg BID, and three patients at 8.5 mg BID and 10.5 mg BID), including five patients who experienced more than one AE-related dose interruption (one patient at 1.5 mg QD and two patients each at 8.5 mg BID and 10.5 mg BID). Of the nine patients receiving RO4987655 at the RP2D (8.5 mg BID), the median duration of dosing was 87.5 days (range 50–194) in the six patients who did not undergo dose modification. Median time to dose modification in the remaining three patients was 37 days (range 14–51).

Eleven patients experienced temporary drug interruptions due to AEs (one each at 1.5 mg QD, 2.0 mg QD, 4.0/3.5 BID and 5.0 mg BID; two at 6.5 mg BID and 10.5 mg BID and three patients at 8.5 mg BID). The median

duration of interruption was 7 days; range 1–21; **Supplementary Table 1**). Seven patients experienced eight treatment-related serious AEs (SAEs; all Grade 2/3; **Table 2**). Five of the SAEs were resolved, two with dose modifications, two without dose modifications and one with treatment discontinuation. The remaining three SAEs were unresolved. Two deaths following disease progression were not considered to be treatment-related.

Anti-tumor activity

Clinical benefit (defined as partial response [PR] or stable disease [SD] lasting ≥16 weeks) was seen in eight of the 38 evaluable patients (21.1%; **Figure 1**), including one confirmed and one unconfirmed PR in patients with skin melanomas (mutational status unknown). These patients received treatment with RO4987655 for 113 and 224 days (mean 168.5 days) and responses lasted for 48 and 168 days (mean 108 days). Six other patients achieved SD lasting >16 weeks: three patients with melanoma, two patients with choroidal melanoma, and one patient with a rectal adenocarcinoma. The median percentage change in tumor size at maximum reduction from baseline in evaluable patients was 9.8% (range –66.9% to 101.4%).

Pharmacokinetics

Pharmacokinetics was assessed in 43 patients (87.8%). Plasma concentrations of RO4987655 increased rapidly following oral administration. For the majority of patients, C_{max} was reached ~30–60 minutes after dosing (**Figure 2A**, **Table 3**). Mean terminal half-life was ~4 hours. Plasma exposure increased approximately dose-proportionally on Day 1 (**Figure 2B**) and increased linearly with dose at steady state (**Figure 2C**). Intra-patient variability in plasma exposure was limited (**Table 3**).

At the MTD, C_{max} and $AUC_{0–12hr}$ were 425 ng/mL and 1660 ng·hr/mL, respectively, at Day 1 and 530 ng/mL and 2577 ng·hr/mL, respectively, at steady state. The mean accumulation index ($AUC_{Day\ 15}/AUC_{Day\ 1}$) was 1.53 (range 1.15–1.96). Increased steady-state plasma exposure was significantly associated with occurrence of Grade 2/3 rash (logistic

regression, p=0.01) and showed a trend towards association with CPK elevation (ANOVA; p=0.07).

Pharmacodynamics

pERK inhibition in peripheral blood lymphocytes

Assessment of target suppression was evaluated by measuring the extent of pERK inhibition in a surrogate tissue, PBMC. The relationship between exposure (RO4987655 plasma concentration) and PD effect (pERK inhibition in PBMCs) was characterized by a direct link PK/PD (effect compartment) model which revealed 70–80% pERK inhibition at plasma concentrations of >200 ng/mL (**Figure 3A**).

Tumor/skin biopsies

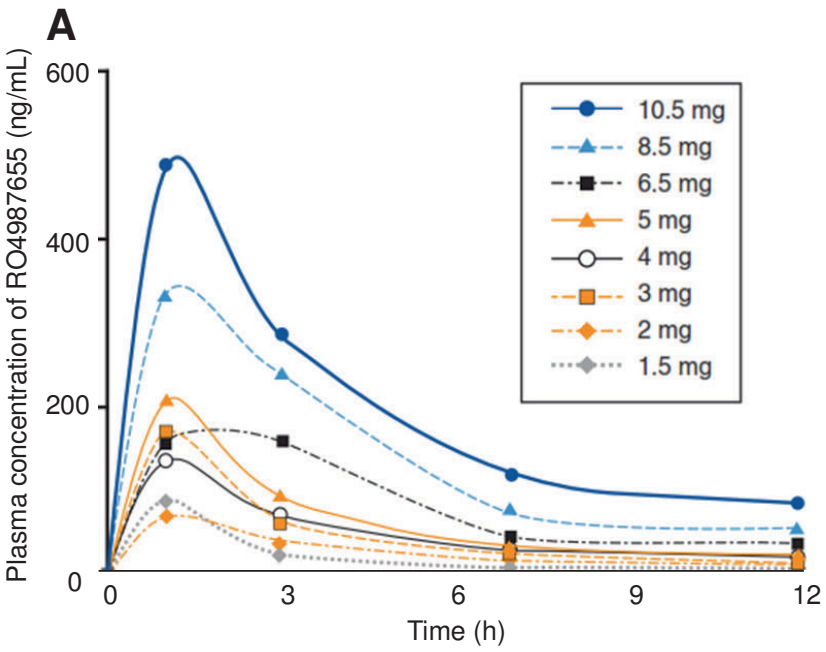
Between baseline and Day 15, pERK expression in tumor biopsies decreased by ≥20% in six of seven evaluable patients and increased by ≥20% in the other (**Figure 3B, Supplementary Table 2**). One tumor demonstrated >90% reduction in pERK expression. Paired pre- and post-treatment normal skin biopsies were available from 20 patients; five showed a decrease in pERK expression of ≥20% by Day 15, 14 showed no change, and IHC failed in one patient (**Figure 3B, Supplementary Table 2**). One skin biopsy demonstrated >90% pERK reduction. Most tumor and skin biopsies showed no change in cell proliferation (Ki67 labeling) between baseline and Day 15 (**Supplementary Table 2**). Three of five paired tumor biopsies demonstrated no change in apoptotic signal between baseline and Day 15 (TUNEL assay; **Supplementary Table 2**). No correlations were observed between changes in biomarker levels and tumor response, mutational status, or exposure.

Mutational analysis

Mutational analyses were performed for tumor samples from a total of 30 patients; 22 samples were suitable for assessment of *BRAF V600* and *KRAS* mutations, 21 were suitable for *NRAS*, 18 for *HRAS* and *PI3K*, and 10 samples were suitable for assessment of *PTEN* loss. Of the 30 tumor

Table 3. Summary of pharmacokinetics of RO4987655 in patients following oral administration.
[a]Accumulation index represents $AUC_{Day\ 15}/AUC_{Day\ 1}$; [b]Maximum tolerated dose. AUC, area under the plasma concentration time curve; BID, twice daily; C_{max}, maximum plasma drug concentration; CV, coefficient of variation; $t_{½}$, plasma half-life.

Regimen	Day 1				Day 15				
	N	AUC_{0-12h} ng•hr/mL (% CV)	C_{max} ng/mL (% CV)	$t_{½}$ hr (% CV)	N	AUC_{0-12h} ng•hr/mL (% CV)	C_{max} ng/mL (% CV)	$t_{½}$ hr (% CV)	Accumulation index[a]
1.5 mg (BID)	4	278 (27%)	88.0 (30%)	4.7 (12%)	4	460 (18%)	105 (30%)	6.0 (19%)	1.65
2.0 mg (BID)	3	321 (7%)	72.9 (64%)	3.7 (50%)	3	629 (12%)	129 (25%)	7.6 (47%)	1.96
3.0 mg (BID)	4	596 (44%)	171 (39%)	3.4 (20%)	4	861 (47%)	176 (58%)	4.0 (20%)	1.15
4.0 mg (BID)	4	608 (38%)	142 (51%)	4.2 (11%)	4	1053 (13%)	183 (61%)	6.1 (16%)	1.90
5.0 mg (BID)	4	797 (64%)	209 (89%)	3.3 (20%)	4	1157 (60%)	201 (66%)	3.8 (8%)	1.45
6.5 mg (BID)	3	1005 (35%)	207 (51%)	3.3 (12%)	4	1458 (21%)	282 (36%)	3.9 (28%)	1.45
8.5 mg (BID)[b]	9	1660 (39%)	425 (50%)	3.4 (25%)	9	2577 (48%)	530 (53%)	8.0 (157%)	1.50
10.5 mg (BID)	4	2282 (43%)	601 (66%)	3.7 (66%)	2	2638 (12%)	399 (45%)	8.1 (87%)	1.16


A
Plasma concentration of RO4987655 (ng/mL)
600
400
200
0
0
3
6
9
12
Time (h)
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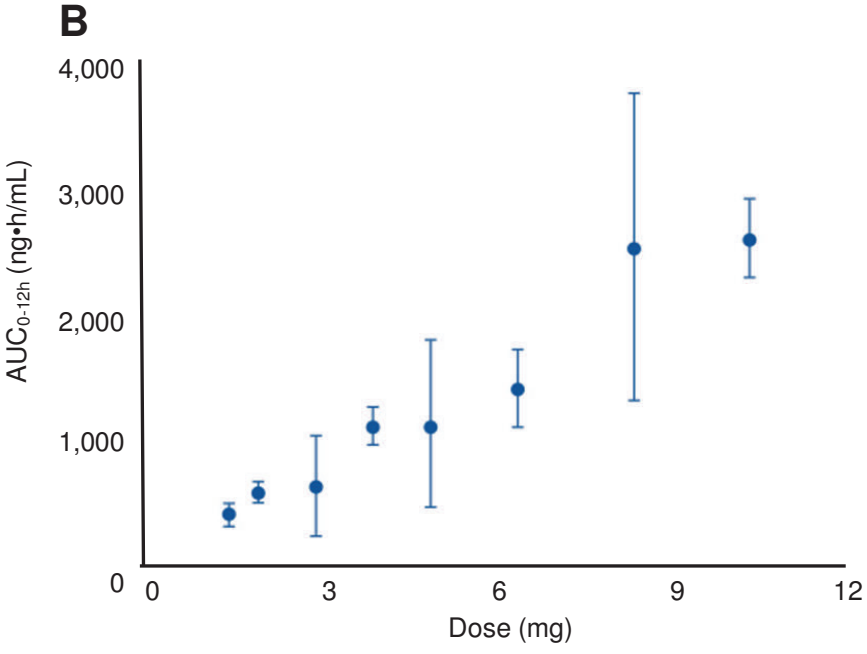
5 mg
4 mg
3 mg
2 mg
1.5 mg


B
AUC0-12h (ng•h/mL)
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Dose (mg)

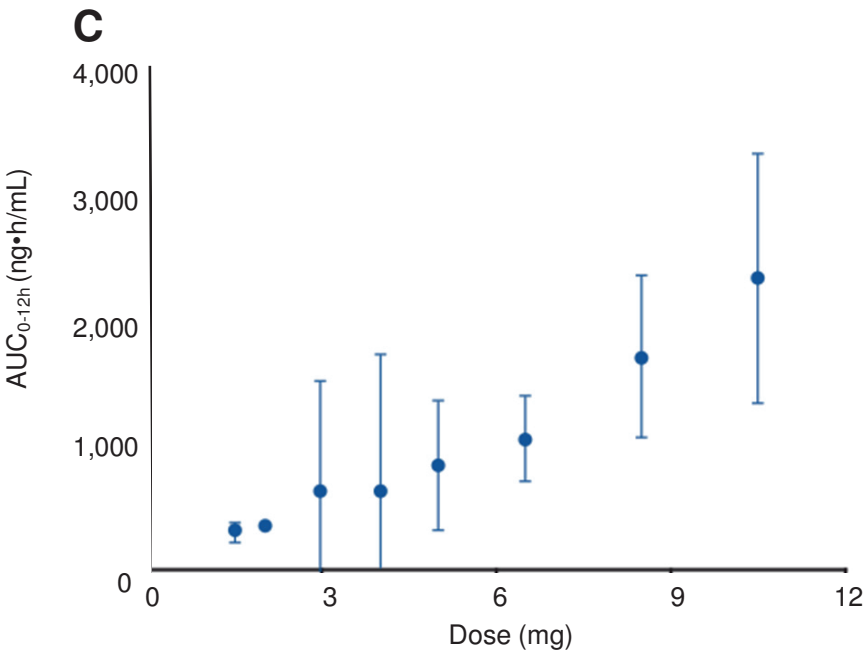
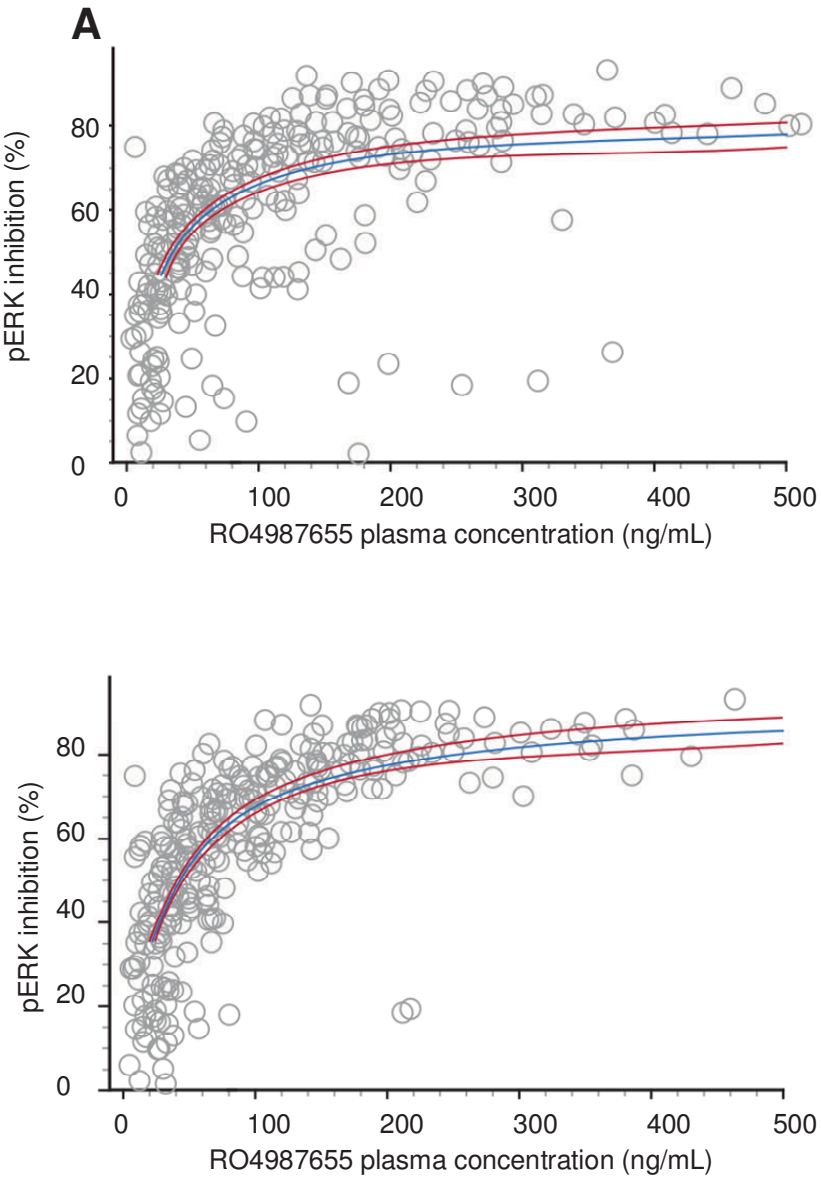


Figure 2. Mean plasma concentration (A) and plasma exposure (B) on Day 1 and plasma exposure on Day 15 (C) of RO4987655 following oral administration.
Blood was collected for PK assessment from 43 patients on Day 1: pre-dose (0 hours) and 1, 3, 7 and 12 hours post-dose. For patients on BID dosing cohorts, PK parameters were measured after the first daily dose. **Figure 2A** (mean plasma concentration): Data points represent the mean plasma concentrations on Day 1. C_{max} was reached approximately 0.5–1 hour after dosing. Mean terminal half-life was ~4 hours. The figure legend shows administered dose, rather than total daily dose. **Figures 2B** (plasma exposure on Day 1) and **2C** (plasma exposure on Day 15): Steady-state conditions were reached by Day 15 and drug accumulations were assessed by the plasma exposure (AUC_{0-12h}). Data points represent mean plasma exposure and error bars represent standard deviation.

Figure 3. (A) RO4987655 plasma and effect concentrations versus PBMC pERK inhibition (all doses); **(B)** Case study: pERK expression in paired tumor (i and ii) and normal skin (iii and iv) biopsies obtained prior to treatment with RO4987655 (baseline) and on Day 15 of Cycle 1; **(C)** Relative change (%) in FDG-PET from baseline to Day 15 of Cycle 1.



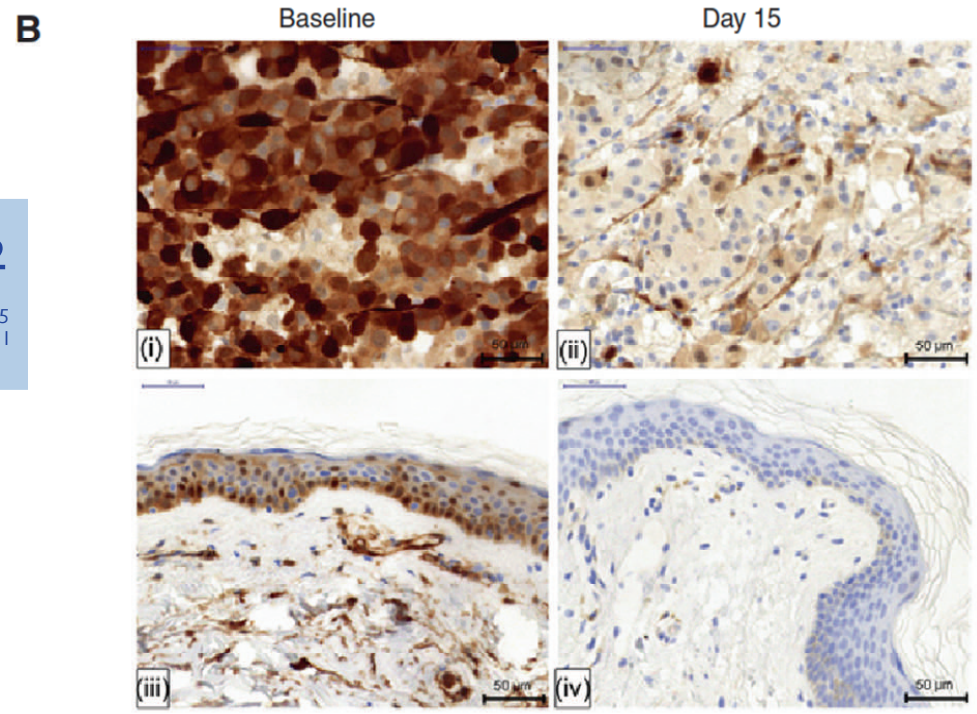


Figure 3A. Measurements were fitted to an effect compartment PK/PD model of pERK inhibition in PBMCs (Supplementary material). 70–80% inhibition of pERK was observed at RO45987655 plasma concentrations of >200 ng/mL. Half maximal inhibitory concentration (IC_{50}), 53 ng/mL; 90% inhibitory concentration (IC_{90}), 480 ng/mL. Average regression is represented by the blue line, and 90% confidence intervals of the mean regression are shown as purple lines. A time delay of ~3 hours was observed between C_{max} and the maximum effect generated by the PK/PD model.

Figure 3B. Representative images depicting immunohistochemistry for pERK in paired tumor biopsies taken from a patient with melanoma. This patient received RO4987655 at a dose of 8.5 mg BID (17.0 mg TDD) and the patient achieved stable disease lasting for 28 weeks. In this case study, reductions in nuclear pERK expression of 65% and 97% were observed in the tumor and skin biopsies, respectively, between baseline and Day 15.

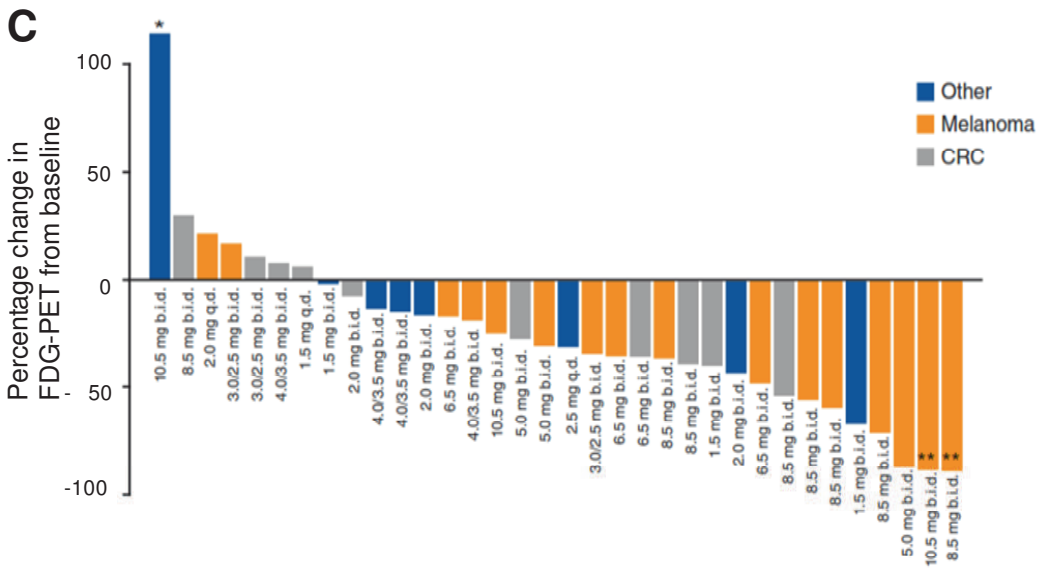


Figure 3C. * +320% change from baseline (PET % change) was trimmed at +125% for aesthetic purposes); **, patients who achieved partial responses (the patient initially treated at 10.5 mg BID underwent a dose reduction to 8.5 mg BID due to a DLT [CPK elevation]). FDG-PET was performed at baseline and on Day 15 of Cycle 1 in 34 patients (69.4%; three patients received RO4987655 QD and 31received RO4987655 BID). BID, twice daily; CRC, colorectal cancer.

samples assessed, eight revealed mutations (**Table 1**) including two melanomas with *BRAF* (V600) mutation, five CRC with *KRAS* mutation and one CRC with both *KRAS* and *PI3K* mutations. **Figure 1** shows the mutational status of tumors that were evaluable for tumor response.

FDG-PET

FDG-PET was performed in 34 patients. Between baseline and Day 15, 27 patients (79.4%, QD, n=1; BID, n=26) demonstrated a reduction in FDG uptake (**Figure 3C**). In most cases, reductions in FDG uptake were maintained until at least Week 8 in patients not progressing between assessments. Excluding one outlier, there was a positive relationship between dose/exposure and change in FDG uptake between baseline and

Day 15 in the overall population. This relationship was more pronounced in melanoma patients, where larger changes in FDG uptake were observed as dose increased. Change in FDG-PET uptake was weakly correlated with tumor response (RECIST). All patients achieving a PR or SD (>16 weeks) demonstrated a reduction in FDG uptake by Day 15 (see Supplementary **Figure 2**). Larger reductions in FDG uptake between baseline and Day 15 were observed with increased pERK inhibition in PBMCs.

Discussion

In patients with advanced solid tumors, oral RO4987655 was moderately tolerated with manageable toxicity and demonstrated a favorable PK/PD profile and encouraging anti-tumor activity. The safety profile of RO4987655 in this study was consistent with data from healthy volunteers with no new safety signals being identified. The management and treatment of safety events was facilitated by the short half-life of RO4987655. The MTD of RO4987655 was 8.5 mg BID (17.0 mg TDD). DLTs were Grade 3 blurred vision (n=1) and Grade 3/4 elevated CPK (n=3); all of which were reversible without treatment.
The most frequent RO4987655-related AEs were skin toxicity and gastrointestinal disorders. MEK inhibitor class effects included rash (91.8%) and eye-related toxicity (26.5%). Previous studies with MEK inhibitors have reported rash, diarrhea, nausea, fatigue and visual disturbances as the most common treatment-related AEs.[5;8;13;21;22] An indirect comparison between RO4987655 and the Phase I published data from other MEK inhibitors suggests that RO4987655 has a comparable safety profile with a higher frequency of rash-related toxicity (92% vs. 38–79%), but a generally lower incidence of diarrhea (32% vs. 32–55%), nausea (14% vs. 29–54%) and eye-related toxicity (27% vs. 33–50%) (5;8;13;21;22),while CPK elevation which was observed regularly in this study, has so far not been observed with other MEK inhibitors. [5;13;21;23]

Ocular toxicity is a known class effect of MEK inhibitors and was also observed in this study. Two episodes of blurred vision even occurred very

early in treatment, after one or two days of dosing. The majority of visual symptoms reported in this study were due to SRD, but OCT was not systematically performed, preventing accurate evaluation of the incidence of associated SRD. Although ocular toxicities can be alarming for both patients and physicians, all cases of SRD reported in this study were reversible without any specific treatment and without long-term damage. This is in line with blurred vision reported in other studies. [5;8;13] Other class-related ocular complications in this study occurred less frequently than SRD and include ocular hypertension, which can be detected by regular measure of intraocular pressure, and RVO, that can be detected early by regular fundus photographs. Since the pathogenesis of MEK-related eye disorders remains unknown and experience with chronic administration of MEK inhibitors is limited, careful monitoring of ocular disorders should be implemented in further clinical trials with MEK inhibitors to ensure adequate management of patients.

CPK elevation was reported in this study and considered related to RO4987655. Systematic measurement of CPK in this study may have generated a higher incidence of reports. Most elevated CPK episodes were asymptomatic and no RO4987655-related cardiovascular disorders or rhabdomyolysis were observed. The mechanism behind the observed CPK elevation remains unknown at present. A direct role of MEK inhibition cannot be excluded as the MAPK pathway plays a key role in regulation of muscle cell signaling. [24-27]

RO4987655 monotherapy demonstrated encouraging anti-tumor activity as measured by RECIST. Like other MEK inhibitors, RO4987655 showed clinical activity against melanoma (13;21-23). Seven of the eight patients who achieved clinical benefit with RO4987655 had melanoma tumors, including two PRs (one confirmed, one unconfirmed; both at 8.5 mg BID). No clear correlation existed between response and mutational status; however, the number of patients with mutation data was limited. Based on the safety and PK/PD profile data presented here, a dose-regimen of 8.5 mg BID (17.0 mg TDD) RO4987655 is recommended for Phase II studies.

Pharmacokinetic analyses showed that RO4987655 was absorbed rapidly, reaching C_{max} 0.5–1 hour after dosing, and that plasma concentration and exposure increased approximately dose-proportionally. The PK of RO4987655 were linear, time-independent, and consistent with an earlier study in healthy volunteers, with the exception of terminal half-life, which was much shorter than previously reported (~4 hours vs. 25 hours, respectively).[16] While the reason for this remains unclear, it may indicate that the longer sampling period employed previously allowed for a more accurate assessment of the terminal phase half-life: RO4987655 was monitored for 72 hours post-dosing in healthy volunteers but only 12 hours in this study due to the inclusion of BID dosing. The influence of food on the absorption of RO4987655 remains to be determined. Data from recent studies with other MEK inhibitors are conflicting; while administration with a high-fat meal was shown to increase exposure to the oral inhibitor CI-1040, exposure to selumetinib was reduced when administered with food.[28;29] Patients in this study were lightly fasted prior to dosing with RO4987655; the effect of food on the exposure to RO4987655 will be investigated during further development of this compound.

Although higher plasma concentrations of RO4987655 were associated with greater pERK inhibition in PBMCs and almost all tumor samples demonstrated a significant decrease in pERK expression by Day 15(Cycle 1), target suppression in skin biopsies was observed in only a quarter of cases. Overall, skin and tumor biopsies showed no change in either tumor cell proliferation or apoptosis between baseline and Day 15, possibly reflecting the RO4987655's cytostatic nature. Data on inhibition of tumor cell proliferation and induction of apoptosis in response to MEK inhibition are limited; decreased tumor Ki67 has been demonstrated in response to treatment with AZD6244 and PD-0325901.[5;13] The exact mechanism by which apoptosis is induced by MEK inhibition has not been identified and better understanding will be important in the development of strategies to overcome treatment resistance.[30;31]
Evidence of biological activity was demonstrated by FDG-PET, particularly in melanoma where decrease in FDG uptake appeared to be associated

with dose and drug exposure. In addition, a weak relationship was observed between change in FDG uptake (between baseline and Day 15) and RECIST tumor assessment. All patients achieving a PR or SD lasting >16 weeks demonstrated a reduction in FDG uptake by Day 15, suggesting that an FDG decrease was necessary, but not sufficient, for later tumor response. Furthermore, FDG-PET data correlated with PBMC pERK inhibition; larger changes from baseline were associated with increased inhibition of pERK. Clinical studies investigating other signal transduction inhibitors like imatinib (in gastrointestinal stromal tumors and soft-tissue sarcomas) [32;33] and erlotinib (in NSCLC) [34;35] and the chemotherapy agent irinotecan (in CRC) [36], have supported the role of FDG-PET as a predictive marker of clinical activity, although this needs large-study confirmation.

There is currently an unmet need for effective treatment of patients with tumors containing *KRAS* mutations and patients with wild-type *BRAF* melanoma [37-41].While the mutational analysis in this study was limited, MEK inhibition may offer a therapeutic option independent of *KRAS* and *BRAF* mutation state, most likely in combination with chemotherapy and/or another targeted agent. Recent preclinical data demonstrate that combined RAF/MEK inhibitors can block ERK activation in resistant cells and may delay emergence of resistance.[42;43] Studies with other MEK inhibitors are investigating combinations with AKT inhibitors, PI3K inhibitors, and chemotherapy agents such as paclitaxel and docetaxel.[44-48] The optimum partners for RO4987655 remain to be determined; however, *in vitro* and *in vivo* data demonstrate that combination withPI3-kinase pathway inhibitors (mTOR, PI3K inhibitors) [15], other targeted agents, or chemotherapy agents (cisplatin, paclitaxel and gemcitabine) may potentiate RO4987655's anti-tumor activity.
Single-agent RO4987655 is currently under investigation in an expansion of this study in four parallel patient cohorts including patients with: (1) melanoma tumors carrying the *BRAF* (V600) mutation, (2) melanoma tumors not carrying the *BRAF* (V600) mutation, (3) NSCLC carrying *KRAS* mutations, and (4) CRC carrying *KRAS* and/or *BRAF* (V600) mutations. The

primary endpoint of this expansion cohort study will be to investigate the efficacy of single-agent RO4987655 in these specific tumor genotypes, using approximately 20 patients per cohort. Further development of RO4987655 will involve combination with chemotherapy or other signal transduction inhibitors.

In summary, oral RO4987655 was reasonably well tolerated in patients with advanced or metastatic solid tumors, but often resulted in skin toxicity (91.8%; primarily facial, and with psychosocial impact reported). RO4987655 revealed a safety profile comparable with other MEK inhibitors. The main DLTs were reversible blurred vision and elevated CPK. At the RP2D, high (~70%) and sustained (>IC_{50} for >90% of time) pERK inhibition was observed in PBMCs, and plasma drug concentrations were in the range predicted to be efficacious in preclinical models. Metabolic and anatomic responses were observed in all tumor types, but particularly in patients with melanoma tumors.

Acknowledgements

The authors thank patients and staff at the study sites, and Stefanie DeSchepper (HistoGeneX), Astrid Heller and Miro Venturi (Roche Translational Research Science), Lucy Lee (ex-Hoffman La Roche) and Yoshitaka Ogita (Chugai Pharmaceutical Co., Ltd.).
Third-party writing assistance was provided by Laura McDonagh, PhD and Jamie Ashman, PhD (Prism Ideas) and funded by F.Hoffmann-La Roche Ltd.

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Supplementary information

Methods

2.2
RO4987655
phase I

1. Patient inclusion/exclusion criteria

Eligible patients had adequate bone marrow (absolute neutrophil count ≥1.5 x 109/L, platelet count ≥100.0 x 10^9/L, hemoglobin ≥9 g/dL), cardiac function (left ventricular ejection fraction ≥50% [or within normal range] as determined by echocardiography or multiple uptake gated acquisition scan), liver function (total bilirubin ≤1.5 the upper limit of normal [ULN], aspartate aminotransferase and alanine aminotransferase ≤2.5 x ULN [≤5 x ULN if liver metastases], alkaline phosphatase <2.5 x ULN [<5 ULN if liver metastases]) and renal function (serum creatine ≤1.5 x ULN or creatine clearance estimate ≥60 mL/min in male and ≥50 mL/min in female [calculated according to Cockroft-Gault formula]). Serum calcium levels, international normalized ratio and partial thromboplastin time were within normal limits.

Exclusion criteria included: patients with known allergies to components of the study drug; a history of ocular disorders or known risk factors; impaired gastrointestinal absorption; active central nervous system lesions; active stone or gallbladder disorders; increased QTc interval (>450 ms for males,>460 ms for females); a family history of long QT syndrome or other risk factors for torsades de pointes; use of concomitant medications that prolong the QT/QTc interval; New York Heart Association Class III/IV cardiac disease; myocardial infarction within the last 6 months; unstable arrhythmias or angina; hypokalemia; active acute or chronic infections (including known infection with HIV, hepatitis B virus and hepatitis C virus); acute or symptomatic bowel disease; and pregnant or lactating female patients. Patients receiving corticosteroids or hormone therapy (unless for prostate cancer) within 14 days of the first planned dose of study drug were excluded. Major surgery, chemotherapy, radiotherapy (other than short cycle of palliative radiotherapy for bone pain), immunotherapy or any investigational agent were not permitted within 28 days of the first planned dose of study drug. Furthermore patients with prior toxicities from

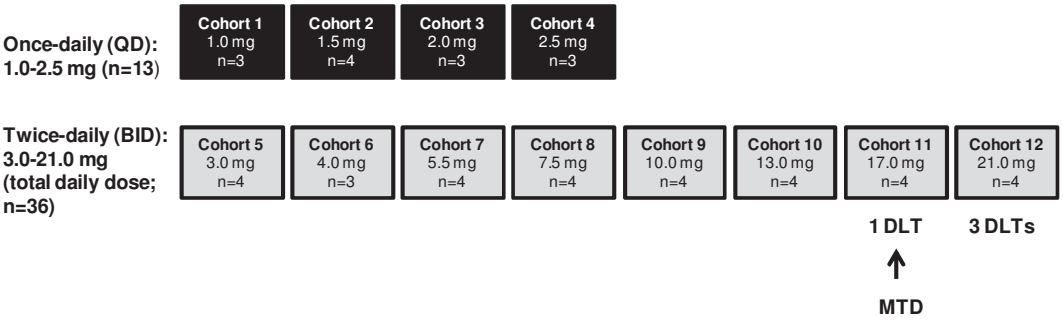
chemotherapy or radio-therapy which had not regressed to Grade ≤1 were also excluded.

Results

Supplementary Table 1. Temporary dose interruptions due to adverse events.

Dose regimen	Number of patients (n)	Total number of interruptions
1.5 mg (QD)	1	3
2.0 mg (QD)	1	1
4.0/3.5 mg (BID)	1	1
5.0 mg (BID)	1	1
6.5 mg (BID)	2	2
8.5 mg (BID)	3	6
10.5 mg (BID)	2	6

BID, twice daily; QD, once daily. Five patients experienced more than one drug interruption (one patient at 1.5 mg QD and two patients at 8.5 mg BID and 10.5 mg BID).



Supplementary Figure 1: Schematic showing dosing cohorts, DLTs and the MTDDLT, dose-limiting toxicity; MTD, maximum tolerated dose. At 8.5 mg BID (17.0 mg total daily dose), one patient experienced isolated Grade 3 elevated CPK. No further DLTs were observed when this cohort was expanded to nine patients. After escalation to 10.5 mg BID (21.0 mg total daily dose), three patients experienced DLTs (Grade 3 CPK elevation, Grade 4 CPK elevation and Grade 3 blurred vision). Accordingly, 8.5 mg BID was defined as the MTD.

2. Pharmacokinetic (PK) and pharmacodynamic (PD) population analysis

PK data available from 43 patients were fitted to a linear two-compartment model with first-order oral absorption and solved using the NONMEM program (version VI, ADVAN4, GloboMax LLC, Maryland, US). Measurements were simultaneously fitted to an effect compartment PK/PD model of inhibited pERK in PBMCs using NONMEM (version VI, ADVAN4 and ADVAN 9). An assumption was made that RO4987655 affects both background and PMA-stimulated phosphorylation of ERK. The percentage inhibition at each time point was:

$$EP_{inh} = \left(\frac{pERK_{0act}}{(pERK_{0baseline})} - 1\right) \cdot 100$$

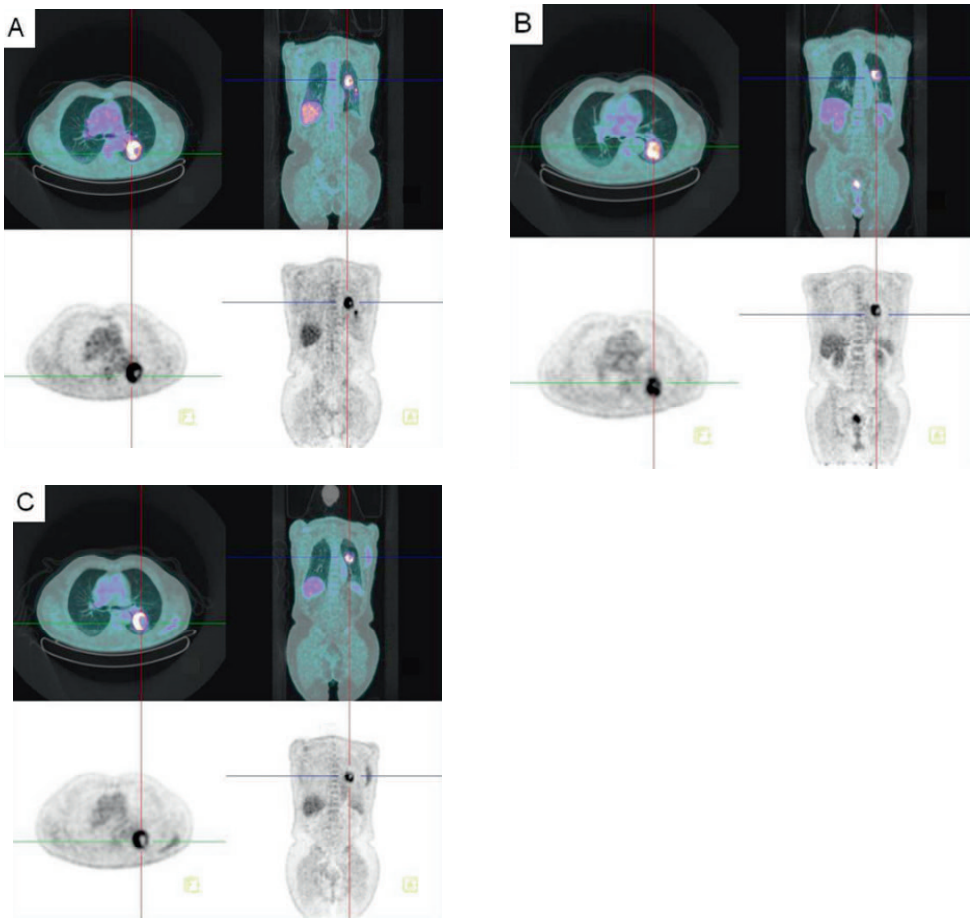
The individual percentage inhibition rate in the presence of an inhibitor (IP_{inh}) is calculated as:

$$IP_{inh} = \left(\frac{(pERK_{baseline} + pERK_{stimulated}) \cdot (1 - EFF)}{(pERK_{baseline} + pERK_{PMA_{stimulated}})}\right) \cdot 100 = EFF \cdot 100$$

Where:

$$EFF = \frac{\mathrm{I_{max}} \cdot \mathrm{RO4987655_{effect}}}{\mathrm{IC_{50}} + \mathrm{RO4987655_{effect}}}$$

Where: $pERK_{Oact}$ is the observed actual time related pERK readout; $pERK_{Obaseline}$ is the observed baseline pERK readout; $pERK_{baseline}$ represents the baseline parameter of pERK; $pERK_{PMA\_stimulated}$ represents the additional stimulation parameter of pERK following PMA stimulation; EFF represents the effect of RO4987655 when using an I_{max} model; Note that $RO4987655_{effect}$ represents the concentration of RO4987655 in an effect compartment (rather than in the plasma). This approach accounts for the delay between the concentration of RO4987655 in the plasma and the pERK inhibitory effect that is observed in the PBMC(Sheiner *et al.,Clin Pharmacol Ther* 1979;25(3):358–371); and IC_{50} represents the half maximal inhibitory concentration. The maximum inhibitory effect (I_{max}) was investigated by objective function profiling.



Supplementary Figure 2. Case-study: FDG-PET uptake at baseline (A) baseline, Day 1 of Cycle 3 (B) and Day 158 (C) in a 62-year-old male with metastatic adenocarcinoma of the rectum with *KRAS* mutation.

This patient had received three previous lines of chemotherapy for metastatic disease (capecitabine + bevacizumab + oxaliplatin; irinotecan; cisplatin + Wee1-kinase inhibitor). RO4987655 was administered at 2.0 mg (BID; total daily dose, 4.0 mg) for a total duration of 31weeks. These images demonstrate a 4.95% reduction in FDG-PET uptake between baseline and Day 1 of Cycle 3 (metabolic stable disease), and a 31.41% reduction in FDG-PET between baseline and Day 158 (metabolic partial response). This patient achieved stable disease for 30 weeks (best overall response [RECIST], 7% reduction in tumor size). Paired skin biopsies demonstrated significant reductions in pERK (cytoplasm) and Ki67 between baseline and Day15.

Supplementary Table 2. Changes in biomarker levels between baseline and Day 15.

	pERK (immuno-histochemistry)	Ki67 (immuno-histochemistry)	Apoptosis (TUNEL assay)
Paired tumor biopsies			
Total number of pairs*	7	7	5
≥20% increase in signal	1	1	1
≥20% decrease in signal	6	1	1
No change	0	5	3
Paired normal skin biopsies			
Total number of pairs*	20[†]	20	0
≥20% increase in signal	0	1	-
≥20% decrease in signal	5	4	-
No change	14	15	-

* Patients from which paired samples were available and of sufficient quality for specific assay;

[†] immunohistochemistry staining failed in one patient.

2.3
Ocular toxicities induced by inhibitors of the MAPK pathway

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Accepted for publication Review

Abstract

By the introduction of molecularly targeted anti-cancer drugs, that are designed to intervene with specific pathways aberrant in cancers with distinct mutations, the type of adverse events encountered has changed greatly compared to the adverse events profile of classical chemotherapeutic agents. Ocular toxicities, such as serous retinal detachment (SRD) and retinal vein occlusion (RVO), are observed in the treatment with several protein kinase inhibitors, such as MEK inhibitors. This review discusses the pathophysiolology, diagnosis and advice for clinical management of these toxicities, and focuses on the current understanding of the underlying molecular mechanisms. Some ocular toxicities can be considered a class effect and a direct result of intervening with the MAPK pathway. Effective recording and monitoring will contribute to increased understanding of the prevalence and of adequate management of these ocular toxicities, but further research is warranted to elucidate the exact underlying mechanisms and to optimize treatment of these undesirable toxicities.

Introduction

Over the past decade the development of anti-cancer drugs has undergone crucial changes. Whereas conventional chemotherapy targets both normal and rapidly dividing cells, newer agents tend to exploit tumor-specific genetic alterations in signal transduction pathways. These targeted therapies, as a result of their mechanism of action, can manifest with a profoundly different toxicity profile.

There are several targeted agents at present widely used in the clinic to treat patients with various types of cancer. Some examples include imatinib (Glivec ®, target: bcr/abl, c-kit, PDGFR, e.g. Ph^+ALL, GIST), trastuzumab (Herceptin ®, target: HER2/NEU, e.g. breast and gastric cancer), erlotinib (Tarceva ®, target: EGFR, e.g. NSCLC) and gefitinib (Iressa ®, target: EGFR, e.g. NSCLC). Many other new drugs are in early stages of clinical development. Each of these agents has shown specific toxicity profiles.

One of the less common toxicities, but all the more important because of its influence on daily life, are the ocular adverse effects. Recently, two reviews described ocular toxicities of both existing chemotherapeutic agents and investigational targeted therapies.[1, 2]

This review focuses on recently observed ocular toxicities of inhibitors of the Mitogen Actived Protein Kinase (MAPK) pathway, most commonly consisting of blurred or impaired vision. Ophthalmologic examinations of this clinical symptom found the most common cause to be serous retinal detachment (SRD).[3] Occasionally, cases of retinal vein occlusion (RVO) have also been encountered. To explain these toxicities, this review attempts to give insight into the pathophysiology of SRD and RVO, both on anatomical and cellular basis, the targeted agents for which these toxicities were encountered (or could be expected) and the possible clinical implications.

Methods

Information for this review was collected by searching PubMed/Medline, ClinicalTrials.gov and American Society of Clinical Oncology (ASCO)

abstract databases. The medical subject heading terms used included (single terms or combinations of the following terms): ocular toxicity, visual impairment, blurred vision, visual disturbances, retina, retinal pigment epithelium (RPE), retinal detachment (RD), serous retinal detachment (SRD), MEK inhibitor, ERK, MAPK, FGF(R), BRAF, EGF(R), PI3K, VEGF(R). Articles published in English before July 2012 were included. In addition, experience was obtained in clinical research with a range of different tyrosine kinase inhibitors.

Anatomy and physiology of the retina

The sclera, choroid and retina are the three main layers that line the back of the eye. The sclera is the white outer coat enclosing the eyeball, containing collagen and elastic fiber. The choroid contains connective tissue and a rich capillary network, providing oxygen and nutrients for the retina.

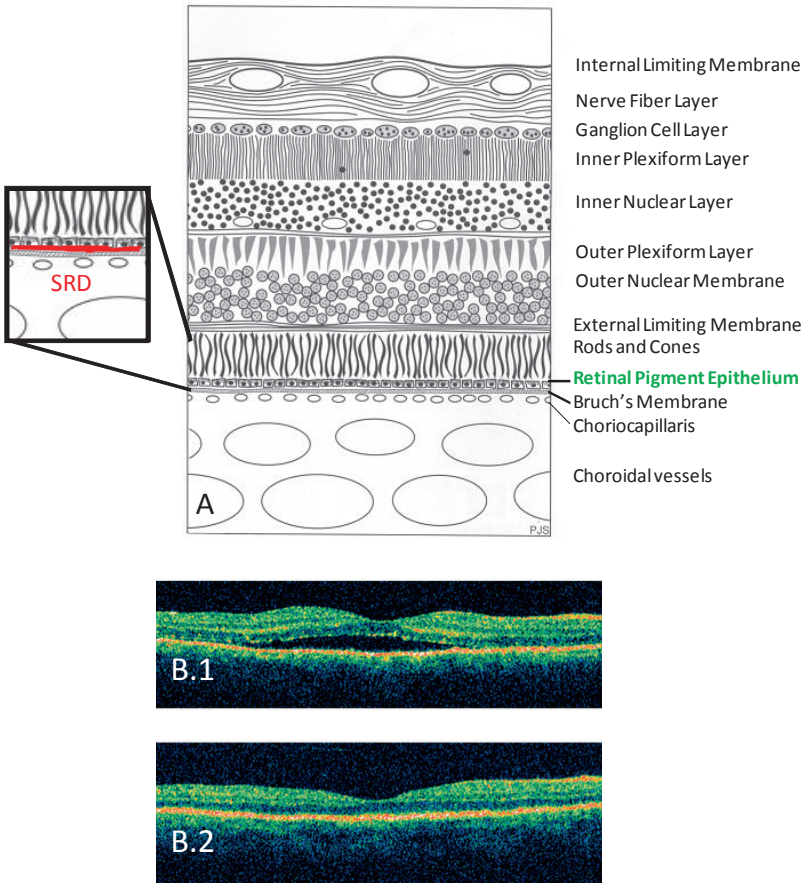
The retina covers about two-thirds of the back of the eyeball and although the retina is no more than 0.5 mm thick, it has a complex structure in which 10 separate layers can be discriminated, including three layers of nerve cells (**figure 1**). Stimulation by light of the rod and cone cells of the retina initiates an electrochemical reaction in which electrical impulses are transmitted to the brain through the optical nerve.

The Retinal Pigment Epithelium (RPE) is a single cell layer of pigmented hexagonal cells firmly attached to the underlying choroid or Bruch's membrane. The RPE and the retinal vascular epithelium together form the blood-retinal barrier (BRB). The RPE protects the retina from excess of incoming light and provides omega-3-fatty acids, glucose and retinol for the photoreceptor cells, which belong to the most metabolically active cells in the human body. Under physiological circumstances water is actively removed to the choroid and a balance of pH is carefully maintained.[4, 5]

Serous retinal detachment

Pathophysiology: retinal detachment (RD) implies fluid accumulation between the normally firmly attached layers of the neural retina and the RPE, while the retina can remain physically intact. Three different

Figure 1. Organization of the retina in a schematic vertical section. [131] **A**. Retinal layers from the vitreous body towards the sclera. Retinal detachment (RD) occurs when fluid accumulates between the neurosensory retina and the retinal pigment epithelium (RPE). Different underlying mechanisms can result in RD. One of these mechanisms is classified as exadutive or serous retinal detachment (SRD); **B.1.** Optical coherence tomography (OCT) images of a patient with serous retinal detachment (SRD); **B.2.** OCT scan images visualizing normal retina.



mechanisms are identified that can lead to RD; 1) the rhegmatogenous form caused by a tear across the retina (most common), 2) tractional detachment, which is a result of cells or adhesions growing in the vitreous and causes mechanical traction, and 3) serous (or exudative) detachment (SRD) which, although normally the most uncommon form, is the type of detachment that will be the focus of this review since this form seems to be of relevance in the treatment with MAPK inhibitors. RD evokes a series of events, including activation of both inflammatory and apoptotic pathways, followed by regeneration processes.[6]

Clinical symptoms in SRD include blurred vision or other signs of visual impairment, often in both eyes - although SRD can also occur in the absence of symptoms, especially when edema is limited to parts outside the fovea (part of the eye responsible for sharp central vision). *Diagnosis* might be performed by a test of visual acuity and confrontational evaluation of visual fields (Amsler grid). More important is slit lamp evaluation and dilated retinal examination. Diagnosis is confirmed by optical coherence tomography (OCT), which can visualize the internal structures of the retina by performing optical sectioning at micron resolution using infra-red light reflectance.[7-9] OCT is an appropriate method to visualize and evaluate intra-retinal edema over time.

Treatment of SRD is expectative as it regularly resolves within a matter of days without requiring treatment. Validated treatments for SRD are currently lacking. Regular monitoring by OCT is recommended.[5, 10]

Retinal vein occlusion (RVO)

Pathophysiology: (RVO) is a vascular disorder of the retina in which blockage of the outflow vessel results in partial obstruction of the blood flow, ischemia, increased intraluminal pressure in the retinal veins, and increased transduction of plasma and blood, causing edema and hemorrhages. Edema secondary to RVO is often accompanied by SRD, probably due to fluid leakage from damaged capillaries into the subretinal space. Other complications include neovascularization of the retina, optic disc, or iris, and neovascular glaucoma. Although the precise pathogenesis of RVO is not understood, it results in ischemia and consequently in

Vascular Endothelial Growth Factor (VEGF) release by Müller cells[11], which increases vascular permeability and in combination with upregulation of other inflammatory factors like interleukin (IL)-6, IL-8 and monocyte chemotactic protein 1 (MCP-1) contributes to disruption of the BRB.[12, 13]

Clinical key symptom is painless visual loss and in some cases even blindness. RVO usually occurs unilaterally and has an acute onset.[5]

Diagnosis is made by an ophthalmologist. Since patients with cardiovascular disease (e.g. hypertension, hyperlipidemia) and diabetes have an increased risk for developing RVO, it is recommended to document this information. Examination includes intraocular pressure, confrontational visual fields, and slit lamp evaluation. Typical findings for RVO in fundoscopic examination include dilated tortuous retinal veins, hemorrhages, cotton wool patches (a sign of ischemia), and edema. Fluorescein angiogram (FAG), a technique using a specialized fundus camera to take rapid-sequence photographs of the retina following intravenous injection of fluorescein sodium [14] often shows delayed filling of retinal veins, leakage of dye and allows quantification of the area of non-perfusion.

Treatment directed at RVO currently does not exist, although different interventions are being applied, partly depending on the localization of the RVO and the presence of macular edema. Most widely used are 1) grid laser photocoagulation and 2) medicinal treatment with VEGF inhibitors or glucocorticoids, both given as intravitreal injections. Ranibizumab is the only VEGF inhibitor approved in Europe and the US for the indication of RVO. Bevacizumab and pegaptanib are other VEGF inhibitors available for clinical use. Glucocorticoids have a risk of increasing intraocular pressure which limits their use. Visual acuity is recommended to be measured and documented at each visit to evaluate progression and/or response to treatment.[15-17]

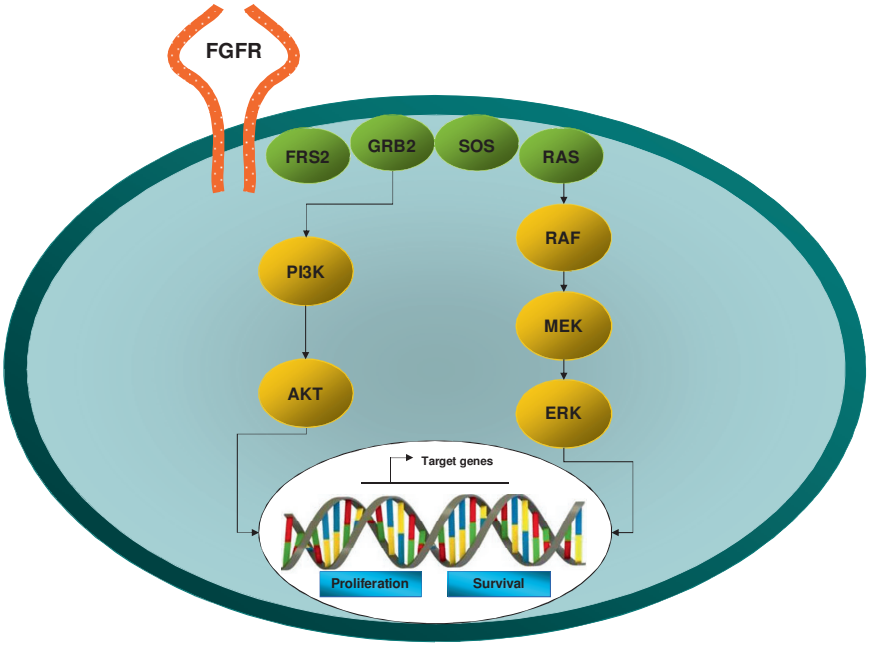


Figure 2. FGFR signaling. When FGF binds to its receptor (FGFR), it activates adaptor protein FGFR Substrate 2 (FRS2), which then acts as a docking site for Growth factor Receptor Bound protein 2 (GRB2). GRB2 is then able to trigger two pathways, mainly the RAS/RAF/MEK/ERK pathway, but also the PI3K/AKT pathway. Both these pathways eventually trigger transcription of genes involved in cell survival and proliferation.[58]

Inhibition of the FGFR–MAPK pathway and ocular toxicity

In trying to understand the cellular signaling involved in the development of the aforementioned toxicities, we first need to understand which factors play significant roles in the normal functioning of the RPE. Review of the available literature points towards a prominent role for the Mitogen Activated Protein Kinase (MAPK) pathway and its activation by the Fibroblast Growth Factor (FGFR) in the maintenance, survival and repair of the RPE (**Figure 2**).

Presence

Basic Fibroblast Growth Factor (bFGF or FGF2) is a neurotrophic factor, expressed throughout the retina and can bind FGFR1 and 2 with high affinity.[18] Expression of bFGF is highest in the nuclei of macroglial cells of the retina and RPE. bFGFis also found in the retinal neurones in rats.[19] Intravitreously injected bFGF in rats and mice showed an upregulation of phosphorylated Extracellular Regulated Kinase (pERK) and c-fos in the inner retina.[20] Inhibition of FGFR by FGFR1 inhibitor PD173074 was able to decrease downstream phosphorylation of ERK and selectively and potently inhibit the neurotrophic effects of bFGF in rat neurons.[21] Furthermore, by inhibiting Ras, Raf-1, ERK and Cyclin D, proliferation of RPE cells is reduced, indicating that this signaling pathway is involved.[22, 23]

Development and maintenance

During development neuroepithelial cells differentiate into RPE cells, which then proliferate to form the RPE. In the mature retina RPE cells have a limited capacity for proliferation.[24] FGF plays an essential role in the proliferation and survival of both developing and mature retinal cells. In young RPE cells, FGF together with Epidermal Growth Factor (EGF), has the most pronounced effect on DNA synthesis and growth, while in mature RPE cells, it appears that activation of FGFR1 is more pronounced and helps to prevent apoptosis in these cells. Indeed, when this receptor is inhibited, more RPE cell death is seen.[25, 26]

The survival of photoreceptors can also be stimulated by FGF and this survival seems to be dependent on ERK activation. When α_2-adrenergic agonists, which can induce FGF especially in photoreceptors, are administered systemically to rats, ERK is activated in Müller cells (the principal glial cells that help maintain the integrity and normal functioning of the retina).

Accordingly, ERK activation is an important early event in photoreceptor protection.[27] When phosphorylation of ERK is abolished *in vitro* by MEK inhibitor U0126 photoreceptor survival is reduced, showing that survival is dependent on the bFGF-MEK-ERK signaling pathway.[28]

The bFGF-MEK-ERK pathway is also essential for survival in mature RPE cells. bFGF stimulates the production of FGF1, which in turn can activate paracrine FGFR1. FGFR1 subsequently activates the MAPK pathway, leading to the production of ERK2 and activation of the anti-apoptotic proteins Bcl-x and -2. Long-term secretion of FGF1 and subsequent activation of downstream ERK2 and Bcl-x and -2 therefore does not lead to proliferation in these mature cells, but instead causes resistance to apoptosis.[29, 30] These anti-apoptotic affects could be abrogated in chick retinal neurons by addition of either an FGFR1 antibody or the MEK inhibitor PD98059, confirming this mechanism.[31]

The fact that FGFR activation of the MAPK pathway promotes cell survival has also been demonstrated *in vivo*. When bFGF was applied to the optic nerve after axotomy in frogs, it increased the amounts of pERK, pCREB and the anti-apoptotic protein Bcl-2, and downregulated the apoptotic proteins Bax and caspase 3.[32, 33] These effects could be reversed by addition of MEK inhibitor U0126.[33]

Protection against injury

In addition to maintaining the integrity of the RPE cells, FGFR-MAPK signaling is able to protect the RPE from injury. Early research showed that bFGF slows the progression of photoreceptor degeneration and protects the retina from light-induced damage in rats, and inhibition of bFGF causes an increase in light-induced damage, both *in vitro* and *in vivo*.[25, 34, 35] bFGF activation of the MAPK pathway and subsequent phosphorylation of ERK is responsible for the protection against damage by overstimulation and oxidative injury.[36, 37]

Similarly, protection of RPE cells against oxidative injury was induced *in vitro* by GTx-822, an estrogen receptor-β agonist. This effect was shown to be mediated by the MAPK pathway but, interestingly, also by the PI3K pathway. Inhibitors of both pathways resulted in increased cell death.[38]

Repair

The role of FGFR activation of the MAPK pathway in the repair of damaged RPE cells has also been well documented. bFGF is clearly upregulated in increase in FGFR1 is also seen *in vitro* in both responses.[39]

MEK-ERK signaling was found to be essential for the first proliferative steps towards the regeneration of RPE cells after injury in newts.[40] An important process in retinal repair is the dedifferentiation of the RPE cells into progenitor-like cells, which are then able to form new retinal cells. *In vivo* experiments in chick embryos and tadpoles show that the promotion of dedifferentiation and regeneration can be triggered by bFGF stimulation and subsequent upregulation of ERK.[41, 42] Furthermore, these processes are abrogated by inhibitors of FGFR and MEK.[43, 44]

Role in ocular disease

Since bFGF and the MAPK pathway appear to play pivotal roles in maintenance, protection and repair of the retina, it can be hypothesized that inhibiting this signaling pathway could lead to disturbances and even degeneration of retinal cells. This hypothesis is strengthened by the fact that bFGF can save photoreceptors in the retina of Royal College of Surgeons (RCS) rats, which are predisposed to retinal degeneration due to a genetic defect in the RPE.[34, 45] Interestingly, bFGF and the expression of FGFRs were downregulated in these rats, indicating that reduced FGF signaling might underlie the primary cause of the retinal dystrophy in these animals.[46, 47]

Inhibiting FGF actively in mice also contributes to retinal damage, including loss of the RPE.[48] An *in vivo* animal model of retinal detachment in cats and rabbits demonstrated that Müller and RPE cells signal via the MAPK pathway, immediately following retinal detachment. bFGF and FGFR1 were identified as possible initiating molecules.[49] This pathway was also activated when ischemia-reperfusion injury was induced in the retina of rats and inhibiting MEK increased ganglion cell death.[50]

After several patients were treated with MEK inhibitor PD0325901 and developed RVO, it was decided to test this drug in RVO animal models. Rabbits received an intravitreous injection of PD0325901 and rats were given this drug orally. Ophthalmologic exams (slit-lamp) were performed at several time points after administration. In rabbits RVO was present within 24 hours after treatment and progression to retinal detachment and edema was observed after 1 week. In rats, these findings were not present,

but their retina did show an increase in gene expression related to oxidative stress response.[51]
An important clinical finding is that bFGF is elevated in the subretinal fluid of patients with retinal detachment (with or without proliferative vitreoretinopathy) suggesting that FGF might play a similar role in protection and survival roles in humans.[52, 53] Although the MAPK pathway seems to be the most important FGFR signaling pathway in the RPE, FGFR can also trigger the Phospho-Inositide-3 Kinase (PI3K) pathway in response to stress, leading to an increase in pAKT and eventually resulting in cell survival.[54] Moreover, the PI3K pathway seems to be involved in response to oxidative injury, since normal antioxidant responses in RPE cells can be abrogated by inhibitors of PI3K, such as LY294002 and wortmannin.[55, 56]

Role in oncology

The family of FGFRs comprises four homologically similar receptor tyrosine kinases (RTKs).[57] In both normal and tumor cells signaling through FGFRs trigger intracellular pathways, mainly the MAPK and PI3K pathway (see **figure 2**).[58] MAPKs mediate intracellular signal transduction and regulate many cellular processes, such as proliferation, differentiation, survival, inflammation and angiogenesis.[59-61] Although the MAPK cascades are often depicted as linear, they are involved in complex signaling networks with cross talk between different MAPK and other pathways.[62, 63] The RAS/RAF/MEK/ERK pathway is probably most extensively studied.
Genetic alterations in this signaling cascade, such as FGFR, RAS and RAF are present in a variety of tumors and can lead to overexpression and aberrant activation of this pathway. These tyrosine kinases are therefore attractive targets for the development of anti-cancer drugs.[64, 65] FGFRs are among the most commonly mutated kinase genes and aberrant FGFR signaling through mutations and/or amplifications has been shown to play a role in several tumor types, such as breast, gastric, bladder and squamous cell lung cancer.[66-69] Also, FGFRs can act as key regulators in angiogenesis.[70]

K-RAS mutations are observed in cancer of the pancreas (~60 -90%), colorectal cancer (CRC) (30-50%), NSCLC (20-30%), thymus, ovarian and endometrial cancer (~15%).[71, 72] B-RAF mutations frequently occur in melanoma (30-60%), cancer of the thyroid (30-50%), ovarian cancer (30%) and CRC (5-20%).[65, 73, 74]

MEK inhibitors in clinical development

As mentioned previously, development of cancer treatment has focused increasingly on targeting specific kinases. One of these targets is the MAPK kinase, also known as MEK. MEK is located downstream of the growth factor receptors and can phosphorylate ERK, which on its turn can then enter the cell nucleus and activate transcription of genes encoding for cell proliferation. All inhibitors currently in development appear to be very selective for MEK.

Efficacy

Reports on efficacy of selective MEK inhibitors vary between studies. While the development of some of these MEK inhibitors has been discontinued due to lack of efficacy (CI-1040, PD0325901), others have shown promising anti-tumor activity. Efficacy has been most pronounced in patients carrying a BRAF mutation.

Selumetinib (AZD6244) did not show a significant difference in progression-free survival when compared with temozolomide in an unselected group of patients with advanced melanoma. However, 5 out of 6 patients showing response were carriers of a BRAF mutation. Further development of selumetinib will focus on combination therapies, either with other targeted agents or chemotherapy.[75]

In contrast, trametinib (GSK1120212) showed promising results in a phase II study in patients with metastatic melanoma carrying a BRAF mutation, either pre-treated with a BRAF inhibitor or chemo- and immunotherapy. The disease control rate (CR + PR + SD) in the whole study was reported as being as high as 81%, with tumor reduction being observed in 64% of the

patients. Clinical anti-tumor activity was minimal in patients pre-treated with a BRAF inhibitor.[76]
Response data from the newer MEK inhibitors vary but also are premature (most of these drugs are still in phase I studies).[3, 77-79]

Ocular toxicity

Ever since the first MEK inhibitor, CI-1040, was clinically evaluated ocular toxicities have been regularly reported for this class of drugs. **Table 1** summarizes these adverse events. Since the reported toxicities of different drugs are very similar, there appears to be a class effect. Furthermore, the observed ocular adverse events appear to be related to the dosing intensity and schedule, with higher and continuous dosing showing more toxicity than lower or intermittent dosing. Although serious, the toxicities seem to be transient in most cases. Furthermore, intervention in the form of dose interruption and/or reduction was required in only a few cases.

BRAF inhibitors in clinical development

The BRAF inhibitors are more advanced in clinical development than MEK inhibitors. RAF is located right upstream of MEK in the MAPK growth signal transduction pathway and is therefore also a frequently targeted kinase in new anti-cancer treatments. Many BRAF inhibitors inhibit mutated BRAF more potently than wild type BRAF, which creates an attractive opportunity for tumor selective inhibition.

Efficacy

The class of BRAF inhibitors has demonstrated significant anti-tumor activity, especially in patients with advanced melanoma carrying a BRAF mutation. Vemurafenib (PLX4032) is the first BRAF kinase inhibitor approved for the treatment of this patient population.[80] It has shown to improve survival when compared with dacarbazine, with response rates being as high as 48%, compared with 5% for dacarbazine.[81]
Dabrafenib (GSK2118436) also showed to be efficacious when given to BRAF mutant metastatic melanoma patients in a phase I study: 69% of

patients showed response, of which 50% were confirmed responses. In addition, the drug showed anti-tumor activity in melanoma patients with brain metastases and other solid tumors carrying BRAF mutations.[82, 83]

Ocular toxicity

An overview of BRAF inhibitors in clinical development and observed ocular toxicities can be found in **Table 2**. Early clinical trials with BRAF inhibitors, such as vemurafenib (PLX4032, Zelboraf®), have not demonstrated any ocular toxicities.[84] However, in a phase III study with vemurafenib, several cases of uveitis, blurred vision, iritis and photophobia were reported. Moreover, another trial showed a case of RVO. This resulted in the advice that patients using vemurafenib should be routinely monitored for ocular abnormalities.[80, 85]

FGFR inhibitors in clinical development

There are several multiple tyrosine kinase inhibitors currently in development that are able to inhibit FGFR. Among these are brivanib (BMS-582664), dovitinib (TKI258), vargatef (BIBF 1120) and lenvatinib (E7080). No ocular toxicities were observed in phase I and II studies with these drugs.[86-93] However, these inhibitors are multikinase inhibitors and all show a similar or even higher potency for inhibition of vascular endothelial growth factor receptor (VEGFR) [IC_{50} in µM: 0.025; 0.010; 0.005; 0.004] compared to FGFR [IC_{50} in µM: 0.148; 0.0085; 0.038; 0.046] and therefore are all able to inhibit angiogenesis to some extent.[94-97]
Interestingly, VEGF inhibitors such as bevacizumab are currently being used by ophthalmologists to treat complaints related to age-related macular degeneration (AMD). It could be that the protective effect of VEGF inhibition on the retinal epithelium actually outweighs the damaging effects of FGF inhibition.[98]
Recently, several specific FGFR inhibitors have entered phase I evaluation, including AZD4547 and BGJ398, which show a much higher specificity for FGFR [IC_{50} in µM: 0.0015; 0.0023 (both average FGFR 1-3)] compared to

Table 1. Ocular toxicities of MEK inhibitors.

Name	Stage of development	Frequency of ocular toxicities	Description of ocular toxicities	IC_{50} for MEK
CI-1040	Discontinued	6 (9%) out of 67 (Phase II)	Blurred vision, altered light perception[110]	17 nM[111]
PD-0325901	Discontinued	9 (14%) out of 66 (Phase I) 5 (38%) out of 13 (Phase II continuous) 1 (5%) out of 21 (Phase II intermittent)	Visual disturbances, including 2 cases of RVO[112] Blurred vision[108] Blurred vision[108]	1nM[113]
Selumetinib (AZD6244)	Phase II	7 (12%) out of 57 (Phase I suspension) 10 (17%) out of 59 (Phase I capsules) 18 (18%) out of 99 (Phase II)	Blurred vision[114] 4 cases of blurred vision (one case grade 3 but transient)[116] 12 cases of blurred vision 4 cases of visual disturbances[75]	14.1 nM[115]
Trametinib (GSK1120212)	Phase I/II	Unknown (Phase I) Unknown (Phase Ib, with gemcitabine) Unknown (Phase II)	2 dose limiting central serous retinopathies[117] 1 dose limiting uveitis[119] 2 cases of central serous retinopathy[76]	3.4 nM[118]
RO4987655	Phase I	26% (Phase I)	1 reversible dose limiting RVO, visual disturbances, some cases of SRD[3]	5.2 nM[120]
MSC1936369	Phase I	23% (Phase I, Schedule 1 - 5/21 days)	Visual disturbances, some cases serous macular detachment 1 dose limiting RVO[78]	NA

		29% (Phase I, Schedule 2 - 15/21 days)	Visual disturbances, some cases serous macular detachment[78]	
GDC-0973/ XL518	Phase I	Unknown (Phase I)	No toxicities reported[79]	< 1 nM[121]
RDEA119	Phase I	Unknown (Phase I)	1 case of chorioretinopathy (recovery after dose reduction), 1 case of hemi-RVO (treated with Intravitreal bevacuzimab)[77]	19 nM[122]
TAK-733	Phase I	Unknown	Unknown	3.2 nM[123]

Table 2. Ocular toxicities of BRAF inhibitors.

Name	Stage of development	Frequency	Description	IC_{50} BRAF mutant	IC50 BRAF Wild type	Off target kinases
Vemurafenib (PLX4032)	Approved (melanoma)	None reported (Phase I)	NA[84]	10 nM	39 nM	Minimal[124]
		None reported (Phase II)	NA[125]			
		21 cases (Phase III - Trial 1)	5 cases of uveitis 5 cases of blurry vision 5 cases of iritis 6 cases of photophobia[80]			
		1 (Phase III - Trial 2)	1 case of RVO[80]			
Dabrafenib (GSK2118436)	Phase III	None reported (Phase I)	NA[82]	0.16 nM	NA	Minimal[126]
		None reported (Phase I/II)	NA[83]			
		None reported (Phase IIa)	NA[127]			
XL281	Phase I	None reported (Phase I)	NA[128]	6 nM	5 nM	NA[129]
RAF865	Phase I	Unknown (Phase I)	2 dose limiting cases of visual disturbances[130]	3-60 nM	14 times less selective	VEGFR2 (30 nM)[129]

VEGFR [IC_{50} in µM: 0.3 (120-fold of FGFR2 – 0.0025); 0.18].[99, 100] To date, no data of these trials have been presented yet.
It is of interest whether preclinical observations of ocular toxicity induced by FGFR inhibition will be predictive of ocular toxicities in patients.

Discussion

There is an ongoing shift in cancer therapy towards more targeted treatment, whereby novel drugs with varying selectivity specifically inhibit transmembrane receptors and intracellular proteins. Since these proteins have important physiological roles in the human body, but display different effects depending on their tissue distribution and intrinsic activity, it is difficult to predict beforehand the range and severity of toxicities that may occur upon inhibition of these proteins. Preclinical *in vitro* and *in vivo* models can help to predict these toxicities, but it should be noted that it will always be difficult to fully approach the human situation. Although there are increasingly specific models for many retinal diseases which can help us to study these targeted therapies in animals with specific genetic aberrations and retinal abnormalities, the treating oncologist should be aware of the signs and symptoms of developing ocular toxicity in new compounds.[101]
In the eye, many signal transduction pathways and endogenous factors need to be in balance in order to support the integrity of this fragile environment. Disturbances of these pathways can potentially lead to detrimental effects. These signaling pathways can be triggered by a range of growth factors (including FGFR, VEGFR and epidermal growth factor receptor [EGFR]), with each growth factor leading to different actions. EGFR inhibitors, such as erlotinib, have already shown specific ocular toxicities, although in a different magnitude than the toxicities mentioned in this review. With EGFR inhibitors the structure and function of the cornea is often disturbed leading to adverse events such as dry eyes and keratitis.[102] As already briefly mentioned in this review, there is also emerging evidence for a role of PI_3K in the repair of retinal cells after

oxidative injury. Inhibiting PI3K or other proteins in this pathway could lead to cumulative injury and loss of these cells, although the currently available data from the first phase I studies with PI3K inhibitors have not yet shown ocular toxicities.[103-105]

The specific retinal toxicities described in this review are likely to be attributed to the inhibition of FGFR/RAS/RAF/MEK pathway. Preclinical data show that these factors play an important role in the survival, maintenance and repair of the retina. Inhibiting these proteins could therefore lead to disturbances in the RPE. As for MEK and BRAF inhibitors, clinical data are available which have confirmed that SRD and/or RVO can also occur in humans. It is of interest to see if inhibiting the upstream FGFR will lead to similar toxicities.

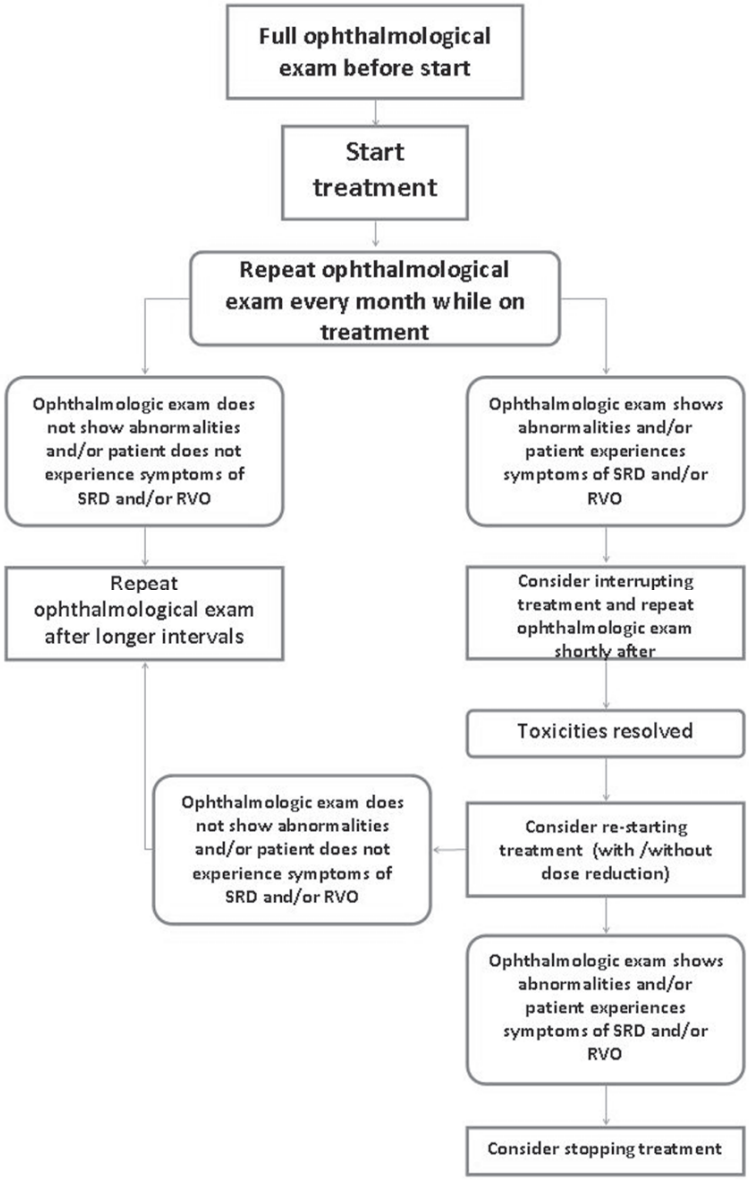
The observed ocular toxicities seem to be mostly reversible (sometimes even when treatment is uninterrupted), but can in some cases cause serious patient discomfort since reduced vision severely affects daily life. However, SRD can also be asymptomatic, which emphasizes the need for effective monitoring, because these toxicities can be detrimental on the long-term.

Advice for clinicians

There are currently no validated treatment options available for SRD and RVO. In SRD, the fluid accumulation is usually re-absorbed, with symptoms recovering and an absent need for treatment. RVO is a more serious adverse event which may require therapeutic intervention. Several studies have attempted to show efficacy of a range of treatments including VEGFR inhibitors, anticoagulants, corticosteroids and thrombolytic agents. Nevertheless, no treatment to date has been validated for RVO because of the lack of large randomized clinical trials.[106, 107] The best advice when encountering these toxicities in the clinic would be effective monitoring, both before and after start of treatment (**figure 3**).[108, 109]

Finally, clinicians should consider not starting these targeted therapies in patients showing possible risk factors for SRD and/or RVO, such as history of RVO or uncontrolled systemic vascular disorders, e.g. hypertension or diabetes.

Figure 3. Flowchart ocular toxicities. A flowchart based on current clinical trials with targeted therapies that could help clinicians make treatment decisions when faced with ocular toxicities. Full ophthalmologic assessments should include visual acuity, Amsler grid, slit lamp, funduscopy (including FAG) and OCT scans.



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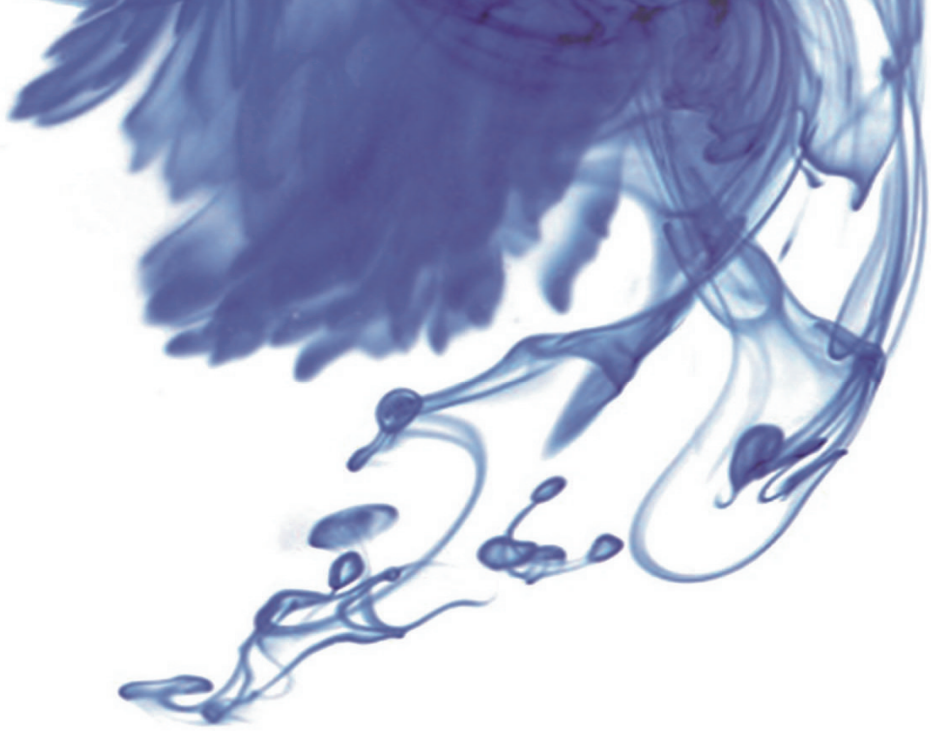

CHAPTER 3

Clinical pharmacological combination studies with Wee1 inhibitor MK-1775

3.1

Abrogation of the G2 checkpoint by inhibition of Wee1 kinase results in sensitization of p53-deficient tumor cells to DNA-damaging agents

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Curr Clin Pharmacol 2010 Aug;5(3):186-91 Review

Abstract

Inducing DNA damage is a well known strategy for attacking cancer, already being used for many years by the application of a variety of anti cancer drugs. Tumor cells and other rapidly dividing cells are more sensitive to DNA damage caused by DNA damaging agents compared to normal cells. While normal cells can rely on various mechanisms for DNA repair in order to protect the integrity of the genome and to promote cell survival, most tumor cells, due to genetic changes, are more challenged when it comes to repair of DNA damage. Wee1 is a tyrosine kinase that phosphorylates CDC2 at Tyr 15 and as such plays a pivotal role in the G2 DNA damage checkpoint. The strategy of inhibition of Wee1 by a tyrosine kinase inhibitor is exploiting the impaired options for DNA damage repair especially in cells with deregulated p53, which results in malfunction of the G1 checkpoint. Tumor cells that are unable to rely on the G1 checkpoint are more sensitive to G2 checkpoint abrogation. Administration of DNA damaging chemotherapy in combination with a Wee1 inhibitor may therefore selectively sensitize p53 deficient cells, while normal cells are spared from toxicity. PD-166285 has been described as a novel G2 abrogator and Wee1 inhibitor, but has also been characterized as a broad-spectrum receptor tyrosine kinase inhibitor. MK-1775 is a specific and potent inhibitor of Wee1 and currently under investigation in a multi-center phase I study in combination with either gemcitabine, carboplatin or cisplatin in patients with advanced solid tumors. Preliminary results show good tolerability and promising anti-cancer activity.

Introduction

The incidence of cancer increases worldwide and therefore the demand for effective and powerful drugs with minor side effects is more urgent than ever. While many conventional anti-cancer drugs kill rapidly dividing cells without discriminating between normal and cancerous cells, the development of new anti-cancer drugs focuses on more specific and individualized treatments by interfering with distinct targeted molecules needed for carcinogenesis and tumor growth. Rationally targeted therapies, interacting with predefined targets on malignant cells, are fundamental to achieving successful future anti-cancer therapies. Advances in cancer cell biology, molecular genetics and pharmaceutical applications, like the ability to design multifunctional and smart drug vehicles, offer intriguing possibilities and potential targets for new anti-cancer drugs.

Important types of targeted anti-cancer drugs include monoclonal antibodies (MAbs) against growth factor receptors expressed on tumor cells or intracellularly expressed ligands of growth receptors, small molecules that inhibit tyrosine kinases (TKIs), cyclin-dependent kinase (CDK) inhibitors, proteasome inhibitors and compounds that interfere more downstream with specific intracellular pathways like the RAS/RAF/MEK/ERK (MAPK) or phosphoinositide-3-kinase (PI3K) pathway. Drugs like imatinib (Bcr-Abl), trastuzumab (HER2), bevacizumab (VEGFR), cetuximab (EGFR) and erlotinib (EGFR), are perfect illustrations of drugs that have largely contributed to the evolution of anti-cancer therapy in the past decades.

The main focus of this article will be on a very new drug within the spectrum of targeted agents: MK-1775, the first Wee1 inhibitor that at present is being evaluated in a clinical setting. This drug specifically interferes with the cell cycle. An attempt has been made to not only provide information about MK-1775, but also to define its (preliminary) role within the context of other targeted drugs. This is thought to be of importance since MK-1775 is the first Wee1 inhibitor that has made it to a clinical evaluation. MK-1775 is an excellent example of a rationally

developed drug with a clearly defined target. Since more and more drugs are being rationally designed, questions that can be easily posed (but more difficultly answered) are: What targets are being selected (or: what makes a good potential therapeutic target)? Which targets or strategies for drug design were more or less successful than others and are good explanations for the difference in effectiveness available at present? Do these new drugs require different strategies for clinical evaluation? In order to be able to discuss these questions, but also to have a better understanding of the mechanism of a Wee inhibitor, it seems inevitable to first briefly address items like the cell cycle, DNA damage response, and the differences and similarities between cancer cells and normal cells regarding these matters.

DNA damage repair in healthy cells and tumor cells and the importance of the G_1 checkpoint

The major mode of action of many conventional anti-cancer agents, including platinum compounds, alkylating agents, anti-metabolites and DNA topoisomerase inhibitors consists of inducing DNA damage. Both platinum compounds and alkylating agents attack DNA directly by the formation of interstrand and intrastrand crosslinks with nucleobases of DNA strands. Anti-folates are anti-metabolites that act specifically during RNA and DNA synthesis (S-phase of the cell cycle). Topoisomerase inhibitors interfere with the enzymes topisomerase I and topoisomerase II respectively, two enzymes involved in the cleavage and binding to the phosphodiesther backbone of DNA strands. Inhibition of topoisomerase I and II results in DNA single and double strand breaks, ultimately leading to apoptosis and cell death. In response to DNA damage, both normal cells and cancer cells rely on activation of cell cycle checkpoints to induce cell cycle arrest, as an opportunity for DNA repair, thus preventing the transmission of damaged DNA to daughter cells.[1;2]
The G1 checkpoint can be considered the first safeguard against genomic stress as it can prevent cells from entering S-phase (the initiation of DNA

replication) and allows for DNA repair prior to DNA replication.(3) When cells have acquired DNA damage in the G2-phase or have escaped the G1 and S checkpoint, the cell cycle can also be arrested at the G2-checkpoint, also named the DNA damage checkpoint.(4;5) The spindle assembly checkpoint (SAC) or mitotic checkpoint normally becomes activated in metaphase, inhibiting the metaphase-anaphase transition of the cell cycle until all chromosomes are properly attached to mitotic spindle and thus ensuring proper segregation of sister chromatids.[6] (See **Figure 1**.)

When repair mechanisms are insufficient, e.g. due to genetic defects or excessive DNA damage, cells can enter a state of senescence (a state in a cell in which it will not divide again, even in the presence of growth factors) or apoptosis (programmed cell death). Alternatively, accumulation of DNA alterations may also lead to genomic instability, transformation and oncogenesis. [7]

A significant difference between normal cells and cancer cells is that one such checkpoint pathway is often abrogated in cancer cells due to loss of function mutations in the p53 tumor suppressor gene.(2) The protein p53 (encoded by the gene TP53) plays a pivotal role in G1 checkpoint control. Many mutagens, oncogenic viruses and inherited factors especially affect the G1 checkpoint. Polyomavirus, adenovirus, and papillomavirus are examples of oncogenic viruses that damage the function of p53 and Rb proteins. For instance human papilloma virus E6 (HPV-E6) can bind and inactivate p53 by proteosomal degradation.[5] Another example is HPV oncoprotein E7, that can bind to Rb protein and hereby can prevent association of Rb with HDAC, which results in a decreased repressing effect of promoter Cyclin E. Normally Rb protein negatively modulates the G1/S transition by silencing specific genes that are active during synthesis (S-phase).[8] Likewise do benzopyrenes of tobacco mutate the p53 gene.(5;9) Malfunctioning p53 or Rb proteins affect the G1 cell cycle checkpoint. The G1 checkpoint can normally be initiated by different mitogenic stimuli, resulting in accumulation of Cyclin D, which binds to CDK4 and CDK6, as well as phosphorylation of Rb. Phosphorylated Rb releases E2F/DP-1 transcription enhancer complexes, which stimulate transcription of downstream genes that are necessary for entry into S-

phase. E2F/DP1 also increases Cyclin E, which forms complexes with CDK2 (CDK2/Cyclin E) that stimulate additional phosphorylation.[2;10] The majority (>50%) of human cancers have impaired G1 cell cycle checkpoint function.[4]

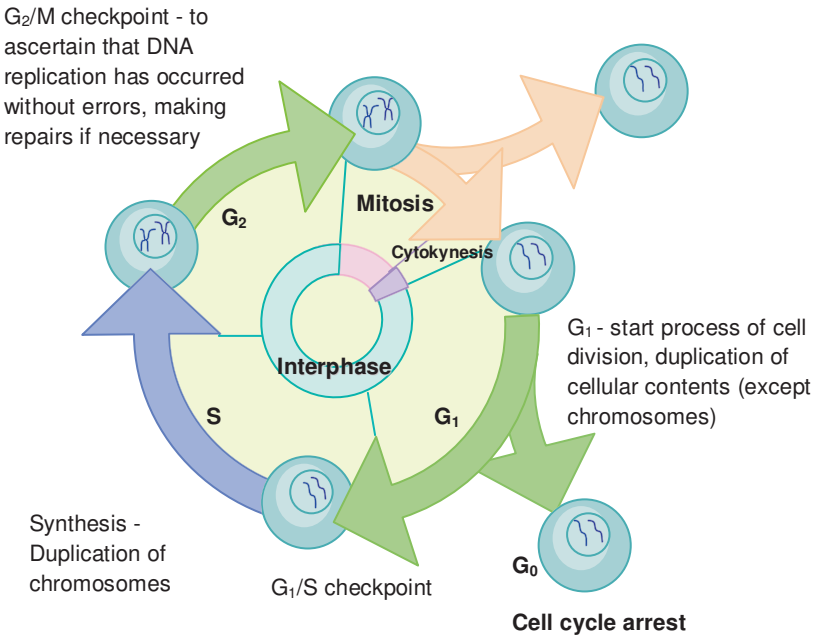

Figure 1. A schematic interpretation of the cell cycle.
The cell cycle consists of mitosis (cell division) and interphase. During interphase the cell grows and duplicates its DNA. Three important phases can be distinguished during interphase: G1 (Gap 1), S (Synthesis) and G2 (Gap 2). **G1** or growth phase, a highly variable period starting from the previous mitosis and lasting until the onset of DNA synthesis, is induced by growth signals or mitogens, and demarcates the onset of the synthesis of various enzymes needed for DNA replication. The **G1 checkpoint** is also referred to as the restriction point, a point of no return (the cell is obligated to divide or to go into apoptosis) and plays a major role in preventing cells from DNA damage. Key player in the G1 checkpoint is p16 which inhibits CDK4/6 preventing interaction with Cyclin D and inhibiting cell cycle progression resulting in apoptosis. Uninhibited p16 CDK4/6 can interact with

Cyclin D and CDK4/6 Cyclin D complexes phosphorylate Rb. Activation of Rb in turn halts the inhibition of E2F transcription factor (unphosphorylated Rb inhibits E2F). Subsequently E2F interacts with DP-1 to form a transcription enhancer complex which ultimately stimulates expression of cyclin E. Cyclin E can interact with CDK2 to form CDK2/Cyclin E complexes that allow for G1-S phase transition. p53 is a mediator of the G1 checkpoint, but can also directly induce apoptosis by several transcriptionally dependent and independent pathways. During **S** phase DNA is replicated and is completed when all chromosomes have duplicated. Then **G2** phase starts, which persists until mitosis. During G2 phase microtubules are formed, which are responsible for the proper segregation of chromosomes and division over the daughter cells. Prior to mitosis DNA damage often occurs. The **G2 checkpoint** allows for DNA repair, preventing DNA damage to daughter cells. CDC2/Cyclin B activity is needed for progression to mitosis. Wee1 and Myt1 catalyze inhibitory phosphorylations of CDC2/Cyclin B complexes. CDC25 can relieve these inhibitory phosphorylations. CDC25 in turn is inactivated by Chk1 and Chk2. During **M** phase the chromosomes are divided between the daughter cells, a process often immediately followed by cytokinesis (cell division). The **spindle checkpoint** ensures correct attachment of chromosomes to the spindles prior to segregation.

CDC2/Cyclin B and Wee1, major players in the G2 checkpoint and the mechanism of action of a Wee1 inhibitor

Because errors in replication may lead to chromosomal alterations, the different stages of the cell cycle are carefully controlled by different surveillance response mechanisms, commonly referred to as cell cycle checkpoints, as already explained above. When observing the G2 checkpoint in more detail, entry into mitosis, G2/M transition, is especially initiated by the activity of the cell cycle regulatory kinase complex CDC2/cyclin B. Phosphorylation of CDC2 (also known as cyclin dependent kinase 1 - CDK1) on threonine 161 (Thr161) in the T-loop allows stable association of CDC2 with cyclin B and is catalyzed by CDC2 activating kinase (CAK), which itself is composed of a CDK complexed with a cyclin (CDC7/cyclin H).[11]

Without CDC2/cyclin B activity cells are unable to progress into mitosis. The synthesis of Cyclin B starts in interphase. Cyclin B then associates with CDC2, but CDC2/cyclin B complexes are kept inactive by inhibitory phosphorylation on two residues in the ATP binding pocket: threonine 14 (Thr14) and tyrosine 15 (Tyr15), a process catalyzed by Myt1 and Wee1 kinases. CDC2 activating dephosphorylation of Thr 14 and Tyr 15 of CDC2 is catalyzed by dual specificity phosphatase CDC25C. Phosphorylation of CDC25C by kinases Chk1 and Chk2 results in inactivation of CDC25C. Hence, both CDC25C inactivation and Wee1/Myt1 activation can activate G2 cell cycle arrest in case of DNA damage.[12-15]

Wee1 phosphorylates CDC2 at Tyr 15, keeping the CDC2/Cyclin B complex inactive and promoting G2/M cell cycle arrest. MK-1775 inhibits phosphorylation of CDC2 at Tyr 15 and therefore results in an inability of keeping the CDC2/Cyclin B complex inactivated and an inability to arrest the cell cycle in case of DNA damage, ultimately leading to apoptosis.(16)

Thus, Wee1 inhibitors exploit the G1 checkpoint deficiency of especially p53 deficient tumor cells to enhance their apoptotic response to DNA damage. In preclinical models, adding a Wee1 inhibitor to DNA damaging agents like carboplatin, cisplatin or gemcitabine, or combining a Wee1 inhibitor with ionizing radiation, resulted in increased cell death. Normal cells, however, can rely on G1 cell cycle arrest and therefore do not seem to be significantly affected.[16]

Wee1 as a therapeutic target

The lack of functional p53 in the majority of cancer cells was hypothesized to be an important therapeutic target. There were different reasons for Wang et al. to focus on development of a Wee1 inhibitor to realize this goal [17]: 1) a Wee1 inhibitor would be a G2 abrogator and therefore expected to preferentially kill p53 mutated cells, because it would deprive these cells from the only opportunity to protect these cells from premature mitosis in response to DNA damage; 2) Wee1 plays a central role in maintaining G2

cell cycle arrest by inhibitory phosphorylation of CDC2; 3) when expressing a mutant of CDC2, Cdc2AF, in HeLA cells this resulted in an ability of Wee1 to phosphorylate CDC2, ultimately leading to premature mitosis[18-20]; 4) while Wee1 is down-regulated in p53 positive cells after DNA damage(21), Wee 1 over-expression can rescue apoptosis.[17;22]

PD-166285

PD-166285 is a Wee1 inhibitor developed by Pfizer. PD-166285 has a pyrido-pyrimidine structure and not only inhibits Wee1 at a nanomolar concentration, but also inhibits (less potently) Myt1 kinase. PD-166285 was tested *in vitro* in 7 different cell lines and 0.5 µM PD-166285 resulted in radiation induced Cdc2 phosphorylation at Tyr15 and Thr14. PD-166285 acts as a radiosensitizer by abrogating G2 cell cycle arrest. The efficacy was higher in cells with inactive p53: PD-166285 radiosensitization was effective in both p53 mutant HT29 cells and E6-transfected p53 null ovarian cancer PA-1 cells, but significantly less effective in p53 wild type PA-1 cells.[17]

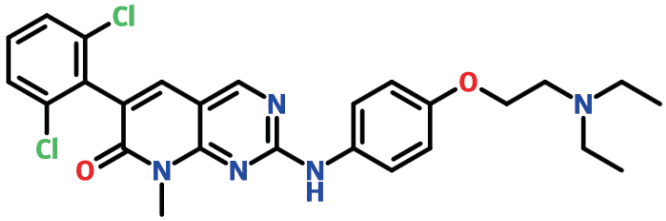

Figure 2. Chemical structure of PD-166285.
(Derived from Wang Y, Li J, Booher RN, Kraker A, Lawrence T, Leopold WR, et al. Radiosensitization of p53 mutant cells by PD0166285, a novel G(2) checkpoint abrogator. *Cancer Res* 2001 Nov 15;**61**(22):8211-7.)

Experiments in B16 mouse melanoma cells showed that PD-166285 abrogated the G2 checkpoint; PD-166285 almost completely abolished Cdc2-Tyr15 phosphorylation and although the expression of CDC2 remained unchanged, the expression of cyclin B decreased significantly. In addition, PD-166285 blocked microtubule stabilization (polymerization failed to occur) and suppressed cyclin D expression. The authors therefore declared that these data suggest that Wee1 may be more than a G2 checkpoint nullifier, since it also affects cell adhesion molecules.(23)

Although PD-166285 is first being described by Wang et al. as a novel G2 abrogator and Wee1 inhibitor (and named PD0166285, with the number '0' after 'PD'), this is not the first article published about this compound. Earlier in time other articles about PD-166285 have already been published (here named PD 166285 without the number '0').(24-28) To our knowledge, the first article about PD-166285 has been published in 1997 by Panek et al.(27) Although PD-166285 was initially designed to inhibit the catalytic domain of PDGFR-ß TK with the intent of inhibiting PDGF signaling and growth of tumor cells, PD-166285 is presented in this article as a multi-targeted kinase inhibitor, inhibiting several kinases. PD-166285 has been assigned the following properties in this article: 1) a novel pyrido-pyrimidine with bicyclic structure; 2) an ATP competitive inhibitor of EGFR, FGFR, c-Src and PDGFR-β and (less potent) to MAPK and PKC; 3) in addition, *in vitro* experiments resulted in prolonged intracellular retention of the drug and in long lasting inhibition of growth-factor mediated cellular functions; 4) finally, the characterization of PD-166285 as a broadly active tyrosine kinase inhibitor was believed to make this compound attractive for use in a number of diseases where growth factor and cytokine signal transduction pathways are aberrantly activated.

In 1999 Dimitroff et al. evaluated PD-166285 for its anti-angiogenic properties in combination with photodynamic therapy (PDT). In microcapillary formation assays PD-166285 inhibited the formation of microcapillaries on Matrigel-coated plastic. *In vivo* mouse models showed dose dependent inhibition of angiogenesis after orally administered PD-166285 (1-25 mg/kg). Prolonged tumor regressions were observed in anti-tumor efficacy studies against a murine mammary 16c tumor with daily

doses of PD-166285 (5-10 mg/kg following PDT compared with PDT alone ($p < 0.001$).(24)

In all results of our literature search, PD-166285 appeared to be a promising compound. After 2006 however, no results were detected in our literature search, and to our knowledge a phase I trial with this compound has never been initiated up to this date. Everything considered, we conclude that although PD-166285 has been described as a Wee1 inhibitor, the compound should be considered as a broadly active tyrosine kinase inhibitor with an uncertain role in clinical settings at present.

MK-1775

Preclinical in vitro experiments with Wee1 inhibitor MK-1775

MK-1775 is a small molecule Wee1 inhibitor from Merck, which inhibits phosphorylation of Cdc2 at Tyr 15, resulting in G2 checkpoint abrogation (see **Figure 3**) .

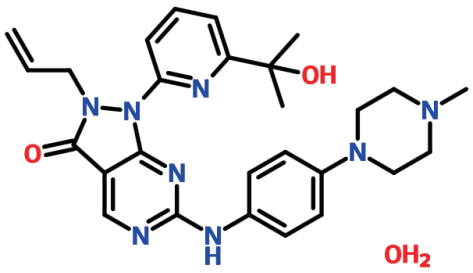

Figure 3. Chemical structure of MK-1775.
(Derived from Hirai H, Iwasawa Y, Okada M, Arai T, Nishibata T, Kobayashi M, et al. Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents. *Mol Cancer Ther* 2009 Nov;**8**(11):2992-3000.

Pre-clinical studies revealed the following biological properties: 1) high potency: in *in vitro* kinase assays a IC_{50} of 5.2 mM was observed with an increasing linear relationship between the IC_{50} value of MK-1775 and ATP

concentration; 2) high selectivity: only 8 of a total of 223 kinases were inhibited by >80% with 1 µmol of MK-1775 and 3) important cellular activity in different cell lines. In gemcitabine pretreated WiDr cells, a human colorectal cancer cell line with mutated p53, MK-1775 abrogated gemcitabine-induced cell cycle arrest. Pre-mature mitotic entry was determined by the use of the immunohistochemical mitosis marker phospho-Histone H3 (*pHH3*). In comparable experiments, in which gemcitabine was replaced by different platinum compounds, similar results were observed. Experiments in other human tumor cell lines with inactive p53, TOV21G (ovarian adenocarcinoma) and H1299 (lung), also showed abrogation of the DNA damage checkpoint induced by MK-1775.[16]

Furthermore, MK-1775 sensitized p53 tumor cells to various anti-tumor agents. In cell viability assays with WiDr cells the IC_{50} of gemcitabine alone was >100 nmol/L, while in combination with 30 and 100 nmol/L of MK-1775 the IC_{50} of gemcitabine was reduced to 21.5 and 7.1 nmol/L respectively. When MK-1775 was administered as a single agent, no significant anti-proliferative effect was observed in doses of 30 to 100 nmol/L. At a dose of 300 nmol/L, in which > 80% of Wee1 was inhibited, anti-proliferative effects were established at 34.1%. Similar results were obtained using H1299 lung cancer cells. MK-1775 did not show enhanced cytoxicity in experiments with paclitaxel or docetaxel. A rational explanation for this is that these agents do not interfere with the G2 DNA damage checkpoint, but target microtubules.[16]

Selective sensitization of p53 deficient tumor cells was confirmed in isogenic matched pair cell lines (TOV1G with wild-type p53 function and TOV1G-shp53 expressing short hairpin RNA resulting in functional loss of p53 respectively). [16]

Preclinical in vivo experiments with Wee1 inhibitor MK-1775

In vivo experiments in nude rats with WiDr (human colorectal) tumors treated with gemcitabine (i.v. bolus) and MK-1775 (oral administration) showed significantly enhanced antitumor effect in case of co-treatment with gemcitabine and MK-1775. Combination treatment did not significantly increase toxicity (measured as body weight, white blood cell

levels and platelet count) compared to treatment with gemcitabine alone. Other mouse models were used to assess and confirm enhancement of the anti-tumor effects of carboplatin and cisplatin by MK-1775.[16]
Inhibition of CDC2 phosphorylation Tyr15 and induction of pHH3 in tumor tissue and skin hair follicles of the animals (hair follicles contain proliferating cells) were used to investigate pharmacodynamic changes and to determine a possible relation between the pharmacodynamic changes and anti tumor effects. Since phosphorylation of CDC2 was indeed inhibited by MK-1775 (reduction at least 50%) in both tumor and hair follicles, skin hair follicle is considered a promising surrogate marker for clinical trials with MK-1775. [16]

Clinical studies with Wee1 inhibitor MK-1775

MK-1775 is currently under phase I clinical trial in combination with either gemcitabine, cisplatin or carboplatin in patients with advanced solid tumors. Patients have received doses as high as 1300 mg of MK-1175 monotherapy and 325 mg of MK-1775 in combination therapy. The preliminary safety analyses demonstrate that MK-1775 is generally well tolerated and shows promising anti-cancer activity. (J.Clin Oncol 27: 15s, 2009 suppl; abstr 3510) Preclinical data suggest that in a clinical setting MK-1775 may increase the response to agents like gemcitabine, carboplatin and cisplatin in patients with p53 mutated tumors. [16]

Other G2 checkpoint abrogators and cell cycle kinases

DNA damage can be introduced by different endogenous and exogenous factors varying from, chemicals, ionizing radiation, chemotherapeutics and by-products arising from normal cellular metabolism. DNA damage is detected by so called sensor proteins, like HUS1, RAD1, RAD9, RAD17, γH2AX, MDC1, P53BP1 and BRCA1.[29-31] Depending upon the nature and origin of the DNA damage, different sensor protein complexes and pathways become activated. Transduction of the majority of the damage signals takes place by ATR, Chk1 and CDC25A or ATM, Chk2 and CdC25C (which activates Cdc2/Cyclin B complex).(32) Some proteins of these sensor complexes are partly used by multiple checkpoints. Chk2 for

instance can both indirectly activate the G1 and G2 checkpoint.(33) The ideal target for G2 abrogation, resulting in an agent with minimal adverse effects on normal cells, would therefore be expected to have its target downstream of these sensor complexes and probably will need to be very selective in its mode of action.[34]

Different substrates have been proposed as potential targets for the development of G2 abrogators and consist of e.g. ATM, ATR, Chk1, Chk2, CDC25, CDK4, CDK6, CDK7 and CDK9.[7;10;34;35] Several CDK inhibitors that have been designed affect multiple targets. The cell cycle is a complex machine and forms a dynamic network of enzymes and proteins that can negatively or positively influence each other, form complexes or interact in other ways. Unpredictability of the effects and toxicities in a clinical setting of these new compounds is an important consequence of this complexity. Another point of attention for designing phase I trials with these compounds is that many of these drugs are chemo-sensitizers and therefore might be more effective in combination with ionizing radiation or certain conventional anti-cancer drugs than as a single agent.

Genetically engineered models may be very useful in obtaining proof of principle. Additionally, the genetic knockout of a particular CDK may have different effects than pharmacological inhibition. The result of genetic knockout of a CDK will in general be more selective than pharmacological inactivation of a CDK with a kinase inhibitor. The following reason to explain this difference has been proposed by Lapenna et al; a CDK inhibitor, especially when ATP competitive, will often inhibit an important function of a CDK, but the CDK will still contain its ability to form complexes with some physiologic substrates or inhibitors, that will in turn not be available anymore for other CDK targets in the cell. [36]

The cell cycle was first investigated in yeast species like *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast) before it was studied in more complex organisms and humans.[37;38] With the increase in complexity of the organism, the number of CDKs has increased during evolution as well. When checkpoints were first investigated it was believed that each part of the cell cycle was driven by specific CDKs. However, there is increasing evidence to believe that reality

is more complex. Against expectation, mouse studies revealed that non-functional CDK2, CDK4, and CDK6 does not result in cell cycle defects in most cells (except highly specialized cell types) and mouse embryos develop normally until mid gestation and failure to thrive was assigned to defective hematopoiesis.[39] Knockout of CDK1 (also known as CDC2) however, prevents that mice embryos can develop beyond a two cell stage, because it causes cell cycle arrest.[7] It has also been optioned that functions of CDKs in tumor cells might sometimes differ from the original function of specific CDKs in normal cells, and that this deserves special attention during drug development.[7]
Examples of kinase inhibitors that interfere with the cell cycle and are being clinically evaluated are UCN-01 (inhibitor of protein kinase C, CDK2, CDK4, CDK6), P276-00 (inhibitor of CDK1, CDK4, CDK9), PD0332991 (inhibitor of CDK4, CDK6) and AZD7762 (inhibitor of CHK1). [7;10;40] For detailed information we would like to refer to existing literature.

Conclusion

Cell cycle deregulation is a common feature of human cancers. The past two decades have significantly contributed to an increased understanding of the cell cycle. CDKs play a pivotal role in control of the cell cycle and therefore are considered important potential therapeutic targets. Nonetheless, while many drugs were already designed to attack these targets, only a limited number have made it to testing in a clinical setting. From all drugs that have entered phase I, only a few have shown clinical advantage and an acceptable toxicological profile. Wee1 inhibitor MK-1775 is an example of a drug that despite its early phase of development shows promising clinical effects. MK-1775 is a selective G2 checkpoint abrogator that inhibits Wee1 that primarily sensitizes p53 deficient cells to DNA damaging agents.

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3.2

A phase I and pharmacologic study evaluating Wee1 inhibitor MK-1775 in both monotherapy and in combination with either gemcitabine, cisplatin, or carboplatin in adult patients with advanced solid tumors

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Preliminary analysis

Abstract

Purpose

MK-1775 is a Wee1-kinase inhibitor inhibiting the G2 checkpoint leading to chemosensitization in p53 deficient tumor cells. In a dose-rising study, safety, tolerability, pharmacokinetics and pharmacodynamics of oral MK-1775 in combination with chemotherapy were evaluated in patients with refractory solid tumors. The Wee1 signature and down regulation of pCDC2 served as biomarkers.

Patients and methods

In part 1 9 patients received one single dose of MK-1775 as monotherapy, 8 of which continued in one of three combination treatment arms. In total 178 patients were treated with MK-1775 in combination with either gemcitabine, carboplatin or cisplatin. In part 2A patients received each cycle one dose of MK-1775 24 h after chemotherapy, and in part 2B 5 BID doses starting simultaneously with chemotherapy. In the gemcitabine arm MK-1775 was also given QD for two days starting simultaneously with gemcitabine. Doses up to 1300 mg were administered in monotherapy, and up to 325 mg in combination therapy. Hair follicles and skin samples were collected for pharmacodynamics. p53 status was determined retrospectively in archival tumor tissue samples.

Results

MK-1775 was well tolerated. Most common adverse events consisted of hematological toxicity, nausea/vomiting and fatigue. The maximum tolerable dose was established in all schedules excluding the MK-1775 QD x2 regimen in combination with gemcitabine. Pharmacokinetics were linear. Target engagement was observed as a predefined reduction of 50% of pCDC2 in mono and combination therapy with cisplatin and carboplatin and changes in Wee1 gene signature in monotherapy. Eighty-four (47.2%) of patients showed stable disease, and 4 (2.2%) partial remission as best response.

Conclusion

Inhibition of Wee1 by MK-1775 was well tolerated and associated with target engagement.

Introduction

One of the main drivers of drug resistance in cancer treatment is p53 mutation.[1-6] A concept to overcome p53 mediated drug resistance is to interfere with the ability of cancer cells to repair DNA damage in the G2 phase of the cell cycle.[7-10] One of the key proteins governing the G2 checkpoint is tyrosine (Tyr) kinase Wee1,[11-13] which inhibits the action of its direct substrate CDC2 by phosphorylation of the Tyr15 residue of CDC2, leading to cell cycle arrest and allowing for DNA repair.[14-17] Wee1 inhibition results in G2 checkpoint abrogation.[18-21] p53 is a key regulator of the G1 checkpoint. p53 deficient tumor cells might be especially sensitive to Wee1 inhibition in combination with DNA damaging chemotherapy because of increased G2 checkpoint dependency for DNA repair [22-25].

Small molecule MK-1775 (2-allyl-1-[6-(1-hydroxy-1-methylethyl)pyridin-2-yl]-6-{[4-(4-methylpiperazin-1-yl)phenyl]amino}-1,2-dihydro-3H-pyrazolo[3,4-d]pyrimidin-3-one) is a potent and specific inhibitor of Wee1.[26;27] In *in vitro* kinase assays MK-1775 showed an IC_{50} value of 5.2 nM.[27] As expected, MK-1775 did not exhibit single agent activity in a preclinical setting.[11;28] MK-1775 induced cell death in combination with chemotherapy and selectively sensitized p53 deficient tumor cell lines to various anticancer agents, including gemcitabine, cisplatin and carboplatin and radiation.[29-33] The effect of MK-1775 monotherapy and gemcitabine on pCDC2 in rat skin and hair follicles was investigated in a Widr xenograft tumor model and enhancement of antitumor effect by MK-1775 was well correlated with inhibition of pCDC2 in a dose dependent manner, suggesting pCDC2 to be a useful PD biomarker.[27]

The objectives of this first in human study with a Wee1 inhibitor were to (1) determine the maximum tolerated doses (MTDs) and dose limiting toxicities (DLTs), to characterize (2) safety, tolerability, (3) the pharmacokinetic (PK) and pharmacodynamic (PD) profile, (4) biomarkers of biological activity and (5) the preliminary anti-tumor activity of oral MK-1775 in combination with either gemcitabine, cisplatin or carboplatin.

Patients and Methods

Patient selection

Patients were ≥ 18 years old, with advanced or metastatic solid tumors for whom no standard therapy was available. All patients had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of 0 or 1, adequate organ function, and evaluable and/or measurable disease according to Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0).[34] (See appendix E1 for additional inclusion/exclusion criteria.) Up to 4 prior cytotoxic chemotherapy regimens were permitted.

Study design and drug treatment

This phase 1, open-label, non-randomized three-arm dose-escalation study was conducted in 8 centers in America, Canada and Europe. The study received approval of the institutional medical ethical review boards and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was given by all patients prior to inclusion in the study.

Patients were enrolled in cohorts and treated at sequentially rising dose levels of oral MK-1775 (**Figure 1**). Dose escalation in combination with chemotherapy was performed according to a modified Toxicity Probability Interval (TPI) scheme that utilized a 30% dose limiting toxicity (DLT) rate. MK-1775 was titrated using a modified Fibonacci design allowing for 50%, 40% and 30% dose increments in subsequent dose levels[35] (see Appendix E2). The MTD of MK-1775 was evaluated for each of the 3 chemotherapy treatment arms separately. MK-1775 monotherapy consisted of a single dose followed by 14 days of observation (Part 1). Eight out of the 9 patients who received monotherapy continued in Part 2A, in which a single dose of MK-1775 was given 24h after standard chemotherapy with either gemcitabine (1000 mg/m^2), cisplatin (75 mg/ m^2) or carboplatin (AUC 5). Part 2B consisted of a multiple dose regimen of MK-1775 (2.5 days twice-daily doses [BID]) starting concomitantly with the chemotherapy. Patients were assigned to a chemotherapy arm according to judgment of the investigator.

Part 1 – 'MK-1775 Monotherapy'	MK-1775 Dose Escalation Levels (no MTD defined)
Day 1: 1 Single Dose of MK-1775 – a single 14 day cycle	325 – 650 – 1300

8/9 patients from Part 1 proceeded to Part 2A

Part 2A –' MK-1775 Single Dose'	MK-1775 Dose Escalation Levels (MTDs in bold)
Day 1: Chemotherapy (one of three arms)	
1. Gemcitabine 1000 mg/m^2 Day 1, 8, 15 – 28 day cycle	100 – **200** mg (Day 2, 9, 16)
2. Cisplatin 75 mg/m^2 Day 1– 21 day cycle	100 – **200** mg
3. Carboplatin AUC 5 Day 1 – 21 day cycle	100 – 200 – **325** mg
Day 2: On single dose of MK-1775	

Figure 1. Study setup.
The first part of the study consisted of monotherapy MK-1775 given as one single dose. Part 2A and 2B consist of three different treatment arms, with gemcitabine, carboplatin, or cisplatin, in two different schedules, with one single dose of MK-1775 administered the day after the chemotherapy (part 2A) or with 5 doses of MK-1775 given BID, the first dose starting concomitantly with chemotherapy. BID = bidaily; MTD = maximal tolerable dose; QD = once daily.

Part 2B – 'MK-1775 Multiple Dose'	MK-1775 Dose Escalation Levels (MTDs in bold)
Day 1: 1 Chemotherapy (one of three arms) + MK-1775 BID (first MK-1775 dose concomitantly with chemotherapy)	
1. Gemcitabine 1000 mg/m^2 Day 1, 8, 15 – 28 day cycle	25 – 50 – 50/25* mg (Day 1, 2, 3, 8, 9, 10, 15, 16, 17)
2. Cisplatin 75 mg/m^2 Day 1– 21 day cycle	50 – 100 – 125 – 150 – **200** – 250 mg
3. Carboplatin AUC 5 Day 1 – 21 day cycle	75 – 150 – **225** – 325 mg
Day 2: MK-1775 BID	
Day 3: MK-1775 QD	
- -	- -
Alternative 'Multiple Dose Gemcitabine' Schedule: **Day 1:** 1 Chemotherapy + MK-1775 QDx2 (first MK-1775 dose concomitantly with chemotherapy) Gemcitabine 1000 mg/m^2 Day 1, 8, 15 – 28 day cycle	100 – 125 – 150 – 175– 200 mg (Day 1, 2; 8, 9; 15, 16) – dose escalation still ongoing
Day 2: MK-1775 QD	

* = 50/25: day 1, 8 and 15 50 mg, day 2, 9, 16 25 mg BID, day 3, 10, 16 25 mg QD

Safety and assessments

Demographic data and medical history were collected during screening. Physical examination, vital signs and other safety assessments (ECOG-PS, 12 lead ECG, hematology/biochemistry and relevant tumor markers) were performed pre-dose and throughout treatment.

Toxicities were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0.[36] DLTs were defined as any grade 4-5 hematological toxicity (with the exception of grade 4 anemia and leucopenia, grade 4 neutropenia lasting for <7 days and grade 4 thrombocytopenia lasting for <4 days [except if a platelet transfusion was required]), and any grade 3, 4, or 5 non-hematologic toxicity (with the specific exception of grade 3 nausea, vomiting, diarrhea, or dehydration occurring in the setting of inadequate compliance with supportive care measures and lasting for less than 48 hrs, alopecia [of any grade] and inadequately treated hypersensitivity reactions).

Tumor assessments (RECIST version 1.0)[37] were performed at screening, every two cycles and whenever there was suspicion of disease progression.

Statistical analyses

PK, PD, safety, and tumor response were analyzed by descriptive statistics. An ANOVA was conducted for each quantitative polymerase chain reaction (qPCR) gene on the log fold-change (post dose to pre dose) scale. The various treatment and dose combinations were included as distinct categorical factors so that all observations were used to estimate a common residual variance; hence, tests were not dependent on variance estimates derived from only a few patients. A Hochberg multiplicity adjustment was applied over the 3 monotherapy doses tested (adjusting for multiple tests within the gene).

Pharmacokinetic assessments

In all parts of the study blood samples for PK analysis were collected in cycle 1 at selected time points up to 48 hrs after administration of the (last) dose of MK-1775 and analyzed by hydrophilic interaction liquid

chromatography (HILIC) coupled with tandem mass spectometry (LC-MS/MS).[38] (See **supplementary information**.)

Pharmacodynamic assessments

Three different PD markers have been assessed in this study: 1) p53 mutation status, 2) inhibition of phospho-CDC2 (pCDC2) relative to CDC2 levels, and 3) the 'Wee1 signature'.

Archival or fresh paraffin embedded baseline tumor samples were collected to correlate p53 mutation status with pharmacodynamic and clinical response. Analysis of p53 status was performed by PCR/sequencing of exons 4-9.

Target inhibition of MK-1775 was assessed as decrease of pCDC2 (Tyr15) relative to CDC2 measured in skin biopsies using multiplex immunohistochemistry (IHC) A significant fold change (FC) of pCDC2 Based on pre-clinical experiments linking PD and efficacy[39], 50% decrease of pCDC2 for post- relative to pre-dose MK-1775 with a 1 sided p-value <0.05 was defined as target engagement.

Hair follicles were also analyzed by qPCR for the 'Wee1 signature',[27] a gene expression-based PD biomarker that consists of a composite score calculated from the average fold change of up- and down regulated genes relative to pre-dose. Gene expression was measured at pre- and post dose time points for 8 genes identified as potential candidates by microarray: CLSPN, FBXO5, MCM10, CCNE1 and CCNE2, EGR1, HIST12BD, MYB; genes closely associated with the G2 checkpoint and commonly modulated in 1) p53-positive and negative cancer cell lines, and 2) skin samples derived from subcutaneous xenograft tumors in rats treated with gemcitabine and MK-1775.[27]

Results

Patient characteristics

Approximately two hundred patients were enrolled between February 2008 and May 2012 and 178 patients received at least one dose of MK-1775

Table 1 . Patient Demographics and Clinical Characteristics.

	Single Dose Gemcitabine		Single Dose Cisplatin		Single Dose Carboplatin		Multi Dose Gemcitabine		Multi Dose Cisplatin		Multi Dose Carboplatin		Total	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Subjects in population	14		13		17		43		45		46		178	
Gender														
Male	4	(28.6)	7	(53.8)	10	(58.8)	19	(44.2)	19	(42.2)	21	(45.7)	80	(44.9)
Female	10	(71.4)	6	(46.2)	7	(41.2)	24	(55.8)	26	(57.8)	25	(54.3)	98	(55.1)
Age (YEARS)														
under 55	7	(50.0)	3	(23.1`)	7	(41.2)	14	(32.6)	22	(48.9)	13	(28.3)	66	(37.1)
55 to less than 65	3	(21.4)	4	(30.8)	4	(23.5)	16	(37.2)	18	(40.0)	18	(39.1)	63	(35.4)
65 to less than 75	3	(21.4)	6	(46.2)	3	(17.6)	10	(23.3)	4	(8.9)	12	(26.1)	38	(21.3)
75 and over	1	(7.1)	0	(0.0)	3	(17.6)	3	(7.0)	1	(2.2)	3	(6.5)	11	(6.2)
Mean	56.5		60.8		58.4		58.7		53.9		58.4		57.4	
SD	14.7		9.6		13.4		12.2		10.4		12.2		12.0	
Range	30 - 83		41 - 70		36 - 79		23 - 79		28 - 76		27 - 78		23 - 83	

Ethnicity														
Hispanic	0	(0.0)	0	(0.0)	1	(5.9)	3	(7.0)	1	(2.2)	1	(2.2)	6	(3.4)
Not Hispanic	14	(100.0)	13	(100.0)	16	(94.1)	40	(93.0)	44	(97.8)	45	(97.8)	172	(96.6)
Prior Treatments														
1	7	(50.0)	9	(69.2)	5	(29.4)	20	(46.5)	26	(57.8)	30	(65.2)	97	(54.5)
2	5	(35.7)	3	(23.1)	6	(35.3)	8	(18.6)	12	(26.7)	10	(21.7)	44	(24.7)
3	1	(7.1)	1	(7.7)	2	(11.8)	7	(16.3)	6	(13.3)	4	(8.7)	21	(11.8)
4 or more	1	(7.1)	0	(0.0)	4	(23.5)	8	(18.6)	1	(2.2)	2	(4.3)	16	(9.0)
ECOG														
0	5	(35.7)	8	(61.5)	8	(47.1)	11	(25.6)	18	(40.0)	24	(52.2)	74	(41.6)
1	9	(64.3)	5	(38.5)	9	(52.9)	31	(72.1)	27	(60.0)	22	(47.8)	103	(57.9)
Not Reported	0	(0.0)	0	(0.0)	0	(0.0)	1	(2.3)	0	(0.0)	0	(0.0)	1	(0.6)

Table 2 . Subjects with Drug Related Adverse Events (≥10% Incidence).

	MK-1775 Single Dose						MK-1775 Multiple Dose						Total	
	Gemcitabine		Cisplatin		Carboplatin		Gemcitabine		Cisplatin		Carboplatin			
	n	(%)	n	(%)	n	(%)	n	(%)	N	(%)	n	(%)	n	(%)
Subjects in population	**14**		**13**		**17**		**43**		**45**		**46**		**178**	
With one or more adverse events	13	(92.9)	13	(100)	13	(76.5)	43	(100)	45	(100)	46	(100)	173	(97.2)
Grade ≥ 3	7	(50)	4	(30.8)	2	(11.8)	25	(58)	20	(44)	31	(67)	89	(50.0)
With no adverse events	1	(7.1)	0	(0)	4	(23.5)	0	(0)	0	(0)	0	(0)	5	(2.8)
Blood and lymphatic system disorders	**6**	**(50.0)**	**2**	**(20.0)**	**6**	**(42.9)**	**24**	**(55.8)**	**18**	**(40.0)**	**34**	**(73.9)**	**90**	**(52.9)**
Grade 3 and above	**5**	**(41.7)**	**1**	**(10.0)**	**1**	**(7.1)**	**17**	**(39.5)**	**6**	**(13.3)**	**26**	**(56.5)**	**56**	**(32.9)**
Anemia	1	(8.3)	0	(0.0)	2	(14.3)	13	(30.2)	5	(11.1)	17	(37.0)	38	(22.4)
Grade 3 and above	0	(0.0)	0	(0.0)	0	(0.0)	7	(16.3)	0	(0.0)	8	(17.4)	15	(8.8)
Leucopenia	3	(25.0)	1	(10.0)	1	(7.1)	5	(11.6)	2	(4.4)	5	(10.9)	17	(10.0)
Grade 3 and above	3	(25.0)	1	(10.0)	1	(7.1)	4	(9.3)	1	(2.2)	4	(8.7)	14	(8.2)
Neutropenia	3	(25.0)	1	(10.0)	1	(7.1)	8	(18.6)	9	(20.0)	16	(34.8)	38	(22.4)
Grade 3 and above	3	(25.0)	0	(0.0)	0	(0.0)	6	(14.0)	5	(11.1)	11	(23.9)	25	(14.7)
Thrombocytopenia	2	(16.7)	0	(0.0)	5	(35.7)	20	(46.5)	8	(17.8)	24	(52.2)	59	(34.7)
Grade 3 and above	2	(16.7)	0	(0.0)	0	(0.0)	9	(20.9)	1	(2.2)	18	(39.1)	30	(17.6)

Gastrointestinal disorders	**9**	**(75.0)**	**11**	**(110)**	**8**	**(57.1)**	**32**	**(74.4)**	**42**	**(93.3)**	**40**	**(87.0)**	**142**	**(83.5)**
Grade 3 and above	**0**	**(0.0)**	**3**	**(30.0)**	**0**	**(0.0)**	**3**	**(7.0)**	**6**	**(13.3)**	**8**	**(17.4)**	**20**	**(11.8)**
Constipation	1	(8.3)	1	(10.0)	1	(7.1)	8	(18.6)	9	(20.0)	10	(21.7)	30	(17.6)
Grade 3 and above	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	1	(2.2)	1	(0.6)
Diarrhea	2	(16.7)	5	(50.0)	3	(21.4)	11	(25.6)	20	(44.4)	31	(67.4)	72	(42.4)
Grade 3 and above	0	(0.0)	2	(20.0)	0	(0.0)	1	(2.3)	1	(2.2)	7	(15.2)	11	(6.5)
Nausea	8	(66.7)	9	(90.0)	6	(42.9)	21	(48.8)	40	(88.9)	31	(67.4)	115	(67.6)
Grade 3 and above	0	(0.0)	1	(10.0)	0	(0.0)	0	(0.0)	1	(2.2)	2	(4.3)	4	(2.4)
Stomatitis	2	(16.7)	0	(0.0)	0	(0.0)	5	(11.6)	4	(8.9)	8	(17.4)	19	(11.2)
Grade 3 and above	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Vomiting	3	(25.0)	6	(60.0)	2	(14.3)	7	(16.3)	23	(51.1)	20	(43.5)	61	(35.9)
Grade 3 and above	0	(0.0)	1	(10.0)	0	(0.0)	0	(0.0)	2	(4.4)	2	(4.3)	5	(2.9)
General disorders and administration site conditions	**12**	**(100)**	**9**	**(90.0)**	**9**	**(64.3)**	**33**	**(76.7)**	**35**	**(77.8)**	**30**	**(65.2)**	**128**	**(75.3)**
Grade 3 and above	**1**	**(8.3)**	**1**	**(10.0)**	**0**	**(0.0)**	**2**	**(4.7)**	**3**	**(6.7)**	**5**	**(10.9)**	**12**	**(7.1)**
Fatigue	9	(75.0)	8	(80.0)	6	(42.9)	24	(55.8)	27	(60.0)	27	(58.7)	101	(59.4)
Pyrexia	5	(41.7)	2	(20.0)	1	(7.1)	14	(32.6)	4	(8.9)	4	(8.7)	30	(17.6)
Grade 3 and above	1	(8.3)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	1	(0.6)
Investigations	**6**	**(50.0)**	**4**	**(40.0)**	**3**	**(21.4)**	**23**	**(53.5)**	**21**	**(46.7)**	**9**	**(19.6)**	**66**	**(38.8)**
Grade 3 and above	**1**	**(8.3)**	**2**	**(20.0)**	**0**	**(0.0)**	**9**	**(20.9)**	**8**	**(17.8)**	**2**	**(4.3)**	**22**	**(12.9)**
Hemoglobin	4	(33.3)	2	(20.0)	2	(14.3)	4	(9.3)	5	(11.1)	2	(4.3)	19	(11.2)

decreased													
Grade 3 and above	0	(0.0)	1	(10.0)	0	(0.0)	0	(0.0)	1	(2.2)	1	(2.2)	3	(1.8)
Neutrophil count decreased	0	(0.0)	3	(30.0)	0	(0.0)	4	(9.3)	8	(17.8)	2	(4.3)	17	(10.0)
Grade 3 and above	0	(0.0)	1	(10.0)	0	(0.0)	0	(0.0)	5	(11.1)	0	(0.0)	6	(3.5)
Metabolism and nutrition disorders	**5**	**(41.7)**	**3**	**(30.0)**	**4**	**(28.6)**	**12**	**(27.9)**	**20**	**(44.4)**	**11**	**(23.9)**	**55**	**(32.4)**
Grade 3 and above	**0**	**(0.0)**	**1**	**(10.0)**	**1**	**(7.1)**	**0**	**(0.0)**	**1**	**(2.2)**	**3**	**(6.5)**	**6**	**(3.5)**
Decreased appetite	4	(33.3)	2	(20.0)	2	(14.3)	10	(23.3)	11	(24.4)	3	(6.5)	32	(18.8)
Grade 3 and above	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	1	(2.2)	1	(0.6)
Musculoskeletal and connective tissue disorders	**2**	**(16.7)**	**2**	**(20.0)**	**1**	**(7.1)**	**9**	**(20.9)**	**3**	**(6.7)**	**4**	**(8.7)**	**21**	**(12.4)**
Grade 3 and above	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**
Nervous system disorders	**2**	**(16.7)**	**2**	**(20.0)**	**1**	**(7.1)**	**15**	**(34.9)**	**13**	**(28.9)**	**18**	**(39.1)**	**51**	**(30.0)**
Grade 3 and above	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**1**	**(2.2)**	**1**	**(2.2)**	**2**	**(1.2)**
Neuropathy peripheral	1	(8.3)	1	(10.0)	0	(0.0)	4	(9.3)	4	(8.9)	10	(21.7)	20	(11.8)
Grade 3 and above	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Respiratory, thoracic mediastinal	**1**	**(8.3)**	**2**	**(20.0)**	**0**	**(0.0)**	**10**	**(23.3)**	**4**	**(8.9)**	**13**	**(28.3)**	**30**	**(17.6)**

disorders													
Grade 3 and above	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**1**	**(2.3)**	**0**	**(0.0)**	**0**	**(0.0)**	**1**	**(0.6)**
Skin and subcutaneous tissue disorders	**6**	**(50.0)**	**1**	**(10.0)**	**1**	**(7.1)**	**17**	**(39.5)**	**5**	**(11.1)**	**11**	**(23.9)**	**41**	**(24.1)**
Grade 3 and above	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**0**	**(0.0)**	**1**	**(2.2)**	**1**	**(0.6)**
Rash	3	(25.0)	0	(0.0)	1	(7.1)	11	(25.6)	1	(2.2)	3	(6.5)	19	(11.2)
Grade 3 and above	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	1	(2.2)	1	(0.6)

Every subject is counted a single time for each applicable specific adverse event. A subject with multiple adverse events within a system organ class is counted a single time for that system organ class.

(**Table 1**).The most common tumor types were melanoma (n=40, 22.8%), ovarian (n=23, 12.9%), breast (n=15, 8.4%), and colorectal cancer (CRC) (n=14, 7.9%). Patients received a median of 2 treatments cycles (range 1-16).

Safety and tolerability

One hundred and seventy three patients (97.2%) experienced at least one treatment-related adverse event (AE). The most common treatment related AEs were hematological toxicities (thrombocytopenia n=59 [34.7%], neutropenia n=38 [22.4%], and anemia n=38 [22.4%]), gastrointestinal disorders (nausea n=115 [67.6%], vomiting n=61 [35.9%], and diarrhea n=72 [42.4%]), and fatigue n=101 (59.4) (**Table 2**). MTDs were obtained in all treatment arms of single and multiple dosing combination therapy with MK-1775 (gemcitabine still ongoing). MK-1775 monotherapy did not meet DLT criteria in the highest dose level (DL) of 1300 mg.

Anti-tumor activity

Four (2.2%) of 178 evaluable patients demonstrated partial remission (PR) as best overall response, eighty-four (47.2%) achieved stable disease (SD) lasting ≥ 6 weeks (**Table 3**).

Pharmacokinetics

Plasma concentrations of MK-1775 increased moderately following oral administration (**Table 4**). For the majority of patients C_{max} was reached ~3 hrs after dosing (range 3.0-6.0 hrs for monotherapy and 1.0-8.0 hrs combination therapy). The mean terminal half-life was ~9 hrs (mean range 8.7-11.6 hrs for monotherapy and 7.9-12.2 hrs for combination therapy). Plasma exposure increased approximately dose-proportionally in monotherapy and combination therapy. In the MK-1775 multiple dose regimen steady state was achieved approximately within 2 days. Accumulation ratios (geometric mean ratio = Day3/Day1) for the area under the plasma-concentration time curve from time 0 to 8 hrs post-dose (AUC_{0-8hr}), C_{max}, and plasma drug concentration observed at 8 hrs post-dose (C_{8hr}) averaged 1.01-3.23, 0.85-2.83 and 1.04-2.96 respectively, across tested MK-1775 doses in the combination with chemotherapy.

Table 3. Number of patients, dose levels, maximal tolerable doses and antitumor activity in each treatment arm.

Total number of patients included	N (178)	DLT	Dose levels (mg) tested	MTD defined	Target engagement	PR (%)	SD (%)	SAE ≥Gr 3 (%)
MONOTHERAPY:	9	No	325 – 650 – 1300	All DLs tolerable	8/9 patient continued in Single Dosing Part			
SINGLE DOSING:	**Day 1 Chemo, Day 2 MK-1775 (1x)**							
- Gemcitabine	14	3	100 – 200	200 mg	Y	0 (0.0)	9 (64.3)	7 (50.0)
- Cisplatin	13	2	100 – 200	200 mg	Y	1 (7.7)	8 (61.5)	4 (30.8)
- Carboplatin	17	2	100 – 200 – 325	325 mg	Y	0 (0.0)	5 (29.4)	2 (11.8)
MULTIPLE DOSING:	**Day 1 Chemo + MK-1775 BID for 2.5 Days**							
- Gemcitabine	43	8	25 – 50 – 50/25 †100 – 125 – 150 - 200	50/25 150 mg*	N N	0 (0.0)	18 (41.9)	25 (58.1)
- Cisplatin	45	7	50 – 100 – 125 – 150 – 200 - 250	200 mg	Y	3 (6.7)	18 (40.0)	20 (44.4)
- Carboplatin	46	13	75 – 150 – 225 - 325	225 mg	Y	0 (0.0)	26 (56.5)	31 (67.4)

† = Day 1 Chemo + MK-1775 QD for 2 Days * = Day 1 Chemo + MK-1775 BID, Day 2, 3 MK-1775 3 doses given BID 25 mg

Table 4A. Preliminary Pharmacokinetic Parameters: following administration of single oral doses of MK-1775 as monotherapy in Part 1 or in combination with either gem = gemcitabine, cis = cisplatin or carbo = carboplatin in part 2A.

Therapy	Dose (mg)	N	C_{max} (µM)		T_{max} (hr)			AUC_{0-8} (µM.hr)		AUC_{0-8} (µM.hr)		AUC_{0-Inf} (µM.hr)		$T_{1/2}$ (hr)		C_{8h} (µM)	
			mean	CV%	median	Min	Max	mean	CV%	mean	CV%	mean	CV%	Mean[a]	SD[b]	mean	CV%
MK 1775	325	3	0.81	44	4	3	6	4.53	44	6.55	40	12.12	29	8.89	1.20	0.59	45
	650	3	2.34	13	3	3	3	13.07	9	17.02	8	29.07	13	8.68	2.17	1.13	7
	1300	3	3.72	28	3	3	6	20.56	35	28.39	37	61.82	57	11.59	4.04	2.19	36
MK-1775 + Cis	100	3	0.21	57	3	3	4	1.14	58	1.51	58	2.61	44	10.92	3.77	0.11	58
	200	9	0.47	36	4	1.5	8	2.53	36	3.60	39	7.85[e]	69	10.17[e]	1.90	0.31	54
MK-1775 + Gem	100	6	0.31	62	2.25	1	6	1.46	49	1.89	44	2.82[d]	35	10.04[c]	0.81	0.13	36
	200	7	0.48	50	3	1.5	6	2.36	52	3.16	51	7.05[e]	33	9.56[e]	2.01	0.24	58
MK-1775 + Carbo	100	3	0.32	47	1.5	1	6	1.43	46	1.89	39	2.76[c]	33	10.10[c]	1.95	0.12	0.6
	200	4	0.46	23	3	3	4	2.41	15	3.26	16	5.87	21	12.16	3.60	0.26	20
	325	8	0.91	45	3	1	8	4.67	50	6.12	47	10.70[f]	35	8.23[f]	1.77	0.43	45

a- Harmonic mean; b-Pseudo standard deviation; c-n=2; d-n=3; e-n=5; f-n=7

Table 4B. Preliminary pharmacokinetic parameters of cisplatin: following administration of multiple oral doses of MK-1775 (bidaily for 2.5 days) in combination with cisplatin in part 2B.

Treatment (Dose)	Day	N	C_{max} (µM)		T_{max} (hr)			AUC_{0-8} (µM.hr)		$T_{1/2}$[c] (hr)		C_{8h} (µM)		C_{trough} (µM)	
			mean	CV%	median	min	max	mean	CV%	mean[a]	SD[b]	mean	CV%	mean	CV%
(50 mg)	1	4	0.16	23	2	1	4	0.74	24	-	-	0.07	36	-	-
	3	4	0.23	29	3	1	4	1.36	43	11.85[d]	1.21	0.11	86	0.09	46
	GMR	-	**1.45**	-	-	-	-	**1.77**	-	-	-	**1.34**	-	-	-
(100 mg)	1	7	0.23	44	4	2	6	1.22	44	-	-	0.14	53	-	-
	3	7	0.56	25	2	2	6	3.66	29	11.78[e]	-	0.39	38	0.29	46
	GMR	-	**2.64**	-	-	-	-	**3.21**	-	-	-	**2.96**	-	-	-
(125 mg)	1	6	0.60	56	3	1	8	3.26	54	-	-	0.37	64	-	-
	3	5	1.35	19	2	2	6	8.16	18	7.91[f]	0.04	0.75	31	0.53	47
	GMR	-	**2.83**	-	-	-	-	**3.08**	-	-	-	**2.38**	-	-	-
(150 mg)	1	8	0.75	42	2	1	4	3.62	38	-	-	0.34	41	-	-
	3	7	1.39	41	2	1	6	8.72	41	11.26[g]	3.25	0.92	48	0.72	62
	GMR	-	**1.80**	-	-	-	-	**2.31**	-	-	-	**2.58**	-	-	-
(200 mg)	1	9	0.76	30	2	1	6	3.84	30	-	-	0.41	37	-	-
	3	8	1.55	41	3	2	8	9.87	34	10.43[f]	0.61	1.21	48	0.69	54
	GMR	-	**2.02**	-	-	-	-	**2.58**	-	-	-	**2.78**	-	-	-
(250 mg)	1	3	1.05	49	4	2	6	6.06	55	-	-	0.72	39	-	-
	3	3	2.52	22	4	4	4	17.60	24	NA	NA	2.01	29	1.63	25
	GMR	-	**2.58**	-	-	-	-	**3.23**	-	-	-	**2.86**	-	-	-

a- Harmonic mean; b-Pseudo standard deviationexit, c-Half lives were determined from the last day of PK observations; d-n=4; e-n=1; f-n=2; g-n=3; NA – Not available due to lack of data in the terminal phase; GMR-Geometric mean ratio

Table 4C. Preliminary pharmacokinetic parameters of carboplatin: following administration of multiple oral doses of MK-1775 (bidaily for 2.5 days) in combination with carboplatin in part 2B

Treatment (Dose)	Day	N	C_{max} (µM)		T_{max} (hr)			AUC_{0-8} (µM.hr)		(hr)		C_{8h} (µM)		C_{trough} (µM)	
			mean	CV%	median	min	max	mean	CV%	mean[a]	SD[b]	mean	CV%	mean	CV%
(75 mg)	1	4	0.22	41	1	1	2	1.10	35	-	-	0.08	39	-	-
	3	4	0.30	66	3	2	8	1.75	80	11.55[e]	5.37	0.18	55	0.11	79
	GMR	-	**1.20**	-	-	-	-	**1.21**	-	-	-	**2.12**	-	-	-
(150 mg)	1	4	0.35	82	3	2	4	1.71	72	-	-	0.13	57	-	-
	3	4	0.65	57	2	1	4	3.61	60	10.04[f]	-	0.30	55	0.21	58
	GMR	-	**2.16**	-	-	-	-	**2.31**	-	-	-	**2.26**	-	-	-
(225 mg)	1	17	0.66	52	4	2	4	3.56	54	-	-	0.39	51	-	-
	3	15	1.41	32	4	1	6	9.06	31	10.87[g]	3.36	0.98	31	0.73	31
	GMR	-	**2.43**	-	-	-	-	**2.94**	-	- $T_{1/2}$[c]	-	**2.81**	-	-	-
(325 mg)	1	12	1.38	41	4	2	6	7.22	43	-	-	0.77	43	-	-
	3	11	2.63	36	4	1	6	17.09	38	11.02[i]	0.94	1.96	33	1.40	43
	GMR	-	**1.93**	-	-	-	-	**2.41**	-	-	-	**2.62**	-	-	-

a- Harmonic Mean; b-Pseudo Standard Deviationexit; c-Half lives were determined from the last day of PK observations; ; e-n=2; f-n=1; g-n=5; h-n=6;

i-n=3; NA – Not available due to lack of data in the terminal phase; GMR-Geometric mean ratio.

Table 4D. Preliminary pharmacokinetic parameters of gemcitabine; following administration of multiple oral doses of MK-1775 (bidaiy for 2.5 days or once a day for 2 Days) in combination with gemcitabine in part 2B.

Treatment (Dose)	Day	N	C_{max} (µM)		T_{max} (hr)			AUC_{0-8} (µM.hr)		$T_{1/2}$[c] (hr)		C_{8h} (µM)		C_{trough} (µM)	
			mean	CV%	median	min	max	Mean	CV%	mean[a]	SD[b]	mean	CV%	mean	CV%
(25 mg)	1	6	0.04	56	3	2	6	0.21	49	-	-	0.02	54	-	-
	3	6	0.09	40	2	1	4	0.50	41	NA	-	0.04	39	0.03	65
	GMR		**2.29**	-	-	-	-	**2.52**	-	-	-	**2.25**	-	-	-
(50 mg)	1	6	0.13	13	3	1	6	0.66	15	-	-	0.08	30	-	-
	3	6	0.25	24	2	1	6	1.49	25	NA	-	0.14	28	0.11	21
	GMR		**1.80**	-	-	-	-	**2.22**	-	-	-	**1.76**	-	-	-
(50/25mg)[d]	1	13	0.16	56	2	1	4	0.79	50	-	-	0.06	37	-	-
	3	13	0.14	37	2	1	4	0.75	38	10.12[f]	0.80	0.07	40	0.05	50
	GMR		--	-	-	-	-	-	-	-	-	-	-	-	-
F1[e] (100 mg)	1	4	0.39	41	2	2	4	1.81	37	-	-	0.17	26	-	-
	2	4	0.32	17	3	2	4	1.79	15	11.21[g]	-	0.19	25	0.04	30
	GMR		**0.85**	-	-	-	-	**1.03**	-	-	-	**1.11**	-	-	-
F2[e] (125 mg)	1	2	0.59	15	2	2	2	3.17	13	-	-	0.27	14	-	-
	2	2	0.72	5	1.5	1	2	3.19	12	NA	-	0.28	30	0.06	19
	GMR		**1.22**	-	-	-	-	**1.01**	-	-	-	**1.04**	-	-	-

a- Harmonic mean; b-standard deviation; c-Half lives were determined from the last day of PK observations for observations; d-50 mg on Day1 and 25 mg on day 2 and 3; e-Treatment groups F1, F2 had QD dosing. All other groups were BID dosing regimen; f- n=3, g- n=2; NA – Not available due to lack of data in the terminal phase, GMR-Geometric mean ratio

The pharmacokinetic target of C_{8hr} = 0.240 µM was achieved at 100 mg MK-1775 in combination with cisplatin and 150 mg MK-1775 in combination with carboplatin on day 3 in the MK-1775 multiple dosing regimen, but not at the MTD of MK-1775 in the multiple dose regimen (BID dosing for 2.5 days) in combination with gemcitabine. An alternate dosing regimen with QD dosing for 2 days at 125 mg achieved the pharmacokinetic target on Day 2 (150 and 200 mg QD of MK-1775 currently being evaluated).

The anti-emetic aprepitant is a substrate of CYP3A4. Although the use of CYP3A4 inhibitors was prohibited, administration of aprepitant was permitted as supportive care according to institutional guidelines. Based on preliminary and exploratory analysis, comparing the pharmacokinetics of MK-1775 in patients who did and did not receive concomitant administration of aprepitant showed ~60% increase in exposure in patients receiving aprepitant, which is statistically significant ($p < 0.0001$ for $AUC_{0\text{-}12hr}$ on Day 1 and Day 3). At the selected MTD, this increase is clinically significant.

Pharmacodynamic analyses

Archival or fresh tumor samples were collected from 105 patients, of which 76 were evaluable. The tumor samples of 21 patients showed p53 mutation by sequencing. One of (1.3%) of these patients showed PR as a best overall response.

MK-1775, by inhibition of Wee1, reduces pCDC2 levels relative to CDC2. Phosphorylation of CDC2 is induced by chemotherapy, especially gemcitabine. Correction for the chemotherapy effect was therefore applied in the analysis of the post dose skin biopsy samples. Nevertheless, significant decreases in pCDC2 were observed across MK-1775 dose levels in monotherapy and chemotherapy combinations; dose-response relationship between MK-1775 dose and pCDC2 was observed, and 125 mg of MK-1775 BID was the threshold dose of MK-1775 for target engagement. In the gemcitabine arm multiple dose regimen however, 50 mg of MK-1775 (BID for 2.5 days) proved to be too toxic and thus the MTD dose was one DL below the 50 mg DL: 25 mg of MK-1775 (BID for 2.5 days) and therefore target engagement was not achieved. An alternate regimen of 50 mg MK-

1775 BID on Day 1, 25 mg BID on Day 2 and 25 mg QD on day 3 was explored and well tolerated. Since the lack of target engagement also at this DL QD multiple day schedules were finally investigated: 100 mg, 125 mg and later 150 mg and 200 mg of MK-1775 QD for 2 days. These regimens were well tolerated. At these DLs target engagement was nearly achieved.

Preclinical data indicated that the hair bulb is the preferable tissue for pCDC2 analysis, this clinical study demonstrated that bulbs are only present in the minority of patient specimens. Therefore (hairy) epidermis was used for pCDC2 analysis, since it is also an actively proliferating tissue and is present in all punch biopsies.

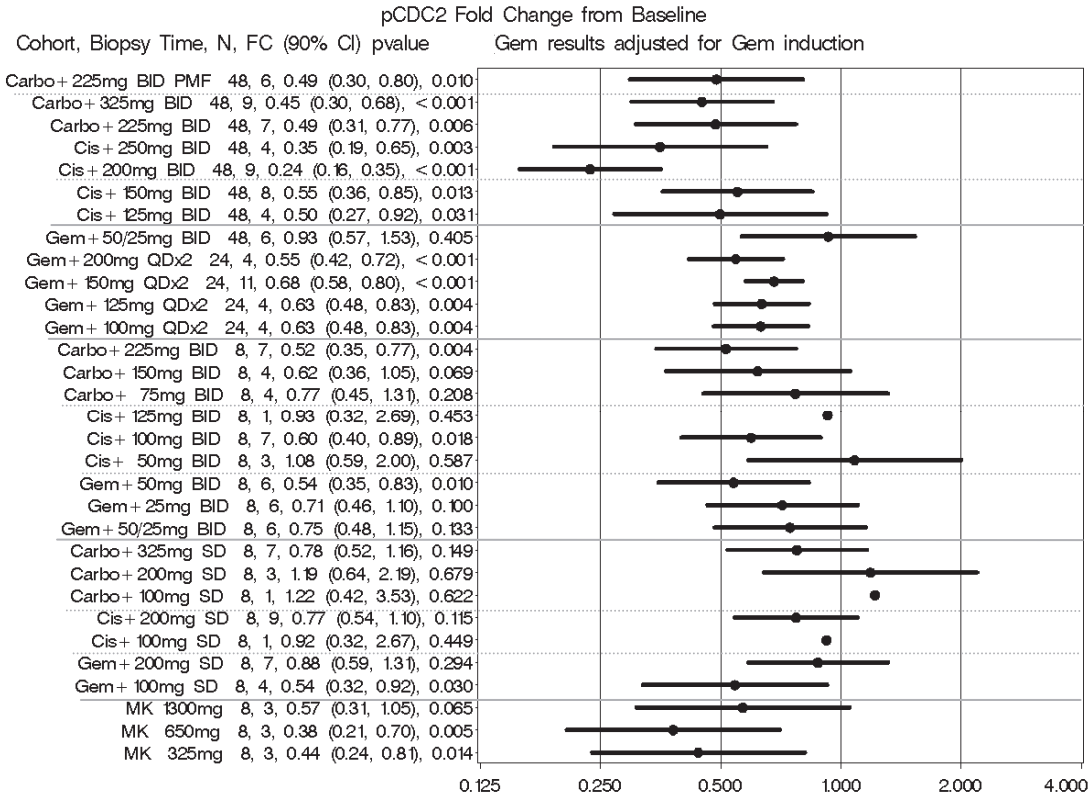

Figure 2. Measurement of pCDC2 (direct substrate of Wee1) in epidermis tissue with hair follicles. pCDC2 levels relative to CDC2 were assessed by IHC in pre- and post-dose skin biopsies (in the hairy part behind the ear). The geometric mean fold

change (FC) was plotted against the MK-1775/chemotherapy combination and the post-dose biopsy time. The number of patients, and 90% confidence interval and p-value were included. PMF= pre-market formulation, QD = once –daily (multiple dosing), BID = bidaily (multiple dosing), SD = single dose.

Gene expression measurements ('Wee1 signature' [27]) demonstrated that 4 (CCNE2, EGR1, CLSPN and HIST12BD) of the 8 selected genes showed significant changes in monotherapy consistent with preclinical expectations ($p<0.05$, unadjusted for multiplicity). Most notable were the effects from EGR1 and CCNE2 ($p < 0.003$ and $p = 0.005$, respectively, after adjustment for multiplicity) in the highest DLs, suggesting a dose-response trend. A composite score derived from the 4 genes that showed direction of effect consistent with that expected based on pre-clinical data (specifically, CCNE2, CLSPN, and MCM10 as down-regulated set and HIST1H2BD as up-regulated) showed a consistent trend indicating target engagement at all monotherapy doses, although a strong dose-response trend is not evident in the limited data available (**Supplementary Table 1.A and 1.B, Supplementary Figure 1, 2**).

Discussion

In this study the tolerability, safety and biological activity of Wee1 inhibitor MK-1775 were explored.
This three chemotherapy arms study investigated MK-1775 administered in different regimens in each chemotherapy, i.e. cisplatin, carboplatin and gemcitabine because preclinically proof of principle with these agents had been reached. Since early *in vitro* experiments examining the sequence of gemcitabine and MK-1775 administration initially demonstrated greatest anti-tumor activity when MK-1775 was given approximately 24 hrs following exposure to DNA damaging agents, [40] patients in part 2A received chemotherapy infusion on Day 1 and one dose of MK-1775 24 hrs (±2) after chemotherapy on Day 2. The relatively short half-life of MK-1775 combined with *in vivo* data obtained while the clinical study was ongoing chemotherapy would increase the efficacy of MK-1775 without affecting

tolerability.[41] In order to maximize checkpoint escape in cancer cells that transition through S-phase during the time of treatment with chemotherapy, the protocol was amended and MK-1775 was given in an additional part of the study (part 2B) in 5 BID doses in all three treatment arms.

In general MK-1775 was well tolerated. In the MK-1775 single dose regimen the observed toxicity was consistent to well known toxicity observed with each chemotherapy alone and there was practically no additional toxicity that could be contributed to MK-1775. However, in the MK-1775 multiple dose regimen toxicity related to MK-1775 was observed, especially bone marrow toxicity, nausea, vomiting, diarrhea, fatigue, and occasionally hiccups and reflux esophagitis. Episodes of vomiting and/or diarrhea mostly occurred at day 2-3, a time point when steady state of MK-1775 in plasma was achieved, suggesting a relationship with dose.

Nausea and vomiting are side effects of MK-1775 and were mainly observed in the multiple dose regimens, of all three chemotherapy arms. During the study a significant difference in exposure between patients treated with and without the anti-emetic aprepitant was found. The observed increase in MK-1775 exposure is likely the result of $CYP3A_4$ inhibition by aprepitant. *In vitro* data suggested that the major pathway of metabolism in humans involve $CYP3A_4$, although FMO_3 and FMO_5 may be involved as well.

Based on safety and target engagement data of this study, the multiple dosing regimens for carboplatin (MK-1775 225 mg BID 2.5 days) and cisplatin (MK-1775 200 mg BID for 2.5 days) and may be the MK-1775 150 mg QD for 2 days adjusted multiple dose regimen for gemcitabine (dose escalation is still ongoing) seem to be the most optimal schedules.

MK-1775 displayed significant anti-tumor activity. Patients included in this study were patients with advanced cancers and treated with up to 4 cytotoxic and targeted treatments (9.0% received 4 or more different prior cytotoxic treatments).

The pharmacokinetics of MK-1775 were linear and increased proportional with increase of dose. Intra-patient variation was moderate, and combination of MK-1775 with chemotherapy did not significantly change the pharmacokinetic profile.

Although p53 mutation status was determined in a large group of patients, p53 pathway mutations were not determined, but are, based on the mechanism of action of MK-1775, equally important. Up to date, no good assay for analysis of p53 pathway mutation was available. However in future studies p53 analysis will be performed in all patients.

The complexity of genes involved in DNA repair and affected by G2 cell cycle checkpoint abrogation is reflected by the results of the Wee1 signature. Insight in the involved pathways will provide better understanding of the observed results.

Preclinical proof of the principal (POP) that MK-1775 is a specific Wee1 inhibitor that inhibits the phosphorylation of CDC2 and results in abrogation of the G2 checkpoint was a strong reason for initiating this first in human clinical trial.

This study also provided pharmacological POP; at the MTD doses target engagement measured as a significant change of pCDC2 relative to CDC2 in pre- and post dose skin biopsies, was observed. Although the observed anti-tumor activity was promising, clinical activity will be further assessed in ongoing Phase II studies: 1) a study with MK-1775 and carboplatin in patients with p53 mutated platinum refractory (relapse > 3 months) or resistant ovarian cancer (NCT01164995), 2) a study with carboplatin and paclitaxel with or without MK-1775 in patients with p53 mutated platinum sensitive ovarian cancer (NCT01357161).

Another focus of interest is glioblastoma (GBM), because of observed overexpression of Wee1 protein in these tumors and promising preclinical results with MK-1775 as radiosensitizer in GBM.[42-46]

Further development with MK-1775 may involve combination with kinase inhibitors like Chk1 inhibitors, together with radiotherapy or cytotoxic agents may result in prolonged anti-tumor activity in a larger population. Preclinically synergy has been observed with Chk1 inhibitors AR458323 and PF-00477736.[47-49] For different tumor types, different regimens with MK-1775 might proof to be most optimal and further investigation of MK-1775 is warranted.

In summary, oral MK-1775 was well tolerated in patients with advanced solid tumors, with hematologic and gastrointestinal complaints being the most common adverse events, especially observed in the multiple dose

regimens. A significant number of patients showed SD (47.2% of patients) or PR (2.2% of patients) as best response. Compared to the single dose regimens, the multiple dose regimens displayed toxicity attributable to MK-1775. MK-1775 revealed a developable pharmacokinetic and pharmacodynamic profile. Target engagement was achieved at the MTDs (MK-1775 single dose regimens: for all 3 arms, MK-1775 multiple dose regimens: BID for 2.5 days for carboplatin and cisplatin).

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Supplementary information

Methods

1. Patient inclusion/exclusion criteria

Eligible patients had adequate bone marrow (absolute neutrophil count ≥ 1,500/mm^3; platelet count 100,000/ mm^3; hemoglobin ≥ 9 g/dL), liver function (serum total bilirubin ≤ 1.5x upper limit of normal [ULN] or direct bilirubin ≤ ULN for patients with serum total bilirubin > 1.5 ULN; ALT and AST ≤ 2.5x ULN or ≤ 5 x ULN for patients with liver metastases, alkaline phosphatase if ≥ 2.5x ULN, the liver fraction had to be ≤ 2.5x ULN;), renal function (serum creatinine ≤ 1.5x ULN or ≥ 60 mL/min for patients with creatinine levels > 1.5x ULN), and adequate coagulation status (International Normalized Ratio [INR] or Prothrombin Time [PT] ≤ 1.5x ULN; Activated Partial Thromboplastin Time [aPTT] ≤ 1.5x ULN). Previous anti-cancer treatment had to be completed at least 4 weeks prior to study entry. Drugs or other products known to be metabolized by CYP3A4, or to inhibit or induce CYP3A4 were not allowed. Patients with Central Nervous System (CNS) metastases were also excluded unless they were clinically stable for 1 month prior to study entry (i.e. no evidence of new enlarging CNS metastasis and off steroids or on a stable dose of steroids for ≥ 2 weeks. Other exclusion criteria included ongoing systemic infections, symptomatic ascites or pleural effusion, pregnancy, and hypersensitivity to the chemotherapy.

2. Study design and treatment

Part 1 of the study (one single dose of MK-1775) used a dose escalation scheme with 100% dose increments and dose level 1 of 325 mg MK-1775. DL1 was calculated based on a dose of 180 mg/m^2 (average Body Surface Area [BSA] of 1.8 m^2) and rounded to the closest multiple of 25. The dose of 180 mg/m^2 MK-1775 was established as the maximum no-effect level in a single dose oral toxicity study in dogs.

Combination therapy with MK-1775 and chemotherapy (Parts 2A and 2B) used a modified Fibonacci scheme. The modified Fibonacci scheme used 50%, 40% and 30% dose increments in subsequent dose levels. The TPI

targets a DLT rate of 30% and allows escalation or de-escalation based on the number of DLT's observed at a given dose level. Upon definition of a preliminary MTD in 6 patients, a cohort expansion for a total of 13 evaluable patients is triggered. During cohort expansion, dose assignment actions continue based on continuous assessment of tolerability information. In case of DLT or toxicity after cycle 1 dose modification to a lower dose level was permitted in individual patients.

3. Pharmacokinetic assessments

Whole blood samples of 4 mL each, for determination of MK-1775 plasma concentrations, were collected at the following time points: Part 1 (monotherapy): pre-dose (0), and then 0.5, 1, 1.5, 3, 4, 6, 8, 24, and 48 hrs after the administration of MK-1775; Part 2A (MK-1775 single dose combination therapy): cycle 1 day 1: pre-dose (0), and then 0.5, 1, 1.5, 3, 4, 6, 8, 24, and 48 hrs after the administration of MK-1775; Parts 2B and 3 (MK-1775 multiple dose combination therapy) cycle 1 day 1, and day 3: pre-dose, 1, 2, 4, 6, and 8 hrs after the first administration of MK-1775 (+ chemotherapy on day 1), cycle 1 day 2: pre-dose (prior to the third administration of MK-1775). Twenty four and 48 hrs after the fifth administration of MK-1775 were optional time points for blood sample collection. In the gemcitabine + QDx2 MK-1775 dosing regimen the time points for plasma collection of day 1 and 2 were similar to day 1 and 3 of the MK multiple dose schedule (part 2B).

4. Pharmacodynamic assessments – Wee1 signature

Plucked hair follicles were obtained pre-dose and post-dose (8 hrs [±2 hrs] after [last] oral administration of MK-1775 in cycle 1). Skin biopsies were obtained pre-dose and post-dose (Part 1 and 2A: 8 hrs [±2 hrs] and part 2B within 2 hrs after last oral administration of MK-1775 in cycle 1).

qPCR assays were performed for all clinical hair follicle samples from the single-dose regimen to analyze gene expression of a selected group of genes, also referred to as the 'Wee1 signature'. A signature responsive to MK-1775 was derived from preclinical experiments and assessed in hair follicles collected at baseline and 8 hrs post dose from patients participating in the monotherapy part of this study. Briefly, a composite

score was calculated as the average fold change of genes down-regulated relative to pre-dose levels subtracted from the average fold change of genes up-regulated relative to pre-dose levels. The initial 8-gene signature (HIST1H2BD, EGR1, CCNE1/2, CLSPN, MCM10, FBOX5, and MYB) was refined based on at an interim analysis that pooled all the treatment groups (not just monotherapy) and determined which genes showed significant effects in a direction consistent with pre-clinical experiments. This lead to a reduced 4-gene signature (up-regulated: HISTH1HSBD; and CCNE2, CLSPN, MCM10 down-regulated).

Individual measurements that fell below the limit of quantification established via an assay validation process (Ct > 34.06) were not used in the analysis. Fold-change (post-dose versus pre-dose) values were calculated using the Comparative Ct Method (ΔΔCt). Statistical tests leading to p-values were conducted using the log (base 2) of fold change. As a QC check, trends in fold-change versus RNA yield were checked and in general there did not appear to be any strong trends for the genes examined. An analysis of the pre/post changes in the house keeping genes was conducted to confirm that significant results were not being driven by effects on those genes.

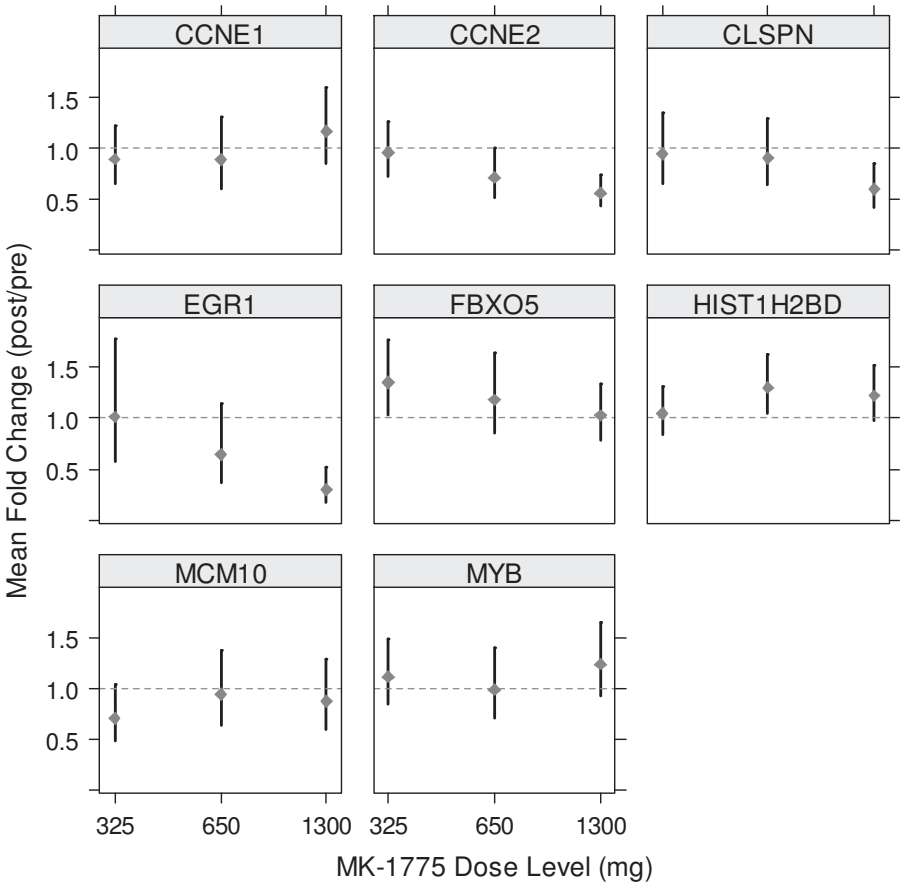
An ANOVA was conducted for each gene to estimate the mean fold-change at each of the combinations of treatment and dose level. All treatments and dose levels were included in a single ANOVA model for each gene as distinct categorical factors so that all observations were used to estimate a common residual variance. However, findings conducted in a separate study of standard of care (SOC) therapies suggested that the natural course of gene expression changes over the time period of interest (24 to 32 hrs) after receipt of SOC is a confounding factor in interpreting the effects of MK-1775 in the combination setting. Hence, statistical inference was restricted to just the monotherapy results. The Hochberg step-up procedure was used to report p-values adjusted for the multiple tests (for different monotherapy dose combinations) within each gene.

Supplementary table 1.A. Gene expression measurements ('Wee1 signature'): unadjusted p-values testing for a non-zero mean log fold-change based on ANOVA.

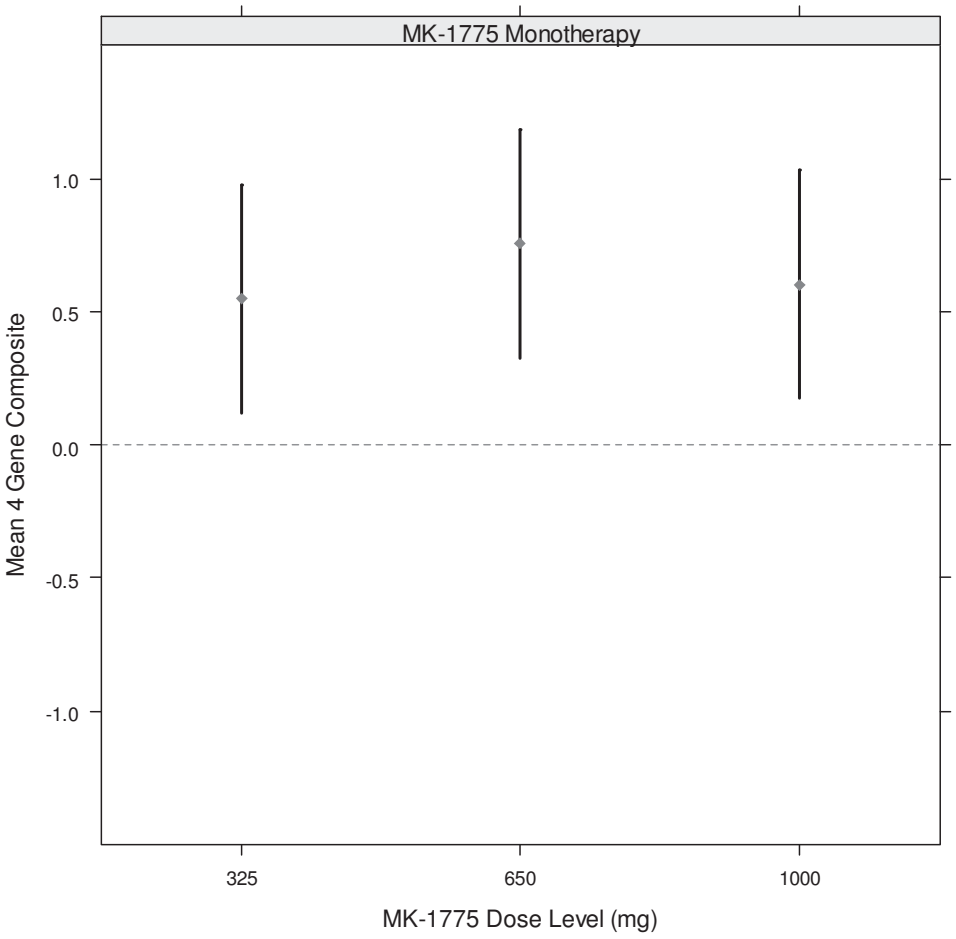
Treatment Group	CCNE1	CCNE2	CLSPN	EGR1	FBXO5	HIST1	MCM10	MYB
Mono dose level 1 (325 mg)	0.576	0.816	0.793	0.963	0.067	0.719	0.127	0.530
Mono dose level 2 (650 mg)	0.628	0.106	0.657	0.205	0.398	0.050	0.767	0.951
Mono dose level 3 (1300 mg)	0.410	0.002	0.020	<0.001	0.865	0.136	0.550	0.212

Supplementary Table 1.B. Adjusted p-values testing for a non-zero mean log fold-change based on ANOVA (Hochberg adjustment applied to all tests within a given gene).

Treatment Group	CCNE1	CCNE2	CLSPN	EGR1	FBXO5	HIST1	MCM10	MYB
Mono dose level 1 (325 mg)	0.628	0.816	0.793	0.963	0.201	0.719	0.381	0.951
Mono dose level 2 (650 mg)	0.628	0.212	0.793	0.410	0.796	0.150	0.767	0.951
Mono dose level 3 (1300 mg)	0.628	0.006	0.060	0.003	0.865	0.272	0.767	0.636



Supplementary Figure 1. Fold Change Means and 90% Confidence intervals (CIs) for MK-1775 monotherapy doses (back-transformed from statistics on Log2 scale).



Supplementary Figure 2. qPCR 4-gene signature score means and 90% confidence intervals (CIs) for monotherapy doses.

3.3

Phase II study with Wee1 inhibitor MK-1775 plus carboplatin in patients with p53 mutated ovarian cancer refractory or resistant (< 3 months) to standard first line therapy

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Preliminary analysis

Abstract

Purpose

This phase II study was designed to test in the clinic the concept that the Wee1 inhibitor MK-1775 in combination with carboplatin is active in patients with p53 mutated platinum refractory or resistant ovarian cancer. MK-1775 abrogates the G2 checkpoint, while p53 deficient tumors are considered to be more dependent on the G2-chekpoint for DNA repair induced by chemotherapeutic agents.

Patients and methods

Twenty-one evaluable patients with epithelial ovarian cancer and early relapse after (< 3 months) or progression during carboplatin containing standard first line treatment and with molecularly proven p53 mutation are to be included and re-exposed to carboplatin at a dose resulting in an area under the curve (AUC) of 5 mg/mL*min in combination with 225 mg of MK-1775 bidaily (BID) for 2.5 days in a 21 day schedule. The dose of MK-1775 was established as the maximal tolerable dose (MTD) in combination with carboplatin determined in a phase I study, and showed target engagement defined by a decrease of phosphorylated CDC2 (pCDC2), which is a direct substrate of Wee1. Applying an A'Hern's Single Stage Phase II Design, a minimum of 6 responses (RECIST 1.0 or CA-125) out of 21 patients will provide a 61% power to demonstrate an efficacy of at least 30% (α=0.05).

Results

At the time of the preliminary analysis fifteen patients were included, 14 patients started study treatment, 13 patients were evaluable for response, of whom 5 patients (38%) demonstrated CA-125 marker response, of which 4 patients (31%) showed a RECIST confirmed partial response (PR), including 3 patients (23%) with a near complete response.

Conclusion

Preliminary analysis shows encouraging activity of MK-1775 in combination with carboplatin in patients with platinum refractory or resistant ovarian cancer and p53 mutation.

Introduction

Resistance to chemotherapy is a limiting factor in the treatment of cancer, with chemotherapeutic agents that exert their function by inducing DNA damage, like platinum compounds (e.g. cisplatin, carboplatin, oxaliplatin), gemcitabine and capecitabine.[1;2] Resistance to DNA damaging agents suggests that cell survival is possible and may be related to the closely linked mechanisms of DNA damage repair and cell cycle regulation, especially at the G1-S transition and G2-M transition.[3-5]
Protein p53, encoded by the gene TP53, plays a key role in the G1 checkpoint, is a tumor suppressor gene and regulates apoptosis.[6] In case of DNA damage, p53 can activate the apoptotic pathway to prevent a defective genome to be conveyed to the next generation of cells. Mutations and deletions of the TP53 gene belong to the most common genetic alterations in human cancer and are associated with limited response to conventional therapies and poor clinical outcome.[7] Tumors harboring p53 pathway mutations are more dependent on the G2 checkpoint for DNA repair due to G1 checkpoint deficiency.[8]

To overcome p53 associated drug resistance the concept has been developed to interfere with the ability of cancer cells to repair DNA damage in the G2 phase of the cell cycle. [9-12] Abrogation of G2 cell cycle arrest can result in mitotic catastrophe when DNA damage is extensive.[13;14]

Wee1 is a key cyclin dependent kinase (CDK) that regulates cell cycle progression by governing the G2 checkpoint.[15-17]. Binding of cyclin B to CDC2 (CDK1) can trigger mitosis, while inhibition of the CDC2/cyclin B complex by inhibitory phosphorylation at tyrosine 15 (Y15) or threonine 14 (T14) of CDC2 by myelin transcription factor 1 (MYT1) or Wee1, respectively, will result in cell cycle arrest. CDC2 activating dephosphorylation of Y15 and T14 is catalyzed by the dual specificity protein CDC25.[18;19] Pharmacological inhibition of Wee1 is a strategy to abrogate G2 cell cycle arrest, and to exploit the G1 checkpoint deficiency of p53 deficient tumor cells, thereby enhancing their apoptotic response to

DNA damage.[20] Since normal cells are not p53 pathway deficient, Wee1 inhibition is considered to influence only p53 pathway deficient cells, and to spare healthy cells.[21]

MK-1775, is a potent and selective inhibitor of Wee1 (IC_{50} = 5.18 nM in kinase screens).[21] *In vitro* experiments demonstrated synergistic anti-tumor effect in combination with various DNA damaging agents, and anti-tumor activity was significantly enhanced by MK-1775 in human xenograft mouse models.[21-23] Additionally, preclinical proof of the concept was obtained for the hypothesis that MK-1775 is active in p53 deficient human xenograft models: MK-1775 potentiated the anti-tumor activity in rats bearing xenografts with the p53 deactivating TOV21G-shp53 mutation.[21]

An ongoing phase I study of MK-1775 in combination with either carboplatin, cisplatin or gemcitabine in patients with different kinds of advanced solid tumors (and with retrospective testing of p53 status in archival tumor tissue) demonstrated an acceptable toxicity profile of MK-1775 and linear pharmacokinetics.[24] Preliminary results show that target engagement, defined by reduction of pCDC2 in surrogate tissue (skin biopsies), was achieved at the applied dose levels, hereby confirming pharmacological proof of the concept in humans.[24]

This study in ovarian cancer patients was designed to determine whether clinical proof can be obtained of the concept that Wee1 inhibitor MK-1775 is active in patients with p53 mutated tumors. The choice to conduct this study in refractory and resistant (defined as progression during or within 3 months after the end of first line chemotherapy) ovarian cancer patients is based on the following: 1) mutations in p53 are frequently observed in platinum resistant and platinum refractory ovarian cancer[25-29]; 2) after standard first line treatment (consisting of carboplatin plus paclitaxel), at present effective treatment options are lacking for this patient group[30-33]; 3) since first line treatment includes carboplatin, reintroduction of carboplatin, but now in combination with Wee1 inhibitor MK-1775, is possible in this patient group; 4) preclinical proof of the concept has been obtained with carboplatin and MK-1775[22]; 5) in the phase I study with

MK-1775 the maximal tolerable dose (MTD) of MK-1775 in combination with carboplatin demonstrated target engagement.[24;34]

Methods

Patient selection

Patients were ≥18 years of age with confirmed histological diagnosis of (all histological subtypes of) epithelial ovarian cancer and p53 mutation determined by polymerase chain reaction (PCR) sequencing of exons 2-10. All patients previously received standard first line therapy (a platinum compound plus paclitaxel) and showed evidence of disease recurrence during or within 3 months after the end of this treatment. Most patients underwent debulking surgery. All patients had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of ≤ 2, evaluable or measurable disease according to Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0)[35] or elevated Cancer Antigen (CA)-125 levels that could be monitored according to CGIG criteria [36], a life expectancy of ≥ 16 weeks, adequate bone marrow function defined as absolute neutrophil count (ANC) ≥ 1500/mm^3 (or ≥ 1.5 x 10^9 /L), platelet count ≥ 100,000/mm^3 (or 100 x 10^9 /L), hemoglobin (Hgb) ≥ 9.0 g/dL (or 5.6 mmol/L), adequate hepatic function defined as alanine transaminase (ALT) and aspartate aminotransferase (AST) ≤ 2.5 upper limit of normal (ULN) (≤ 5 times ULN in case of liver metastases), adequate renal function defined by serum creatinine ≤ 1.5 times ULN or creatinine clearance (estimated using the formula of Cockcroft and Gault) ≥ 60 mL/min for patients with creatinine levels > 1.5 times ULN. Exclusion criteria included cerebral or leptomeningeal metastases, radio- or chemotherapy within the last 4 weeks prior to study entry (limited palliative radiation for pain reduction was allowed) and drugs or other products known to be metabolized by, to inhibit or induce CYP3A4, and included the use of aprepitant anti-emetic treatment.

Treatment plan and study design

This phase II, open label, non-randomized, proof of concept (POC) study is being performed at the Netherlands Cancer Institute (NKI) in Amsterdam, the Netherlands. The study received approval of the institutional medical ethical review boards and is conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent (IC) was given by all patients prior to inclusion in the study.

Patients received carboplatin intravenously (i.v.) at a dose resulting in an area under the curve (AUC) of 5 mg/mL*min in a 30 minutes infusion, combined with 225 mg oral MK-1775 bidaily (BID) for 2.5 days in a 21 day cycle. MK-1775 was administered with 12 h dose intervals and the first dose started concomitantly with the start of the chemotherapy infusion. Oral intake was not allowed 2 h before and up to 1 h after intake of MK-1775. Prophylactic anti-emetic treatment was applied: day 1: granisetron 1 mg BID i.v. and dexamethasone 10 mg QD (once daily) i.v.; days 2 and 3: granisetron 1 mg BID p.o. (orally) and dexamethasone 3 mg BID p.o., days 4 and 5: dexamethasone 1.5 mg QD p.o. and metoclopramide 10 mg QID (4 times daily) p.o. or 20 mg TID (3 times daily) supp. (as suppository) on indication.

Safety and assessments

Demographic data and medical history were collected during screening. Physical examination, vital signs and other safety assessments (ECOG-PS, registration of concomitant medication, hematology/biochemistry, and urine analysis) were performed at baseline and throughout treatment (day 1, 8, 15 and 22 of every cycle and at end of treatment).

The incidence and severity of adverse events (AEs) were evaluated and coded according to the National Cancer Institute Common Terminology Criteria (CTC) version 4.0.[37]

In case of toxicity, treatment was postponed for 1 week until recovery to CTC grade ≤ 1. Re-administration of study treatment occurred at a reduced dose level: 1) nausea and vomiting during optimal anti-emetic treatment resulted in dose reduction of MK-1775 of 225 mg to 175 mg (both BID for

2.5 days); 2) in case of hematologic toxicity: decreased platelet count grade 4 (< 25,000/mm^3 or < 25 x 10^9 /L) or decreased neutrophil count grade 4 (<500/mm^3 or 0.5 x 10^9 /L) carboplatin was reduced to a dose resulting in exposure to AUC 4 mg/mL*min; 3) if recurrent hematologic toxicity was encountered, MK-1775 dose was reduced with one dose level to 175 or 125 mg (BID for 2.5 days).

Pharmacokinetics/pharmacodynamics

To determine the plasma concentrations of MK-1775 in plasma, blood samples of 4 mL venous blood were collected in K2EDTA tubes (tubes coated on the interior with spray dried di-potassium ethylene diamine tetraacetic acid) on cycle 1 day 1 at pre-dose, and on cycle 1 day 3 prior to, 3 h and 8 h after administration of the fifth and last dose of MK-1775. Plasma was obtained by immediate centrifugation (10 minutes; 4 °C 1,500x *g*). Samples were stored in 3.6 mL internally-threaded NUNC cryotubes at -20 °C until analysis. Analysis was performed through hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectometry (LC-MS/MS).[38]

Dry blood spot sampling was part of this study, for clinical validation of an assay developed for DBS sampling and to compare plasma PK analysis with DBS of MK-1775. From all K2EDTA tubes obtained for MK-1775 plasma PK, prior to centrifugation, 200-300 µL of blood was removed from the tube for dry blood spot (DBS) sampling of MK-1775 on Whatman FTA® DMPK-A cards (DMPK = drug metabolism pharmacokinetics). Without touching the card, ~40 µL of blood was gently placed on each of 4 circles with a bulb pipette, after which the card was placed on a drying rack and allowed to air dry for t least 4 h. Each card was stored in a glassy envelope, placed in a gas impermeable bag with one or more desiccant packs and stored at room temperature (RT) until analysis by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectometry (LC-MS/MS).[38]

For carboplatin pharmacokinetic analysis blood samples of 4 mL venous blood were collected in sodium-heparin tubes on day 1 at pre-dose, end of infusion (EOI), EOI + 1 h, EOI + 5 h, and after 24 h. Plasma was obtained by

immediate centrifugation (10 minutes; 4 ºC 2000x *g*). Plasma was transferred directly to a Centrifree® UF device with an Ultracel YM-T membrane filter (Millipore® Ireland Ltd, Co.Cork, Ireland) and centrifuged at 1500xg for 35 minutes at room temperature (RT). The resulting plasma ultrafiltrate (pUF), representing the non-protein bound carboplatin fraction, was stored at -80ºC until analysis. Free platinum was determined using a validated inductively coupled plasma mass spectrometry (ICP-MS) method.[39]

To count the number of circulating tumor cells (CTCs), CTCs and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood with the use of cell preparation tubes (CPT), a method based on density gradient centrifugation. Whole blood was collected in 8 mL BD Vacutainer® CPT™ Mononuclear Cell Preparation tubes with sodium citrate during cycle 1: pre-dose on day 1 and 3 h after the fifth and last administration of MK-1775 on day 3, and during cycle 2: 30 minutes after carboplatin infusion on day 1. In order to avoid possible contamination by epithelial cells during skin puncture, the first 4 mL of blood were collected in a separate tube and discarded. The blood was processed directly after it was obtained. After centrifugation for 25 minutes at 1500x *g* at RT, the upper phase was transferred to a 50 mL Falcon tube. Three mL of NaCL 0.9% was added to the empty CPT tube and this was also transferred to the Falcon tube, which was followed by incubation for 15 minutes at RT after addition of 1 mL of 40% formaldehyde. Next, 40 mL of 0.9% NaCl was added to the Falcon Tube, which was subsequently centrifuged for 10 minutes at 4°C at 1000x g. The supernatant was removed and the tube placed on ice. The pellet was resuspended in 1 mL ice cold MeOH/PBS (50/50% v,v%) and vortexed for 10 seconds. After incubation on ice for 10 minutes the CTC samples were stored at -80ºC until analysis. Finally, antibodies against epithelial marker EpCAM coupled to magnetic microbeads, which specifically bind to CTCs, were applied in magnetic cell sorting (MACS®, Miltenyi Biotec) to purify CTCs from the remaining PBMC background and detected and counted by fluorescence-activating cell sorting (FACS) in a method previously described.[40]

Skin biopsies (in the hairy part behind the ear) were collected with a 4 mm punch biopsy pre-dose and on day 3 of cycle 1 within 2 h after the fifth and last dose of MK-1775 to measure phosphorylated CDC2 (pCDC2). pCDC2 levels relative to CDC2 were assessed by immunohistochemistry (IHC). Subsequently, the fold change (FC) between pre- and post-dose was calculated. Target engagement was defined as 50% reduction.

p53 status analysis

To select patients with a high likelihood of tumors with mutations in p53 leading to loss of function, p53 mutation status was analyzed in formalin-fixed, paraffin-embedded pre-treatment tumor samples, mostly obtained during debulking surgery. Standard immunohistochemistry (IHC) and mutation analysis by PCR and direct sequencing as routinely performed in our laboratory were performed prior to inclusion with proven TP53 mutation as a mandatory inclusion criterion. All samples were also analyzed by the AmpliChip p53 test at a later time point.
A minimum of a tumor cell percentage of 50% of tumor cells was used for evaluation. For p53 IHC staining the standard antibody DO-7 (DAKO M7001) was used. Strong staining of a minimum of 50% of cells was required for a positive p53 scoring. Co-scoring by a central pathologist was performed to support scoring consistency.

For sequencing analysis, TP53 exons 2-10 were amplified by PCR from genomic DNA, as previously described.[41] Subsequently, electrophoresis in 2% agarose gels with 0.5X Gelred (Biotium®) was used to assess PCR products. Sequencing was performed using the automatic ABI PRISM 3730 DNA genetic analyzer (Applied Biosystems®). Sequence data were compared with wild type (WT) sequences and with Mutation Surveyor software (Softgenetics®).

The AmpliChip p53 test is a micro-array based sequencing test that allows sequencing of the entire coding region of the TP53 gene, including the flanking splicing regions of exons 2-11, and detection of single nucleotide substitutions and one base pair (bp) deletions. This is accomplished by comparative analysis of the hybridization pattern of a series of probes to

sample, and WT reference DNA. Each probe contains multiple copies of a specific nucleotide sequence, and a total of 1,300 nucleotide positions of coding regions are tiled on the AmpliChip array. Another advantage would be the ability to identify p53 mutations in samples that contain a mixture of normal and tumor cells, without the need for microdissection. Main steps of AmpliChip TP53 array consist of extraction of genomic DNA extraction, PCR amplification of purified DNA, fragmentation and labeling of PCR products, followed by hybridization to the microarray, staining, scanning and determination of the sequence of the p53 gene.[42-44]

Clinical implications of encountered mutations and deletions with both methods were compared with results on the IARC TP53 database (http:/www.p53.iarc.fr/index.html). Functional classification of the mutations in the IARC TP53 database has been based on the overall transcriptional activity on 8 different promoters. For each mutant the median of the 8 promoter-specific activities (expressed as the percentage of wild-type protein) has been calculated. Missense mutations are classified as 'non-functional' if the median is < 20%, partially functional' if the median is >20% and ≤ 75%, while 'functional' if the median is >75%. In case of missense mutations dominant-negative effect (DNE) has been established according to the results of Kato et al.[45] DNE over wild-type p53 was established as 'yes' in case of DNE on both WAF1 and RGC promoters, or on all promoters in a large study (by Dearth et al [46]), 'moderate' in case of DNE on some promoters and not others, and 'no' in case of no DNE. Protein p21, encoded by the gene named WAF1, localized on chromosome 6 (6p21.2), is a potential mediator of p53 suppression.[47] Heterogeneity in transcriptional activity is observed between WT and mutant p53 in different target sequences, one of them is named ribosomal gene cluster (RGC).[48]

Activity assessment

Radiological disease assessments were performed by computer tomography (CT) scan or magnetic resonance imaging (MRI) at baseline and every 2 cycles. Tumor response was evaluated using RECIST 1.0.[35] Additional analysis was performed by external experts blinded to all clinical

Table 1. Baseline patient characteristics.

	Total number of patients
	14
Age at registration	
Median (years, range)	58 (25-74)
FIGO stage of cancer*	
IIA	0 (0%)
IIB	1 (8%)
IIIA	1 (8%)
IIIB	0 (0%)
IIIC	4 (31%)
IV	8 (57%)
WHO Performance Status	
WHO 0: Normal Activity; asymptomatic	8 (57%)
WHO 1: Symptomatic; but fully ambulatory	5 (38%)
WHO 2: Symptomatic; in bed less than 50% of the day	1 (8%)
WHO 3: Symptomatic; in bed more than 50% of the day	0 (0%)
WHO 4: Unable to get out of bed	0 (0%)

* FIGO = International Federation of Obstetricians and Gynecologists Staging System for Ovarian Cancer

features surrounding the cases and who performed volumetric imaging analysis, by using semi- automated algorithms, in an attempt to quantify the volumes and densities of measurable tumor masses.

Response of CA-125 was defined as 50% reduction during treatment and confirmed after 4 weeks according to the CGIG criteria.[36]

Statistical analyses

The primary endpoint of the study was to assess the response rate of MK-1775 225 mg (BID for 2.5 days) in combination with carboplatin at a dose resulting in exposure to an AUC 5 mg/mL*min in a 21 day schedule in epithelial ovarian cancer patients with p53 confirmed by PCR/direct sequencing in archival tumor tissue. To test the hypothesis that MK-1775 is

effective in patients with tumors harboring p53 mutations, an A'Herns single stage phase II design is being applied, in which a total of 21 evaluable patients will be collected. A minimum of 6 responses (defined by RECIST 1.0 criteria or a CA-125 marker response) is needed to provide a 61% power to declare an efficacy of at least 30% (α=0.05). A response of 13% or less would definitely indicate no efficacy of interest.
Secondary objectives consisted of 1) determining the pharmacokinetics of MK-1775 in plasma and DBS, and of carboplatin in plasma and ultrafiltrates; 2) determining the pharmacodynamic changes induced by MK-1775 and carboplatin in surrogate skin tissue, 3) determining the safety of MK-1775 plus carboplatin in p53 mutated ovarian cancer patients. 4) time to progression (TTP).

Results

Patient recruitment

A total of 15 patients were included in the study at the time of the interim analysis. Fourteen patients started study treatment. Patient characteristics are presented in **Table 1**. Median age of the patients was 58 years (range 25-74), majority of patients had extensive disease progression (International Federation of Obstetricians and Gynecologists Staging System for Ovarian Cancer [FIGO] stage IV), and WHO performance status. Fourteen patients were evaluable for toxicity; one patient never stated study treatment, because of early progression in the period after registration and prior to study start. The preliminary main treatment related and clinically significant adverse events per patient are presented in **Table 2**. Bone marrow toxicity, fatigue, diarrhea, nausea, vomiting, and were the most common adverse events. Preliminary results show that thrombocytopenia grade 4 and/or neutropenia grade 2-4 resulted in dose holds and/or dose reductions in 7 patients or 10 times. Toxicity results are preliminary and can be underestimated as this concerns an interim analysis based on available data of 11 out of the 14 patients at the moment of the interim analysis.

Table 2. Main related adverse events, scored by highest grade per patient.
Only clinically significant adverse events are included (i.e. neutropenia grade 4 in 'the rest week' has not been reported in this table, because of lack of clinical significance, while grade 2 neutropenia resulting in clinically significant dose delay has been reported). Grade 1 toxicities that were encountered only in one patient have been excluded. Main hematological adverse events consisted of grade 4 thrombocytopenia and neutropenia, and grade 2 anemia. Most observed non-hematological adverse events were fatigue, diarrhea, nausea and vomiting. Of note, the adverse events profile might be underestimated, because this concerns an interim analysis with data at the time of interim analysis of 11 out of the 14 patients.

Toxicity	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Total**	(%)
Abdominal pain	1	1			2	(14%)
Allergic reaction carbo		2			2	(14%)
ALT increase		1	1		2	(14%)
Anemia		8			7	(57%)
AST increase		1			1	(7%)
Diarrhea	6	2			8	(57%)
Dry mouth	2				2	(14%)
Dyspnea	3	1			4	(29%)
Exanthema	1				1	(7%)
Fatigue	3	5	1		9	(64%)
Gastroenteritis		1			1	(7%)
Heartburn		1			1	(7%)
Hypertension		1			1	(7%)
Hypokalemia	1		1		2	14%)
Hypomagnesemia	1	2	1		4	(29%)
Hypophosphatemia			1		1	(7%)
Leucopenia				1	1	(7%)
Mucosal infection		1			1	(7%)
Nausea	7	1	1		9	(29%)
Neuropathy	2				2	(14%)
Neutropenia			1	4	5	(36%)
Thrombocytopenia				7	8	(57%)
Vomiting	3	2			5	(36%)
Number of patients and maximal grade of toxicity			3	1	7	11

Anti-tumor activity

One patient (the first patient) was considered not evaluable for response, because after a positive IHC staining, p53 mutation could not be confirmed by PCR/direct sequencing. The protocol was amended; not a positive IHC staining for p53, but a mutation confirmed by PCR/direct sequencing of exons 2-10 was mandatory for inclusion. Thirteen patients were therefore evaluable for response evaluation according to RECIST and CA-125 marker response. Six patients showed progressive disease (PD) after the first evaluation after 2 cycles. Two patients experienced stable disease (SD) as best response (progression was observed after 4 and 6 cycles respectively). Five patients (38%) had a CA125 marker response according to GCIG criteria (see also **Figure 1**). In addition, four of these patients (31%) showed a partial response (PR) according to RECIST as best response, including 3 patients (23%) with a near complete response.

It is too early to calculate TTP at this stage. Two patients with near complete response were still on study at time of the preliminary analysis: one patient in cycle 12 (see also **Figure 2**) and one patient in cycle 6 of treatment. One patient with a CA-125 marker response and near complete response went off study after 8 cycles in, but reported progression of disease with increase of CA-125 9 weeks after study stop (PFS of 6 months). One patient with a partial remission, showed an initial decrease of CA-125 and significant changes on the CT scan after 4 cycles, but progressed after 6 cycles (PD on CT scan and increasing CA-125 levels). The one patient with a CA-125 marker response that showed SD as best response (see also **Figure 2**), went off study after 4 cycles due to ileus and PD.

Figure 1. Preliminary CA-125 levels. Marker responses have been encountered in 5 patients (marked with 'x'). Patient number 10 displayed an initial drop, but later in increase, and progressive disease (PD) on the computed tomography (CT) scan. Right top of figure is an enlargement of the left bottom part of the figure. CA-125 levels prior to the zero time point, represent CA-125 levels obtained prior to study start.

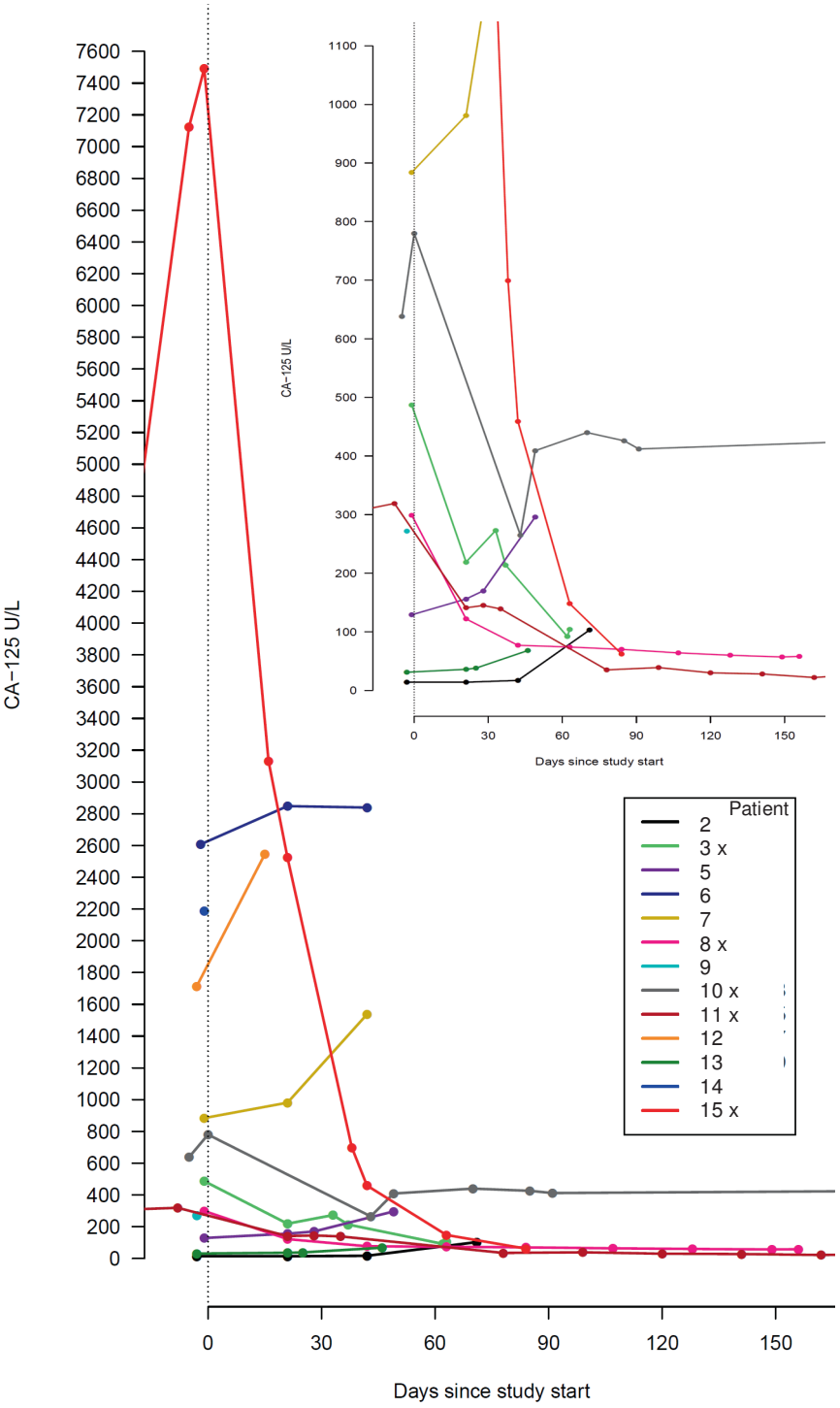
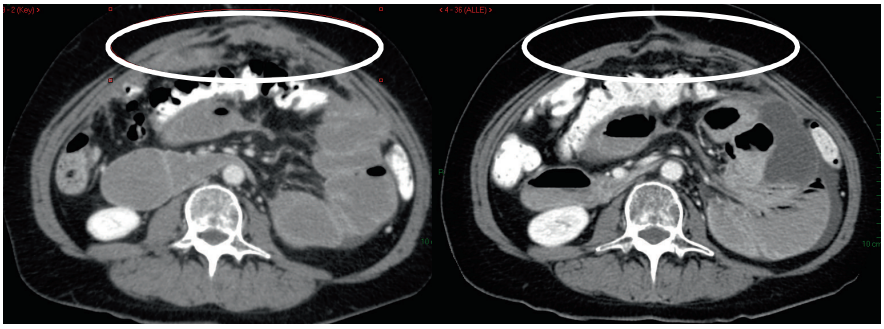

CA−125 U/L
Days since study start
Patient
2
3 x
5
6
7
8 x
9
10 x
11 x
12
13
14
15 x

Figure 2. Computed tomography (CT) scan images of two patients obtained prior to start and after 2 and 5 cycles, respectively.
The first patient is the patient with initially a CA-125 marker response (from 525 U/L to 26 U/L) and SD (or an unconfirmed PR) according to RECIST, but who developed ileus and increasing CA-125 levels (to 487 U/L) and therefore was taken off study after 4 cycles. The CT scan images show significant changes in the aspect of the omental cake (upper images).
The last 4 images are CT scan images are from the patient who was still on study (cycle 12) at the time of the preliminary analysis and who shows a near complete response. CA-125 levels at study start were 7492 U/L and had dropped to 62 U/L at time of the CT scan images after 5 cycles.

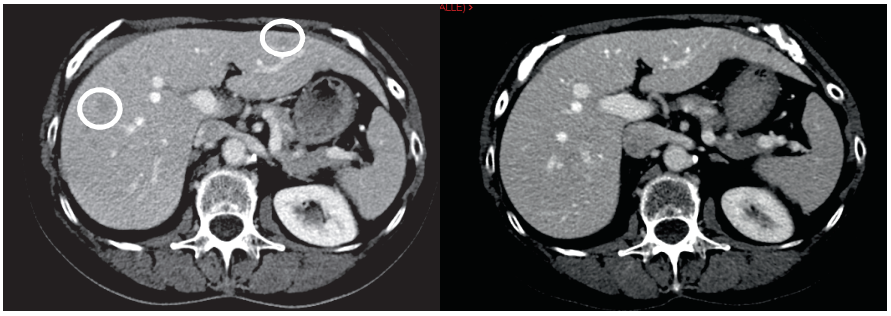
Patient case I



Prior to start

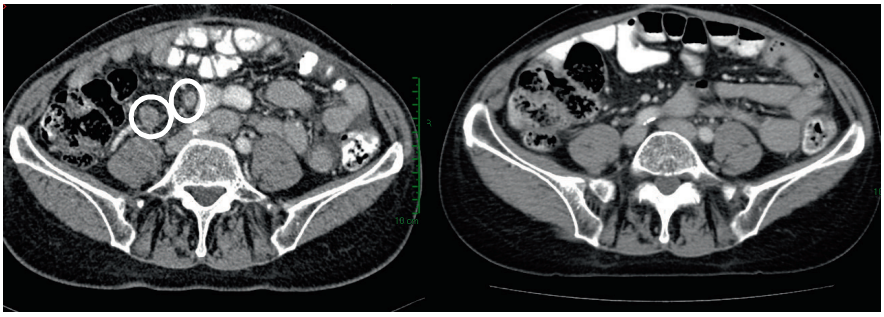
After 2 cycles (= 6 wks)

Patient case II



Prior to study start

After 5 cycles



Prior to study start After 5 cycles

p53 status analysis

Results from the p53 status analysis by IHC, direct sequencing and AmpliChip p53 array are presented in **Table 3**. In two patients with negative IHC staining for p53, a mutation and a deletion, respectively, were found with PCR/direct sequencing and AmpliChip p53 array. All p53 mutations found were in exons 5-8, which is in line with results published in literature (IARC TP53 database). AmpliChip p53 and direct sequencing found similar aberrations, except for 3 patients. AmpliChip p53 array identified in two patients one and two additional mutations in the TP53 gene, respectively. The first patient (patient number 7) had been included based on a missense mutation in exon 6 (c.643A>G;p.Ser215Gly) known to result in a non functional protein. With AmpliChip p53 analysis one additional missense mutation in exon 5 (c.523C>T;p.Arg175Cys, known to result in a partially functioning protein) and one frameshift mutation in exon 4 (c.293delC;p.Prp98fs) were identified. In the second patient (patient number 12) two missense mutations were identified: one missense mutation in exon 6 (c.587G>A;p.Arg196Gln - known to result in a partially functioning protein) and a missense mutation in exon 8 (c.817C>T;p.Arg273Cys - known to result in a non-functional protein). With AmpliChip TP53 analysis an additional missense mutation in exon 8 (c.799C>T;p.Arg267Trp - also known to result in a non-functional protein) was identified. In the third patient (patient number 15) PCR/direct sequencing and AmpliChip TP53 analysis both identified a different mutation. PCR/direct sequencing identified a frameshift mutation in exon 5

Pt	IHC	Direct PCR/ Sequencing	Detected mutations	Amplichip p53 array	Detected mutations	Exon	Codon	Mutation	Mutation type	Transactivation class (IARC TP53 dbase)	DNE (dominant negative effect)
1	30-40% pos.	Neg.	NA	Neg.	NA	NA	NA	NA	NA	NA	NA
2	100% pos.	Pos.		Pos.		5	175	c.524G>A;p.Arg175His	Missense	Non-functional	yes
3	100% pos.	Pos.		Pos.		7	238	c.713G>T;p.Cys238Phe	Missense	Non-functional	yes
5	70% pos.	Pos.		Pos.		5	164	c.490A>G;p.Lys164Glu	Missense	Non-functional	NA
6	90% pos.	Pos.		Pos.		8	275	c.824G>A;p.Cys275Tyr	Missense	Non-functional	NA
7	70% pos.	Pos.	no.1	Pos.	no.1, 2, 3	1) 6 2) 5 3) 4	1) 215 2) 175 3) 98	1) c.643A>G;p.Ser215Gly 2) c.523C>T;p.Arg175Cys 3) c.293delC;p.Prp98fs	1) Missense 2) Missense 3) Frameshift	1) Non-functional 2) Partially functional 3) NA	1) NA 2) NA 3) -
8	100% pos.	Pos.		Pos.		7	248	c.742C>T;p.Arg248Trp	Missense	Non-functional	Yes
9	100% pos.	Pos.		Pos.		5	179	c.535C>G;p.His179Asp	Missense	Partially functional	NA
10	Neg.	Pos.		Neg.		8	298	c.892G>T;p.Glu298X	Nonsense	NA	-
11	100% pos.	Pos.		Pos.		8	273	c.818G>T;p.Arg273Leu	Missense	Non-functional	Yes

12	100% pos.	Pos.	no. 1, 2	Pos.	no.1, 2, 3	1) 6 2) 8 3) 8	1) 196 2) 273 3) 267	1) c.587G>A;p.Arg196Gln 2) c.817C>T;p.Arg273Cys 3) c.799C>T;p.Arg267Trp	1) Missense 2) Missense 3) Missense	1) Partially functional 2) Non-functional 3) Non-functional	1) NA 2) Yes 3) Moderate
13	50-60% pos	Pos.		Neg.		7	255	c.764_766delTCA: p.Ile255del	Deletion	NA	-
14	70-80% pos.	Pos.		Pos.		8	282	c.844C>T;Arg282Trp	Missense	Non-functional	No
15	Neg.	Pos.	no. 1	Pos.	no.2	1) 5 2) 4	157 105	1) c.469_473delGTCCG; p.V157fs*22 2) c.313G>T;p.Gly105Cys	1) Frameshift 2) Missense	1) NA 2) Non-functional	1) - 2) NA

Table 3. Results from the p53 status analysis. Analysis was performed by immunohistochemistry (IHC), polymerase chain reaction (PCR)/direct sequencing and AmpliChip p53 array. If not the same mutations have been detected by both detection methods PCR/ direct sequencing and AmpliChip p53 array, the encountered mutations per method have been specified in the column 'detected mutations'. Patient 4 never started study treatment due to early PD.
IARC TP53 Mutation Database http:/www.p53.iarc.fr/index.html; IHC = immunohistochemistry; neg. = negative; no. = number; PCR = polymerase chain reaction; pos. = positive, pt = patient.

(c.469_473delGTCCG;p.V157fs*22), while AmpliChip TP 53 array identified a missense mutation in exon 4 (c.313G>T;p.Gly105Cys - known to result in a non-functional protein). This is one of the patients with negative p53 IHC results. See also **Table 3**.

In addition to the first patient with a 30-40% positive IHC, but negative result in PCR/direct sequencing of TP 53 (after which the protocol was amended and a mutation based on PCR/direct sequencing was made mandatory for inclusion in the study), 5 additional patients were excluded from the study based on a negative PCR/direct sequencing result for TP 53. Three out of these 5 patients were analyzed negative for IHC, 1 patient showed <5% positive and 1 patient was 100% positive for IHC p53 mutation staining. The archival tumor samples of these 5 patients were not tested for AmpliChip TP53 analysis because these patients did not participate in the study.

Pharmacokinetics and pharmacodynamics

Blood samples for the measurement of total and unbound ultrafilterable carboplatin and MK-1775 were obtained in all patients.

The sampling schedule for MK-1775 used in this study was sparse compared to the sampling schedule used in the Phase I study with MK-1775. However, PK parameters C_{8h}, C_{max}, T_{max} and AUC_{0-8h} are consistent and presented in **Figures 3A** and **3B**, respectively. C_{8h} was 8 h post dose on day 3 (after the last intake of MK-1775) both in the phase I study (n=13) and this phase II ovarian study (n=12). In the phase I study T_{max} varied between 1 to 6 h (n=15), while in this study T_{max} was 3 h for all patients (n=12). AUC_{0-8h} in the phase I study was based on 6 points at 0, 1, 2, 4, 6, 8 h on day 3 (n=13), while in this study it was based on only 3 points at 0, 3, 8 h on day 3 (n=12).

Although CYP3A4 modulating drugs were not allowed per protocol, an exception was made for aprepitant as part of anti-emetic treatment. The first 3 patients received aprepitant and these PK results, together with the PK results of patients treated in the phase I study that received aprepitant, showed significant increased MK-1775 plasma concentrations. Based on (unpublished) preclinical data it was anticipated that CYP3A4 modulating drugs could influence uptake of MK-1775 and result in different plasma

concentrations. After these results the protocol was amended and, like other CYP3A4 modulating drugs, the use of aprepitant was prohibited.

The results of MK-1775 DBS sampling correlated to the MK-1775 plasma concentrations. The results and details of the assay are published in a separate article.[38]

At the time of the preliminary analysis carboplatin samples were analyzed for 12 patients. Carboplatin concentration measurements in ultrafiltrate revealed AUCs that approximated the target area under the free carboplatin curves that were calculated using the modified Calvert formula. Following administration free and total carboplatin concentrations dropped rapidly, followed by a slower terminal elimination phase (**Figures 4A** and **4B**).

CTCs were obtained in all patients, except in one patient the last CTC sample was not obtained. In four out of twelve patients a correlation between CTC count and CA-125 and response was observed, in the additional patients baseline values were too low to determine a pharmacodynamics effect. However, the sample size is still too small to draw conclusions.

Skin biopsies were collected in all patients. Since MK-1775 inhibits Wee1, which phosphorylates CDC2, a decrease of pCDC2 on day 3 compared to day1 was expected, and indeed a decrease in pCDC2 was observed. Compared to results obtained in the Phase I study with MK-1775 and carboplatin (or cisplatin or gemcitabine) the decrease (FC) in the patients included in this study is slightly lower: the mean decrease was 21%, or 27% after removing one possible outlier. This difference can be explained by the fact that the baseline levels in the Phase I study were higher compared to the baseline pCDC2 levels in this study (~35% versus ~24% respectively). The post baseline levels of both studies were comparable.

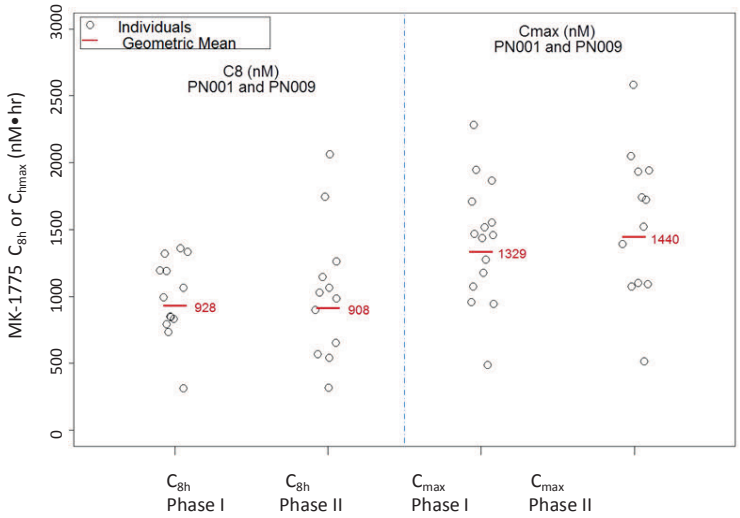


Figure 3A. Phase I versus Phase II ovarian study MK-1775 pharmacokinetics comparison of C_{8h} (nM) and C_{max} (nM) on day 3 (after the last intake of MK-1775).

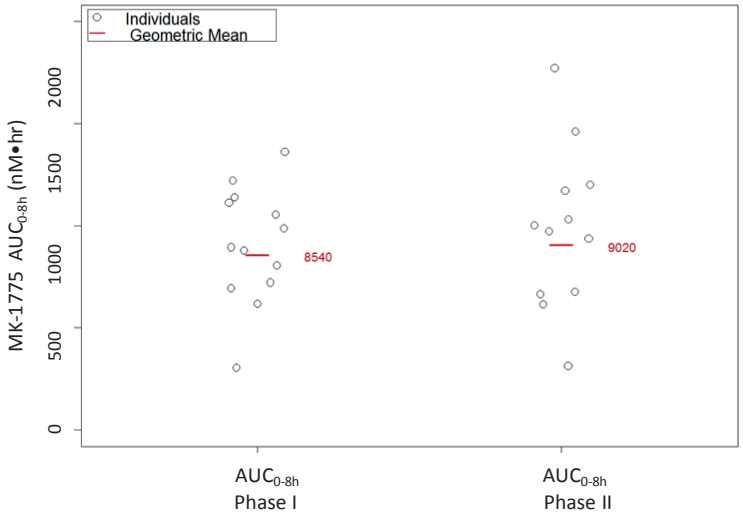


Figure 3B. Phase I versus Phase II ovarian study MK-1775 pharmacokinetics comparison AUC_{0-8h} (nM•h) on day 3 (after the last intake of MK-1775).

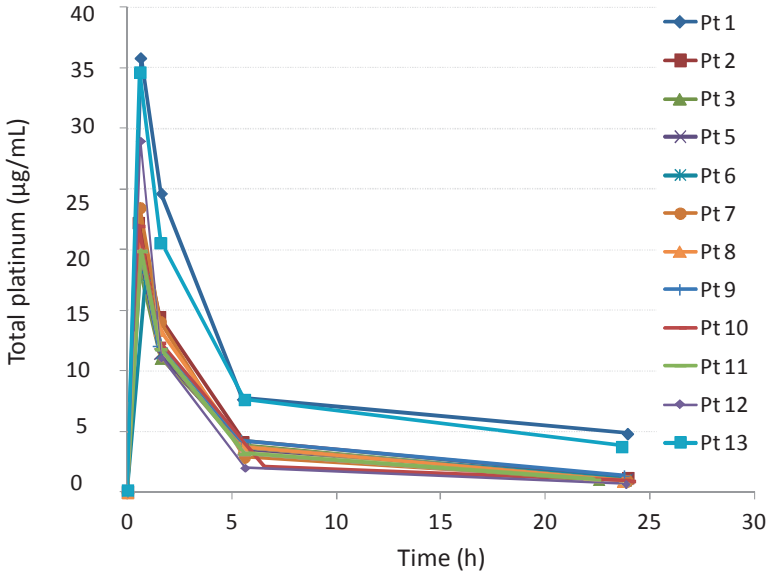


Figure 4A. Pharmacokinetics of total carboplatin, (n= 12). Carboplatin dose target of area under the curve AUC 5 mg/mL*min was calculated using the modified Calvert formula. The graph demonstrates carboplatin decay, in a biphasic manner after a 30 minutes intravenous infusion.

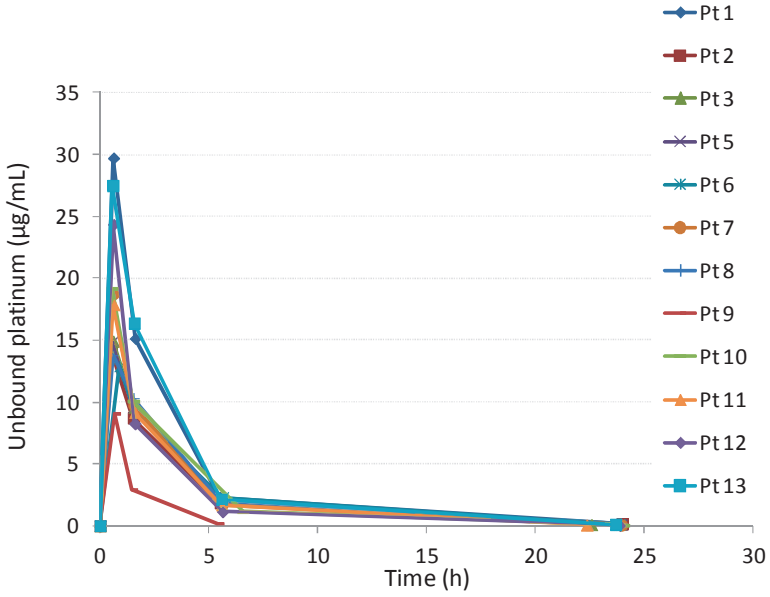


Figure 4B. Pharmacokinetics of unbound carboplatin (measured as unbound platinum in ultrafiltrates), (n=12).

Discussion

Testing the hypothesis that the Wee1 inhibitor MK-1775 plus carboplatin is active in patients with platinum resistant or refractory ovarian cancer harboring p53 mutations was the main reason for conducting this phase II study with MK-1775 plus carboplatin in patients with refractory or resistant (defined as progression within 3 months after the end of first line treatment) ovarian cancer.

At the time of this preliminary analysis a substantial significant number of patients experienced response: 5 patients (38%) demonstrated a CA-125 marker responses, 4 of these patients (31%) experienced RECIST confirmed PR as best response, of which 3 patients (23%) even demonstrated a near complete response. As all patients were platinum resistant or refractory, these results would not have been expected with carboplatin alone. Of course conclusions can only be made at the end of this study, but these preliminary results are encouraging. The statistical barrier set for this study is very high and still it seems reasonable to expect that the predefined number of patients needed for response will eventually be achieved.

Nevertheless, 6 patients did not respond and showed progression of disease, and two patients showed SD for a limited period (2 and 4 cycles). It is unclear at present how long the anti-tumor response remains and it will be interesting to estimate the PFS in all patients and in patients that will have benefitted from the treatment and that have shown significant tumor regression and/or normalization of CA-125 levels. In addition, it should be realized that there is wide inter-patient variability in the number of cycles of carboplatin -or other platinum compounds- that can be administered, as a result of adverse effects due to platinum accumulation, like neurotoxicity and development of bone marrow suppression. Furthermore, the patients with a temporary response demonstrate that adding MK-1775 to DNA damaging chemotherapy is not effective for a subgroup of patients with p53 deficient tumors.

The results of p53 analysis are in line with the results in literature, and encountered in exon 5-8 of TP53. AmpliChip TP53 array is a relatively new

test and a comparison with direct PCR/ direct sequencing in this stage is valuable. The results of the AmpliChip TP53 array on the current preliminary dataset seem at least in line with PCR/direct sequencing. A slight increase might be present with the AmpliChip TP53 array. In contrast, deletions (and insertions) larger than 1 bp are missed by the AmpliChip TP 53 array. In our patient group one patient would have been excluded as result of this limitation of the AmpliChip results, while PCR/direct sequencing identified a 3bp deletion (patient 13).

The toxicity profile of the MK-1775 carboplatin combination with nausea/vomiting, diarrhea, fatigue and bone marrow depression as major adverse events, is in line with the toxicity profile observed in the phase I study with MK-1775 and carboplatin (or cisplatin or gemcitabine) in patients with different kind of advanced solid tumors.[24] In this study however, thrombocytopenia was more pronounced in the first cycle and resulted more often in dose reduction of carboplatin (decrease of a dose resulting in AUC 5 to 4) in cycle 2. All patients were previously treated with bone marrow toxic chemotherapy in first line therapy and the increased bone marrow toxicity observed in this study might be explained by a bone marrow sensitivity induced by first line therapy.

Altogether, preliminary results are promising, and although this study is designed to proof a principle and the statistical barrier set is high, twenty-one patients is a very small group and additional studies will be needed to further investigate the value of MK-1775.

At this stage of development it is also considered important to think beyond the 'MK-1775 + DNA damage inducing chemotherapy'-strategy and to investigate other drug combinations. These combinations should preferably have a solid rational and first be investigated preclinically prior to initiating clinical trials.

One option that seems to be worth investigating is synergistic induction of apoptosis by for instance adding a Chk1 inhibitor to MK-1775 with/or without a DNA damage inducing drug. Chk1 inhibition is able to induce catastrophic chromosomal instability, resulting in mitotic catastrophe.

Combination of Wee1 and Chk1 inhibition might be able to potentiate this effect. Promising results have already been obtained *in vitro* with the combination of MK-1775 with Chk1 inhibitor AR458323 [49] and CHK1 inhibitor PF00477736.[50;51]

Another option worthwhile studying is the combination of MK-1775 with a Poly-ADP Ribose Polymerase (PARP) inhibitor and possibly other DNA damaging therapy. MK-1775 (and PD-166285) was shown to inhibit homologous recombination repair (HRR) *in vitro*.[52] [53] Thus, combined Wee1 and PARP inhibition may be able to induce a strong anti tumor effect, especially in p53 deficient tumor cells. This concept was explored *in vitro* using a combination of Chk1 inhibitor AZD7762, PARP1 inhibitor olaparib and radiation.[54;55] Recently, results have also become available with MK-1775 and olaparib, again in combination with radiation *in vitro* demonstrating increased radiosensitization. Additionally, MK-1775 was shown to have an effect on HRR.[52] Moreover, inactivation of HRR as a result of Wee1 inhibition by MK-1775 (and PD-166285) was also demonstrated *in vitro*.[53] Altogether, these preclinical results suggest that MK-1775 and PARP might be a successful combination strategy, and deserves further investigation.

In conclusion, the preliminary data of this study are encouraging, and if the observed trend continues in the additional patients to be recruited in the study, a next step in confirming the hypothesis that MK-1775 is active in platinum resistant or refractory ovarian cancer patients with p53 mutation will have been made. However, additional studies in a larger patient population are needed to establish the true value of MK-1775 as a novel anti-cancer drug in combination with existing DNA damage inducing drugs.

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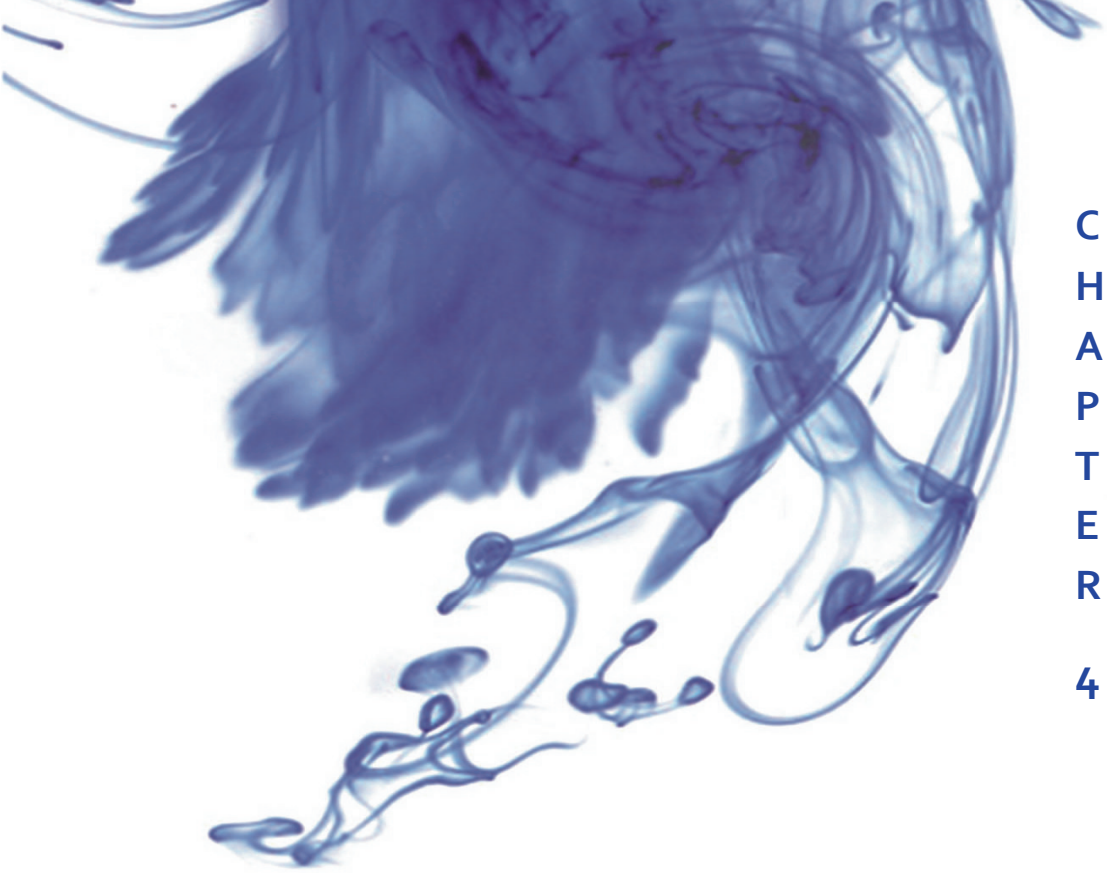

CHAPTER 4

Clinical pharmacological combination study with fixed dose rate gemcitabine

4.1

Phase I dose escalation study and population pharmacokinetic analysis of fixed dose rate gemcitabine plus carboplatin as second-line therapy in patients with ovarian cancer

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Abstract

Purpose

This Phase I study of Fixed Dose Rate (FDR) gemcitabine and carboplatin assessed the maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), safety, pharmacokinetic (PK)/pharmacodynamic (PD) profile and preliminary anti-tumor activity in patients with recurrent ovarian cancer (OC).

Patients and methods

Patients with recurrent OC after first line treatment were treated with carboplatin and FDR gemcitabine (10 mg/m^2/min) on days 1, 8 and 15, every 28 days. Pharmacokinetics included measurement of platinum concentrations in plasma ultrafiltrate (pUF) and plasma concentrations of gemcitabine (dFdC) and metabolite dFdU. Intracellular levels of dFdC-triphposphate (dFdC-TP), the most active metabolite of gemcitabine, were determined in peripheral blood mononuclear cells (PBMCs). Population pharmacokinetic modelling and simulation was performed for the further investigate the optimal schedule.

Results

Twenty three patients were enrolled. Initial dose escalation was performed using FDR gemcitabine 300 mg/m^2 combined with carboplatin AUC 2.5 and 3 mg/mL*min. Excessive bone marrow toxicity led to a modified dose escalation schedule: carboplatin AUC 2 mg/mL*min and dose escalation of FDR gemcitabine (300 mg/m^2, 450 mg/m^2, 600 mg/m^2 and 800 mg/m^2). DLT criteria as defined per protocol prior to the study were not met with carboplatin AUC 2 mg/mL*min in combination with FDR gemcitabine 300-800 mg/m^2 because of myelosuppressive dose-holds (especially thrombocytopenia and neutropenia).

Conclusion

FDR gemcitabine in combination with carboplatin administered in this 28 days schedule resulted in increased grade 3/4 toxicity compared to

conventional 30-minute infused gemcitabine. A two weekly schedule (chemotherapy on day 1 and 8) would be more appropriate.

Introduction

Ovarian cancer is the second most common gynecologic malignancy in women, after endometrial cancer, and associated with a high mortality rate.[1;2] Clinical symptoms reported by patients with ovarian cancer (OC) include abdominal discomfort, swelling and bloating and can often be attributed to many different conditions.[3-5] This contributes to the fact that the majority of patients are diagnosed at an advanced stage of disease: 75% of patients present with stage III (disease spread to lymph nodes or peritoneal cavity) or stage IV (disease spread to more distant sides) at time of diagnosis.[6;7]

Although first line treatment with carboplatin and paclitaxel results in clinical remission in most women, over 50% of women experience relapse at some point after first line treatment.[1;8-12] Depending on the time of the relapse after initial therapy, which is also referred to as the platinum free interval, patients are considered either platinum resistant (relapse within 6 months of initial therapy) or platinum sensitive (relapse more than 6 months after initial therapy).[13;14] Treatment of chemotherapy-sensitive recurrent OC usually consists of platinum based combination therapy. Frequently used combinations are carboplatin/paclitaxel, carboplatin/gemcitabine and carboplatin/doxorubicin.[15] Etoposide, topotecan, liposomal doxorubicin and gemcitabine are sometimes used as single agent therapy. Newer combinations and treatment options that are being explored include addition of vascular endothelial growth factor (VEGF) inhibitors (e.g. bevacizumab) or poly (ADP-ribose) polymerase (PARP) inhibitors (e.g. olaparib) to for instance carboplatin and/or gemcitabine.[16] Hence, gemcitabine has single agent activity in recurrent OC, but is also commonly used in combination therapy in the treatment of OC.

Gemcitabine (2′,2′-difluorodeoxycytidine or dFdC) is a nucleoside analogue and antimetabolite with anti-tumor activity against different solid tumors including ovarian, pancreatic, breast, bladder and non-small cell lung cancer (NSCLC).[17-21] Gemcitabine is an intravenously administered pro-drug and metabolism into active forms occurs in the body. The anti-

proliferative effect of gemcitabine is mediated by incorporation of gemcitabine triphosphate (2′,2′-difluorodeoxycytidine triphosphate or dFdC-TP), the active metabolite of gemcitabine into the DNA, thereby replacing the nucleic acid cytidine, and eventually resulting in apoptosis. Gemcitabine also binds to the enzyme riboncleotide reductase (RNR) which is required for DNA repair and replication.[22;23] Human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNT) largely account for uptake of dFdC into cells. Cellular uptake is followed by multiple intracellular phosphorylation steps.[24-27] The enzyme deoxycytidine kinase (dCK) mediates phosphorylation of dFdC into difluorodeoxycytidine monophosphate (dFdC-MP) which is the rate limiting step[28;29], while subsequent phosphorylation results in the active difluorodeoxycytidine diphosphate (dFdC-DP) and difluorodeoxycytidine triphosphate (dFdC-TP) metabolites.[30] However, in plasma and several organs (e.g. liver) gemcitabine can be metabolized by the enzyme cytidine deaminase (CDA) into the less pharmacologically active metabolite 2′,2′-difluoro-deoxyuridine (dFdU).[31]

Recommended doses of gemcitabine 1000-1200 mg/m^2 given as a standard 30-minute infusion (~33 mg/m^2/min) result in dFdC plasma concentrations of 20-60 µM, while saturation of dCK is already achieved at dFdC plasma concentrations of 15-20 µM.[32-35] To avoid saturation of dCK and subsequent deamination to dFdU, and to increase intracellular formation of dFdC-TP, prolonged gemcitabine infusion at a fixed dose rate (FDR) of 10 mg/m^2/min has been proposed, resulting in steady state plasma levels of 15-20 µM and with the aim to enhance anti-tumor activity.[35;36]

This study was designed to identify the maximum tolerated dose (MTD), toxicity profile, pharmacokinetic profile and the preliminary response rate of FDR gemcitabine plus carboplatin in patients with OC after first line treatment with platinum containing therapy.

Methods

Patient selection

Patients were ≥18 years of age with confirmed histologic diagnosis of epithelial ovarian cancer that had relapsed after first line treatment with platinum containing therapy. All patients had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of ≤2, evaluable or measurable disease according to Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0)[37], a life expectancy of ≥ 12 weeks, and adequate hematological function defined as an absolute neutrophil count (ANC) of ≥ 1.5 x 10^9/L or 1500/mm^3, platelet count of ≥ 100 x 10^9/L or 100,000/mm^3 and hemoglobin ≥ 6.2 mmol/L or 10.0 g/dL, adequate hepatic function defined as bilirubin ≤ 1.5 the upper limit of normal (ULN), alanine transaminase (ALT) and aspartate aminotransferase (AST) ≤ 2.5 ULN (≤ 5 times ULN in case of liver metastases, adequate renal function defined as creatinine clearance (estimated using the formula of Cockcroft and Gault) ≥ 60 mL/min.

Study design and dose escalation

This phase I, open label, non-randomized, dose escalation study was performed at the Netherlands Cancer Institute (NKI) in Amsterdam, the Netherlands. The study received approval of the institutional medical ethical review boards and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent (IC) was given by all patients prior to inclusion in the study.

Patients were enrolled in sequentially rising dose levels. Initial dose escalation was performed with carboplatin, starting at a dose resulting in AUC 2.5 mg/mL*min, and the second dose level AUC 3 mg/mL*min, both in combination with FDR gemcitabine 300 mg/m^2 (as a 30 minutes infusion). The carboplatin dose was calculated for each carboplatin administration, using the target area under the free carboplatin plasma concentration versus time curve (AUC in mg/mL*min) and the modified Calvert formula.[38] The glomerular filtration rate (GFR) was estimated using the formula of Cockcroft and Gault.[39]

During conduct of the study, the dose levels (DLs) with carboplatin AUC 2 .5 and 3 mg/mL*min was considered too toxic and resulted in frequent dose holds due to bone marrow toxicity (13% and 27% dose-holding neutropenia and 15% and 11% dose holding thrombocytopenia respectively), an alternative dose escalation schedule was designed to allow dose escalation of FDR gemcitabine. The following DLs of gemcitabine FDR were explored: 300 mg/m^2 (in 30 minutes), 450 mg/m^2 (in 45 minutes), 600 mg/m^2 (in 60 minutes) and 800 mg/m^2 (in 80 minutes), all in combination with a dose of carboplatin resulting in AUC 2 mg/ml*min (**Table 1**). A traditional 3+3 design was applied.[40]

Table 1. Baseline patient characteristics.

	Total no. of patients 22	
Age at start treatment		
Median (range)	59.5	(30-73)
ECOG performance status		
0	6	(27%)
1	12	(55%)
2	4	(18%)
Prior lines of chemotherapy		
Median (range)	2	(1-3)
1 line	10	(45%)
2 lines	5	(23%)
3 lines	2	(9%)
Prior lines of hormonal therapy		
Median (range)	0	(0-3)
Prior radiotherapy		
No	19	86%
Yes	3	14%

ECOG = Eastern Cooperative Oncology Group performance status.

Safety and assessments

Demographic data and medical history were collected during screening. Physical examination, vital signs and other safety assessments (ECOG-PS, hematology/biochemistry) were performed at baseline and throughout treatment (day 1, 8, 15, 22 of every cycle).

The incidence and severity of adverse events (AEs) were evaluated and coded according to the National Cancer Institute Common Terminology Criteria (CTC) version 3.0.[41] DLT definition included the following during the first treatment cycle: grade 4 neutropenia (ANC < 0.5 x 10^9/L or 500/mm^3) lasting over 7 consecutive days, grade 3 febrile neutropenia, grade 4 thrombocytopenia (< 25 x 10^9/L or 25,000/mm^3) or grade 3 thrombocytopenia (platelets < 50.0 x 10^9/L or < 50,000/mm^3) with a bleeding episode and/or requiring platelet transfusion, grade ≥ 3 non-hematological toxicity directly related to the study medication (excluding nausea, vomiting and diarrhea), and neurotoxicity ≥ grade 2.

In case of toxicity or DLT, treatment was postponed for 1 week until recovery to CTC grade ≤1. Re-administration of study treatment occurred at a reduced DL.

Pharmacokinetics/pharmacodynamics

Regular blood sampling was performed to assess the pharmacokinetics of carboplatin and gemcitabine. For carboplatin pharmacokinetic analysis blood samples of 4 mL venous blood were collected in sodium-heparin tubes on day 1 at pre-dose, end of infusion (EOI) and 4.5h after EOI. Plasma was obtained by immediate centrifugation (10 minutes; 1,600 *g*). Plasma was transferred directly to a Centrifree® UF device with an Ultracel YM-T membrane filter (Millipore® Ireland Ltd, Co.Cork, Ireland) and centrifuged for 20 minutes at 1,500x *g*. The resulting plasma ultrafiltrate (pUF), representing the non-protein bound carboplatin fraction, was stored at -80ºC until analysis. Free platinum from patient 1-12 was determined using a validated Zeeman atomic absorption spectrometry method.[42] For the samples of patients 13 -23 a validated inductively coupled plasma mass spectrometry (ICP-MS) method was applied.[43]

To analyze plasma concentrations of dFdC and metabolite dFdU, blood samples of 4 mL venous blood were collected in sodium-heparin tubes on day 1 at pre-dose, after 1/3 of infusion, after 2/3 of infusion, EOI, 1h, 2h, 8h and 24h after EOI). Immediately after sampling, blood was transferred to propylene tubes containing 0.03 mL Calbiochem® tetrahydrouridine (THU), a potent competitive inhibitor of CDA. Plasma was obtained by immediate centrifugation (5 minutes; 1,600x *g*). Analytes were quantified using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS).[44]

Intracellular levels of gemcitabine triphosphate (dFdC-TP), the active metabolite of gemcitabine, were determined by LCMS/MS in peripheral blood mononuclear cells (PBMCs), which were isolated from venous blood samples[45] collected in 4 mL sodium-heparin tubes on day 1 at pre-dose, end of infusion, 1h, 2h, 8h and 24h after EOI. In short, buffy coats were collected from whole blood, and PBMC's were separated using Ficoll-Paque™ PLUS density gradient (GE Health Care Life Sciences, UK). A volume of 10 µL of cell suspension was used for the analysis of protein concentrations. The remainder of the cell suspension was used for protein precipitation by $HClO_4$ and extraction of the acid soluble dFdC-TP. The amount of protein was used for the calculation of dFdC-TP concentration in ng per mg protein.

Non-compartmental analysis has been employed for dFdC. Because of the sampling design the AUC could not be determined for dFdU and dFdC-TP only the maximal concentration (C_{max}) has been used.

For carboplatin a limited sampling design was employed and, therefore, PK parameters could not be estimated by non-compartmental models. Maximum a posteriori Bayesian estimates were generated for each patient using the POSTHOC option of NONMEM (version 7.2, ICON, Ellicot City, MD, USA) and a previously developed population PK model of carboplatin.[46]

Tumor response

Radiological disease assessments were performed by computed tomography (CT) scan or magnetic resonance imaging (MRI) at baseline and every 2 cycles. Tumor response was evaluated using RECIST 1.0.[47] CA-125 levels were monitored monthly and evaluated by the GCIG CA-125 response criteria.[48]

Statistical analyses

The primary endpoint of the study was to assess the safety, DLT and MTD of FDR gemcitabine in combination with carboplatin in patients with OC after first line treatment. Secondary endpoints consisted of assessing the concentration of dFdC-TP concentrations in PBMCs and pharmacokinetics of dFdC and dFdU in plasma, and to characterize the population pharmacokinetics (PopPK) of FDR gemcitabine plus carboplatin through the assessment of PK data of this study.

Non-linear mixed effects modeling and simulation

Population pharmacokinetic (PK)/pharmacodynamic (PD) modeling was performed using the non-linear mixed effect modeling software NONMEM (version 7.2, ICON, Ellicot City, MD, USA). For gemcitabine FDR a 2-compartment PK model was developed based on data of this clinical study. For carboplatin a previously developed PK model was used.[49] For the development of the PD part of the model, all PK parameters (random and fixed effects) were fixed while PK data were retained in the dataset according to the method for sequential PK/PD analysis described by Zhang et al.[50]

All data from all treatment cycles (dosing data, PK data, neutrophil and thrombocyte counts) were analyzed using the semi-physiological model for hematological toxicity as introduced by Friberg et al.[51] The effect of gemcitabine and carboplatin on the proliferation of neutrophils and thrombocytes was introduced using a slope model. Interindividual variability was estimated on the PD parameters mean transit time (MTT), drug effects and baseline counts. For each set of parameters related to neutrophil counts and thrombocyte counts covariance was estimated.

A simulation study was performed to assess the feasibility of different combinations of FDR gemcitabine and carboplatin. The parameters of the final PK/PD model were used to simulate 36 different DLs by combining 6 dose levels of carboplatin (AUC 1.5, 2, 2.5, 3, 3.5 and 4 mg/mL*min) with 6 dose levels of gemcitabine (300, 400, 500, 600, 700 and 800 mg/m^2). Covariance between MTT neutrophils and thrombocytes and between drug effect carboplatin on neutrophils and thrombocytes could not be estimated and, therefore, a 50% positive correlation was assumed. Body surface area and creatinine clearance were sampled from a log-normal distribution with a typical value of 1.8 m^2 (omega 15%) and 111 ml/min (omega 15%). For each DL 1000 patients were simulated for a period of 2 complete treatment cycles. Identical to the clinical trial, the ANC and platelet count were assessed prior to dosing, and in case of ANC < 1.5 x 10^9/L or 1500/mm^3) and/or platelets < 100 x 10^9/L or 100,000/mm^3 the dose was withheld. Subsequently, the means for the following parameters were calculated: dose intensity (defined as the percentage of the ratio administered dose versus intended dose), percentage of the 1st to 6th administered doses, the final carboplatin dose (AUC mg/mL*min) and FDR gemcitabine (mg/m^2) dosed in two complete cycles.

Results

Patients

In total 23 patients were included in the study, of which 22 patients were considered evaluable (one patient refused further treatment after a single dose, due to side effects of nausea and vomiting).

Baseline patient characteristics are presented in **Table 1**.The median age was 59.5 years (range 30-73 years). The majority of patients had an ECOG PS ≤ 1: PS 0: 6 patients (26%), PS 1: 12 patients 55%). Ten (43%) patients had received one line of prior therapy, five (22%) and eight (35%) patients received two or three prior lines of therapy respectively. Three patients (14%) had received radiation as part of treatment for OC (> 4

weeks) prior to study start. .

Patients received a median of 4 cycles per patient (range 1-9). The maximal number of cycles to be in best interest of the patient was decided by the treating physician and varied from 6-9 cycles. Seven patients received < 4 cycles, while 15 patients received > 4 cycles (of which seven patients received ≥ 6 cycles).

Safety

Of 23 patients, five (22%) patients received carboplatin resulting in AUC 2.5 mg/mL*min (including the one patient that received only a single dose and withdrew informed consent) and three (13%) patients a dose resulting in AUC 3 mg/mL*min, both in combination with FDR gemcitabine 300 mg/m^2. Fifteen (65%) patients were treated with carboplatin AUC 2 mg/mL*min and increasing DLs of gemcitabine FDR varying form 300 - 800 mg/m^2 (**Table 2**).

Adverse events that met the pre-defined DLT criteria were not encountered at any DL. However, frequent dose holds and dose reduction occurred at all DLs. To allow gemcitabine dose escalation the dose of carboplatin was de-escalated to a dose resulting in AUC 2 mg/mL*min. The main toxicity was bone marrow depression, especially neutropenia and thrombocytopenia (**Table 3**). Neutropenia (80% of the patients) and/or thrombocytopenia (40% of the patients) were the most prominent reasons for dose holds. A clear relationship between toxicity (and subsequent dose holds) and DL was absent (**Table 4**).

Seven patients required one or more blood transfusions (although not prohibited, growth factors were not administered).

Eight of 23 patients discontinued study treatment as a result of an AE: one patient responded well to treatment (unconfirmed PR), but tolerated the study medication poorly (nausea and vomiting) and discontinued treatment after 4 cycles, one additional patient stopped after a single dose because off of nausea and vomiting and withdrew informed consent, two patients developed ileus (which was interpreted as clinical PD), one patient

developed non-treatment related pneumonia, 3 patients developed an allergic reaction to carboplatin (1 patient after 4 cycles and two patients during the fifth cycle) and discontinued (reintroduction of study treatment with anti-allergic medication and/or carboplatin at a low infusion speed was not explored).

Anti-tumor activity

Four (17%) of 21 patients evaluable for response experienced a RECIST confirmed partial response (PR) as best response. All these patients also had a CA-125 response (defined as ≥ 50% reduction of CA-125). Eight additional patients reported a CA-125 response but had SD as best response according to RECIST. Twelve (57%) patients in total experienced stable disease according to RECIST. Four (17%) patients did not benefit from treatment and showed progressive disease (PD) at the first evaluation after 2 cycles.

Table 2. Dose levels. After initial dose escalation of carboplatin, the dose escalation scheme was adjusted. The carboplatin dose was reduced to a dose resulting in AUC 2 mg/mL*min, to allow dose escalation of FDR gemcitabine.

Carboplatin dose* (day 1, 8 and 15)	**FDR gemcitabine dose** (day 1, 8 and 15)**	**Patients (%) (n=23)**
AUC = 2.5 mg/mL*min	300 mg/m^2 (in 30 min)	5†(22%)
AUC = 3 mg/mL*min	300 mg/m^2 (in 30 min)	3 (13%)
AUC = 2 mg/mL*min	300 mg/m^2 (in 30 min)	6 (26%)
AUC = 2 mg/mL*min	450 mg/m^2 (in 45 min)	3 (13%)
AUC = 2 mg/mL*min	600 mg/m^2 (in 60 min)	3 (13%)
AUC = 2 mg/mL*min	800 mg/m^2 (in 80 min)	3 (13%)

* All carboplatin doses were administered as a 30 minute infusion

**The dose of FDR gemcitabine was administered 1h after the end of the carboplatin infusion

† 1 patient received only cycle 1 day 1 and then decided to discontinue study treatment

Table 3. Adverse events. Number of patients with related toxicity by highest grade per patient. Hematological toxicity was most frequently observed, especially thrombocytopenia, neutropenia and anemia. Most non-hematological toxicities consisted of alanine amino-transferase (ALT) and aspartate amino-transferase (AST) elevation. GGT = gamma glutamyl transpeptidase.

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Hematological toxicity				
Anemia		5	2	
Leucopenia			5	1
Lymphopenia			1	
Neutropenia		6	5	7
Thrombocytopenia	4	3	4	1
Non-hematological toxicity				
ALT increase	1	4	1	
Allergic reaction		3	2	
AST increase	1	3	1	
Atrial fibrillation		1		
Constipation			1	
Fatigue		1	1	
GGT			2	1
Ileus			2	
Nausea	1		1	
Pneumonia			1	
Pulmonary embolism				1
Vomiting	1		1	

Table 4. Dose intensity. Overview of the dose intensity per fixed dose rate (FDR) gemcitabine dose level, specified by the number of cycles per dose level (total number of patients), the percentage of planned doses and the reasons why treatment was not given. Upper part of table displays the reasons for day 1 dose not given, middle part: reasons for day 8 not given, down part: reasons for day 15 not given. PLT = platelets (thrombocytopenia); ANC = absolute neutrophil count (neutropenia); Liver refers to alanine amino-transferase (ALT) and aspartate amino-transferase (AST) elevation.

FDR gemcitabine	Carboplatin	Patients	Total number of cycles	Doses not given	**Not given day 1**	PLT	ANC	Liver	Other
(mg/m2)	(AUC mg/mL*min)	(n)	(n)	(%)	(n [%])	(n [%])	(n [%])	(n [%])	(n [%])
300	2.5	4	19	24%					
300	3	3	21	41%		1 (5%)	3 (14%)		
300	2	6	27	25%	4 (19%)		1 (4%)		
450	2	3	9	41%	1 (4%)				
600	2	3	12	28%					
800	2	3	12	33%					

FDR gemcitabine (mg/m2)	Carboplatin (AUC mg/mL*min)	Patients (n)	Total number of cycles (n)	Doses not given (n [%])	**Not given day 8** (n [%])	PLT (n [%])	ANC (n [%])	Liver (n [%])	Other (n [%])
300	2.5	4	19	24%	3 (16%)	1 (5%)	3 (16%)		1 (5%)
300	3	3	21	41%	14 (67%)	2 (10%)	12 (57%)		8 (38%)
300	2	6	27	25%	6 (22%)		4 (15%)	1 (4%)	1 (4%)
450	2	3	9	41%	8 (89%)		6 (67%)	1 (11%)	3 (33%)
600	2	3	12	28%	5 (42%)	2 (17%)		2 (17%)	1 (8%
800	2	3	12	33%	9 (75%	1 (8%)	8 (67%)	1 (8%)	

FDR gemcitabine (mg/m2)	Carboplatin (AUC mg/mL*min)	Patients (n)	Total number of cycles (n)	Doses not given (n [%])	**Not given day 15** (n [%])	PLT (n [%])	ANC (n [%])	Liver (n [%])	Other (n [%])
300	2.5	4	19	24%	10 (53%)	7 (37%)	4 (21%		1 (5%)
300	3	3	21	41%	8 (38%)	4 (19%)	6 (29%)		
300	2	6	27	25%	13 (48%)	5 (19%)	10 (37%)		3 (11%)
450	2	3	9	41%	3 (33%)		1 (11%)		1 (11%)
600	2	3	12	28%	5 (42%)	4 (33%)	2 (17%)		1 (8%)
800	2	3	12	33%	3 (25%)	3 (25%)	2 (17%)		

developed non-treatment related pneumonia, 3 patients developed an allergic reaction to carboplatin (1 patient after 4 cycles and two patients during the fifth cycle) and discontinued (reintroduction of study treatment with anti-allergic medication and/or carboplatin at a low infusion speed was not explored).

Anti-tumor activity

Four (17%) of 21 patients evaluable for response experienced a RECIST confirmed partial response (PR) as best response. All these patients also had a CA-125 response (defined as ≥ 50% reduction of CA-125). Eight additional patients reported a CA-125 response but had SD as best response according to RECIST. Twelve (57%) patients in total experienced stable disease according to RECIST. Four (17%) patients did not benefit from treatment and showed progressive disease (PD) at the first evaluation after 2 cycles.

Pharmacokinetics/pharmacodynamics

Plasma samples were obtained from 22 patients (in one patient blood sampling was discontinued due to difficulties in obtaining blood). Pharmacokinetics of carboplatin was not altered by the addition of FDR gemcitabine and measurements of total platinum concentrations in plasma ultrafiltrate revealed AUCs that approximated the target area under the carboplatin curves that were calculated using the Calvert formula (**Figure 1**).

Pharmacokinetics of dFdC and dFdU showed proportional increase of C_{max} and AUC with increase of the dose and this finding conform results described in literature. (See also **Table 5** and **Figure 2A** and **2B** and **Figure 3**.)

Mean maximal concentrations of active gemcitabine metabolite dFdC-TP concentrations in ng per mg protein measured in PBMC lysate of individual patients at different dose levels measured in PBMCs are presented in **Figure 4.** No obvious relationship between dFdC-TP concentrations and gemcitabine dose was observed.

Figure 1. Carboplatin pharmacokinetics. Distribution of measured carboplatin area under the curve (AUC) mg/mL*min, measured as platinum concentrations in ultrafiltrates, compared to calculated carboplatin AUC dose using the modified Calvert formula. Carboplatin dose levels consisted of (calculated) carboplatin dose level AUC 2 mg*mL/min (n=14), AUC 2.5 mg*mL/min (n=6), and AUC 3 mg*mL/min (n=3).

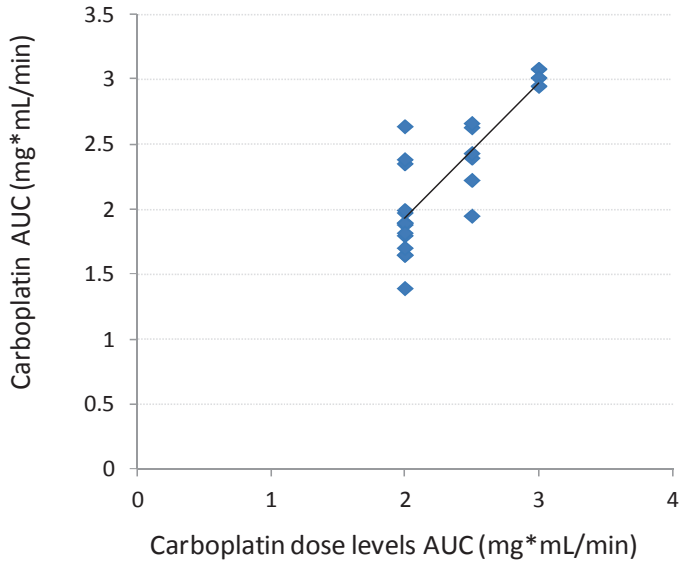

Table 5. Gemcitabine pharmacokinetics. Gemcitabine (2',2'-difluorodeoxy-cytidine, dFdC) and metabolite 2',2'-difluorodeoxyuridine (dFdU). Maximal concentration (C_{max}) and area under the curve (AUC) are presented per dose level.

	dFdC			
FDR gemcitabine dose level	**300 mg/m²**	**450 mg/m²**	**600 mg/m²**	**800 mg/m²**
Patients (n)	14	3	2	3
C_{max} (ng/mL)	4059 (2220 - 7270)	4310 (2290 - 6000)	5560 (4510 - 6610)	7047 (5320 - 8490)
AUC_{0-inf} (ng*h/mL)	2388 (919 - 3325)	3690 (2656 - 4698)	6765 (6744 -6786)	8935 (7097 - 10130)
	dFdU			
FDR gemcitabine dose level	**300 mg/m²**	**450 mg/m²**	**600 mg/m²**	**800 mg/m²**
Patients (n)	14	3	2	3
C_{max} (ng/mL)	9680 (5180 - 14900)	13833 (10800 -18600)	19100 (18700 - 19500)	24700 (23400 - 27000)

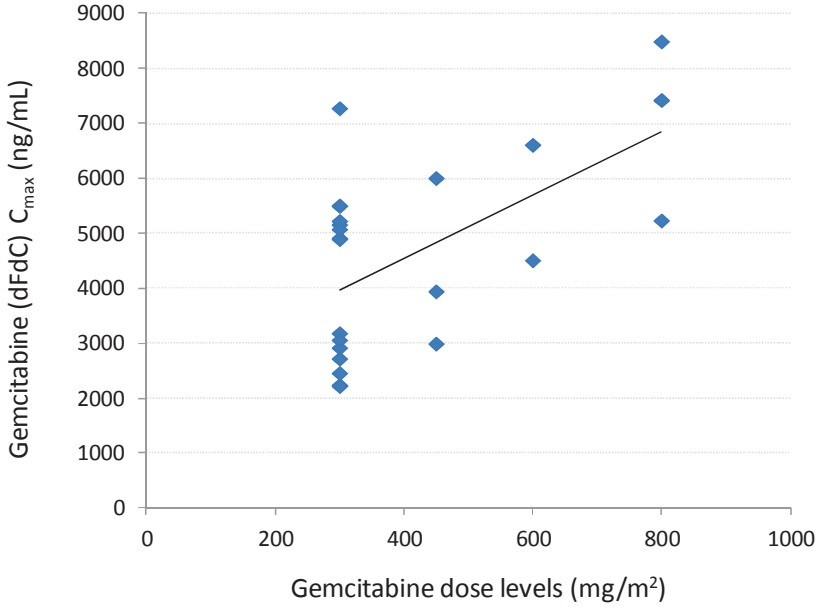

Figure 2A. Gemcitabine pharmacokinetics. Maximal concentration (C_{max}) of gemcitabine (2',2'-difluorodeoxycytidine, dFdC) per gemcitabine dose level (300, 450, 600 and 800 mg/m^2).

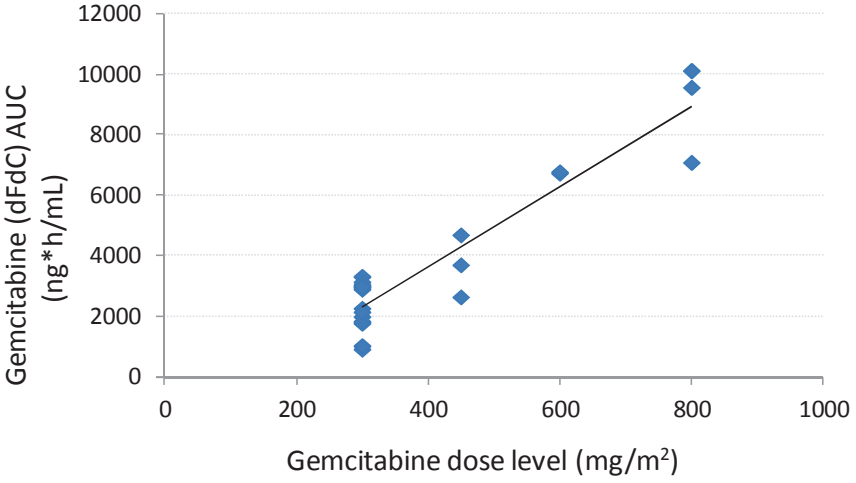

Figure 2B. Gemcitabine pharmacokinetics. Area under the curve (AUC) of gemcitabine (2',2'-difluorodeoxycytidine, dFdC) per gemcitabine dose level (300, 450, 600 and 800 mg/m^2).

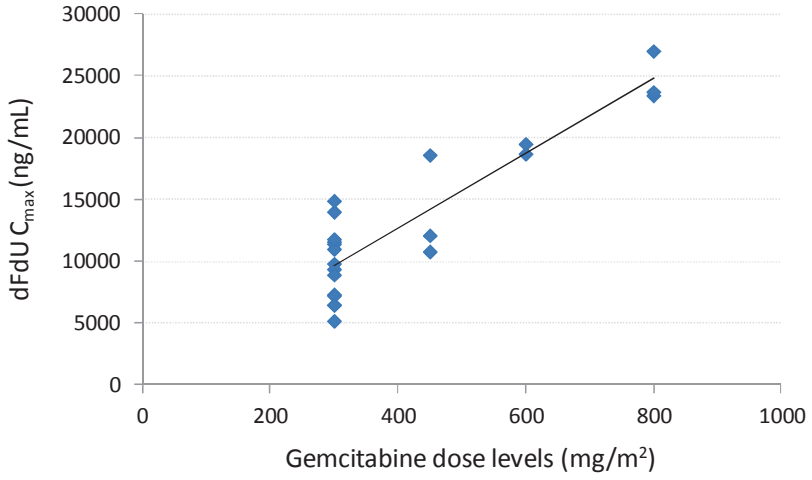

Figure 3. Gemcitabine metabolite pharmacokinetics. Maximal concentration (C_{max}) of gemcitabine metabolite 2',2'-difluorodeoxyuridine (dFdU) per patient per gemcitabine dose level: 300 mg/m^2 (n=14), 450 mg/m^2 (n=3), 600 mg/m^2 (n=2), and 800 mg/m^2 (n=3).

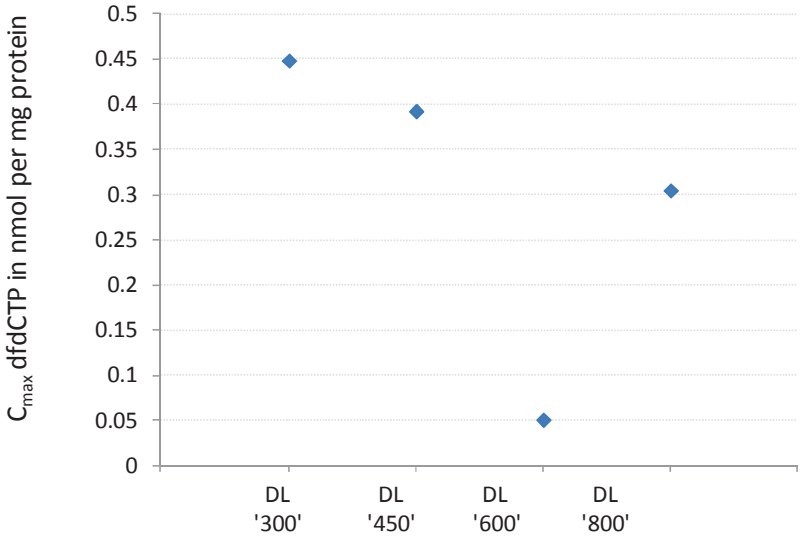

Figure 4. Mean maximal concentrations of gemcitabine metabolite gemcitabine triphosphate (dFdC-TP) lysate suspension in ng dFdC-TP per mg protein, presented per patient per gemcitabine dose level: 300 mg/m^2 (n=7), 450 mg/m^2 (n=2), and 800 mg/m^2 (n=3).

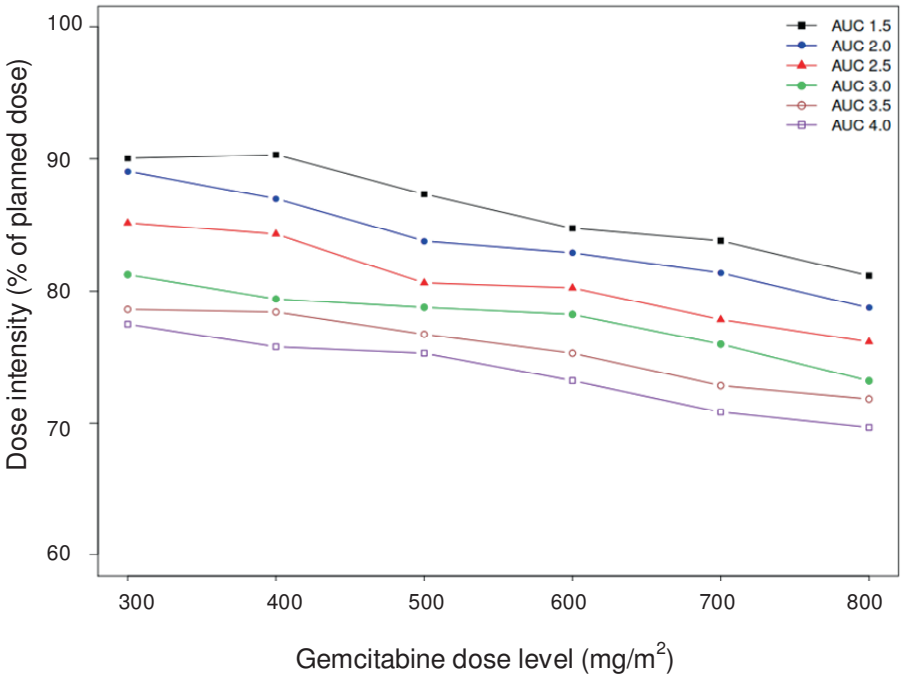

Figure 5. Non-Linear Mixed-Effects Modeling (NONMEM) population pharmacokinetics (PopPK) analysis of the schedule with fixed dose rate (FDR) gemcitabine plus carboplatin administered on days 1, 8 and 15 of a 28 days schedule performed in 1000 simulated patients. Dose intensity is quantified as the percentage of the administered versus the planned doses and calculated for different dose combinations of FDR gemcitabine and carboplatin.

Non-linear mixed effects modeling and simulation

Population PK/PD modeling and simulation demonstrated that the combination of gemcitabine FDR plus carboplatin both given on day 1, 8 and 15 at a 28 day cycle is not feasible in ovarian cancer patients after first line treatment within all potential DLs explored: different doses of carboplatin resulting in AUCs varying from 1.5 to 4 and FDR gemcitabine DLs 300 to 800 mg/m^2 (10 mg/m^2/min) (**Figure 5**) due to neutropenia and thrombocytopenia. The percentage of the planned dose is higher at the lowest FDR gemcitabine DL compared to the higher FDR gemcitabine DLs: a linear correlation is observed between the dose intensity and the gemcitabine FDR DL in combination with different DLs of carboplatin.

Discussion

Since the pharmacokinetics of FDR gemcitabine were investigated in the early nineties[35;52], the hypothesis held a promise of improved efficacy by prolongation of dCK saturation and increased intracellular dFdC-TP concentrations, and therefore the concept of FDR gemcitabine has been explored in many different settings since then.

In the late nineties, one of the first clinical studies published about FDR gemcitabine as a single agent for the treatment of solid tumors (by Brand et al.) showed a MTD of FDR gemcitabine of 1,500 mg/m^2 when administered on day 1, 8 and 15 of a 28 day cycle.[53] The schedule resulted in more severe myelosuppression (especially neutropenia and thrombocytopenia) than would be expected with gemcitabine administered as a conventional 30 minute infusion.[53]

Consistently increased hematologic toxicity with FDR gemcitabine was also observed by Tempero et al. in 2003 in a phase II study performed in advanced pancreatic adenocarcinoma patients, which compared FDR gemcitabine (1,500 mg/m^2 in 150 minutes) to gemcitabine as a 30 minutes infusion (at a dose of 2,200 mg/m^2) in a two arm study.[54] However, the study also showed an increased overall survival in favor of FDR gemcitabine of 8 versus 5 months, p = 0.013), which has encouraged the design of additional studies.

In addition, several studies have at present been performed with FDR gemcitabine as a single agent, especially in patients with ovarian,[55];[56] pancreatic, biliary and hepatocellular cancer, and these studies actually demonstrate 1) a varying degree of toxicity due to FDR gemcitabine: from tolerable and no additional toxicity to increased myelosuppression; 2) varying results regarding efficacy: from no additional effect to the majority of studies reporting promising effects with increased overall survival [OS] rates and the correct additional remark that further studies are warranted (because these studies were predominantly phase I and II studies, and therefore not designed to evaluate efficacy); 3) if PK sampling was

performed, an intracellular increase of active metabolite dFdC-TP was reported.[57-63] Especially chemonaive patients with unresectable pancreatic cancer seem a patient group that might benefit from FDR gemcitabine as a single agent.

Because of the therapeutic indications, the combination of FDR gemcitabine with carboplatin, the combination used in this study has predominantly been studied in non-small cell lung cancer (NSCLC) patients and ovarian cancer.

Gajra et al. published in 2001 data from a phase I study in chemonaive patients with advanced NSCLC treated with FDR gemcitabine plus carboplatin on day 1 and 8 in a 21 day cycle. The MTD was defined at FDR gemcitabine 800 mg/m^2 and carboplatin AUC 4 mg/mL*min with neutropenia and thrombocytopenia being DLTs. Grade 3-4 hematological toxicity was the most commonly observed AE: leucopenia (28%), neutropenia (22%) and thrombocytopenia (22%).[64]

4.1
FDR
NSCLC

Soo et al. (2003) performed a phase I study in chemonaive patients with advanced NSCLC treated with FDR gemcitabine on day 1 and 8 and carboplatin AUC 5 mg/mL*min on day1 in a 21 day cycle. Although the MTD was reached at 900 mg/m^2 the recommended phase II dose (RP2D) was 750 mg/m^2. Thrombocytopenia, liver failure and repeated dosing neutropenia were DLTs.[65] In addition, Soo et al. also published in 2006 about a phase II study in a similar patient group and treated with either FDR gemcitabine 750 mg/m^2 or standard gemcitabine 1000 mg/m^2 on day 1 and 8 and carboplatin AUC 5 mg/mL*min on day 1 in a 21 day cycle. Although PK analysis showed increased intracellular delivery of dFdC-TP for the FDR rate arm, response rates, toxicity and quality of life scores were comparable for both treatment arms. [66]

Finally, in 2007 Wang et al. reported about a phase II study in chemonaive advanced NSCLC patients treated with either FDR or standard gemcitabine 1,200 mg/m^2 on day 1 and 8 of a 21 day cycle. No statistical difference in toxicity between the two arms ($p > 0.05$) was observed. The incidence of overall grade 3-4 hematological toxicity consisted of: thrombocytopenia

43% and 38%, neutropenia 33% and 24%. No important differences in survival were observed.[67]

Altogether it can be concluded that studies performed in NSCLC patients with FDR gemcitabine and carboplatin show increased toxicity compared to standard dose gemcitabine, but no improved efficacy.

In 2009 Alvarez et al. published the results of a phase I study in platinum sensitive ovarian cancer patients treated with different schedules of FDR gemcitabine plus carboplatin.[68] The MTD for FDR gemcitabine was 1000 mg/m^2 (in 100 minutes) given on day 1 and 8 in combination with carboplatin AUC 5 mg/mL*min on day 1 every 21 days. In a slightly different schedule, with carboplatin AUC 2.5 mg/mL*min administered both on day 1 and 8, the MTD for FDR gemcitabine was also 1000 mg/m^2 (in 100 minutes), which is not surprising because the total amount of carboplatin administered per cycle at these MTD DLs is the same for both schedules. FDR gemcitabine 1000 mg/m^2 on day 1 in combination with a single dose of carboplatin AUC 2.5 mg/mL*min on day 1 every 14 days was not considered feasible due to toxicity. DLTs consisted of myelosuppression. Grade 3-4 AEs consisted of neutropenia (82%), thrombocytopenia (50%) and grade 3-4 fatigue (28.4%). Taking into account the limited size of the study, preliminary efficacy results seemed to be comparable to standard-dose gemcitabine results.[69]

Our study proofed that a 28 day cycle with FDR gemcitabine and multiple carboplatin doses is not feasible because of dose holds due to bone marrow toxicity, and this has been demonstrated in actual patients by dose escalation and confirmed by PopPK. PopPK used the data of the patients included and gathered information about virtual additional patients by using simulations. Without actually including additional real patients it could be determined with significantly increased precision that this combination of drugs in this applied schedule (3 weeks both drugs followed by one rest week) was not feasible, whatever combination of dose would have been chosen. If the dose of carboplatin is increased, the dose of gemcitabine as a consequence will need to be decreased and vice versa.

Because of the potentiating effect of FDR gemcitabine, administering FDR gemcitabine three-weekly is too ambitious when combined with another myelotoxic drug like carboplatin. Even with gemcitabine administered as a standard 30 minute infusion, the original day 1-8-15 schedule in daily practice often results in a day 1-8 schedule due to bone marrow depression or transient elevated liver enzymes. Although the study from Alvarez et al. also used the FDR gemcitabine and carboplatin combination, similar to this study, but in three different schedules, the two feasible schedules both used 21 day cycles with FDR gemcitabine administered on day 1 and 8. But even in such a schedule, increased hematological toxicity can be expected.

Overall, the concept of FDR gemcitabine compared to standard 30 minute infusion results in increased toxicity. A logical explanation would be a more efficient metabolism of dFdC and higher intracellular dFdC-TP levels. However, this has not been observed.

A factor contributing to toxicity in this patient group might be that first line therapy of ovarian cancer consists of carboplatin plus paclitaxel, which also induces significant myelosuppression, and might contribute to a permanent or long lasting increased sensitivity to bone marrow toxicity. The pancreatic studies with FDR gemcitabine were mostly performed in chemonaive patients, and might explain the tendency to an improved tolerability of FDR gemcitabine compared to ovarian cancer patients.

The preliminary efficacy results from this study, like the study from Alvarez et al. are not drastically altered by the use of FDR gemcitabine compared to standard gemcitabine given as a 30 minute infusion, but the size of the studies is small and the studies were not designed to evaluate efficacy. A gradual increase with a peak concentration a bit later due to the extended infusion time and a higher maximal concentration over a prolonged time period would have been expected with FDR gemcitabine, compared to gemcitabine administered as a 30 minute infusion. Especially, an increase of maximal concentration at the higher dose levels was anticipated. The reason for these results is unclear at present, but since the pharmacokinetic results of dFdC and dFdU are normal, it is likely that the cause will be found

in the method used to determine protein in PBCMCs, used for the measurement of dFdC-TP. Variable contamination with red blood cells interferes with the accurate determination of protein levels. The dFdC-TP levels are calculated relative to the protein dFdC-TP levels, which are expressed in mg of protein. Further investigation is warranted.

Nevertheless, the concept of FDR gemcitabine still remains interesting and promising results with FDR gemcitabine have been obtained in combination with other anti-cancer agents and include: FDR gemcitabine as a single agent or plus for instance capecitabine in pancreatic cancer[70;71], FDR gemcitabine plus erlotinib in pancreatic cancer[72;73], FDR gemcitabine plus paclitaxel in breast cancer patient (pretreated with anthracyclins)[74], and FDR gemcitabine plus pemetrexed in advanced solid tumors.[75] In ovarian cancer the combination of FDR gemcitabine and pegylated liposomal doxorubicin has been explored and the patients with higher baseline levels of dCK reported longer progressive-free survival.[76]

In conclusion: this study showed that the combination of FDR gemcitabine plus carboplatin on day 1, 8 and 15 is not feasible because of increased myelosuppression and subsequent repetitive dose holds, due to the same potentiating effect of FDR gemcitabine. PopPK demonstrated that other dose combinations of gemcitabine and carboplatin in this schedule are also not feasible: increasing carboplatin or gemcitabine dose will result in excessive toxicity. A two weekly schedule (chemotherapy on day 1 and 8) instead of a three weekly schedule might be more feasible.

Acknowledgements

The authors thank patients and staff at the NKI, along with Eli Lilly for kindly providing the gemcitabine, and the people from the department of Pharmacy & Pharmacology in the Slotervaart Hospital in Amsterdam, the Netherlands, who performed the pharmacokinetic and pharmacodynamics analyses: Hilde Rosing, Ciska Koopman (carboplatin), Lianda Nan en Abadi Gebretensae (dFdC, dFdU), Ellen J. Derissen and Robert S. Jansen, and Matthijs Tibben (dFdC-TP and Bradford).

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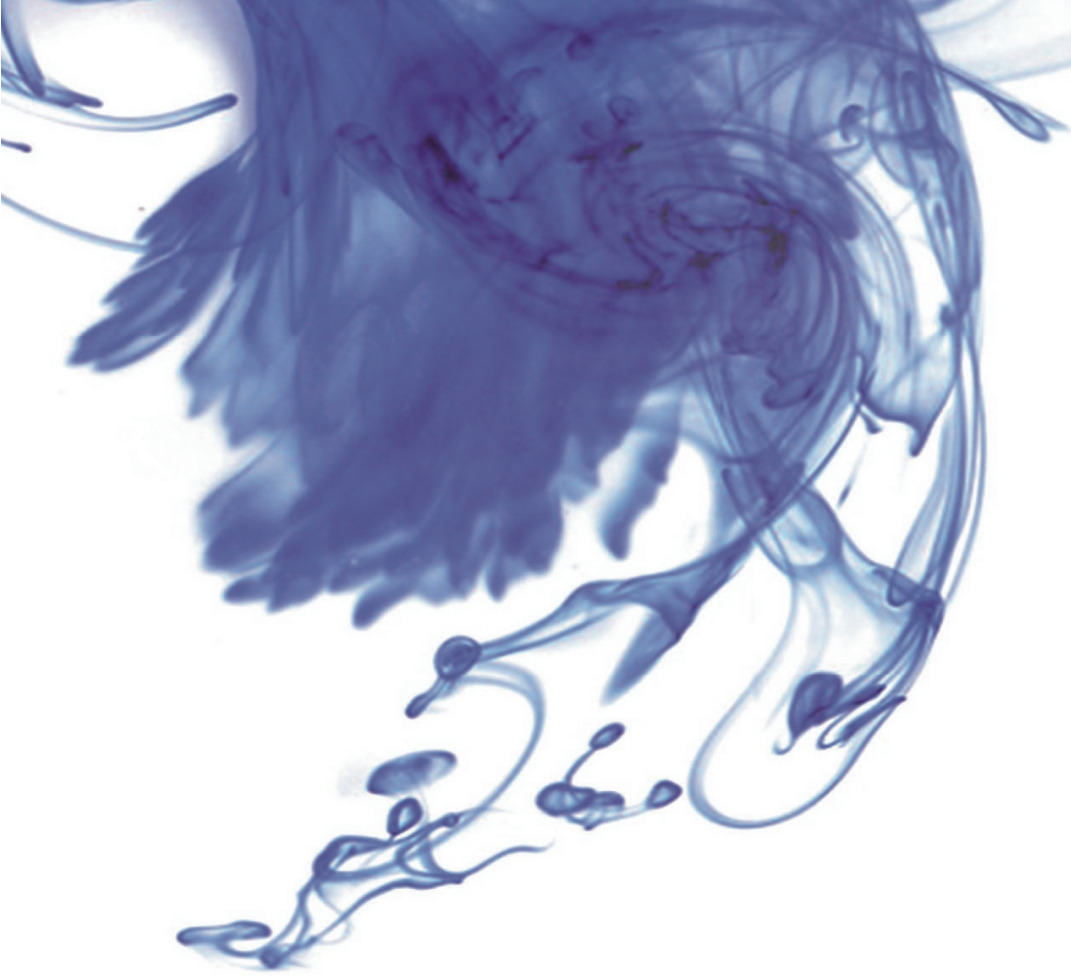

CHAPTER 5

Clinical pharmacological combination study with ruthenium anti-cancer agent NAMI-A

5.1

Phase I/II Study with ruthenium compound NAMI-A and gemcitabine in patients with non-small cell cung cancer after first line therapy

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Preliminary analysis

Abstract

Purpose

This phase I/II study was designed to determine the maximal tolerable dose (MTD), dose limiting toxicities (DLTs), antitumor activity, the pharmacokinetics (PK) and pharmacodynamics (PD) of ruthenium compound NAMI-A in combination with gemcitabine in Non-Small Cell Lung Cancer (NSCLC) patients after first line treatment. This patient group was selected based on promising antimetastatic results with NAMI-A and gemcitabine in solid lung tumor mouse models.

Patients and methods

Initial dose escalation of NAMI-A was performed in a 28 day cycle: patients received NAMI-A as a 3 h infusion through a port-a-cath at a starting dose of 300 mg/m^2 at day 1, 8 and 15, in combination with gemcitabine 1000 mg/m^2 at days 2, 9 and 16. Subsequently, dose escalation of NAMI-A in a 21 day schedule was explored: patients received NAMI-A as a 3 h infusion through a port-a-cath at a starting dose of 450 mg/m^2 at day 1 and 8, in combination with gemcitabine 1000 mg/m^2 at days 2 and 9. At the MTD dose level of this schedule an expansion group was enrolled consisting of 19 patients, of which 15 patients were evaluable for response according to Response Evaluation Criteria In Solid Tumors (RECIST) version 1.0.

Results

Due to frequent neutropenic dose interruptions in the third week, the 28 day schedule was amended into a 21 day schedule. The MTD for the 28 day schedule was 300 mg/m^2 of NAMI-A and for the 21 day schedule it was higher: 450 mg/m^2 of NAMI-A. Main adverse events consisted of neutropenia, anemia, elevated liver enzymes, transient creatinine elevation, nausea, vomiting, constipation, diarrhea, and fatigue. In the dose expansion cohort 9 patients (60%) out of 15 patients evaluable for response showed stable disease (SD) for at least 6 weeks as best response, whereas 6 patients (40%) showed progressive disease (PD).

Conclusion

NAMI-A administered as a 3 h infusion at a dose of 450 mg/m^2 at day 1, and 8 in combination with gemcitabine 1000 mg/m^2 at days 2 and 9 of a 21 day schedule is moderately tolerated and moderately active in NSCLC patients after first line treatment.

Introduction

Platinum compounds like cisplatin, carboplatin and oxaliplatin are powerful anticancer drugs, active against a variety of tumor types and widely used, but also associated with substantial side effects and primary or secondary developed resistance.[1-5] This has encouraged the search for new metal-based anti-cancer drugs with increased efficacy besides a more favorable toxicity profile and with the aim to overcome platinum resistance.[6-8] For the past decades, ruthenium (Ru), a transition metal of group 8 of the periodic table, has been considered an attractive candidate for this purpose,[9-11] because of some unique biochemical properties that theoretically might apply to ruthenium derived anti-cancer drugs when used in the human setting: 1) transferrin transport, 2) activation by reduction (when in the 3+ oxidation state), 3) slow ligand exchange kinetics, and 4) DNA binding.[12-15]

By mimicking iron, ruthenium can bind to serum transferrin and albumin, which are proteins involved in the solubilization and transport of iron in plasma.[16-19] As a result of increased metabolism and a higher mitotic activity, cancer cells often require more iron and therefore the expression of transferrin receptors on the cell surface of cancer cells is generally increased compared to healthy cells.[20] The upregulated receptor density would result in enhanced uptake and accumulation of ruthenium complexes, especially in tumor masses.[21]

Another mechanism is believed to contribute to preferential uptake in tumor tissue is called activation by reduction.[22] Tumor masses are often characterized by poorly organized blood circulation and low oxygen levels, leading to glycolysis as the preferred metabolic route (instead of Kreb's cycle) and an environment with a low pH.[23-26] Ruthenium complexes could be considered pro-drugs: in the relative inert +3 oxidation state (Ru(III)) they are supposed to circulate almost intact in the blood, until they are reduced to the more reactive +2 oxidation state.[27] In tumor tissue, due to the more reducing environment, re-oxidation of Ru(II) to Ru(III) is less likely to occur, thus leading to an accumulation of active species.

[17;28] This would not only imply selective efficacy, but also selective toxicity.[16;17;29]

Most administered metal drugs undergo spontaneous modifications prior to reaching the target (typically, some ligands are released) and therefore ligand exchange kinetics in ruthenium compounds is an important factor. Similar to platinum drugs, the ligand exchange kinetics is relatively slow (in the range of minutes to days, instead of microseconds to seconds) contributing to their general inertness and preventing rapid equilibration reactions.[30-33]

Although both platinum and ruthenium compounds bind to DNA, the binding mode differs substantially.[17] Platinum anti-cancer drugs exhibit square-planar geometry and are able to form various crosslinks with DNA. Ruthenium(II) and ruthenium(III) complexes often display octahedral or pseudo-octahedral structures, something that was initially considered to sterically hinder stable DNA binding.[34-36] Nevertheless, it was commonly believed that DNA damage –as a consequence of DNA binding– was the main target for ruthenium based drugs.[37-39] The *in vitro* binding capacity of some ruthenium compounds is strong, (e.g. RM175) and the formed adducts sometimes quite resistant to cell repair mechanisms, while other ruthenium compounds are hardly capable of binding to DNA (e.g. RAPTA-T and DW1/2).[40-42] In other words, different ruthenium compounds bind differently to DNA and, despite intensive research, it still remains to be elucidated to what extend DNA binding is responsible for their mechanism of action.[43]

Imidazolium-*trans*-tetrachloro(dimethylsulfoxide)imidazoleruthenium(III) ($C_8H_{15}Cl_4N_4ORu(S)$ or NAMI-A (acronym for Novel Anti-tumor Metastasis Inhibitor A) is the first ruthenium derived anti-cancer drug to have entered clinical evaluation.[44]
NAMI-A is an imidazolium salt (replacement of Na^+ by ImH^+) of the earlier developed NAMI ($Na[trans\text{-}RuCl_4(DMSO)\text{-}(Im)]$; NAMI-A is a non-hygroscopic compound with improved stability in solid state and good water solubility.

Properties, effects and proposed mechanisms of action of NAMI-A include the following:
Metastasis control: a) limiting actin dependent adhesion *in vitro* [45;46]; b) limiting *in vitro* tumor cell motility by cytoskeleton remodeling: activation of collagen receptor integrin β1 on the cell surface results in RhoA activation and subsequently to rearrangement of the cytoskeleton *in vitro*.[47-50]; c) anti-invasive effect *in vitro* and *in vivo* by promoting capsule formation: NAMI-A increases the extracellular matrix around tumor cells and tumor vasculature by triggering fibrotic reactions, regulates TGFβ1 expression, binds to collagen and stimulates collagen production. [51-55]; d) anti-angiogenic effect (e.g. NAMI-A inhibits *in vitro* the angiogenic effect induced by vascular endothelial growth factor [VEGF])[56;57];
It transiently blocks cell cycle progression *in vitro* at G_2M phase.[58-60] The mechanism might be activation of Chk1, resulting in inhibition of CDC25 and subsequently in inactive phosphorylated CDC2 thereby preventing mitotic entry.[61];
In vitro it inhibits the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway and c-myc transcription. [62-64]

DNA binding: although intrastrand adduct formation of NAMI-A is significantly less than of cisplatin, Ru-G and Ru-AG intrastrand adducts were observed *in vitro*.[65] The AG:CG adduct ratio was four times higher for NAMI-A compared to cisplatin. NAMI-A sporadically forms interstrand crosslinks, whereas the formation of DNA protein crosslinks is comparable to cisplatin.[66] Although the cytotoxic effect of NAMI-A (contrary to cisplatin) is not remarkable (on average 1053 times less than cisplatin) [67-69], the cytotoxicity has been found to be correlated with DNA binding (which is also the case for cisplatin).[70]

Impressive pre-clinical results were observed with NAMI-A in lung tumor mouse models in which NAMI-A was especially active against tumor metastases and reduction of lung metastases was followed by increased life-expectancy. [71-75] Based on the clinical results of gemcitabine in

combination with platinum containing regimens in non small cell lung cancer (NSCLC) patients, preclinical mouse studies with intravenously administered NAMI-A and gemcitabine were performed (data on file). Based on these promising preclinical results this clinical phase I study with NAMI-A and gemcitabine in NSCLC patients was initiated.

Because painful thrombophlebitis with scar formation was a known adverse event in the previously conducted phase I study with NAMI-A administered as a single agent,[76] and because the first patient in this study also developed phlebitis while NAMI-A was administered as a peripheral infusion, it was decided that all additional patients in this study would receive NAMI-A by infusion through a port-a-cath.

Methods

Patient selection

Patients were ≥18 years of age with confirmed histologic diagnosis of advanced NSCLC and previously treated with platinum containing therapy (i.e. cisplatin or carboplatin). All patients had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of ≤ 2, evaluable or measurable disease according to Response Evaluation Criteria In Solid Tumors (RECIST, version 1.0)[77], a life expectancy of ≥ 16 weeks, adequate hepatic function defined as alanine transaminase (ALT) and aspartate aminotransferase (AST) ≤ 2 upper limit of normal (ULN) (≤ 5 times ULN in case of liver metastases), adequate renal function defined as creatinine clearance (estimated using the formula of Cockcroft and Gault) ≥ 50 mL/min.

Treatment plan and study design

This phase I, open label, non-randomized, dose escalation study was performed at the Netherlands Cancer Institute (NKI) in Amsterdam, the Netherlands. The study received approval of the institutional medical ethical review boards and was conducted in accordance with the

Declaration of Helsinki and Good Clinical Practice. Written informed consent (IC) was given by all patients prior to inclusion in the study.
Initial dose escalation, in a traditional 3+3 design[78], was performed with NAMI-A administered as a 3 h infusion through a port-a cath at a starting dose of 300 mg/m^2 at day 1, 8 and 15, in combination with gemcitabine 1000 mg/m^2 as a 30 minutes infusion at days 2, 9 and 16. Subsequently, dose escalation of NAMI-A in a 21 day schedule was explored: patients received NAMI-A as a 3h port-a-cath infusion at a starting dose of 450 mg/m^2 at day 1 and 8, in combination with gemcitabine 1000 mg/m^2 at days 2 and 9. The phase II part of the study consisted of expansion of the 450 mg/m^2 MTD dose level of NAMI-A with gemcitabine 1000 mg/m^2 (as the 21 day schedule) by 15 evaluable patients.
Preparation of NAMI-A was consistent with the phase I study (NAMI-A monotherapy).[79]

Safety and assessments

Demographic data and medical history were collected during screening. Physical examination, vital signs and other safety assessments (ECOG-PS, registration of concomitant medication, hematology/biochemistry, and urine analysis) were performed at baseline and throughout treatment: day 1, 8, 15 –and 22 in the 28 day cycle– of every cycle. (During the study, based on toxicity results, the protocol was amended and the schedule changed from a 28 to 21 day cycle.)

The incidence and severity of adverse events (AEs) were evaluated and coded according to the National Cancer Institute Common Terminology Criteria (CTC) version 3.0.[80] DLT definition included: 1) any grade 3 or higher hematological or non-hematological toxicity considered to be directly related to the study drug, 2) any repeated grade 2 hematological or non-hematological toxicity considered to be directly related to the study drug and requiring dose reduction, and 3) failure to administer > 75% of the planned dosage of the study drug during cycle one as a result of treatment-related toxicity.
In case of toxicity or DLT, treatment was postponed for 1 week until recovery to CTC grade ≤1. Re-administration of study treatment occurred at a reduced dose level.

Pharmacokinetics and pharmacodynamics

Regular blood sampling was performed to assess the pharmacokinetics of NAMI-A and gemcitabine. For NAMI-A pharmacokinetic analysis blood samples of 4 mL venous blood were collected in sodium-heparin tubes on day 1 at pre-dose, 1.5 h after start of infusion, end of infusion (EOI), EOI + 15 min, EOI + 30 min, 4 h, 6 h and 8 h, 24 h and 48 h after start of infusion. Plasma was obtained by immediate centrifugation (10 minutes at 4˚C, 1,500 *g*). Part of the plasma was transferred directly to a Centrifree® UF device with an Ultracel YM-T membrane filter with 30,000 Da molecular weight cut off (MWCO) (Millipore® Ireland Ltd, Co.Cork, Ireland) and centrifuged for 30 minutes at room temperature (RT) 1,500 *g*. The resulting plasma ultrafiltrate (pUF), representing the non-protein bound Ru fraction, and the plasma representing the total Ru concentration, were stored at -80ºC until analysis. Total and unbound Ru were determined using a validated Zeeman atomic absorption spectrometry method. Graphite furnace atomic absorption spectrometry (GF-AAS) with Zeeman correction actually measures ruthenium, but with the use of NAMI-A calibration curves the concentrations of total and unbound Ru concentrations are presented as NAMI-A levels in plasma and ultrafiltrates, respectively.[81]

To analyze plasma concentrations of gemcitabine (2',2'-difluorodeoxy-cytidine, dFdC) and metabolite 2',2'-difluorodeoxyuridine (dFdU), blood samples of 4 mL venous blood were collected in sodium-heparin tubes on day 1 at pre-dose, EOI, EOI + 30 min, 2h, 4h, and 6h after EOI, and 24h after start of infusion). Immediately after sampling, blood was transferred to propylene tubes containing 0.03 mL Calbiochem® tetrahydrouridine (THU), a potent competitive inhibitor of CDA. Plasma was obtained by immediate centrifugation (5 minutes; 1,600x *g*). Analytes were quantified using validated liquid chromatography tandem mass spectrometry (LC-MS/MS).[82]

Intracellular levels of gemcitabine triphosphate (dFdC-TP), the active metabolite of gemcitabine, were determined by LCMS/MS in peripheral blood mononuclear cells (PBMCs), which were isolated from venous blood samples collected in 4 mL sodium-heparin tubes on day 1 at pre-dose, EOI,

EOI + 2h according to a method previously described.[83] In short, buffy coats were collected from whole blood, and PBMC's were separated using Ficoll-Paque™ PLUS density gradient (GE Health Care Life Sciences, UK). A volume of 10 µL of cell suspension was used for the analysis of protein concentrations. The remainder of the cell suspension was used for protein precipitation by $HClO_4$ and extraction of the acid soluble dFdC-TP. The amount of protein was used for the calculation of dFdC-TP concentration in nanograms per milligram of protein.

Tumor response

Radiological disease assessments were performed by computed tomography (CT) scan or magnetic resonance imaging (MRI) at baseline and every 2 cycles, i.e. every eight or six weeks. Tumor response was evaluated using RECIST 1.0.[84] Although CT-scans were performed in all patients to evaluate response to treatment, per protocol, anti-tumor activity assessment was limited to patients in the expansion cohort (in which patients were treated with the MTD of NAMI-A plus gemcitabine). In the dose expansion cohort 15 patients were required with tumors that could be evaluated by Response Evaluation Criteria In Solid Tumors (RECIST) version 1.0. Patients with tumors that could not be evaluated according to the RECIST criteria were declared not evaluable for response evaluation.

Statistical analyses

The primary endpoint of the phase I part of the study was to establish the optimal dose of the combination of NAMI-A and gemcitabine for second line treatment of NSCLC. Secondary objective was to assess the pharmacokinetic profile of the combination of NAMI-A and gemcitabine and measuring the active gemcitabine metabolite dFdC-TP concentrations in PBMCs.

The primary endpoint of the phase II part of the study was to assess the antitumor activity and secondary endpoints were the safety, DLT and MTD of NAMI-A in combination with gemcitabine in patients with NSCLC after first line treatment. A Simon two-stage design with a stopping rule has been applied for this study [85], implicating that the first (phase I) stage of

the study was designed to establish the best tolerated dose of the treatment
combination in both the 21 and 28 day schedules, while in the second (phase II) stage patients were treated with the MTD defined in the first stage in order to collect activity data and additional information about the toxicity profile. The second stage, consisted of an initial 15 patients treated at the MTD. Based on response, the cohort could be expanded with 12 additional patients to a total of 27 patients. Expansion would occur in case of at least 1 response at the MTD cohort (activity of 5% or more). Prior to the study a cutoff point of at least 15% response rate in the second stage of the study obtained with this treatment administered at the MTD (in a group of 27 patients) was considered mandatory to consider this treatment of interest for further use: with 3 or less responses the treatment would be declared of insufficient activity, while with 4 or more responses the study would be declared of sufficient activity. This design has 80% power to retain the treatment as active if the response rate associated with this treatment would be 20% or more. Simon's minimax design has been used with parameters p_0=0.05, p_1=0.20, alpha=0.05 and beta=0.20.

Results

Patients

A total of 32 patients were included in the study. One patient included in the 450 mg/m^2 NAMI-A and gemcitabine 28 day dose escalation cohort never initiated study treatment, and was replaced. This patient was not included in any of the analyses. Patient characteristics are presented in **Table 1**. Median age of the patients was 57 years (range 40-73). The majority of patients had received 1 line of previous therapy (68%) prior to the study and all patients had an ECOG- PS of 0 or 1. A total of 31 cycles were administered. The number of patients per dose level and the number of cycles administered are summarized in **Table 2**.

Table 1. Baseline patient characteristics.

Total no. of patients	31	
Age		
Median (range)	57	(40-73)
Gender		
Male	18	58%
Female	13	42%
Race		
White or Caucasian	28	90%
Black	2	6%
Asian	1	3%
ECOG performance status		
0	14	45%
1	17	55%
2	0	0%
Tumor type		
Adenocarcinoma	19	61%
Squamous cell	5	16%
Large cell	7	23%
Diffuse bronchoalveolar	0	0%
Disease stage at diagnosis		
I	1	3%
II	4	13%
III	11	35%
IV	15	48%
Disease stage prior to study start		
III	1	3%
IV	30	97%
Previous lines of chemotherapy		
1	21	68%
2	8	26%
3	2	6%
Previous radiotherapy		
Yes	16	57%
No	12	43%

Unknown	3	
Previous surgery		
Yes	8	26%
No	23	74%
Tobacco Use		
Never	4	13%
Former	24	77%
Cigar	0	0%
Pipe	1	3%
Cigarette	2	6%

Table 2. Dose Levels. NAMI-A administration infusion through a port-a-cath was followed by intravenous gemcitabine administration in 30 minutes the next day. The gemcitabine dose was fixed at 1000 mg/m^2. In the 28 day schedule NAMI-A was administered on day 1, 8 and 15, and gemcitabine on day 2, 9 and 16. In the 21 day schedule NAMI-A was administered on day 1, and 8, and gemcitabine on day 2 and 9.

	Dose level			
	300 mg/m^2 28 days	**450 mg/m^2** 28 days	**450 mg/m^2** 21 days	**600 mg/m^2** 21 days
Study stage	1	1	1 and 2*	1
No. of patients	3	6	19	3
No. of cycles				
1	-	1 (17%)	4 (21%)	-
2	-	3 (50%)	8 (42%)	1 (33%)
3	-	-	2 (11%)	-
4	2 (67%)	2 (33%)	4 (21%)	2 (67%)
6	1 (33%)	-	1 (5%)	-

* =includes the 450 mg/m^2 21 days schedule dose escalation patients (stage 1) and 450 mg/m^2 21 days schedule expansion cohort patients (stage 2) of the study: 19 patients in total. Patients that participated in the dose escalation part were also included in the expansion cohort. Fifteen out of 19 patients had tumors that were evaluable according to the RECIST criteria.

Table 3. Adverse events per worth grade per patient. AEs that were clinically significant have been presented in this overview.

	Dose level				
	300 mg/m² 28days	450 mg/m² 28days	450 mg/m² 21days	600 mg/m² 21days	Total
Patients (n)	3	6	19	3	**31**
Hematology					
Anemia					**16 (52%)**
Grade 2			12 (63%)	2 (67%)	14 (45%)
Grade 3			2 (11%)		2 (6%)
Grade 4			1 (5%)		1 (3%)
Leucopenia					**12 (39%)**
Grade 2			7 (37%)		7 (23%)
Grade 3			4 (21%)	1 (33%)	5 (16%)
Neutropenia					**18 (58%)**
Grade 2	1 (33)%)	2 (33%)	5 (26%)	1(33%)	9 (29%)
Grade 3		2 (33%)	2 (11%)	1 (33%)	5 (16%)
Grade 4			3 (16%)		3 (16%)
Thrombocytopenia					**2 (6%)**
Grade 2		1 (17)%)	1 (5%)		2 (6%)
Chemistry					
ALT elevation					**17 (55%)**
Grade 2	1 (33%)	1 (17)%)	5 (26%)	2 (67%)	9 (29%)
Grade 3	2 (67%)	1 (17)%)	4 (21%)	1 (33%)	8 (26%)
AST elevation					**8 (26%)**
Grade 2	1 (33)%)		5 (26%)		6 (19%)
Grade 3		1 (17)%)		1 (33%)	2 (6%)
Creatinine elevation					**4 (13%)**
Grade 1		1 (5%)	1 (5%)		2 (6%)
Grade 2		1 (17)%)			1 (3%)
Grade 3			1 (5%)		1 (3%)
Gastrointestinal symptoms					
Constipation					**10 (32%)**

Grade 1		2 (33%)	6 (32%)		8 (26%)
Grade 2			1 (5%)		1 (3%)
Grade 3	1 (33%)				1 (3%)
Diarrhea					**6 (19%)**
Grade 1	1 (33%)	3 (50%)	1 (5%)	1 (33%)	6 (19%)
Nausea					**15 (48%)**
Grade 1	2 (67%)	2 (33%)	8 (42%)	1 (33%)	13 (42%)
Grade 2		2 (33%)		1 (33%)	3 (10%)
Vomiting					**12 (39%)**
Grade 1	1 (33%)	2 (33%)	7 (37%)	2 (67%)	12 (39%)
General symptoms					
Blisters fingers					**1 (3%)**
Grade 2				1 (33%)	1 (3%)
Fatigue					**15 (48%)**
Grade 1		2 (33%)	3 (16%)	1 (33%)	6 (19%)
Grade 2	1 (33%)	2 (33%)	3 (16%)	1 (33%)	7 (23%)
Grade 3			2 (11%)		2 (6%)
Pyrexia					**3 (10%)**
Grade 1			1 (5%)	1 (33%)	2 (6%)
Grade 2			1 (5%)		1 (3%)
Neurological symptoms					
Neuropathy					**6 (19%)**
Grade 1		1 (17%)	2 (11%)		3 (10%)
Grade 2		1 (17%)	1 (5%)		2 (6%)
Grade 3				1 (33%)	1 (3%)
Respiratory symptoms					
Pneumonitis					**1 (3%)**
Grade 3			1 (5%)		1 (3%)
Vascular and PAC related					
Thrombosis					**1 (3%)**
Grade 3			1 (5%)		1 (3%)
Thrombus PAC					**2 (6%)**
Grade 3			2 (11%)		2 (6%)
Obstruction PAC					**4 (13%)**

Grade 2	2 (11%)	2 (6%)
Grade 3	2 (11%)	2 (6%)
Allergic reactions to chemotherapy		
Allergic reaction to gemcitabine		**2 (6%)**
Grade 1	1 (5%)	1 (3%)
Grade 2	1 (5%)	1 (3%)

Safety

Thirty-one patients were evaluable for toxicity. The main treatment related adverse events per patient are presented in **Table 3**. In the highest dose level with 600 mg/m^2 of NAMI-A, and gemcitabine administered as a 21 day cycle neutropenia grade 3 was observed in all three patients and reason for dose holds in the second week. These toxicities fulfilled the DLT criteria. In the lower 450 mg/m^2 dose level of NAMI-A with gemcitabine given in a 28 day schedule, neutropenia was also frequently observed and reason for dose interruptions, especially in the third week. This observation was reason for an amended 21 day study schedule. Neutropenic dose interruptions were DLTs in the 450 mg/m^2 of NAMI-A with gemcitabine 28 day schedule that declared 300 mg/m^2 of NAMI-A with gemcitabine the MTD for the 28 day schedule, and in the 600 mg/m^2 of NAMI-A with gemcitabine 21 day schedule that declared the 450 mg/m^2 21 day schedule the MTD.

Overall, mild clinically significant hematologic toxicity occurred, mainly consisting of neutropenia (grade 2-4) and anemia (grade 2-4). Neutropenia grade 2-4 resulted in dose interruptions and dose reductions (and mostly occurred in the 600 mg/m^2 of NAMI-A with gemcitabine 21 day schedule and 450 mg/m^2 of NAMI-A with gemcitabine 28 day schedule).

The main non-hematological adverse events consisted of nausea or vomiting, constipation, diarrhea, transient creatinine elevation, elevated liver enzymes, and fatigue. CTC grade 3 creatinine increase occurred in four patients, all included at the 450 mg/m^2 of NAMI-A dose levels and was

transient in all patients. ALT and AST elevations were reason for frequent dose holds and dose reductions. Blister formation on the fingers was observed in one patient at the highest dose level consisting of 600 mg/m^2 of NAMI-A and gemcitabine in a 21 day schedule. (See also **Table 4**).

Not presented in table 3, but observed in a significant number of patients, were flushing after the NAMI-A infusion, the remarkable sudden and simultaneous onset of vomiting and diarrhea, and transient change in color of urine a few hours after the NAMI-A infusion. In contrast to urine discoloration observed with KP1339 (the more soluble sodium salt of K1019), where greenish urine discoloration was observed, the color of urine turned reddish, orange and pink after NAMI-A infusion.[86]

Anti-tumor activity

Anti-tumor activity was evaluated in patients in the phase II expansion cohort. In total 19 patients needed to be included to have 15 patients evaluable for response evaluation according to RECIST.[87] Four patients were not considered evaluable for response. One patient died of acute heart failure, not related to the study drugs, a week after having received one dose of both drugs. Two patients went off study prior to the first tumor evaluation due to (transients) creatinine elevation, and one patient had no tumor that was evaluable by RECIST. Of the 15 patients, 9 (60%) experienced stable disease (SD) as best response. The other 6 (40%) showed progressive disease (PD) after the first tumor evaluation.

In all patients that participated in the study (i.e. patients included in the phase I and II part) anti-tumor activity was observed in 15 (56%) out of 27 patients evaluable for response, consisting of partial remission (PR) in 1 patient (4%) and stable disease for at least 6-8 weeks in 10 patients (37%). The patient with PR was treated at the 300 mg/m^2 dose level of NAMI-A in the 21 day schedule. (See also **Table 5**).

Table 4. Dose delay, dose reduction and study discontinuation. Most common adverse events independent of grade are presented and the action that was taken regarding the study drugs NAMI-A and gemcitabine. One adverse event can be listed multiple times for one patient.

		Action									
		Action	Dose delayed				Dose Reduced				Discontinuation
Toxicity	**Total**	unknown	NAMI-A	Gemcitabine	Both	Total	NAMI-A	Gemcitabine	Both	Total	study
Anemia	**41**	40			1	**1**					
ALAT	**39**	32		1	4	**5**		2		**2**	
ASAT	**8**	7						1		**1**	
Creatinine	**4**	0			2	**2**					**2**
Constipation	**35**	34			1	**1**					
Diarrhea	**23**	23									
Fatigue	**58**	57			1	**1**					
Neutropenia	**30**	26			4	**4**			1	**1**	
Thrombocytopenia	**4**	2			2	**2**					

Table 5. Response evaluation. To assess anti-tumor activity of the combination NAMI-A and gemcitabine in non-small cell lung cancer (NSCLC) patients, response evaluation was limited to the phase II dose expansion cohort, in which patients were treated with the maximal tolerable dose (MTD) established in the phase I part of the study: 450 mg/m^2 NAMI-A and 1000 mg/m^2 gemcitabine administered in a 21 day schedule. The table contains the tumor responses of all patients (phase I and phase II).

	Dose level				
	300 mg/m^2 28 days	**450 mg/m^2** 28 days	**450 mg/m^2 21 days**	**600 mg/m^2** 21 days	Total
No. of patients	3	6	**15**	3	27
Partial response	1(33%)	-	**-**	-	**1 (3%)**
Stable disease	2 (67%)	2 (33%)	**9 (60%)**	2 (67%)	**16 (59%)**
Progressive disease	-	4 (67%)	**6 (4%)**	1 (33%)	**10 (37%)**
Not evaluable or not according to RECIST	Unknown	Unknown	4	Unknown	4 (12%)

Table 6. NAMI-A pharmacokinetic parameters. Maximal concentration (C_{max}), elimination half-life ($T_{1/2}$), area under the curve (AUC), clearance (Cl) and volume of distribution (V) at different dose levels for total NAMI-A in plasma and unbound NAMI-A in ultrafiltrates.

Dose level (mg/m^2)	n	C_{max} (µg/mL)	$T_{1/2}$ (h)	AUC_{0-48} (µg·h/mL)	Cl (L/h)	V (L)
Total NAMI-A concentrations						
300 mg/m^2 NAMI-A	3	**59.5** (46.2 - 71.1)	**65.3** (60.8 - 67.2)	**1870** (1370 - 1856)	**0.33** (0.27 - 0.44)	**30.8** (23.5 - 42.3)
450mg/m^2 NAMI-A	25	**95.4** (65.0 - 135.9)	**51.7** (41.6 - 73.8)	**2948** (2102 - 4078)	**0.30** (0.17 - 0.43)	**22.3** (12.2 - 32.8)
600 mg/m^2 NAMI-A	3	**114.3** (90.5 - 129.0)	**68.7** (60.0 - 80.0)	**3592** (3215 - 3841)	**0.30** (0.25 - 0.37)	**30.2** (22.6 - 44.2)
Unbound NAMI-A concentrations						
300 mg/m^2 NAMI-A	3	**0.98** (0.80 - 1.33)	**14.5** (13.7 - 16.0)	**7.61** (6.60 - 8.99)	**75.4** (66.7 - 90.8)	**1566** (1319 - 1791)
450mg/m^2 NAMI-A	25	**1.78** (1.1 - 2.6)	**15.2** (7.0 - 25.9)	**14.66** (8.65 - 25.66)	**62.1** (29.6 - 87.1)	**1342** (583 - 2477)
600 mg/m^2 NAMI-A	3	**2.78** (2.53 - 3.14)	**12.6** (7.7 - 16.8)	**19.58** (15.83 - 23.54)	**56.2** (43.3 - 75.8)	**1075** (836 - 1838)

Table 7. Gemcitabine pharmacokinetic parameters. Maximal concentration (C_{max}), area under the curve (AUC) for gemcitabine (2',2'-difluorodeoxycytidine, dFdC) and metabolite 2',2'-difluorodeoxyuridine (dFdU) in plasma.

Gemcitabine 1000 mg/m^2 (n = 31)	C_{max} (µg/mL)	AUC_{024} (µg·h/mL)
dFdC	12.3 (1.1 - 21.5)	7 (1.4 - 13.01)
dFdU	32 (23.0 - 45.2)	219.2 (137.1 - 359.0)

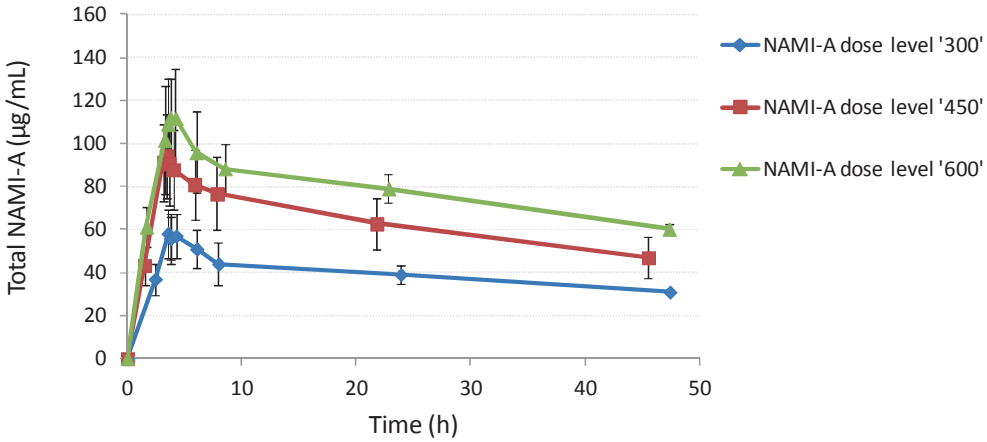


Figure 1A. NAMI-A pharmacokinetics. Mean concentration-time curves of total NAMI-A concentration measured in plasma at different dose levels. Blue line: 300 mg/m^2 NAMI-A dose level (28 day schedule), n=3. Red line: 450 mg/m^2 NAMI-A dose level (28 and 21 days schedule), n= 25. Green line: 600 mg/m^2 NAMI-A dose level (21 days schedule), n= 3. In all dose levels, gemcitabine was administered at a fixed dose of 1000 mg/m^2.

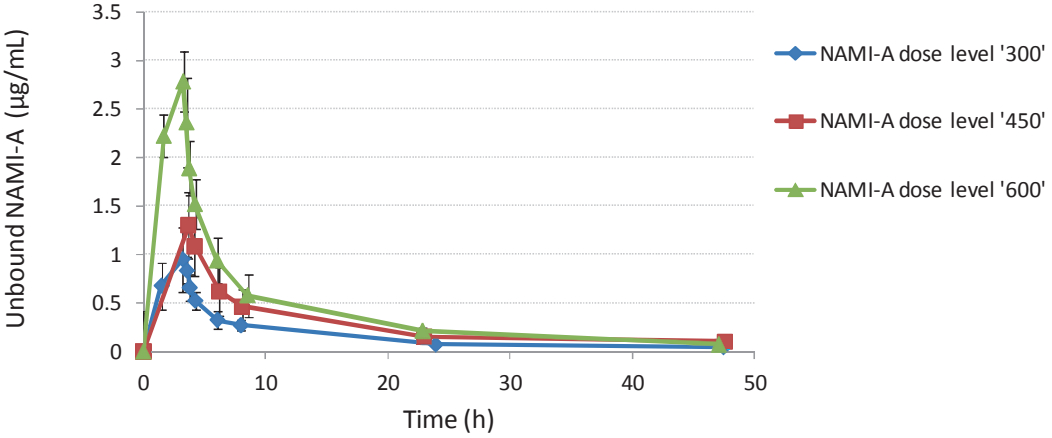


Figure 1B. NAMI-A pharmacokinetics. Mean concentration-time curves of unbound NAMI-A concentration measured in ultrafiltrates at different dose levels. Blue line: 300 mg/m^2 NAMI-A dose level (28 day schedule), n=3. Red line: 450 mg/m^2 NAMI-A dose level (28 and 21 days schedule), n= 25. Green line: 600 mg/m^2 NAMI-A dose level (21 days schedule), n= 3. In all dose levels, gemcitabine was administered at a fixed dose of 1000 mg/m^2.

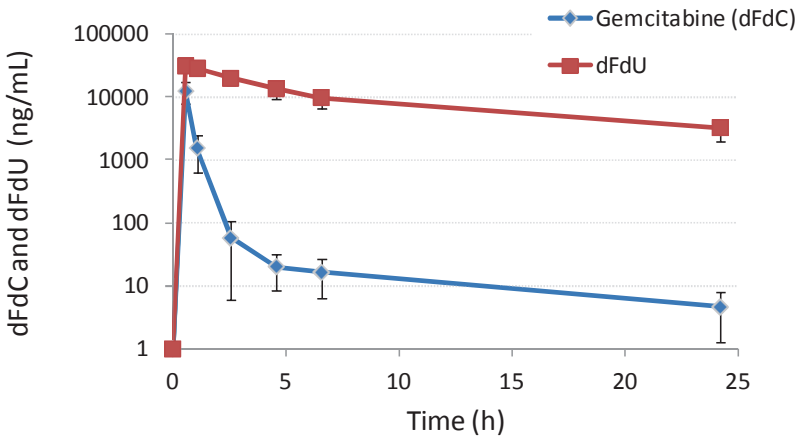
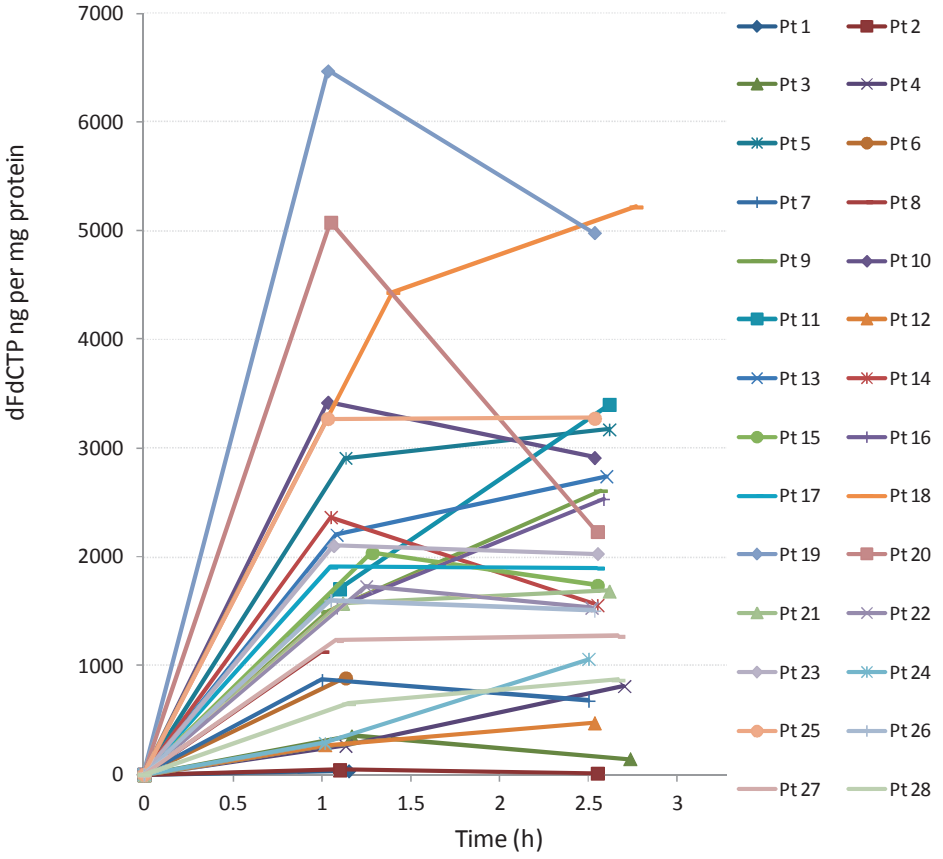


Figure 2. Gemcitabine pharmacokinetics. Mean concentration-time curves of gemcitabine (2',2'-difluorodeoxycytidine, dFdC) and metabolite 2',2'-difluorodeoxyuridine (dFdU) in plasma. In all different NAMI-A dose levels, gemcitabine was administered at a fixed dose of 1000 mg/m^2.

Figure 3. Gemcitabine triphosphate (dFdC-TP) concentration-time curves of individual patients (n= 28). Measurements of dFdC-TP were performed in peripheral blood mononuclear cells (PBMCs).



Pharmacokinetics/pharmacodynamics

Blood samples for the measurement of total and unbound ultrafiltrable ruthenium, dFdC, dFdU, and dFdC-TP in PBMCs were obtained in all patients.

Figures 1A and **1B** represent the plasma concentration time curves of total ruthenium in plasma and ultrafiltrate respectively during cycle 1 in patients receiving 300, 450 and 600 mg/m^2 of NAMI-A (also for additional parameters). $AUC_{0\text{-}48\ h}$ of bound and unbound NAMI-A was proportional to dose. Mean plasma clearance (Cl) of total and unbound NAMI-A overall dose levels was 0.31 L/h and 64.6 L/h respectively, and the mean terminal half life over all dose levels was 61.9 h and 14.1 h respectively (see **Table 6** for parameters of each individual dose level). These data are all in line with the results reported in the phase I single agent study with NAMI-A.[88]

Gemcitabine (dFdC) and metabolite dFdU time-concentration curves and parameters are presented in **Figure 2** and **Table 7**. Gemcitabine pharmacokinetics is not altered by co-administration of NAMI-A the previous day.

Active gemcitabine metabolite dFdC-TP concentration-time curves of the individual patients (n= 28) measured in PBMC lysates as dFdC-TP concentrations in ng per mg of protein are presented in **figure 3** and show a wide variability. Although sparse sampling has been performed (pre-dose, EOI + 30 min, 2h after start), mean C_{max} of 2500 ng dFdC-TP in PBMC lysate per mg of protein occurred after approximately 1 h, and lower values were found after 2 h. Overall, all values are higher than expected. Since the dFdC-TP values are correlated to the protein fraction (amount of dFdC-TP is expressed per amount of protein), low protein values will directly result in high dFdC-TP values. The reason for the wide variability with many low values in the protein values is unclear at present. One option that should be investigated is loss of cells due to clotting during the preparation process. Clotting might be caused by processing the samples at low temperature and/or the use of ice cold phosphate buffered saline (PBS) and cold Ficoll-Paque™ PLUS. Varying the temperature has resulted in better protein levels (data on file) but should be looked into deeper. A reason in the

processing is at this stage considered most likely, especially since plasma concentrations of dFdC and dFdU seem to be in the expected range.

Discussion

The efficacy results were not met in the second stage of the study. Although one patient in the dose escalation part of the study experienced a PR (300 mg/m^2 of NAMI-A 21 day schedule), no patients treated at the MTD in the expansion phase II part of the study experienced PR as a best response. In the extension cohort, at least one patient with a response was required to expand the cohort to 27 patients. As per protocol, the expansion with an additional 12 patients was therefore not performed and the treatment is declared to be insufficiently effective for further use. One patient in the dose escalation part (450 mg/m2 of NAMI-A 28 day schedule) experienced an unconfirmed PR with significant regression of lung lesions, however brain metastases increased. This is line with the assumption that NAMI-A does not cross the blood brain barrier.[89]
The concept of ruthenium and other non-platinum metal anticancer drugs has intrigued scientists for over 25 years. NAMI-A is a ruthenium compound that has been extensively studied in the preclinic and showed very promising anti-metastatic results in several mouse models.[90] Activity was especially detected against metastases and more prominent than the effect on the primary tumor.[91] Connective tissue formation around tumor metastases was observed and considered to be an important explanation for the effect of NAMI-A.[92] Furthermore, NAMI-A exhibited preclinically a mild toxicity profile, superior to cisplatin, and was well tolerated by beagle dogs and mice.[93;94]

Successful pharmaceutical formulation enabled to apply NAMI-A in the clinic: although NAMI-A is stable in solid state, in solution the compound degrades rapidly upon increasing the pH (relative stability with an estimated loss of 2% per hour is observed at pH 2-5) and hydrolysis of two chlorides from NAMI-A occurs within minutes at pH 7.4. A lyophilized formulation proved to be most suitable for parenteral use in the clinic.[95-

99] Fluid prepared for infusion is stable for 3.5 h at room temperature when protected from light.[100]

A phase I study with NAMI-A monotherapy given intravenously (as a 3 h peripheral infusion) for 5 consecutive days in a 21 day schedule showed blister formation on hands, fingers and toes to be DLTs. The MTD for this schedule was defined at 300 mg/m^2/day. Other main AEs included peripheral phlebitis at the infusion site, sensitivity reactions to NAMI-A, significantly disabling nausea and vomiting, diarrhea, grade 1 and 2 renal dysfunction (defined as increased creatinine levels) and fever. A pre- and post-hydration schedule was used to minimize nephrotoxicity. A linear relationship between dose and AUC was observed for total and unbound ruthenium, and the $t_{1/2}$ was 50 h (±19 h).

The toxicity profile of NAMI-A in combination with gemcitabine in this study is consistent with the single agent phase I study, with nausea, vomiting and diarrhea being the most prominent AEs. Transient nephrotoxicity (grade 3 creatinine elevation without ultrasound abnormalities) was observed in 4 patients and the reason to discontinue study treatment in 3 patients. Preclinically accumulation of NAMI-A has been observed in collagen rich tissue (e.g. lungs), liver but also kidney, and nephrotoxicity was observed with increased creatinine, and histological lesions of glomeruli and tubuli of mice, which fully recovered within 15-30 days.[101-105]

Fatigue 2-3 was also commonly observed, and in combination with the disabling nausea/vomiting and diarrhea, which often happened to occur simultaneously and with a sudden onset, this resulted in many patients experiencing the study as very exhausting and conflicting with the quality of life (QoL). AEs were scored by CTC grade and no QoL questionnaires were collected, which probably has underestimated the impact of the study on patients in the official study results, but is generally confirmed by the treating physicians and all the study personnel that had regular contact with the patients. The intense study schedule, of weekly administration of chemotherapy with the gemcitabine one day after the administrations of

NAMI-A contributed to the subjective intensity of the study. Preclinical studies with gemcitabine and NAMI-A used this schedule, and it was considered that additional preclinical studies (demonstrating the safety of applying both agents the same day in the preclinic) were needed to administer both agents on the same day (data on file).

Elevated liver enzymes and neutropenia, which are commonly encountered adverse events with gemcitabine therapy, were also reported in this study. In the initial 28 day cycle neutropenia grade 3 often occurred in the third week leading to dose interruptions followed by the rest week. The three weeks on, one week off 28 day schedule in practice resulted in a two weeks on, two weeks off schedule. Therefore the protocol was amended into a 21 days schedule. In the 21 day two weeks on, one week off schedule the neutropenic events often coincided with the rest week and the bone marrow was generally sufficiently recovered to receive the next cycle. This schedule not only allowed intensified dosing but also allowed a higher dosing, reflected in the higher MTD (450 mg/m^2 of NAMI-A compared to 300 mg/m^2 both in combination with gemcitabine 1000 mg/m^2). The 21 day schedule is therefore considered the better, more practical, more patient friendly and therefore the preferred schedule.

A significant number of patients experienced (multiple events of) upper extremity deep vein thrombosis (UEDTV) or PAC problems due to thrombosis or blood clot obstruction. It is unclear to what extend these events are related to NAMI-A, or if they can be fully attributed to common risk factors. Examples of well known risk factors include a PAC, immobility, cancer, advanced age, a recent transfusion, a history of thrombosis and co-morbidities like renal disease or infection.[106;107] A partial effect of NAMI-A seems to be possible, but further research is needed to establish the exact contribution of NAMI-A. After a few patients were treated with antithrombotic therapy for thrombosis, from patient 11 onwards all patients received prophylactically Fraxodi® low molecular weight heparin (LMWH) 0.3 mL daily (containing 19,000 IU anti-Xa/mL) as a subcutaneous injection.

In conclusion, NAMI-A in combination with gemcitabine is well tolerated according to the CTC criteria, but experienced by patients as a very intense treatment. Although NAMI-A should be considered a very elegant antimetastatic drug with a variety of mechanisms of action in the preclinic, a future role of NAMI-A as part of the arsenal of drugs available for physicians remains at present uncertain, due to the toxicity profile and the lack of convincing preliminary efficacy results. Nevertheless, additional trials in larger populations are needed to be able to draw definitive conclusions.

Acknowledgements

We would like to thank Fondazione CRTrieste and Commissariato del Governo – Fondo Trieste for providing financial support. We are greatly indebted to Ms Lidwina Wever and Dr. O. Dalesio of the trial office of The Netherlands Cancer Institute. Finally, we are thankful for the support of G. Dastoli MD, Eudax SrL, Pavia Italy and R. Bianchi, Aquisitio S.p.A., Milan Italy.

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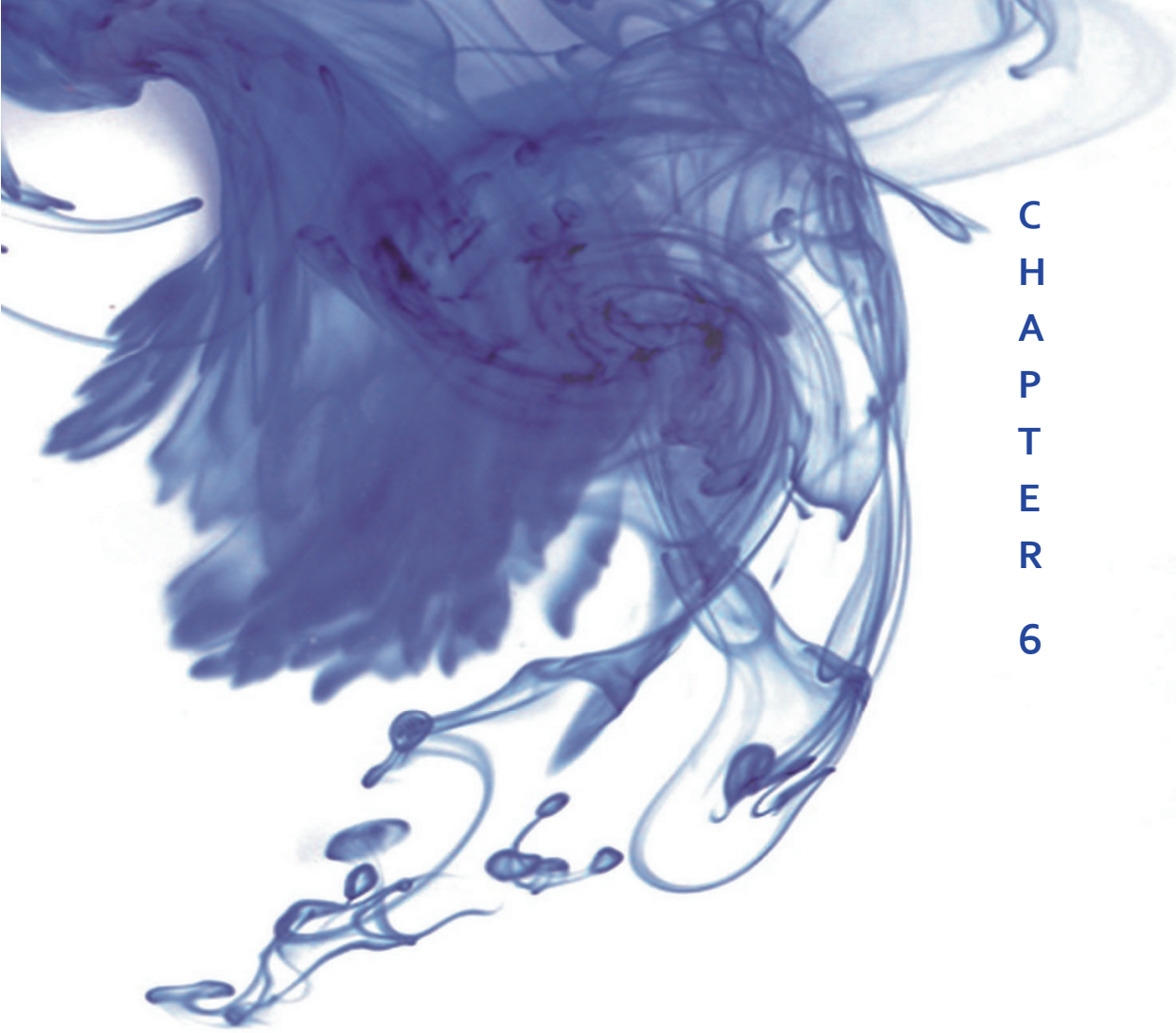


CHAPTER 6

Conclusions and Perspectives

6
Conclusions and Perspectives

Conclusions

The different studies described in this thesis consist of drug combinations, or drugs that in the future are expected to display increased efficacy in combination with other anti-cancer agents. As explained in chapter 1, with the introduction of new targeted anti-cancer drugs, the design of early clinical trials and approach for further development is changing rapidly. This change can also be observed by taking a closer look at the studies discussed in this thesis, by looking at the studies in the chronological order in which the set-up has taken place.

The first studies for instance, although with very interesting mechanisms of action are not typical molecularly targeted agents (NAMI-A study and the prolonged infusion study at fixed dose rate [FDR] with gemcitabine), while the newer ones are (MEK inhibitors selumetinib and RO4987655, and Wee1 inhibitor MK-1775). Furthermore, realizing the importance of trying to understand tumor biology and the changes that can be induced by anti-cancer drugs on a molecular level, including those that occur as a result of resistance needed some time to 'sink in' and become part of regular 'clinical practice' in early phase clinical trials, which is reflected in the studies. The earlier studies did not include fresh tumor biopsies (NAMI-A, and FDR gemcitabine study), for the phase I trial with MK-1775 tumor biopsies were optional, for MEK inhibitor RO4987655 study tumor biopsies were mandatory in the phase II part.

Nevertheless, all studies include excellent examples of key characteristics of modern early phase clinical trials: 1) intensive pharmacokinetic analysis (e.g. FDR gemcitabine and NAMI-A study); 2) modeling and simulation (FDR gemcitabine study); 3) The use of pharmacological biomarkers in multiple surrogate tissues (e.g. measurement of pCDC2 -direct substrate of Wee1- in the MK-1175 studies, and measurement of pERK inhibition in peripheral blood lymphocytes and tumor tissue in the RO4987655 MEK inhibitor study); and 4) measurement of metabolic activity with FDG-PET (RO4987655 MEK inhibitor study).

To evaluate the key findings of all the studies included in this thesis, an overview of the main conclusions is provided per study, followed by the perspectives for each investigational drug.

MEK inhibitor studies

The food effect study with MEK-inhibitor selumetinib demonstrated that the presence of food decreases the extend and rate of absorption of selumetinib hyd-sulphate capsule formulation, which lead to the recommendation that intake in further development should occur on an empty stomach.

In the phase I dose-escalation study, RO5987655 displayed a manageable safety profile with class effects similar to other MEK inhibitors, consisting of skin toxicity, diarrhea, and ocular toxicity. Predominantly asymptomatic creatinine phosphokinase (CPK) elevation was also observed. The pharmacokinetic and pharmacodynamic profile were favorable with confirmed target engagement measured as pERK inhibition in peripheral blood lymphocytes). The preliminary anti-tumor activity was promising. The ongoing analysis of the expansion part of the study conducted in specific cohorts with patients with RAS and/or RAF mutations will provide additional information regarding anti-tumor activity.

Wee1 inhibitor studies with MK-1775

The first in human study with Wee1 inhibitor MK-1775 in combination with gemcitabine, carboplatin or cisplatin showed that MK-1775 was well tolerated, with hematologic and gastrointestinal toxicity mainly observed in the multiple dosing regimen of combination therapy. The pharmacokinetic profile was favorable. Target engagement, measured as pCDC2 reduction, in skin biopsies was observed at the applied dose levels (DLs).

The preliminary analysis of the proof of principle study with MK-1775 and carboplatin in p53 mutated ovarian cancer and recurrence during or within 3 months of standard first line therapy showed promising results with a 31% response rate. Normalizations of CA-125 levels were observed in combination with partial remissions and near complete responses on CT-scans.

FDR gemcitabine

Phase I dose escalation study with fixed dose rate (FDR) gemcitabine and carboplatin as second line therapy in ovarian cancer patients showed that a schedule with both agents administered at day 1, 8 and 15 is too toxic, which was confirmed by modeling and simulation.

Ruthenium derivative NAMI-A

The NAMI-A and gemcitabine combination was explored in a phase I study in non-small cell lung cancer (NSCLC) patients after first line therapy and was moderately tolerated in a 3 week schedule. Gastrointestinal toxicities, hematologic toxicity, transient creatinine and liver enzyme elevation, and fatigue were most commonly observed adverse events. Pharmacokinetic evaluation showed that both gemcitabine and NAMI-A PK was unaffected by the combination. Anti-tumor activity was modest.

Perspectives

MEK inhibitors

Many MEK inhibitors display similar type of adverse events. Ocular toxicities are a known class effect, and were regularly observed with MEK inhibitor RO4987655. Further studies are therefore needed to investigate the long term safety.

Best preliminary efficacy results with MEK inhibitors were observed in melanoma patients, and although analysis of the phase II part of the study with RO4987655 is still ongoing, a similar trend is awaited. For this reason, further studies with MEK inhibitor RO4987655 are indicated and needed to develop this drug for the indication of melanoma, especially melanoma with N-RAS mutation. To do so a biomarker driven phase III study is of utmost importance. The set-up of the study could consist of a comparison with best supportive care if RO4987655 will be administered as last line of treatment, while comparison with registered treatment could be used in comparison to first or second line treatment. Interaction with anti CTLA-4 monoclonal antibody ipilimumab is currently unknown and should be investigated. In addition, potential benefit of (other) combinations needs to be demonstrated. If possible, preference is given to preclinical investigation of drug combinations in order to allow targeted clinical development.

Other options for a potentially favorable combination therapy that will be of interest to explore include a MEK inhibitor with addition to a BRAF inhibitor or with addition to an AKT/mTOR inhibitor; in melanoma and possibly other tumor types with RAS/RAF mutations. Again, the basis for the rationale should first be found preclinically and then followed by biomarker driven proof of principle studies in the clinic.

Wee1 inhibitor MK-1775

Different early stage biomarkers have been applied in the three-arm phase I study with MK-1775: the Wee1 signature and pCDC reduction in surrogate tissue. These were examples of pharmacological biomarkers. Further studies should address biomarker profiles for selection of patients for

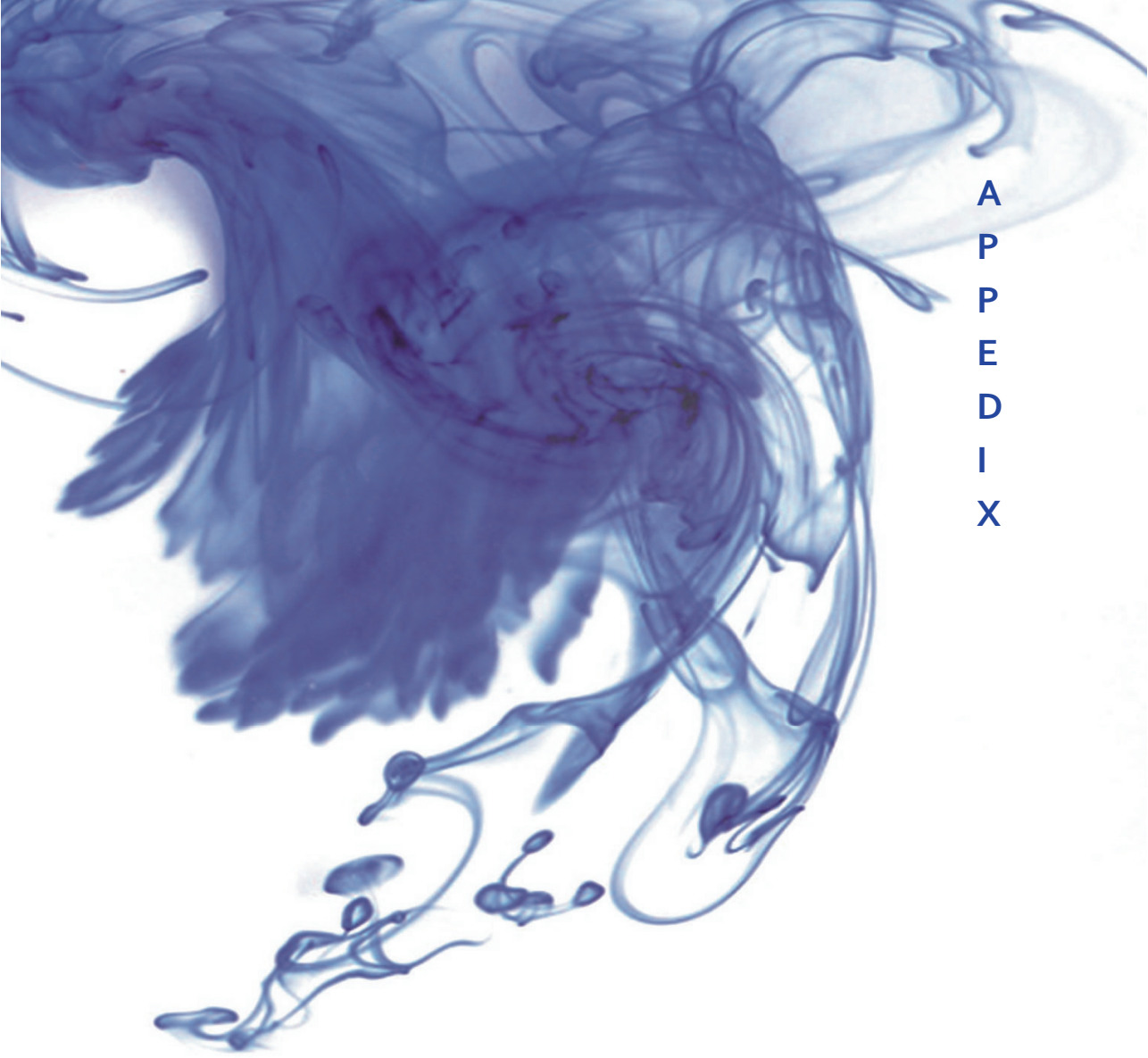
pivotal studies. For the proof of principle phase II study p53 mutation was a mandatory inclusion criterion. However, integrity of p53 pathway is not yet sufficiently determined and a functional assay would be of great benefit. The signal obtained with MK-1775 plus carboplatin in ovarian cancer is promising. Further development is therefore warranted.

FDR gemcitabine

The idea for FDR gemcitabine was studied preclinically prior to testing the hypothesis in the clinic. The applied schedule in combination with carboplatin showed too much toxicity in ovarian cancer patients. By using the obtained data and model all possible dose levels in a virtual, but large group of patients confirmed that exploring different dose levels in the same schedule, would not result in a tolerable therapy. This study demonstrated that modeling and simulations can be of great benefit in directing development of drug combinations based on toxicity and PD endpoints and therefore should be encouraged in other clinical trials with different compounds.

Ruthenium derivative NAMI-A

The scheme as used in the NSCLC study in combination with gemcitabine is toxic and not active enough for further development. Lack of an appropriate pharmacologic biomarker prohibits targeted clinical development which is considered to be a high risk of failure. Besides biomarker exploration additional preclinical studies are needed to address activity in combination with other agents.



APPENDIX

Chemical structures of investigated molecules

Summary

Nederlandse samenvatting (Dutch summary)

Dankwoord (acknowledgments)

Curriculum vitae

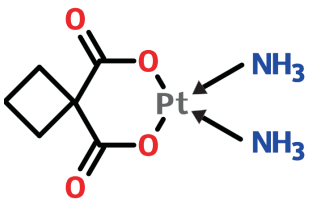
List of publications

Appendix

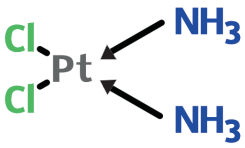
Chemical structures of investigated molecules

The chemical structures of the compounds used in this thesis are listed in alphabetical order.

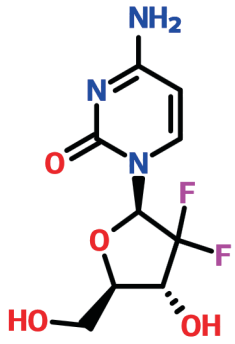
Carboplatin



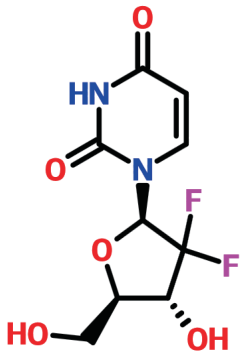
Cisplatin



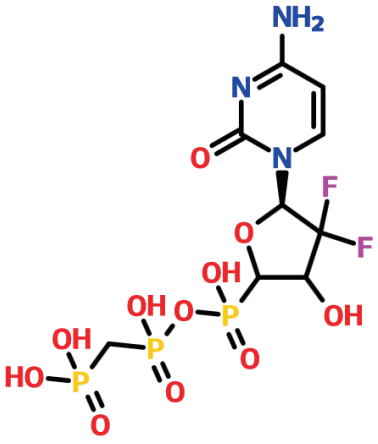
Gemcitabine (dFdC)



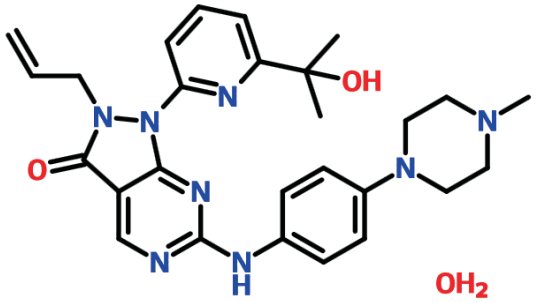
Gemcitabine metabolite dFdU



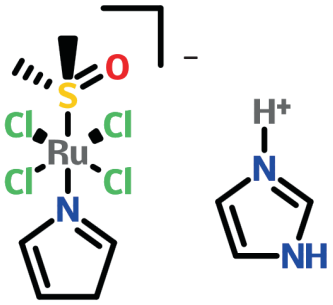
Gemcitabine triphosphate (dFdC-TP)



MK-1775



NAMI-A



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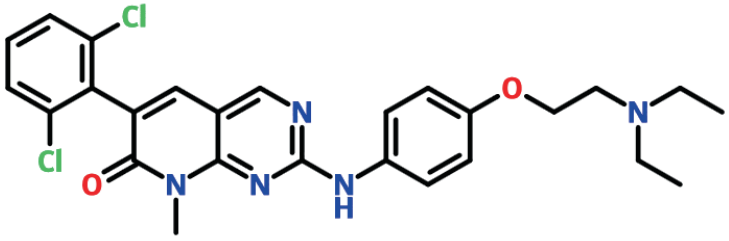
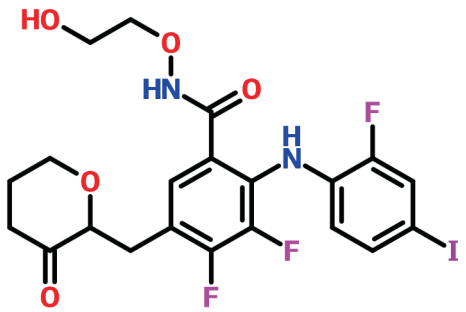
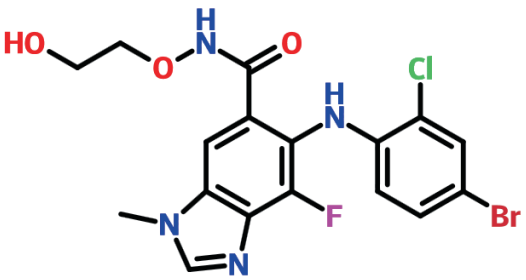


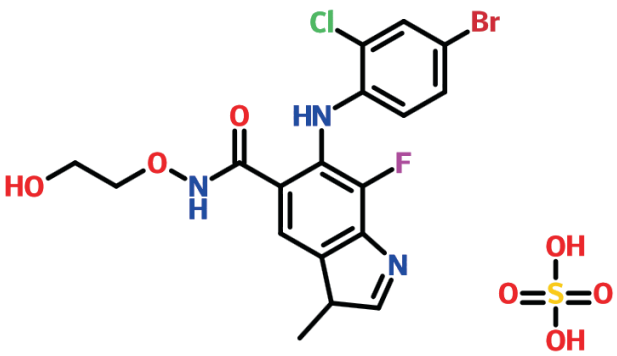
RO4987655



Selumetinib (AZD6244 Hyd-Sulfate)



Selumetinib (AZD6244 free-base)



Summary

Chapter 1 starts with a brief introduction of this thesis and is followed by a literature overview that discusses the shift in the development of new anti-cancer drugs to molecularly targeted agents (MTAs) as a result of the recent advances in tumor biology and the challenges in how to conduct early clinical trials with MTAs (or combinations of MTAs and/or traditional chemotherapeutic agents). Advantages and disadvantages of phase 0 trials, of alternative phase I trials and the trend of extensive use of biomarkers and tumor biopsies in early clinical trials is being discussed.

Chapter 2 describes the results of two studies with two different MEK inhibitors in different stages of development; selumetinib and RO4987655. Mitogen-activated protein kinase kinase (MEK) is part of a signaling pathway that regulates cellular activities including proliferation, survival, and cell cycle regulation. This pathway consists of a protein kinase cascade in which Rapidly Activated Fibrosarcoma (RAF), MEK, and Extracellular signal-Regulated Kinase (ERK) are in a sequential order. Genetic alterations in this pathway e.g. in RAS and RAF genes can result in overexpression and aberrant activation. Inhibition downstream of a mutation, by pharmacological inhibition of MEK, has the potential to block inappropriate signal transduction and tumor growth.
Selumetinib is a relatively older, second generation MEK inhibitor and with currently over 20 clinical phase I and II studies ongoing, most of the studies in combination with targeted or cytotoxic agents. Selumetinib was first developed as a mix and drink formulation, and initial clinical studies were conducted using this formulation. A new capsule formulation was developed with improved pharmacologic properties. **Chapter 2.1** describes the multi-center, open-label, food effect study that has been performed with the new hyd-sulphate capsule formulation to determine the effect of food on the absorption of selumetinib. It has been shown that the presence of food decreased the extent and rate of absorption of selumetinib and therefore it is recommended for further clinical trials that intake of selumetinib occurs on an empty stomach.

RO4987655 is a newer MEK inhibitor, and in **Chapter 2.2** the results are presented of the first in human multi-center phase I study with RO4987655 as a single agent. The study consists of two parts. In the first (phase 1) part of the trial patients with different kind of solid tumors were included and through dose escalation in different patient groups the maximal tolerable dose (MTD) was defined and established at 8.5 mg BID. Main adverse events were skin rash, creatinine phosphokinase (CPK) elevation, and gastro-intestinal disorders. Ocular toxicity was also observed. The second (expansion) part of the study consists of dose expansion in four parallel patient cohorts (n=20) to investigate efficacy of single agent RO4987655: in melanoma tumors with BRAF (V600) mutation, melanoma tumors not carrying BRAF (V600) mutation, non-small cell lung cancer (NSCLC) with KRAS mutations, and colorectal cancer (CRC) carrying KRAS and/or BRAF(V600) mutations. Analyses of this part of the study are currently ongoing.

MEK inhibitors belong to the group of targeted drugs and intervene with a pathway important in many physiological processes in the body, making it difficult to predict the nature and severity of encountered toxicities. Ocular toxicities are observed with different MEK inhibitors and other targeted agents and have important implications for patients. **Chapter 2.3** discusses the diagnosis and advice for clinical management of these toxicities and explains that, because the fibroblast growth factor (FGFR) and RAS/RAF/MEK/ERK (MAPK) pathways play pivotal roles in maintenance, protection and repair of the retina, the ocular toxicities of MEK inhibitors are likely to be attributed to the inhibition the FGFR of and MAPK pathway.

The theme of **Chapter 3** is MK-1775, a small molecule and specific inhibitor of Wee1. Wee1 is a key player in the G1 checkpoint. Cell cycle checkpoints are involved in the repair of DNA damage. Cancer cells often harbor p53 mutations. Since p53 is a key player in the G1 checkpoint, cancer cells are more dependent on the G2 checkpoint for DNA repair. Pharmacological inhibition of Wee1 in combination with DNA damage inducing

chemotherapy will result in mitotic catastrophe in p53 deficient tumor cells while spearing normal cells.

Chapter 3.1 describes the mechanism of action of G2 checkpoint abrogation by Wee1 inhibition and discusses two compounds: MK-1775 and PD-166285. Of the two compounds, MK-1775 specifically inhibits Wee1. PD-166285 is a more broadly active tyrosine kinase inhibitor, which also seems capable of inhibiting Myt1, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR) and platelet derived growth factor receptor beta (PDGFR-ß). Development of PD-166285 seems discontinued, while development of MK-1775 is currently ongoing.

Chapter 3.2 consists of a preliminary analysis of the first in human multi-center phase I study with a Wee1 inhibitor: oral MK-1775 in combination with gemcitabine, carboplatin or cisplatin, is investigated in patients with advanced solid tumors. Strictly speaking, this study consists of three phase I studies in one. The focus of the preliminary analyses is on the two arms cisplatin and carboplatin, since not all the results with gemcitabine were available at the time of analysis due to ongoing dose escalation in the gemcitabine arm. MK-1775 has shown an acceptable and developable toxicity profile, with mild additional hematologic toxicity, nausea, vomiting and fatigue being the most common adverse events in combination therapy. Pharmacokinetics were linear and showed a half-life of approximately 9 hrs. Target engagement measured as a reduction of pCDC2 (CDC2 is the direct substrate of Wee1) was observed in both arms for which data were available.

Although preclinical proof of the concept, and in the phase I study also pharmacological proof of the concept were obtained, clinical proof of the concept that Wee1 inhibitor MK-1775 in combination with DNA damage inducing chemotherapy is active in tumors with p53 deficient tumors is needed to decide if further clinical development of MK-1775 is useful or not. Therefore the study described in **Chapter 3.3** was designed. In this chapter the results of a preliminary analysis are presented of the investigator initiated phase II proof of concept study with MK-1775 plus

carboplatin administered to p53 mutated ovarian cancer patients refractory (disease progression during treatment) or resistant (disease progression within 3 months after treatment) to standard first line therapy (carboplatin and paclitaxel). Although the preliminary results are promising, additional studies in a larger patient population are needed to establish the true value of MK-1775 as a novel anti-cancer drug in combination with existing DNA damage inducing drugs.

Chapter 4 describes a phase I dose-escalation study with fixed dose rate (FDR) gemcitabine plus carboplatin as second line therapy in patients with ovarian cancer. Standard doses of gemcitabine are given as a 30-minute infusion (~33 mg/m^2/min). The enzyme deoxycytidine kinase (dCK) is involved as a rate limiting step in the metabolism of (inactive) gemcitabine to active forms. Fixed dose rate gemcitabine (10 mg/m^2/min) takes into account the saturation of dCK and was designed with the aim to enhance tumor activity. This study demonstrated that FDR gemcitabine in combination with carboplatin in the applied schedule results in increased grade 3/4 toxicity compared to conventional 30-minute infused gemcitabine. Population pharmacokinetics using the nonlinear mixed-effects model software (NONMEM) confirmed these observations.

Finally, **Chapter 5** presents the results of a phase I/II study with novel anti-tumor metastasis inhibitor-A (NAMI-A) in combination with gemcitabine in non-small cell lung cancer patients (NSCLC). NAMI-A was given as a port-a cath infusion to avoid painful phlebitis. Research on the concept of non-platinum metallobased drugs has been ongoing for over 25 years and NAMI-A is the first ruthenium derived anti-cancer drug to have entered clinical evaluation. The effects of NAMI-A in the pre-clinic were mainly detected against metastases instead of on the primary tumor. The phase I study with NAMI-A as a single agent was performed in a different schedule (5 consecutive days of NAMI-A, in a 21 day cycle) than used in the study described in chapter 5. Initial dose escalation of NAMI-A in the study with gemcitabine in NSCLC was performed in a 28 day cycle. Subsequently, dose escalation of NAMI-A in a 21 day schedule was explored, followed by an expansion group treated with the MTD (450 mg/m^2 of NAMI-A and

1000 mg/m^2 of gemcitabine) of the 21 day schedule. Main adverse events consisted of nausea, vomiting, diarrhea, transient creatinine elevation, elevated liver enzymes, neutropenia, and fatigue. Altogether, NAMI-A was moderately tolerated. Expansion of the phase II part was not conducted because the predefined number of responses was not reached.

Samenvatting

Hoofdstuk 1 begint met een korte introductie van het proefschrift en wordt gevolgd door een literatuuroverzicht over de veranderingen in de ontwikkeling van anti-kankermiddelen naar specifiek werkende anti-kankermiddelen als gevolg van de recente ontdekkingen in de tumorbiologie en over uitdagingen die het uitvoeren van vroege klinische studies met specifiek werkende anti-kankermiddelen (of combinaties van verschillende specifiek werkende anti-kankermiddelen en/of traditionele chemotherapeutica) met zich mee-brengen. Voor- en nadelen van fase o studies, van alternatieve fase I studies en de trend van het intensieve gebruik van biomarkers en tumorbiopten worden besproken.

Hoofdstuk 2 beschrijft de resultaten van twee studies met verschillende MEK remmers in verschillende stadia van ontwikkeling; selumetinib en RO4987655. Mitogen-activated protein kinase kinase (MEK) maakt deel uit van een signaaltransductiepathway die cellulaire activiteit reguleert, waaronder proliferatie en overleving van cellen, en regulatie van de celcyclus. Deze pathway bestaat uit een cascade van proteine kinases, waarin Rapidly Activated Fibrosarcoma (RAF), MEK, and Extracellular signal-Regulated Kinase (ERK) in volgorde voorkomen. Genetische veranderingen in deze pathway bijvoorbeeld in de genen RAS en RAF kunnen resulteren in overexpressie en onjuiste activatie. Inhibitie van componenten van de cascade na de mutatie, door farmacologische inhibitie van MEK, heeft de potentie om de afwijkende signaaltrtansductie te blokkeren en daardoor tumorgroei te remmen.

Selumetinib is een relatief oudere, tweede genereatie MEK remmer waarmee op dit moment meer dan 20 fase I en II onderzoeken uitgevoerd worden, waarvan de meeste bestaan uit combinatiestudies met hetzij speciefiek werkende anti-kankermiddelen of cytotoxische middelen. Selumetinib was eerst ontwikkeld als mix-en-drink formulering. Later is een capsule formulering ontwkkeld met verbeterde farmacologische eigenschappen. **Hoofdstuk 2.1** beschrijft een multi-center, open label, voedsel-effectstudie met de nieuwe hyd-sulfaat capsule formulering om

het effect van voedsel te bepalen op de absorptie van selumetinib. Het is gebleken dat de aanwezigheid van voedsel de mate en de snelheid van de absorptie van selumetinib verminderd. De aanbeveling is daarom dat in verdere klnische studies de inname van selumetinib plaatsvindt op een lege maag.

RO4987655 is een nieuwere MEK remmer en **hoofdstuk 2.2** presenteert de resultaten van de voor het eerst in de mens uitgevoerde, multi-center, fase I studie met RO4987655 als enig middel. De studie bestaat uit twee delen. In het eerste (fase I) gedeelte van de studie zijn patiënten met veschillende solide tumoren geincludeerd en door middel van dosisecalatie in verschillende patiëntengroepen is de maximaal tolereerbare dosis (MTD) bepaald op 8,5 mg twee maal daags. De voornaamste bijwerkingen waren huiduitslag, stijging van kreatininefosfaat en maag-darmklachten. Oogtoxiciteit kwam ook voor. Het tweede (expansie) deel van de studie betreft een dosisexpansiecohort in vier parallelle groepen (n=20) om het effect van RO4987655 te onderzoeken: in melanoompatiënten met BRAF (v600) mutatie, melanoompatiënten zonder BRAF (v600) mutatie, melanoom-patiënten met BRAF (v600) mutatie, niet-kleincellig longcarcinoom met KRAS mutatie, en coloncarcinoom met KRAS en/of BRAF (v600) mutaties. Analyses van dit deel van de studie zijn op dit moment nog in gang.

MEK remmers behoren tot de groep van specifiek werkende anti-kankermiddelen en die interveniëren in belangrijke fysiologische processen in het lichaam, die het moeilijk maken om de exacte aard en ernst van de bijwerkingen van MEK remmers te voorspellen. Oogklachten zijn gereapporteerd bij verschillende MEK remmers en andere specifiek werkende anti-kankermiddelen en hebben belangrijke implicaties voor patiënten. **Hoofdtuk 2.3** behandelt de diagnose en geeft advies voor de klinische behandeling van deze oogklachten en legt uit, dat omdat de fibroblast groei- factor (FGFR) en RAS/RAF/MEK/ERK (MAPK) pathways een cruciale rol spelen bij de instandhouding, bescherming en herstel van de retina, oogklachten van MEK remmers waarschijnlijk toegeschreven kunnen worden aan inhibitie van de FGR en MAPK pathway.

Het thema van **hoofdstuk 3** is MK-1775, een small molecule en specifieke remmer van Wee1. Wee1 is een key player van het G1 checkpoint. Celcyclus checkpoints zijn betrokken bij het herstel van DNA schade. In kankercellen is p53 mutatie vaak gemuteerd. Omdat p53 een key player is van het G1 checkpoint, zijn kankercellen meer afhankelijk van het G2 checkpoint voor herstel van DNA schade.Farmacologische remming van Wee1 in combinatie met chemotherapie die DNA schade induceert, zal resulteren in mitotische catastrofe (apoptose) in met name p53 deficiënte tumorcellen, terwijl normale cellen gespaard blijven.

Hoofdstuk 3.1 beschrijft het principe van remming van het G2 checkpoint door Wee1 inhibitie en beschrijft twee Wee1 remmers: MK-1775 en PD-166285. Van deze twee middelen is MK-1775 een hele specifieke remmer van MK-1775. PD-166285 lijkt daarentege een bredere tyrosine kinase remmer, want het remt ook Myt1, de epidermale groeifactor receptor (EGFR), de fibroblast groei- factor receptor (FGFR) en de platelet derived groeifactor receptor beta (PDGFR-ß). De verdere ontwikkeling van PD-166285 lijkt gestopt, terwijl de ontwikkeling van MK-1775 vooralsnog in volle gang is.

Hoofdttuk 3.2 bestaat uit een voortijdige analyse van de multi-center, fase I studie met de voor het eerst in mensen toegepaste Wee1 inhitor: oraal MK-1775 in combinatie met gemcitabine, carboplatine or cisplatine, en is onderzocht in patiënten met solide tumoren. Strikt gesproken bestaat deze studie uit drie fase I studies in één. De focus van de voortijdige analyse ligt op de cisplatine- en carboplatine-armen van de studie, omdat nog niet alle resultaten van de gemcitabine-arm beschikbaar waren ten tijde van de analyse als gevolg van nog lopende dosisescalatie in de gemcitabine-arm.

MK-1775 beschikt over een acceptabel bijwerkingsprofiel, geschikt voor verdere ontwikkeling, met milde additionele hematologische toxiciteit, misselijkheid, braken, overgeven en moeheid als meest voorkomende bijwerkingen in combinatietherapie. De farmacokinetiek was lineair en liet

een halfwaardetijd zien van ongeveer 9 uur. Target engagement werd gemeten als een reductie van pCDC2 (CDC2 is het directe substraat van Wee1) en werd gezien in de twee armen waarvoor data beschikbaar waren.

Ondanks dat er al preklinisch bewijs van het concept was, en ondanks dat de fase I studie ook farmacologish bewijs van het concept aantoont, klinisch bewijs van het concept dat Wee1 inhibitor MK-1775 in combinatie met DNA schade inducerende chemotherapie actief is in p53 deficiënte tumoren is noodzakelijk om te beslissen of verdere klinsiche ontwikkeling van MK-1775 nuttig is of niet.
Om deze reden is de studie beschreven in **hoofdstuk 3.3** opgezet. In dit hoofdstuk zijn de resultaten van een voortijdige analyse weergegeven van de investigator initiated, 'bewijs van het concept'- fase II studie met MK-1775 in combinatie met carboplatine uitgevoerd in patiënten met p53 gemuteerd ovariumcarcinoom refractair (ziekteprogressie gedurende behandeling) of resistent (ziekteprogressie binnen 3 maanden na behandeling) voor eerste lijns behandeling (bestaande uit carboplatine en paclitaxel).
Ondanks dat de voortijdige resultaten gunstig zijn, zijn additionele studies in een grotere patiëntenpopulatie nodig om de werkelijke waarde van MK-1775 als nieuw anti-kankermiddel in combinatie met bestaande DNA schade inducerende chemotherapie aan te tonen.

Hoofdstuk 4 beschrijft een fase I dosisescalatiestudie met gemictabine gegeven op een vaste (en verlaagde) doseringssnelheid (fixed dose rate = FDR) in combinatie met carboplatine en als tweede lijns behandeling aan patiënten met ovariumcarcinoom. Standaarddoseringen van gemcitabine worden doorgaans gegeven als 30 miuten durend infuus (~33 mg/m^2/min). Het enzym deoxycytidine kinase (dCK) is als snelheidsbepalende stap betrokken in de omzetting van (inactief) gemcitabine naar actieve metabolieten. FDR gemcitabine (10 mg/m^2/min) houdt rekening met saturatie van dCK en was ontwikkeld met het doel om de anti-tumoractiviteit te vergroten.

Deze studie heeft aangetoond dat FDR gemcitabine in combinatie met carboplatine in het toegepaste schema resulteert in meer graad 3/4 toxiciteit in vergelijking met gemcitabine gegeven als conventioneel 30 minuten durend infuus. Deze observeringen werden bevestigd met populatie-farmacokinetiek door middel van nonlinear mixed-effects model software
(NONMEM).

Tenslotte worden in **hoofdstuk 5** de resultaten gepresenteerd van een fase I/II studie met novel anti-tumor metastasis inhibitor-A (NAMI-A) in combinatie met gemcitabine in patiënten met niet-kleincellig long-carcinoom. NAMI-A werd hierbij gegeven via een port-a cath om pijnlijke flebitisklachten te voorkomen. Onderzoek naar het concept van niet-platinum metaalgeneesmiddelen is al meer dan 25 jaar in gang en NAMI-A is het eerste rutenium anti-kankermiddel dat is onderzocht in de kliniek. De effecten van NAMI-A in preklinisch onderzoek waren vooral gericht tegen de metastase in plaats van tegen de primaire tumor. De fase I studie met NAMI-A als enkel middel was onderzocht in een ander schema (5 aangegesloten dagen met NAMI-A, in een 21 dagen durende cyclus) dan het schema dat gebruikt is in de studie beschreven in hoofdstuk 5. Aanvankelijk vond dosisescalatie van NAMI-A in de studie met gemcitabine in niet-kleincellig longcarcinoom plaats in een 28 dagen durende cyclus. Vervolgens is dosisescaltie in een 21 dagen durende cyclus onderzocht, gevolgd door een esxpansiegroep die behandeld werd met de MTD (450 mg/m^2 of NAMI-A and 1000 mg/m^2 of gemcitabine) van het 21 dagen durende schema. De meest voorkomende bijwerkingen bestonden uit misselijkheid, overgeven, diarree, creatininestijging, stijging van de leverenzymen, neutropenie en moeheid. Alles samengenomen werd NAMI-A matig verdragen. Expansie van het fase II deel is niet uitgevoerd omdat het van tevoren vastgestelde aantal responsen niet was bereikt.

Dankwoord (acknowledgements)

Promoveren is een proces waarbij heel veel mensen zijn betrokken. Ik ben iedereen erg dankbaar.

Allereerst wil ik de patiënten, hun familieleden en dierbaren bedanken voor deelname aan de klinische studies.

De leescommissie bestaande uit prof. dr. E.E. Voest, prof. dr. A. de Boer, prof. dr. B. Olivier, prof. dr. S.M. Imhof, prof. dr. P. Baas, ben ik zeer erkentelijk voor het lezen en beoordelen van mijn proefschrift.

Beste Jan, mijn promotor, je uitgebreide kennis, je gedrevenheid en doelmatigheid blijven indrukwekkend. Net als de gigantische hoeveelheid werk die je dag in dag uit met veel toewijding en soms moeiteloos lijkt te verzetten. Eén van je talenten als begeleider is dat je het beste uit iemand weet te halen, en daarbij rekening houdt met individuele verschillen. Daarom was je voor mij een uitstekende promotor.

Jos, hoofd van de apotheek van het Slotervaart ziekenhuis en mijn andere promotor, dank voor je kritische review van artikelen en ik wist, dat als het nodig was, ik altijd bij je terecht kon.

Lieve Serena, oncoloog in het NKI en klinisch beoordelaar bij het CBG, maar 56 uur eerder geboren dan ik in Italië, maar op zoveel punten zoveel verder en wijzer dan ik. Je hebt me non-stop gesteund en me zelf-vertrouwen gegeven door altijd in mijn kunnen te geloven. Echt super!

Alwin, Hilde, Joets, Bastiaan en alle anderen mensen van de Slotervaart apotheek, inclusief de analisten zoals Abadi, Matthijs en Joke. Dank voor jullie belangrijke ondersteuning van de klinische studies, het meten van samples en kennis op gebied van o.a. PK en PD waar ik een beroep op mocht doen.

Jolanda, personal assistant van Jan, je haalde me op in de hal van het NKI bij mijn eerste bezoek en maakte dat het meteen als een warm bad voelde. Niet altijd even zichtbaar, maar wat doe je belangrijk werk. Je contact met mensen (patiënten, oio's etc.) is een grote aanwinst.

Marja, arts en clinical trial manager, betrokken bij patiëntenzorg en in nauw contact met de farmaceutische bedrijven voor de klinische studies, je

was er niet alleen met de teleconferenties, maar voor zo veel meer. Het leukste vind ik dat je zo kan genieten van de schijnbaar kleine dingen in het leven.

Het team van 4C, het farmacologie team, iedereen bedankt voor jullie onmisbare bijdrage aan de studies: Brenda, Marja's rechterhand, VS-en Jana, Emmy, Annemarie, Marianne, Karina en Annemiek, Wendy en Mariska, secretaresses Marijke, Magda, Hilleke. En uiteraard ook alle artsen, in het bijzonder Neeltje en Frans. Sjaak en Paul veel dank m.n. voor jullie belangrijke betrokkenheid bij de NRA studie. Gabe, jouw inzet voor de MKO studie is van groot belang en wordt enorm gewaardeerd.

Angela, teamleider van 4C. Ik heb pas later ontdekt (en ervaren) dat je ook een hele goeie coach bent!

Alle verpleegkundigen heel veel dank voor de prettige en gezellige samenwerking, in het bijzonder Thea, mijn lieve steun en toeverlaat, maar ook Noëlle, Dees, Maaike, Bregje, Annelies, José, Marjolein, Saskia, Sandra, en toch ook weer Karina, Annemiek, Wendy en Mariska B. (inmiddels gepromoveerd tot VS/VS i.o.). Mariska Z., samenwerken als je op de afdeling stond was altijd heel erg plezierig, maar ik ben je vooral ook erg dankbaar voor het inplannen van de patiënten (hoe lastig soms, 't lukt je toch altijd!).

Veel dank voor de afdeling (algemene en moleculaire) pathologie, die in de jaren steeds belangrijker is geworden bij het uitvoeren van klinische studies (o.a. screening van mutaties). In het bijzonder ben ik veel dank verschuldigd aan Daphne, Efraim (p53 analyses!!) en Petra.

Het trial bureau waar de vrouwen goed vertegenwoordigd zijn, is toch wel één van de gezelligste plekjes van het NKI. Iedereen enorm bedankt, maar met name Frauwkje, Ludy, Lidwina en Aarti voor o.a. het invullen van de CRFs. Maar natuurlijk ook Josien, Christiane, Marjolein, en de rest! Frauwkje, jij hebt me op persoonlijk vlak ook menigmaal met raad en daad bijgestaan, en dat zal ik nooit vergeten.

Otilia, hoofd van de wetenschappelijke administratie, you helped me really well with some practical issues in the latest phase of my PhD. Thanks!

Paula, voor mij de dame van de contracten en het geld, altijd vrolijk en erg punctueel.

Erik, je maakt statistiek begrijpelijk. Dat is een groot talent. Het was een flinke klus, maar we hebben toch gezellig gepuzzeld bij de analyses van de betreffende studies.

Yvonne, monitor van huisstudies, je hebt me ondanks je drukke agenda altijd enorm goed geholpen! Je ervaring van andere banen komt hier goed van pas.

Cecile en later Ineke, kwaliteitsmanagment is onlosmakelijk verbonden aan good clinical practice (GCP), aan jullie de eer om te zorgen dat iedereen dat blijft naleven! Iets wat jullie beiden met overgave doen.

Song, Tony , Ernesto, Jeroen, Brigitte en Harriet, voor mij soms de bijna onzichtbare afdeling, maar zonder jullie wordt het toch echt lastig. Bedankt voor jullie hulp!

Dick, officieel analist, maar in werkelijkheid veel meer. Dank voor al je hulp gedurende alle jaren. En natuurlijk voor al je werk voor de NRA en MKO studie.

Kamergenoten en mede-oio's: Bojana, ga je dromen achterna zonder te twijfelen, dan komen ze volgens mij allemaal uit! Geert, Ruud, Didier, succes met de rest van jullie promotie! Bart, Robin jullie zijn echte aanwinsten als 'nieuwe' oio's. R-Rik dank voor het R-en (programma gebruikt voor kinetiekanalyses), Artur, je bent een geval apart :-), het allerbeste! Ook oud-oio's, zoals Maarten en Lot enorm bedankt voor alles. David, je hebt me eigenlijk bijna alles geleerd, je was mijn grote voorbeeld.

Dear Johnny, it has been such a great pleasure working with you on MK-1775! You do your job as a senior clinical lead in an amazing manner!! I would love to catch up with you during a dinner. May be we can arrange something with Tim in Milano..?! ;-)Tim, thank you so much for all your support. Rose, you joined the MK-1775 team at Merck in a later stage, does your offer to cook a south Indian dinner with idli and dosa for me whenever I will be around in NJ still stand? Mariëlle, jij was een keigave supermonitor, en samen met Frauwkje vond ik de samenwerking echt top! Marja v. B., jij ook bedankt voor je bijdrage.

Valerie, merçi beaucoup pour tout! Bas, je was een leuke monitor en altijd bereid om mee te denken.

De basis voor het promoveren, voor onderzoek doen, is gelegd in Johns Hopkins, in het lab van Alan Friedman. Elsken van der Wall, toen nog geen professor in Utrecht, maar werkzaam in de VU, je was mijn fantastische begeleider in NL tijdens deze stage. Daar ben ik je nog steeds enorm dankbaar voor! Ook liet je me zien, dat je er gewoon voor moet gaan als je iets wil.

Privé zijn o.a. de volgende mensen erg belangrijk geweest tijdens het promotieonderzoek:

Lieve Clara, oud klasgenootje van de lagere én middelbare school, inmiddels een zeer bekwame hematoloog, jij hebt me het laatste zetje gegeven om te gaan promoveren en daar ben ik erg blij om!

Wieteke en Joeno, Tenny en André, Mahtab en Evert, oorspronkelijk kennen de 'meiden' elkaar van de studie, nu dus met 'aanhang' , wat leuk dat we nog steeds zulk goed contact hebben (en wat hebben jullie allemaal leuke kinderen)!

Lieve Anouk, je bent toch echt een beetje mijn zusje, hoor. Soms zien we elkaar tijden niet, maar als we elkaar zien gaan we gewoon weer verder. Marianne, moeder van Anouk, en ook echt 'familie', ik waardeer ons fijne contact. En toen was je opeens patiënt in het AVL… Je hebt je er kranig doorheen geslagen en het gaat nu gelukkig heel goed met je.

Abdlakrim, lieve schat, je bent 'asmoun inou'.

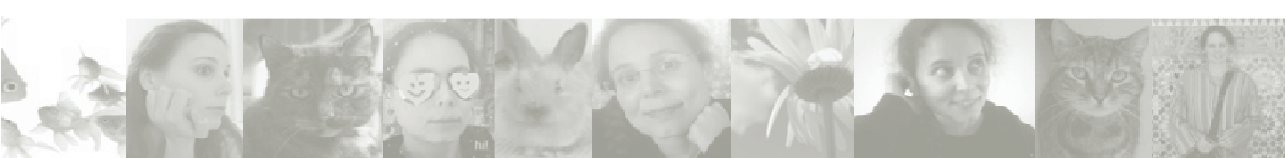
Lieve Marjolijn en lieve Jan, wat ben ik intens blij met jullie als mijn ouders. Jullie onvoorwaardelijke liefde en steun is ongelofelijk waardevol, altijd, maar ook zeker in deze afgelopen periode. Marjolijn al woon je in Portugal, je bent er toch ALTIJD voor me en dat is super. Jan, dichterbij in Amsterdam, wat verwen je toch met alles, en je bent werkelijk een ontzettend superlieve & leuke vader! Ik hou van jullie!

Curriculum Vitae (Nederlands)

Suzanne Leijen werd geboren op 11 juni 1974 in Amsterdam. In 1992 behaalde zij het eindexamen aan het St. Ignatius Gymnasium in Amsterdam, met een vakkenpakket zonder natuur- en scheikunde. Daarna bracht zij een jaar door in Leonidion, een prachtig dorpje op de Peloponnesos in Griekenland, en leerde zij Grieks, gevolgd door een vierjarige opleiding voor alternatieve geneeskunde aan het Nederlands College voor Natuurgeneeswijzen in Hilversum. Na het alsnog behalen van een natuur- en scheikunde diploma op VWO niveau, begon zij de studie Geneeskunde aan de Vrije Universiteit in Amsterdam, alwaar zij eind 2007 als basisarts afstudeerde. De interesse voor de oncologie werd gewekt tijdens de vergpleeghulpstage, die zij volgde in het BOC Oncology Center, Nikosia (Cyprus). Gedurende de studie geneeskunde volgde zij twee keer een wetenschappelijke stage. De eerste was in 2002-2003 gedurende een periode van ongeveer een schooljaar in het laboratorium van dr. Alan D. Friedman van het Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins, University in Baltimore (Verenigde Staten van Amerika). Het onderwerp van deze stage en tevens van haar afstudeerscriptie van de masterfase was: 'Study of phosphorylation mutant AML1 S249A/S266A, in 32D cl3 myeloid murine cells' (zie ook publicatielijst). De scriptie werd cum laude beoordeeld aan beide universiteiten. Haar stagebegeleider aan de Vrije Universiteit gedurende deze periode was dr. Elsken van der Wall. Een tweede, extra wetenschappelijke stage volgde zij als onderbreking van de co-schappen in 2006-2007 aan de Vrije Universiteit op het laboratorium bij prof. dr. Gerd Bouma onder begeleiding van dr. Anton Zwiers en betrof onderzoek naar de rol van toll like receptors (TLRs) en tight junction molecuul Claudine 18 bij inflammatoire darmziekten (zie ook publicatielijst). Na de studie geneeskunde werd vanaf april 2008 het in dit proefschrift beschreven onderzoek uitgevoerd onder begeleiding van prof.dr. Jan H.M. Schellens en prof. dr. Jos H. Beijnen.

Curriculum Vitae (English)

Suzanne Leijen was born on June 11, 1974 in Amsterdam (the Netherlands). In 1992 she completed her final exam at the St. Ignatius Gymnasium (grammar school) in Amsterdam, with chosen subjects that did not include physics and chemistry. She then spent one year in Leonidion, an idyllic village on the Peloponnese in Greece, where she learned Greek, followed by a four year education in alternative medicine at the Dutch College for Alternative Medicine in Hilversum (the Netherlands). After graduating in physics and chemistry at VWO (grammar school) level, mandatory for medical school in the Netherlands, she started the study of Medicine at the VU University in Amsterdam. She graduated end of 2007 as an MD. Her first interest for oncology was triggered during a curricular internship as a nursing help, which she completed at the BOC Oncology Center, Nikosia (Cyprus). She also completed two research internships during the period she attended medical school. The first internship she completed during an 8 month period as a visiting student in the laboratory of dr. Alan Friedman at the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University in Baltimore (USA) in 2002-2003. The subject of this research and of her master's thesis was: Study of phosphorylation mutant AML1 S249A/S266A, in 32D cl3 myeloid murine cells (see also list of publications). The thesis was evaluated cum laude at both universities. Her supervisor at the VU University, Amsterdam, during this period was Elsken van der Wall, MD, PhD. The second, extra-curricular internship she completed in 2006-2007 at the laboratory of prof. Gerd Bouma, MD, PhD, under supervision of Anton Zwiers, MSc, PhD, and involved investigation of the role of toll like receptors (TLRs) and tight junction molecule Claudin-18 in colitis (see also list of publications). After medical school, research described in this thesis was initiated from April 2008 onwards under supervision of prof. Jan H.M. Schellens, MD, PhD en prof. Jos H. Beijnen PharmD, PhD.



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