π - π Stacked Polymeric Micelles as Carriers of Anticancer Therapeutics for Hematological Malignancies

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Aida Alexandra Varela Moreira

Department of Clinical Chemistry and Hematology, University Medical Centre Utrecht and Department of Pharmaceutics, Utrecht University, Utrecht, the Netherlands

Author: Aida Varela Moreira

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**Pi-pi gestapelde polymere micellen als dragers voor anti-kanker
therapeutica voor hematologische maligniteiten**

(met een samenvatting in het Nederlands)

Proefschrift

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Aida Alexandra Varela Moreira

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te Porto, Portugal

Promotoren:

Prof. dr. R.M. Schiffelers

Prof. dr. W.E. Hennink

Copromotoren:

Dr. M.H.A.M. Fens

Dr. M. Buchner - Mayr

To my father

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1

General Introduction

1.POLYMERIC MICELLES

1.1. Polymeric micelles as nanocarriers

Polymeric micelles (PM) based on amphiphilic block copolymers are versatile nanocarriers that have been extensively explored in the last decades [1-3]. PM are highly tailorable nanocarriers since the chemical composition, molecular weight of the polymer blocks and the ratio between the blocks can be tuned to design formulations with favorable size, stability, drug loading and release [1]. PM prepared from amphiphilic block copolymers form core-shell structures in aqueous media with the core formed by a hydrophobic block and the shell by a hydrophilic block, often poly(ethylene glycol)–(PEG) [4]. PM spontaneously form when the copolymer is brought into an aqueous environment and is present above a certain concentration, known as the critical micelle concentration. PM can accommodate hydrophobic compounds in their core, while the hydrophilic shell is responsible for colloidal stability and prevention of rapid clearance *in vivo* [5, 6]. The shell also provides the possibility for decoration of the PM surface with specific peptides or antibodies to target them to a certain cell type. The small average diameter of PM, 10-100 nm, and a near neutral surface charge are generally regarded as advantageous for long circulation and accumulation in target tissues by means of the enhanced permeability and retention (EPR) effect [7], provided that they are sufficiently stable in the circulation.

1.2. Solubilizers versus carriers

PM were first investigated to improve the aqueous solubility of poorly water-soluble drugs. Such drugs were conventionally solubilized in excipients as Cremophor EL and Polysorbate 80, which however, are associated with significant toxicity and complement activation [8, 9]. PM have advantages over these excipients as they show good biocompatibility and can greatly increase water solubility of hydrophobic drugs [10, 11]. Genexol™ was the first drug-loaded polymeric micelle-based formulation approved for clinical practice. This formulation is based on mPEG-b-poly(D,L-lactic acid) with paclitaxel (PTX) loaded in the hydrophobic core [12, 13]. Genexol™ has a poor pharmacokinetic (PK) profile, as upon intravenous (i.v.) administration PTX is rapidly cleared from blood circulation [12]. Nevertheless, the solubilization of PTX in the Genexol™ formulation remains valuable and showed improved biocompatibility over PTX solubilized in the classical Cremophor EL excipient in the marketed Taxol formulation. The improved solubilization of PTX in PM in Genexol™ has allowed the administration of higher doses, that otherwise were not possible due to toxicity associated with Cremophor EL administration. This benefit resulted in the approval of Genexol™ as a drug solubilizer. This means that the development of PM-based systems that perform as true drug

carriers which are stable in the circulation after i.v. administration and exploiting the mentioned EPR effect is the major challenge of the field [2]. The poor *in vivo* stability and drug retention upon systemic administration of drug-loaded PM hinders the distribution towards target tissues, by the EPR effect.

PM are dynamic systems and therefore after i.v. administration they encounter many challenges that could lead destabilization [14, 15], as :

- the micellar dilution in the bloodstream, that can decrease polymer concentration below the CMC;
- the binding of monomers to solubilizers of hydrophobic compounds like albumin and apolipoproteins.

Therefore, to act as true drug carriers PM should exhibit a colloidal stability in biological environments that promotes extended circulation times of the carrier after administration. Such long circulating PM exploit the EPR effect and thus have higher chances to deposit their payload at the target site to result in increased tissue concentrations. Naturally, more drug-loaded PM at the target site would be expected to show improved therapeutic efficacy.

Equally important is good retention of the loaded drug in the core of PM to take full advantage of the circulation and tissue distribution profile of PM. When optimal retention is achieved, the encapsulated drug and the drug carrier display identical circulation times after administration in the bloodstream [16, 17]. Correspondingly, this is expected to improve the accumulation of drug molecules at the target site [18].

1.3. Loading methods

Drug molecules can be physically loaded in [19] or chemically conjugated [20, 21] to the core of PM. Chemical conjugation of drugs to the micellar core is generally exploited to improve drug retention after i.v. administration when, for instance, the drug is extracted by plasma proteins such as albumin and apolipoproteins. However, this strategy can be challenging as careful optimization for every drug-polymer combination is required and the pharmacological action of a drug might be adversely affected due to modification of the parent drug and/or due insufficient drug release from PM [22].

Physical methods for drug loading include, amongst others, coordination interactions, electrostatic interactions and π - π stacking [17, 23]. Particularly π - π interactions have received increasing attention and successfully employed in the development of nanocarriers [23] loaded with various low molecular weight anticancer drugs [24], and even nucleic acids, peptides and photosensitizers. Drug loading of PM via π - π

interactions is favorable because chemical modification of both the polymer and the active ingredient are not necessary. Importantly, in aqueous environments π - π interactions are fairly strong based on the arrangement of the electrons in the π systems [25]. Also, it was shown that π - π stacking between polymer and drugs improved the *in vivo* behavior of drug-loaded nanoparticles and resulted in increased tumor accumulation and better antitumor efficacy [24].

1.4. Polymeric micelles for cancer therapy

Polymeric micelles have been primarily investigated for cancer therapy. Cancer is a life-threatening disease that affects millions of people worldwide, and as a result many new therapies have been developed and are under evaluation for treatment of this class of diseases [26]. This is clearly demonstrated by the number of PM-based formulations for cancer treatment that have entered clinical trials [3]. In **Chapter 2** we review the PM-based formulations that have been in clinical evaluation for cancer treatment [27].

Apart from the unmet medical need, a substantial number of anticancer drugs used in clinical practice and under development are poorly water-soluble which poses a challenge to their formulation [28]. Also, in this respect PM are attractive drug carriers for solubilization and targeted delivery of poorly water-soluble drugs and drug candidates. Moreover, when PM are able to act as a true drug carrier, and not only as solubilizer, the PM formulation may improve accumulation of anticancer drugs at the target site by exploiting the EPR effect [29]. The EPR effect was first described by Maeda in the late 1980s [7] and demonstrates that nanocarriers with long circulating properties *in vivo* can effectively accumulate in tumor tissues due to the enhanced permeability of the tumor vasculature. Once within the tumor, their retention is also enhanced due to impaired lymphatic drainage. Both effects combined result in an increased dose of nanocarriers at the tumor site. Recent reports however emphasize the high variability of the EPR effect in human patients [30, 31]. This fact would argue for more ‘personalized’ therapies for nanocarriers, where the EPR ‘status’ of the patient is established before treatment commences [32].

2. HYDROPHOBIC COMPOUNDS FOR CANCER TREATMENT

Many anticancer drugs have poor aqueous solubility, representing a challenge for drug formulation development [33]. Systemic administration of these drugs often leads to binding to proteins like albumin and apolipoproteins limiting therapeutically effective drug concentrations [15]. Moreover, these drugs often have a poor PK profile as they suffer from rapid clearance that further limits target tissue accumulation. PM based

on amphiphilic block copolymers are therefore attractive candidates for solubilization and targeting of poorly water-soluble drugs [6, 34, 35]. In the next section we highlight several drugs and drug candidates that due to poor water-solubility, poor PK properties and/or off target adverse effects are good candidates to accommodate in the core of PM.

2.1. Proteasome inhibitors

Proteasome inhibitors represent an important class of targeted therapies [36]. These inhibitors downregulate the proteasome activities that represent a core element in the protein degradation machinery in eukaryotic cells [36, 37]. Both normal and malignant cells rely on the proteasome activity to regulate the expression of proteins associated with survival and proliferation, but malignant cells are more sensitive to its inhibition [38, 39]. Three proteasome inhibitors – bortezomib (Velcade®), carfilzomib (Kyprolis®) and ixazomib (Ninlaro®) - gained clinical approval for the treatment of multiple myeloma (MM) or mantle-cell lymphoma (MCL). Carfilzomib (CFZ) is a second-generation proteasome inhibitor currently approved for the treatment of MM [40, 41]. Due to poor water solubility, the approved formulation - Kyprolis® - contains CFZ solubilized in sulfobutylether β -cyclodextrin to allow systemic administration. Still, CFZ in Kyprolis® has a poor PK profile with short half-lives (around 30 minutes), rapid clearance from the body and a large volume of distribution [42, 43].

2.2. Bruton's tyrosine kinase inhibitors

Ibrutinib (Imbruvica®) is the first in class Bruton's tyrosine kinase (BTK) inhibitor, approved for the treatment of mantle cell lymphoma, Waldenström's macroglobulinemia, marginal zone lymphoma, chronic graft *versus* host disease and chronic lymphocytic leukemia [44]. Ibrutinib inactivates BTK through covalent and irreversible binding to a cysteine residue of the BTK. Also, downstream of BTK several survival pathways are inactivated by ibrutinib, as ERK1/2, PI3K and NF- κ B [45]. Ibrutinib has a very low solubility in water (0.003 mg/mL [46]) and was shown to have major hepatic first pass effect resulting in a low oral bioavailability [47, 48]. Consequently, this drug is dosed in capsules at very high doses which, however, leads to several adverse off target effects [49].

2.3. Negative feedback inhibitors

Dual specificity phosphatase 6 (DUSP6) of the mitogen-activated protein kinase (MAPK) pathway is overexpressed in several types of cancers and has been associated with the maintenance of cancer cell survival [50-52]. DUSP6 reduces hyperactive MAPK signaling triggered by upstream activation of the pathway. Studies have shown that the depletion of DUSP6 can reduce tumor load, suggesting that hyperactive MAPK signaling induces

cell death [53, 54]. Therefore, inhibitors of the DUSP6 phosphatase may represent a novel treatment strategy.

BCI, a DUSP6 inhibitor, has shown to hinder DUSP6 activity by binding to its phosphatase domain [55]. For this category of therapeutic compounds, dosing and timing are essential. BCI is a drug candidate in early pre-clinical studies and little is known about its PK profile, yet the physicochemical properties of BCI anticipate that systemic administration of the free compound might be challenging because of its low aqueous solubility.

3. HEMATOLOGICAL MALIGNANCIES

Hematological malignancies (HM) are cancers that originate from the hematopoietic lineage and affect the blood, bone marrow, liver and lymphoid organs. These organs are part of the immune system and thus, HM usually involves in more than one of these sites. HM derive from two blood cell lineages: myeloid and lymphoid lineage. The first produces granulocytes, erythrocytes, thrombocytes, macrophages and mast cells; the lymphoid lineage further differentiates into B, T, NK and plasma cells. Malignancies that originate from the myeloid lineage are acute and chronic myelogenous leukemia, myelodysplastic syndromes and myeloproliferative diseases. While lymphomas, lymphocytic leukemias and myeloma arise from the lymphoid lineage.

HM have been targeted to a lesser extent by nanocarriers than solid tumors which may be partly ascribed to the less localized character of HM [56-58]. As mentioned above, cancer cells from HM originate/accumulate in the bone marrow, spleen [59], lymph nodes, peripheral blood and liver [60]. From a drug delivery point of view, hepatosplenic sites of HM are extremely advantageous targets as the typical (and usually unwanted) accumulation sites of PM (and nanocarriers in general) are the liver and spleen.

At the same time, the other HM-involved tissues may be less straightforward to target [60, 61]. Still, PM may have advantageous characteristics to also improve delivery there. For the peripheral blood, PM with prolonged circulation times may increase chances of targeting these circulating cells. Also, the small size of PM (generally below 100 nm) is advantageous to target bone marrow [62]. Previous reports have shown that nanomedicines with a size below 100 nm can penetrate into bone marrow interstitium [63]. Finally, as widely recognized for solid tumors, the EPR effect can also be explored in HM. In solid tumors, angiogenesis is regarded as an important driver of the EPR effect [64-66]. In bone marrow biopsies of patients with acute myeloid leukemia increased

angiogenesis has also been observed [67]. This represents an opportunity to further explore the delivery of drugs with nanocarriers to improve the treatment of HM. In **Chapter 3** we summarize nanocarriers currently in clinical trials or approved for HM treatment; also, key aspects of nanocarriers that make them beneficial to target HM are highlighted [62].

4. AIM AND OUTLINE OF THE THESIS

The aim of this thesis is to explore the potential of PM as drug carriers of hydrophobic drugs for the treatment of hematological malignancies.

In **Chapter 2** we review PM-based formulations currently in clinical evaluation for the treatment of cancer. We described different formulations and summarize the preclinical and clinical data available for PM-based formulations along with the strategies to translate PM to the clinical practice.

Chapter 3 links/ highlights the development of PM for the treatment of hematological malignancies (HM). This comprehensive work summarizes different types of HM, currently available therapies and their shortfalls. Various nanomedicines, including PM, are presented as an attractive approach to ameliorate treatment, and an overview of investigational and approved formulations are presented. Finally, challenges encountered in the development of novel nanomedicine formulations and their clinical translation for HM treatment are discussed.

In **Chapter 4** we assessed the tolerability of carfilzomib-loaded PM (CFZ-PM) in a “humanized” BM-like scaffold (huBMsc) model. Further, circulation and tumor & tissue distribution of fluorescently labelled micelles was determined in this human-mouse hybrid mouse model of multiple myeloma.

In **Chapter 5** we explore the potential of PM based on mPEG-b-p(HPMA-Bz) as ibrutinib nanocarrier for the treatment of CLL in a preclinical mouse model. We examined circulation kinetics and tissue distribution of labeled micelles in wild type and protein kinase C- β II knock out mice (insusceptible to CLL cells engraftment), transplanted with CLL cells. Finally, therapeutic efficacy of ibrutinib-loaded micelles was studied in wild type mice engrafted with CLL cells.

In **Chapter 6** we explored the formulation of a DUSP6 inhibitor, BCI, in mPEG-b-p(HPMA-Bz)-based micelles. A co-culture of primary CLL patient cells with bone marrow stromal

Chapter 1

cells was set up and the cytotoxicity of BCI formulations had been evaluated. The circulation times of BCI and labeled PM were evaluated in a solid tumor xenograft of neuroblastoma and in CLL-engrafted mice. Finally, the therapeutic potential of BCI-PM was assessed in both models.

In **Chapter 7** we disclose which physicochemical properties of drugs influence their loading and retention in PM based mPEG-b-p(HPMA-Bz) block copolymer. PM were loaded with drugs containing different number of aromatic rings and characterized for size, loading efficiency and release profile in different media. Correlations between physicochemical properties of drugs and loading capacity or drug retention were presented. Finally, circulation times of labeled PM and two selected drugs loaded in PM were evaluated.

Finally, **Chapter 8** summarizes the results of this thesis followed by a discussion and future perspectives for further development of PM.

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Clinical Application of Polymeric Micelles for The Treatment of Cancer

Aida Varela-Moreira^{a,b}, Yang Shi ^c, Marcel H. A. M. Fens^a,
Twan Lammers^{b,c,d}, Wim E. Hennink^b, Raymond M. Schiffelers ^{a*}

^a Department of Clinical Chemistry and Haematology, University
Medical Centre Utrecht, Utrecht, the Netherlands

^b Department of Pharmaceutics, Utrecht Institute for Pharmaceutical
Sciences, Utrecht University, Utrecht, the Netherlands

^c Department of Nanomedicine and Theranostics, Institute for Experimental
Molecular Imaging, RWTH Aachen University Clinic, 52074 Aachen, Germany

^d Department of Targeted Therapeutics, MIRA Institute for Biomedical
Technology and Technical Medicine, University of Twente,
Enschede, 7522 NB, the Netherlands

ABSTRACT

The *in vivo* administration of chemotherapeutic drugs is a challenge due to their poor pharmacokinetic (PK) and biodistribution profile. For this reason, the development of delivery systems capable of targeting these compounds to pathological sites is of great importance. Polymeric micelles (PM) are good systems for the encapsulation of hydrophobic compounds because their hydrophobic core can accommodate these type of drugs whereas their hydrophilic corona, usually poly(ethylene glycol), enables PM to circulate for extended periods of time in the bloodstream which allows them to reach tumour tissues by means of the enhanced permeability and retention (EPR) effect. The first generation of PM were rather unstable and essentially used to solubilize hydrophobic drugs for intravenous (i.v.) administration. More recently, next-generation of PM have been developed to achieve high encapsulation and retention of drugs while maintaining prolonged circulation after i.v. administration. These systems are suitable for both passive and active drug targeting. Different approaches have been employed to achieve the abovementioned goals: both non-covalent (hydrophobic and π - π interactions) and chemical (covalently binding of the drug to the polymer backbone and/or crosslinking of the core/shell) strategies have been used to improve the stability in the circulation and to retain the loaded drug in the PM. This will result in accumulation of the drug at the target site at a greater extent than in healthy tissues and will, in principle, lead to improved therapeutic outcome.

Several PM-based formulations are currently being evaluated in clinical trials. In this review, the pre-clinical and clinical outcome of these PM are summarized, as well as the strategies to translate PM to the patient.

1. INTRODUCTION

Nanoparticulate systems for the delivery of drugs to tumours have been extensively studied to facilitate drug accumulation at target site and simultaneously reducing the toxicity related to the systemic delivery of the free drugs[1, 2]. In other words, these strategies aim to improve the therapeutic outcome of anticancer drugs. PM are attractive systems as they can accommodate a great variety of hydrophobic cytostatic drugs[3, 4] PM are nano-sized colloidal particles with a core-shell structure, which are spontaneously formed by self-assembly of amphiphilic macromolecules in a block-selective solvent into nanoaggregates above a certain concentration, the critical micelle concentration (CMC). PM contain several features that endow them for the delivery of drugs[5-8]. Namely, PM present small and narrow sizes, usually below 100 nm which enables them to accumulate in tumours/inflamed tissues by means of extravasation as a result of the so-called enhanced permeability and retention (EPR) effect [9, 10]. The EPR effect is based on the fact that tumours, and inflamed tissues in general, comprise abnormal, angiogenic blood vessels with a wide fenestrated endothelium. Therefore, if PM circulate long enough in the blood stream they will accumulate in tumour/inflamed tissues to a higher extent than in healthy tissues. Moreover, the lymphatic drainage is impaired in these tissues, which also contributes to the retention of the PM at the target site. PM present a unique core-shell structure with hydrophobic segments of amphiphilic block copolymers forming the inner core of the nanoparticles while the hydrophilic segments are positioned in the aqueous milieu to form the shell. Hydrophobic molecules can be solubilized in the core while the hydrophilic corona stabilizes the PM and provide prolonged circulation time in the blood. The shell of the PM further provides the possibility of decorating the surface with targeting moieties, exploited for specific targeting to cancer cells[11, 12]. Finally, the chemical composition and molecular weight of the blocks can be altered in order to obtain PM with tailorable size, loading and retention of the drug and drug release profile.

The core segregation that occurs in an aqueous solution is the driving force for micelles formation and is accompanied by intermolecular forces, as hydrophobic interaction, electrostatic interaction, metal complexation and hydrogen bonding. Consequently, a myriad of bioactive molecules can be entrapped in the micellar core by means of a strong interaction between the core-forming block and the drug molecules. Besides hydrophobic drugs also, nucleic acids [13, 14], antisense oligonucleotides [15] and diagnostic agents [16, 17] can be entrapped in the core. As mentioned before, PM are most frequently used for the delivery of hydrophobic molecules that are physically entrapped in their core or chemically attached to micelle forming block copolymer.

In vivo, the administration of free chemotherapeutic drugs presents a low therapeutic value. On one hand, the rapid extraction of the drug from the systemic circulation reduces the tumour accumulation and retention of the drug. On the other hand, these drugs are i.v. administered in relatively high doses and accumulate in healthy tissues/organs causing severe toxic effects. Also, the large volume of distribution of these drugs lead to their accumulation in healthy tissues/organs. In other words, the poor PK profile of chemotherapeutic drugs after systemic administration lead to their poor therapeutic outcome[18, 19].

Cremophor® is one of the most frequently used vehicles for the solubilization of hydrophobic drugs[20, 21]. This excipient is based on a polyoxyethylated castor oil and is clinically used for the solubilization of anaesthetics, photosensitizers, sedatives and anticancer drugs, as paclitaxel. The drugs are solubilized with Cremophor® to be injected i.v., however the drugs are rapidly transferred to the blood proteins and subsequently cleared from the circulation[22-24]. Moreover, the use of Cremophor is associated with several side effects as dyspnoea, flushing, rash and generalized urticarial[25-27]. As an example, Taxol is a formulation of paclitaxel in a 1:1 mixture of Cremophor and ethanol [28]. Many complications have been reported after administration of this formulation. The main problem, as mentioned before, is the toxicity associated with the administration of paclitaxel in presence of the vehicle. Moreover, a large volume of Cremophor is required to deliver a therapeutically effective dose of the paclitaxel. Given this, alternative formulations that do not require Cremophor and could be i.v. injected have been explored. The first generation of PM aim at solubilizing hydrophobic compounds. PEG-b-poly(D,L-lactic acid) (PEG-PLA) is an example of block copolymer used for solubilization of hydrophobic compounds. Paclitaxel loaded in PEG-PLA (Genexol™-PM) is a micellar formulation able to solubilize paclitaxel in its core, and that decreases the toxicity associated with Cremophor administration[29-32].

Although such studies support the pharmaceutical potential of PM-based formulations, there are still some challenges regarding the clinical translation of these nanomedicines. Currently, the main challenges in the PM field are the *in vivo* stability and drug retention upon i.v. administration, which are both essential for an EPR effect-mediated passive drug delivery. As PM are dynamic systems, upon i.v. administration and concomitant micellar dilution in the bloodstream, the copolymer concentration will decrease below the critical micelle concentration (CMC). Consequently, there will be a shift in the equilibrium towards the unimer state that lead to (partial) micellar dissolution. The binding of the block copolymers to blood components such as albumin and apolipoproteins can also initiate micelle dissociation and premature drug loss [33].

Therefore, to be more than a solubilisation vehicle, PM should be able to retain the drug for a certain period of time and also present long circulation time in blood to guarantee that the PM with the loaded drug will reach the target site at reasonable concentrations to exert its function. Different strategies can be employed to enhance drug retention in the PM core and to improve their circulation time in blood. In the first generation of PM the drug was formulated mainly by physical (hydrophobic) interactions between the core-forming block and the loaded molecules. In recent years, methods have been developed to chemically crosslink either the core or the shell of PM to enhance their stability in the circulation. Also the chemical coupling of the drug via reversible bonds to the block copolymer has been exploited to retain the drug in the micelles during transport and gradually release the drug at the site of action[34-37].

In 2007, the first micellar formulation was approved in South Korea for the treatment of breast and non-small cell lung cancer (NSCL)[38]. Genexol™-PM is a formulation based on the block copolymer mPEG-b-poly(D,L-lactic acid) with paclitaxel encapsulated in the core of the micelles. Following the approval of Genexol™-PM, several PM have entered clinical trials. Poly(ethylene glycol)-block-poly(aspartic acid) (PEG-b-pAsp) loaded with doxorubicin (NK911) or with paclitaxel (NK105) and poly(ethylene glycol)-block-poly(glutamic-acid) (PEG-b-pGlu) entrapped with cisplatin (NC-6004), SN-38 (NK012) or oxaliplatin (NC-4016) are some examples of formulations that are under clinical investigation.

In this review, we summarize the PM formulations that are in clinical evaluation for the treatment of cancer.

2. FIRST GENERATION OF POLYMERIC MICELLES

2.1. SP1049C

SP1049C is a micellar formulation composed of doxorubicin physically loaded in a blend of two micellar forming non-ionic Pluronic® block copolymers, namely Pluronic® L61 and L127 [39]. The formulation is prepared by reconstitution of freeze dried doxorubicin in 0.9% sodium chloride solution with 0.25 % (w/v) of L61 and 2% (w/v) of L127. The formulation has an average particle size of 30 nm and the drug loading capacity is 8.2%.

In vitro data demonstrated that SP1049C was effective against multidrug resistant cells and presented superior activity than free doxorubicin [40]. *In vivo* PK show an area under the curve (AUC) of the PM two times higher than free doxorubicin, 14.6 µg.h.ml⁻¹ and 7.1 µg.h.ml⁻¹ respectively, in mice bearing Lewis lung carcinoma 3LL M-27 cells. SP1049C

formulation inhibited tumour growth in more than 50% in nine different tumour models, which represents a superior antitumor activity when compared to free doxorubicin. Antitumor activity revealed that the inhibition of tumour growth was maintained longer when mice were treated with the micelle formulation when compared to the free drug at the same dose. Moreover, the antitumor activity appeared to be dependent on the ratio between hydrophilic/hydrophobic blocks. Higher activity was observed when the copolymer had longer hydrophobic blocks[41]. SP1049C antitumor activity was also assessed in mice bearing leukaemia cells intraperitoneally inoculated and showed to decrease the tumorigenesis and aggressiveness of tumour cells *in vivo*[42].

This formulation proceeded to phase I clinical trials in patients with advanced solid tumours[43]. The starting dose was 5 mg.m⁻² up to 90 mg.m⁻² administered every three weeks until a maximum of six cycles. From the total number of patients that enrolled this trial, 11.5% had a partial or complete response after SP1049C treatment and 30.8% of the treated patients had stable disease with median time for disease progression of 17.5 weeks. The dose limiting toxicity was myelosuppression which was reached at a dose of 90 mg.m⁻². The maximum tolerated dose was 70 mg.m⁻² which was the recommended dose for following clinical trials.

A phase II trial was conducted in patients with advanced adenocarcinoma of the oesophagus or gastroesophageal junction. After SP1049C reconstitution in 0.9% sodium chloride, it was administered at a dose of 75 mg.m⁻². Each patient received a median number of four cycles, ranging from one to six. The most commonly observed toxicity was neutropenia of grade 3 or 4 in 62% of the patients. The antitumor activity of this formulation resulted in nine patients with partial response, whereas eight patients showed minor response or stable disease and two had progressive disease. The median overall survival and median progression-free survival were of 10 and 6.6 months, respectively[44]. This formulation obtained recently FDA approval for a pivotal phase III trial in patients with cancer of the gastrointestinal tract.

2.2. NK911

Kataoka and colleagues developed a hybrid drug delivery system based on a covalent-physically loading strategy. A block copolymer of poly(ethylene glycol)-b-poly(α,β -aspartic acid) was synthesised to target solid tumours. To increase the hydrophobicity of the core forming block, doxorubicin was covalently bound to 50% of carboxylic groups of the poly-aspartate block (PEG-b-p(Asp-Dox), see table 1. As chemically conjugated doxorubicin did not exert any antitumor activity, likely because it was coupled via the polymer backbone via a hydrolytically stable amide bond, doxorubicin was also physically loaded into the PM. Free doxorubicin molecules interact with covalently bound doxorubicin essentially through π - π interactions. This results in stabilization of the micellar core and retention of the physically loaded doxorubicin into nano-sized PM with an average diameter of 40 nm[45]. Therefore, both chemically and physically loaded doxorubicin contributed to the low leakage of doxorubicin in the circulation, leading to a reduction of side effects and prolonged antitumor activity due to sustained release of physically entrapped doxorubicin upon disposition of the PM in the tumour.

In preclinical studies, these PM displayed a prolonged circulation time in a mouse model with subcutaneously implanted colon 26 tumour cells, with 29-fold higher AUC in blood when compared to free doxorubicin ($120 \mu\text{g.h.ml}^{-1}$ versus $4 \mu\text{g.h.ml}^{-1}$, respectively). As a result, these PM accumulate 3.4 times more in the tumour than the free drug; tumour AUCs of $1605 \mu\text{g.h.g}^{-1}$ versus $474 \mu\text{g.h.g}^{-1}$, respectively. PM showed good antitumor activity in mouse models of sarcoma, lung, colon and breast cancer[45].

In 2001, it was the first micellar formulation to progress into clinical trials. A phase I clinical trial to study PEG-b-(pAsp-Dox) polymeric micelles (NK911) toxicity was performed with twenty three patients that previously received chemotherapeutic treatment. Treatment started with a dosage of 6 mg.m^{-2} doxorubicin and increased up to 67 mg.m^{-2} , comprising 6 escalating doses in total[46]. NK911 administration was performed every 3 weeks using an infusion pump at a rate of 10 mg.min^{-1} . NK911 injection was in general well tolerated and the non-haematological toxicities reported were nausea, vomiting and anorexia. However, neutropenia was the predominant haematological toxicity, with grade 3 and 4 neutropenia at dosing level 5 (50 mg.m^{-2}). At dose level 6 of 67 mg.m^{-2} , all patients developed neutropenia, which in half of the patients lasted for more than 5 days. Therefore, 67 mg.m^{-2} was considered the maximum tolerated dose due to grade 4 neutropenia dose limiting toxicity and 50 mg.m^{-2} was the recommended dose to proceed with phase II clinical trials.

Chapter 2

The AUC of NK911 at the recommended dose was $3.2 \mu\text{g.h.ml}^{-1}$ whereas in case of free doxorubicin at equivalent dose the AUC was $1.6 \mu\text{g.h.ml}^{-1}$ (table 2). At the end of this study, eight patients exhibited stable disease for more than 4 weeks. One patient with metastatic pancreatic cancer showed partial response, with 50% decrease of the liver metastasis. This formulation proceeded to Phase II clinical trials, but the results are pending.

Table I. Polymeric micelles in clinical trials

Name	Polymer structure	Compound	Indication	Phase	Company
NK911	<p>PEG-b-p(Asp-DOX)</p> <p>R=H or DOX</p> <p>DOX=</p>	Doxorubicin	Pancreatic and Colorectal Cancer	II	Nippon Kayaku, Japan
NK105	<p>PEG-b-p(Asp-4-phenyl-1-butanol)*</p>	Paclitaxel	Stomach, Breast Cancer	III	NanoCarrier/Nippon Kayaku, Japan

Table I. Continued

Name	Polymer structure	Compound	Indication	Phase	Company
NK012	PEG-b-p(Glu-SN-38)	SN-38	Breast, Lung, Colorectal Cancer	II	Nippon Kayaku, Japan
NC-6004	PEG-b-p(Glu)	Cisplatin	Pancreatic, Head and Neck, Lung, Bladder and Bile Duct Cancer	III	NanoCarrier, Japan/Orient EuroPharma (co-development)
NC-6300	PEG-b-p(Asp-hydrazone)	Epirubicin	Solid tumors	I	NanoCarrier, Japan/Kowa (co-development)

Table I. Continued

Name	Polymer structure	Compound	Indication	Phase	Company
NC-4016		DACH-Platinum	Solid tumors and Lymphoma	I	NanoCarrier, Japan
CriPec® docetaxel		Docetaxel	Solid tumors	I	Cristal Therapeutics, The Netherlands
SP1049C		Doxorubicin	advanced adenocarcinoma of the esophagus or gastroesophageal junction	II	Supratek Pharma Inc.

Table II. Pharmacokinetic parameters of phase I clinical trials polymeric micelles

Pharmacokinetic parameters	Formulation									
	NK911 [46]		NK105		NK012 [47]		NC-6004		SP1049C [43]	
	Free doxorubicin	Micelles	Free paclitaxel [48]	Micelles [49]	Free SN-38	Micelles	Free cisplatin [50, 51]	Micelles [52]	Free doxorubicin	Micelles
Dose (mg m ⁻²)	50	50	210	150	28	28	80-100	90	50	70
t _{1/2} (h)	0.8 ± 1.1	2.8 ± 0.3	13.3 ± 1.5	10.6 ± 1.3	209 ± 25	137 ± 19	0.5	129 ± 40	0.8 ± 1.1	28.95 ± 52.03
AUC (µg h ml ⁻¹)	1.6 ± 1.1	3.2 ± 0.4	23.2 ± 10.7	369.8 ± 35.2	2.12 ± 0.83	294 ± 62	45.89 ± 4.62	2,836 ± 554	1.6 ± 1.1	3.42 ± 0.93
Max. Concentration in blood (µg ml ⁻¹)	n.a.	3.9 ± 0.7	6.74 ± 2.7	40.2 ± 5.5	0.114 ± 0.031	19.1 ± 3.9	3.31 ± 0.29	60.8 ± 12.5	n.a.	n.a.
Total Clearance (ml h ⁻¹ m ⁻²)	33,600 ± 13,066 ^a	15,633 ± 2,566 ^a	10,740 ± 4,860	408.6 ± 37.3	n.a.	98.8 ± 20.6	20,700 ± 5820	33.8 ± 11.1 ^a	33,600 ± 13,066 ^a	2,7638.9 ± 10712.72 ^a
Volume of distribution (ml m ⁻²)	933,333 ± 466,666 ^a	579,444 ± 140,00 ^a	58,900 ± 24,700	4,527.1 ± 639.5	n.a.	2,020 ± 530	12,000	6,555 ± 3,833 ^a	933,333 ± 466,666 ^a	1,077.1 ± 759.8 ^a

a – calculated by considering the body surface area equal as 1.8 m²

2.3. NK105

Paclitaxel is one of the most effective anticancer compounds and it has been shown to be efficacious in different types of solid tumours, like breast and ovarian cancers[53-55]. Due to its poor water solubility, however, the use in clinical practice is hampered by the adverse effects of the solubilizing agents needed for i.v. injection of this drug. The development of a biocompatible drug delivery system could reduce these adverse effects and potentially increase the therapeutic potential of paclitaxel.

NK105 is a polymeric micellar nanoformulation composed of an amphiphilic block copolymer, containing PEG as hydrophilic block and modified pAsp as hydrophobic block (figure 1). Half of the carboxylic groups of pAsp were modified with 4-phenyl-1-butanol by an esterification reaction to render this block hydrophobic and make micelle formation possible. Paclitaxel was physically loaded in the PM through hydrophobic interactions and the formulation was subsequently freeze dried. Upon reconstitution, PM had an average diameter of 85 nm and had a paclitaxel loading of 23% (w/w).

Preclinical data on the PK of NK105 in HT-29 colon tumour-bearing mice showed that paclitaxel was present in the circulation for up to 72 h after injection, whereas free paclitaxel could not be detected 24 h post administration. The i.v. injection at a dose of 50 mg.kg⁻¹ the plasma AUC was of 7860.9 and 90.2 $\mu\text{g.h.ml}^{-1}$ for NK105 and free paclitaxel, respectively[56]. In terms of tumour accumulation, the NK105 formulation achieved a 25-fold higher tumour AUC and 3-fold higher maximum concentration of paclitaxel when compared to free paclitaxel. Moreover, NK105 was retained in tumours at least until 72 h after injection.

Figure 1. Illustration of NK105 formulation. NK105 micelles are formed by the assembly of the amphiphilic copolymer (composed of polyethylene glycol and polyaspartate modified with 4-phenyl-1-butanol) and Paclitaxel. Paclitaxel is loaded in the hydrophobic core of the micelles via physical interaction, mainly π - π stacking. Image reproduced from [56], with permission of Nature Publishing Group, 2005.

The therapeutic efficacy of the micellar formulation in mice bearing subcutaneous HT-29 colon tumours showed reduced tumour growth rates for both free paclitaxel and NK105. Still, the antitumor activity of NK105 was superior when compared to free drug. Antitumor efficacy at a dose of 25 mg.kg^{-1} of NK105 was equivalent to 100 mg.kg^{-1} of free dosed paclitaxel. Tumour suppression was dose dependent and mice were cured after a single injection of NK105 at a dose of 100 mg.kg^{-1} . Following the promising outcome of preclinical studies, phase I clinical trials were conducted in patients with advanced solid tumours to determine the PK, the maximum tolerated dose and the recommended dose for phase II studies. NK105 was supplied as a freeze-dried formulation containing 30 mg of paclitaxel. After reconstitution in 5% glucose, the formulation was stable for 24 h at room temperature. NK105 was infused i.v. once every three weeks at a speed of 250 ml.h^{-1} , for 1 h. The starting dose was 10 mg.m^{-2} escalating up to 180 mg.m^{-2} . A total of nineteen cancer patients who had previously received chemotherapy (not including Taxanes) were recruited. Generally, no major toxicity was noted upon NK105 administration, apart from grade 1 or 2 neuropathy, irrespective of the dosing schedule. No haematological toxicity was observed until dose level 4 (80 mg.m^{-2}). Dose-limiting

toxicity was reached at level 7 (180 mg.m^{-2}) in two out of five patients, who showed grade 4 neutropenia for more than five days. Therefore, 180 mg.m^{-2} was considered the maximum tolerated dose, with neutropenia as the dose-limiting toxicity. In subsequent phase II trials, a dosage of 150 mg.m^{-2} was selected as the recommended dose. NK105 PK were assessed in patients that received the 150 mg.m^{-2} dose. The AUC of NK105 was 15-fold higher than of free paclitaxel given at the conventional dose of 210 mg.m^{-2} [48]. Six patients had stable disease for more than four weeks after completion of the study. A partial response was observed in a patient with metastatic pancreatic cancer, in whom liver metastasis decreased more than 90%. A patient with stomach cancer experienced a reduction of 40% in a peritoneal metastasis.

A phase II trial was performed from 2007 to 2010 to assess the efficacy and safety of the NK105 formulation in patients with advanced gastric cancer who did not respond to first-line chemotherapy consisting of fluoropyrimidine and/or cisplatin. NK105 was administered to fifty seven patients every three weeks at a dose of $150 \text{ mg paclitaxel.m}^{-2}$ [49]. The median treatment duration was 2.8 months and patients received an average of four cycles of treatment.

The overall response rate was 25% with two patients showing complete response and twelve patients showing partial responses (out of fifty six evaluable patients). Additionally, ~30% of the patients (seventeen out of fifty six) experienced stable disease for several months. The disease control rate of 55% is encouraging in patients that had previously received chemotherapy. The median time to response was 1.8 months and the duration of the response was 3.7 months. The progression free survival, overall survival and time to treatment failure were 3.0, 14.4 and 2.8 months, respectively [57].

The major adverse effects were neurotoxicity of grade 1 or 2 and the most common haematological toxicity was grade 4 neutropenia. The PK profile of NK105 (table 2) was also assessed at dose of 150 mg.m^{-2} . The AUC was found to be 9-fold higher than that of free paclitaxel at the conventional dose (210 mg.m^{-2}).

A multi-national phase III clinical study to confirm NK105 therapeutic efficacy in patients with metastatic or recurrent breast cancer has started in 2012 and is estimated to be completed at the end of 2017 (Study NCT01644890). A recent press release stated the Phase III clinical trial failed to provide substantial benefit for patients. This was because one of the primary endpoints - progression free survival (PFS) – was not achieved, exemplifying the need for improved PM formulations.

3. NEXT GENERATION OF POLYMERIC MICELLES

3.1. NC-6300

Progress in the drug delivery field has led to the development of systems capable of “sensing” and responding to stimuli such as pH. Taking this into consideration, pH sensitive PM were developed by Kataoka and colleagues by modification of an existing formulation, NK911. NK911 is formed by the self-assembly of the block copolymer with doxorubicin covalently bound and doxorubicin physically loaded, only the latter presents cytotoxic effect. In NC-6300 doxorubicin is covalently conjugated to the carboxylic acid groups of PEG-b-p(b-Asp) via an hydrolysable linker, hydrazone bond, to enable drug release upon a pH stimuli (pH <5). This block copolymer was synthesized by means of ring-opening polymerization of β -benzyl-L-aspartate N-carboxyl-anhydride using α -methoxy- ω -amino poly(ethylene glycol) as initiator [58]. The benzyl groups were subsequently removed in order to attach the hydrazone groups. The resulting polymer, PEG-p(Asp-Hyd) had an average of 37 repeating units of aspartate of which 28 were modified with hydrazone. Finally, doxorubicin was conjugated to the polymer backbone via a hydrazone bond. Although the starting poly(ethylene glycol)-poly(β -benzyl-L-aspartate) is fully water-soluble, after derivatization with doxorubicin the PEG-p(Asp-Hyd-dox) block is hydrophobic to make micelle formation possible. PM were prepared via a dialysis method and had a mean size of 65 nm. Release experiments showed that the PM were stable at neutral pH and released 30% of their payload at pH 5 in 72 h due to the acid sensitivity of the hydrazine bonds.

The stability of the PM at neutral pH enabled a 4-fold higher dosing of doxorubicin when i.v. administered in mice bearing C26 tumour cells, compared to the free drug [59]. The PK profile of doxorubicin either administered as free drug or loaded in the PM at the same dose (10 mg.kg^{-1}) showed that PM circulated 15-fold longer in blood than free doxorubicin (the AUC was $58.86 \text{ }\mu\text{g.h.ml}^{-1}$ and $858.54 \text{ }\mu\text{g.h.ml}^{-1}$, respectively). This was paralleled by a 4 times higher tumour accumulation of doxorubicin administered as PM compared to free doxorubicin. C26-tumour bearing CDF1 mice were treated with free doxorubicin at doses ranging from 5 to 15 mg.kg^{-1} whereas the micellar formulation was administered at starting dose of 5 mg.kg^{-1} up to 60 mg.kg^{-1} .

The antitumor activity of free doxorubicin was demonstrated at a dose of 10 mg.kg^{-1} however mice treated at 15 mg.kg^{-1} died due to toxicity. The PM antitumor effect was observed for doses $\geq 20 \text{ mg.kg}^{-1}$. Complete cure was obtained for 2/6 and 3/6 of the treated mice with a dose of 20 and 40 mg.kg^{-1} of doxorubicin-loaded PM, respectively[59]. Mice treated at 60 mg.kg^{-1} of doxorubicin in the PM showed a toxic

death. Additionally, at therapeutic dose for the free doxorubicin (at 10 mg.kg⁻¹) or the micellar formulation (at 20 and 40 mg.kg⁻¹), the decrease in tumour volume was more pronounced in mice treated with PM compared to the free drug treatment. In terms of toxicity, the maximum tolerated doses were established at 40 mg.kg⁻¹ and 10 mg.kg⁻¹, for the micellar and the free doxorubicin, respectively. Taking into account that the therapeutic window for the micellar formulation is broader than for the free drug and the fact that doxorubicin can be dosed higher when formulated in the PM without showing toxic effects, it presents an advantage towards the application of these PM. For further development of this nanoformulation, doxorubicin was substituted by epirubicin which differs from doxorubicin on the spatial orientation of the hydroxyl group at the 4' carbon of the sugar moiety. Epirubicin presents the same efficacy as doxorubicin, however, the toxicity is lower for epirubicin[60, 61]. Several clinical trials compared equimolar administration of doxorubicin and epirubicin in patients with breast cancer and showed that the percentage of patients with cardiotoxicity, nausea, vomiting and neutropenia was higher when treated with doxorubicin than when patients were treated with epirubicin[61].

Additionally, the block copolymer was partially substituted with benzyl groups in order to increase micellar stability, circulation kinetics and ultimately the therapeutic efficacy[62]. The block copolymer with epirubicin covalently coupled via a hydrazone bond, known as NC-6300 (figure 2), also spontaneously assembles into micellar structures with a diameter of 40-80 nm in an aqueous milieu.

In preclinical experiments, NC-6300 was administered to mice bearing human hepatic Hep3B tumours at doses of 15 or 20 mg.kg⁻¹ epirubicin compared to 7 mg.kg⁻¹ of the free drug, which is the maximum tolerated dose[63]. The formulation was able to decrease the tumour mass by 95% and 99% at doses of 15 and 20 mg.kg⁻¹, respectively, whereas free epirubicin decreased tumour weight by a mere 53%, when compared to untreated control mice.

PK analyses performed in healthy male Wistar rats showed that the half-life of epirubicin when dosed in NC-6300 formulation was substantially prolonged to 4 h, whereas free epirubicin was completely cleared within 0.4 h. The plasma AUC was 2200-fold increased for NC-6300 as compared to free epirubicin. In nude mice bearing MDA-MB-231 breast tumours, free epirubicin was detected up to 48 h after i.v. injection whereas micelle-loaded epirubicin was detected at higher concentrations and for up to 168 h after administration. Additionally, 24 h after injection 74% of the total epirubicin found was in its free form in tumour tissue, while in the case of other tissues (spleen, kidney, liver,

lung, heart), only around 20-40% was found as free drug. This indicates that epirubicin is efficiently released in the tumour tissue likely due to the slight acidic pH in tumour [64].

Cardiotoxicity is known as the major adverse effect related to the use of epirubicin[65]. Mice treated with free epirubicin indeed showed a weakened cardiac function after 9 weeks of treatment. Importantly, mice given the NC-6300 formulation, did not show any cardiac malfunction which confirms the ability of PM to reduce drug accumulation in toxicity-sensitive tissues.

Figure 2. NC-6300 formulation is spontaneously formed when poly(ethylene glycol)-b-poly(benzyl-aspartate-hydrazone) copolymer containing an acid-labile linker is disposed in aqueous media; epirubicin is covalently bound to the polymer via hydrazone bond. The micelles present an average diameter of 40-80 nm and are stable under physiological conditions. In acidic conditions, the hydrazone bond dissociates and the drug is released [65].

To further improve the selectivity of the targeting approach, NC-6300 was conjugated with an antibody against tissue factor (TF), which is overexpressed in various cancer cells[66]. *In vitro* studies demonstrated that the extent of cell binding of the targeted versus the non-targeted (control) formulation to a gastric cancer cell line (44As3) and a pancreatic cancer cell line (BxPC3) was two-fold higher. Moreover, targeted PM were internalized faster and more efficiently than non-targeted PM.

In mice bearing tumours with high TF expression, the efficacy of anti-TF-NC-6300 formulation was better than that of the control formulation (tumour size at day thirty five was 1200 mm² and 2000 mm², respectively). However, the untargeted formulation did not perform better than the free epirubicin; free epirubicin presented higher therapeutic effect than the control mice (untreated). In tumours with low TF expression, the efficacy of both formulations was comparable. Meaning that the superior efficacy of

the targeted formulation in high TF expressing tumours is due to the targeting ligand. The AUC of free epirubicin in the tumour after administration of the targeted or non-targeted PM was also similar irrespective of the expression level of TF. This points that the formulations had the same circulation kinetics resulting in the same dose of tumour deposited PM. The intratumoral distribution of the PM was highly affected by level of TF expression. Anti-TF-NC-6300PM reached most of the tumour cells in the malignant tissue and were homogeneously distributed over tumours with high TF expression. In the case of low TF expressing tumours, the targeted PM were mostly located in the areas surrounding the tumour blood vessels. The distribution of the non-targeted formulation in high or low TF-expressing tumours was not studied.

NC-6300 entered a phase I clinical trial in 2013 to assess tolerability and the recommended dose in patients with advanced solid tumours[38]. To date, no results of this study are available.

3.2. NC-6004

Platinum based anticancer drugs are widely used in the clinic as part of the treatment of a variety of tumours, such as melanoma, ovarian cancer and lymphomas [67]. Cisplatin is one of the most extensively used drugs of its class. This drug is a platinum chelate complex that targets DNA, forming platinum-DNA adducts that change the structure of the DNA molecule by unwinding and bending it, and ultimately destabilizing DNA[68]. Platinum based drugs present as major limitation systemic toxicities, like nephrotoxicity [69] and neurotoxicity [70] featured mainly by peripheral neuropathy.

Kataoka and colleagues developed the first PM loaded with cisplatin. PM were formed via polymer-metal complex formation between the carboxylic groups of PEG-b-p(α,β -aspartic acid) and cisplatin in water [71]. Cisplatin-loaded PM had an average diameter of 16 nm at a molar ratio between cisplatin and aspartic acid residues of the polymer of 1:1 [72]. PM were stable in distilled water for more than 24 h of incubation. When incubated in an aqueous buffer containing NaCl, the PM were stable for 10 h, after which the drug was gradually released. This suggests that the core of the PM is not fully packed and the chloride ions can permeate the core and cause disassembly of the polymer-metal complex[71, 73]. Cisplatin-loaded PM were administered i.v. in lung carcinoma bearing mice to assess their PK profile. The AUC for micellar cisplatin was 5.2 and 4.6-fold higher in plasma and tumour, respectively, in comparison to the free drug [74]. Even though, the fast structural decay of the micelles led to the accumulation of the drug in liver and spleen at high levels which resulted in a similar antitumor efficacy as the free drug. To improve both the stability of the PM and the drug release profile, a modified

carrier system composed of PEG-b-p(L-glutamic acid) (PEG-p(Glu)) was developed [75]. Cisplatin and PEG-p(Glu) formed PM with an average diameter of 30 nm and a drug content of 39% (w/w). These PM were very stable in water. In physiological saline, the PM size was stable (25 nm) for 50 h and the drug release followed gradually over 150 h. Preclinical data demonstrated that the drug level in plasma was over 50% of the injected dose (I.D.) at 8 h, and at 24 h after injection 13% I.D. was still in the circulation. This is a significant improvement compared to the pAsp PM, for which only 1.5 % of the I.D. was recovered in the circulation at 24 h after administration [74]. This favourable PK profile was ascribed by the authors to the higher stability of the pGlu based formulation compared to that based on pAsp.

Cisplatin delivered by PEG-pGlu PM accumulated in the tumour 20-fold more than free cisplatin. In C26-tumour bearing mice treated with 4 mg.kg⁻¹ cisplatin, four out of ten mice showed complete tumour regression[75]. Similarly, in human gastric cancer (MKN-45) [76] and in pancreatic adenocarcinoma (BxPC3)-bearing mice [77], administration of cisplatin in PEG-pGlu PM resulted in improved antitumor activity. Moreover, PM were able to overcome toxicities related to administration of free drug, such as nephrotoxicity, decrease in nerve conduction velocity, and degeneration of the sciatic nerve[76]. In a guinea pig model, cisplatin-loaded PM displayed less ear toxicity in comparison to free cisplatin[78].

Due to the favourable outcome in preclinical studies, cisplatin loaded in PEG-pGlu PM entered phase I clinical trials under the name NC-6004. A phase I clinical trial started in the UK with seventeen patients[52]. The administration of NC-6004 commenced at a dose of 10 mg.m⁻² escalating to 120 mg.m⁻². A sterile formulation of NC-6004 with an equivalent dose of 2.5 mg.ml⁻¹ of cisplatin was i.v. injected once every 3 weeks. Plasma AUC was more than 60-fold higher for NC-6004 than the AUC for the free drug at equivalent dose. In general, NC-6004 was well tolerated with no occurrence of haematological toxicities. Regarding non-haematological toxicities, the most common were fatigue, nausea, anorexia and vomiting. From six patients treated at 90 mg.m⁻², one presented grade 3 fatigue as dose limiting toxicity. In addition, renal toxicity of grade 2 was observed at 90 and 120 mg.m⁻² even though prophylaxis was employed. Therefore, 120 mg.m⁻² was concluded to be the maximum tolerated dose and the recommended dose for Phase II studies was 90 mg.m⁻². At the recommended dose for phase II, 50% of the patients presented stable disease. The median progression-free survival time was forty nine days and fourteen patients (82%) patients died or presented tumour progression.

A phase I/II study (Study NCT00910741) was conducted in Taiwan and Singapore to determine the maximum tolerated dose and recommended dose of NC-6004 in combination with gemcitabine in patients with pancreatic cancer [79]. In total nineteen patients received NC-6004 at doses of 30 up to 120 mg.m⁻² once every three weeks, and gemcitabine at 1000 mg.m⁻² twice every 3 weeks. Dose limiting toxicity occurred at 120 mg.m⁻², and therefore 120 mg.m⁻² was considered the maximum tolerated dose and 90 mg.m⁻² the recommended dose of NC-6004 in combination with gemcitabine. From seventeen patients evaluated, one patient had a partial response and ten patients experienced stable disease. No cases of complete response were noted. Overall the disease control percentage in this study was 65%.

A phase III trial started in 2014 to assess the efficacy of the combined therapy of NC-6004 with gemcitabine versus gemcitabine alone in patients with locally advanced or metastatic pancreatic cancer (Study NCT02043288). The results have not been published yet. A number of phase I trials are currently running to determine the maximum tolerated dose and recommended dose of NC-6004 in combination with several other drugs. NC-6004 in combination with 5-FU and cetuximab is being tested in patients with recurrent or metastatic squamous cell carcinoma of the head and neck, in Taiwan (Study NCT02817113). Also, in Japan and the U.S.A., NC-6004 safety and tolerability is being tested in patients with head and neck cancer. A Phase I/II trial of NC-6004 in combination with gemcitabine for the treatment of advanced solid tumours or non-small cell lung, biliary tract, and bladder cancer is being conducted in the U.S.A. (Study NCT02240238). A phase III clinical trial of the combination of NC-6004 and gemcitabine versus gemcitabine alone in patients with locally advanced metastatic pancreatic cancer started in 2014 (Study NCT02043288).

3.3. NC-4016

Cisplatin-loaded PM, as mentioned above, showed good stability and efficient disposition of the drug in the tumour. However, cisplatin administration is associated with acute toxicity and acquired resistance. Therefore, other platinum based drugs, like dichloro (1,2-diaminocyclohexane)platinum(II) (DACHPt), the oxaliplatin parent complex, were developed to enhance drug accumulation in the tumour, DACHPt is more hydrophobic than cisplatin and has lower toxicity[80]. DACHPt was loaded in PEG-poly(γ -benzyl-L-glutamate) block copolymer via polymer-metal complexation between the platinum of DACHPt and the carboxylic groups of the block copolymer (figure 3). The PM with an average size of 40 nm showed platinum loading of 75% (w/w). No drug release occurred in water, whereas in phosphate buffered saline the drug was released in a sustained manner with 60% being released after 96 h of incubation at 37°C.

The good stability of DACHPt-loaded PM resulted in good circulation kinetics with 80% of the I.D. in the circulation 9 h post administration and 16% of I.D., 27 h after injection in CDF₁ mice bearing C-26 tumours[80]. In these mice, DACHPt-loaded PM accumulation in the tumour peaked at 11% of ID 48 h post injection[81]. Compared to free oxaliplatin, this represents a 25-fold increase in tumour accumulation. Antitumor activity of DACHPt-loaded PM was achieved at doses of 2 up to 6 mg.kg⁻¹ whereas free oxaliplatin at doses up to 10 mg.kg⁻¹ did not show any reduction in tumour growth rate. Noticeably, no body weight loss was detected at the therapeutic effective doses of 2 and 4 mg.kg⁻¹ of the micellar formulations. Mice treated with 10 mg.kg⁻¹ of free oxaliplatin experienced toxic death after the fourth injection. Mice were inoculated intraperitoneally with bioluminescent HeLa (HeLa-Luc) cells and subsequently treated with either free oxaliplatin or DACHPt-loaded PM. The therapeutic efficacy, determined by bioluminescence, point to a 10- to 50-fold decrease in fluorescence in the group of mice treated with DACHPt-loaded PM, meaning that these PM are able to reduce the metastatic character of tumours[81].

Figure 3. NC-4016 micellar formulation. Micelles are formed by coordination interaction between poly(ethylene glycol)-*b*-poly(glutamic acid) and DACHPT in an aqueous environment. Image reproduced from [82], with permission of Nature Publishing Group, 2011.

Antitumor efficacy was demonstrated in tumour models of colon carcinoma[81], orthotopic scirrhous gastric cancer[83], melanoma[84], lung metastasis[84], intraperitoneal metastases[81], and lymph node metastasis[83].

The effect of combined treatment with epirubicin and oxaliplatin-loaded micelle formulations (NC-6300 and NC-4016, respectively) was assessed in a gastric cancer xenograft model[85]. The antitumor effect was superior when both formulations were administered, as compared to the combination of the free drugs. The combination of epirubicin, oxaliplatin and capecitabine is one of the standard treatments in Europe for gastric cancer. The good outcome of NC-6300 and NC-4016 formulations is encouraging to further study this combination in gastric cancer patients. Regarding NC-4016, a phase I clinical trial started in the U.S.A. for the treatment of patients with advanced solid tumours and lymphoma (Study NCT01999491).

3.4. NK012

NK012 is a polymeric prodrug of SN-38 forming a core-shell structure. SN-38 (7-ethyl-10-hydroxycamptothecin) is covalently conjugated to PEG-*b*-poly(L-glutamic acid) by esterification of the drug's phenol groups and the carboxylic acid groups of the polymer[86].

NK012 is a freeze dried formulation containing 20% (w/w) of SN-38 with an average size of 20 nm. The drug release in phosphate buffered saline pH 7.4 is reported to be 57% over 24 h and in 5% glucose (pH 4.6) only 1%, indicating that the release is likely dictated by the pH of the solution.

PK of NK012 in HT-29 tumour-bearing mice showed a much slower plasma clearance of SN-38 when compared to free drug, presenting an AUC that was 200-fold higher, even when the dose of SN-38 was more than two-fold lower for the micellar formulation (30 mg.kg⁻¹ versus 66.7 mg.kg⁻¹, respectively). The prolonged circulation time of NK012 enabled increased drug accumulation in tumour tissue, which in mice bearing HT-29 tumours was 23-fold higher 24 h after administration when compared to the free drug. Moreover, the tumour-to-plasma concentration ratio of the drug was found to be 100 times higher for NK012 than for the free drug, at 168 h after injection. These PK characteristics resulted in a successful pharmacodynamic antitumor activity of the NK012 formulation. A significant decrease in HT-29 tumour volume was observed 60 days after the first injection, at doses of 15 and 30 mg.kg⁻¹ for NK012[86].

The NK012 formulation was also tested in VEGF-secreting tumours (SBC-3/VEGF) to evaluate the antitumor effect in hypervascularized tumours. In mice bearing slowly vascularising SBC-3/Neo tumours, administration of this drug at 10 and 20 mg.kg⁻¹, led to a significant decrease in tumour volume in comparison to the free drug at higher dose (15 and 30 mg.kg⁻¹). For mice with hypervascularized SBC-3/VEGF tumours, the tumours were completely eradicated after 20 days of the first injection and for a total of three injections (10 and 20 mg.kg⁻¹). This indicates that high vascularization enhanced the activity of the nanoformulation, likely through increased EPR effect. This formulation was additionally tested in several human tumour xenografts models, such as glioma [87, 88], renal cancer [89], gastric cancer [90], pancreatic cancer [91] and small cell lung cancer [92]. In all these models superior antitumor activity over the free drug was demonstrated. NK012 was employed in combination with different conventional drugs to further improve the efficacy of the monotherapy regimen. In combination with 5-fluoruracil (5-FU) improved efficacy in the treatment of colorectal cancer was shown[93]. Similarly, with cisplatin the treatment of small cell lung cancer was improved[94]. NK012 was also evaluated in a mouse model of multiple myeloma. The combination of this formulation with the clinically approved proteasome inhibitor bortezomib [95] showed improved antitumor efficacy when compared with bortezomib alone[96]. These pre-clinical studies provided a strong support for clinical testing of the NK012 formulation.

Two independent phase I clinical trials, one in Japan [47] and the other in the United States (study NCT00542958) [97], were conducted in patients with solid tumours refractory to standard therapy or in patients for whom no alternative therapies were available. NK012, containing 20% (w/w) of SN-38, was diluted in 5% glucose solution and administered as a 30 minute infusion every twenty one days.

In one of the studies [47], the starting dose was 2 mg.m^{-2} equivalent of SN-38 and increased up to 28 mg.m^{-2} . Plasma AUC of SN-38 in NK012 was approximately 150-fold higher than that of free SN-38, being 294 ug.h.ml^{-1} and $2.12 \text{ ug.h.ml}^{-1}$ for NK012 and SN-38 at a dose of 28 mg.m^{-2} , respectively. The total clearance and the volume of distribution was 150- and 80- fold lower than for the free drug[47, 98], respectively.

Out of twenty three patients that received the formulation two patients presented a partial response and nine patients had stable disease. Moreover, from twelve patients who had colorectal cancer, five presented partial response. The median number of cycles administered was of 3.5, ranging from one to twelve cycles. Twenty-two patients received more than two administrations. The common toxicities were grade three/four leukopenia and neutropenia, which was reduced to grade one in four to sixteen days. Dose limiting toxicity was neutropenia at 28 mg.m^{-2} . The maximum tolerated dose was not determined due to haematological toxicities. The recommended dose for phase II was 28 mg.m^{-2} .

On the other study [97], NK012 was administered at a dose starting from 9 to 37 mg.m^{-2} . The NK012 formulation at a dose of 28 mg.m^{-2} and the free drug at a dose of 250 mg.m^{-2} presented an AUC of 287 and $27.86 \text{ ug.h.ml}^{-1}$, respectively, which accounts for a 10-fold higher AUC of NK012 compared to the free drug and at an almost ten times lower concentration of I.D. Of a pool of thirty seven patients, six patients had partial response, eighteen presented stable disease and twelve patients showed disease progression; the median number of cycles administered per patients was four; and six or more cycles were administered to sixteen patients. In terms of toxicity, NK012 was generally well tolerated and the dose limiting toxicity was grade three neutropenia. The maximum tolerated dose was 28 mg.m^{-2} . Therefore, the recommended dose for phase II was set at 28 mg.m^{-2} . These combined data demonstrate that the NK012 formulation is stable upon systemic injection and the PK data support EPR-mediated accumulation at the target site[38].

Given the promising outcome in preclinical studies in which the combination of NK012 with 5-FU was examined[93], a phase I trial (study NCT01238939) of NK012 in combination with 5-FU and leucovorin (or folinic acid, is a compound with the same chemical structure as vitamin B₉ and also exert the same vitamin activity) was conducted. Leucovorin is used in combination with chemotherapeutic drugs to either enhance their therapeutic efficacy or decrease their toxic side effects. The number of patients that enrolled in this trial is estimated to be thirty five. The primary outcome measures were set as the number of patients with dose limiting-toxicity, which will determine the maximum tolerated and recommended dose. The outcome has not been yet reported.

Another phase I study of NK012 in combination with carboplatin in patients with advanced solid tumours, following a dose expansion in patients triple negative metastatic breast cancer (study NCT01238952) started at the end of 2010. Thirty-five patients were expected to enroll in this trial that ended in 2014. Also in this case, results are not yet available. A phase II trial was performed in the U.S.A. to assess efficacy of NK012 in patients with relapsed small cell lung cancer (study NCT00951613). Patients with sensitive relapsed and refractory relapsed lung cancer were enrolled in this study. In patients with sensitive relapsed, the overall response rate was of 22%, with two patients with complete response; the disease control rate (sum of complete response, partial response and stable disease) was of 68% [99]. The PM were well tolerated, with neutropenia as the dominant toxicity observed (44%). NK012 was also evaluated in a phase II trial, in the U.S.A., in patients with locally advanced non-resectable and metastatic breast cancer (study NCT00951054). This study was completed in 2015, but no results are available at present.

In Japan, a phase II trial was conducted in fifty-eight patients with recurrent or metastatic colorectal cancer who had previously received oxaliplatin-based chemotherapy (Study JapicCTI-090780). The overall response rate was 3.8% and disease control rate was of 56%. The median for progression free survival and the overall survival was of 99 and 451 days, respectively [100].

The phase I trials discussed above show that NK012 formulation presents a satisfactory safety profile. NK012 was well tolerated for doses up to 28 mg.m⁻² and the toxicity profile was markedly different from the one exhibited by the free drug. Grade 3 and 4 diarrhea, nausea and vomiting, which are common toxicities of SN-38 systemic administration were not reported in patients that received NK012 formulation. To note that the maximum tolerated dose was not determined due to hematological toxicities.

In a phase II trial, NK012 shown to be efficacious after administration in patients with lung cancer and showed an overall response rate of 22% and two patients had complete response. In this case the toxicity was mild and as expected.

Another phase II study conducted in patients with colorectal cancer NK012 showed similar efficacy as the free compound. In terms of toxicity, gastrointestinal toxicity and myelosuppression were observed. The results presented are encouraging to pursue with the development of this formulation; however, further optimization of the formulation is necessary to decrease toxicity.

3.5. CriPec docetaxel

NC-6300 makes use of an acid labile bond to better control drug release after *in vivo* administration. However, these PM rely on hydrophobic interactions for stability. Hennink and co-workers proposed chemical crosslinking of the micellar core in order to prevent disintegration of the PM in the circulation (figure 4)[101]. Partially methacrylated mPEG-b-pHEMAmLac_n block copolymers rendered core-crosslinked CCPM after UV-induced photo-crosslinking. These CCPM demonstrated superior physicochemical stability when compared to non-crosslinked NCPM. CCPM showed prolonged circulation time with concomitant increased tumour accumulation. Half of the I.D. was still in the circulation at 6 h post-injection and which resulted in a high tumour accumulation, as 6% I.D. accumulated in the tumour whereas only 1% I.D. of the non-crosslinked PM was present at tumour site, at 48 h post injection[101]. Even though CCPM demonstrated an excellent PK and tissue distribution profile, the encapsulated drug was rapidly released from the micellar core. Already at 30 min after administration, 95% of the initially loaded drug was released [102]. This study clearly indicates that prolonged blood circulation and target tissue accumulation of PM does not necessarily represent the same kinetics for the encapsulated drug.

Figure 4. Schematic representation of the formation of core-crosslinked PM based on mPEG-b-PHEMA-Lac_n. The amphiphilic block copolymer assembles into micelles when above the CMC; crosslinking of the micellar core is performed subsequently and to prevent destabilization and drug release. Image reproduced from [101], with permission of Elsevier, Copyright 2007.

To overcome premature drug release, Hennink's group covalently coupled the drug in the micellar core to warrant an efficient delivery of the drug to the target site. For this, mPEG-b-p(HPMAM-Lac_n) was co-crosslinked with doxorubicin functionalized with a methacrylamide group (DOX-MA)[103] through a pH sensitive linker – hydrazone – that degrades under acidic conditions[104]. DOX-MA was loaded into the PM and then copolymerized with the methacrylate groups of the thermosensitive block. In principle, doxorubicin covalently entrapped through the hydrazone linker, is preferentially released in the tumour microenvironment or intracellularly in lysosomes where the pH is below 5. PMs had an average size of 80 nm and 30-40% (w/w) of the added drug was covalently entrapped. Due to the proton catalysed hydrolysis of the hydrazine bond, at pH 7.4 only 5% release was observed whereas 100% of drug was released within 24 h whereas at pH 5. The therapeutic efficacy of this micellar nanoformulation was assessed on mice bearing B16 melanoma carcinoma and compared to the free drug at equivalent dose. A significant decrease in tumour growth was observed in mice treated with free and micellar doxorubicin compared to control (untreated) mice. Importantly, the mice treated with doxorubicin loaded PM showed a slower rate of tumour growth when compared to mice treated with free doxorubicin[103]. The same formulation was coupled with an anti-EGFR nanobody EGa1 to specifically target and increase the uptake of the PM by cancer cells. An *in vitro* binding assay was performed with rhodamine labelled PM showing that after 1 h of incubation with EGFR overexpressing tumour cell lines (A431 and UM-SCC-14C) there were significantly more PM associated

with the cell surface in the case of EGa1-coupled PM than in the case of untargeted PM[105]. This micellar formulation was subsequently evaluated in mice bearing 14C tumour xenografts and both untargeted and targeted micellar formulation showed to be more effective than free doxorubicin in inhibiting tumour growth and increase animal survival[106]. Moreover, targeted micelles were able to inhibit tumour growth even in the absence of the drug due to the intrinsic anticancer activity of the nanobody. The release kinetics of the drug can be tailored through the derivatization of the drug coupled to the polymer backbone with different linkers. For instance, dexamethasone was derivatized with three different methacrylated spacers via ester bonds to create dexamethasone derivatives capable of polymerization. The sulphide, sulfoxide and sulfone linkers presented different hydrolysis rates, enabling control of the release rate of the drug from the PM[107].

Recently, docetaxel was methacrylated via an ester bond and chemically loaded into core-crosslinked PM composed of mPEG-b-p(HPMAm-Lac_n)[108]. PM were formed with a mean diameter of 66 nm and had a docetaxel encapsulation efficiency of 75% (w/w) at a loading capacity of 12% (w/w). In mice with MDA-MB-231 breast tumour model, the half-life of docetaxel in CCPM in the circulation was 16.2 h[108] whereas that of free docetaxel was only 0.88 h[109]. The tumour accumulation of docetaxel was strongly enhanced in the case of CCPM. After a single injection of 30 mg.kg⁻¹ docetaxel-loaded CCPM, docetaxel concentration in the tumours of MDA-MB-231 tumour bearing mice, were 20- and 50-fold higher after 2 and 4 days, respectively, when compared to an equivalent dose of the free docetaxel formulation Taxotere (docetaxel in polysorbate 80). Therapeutic efficacy of docetaxel-loaded CCPM was assessed after a single i.v. injection at a dose of 30 or 60 mg.kg⁻¹. At a dose of 30 mg.kg⁻¹ the decrease in tumour growth as compared to the saline control was the same for either the docetaxel in CCPM as for Taxotere. At a dose of 60 mg.kg⁻¹ CCPM significantly inhibited the tumour growth whereas Taxotere formulation did not show any improvement compared to the 30 mg.kg⁻¹ concentration. Importantly, a dose of 125 mg.kg⁻¹ of docetaxel in the micellar formulation led to the complete eradication of the tumour growth in mice and to the survival of all mice after 62 days. This formulation, CriPec® docetaxel, was the first in Europe to enter phase I clinical trials, starting in 2015 (study NCT02442531). The same micellar strategy was applied for the formulation of a therapeutic peptide, leuprolide. Leuprolide was covalently coupled to CCPM through hydrolysable ester bonds[110]. Leuprolide entrapped in CCPM showed a substantially improved (14.4 h) half-life in circulation in comparison to the free peptide, leading to an AUC that was 100-fold higher for leuprolide-CCPM than for free leuprolide. Importantly, the drug released from the PM was still biologically active.

The conjugation of drug molecules to the polymer backbone by chemical bonds results in a good retention of the drug in the PM during circulation and allows tailoring of drug release kinetics by means of different linkers. Although this approach shows promising results, it also demands extra steps in the formulation of the micelles. Moreover, by crosslinking either the copolymer and/or the drug the principle of the micellar formation as a dynamic equilibrium between unimers is compromised.

4. CONCLUSIONS

PM have been extensively studied in the last decades due to their ability to efficiently and stably load hydrophobic drugs in their core. As many of the anticancer drugs have a hydrophobic character, much attention has been paid to the development of PM-based therapies focusing on the treatment of cancer. This resulted in several formulations being investigated in clinical studies and to date one of them has been FDA approved (Genexol™-PM). The first class of PM aimed to be used as solubilizers of hydrophobic compounds. However, these formulations lack good stability when present in the circulation and, importantly, the loaded drug is rapidly extracted from the PM' core which compromised the therapeutic efficacy of these formulations. Different strategies have been successfully employed to achieve improved retention and stability of drug-loaded PM upon i.v. injection. These strategies include chemical crosslinking of the PM core and/or drug, covalent coupling of the drug to the polymer chains, and hydrophobic interactions between the drug and the hydrophobic block of the copolymer, as π - π interactions. Promising preclinical studies performed with these PM have translated into several formulations that are currently under clinical investigation for the treatment of different types of cancer with encouraging results. Depending on the pharmacodynamic profile of the incorporated drug, triggered release, e.g. by exogenous light or sound, or endogenous microenvironmental cues, like pH or enzymatic activity could be envisioned to further improve the therapeutic efficacy. Also, modulation of cell specificity by introduction of targeting ligands may be an avenue to steer the formulation within the tumour environment, which is especially important for drug molecules that have difficulty in crossing cellular membranes. These developments could render new classes of pathway-specific therapeutic molecules to be attractive candidates for PM encapsulation.

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3

Nanomedicines for The Treatment of Hematological Malignancies

Anil K. Deshantri^{1,2}, Aida Varela-Moreira^{1,3}, Veronika Ecker⁴,
Sanjay N. Mandhane², Raymond M. Schiffelers^{1,3},
Maike Buchner^{4*} and Marcel H.A.M. Fens^{1,3*\$}

¹Department of Clinical Chemistry and Hematology, University
Medical Center Utrecht, Utrecht, The Netherlands

²Biological Research Pharmacology Department, Sun Pharma
Advanced Research Company Ltd. Vadodara, India

³Department of Pharmaceutics, Utrecht Institute for Pharmaceutical
Sciences, Utrecht University, Utrecht, The Netherlands

⁴Institute for Clinical Chemistry and Pathobiochemistry, Klinikum
rechts der Isar, Technical University Munich, Munich, Germany

ABSTRACT

Hematological malignancies (HM) are a collection of malignant transformations originating from cells in the primary or secondary lymphoid organs. Leukemia, lymphoma, and multiple myeloma comprise the three major types of HM. Current treatment consists of bone marrow transplantation, radiotherapy, immunotherapy and chemotherapy. Although, many chemotherapeutic drugs are clinically available for the treatment of HM, the use of these agents is limited due to dose-related toxicity and lack of specificity to tumor tissue. Moreover, the poor pharmacokinetic profile of most of the chemotherapeutics requires high dosage and frequent administration to maintain therapeutic levels at the target site, both increasing adverse effects. This underlines an urgent need for a suitable drug delivery system to improve efficacy, safety, and pharmacokinetic properties of conventional therapeutics. Nanomedicines have proven to enhance these properties for anticancer therapeutics. The most extensively studied nanomedicine systems are lipid-based nanoparticles and polymeric nanoparticles. Typically, nanomedicines are small sub-micron sized particles in the size range of 20-200 nm. The biocompatible and biodegradable nature of nanomedicines makes them attractive vehicles to improve drug delivery. Their small size allows them to extravasate and accumulate at malignant sites passively by means of the enhanced permeability and retention (EPR) effect, resulting from rapid angiogenesis and inflammation. Moreover, the specificity to the target tissue can be further enhanced by surface modification of nanoparticles. This review describes currently available therapies as well as limitations and potential advantages of nanomedicine formulations for treatment of various types of HM. Additionally, recent investigational and approved nanomedicine formulations and their limited applications in HM are discussed.

Key words

Hematological malignancies, drug delivery, nanomedicines, liposomes, micelles, bone marrow microenvironment

Graphical Abstract

1. INTRODUCTION

Presently, multiple chemotherapeutic and molecular targeted agents are available for the treatment of hematological malignancies (HM). Nevertheless, only a subset of patients will achieve long-term remission or complete cure of the disease. This is at least partly due to the lack of specificity of these agents for the disease site and their short biological half-lives in the circulation [1]. Lack of specificity results in exposure of healthy organs to the drugs that gives off-target adverse effects. High doses and frequent dosing to maintain the therapeutic levels at the malignant site further increase the magnitude of these adverse effects [2, 3]. Therefore, drug delivery systems could be essential not only to decrease exposure of healthy tissues to the drug, but also to retain the active substance in the circulation and deliver it to the malignant cells. Thereby, nanomedicines can significantly improve efficacy and safety profiles of encapsulated chemotherapeutic agents. In past few decades, nanocarriers such as liposomes and polymeric nanoparticles received considerable attention for the treatment of various types of solid tumors, leading to several successful formulations that entered the clinic [2, 4]. However, less work has been done to develop nanomedicines for the treatment of hematological malignancies [1]. In this review, currently available (chemo)therapies for HM and their shortfalls are discussed. Nanomedicines are presented as an attractive approach to improve treatment, which is elucidated by an overview of investigational and approved drug formulations. Finally, challenges associated with development of novel nanomedicine formulations and their clinical translation, are discussed.

2. HEMATOLOGICAL MALIGNANCIES

Hematological malignancies (HM) comprise a variety of cancers derived from the blood, bone marrow (BM) and lymphatic system. Of all cancers diagnosed in the United States in 2017, 10.2% was estimated to be categorized as a hematological malignancy [5]. In children, adolescents and young adults, leukemia causes more deaths than any other cancer [6]. In majority of HM, bone marrow is the predominant site of tumor localization together with peripheral blood and secondary lymphoid organs, such as spleen and lymph nodes. [7, 8]. In BM, normal hematopoietic stem cells differentiate into cells of the myeloid or lymphoid lineage. Granulocytes, monocytes, mast cells, erythrocytes and thrombocytes differentiate from myeloid precursor cells (myelopoiesis), whereas T cells, B cells, Natural Killer (NK) cells and plasma cells are produced by the lymphoid lineage (lymphocytopoiesis). Hematological malignancies can be subdivided into leukemia, lymphoma, and multiple myeloma, based on the cell-of-origin. Table 1 provides an overview of different types of HM.

Table 1. An overview of different types hematological malignancies. The classification is based on origin of the disease and cell types involved. Chromosomal abnormalities and/or mutations frequently associated are highlighted but not limited to the list. Due to the scope of this review the somewhat rarer forms are not discussed here.

Type of HM	Subtype	Origin	Cancer statistics 2017 (USA)[5] Estimated new cases / deaths Epidemiology	Cell types involved and infiltrated sites	Frequently associated chromosomal abnormalities/mutations/ translocations (t)	Ref
1. Leukemia (62 130 / 24 500) <i>Originate from bone marrow precursor cells from either the myeloid or the lymphoid lineage.</i>	(i) Acute Lymphoblastic Leukemia (ALL)	Hematopoietic precursors of the lymphoid lineage.	5970 / 1440 Most common leukemia in people younger than 20 years.	Clonal population of lymphoid cells. 85% of ALL arise from the B cell lineage and 15% from T cell precursors.	t(12;21): <i>ETV6-RUNX1</i> t(1;19) (q23;p13): <i>E2A-PBX1</i> t(9;22) (q34;q11): <i>BCR-ABL1</i> t(4;11) (q21;q23): <i>MLL-AF4</i>	[5, 7, 9-11]
	<i>For detailed information see reference 4</i>			Infiltration of bone marrow, blood and extramedullary sites		
	(ii) Acute Myeloid Leukemia (AML)	Hematopoietic precursors of the myeloid lineage.	21,380 / 10,590 Median age of diagnosis is 64 years.	Abnormally or poorly differentiated blasts of the myeloid system.	Chromosomal mutations in 97% of cases inv(3)(q21;q26.2) t(3;3)(q21;q26.2) occasionally t(9;22)(q34;q11)	[7, 12] [5, 13, 14]
	<i>For detailed information see reference 8</i>			Infiltration of the bone marrow, peripheral blood and other organs.	t (8;21), inv (16), t (15;17): part of diagnosis	
	(iii) Chronic myeloid Leukemia (CML)	Hematopoietic stem cells	8950 / 1080 Median age of diagnosis is 60 years.	Myeloid cells and their precursors in the bone marrow.	Driven in almost all cases by the Philadelphia chromosome: t(9;22) (q34;q11): <i>BCR-ABL1</i>	[5, 15-17]
	<i>For detailed information see reference 11</i>			Involvement of bone marrow, peripheral blood and spleen.		

Table 1. Continued

Type of HM	Subtype	Origin	Cancer statistics 2017 (USA)[5] Estimated new cases / deaths Epidemiology	Cell types involved and infiltrated sites	Frequently associated chromosomal abnormalities/mutations/ translocations (t)	Ref
2. Lymphoma (80 500 / 21 210) <i>Lymphomas are defined as a group of malignancies derived from mature lymphoid cells. The majority arises from B cells and are therefore called B cell lymphomas.</i>	(i) Hodgkin's Lymphoma (HL)	B cells (majority of cases)	8260 / 1070	Giant cells termed Hodgkin and Reed-Sternberg (HRS) cells in classical HL (95% frequency) and lymphocytic and histiocytic (L&H) cells in LPHL.	Rare events affecting <i>p53</i> , <i>Fas</i> , <i>IkBα</i> Frequent microsatellite instability	[5, 7, 18-21]
	<i>For detailed information see reference 15</i>		Two age groups: early adulthood (age 15–40, usually 25–30) and late adulthood (after age 55).		Novel translocation: t(2;14)(p13;q32.3): <i>BCL11a</i>	
	(ii) Non-Hodgkin's Lymphoma (NHL)	Arise from lymphocytes that are at various stages of development	72,240 / 20,140			[2, 5, 7, 8, 22, 23]
	<i>For detailed information see reference 19</i>					
	(a) Chronic Lymphoid Leukemia (CLL)	B cells	20,110 / 4660	Monoclonal CD5 positive B cells.	del13q14	[5, 7, 24-26]
	<i>For detailed information see reference 22</i>		Average age of diagnosis at 70 years.	Accumulation of malignant B cells in the blood, bone marrow and lymphocytic tissue	trisomy 12 del11q22-q23 del17p13.	
	(b) Follicular Lymphoma (FL)	B cells	n.d.	Germinal center (GC) B cells.	t(14;18)(q32;q21): ectopic <i>Bcl2</i> expression; in 85% cases of FL.	[6, 7, 27]
	<i>For detailed information see reference 23</i>		Follicular lymphomas account for 20% of NHL Average age of diagnosis at 60 years.	Infiltration of cervical, axillary, inguinal and femoral lymph nodes. Bone marrow is involved in 50% of patients.	Inactivating mutations of the <i>MILL2</i> gene is found in over 80% of FL.	
	(c) Diffuse large B cell lymphoma (DLBCL)	B cells	n.d.	Heterogeneous group of B cell malignancies, characterized by large cells.	3q27: <i>Bcl6</i> t(14;18): <i>Bcl2</i>	[7, 28]
	<i>For detailed information see reference 25</i>		Most common subtype of NHL (40%) Middle age	Develop at either nodal or extra-nodal sites.		

Table 1. Continued

Type of HM	Subtype	Origin	Cancer statistics 2017 (USA)[5] Estimated new cases / deaths Epidemiology	Cell types involved and infiltrated sites	Frequently associated chromosomal abnormalities/mutations/ translocations (t)	Ref
3. Multiple myeloma 30 280 / 12 590	<i>For detailed information see reference 32</i>	B cells	30,280 / 12,590 Median age of disease onset is 65–70 years	Monoclonal post-GC plasma cells. Infiltration of bone marrow and extramedullary sites.	Trisomies: trisomic MM 14q32: IgH translocated MM Or combination of both Secondary cytogenetic abnormalities: gain(1q), del(1p), del(17p), del(13), <i>RAS</i> mutations, and secondary translocations involving <i>MYC</i>	[5, 7, 29–35]

n.d. = not determined

3. TARGET SITES IN HEMATOLOGICAL MALIGNANCIES

To improve therapeutic intervention of hematological malignancies, delivery of drugs at the site of disease is one of the goals. Figure 1 illustrates various target sites in HM.

Figure 1. Target sites in hematological malignancies. The peripheral blood (1), secondary lymphoid organs (2 & 3) and bone marrow (4) are the primary target sites for drug delivery in hematological malignancies.

3.1 Bone marrow microenvironment

The BM microenvironment consists of cellular and non-cellular compartments. The importance of bone marrow vasculature was recently discussed [36-39]. The cellular compartment includes bone marrow stromal cells (BMSC), endothelial cells, osteoclasts, and osteoblasts, whereas noncellular compartment includes extracellular matrix and important cytokines like interleukin (IL)- 6, IL-21, and tumor necrosis factor alpha (TNF α), and growth factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) [40]. Interaction of BMSC and neoplastic cells plays a crucial role

in proliferation and survival of neoplastic cells and progression of the disease [37, 38]. This interaction can also lead to drug resistance [39, 41]. The interaction of malignant cells with BMSC and other cells in the bone marrow microenvironment is depicted in Figure 2 together with their key signaling factors. BMSC can interact with malignant cells via cell surface molecules; for example, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on BMSC connect to lymphocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4) on malignant cells, respectively. These interactions trigger activation of the nuclear factor kappa B (NF- κ B) pathway, and secretion of IL-6 from BMSC, which enhances the production and secretion of VEGF from malignant cells. The abnormal proliferation of osteoblasts and osteoclasts results from the progression of the disease. In MM, for instance, the balance between osteoclast activation and osteoblast proliferation is lost, causing bone lesions. Bone formation and resorption is normally a controlled process regulated by mainly two molecules, receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG) [42]. RANKL produced by BMSC and macrophage inflammatory protein 1 alpha (MIP-1a) produced by MM cells stimulates osteoclast activation in the disease. OPG would limit osteoclast formation, but OPG is decreased by high local IL-3, Dickkopf-related protein 1 (DKK-1) and hepatocyte growth factor (HGF) concentrations [32]. Notch signaling induces production of IL-6, VEGF, and IGF, leading to malignant cell proliferation and survival [38]. The intensive signaling via direct cell-cell interaction and through soluble mediators leads high vascularization and angiogenesis in leukemic bone marrow, which is responsible for enhanced proliferation and survival of malignant cells and hence increased resistance [43].

Figure 2. Bone marrow microenvironment (BM) in hematological malignancies (HM). Four different types of malignancies are chosen as examples to show the complexity of BM microenvironment in HM.

Cross talk of malignant cells with BMSC and other cells in the BM microenvironment is shown. The interaction between VCAM1 on BMSC and VLA-4 on malignant cell is found in all kind of HM. In multiple myeloma and in CLL, ICAM1 on BMSC interacts to the LFA-1 present on myeloma cell and CLL cell. In MM, Notch-1 binds to DLL1. Soluble factors such as VEGF, bFGF, Ang-1, TGF- β , PDGF, HGF, IL-1, and IL6 secreted by BMSC; IL-6 and OPG by osteoblasts; IL17 by Th17 cells; VEGF and IGF-1 by myeloma cells increase proliferation of myeloma cells and increase angiogenesis. VCAM1-VLA-4 and ICAM1-LFA-1 bindings take place between BMSC and ECs in AML. Soluble factors IL-6, IL-8 and CXCL12 from BMSC increase proliferation and survival of leukemic cells in AML through binding to IL-6R, CXCR2 and CXCR4, respectively. RANK present on osteoclasts reacts with RANKL on AML cells and myeloma cells. Osteoblasts increase activation and survival of CML cells via Ang-1-Tie2 interaction. CD44 and VLA-4 on CML cells help homing and adhesion of these cells by interacting to HA and fibronectin in the extracellular matrix in BM microenvironment. In CLL, NLCs and T cells increase proliferation of the leukemic cells. NLCs secrete CXCL12 and CXCL13 that binds to CXCR4 and CXCR5 respectively; BAFF/APRIL on NLCs interacts with BCMA/TACI/BAFFR on CLL cells. T cells react via CD40-CD40L interaction. Together these complex interactions between malignant, stromal and other cells in the BM result in enhanced proliferation and survival of malignant cells, resistance to apoptosis, and CAM-DR.

Abbreviations: BMSC = Bone marrow stromal cell, BM = bone marrow, VCAM1 = Vascular cell adhesion molecule 1, VLA-4 = Very late antigen-4, HM = hematological malignancy, CLL = Chronic lymphocytic leukemia, ICAM1 = Intercellular adhesion molecule 1, VEGF = Vascular endothelial growth factor, bFGF = basal fibroblast growth factor, Ang-1 = Angiopoietin 1, TGF- β = Transforming growth factor beta, PDGF = Platelet-derived growth factor, HGF = Hepatocyte growth factor, IL = interleukin, OPG = Osteoprotegerin, IGF-1 = Insulin-like growth factor 1, LFA-1 = Lymphocyte function-associated antigen 1, ECs = Endothelial cells, AML = Acute myeloid leukemia, CXCL = Chemokine (C-X-C motif) ligand, CXCR = Chemokine (C-X-C motif) receptor, RANK(L) = Receptor activator of nuclear factor kappa B (ligand), CML = Chronic myeloid leukemia, MM = Multiple myeloma, NLCs = Nurse-like cells, BAFF(R) = B cell activating factor (receptor), APRIL = A proliferation-inducing ligand, BCMA = B cell maturation antigen, TACI = Transmembrane activator and CAML interactor, CAM-DR = Cell adhesion-mediated drug resistance.

3.2 Secondary lymphoid organs

Secondary lymphoid organs such as lymph nodes and spleen provide a distinct microenvironment for tumor cells in hematological malignancies.

3.2.1 Lymph nodes

Lymph nodes are the primary organ where immune responses are initiated and are therefore rich in immune cells such as dendritic cells, B, T and NK cells as well as macrophages. Notably, immune responses can prevent malignant transformation. For example, downregulation of MHC class I molecules on the surface of transforming cells is detected by NK cells and induces lysis of the respective cells. However, transformed cells have developed mechanisms to circumvent effective immune responses and often mediate immune suppression. Functional NK cells in lymph nodes of patients, which produce antitumor cytokines like TNF- α and interferon gamma (IFN- γ), correlate with a favorable prognosis of multiple types of HM [44, 45]. This indicates that anti-tumor immune responses are still active in some malignancies, impacting progression and outcome. On the other hand, a proinflammatory microenvironment can also support the expansion of HM. Follicular dendritic cells (FDC) are key players in secondary lymphoid organs comprising approximately 1% of all germinal center (GC) cells. FDC interfere with apoptosis and promote survival of B cells in GC by secretion of cytokines such as IL-15, thereby supporting the proliferation and expansion of the malignant cells [46].

3.2.2 Spleen

As the largest lymphoid organ of the body, the spleen plays an important role in immunological defenses. Involvement of spleen is seen in all types of HM, most prominently in lymphomas. Spleen involvement is found in approximately 30-40% cases of NHL and in one third of HL [47]. In HL, spleen involvement can also upstage the disease. Splenomegaly is also found in other HM such as CML. The spleen is organized in two regions; the red pulp and the white pulp, which are separated by the marginal zone [48]. Filtration of blood and iron recycling take place in the red pulp. The white pulp contains T and B cells zones. Spleen is responsible to regulate innate as well as adaptive immune responses and plays a key role in tumor immunity by recruiting monocytes and macrophages to the tumor tissues [48]. Because of the phagocytic activity of monocytes and macrophages, a significant amount of intravenously administered nanoparticles tends to accumulate in the spleen [49]. Though this property of spleen “eating” the nanoparticles could be advantageous [50]. Number of inflammatory monocytes has been shown to have negative correlation with lymphoma patient survival [51]. As inflammatory and noninflammatory monocytes use distinct mechanisms for recruitment, targeting inflammatory monocytes in spleen and bone marrow by using

siRNA containing nanoparticles, and thereby altering their recruitment to the tumors has been evident to reduce tumor growth in vivo [50]. The spleen can be considered as one of the major target sites in HM [47, 52].

3.3 Peripheral blood

The cellular compartment of peripheral blood consists normally of leukocytes, red blood cells (RBC) and platelets. Detection of circulating malignant cells by complete blood count (CBC), flow cytometry and blood smears is applied in the diagnosis of HM as well as in monitoring therapeutic efficacy [53-55]. Moreover, peripheral cytopenias such as anemia, thrombocytopenia, and pancytopenia are common in HM. The mature blood cells are replaced by immature blast cells. In multiple myeloma, for instance, the plasma cells are replaced by plasmablasts, which are rapidly dividing cells that secrete high amounts of immunoglobulins [31]. There is also an increase in calcium levels in the blood because of the bone resorption due to local osteoclast activation (section 3.1). The accumulation of immunoglobulins and calcium in the kidneys can cause inflammation and subsequent renal failure [56]. In leukemias including CML, presence of >20% blast cells in peripheral blood (and bone marrow) is classified as a blast crisis, which is difficult to treat [57].

3.4 Liver

The liver is the second largest organ of the body and a part of reticuloendothelial system. In hematological malignancies, liver is a site of malignant cells infiltration which produce hepatomegaly or formation of multiple nodules in the liver [58, 59]. Although life-threatening complications such as acute liver failure in patients with HM is rare [60-62], clinical manifestations are often seen due to hepatic involvement [58]. Lymphomatous infiltration of the liver has been seen in lymphomas, more commonly in non-Hodgkin lymphoma (NHL) (16–43% patients). In Hodgkin disease, malignant infiltration of Reed-Sternberg cells have been described in up to 14% of patients [58, 59]. Jaundice and Cholestasis are also sometimes found in lymphoma patients [59]. In ALL and AML hepatic involvement has been described to be approximately 95% and 75% cases, respectively [58]. Liver infiltration and mild to moderate hepatomegaly can also be seen in chronic phase as well as blast crisis in CML. Chemotherapeutic-induced hepatotoxicities are also common in HM patients [59]. This makes liver an important target site in HM.

4. CURRENT AND NOVEL DRUGS FOR THE TREATMENT OF HEMATOLOGICAL MALIGNANCIES

In many solid tumors, the malignant tissue, including infiltrated surrounding tissue, can often be removed surgically. Excision can be further combined with radio- and chemotherapy to eradicate potential residual cancer cells. However, in case of HM, the malignant cells are not restricted to a specific site and treatment needs to be effective in multiple tissues, including bone marrow, secondary lymphoid organs and the blood stream. Treatment is variable and dependent of the specific type and stage of HM that the patient is diagnosed with. Treatment strategies consist of bone marrow transplantation, radiotherapy and chemotherapy. Recently, the potential of immunotherapeutic approaches has been demonstrated for the first time in HM [63-65]. More than 70 drugs are currently available for the treatment of HM. however despite these therapeutic options not all HM are curable up to now.

4.1 Currently available drugs and treatment combinations

4.1.1 Radiotherapy

With radiotherapy, the patient is exposed to a specific dose of ionized radiation targeted to the region of the malignant neoplasm. The DNA of the cells exposed to the radiation is damaged. Radiotherapy is often used prior to stem cell transplantation as a part of a conditioning regimen or in combination with chemotherapy for a synergistic effect [66]. There are several side effects associated with radiotherapy. Damage to healthy tissue that is in proximity to the target site is the most frequently encountered adverse effect causing dose-limiting toxicity. Based on the diffuse distribution of the malignant cells in HM, radiotherapy is only successful in a small subset of patients.

4.1.2 Hematopoietic stem cell transplantation (HSCT)

Stem cell transplantation is a treatment option for durable remissions of HM [67]. After conditioning via high dose therapy with cytotoxic drugs and/or radiation, which aim for temporary yet complete destruction of the hematopoietic system, the patient's bone marrow is restored with healthy hematopoietic stem cells by autologous or allogeneic stem cell transplantation [68]. In autologous HSCT the patient's own healthy cells are collected prior to the conditioning regimen and re-infused afterwards. Although autologous HSCT has become a treatment choice for HM such as MM and some lymphomas, however, relapse is often inescapable. Relapse may come from sporadic tumor cells that remain after chemotherapy or by contamination of cancer cells in the transplanted cells. The latter can be avoided by using another approach of HSCT, i.e. allogeneic HSCT. In allogeneic HSCT, stems cells from a human leukocyte antigen (HLA)-

matched healthy donor are transplanted after a conditioning regiment designed to completely eradicate the recipient's hematopoietic system. However, allogeneic HSCT is associated with an increased risk of graft-versus-host disease, which may also induce anti-tumor immune responses, known as the 'graft-versus-leukemia' effect.

4.1.3 Chemotherapy and targeted therapies

To date, multiple chemotherapy and antibody-based drugs are available for the treatment of hematological malignancies. These include various classes of cytotoxic agents, small molecules, immune-modulators, and immunotherapeutics (Table 2). Combination therapy is often applied with multiple drugs and/or other treatment modalities such as radiation. The rationale is that it will improve treatment of HM by simultaneously targeting multiple pathways. Advances in understanding the pathogenic pathways have led to the development of several targeted approaches in HM. These include modulators of epigenetic alterations, i.e. histone deacetylase inhibitors (HDI), proteasome inhibitors, immune modulators and small molecules such as tyrosine kinase inhibitors (TKI, Table 2). TKI inhibit active signaling pathways and aim to target the malignant cells' growth and survival pathways. With imatinib mesylate the proof-of-principle for the successful treatment using TKI in HM was provided. By directly binding to and inhibiting the constitutively active kinase Abelson murine leukemia viral oncogene homolog 1 (ABL1), imatinib treatment resulted in long-term remission and almost doubled the 5-year survival rate in CML. However, in CML and breakpoint cluster region-ABL1-driven ALL, resistance towards imatinib frequently develops via mutations in ABL1 that prevent binding of the TKI. For this reason, several second generation TKI have been developed to target imatinib resistant clones. Second generation TKI such as nilotinib and bosutinib show better potency against most mutated forms of ABL1 but still failed to overcome the T315I gatekeeper mutation [69]. Recently, a third generation TKI, ponatinib, has been developed to successfully treat the T315I mutation containing disease [69]. Furthermore, several TKI target more than one kinase and can be used for the treatment of additional kinase-driven malignancies. One example is dasatinib, initially designed to target ABL1, which also inhibits the function of Src, c-Kit and PDGFR kinases. Based on this effect, dasatinib therapy for the treatment of other (solid) malignancies is under investigation [70].

Table 2. Currently available treatments for hematological malignancies.

Category (MoA)*	Drug	Trade name	Indication [66, 67]
Antitumor antibiotics	Bleomycin	Cerubidin®	HL, NHL
	Daunorubicin	Adriamycin®, Rubex®	AML, ALL
	Doxorubicin	Blenoxane®	AML, ALL, HL, NHL
	Idarubicin	Idamycin®	AML
	Mitoxantrone	Novantrone®	AML, NHL
Antimetabolites	Azacitidine	Vidaza®	CML
	Cladribine	Leustatin®	CLL
	Clofarabine	Clolar®	AML, ALL
	Cytarabine	Cytosar-U®	AML, ALL, HL, NHL
	Decitabine	Dacoge®	AML
	Fludarabine	Fludara®	CLL, NHL
	Hydroxyurea	Hydrea®, Droxia®	CML
	Mercaptopurine	Purinethol®	ALL
	Methotrexate	Emthexate®	AML, ALL, CLL, CML, HL, NHL
	Pralatrexate	Foloty®	NHL
Biphosphonates**	Thioguanine	Thioguanine®, Tabloid®	AML, ALL, CML
	Pamidronate	Aredia®	MM
	Zoledronic acid	Zometa®	MM
Cell-maturing agents	Arsenic trioxide	Trisenox®	AML
	Tretinoin	Vesanoid®	AML
	Bendamustine	Treanda®	CLL, HL, NHL
	Busulfan	Myleran®, Busulfex®	AML, ALL, CLL, CML, HL, NHL
	Carboplatin	Paraplatin®	HL, NHL
	Carmustine	BiCNU®	MM, HL, NHL
	Chlorambucil	Leukeran®	CML, NHL
	Cisplatin	Platinol®	AML, ALL, CLL, CML, HL, NHL
DNA-damaging drugs	Cyclophosphamide	Cytosan®, Neosar®	AML, ALL, CLL, CML, HL, NHL
	Dacarbazine	DTIC-Dome®	HL
	Ifosfamide	Ifex®	HL, NHL
	Lomustine	CeeNU®	HL
	Mechlorethamine	Mustargen®	CLL, CML
	Melphalan	Alkeran®	MM
	Nelabrine	Arranon®, Atrience®	ALL, NHL
	Procarbazine	Matulane®	HL
DNA-repair enzyme inhibitors	Etoposide	VePesid®, Etopophos®, Toposar®	AML, ALL, CLL, CML, HL, NHL
	Teniposide	Vumon®	ALL

Table 2. Continued

Category (MoA)*	Drug	Trade name	Indication [66, 67]
Glucocorticoids	Dexamethasone	Decadron®	MM
	Methylprednisolone	Medrol®	ALL
	Prednisone	Deltasone®	ALL, HL, NHL, MM
Histone deacetylase inhibitors	Belinostat	Beleodaq®	NHL
	Panabinostat	Farydak®	MM
	Romidepsin	Istodax®	NHL
	Vorinostat	Zolinza®	NHL
Immune Modulators	Lenalidomide	Revlimid®	MM
	Pomalidomide	Imnovid®, Pomalyst®	MM
	Thalidomide	Thalmid®	MM
Mitotic inhibitors	Paclitaxel	Taxol®	ALL
	Vinblastine	Velban®	HL
	Vincristine	Oncovin®	ALL, HL, NHL
Monoclonal antibodies	Alemtuzumab	Campath®, Lemtrada®	CLL, NDL
	Blinatumomab	Blinicyto®	ALL
	Brentuximab vedotin	Adcetris®	HL, NHL
	Daratumumab	Darzalex®	MM
	Gemtuzumab ozogamicin	Mylotarg®	AML
	Ibritumomab	Zevalin®	NHL
	Obinutuzumab	Gazyva®, Gazyvaro®	CLL
	Ofatumumab	Arzerra®	CLL
	Rituximab	Rituxan®	AML, ALL, CLL, CML, HL, NHL
	Tositumomab	Bexxar®	FNHL
Phosphoinositide 3-kinase inhibitors	Idelalisib	Zydelig®	CLL, NHL
Proteasome inhibitors	Bortezomib	Velcade®	MM, NHL
	Carfilzomib	Kyprolis®	MM
	Ixaomib	Ninlaro®	MM
Tyrosine kinase inhibitors	Bosutinib	Bosulif®	CML
	Dasatinib	Spryce®	ALL, CML
	Ibrutinib	Imbruvica®	CLL
	Imatinib mesylate	Gleevec®, Glivec®	ALL, CML
	Nilotinib	Tasigna®	CML
	Omacetaxine mepesuccinate	Synribo®	CML
	Ponatinib	Iclusig®	CML

Table 2. Continued

**Some Chemotherapeutic agents possess multiple mechanisms of action. Classification here is based on their primary mechanism.*

***Bisphosphonates are not part of cytotoxic treatment protocols, but are used as part of supportive care in selected malignancies, multiple myeloma for instance.*

MoA = mechanisms of action, HL = Hodgkin's lymphoma, NHL = non-Hodgkin's lymphoma, AML = acute myeloid leukemia, ALL = acute lymphoid leukemia, CML = chronic myeloid leukemia, CLL = chronic lymphoid leukemia, MM = multiple myeloma, FNHL = follicular non-Hodgkin lymphoma;

4.1.4 Antibody and immunotherapy

4.1.4.1 Monoclonal antibodies

Monoclonal antibodies naturally possess a long circulatory half-life. This property helps to overcome the pharmacokinetic challenges of many small molecules. By humanizing antibodies, immune responses against subsequent doses can be minimized so that the long-circulating property is maintained. In general, monoclonal antibodies target specific surface molecules of the malignant cell population although frequently the surface molecules are also expressed, albeit to a lesser extent on healthy counterparts. After binding, cells can be killed via direct cytotoxic effects as well as complement-mediated cell lysis (CDC) and antibody-dependent cytolytic effects (ADCC) by NK cells. Modifications can further increase the efficacy as described in the following section. Rituximab was the first monoclonal antibody approved for treatment of HM. It binds to the large loop of CD20 and causes cell polarization as well as CDC and ADCC. In addition, a number of other CD20 targeting antibodies are used for the treatment of HM, including ofatumumab. Ofatumumab is a fully humanized CD20 antibody with increased cytolytic effect and a slow dissociation rate, and is particularly suitable for cells with low target expression [73]. Obinutuzumab is another humanized anti-CD20 antibody with increased binding affinities towards FcγR on NK cells by afucosylated Fc segments that may be beneficial as compared to Rituximab [74]. Based on their (semi-)specific binding to the malignant cells, monoclonal antibodies can also be used for specific site delivery of coupled cytotoxic agents or radioisotopes: For example, ibritumomab targets the same epitope on the CD20 molecule as rituximab but that antibody is of murine origin. It is covalently bound to tiuxetan, which is a chelator bound to the radioactive element yttrium-90. Due to its high beta energy, the radiation can kill even bulky lymphomas [75, 76]. Tositumomab also targets CD20 and is often used in combination with tositumomab labeled with the radioisotope iodine-131 [77]. However, despite promising response rates, tositumomab was discontinued in 2014 due to the decline in usage [78].

4.1.4.2 Bispecific antibodies

Blinatumomab is the first bispecific T-cell-engager antibody (BiTE) approved for the treatment of relapsed/refractory Ph-negative ALL [79]. It is a single chain protein that targets CD3 and CD19. Thereby, it brings CD3+ T cells and (malignant) CD19+ B cells in close proximity. This activates T cells that subsequently lyse the target B cells via release of cytotoxic granules and activation of the perforin–granzyme pathway in the B cell. Although the activated T cells are able to engage multiple leukemic cells, the optimal administration schedule requires continuous infusion because of the short plasma half-life of the single chained antibody derivative. Potential improvements of this therapeutic strategy include bispecific antibody-drug conjugates that could enhance the T-cell activity. Alternatively, the physical connection between the two epitopes could be made through a coupling of both epitopes to a nanoparticle surface. However, identification of two expressed targets on the same tumor cell, different tumor cells or cells in the microenvironment, for which bispecificity is actually beneficial, remains challenging.

4.1.4.3 Chimeric antigen receptor (CAR) T-cell therapy

Ex vivo engineering of T cells is a highly promising treatment option for HM. In this approach, T cells from the patient are isolated and engineered to recognize the target tumor cells via a chimeric antigen receptor (CAR) that binds characteristic surface receptors on these tumor cells such as CD19. The genetic information for the expression of chimeric receptor is delivered via lenti- or retroviral transduction of the T cells. These CAR T cells are reinfused into the patient to destroy their target cells [80]. Despite the impressive clinical responses found in initial trials, serious therapy-associated toxicities can occur. A recent report provided evidence that CAR T cells could also act as targeted delivery vehicles for precise delivery of therapeutic cargoes, such as herpes virus entry mediator (HVEM). HVEM is a protein that binds and activates the negative regulator B-and T-lymphocyte attenuator (BTLA) in normal B cells that limits proliferation. However, its function is frequently blocked in B cell lymphoma. Using CD19-directed engineered CAR T cells, functional HVEM protein can be delivered directly to lymphomas *in vivo*. In addition to directly attacking malignant cells, CAR T cells can thereby be used as ‘micro-pharmacies’ for precise therapeutic delivery [81].

4.1.4.4 Anti-angiogenic therapies

Angiogenesis is a hallmark of cancer. However, also in HM a role of angiogenesis has been strongly evident. Antibodies and small molecules interfering vessel formation are in clinic for solid tumors as well as HM [82, 83]. The role of VEGF in angiogenesis is very well documented. Approaches to inhibit VEGF or VEGF receptors (VEGFRs) have proven

successful [84]. Anti-VEGF antibody bevacizumab is approved for solid tumor treatments and is currently in clinical trials for HM, including AML, CLL, CML, NHL, and MM [82]. Midostaurin is an inhibitor of VEGFR2, PKC, PDGFR, Flt3, and c-Kit. It is recently approved for FLT3-mutated AML [85]. Other receptor tyrosine kinase (including VEGFRs) inhibitors such as vatalanib, semaxinib, sorafenib, sunitinib, cediranib are approved for various solid tumor treatments and are currently being investigated for HM [82, 86]. Proteasome inhibitors have been shown to have antiangiogenic properties by downregulating VEGF expression via p53 induction and inhibition of NFκB pathway [82, 86, 87]. Proteasome inhibitors bortezomib, carfilzomib and ixazomib are approved for multiple myeloma. Immune modulatory drugs thalidomide and its analogs lenalidomide and pomalidomide are in clinic for treatment of multiple myeloma. They have antiangiogenic properties by downregulating VEGF secretion and inhibiting PI3K-Akt signaling pathway [86, 88]

4.2 Novel drugs

Novel treatment approaches based on recent insights into the molecular mechanisms of malignant transformation is used to design molecules that specifically target specific steps in disease pathogenesis. On average, only 1 in 1000 of these molecules will make it to human testing. This high attrition rate is primarily due to tox/PK/PD and is eventually reviewed by regulatory authorities. Of those, that do make it this far, only a 1 of 5 will be approved, (as a result of) patient variability, disappointing efficacy, and limited predictability of *in vitro* and *in vivo* models. Therefore, new molecules have only a 1:5000 chance of reaching regulatory approval [89].

4.2.1 Novel kinase inhibitors

Second and third generations of kinase inhibitors aim to overcome the development of resistance generated by the first line drugs. MK-0457 (tozasertip/VX680) is an example [90, 91]. MK-0457 not only overcomes mutant T315I of ABL1 but also potently inhibits aurora kinases. Unfortunately, this drug exhibited strong cardiac toxicity [92]. Danusertib (PHA-739358), is a potent inhibitor of all three Aurora kinases with additional inhibition of both wild type and mutant ABL as well as several other kinases. The drug has been tested in a phase II study in patients with CML who relapsed on imatinib. The study recorded two complete hematologic responses in patients carrying the T315I mutant. Dosing was via a 6h infusion, once weekly, which was well tolerated. In a phase I study, adults with either accelerated or blastic phase CML or BCR-ABL1+ ALL resistant cases were enrolled. 20% of the patients responded to the treatment, however, adverse events included anemia, diarrhea, and febrile neutropenia [93]. PIM kinases promote proliferation and accelerate downstream cytokine and growth factor signaling networks and are frequently upregulated in leukemia and lymphoma. [94]. AZD1208 is a potent

and highly selective pan-PIM kinase inhibitor that showed efficacy in AML in preclinical models. The phase I study in AML patients was, however, terminated before completion because of dose limiting toxicities and no clear evidence of antitumor activity [95-97].

4.2.2 Negative feedback inhibitors

Insights into regulatory mechanisms have revealed new treatment targets: signals from the B cell receptor (BCR) are essential for survival and proliferation of normal B cells. This would argue for inhibition of the BCR as therapeutic strategy. Surprisingly, however, in specific B-cell malignancies this signaling is disrupted. Depending on the developmental stage and (costimulatory) context, also hyperactive signaling from a self-reactive BCR by the ubiquitous presence of a self-antigen can induce negative selection and cell death. We have recently shown that in BCR-ABL1-driven ALL, not only a reduction of signaling strength in pre B ALL (i.e. by TKI) results in significant cell death but also an increase of (pre-)BCR signaling [98]. Therefore, agonist and antagonists of this signaling pathway may represent novel treatment targets. Careful dosing and timing is of particular relevance for this class. Improved drug delivery that promoted optimal concentration and sufficient serum half-life may be critical for this strategy.

4.2.3 Targeting apoptosis

The hematopoietic system has a high turnover rate and strict control of cellular apoptosis is essential for homeostasis. In HM the apoptosis program is frequently deregulated resulting in uncontrolled proliferation and accumulation of malignant cells. In addition, resistance to apoptosis is a major cause for treatment failure. Thus, the deregulated apoptosis pathway in HM is a promising therapeutic target. A variety of cell death promoting and inhibiting proteins that constitute potential targets comprise death receptors and ligands, including DR4/5, TRAIL, the inhibitor of apoptosis (IAP) family or the second mitochondria-derived activator of caspase (SMAC). Furthermore, the pro-survival Bcl-2 protein family, provide attractive targets. Death receptor agonists and ligands include mapatumumab a monoclonal antibody targeting DR4 (Glaxo Smith Kline/ Human Genome Science), drozitumab, a monoclonal antibody for DR5 (Genentech), and the soluble ligand rhApo2L/TRAIL for DR4/5 (Genentech and Amgen). Furthermore, the IAP survivin can be inhibited by the small molecule antagonist YM155 (Astellas Pharma) [99, 100]. All compounds are in Phase II clinical trials. YM155 competes for the binding sites of the transcription factor Sp1 on the survivin promoter and thereby prevents its transcription. Additionally, SMAC mimetics, such as LCL161 by Novartis, are also under clinical investigation. Different strategies to inhibit Bcl-2 proteins have been developed and comprise small molecule inhibitors, drugs targeting Bcl-2 mRNA and BH3 peptidomimetics, the natural inhibitor of Bcl-2 proteins. Despite overall encouraging

progress, the therapeutic window is small. For example the poor pharmacological properties of BH3 mimetics has prevented their entry in clinical trials [101]. A small molecule inhibitor obatoclax mesylate, in phase 1-2 (Gemin X/Teva Pharmaceutical Industries) is limited in dose through transient neurotoxicity as most common adverse event [102]. Another small molecule inhibitor ABT-199 showed efficacy in ALL and lymphoma mouse models and also a clinical trial with CLL patients has been initiated, with success in reduction of lymphadenopathy and peripheral blood lymphocytes, however scheduling and dosing of ABT-199 needs to be improved, as the tumor lysis syndrome frequently occurred [103]. Approaches to activate caspases are under pre-clinical evaluation but have not yet entered clinical trials.

4.2.4 Other agents

Screening of approved drugs for activity in HM has resulted in surprising activities [104]. For example a NO modification to the first approved HIV protease inhibitor saquinavir (Saq-NO) resulted in activity against ALL, AML. The intracellular p70S6 kinase of the mTOR pathway might be the target of Saq-NO [105]. The nuclear export receptor, exportin 1 (XPO1) is hyperactive in aggressive lymphomas, AML and CLL. It mediates, for example, the transport of tumor suppressors. KPT-330 (selinexor), an XPO1 blocker, entered clinical phase I/II, but systemic toxicities occur. A next-generation XPO1 inhibitor, termed KPT-330 was designed, possessing similar potency in vitro and increased tolerability, even when dosed daily. Also enhanced survival in AML and CLL mouse models compared to KPT-300 was observed [106]. Taken together, the newer generation chemotherapeutics are potent drugs, however, their offtarget kinase inhibiting activity frequently lead to severe adverse effects. The balance between on- and off-target is of crucial importance in order to achieve a wide therapeutic window. Most conventional therapies, including chemotherapy, generally lack specificity and selectivity towards the molecular process of transformation or towards the site of the disease. Therefore, healthy organs are exposed with the cytotoxic drugs, which result in off-target adverse effects. Moreover, most of the chemotherapeutic drugs as well as several small molecules are associated with pharmacokinetic challenges, i.e. poor solubility, short biological half-life, large volume of distribution, and rapid clearance. Furthermore, binding to plasma proteins such as albumin and α -1 acid glycoprotein (AGP) results in low bioavailability of the drugs [44]. This may result in resistance to the therapy and disease relapse. Resistance may also occur by drug efflux by e.g. pglycoproteins [45]. Consequently, escalated doses and more frequent dosing are often required to maintain the therapeutic concentrations of the drug in the target tissue. To overcome these challenges associated with conventional chemotherapy and to improve the safety profiles, there is a currently unmet need for better therapy

selectivity: either based on molecular pathways or based on targeted localization, or both. Using drug delivery systems, which are more specific to the tumor site(s) and minimally affect the healthy tissues, therefore offer great potential in the treatment of HM. Drug delivery approaches by using nanomedicines offers a tool to ameliorate the therapeutic index of the potent anticancer drugs by improving overall pharmacological properties i.e. tox/PK/PD. Nanomedicines have proven to be successful in overcoming the shortfalls of conventional therapy. The following section focuses on the rationale of using nanomedicines in HM. Additionally, an overview is given of the different types of nanoparticles that currently are being used in the clinic or that are in various stages of the (pre-) clinical development.

5. NANOMEDICINES FOR DRUG DELIVERY IN HEMATOLOGICAL MALIGNANCIES

3

Nanomedicines are small sub-micronized particles that, when delivered intravenously, tend to accumulate passively at the site of inflammation. Solid tumors display a chronic inflammatory phenotype featured by angiogenesis and infiltration of immune cells. Tumors require angiogenesis to fulfill high nutrition and oxygen demands to be able to multiply. The ability of tumors to stimulate this process leads to rapid and uncontrolled neovascularization resulting in an irregular shape, dilated lumen, and leaky architecture of the blood vessels walls. Basemen membrane in these vessels is also abnormal and poorly organized. In addition, the deranged inflammatory signaling in and around tumors leads to a chronic pro-inflammatory state further increasing capillary permeability. These abnormal anatomical features allow macromolecules and nanoparticles below a size of several hundreds of nanometers to passively extravasate into tumor tissue (enhanced permeability). At the same time, poor lymphatic drainage in tumors and extracellular matrix hindrance limits clearance (enhanced retention). The EPR effect in solid tumors was described more than 30 years ago by Maeda and colleagues [107]. In contrast to solid tumors, this EPR phenomenon is not very much appreciated in hematological malignancies. The diffuse localization of HM and its vascular phase might appear to make EPR less relevant. However, there is strong evidence of increased angiogenesis in these “liquid” tumors as well. Increased microvessel density and/or infiltration of inflammatory cells is seen in bone marrow of patients suffering from virtually all types of HM [84, 108-114]. Increased microvessel density and endothelial cell mass plays an important role in providing nutrients and oxygen to malignant cells. In addition, malignant cells also produce angiogenic factors and express cognate receptors that support angiogenesis and tumor cell proliferation and expansion in HM [115]. CLL is one example where proliferation is occurring primarily in secondary lymphoid organs

with an inflammatory signature. CLL cells mediate activation of the proinflammatory transcription factor NF κ B and this activation is critical for CLL engraftment and disease progression in mouse models [116]. Since HM also rely on angiogenesis to rapidly develop and expand, antiangiogenic therapy, which is extensively studied for solid tumor treatment, has also been proved successful for the treatment of HM [84]. Apart from accumulating at the tumor site, nanomedicines are also inclined to accumulate in organs of the mononuclear phagocytic system (MPS) such as liver, spleen and bone marrow. These organs contain large numbers of phagocytes and interestingly also are primary and/or secondary target sites in most of the hematological malignancies (see Section 3). Hence, targeting these organs can further improve the therapeutic efficacy. As described in previous sections, peripheral blood is one of the prominent target sites in HM. Long circulation properties would enhance the probability of NPs to encounter malignant cells in peripheral blood. Furthermore, most NPs are less than 200 nm in diameter [1]. Due to their small size, accumulation of NPs increases in the bone marrow, the site of origin in most

HM [36, 117]. Several reports have shown accumulation of small, long circulating liposomes in bone marrow by passive diffusion [118, 119]. A more detailed overview of specific properties of NPs for BM targeting is discussed in Section 6. The next sections describe nanomedicines.

5.1 Liposomes

Liposomes were first described by Alec Bangham in 1965 [120]. In the early 1970s, it was hypothesized that liposomes could have potential as drug delivery systems. Since then, several liposomal drugs have received regulatory approval for treatment of solid tumors as well as HM. Liposomes are composed of membrane forming phospholipids and cholesterol that form, in aqueous solutions, metastable spherical structures with a lipid bilayer surrounding an internal aqueous compartment. The structure is stabilized by hydrophobic interactions and additionally by hydrogen bonds, van der Waals forces, and electrostatic interactions. The amphiphilic property of liposomes offers an excellent opportunity to encapsulate a wide range of hydrophobic and hydrophilic drug molecules in the lipid bilayer and in the aqueous compartment, respectively. Conventional liposomes contain a phospholipid bilayer, which often includes cholesterol. Use of cholesterol improves the stability of the membrane bilayer and prevents leakage of drugs encapsulated in the aqueous core. Second generation liposomes provide a longer circulation time by reducing opsonization by poly(ethylene glycol) (PEG) coating. PEG is a hydrophilic polymer that provides steric stabilization to liposomes that prevents opsonin adherence to the surface. A third generation of liposomes is additionally decorated with

targeting ligands specific to proteins or receptors present on the target cell surface (Figure 3). Many malignant cells express increased levels of certain receptors such as growth factor receptors, as compared to normal cells, which can be targeted by specific peptides and/or antibodies.

Figure 3. Schematic representation of a nanoparticle showing liposomes and polymeric micelles

5.1.1 Clinically available liposomal formulations for the treatment of hematological malignancies

Currently there are four liposomal formulations available in the clinic that are used for the treatment of HM (Table 3).

5.1.1.1 Liposomal doxorubicin (Doxil®/Caelyx®/LipoDox®)

Doxorubicin is an anthracycline used as a first line therapy in many cancers. Doxorubicin shows antitumor activity by intercalating into the DNA double helix. It interferes with DNA and RNA synthesis and inhibits the DNA topoisomerase II enzyme in tumor cells. Doxorubicin treatment is limited by side effects; the dose-limiting toxicity is cumulative dose-related cardiotoxicity. Other side effects include myelosuppression, nausea, vomiting, and mucocutaneous effects. The safety profile of doxorubicin has been improved by liposomal encapsulation. Liposomal doxorubicin shows reduced cardiotoxicity allowing intensified treatment schedules. The pharmacokinetic profile has been improved distinctly with extended circulation time and small volume of distribution limited to the plasma volume, as compared to free drug. The EPR effect can result

in enhanced accumulation in tumor tissue. Nevertheless, the ground for regulatory approval was mainly based on reduced cardiotoxicity. A recent case report described a patient that has received 115 cycles of liposomal doxorubicin without apparent cardiotoxicity. The basis for this improvement is the non-permeable vasculature in the heart, reducing exposure. Although cardiotoxicity is reduced, new toxicities can emerge as a result of the changed tissue distribution e.g. liver, spleen, bone marrow toxicities and hand-foot syndrome etc. Although tumor accumulation is higher, this does not automatically translate into improved efficacy. The liposomal membrane prevents toxicity but also prevents bioactivity of the doxorubicin. Therefore, the doxorubicin is not active until the membrane integrity is compromised. This can happen in the tumor interstitium, in the tumor cells or in stromal cells. As a result the peak concentrations of doxorubicin may be substantially lower than for free doxorubicin, which may impact its therapeutic effect. Two types of liposomal doxorubicin have been developed and approved for clinical use, PEGylated liposomal doxorubicin (PLD) and Non-PEGylated liposomal doxorubicin (NPLD). PLD is indicated for the treatment of AIDS-related Kaposi's sarcoma and hematological malignancies whereas NPLD (Myocet®) is approved in Europe and Canada for the treatment of metastatic breast cancer in combination with cyclophosphamide. Doxil®, PEGylated liposomal doxorubicin hydrochloride (PLD) received accelerated approval in 1995 for the treatment of AIDS-related Kaposi's sarcoma. In 2007, Doxil® received approval in combination with proteasome inhibitor bortezomib for the treatment of multiple myeloma patients who have received at least one prior therapy not including bortezomib. This approval was based on a randomized phase III clinical trial with 646 patients having progressive multiple myeloma [121]. PLD with bortezomib was found to be superior to bortezomib monotherapy in this study effectuating increased median time to progression (9.3 months vs. 6.5 months), improved 15-month survival rate (76% vs. 65%), and increased median duration of response (10.2 vs. 7.0 months). However, Grade 3/4 adverse events were more frequent in the combination group (80% vs. 64%). The safety profile was consistent with the known toxicities of the two drugs. An increased incidence in the combination group was seen of grade 3/4 neutropenia, thrombocytopenia, asthenia, fatigue, diarrhea, and hand-foot syndrome. Despite these toxicities, PLD has been successfully used for the treatment of multiple myeloma. Interestingly, only liposomal doxorubicin is used for the treatment of multiple myeloma but not the free drug.

5.1.1.2 Liposomal daunorubicin (DaunoXome®)

Daunorubicin is another antineoplastic anthracycline closely related to doxorubicin with a similar activity profile. Daunorubicin, in combination with other drugs, is given for the treatment of hematological malignancies including acute non-lymphocytic

leukemia (myelogenous, monocytic, erythroid) in adults and acute lymphocytic leukemia in children and adults. DaunoXome® is a non-PEGylated liposomal formulation of daunorubicin. Similar as for doxorubicin, liposomal encapsulation of daunorubicin improved the therapeutic index of this drug. A favorable pharmacokinetic profile was observed for DaunoXome®. A recent phase III clinical trial demonstrated improved long term survival with DaunoXome® in comparison to the free drug in patients with AML aged 60 and above [122]. Also in pediatric relapsed AML patients, DaunoXome® showed improved therapeutic outcome when combined with fludarabine, cytarabine and granulocyte colony-stimulating factor (FLAG) compared to FLAG treatment alone [123].

Table 3 Approved and (pre-)clinical liposomal formulations for the treatment of hematological malignancies.

Product [Company]	Type of nanomedicine	Drug	Indication (Hematological malignancy(ies))	Development stage	Reference(s)
Doxil®/Caelyx® [Johnson & Johnson]	Liposome	Doxorubicin	Kaposi's sarcoma, ovarian cancer, breast cancer, multiple myeloma, lymphoma	Market	[1, 116-118]
DaunoXome® [Gilead Sciences, Inc.]	Liposome	Daunorubicin	Acute myeloid leukemia, non-Hodgkin lymphoma, leukemia and solid tumors	Market	[116]
Marqibo® [Talon] [Spectrum Pharmaceuticals:]	Liposome	Vincristine	Acute lymphoid leukemia, non-Hodgkin lymphoma	Market	[116, 118-120]
CPX-351 [Celator]	Liposome	Cytarabine + daunorubicin	Acute myeloid leukaemia	Market	[1, 2, 118, 121]
Onco-TCS [Inex/Enzon]	Liposome	Vincristin	Non-Hodgkin lymphoma	Phase II/III	[4, 122]
Oncocort® [Enceladus Pharmaceuticals]	Liposome	Dexamethasone phosphate	Multiple myeloma	Phase I/IIa	[123]
L-Annamycin [Callisto]	Liposome	Annamycin	Acute lymphoid leukemia, acute myeloid leukemia,	Phase I/II	[116, 124]
Liposomal tretinoin (ATRA-IV) [MD Anderson/NCI]	Liposome	Tretinoin	Refractory Hodgkin disease, kidney cancer, solid tumors, Acute promyelocytic leukemia	Phase I/II	[116, 125]
DCR-MYC (DCR-M1711) [Dicerna Pharmaceuticals]	Lipid nanoparticle*	Dicer substrate RNAi (DsiRNA) targeting MYC oncogene	Solid tumors, multiple myeloma, lymphoma	Phase I	[4, 126]
LEM-ETU [NeoPharm/Insys]	Liposome Cationin liposome	Mitoxantrone	Solid tumors, lymphoma, acute myeloid leukemia, multiple sclerosis, and prostate cancer	Phase I	[127-129]
MRX34 [Mirna Therapeutics]	Liposome	miR-RX34	Liver cancer, solid tumors, lymphoma, myeloma	Phase I	[130-132]

Table 3 Continued

Product [Company]	Type of nanomedicine	Drug	Indication (Hematological malignancy(ies))	Development stage	Reference(s)
PNT2258 [ProNAI Therapeutics]	Liposome	DNAi targeting BCL-2	Non-Hodgkin lymphoma, solid tumors	Phase I	[133, 134]
Liposomal Grb-2 [MD Anderson / Bio-Path]	Liposome	Grb2 antisense nucleotide	Leukemia	Pre-clinical	[4, 135]
Nanobins	Liposome	Arsenic trioxide	Lymphoma	Pre-clinical	[1, 136]
-	Liposome	Bortezomib	Chronic myeloid leukemia, , neuroblastoma	Pre-clinical	[137-140]
-	Liposome	Carfilzomib	Multiple myeloma	Pre-clinical	[141-143]

*Not a liposomal formulation.

5.1.1.3 Liposomal vincristine (Marqibo®)

Vincristine is a semisynthetic vinca alkaloid. Vincristine induces apoptosis, primarily by inhibition of mitosis at the metaphase through its binding to tubulin [152]. Marqibo®, the liposomal formulation of vincristine, also termed vincristine sulphate liposome injection (VSLI), comprises a sphingomyelin and cholesterol bilayer, encapsulating vincristine sulfate in the aqueous core. Marqibo® received accelerated approval by the FDA in 2012 for the treatment of patients with Philadelphia chromosome-negative ALL [153]. This approval was based on the results of a single-arm trial (HBS407 trial). In this trial, 65 ALL patients older than 18 years received Marqibo® [154]. The complete remission was 4.6% (3/65 patients), and the percentage of complete remission with incomplete blood count recovery was 10.8% (7/65 patients). A recent phase II clinical trial also supports Marqibo® as a better treatment choice for the treatment of ALL in adults [155]. In a phase 1 study using Marqibo® in pediatric ALL patients, an improved therapeutic outcome was also found [156]. Marqibo®, as part of a regimen containing cyclophosphamide, doxorubicin, prednisolone and rituximab, or just with or without rituximab, for treatment of patients with (relapsed and refractory) aggressive Non-Hodgkin lymphomas, also showed favorable phase II results [157, 158].

5.1.1.4 Liposomal cytarabine + daunorubicin (CPX-351/VYXEOSTM)

VYXEOSTM or CPX-351 has been recently approved after positive results in a Phase III trial. Cytarabine and daunorubicin combination therapy, known as “7+3” in the field, is a standard regimen for the treatment of AML. CPX-351, a liposomal formulation co-encapsulating cytarabine:daunorubicin in a synergistic molar ratio of 5:1 showed the largest therapeutic index over a range of free drug combinations in preclinical in vivo experiments [159]. A phase III randomized open label study of CPX-351 in 309 elderly patients with newly diagnosed high-risk secondary AML showed significantly improved overall survival, event free survival, and response. There were equal Grade 3-5 adverse events with similar frequency and severity in both CPX-351 and “7+3” arms [160-162].

5.1.2 Liposomal formulations in (pre-)clinical stages for the treatment of hematological malignancies

Apart from the successful clinical translation of above-mentioned liposomal formulations, there are several other liposomal formulations being tested in pre-clinical and clinical stages (Table 3). Glucocorticoids (GCs) are a class of molecules that inhibits tumor growth by multiple mechanisms of action including antiangiogenic, anti-inflammatory and antitumor activities [163-166]. The clinical outcome of GCs treatment is limited due to their short half-life in circulation and serious off-target adverse effects [167]. We have previously shown that liposomal encapsulation of (GCs)

substantially improved the therapeutic index in various solid tumor models. Liposomal prednisolone phosphate (LCL-PLP) showed tumor growth inhibition in melanoma and colon carcinoma mouse models [168-170]. In a spontaneous breast carcinoma model, LCL-PLP showed enhanced accumulation into tumors, and superior efficacy compared to free drug [165]. Oncocort® (Enceladus Pharmaceuticals), a liposomal formulation of dexamethasone phosphate is currently in phase I/IIa as a monotherapy in patients with progressive multiple myeloma [131]. Proteasome inhibitors induce apoptosis in the malignant cells by inhibiting 26S proteasome activity [171]. Moreover, they also possess antiangiogenic properties [87]. Bortezomib, carfilzomib, and ixazomib are approved proteasome inhibitors for MM treatment. Although these small molecules have improved overall responses, dose-related toxicity often remains a challenge. Bortezomib treatment resulted in minimal benefits and considerable toxicity in a pilot study in patients with imatinib-refractory CML [172]. Liposomally encapsulated bortezomib, however, showed prolonged blood circulation and decreased clearance compared to the free drug, and better efficacy/toxicity profiles in CML animal model [145]. In MM and neuroblastoma, liposomal bortezomib showed remarkable tumor growth inhibition with reduced systemic toxicity compared to free drug in vivo [146, 147]. Similarly, liposomal carfilzomib showed significant tumor growth inhibition and less toxicity in MM animal models. Synergy was observed when liposomal carfilzomib was combined with free doxorubicin, or co-encapsulated liposomal doxorubicin [149, 150]. This data strongly suggest that liposomal encapsulation would increase the therapeutic index of potent chemotherapeutics by improving their tox/PK/PD profiles.

5.2 Polymer-based nanomedicines

Polymer-based nanomedicines offer an infinite variety of building blocks to tailor the system's characteristics to meet the needs of different compounds. Polymers can be biocompatible, the release of the active compound can be tailored and they can respond to specific stimuli, such as pH or temperature. Polymeric micelles (PM) made of block copolymers with an amphiphilic nature, enable them to load hydrophobic compounds in the core. The development of PM as drug delivery systems started more than three decades ago with seminal work of Bader and coworkers [173]. PM form spontaneously when amphiphilic block copolymers are brought in an aqueous environment. During PM formation, the hydrophobic interactions between the hydrophobic building blocks will form the core whereas the hydrophilic chain engages with H-bridges with water to form the shell of the micelles. The core-shell structure enables the segregation of hydrophobic compounds in the core of the micelles while the hydrophilic shell guarantees colloidal stability and a prolonged circulation in vivo; the small average diameter of PM, 10-100 nm, and a near neutral surface charge are advantageous for efficient accumulation in

target tissues by means of the EPR effect. The chemical composition, molecular weight of the polymer blocks and the ratio between the blocks can be tailored in order to design formulations with favorable size, drug loading and release. The shell of the PM provides the possibility of decoration with specific antibody or peptide ligands to target the particles towards specific cell population.

5.2.1 Polymeric nanomedicines in (pre-)clinical stage for the treatment of hematological malignancies

HM are not often investigated first when developing polymeric nanomedicine therapies. For solid tumors, two polymeric micelles-based formulations are currently on the market. Genexol®-PM is a paclitaxel-encapsulating micelle for the treatment of various types of cancers such as ovarian, small-cell lung, pancreatic, breast and bladder cancer [174, 175], based on enabling work by Kataoka and co-workers [176]. Several other PM nanoparticle formulations are currently under clinical evaluation for the treatment of solid tumors such as breast cancer (phase III) and colorectal cancer (phase II) [177-181]. PM formulations showed promising results in pre-clinical studies and are presently being evaluated clinically for the treatment of hematological cancers (Table 4). SP1049C is a formulation composed of doxorubicin loaded in a blend of two non-ionic Pluronic® block copolymers. SP1049C has an average diameter of 30 nm and a drug loading capacity of 8%. In a murine leukemia model animals treated with the SP1049C formulation showed a decreased ability of the tumor cells to form colonies, and decreased tumor growth rate [182]. Moreover, SP1049C also prevented multidrug resistance in leukemic cells [183]. NK012, is a PM loaded with the prodrug of SN-38 (7-ethyl-10-hydroxycamptothecin) [184]. SN-38 is chemically conjugated to the block copolymer, PEG-b-poly(L-glutamic acid), via esterification of the phenol group present in the drug molecule and the carboxylic acid group in the polymer backbone. The freeze-dried product contains 20% (w/w) of SN-38 and has an average diameter after reconstitution of 20 nm. The anti-myeloma activity of this formulation was assessed in an orthotopic model of multiple myeloma in immunodeficient mice [185]. Mice were treated with NK012 at 9.4 mg/kg/day every week for 6 weeks. Bortezomib was also included in this study and was injected in combination with NK012 at a dose of 0.5 mg/kg/day every 4 days in a total of 8 injections. The dosing schedule of NK012 and bortezomib was selected based on their clinically recommended dose. NK012 decreased the percentage of myeloma cells in a dose-dependent manner. Plasma levels of M protein in untreated animals were significantly higher. In animals treated with NK012 M protein concentrations in plasma were diminished and were similar to those of animals treated with bortezomib. Both treatments also decreased bone destruction compared to untreated mice. The combination of NK012 with bortezomib

prolonged survival to 83.5 days, while survival of untreated animals was 42.5 days on average. NK012 is currently clinically evaluated for the treatment of breast, lung and colorectal cancer [186-188]. Also a phase I/II clinical trial is initiated in patients with relapsed or refractory MM in Japan [189]. NC-4016 is a polymeric micelle formulation loaded with the platinum-based drug (1,2-diaminocyclohexane)platinum(II) (DACHPt) [190]. Platinum-based drugs are extensively used for ovarian cancer, melanoma and lymphoma. Their cytotoxic effect is due to the formation of a chelate-complex with DNA, forming platinum-DNA adducts that alter the conformation of DNA. The administration of the free drug leads to systemic toxicity including nephro- and neurotoxicity. DACHPt is a platinum drug with less cytotoxicity than its parent compound oxaliplatin. NC-4016 is formulated by loading DACHPt in PEG-poly(γ -benzyl-L-glutamate) block copolymers via metal-complex formation between the drug and the carboxylic group of the polymer backbone. The micelles have an average diameter of 40 nm and a drug loading capacity of 75% (w/w). NC-4016 efficiently targets the primary tumor in an orthotopic model of scirrhus gastric cancer as well as the lymphatic metastases. NC-4016 efficiently inhibited tumor growth. This formulation is currently being tested in a phase I trial in patients with advanced solid tumors or lymphoma, [191].

Table 4. Investigational polymer-based nanomedicines for the treatment of hematological malignancies.

Product [company]	Type of nanoparticle	Drug	Indication (Hematological malignancy(ies))	Development stage	Reference
AB1-011 (nab5404) [Abraxis BioScience/Celgene]	Albumin nanoparticle	Thiocolchicine dimer (IDN 5404)	Solid tumors, lymphoma	Phase 1	[184, 185]
Abraxane® [Mayo Clinic]	Albumin-stabilized nanoparticles	Paclitaxel	Multiple Myeloma	Phase 2	[186]
CD19-DOX-NPs	Polymeric nanoparticle	Doxorubicin	ALL	Pre-clinical	[187]
SP1049C [Supratek Pharma]	Pluronic-based micelles	Doxorubicin	AML	Phase 3	[174, 175]
CFZ-PM	Polymeric micelle	Carfilzomib	Lung cancer, Multiple Myeloma	Pre-clinical	[188]
Folate and retinoic acid grafted/dextran (FA-RA/DEX)	Polymeric micelle	Doxorubicin	AML	Pre-clinical	[189]
NK012 [Nippon Kayaku Co., Ltd.][180]	Polymeric micelle	Prodrug of 7-ethyl-10-hydroxy camptothecin (SN-38)	Multiple Myeloma	Phase 1/2 JapicCTI-111652	[177]
NC-4016 [NanoCarrier]	Polymeric micelle	Oxaliplatin	Solid tumors, lymphoma	Phase 1 NCT01999491	[190]
Polybutylcyanoacrylate nanoparticles	Polymeric nanoparticle	Imatinib mesylate	Leukemia	Pre-clinical	[191]
PEG-PCL bearing pendant cyclic ketals	Polymeric nanoparticle	Dexamethasone	ALL	Pre-clinical	[192]
Alendronate-modified PEG-PLA	Polymeric micelle	Ponatinib and SAR302503	CML	Pre-clinical	[193]

5.3 Other drug delivery systems for the treatment of hematological malignancies

Drug conjugates include protein-drug conjugates, antibody-drug conjugates, lipid-drug conjugates, PEG-protein conjugates, and PEG-drug conjugates. Abraxane® is an albumin-based protein-drug conjugate formulated with paclitaxel. Paclitaxel is an antimitotic agent that exerts its function by stabilizing the microtubules and preventing their disassembly during cell division. Abraxane® is approved for the treatment of advanced non-small cell lung cancer, metastatic breast and pancreatic cancers and is currently under clinical evaluation for the treatment of multiple myeloma and lymphomas [202]. The formulation was tested in a clinical phase II trial in relapsed or refractory MM patients [203]. Patients received the formulation intravenously at day 1, 8 and 15. Treatment was repeated every 28 days up to 12 dosages when no adverse effects were present. To date, no results are available as the study is still ongoing. The same formulation was tested in MM patients in combination with lenalidomide in a phase I/II trial [202]. Abraxane® was dosed weekly at 100 mg·m⁻² for 3 weeks together with 10 mg of lenalidomide daily for 21 days, with a dose escalation for lenalidomide up till 25 mg. Also here results on efficacy have not been published. Nab5404 (formerly ABI-011) is a formulation of albumin nanoparticles loaded with a thiocolchicine dimer (IDN 5404). IDN 5404 was selected for its dual activity as an anti-tubulin agent and topoisomerase-I inhibitor. It acts as a vascular disrupting agent (VDA) and is able to lead to rapid collapse of vascular tissues. This formulation was tested pre-clinically in tumor xenograft models of ovarian and colon cancer showing promising results [98, 99]. A phase I trial started in 2010 for the treatment of advanced solid tumors and lymphomas [204]. The study has been completed, however no results are available yet. Antibody-drug conjugates like, brentuximab vedotin (Adcetris®), and ibritumomab tiuxetan (Zevalin®) are approved for the treatment of HL and NHL, respectively [205, 206]. Brentuximab vedotin is an anti-CD30 antibody conjugated via a valine-citrulline peptide linker to the antimitotic agent monomethyl auristatin E (MMAE). After CD30-mediated internalization, proteolytic enzymes cleave off MMAE, leading to tubulin polymerization and G2/M-phase growth arrest followed by apoptosis [205, 207, 208]. Similarly, ibritumomab tiuxetan is an anti-CD20 antibody conjugated to a radioactive isotope: either Yttrium-90 or Indium-111 [206]. Finally, Oncaspar™ is a PEG conjugate of asparaginase, which is approved for first line treatment of children with ALL [209]. Table 5 depicts approved and investigational drug conjugates for HM.

Table 5. Clinically available and in (pre-)clinical development drug-conjugates nanomedicines for HM.

Product [Company]	Type of conjugate	Drug	Indication (Hematological malignancy(ies))	Development stage	Reference(s)
Brentuximab vedotin (Adcetris®) [Seattle Genetics]	Antibody-drug conjugate	MMAE	Hodgkin lymphoma	Market	[197]
(Ibritumomab tiuxetan Zevalin®) [IDEC/Spectrum]	Antibody-drug conjugate	Yttrium-90 or Indium- 111	Non-Hodgkin lymphoma	Market	[198]
Oncaspar™ (PEG) [Baxalta]	PEG protein conjugate	L-asparaginase Asperginase paclitaxel	ALL	Market	[2, 118, 201]
Elacytarabine (Clavis Pharma)	Lipid-drug conjugate	Cytarabine	AML	Phase III	[1, 202-205]
PegAsys [Hoffmann-La Roche]	PEG protein conjugate	IFNα2a/-IFNα2b	CML	Phase III	[2, 206]
PEG-SN38 (EZN-2208) [Belrose Pharma/Enzon]	PEG drug conjugate	SN38 (irinotecan derivate)	lymphoma, solid tumors, breast cancer, colorectal cancer	Phase II	[207, 208]

6. TARGETING THE BONE MARROW IN HEMATOLOGICAL MALIGNANCIES WITH PARTICULATE DELIVERY SYSTEMS

It is clear that the bone marrow microenvironment is a crucial site in the development and progression of hematological malignancies. Targeting the bone marrow microenvironment can be improved by drug delivery systems and can be achieved passively or actively. Various factors play roles in targeting of nanomedicines to the bone marrow, including particle size, composition, and surface charge of nanoparticles.

6.1 Particle size

Nanomedicines are naturally inclined to accumulate in the bone marrow. Reticuloendothelial sinusoidal blood capillaries consist of pores as large as 60 nm in diameter. Therefore, nanoparticles below this size can penetrate and distribute into the bone marrow interstitial space [217]. Notably, like in solid tumors, the bone marrow also displays local inflammation and angiogenesis during HM [108]. Also here, tumor development is dependent on growth factors such as VEGF that facilitate rapid formation of angiogenic blood vessels. However, these blood vessels are abnormally structured and tortuous causing blood flow to be heterogeneous [218]. Additionally, the endothelial cell-cell junctions are less tightly coupled leading to increased permeability and leakiness [219]. As a result, the abnormal vessel wall structure is characterized by a large diversity in inter-endothelial junction widths with maximum pore diameters as large as several hundred nanometers, allowing even nanoparticles of this size to extravasate into the tumor [218, 220, 221]. Size is also important because of the fact that smaller liposomes <100 nm in diameter circulate longer in the circulation, have less interaction with plasma proteins, and escape uptake by MPS. Larger liposomes would be cleared more rapidly from the circulation. On the other hand liposomes smaller than 50 nm will limit the drug encapsulation efficiency [222].

6.2 Composition

Lipid composition affects the uptake of liposomes by bone marrow. Cholesterol plays an important role in the stability of liposomal formulations. Liposomes containing a molar cholesterol content <30% are unstable in the circulation resulting in rapid release of encapsulated drug [118, 223]. Apart from its direct role in membrane stability, cholesterol content also affects the uptake of liposomes by various tissues including bone marrow. High cholesterol content in hydrogenated egg phosphatidylcholine (HEPC) and egg phosphatidylcholine (EPC) liposomes markedly increased accumulation in bone marrow. Different uptake mechanism and involvement of serum components was hypothesized for cholesterol-dependent uptake of HEPC liposomes by bone marrow.

It is likely that selective opsonization by serum components such as C3 complement protein plays a role in the increased uptake of cholesterol-rich liposomes by phagocytic cells in the bone marrow. This also fits with the earlier findings that high cholesterol containing liposomes activate complement system [224, 225]. Regarding polymer-based nanoparticles, the polymer molecular weight has been shown to influence the bone targeting capacity. For instance, in vivo behavior of aspartic acid-HPMA copolymers was evaluated by Wang and co-workers and they found that higher molecular weight leads to higher bone accumulation, which also correlated to prolonged circulation times of the conjugates [226].

6.3 Surface charge

Surface charge of liposomes plays a major role in bone marrow uptake. Negatively charged phospholipids like phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidic acid (PA) increased the uptake of liposomes by bone marrow macrophages [227]. Targeting and/or depleting bone marrow macrophages has a great therapeutic potential in HM (see section 7.2.1) but also means indirect competition with organs such as liver and spleen that are also rich in macrophages. Additionally, receptors on the macrophages can be targeted to further enhance the uptake by active targeting approaches. These receptors include, for instance, mannose receptors, galactose receptors, folate receptors, nicotinic acetylcholine receptors, and scavenger receptors A and B (CD36). These receptors can be targeted by specific ligands coated on the surface of nanoparticle. Negatively charged liposomes found to be accumulated in the bone marrow. The presence of 20% DSPG in the bilayer of CPX-351 liposomes resulted in higher accumulation in the mouse bone marrow [118]. In recent studies, succinic acid-coated liposomes have been shown to accumulate in the bone marrow. It is believed that succinic acid and other anions are ligands of scavenger receptors of the macrophages. Also degree of PEGylation plays a major role in uptake by bone marrow. They found optimal amount of PEG to be 0.6% to get the highest uptake by rabbit and monkey bone marrow. Another important factor is the extent of total lipids. Above the dose of 50 mg/kg of lipids, bone marrow was found to be the first organ to be saturated, and most of the liposomes are taken up by liver and spleen. In later studies it was revealed that uptake of succinic acid liposomes by bone marrow is species dependent as only large animals such as rabbit and monkey were found to accumulate major portion of liposomes to the bone marrow while in small animals such as mouse and rat the results were opposite [119]. Unfortunately, similar studies have not been performed in humans yet. Another strategy to target bone marrow is the use of bisphosphonates. Molecules from this category are known to be calcium-chelating agents. Since bone marrow, especially sites that display high bone turnover,

actively recruit calcium. This feature of bone marrow, found in HM malignancies such as multiple myeloma, provides opportunity to target by bisphosphonates as has been shown for polymeric-based particulate systems. N-(2-Hydroxypropyl) methacrylamide (HPMA) is a hydrophilic polymer that has been extensively used for bone targeting purposes. For instance, HPMA copolymer-PTX-alendronate (ALN) conjugate showed antitumor efficacy against mammary adenocarcinoma inoculated into the tibia, when compared to PTX alone or in combination with ALN [228]. Instead of PTX, TNP-470, which is a potent anti-angiogenic agent used as a therapeutic drug. HPMA copolymer-ALN-TNP-470 conjugate showed 65% tumor inhibition in a murine osteosarcoma model, when compared to 50% inhibition for free TNP-470 together with ALN [229]. PLGA nanospheres have been used for bone targeting via alendronate functionalization of the nanoparticle surface. A PEG-PLGA copolymer was functionalized with ALN and was demonstrated that by increasing the ALN density the adsorption to Hap increases, as expected. The effect of the molecular weight of PEG used was also assessed, and by increasing PEG molecular weight the adsorption of targeted nanoparticles to Hap decreased, probably due to the shielding of the targeting moieties by the longer PEG chains [230]. In another study, ALN-PEG-PLGA nanoparticles loaded with bortezomib were tested in a MM mouse model. The optimal ALN percentage at the NPs surface was found to be 20%, and further increase in ALN content up to 60% led to a plateau on the binding capacity to HA. Bortezomib loaded untargeted and targeted NPs were incubated with MM1S cells to assess the ability to induce apoptosis. It was shown that ALN did not show any improvement on the ability of bortezomib NPs to induce apoptosis. This result demonstrates that the setup of the experiment does not translate the *in vivo* scenario where different types of cells are present and the targeted NPs are of great value in specifically deliver the content to the multiple myeloma cells. Fluorescently labeled NPs were intraperitoneally injected and 24h after mice injected with targeted NP showed increased retention at spleen, femur, skull and lymph nodes, compared to untargeted formulation. Contrariwise to *in vitro* results, the bone accumulation of targeted nanoparticles was 9.6-fold higher than in the untargeted counterpart [231]. PLGA nanoparticles functionalized with ALN were also prepared by others and evaluated their toxicity profile, which showed not to have any cytotoxicity and satisfactory blood compatibility [232]. *In vivo* efficacy of these nanoparticles was evaluated in an orthotopic mouse model of breast cancer with bone metastases. Drug loaded and unloaded targeted nanoparticles decreased the number of osteoclast in the tumor area, which was attributed to be due to the alendronate activity [233]. PLGA-PEGALN micelles were also applied for vancomycin delivery to bone with osteomyelitis [234]. Similarly, PEG-PLA nanoparticles functionalized with different percentages of ALN (ranging from 0 to 40%) were prepared. The higher the ALN percentage, higher was the accumulation

in bones. At 40% of ALN the accumulation was 3.2 fold higher compared to untargeted nanoparticles. However, the targeting moiety only contributed to a higher accumulation in bone when was present at the nanoparticle surface at a ration higher than 10% [201]. Poly(γ -benzyl-L-glutamate) nanoparticles, with average size below 80 nm, were also functionalized with PEGALN to target bone marrow showed adequate delivery towards the bones [235].

6.4 Targeting moieties

Active targeting increases specific delivery to the tumor tissues. Many cell surface biomarkers which are specifically or extensively expressed to the malignant cells surface such as receptors and other proteins, peptides, antibodies, and polysaccharides can be specifically targeted by coupling ligands to the surface of liposomes and other nanoparticles for drug delivery [236]. Several types of ligands can be used for active targeting of tumor cells, including antibodies, peptides, and oligonucleotide aptamers being the mostly used for hematological malignancies. Targeting via antibodies is a very straightforward approach. Antibody conjugated liposomes are called immunoliposomes. Various techniques can be used to modify antibodies and subsequently to couple them to stealth liposomes. These techniques include the use of antibodies modified with several reactive groups such as 2-Iminoethiolane (Traut's reagent), N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP), Succinimidyl oxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene (SMPT), N-hydroxysuccinimide Sacetylthioacetate (SATA), S-Acetylmercaptosuccinic anhydride (SAMSA), and Succinimidyl acetylthiopropionate (SAP) [237]. Monoclonal antibodies against specific biomarkers on malignant cells can be used to target liposomes for HM. An additional advantage of immunoliposomes is that in most cases the antibody itself has cytotoxic effects to the malignant cells. For example CD38 is uniformly expressed antigen on myeloma cells [238]. Daratumumab, an anti-CD38 antibody is effective against multiple myeloma and was approved as a monotherapy last year by FDA [239]. Coupling anti-CD38 antibody to liposomes could not only provide the specificity to the malignant B cells but could also be used as a combination therapy. Immunoliposomes coated with specific antibody and loaded with a chemotherapeutic drug against specific cell surface marker might be a promising targeted combination therapy for HM. CD19-targeted and DOX loaded micelles showed increased survival time, compared to controls, when injected in a human ALL xenograft model [195]. Transferrin targeted and DOX loaded polymeric nanoparticles composed of Pluronic85/lipid were developed for the treatment of childhood leukemia [240]. These nanoparticles were able to inhibit tumor growth up to 90%, whereas control groups inhibit tumor growth in around 10%. Transferrin was also conjugated to PEG-PLL-PLGA for the preparation of targeted micelles loaded with edelfosine for the treatment of

leukemia [241]. Micelles size was of 120 nm and when functionalized with transferrin 190 nm. Circulation time of the loaded drug was assessed and found to be similar for targeted and untargeted formulations. Targeting nanoparticles using peptides is another strategy investigated extensively in drug delivery research. In case of HM, bone marrow is the primary target. Interaction with specific adhesion molecules expressed by human bone marrow endothelial cells (HBMEC) is a potential method for bone marrow homing of nanoparticles. Therefore, targeting bone marrow endothelium by using peptides that bind to these molecules offers a useful tool for drug delivery. To target molecules that are overexpressed on angiogenic endothelial cells such as $\alpha v \beta 3$ integrins, liposomes can be coated with Arg-Gly-Asp (RGD) peptides [242, 243]. Another well-known example of endothelial targeting is very late antigen-4 (VLA-4), also known as $\alpha 4 \beta 1$ integrin. Interestingly, VLA-4 is a heterodimer not only expressed on the surface of angiogenic endothelial cells but also by abnormal white blood cells in many hematological malignancies including lymphomas, leukemias, and multiple myeloma [244-248]. It has been shown that liposomes conjugated with a cyclic pentamer peptide, called VLA-4 peptide, can be used to target HM [149, 245]. Other targets also facilitate targeting of bone marrow endothelium, for instance, E-selectin, a cell adhesion molecule [249] which is expressed in response to inflammatory stimuli but absent in normal cells [250, 251]. Targeting bone marrow by use of a thioaptamer that is specific to E-selectin increased bone marrow accumulation of nanoparticles [250, 252].

7. CHALLENGES IN THE DEVELOPMENT OF NANOMEDICINE FORMULATIONS AND THEIR CLINICAL TRANSLATION

Despite the acknowledgment that nanomedicines offer many advantages in drug delivery over free drugs, it remains difficult to develop successful nanoparticle-based formulations that are approved and used in the clinic. For example, to date only 11 liposomal formulations are approved for clinical use [38]. With regard to polymeric-based nanomedicines, only two formulations have received approval for use in clinical practice – one micellar formulation and one albumin-based nanoparticle.

7.1 Technical challenges

7.1.1. Liposomal nanomedicines

Loading therapeutic compounds efficiently and stably in liposomes is one of the major challenges. Working on a small-scale formulation in a laboratory is rather simpler approach than large-scale production to fulfill the formulation demand at industrial level. Furthermore, the formulation must be stable to allow long term storage, while at the same time, once administered, the encapsulated drug must be available at the

right site and at right time to exert therapeutic effects. Non-PEGylated conventional liposomes have shorter half-lives than their stealthy counterparts, conceivably narrowing their therapeutic window. Slow release from long circulating PEGylated liposomes could contribute to systemic effects, however, it also diminishes drug concentrations at the (primary) site of disease. Moreover, long circulation properties may result in non-specific accumulation in the skin causing serious side effects such as hand-foot syndrome, or palmar-plantar erythrodysesthesia, as reported for Doxil® [253]. Particle size is one of the very important characteristics that defines the opportunity of nanomedicines to be used as drug delivery vehicles. Conventional small scale, batch-based laboratory methods for production of nanoparticles are usually highly dependent on the operator's experience and are not suitable for reproducible large-scale production [254]. The use of advanced techniques, however, enables to not only produce nanoparticles of narrow size distribution but also results in precise control over chemical composition, drug loading and surface properties of nanoparticles that is operator independent. Examples of such techniques include microfluidics technologies and particle replication in non-wetting template (PRINT) technology [255-258]. In general, in order to develop a successful liposomal formulation optimization of loading efficiency and stability must be carefully monitored.

7.1.2 Polymer-based nanomedicines

One key aspect in the development of polymeric nanomedicines is the stability of the loaded drug inside the nanoparticle. In the design of polymeric drug delivery systems one can tune the polymers properties and introduce specific side groups for instance to increase the compatibility between polymer and drug to be loaded. Therefore, the properties of a nanocarrier such as molecular weight, ratio of hydrophobic/hydrophilic block, concentration of drug carrier in relation to the drug will all influence its performance as a drug delivery system. These properties should be tuned accordingly to each drug and purpose.

7.2 Biological challenges

7.2.1 Circulation times and clearance

Besides the technical challenges, there are some biological hurdles associated with drug delivery. Once nanomedicines are administered, the defense system of body recognizes them as foreign. Opsonins present in plasma, such as immunoglobulins (Ig), fibronectin, lipoproteins and complement proteins adsorb to the surface of nanocarriers [222, 259]. The opsonized nanoparticles are subsequently taken up by the MPS (previously called reticuloendothelial system (RES)). The organs involved in clearance are liver, spleen, kidney, lung, and bone marrow with liver and spleen

being the major source of particle uptake and clearance [260]. Macrophages and other phagocytes present in these organs are responsible for both opsonization-dependent and opsonization-independent clearance of nanoparticles. For this reason, various surface coatings have been developed to decrease recognition by host cells and thereby initiating increasing circulation times in blood. The most abundantly applied coating is PEG. This highly hydrophilic molecule makes the particles more 'stealthy' by reducing protein absorption and affecting the composition of the proteins that are absorbed on the surface, thereby evading immune cells [261, 262]. Although, the stealth property of PEGylated nanoparticles can delay and partly reduce the clearance by the MPS, repeated injections of PEGylated nanoparticles may also trigger anti-PEG IgM production, altering the pharmacokinetics and biodistribution [263, 264]. This process, also known as accelerated blood clearance (ABC) phenomenon, depends on several factors including composition, coating/PEG density and the chain length, size and surface charge of nanoparticles, route and time interval of administration, and animal species [263-268]. Apart from the IgM production, PEGylated nanoparticles may also activate innate immune responses leading to immediate, non-IgE mediated complement activation resulting in faster blood clearance and pseudoallergic reactions. This complement activation related-pseudoallergy (CARPA) is associated with clinical symptoms such as anaphylaxis, facial flushing and swelling, headache, chills, and cardiopulmonary distress [269]. To circumvent all these biological challenges and in order to make a clinically translational formulation, careful optimization of various aspects such as size, composition, density and length of coating/PEG moiety, dosing frequency, total dose and animal model (and strain), is required. Additionally, a different strategy to avoid early clearance of nanomedicines from the peripheral blood has been studied; depletion of phagocytes. Temporary (partial) depletion of the immune cells that are mainly responsible for nanoparticle clearance could be facilitated by clodronate-loaded liposomes. Using clodronate liposomes significantly increased plasma residence times and changed the biodistribution of nanomedicines in animal models [78, 270]. Clodronate is a first generation bisphosphonate that is approved for the prevention and treatment of osteoporosis. When encapsulated in liposomes it specifically kills tissue macrophages of the MPS [271, 272]. Macrophage depletion in the organs from the MPS by clodronate liposomes not only supports prevention of nanoparticle clearance from the peripheral blood; it also is a tool to study the role of macrophages and other phagocytes in health and disease [273-275]. Finally, administration of clodronate liposomes has therapeutic potential in a variety of diseases including CLL [276-280].

7.2.2 Animal models and clinical translation

The first step to evaluate novel pharmaceuticals including nanomedicines is *in vitro* testing in order to identify the biocompatibility and efficacy towards cancer cells. Conventional *in vitro* cell culture models, however, lack the complexity of the tumor microenvironment [281]. To address this issue, some advanced *in vitro* models have been recently developed including 3D co-cultures and organ-on-a-chip technologies. In 3D co-culture systems, tumor cells are cultured together with supporting cells, such as bone marrow stromal cells, that have direct cell-to-cell interaction and/or paracrine interactions with the tumor cells and mimic the bone marrow microenvironment [281]. This allows evaluation of therapeutics in a microenvironment, which is somewhat closer to the actual disease situation. However, several important aspects such as vascularization, role of immune system, and perfusion are still lacking in these 3D culture models. Organ-on-a-chip technology is another advanced tool to study *in vitro*, which overcomes the disadvantage of lack of perfusion [282]. Nevertheless, In order to establish circulation, biodistribution, safety and efficacy profiles *in vivo*, animal models are required before first-in-human trial of nanomedicines. Good animal models are essential for pre-clinical testing in order to obtain predictable and translational results. Tumor models in general essentially lack the predictive power that is required to translate preclinical efficacy into clinical activity [283]. Hence, animal models used to study drug delivery to HM should preferably be orthotopic, accurate and clinically more relevant making translation of results towards patients more reliable. To improve animal models to study drug delivery and therapeutic efficacy in HM a number of factors should be considered. Firstly, use of a patient-derived xenograft model, rather than a cell line-derived xenograft or murine models, prevents the clonal selection introduced by *in vitro* culturing of tumor cells [283] and additionally allows studying patient-specific sensitivity to certain drugs ('personalized medicine'). Secondly, animal species used for *in vivo* testing models are physiologically and anatomically very different from humans. Introducing tumors by bulk inoculation or implantation of exogenous tumor cells also deviates in various ways drastically from the normal pathobiology of cancer. Therefore it is realistic that growth and development of these tumors in animal models is also different than in humans. EPR effect is the gold standard of nanomedicine delivery to the tumor tissues [284]. However, there appears a clear difference in the magnitude of the EPR effect between animal models and the human disease. This makes translational studies based on this effect more challenging [285]. Recent reports highlighted the variable significance of EPR phenomena in patients [286]. The EPR effect in patients varies greatly between cancer types, between tumors of same cancer type, and even within a tumor itself [254] [285]. On the other hand, even a small EPR effect might offer considerable increased accumulation of a nanomedicine formulation over the

conventional drug. Also there are advantages beyond the EPR effect such as tailored pharmacokinetics/pharmacodynamics profiles, improving cellular uptake, balancing off- and on-target accumulation, and local release kinetics of the active compound. These should also be considered in the activity of nanomedicines [285] [95]. Nevertheless, spontaneous tumor models and humanized models carrying human genes, cells and/or tissues, likely simulate the conditions in humans during cancer development far closer [165, 287]. Lastly, to allow xenograft models (using either cell lines or patient cells) to grow in mice, immunodeficient strains are used (possibly in combination with sublethal irradiation). Dependent on the mouse strain, these mice lack all or parts of their (innate) immune system. This likely introduces critical deviations compared to patients when studying nanomedicines in these models, such as drastically altered pharmacokinetics and pharmacodynamics [288]. It is important to understand the 'translational value' of used models towards cancer in humans. For instance, in recent debate regarding development and testing of nanomedicines, the actuality of the EPR effect in the clinic and the translational value of animal models in (solid) cancer research has been discussed [254, 289, 290]. Better models provide more accurate information, which is crucial for screening of potential new drugs and nanomedicines, not only to make it to the clinic faster but also to be able to dismiss unsuccessful formulations in a much earlier stage of development.

8. CONCLUSIONS AND FUTURE PERSPECTIVE

Several approaches are employed in the clinic for the treatment of HM, including radiotherapy, stem cell transplantation, immunotherapy and chemotherapy. Many (chemo)therapeutic drugs have been developed to inhibit various cellular and/or molecular pathways. Combination of two or more drugs is often used to attack the tumor cells by hampering multiple oncogenic pathways. This frequently results in improved overall survival, and in some cases higher cytogenetic and molecular responses. However, (chemo)therapeutic drugs are associated with various limitations. First, most of these drugs are (semi)synthetic chemical entities which have short half-lives and rapid clearance from the circulations, resulting in a poor pharmacokinetic profile and reduced bioavailability. Lower bioavailability requires high dosing schedules, which cause dose-related and thus dose-limiting toxicities. Second, lack of specificity of these drugs towards the malignant organ/tissue affects healthy tissues leading to off-target toxicities. Third, resistance to drugs is often seen. This acquired resistance to one or more (multi-drug resistance or MDR) (chemo)therapeutic drugs may occur due to several micro-environmental and cellular phenomena [291, 292]. The latter includes, but is not limited to: induction of cell survival pathways, inability to induce

apoptosis, overexpression of certain membrane-embedded drug efflux pumps, such as P-glycoproteins and MDR proteins. Resistant malignant cells express elevated amounts of these proteins on their membranes [293-295]. Nanomedicines such as liposomes and polymeric micelles will increase the statistical chances of active compounds to be accumulated at the malignant sight, by changing pharmacokinetic properties of drug molecules leading to enhanced retention in the circulation. The net effect is higher local drug concentrations at the site of interest while healthy organs remain minimally exposed. The fact that hematological malignancies are also characterized by increased microvessel density as a result of angiogenesis, provides a rationale to use these nanocarrier systems for this class of cancer as well. Lessons learned from the success of nanoformulations such as Doxil® and VYXEOS® have proven the therapeutic value of nanomedicines in HM where liposomal encapsulation improved safety and efficacy of existing chemotherapeutics. Moreover, nanomedicines may partly, if not completely, overcome MDR. Since transport of free small drug molecules into malignant cells is mainly by passive diffusion through the cell membrane, they encounter membrane proteins including drug efflux pumps that potentially reduce their net concentration in the cytoplasm, consequently leading to suboptimal concentrations in the target cell, resulting in MDR. Nanomedicines, on the other hand, due to their large size, are generally taken up by endocytosis. Hence, they bypass this mechanism of drug efflux and the concentration within the cell is much higher as compared to free drug [291, 294, 295]. Although, results from in vitro experiments showed only a slight improvement in overcoming MDR by using classical nanocarrier systems [294]. This data should be interpreted carefully, as in vitro models lack several important aspects of tumor, for example angiogenesis, EPR effect, complex tumor microenvironment etc. Despite a reasonable understanding of pathology, molecular mechanisms behind disease progression and resistance, and extensive research in the field, only a few of formulations could successfully reach to clinic for treatment of HM (as is true for the treatment of solid tumors). The explanation lies in the reality that even though nanoparticle systems show substantial benefits in preclinical settings, their clinical translation still remains difficult. Loading drug molecules into nanoparticles, stability of nanoparticle formulations, and in vivo release of encapsulated drugs at the target site are some challenges met in development of successful nanomedicine formulations. Moreover, lack of appropriate animal models that mimic the actual clinical situation (etiology/pathology/progression) in HM makes it difficult to predict favorable outcomes for clinical trials. Nevertheless, in last few decades nanomedicines have been appreciated and proven as potential drug delivery systems. Several formulations are successfully developed and clinically approved not only for treatment of many types of malignancies but also for chronic inflammatory diseases. More research in the field

could in the future lead to novel nanoparticulate formulations for treatment of various types of HM in patients.

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4

Polymeric Micelles Loaded with Carfilzomib Increase Tolerability in a Humanized Bone Marrow-Like Scaffold Mouse Model

Aida Varela-Moreira^{a,b}, Demian van Straten^a, Heleen van Leur^a,
Ruud W.J. Ruiter^c, Anil K. Deshantri^{a,d}, Wim E. Hennink^b, Marcel H.A.M.
Fens^b, Richard W.J. Groen^c, and Raymond M. Schiffelers^{a,b*}

^a Department of Clinical Chemistry and Hematolog, University Medical Center
Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, The Netherlands.

^b Department of Pharmaceutics, Utrecht Institute for Pharmaceutical
Sciences (UIPS), Faculty of Science, Utrecht University,
Universiteitsweg 99, 3584 CG Utrecht, The Netherlands

^c Department of Hematology, Amsterdam UMC, VU University Medical Center,
Cancer Center Amsterdam, De Boelelaan 1118,
1182 DB Amsterdam, The Netherlands

^d Biological Research Pharmacology Department, Sun Pharma
Advanced Research Company Ltd. Vadodara, India

ABSTRACT

Carfilzomib-loaded polymeric micelles (CFZ-PM) based on poly(ethylene glycol)-b-poly(N-2-benzoyloxypropyl methacrylamide) (mPEG-b-p(HPMA-Bz)) were prepared with the aim to improve the maximum tolerated dose of carfilzomib in a “humanized” bone marrow-like scaffold model. For this, CFZ-PM were prepared and characterized for their size, carfilzomib loading and cytotoxicity towards multiple myeloma cells. Further, circulation and tumor & tissue distribution of fluorescently labeled micelles were determined. Tolerability of CFZ-PM versus the clinical approved formulation – Kyprolis® – was assessed. CFZ-PM presented small diameter below 55 nm and low PDI < 0.1. Cy7-labeled micelles circulated for extended periods of time with over 80% of injected dose in circulation at 24 h after intravenous injection and 1.3% of the injected dose of Cy7-labeled micelles accumulated in myeloma tumor-bearing scaffolds. Importantly, CFZ-PM were well tolerated whereas Kyprolis® showed adverse effects. Kyprolis® dosed at the maximum tolerated dose, as well as CFZ-PM, did not show therapeutic benefit, while multiple myeloma cells showed sensitivity *in vitro*, underlining the importance of the bone marrow crosstalk in testing novel formulations. Overall, this work indicates that PM are potential drug carriers of carfilzomib.

Key words: Polymeric micelles, Carfilzomib, Drug delivery, Proteasome inhibitor, Multiple Myeloma, Bone Marrow Microenvironment

1. INTRODUCTION

Multiple Myeloma (MM) is one of the most common types of cancer, accounting for 1% of all cancers and 13% of blood cancers [1]. The median age at diagnosis is 70 years. MM is a hematologic cancer resulting from a clonal expansion of plasma cells in the bone marrow (BM) [2] and clinically characterized by the following symptoms: high Calcium levels, Renal damage, Anemia and Bone lesions – collectively known as the CRAB features.

Conventional treatments include combination of melphalan and prednisone or high dose chemotherapy combined with stem cell transplantation [3]. Although MM remains an incurable disease, progress has been made with the introduction of novel therapeutic agents that improve the median recurrence free survival. New targeted treatments such as immunomodulatory agents (*e.g.* thalidomide, lenalidomide and pomalidomide) and proteasome inhibitors (*e.g.* bortezomib, carfilzomib and ixazomib) [4, 5] given in combination with classical chemotherapeutics such as melphalan and cyclophosphamide have further increased the survival rate and in general improve patients' quality of life [3,6]. Particularly proteasome inhibitors have shown an added advantage. This class of agents play a role in the disruption of the ubiquitin proteasome pathway thereby causing accumulation of damaged/misfolded proteins, pro-apoptotic proteins, cyclins and inhibition of NF- κ B signaling among others [4]. This leads to a cell cycle arrest and eventually apoptosis. Interestingly, malignant cells were shown to be more sensitive to proteasome inhibitors than healthy cells which make them a valuable therapeutic option [6].

Carfilzomib (structure shown in Figure 1 A), a second generation proteasome inhibitor, is a tetrapeptide bearing an epoxyketone that covalently and irreversibly binds to the beta- 5 subunit of the proteasome [7]. Clinical studies showed that relapsed or refractory patients, including those that failed to respond to the CFZ-analogue bortezomib, would still benefit from treatment with CFZ [8, 9]. Due to its limited solubility in aqueous solutions, CFZ depends on a vehicle to solubilize and enable systemic administration. In 2012, a formulation of CFZ (Kyprolis®) was approved by the FDA for the treatment of relapsed or refractory MM patients as single agent [10]. Kyprolis® consists of CFZ complexed in sulfobutylether beta-cyclodextrin (Captisol®) to allow systemic administration. However, Kyprolis® has a poor pharmacokinetic profile with a half-life of 30 min after intravenous (i.v.) injection, and is also rapidly metabolized mainly via extrahepatic peptidase cleavage and inactivated by epoxide hydrolysis [8,

11, 12]. As a result, high dosing and multiple administration are needed to achieve therapeutic benefits.

We have previously identified π - π stacked polymeric micelles based on poly(ethylene glycol)-b-poly(N-2-benzoyloxypyrrol methacrylamide) (mPEG-b-p(HPMA-Bz)) (structure shown in Figure 1B) that allow long-circulation of paclitaxel and showed tumor regression in two solid tumor models [13]. This micellar system has also shown therapeutic advantages over liposomes and lipoprotein-based nanoparticles in a mouse model of atherosclerosis, leading to a decrease of macrophage burden within atherosclerotic plaques [14]. Similar to paclitaxel, CFZ contains aromatic moieties and we therefore hypothesized that CFZ can be efficiently accommodated and retained in π - π stacked polymeric micelles.

Figure 1. Chemical structure of (A) carfilzomib and (B) mPEG-b-p(HPMA-Bz) copolymer (Total Mn = 22 kDa, mPEG of 5 kDa) prepared with HPMA-Bz as monomer and mPEG₂-ABCPA as initiator

Importantly, a suitable MM model is needed to evaluate the therapeutic potential of CFZ-loaded micelles (CFZ-PM). In MM, the BM microenvironment is of utmost importance for the support and maintenance of myeloma cells [15]. Malignant plasma cells interact with cellular and non-cellular components of the BM microenvironment, which leads to the release of soluble factors that in turn result in survival and/or proliferation of these cells. Taking this into account, the development of suitable mouse models that review the alliance of tumor cells and supporting/surrounding cells for drug testing is key.

Here, we used a “humanized” BM-like scaffold (huBMsc) model, an advanced mouse model of MM dependent on tissue-engineering of bone on ceramic material in immunodeficient RAG2^{-/-}γc^{-/-} mice [16, 17]. In short, calcium phosphate scaffolds are seeded with human mesenchymal stem cells (hMSCs), which after *in vitro* osteogenic

differentiation and subcutaneous implantation develop into a BM-like environment that is well vascularized and has shown to support MM outgrowth. This human-mouse hybrid animal model has shown to closely resemble the actual disease and accurately predict individual patient response in a personalized medicine set-up [16]. In the present work, we investigated loading and retention of carfilzomib in π - π stacked polymeric micelles and compared it to the clinical approved formulation - Kyprolis®. Subsequently, circulation time and tissue disposition profile of labeled polymeric micelles was assessed. Finally, the therapeutic potential and tolerability of CFZ-PM were evaluated.

2. MATERIALS AND METHODS

2.1. Preparation and characterization of carfilzomib-loaded polymeric micelles

CFZ-PM were prepared by nanoprecipitation. In brief, mPEG-b-p(HPMA-Bz) copolymer (total M_n of 22 kDa, mPEG of 5 kDa) prepared with HPMA-Bz as monomer and mPEG₂-ABCPA as initiator [18] and carfilzomib (LC laboratories; Woburn, USA) were dissolved in THF (Biosolve BV, Valkenswaard, the Netherlands). Briefly, 30 mg of copolymer and 1 mg of carfilzomib were dissolved in 1 mL of THF and subsequently added dropwise to 1 mL of water while stirring. The sample was placed in the fume hood overnight to allow organic solvent evaporation. After 16 hours, the volume of the micellar dispersion was adjusted to 1 mL with a concentrated solution of HEPES Buffered Saline (HBS), 200 mM HEPES, 1500 mM NaCl, pH 7.4, in order to obtain a micellar dispersion with a final concentration of 20 mM HEPES, 150 mM NaCl, pH 7.4. Subsequently, the micelles were filtered through a 0.45 μ m pore size filter (Phenomenex Inc, USA) to remove non-entrapped/precipitated drug. Cy7-labeled micelles (Cy7-PM) were prepared as described above by blending Cy7-labeled mPEG-b-p(HPMA-Bz) (1.5% in weight) [13] with non-labeled mPEG-b-p(HPMA-Bz). Size of CFZ-PM was determined by Dynamic Light Scattering after 10x dilution in water at 25°C (ZetaSizer Nano ZS 90, Malvern Analytical, UK). Mean Z-averaged particle size and polydispersity index (PDI) were obtained from analysis of supplier's software. To assess CFZ content, micelles were diluted 10x in acetonitrile and briefly vortexed. CFZ content was determined by ultra-high-performance liquid chromatography (UPLC) using a Waters ACQUITY system (Waters Associates Inc., Milford, MA, USA) equipped with a C18 column (ACQUITY UPLC BEHC18 1.7 μ m, 2.1 x 50 mm). In brief, a gradient was run starting with 35% of eluent B and 65% of eluent A and increasing to 70% B and 30% A in 3 minutes. This fraction was kept until 4.7 min. Then, the volume fraction of eluent B decreased again to 35% from 4.7 to 6.2 minutes. Eluent A was 2 mM ammonium formate in water and eluent B acetonitrile [19]. Run time was 7 minutes, injection volume was of 7.5 μ L and wavelength for detection was 220 nm. Flow rate was set to 0.4 mL/min and column temperature

was 30°C. CFZ calibration was done in water/acetonitrile (1:1, v/v) at concentrations ranging from 0.25-250 µg/mL. Chromatograms were analysed using Empower Software.

2.2. Retention of carfilzomib in polymeric micelles

CFZ-PM samples (1 mL) were transferred inside a dialysis cassette (Float-a-Lyzer G2 100 kDa MWCO, Repligen Corporation, Boston, MA, USA). Dialysis was performed against a solution of 2% (v/v) of Triton X-100 (Sigma-Aldrich Zwijndrecht, the Netherlands) in PBS at 4 or 37°C for 24 h [20]. As control, release was also conducted only in PBS at 37°C for 24 h. Dialysis samples were treated the same as the micelles, and diluted 10x with acetonitrile. Release of CFZ-PM in bovine serum albumin (Sigma-Aldrich Zwijndrecht, the Netherlands) (40 mg/mL) was conducted similarly, but with a Float-al-Lyzer of 300 kDa MWCO (Repligen Corporation, Boston, MA, USA). An excess of albumin compared to CFZ was used, at a molar ratio of 1300:1, albumin:CFZ. The collected samples were diluted 10x with acetonitrile and centrifuged at 12,000 g for 10 min to precipitate and separate albumin from CFZ. The supernatant was collected, and water added to have a final concentration of 50% acetonitrile in the sample for injection in UPLC.

2.3. Effect of carfilzomib (free and formulations) on multiple myeloma cells viability *in vitro*

Cytotoxic effect of CFZ-PM, CFZ-CD and CFZ free was assessed on different MM cell lines. Luciferase transduced MM cell lines were generated as described previously [16]. MM.1S, L363 and UM-9 were cultured in Greiner CELLSTAR® T75 flasks with RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin). Cells were kept in culture at 37°C in a humidified atmosphere containing 5% CO₂. Cells were seeded in Greiner CELLSTAR® 96-well plates at a density of 6250 cells per well. On the same day, cells were incubated with solutions of increasing concentrations (0.8 – 20 nM) of carfilzomib in DMSO (CFZ free), in sulfobutylether β-cyclodextrin (CFZ-CD) or in polymeric micelles (CFZ-PM) for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. As control, empty PM prepared at polymer concentrations ranging from 1 to 100 µg/mL were also added to cells. After incubation, beetle luciferin (Promega, US) was added to each well at final concentration of 3 mM. After 10 minutes, luminescence was detected (SpectraMax M2e, Molecular Devices, Canada). The percentage of viable cells was calculated and compared to control (untreated) wells. All products, otherwise mentioned, were supplied by Sigma-Aldrich (Zwijndrecht, the Netherlands).

2.4. Humanized BM-like scaffold (huBMsc) xenograft model

Female RAG2^{-/-}γc^{-/-} mice were kept in standard housing on a 12 hours light/dark cycle with free access to water and food. Human bone-like scaffolds (four per mice) of 2- to 3-mm biphasic calcium phosphate particles were prepared as previously described [17, 21]. Scaffolds were implanted subcutaneously into the back side of RAG2^{-/-}γc^{-/-} mice [16] and loaded with hMSCs injected directly into the bone structures (Figure 2). After at least eight weeks of implantation, mice were intraperitoneally injected with Busilvex® (18 mg/kg) to prevent rejection of cell transplantation. Next day, scaffolds were injected with luciferase expressing MM.1S cells (1x 10⁶ cells/scaffold). Treatment started 12 days after tumor cells inoculation. The animal experiments were performed with permission from the local ethical committee for animal experimentation and were following the Dutch Animal Experimentation Act.

Humanized BM-like scaffold (huBMsc) xenograft model

Figure 2. Schematic representation of “humanized” BM-like scaffold (huBMsc) xenograft model and treatment schedule. Eight weeks after implantation of bone-like scaffolds (4 per mouse) seeded with hMSCs, MM.1S cells were inoculated into the scaffolds. Treatments started twelve days after MM.1S cells inoculation. Formulations were i.v. administered twice a week, with a total of five injections per mouse. Bioluminescence imaging was performed every week up to four weeks from start of the treatment.

2.4.1 In vivo circulation kinetics and biodistribution of Cy7-labeled polymeric micelles

For circulation and biodistribution studies, mice were i.v. injected with 100 μL of empty Cy7-PM (30 mg/mL of mPEG-b-p(HPMA-Bz) containing 1.5 wt % of Cy7-labeled polymer) via the tail vein. Blood was withdrawn at 1 min, 1 and 2 h after injection, by puncture of the submandibular vein and at 4 and 24 h via cardiac puncture when mice were sacrificed via CO₂ asphyxiation. Blood collected in heparinized tubes was centrifuged

at 2000 g, 20°C for 5 minutes. Plasma was collected and stored at -80°C until further analysis. Tumor-bearing scaffolds and organs (liver, spleen, kidneys, heart, lungs, femurs, sternum and brain) were excised, and imaged for fluorescence (Biospace Lab Photon Imager, Meyer instrument, USA), snap-frozen in liquid nitrogen and stored at -80°C until further use. Fluorescence of Cy7-PM was detected in plasma and tissue homogenates by a fluorescence scanner Odyssey® (LI-COR Westburg, the Netherlands). Plasma was diluted with PBS prior to fluorescence detection. Frozen tissues were transferred to tubes with ceramic beads, weighed and homogenized in RIPA lysis buffer (20-188, Merck Millipore) (100 µL of buffer per 100 mg of tissue) using a Precellys 24 bead mill homogenizer (Bertin Instruments, France) for 60 seconds at 6000 rotation per minute (rpm). Homogenates were then centrifuged at 12,000 x g for 10 minutes and supernatants were analyzed for fluorescence. A calibration curve was prepared with Cy7-labeled polymer dissolved in DMSO and subsequently diluted in PBS or RIPA buffer at concentrations between 0.01-100 µg/mL.

2.4.2 Therapeutic efficacy of carfilzomib formulations

RAG2^{-/-}γc^{-/-} mice bearing human bone-like scaffolds, described in section 2.4, were treated with CFZ formulations. Bioluminescence imaging (BLI) (Biospace Lab Photon Imager, Meyer instrument, USA) was performed before start of the treatment (day 0). Based on BLI signals, mice were randomized into the following groups: phosphate buffered saline (PBS) (n=4), CFZ-CD 4 mg/kg (n=4) and CFZ-PM 4 mg/kg (n=4). All treatments were administered i.v. via tail vein twice a week for a total of five injections. BLI was performed once weekly up to four weeks from start of the treatment. Body weight was monitored throughout the study. Animals were sacrificed when humane endpoint was reached, *i.e.* 20% decrease in body weight compared to the start of treatment, or when cumulative tumor volume > 10% of body weight (measured by digital caliper, Mitutoyo, Japan).

2.5 Statistical analysis

Data of *in vitro* cytotoxicity experiments were analyzed with GraphPad Prism 7.04 non-linear regression (GraphPad Software, Inc., La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

3.1 Micelles characteristics: loading and release

CFZ was loaded at feed concentration of 1 mg/mL in 30 mg/mL PM using a nanoprecipitation method. CFZ-PM had an average diameter of 55 nm and polydispersity index (PDI) below 0.1, (Table 1). Bagheri et al. also reported average diameter of 55 nm

for empty PM based on mPEG-b-p(HPMA-Bz) at the same polymer concentration [18]. A similar size range was reported by Park et al. for micelles based on poly(ethylene glycol)-poly(caprolactone) (PEG-PCL) copolymers [22]. The small diameter of CFZ-PM is beneficial for long circulation in the blood stream [23], as well as for tumor penetration [24, 25]. Further, the PEG shell creates a protective layer able to delay opsonization by plasma proteins and as a result can evade recognition by the mononuclear phagocytic system. Importantly, the size of CFZ-PM described here is reported to be advantageous for BM targeting [26]. The reticuloendothelial sinusoidal blood capillaries contain pores with diameters of 60 nm, indicating that nanomedicines below this size can permeate into the BM interstitium.

CFZ was efficiently loaded in PM at feed concentration of 1 mg/mL of CFZ, the encapsulation efficiency was close to 90% and the loading content was around 3% in weight (Table 1). Ao *et al.* prepared CFZ micelles with a PEG-PCL block copolymer of different molecular weight of PCL block (2.3 or 5.5 kDa) and a fixed molecular weight of PEG (5 kDa). The best loading content (4% in weight) was achieved with the small PCL block (2.3 kDa) at 1: 10 feed weight ratio of drug and polymer, respectively [27]. In this work, best loading content found for CFZ-PM was of 4% (data not shown) in weight at a feed ratio 1.5:30 between drug and polymer.

Table 1 - Characteristics of polymeric micelles loaded with carfilzomib

	Size (d.nm) ^a	PDI ^a	Encapsulation efficiency (%) ^b	Loading Content (%) ^b
carfilzomib micelles	54.7 ± 1.4	0.08 ± 0.02	88.4 ± 16.7	2.9 ± 0.5

a – determined by DLS; b – determined by UPLC

Characteristics presented for CFZ-PM prepared at feed concentration of 1 mg/mL of carfilzomib; Data are presented as mean ± SD, n≥3

The *in vitro* retention of CFZ in the core of PM was assessed with a method previously reported [20]. A dispersion of CFZ-PM was placed inside a dialysis cassette and dialyzed against PBS containing 2% (v/v) Triton™ X-100. This surfactant forms micelles in water which solubilize hydrophobic molecules and thus act as a sink for the released drug [20]. Furthermore, Triton™ X-100 micelles did not mix with polymeric micelles [20]. At 24 h after incubation with Triton™ X-100 at 4 and 37°C, 67 and 40% of CFZ was retained in the micelles, respectively (Table 2). The highest release was observed at 37°C which can be attributed to higher solubility of CFZ molecules in water at higher temperatures which aids to the transfer of CFZ molecules from the hydrophobic core of the micelles to the aqueous environment containing the Triton™ X-100 micelles. Curiously, retention

in PBS at 37°C (without the presence of any acceptor for hydrophobic compounds in the aqueous medium) revealed that only 50% of the micellar content was retained in the micelles. Release (based on dialysis) was also performed in the presence of albumin since this is the most abundant protein in plasma and has hydrophobic pockets to accommodate hydrophobic drugs [28, 29]. Upon 24 h incubation with albumin at 37°C, 22% of the initial CFZ loading was retained by micelles (Table 2) which is half of the percentage found after incubation with Triton™ X-100 at the same temperature. These figures lead us to conclude that albumin is expected to better predict the release profile after i.v. injection of these formulations.

Table 2 - *In vitro* retention of carfilzomib in polymeric micelles after incubation in different media for 24h

	PBS 37°C	2% (v/v) TritonX 4°C	2% (v/v) TritonX 37°C	4% (w/v) albumin 37°C
carfilzomib micelles	50 ± 13	67 ± 11	40 ± 17	22 ± 1

Data are presented as mean ± SD, n=3 for Triton™ X-100, n=2 for albumin and PBS

3.2 *In vitro* cytotoxicity of carfilzomib-loaded polymeric micelles

The effect of CFZ-PM on the cellular viability of MM cell lines (MM.1S, L363 and UM-9) was evaluated. CFZ-CD and CFZ free (solubilized in DMSO) were also tested. CFZ-PM, CFZ-CD and CFZ free showed a dose dependent cytotoxic effect towards MM cell lines at the nanomolar range, which indicates the potency of CFZ (Figure 3 A, B and C). The IC₅₀ of CFZ-PM was of 6.8, 8.8 and 8.1 nM in MM.1S, L363 and UM-9 cells, respectively. For CFZ-CD and free CFZ, IC₅₀ values were slightly lower (Table 3). The higher IC₅₀ of CFZ-PM, compared to CFZ-CD and free CFZ, can be attributed to CFZ retention in the core of the PM and thus leads to a delayed/minor cytotoxic effect of the formulation. As a control, the effect of empty PM on the viability of MM cells was also determined (Figure 3 D). For the concentrations tested, more than 80% of cells remained viable which is in line with the observed cytocompatibility of empty micelles upon incubation with adherent cell lines [13].

Figure 3. Viability of MM cell lines after incubation with free carfilzomib, carfilzomib formulations and empty formulations for 48 h at 37°C and 5% CO₂. **(A)** MM.1S, **(B)** L363 and **(C)** UM-9 cells were treated in triplicates with increasing concentrations of carfilzomib solubilized in DMSO (CFZ free), in sulfobutylether β-cyclodextrin (CFZ-CD) or in polymeric micelles (CFZ-PM); **(D)** MM.1S, L363 and UM-9 cells incubated with empty polymeric micelles, at polymer concentrations between 0 and 100 μg/mL. After incubation, beetle luciferin was added to the wells at a final concentration of 3 mM. Ten minutes later, luminescence was detected. Percentage of viable cells was calculated compared to control (untreated) wells. Data is expressed as mean ± SD, n≥3.

Table 3 - IC₅₀ (in nM) of carfilzomib formulations upon 48 h incubation with MM cells

	MM.1S	L363	UM-9
CFZ-PM	6.8	8.8	8.1
CFZ-CD	3.8	4.9	4.2
CFZ free	2.9	4.1	3.6

3.3 *In vivo* circulation and biodistribution of Cy7-labeled PM

To investigate the potential of PM as a delivery system with extended circulation time after systemic administration and targeted tissue distribution, huBMsc mice xenografted with MM.1S cells were i.v. injected with empty Cy7-PM. Blood concentrations of Cy7-PM were assessed at 1, 2, 4 and 24 h after injection whereas the tissue distribution of the labeled micelles was quantified 4 and 24 h post-injection. Cy7-PM showed a long circulation time with 80% of the injected dose (ID) in circulation 24 h after injection (Figure 4 A). With the same micellar system, Shi et al. reported around 20% ID in a solid tumor mouse model (CD-1 mice) [13] and Alaarg et al. reported 10%ID/g of tissue on a mouse model of advanced atherosclerosis (Apoe^{-/-} mice) [14], at the same time point post-injection. These differences in circulation times can be attributed to the different mouse strains used in the different models applied in the different studies. Moreover, PM prepared here present average hydrodynamic diameter of 50 nm whereas in the above mentioned studies, mPEG-b-p(HPMA-Bz) based micelles had sizes of 100 [13] or 80 nm [14]. Additionally, the RAG2^{-/-}γc^{-/-} mice used in the present work have an almost absent immune system [30] – i.e. no T, B or NK cells and only tissue macrophages were present in this model. The absence of these immune cells likely limits recognition and subsequent clearance of the polymeric micelles by the mononuclear phagocyte system. Similarly, we recently reported prolonged circulation times of liposomes in RAG2^{-/-}γc^{-/-} mice compared to other mouse models (involving different mouse strains) [31] indicating that this phenomenon also applies to other nanoparticle types.

Scaffolds and organs were imaged for fluorescence analysis (Figure 4 B). Tumor-bearing scaffolds showed accumulation of Cy7-PM at both time points evaluated (4 and 24 h) with around 1% of the ID in the tumors (Figure 4 C). In kidneys and lungs, Cy7-PM were also detected, however, in these organs the signal might indicate that the localization can be ascribed to circulating PM still present in blood. Highest accumulation of Cy7-PM was found in liver and spleen (10 and 11 % ID, respectively) which are macrophage-rich organs, responsible for the clearance of nanosized particles [32]. Besides this, liver is a highly vascularized organ and thus part of the PM's disposition can be attributed to micelles still in blood circulation inside the liver. Although the spleen is not the primary target organ in MM, spleen involvement is observed in 30% of MM patients [33]. Therefore, one can take advantage of high accumulation of Cy7-PM in the spleen. The presence of fluorescence from Cy7-PM in the kidney can be due to accumulation of polymer chain unimers with which micelles are in equilibrium [13, 34]. An alternative explanation is that renal damage occurred in this model, a typical complication also in clinical MM, due to the accumulation of light chains of antibodies leading to local inflammation [35].

Figure 4. Blood circulation kinetics and distribution in tumor-bearing scaffolds & tissues of Cy7-labeled polymeric micelles on a “humanized” bone marrow-like scaffold model. **(A)** Circulation of Cy7-PM in blood at different timepoints after injection (1, 2, 4 and 24 h), expressed as % of Injected dose (ID) present in circulation. Blood collected immediately after injection was considered as 100% ID. Fluorescence intensity in plasma samples was measured in Odyssey® imager; **(B)** Images of scaffolds and tissues 4 and 24 h after i.v. injection of Cy7-PM. Tissues were excised and imaged using BiospaceLab Photon Imager; **(C)** Scaffolds and tissue distribution of Cy7-PM at 4 and 24 h after i.v. injection. Cy7-PM accumulation was determined after homogenization of the scaffolds and tissues. Fluorescence present in the homogenates was measured with Odyssey® imager. Data is expressed as mean $\pm$ SD, $n=3$ per time point, $n(\text{scaffolds}) > 5$ per time point.

3.4 Tolerability and therapeutic potential of carfilzomib-loaded polymeric micelles

The tolerability and therapeutic potential of CFZ-PM were evaluated in the huBMsc model xenografted with MM.1S cells. Twelve days after myeloma tumor cells inoculation into the huBMsc, treatment was started. Each mouse received two injections per week for a total of five injections. In this study, CFZ-PM was compared with the clinically approved formulation of carfilzomib - which is CFZ loaded in sulfobutylether β -cyclodextrin, i.e. Kyprolis® - at a dose close (and below) to previously reported MTD for CFZ in mice [36]. To evaluate peripheral toxicity due to the administered treatments, we monitored the body weight of the treated mice throughout the study (Figure 5 A).

The PM-treated group showed a moderate reduction in body weight (<5%) during the first week after which no further weight reduction was observed during the remainder of the experiment. On the other hand, Kyprolis® was not well tolerated and resulted in half of the mice in this group with more than 20% weight loss after one week of treatment (2 injections). These mice needed to be humanely killed since the humane end point was reached. This indicates that the maximum tolerated dose for Kyprolis® was met in this huBMsc mouse model whereas CFZ-PM formulation might have been administered in a higher dose than the one applied. Tumor growth was followed by weekly bioluminescence imaging (BLI) and showed growth in all treatment groups (Figure 5 B and C). For BLI of all scaffolds and percentage of tumor growth of individual scaffolds we refer to Figure S1 and S2, respectively. As in the present study, Park et al. did not find superior antitumor activity of CFZ loaded in PEG-PCL polymeric micelles over CFZ-CD in a human lung cancer xenograft mouse model [22]. Furthermore, they also reported systemic toxicity in mice treated with 6 mg/kg of CFZ-CD, where 4 out of 6 mice died, whereas no toxicity was found for mice treated with CFZ-PM at the same dose.

Figure 5. Effect of carfilzomib loaded polymeric micelles (CFZ-PM) versus carfilzomib in sulfobutylether β -cyclodextrin (CFZ-CD) on tumor growth of a “humanized” bone marrow-like scaffold model. Mice bearing four human bone containing scaffolds were inoculated with luciferase transduced MM.1S cells into the scaffolds. Twelve days after inoculation of tumor cells, animals were treated with PBS, CFZ-CD or CFZ-PM, both at 4 mg/kg. Arrows represent i.v. injections. Bioluminescence imaging (BLI) was performed weekly. **(A)** Relative body weight of the mice throughout the experiment; **(B)** Percentage of tumor growth compared to the day when treatment started. Tumor growth % was determined through analysis of BLI of each scaffold as counts per minute (cpm), and 100% tumor growth considered on day 0 – start of the treatment; **(C)** BLI images of representative mice 7 days (top panels), 14 days (middle panels) and 21 days (bottom panels) after treatment initiation. Fifty percent of animals treated with CFZ-CD needed to be removed from the experiment due to toxicity. Data are presented as mean $\pm$ SEM, $n=4$ per group.

The improvement of the tolerability is an important step in the treatment of MM as it may allow the use of higher doses that can be expected to be more efficacious. Importantly, whereas the *in vitro* experiments indicate sensitivity of the MM cell line, this sensitivity is apparently lost *in vivo*. This underlines the importance of drug screening in advanced models that resemble the complexity of human tumors to fully appreciate the complexity of treatment.

4. CONCLUSIONS

In the present work, CFZ was efficiently loaded in the core of polymeric micelles. The micelles have a favorable size (55 nm) for long circulation upon systemic injection and for passive targeting of tissues with increased capillary permeability. This was confirmed by evaluation of the circulation times of labeled micelles, with more than 80% of ID still present in the circulation after 24 h, which might partly be explained by the immune status of the RAG2^{-/-}γC^{-/-} mouse strain used in this study. Increase in PM accumulation over time was observed in tumor-bearing scaffolds as well as in macrophage-rich organs such as the liver and spleen. The effects on tumor growth delay in a “humanized” BM-like scaffold model were absent, but similar to what was found for the clinically approved formulation - Kyprolis® - dosed at the MTD. In addition, and importantly, the tolerability of the formulation presented in this work was substantially better than that of Kyprolis®, which indicates that higher doses and/or more frequent treatment could be pursued.

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5

Micellar Encapsulation Improves the Therapeutic Efficacy of Ibrutinib in a Mouse Model of Chronic Lymphocytic Leukemia

Aida Varela-Moreira^{a,b}, Veronika Ecker^c, Martina Braun^c,
Dave J. van de Heuvel^d, Wim E. Hennink^b, Raymond M. Schiffelers^{a,b},
Maïke Buchner^c and Marcel H. Fens^c

^a Department of Clinical Chemistry and Haematology, University
Medical Centre Utrecht, Utrecht, the Netherlands

^b Department of Pharmaceutics, Utrecht Institute for Pharmaceutical
Sciences, Utrecht University, Utrecht, the Netherlands

^c Institute for Clinical Chemistry and Pathobiochemistry, Klinikum
rechts der Isar, Technical University Munich, Munich, Germany

^d Soft Condensed Matter and Biophysics, Debye Institute for Nanomaterials
Science, Utrecht University, Utrecht, the Netherlands

ABSTRACT

With the aim to decrease ibrutinib doses and yet achieve adequate therapeutic efficacy, in the present work, we prepared poly (ethylene glycol)-b-poly (N-2-benzoyloxypropyl methacrylamide) (mPEG-b-p(HPMA-Bz)-based micelles to accommodate ibrutinib in their hydrophobic core. Circulation and tissue distribution of Cy7-labeled polymeric micelles (Cy7-PM) were assessed in wild type and Protein kinase C- β II knockout mice, with and without CLL engraftment. Finally, therapeutic efficacy of ibrutinib-loaded micelles was studied in wild type mice engrafted with CLL cells. The present work demonstrates that mPEG-b-p(HPMA-Bz)-based micelles loaded with ibrutinib show therapeutic value in the treatment of CLL. The ibrutinib-PM showed good encapsulation efficiency (80%) and high drug loading (8 mg/mL). Between 20 and 50% of the injected dose of Cy7-PM was found in blood 24 h after i.v. administration, indicating good colloidal stability. Highest tissue disposition of Cy7-PM was found in liver and spleen, as frequently reported for nanomedicines. Thus, the observed accumulation of Cy7-PM in these organs can be advantageous to target infiltrated CLL cells. Treatment of CLL-recipient wt mice with ibrutinib-PM at 5 mg/kg display transient decrease of CLL cells in peripheral blood and a significant decrease of CLL cells in the spleen. These data indicate that ibrutinib loaded mPEG-b-p(HPMA-Bz)-based micelles are promising drug delivery systems for the treatment of CLL.

Key words

Drug Delivery; Nanomedicine; polymeric micelles; Ibrutinib; Chronic lymphocytic leukemia; π - π stacking

Chronic lymphocytic leukemia (CLL) is one of the most prevalent B-cell malignancies in Western countries. [1, 2]. CLL is characterized by the monoclonal proliferation and accumulation of B-lymphocytes mainly in the peripheral blood (PB) and lymphoid organs, such as lymph nodes and spleen. Although most B-lymphocytes circulating in PB have low proliferation rates, a small fraction is actively proliferating [3]. They are replenished from a fraction localized in the proliferation centers in lymphoid organs [4]. In these centers, survival and proliferation of CLL cells occurs through signaling by accessory cells, namely T and stromal cells [5, 6]. One of the prominent pathways activated by the CLL microenvironment is B cell receptor (BCR) signaling. [7-9]. Activation of the BCR induces a signaling cascade which in turn initiates activation of spleen tyrosine kinase (SYK), Bruton tyrosine kinase (BTK), and phosphoinositide 3-kinase (PI3K). Hence, kinase inhibitors are attractive for the treatment of CLL. Ibrutinib is the first in class oral covalent inhibitor of BTK approved for the treatment of CLL and is currently first line therapy also in relapsed CLL. Unfortunately, ibrutinib only leads to a small number of complete responses. As a result is ibrutinib given for prolonged periods of time to partial responders or stable disease patients [10, 11], which poses high costs and increases the chances of development of resistance [12]. Recent studies have highlighted potential advantages of delivering ibrutinib directly to the CLL microenvironment to intensify impact of treatment [13, 14].

Ibrutinib has low water solubility and poor oral bioavailability of less than 10% [15]. Due to this, oral ibrutinib capsules have to be taken at high doses, leading to frequent gastro-intestinal side effects [16]. An approach to circumvent the limitations of oral ibrutinib administration and intensify its therapeutic effects, is the encapsulation of ibrutinib in nanocarriers. The use of nanocarriers to deliver therapeutics can offer several advantages over the administration of free compounds. Nanocarriers, such as polymeric micelles (PM), can prevent the rapid clearance, as seen for free compounds, from blood and consequently increase drug exposure at the target sites [17]. In the treatment of solid tumors, nanocarriers can also increase target tissue accumulation of drugs based on the enhanced permeability and retention effect [18]. For hematological cancers this effect has been investigated to a limited extent [19]. It may be hypothesized that this approach is also feasible for these types of cancer, as they also induce angiogenesis to supply nutrients and oxygen to the proliferation centers. These blood vessels also tend to be leaky, due to fast development but also due to local inflammation [20-24]. In CLL, the engraftment of CLL cells in lymphoid organs of mice is driven by the expression of protein kinase C- β II and subsequent activation of NF- κ B [25], a proinflammatory transcription factor, highlighting the role of inflammation in the development and progression of CLL. Given ibrutinib's hydrophobicity, polymeric

micelles are attractive nanocarriers to accommodate this drug. PM present a core-shell structure [26]. The hydrophilic shell stabilizes PM in aqueous environments and delays nanoparticle recognition by plasma proteins which allow them to circulate longer in blood [27].

In present study, we have explored the potential of polymeric micelles based on mPEG-b-p(HPMA-Bz) as nanocarriers for ibrutinib in the treatment of CLL in a preclinical mouse model. We examined the circulation kinetics and tissue distribution of Cy7-labeled micelles (Cy7-PM) in wild-type (*wt*) and protein kinase C- β II knockout (*Prkcb*^{-/-}) mice transplanted with CLL-cells derived from the TCL1 transgenic (TCL1tg) CLL mouse model [28]. Finally, therapeutic efficacy of ibrutinib-PM was tested *in vitro* and assessed in CLL-bearing mice.

PM were prepared using mPEG-b-p(HPMA-Bz) copolymer [29, 30] (Scheme 1), and were either empty or loaded with ibrutinib at 10 mg/mL. Ibrutinib-PM showed an average hydrodynamic size of 56 nm with low polydispersity index (PDI < 0.1) (Figure 1 A). The average diameter of empty PM was in the same range of what was found for ibrutinib-PM (54 nm $\pm$ 1.0, PDI = 0.07 $\pm$ 0.005).

Scheme 1. Schematic representation of the preparation of ibrutinib-loaded polymeric micelles. Ibrutinib-PM were prepared by nanoprecipitation. Ibrutinib and mPEG-b-p(HPMA-Bz) copolymer (Mn=22 kDa with HPMA-Bz as monomer and mPEG₅₀₀₀ as initiator) were dissolved in THF and subsequently added dropwise, while stirring, to an equal volume of water. After solvent evaporation for 16 h at room temperature, micelles were characterized based on size/polydispersion and loading content.

The small size of mPEG-b-p(HPMA-Bz)-based micelles is expected to favor long circulation times after intravenous (i.v.) administration and facilitate vascular escape [19, 31]. To assess ibrutinib content in the core of PM, one volume of PM was added to nine volumes of acetonitrile to disrupt the micelles and release their content. Ibrutinib concentration was determined by UPLC. Ibrutinib was loaded in high concentrations in the core of PM as 80% of the added drug (10 mg/mL) was accommodated in the core of PM (Figure 1 A). The retention of ibrutinib in PM was assessed based on a method that we previously described [32], in which PM were incubated with a solution of TritonX™-100 (2% v/v in PBS) that functioned as an acceptor of the released hydrophobic drug molecules. In brief, ibrutinib-PM were added to a dialysis cassette (Float-A-Lyzer MWCO of 100 kDa, Repligen Corporation, MA, USA) and dialyzed against an aqueous solution of TritonX™-100. At predetermined time points, samples were collected and analyzed by UPLC to measure ibrutinib concentration. Twenty-four hours after incubation, 83 and 31% (Figure 1 A) of the initial loaded ibrutinib amount was retained in the micelles at 4 and 37°C, respectively. Zhao et al. also found faster release of hydrophobic model drugs from PEG-PLGA nanoparticles at higher temperatures [33]. Additionally, as PM were stabilized by hydrophobic interactions, thermal fluctuations can lead to destabilization of the loaded drug [34]. Drug-mediated effects on primary CLL patient cells can occur directly or via signaling of accessory cells, such as bone marrow stromal cells. To test whether PM internalize in primary CLL patient cells or in their accessory cells, we prepared fluorescently labeled PM [29] (Cy3 for microscopy and Cy5.5 for FACS analysis) and examined internalization kinetics on a co-culture of the murine bone marrow stromal cell line ST-2 with primary CLL patient cells or on a monoculture of either cell type. Between 0 and 1 h, an increase in fluorescence signal on the cell membrane of ST-2 cells is observed (Figure 1 B). At 4 h, the signal of Cy3-PM at ST-2 cell membrane increased further and punctuated signals within the cell indicated internalization via endocytosis. Primary CLL patient cells hardly internalized Cy3-PM. This was confirmed by flow cytometric analysis that showed Cy5.5-PM mainly associated with ST-2 cells, alone and in co-culture with primary CLL patient cells (Figure 1 C). After 4 h, more than 80% of ST-2 cells showed Cy5.5-PM association whereas this was less than 10% of primary CLL patient cells. Low association of PM with primary CLL patient cells was possibly related to the quiescent state of these cells when cultured *in vitro*. To test the cytotoxic effect of ibrutinib-PM on primary CLL patient cells and to investigate the contribution of accessory cells, a co-culture of primary CLL patient cells with ST-2 cells was set up. In brief, ST-2 cells were seeded in 96-well plates at 10,000 cells/well. Next day, primary CLL patient cells were thawed and added to attached ST-2 cells at a ratio of 1:10 ST-2:CLL cells. A monoculture of primary CLL patient cells served as control. Ibrutinib was added at concentrations between 5 and 20 µM per well. As

expected, the viability of a monoculture of CLL patient cells was lower when compared to the co-culture with accessory cells (Figure 1 D) – as only 30 and 4% of untreated CLL cells (black bars) were viable for patient 1 and 2, respectively. When co-cultured with ST-2 cells, more than 80% of primary CLL patient cells remained viable for both patient samples [35] (Figure 1 D), which emphasizes the role of accessory cells in CLL cell survival [6]. Empty PM did not show any cytotoxicity on primary CLL patient cells whereas the cytotoxic effect of ibrutinib-PM was dose dependent for most of primary CLL patient cells.

Figure 1. Characteristics of ibrutinib-loaded polymeric micelles, cellular association with la-
beled-polymeric micelles and viability of primary CLL patient cells after contact with ibruti-
nib-polymeric micelles. Ibrutinib-PM (10 mg/mL of ibrutinib; 30 mg/mL polymer) were char-
acterized based on: **A** - Size and PDI. Size of ibrutinib-PM was measured after (10x) dilution in
water by DLS at 25°C (ZetaSizer Nano ZS 90, Malvern Analytical, UK). Encapsulation Efficiency
and Loading Content of ibrutinib-PM were calculated after disruption of the micellar dispersion
with acetonitrile (10x in volume) and subsequent analysis by UPLC.

Figure 1: Continued

Ibrutinib retention in PM after 24 h incubation with TritonX™-100 (2% v/v) at 4 and 37°C; **B** and **C** – Cellular association of labelled PM (Cy3 for microscopy and Cy5.5 for FACS analysis depicted in **B** and **C**, respectively) with a co-culture of primary CLL patient cells and ST-2 cells. Labeled PM were added to cells and 1 and 4 h after incubation cells were (B) imaged at 40x magnification (Cell Voyager CV7000s), after nuclei staining with Hoechst (33342, blue) for 30 min.; (C) the percentage of Cy5.5⁺ cells was determined by flow cytometry; **D** - Cell viability of primary CLL patient cells (with and without ST-2 cells) after incubation with empty PM, free ibrutinib or ibrutinib-PM. Cells were incubated with ibrutinib at concentrations between 5 and 20 μ M for 48 h. Empty PM were added at polymer concentration equal as for ibrutinib-PM at 10 μ M. Percentage of viable cells was determined by flow cytometric analysis after excluding DAPI (4',6-Diamidin-2-phenylindol) stained cells. Data are presented as mean $\pm$ SD, n=3.

We then set off to investigate the circulation and tissue distribution profiles of Cy7-labeled PM *in vivo* in a mouse model of CLL. To examine whether the PM *in vivo* distribution is impacted by the microenvironment inflammatory signaling induced by CLL, we compared circulation and tissue distribution profiles of Cy7-labeled PM in wild-type (*wt*) and Protein kinase C – β II knockout (*Prkcb*^{-/-}) mice. *Wt* and *Prkcb*^{-/-} recipient mice were transplanted with 2x10⁷ CLL cells from TCL1tg mice. *Wt* and *Prkcb*^{-/-} mice without CLL cells injection were used as controls. *Prkcb*^{-/-} mice showed to be insusceptible to CLL cell transplantation [25], since less than 2% of CLL cells were detected in PB at the time of Cy7-PM injection (Figure 2 A and Figure S1). The lack of expression of protein kinase C (PKC)- β II by accessory cells causes poor survival of CLL cells, underlining the importance of the microenvironment. *Wt* mice were susceptible to CLL engraftment (Figure 2 A). CLL-engrafted mice were divided according to disease severity with either low (20%-50%) and high (>50%) percentage of CLL cells in PB (Figure 2 A).

Upon i.v. injection, Cy7-PM concentration found in the blood stream after 1 minute was considered as 100% of injected dose (ID). At 24 h after PM administration, between 24-40% of the ID was found in circulation in *wt*, *wt* low CLL and *Prkcb*^{-/-} mice (Figure 2 B). Whereas in *wt* high CLL and *Prkcb*^{-/-} with CLL mice, lowest percentages of Cy7-PM at 24 h were recovered in blood: 17 and 11% of the ID, respectively.

Tissue distribution profile of Cy7-PM was analyzed 24 h after i.v. injection. Tissues were grinded and fluorescence quantified, based on a Cy7-labeled polymer calibration curve. Livers of mice engrafted with CLL contained higher Cy7-PM concentrations than livers of mice without CLL (Figure 2 C). Interestingly, livers of *wt* and *wt* low CLL mice showed similar concentrations of Cy7-PM (Figure 2 C and G). This was supported with no signs of CLL infiltration into their livers (Figure 2 J and S2A). In the liver of *wt* high CLL mice,

we observed visible engraftment of CLL cells (Figure 2 J and S2 B), also supported by the increased organ size. Here, the accumulation of Cy7-PM was 2.5-fold higher when compared to *wt* mice (Figure 2 G), which could also be clearly observed in the whole organ fluorescence images (Figure 2 F, middle panels). Livers of *Prkcb*^{-/-} mice engrafted with CLL also showed 2-fold higher Cy7-PM accumulation than *Prkcb*^{-/-} mice without CLL (Figure 2 G). This is in accordance with observed fluorescence in the IVIS scans of these livers (Figure 2 F, bottom panel). In addition, CLL infiltration was observed in *Prkcb*^{-/-} mice with CLL, indicating a delayed clearance from the liver as compared to the spleen and PB (Figure 2 J). Several articles previously reported infiltration of CLL cells in the liver [36-38]. The fact that PM accumulation in liver increases with CLL engraftment is expected to be advantageous for therapeutic targeting strategies.

An even more pronounced display of fluorescence was seen for the spleen. *Wt* mice with low CLL infiltration showed moderately higher Cy7-PM accumulation in the spleen, compared to the *wt* without CLL (Figure 2 D), which was also observed in representative fluorescence images of spleens (Figure 2 F, top panel). Due to splenomegaly in both *wt* mice with high CLL and *Prkcb*^{-/-} mice with CLL, the concentration of Cy7-PM per gram organ tissue is difficult to interpret (Figure 2 D). However, at the total organ level an increase in accumulation is clearly observed for *wt* mice with high CLL (Figure 2 H). Splenomegaly is often seen in CLL due to infiltration and accumulation of CLL cells in this organ [2, 39]. The fact that PM show increased accumulation in typical proliferative tissue like the spleen, in the presence of CLL is an important asset for determination of a therapeutic strategy [40, 41]. Remarkably, only one out of three *Prkcb*^{-/-} with CLL mice showed increase in splenic Cy7-PM accumulation when compared to *Prkcb*^{-/-} mice without CLL (Figure 2 H), which is likely due to the impaired survival of CLL cells in the spleen of *Prkcb*^{-/-} mice, although previously challenged with CLL cells [25]. This is in agreement with the negligible percentage of CLL cells found in PB at the time of Cy7-PM injection (Figure 2 A). We detected low amounts of Cy7-PM in the kidneys of all mice tested (Figure 2 E and I), suggesting good colloidal stability of the micelles in PB and therefore low accumulation of polymer chain unimers resulting from destabilized micelles.

Figure 2. Circulation and tissue distribution of Cy7-labeled micelles 24 hours after intravenous administration on wild type and protein kinase C- β II knockout (*Prkcb*^{-/-}) mice, with and without CLL. Mice were i.v. injected with 2×10^7 splenocytes from diseased TCL1tg mice. Twenty-one days after injection, mice were treated with Cy7-labeled micelles. A – Percentage of CLL cells (CD19⁺CD5⁺ cells) in peripheral blood at the start of the treatment, quantified by flow cytometric analysis; B – Cy7-PM present in circulation 24 h after PM i.v. injection; C, D, E – Accumulation of Cy7-PM in liver, spleen and kidney, expressed as μ g Cy7-polymer per g of tissue; F – Representative fluorescence images of liver, spleen and kidney. Excised organs were imaged using an IVIS imager (IVIS Spectrum In Vivo Imaging System; Perkin Elmer); G, H, I – Quantification of Cy7-PM accumulation in liver, spleen and kidney.

Figure 2. Continued

Fluorescence intensity per total organ (as determined by analysis of IVIS images) was plotted as fold change, compared to the respective group without CLL (considered as 1); J – Immunohistochemistry of liver, spleen and kidney of mice treated with Cy7-PM. Slides were stained with B220 with hematoxylin as counterstaining. Data are presented as Mean $\pm$ SD, $n \geq 3$. Unpaired t-test was performed, * $p < 0.05$, ** $p < 0.01$.

To further explore the promising characteristics of Cy7-PM accumulating in relevant tissues for CLL treatment, we tested the therapeutic effect of ibrutinib-PM in CLL-bearing mice. Treatment started twenty-one days after splenocytes transplantation into recipient C57BL/6 mice (Figure 3 A). Mice were i.v. injected with ibrutinib free or ibrutinib-PM at 5 mg/kg, the control group was treated with saline. The percentage of CLL cells in PB was monitored throughout the study (Figure 3 B). The number of CLL cells in PB does not reflect tumor burden. Increased CLL counts in PB may reflect a redistribution of CLL cells from infiltrated tissues, such as lymph nodes, spleen and liver to the circulation. A transient increase in CLL cells in PB followed by a decrease after the end of the treatment with ibrutinib was previously also observed when using TCL1tg mice as CLL donors and SCID mice as recipient of the CLL cells [42]. The same pattern of response to ibrutinib treatment is observed in CLL patients treated with ibrutinib [43, 44]. At day 32 after start treatment, the percentage of CLL cells in the spleen was assessed by flow cytometry (Figure 3 C). Ibrutinib-PM treated group showed significantly less CLL cells in the spleen when compared to control group, whereas no significant difference was found between ibrutinib free and control group (Figure 3 C). Body weight of treated mice was measured to monitor toxicity. No significant differences were noted for any of the formulations throughout the study, indicating good compatibility of the micelles (Figure 3 D). Ponader et al. evaluated free ibrutinib treatment at 2.5 or 25 mg/kg dosed orally and daily for 3 consecutive weeks using a similar CLL mouse model. The authors found a decrease in circulating CLL cells and reduced spleen weights when mice were treated at 25 mg/kg/day. When treated at lower dose (2.5 mg/kg/day), no differences were observed compared to untreated mice [42]. In our study, treatment with a dose of 5 mg/kg administered i.v., although with less frequent and a lower total number of injections, we found fewer CLL cells in the spleen compared to controls. The improved delivery of ibrutinib to the CLL microenvironment in the spleen, through encapsulation into PM, likely has contributed to this effect.

Figure 3. Therapeutic efficacy of ibrutinib-loaded micelles in CLL-bearing mice. **A** – Schematic representation of the CLL model used and treatment schedule performed. Splenocytes (> 70% CD19⁺CD5⁺ cells) from diseased TCL1tg mice were transplanted into recipient C57BL/6 mice (2×10^7 splenocytes per mouse) 21 days before the start of treatment. The percentage of CD19⁺CD5⁺ cells in blood was evaluated 4 days before first injection. Mice were i.v. injected with ibrutinib-PM (n=6) or ibrutinib free (n=6) both at 5 mg/kg of ibrutinib or saline as control (n=7). Treatments were administered *via* the tail vein 3 times per week for 3 consecutive weeks, for a total of 9 injections. The percentage of CD19⁺CD5⁺ cells in blood was monitored throughout the study.

Figure 3. Continued

Two weeks after the last injection mice were sacrificed and the percentage of CD19⁺CD5⁺ cells in spleen quantified by flow cytometry. **B** - Percentage of CD19⁺CD5⁺ cells in blood during the experiment; **d7** and **d32** results are depicted as individual graphs to stress the differences between percentage of CD19⁺CD5⁺ cells 7 days (after 3 injections) and 32 days (16 days after last injection) after start of the treatment, respectively. **C** – Quantification of the percentage of CLL cells (CD19⁺CD5⁺ cells) in spleens of treated mice two weeks after last injection. **D** – Body weight changes in the treatment groups throughout the experiment, plotted as percentage relative to day -4 (considered as 100%). Data is expressed as mean $\pm$ SEM. In C, unpaired t-test was performed, * means p value < 0.05.

In summary, we prepared ibrutinib-loaded polymeric micelles with high ibrutinib loading. Around 20-40% of the injected dose of Cy7-labeled micelles was still present in plasma 24 h after administration. Tissue distribution of Cy7-PM was mainly towards liver and spleen. Infiltrated CLL cells were found predominantly in the spleen and liver, leading to augmented accumulation of PM in these organs. Increased circulation and target site accumulation both are favorable to target and treat CLL in better way and more specifically. Interestingly, in a mouse model where CLL engraftment is impaired, we observed low Cy7-PM accumulation in the spleen, indicating that the level of CLL engraftment can likely affect the level of micellar accumulation. Finally, therapeutic studies with ibrutinib-loaded micelles revealed a significant decrease in the percentage of CLL cells in mouse spleens. These data indicate that ibrutinib loaded mPEG-b-p(HPMA-Bz)-based micelles are promising to more successfully treat CLL.

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SUPPLEMENTARY INFORMATION

Methods

Micelles preparation and characterization

PM were prepared as described in Chapter 4. Briefly, 30 mg of polymer and 10 mg of ibrutinib were dissolved in 1 mL of THF (HPLC grade, BioSolve, Valkenswaard, the Netherlands) and subsequently added drop wise to an equal volume of water under agitation. The micellar formulation was kept overnight in the fume hood to allow solvent evaporation. On the next day, the volume was added up to a final 1 mL with a concentrated solution of HBS to have a final concentration of 20 mM HEPES and 150 mM NaCl, pH 7.4. PM were filtered through a 0.45 μm pore size filter and subsequently their average size was determined with a ZetaSizer (Nano ZS 90, Malvern Analytical, UK) after 10x dilution in water. The drug loaded in the micellar core was assessed by UPLC by disrupting the micelles with acetonitrile at a ratio of 1:9 (v/v) micelles to acetonitrile, respectively.

Ibrutinib concentration was determined by UPLC with a gradient of eluent A (10% ACN with 0.1% phosphoric acid) and eluent B (acetonitrile with 0.1% phosphoric acid). Eluent B increased from 0% to 95% in 2 minutes and remained stable until 4 min; then, the eluent B decreased to 10% from 4 to 4.5 min. Total run time was of 5 minutes with a flow rate of 0.5 mL/min. An Acquity BEH C18 column was used, column temperature was of 50°C during detection and ibrutinib was detected at 254 nm. The injection volume was of 5 μL . Calibration was done with ibrutinib samples at concentrations ranging from 1 to 100 $\mu\text{g/mL}$ prepared in 100 % ACN. Both standards and samples were injected in 100% Acetonitrile.

In vitro release of the hydrophobic drugs was determined by a method previously described [32], where the micelles were placed inside a dialysis membrane and dialysed against 2% (v/v) TritonX-100 in PBS. The release experiment was conducted either at 4°C or 37°C under constant agitation. Samples from inside the dialysis bag were collected 24 h after and analysed by UPLC, as described above.

A co-culture of Bone Marrow Stromal Cells (ST-2 cells) and patient-derived CLL cells was set up to test the cytotoxic effect of empty PM, ibrutinib-PM and free ibrutinib. Briefly, ST-2 cells were seeded on 96-well plates at a density of 1×10^4 cells per well. On the day after, 1×10^5 patient derived CLL cells were seeded on top of the accessory cells and incubated for at least 4h before treatment started. Cells were treated with concentrations ranging from 5 to 20 μM of ibrutinib. Empty PM were added at polymer

concentration equal as for ibrutinib-PM at 10 μ M. Cells were incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. Cell viability was assessed by 4',6-diamidino-2-phenylindole (DAPI) exclusion staining and analysed on a FACS Canto II (BD, BioSciences, Allschwil, Switzerland). Primary CLL patient cells were discriminated from ST-2 cells based on size in Flow Cytometry. Contamination with ST-2 cells < 2% was considered acceptable.

Cellular association of fluorescently labelled polymeric micelles was assessed by Flow Cytometry on the co-culture of patient derived CLL cells and ST-2 or on either cell alone. Empty PM were prepared with 1.5% (w/w) of Cy3 or Cy5.5 labelled polymer [29]. Labelled micelles were added to the wells at a final concentration of 750 μ g total polymer/mL. For Flow Cytometry, after 1 or 4h incubation at 37°C, cells were trypsinized, washed gently with 0.1% (w/v) BSA in PBS and fixed in 4% (v/v) Formalin. Ten thousand gated events were acquired and the percentage of Cy5.5 positive cells was assessed by comparison to untreated cells. The kinetics of internalization of Cy3-labelled micelles in the same co-culture set up was evaluated by a high throughput confocal microscopy (Cell Voyager CV7000s, Yokogawa). Cells were seeded as explained above, but the number of CLL cells was 50x10³ cells per well. Cells were incubated for 30 minutes with Hoechst (33342) for nuclei staining. Subsequently, PM were added to each well and images were acquired every 15 minutes until 4h.

Animal studies

Mouse model of CLL

TCL1 transgenic (TCL1tg) mouse model of CLL was used [28, 45]. As in this model CLL takes between 8 to 12 months to develop and the disease development is relatively heterogeneous among mice, we used a transplantation method [28, 46, 47]. In brief, TCL1tg mice were grown for 8-12 months until disease development. Then, 2 x 10⁷ splenocytes (with more than 70% of splenocytes positive for CD19⁺CD5⁺) were i.v. injected *via* the tail vein into C57BL/6 recipient mice in order to obtain uniform disease development. Blood was withdrawn periodically to monitor the percentage of circulating CLL cells (analyzed by flow cytometric analysis as the percentage of CD19⁺CD5⁺ cells) in the peripheral blood. The animal studies were performed in compliance with guidelines set by national regulations and were approved by the local animal experiments ethical committee.

Circulation and tissue distribution of Cy7-PM

To determine circulation time of PM, mice were i.v. injected with empty Cy7-labeled PM (Cy7-PM). Cy7-PM were prepared with 30 mg/mL polymer mPEG-b-p(HPMA-Bz)

polymer in which 1.5 % in weight was of Cy7-labelled polymer [29], and i.v. injected *via* the tail vein. Blood samples were collected after 1 min (set as 100% injected dose) via submandibular puncture and after 24 h via cardiac puncture when mice were humanely killed via isoflurane anesthesia followed by cervical dislocation. Blood collected in Lithium-heparin-coated tubes was centrifuged at 2000 g, 20°C, 5 minutes and collected plasma was stored at -80°C. Liver, spleen and kidneys were collected, immediately imaged using an IVIS imager (IVIS Spectrum In Vivo Imaging System; Perkin Elmer). Pieces of organs were diluted in RIPA lysis buffer (20-188, Merck Millipore) (100 µL buffer per 100 mg organ) on tubes with ceramic beads and grinded on a tissue homogenizer (PreCellys 24, Bertin Instruments, France) at 6000 rpm for 60s. Next, homogenized tissues were centrifuged at 12000 g for 10 minutes and supernatant collected for fluorescence determination. Fluorescence detection in plasma and tissue homogenates was performed on a fluorescence scanner, Odyssey® (LI-COR, Westburg, the Netherlands). Plasma samples were diluted in PBS for fluorescence detection. A calibration curve of Cy7-labelled polymer (dissolved in DMSO) in PBS or RIPA buffer was prepared at polymer concentrations ranging from 0.1-60 µg/mL.

Therapeutic efficacy of Ibrutinib on a Leukemia mouse model

Mice were treated with 5 mg/kg of ibrutinib loaded PM or free ibrutinib. Mice injected with saline were used as control. All treatments were given i.v. via tail vein three times per week, for a total of 9 injections. Blood was drawn throughout the study every 2-3 days to assess the percentage of circulating CLL cells (percentage of CD19⁺CD5⁺ cells). Body weight of mice was monitored during the experiment to check for systemic toxicity of the given treatment. Two weeks after last injection, mice were humanely killed, and spleens collected to quantify the percentage of leukemic cells.

Figure S1. Percentage of CD19⁺CD5⁺ cells in peripheral blood 7 and 14 days after i.v. injection of diseased splenocytes from TCL1tg mice in recipient *wt* and *Prkcb*^{-/-} mice.

Figure S2. Representative images of liver and spleen tissue sections after H&E staining and fluorescence detection in **A** - wt mice with low CLL, **B** – wt mice with high CLL and **C** - *Prkcb*^{-/-} mice with CLL, 24 h after administration of Cy7-PM. Collected tissues were immediately frozen. Cryosections 5 μ m thick were obtained, cell nucleus counterstained with Hoechst 33342 (blue) and actin cytoskeleton with Phalloidin-Alexa488 (green). Cy7-PM are shown in red. H&E images were taken at 10x magnification.

C

Figure S2: Continued

Formulation of a DUSP6 Inhibitor - BCI - in Polymeric Micelles to Target Solid and Hematological Cancers

Aida Varela-Moreira^{a,b}, Veronika Ecker^c, Martina Braun^c, Wim E. Hennink^b,
Marcel H. Fens^b, Maike Buchner^c, Raymond M. Schiffelers^{a,b}

^a Department of Clinical Chemistry and Hematology, University
Medical Center Utrecht, Utrecht, the Netherlands

^b Department of Pharmaceutics, Utrecht Institute for Pharmaceutical
Sciences, Utrecht University, Utrecht, the Netherlands

^c Institute for Clinical Chemistry and Pathobiochemistry, Klinikum
rechts der Isar, Technical University Munich, Munich, Germany

ABSTRACT

In the present study, we prepared polymeric micelles (PM) based on poly (ethylene glycol)-b-poly (N-2-benzoyloxypropyl methacrylamide) (mPEG-b-p(HPMA-Bz)) block copolymers loaded with the DUSP6 inhibitor BCI. The circulation times of BCI and Cy7-labeled PM were evaluated in a solid tumor of neuroblastoma and in a hematopoietic-derived tumor of chronic lymphocytic leukemia (CLL). Finally, the therapeutic potential of BCI-PM was assessed in both mouse models.

BCI-PM presented an average diameter of 60 nm with low polydispersion (< 0.1). BCI loaded at a feed concentration of 6 mg/mL rendered PM with encapsulation efficiency close to 100% and drug loading content of 16% (in weight). Drug retention experiments at 37°C revealed that 40% of BCI remained in PM after 24 h. Cy7-labeled PM were detected in plasma 24 h after PM administration, with 46 and 24% of the injected dose found in solid tumor- and CLL-bearing mice, respectively. At the same timepoint, 5.5% of BCI injected dose in PM was found in the circulation of CLL-bearing mice. Effects on tumor growth evaluated in the solid tumor model were inconclusive. In CLL-bearing mice, a decrease of about 40% of CLL cells in peripheral blood was observed throughout the experiment. Still, superior therapeutic efficacy of BCI-PM over the free compound was not achieved possibly due to premature drug loss in circulation. In conclusion, BCI-loaded mPEG-b-p(HPMA-Bz)-based micelles hold potential for nanomedicine-based delivery of BCI for the treatment of CLL.

Key words: Nanomedicines, Polymeric micelles, Cancer, DUSP6 inhibitor, Erk signaling, CLL.

1. INTRODUCTION

Mitogen-activated protein kinases (MAPK) are a family of highly conserved enzymes that transmit information from extracellular signals to the intracellular effectors via cell surface receptors [1]. The MAPK family includes extracellular signal-regulated kinase (ERK 1/2), c-Jun N-terminal kinase (JNK) and the protein kinase p38 [2]. MAPK are activated through phosphorylation of both threonine and tyrosine residues in the activation loop of the kinase. MAPK signaling activation and subsequent downstream targets in the nucleus are known to control processes like proliferation, differentiation, migration and apoptosis. Abnormalities in the MAPK signaling pathways are therefore often connected with tumorigenesis. From all MAPK, the ERK pathway has been classically associated with cancer cell proliferation and is dysregulated in about 30% of cancers [3]. Upon ERK activation, it can translocate to the nucleus to modulate gene transcription [1]. MAPK phosphatases can inhibit the MAPK pathways [4]. The dual-specificity phosphatases (DUSPs) are the largest group of phosphatases that can regulate the phosphorylation of MAPK [3]. They dephosphorylate both threonine and tyrosine residues, however, the dephosphorylation of one residue is sufficient for kinase downregulation. To oppose oncogenic signaling due to activation of MAPKs, cells can activate negative feedback signaling by increasing the concentration of DUSPs. DUSPs generally have broad substrate specificity and most of them can inactivate ERK, JNK as well as p38 MAPKs. Interestingly, DUSP6 has high selectivity towards ERK and almost no activity to JNK and p38. Several studies report DUSP6 activation/expression as a marker of improved prognosis due to its tumor suppressive role via negative-feedback regulation of ERK1/2 in solid tumors such as pancreatic, lung and ovarian cancer [5-9]. Others indicate that overexpression of DUSP6 was found to be associated with tumor progression [10-16]. Also in hematopoietic-derived cancers this dual role was seen, DUSP6 was reported for its role as tumor suppressor in acute myeloid leukemia [17, 18] whereas in acute lymphoblastic leukemia DUSP6 overexpression was found as a predictor of poor clinical outcome in patients [18].

Recently, a small-molecule inhibitor of DUSP6 - (E)-2- benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI) - was identified using a chemical screen in transgenic zebrafish [19, 20]. Following its discovery, pharmacological inhibition of DUSP6 by BCI was studied in solid tumors [13, 21-23] as well as in hematopoietic-derived cancers [18]. Although BCI has shown its potency in downregulation DUSP6 phosphatase activity *in vitro*, systemic administration of BCI is challenging due to its poor water solubility [20]. Moreover, the *in vivo* administration of free small molecular weight drugs often leads to poor target tissue distribution which, in turn, will compromise the therapeutic outcome.

To overcome this, the encapsulation of poorly water-soluble drugs in nanomedicines is potentially advantageous. Particularly polymeric micelles (PM) with a hydrophilic shell and a hydrophobic core are attractive drug delivery systems to formulate these type of drugs [24-26]. The hydrophobic core can accommodate hydrophobic drugs and the hydrophilic shell can protect against opsonization, which aids extended circulation of the PM in the body. Importantly, PM increase the solubility of drugs and enables intravenous (i.v.) dosing without the need of high concentrations of organic solvents.

PM based on poly (ethylene glycol)-*b*-poly(N-2-benzoyloxypropyl methacrylamide) (mPEG-*b*-p(HPMA-Bz)) block copolymers (Figure 1 A) have previously shown to successfully accommodate and retain paclitaxel (PTX) [27]. These PM also displayed prolonged circulation times in blood upon i.v. administration. For PTX a half-life of about 8 hours was seen in immunocompromised mice. Like PTX, BCI is poorly water soluble and contains aromatic moieties (Figure 1 B), which is hypothesized to be an advantage for good encapsulation and retention in mPEG-*b*-p(HPMA-Bz)-based micelles.

In the present study, we investigated and characterized the formulation of BCI in mPEG-*b*-p(HPMA-Bz)-based micelles. The circulation times of BCI and labeled PM were evaluated in a solid tumor of neuroblastoma and in chronic lymphocytic leukemia-bearing mice. Finally, the therapeutic potential of BCI-PM was assessed in these mouse models.

Figure 1. Structure of (A) mPEG-*b*-p(HPMA-Bz) block copolymer (total Mn= 22 kDa, prepared with HPMA-Bz as monomer and mPEG₂-ABCPA as initiator [28]) and (B) BCI.

2. MATERIALS AND METHODS

2.1 Formulation and characterization of BCI-loaded micelles

Polymeric micelles were prepared as described elsewhere [27, 28]. Formulations were prepared at polymer concentration of 30 mg/mL, and BCI concentration of 6 mg/mL. Briefly, BCI and polymer were dispersed in 1 mL of tetrahydrofuran (THF) and this solution was added dropwise while stirring to 1 mL of water. After overnight solvent evaporation, micelles volume was adjusted to 1 mL with a concentrated stock solution of HBS, to obtain the final formulation dispersed in HBS buffer 20 mM HEPES, 150 mM NaCl, pH 7.4.

Average hydrodynamic diameter and the polydispersity index (PDI) of the prepared formulations were measured at 25°C by dynamic light scattering (DLS) with a ZetaSizer Nano ZS90 (Malvern Instruments, Malvern, UK) with an angle of 90°. PM size was measured at a final polymer concentration of 3 mg/mL, i.e. ten times dilution in deionized water of the original formulation. Release of BCI from PM was conducted as described in Chapter 4.

To disrupt the micelles, one volume of BCI-PM was added to nine volumes of acetonitrile and briefly vortexed. BCI was quantified by HPLC. The mobile phase was run on a gradient of eluent A (Milli-Q water with 0.1% perchloric acid) and eluent B (acetonitrile with 0.1% perchloric acid) with the volume fraction of eluent B increasing from 50% to 100% from 0 to 5 minutes. Subsequently, volume fraction of eluent B decreased from 100% to 50% between 5 and 6 minutes after start run. Total run time was 7 minutes with a flow rate of 1 mL/min. The injection volume was 20 µL, column temperature 40°C and the detection wavelength was 320 nm. Samples were injected at a final concentration of 50% acetonitrile in water. Calibration was done using samples of BCI in 50% ACN with concentrations ranging from 0.1 to 100 µg/mL.

2.2 Cytotoxic effect of BCI and BCI-loaded micelles

Compatibility of empty polymeric micelles and the cytotoxicity of free BCI and BCI-PM were evaluated using adherent solid tumor cells (Neuro2A, A431, A549 and B16F10) and a co-culture of primary CLL patient cells with murine bone marrow stroma cells (ST-2). Murine neuroblastoma (Neuro2A) cells were cultured in RPMI-1640 supplemented with 10% (v/v) FBS and 1x Non-Essential Amino Acids (NEAA). Human epidermoid carcinoma cell line A431 was cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Human lung cancer cell line A549 was cultured in DMEM/F12 supplemented with 10% (v/v) FBS. B16F10 murine melanoma cells were cultured with DMEM high glucose

complemented with 10% (v/v) FBS and 1% (v/v) sodium pyruvate. Cells were kept in culture at 37°C in a humidified atmosphere containing 5% CO₂. Cells were seeded into 96-well plates at a density of 1x10³ cells/well and incubated for 24 h at 37°C in a 5% CO₂ humidified atmosphere. Empty micelles (1 to 1000 µg/mL of polymer concentration per well) and free BCI or BCI-PM (2 to 10 µM final BCI concentration per well) were prepared as described above. Free drugs and formulations were diluted in the corresponding cell culture media to yield different concentrations of vehicles and drugs. Cell viability was determined after 48 h using MTS colorimetric assay. Untreated cells were considered as 100% viable.

Co-culture of primary CLL patient cells with ST-2 cells was set up as follows. ST-2 and primary CLL patient cells were cultured in RPMI-1640 supplemented with 10% (v/v) FBS, 1% (v/v) of sodium pyruvate, NEAA, L-glutamine and sodium bicarbonate. ST-2 cells were seeded in 96-well plates at a density of 1 x 10⁴ cells/well. Next day, primary CLL patient cells were thawed and added to ST-2 cells at a concentration of 1 x 10⁵ cells/well. As a control, primary CLL patient cells were cultured alone at the same cell density. BCI, free or in PM, was added at final concentrations ranging from 0.625 to 10 µM per well. Empty PM were added at same polymer concentration used for BCI-PM at highest drug concentration (10 µM). Cell viability was assessed after 48 h incubation at 37°C by 4',6-diamidino-2-phenylindole (DAPI) exclusion staining. Primary CLL patient cells were discriminated from ST-2 cells based on size in Flow Cytometry. Contamination with ST-2 cells < 2% was considered acceptable.

2.3 Mouse models

Female A/J mice weighing between 20-23 g had free access to water and food. Neuro2A tumor xenografts were established by subcutaneous inoculation of the mice in the right flank with 1 × 10⁶ Neuro2A cells suspended in 100 µL PBS pH 7.4 [29-31]. Tumors were measured using a digital caliper. The tumor volume V (in mm³) was calculated using the equation $V = (\pi/6) LS^2$, where L is the largest and S is the smallest superficial diameter.

A TCL1 transgenic (TCL1tg) mouse model of CLL was used [32, 33]. As in this model CLL takes between 8 to 12 months to develop and the disease development is relatively heterogenous among mice, we used a transplantation method [32, 34, 35]. In brief, TCL1tg mice were grown for 8-12 months until disease development. Then, 2 x 10⁷ splenocytes (with more than 70% of splenocytes positive for CD19⁺CD5⁺) were i.v. injected *via* the tail vein into C57BL/6 recipient mice in order to obtain similar disease development. Blood was withdrawn periodically to monitor the percentage of circulating

CLL cells (analyzed by flow cytometric analysis as the percentage of CD19⁺CD5⁺ cells) in the peripheral blood.

The animal studies were performed in compliance with guidelines set by national regulations and were approved by the local animal experiments ethical committee.

2.3.1 Circulation kinetics of BCI and Cy7-labeled micelles

To determine circulation time of PM, mice were i.v. injected with BCI loaded in Cy7-labeled PM (Cy7-PM). Cy7-PM were prepared with mPEG-b-p(HPMA-Bz) polymer in which 1.5 % in weight was of Cy7-labelled polymer [27]. BCI-loaded in Cy7-PM (6 mg/mL of BCI, and 30 mg/mL polymer) were i.v. injected *via* the tail vein. Blood samples were collected after 1 min (set as 100% injected dose), 1, 3, and 6 h by submandibular vein puncture, and after 24 h via cardiac puncture when mice were killed via CO₂ asphyxiation or isoflurane anesthesia followed by cervical dislocation. Blood collected in EDTA- or Lithium-heparin-coated tubes was centrifuged at 2000 g, 20°C, 5 minutes and plasma was stored at -80°C. Fluorescence of Cy7-PM was detected in plasma using a fluorescence scanner (Odyssey®, LI-COR, Westburg, the Netherlands). Plasma was diluted (5x) with PBS prior to fluorescence detection. A calibration curve was prepared with Cy7-labelled polymer (dissolved in DMSO) diluted in PBS at concentrations ranging between 0.05 and 60 µg/mL. For BCI detection in plasma, one volume of plasma was mixed with nine volumes of acetonitrile and vortexed for one minute followed by centrifugation at 12,000 g for 10 minutes. The BCI concentration in the supernatant was measured by HPLC analysis as described in section 2.1.

2.3.2 Therapeutic value of BCI-loaded micelles for cancer treatment

Neuro2A cell culture conditions, mouse strain, housing conditions and inducement of the subcutaneous tumor are described above. When the tumors reached a volume of 100-200 mm³, mice were included in the study. The mice received 100-200 µL i.v. injections in the tail vein 3 times per week for a total of 6 injections. Each injected dose corresponded to 40 mg/kg of BCI (n=6) (dissolved in DMSO and then diluted in PBS; final DMSO 10% v/v) or mPEG-b-p(HPMAm-Bz)-based micelles (n=6). Control group (n=6) were injected with 200 µL of PBS (with DMSO 10% v/v) pH 7.4.

For CLL treatment, on the basis of the percentage of CLL cells (CD19⁺CD5⁺) in PB of C57BL/6 on day 0, animals were randomized into different treatment groups: phosphate buffered saline (PBS) (n=7), free BCI (n=7) and BCI-PM at 20 mg/kg (n=7). All treatments were given i.v. *via* the tail vein three times per week, for a total of nine injections. Blood was drawn regularly during the study to assess the percentage of circulating CLL cells in

PB. In both studies, body weight of all mice was monitored throughout the experiment to check for systemic toxicity of the given treatments.

3. RESULTS AND DISCUSSION

3.1 Characterization of BCI formulations

BCI-PM were prepared based on mPEG-b-p(HPMA-Bz) copolymers [27, 28] by nanoprecipitation at a feed drug concentration of 6 mg/mL. BCI-PM showed an average hydrodynamic diameter of 60 nm and low PDI of 0.08 (Table I). Essentially all BCI added to the polymer was accommodated in the core of PM, as we found an encapsulation efficiency of 96% at this feed drug ratio. Retention of BCI in PM was evaluated with a method we developed in which drug loaded PM are inserted in a dialysis cassette and incubated with an aqueous solution of TritonX-100 (2% v/v: forming micelles of ± 20 nm) as acceptor [36]. At 4°C we found 86% of BCI was retained in PM whereas at 37°C only 40% of the initial BCI added was retained in the core of PM. The higher release at 37°C might be attributed to higher flexibility of drug molecules at the PM interface [37].

Table I – Characteristics of BCI-loaded micelles

	Size (d. nm) ^a	PDI ^a	EE (%) ^b	LC (%) ^b	Retention 4°C ^b	Retention 37°C ^b
BCI-PM	60.1 $\pm$ 1.3	0.08 $\pm$ 0.01	96.6 % $\pm$ 7.9	16.2 % $\pm$ 3.04	86.4% $\pm$ 6.9	40.9% $\pm$ 5.6

a – determined by DLS; b – after 24h incubation, determined by HPLC

BCI-PM were prepared at 6 mg/mL of BCI; Retention of BCI-PM was assessed after 24 h incubation with TritonX-100 (2% v/v) in PBS

All results are $n \geq 3$

3.2 *In vitro* cytotoxicity of BCI-loaded micelles

A range of concentrations of free BCI and BCI-PM was added to different tumor cell lines for 48 h. Viability of the cells exposed to the formulations was determined by MTS assay, detecting the metabolic activity. Neuro2A cells showed a dose dependent cell killing when treated with either free BCI or BCI-PM (Figure 2 A), indicating that the bioactivity of BCI encapsulated in the polymeric micelles was preserved. BCI also showed moderate cytotoxicity towards A549 cells (Figure 2 B), with less than 50% of viable cells at the highest dose of BCI tested (10 μ M). Interestingly, A431 and B16F10 cells (Figure 2 C and D, respectively) were not sensitive to either free BCI or BCI-PM at any tested concentration. Empty PM at polymer concentrations between 1 and 1000 μ g/mL did not show any cytotoxicity towards any cell line tested, as cell viability was close to 100% for all concentrations evaluated (Figure 2 E).

Figure 2. Viability of (A) Neuro2A, (B) A549, (C) A431 and (D) B16F10 tumor cells after exposure to BCI. For 48 h, cells were treated with free BCI or BCI-PM solutions at concentrations between 0 and 10 μM per well - (A), (B), (C) and (D) – and with empty PM at polymer concentrations between 1 and 1000 $\mu\text{g/mL}$ (E). The percentage of viable cells was expressed relative to untreated cells. Data expressed as Mean $\pm$ SD.

The cytotoxic effect of BCI-PM on primary CLL patient cells was tested in co-culture with ST-2 cells, as these stromal cells have been described to reduce CLL cells sensitivity to treatments, which is also seen *in vivo* [38]. Unfortunately, the percentage of viable primary CLL patient cells was already low when cultured in the absence of drug (Figure 3 top panels, black bars). The initial cell viability was variable (4.3 and 26% for patient 1 and 2, respectively) depending on the patient. Primary CLL patient cells in monoculture were highly sensitive to BCI, either free or loaded in PM, as essentially all cells were dead when incubated with concentrations from 0.625 μM BCI and higher (Figure 3 top panels).

On the contrary, when co-cultured with ST-2 cells, more than 80% of primary CLL patient cells were viable for the two patient samples tested (Figure 3, bottom panels, black bars), which underlines the role of stromal cells in CLL cell survival [39, 40]. Here, we found a dose dependent cytotoxic effect of BCI (Figure 3, bottom panels), with more than 50% cell killing at BCI concentrations above 2.5 μM . Empty PM did not show cytotoxicity, neither on primary CLL patient cells nor in the co-culture with ST-2 cells. Cells isolated

from different patients showed different sensitivity to BCI treatment, illustrating CLL heterogeneity and potentially predicts inter-patient response to treatment variations.

Figure 3. Effect of BCI or BCI-PM on the viability of primary CLL patient cells. Free BCI or BCI-PM at concentrations between 0.625 and 10 μ M were added to primary CLL patient cells when cultured alone (top panels) or in co-culture with ST-2 cells (bottom panels) for 48 h. Cells were also treated with empty PM at same polymer concentration used at 10 μ M of BCI-PM. The percentage of viable cells was plotted as the percentage of viable CD19⁺CD5⁺ cells. Data are presented as Mean $\pm$ SD, n=3

3.3 Circulation kinetics of BCI and Cy7-labeled micelles

The circulation kinetics of Cy7-labeled and BCI-loaded PM were evaluated in neuroblastoma- and in CLL-bearing mice. Figure 4 A and B show that Cy7-PM were detected in circulation for extended periods of time in blood with 46 and 24% of the injected dose (ID) present in circulation 24 h after i.v. administration in Neuro2A- and CLL-bearing mice, respectively. The difference in circulation time between the mouse models may be due to the immune status of the different mouse strains. C57BL/6 mice are immunocompetent which is compatible with faster clearance of

PM observed. A/J mice have a defect in macrophage function which is expected to lead to slower clearance of nanoparticles [41, 42]. BCI-loaded in PM showed markedly shorter circulation times in plasma than the PM, which might likely be due to the rapid extraction of BCI by blood components containing hydrophobic pockets, such as albumin [43, 44]. BCI was recovered from plasma at 24 h at 5.5 % ID in CLL-bearing C57BL/6 mice (Figure 4 B) whereas in Neuro2A-bearing A/J mice BCI was only detected up to 6 h after administration (Figure 4 A). Although this rapid extraction is not ideal to take full advantage of encapsulation into PM, it still presents benefit when compared to the pharmacokinetics of free BCI: 5 minutes after i.v. injection the drug could hardly be detected in blood (Figure 4 B).

Figure 4. Circulation kinetics of BCI (free and loaded in PM) and Cy7-labeled micelles. Cy7-PM loaded with BCI (1.5% wt of Cy7-labelled polymer [27], 6 mg/mL of BCI and 30 mg/mL polymer) were i.v. injected (100-200 μ L) in **(A)** neuroblastoma-bearing mice and **(B)** CLL-bearing mice. At predefined time points blood was withdrawn to determine drug and polymer concentrations in circulation. Percentage of ID of BCI and Cy7-PM in blood were calculated considering the concentration detected after 1 minute injection as 100% ID. Data are presented as mean $\pm$ SD, $n \geq 3$ mice per group.

3.4 Therapeutic value of BCI-loaded micelles for cancer treatment

Effects on tumor growth of BCI-PM evaluated in the neuroblastoma tumor model were inconclusive (data not shown). In the neuroblastoma tumor model, difficulties related with uniform tumor growth in the different groups became challenging to draw conclusions.

Therapeutic value of BCI-PM was evaluated in CLL-bearing mice. The relative expansion of CLL cells (CD19⁺CD5⁺ cells) in PB was assessed during the time course of the study (Figure 5 A). For BCI-PM treated mice, the relative expansion of CLL cells was,

throughout the study, consistently below the cell numbers at the start of treatment. We calculate the area under the curve (AUC) for the three treatment groups (AUCs of 30, 29 and 20 for PBS, free BCI and BCI-PM, respectively) that revealed to be significantly different between mice treated with PBS and BCI-PM (p -value = 0.025). Between free BCI and BCI-PM treated mice, the AUC difference was not significant (p -value = 0.055). Two weeks after the last injection, mice were humanely killed and organs collected. Percentage of CLL cells in spleens was determined and no significant differences between treatment groups was found (Figure 5 B). Body weight of treated mice was evaluated throughout the experiment and no differences were found compared to the weight at the beginning of the treatment, indicating that the treatments were well tolerated by all the animals (Figure 5 C).

Figure 5. Therapeutic efficacy of BCI-PM on CLL-bearing mice. Mice were treated with BCI or BCI-PM at 40mg/kg of BCI. **(A)** Relative expansion of CLL cells (CD19⁺CD5⁺ cells) in peripheral blood was quantified throughout the experiment; **(B)** Percentage of CLL cells (CD19⁺CD5⁺ cells) in the spleen two weeks after the last injection; **(C)** Relative body weight of treated mice during the course of the experiment. Data are presented as Mean $\pm$ SEM, $n=7$ per group.

4. CONCLUSIONS

In the present study, mPEG-b-p(HPMAm-Bz)-based polymeric micelles were loaded with the DUSP6 inhibitor – BCI. To our knowledge, this was the first time that the investigational small molecule inhibitor of DUSP6, BCI, was formulated in polymeric micelles. The prepared micelles are characterized by high loading content of BCI. Cell viability studies indicated that polymeric micelles were well tolerated by cells of several tumor cell lines. Importantly, the same was observed on primary CLL patient cells (co-cultured with and without stromal cells). *In vitro*, BCI loaded in the polymeric micelles displayed comparable therapeutic effects as free BCI. Strikingly, primary patient CLL cells when cultured with stromal cells exhibited markedly lower cytotoxicity, for the same BCI concentration, compared to monoculture of CLL cells.

After i.v. injection, Cy7-labeled micelles displayed prolonged blood circulation in neuroblastoma- and in CLL-bearing mice. However, circulation kinetics of encapsulated BCI was significantly shorter, indicating high extraction of BCI in plasma. BCI-loaded PM were well tolerated in both mouse models. In the CLL mouse model, BCI-loaded mPEG-b-p(HPMAm-Bz) micelles induced a persistent decrease in circulating CLL cells which was not seen for free BCI treated mice.

These preliminary experiments suggest that modifications are required to potentially improve therapeutic outcome. For instance, the extraction of BCI from the PM is fast in plasma. Better micellar retention, for example by lowering the feed ratio of drug to polymer, may improve this. Additionally, the composition of the PM may be changed to improve BCI retention even in challenging, but physiological, environments. Finally the dose and dosing frequency we studied here might not have been sufficient to induce downregulation of DUSP6 phosphatase activity and subsequent hyperactivation of ERK [18]. In future experiments, *in vivo* evaluation of the therapy-induced effects on the molecular pathways should be performed to ensure that local drug levels are adequate to affect designated signaling routes in target tissues.

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In Vitro Retention of Hydrophobic Drugs in Polymeric Micelles Containing Aromatic Moieties Predicts In Vivo Circulation

Aida Varela-Moreira^{a,b}, Heleen van Leur^b, Danielle Krijgsman^b,
Veronika Ecker^c, Martina Braun^c, Maike Buchner^c, Marcel H. A. M. Fens^b,
Wim E. Hennink^b and Raymond M. Schiffelers^{a,b*}

^a Department of Clinical Chemistry and Hematology, University Medical
Center Utrecht, Heidelberglaan 100,
3584 CX, Utrecht, The Netherlands

^b Department of Pharmaceutics, Utrecht Institute for Pharmaceutical
Sciences (UIPS), Faculty of Science, Utrecht University, Universiteitsweg 99,
3584 CG Utrecht, The Netherlands

^c Institute for Clinical Chemistry and Pathobiochemistry, Klinikum
rechts der Isar, Technical University Munich, Munich, Germany

ABSTRACT

In the present work, we investigated which physicochemical properties of drugs influence their loading and retention in polymeric micelles (PM) based on poly(ethylene glycol)-b-poly (N-2-benzoyloxypropyl methacrylamide) (mPEG-b-p(HPMA-Bz)) block copolymer. PM were loaded with hydrophobic drugs containing different number of aromatic rings (between two and five) by nanoprecipitation and characterized for size, loading efficiency and release profile in different media. PM were prepared at weight ratios ranging from 0.5:30 to 20:30 of drug:polymer. BCI and PTX-loaded micelles showed an increase in size (from 55 to 75 nm and from 80 to 100 nm, respectively) as the feed drug increased, whereas the size of the micelles loaded with the other drugs remained constant. Loading content varied between 0.8 and 34 % in weight. Release of the drugs was evaluated over seven days in buffer containing Triton X-100 or albumin, both at 37°C. We found lower retention of drugs when in contact with albumin and after 24 h of incubation between 10 and 55% of the loaded drug was retained in PM.

We found high log P is essential for a good loading efficiency but is not correlated with a high drug loading. Similarly, the number of aromatic moieties of the drugs is not correlated with the loading capacity. Drug loading showed a negative correlation with the compound's molecular weight and flexibility. Interestingly, drug retention in PM was strongly positively correlated with the number of aromatic moieties in drugs. For two selected drugs (ABT-737 and BCI) circulation time after intravenous administration was examined *in vivo*. A similar drug retention was found *in vivo* (19 and 6% of the injected dose) compared to *in vitro* release patterns indicating that these assays can be used to predict the plasma retention of drug loaded PM.

Key words

Polymeric micelles; Drug delivery; Hydrophobic Drugs; Aromatic moieties; Hydrophobic interaction; π - π stacking

1. INTRODUCTION

Many drugs and drug candidates have a poor aqueous solubility [1-3]. This is a challenge for drug development, as these drugs are often difficult to formulate. To improve aqueous solubility of these drugs, the formulation in nanoparticles is a frequently investigated method [1, 4]. Moreover, administration of free drugs often leads to poor pharmacokinetic and tissue distribution profiles after systemic administration.

One class of nanoparticles able to solubilize these drugs is polymeric micelles (PM). PM are composed of amphiphilic macromolecules that self-assemble in a core-shell structure and allow accommodation of drugs in their hydrophobic core [5-8]. This increases the drug's solubility and can also improve circulation times in blood after systemic administration. Prolonged circulation can be achieved by the PM's hydrophilic shell, generally composed of poly(ethylene glycol), that sterically stabilizes the PM surface in biological milieu and avoids recognition by the mononuclear phagocyte system. This prolonged circulation paves the road for target site accumulation through the enhanced permeability and retention (EPR) effect [9, 10]. Improved target tissue accumulation and/or reduced accumulation in healthy tissues, widens the therapeutic window.

The clinical translation of PM remains challenging with few formulations having reached approval to use in patients [6, 11]. The main challenges in the PM field are the limited *in vivo* stability and drug retention after intravenous (i.v.) administration. Both stability and retention are essential for EPR effect-mediated drug delivery [12, 13]. To this end, PM are stabilized by various methods, based on non-covalent [14, 15] and covalent interactions [16]. Most employed covalent stabilization is based on chemical crosslinking or covalent drug conjugation to the polymer backbone. However, chemical conjugation can be challenging as it needs careful optimization for every drug-polymer combination and drug efficacy can likely be affected due to insufficient drug release from PM [17-19]. Regarding non-covalent methods, π - π stacking appears a very attractive method due to the strength of the interaction [20].

Concerning the drugs, hydrophobicity is regarded the main parameter responsible for efficient loading in PM. Degree of hydrophobicity is usually expressed as octanol/water partition, known as the log P value, with higher values for hydrophobic drugs. However, other physicochemical properties may also play a role in the loading and retention of drugs in PM. Lübtow and colleagues tested the solubilization of five poorly water-soluble drugs using a library of 18 triblock copolymers poly(2-methyl-2-oxazoline) as hydrophilic

block and poly(2-oxazoline) or poly(2-oxazine) as hydrophobic block and argued that rigidity and absence of hydrogen donating groups hamper drug loading [21].

Particularly for π - π stacking based interactions, the presence of aromatic moieties in the structure of the hydrophobic drugs and in the hydrophobic domains of the amphiphilic block copolymers is likely important for drug loading. Hahn et al. tested different poly(2-oxazoline)-based amphiphiles with an increasing number of aromatic rings and loaded drugs with different number of aromatic moieties. However, they did not find a clear correlation. For example, for paclitaxel (PTX), a drug with three aromatic rings, its loading decreased with increasing number of aromatic groups present in the polymers studied. The authors suggested that a case-by-case evaluation is necessary [22]. We have previously shown that PM based on methoxy poly(ethylene glycol)-block-(N-(2-benzoyloxypropyl) methacrylamide) (mPEG-b-p(HPMA-Bz)) attain good loading and retention of PTX [23]. It was suggested that the major stabilizing force is π - π stacking between the aromatic moieties in the hydrophobic drug and aromatic moieties in the p(HPMA-Bz) block.

In the present work, we investigated which molecular properties of drugs influence not only their loading efficiency but also retention in mPEG-b-p(HPMA-Bz)-based PM. Six drugs containing aromatic rings were selected and loaded into PM (figure 1 and table I). Four drugs and two 'drug candidates' were loaded in PM, namely bortezomib (Velcade®), carfilzomib (Kyprolis®), paclitaxel (Taxol®) and ibrutinib (Imbruvica®), BCI and ABT-737, respectively. To simplify we will name both drug and drug candidates as 'drugs' throughout this study. Drug-loaded PM were characterized for size and loading efficiency/loading content. Release profiles of tested drugs were determined in different release media. Finally, the circulation times after i.v. injection in mice of two selected drugs loaded in PM were assessed and compared to their release profiles found *in vitro*.

Figure 1. Chemical structure of drugs loaded in polymeric micelles. (A) BCI, (B) bortezomib, (C) carfilzomib, (D) ibrutinib, (E) paclitaxel and (F) ABT-737.

Table I – Physicochemical properties of hydrophobic drugs loaded in polymeric micelles

Hydrophobic drugs	Molecular Weight* (g/mol)	Number of Aromatic Groups*	Log P*	Number of Rotatable Bonds*	Charge* (pH 7.4)
BCI	317	2	4.94	3	0.89
bortezomib	384	2	1.53	9	-0.05
carfilzomib	720	2	4.20	20	0.00
paclitaxel	854	3	3.54	14	0.00
ibrutinib	441	4	3.63	5	0.00
ABT-737	813	5	7.48	15	0.88

*molecular properties retrieved from Chemicalize (ChemAxon, Budapest, Hungary).

2. MATERIALS AND METHODS

2.1 Materials

All organic solvents were from Biosolve Ltd. (Valkenswaard, the Netherlands), all chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) unless mentioned otherwise. Water was purified to Milli-Q water by Synergy UV water purification system (Merck Millipore, Darmstadt, Germany). Dialysis cassettes Float-A-Lyzer G2 purchased from Repligen Corporation (Boston, MA, USA). Filters of 0.45 μm regenerated cellulose membrane were purchased from Phenomenex, Utrecht, the Netherlands. HPLC and UPLC columns and systems were supplied by Waters Associates Inc., Milford, MA, USA. Bortezomib, carfilzomib, ibrutinib and paclitaxel were obtained

from LC Laboratories (Woburn, MA, USA), BCI from Axon Medchem (Groningen, the Netherlands) and ABT-737 from AdooQ BioScience (Irvine, CA, USA).

2.2 Preparation and characterization of polymeric micelles

PM were prepared using mPEG-b-p(HPMA-Bz) copolymer (M_n of 22 kDa, mPEG of 5 kDa) prepared with HPMA-Bz as monomer and mPEG₂-ABCPA as macroinitiator (figure 2) [24]. In brief, 30 mg of polymer and drug (amounts varied from 0.5 to 20 mg) were dissolved in 1 mL of tetrahydrofuran (THF) [23]. Then, the polymer/drug solution was added dropwise to 1 mL of water under agitation for 1 minute at room temperature. The micellar dispersion was incubated overnight in a fume hood to allow evaporation of THF. Next, the volume was adjusted to 1 mL with 10x concentrated HEPES-buffered saline (HBS) (200 mM HEPES, 1500 mM sodium chloride, pH 7.4) to yield micellar dispersions in HBS at final concentration of 20 mM HEPES, 150 mM sodium chloride, pH 7.4. PM were filtered through an 0.45 μ m filter membrane to remove any precipitated/non encapsulated drug. The size of PM was determined by Dynamic Light Scattering after 10x dilution in water at 25°C (ZetaSizer Nano ZS 90, Malvern Analytical, UK). Mean Z-averaged particle size and polydispersity index (PDI) were obtained from analysis of supplier's software.

To determine the amount of the loaded drug, one volume of PM was added to nine volumes of acetonitrile (ACN), unless mentioned, to disrupt the PM and solubilize the drug content. The samples were subsequently analyzed by HPLC (Waters Alliance System), with a SunFire C18 column (5 μ m, 4.6 x 150 mm) or UPLC (Waters ACQUITY System) using an ACQUITY BEH C18 column (1.7 μ m, 2.1 x 50 mm). HPLC or UPLC methods for detection and quantitation of each drug can be found as Supporting Information. Loading efficiency and loading content were calculated as follows:

$$\begin{aligned}\text{Loading efficiency \%} &= \text{Weight loaded drug} / \text{Weight feed drug} \times 100\% \\ \text{Loading content \%} &= \text{Weight loaded drug} / \text{Weight (loaded drug+ polymer)} \times 100\%\end{aligned}$$

Loading capacity value for each drug is defined as the highest loading content, meaning the maximum weight of drugs that can be loaded in PM.

Figure 2. Structure of mPEG-b-p(HPMA-Bz) used to prepare polymeric micelles. Total $M_n = 22$ kDa, mPEG of 5 kDa) prepared with HPMA-Bz as monomer and mPEG2-ABCPA as initiator.

2.3 Drug release from polymeric micelles

The drug release assay was performed in Float-A-Lyzer G2 dialysis cassettes of 100 kDa molecular weight cut-off (MWCO) using 2% v/v Triton X-100 in phosphate buffered saline (PBS, containing 11.9 mM phosphate, 137 mM sodium chloride and 2.5 mM potassium chloride) as the releasing medium [25]. In brief, 30 mL of 2% v/v Triton X-100 in PBS was added to 50 mL tubes and the dialysis cassette subsequently loaded with 1 mL of drug-loaded PM (below each drug's highest loading content) was put inside the tube. These were well sealed and let rotating at 4 or 37°C. At predefined time points - 1, 2, 4, 6, 8, 24, 48, 72, 96, 120, 144 and 168 hours - aliquots of 10 μ L were collected from inside the dialysis cassette and analyzed by HPLC or UPLC as described in supporting information (SI).

Drug release in bovine serum albumin (40 g/L in PBS) was conducted similarly, unless stated here. The molar ratio between albumin:drug was calculated based on the amount of drug loaded in PM used in this assay. The molar ratio albumin:drug was between 95 and 1500 mol/mol. Since one albumin molecule binds to two drug molecules [26, 27], the albumin:drug ratios used are more than sufficient to solubilize all released drug molecules. The dialysis cassette used had a MWCO of 300 kDa to allow albumin molecules to freely distribute/diffuse between the inside of the dialysis cassette and the external releasing medium. To maintain osmotic equilibrium, 0.5 mL of drug-loaded PM and 0.5 mL of albumin solution were added to the dialysis cassette. To separate the drug from albumin, 90 μ L of ACN was added to the 10 μ L aliquots, vortexed for 1 min and subsequently the precipitated albumin was spun down at 12,000 g for 10 min. The supernatant was collected and analyzed by HPLC/UPLC according to analytical methods for each drug described in SI. This method was validated by spiking known amounts of drug with albumin, followed by precipitation of albumin and determination of the drug concentration in the supernatant. Full recover of the added drugs was achieved.

2.4 Circulation time of drug loaded in Cy7-labeled micelles

Circulation times of ABT-737 and BCI loaded Cy7- labelled PM were assessed using immunocompetent C57BL/6 mice. Mice were i.v. injected with 100-200 μ L of ABT-737-PM (1 mg/mL of drug; 30 mg/mL of polymer) and BCI-PM (6 mg/mL of BCI; 30 mg/mL of polymer) via the tail vein. Blood was withdrawn via submandibular puncture at 1 min (considered as 100% of the injected dose), 1 and 24 h after injection and collected in lithium-heparin coated tubes. Plasma was obtained by whole blood centrifugation (2000x g, 5 min, 20°C) and stored at -80°C until further use.

To extract ABT-737 from plasma, 1 volume of plasma was mixed with 9 volumes of methanol:acetonitrile (1:1 v/v) and vortexed for 1 min for plasma proteins precipitation. Next, the samples were centrifuged at 12,000 g for 15 min and the supernatant was collected to determine ABT-737 concentration by HPLC as described in SI. For BCI quantification, 1 volume of plasma was mixed with 9 volumes of ACN and vortexed for 1 min for plasma proteins precipitation. Samples were centrifuged at 12,000 g for 15 min and the supernatant was collected for quantitation of BCI concentration in plasma by HPLC as described in SI. The method to extract ABT-737 and BCI from plasma was validated by spiking known amounts with drug with plasma, followed by precipitation of albumin and determination of the drug concentration in the supernatant. Full recovery of the drugs was obtained.

Fluorescence of Cy7-PM was detected in plasma by a fluorescence scanner Odyssey® (LI-COR Westburg, the Netherlands). Plasma was diluted in PBS prior to fluorescence detection. A calibration curve was prepared with Cy7-labeled polymer (synthesized as previously described [23]) dissolved in DMSO and subsequently diluted in PBS at concentrations between 0.01-100 μ g/mL.

2.5 Statistical Analysis

Statistical analysis was performed by GraphPad Prism software version 7.04 (GraphPad Software, Inc., La Jolla, CA, USA). Correlation between the variables was assessed with linear regression, exhibiting the r^2 as a measure of the degree of correlation between the analyzed data.

3. RESULTS AND DISCUSSION

3.1 Characterization of drug-loaded polymeric micelles

The average diameter of PM loaded with different drugs and prepared at different feeds was determined by dynamic light scattering. BCI-PM (figure 3 A) showed a dose-

dependent increase in average size from 55 to 75 nm with a concomitant slight increase in PDI. PTX-PM, as previously reported, also showed an increase in size from 80 to 100 nm [23]. In contrast, PM loaded with bortezomib, carfilzomib, ibrutinib and ABT-737 presented very stable sizes around 55 nm (PDI < 0.10) for all feed drug ratios tested (figure 3 B, C, D, E).

Figure 3. Size and Polydispersity Index of polymeric micelles loaded with hydrophobic drugs. The size is the Z-average diameter as determined by DLS after 10x dilution of the micellar dispersion in water. PDI is the polydispersity index of the polymeric micelles. Data are shown as mean $\pm$ SD, $n=3$.

Loading efficiency and content are shown in figure 4. BCI and ibrutinib (figure 4 A and 4 D) showed good loading efficiencies close to 100% for all feed ratios investigated. Although BCI is charged at pH7.4, this does not negatively influence its loading in PM (table I). Obviously, despite its charge, the drug molecule is sufficiently hydrophobic for solubilization in the core of the micelles. In addition, the hydrophobic environment of the core might change the pKa of the amine in this drug. figure 4 A and 4 D show that the loading content increased with increasing drug feed, reaching up to 21 and 34 wt % for BCI and ibrutinib, respectively. In a previous study it was shown that PTX had a good loading efficiency with high loading content up to 23 wt % [23]. Bortezomib (figure 4 B) displayed low loading efficiency (< 20%) and consequently low loading capacity – 5 wt %. Carfilzomib was efficiently loaded in the core of PM up to a feed drug concentration of 1.5 mg/mL (figure 4 C). At concentrations of carfilzomib higher than this concentration,

the relative amount of loaded drug dropped, indicating saturation and resulting in a loading capacity of 4 wt %. Similarly, ABT-737-PM showed a loading content < 10 wt % for all feed drug ratios tested (figure 4 E). Although carfilzomib and ABT-737 were not accommodated in high payloads in the core of PM, i.e. showed low loading capacity, at low feed drug concentrations (up to 2 mg/mL) these were well encapsulated in PM (> 80%), indicating that these drugs are good candidates for further testing.

Figure 4. Loading efficiency and content of polymeric micelles loaded with hydrophobic drugs. The loaded drug in the micelles was quantified by HPLC/UPLC as described in the SI. Data are given as mean $\pm$ SD, $n=3$.

3.2 Effect of log P, aromatic content, rotatable bond count and molecular weight of drugs on their loading in polymeric micelles

Poor drug loading and retention in PM are regarded as one of the major drawbacks hampering their clinical application [11, 28]. To investigate this aspect, PM at a fixed polymer concentration (30 mg/mL) were loaded with increasing amounts of the drugs listed in Table I. Loading content was determined (figure 4). When the loading content reached a plateau, this value was considered as the loading capacity (LC). LC is plotted as a function of log P, number of aromatic rings, molecular weight and number of rotatable bounds of the drugs (figure 5).

Log P is the parameter commonly used to define the hydrophobicity of a drug. However, figure 5 A shows that there is no correlation between the loading capacity and log P

of the tested drugs. However, it is logical to assume that drugs with low log P do not partition efficiently in the core of PM. This was indeed the case for bortezomib, with a log P of 1.5 showed loading efficiency below 20 wt % for all feeds tested (figure 4 B). Even though bortezomib has two aromatic rings, the molecule obviously lacks hydrophobicity to be solubilized in PMs' hydrophobic core. Luxenhofer and colleagues found similar results with dexamethasone (log P 1.68) loaded in triblock copolymers-based micelles containing benzyl or phenyl moieties in the core (loading efficiency < 30% and loading content < 5.6%) [21]. Carfilzomib and ABT-737 with log P > 3.5 have loading capacity below 10% (figure 4 C and E), which suggests that high log P value does not necessarily result in high payloads in PM. Yet, at feed drug concentrations up to 2 mg/mL these drugs showed high loading efficiency (>90%), which indicates a good solubilization in PM. Thus, high log P of drugs is an important factor for good accommodation in PM that might not translate in high loading capacity.

The number of aromatic rings is also considered as an important factor for loading of drugs in mPEG-b-p(HPMA-Bz)-based PM because these moieties could be involved in π - π stacking [20, 29, 30]. This was previously hypothesized as the driving force for drug loading in mPEG-b-p(HMPA-Bz)-based PM [23]. The correlation between the number of aromatic rings and loading capacity is depicted in figure 5 B. Although ABT-737 has five aromatic rings, this did not translate in high loading capacity (figure 5 B). Bortezomib and carfilzomib, both drugs with two aromatic moieties, showed low loading capacity. BCI, PTX [23] and ibrutinib with 2, 3, and 4 aromatic moieties, respectively, showed the highest and increasing loading capacity (21, 23 and 34 % in weight, respectively). BCI, bortezomib and carfilzomib, all with two aromatic rings, display different loading capacities. Thus, the aromatic content of drugs is probably not the major property of drugs to influence the loading in PM. Analysis of the physicochemical properties of BCI, bortezomib and carfilzomib (table I), we can hypothesize that low loading capacity of bortezomib is likely due to low log P value, as discussed above. BCI and carfilzomib largely differ in molecular weight and number of rotatable bounds (molecular weight of 317 and 720 g/mol and number of rotatable bounds 3 and 20, respectively). Consequently, these properties can also have an effect in the loading of these drugs in PM.

Linear regression analysis showed a modest negative correlation between loading capacity and molecular weight ($r^2=0.47$, figure 5 C). Drugs with large molecular weights (ABT-737 and carfilzomib) showed lowest loading capacity, whereas BCI and ibrutinib, that present roughly half of that molecular weight, showed highest loading capacity. Plotting loading capacity expressed in moles of drug per gram of polymer,

the correlation between loading capacity and molecular weight of drugs becomes more evident ($r^2=0.60$, figure S 1) Thus, PM can solubilize more drug molecules of low molecular weight drugs per gram of polymer as compared to high molecular weight drugs.

The number of rotatable bonds is a measure of molecular flexibility of hydrophobic drugs [31]. This is defined as any single bond, that is not present in a ring structure, bound to a nonterminal non-hydrogen atom [32]. Linear regression analysis showed a modest negative correlation between loading capacity and number rotatable bonds ($r^2=0.49$, figure 5 D). Drugs with a relatively high number of rotatable bounds, i.e., ABT-737 and carfilzomib, presented the lowest loading capacity. With an increase in number of rotatable bonds, the molecular flexibility also increases and consequently the association strength between molecules is reduced [33]. Since high molecular weight drugs are more likely to have higher numbers of rotatable bonds (figure S 2) these parameters are interrelated and should be cautiously interpreted as separate parameters.

Figure 5. Loading capacity of drug-loaded polymeric micelles as a function of (A) log P, (B) number of aromatic rings, (C) molecular weight and (D) number of rotatable bounds of the drugs – BCI, ibrutinib, bortezomib, carfilzomib, ABT-737 and paclitaxel.

3.3 Drug retention in polymeric micelles

Prolonged circulation times in blood and targeted tissue distribution are two features that characterize PM. To benefit from these favorable characteristics and display improved therapeutic effects, not only good accommodation of hydrophobic drugs in their core is essential, but also drug retention is crucial [13]. Here we investigated the release of drugs from PM in buffer containing Triton X-100 (2%, v/v) [25]. Triton X-100 is a surfactant able to form small (± 10 nm) micelles in water (figure S 3 A) which can solubilize released drugs in the aqueous acceptor phase acting as a sink. Importantly, Triton X-100 micelles did not mix/fuse with drug-loaded PM (figure S 3 B and C) in agreement with previous findings [25]. Dialysis cassettes of 100 kDa MWCO were used to allow Triton X-100 micelles to freely flow between inside and outside of the dialysis cassette. We showed that when the used dialysis membrane is impermeable for Triton X-100 micelles, the retention appears substantially higher (figure S 4 A) [34].

Figure 6 shows the retention of drugs in PM over 168 h. BCI, carfilzomib, ibrutinib, ABT-737 and PTX were loaded in PM at two concentrations below the maximum loading content, which were 6, 1, 5, 1 and 6 mg/mL, respectively. Bortezomib was excluded from these experiments as it showed poor drug loading in PM and thus does not represent a promising candidate to formulate in these PM. When drug-loaded PM were incubated in buffer containing Triton X-100 micelles at 4°C they showed best retention of the payload, with more than 60% of the initially added drug retained in PM 24 h after incubation (figure 6 A). At body temperature - 37°C - and in buffer containing Triton X-100, drug release was considerably faster (figure 6 B). This figure shows that under these conditions and after 24 h of incubation, more than 50% of drug was released for the majority of the drugs tested. The lower retention observed at 37°C than at 4°C can be attributed to the higher solubility of drug molecules at elevated temperatures. The increase in solubility aids the transfer of drug molecules from the hydrophobic core of PM to the aqueous environment [35]. Faster release of drugs from PM at higher temperatures was also previously reported [36]. Interestingly, no striking differences in the release profile of the different drugs tested were observed (figure 6 B).

In addition, drug retention was tested in the presence of albumin, at concentration present in human plasma (40 g/L in PBS pH 7.4), since this is the most abundant protein in plasma and containing hydrophobic pockets to accommodate hydrophobic drugs [12, 37]. For BCI and carfilzomib, rapid drug release/extraction (> 60%) from PM was observed after 1 h of incubation in the presence of albumin (figure 6C). After 24 hours incubation of PM in albumin, 10-20% of the loaded drug was retained in the core of the micelles loaded with BCI and carfilzomib, respectively. Figure 6 C shows that for PTX,

50% was released at the time the first sample was taken, while thereafter no further release occurred. Shi *et al.* showed high stability of PTX-PM in the circulation [23]. In the study of Shi *et al.*, PTX-PM were prepared at 3.2 mg/mL of PTX (27 mg/mL of polymer) while in the present study the release of the same micellar formulation in albumin was evaluated at 6 mg/mL of PTX (30 mg/mL of polymer). Thus, the initial fast extraction of 50% of the loaded PTX is likely due to overloading of PM. ABT-737-PM displayed a more sustained retention of the loaded drug as no burst release was observed and after 24 h incubation with albumin 55% of the drug was still retained in PM.

Figure 6. Retention of hydrophobic drugs in polymeric micelles over 168 hours. Drug loaded polymeric micelles were prepared at 6, 1, 5, 1 and 6 mg/mL of BCI, carfilzomib, ibrutinib, ABT-737 and PTX, respectively – that rendered loading efficiencies close to 100%. Drug-loaded PM (1 mL PM for Triton and 0.5 mL PM plus 0.5 mL albumin for the latter) were transferred into a dialysis cassette and incubated either with Triton X-100 (2% v/v in PBS; 30 mL) at 4°C (A) and at 37°C (B) or with bovine serum albumin (40 g/L in PBS; 30 mL) at 37°C (C). At several timepoints ranging from 1 to 168 h, samples of 10 μ L of the dispersion inside the dialysis tube were collected and analyzed by HPLC or UPLC to determine the percentage of drug retained in the micelles compared to the amount added at the start of the experiment. Data are shown as mean $\pm$ SD, n=3 for the assay using Triton X-100 and n=2 for the assay using albumin as solubilizer.

3.4 Effect of log P, number of aromatic groups, numbers of rotatable bonds and molecular weight on drug retention in PM upon incubation in buffer with albumin

The percentage of drug retained in PM after 8 h incubation with albumin was plotted as a function of physicochemical properties of the drugs tested (figure 7). This time point

was chosen as it showed the highest discriminatory value. Log P value was positively correlated to the drug retention in PM (figure 7 A). This indicates that the (lack of) water solubility is important for drug retention in PM. Strikingly, the number of aromatic moieties almost perfectly correlates ($r^2 = 0.98$) with the percentage of drug retained in the core of PM (figure 7 B), suggesting that π - π interactions between polymer and drug molecules play a key role in this process. The molecular weight is also positively correlated to drug retention in PM, although to a lesser extent ($r^2 = 0.43$, figure 7 C). Again, aromatic content, molecular weight and log P could be considered as confounding factors, but the near perfect correlation with the number of aromatic moieties indicates this is the dominant parameter. No correlation observed between drug retention and number of rotatable bonds (figure 7 D).

Figure 7. Drug retention after 8 h incubation of drug loaded polymeric micelles (1-6 mg/mL of drugs and 30 mg/mL polymer; 0.5 mL PM with 0.5 mL albumin) with bovine serum albumin (40 g/L albumin in PBS; 30 mL) at 37°C as a function of (A) log P, (B) number of aromatic rings, (C) molecular weight and (D) number of rotatable bounds of the drugs – BCI, carfilizomib, ABT-737 and paclitaxel. Molar ratio between albumin and drugs was between 95-1500 excess of albumin, compared to the number of moles of the drugs here tested.

3.5 Circulation time of drug-loaded polymeric micelles

Often *in vitro* retention assays fail to predict and recapitulate the performance of drug-loaded PM *in vivo*. We measured circulation time of Cy7-labeled PM loaded with either ABT-737 or BCI (at 1 and 6 mg/mL, respectively, that rendered loading efficiencies above 97% for both drugs, 30 mg/mL polymer – figure 4 A and E) in healthy immunocompetent mice and compared it to the release found in *in vitro* tests (ABT-737 or BCI showed 55 and 10 % retention in albumin after 24 h incubation, respectively). Figure 8 shows that Cy7-labeled PM showed prolonged blood circulation time with 30% of ID still in the circulation 24 h after i.v. injection, which was similar to what was previously reported for the same micellar system [23]. When drugs were loaded in PM, ABT-737 was better retained than BCI after i.v. injection. ABT-737 displayed prolonged circulation time in plasma with 62 and 19% of ID in circulation at 1 and 24 h post-injection, respectively (figure 8). BCI-PM showed faster drug loss/extraction *in vivo*, as at 1 and 24 h after PM injection only 32 and 6% of ID, respectively, was detected in the circulation. Both drugs when i.v. injected as free drugs were rapidly extracted and 1 h after injection no drug was detected in the analyzed plasmas. When compared to the albumin release assay *in vitro*, we found that drug retention was slightly higher compared to the *in vivo* experiment. One of the differences between both experimental set-ups is that plasma constitution in terms of total protein content and composition is different than albumin dissolved in buffer [36]. Also, part of PM leave systemic circulation and accumulate in tissues contributing to an apparent loss of retention. Taking this into account, we recalculated drug retention *in vivo* considering the % ID of Cy7-labeled PM at 24 h set as 100% of micelles in circulation (figure 8). Thus, from those PM that remained in circulation, 68% of the initial loading of ABT-737 was still retained in the PM. This is considerably closer to the percentage of ABT-737 retained *in vitro* in the presence of albumin, i.e., 55% (figure S 5). The same calculation was done for PTX-PM, given the Cy7-PM and PTX circulation in blood 24 h after i.v. administration, as reported in [23], which amounted to 20 and 10% of ID, respectively. Thus, from the micelles in circulation at 24 h, 50% of the loaded PTX was retained in the PM. Remarkably, this was the same figure we found for *in vitro* retention with albumin (figure 6 C). This indicates that with *in vitro* release assays based on albumin as an acceptor molecule for hydrophobic drugs, as described in the present work, a good estimation of the behavior of formulations upon i.v. administration can be achieved.

Figure 8. Circulation times in blood of ABT-737, BCI and Cy7-labelled PM. Immunocompetent mice were i.v. injected with 100-200 μ L of free ABT-737 and ABT-737-PM (1 mg/mL of ABT-737, 30 mg/mL polymer) and free BCI and BCI-PM (6 mg/mL of BCI, and 30 mg/mL polymer) via the tail vein. At 1 min, 1 and 24 h blood was withdrawn to determine the percentage of Injected dose (%ID) present in the circulation. Blood collected shortly after injection (1 min) was considered as 100% ID. Fluorescence intensity of Cy7-PM in plasma was measured in Odyssey[®] imager. Drug concentrations in plasma were determined by HPLC. Data are presented as mean $\pm$ SD, n=3 for Cy7-PM and BCI, n=2 for ABT-737.

4. CONCLUSIONS

In this study, we evaluated the *in vitro* loading and retention as well as the *in vivo* pharmacokinetics of drugs containing aromatic moieties loaded in mPEG-b-p(HPMA-Bz)-based micelles. The data were correlated to physicochemical properties of the drugs to determine which molecular properties influence their loading and retention in PM. Interestingly, we found that a number of parameters influence loading and retention of hydrophobic drugs in PM.

High log P is an important parameter to attain good loading efficiency, but the same is not true to obtain high loading capacity of drugs in PM. As expected, high log P is also important to retain the drug in the core of PM. Strikingly, *in vitro* drug retention in PM shows high correlation with the aromatic content of the tested drugs. It appears that the retention of the drugs in the core of PM positively correlates with the number aromatic groups in the drug molecules investigated. This indicates that the aromatic content of drugs is key to preserve drug retention when PM are in physiologic environments.

Finally, pharmacokinetic data of two selected drugs exhibit the same drug retention as found *in vitro*, which supports the relevance of our findings. Overall, we demonstrate that parameters that play a role in drug loading are not necessarily the same that determine drug retention. Moreover, we show that our *in vitro* findings are comparable with the circulation times of the tested drugs after intravenous administration. Thus, *in vitro* release assays particularly the one based on albumin as acceptor of hydrophobic drugs appear to properly translate the circulation times of drugs *in vivo*. The approach proposed here can serve as a first screening *in vitro* to test potential drugs to be loaded in PM. Consequently, this can help to reduce the number of animal experiments and the costs involved.

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SUPPLEMENTARY INFORMATION

BCI HPLC method

The mobile phase was a gradient of eluent A (Milli-Q water with 0.1% perchloric acid) and eluent B (acetonitrile (ACN) with 0.1% perchloric acid) with a volume fraction of eluent B increasing from 50% to 100% from 0 to 5 min and subsequently decreasing to 50% from 5 to 6 min. Total run time was 7 min with a flow rate of 1 mL/min. The injection volume was 20 μ L, column temperature 40°C and the detection wavelength was 320 nm. Micelles were destabilized with a 10-fold volume of ACN. The samples were injected at a final concentration of 50% acetonitrile (ACN) in water. Calibration was done using samples of BCI in 50% ACN with concentrations ranging from 0.1 to 100 μ g/mL.

ABT-737 HPLC method

A gradient was run with a volume fraction of eluent B (100% acetonitrile with 0.1% perchloric acid) increasing from 0% to 95% in 3.5 minutes. This ratio was kept until 5 minutes and then the volume fraction of eluent A (10% acetonitrile with 0.1% perchloric acid) increased from 5% to 100% at 6 minutes. Running time was 7 minutes at a flow rate of 1 mL/min. The injection volume was of 50 μ L and ABT-737 was detected at 300 nm. Samples were prepared by destabilizing the micelles in at least a 10-fold volume ACN/MeOH (1:1). Calibration was done using samples of ABT-737 mL in ACN/MeOH (1:1) with concentrations ranging from 0.1 to 100 μ g/mL.

Bortezomib UPLC method

Free bortezomib was removed from the dispersion of bortezomib-loaded micelles (bortezomib-PM) by gravity flow filtration. Disposable PD-10 desalting columns with Sephadex G-25 resin (GE Healthcare Life Sciences) were used and the gravity protocol performed as described in the manufacturer's manual. Briefly, 1 mL of bortezomib-PM was added, and 3.5 mL eluate was collected. Then 10 μ L of bortezomib-PM was disrupted with a 10-fold excess of ACN and subsequently diluted with Milli-Q water to get a final concentration of approximately 33% ACN. The samples were run using UPLC by isocratic elution with the mobile phase ACN/Milli-Q water = 35/65 (% v/v) containing 0.1% formic acid. Total run time was 1 minute with a flow rate of 0.6 mL/min. The injection volume was 7.5 μ L, column temperature 50°C and the detection wavelength was 270 nm. Calibration was done using samples of bortezomib in the mobile phase at concentrations ranging from 1-100 μ g/mL.

Carfilzomib UPLC method

The elution was isocratic with a mobile phase of ACN/Milli-Q water = 50/50 (v/v) with 0.1% trifluoroacetic acid. Total run time was 1 minute with a flow rate of 0.6 mL/min. The injection volume was 7.5 μ L, column temperature 50 °C and the detection wavelength was 210 nm. Samples were prepared by destabilizing the micelles in at least a 10-fold volume ACN and further diluted with Milli-Q to get a final concentration of 50% ACN. Calibration was done using samples of carfilzomib in 50% ACN at concentrations between 1-100 μ g/mL.

Ibrutinib UPLC method

The mobile phase was a gradient of eluent A (10% ACN with 0.1% phosphoric acid) and eluent B (acetonitrile with 0.1% phosphoric acid) with the volume fraction of eluent B increasing from 0% to 95% from 0 to 2 min, then kept stable from 2 to 4 min and subsequently decreased back to 0% B from 4 to 4.5 min. Total run time was 5 minutes with a flow rate of 0.5 mL/min. The injection volume was 5 μ L, column temperature 50°C and the detection wavelength was 254 nm. The samples and calibration curve were injected in 100% ACN. Calibration was done using samples of ibrutinib at concentrations between 1-100 μ g/mL.

Paclitaxel UPLC method

The elution was isocratic with ACN/Milli-Q water = 45/55 (v/v) with 0.1% formic acid. The total run time was 1.5 minute with a flow rate of 1 mL/min. The injection volume was 2 μ L, column temperature 50 °C and the detection wavelength was 227 nm. The samples were in 100% ACN. Calibration was done using samples of paclitaxel at concentrations between 5-100 μ g/mL.

Figure S1. Correlation between loading capacity (expressed in moles of drug per gram of polymer) and molecular weight of drugs loaded in PM.

Figure S2. Correlation between molecular weight and number of rotatable bonds of drugs loaded in PM.

Figure S3. Size of Triton X-100 micelles (A) and PM micelles (B) separately and its mixture of both (C). A – Triton X-100 micelles are spontaneously formed in a dispersion of 2% (v/v) of Triton X-100 in PBS; B - Polymeric micelles (10-fold dilution in water; 3mg/mL); C - Mixture of 2% (v/v) of Triton X-100 in PBS and polymeric micelles (100-fold dilution in PBS with TritonX-100; 0.3mg/mL) .

Figure S4. Retention drug in loaded polymeric micelles using dialysis cassettes of different MWCO: (A) 10 kDa (B) 50 kDa and (C) 100 kDa. Data expressed as mean $\pm$ SD

Figure S5. Comparison of circulation time in plasma with drug retention found *in vitro* with bovine serum albumin for (A) ABT-737 polymeric micelles and (B) BCI polymeric micelles. Mice were i.v. injected with 100-200 μ L of ABT-737-PM (1 mg/mL of ABT-737, 30 mg/mL polymer) and BCI-PM (6 mg/mL of BCI, and 30 mg/mL polymer). Based on the %ID of Cy7-PM in circulation at each time point (1 and 24 h), the expected retention (%) *in vitro* was calculated.

8

Summary and Discussion

In the past decades, polymeric micelles (PM) have been extensively investigated as drug carriers, primarily for cancer treatment [1, 2]. PM are nano-sized drug carriers (with a diameter of 10-100 nm) with a core-shell structure, that can accommodate hydrophobic compounds in their hydrophobic core. These colloidal particles provide opportunities for the pharmaceutical development of formulations for drugs that are poorly water-soluble [3].

Abundant pre-clinical evaluation of PM loaded with anticancer drugs led to the clinical approval of Genexol™-PM [4, 5]. Genexol™-PM functions as paclitaxel (PTX) solubilizer and not as a true drug carrier. This is due to the poor stability of the formulation after systemic injection that leads to disintegration of the micelles and subsequent rapid clearance of the drug from the circulation. After successful development of Genexol™-PM, several PM-based formulations have entered clinical trials [6]. In **Chapter 2** we summarize PM-based formulations in clinical trials for cancer treatment. Different strategies were employed to improve stability of PM and drug retention upon intravenous (i.v.) injection. These strategies include chemical crosslinking of the PM core, covalent coupling of the drug to the polymer chains, and hydrophobic interactions between the drug and the hydrophobic block of the copolymer, such as π - π interactions [7-10]. Encouraging preclinical studies performed with these PM have resulted into numerous formulations under clinical evaluation.

Still, regardless of the number of PM in (pre-)clinical studies, the translation into the clinic remains difficult. Outcomes of clinical tests of these formulations were frequently disappointing showing a lack of therapeutic superiority of the PM-based formulations compared to the standard treatment. This can, probably, partially be attributed to the low translational potential of many pre-clinical animal (mouse) models used. Frequently, promising therapeutic effects are observed in pre-clinical animal models, which however do not represent the pathophysiological status of the disease found in humans. A large number of tumor models lack the supportive stromal cells that are important components of the tumor microenvironment and dictate the sensitivity of tumor cells to therapy [11]. Many pre-clinical studies also make use of immunodeficient animals (in particular, engineered mice) to allow the inoculation/transplantation of human-derived tumor cells to evaluate therapeutic effects. In these mice, the immune system is altered in a way that whole lineage of immune cells is depleted, which is not observed in patients. In patients, changes in the immune system can be expected due to administered drugs or the disease itself [12].

The fact that the pathophysiology of the disease in humans is often not fully recapitulated in pre-clinical animal models, is also in line with the current debate regarding the heterogeneity seen for the EPR effect [13, 14]. Since Maeda's description of the EPR effect, the drug delivery field attributes the accumulation and retention of nanocarriers in target tissues to this phenomenon [15-17]. Nevertheless, recently more and more emphasis is put on the fact that the EPR effect is highly variable between tumor types and between patients and even within different regions of the same tumor [18]. Taken together, these aspects make development of PM as carriers of anticancer therapeutics challenging.

Up to now, PM and other nanocarriers received considerable attention for the treatment of various types of solid tumors [2,4]. However, less work has been done to develop nanomedicines for the treatment of hematological malignancies (HM) [19].

HM are a collection of malignant transformations originating from cells in the primary or secondary lymphoid organs. Leukemia, lymphoma, and multiple myeloma comprise the three major types of HM. Current treatment consists of hematopoietic stem cell transplantation [20], radiotherapy [21], immunotherapy [22] and chemotherapy [23]. Although many chemotherapeutic drugs are clinically available for the treatment of HM, the use of these agents is limited due to dose-related toxicity and lack of specificity to tumor tissue. Moreover, the poor pharmacokinetic profile of most of the chemotherapeutics requires high dosage and frequent administration to maintain therapeutic levels at the target site. Both aspects are responsible for increasing adverse effects and underlines the need for suitable drug delivery systems to improve efficacy, safety, and pharmacokinetic properties of conventional therapeutics. Nanomedicines have proven to enhance these properties for anticancer therapeutics [24]. The most extensively studied nanomedicine systems are lipid-based and polymeric nanoparticles. **Chapter 3** describes currently available therapies as well as limitations and potential advantages of nanomedicine formulations for treatment of various types of HM. Additionally, recent investigational and approved nanomedicine formulations and their limited applications in HM are discussed.

Multiple myeloma (MM) is a type of hematological cancer characterized by the clonal proliferation of plasma cells [25]. A remarkable feature of MM is the infiltration of the bone marrow with malignant plasma cells that replace the hematopoietic tissue [26]. Previous reports showed an increase in angiogenesis in the bone marrow (BM) of MM patients, which represents an opportunity to explore the EPR effect in HM [27, 28]. Additionally, nano-sized drug carriers with diameter below 100 nm can extravasate

bone interstitium and accumulate in the bone marrow [29]. Therefore, PM with a long circulation time profile in blood can potentially accumulate in the bone marrow of MM. To study this, a mouse MM model was used that despite its immunocompromised status has shown predictive value for patient responses [30]. In **Chapter 4**, carfilzomib-loaded polymeric micelles (CFZ-PM) based on mPEG-b-p(HPMA-Bz) copolymer [31] were prepared with the aim to improve the maximum tolerated dose in an humanized BM-like scaffold (huBMsc) xenograft model of multiple myeloma. CFZ was efficiently loaded in the core of polymeric micelles (90% loading efficiency). The micelles had a favorable size (55 nm) for long circulation upon systemic injection and to target tissues with increased capillary permeability [17, 32]. This was confirmed by evaluation of the circulation times of labeled micelles, with more than 80% of injected dose (ID) present in the circulation after 24 h, which might partly be explained by the immune status of the RAG2^{-/-}γc^{-/-} mouse strain used in this study. PM accumulation was observed in tumor-bearing scaffolds (1% of ID after 24 h of i.v. injection) as well as in macrophage-rich organs such as liver and spleen. However, both CFZ-PM and the clinical approved formulation Kyprolis[®] dosed at the maximum tolerated dose (for Kyprolis[®]) did not show therapeutic efficacy. Importantly, CFZ-PM were well tolerated whereas Kyprolis[®] showed severe adverse effects in 2 out of 4 mice tested. In Kyprolis[®], the half-life of CFZ is about 30 minutes. Thus, the adverse effects were, probably, a result of the increased CFZ concentration in the circulation due to fast release of CFZ from the Kyprolis[®] formulation. We hypothesized that the lack of therapeutic efficacy was either due to the premature extraction of CFZ from PM to (hydrophobic pockets of) blood components as albumin, or due to the low CFZ dose. The former may be overcome by decreasing feed drug:polymer ratio used for *in vivo* therapeutic tests to minimize drug extraction upon i.v. administration, or by conjugation of the drug to the polymer backbone by covalent bonds. The latter indicates that in future experiments higher and/or more frequent dosing of CFZ loaded in PM should be pursued.

The MM mouse model used here is similar to the human BM niche. Also, it was shown that this model has clinical predictive value for therapy [30]. Still, one drawback of this model is the immune status of the mice. They are severely immunocompromised as they lack functional B- and T-lymphocytes and NK-cells, which can lead to biased results particularly when studying plasma residence of nanocarriers. For instance, blood circulation of Cy7-labeled PM (Cy7-PM) was considerably prolonged in this mouse strain when compared to what is usually observed for PM with excellent stability profiles. This phenomenon is, most likely, an overestimation of the circulatory half-life due to the lack of a functional immune system. In figure 1 we show the circulation of Cy7-PM in the different mouse models tested in this thesis. It appears that the circulation times

of PM are affected by the mouse strain selected which may impact interpretation of therapeutic data.

Figure 1. Circulation times in blood of Cy7-PM in three mouse strains tested in this thesis. Cy7-PM (30 mg/mL of polymer in which 1.5% (w/w) was Cy7-labelled polymer) were i.v. injected (100 - 200 μ L) in mice; Cy7-PM diameter was 50-60 nm. RAG2^{-/-}γc^{-/-} mice were used to inject bone-like scaffolds to mimic multiple myeloma microenvironment, as explored in **Chapter 4**; A/J mice were xenografted with neuroblastoma cell line (Neuro2A) explored in **Chapter 5**; C57BL6 were recipient mice of CLL splenocytes transplanted from diseased TCL1tg mice and used in **Chapters 5 and 6**, and C57BL6 without tumors were employed in **Chapter 7**.

Chronic lymphocytic leukemia (CLL) is also an HM derived from the lymphoid lineage and is characterized by the monoclonal proliferation and accumulation of B-lymphocytes in the peripheral blood (PB) and lymphoid organs, such as the spleen [33]. In CLL, the tumor microenvironment plays an important role in malignant transformation and survival [34, 35]. To examine whether the PM *in vivo* fate is impacted by the microenvironment inflammatory signaling induced by CLL, we compared circulation and tissue distribution profiles of Cy7-PM in wild-type (wt) and Protein kinase C – β II knockout (*Prkcb*^{-/-}) mice in **Chapter 5**. *Prkcb*^{-/-} mice were used as they previously showed to be insusceptible to CLL cell transplantation [36]. In control mice (either wt or *Prkcb*^{-/-}) or low CLL, we found around 30-40% of ID of Cy7-PM in circulation 24 h after micelles injection. Whereas in mice bearing high percentage of CLL cells, less Cy7-PM were found in circulation 24 h after administration (20%). Tissue distribution of Cy7-PM was mainly towards spleen and liver. Infiltrated CLL cells were found predominantly in the spleen and liver, leading to augmented accumulation of PM in these organs. Increased circulation and target site accumulation both are favorable to target and treat CLL in a superior and specific manner. Interestingly, in a mouse model where CLL engraftment is impaired (*Prkcb*^{-/-}), we observed low Cy7-PM accumulation in the spleen, indicating that the level of CLL

engraftment can likely affect the level of micellar accumulation. In this study we clearly demonstrate that PM hold promise in targeting the inflammatory microenvironment induced by infiltrated CLL cells. Finally, therapeutic studies with ibrutinib-loaded micelles revealed a significant decrease in the percentage of CLL cells in mouse spleens.

In this chapter we tested circulation and biodistribution of labeled PM and therapeutic efficacy of ibrutinib-PM in a CLL mouse model. The utility of using such model is dependent on the degree of relationship of CLL characteristics between the model and patients. The model should resemble several features related to the genetic, histological, phenotypic and clinical status as observed in human CLL. This is the case for the TCL1tg mouse model of CLL used in **Chapter 5** and **6**. This model was reported as a tool to investigate pathological mechanisms as well as for drug screening tests [37-40]. Also, this model was shown to be a suitable pre-clinical model by revealing that the response of mice to treatment with fludarabine (drug used as first-line treatment of CLL) was similar as observed in humans [40]. In **Chapter 6**, mPEG-b-p(HPMAm-Bz)-based polymeric micelles were loaded with the DUSP6 inhibitor – BCI. To our knowledge, this was the first time that the investigational small molecule inhibitor of DUSP6, BCI, was formulated in polymeric micelles. *In vitro*, BCI loaded in the PM displayed comparable therapeutic effects as free BCI. Strikingly, primary patient CLL cells when cultured with stromal cells exhibited markedly lower cytotoxicity, for the same BCI concentration, compared to monoculture of CLL cells highlighting the protective role of stromal cells on the survival of CLL cells [34, 41]. Cy7-PM displayed prolonged blood circulation in neuroblastoma- and in CLL-bearing mice (46 and 24% of ID, respectively). However, circulation kinetics of BCI initially loaded in PM were significantly shorter, indicating high extraction of BCI in plasma. BCI administered as free drug showed fast clearance and was not detected at the first time point analyzed (1 h post injection). Despite the poor pharmacokinetic profile of the loaded drug, we could still observe a persistent decrease in circulating CLL cells which was not seen for free BCI treated mice. These data suggest that adjustments are required to further improve therapeutic outcome. For instance, the extraction of BCI from PM was fast in plasma, indicating that here PM functioned as solubilizer of BCI. Better micellar retention by lowering the feed ratio of drug to polymer, may improve this. Additionally, the composition of the PM may be changed to improve the BCI retention for example by chemical conjugation of the drug to the polymer backbone and/or crosslinking the core of the micelles. Also, the dose and dosing frequency we studied here might not have been sufficient to induce downregulation of DUSP6 phosphatase activity and subsequent hyperactivation of ERK [42].

PM prepared from mPEG-b-p(HPMA-Bz) copolymers have previously shown good colloidal stability and high retention of PTX *in vivo*, which translated in strong therapeutic effects [31]. The excellent stability and drug retention noted was attributed to the fact that both copolymer and drug have aromatic rings in their structure and thus PM were stabilized by π - π interactions. Following these promising data with PTX, in **Chapters 4, 5 and 6** we aimed at loading drugs that also contained aromatic moieties in their structures and that were expected to also show good drug retention *in vivo*. As shown in previous chapters, this was not the case and fast drug extraction from PM was observed *in vivo* (**Chapter 6**) which, less surprisingly, led to negligible therapeutic outcome. This result made us get back to the formulation phase. In **Chapter 7** we explored which physicochemical properties of drugs influence their loading and retention in polymeric micelles (PM) based on poly(ethylene glycol)-b-poly(N-2-benzoyloxypropyl methacrylamide) (mPEG-b-p(HPMA-Bz)) block copolymer. PM were loaded with hydrophobic drugs containing different number of aromatic rings (between two and five). Besides the drugs explored in **Chapters 4, 5 and 6** we included bortezomib, ABT-737 and PTX. We found that high log P is an important parameter to attain good loading efficiency, but the same is not true for obtaining a high drug loading capacity in PM. As expected, high log P is also important to retain the drug in the core of PM. Strikingly, *in vitro* drug retention in PM showed high correlation with the aromatic content of the tested drugs. It appears that the retention of the drugs in the core of PM correlates with the number aromatic groups in the drug molecules investigated, indicating that the aromaticity of drugs is key to preserve drug retention when PM are in physiologic environments. Finally, pharmacokinetic data of two selected drugs, BCI and ABT-737, exhibit comparable drug retention as found *in vitro*, which supports the relevance of our findings. Similarly, we found the same retention *in vitro* for PTX compared to the *in vivo* behaviour previously reported [31]. Interestingly, we demonstrate that parameters that play a role in drug loading are not necessarily the same that determine drug retention. The results presented here can be used to predict the suitability of a drug for loading in this micellar system. *In vitro* release assays based on albumin as acceptor of hydrophobic drugs appear to properly translate the circulation times of drugs *in vivo*. In future experiments albumin substitution by plasma is likely to better resemble the systemic circulation environment. The approach proposed here can serve as a first screening *in vitro* to test potential drugs to be loaded in PM and, at the same time, significantly reduce the number of experimentation animals.

CONCLUSIONS

The research presented in this thesis explores mPEG-b-p(HPMA-Bz)-based micelles as nanocarriers for cancer treatment, with focus on hematological cancers. PM showed good circulation and attractive tissue distribution in clinically relevant models of multiple myeloma and chronic lymphocytic leukemia. However, therapeutic outcome of the developed formulations was minimally improved. This is most likely due to the poor pharmacokinetic profile of drug-loaded PM in blood and consequently advantages attributed to nanocarriers, such as target tissue accumulation, could not be fully explored. Finally, physicochemical parameters of drug molecules that influence drug loading and retention *in vivo* were examined. Further *in vivo* studies to evaluate therapeutic effect of drugs with favorable drug retention can elucidate the potential of this micellar system.

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Appendices

NEDERLANDSE SAMENVATTING EN DISCUSSIE

In de laatste decennia zijn polymere micellen (PM) nadrukkelijk onderzocht als dragers voor geneesmiddelen, voornamelijk gericht op de behandeling van kanker [1, 2].

PM zijn zogeheten nanodeeltjes (met een diameter tussen de 10-100nm) met een kern-schil structuur, die hydrofobe moleculen kunnen bergen in hun hydrofobe kern. Deze colloïdale deeltjes maken de farmaceutische ontwikkeling van formuleringen mogelijk van geneesmiddelen die slecht in water oplosbaar zijn [3].

Verscheidene preklinische studies met PM die zijn geladen met medicijnen tegen kanker hebben ertoe geleid dat Genexol™-PM zijn goedgekeurd voor klinische toepassing [4, 5].

Genexol™-PM fungeert echter meer als een oplosmiddel voor Paclitaxel (PTX) dan dat het een daadwerkelijke drager is. De Genexol-PTX formulering is niet stabiel waardoor de micellen na injectie vrij snel uit elkaar vallen en het vrije geneesmiddel wordt geklaard. Na de succesvolle ontwikkeling van Genexol™-PM zijn er verschillende PM gebaseerde formuleringen klinisch getest [6].

In **Hoofdstuk 2** bespreken we de PM gebaseerde formuleringen die op dit moment klinisch worden getest voor de behandeling van kanker. Er worden verschillende strategieën toegepast om de stabiliteit van micellen, en daarmee de retentie van het geneesmiddel, te verbeteren na intraveneuze (i.v.) injectie. Deze strategieën omvatten onder andere het chemisch versterken van de micel kern, het covalent binden van het geneesmiddel aan de polymere ketens en het introduceren van hydrofobe interacties tussen het geneesmiddel en het hydrofobe deel van de copolymeer, zoals π - π interacties. [7-10]. Bemoedigende resultaten in preklinisch onderzoek hebben geleid tot klinisch onderzoek met verscheidene van dit soort PM.

Toch blijkt de vertaling naar een klinisch toepasbaar product ingewikkeld, ondanks de positieve resultaten in (pre-)klinisch onderzoek. Uitkomsten van klinische testen laten vaak zien dat de PM formulering niet beter werkt dan de huidige, standaard toegepaste behandeling. Dit kan waarschijnlijk worden verklaard doordat de resultaten in muismodellen vaak lastig te vertalen zijn naar de mens. Vaak worden de veelbelovende resultaten behaald in een muismodel, dat de pathofysiologische situatie in de patiënt niet adequaat vertaalt. Veel van de tumormodellen missen bijvoorbeeld de ondersteunende stroma cellen, welke juist belangrijk zijn voor de tumor micro-omgeving en een cruciale rol spelen in de gevoeligheid van de tumor voor een geneesmiddel [11].

In veel preklinische onderzoeken wordt gebruikt gemaakt van immuun gecompromitteerde muizen (vaak genetisch gemodificeerd) zodat er humane tumorcellen kunnen worden getransplanteerd. In deze muizen is het immuunsysteem zodanig aangepast dat een complete lijn immuuncellen is verwijderd, een situatie die men niet ziet in patiënten. In patiënten kan men veranderingen verwachten in het immuunsysteem, enerzijds door het geneesmiddel, anderzijds door de ziekte [12].

Het feit dat de pathofysiologie van de ziekte in de mens niet volledig kan worden nagebootst in preklinische diermodellen komt ook terug in het debat omtrent de heterogeniteit wat betreft het “enhanced permeation and retention” ofwel het verhoogde permeabiliteit en retentie (EPR) effect [13, 14].

Sinds beschrijving van het EPR effect door Maeda wordt in het drug delivery veld de accumulatie en retentie van nanopartikels in tumoren door dit effect verklaard [15-17]. Tegenwoordig wordt echter steeds meer de focus gelegd op het feit dat dit EPR effect erg heterogeen is tussen verschillende tumoren, tussen verschillende patiënten en zelf binnen dezelfde tumor [18]. Deze aspecten maken het ontwikkelen van PM als dragers van kankermedicijnen uitdagend.

Tot nu toe zijn PM en andere nanodeeltjes voornamelijk onderzocht met de gedachte om solide tumoren te behandelen. [2,4]. Er is echter minder aandacht besteedt aan het ontwikkelen van nanodeeltjes voor hematologische aandoeningen (HA) [19].

HA is een verzameling van maligne transformaties die ontstaan in cellen van de primaire of secundaire lymforganen. Leukemie, lymfomen en multiple myeloom omvatten de drie voornaamste vormen van HA. De huidige behandelopties zijn hematopoëtische stamcel transplantatie [20], radiotherapie [21], immunotherapie [22] en chemotherapie [23].

Hoewel er verscheidene chemotherapieën beschikbaar zijn voor HA, is het gebruik van deze middelen beperkt vanwege de dosis-gelimiteerde toxiciteit en gebrek aan specificiteit voor tumorweefsel. Daar komt bij dat veel chemotherapieën een ongunstig farmacokinetisch profiel hebben en hoge en frequente doseringen vereisen om effectief te zijn. Beiden aspecten zorgen voor toenemende ongewenste bijeffecten en onderstrepen de behoefte aan betere manieren om geneesmiddelen toe te dienen en de effectiviteit, veiligheid en farmacokinetische eigenschappen van geneesmiddelen te verbeteren. Nanodeeltjes hebben laten zien deze eigenschappen te kunnen verbeteren voor kankermedicijnen [24].

De meest uitgebreid onderzochte deeltjes zijn gebaseerd op lipiden en/of polymeren. **Hoofdstuk 3** beschrijft zowel de op dit moment beschikbare therapieën als de beperkingen en potentiële voordelen van nanopartikel gebaseerde formuleringen voor de behandeling van verschillende HA. Daarnaast worden nanoformuleringen besproken die recent zijn onderzocht dan wel goedgekeurd voor de behandeling van HA.

Multiple myeloom (MM) is een vorm van hematologische kanker die bekend staat om de ongecontroleerde deling van plasmacellen. [25]. Een buitengewoon kenmerk van MM is dat maligne plasmacellen het beenmerg infiltreren en daar de hematopoëtische weefsels vervangen [26]. Eerdere studies beschrijven een toegenomen angiogenese in het beenmerg van MM patiënten wat de mogelijkheid biedt om van het EPR effect gebruik te maken in HM [27, 28]. Daarnaast is bekend dat nanodeeltjes kleiner dan 100nm uit het bot interstitium kunnen extravaseren en in het beenmerg kunnen accumuleren [29].

Het is daarom mogelijk dat lang circulerende PM kunnen accumuleren in het beenmerg van MM patiënten. Om dit te bestuderen is er een MM muismodel gebruikt dat, ondanks het aangetaste immuunsysteem, kon voorspellen hoe patiënten reageren op de behandeling [30]. In **Hoofdstuk 4** wordt de ontwikkeling van carfilzomib (CFZ) geladen PMs beschreven die zijn gebaseerd op het mPEG-b-p(HPMA-Bz) copolymeer [31].

Deze PM formulering is ontwikkeld met de gedachte de maximaal getolereerde dosis van CFZ te verbeteren. Dit is getest in een xenograft MM muismodel met behulp van een gehumaniseerd, beenmerg-modulerend implantaat (huBMsc). CFZ kon efficiënt worden geladen in de kern van de micellen (90% lading efficiëntie). De PM hadden een gunstige grootte van 55nm waardoor ze langer circuleren na systemische injectie en kunnen accumuleren in weefsels met permeabele bloedvaatjes [17, 32].

Met behulp van fluorescente micellen is een verlengde circulatietijd geconstateerd, waarbij 80% van de geïnjecteerde dosis nog kon worden gedetecteerd na 24h. Mogelijk kan dit deels worden verklaard door het aangetaste immuunsysteem van de RAG2-/- γ c-/- muizen die voor deze studie zijn gebruikt. De micellen accumuleerden in de tumor dragende implantaten (1% van de geïnjecteerde dosis na 24 uur) en in macrofaagrijke organen als de lever en milt. Zowel de CFZ geladen PM als de klinisch toegepaste CFZ formulering Kyprolis hadden geen therapeutisch effect met de maximaal getolereerde dosering van Kyprolis. Er zijn geen bijwerkingen geconstateerd in muizen die zijn behandeld met CFZ- PM. Dit in tegenstelling tot Kyprolis behandelde muizen, waarbij

twee van de vier muizen last hadden van bijwerkingen. De halfwaardetijd van Kyrpolis is ongeveer 30 minuten waardoor mogelijk de concentratie van vrij CFZ te snel stijgt in de circulatie, met bijwerkingen als gevolg. De hypothese is dat het uitblijven van therapeutische effectiviteit komt omdat CFZ vroegtijdig uit de micellen wordt vrijgelaten en aan hydrofobe delen van bloedcomponenten zoals albumine gaat zitten, of dat de dosis van CFZ te laag is geweest. Het eerste probleem kan mogelijk worden opgelost door de ratio tussen geneesmiddel en polymeer te verkleinen of door het geneesmiddel covalent aan het polymeer te conjugeren. De te lage dosering kan worden opgevangen door ofwel een hogere dosering, ofwel door vaker een dosis toe te dienen.

Het MM muismodel dat hier is gebruikt heeft een beenmerg niche dat vergelijkbaar is met die van de mens. Het model kon worden gebruikt om de respons van patiënten op behandeling te voorspellen [30]. De immuun status van deze muizen is echter een nadeel. Ze hebben een ernstig aangedaan immuunsysteem en missen functionele B- en T-lymfocyten en NK-cellen, wat de resultaten, zeker wat betreft de circulatietijd van nanodeeltjes, kan beïnvloeden. De circulatie van Cy7 gelabelde PM (Cy7-PM) was bijvoorbeeld langer in dit muismodel dan wat normaal wordt geobserveerd voor vergelijkbare stabiele PM. Deze overschatting van de halfwaardetijd kan dus waarschijnlijk worden verklaard door het ontbreken van een functioneel immuunsysteem. In Figuur 1 is de circulatie van Cy7-PM in meerdere muismodellen weergegeven. Het lijkt erop dat de circulatie van de PM wordt beïnvloed door het ras van de gebruikte muizen, wat op zijn beurt de interpretatie van therapeutische data weer kan beïnvloeden.

Figuur 1. De bloedcirculatielijden van Cy7-PM in de drie muizenrassen die zijn gebruikt in deze thesis. Cy7-PM (30 mg/mL polymer waarvan 1.5% (w/w) Cy7-gelabeld polymeer) zijn i.v. geïnjecteerd (100-200μl) in muizen; de diameter van Cy7-PM was 50-60nm. RAG2^{-/-}γc^{-/-} muizen zijn gebruikt voor de plaatsing van botachtige implantaten om de MM micro-omgeving na te bootsen, zoals onderzocht in **Hoofdstuk 4**. A/J muizen werden getransplanteerd met Neuro2A neuroblastoomcellen in **Hoofdstuk 5**. In **Hoofdstuk 5** en **6** is gebruikt gemaakt van C57BL6 muizen die werden getransplanteerd met CLL splenocyten van overleden TCL1tg muizen. In **Hoofdstuk 7** zijn tumorvrije C57BL6 muizen gebruikt.

Chronische lymfatische leukemie (CLL) is ook een HM met een lymfatische oorsprong en wordt gekarakteriseerd door monoklonale deling en toename van het aantal B-lymfocyten in het perifere bloed (PB) en in lymfatische weefsels als de milt [33]. In CLL speelt de tumor micro-omgeving een belangrijke rol in de maligne transformatie en overleving van deze cellen [34, 35]. Om het effect van de inflammatoire micro-omgeving in CLL op de circulatie van PM te bestuderen, zijn Cy7-PM i.v. toegediend aan wild-type (wt) en Proteïne kinase C – βII knockout (Prkcb^{-/-}) muizen in **Hoofdstuk 5**. Deze Prkcb^{-/-} muizen zijn gebruikt omdat ze niet vatbaar zijn voor CLL transplantatie [36]. In controle (wt of Prkcb^{-/-}) muizen of muizen met een lage CLL last, was 30-40% van de geïnjecteerde micellen te detecteren na 24 uur. In muizen met een hogere CLL last kon maar 20% van de geïnjecteerde micellen gedetecteerd worden. De Cy7-PM lokaliseerden voornamelijk naar de lever en milt. Geïnfiltreerde CLL cellen werden ook voornamelijk teruggevonden in de lever en milt, wat de toegenomen accumulatie van micellen in deze organen verklaart. Voor de behandeling van CLL is de lange circulatie en accumulatie van PM in lever en milt gunstig. Opvallend is dat in muizen waar de manifestatie van CLL wordt bemoeilijkt (Prkcb^{-/-}), de accumulatie van PM in de milt is afgenomen. Dit suggereert dat de ernst van CLL bepaalt in welke mate de micellen in het doelgebied accumuleren. In deze studie wordt gedemonstreerd dat een PM formulering een veelbelovende manier kan zijn om de inflammatoire micro-omgeving die wordt

gevormd door CLL cellen te bereiken. Tenslotte laten studies met ibrutinib geladen PM een significante afname zien van CLL cellen in de milt van CLL muizen.

In dit hoofdstuk hebben we de circulatie en distributie van gelabelde PM en therapeutische effectiviteit van ibrutinib-PM getest in muizen met CLL. De bruikbaarheid van een dergelijk model is afhankelijk van de overeenkomsten tussen patiënt en het model. Een goed model heeft dezelfde genetische, histologische, fenotypische en klinische manifestatie zoals die in de patiënt te zien is. Een dergelijk model is te vinden in de vorm van de TCL1g muizen die gebruikt zijn in **Hoofdstuk 5** en **6**. Dit model is gebuikt als een middel om zowel de pathologische mechanismen van CLL te onderzoeken, als voor het vinden van nieuwe medicijnen [37-40].

Daarnaast is het een geschikt preklinisch model gebleken doordat de muizen hetzelfde op een fludarabine (eerstelijns medicijn voor CLL) behandeling reageerden als menselijke patiënten [40]. **Hoofdstuk 6** beschrijft de ontwikkeling van mPEG-b-p(HPMAm-Bz)-PM geladen met de DUSP6 inhibitor genaamd BCI. Voor zover bekend is dit de eerste keer dat BCI is geformuleerd in een PM. *In vitro*, was de PM formulering van BCI even effectief als het vrije molecuul. Opvallend was dat primaire CLL cellen, geïsoleerd uit patiënten, minder gevoelig werden voor BCI wanneer ze samen met stromale cellen werden gekweekt, wat de beschermende rol van tumorstroma onderschrijft in CLL [34, 41].

Cy7-PM lieten een respectievelijk 46% en 24% verlengde halfwaardetijd zien in muizen met neuroblastoom of CLL. De circulatie kinetiek van BCI was echter significant korter, wat suggereert dat BCI vrij snel uit de kern van de micel getrokken wordt in plasma. Vrij BCI wordt snel geklaard uit het bloed en was 1 uur na injectie niet meer te meten. Echter, ondanks het tegenvallende farmacokinetische profiel van PM geladen BCI, was er een aanhoudende afname van CLL cellen te meten in BCI-PM behandelde muizen, terwijl dit niet te zien was wanneer het vrije molecuul werd toegediend. Deze data suggereert dat dit resultaat verbeterd kan worden met bepaalde aanpassingen aan de formulering. Aangezien BCI snel uit de PMs werd getrokken door bepaalde bloedcomponenten, lijkt de PM meer als oplosmiddel te fungeren dan een drager. Betere retentie van BCI in de PMs zou kunnen worden bewerkstelligt door de ratio van medicijn tot polymeer te verlagen. Anderzijds zou de compositie van de PM formulering kunnen worden veranderd. Dit kan bijvoorbeeld door BCI chemisch aan de polymeren te conjugeren, of de polymeren onderling te crosslinken, om de retentie van BCI te verhogen. Verder zouden de toegediende dosis en de frequentie van dosering kunnen worden geoptimaliseerd om de inhibitie van DUSP6 fosfatase activiteit, en daarmee de hyperactivatie van ERK, voldoende te verlagen [42]. Micellen gebaseerd op mPEG-b-

p(HPMA-Bz) hebben eerder aangetoond colloïdaal stabiel te zijn en hebben een hoge retentie van PTX *in vivo*, wat zich vertaalde tot een hoge therapeutische activiteit [31].

De hoge stabiliteit van de formulering en de sterke retentie van het geladen medicijn zijn gebaseerd op het feit dat zowel het polymeer als het medicijn aromatische ringen bevatten in hun chemische structuur, waardoor de componenten zogenaamde π - π interacties ondergaan. In **Hoofdstuk 4, 5 en 6** is gekeken naar andere medicijnen met aromatische ringen om te laden in deze PM, met de gedachte dat, vanwege de π - π interacties, de medicijnen stabiel in de micellen zou blijven *in vivo*. Dit was echter niet het geval en de PM bleken snel hun inhoud te verliezen *in vivo* (**Hoofdstuk 6**), wat, minder verrassend, teleurstellende therapeutische resultaten gaf.

Vanwege dit resultaat is er gekeken naar de formulering. In **Hoofdstuk 7** is onderzocht hoe de fysicochemische eigenschappen van medicijnen, hun lading en retentie in mPEG-b-p(HPMA-Bz) micellen beïnvloed. Om dit te bepalen zijn de micellen geladen met farmacons met verschillende hoeveelheden aromatische ringen (tussen de twee en vijf ringen). Naast de medicijnen die zijn besproken in **Hoofdstuk 4, 5 en 6** is er gekeken naar bortezomib, ABT-737 en PTX. Log P blijkt een belangrijke parameter voor een goede laad efficiëntie, maar niet voor de ladingcapaciteit in deze PM. Zoals verwacht is Log P ook belangrijk voor de retentie van het farmacon in de kern van de PM. Het is opvallend dat de *in vitro* retentie van medicijnen sterk afhangt van de aromatische ringen in hun chemische samenstelling. Het blijkt dat de hoeveelheid aromatische ringen van een farmacon sterk correleert aan hoe goed het in de kern van de PM blijft onder fysiologische omstandigheden. De *in vitro* farmacokinetische data van BCI en ABT-737 laten een vergelijkbare retentie van de stoffen zien als *in vivo*, wat het belang van deze bevindingen bevestigt. De *in vitro* resultaten met PTX zijn ook vergelijkbaar met de gepubliceerde *in vivo* resultaten [31].

Het is interessant dat de parameters die belangrijk zijn voor het laden van het medicijn niet perse belangrijk zijn voor de retentie van het medicijn in deze PM. De resultaten die hier worden beschreven kunnen worden gebruikt om de compatibiliteit tussen een farmacon en HPMA-(PEG-Bz) micellen te voorspellen. *In vitro* onderzoeken naar afgifte van hydrofobe medicijnen kunnen met behulp van albumine als acceptor, resultaten geven die vertaalbaar zijn naar de afgiftekinetiek *in vivo*. In vervolgonderzoeken zou het vervangen van albumine-houdende media door humaan plasma mogelijk nog relevantere resultaten geven. De aanpak die hier beschreven wordt kan dienen als een eerste *in vitro* screening om potentiële PM-medicijn combinaties te testen en kan tegelijkertijd mogelijk het aantal dierproeven laten afnemen.

CONCLUSIES

De onderzoeken die in deze thesis worden beschreven toetsen polymere micel formuleringen gebaseerd op mPEG-b-p(HPMA-Bz) als dragers van kankermedicijnen, met hematologische kankers als primair doelwit. De PM laten een verlengde circulatie en aantrekkelijke weefseldistributie zien in klinisch relevante modellen voor multiple myeloom en chronisch lymfatische leukemie. De therapeutische uitkomsten van de geteste formuleringen was echter nauwelijks verbeterd ten opzichte van de klinisch toegepaste formuleringen. Dit komt hoogstwaarschijnlijk door de farmacokinetische profielen van de medicijn geladen PM in bloed, waardoor er niet van de voordelen van nanodeeltjes als medicijndrager, zoals accumulatie in doelwit weefsels, kon worden geprofiteerd. Verder is er gekeken naar de fysicochemische eigenschappen van medicijnen die de lading en retentie van deze moleculen in PM beïnvloeden. Medicijnen met aantrekkelijke retentie profielen kunnen *in vivo* verder onderzocht worden om hun therapeutische potentie te bepalen.

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CURRICULUM VITAE

Aida Varela Moreira was born on April 18, 1989 in Porto, Portugal. After obtaining her high school diploma, she started the bachelor's degree in Biology at the Faculty of Sciences, Porto University. In 2012 she enrolled the master's program of Medicine and Molecular Oncology at Faculty of Medicine, Porto University. Her master's internship was performed at the Institute of Biomedical Engineering (INEB) under the supervision of Dr. Ana Paula Pego. Her project aimed to develop chitosan-based carriers for nucleic acid delivery. In 2015 she started her PhD at the Department of Clinical Chemistry and Hematology at University Medical Centre Utrecht in collaboration with the Department of Pharmaceutics, Utrecht University under the supervision of Prof. dr. Raymond Schiffelers, Prof. dr. Wim Hennink, Dr. Marcel Fens and Dr. Maike Buchner-Mayr. Her PhD project aimed to explore π - π stacked polymeric micelles as drug carriers of hydrophobic drugs for the treatment of hematological malignancies. The results of this project are presented in this thesis. She continued her career as a post-doctoral researcher at Dr. Ronald Stam group at Prinses Maxima Centrum for pediatric oncology in Utrecht.

LIST OF PUBLICATIONS

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