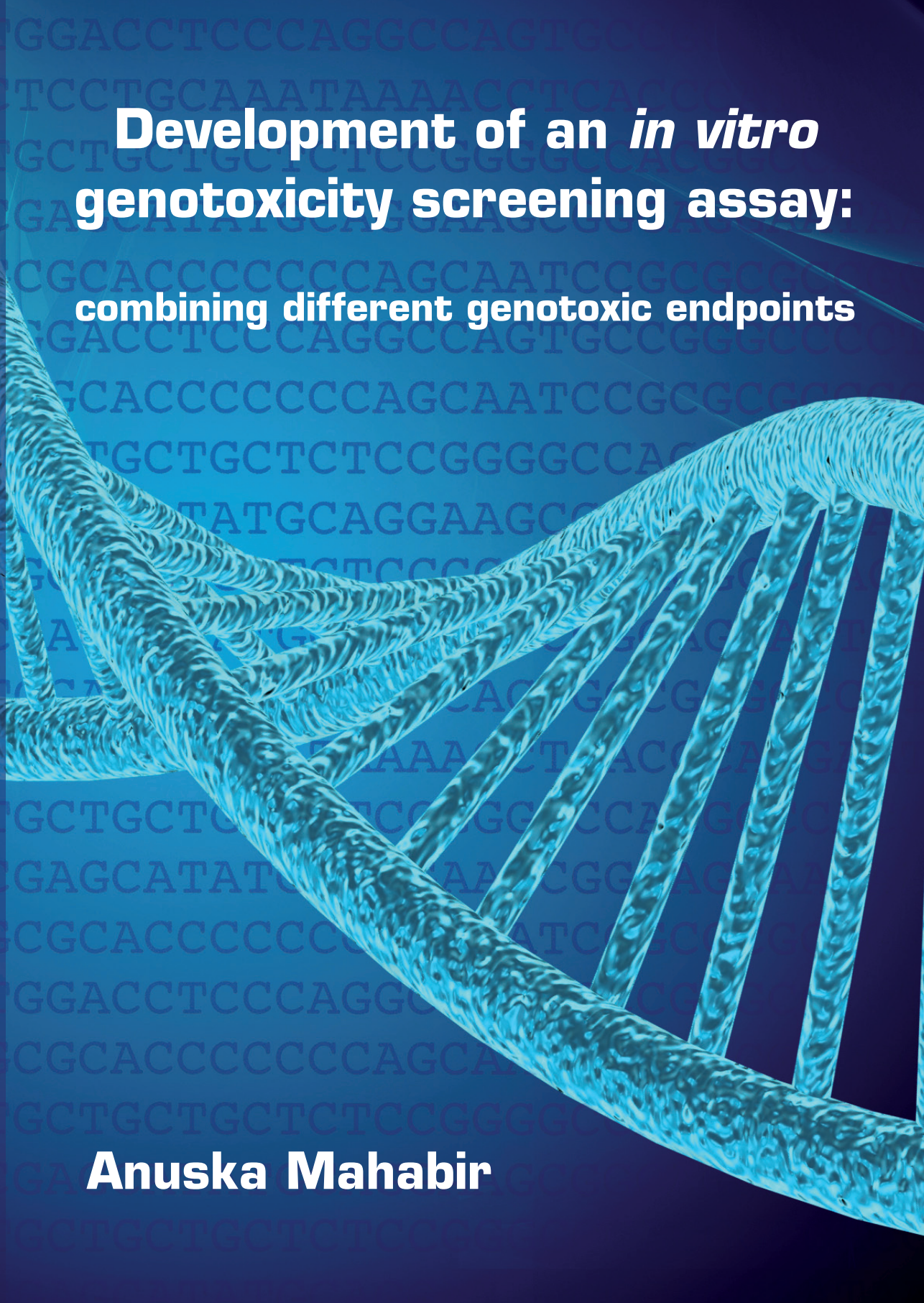
Development of an *in vitro* genotoxicity screening assay:

combining different genotoxic endpoints



Anuska Mahabir

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Development of an *in vitro* genotoxicity screening assay: combining different genotoxic endpoints

Ontwikkeling van een *in vitro* genotoxiciteitstest:
combinatie van verschillende genotoxische eindpunten
(met een samenvatting in het Nederlands)

Proefschrift

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Voor Radjen

Aan mama, papa en Rohied

Contents

List of Abbreviations

AGMT	Alkylguanine-DNA methyltransferases
BER	Base Excision Repair
BLM	Bleomycin
CAT	Chromosome Aberration Test
Clastogen	Chemical causing predominantly chromosome aberrations
CS	Cockayne syndrome
DNA	Deoxyribonucleic acid
DNA-PK_{CS}	DNA-dependent protein kinase catalytic subunit
DS	Double-strand
DSBs	Double-strand breaks
E. coli	*Escherichia coli*
GGR	Global Genome Repair
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRR	Homologous Recombination Repair
ICH	International Conference on Harmonization
Kb	Kilobase
LacZ MF	*LacZ* Mutant Frequency
MEFs	Mouse Embryonic Fibroblasts
MLA	Mouse Lymphoma Assay
MMC	Mitomycin C
MMR	MisMatch Repair
MNi	Micronuclei
MNT	Micronucleus test
Mutagen	Chemical causing predominantly gene mutations
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End-Joining
PCNA	Proliferating cell nuclear antigen
P-Gal	Phenyl-ß-D-galactoside
REACH	Registration, Evaluation, Authorization and restriction of Chemicals
RFC	Replication factor C
RNA	Ribonucleic acid

RPA	Replication protein A
TCR	Transcription Coupled repair
TFIIH	Transcription factor IIH
TFT	Trifluorothymidine
TK	Thymidine kinase
6-TG	6-thioguanine
UV	Ultra Violet
WT	Wild-Type
X-Gal	5-bromo-4-chloro-3-indolyl ß-D-galactopyramoside
XP	Xeroderma Pigmentosum
XRCC4	X-ray-repair-cross-complementing defective repair in Chinese hamster mutant 4

Chapter 1

General Introduction

1.1 Introduction

Humans are daily exposed to numerous chemicals, like those present in food/feed additives, packing material, drugs, cosmetics and pesticides. It is of vital importance that before marketing, chemicals present in those products are evaluated for their potential adverse health effects for humans. Many of these chemicals are genotoxic and can cause DNA damage, which can form a major threat to the integrity of chromosomes and viability of cells. Fortunately, cells are equiped with several DNA repair mechanisms, which can repair/remove the different types of DNA lesions efficiently and accurately maintain the integrity of the genome (5,21,51). There are at least 5 major DNA repair pathways, which will be discussed in more detail in section 1.3 and 1.4. Defects in DNA repair give rise to an increase in sensitivity to DNA-damaging agents, accumulation of mutations, various metabolic disorders, apoptosis, cell death, accelerated ageing, genetic diseases or development of cancer (5,6,28).
Through the years several well-defined tests have been developed for the assessment of harmfull effects of chemicals and drugs. The focus in this thesis lies in the genotoxic properties of chemicals. Over the past decades genetic toxicology testing has demonstrated that no single test is capable to detect both types of genotoxic effects, chromosome aberrations and gene mutations. Therefore, the potential genotoxic effects of chemicals are assessed in a battery of *in vitro* and *in vivo* tests. Genotoxic tests are usually performed in the early development of a chemical, since these are comparatively short in duration, relatively inexpensive and help to identify potential genotoxic carcinogens, which otherwise would only be known after completion of the 2-year cancer bioassay (59).
There are several strategies for genotoxicity testing of chemicals (new and existing chemicals, food/feed additives, packing material, cosmetics and pesticides) and drugs. The guidance for drugs and chemicals is different from each other. In 1981 in Europe a law was introduced which made it compulsory to test both new and existing chemicals for their potential harmful effects for humans. Little information is known on toxicity, however, for 99 percent of the enormous number of existing chemicals placed on the market before 1981. These chemicals are still being used and at very low levels they are not considered to be harmful to human health. However, if these substances bioaccumulate in the human body, dangerous concentrations can be reached and consequently may have adverse effects on human health. These compounds can also chemically interact with each other, producing new substances with new risks. In June 2007 a new European Chemicals Legislation on testing of chemicals

was implemented, REACH, the Registration, Evaluation, Authorisation and restriction of CHemical substances (19). The main objective of REACH is to ensure the protection of human health and the environment through safety assessment for new and existing (developed before 1981) chemicals. Other objectives are stimulation of the use of alternative test strategies, the development of new *in vitro* tests and thereby reducing the number of experimental animals and minimalization of test strategies (22,30). The actual number of tests required for chemicals under REACH depends on the production volume of chemical (tons per year).

As for chemicals, there is also guidance for testing of pharmaceuticals (drugs) by the International Conference on Harmonization (ICH). This guidance is in use since 1997 (31) using a battery approach consisting of 3 or 4 tests to provide a full genotoxic profile of a drug. In case of positive results, additional testing can be necessary. The purpose of this guidance is to maintain the quality, safety and efficacy of drugs and to protect public health (60).

The different *in vivo* tests developed and implemented through the years for genotoxicity testing of chemicals resulted in an increased use of laboratory animals. The last years a trend towards the use of *in vitro* testing has been initiated, which should lead to a reduction in laboratory animal use. An example is the ban of cosmetic products with ingredients tested on animals, by the European Union in March 2009, with a complete sales ban that will be effective in 2013 (17,18).

Through the years, several *in vitro* and *in vivo* tests have been developed for genotoxic screening of chemicals. The most commonly used *in vitro* tests to detect chemicals inducing gene mutations are the Ames test, the Mouse Lymphoma Assay (MLA) and the *hprt* test (29,58,61); while the *in vitro* chromosome aberration test (CAT) and the *in vitro* micronucleus test (MNT) are used to detect chemicals causing chromosomal aberrations (20,25,29). Under *in vivo* conditions there are several transgenic animal models to detect chemicals causing gene mutations, and the *in vivo* MNT and the *in vivo* CAT tests to detect chemicals causing chromosomal aberrations. However, none of these *in vivo* and *in vitro* genotoxicity tests cover both endpoints: gene muations and chromosomal aberrations. Therefore, the development of a test system that detects both endpoints simultaneously may be of great advantage, since this can lead to a reduction in the number of tests needed (both *in vitro* and *in vivo*) and consequently a reduction of the number of laboratory animals used (for the *in vivo* conditions). With the transgenic mouse model we used, the pUR288 mouse model, we were able to detect both endpoints of genotoxicity simultaneously. Moreover, mouse embryonic fibroblasts (MEFs) derived from this transgenic mouse model, were capable to detect both

endpoints of genotoxicity as well. Using the pUR288 model *in vivo* and MEFs derived from this model it is possible not only to detect both genotoxic endpoint simultaneously, but also to detect these endpoints in tests with the same read-out system.

1.2 Genotoxicity

Genotoxicity is assessed in a small number of *in vitro* and *in vivo* tests, which cover the two endpoints of concern (gene mutations and chromosome aberrations). Most genotoxic chemicals lead to gene mutations and chromosome aberrations with a preference for one endpoint. In order to get reliable insight into the genotoxic properties of chemicals, both genotoxic endpoints need to be tested (58).

1.3 Gene mutations and repair

One of the endpoints of genotoxicity are gene mutations. Mutagenic chemicals (mutagens[1]) cause predominantly gene mutations (Figure 1), which are generally not lethal but can form a major threat to the integritiy of chromosomes and viability of cells.

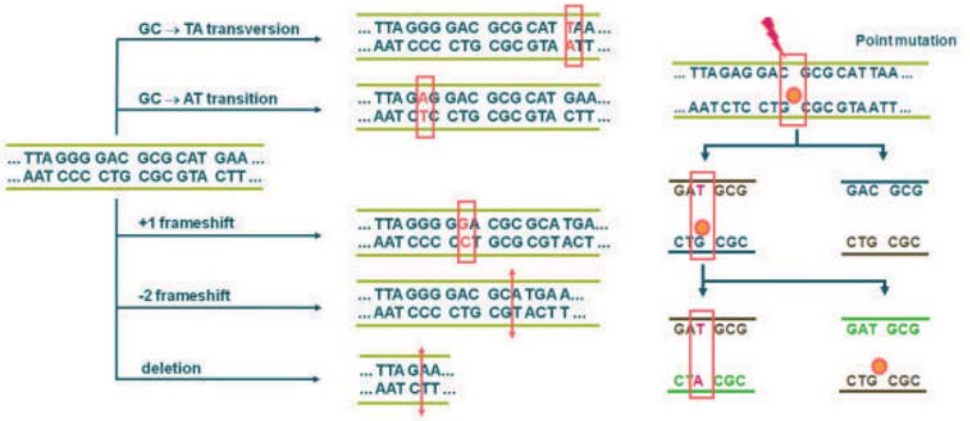

Figure 1. Different types of gene mutations including transversion, frameshifts, deletions and point mutations.

A transversion is a substitution of a purine (A or G) for a pyridine (C or T) or vice versa (*e.g.* GC => TA). A transition is a point mutation that changes a purine nucleotide to another purine (*e.g.* GC => AT). A frame shift is a genetic mutation such as insertion (+1 frameshift) or deletion (-2 frameshift) of a number of nucleotides. A point mutation is a single base substitution (*e.g.* CG => TA).

[1]For the simplicity we use the term mutagens for chemicals causing predominantly gene mutations.

Fortunately, cells are equiped with several DNA repair systems. Depending on the specific classes of DNA lesions, one or more DNA repair pathways become active (21). Four of the 5 major DNA repair pathways are involved in the repair of DNA lesions leading to gene mutations: direct repair, base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR; Ref. 64). The 5th major repair pathway, involved in single/double-strand break repair, will be discussed in more detail in section 1.4.

1.3.1.1 Direct repair

Direct repair acts by removing or reversing the DNA lesions by a single enzyme reaction in a basically error-free manner and with high substrate specificity. This mechanism does not require a template, since the damage they restore only occurs in one base and there is no involvement of incision of the sugar-phosphate backbone or base excision. These lesions can occur due to alkylating agents. Direct repair is carried out by specific enzymes called alkylguanine-DNA methyltransferases (AGMT), which remove the alkyl group from the guanine residue of DNA and transfers it to one of its own cysteine residues. Next to AGMT, in bacteria and yeast, photolyases can directly reverse UV-induced DNA damage (14,41,48).

1.3.1.2 Base excision repair (BER)

Base excision repair (BER) is a cellular mechanism that repairs damaged DNA throughout the cell cycle. This mechanism protects cells from the deleterious effects of endogenous DNA damage induced by hydrolysis, reactive oxygen species and other intracellular metabolites, and is also responsible for the removal of many lesions induced by ionizing radiation and strong alkylating agents. The main enzymes involved in BER are DNA glycosylases and AP endonucleases. The DNA glycosylases are involved in excision of the damaged base, whereafter the remaining a-basic site is further processed by AP endonucleases. BER is divided into short-patch repair (where a single nucleotide is replaced) or long-patch repair (where 2-10 nucleotides are replaced, Refs. 35,41,42,48).

1.3.1.3 Nucleotide excision repair (NER)

Nucleotide excision repair (NER) is a repair pathway that is involved in the removal of several kinds of DNA lesions which mainly originate from exogenous sources like UV light or genotoxic chemicals producing bulky adducts and DNA crosslinks (51,57). NER consists of two different sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). These two subpathways are only different in the first step of DNA damage

recognition. The first pathway (GGR) eliminates DNA damage present in the genome overall. The DNA recognition is accomplished by a complex of protein factors (XPC-HR23B and XPE). The second pathway (TCR) removes lesions from active genes. Hereby, the primary trigger in the DNA damage recognition is a stalled RNA polymerase II, which is accompanied by Cockayne syndrome (CS) proteins (*i.e.* CSA and CSB; Refs. 24,39,51,57). The next stages involved in DNA repair are mostly studied for GGR, but are identical in the TCR pathway. After binding of the XPC-HR23B complex to the damaged DNA in GGR, several other proteins are bound such as a complex called transcription factor IIH (TFIIH) and the endonuclease XPG. TFIIH contains two DNA helicase activities with opposite polarity (XPB and XPD) that unwind the DNA duplex. After binding of the replication protein A (RPA), the damage is verified by XPA, whereafter the endonucleases XPG and ERCC1/XPF cleave the 3' and the 5' of the DNA lesion. This results in the release of a fragment, containing the DNA damage, of 27-30 nucleotides. The remaining gap is filled in by a complex formed by DNA polymerase d or e, the accessory replication proteins, the proliferating cell nuclear antigen (PCNA), RPA and the replication factor C (RFC; Figure 2; Refs. 27,51,57).

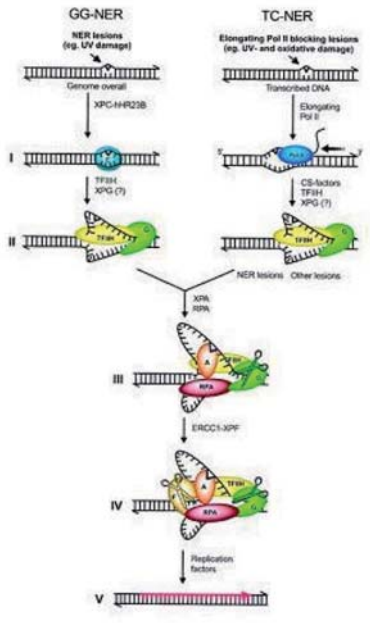

Figure 2. The two different repair mechanisms of NER; TCR and GGR.
The only difference between these pathways is the DNA damage recognition step. Adapted from Hoeijmakers 2001 (27).

1.3.1.4 Mismatch repair (MMR)

Mismatch repair (MMR) is a system that recognizes and repairs erroneous insertions, deletions and mis-incorporation of bases. These can arise during DNA replication and recombination. MMR is a strand-specific repair. During DNA synthesis, the newly synthesized (daughter) strand may include incorrect bases. Examples of mismatch bases include base pairs like G/T or A/C. To repair these mismatched base pairs in the correct manner, it is very important to discriminate between the newly synthesized (mismatched) strand and the parental strand. The first step in MMR is recognition of the deformity caused by the mismatch. Thereafter, the template and the non-template strand are determined and the incorrect incorporated base is excised and replaced with the correct nucleotide. During the repair process not only the mismatched nucleotide is removed, but a few or up to thousands of bases of the newly synthesized DNA strand can be removed and replaced (62).

1.3.2 *In vivo* gene mutation tests

Originally it was quite difficult to measure *in vivo* gene mutations. Existing tests such as the mouse spot assay were insensitive and required many laboratory animals. In the early 90's several new *in vivo* gene mutation tests have been developed including the $tk^{+/-}$, *hprt*, *dlb-1* and several transgenic mouse (and rat) models including Muta^{TM}Mouse (*lacZ*), Big Blue® mice (*lacI*), Big Blue® rats (*lacI*), pUR288 (*lacZ*), λ*supF* mice and *gpt*Δ mice (58). An OECD guideline for all these models is currently under development (Douglas, personal communication). The focus in this thesis lies on transgenic mouse models based on *lacZ*/*lacI*; therefore the other models will not be discussed in further details. The main difference between these models is the type of reporter gene incorporated, *e.g. lacZ* or *lacI,* and the type of shuttle vector used, *e.g.* bacteriophage λ or plasmid (10,11,54,58).

There are two commercially available mouse models, Muta^{TM}Mouse and Big Blue®, for the detection of gene mutations and small deletions (52,53). The Muta^{TM}Mouse model consists of a λgt10*lacZ* shuttle vector which can be excised from genomic DNA and packaged into phage heads using an *in vitro* packaging extract. The phage is absorbed onto *Escherichia coli C* ($galE^-$ $lacZ^-$). The mutation selection is performed using a selective and a non-selective culture medium. Only *E.coli* bacteria that harbour a mutated *lacZ* will be able to form plaques in the selective medium. The *lacZ* mutant frequency (*lacZ* MF), based on the plaques formed on the selective versus the non-selective plates, is an indicator for the mutagenic properties of chemicals tested (29).

In contrast to the Muta™Mouse model, the Big Blue® mouse model is based on the bacterial *lacI* gene. The Big Blue® model consists of a λLIZα shuttle vector, carrying the bacterial *lacI* gene as a mutational target and the α-*lacZ* gene. The *lacI* gene codes for a homotetrameric protein that binds to the *lacO* operator sequence, which negatively regulates *lacZ* expression. The α-*lacZ* gene codes for the α-amino portion of β-galactosidase. The phage is absorbed to *E. coli* SCS-8 cells (*lacZΔM15*), whereafter the bacteria are seeded on a selective medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). The *lacI* gene allows the *lac* repressor to bind to the *lac* operator which inhibits α-*lacZ* expression and thus β-galactosidase activity resulting in white (colourless) plaques. However, mutations in *lacI* will produce a *lac* repressor that is unable to bind to the *lac* operator. Hereby, α-*lacZ* transcription will be depressed and β-galactosidase will cleave X-Gal, producing a blue plaque. The ratio between white and blue plaques is a measure for the mutant frequency, which is an indicator for the mutagenicity of chemicals (29,58).

These commercial bacteriophage-based models have no tissue restriction, which makes it possible to measure gene mutation in any organ. However, they do not recognize large deletions. This restriction is caused by the fact that these models use a bacteriophage to infect *E. coli* and thus the length of the insert as well as the presence of flanking *cos*-sites are essential. Moreover, low rescue efficiency is associated with these models (3,52).

In 1989 the *lacZ* plasmid-based transgenic mouse model, the pUR288 model, was developed by Gossen *et al.* to measure gene mutations (23). The *lacZ* gene consists of 3282 base pairs. The pUR288 model carries approximately 20 copies of the *lacZ* reporter gene (5.5 Kb), chromosomally integrated head to tail, which can be isolated from genomic DNA for the purpose of mutagenic testing (3,9,37). *LacZ*-containing plasmids are excised from genomic DNA and incorporated into *E. coli C* (*lacZ⁻, galE⁻*). Mutation selection is performed by using a selective and a non-selective culture medium. The selective medium consists of P-Gal (phenyl-β-D-galactoside), which is metabolized into phenol and galactose by β-galactosidase, the product of *lacZ*. The galactose is thereafter metabolized into the highly toxic UDP-galactose which kills WT cells. Only mutants lacking β-galactosidase can grow and will form colonies on the selective medium. The ratio between the colonies formed on the selective and the non-selective medium is a measure for mutagenicity of chemicals (Figure 3; Ref. 58).

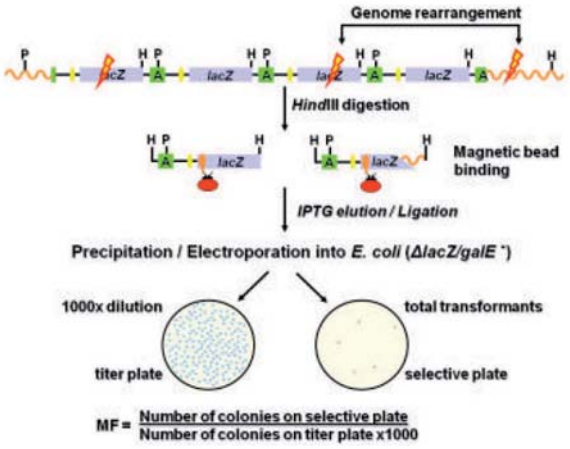

Figure 3. The *lacZ* plasmid assay (Boerrigter *et al.* 1995, modified (3)).

There are two major advantages of this plasmid model compared to the bacteriophage-λ models: high rescue efficiency and the possibility to detect different types of mutations including small and large deletions (>500 base pair; Refs. 10,11,33). Large deletions may result in chromosomal rearrangements (when the chromosomes are repaired, see Ref. 4). Consequently, this model is able to detect both gene mutations as well as chromosomal rearrangements.

LacZ mutants that originate from large deletions (chromosomal aberrations) carry mouse DNA fragments, which can be detected through hybridization using total mouse DNA. All mutants that hybridize with total mouse DNA carry chromosome rearrangements. Although this is an extra step, hybridization can be used to confirm the clastogenicity of less potent chemicals.

1.3.3 *In vitro* gene mutation tests

There are several *in vitro* gene mutation tests available for the detection of mutagens. The most commonly used tests include the Ames test (gene mutation test in bacteria), the mouse lymphoma assay (gene mutation test in mammalian cells using the *tk* locus) and the *hprt* assay (gene mutation test in mammals using the *hprt* locus; Ref. 36).

In the Ames test, a reverse gene mutation test, mutant bacteria carrying a frame shift or a base pair substitution are used. Compounds reversing this mutation are considered mutagenic. The

Ames test is the oldest and most widely used test and always the first test in a strategy. It is still considered the most sensitive test for mutagenic chemicals.

The mouse lymphoma assay (MLA) is a gene mutation assay using the *tk* (thymidine kinase) locus. Mammalian cells normally have two copies of the *tk* gene, but the mouse lymphoma cell line has only one functional copy ($tk^{+/-}$). TK is not an essential enzyme, but is part of a system that recycles free thymidine that is eventually incorporated into DNA. Mutants are selected by culture in the presence of trifluorothymidine (TFT), a substrate for the enzyme. TFT is a toxic analogue of thymidine and interferes with DNA metabolism, thereby killing the cell. *Tk* mutant cells, containing no functional copy of the *tk* gene, are resistant to TFT and will grow relatively well and form normal large colonies. However, when the *tk* gene is lost due to a greater genetic injury such as chromosomal breaks, adjacent genes are also lost and the cells poorly grow, resulting in small colonies. The ratio between the large and the small colonies is a measure for mutagens (large colonies) and clastogens (small colonies; Refs. 58,61).

Hprt (hypoxanthine-guanine phosphoribosyl transferase) is a non-essential enzyme for cells in culture. *Hprt* is an endogenous and housekeeping gene present in all tissues. Mutant are selected by culture in the presence of 6-thioguanine (6-TG), which is a substrate for *hprt*. 6-TG is converted into monophosphate, which is cytotoxic and when accumulated in the cell, inhibits proliferation and in the end the cell dies. *Hprt* mutants, who have lost this enzyme activity, are resistant to the toxic effects of 6-TG and can grow in medium containing 6-TG. The mutant frequency calculated by the ratio between the selective and the non-selective plates is an indication for the mutagenicity of chemicals (29,58).

1.4 Chromosomal aberrations and repair

The other endpoint of genotoxicity, chromosomal aberrations, is caused by clastogenic chemicals (clastogens[2]; Ref. 38). Chromosome aberrations can either be structural (clastogenic) or numerical (aneugenic). DNA damages like double-strand breaks (DSBs), threaten the integrity of chromosomes and viability of cells. Unrepaired or misrepaired DSBs can lead to mutations, chromosome rearrangements, cell death and cancer (12,13,32,55). Numerical chromosome aberrations (aneuploidy) can be either loss or gain of chromosomes per cell (like trisomy 21 in Down Syndrome) and can be lethal or cause genetic diseases.

Fortunately we also possess systems to repair DSBs, the last of the earlier mentioned repair systems.

In mammalian cells, DSBs are mainly repaired by either homologous recombination repair (HRR) or non-homologous end-joining (NHEJ) repair, respectively (Figure 4; Refs. 13,16,49).

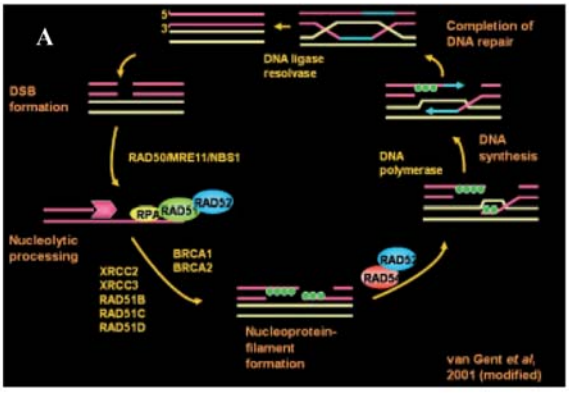

Figure 4. Homologous recombination repair (van Gent *et al.* 2001, modified (49)).

[2]For the simplicity we use the term clastogens for chemicals causing predominantly chromosomal aberrations.

The main difference in HRR and NHEJ is the requirement of a homologous DNA sequence in HRR, which is therefore an error-free mechanism. In contrast, NHEJ, which does not use sequence homology is an error-prone mechanism (8,13,44). Another difference is their dependency of the cell cycle. HRR depending on the presence of an intact sister chromatid is more efficient during late S and G_2 phase of the cell cycle, when sister chromatids are active in dividing cells. NHEJ not depending on a homologous DNA strand can repair DSBs in all cell cycle stages, G_1, S and G_2 phase (1,2,15,56).

Finally, the contribution of HRR and NHEJ repair can also depend on the stage of mammalian development. It has been shown that HRR acts at the embryonic stage, where the embryonic cells were sensitive towards ionizing radiation, but its action in adults was not detected, unless NHEJ is disabled (16). It was concluded that the contribution of HRR and NHEJ can differ depending on mammalian developmental stage (*i.e.* cell type) and on the specific type of DNA damage.

1.4.1.1 Homologous recombination repair

Homologous recombination repair is an error-free repair system. The RAD52-group of proteins, including RAD50, RAD51, RAD52 and RAD54, and MRE11 play a major role in HRR (Figure 5; Refs. 26,44,46,49). In the case of a DSB, the initial cellular response is the recognition of this break through the RAD50/MRE11/NBS1 complex. Subsequently, followed by nucleolytic processing of the broking ends of DNA into 3'-end single-stranded DNA. The single-stranded DNA is bound by RPA (replication protein A). After RPA is removed and replaced by RAD51, the RAD51 nucleoprotein filament mediates the search for a homologous duplex template DNA whereafter the complex of joint molecules between the broken DNA ends and the intact ds DNA repair template is formed. The *Rad52, Rad54, Rad50* paralogues (such as *Rad51B, Rad51C, Rad51D*), *Xrcc2*, *Xrcc3* and *Dmc1* are accessory to *Rad51* at various stages of HRR. After polymerisation of nucleotides to restore degraded DNA strands and resolution of the recombination intermediates, the HRR is completed resulting in an error-free double-stranded DNA. The breast-cancer-susceptibility proteins BRCA1 and BRCA2 are involved in HRR as well, however, their role is not well understood (26,43,45,49). Lost of most HRR factors can lead to early or mid-embryonic lethality in mice (8,13,47). This suggests that HRR plays an important role in development, presumably to repair spontaneously arising DNA damage (8,13), which is in agreement with the findings that HRR and NHEJ can play different roles during the mammalian developmental stages (16).

1.4.1.2. Non-homologous end-joining repair

Non-homologous end-joining (NHEJ) is an error-prone repair mechanism. There are at least 3 steps involved in NHEJ (Figure 5).

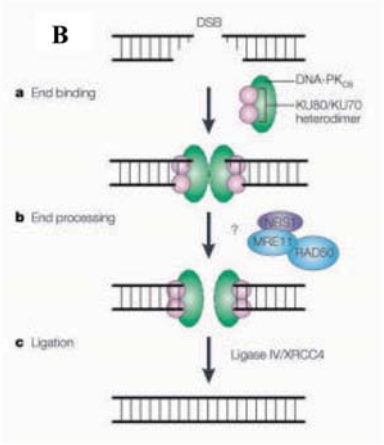

Figure 5. Non-homologous end-joining repair (van Gent *et al.* 2001, modified (49)).

The first step is the detection of the strand break and the end-binding mediated by DNA-PK consisting of the three subunits DNA-PK_{CS} (DNA-dependent protein kinase catalytic subunit) and the KU80/KU70 heterodimer, which are involved in the formation of a molecular bridge that holds the broken DNA together. Hereafter, the NBS1/MRE1/RAD50 complex is involved in the processing prodecure that modifies non-matching and/or damaged DNA ends into incompatible and ligatable ends. Finally, in the ligation step, a complex consisting of DNA ligase IV and XRCC4 (X-ray-repair-cross-complementing defective repair in Chinese hamster mutant 4) ligates the two DNA ends together forming an intact double-strand DNA molecule (46,49,50,56). Recently, Cernunnos-XLF was discovered, which is also involved in NHEJ (40). Cernunnos-XLF interacts and stimulates the DNA ligase IV-XRCC4 (LX) complex, which acts in the final ligation step in NHEJ (40,63).

1.4.2 *In vivo* chromosomal aberration tests

The chromosome aberration test (CAT) and the micronucleus test (MNT) are most commonly used and well validated *in vivo* chromosome aberration tests.

The CAT measures the occurrence of chromosome aberrations generally in bone marrow or peripheral blood cells. In the CAT the mitosis is arrested in the metaphase stage with a mitotic inhibitor colchicine. Metaphase preparations are examined for chromosome breaks and/or

chromosomal rearrangements. The number of cells with chromosomal breaks is a measure for clastogenicity of chemicals for this test (29).

The MNT measures the number of cells containing a micronucleus again in dividing cell populations such as bone marrow or peripheral blood erythrocytes. A micronucleus is formed during the metaphase/anaphase transition of mitosis (cell division). As chromosome fragments (acentric fragments) and/or whole lagging chromosomes are not dragged to the nuclear poles either because there is no centromere to bind microtubules or because the spindle figure itself is spoiled. Consequently, these fragments or chromosomes lag behind at the equatorial plane and cannot integrate into the daughter nuclei. During telophase, a nuclear envelope forms around the lagging chromosomes and fragments. This small nucleus is smaller than the main nucleus in the cell, hence the name "micronucleus". Micronuclei (MNi) carrying a chromosomal fragment are considered a reliable measure for chromosomal breaks and thus for clastogenicity; MNi with a lagging chromosome a measure for aneuploidy (numerical chromosome aberrations; Refs. 20,25,53).

1.4.3 *In vitro* chromosomal aberration tests

The CAT and the MNT are also used under *in vitro* conditions. The *in vitro* CAT test is performed according to the procedures previously described in section 1.4.2., but performed in either primary cells (*e.g.* human blood lymphocytes) or cell-lines (*e.g.* CHO, V79 cells). The same accounts for the *in vitro* MNT. However, in the *in vitro* variant of this test MNi are sometimes scored in bi-nuclear cells. Bi-nuclear cells are induced by treatment with cytochalasin B, which is an inhibitor of actin. Cytochalasin B blocks cytokinesis but not nuclear division resulting in bi-nuclear cells. The cytokinesis-block assures detection of cells containing a MNi due to treatment with clastogens and not to altered cell division kinetics caused by cytotoxicity of the used chemicals or cell culture conditions (7,20).

1.5 Outline of the thesis

Exposure to genotoxic chemicals can cause several types of DNA lesions, which are a major threat to the integritiy of chromosomes and viability of cells and may lead to carcinogenesis, accelerated ageing or cell death. Genotoxicity can be divided into two endpoints, gene mutations and chromosomal aberrations (28). There are several *in vivo* and *in vitro* genotoxicity tests for the assessment of genotoxic properties of chemicals. However, none of these tests are capable to detect both endpoints of genotoxicity simultaneously. This implies that at least two separate tests are necessary to get reliable insight in the genotoxic properties of chemicals. For the *in vivo* conditions this leads to an increase in the number of laboratory animals used. The aim of this thesis was the development of a system that can detect both gene mutations as well as chromosome aberrations simultaneously. For the *in vivo* conditions we used the pUR288 mouse model, containing the *lacZ* gene, developed by Gossen *et al.* (23); for the *in vitro* conditions mouse embryonic fibroblasts derived from the pUR288 mouse model. Using the pUR288 model *in vivo* and cells derived from this model *in vitro* enables us not only to detect both genotoxic endpoints simultaneously, but also in systems with the same read-out system.

The *in vivo* studies described in **Chapter 2** were performed to investigate if the clastogenic effects of chemical compounds could be detected in the pUR288 and the Muta^{TM}Mouse mouse models. Of all the used clastogens, only bleomycin (BLM) showed a slight increase in the *lacZ* mutant frequency (*lacZ* MF) in pUR288 mice, but not in the Muta^{TM}Mouse. Further analysis using hybridization studies with mouse DNA showed that all clastogens induced chromosomal rearrangements in several tissues of the pUR288 mouse model. These results, *lacZ* MF combined with hybridization, indicated that the pUR288 mouse model was able to detect the clastogenic properties of compounds, but the system is not optimal for routine testing. The low or lack of sensitivity of the Muta^{TM}Mouse mice towards clastogens was further analyzed in **Chapter 3** by using different routes of administration and higher doses. It appeared that all compounds administered *i.p.* showed a better response but at higher doses as compared to that upon oral exposure. These results show that the route of administration and dosing are very important to measure the effect of genotoxic compounds.

The possibilities of the pUR288 model in genotoxicity testing were further explored in **Chapter 4** by using different clastogens. Since we had doubts on the sensitivity of the pUR288 model to detect clastogenic effects, we used mice with a defect in homologous

recombination repair, *i.e. Rad54/Rad54B* mice. RAD54, belongs to the RAD52 epistatis group, is a member of the SWI2/SNF2 family of double-strand DNA-stimulated ATPases and DNA/RNA helicases (13,15,55). RAD54B, a homologue of RAD54, has been identified in human and mouse cells and is a DNA-dependent ATPase and a DNA-binding protein (1,13,34). The rationale behind the use of *Rad54* and *Rad54B* double knock-out mice (*Rad54/Rad54B* mice) is that they are supposed to be more sensitive towards clastogens compared to repair-proficient mice. This may lead to either unrepaired DNA breaks or to a shift in repair to NHEJ also leading to chromosomal rearrangements since NHEJ is an error-prone DNA repair system.

Unexpectedly, however, the *lacZ* MF and the micronucleus test (used as a control for clastogenicity) showed that the *Rad54/Rad54B/lacZ*$^+$ mice were only moderately more sensitive towards the clastogens BLM and γ-radiation, but not mitomycin C (MMC). It was assumed that, although still a promising test, under the present non-optimal conditions both *in vivo* models were not sensitive enough to detect the effect of exposure to clastogens.

This issue was further investigated by performing *in vitro* studies using mouse embryonic fibroblasts, derived from both pUR288 *lacZ*$^+$ mice (wild-type mice) and the *Rad54/Rad54B/lacZ*$^+$ mice (**Chapter 5**). Both mutagens as well as clastogens showed a dose-dependent induction in the *lacZ* MF in MEFs of both genotypes. Moreover, a genotype effect was observed after MMC treatment. Exclusively, MMC-treatment resulted in a higher *lacZ* MF in *Rad54/Rad54B/lacZ*$^+$ MEFs compared to wild-type (WT) MEFs, which was confirmed in hybridization studies. These results show that there are clastogens which cause different types of DNA damage, *i.e.* single/double-strand breaks (BLM) which can be removed by both HRR and NHEJ and DNA interstrand crosslinks (MMC) which at least in part depend on intact HRR.

This hypothesis was further investigated in a transcription (microarray) study described in **Chapter 6**. It was shown that the genotype effect of MMC described in **Chapter 5** was due to a weaker response through the *p53* signalling pathway in *Rad54/Rad54B/lacZ*$^+$ MEFs, which can provide an explanation for the increased *lacZ* MF after MMC exposure. An impaired DNA damage response in MMC-treated *Rad54/Rad54B* MEFs will lead to a weaker DNA repair response and therefore a larger percentage of the cells will carry a *lacZ* mutation, hence a higher *lacZ* MF in the *Rad54/Rad54B* MEFs compared to WT MEFs.

This suggests that the *Rad54* and/or *Rad54B* genes are involved in DNA crosslink damage recognition and repair.

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Chapter 2

Detecting the genotoxic effects of potential clastogens: an *in vivo* study using the *lacZ* plasmid and the Muta™Mouse model

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Abstract

In the present paper the capacity of the pUR288 plasmid mouse model and the Muta^{TM}Mouse model to detect the clastogens bleomycin, *m*-AMSA, *o*-AMSA and camptothecin, was investigated. Ethylnitrosourea (ENU) served as a positive control, methylcellulose as a negative control. Only bleomycin induced a slight but significant increase in *lacZ* mutant frequency (MF) in bone marrow of pUR288 plasmid mice. Exposure to the other compounds did not result in an increase in the MF in bone marrow and liver in both mouse models. For the Muta^{TM}Mouse this result was expected, for the plasmid mouse an increase in MF after clastogen exposure was expected. The positive control ENU induced statistically significant increases in MF compared to the negative control in both models and in both tissues analyzed. Hybridisation of DNA of mutant colonies derived from plasmid mice with labelled total mouse DNA (Hybridisation Assay) demonstrated an increase in the percentage of colonies hybridised with total mouse DNA as compared with the negative control, which suggests that there was indeed a biological response associated with treatment. These latter results indicate that the plasmid mouse assay may still be a promising model for the detection of clastogens.

Introduction

Regulatory agencies are frequently confronted with the legislation of new chemicals, re-evaluation of existing chemicals and with the risk estimation of putative genotoxic or carcinogenic compounds. Newly developed chemicals may have genotoxic liabilities for human exposures, which require assessment. It is important to pay attention to the effects of genotoxic compounds, since DNA damage induced by these agents may lead to cancer and other genetic diseases. Genotoxicity has two different endpoints: gene mutations such as base pair substitution, frame shifts, deletions and insertions, and chromosome aberrations, which in turn can be structural (clastogenic effect) and/or numerical (aneugenic effect; Ref. 14).

Initially, genotoxicity is assessed in a small number of *in vitro* tests, which cover the two endpoints of concern. A positive *in vitro* response triggers *in vivo* testing, ideally covering the same endpoint. *In vivo* tests take into account whole animal processes like absorption, tissue distribution, metabolism and excretion of the chemical and its metabolites. Problems occur when a chemical induces gene mutations *in vitro*, because comparable endogenous gene mutation assays *in vivo*, which are well validated and reliable, are lacking. Therefore, a gene mutation test with transgenic animals may be a justified alternative for the assessment of mutagenesis *in vivo* (23).

There are two commercially available transgenic mouse models, Muta^{TM}Mouse and Big Blue®, for the detection of gene mutations and small deletions. A disadvantage of these models is the very low response to clastogens because chromosomal rearrangements will not be detected (25). To overcome this restriction, a *lacZ* plasmid-based transgenic animal model (pUR288 plasmid mouse model) was designed by Boerrigter *et al.* (2). This model detects large deletions (>500 base pair) in addition to small deletions and point mutations (7,18,26). The majority of the size-change mutations detected by the *lacZ* plasmid mouse model are chromosomal rearrangements (3).

In the present paper, we investigated the capacities of the pUR288 plasmid mouse and Muta^{TM}Mouse mouse models to detect clastogens. The clastogens used were bleomycin, *m*-AMSA, *o*-AMSA and camptothecin. The selection of these compounds was based on (a) their mechanism of action and (b) their putative clastogenicity based on results of *in vitro* studies (15). Bleomycin induces single- and double-strand breaks by direct cleavage of DNA *in vitro* (19,28) whereas camptothecin, *m*-AMSA and *o*-AMSA are topoisomerase inhibitors that have not been tested in transgenic mouse models before. Exposure to *m*-AMSA and *o*-AMSA

has previously been shown to result in chromosome breaks (4), whereas *m*-AMSA and camptothecin has been shown to induce micronuclei in non-transgenic mice (12,13,28). ENU, a direct acting alkylating agent and point mutagen, was used as a positive control in both transgenic mouse models (27). The *in vivo* micronucleus test was used as a control for detection of clastogenic properties of the compounds used. Based on their specific features, it was expected that both models would detect ENU whereas only the *lacZ* plasmid mouse model would be more sensitive to the clastogens.

Materials and methods

Animals and treatments

Male C57BL/6J transgenic mice (6-12 weeks old) harbouring pUR288 plasmids were bred and maintained under specific pathogen-free conditions at the RIVM. Mice were weighed before treatment and on the day of necropsy. Mice were treated for five successive days by oral gavage with bleomycin (2.5 mg/kg bw; CAS no. 9041-93-4), *m*-AMSA (4'-(9-acridinylamino)-methanesulfon-*m*-anisidide; 3.0 mg/kg bw; CAS no. 51264-14-3), *o*-AMSA (4'-(9-acridinylamino)-methanesulfon-*o*-anisidide; 3.0 mg/kg bw; CAS no. 51264-14-3) and camptothecin (0.5 mg/kg bw; CAS no. 7689-03-4) in a volume of 100 µl methylcellulose (1%). Published data for toxicity in the mouse were used to set dose levels. *o*-AMSA is a much less potent congener of *m*-AMSA and was used as a negative control in the present study. ENU (*N*-ethyl-*N*-nitrosourea, 50% ENU + 47% H_2O + 3% acetic acid, 50 mg/kg bw; CAS no. 759-73-9), dissolved in 10% dimethyl sulfoxide (DMSO), was used as a positive control and injected intraperitoneally (*i.p.*). The negative control mice received the vehicle methylcellulose by oral gavage. Twenty four hours after the final treatment, 25 µl of peripheral blood was collected by orbital punction in EDTA coated tubes for the micronucleus assay. Thirty-five days after final treatment, mice were sacrificed by cervical dislocation. Tissues (bone marrow and liver) were collected, snap-frozen in liquid N_2 and stored at -80°C until used for DNA isolation. Each treatment group consisted of 4-6 mice, which is sufficient to obtain adequate statistical power (~2 fold increase with 80% power and 0.05 alpha).

Male Muta™Mouse mice (6-12 weeks old) were obtained from Covance Research Products Inc. (USA) and maintained under specific pathogen-free conditions at GlaxoSmithKline as previously described (20). Mice were weighed daily during dosing and on the day of necropsy. Mice were treated in the same way with the same compounds and identical tissues were collected as described for the *lacZ* plasmid mice. Mice were killed 35 days after the final treatment by exposure to a rising concentration of CO_2 gas. Twenty-four hours after the final treatment, 160 µl of peripheral blood was collected from the tail vein for the micronucleus assay. Animal treatment and husbandry were in accordance with approved procedures of the Animals (Scientific Procedures) Act, UK, 1986. Each treatment group consisted of 4-6 mice, which is sufficient to obtain adequate statistical power.

All animal experiments were approved by the Institute's Animal Ethics Committee.

***LacZ* gene mutation assay**

To determine the mutant frequency in *lacZ* plasmid mice, total genomic DNA was isolated from bone marrow and liver using a procedure described by Dollé *et al.* (6). Briefly, pUR288 plasmids were rescued from total genomic DNA (20–50 μg) with magnetic beads coated with the *lacZ*/*lacI* fusion protein. These plasmids were subsequently transfected into the electro-competent *E. coli* strain C (*lacZ*$^-$ *galE*$^-$). A fraction (2 μl of the 2 ml total) of the bacterial sample was plated on non-selective 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) plates to determine the rescue efficiency. The remainder was plated onto selective phenyl-β-D-galactoside (P-gal) plates to select for mutants. The *lacZ* mutant frequency was calculated by dividing the number of mutants by the total number of rescued colonies x 1000.

In the Muta^{TM}Mouse assay, high molecular weight genomic DNA was isolated by use of the Stratagene RecoverEase™ DNA isolation kit. A bacteriophage λ packaging extract (Stratagene) was used to excise and package the λgt10-*lacZ* shuttle vector. The resulting phage particles were used to transfect *E. coli* C [Δ*lacZ*, *galE*$^-$, *recA*$^-$, pAA11]. Phage-adsorbed bacteria were plated in the presence of P-gal to select for plaques containing phage with mutant *lacZ*. In parallel, a small amount of phage-adsorbed bacteria was plated under non-selective conditions to determine the rescue efficiency. The mutant frequency for each animal was determined from the ratio of the number of plaque forming units (pfu) produced under positive selection to the total number of pfu estimated from the non-selective plates.

Hybridisation of mutant colonies rescued from the *LacZ* plasmid mouse

For hybridisation, restricted mouse DNA fragments were labelled. For the digestion 250 ng DNA in milliQ-UF water was incubated with NaCl, *HindIII* and digestion mix (100 mM Tris pH 7.6, 80 mM $MgCl_2$ and 10 mM DTT). After digestion, the DNA was stored at -20°C. A ^{T7}Quickprime Kit (Pharmacia) was used containing reagent mix and T7 DNA-polymerase. A maximum of 25 ng total mouse DNA was denaturated at 100°C and put on ice immediately. After centrifugation, the DNA was collected and a mixture of a reagent mix, [α-^{32}P]dCTP and T7 DNA polymerase, was added to the denaturated DNA before incubation at 37°C. The DNA was separated on a Sephadex G50 column to obtain labelled DNA free of any non-incorporated [α-^{32}P]dCTP. The reaction was stopped with TES (containing 10 mM Tris pH 8.0, 1 mM EDTA and 0.1% SDS). Labelled DNA was eluted from a Sephadex G50 column by adding 1 ml TES. The first 600 μl was discarded; the remaining 400 μl, containing the

labelled DNA, was collected. This labelled DNA was used as a probe for hybridisation of the mutant pUR288 clones.

Mutant colonies were grown on selective plates containing P-gal overnight at 37°C and the individual colonies were grafted on a Hybond-N^+ filter (Amershan). To obtain exclusively DNA bound to the filter, the cells were lysed and washed twice with a denaturation buffer and twice in a neutralisation buffer to wash cell debris and unbound DNA from the filter. The filter was washed with 2xSSC (3 M NaCl and 0.3 M $Na_3C_6H_5O_7$) and dried avoiding contact with other DNA sources. For the hybridisation the pre-hybridisation mix (1 M NaCl, 10% dextran sulphate and 1% SDS) was pre-warmed at 65°C. Herring sperm DNA solution was denaturated at 100°C for 5 minutes and added to the pre-hybridisation mix (1/100 volume). The filter was pre-hybridised at 65°C while shaking carefully. The ^{32}P-probe was denaturated at 100°C. After adding the probe to the hybridisation mixture and the filter, it was allowed to cool on ice. The filter was hybridised overnight at 65°C while shaking carefully. After washing several times with 2xSSC, the filter was packed in Saran wrap foil, put against a Phosphor imager and illuminated. The clones positive for mouse DNA were quantified.

The peripheral blood micronucleus test (MN test)

The peripheral blood samples of *lacZ* plasmid mice were analysed using acridine orange staining (11). Microscope slides were coated with acridine orange (10 µl, 1 mg/ml) on a pre-heated plate (65°C). Four microliters fetal calf serum (FCS) and 1 µl blood was pipetted onto the slides and covered with a coverslip. The slides were analysed under a Zeiss Axioscope fluorescence microscope. Per animal the number of micronuclei in 500 polychromatic erythrocytes (PCE) was analysed. The percentage PCEs in 1000 normochromatic erythrocytes (NCE) was calculated as an indicator of bone-marrow cell toxicity.

Each blood sample of Muta^{TM}Mouse was fixed according to the µicroFlow™ mouse micronucleus protocol (Stratagene) and shipped on dry ice to Litron Laboratories (Rochester, NY, USA). The samples were analysed using flow cytometry (FacStarPLUS, Becton Dickinson) according to published methods (5). The number of micronucleated reticulocytes (MnRET) was determined and expressed as a percentage of the total number of reticulocytes (RET) analysed per animal. The ratio of RET relative to the total number of erythrocytes (1,000,000) was calculated to provide an indicator of stem-cell toxicity.

Statistical analysis

The mean *lacZ* plasmid mutant frequency and the micronucleus frequency for the different treatment groups were compared with the Student's *t*-test. The percentages micronucleated PCEs and the size-change mutations (chromosomal rearrangements) were tested for significance with the χ-square test ($\alpha = 0.05$).

Data (unpaired mutant frequency, and paired micronucleus frequency) obtained with the Muta^{TM}Mouse were evaluated initially using analysis of variance following transformation to determine whether there was a statistically significant difference between treatment groups, and to provide an estimate of between-animal variability. Dunnett's method was used to compare the control and treatment groups (10).

Results

Gene mutation assay with transgenic animals

The *lacZ* mutant frequency (MF) was determined in bone marrow and liver of the *lacZ* plasmid and Muta^{TM}Mouse model (Tables 1A and 1B). A consistent increase in the MF after treatment with the different clastogens was neither observed in the *lacZ* plasmid model nor in the Muta^{TM}Mouse. Only a slight but statistically significant increase was seen for bleomycin in the bone marrow of the *lacZ* plasmid model. As expected, the positive control ENU showed a statistically significant increase in all tissues analyzed of both transgenic mouse models.

Table 1A. The *lacZ* MF in bone marrow and liver of the *lacZ* plasmid mouse model.

	Dose (mg/kg.bw)	Animal number	Bone marrow			Liver		
			Total number of plasmids	Number of mutants	MF x 10^{-5}	Total number of plasmids	Number of mutants	MF x 10^{-5}
Methyl cellulose	0	1	666	16	4.8	594	37	6.1
		2	472	20	3.5	468	18	3.7
		3	367	7	1.9	267	14	5.2
		4				314	10	3.3
		5				823	48	5.8
		6				449	44	9.8
		7				351	20	5.7
		8				217	12	5.5
		9				580	35	6.0
		10				460	19	4.1
					3.4 ± 1.5			**5.5 ± 1.8**
ENU	50	1	863	186	32.3	232	54	23.1
		2	361	74	30.7	787	219	27.9
		3	1512	348	24.6	633	91	14.4
		4	710	140	29.6	166	47	28.3
		5	1038	172	24.9	332	66	19.9
					28.4 ± 3.5**			**22.7 ± 5.8****
Bleomycin	2.5	1	520	32	6.2	328	16	4.7
		2	273	17	6.2	527	27	5.0
		3	619	24	3.9	326	13	3.8
		4	389	31	8.0	504	19	3.8
		5	806	28	3.8	455	23	5.1
		6				662	17	2.5
					5.6 ± 1.8*			**4.2 ± 1.0**
m-AMSA	3.0	1	719	15	3.1	936	72	7.7
		2	878	16	2.7	948	54	5.6
		3	1512	29	2.1	447	35	7.7
		4	1116	25	3.4	566	26	4.5
		5				131	6	4.2
					2.8 ± 0.6			**6.0 ± 1.7**
o-AMSA	3.0	1	1048	16	2.3	778	33	4.2
		2	408	14	5.1	854	31	3.7
		3				469	13	2.8
		4				184	9	4.9
					3.7 ± 2.0			**3.9 ± 0.9**
Camptothecin	0.5	1	661	19	2.9	411	17	4.0
		2	1610	130	8.1	838	26	3.0
		3	768	17	2.2	660	22	3.3
		4	1524	66	4.2	1104	32	2.9
		5	861	13	1.6	865	29	3.4
		6				765	26	3.4
					3.8 ± 2.6			**3.3 ± 0.4**

* $p<0.05$

** $p<0.001$

Table 1B. The *lacZ* MF in bone marrow and liver of the Muta^{TM}Mouse model.

	Dose (mg/kg bw)	Animal number	Bone marrow			Liver		
			Total number of plasmids	Number of mutants	MF x 10^{-5}	Total number of plasmids	Number of mutants	MF x 10^{-5}
Methyl cellulose	0	1	113220	25	22.1	740520	12	16.2
		2	832830	19	22.8	279480	9	32.2
		3	191250	6	31.4	572730	25	43.7
		4	256530	8	31.2	549780	26	47.3
		5	222870	16	71.8	450330	33	73.3
		6	270810	7	25.9	775200	25	32.3
		7	83130	1	12.03	355470	39	109.7
		8	293250	31	105.7	315333	14	44.4
		9	283050	1	3.53	424830	21	49.4
		10				188700	18	95.4
					36.3 ± 32.2			**54.4 ± 29.5**
ENU	50	1	204000	294	1441.2	163200	36	220.6
		2	339150	428	1262.0	665550	159	238.9
		3	267750	300	1120.5	687990	238	354.9
		4	371790	449	1207.7	435030	202	464.3
		5	175440	305	1738.5	387090	150	387.5
					1354.0 ± 244.9**			**333.3 ± 102.8**
Bleomycin	2.5	1	427890	15	35.1	249900	3	12.0
		2	308040	23	74.7	435030	19	43.7
		3	397290	6	15.1	366690	23	35.7
		4	334050	22	65.9	368730	30	81.4
		5	314670	9	28.6	364650	35	96.0
					43.9 ± 25.4			**53.7 ± 34.3**
m-AMSA	3.0	1	431970	13	30.1	384540	18	46.8
		2	313242	35	111.7	302940	11	36.3
		3	69870	3	42.9	175950	13	73.9
		4	759900	17	22.4	138210	9	65.1
		5				168810	7	41.5
					51.8 ± 40.9			**52.7 ± 16.1**
o-AMSA	3.0	1	409020	21	51.3	1483080	16	10.8
		2	496740	26	52.3	916470	9	9.8
		3	177480	11	62.0	415650	15	36.1
		4	1945600	12	6.2	189210	12	63.4
					43.0 ± 25.0			**36.4 ± 26.8**
Camptothecin	0.5	1	290700	3	10.3	341230	8	33.2
		2	174420	5	28.7	1048800	19	18.1
		3	201450	19	94.3	554370	21	37.9
		4	154530	3	19.4	310080	16	51.6
		5	230010	19	82.6	316200	22	69.6
					47.1 ± 38.6			**42.1 ± 19.5**

** $p<0.001$

Hybridisation of mutant colonies of the *lacZ* plasmid mice

Mutant colonies of bone marrow of the *lacZ* plasmid mice treated with different compounds were analyzed to determine the percentages of mouse DNA positives clones after hybridisation with labelled total mouse DNA (Figs. 1 and 2 and Table 2).

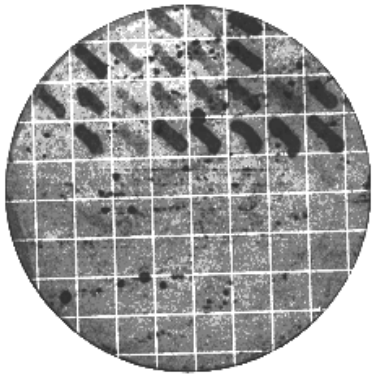

Figure 1: Hybridisation of mutant colonies with total mouse DNA. *LacZ*-negatives mutant clones were collected and grown overnight on a separate plate. Colonies were lifted and hybridised with a probe containing total mouse DNA (for details see material and Section 2). All clones containing (fractions of) mouse DNA stain black.

In the mutant colonies derived from bone marrow of clastogen-treated mice, an increase in the percentages of mouse DNA-positives clones as compared to concurrent controls was seen for all clastogens. As expected such an increase was not observed in ENU-treated mice. Camptothecin was the most positive clastogen, showing the highest increase in the percentage of mouse DNA-positives clones compared with the negative control.

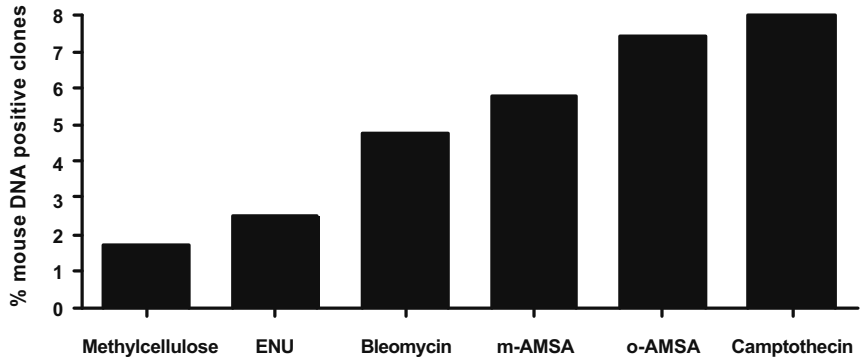

Figure 2: Quantification of mouse DNA-positive *lacZ* mutant clones upon treatment with different clastogens. The percentages of mutant colonies hybridised with total mouse DNA in the bone marrow of the *lacZ* plasmid mouse model after treatment with methylcellulose (negative control), ENU (positive control), bleomycin, *m*-AMSA, *o*-AMSA and camptothecin

Table 2. The percentages of mutant colonies hybridised with total mouse DNA in the bone marrow of the *lacZ* plasmid mice.

Treatment (mg/kg.bw)	Dose (mg/kg.bw)	% of colonies hybridised with total mouse DNA
Methyl cellulose	-	1.72
ENU	50	2.50
Bleomycin	2.5	4.76
m-AMSA	3.0	5.80
o-AMSA	3.0	7.40
Camptothecin	0.5	8.00

Micronucleus test (MN test)

The induction of micronuclei (MN) in both *lacZ* plasmid mice and Muta^{TM}Mouse is shown in Table 3. Rather similar results were observed in both mouse models. Clastogen treatment of *lacZ* plasmid mice and Muta^{TM}Mouse resulted in a slight increase in MN induction in blood cells compared to the negative control methylcellulose. Only camptothecin treatment resulted in a statistically significant increase in the number of micronucleated blood cells.

Strikingly ENU, considered as a compound that predominantly induces point mutations and thus was expected to be negative in these micronucleus tests (1), showed a high and statistically significant increase in micronucleus induction in both transgenic models.

Bone-marrow toxicity, measured as PCE/NCE ratio in plasmid mice or RET ratio in Muta^{TM}Mouse, was decreased in the blood of almost all treated mice and is indicative of sufficient exposure in the target cells (Table 3).

Table 3. Micronucleus induction 24 h after the last treatment in the *lacZ* plasmid mouse and the Muta^{TM}Mouse model.

Compound	Dose (mg/kg bw)	Animal number	%MNPCE	%PCE/NCE	%MnRET	%RET
Methyl cellulose	0	1	0.63	10.0	0.30	3.28
		2	0.35	12.0	0.32	3.54
		3	0.18	27.0	0.38	2.75
		4	0.31	11.2	0.37	2.14
		5	0.61	13.1	0.34	1.48
		6		53.6	0.43	2.05
		7			0.36	2.36
		8			0.37	2.12
		9			0.33	2.99
		10			0.32	10.97
			0.42 ± 0.19	**21.2 ± 17.1**	**0.35 ± 0.04**	**3.37 ± 2.74**
ENU	50	1	3.63	6.7	5.66	0.63
		2	5.56	10.3	4.99	0.56
		3	6.74	11.4	4.96	0.48
		4	6.54	11.0	4.67	0.29
		5	3.26		4.42	0.31
			5.62 ± 1.42**	**9.9 ± 2.1****	**4.94 ± 0.46***	**0.45 ± 0.15****
Bleomycin	2.5	1	1.40	16.1	0.37	3.51
		2	0.21	15.4	0.40	12.79
		3	0.24	13.1	0.32	2.56
		4	0.72	12.0	0.35	13.11
		5	1.20	10.5	0.39	2.02
			0.75 ± 0.55	**13.4 ± 2.3**	**0.37 ± 0.03**	**6.80 ± 5.64**
m-AMSA	3.0	1	0.76	15.7	0.25	1.61
		2	0.68	16.9	0.20	1.76
		3	0.73	15.7	0.22	2.02
		4	0.71	12.4	0.25	1.99
		5	0.98	13.3	0.24	2.09
			0.77 ± 0.14	**14.8 ± 1.9**	**0.23 ± 0.02**	**1.89 ± 0.20**
o-AMSA	3.0	1	0.64	13.4	0.30	2.14
		2	0.70	14.7	0.23	2.62
		3	1.00	13.3	0.22	2.18
		4	0.67	18.2	0.21	2.71
		5	0.80	15.0		
			0.76 ± 0.14	**14.9 ± 2.0**	**0.24 ± 0.04**	**2.41 ± 0.29**
Camptothecin	0.5	1	2.01	15.4	0.32	1.63
		2	0.98	11.6	0.33	1.85
		3	1.75	17.2	0.32	1.50
		4	0.98	11.0	0.34	1.73
		5		9.2	0.35	1.25
		6		7.3		
			1.43 ± 0.53*	**11.3 ± 2.5***	**0.33 ± 0.01**	**1.59 ± 0.23**

* $p<0.05$

** $p<0.001$

Discussion

In the present paper, we studied the capacities of the *lacZ* plasmid mouse model and the Muta™Mouse model to detect clastogens. In addition, we wanted to verify whether the *lacZ* plasmid mouse was a more sensitive model for the detection of clastogenicity compared with the Muta™Mouse model.

The *lacZ* MF for the negative control in both mouse models were comparable with the spontaneous MF similar to those previously reported (2,22). There was no consistent increase in the *lacZ* MF for all tested putative clastogens in either mouse model. Only bleomycin showed a slight but statistically significant increase in MF in bone marrow of *lacZ* plasmid mice. In the accompanying paper, Lynch *et al.* show also a significant increase in mutant frequency and peripheral blood micronuclei after *i.p.* administration of bleomycin (50 mg/kg bw). These results suggest that bleomycin has both mutagenic and clastogenic properties as discussed in the accompanying paper. The plasmid mouse and Muta™Mouse have different genetic backgrounds, which could have an effect on the responses of the different compounds. The plasmid mouse is derived from a B6 background (like the Big Blue mouse), whereas the Muta™Mouse is from a DBA background. Whereas it is known that the DBA strain has different metabolic competencies compared with B6, Lambert *et al.* did not find differences in *lacZ* MF between the DBA and B6 strains (17). Due to phage-packaging constraints, it was expected that clastogenicity would not be detected (*i.e.* would score negative) in the Muta™Mouse whereas for the plasmid mouse an increase in MF was expected after clastogen exposure (8,26). The present findings for ENU and bleomycin, but not for the other clastogens, therefore confirm this expectation. The low induction in *lacZ* MF for the different clastogens cannot be ascribed to the performance of the assay, because ENU induced statistically significant increases in *lacZ* MF compared with the negative control in both models and in both tissues analyzed. In Muta™Mouse there was a 6-40 fold increase in *lacZ* MF (depending on tissue), which is in line with published data (16). The low induction could also be due to the tissue selection and the duration of treatment for the different compounds. The duration of the studies do not meet those considered optimal in the IWGT guidance documents, but the in-life phases of the studies described in this paper were conducted prior to publication of these recommendations (12,23). There is however, clear precedence for a 5-day dosing regimen in the literature and although the effect may not be optimal, there is ample evidence that a mutagenic signal can be observed after this treatment

period (17). The bone marrow and liver were evaluated, as they tend to be default tissues for study in genotoxicity assays (*cf*. comet assay).

Strikingly, also in the MN test the clastogenic effects of the different compounds were not obvious. Although in the plasmid mouse, but not in Muta^{TM}Mouse, a slight increase was seen for all clastogenic compounds, only the exposure to camptothecin resulted in a statistically significant increase in micronucleated erythrocytes in both models. As the PCE/NCE as well as the RET ratio, which are indicative of bone marrow cell toxicity and thus indirectly of exposure, were mostly decreased in all treatment groups compared with the negative control, the absence of genotoxicity (*i.e.* the low MN induction and/or lack of increased MF after treatment with clastogens) is unlikely to be due to lack of exposure of the target cells, although this cannot be excluded. Thus the absence of a response is most likely due to other reasons, *e.g.* effect of cell death (see accompanying paper Lynch *et al.*). The different protocols used for plasmid mouse and Muta^{TM}Mouse blood cells cannot explain the different findings since the MN background frequency in the *lacZ* plasmid mouse (0.42% ± 0.19) was comparable to that observed in the Muta^{TM}Mouse model (0.35 ± 0.04) in the current study, and is consistent with published data (0.42%; Ref. 21).

ENU treatment showed a statistically significant increase in MN frequency compared with the negative controls in both mouse models. This notable result could be explained by the fact that ENU is able to form different types of adducts like O^6-guanine, which may lead to chromosomal damage (9).

The low MF induction levels by the different clastogens investigated in the present study could be due to unsuitable route of administration of the clastogens resulting in an insufficient exposure of the target cells. The results in the accompanying paper (Lynch *et al.*) show that bioavailability can play an important role. For example, when bleomycin is administered orally, there is no induction of micronuclei at different doses, while bleomycin given by *i.p.* route shows a significant induction of micronuclei. However, the toxicity data observed in the MN test imply that there was sufficient bone-marrow exposure in the animals. Another explanation could be the use of inappropriate doses in the present study. Of all clastogens used, only the dose of camptothecin used showed statistically significant increases in the micronucleus frequency compared with the negative control. To explore this further, a dose-effect study with the compounds was performed and is presented in the accompanying paper (Lynch *et al.*).

Although the *lacZ* MF in the plasmid mouse did not increase, the mutation spectrum was characterised to determine whether treatment with the various putative clastogens altered the

"mutational fingerprint" compared with the negative controls. To investigate this, the mutant colonies were analyzed for mouse DNA positives clones by hybridisation with labelled total mouse DNA. In this hybridisation study, all clastogens showed an increase in mouse DNA-positives clones (Table 2). As expected such an increase was not observed in ENU-treated mice. This suggests that there was indeed a biological response associated with treatment; however, the frequency was near or at the limit of detection (*i.e.* background). Whether or not this is biologically meaningful is too early to conclude (but single chromosome rearrangements are clearly associated with carcinogenesis, *e.g.* Philadelphia chromosome on CML). These results indicate that the plasmid mouse assay as a test for chromosomal aberrations does not need to be dismissed yet; increasing the sensitivity, optimisation of the protocol, or the use of more relevant doses of test compounds may lead to more promising results. Clearly, further studies are needed. Increasing the sensitivity of the assay may also be attained through inactivation of DNA repair pathways, especially those involved in the repair of double-strand breaks. Inactivation of homologous recombination, pushing the repair of double-strand breaks towards non-homologous end joining repair, which is an error-prone system, may lead to a better detection of clastogenic compounds.

Despite the unexpected results in the *lacZ* plasmid mice described above, the model may still hold promise for the detection of clastogens. In particular, we consider the finding that the number of mutant colonies with murine chromosomal rearrangements increased after treatment with clastogens justifies further investigations of plasmid mice to detect chromosomal rearrangements and gene mutations in the same *in vivo* system.

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Chapter 3

Is Muta™Mouse insensitive to clastogens?

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Abstract

Several studies suggest that Muta™Mouse is insensitive to clastogens, including the accompanying paper by Mahabir *et al*., which describes a study with bleomycin, camptothecin, *m*-AMSA (4'-(9-acridinylamino)-methanesulfon-*m*-anisidide) and its *ortho*-analogue, *o*-AMSA (4'-(9-acridinylamino)-methanesulfon-*o*-anisidide). Only camptothecin was clastogenic in Muta™Mouse and none of these four compounds induced mutations at the *lacZ* locus. However, to improve exposure, dose range-finding studies were performed in CD2F1 mice, the parental strain of Muta™Mouse. Male CD2F1 mice (n=3) were treated with bleomycin (25-100 mg/kg bw, *p.o.* and *i.p.*), camptothecin (1-10 mg/kg bw *p.o.*) and *m*-AMSA (10-50 mg/kg bw, *p.o.* and 1-5 mg/kg bw *i.p.*) for 5 days and blood was sampled on day 3 and/or day 6 for analysis by flow cytometry to determine %MN-RETs. Camptothecin (1mg/kg bw, day 6) induced a 3.6-fold increase in %MN-RET ($p<0.05$) but was toxic at higher doses. All day-3 camptothecin samples were positive ($p<0.05$). Bleomycin was negative when administered *p.o.* but positive at all doses on both days when given *i.p.* ($p<0.05$) whereas *m*-AMSA was negative when given *i.p.* or orally. Based on these results, male Muta™Mouse mice (5 per group) were dosed daily with bleomycin (50 mg/kg bw) for 5 days or with camptothecin (5 mg/kg bw) for 2 days. Peripheral blood was sampled 24 h after the final dose in each group and tissues were sampled 37 days later. Both compounds induced significant increases in %MN-RET, but only bleomycin induced a significant increase in MF (6-fold in liver, 4.5-fold in kidney and 2-fold in lung) compared with the untreated control. These studies support the view that Muta™Mouse is insensitive to compounds where the genotoxic mechanism of action is predominantly clastogenesis but demonstrates that the peripheral blood micronucleus test is a useful adjunct to the transgenic gene-mutation assay.

Introduction

Clastogens are chemical or physical agents that induce chromosomal breaks, which may result in large deletions in the DNA. The Muta™Mouse assay is an *in vivo* mutagenicity test that allows organ-specific detection of gene mutations in a *lacZ* reporter gene, first described by Gossen *et al.* (1). A number of clastogens have previously been tested in the *lacZ* transgenic gene-mutation assay e.g. MMS and mitomycin-C, with mixed results (2-4). In the accompanying paper by Mahabir *et al* (5), we reported that exposure to a series of putative clastogens (bleomycin, camptothecin, *m*-AMSA and *o*-AMSA) did not result in an increase in the mutant frequency (MF) in bone-marrow and liver in either the pUR288 plasmid mouse model or in Muta^{TM}Mouse. Moreover, only camptothecin treatment induced a statistically significant increase in clastogenic effects in the pUR288 plasmid mouse and Muta^{TM}Mouse when assessed with the peripheral blood micronucleus (PB-MN) assay, although bleomycin treatment resulted in a significant increase in mutation frequency in the liver of the pUR288 plasmid mouse.

The *in vivo* micronucleus test is used to screen compounds for clastogenic (leading to chromosome breakage) and aneugenic (resulting in chromosome loss) activity (6,7). In the flow-cytometry PB-MN assay, micronuclei are evaluated in new erythrocyte cell populations, which are identified by immunocyto-chemistry with antibodies against the transferrin receptor (CD71; Ref. 8). Given the negative results of our original studies, particularly in the PB-MN assay, we were concerned that the animals had not been adequately exposed, particularly as doses were selected based on toxicity data reported in the literature. Therefore, we concluded that additional studies were required to see whether the lack of a response in our original study was due to a lack of sensitivity to clastogens in the Muta^{TM}Mouse model or to inadequate exposure to the test compounds. To address these issues, dose range-finding studies were conducted using the same set of compounds in non-transgenic animals with the same genetic background as Muta™Mouse. Clastogenicity was determined using the flow-cytometry PB-MN assay. Based on the results of the dose-finding studies, further Muta™Mouse gene-mutation studies were conducted with camptothecin and bleomycin. The results of these studies are reported below.

Materials and methods

Animal supply, husbandry and treatment

Male CD2F1 mice (age, approximately 7 weeks) were obtained from Charles River (France) and male Muta™Mouse mice (age, approximately 10 weeks) from Covance Research Products Inc. (USA). Animals were allowed to acclimatise for at least 5 days prior to treatment and were housed 3-5 mice per cage according to treatment group. The animals had free access to food (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan, UK) and water (Three Valleys Water plc). Environmental controls were set at 21 ± 2°C, 55 ± 10% relative humidity and fluorescent lighting between 06.00 to 18.00 h GMT. All animal treatment and husbandry were in accordance with approved procedures of the Animals (Scientific Procedures) Act, UK, 1986.

Dose range finder studies

Study 1: male CD2F1 mice (3 animals per group) were dosed orally for 5 consecutive days with bleomycin (25, 50 and 100 mg/kg bw/day), camptothecin (1, 5 and 10 mg/kg bw/day), *m*-AMSA (10, 25 and 50 mg/kg bw/day) and benzo[a]pyrene (150, 300 and 500 mg/kg bw/day). Negative control animals received 1% w/v aqueous methylcellulose. Blood (120µl) was collected via the retro-orbital sinus route 24 h after the last treatment.

Study 2: The route of administration for bleomycin and *m*-AMSA was changed to improve exposure. Male CD2F1 mice (3 animals per group) were dosed *i.p.* for 5 days with bleomycin (25, 50 and 100 mg/kg bw/day) and *m*-AMSA (1, 3 and 5 mg/kg bw /day) and orally for 2 days with camptothecin (1, 5 and 10 mg/kg bw/day). Six untreated negative control animals were included. Blood (120µl) was collected as above 24 h after the second dose (day 3) and the last dose (day 6).

Muta^{TM}Mouse assay

Male Muta^{TM}Mouse mice (5 animals per group) were dosed with bleomycin 50 mg/kg bw/day *i.p.* for 5 consecutive days, or with camptothecin at 5 mg/kg bw/day *p.o.* for 2 consecutive days. Negative control animals received saline and positive control animals were treated with MNU, 50 mg/kg/day *i.p.* for 5 days. Blood (120µl) was collected as above approximately 24 h after the final dose (day 3 for camptothecin and day 6 for bleomycin and MNU). All animals were humanely killed 37 days after the last dose. Various somatic tissues

were removed at necropsy and flash frozen in liquid nitrogen before storage at -80°C. Gene mutations at the *lacZ* locus were analysed in kidney, liver and lung (bleomycin only).

The peripheral blood micronucleus test (MN test)

Blood samples were fixed according to the µicroFlow™ mouse micronucleus protocol (Stratagene, La Jolla, CA). The samples were analysed by use of flow cytometry (FacStarPLUS, Becton Dickinson) as described by Dertinger *et al.* (8). Blood samples infected with a malarial parasite were used as an internal control (9). The frequency of micronucleated reticulocytes (MN-RETs) and micronucleated normochromatic erythrocytes (MN-NCEs) were calculated to provide an indication of genotoxic potential. The frequency of RETs, relative to the number of total red blood cells was measured to provide an indication of stem-cell toxicity. Up to 20,000 RETs were analysed per sample.

***LacZ* gene mutation assay**

High molecular weight genomic DNA was extracted from the Muta™Mouse tissues using the RecoverEase™ DNA isolation kit (Stratagene). The λgt10-*lacZ* shuttle vector was recovered from the genomic DNA using Transpack® Packaging extract (Stratagene) and the lambda phage particles were used to transfect *Escherichia coli* C [*ΔlacZ*, *galE*$^-$, *recA*$^-$, *Kan*^{r}] cells. Phage-adsorbed bacteria were plated in the presence of phenyl-β-D-galactoside (P-gal) to select for plaques containing phage with mutant *lacZ*. In parallel, a small number of phage-adsorbed bacteria were plated under non-selective conditions to determine the rescue efficiency. The mutation frequency for each animal was determined from the ratio of the number of plaque-forming units (pfus) produced under positive selection to the total number of pfu estimated from the non-selective plates.

Statistical analysis

Micronucleus data were evaluated using Dunnett's method to compare the control and treatment groups (10).

Results

Peripheral blood micronucleus test (PB-MN test)

CD2F1 mice

The results of the assay to test micronucleus (MN) induction in CD2F1 mice are shown in Table 1.

Following oral exposure, benzo[a]pyrene induced statistically significant ($p<0.05$) increases in %MN-RETs and decreases in %RETs in CD2F1 mice, although the reduction in reticulocyte frequency was not statistically significant. Oral treatment with bleomycin and *m*-AMSA did not induce significant increases in %MN-RETs at any of the doses tested in CD2F1 mice. There were significant decreases in %RETs in all bleomycin-treated mice, consistent with target-tissue toxicity, but not in *m*-AMSA treated mice. Camptothecin induced a statistically significant ($p<0.05$) increase in %MN-RETs at 1 mg/kg bw/day orally but higher doses caused excessive levels of bone-marrow cytotoxicity and samples could not be scored. As the data for bleomycin and *m*-AMSA were negative, additional dose-finding studies were conducted using an intra-peritoneal route of administration to improve exposure. In the repeat study, bleomycin (25-100 mg/kg bw/day) given by the *i.p.* route induced dose-dependent and statistically significant increases in %MN-RETs in day-3 blood samples and in day-6 blood samples, with concomitant decreases in %RETs (*i.e.* indicating stem cell cytotoxicity). In contrast, *m*-AMSA (1-5 mg/kg bw/day, *i.p.*) was negative for MN induction at all doses, both on day 3 and day 6. The lack of cytotoxicity for *m*-AMSA suggested inadequate exposure despite the change in route of administration. Camptothecin (given orally at 1-10mg/kg bw for 2 days) induced statistically significant ($p<0.05$) increases in %MN-RETs at all doses on day 3.

Table 1. Summary of peripheral blood micronucleus data in CD2F1 mice.

Compound	route of admin.	Dose (mg/kg/day)	Blood sample day	*Mean %MN-RET ± S.D.*	*Mean %RET ± S.D.*
Study 1					
Vehicle control	*p.o.*	0	6	0.19 ± 0.04	1.41 ± 0.16
Bleomycin	*p.o*	25	6	0.23 ± 0.06	0.94 ± 0.28 *
		50	6	0.21 ± 0.04	0.96 ± 0.02 *
		100	6	0.19 ± 0.04	0.66 ± 0.05 *
Camptothecin	*p.o*	1	6	0.70 ± 0.35 *	0.92 ± 0.14 *
		5	6	Cytotoxicity	0.13 ± 0.03 *
		10	6	Cytotoxicity	0.13 ± 0.05 *
***m*-AMSA**	*p.o*	10	6	0.24 ± 0.1	1.52 ± 0.28
		25	6	0.25 ± 0.04	1.57 ± 0.33
		50	6	0.25 ± 0.06	1.52 ± 0.32
Benzo[a]pyrene	*p.o*	150	6	0.45 ± 0.05 *	0.95 ± 0.32
		300	6	0.48 ± 0.08 *	1.00 ± 0.12
		500	6	0.66 ± 0.06 *	0.95 ± 0.42
Study 2					
Untreated Control	-	-	3	0.27 ± 0.04	1.93 ± 0.4
			6	0.21 ± 0.04	2.13 ± 0.74
Bleomycin	*i.p*	25	3	0.50 ± 0.05 *	1.20 ± 0.36 *
			6	0.48 ± 0.06 *	1.27 ± 0.43
		50	3	0.88 ± 0.04 *	1.03 ± 0.14 *
			6	0.69 ± 0.14 *	0.51 ± 0.14 *
		100	3	1.06 ± 0.26 *	0.73 ± 0.06 *
			6	0.86 ± 0.08 *	0.38 ± 0.2 *
Camptothecin	*p.o*	1	3	0.91 ± 0.22 *	1.55 ± 0.7
		5	3	2.86 ± 0.21 *	0.28 ± 0.04 *
		10	3	1.73 ± 0.11 *	0.30 ± 0.1 *
***m*-AMSA**	*i.p*	1	3	0.29 ± 0.05	1.64 ± 0.2
			6	0.22 ± 0.04	1.7 ± 0.51
		3	3	0.28 ± 0.05	1.98 ± 0.28
			6	0.27 ± 0.06	1.72 ± 0.1
		5	3	0.32 ± 0.01	2.04 ± 0.24
			6	0.31 ± 0.1	2.28 ± 0.39

*Statistical significance * $p<0.05$, Dunnett's test (1955).*

Muta™Mouse mice

On the basis of the range-finding studies in CD2F1 mice described above, Muta™Mouse studies were only conducted with bleomycin and camptothecin. MNU treatment was used as a positive control. All of the compounds tested induced an increase in micronucleus induction in immature (CD71-positive) erythrocytes in peripheral blood compared with the negative control (Table 2). There was a concomitant decrease in mean frequency of reticulocytes in each treatment group compared with the negative control, demonstrating bone-marrow cytotoxicity consistent with target-tissue exposure. Overall, bleomycin (50 mg/kg bw/day, *i.p.* for 5 days) induced a 4-fold increase, camptothecin (5 mg/kg bw/day, *p.o.* for 2 days) induced a 4.6-fold increase and MNU (50 mg/kg bw/day, *i.p.* for 5 days) a 2-fold increase in % MN-RETs compared with the vehicle control, consistent with bone-marrow clastogenicity.

Table 2. Summary of peripheral blood micronucleus data in Muta™Mouse**.**

Treatment	%MnRETs (Mean ± SD)	Fold Change	No. RET (Mean ± SD)	Relative Cytotoxicity
Control[1]	0.15 ± 0.07	-	6259 ± 1140	100%
Bleomycin[1] (50 mg/kg/day *i.p.*)	0.61 ± 0.33 *	4	4788 ± 1153	76.5%
Camptothecin[2] (5 mg/kg/day *p.o.*)	0.69 ± 0.17 *	4.6	2363 ± 532	37.7%
MNU[1] (50 mg/kg/day *i.p.*)	0.29 ± 0.12 *	2	3363 ± 112	53.7%

SD: Standard deviation. Statistical significance.

[1] *5 days treatment and blood sampled on day 6.*

[2] *2 days treatment and blood sampled on day 3.*

(*) Based on Dunnett's test ($p<0.05$).

Gene mutation assay with Muta™Mouse

Gene mutation frequencies (MF) were determined in the liver, kidney and lung (bleomycin only) sampled on day 42 after the start of treatment. A summary of the results is shown in Table 3.

Bleomycin (50 mg/kg bw/day, *i.p.* for 5 days) induced a significant increase in MF compared with the untreated control in the liver (6-fold change), the kidney (4.5-fold change) and the lung (2-fold change). In contrast, camptothecin treatment (5 mg/kg bw/day, *p.o.* for 2 days) did not bring about any changes in MF compared with the untreated control in any of the tissues examined (liver and kidney). The positive control MNU (50 mg/kg bw/day, *i.p.* for 5 days), induced a significant increase in MF in the kidney (4-fold) compared with the vehicle control, but not in the liver.

Table 3. Summary of mutation frequency data at the lacZ locus in Muta™Mouse.

Compound (route)	Dose (mg/kg/day)[1]	Animal Number	Liver			Kidney			Lung		
			Total pfu	No. of mutants	MF x 10^{-6}	Total pfu	No. of mutants	MF x 10^{-6}	Total pfu	No. of mutants	MF x 10^{-6}
Control	**0**	1	16,665	2	42.8	48,807	2	40.9	82,467	7	84.8
		2	47,583	1	21	42,840	1	23.3	360,774	24	66.5
		3	121,788	5	41	184,212	4	21.7	89,658	7	78
		4	52,938	2	37.7	32,283	2	61.9	462,060	34	73.5
		5	100,674	6	59.5	66,402	4	60.2	268,974	15	55.7
					40.4 ± 13.7			**41.6 ± 19.3**			**71.7 ± 11.2**
Bleomycin[2] (*i.p.*)	**50**	1	110,619	30	271.2	147,492	42	284.7	226,440	30	132.4
		2	376,533	104	276.2	120,870	17	140.6	59,823	6	100.2
		3	-	-	-	134,793	23	170.6	336,588	52	141.8
		4	52,479	10	190.5	48,654	7	143.8	-	-	-
					245.9 ± 48.1			**184.9 ± 67.9**			**124.8 ± 21.8**
Camptothecin (*p.o.*)	**5**	1	71,910	3	41.7	331,092	6	18.1			
		2	68,850	2	29.1	105,723	2	18.9			
		3	119,646	3	25.1	*	*	*			
		4	125,460	4	31.8	58,446	3	51.3			
		5	632,196	3	4.7	101,286	4	39.4			
					26.5 ± 13.6			**31.9 ± 16.3**			
MNU (*i.p.*)	**50**	1	156,978	9	57.3	121,941	24	196.8			
		2	93,024	7	75.2	226,746	45	198.4			
		3	54,009	3	55.5	64,260	10	155.6			
		4	98,226	4	40.7	63,342	12	189.4			
		5	63,648	4	62.8	99,450	12	120.6			
					58.3 ± 12.5			**172.2 ± 33.6**			

pfu: plaque-forming units; MF: mutation frequency; bold: mean ± standard deviation.

[1] Treatment was for 5 days except camptothecin (2 days).

[2] One animal died.

Discussion

In the present paper we investigated whether the lack of a response seen in the study described in our accompanying paper by Mahabir *et al.* (5) was due to a lack of sensitivity to clastogens in the Muta^{TM}Mouse model or to inadequate exposure to the test compounds. To address these issues, dose range-finding studies were conducted using the same set of compounds in non-transgenic animals with the same genetic background (CD2F1) as Muta™Mouse. Clastogenicity was determined using the flow-cytometry PB-MN assay. Based on the results of these studies, further Muta™Mouse gene-mutation studies were conducted.

Background MN-frequencies (mean ± S.D.) in the peripheral blood of negative controls were similar in CD2F1 mice (0.19 ± 0.04) and Muta^{TM}Mouse (0.15 ± 0.07) in the current study. However, these were slightly lower than seen previously in the study described by Mahabir *et al.* (0.35 ± 0.04; see accompanying paper). In the *lacZ* gene-mutation assay control MF in the liver (41 x 10^{-6}) and kidney (41 x 10^{-6}) were in accordance with the published literature (11), whereas the data for lung (72 x 10^{-6}) was slightly higher than previously reported (12).

In the PB-MN dose range-finding study in CD2F1 mice, the positive control B(a)P induced dose-dependent and statistically significant increases in %MN-RETs when given orally at doses of 150-500 mg/kg bw/day for 5 days. B(a)P has been shown to induce gene mutations in various organs (fore-stomach, spleen, colon and glandular stomach) in Muta^{TM}Mouse when given orally at doses of 75 and 125 mg/kg bw/day for 5 consecutive days (13). These B(a)P doses were also shown to be carcinogenic in Muta^{TM}Mouse (14). In contrast, *m*-AMSA, a topoisomerase II inhibitor and cytotoxic agent used in cancer therapy, was negative in the dose range-finding studies in CDF1 mice using the PB-MN assay, when given orally at doses of 1-50 mg/kg bw/day or of 1-5 mg/kg bw/day via the intra-peritoneal route for 5 consecutive days, respectively. These doses were not associated with stem-cell toxicity as determined by % RETs. Because of the negative results, *m*-AMSA was not tested further in the Muta^{TM}Mouse assay.

Bleomycin, a glycopeptide antibiotic, is an anti-tumour agent that can cause intracellular double-strand breaks in DNA. Bleomycin induced significant increases in peripheral blood micronucleus frequencies in both transgenic and CD2F1 mice, the parental strain of Muta^{TM}Mouse. These data confirm that bleomycin is both clastogenic and cytotoxic to the bone-marrow of mice when administered at doses of 25 mg/kg or above by the intra-

peritoneal route but not when given orally. Bleomycin given by the *i.p.* route also induced a significant increase in hepatic, renal and pulmonary mutation frequencies in Muta™ Mouse compared with the vehicle control. These studies suggest that route of administration, and therefore systemic bio-availability is an important factor in the genotoxicity of bleomycin *in vivo* (indeed, both dose and route should be given careful consideration in the design of all *in vivo* studies). The results, reflecting both mutagenic and clastogenic activity, are consistent with the known mechanisms of action and types of DNA damage induced by bleomycin (15), including gene mutations (via oxidative damage) and single- and double-strand DNA breaks. Bleomycin is also a well-characterised lung toxin that causes pulmonary fibrosis in both rodents and humans, through the generation of reactive oxygen species (16). To our knowledge, this is the first report described in the literature of pulmonary mutagenesis induced by bleomycin *in vivo.*

Camptothecin is a cytotoxic anti-tumour agent that specifically inhibits DNA topoisomerase I and indirectly induces DNA single-strand breaks, probably by drug-induced stabilisation of topoisomerase I enzyme-DNA cleavage complexes, resulting in chromosome aberrations *in vitro* (17,26) and *in vivo* (18,19). Camptothecin induced a 4.6-fold increase in %MN-RETs and a decrease in %RETs in the peripheral blood micronucleus assay, confirming the bone-marrow clastogenicity and cytotoxicity observed in our original study (Mahabir *et al*, accompanying paper). However, under the present experimental conditions, camptothecin was negative for the induction of gene mutations at the *lacZ* locus in Muta™ Mouse. This may be due to the mechanism of action of camptothecin and in particular, the nature and size of the DNA deletions induced by this compound. The λgt10-*lacZ* shuttle vector is approximately 47kb in length and is flanked by *cos* sites. Within the Muta™ Mouse genome, the λgt10-*lacZ* shuttle vector resides as a concatamer of approximately 40 copies (20), which is located on chromosome 3 (21). The *cos* sites are recognised by the Transpack® Packaging extract used to construct viable phage. However, phage particles are unable to package vectors less than 40 kb or greater than 52 kb in size (22) and therefore will not rescue vectors containing DNA deletions of 5-10 kb or more. Thus, if the majority of DNA lesions induced by camptothecin are a result of large DNA deletions (*i.e.* >10 kb), it is unlikely that they would be detected in the Muta™ Mouse assay. The negative result in the gene mutation arm of the current study suggests that camptothecin is not a mutagen, but the positive peripheral blood micronucleus data confirms that camptothecin is a mammalian clastogen *in vivo*. The

observation of mouse DNA-positives clones in the *lacZ* plasmid mouse assay (Mahabir *et al.*, accompanying paper) would appear to support this conclusion.

MNU was chosen as the positive control in the present Muta™ Mouse study because it is a direct-acting carcinogen (alkylating agent) and has previously been reported to increases the mutation frequency in various tissues in Muta™ Mouse (23) and in other transgenic models, for example, *rspL* transgenic mice (24). MNU induced mutations in the kidney but was, unexpectedly, negative in the liver. MNU induced a 2-fold increase in %MN-RETs and a decrease in %RETs, which is consistent with bone-marrow clastogenicity and cytotoxicity.

The studies reported here support the view that Muta™Mouse gene-mutation assay is insensitive to compounds whose genotoxic mechanism of action is predominantly clastogenesis. It is interesting to note, however, that despite the suboptimal assay design both bleomycin and MNU were positive for clastogenicity and mutagenicity in Muta™ Mouse (although MNU was not positive for mutagenicity in all tissues tested). It is possible that with extended dosing and tissue sampling, as recommended by Thybaud *et al.* (25), all of the compounds under investigation would have come out positive. This requires further investigation. Nevertheless, this study demonstrates that the peripheral blood micronucleus assay is a useful adjunct to the transgenic gene-mutation assay, since potential clastogenicity and cytotoxicity may be determined in the same animals without much additional effort. Furthermore, because of the discrepancies with regard to compound toxicity observed in the current study and in those previously reported in the literature, our studies suggest that the CD2F1 genetic background may be a-typical for mouse strains commonly used in genetic toxicology.

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Chapter 4

DNA-repair-deficient *Rad54/Rad54B* mice are more sensitive to clastogens than wild-type mice

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Abstract

The sensitivity of DNA-repair-deficient *Rad54/Rad54B* mice for clastogens was studied and compared to that of wild-type mice. *LacZ* mutant frequencies (MF) in *Rad54/Rad54B* mice, after treatment with mitomycin C (MMC), bleomycin (BLM) and γ-irradiation, were compared to those of the wild-type mice following the same treatment. While none of the clastogens showed an induction of the *lacZ* MF in the wild-type mice, there was a significant increase of the *lacZ* MF in the bone marrow of the *Rad54/Rad54B* mice after treatment with BLM and γ-irradiation and in the spleen after MMC treatment. As expected, the positive control ENU showed a significant increase in the *lacZ* MF in all tested organs in the wild-type mice. Mutant colonies were hybridized with total mouse DNA in order to discriminate between small gene mutations and large DNA rearrangements and translocations (size-change mutations). The hybridization studies showed a significant increase in mouse DNA positive clones 4 days after treatment with MMC and BLM in the bone marrow of the wild-type mice, which is indicative for chromosomal rearrangements and translocations to occur. An even more pronounced increase was seen 28 days after treatment with the same compounds in the *Rad54/Rad54B* mice.

Introduction

Humans are exposed, on a daily basis, to numerous chemicals such as those present in food/feed additives, packing material, drugs, cosmetics and pesticides. It is of vital importance that before marketing, chemicals present in those products are evaluated for their potential adverse health effects. Genotoxic agents are a major threat to the integritiy of chromosomes and viability of cells, specially if the damage is not repaired, because it can lead to chromosome instability, cell cycle arrest, cell dysfunction, induction of apoptosis or carcinogenesis (10). For genotoxicity, two main endpoints are gene mutations and chromosome aberrations; the latter can either be structural (clastogenic) or numerical (aneugenic). The strategy for the assessment of the potential genotoxicity of chemicals is assessed in short-term *in vitro* and *in vivo* genotoxicity tests covering these endpoints.

For all genotoxic endpoints reliable and relatively validated *in vitro* tests exist. A positive *in vitro* test triggers *in vivo* testing for the same genotoxic endpoint. In contrast to chromosomal aberration inducers, when a chemical induces gene mutations *in vitro,* there is not a validated and reliable *in vivo* gene mutation assay. The *in vivo* gene mutation assay with transgenic animals may be a suitable and justified alternative *in vivo* test (11,16). Lac operator-based transgenic reporter mice and rats contain many copies of a bacterial reporter gene. These transgenic, mainly the bacterial *lacI* or *lacZ* genes, are present in a shuttle vector and are transmitted by the germ cells, and thus presented in multiple copies of every cell including the germ cells. The two commercially available mouse models, Muta^{TM}Mouse and Big Blue® are able to detect point mutations and small deletions or insertions. The main restriction of these models is that large deletions cannot be detected because in order for the bacteriophage to infect *Escherichia coli* for the recovery of a lengthy insert, flanking cos-sites are essential. The transgenic mouse model with the *lacZ* transgene on a plasmid vector, the pUR288 plasmid mouse model designed by Boerrigter *et al.* (2) can surpass this problem and is capable of detecting large deletions in addition to point mutatios (>500 base pairs; Refs. 5,14,17) Large deletions or translocations may lead to the *ad random* fusion of the *lacZ* transgene with mouse chromosomal DNA, such mutations are referred to as “size-change mutations” (5,6). The majority of these size-change mutations detected by the lacZ plasmid mouse model are chromosomal rearrangements (3). These include translocations, resulting from breaks and incorrect repair or DNA fusion. Therefore, both genotoxic endpoints may be assessed in one instead of two separate tests reducing the number of laboratory animals.

Previous studies investigating the capacity of the pUR288 plasmid mouse model to detect clastogens demonstrated some sensitivity towards clastogens, although the response was not very robust (13). A possible reason for this maybe that these mice are repair-proficient and are able to counteract clastogenic actions of compounds. In the present study we investigated the effect of DNA-repair on the sensitivity of pUR288 mice towards clastogenic compounds. Wild-type (WT) and *Rad54/Rad54B* repair-deficient (*Rad54/Rad54B*) mice (8,19), both harboring the *lacZ* gene, were exposed to three clastogens, mitomycin C (MMC), bleomycin (BLM) or γ-irradiation. The *Rad54/Rad54B* mutations, introduced to increase the number of chromosomal rearrangements, were compared to WT mice. The *Rad54* and *Rad54B* genes are involved in homologous recombination (HR) repair. Deletion of *Rad54* and *Rad54B* results in defective HR, and might result in a shift towards other repair systems for chromosomal breaks like non-homologous end-joining (NHEJ) repair. As NHEJ is error-prone, it may repair the initial damage erroneously, leading to detectable chromosome rearrangements, as compared to the wild-type situation. The *in vivo* micronucleus test (MN test) was used as a classical control for detection of clastogenic properties of the compounds used.

Animals, materials and methods

Animals and treatments

Animal protocols were approved by the institutional animal ethics committee. Eight- to 12-week-old wild-type (WT) and *Rad54/Rad54B* repair-deficient (*Rad54/Rad54B*) mice both harbouring pUR288 plasmids (*lacZ*) were bred and maintained under specific pathogen-free conditions at the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Mice were weighed before dosing and on the day of necropsy. Mice were *intraperitoneally* (*i.p.*) treated with 1.25 mg/kg body weight (bw) MMC (CAS no. 50-07-7), 2.5 mg/kg bw BLM (CAS no. 9041-93-4), 100 mg/kg bw ENU (only in WT mice, CAS no. 759-73-9), all in a volume of 100 µl phosphate buffered saline (PBS) or irradiated with 5 Gy γ-irradiation. Untreated control mice of both genotypes received (through *i.p.*) the vehicle PBS in a volume of 100 µl.

Both at 4 and 28 days after treatment, mice were sacrificed by cervical dislocation. Tissues (bone marrow, liver and spleen) were collected, snap frozen in liquid N_2 and stored at -80 ◦C until used for DNA isolation. At the same time points three drops of peripheral blood were collected by orbital punction in EDTA coated tubes for the micronucleus test. The remaining peripheral blood was collected in EDTA coated tubes for histological analysis.

***LacZ* gene mutation assay with transgenic animals**

The mutant frequency (MF) in WT and *Rad54/Rad54B* mice was determined using a procedure described by Dollé *et al.* (4). Briefly, pUR288 plasmids were rescued from total genomic DNA (20-50 µg) using magnetic beads coated with the *lacZ*/*lacI* fusion protein. After detachment from the beads, these plasmids were subsequently transfected into electrocompetent *E. coli* strain C (*lacZ*$^-$ *galE*$^-$). A fraction (2 µl of the 2 ml total) of the bacterial sample was plated on non-selective 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) plates to determine the rescue efficiency; the remainder onto selective phenyl-β-D-galactoside (P-gal) plates to select for mutants. The *lacZ* mutant frequency was calculated by dividing the number of mutants by the total number of rescued colonies × dilution factor (1000).

Hybridization of mutant colonies with total mouse DNA

LacZ mutants that originate from aberrant translocation events and that carrying "ad random" mouse DNA fragments were made visible through hybridization using total mouse DNA as a probe. Hereto total mouse DNA (250 ng in MilliQ-UF) was incubated with *Hind*III in digestion mix (100 mM Tris pH 7.6, 80 mM $MgCl_2$ and 10 mM DTT). After digestion, the DNA was stored at - 20°C. A Gene Images Random Prime Labelling kit was used containing nucleotide mix, primer and enzyme solution (Klenow). A maximum of 50 ng total mouse DNA was denaturized at 100°C and put on ice. After centrifugation, the DNA was collected and a mixture of nucleotide mix, primer and enzyme solution, was added before incubation at 37°C for 1 h. The reaction was stopped by adding 0.5 M EDTA pH 8.0 and the mixture was put on ice. This labelled DNA was used as a probe for hybridization of the mutant pUR288 clones.

LacZ mutant colonies were grown overnight on selective P-gal plates at 37°C and the individual colonies were grafted on a Hybond-N^+ filter (Amershan). To obtain exclusively DNA bound to the filter, the cells were lysed and washed twice with a denaturation buffer and twice in a neutralization buffer to remove cell debris and unbound DNA. The filter was washed with 2×SSC (3 M sodium chloride and 0.3 M sodium citrate) and dried avoiding contact with other DNA sources. For the hybridization the pre-hybridization mix (1 M NaCl, 10% dextran sulphate and 1% SDS) was pre-warmed at 65°C. Herring sperm DNA solution was denaturized at 100°C for 5 min and added to the pre-hybridization mix (1/100, v/v) solution. The mixture was pre-hybridized at 65°C for approximately 3 h. The probe was denaturized at 100°C for 5 min. Before adding the probe to the hybridization mixture and the blot, it was allowed to cool on ice. After adding the probe to the hybridization mixture, the blot was hybridized overnight at 65°C while shaking carefully. After washing once with 1×SSC (3 M sodium chloride and 0.3 M sodium citrate) and 0.1% SDS, and once with 0.5× SSC (3 M sodium chloride and 0.3 M sodium citrate) and 0.1% SDS, the blot was packed in wrap foil (Saran), put against a Phosphor imager (Storm 860, Molecular Dynamics, GE Healthcare Bio-Sciences) and illuminated. Mutants that hybridized with mouse DNA were counted.

The peripheral blood micronucleus test

The peripheral blood samples of treated and control WT and *Rad54/Rad54B* mice were analyzed using acridine orange staining (9). Slides were coated with acridine orange (10 µl, 1 mg/ml) on a pre-heated plate (65°C). Eight microliters of FBS (fetal bovine serum) and 3 µl blood was pipette on slides and covered with a cover slip. The slides were analyzed under a Zeiss Axioscope fluorescence microscope. Per animal, the frequency of MN was analyzed in 1000 polychromatic erythrocytes (PCE) in both WT mice and *Rad54/54B* mice. The percentage PCEs in 2000 normochromatic erythrocytes (NCE) was determined as an indicator of cytotoxicity in both mouse models.

Statistical analysis

The mean *lacZ* plasmid mutant frequency, the percentages of micronucleated PCEs and the size-change mutations (chromosomal rearrangements) for the different treatment groups were tested for significance with the Student's *t*-test.

Results

LacZ gene mutation assay with transgenic animals

WT and *Rad54/Rad54B* mice were treated *i.p.* once with PBS (negative control) and three different clastogens, MMC, BLM or γ-irradiation. Only WT mice were treated with the mutagen ENU, which served as a positive control for the *lacZ* plasmid rescue procedure.

The *lacZ* mutant frequency was determined 4 and 28 days after treatment in bone marrow, liver and spleen (Figs. 1 and 2). In the WT mice, no consistent increase in the *lacZ* MF was found in all tissues tested after treatment with the clastogens compared to the untreated control. However, in *Rad54/Rad54B* mice treated with BLM and γ-irradiation a slight but statistically significant ($p<0.05$) increase in the *lacZ* MF in the bone marrow compared to the untreated control was found (Fig. 2A). This increase was already observed 4 days after treatment with BLM. This result may indicate that the *Rad54/Rad54B* mice are more sensitive to clastogens compared to the WT mice. Although there was no consistent increase in the *lacZ* MF of the liver in the *Rad54/Rad54B* mice after treatment with all compounds, there was a rather high background *lacZ* MF in the untreated control compared to the untreated control in the liver of WT mice.

As expected, the positive control compound ENU, tested in WT mice only, showed a statistically significant ($p<0.05$) increase in *lacZ* MF in all tissues analyzed compared to the untreated control.

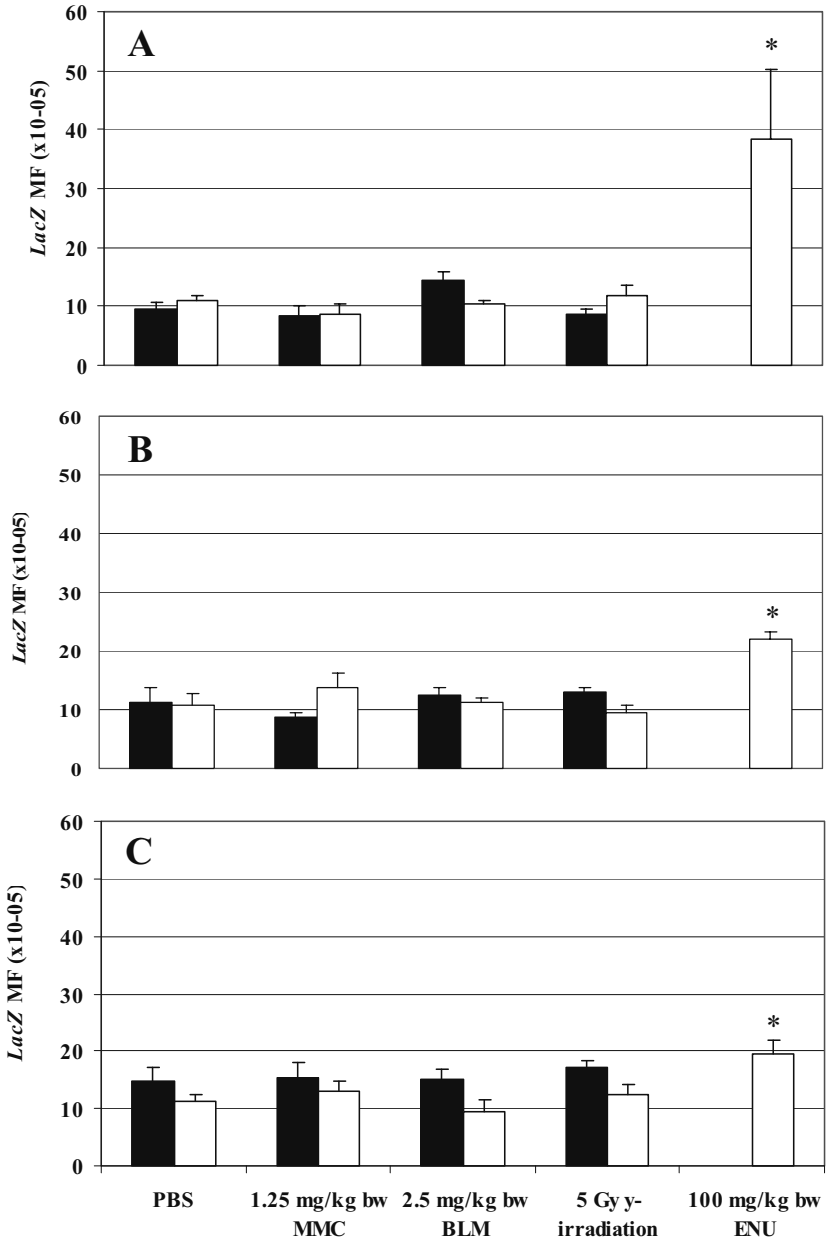

Figure 1: The *lacZ* MF in WT mice 4 (black bars) and 28 days (grey bars) after treatment with PBS (negative control), ENU (positive control), clastogens MMC, BLM and γ-irradiation, were given at the indicated doses.

A: Bone marrow, **B:** liver, **C:** spleen

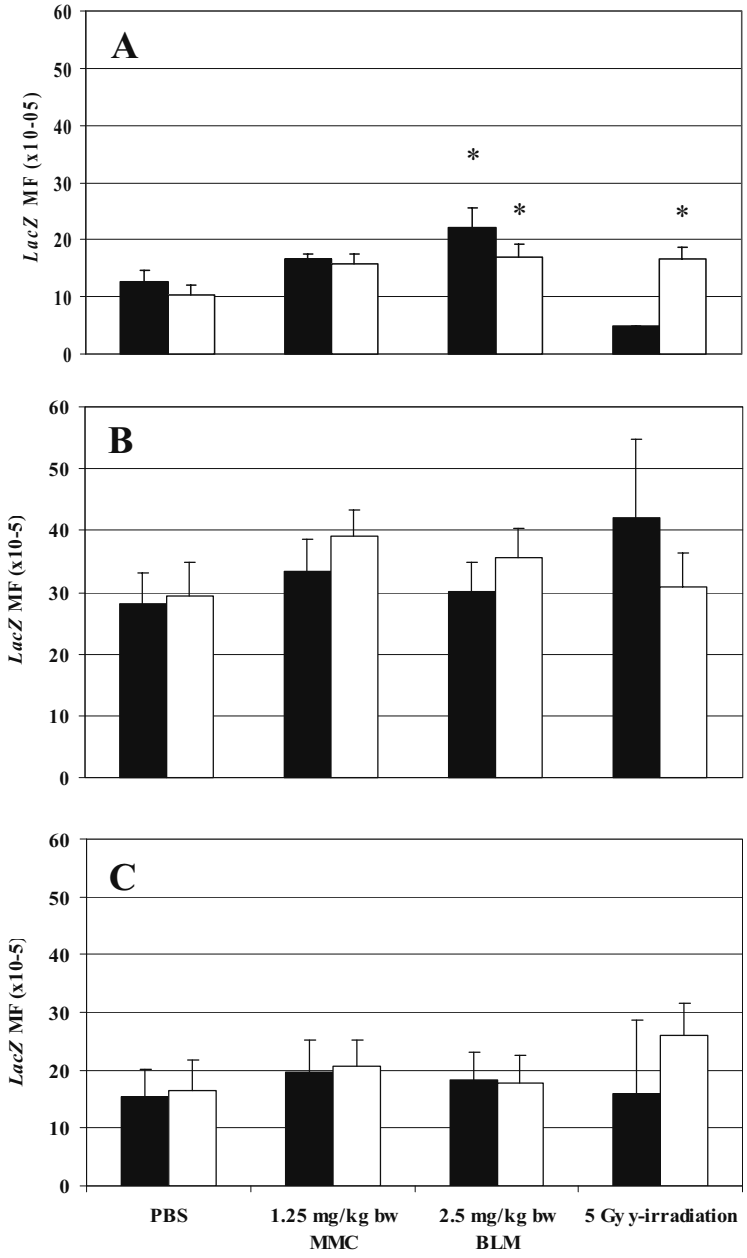

Figure 2: The *lacZ* MF in *Rad54/Rad54B* mice 4 (black bars) and 28 days (grey bars) after treatment with PBS (negative control), MMC, BLM and γ-irradiation.

A: Bone marrow, **B:** liver, **C:** spleen

Hybridization of mutant colonies with total mouse DNA

The percentages of mutant colonies with size mutations and chromosomal rearrangements were determined by hybridization with total mouse DNA in the bone marrow of WT and *Rad54/Rad54B* mice after treatment with the different compounds (Fig. 3). In WT mice there was an increase in the percentages of mouse DNA positive colonies in the bone marrow only 4 days after treatment with MMC and BLM compared to the untreated control. Interestingly, an almost similar increase was seen 28 days after treatment with MMC and BLM in the bone marrow of the *Rad54/Rad54B* mice with an intermediate result 4 days after treatment with MMC only. In contrast, no significant increase in the percentages of mouse DNA positive clones were found 28 days after γ-irradiation in both mouse models nor, as expected, after ENU treatment in WT mice (data not shown).

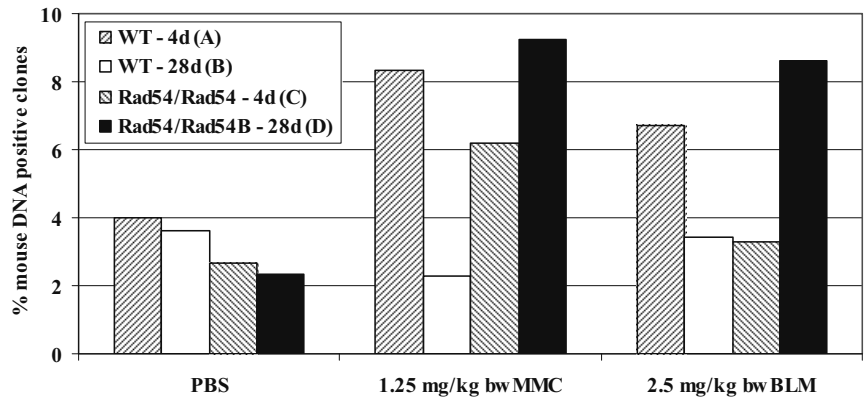

Figure 3: Quantification of mouse DNA positive *lacZ* mutant clones from the bone marrow of WT and *Rad54/Rad54B* mice 4 and 28 days upon treatment with PBS (negative control), MMC and BLM.

A: WT mice 4 days after treatment

B: WT mice 28 days after treatment

C: *Rad54/Rad54B* mice 4 days after treatment

D: *Rad54/Rad54B* mice 28 days after treatment

Micronucleus test

The MN test was performed as a classical control for detection of clastogenic properties of the compounds used. The percentages of PCEs (Tables 1 and 2), which is an indication of cytotoxicity, was decreased statistically significant in the blood of all treated mice, except for the *Rad54/Rad54B* mice 4 days after treatment with BLM.

This decrease is an indication of sufficient exposure of the target cells of both genotypes.

A statistically significant increase in the percentages of MN was seen after treatment with all clastogens 4 and 28 days after treatment in both genotypes. There were no significant differences in the induction of MN between the two genotypes.

Unexpectedly, ENU, considered as a compound which predominantly induces point mutations and thus expected to be negative in the MN test (1), showed a higher and significant increase in cells with MN in WT mice.

Table 1. Micronucleus induction in peripheral blood of WT mice 4 and 28 days after treatment.

			WT mice (4 days)		WT mice (28 days)	
Compound	**Dose (mg/kg.bw)**	**Animal number**	**%MnPCE (mean ± SEM)**	**%PCE (mean ± SEM)**	**%MnRET (mean ± SEM)**	**% PCE (mean ± SEM)**
PBS	-	1	2.60	3.50	2.20	5.00
		2	3.80	3.10	4.20	4.35
		3	2.20	2.60	3.10	3.70
		4	2.40	3.80	2.80	3.40
		5	2.40	3.35	3.00	3.00
		6	3.20	3.00	4.60	2.80
			2.77 ± 0.25	3.23 ± 0.17	3.23 ± 0.17	3.71 ± 0.34
ENU	100	1	ND[a]	ND	5.40	2.95
		2			3.90	2.95
		3			6.00	2.10
					5.10 ± 0.62*	2.67 ± 0.28*
MMC	1.25	1	6.40	1.30	7.00	1.90
		2	7.00	1.70	6.20	2.20
		3	6.20	1.55	5.50	3.00
		4	6.80	1.50	7.60	1.70
		5	4.20	1.60	7.40	2.00
		6	4.00	1.80	7.00	2.10
			5.77 ± 0.54**	1.58 ± 0.07**	6.78 ± 0.32**	2.15 ± 0.18**
BLM	2.5	1	5.00	1.80	6.00	2.10
		2	5.70	2.65	4.60	3.00
		3	4.80	2.20	5.50	3.20
		4	6.60	1.70	7.00	2.50
		5	3.70	4.75	5.90	2.75
		6	3.30	2.35	4.00	2.40
			4.85 ± 0.50**	2.58 ± 0.46*	5.50 ± 0.44*	2.66 ± 0.17*
γ-irradiation	5 Gy	1	ND	ND	2.80	2.90
		2			7.40	3.15
		3			8.00	2.70
		4			5.10	4.15
		5			6.60	2.00
		6			4.30	4.00
					5.70 ± 0.81*	3.15 ± 0.33*

[a]ND = not determined

* $p<0.05$

** $p<0.001$

Table 2. Micronucleus induction in peripheral blood of *Rad54/Rad54B* mice 4 and 28 days after treatment.

			***Rad54/Rad54B* mice (4 days)**		***Rad54/Rad54B* mice (28 days)**	
Compound	**Dose (mg/kg.bw)**	**Animal number**	**%MnPCE (mean ± SEM)**	**%PCE (mean ± SEM)**	**%MnRET (mean ± SEM)**	**%PCE (mean ± SEM)**
PBS	-	1	2.30	3.40	2.70	2.85
		2	2.90	3.05	2.00	3.05
		3	2.20	2.60	2.40	3.25
		4	2.30	4.10	1.70	3.60
		5	2.10	3.15	8.80	2.20
		6	3.30	3.30	2.00	3.10
		7			2.20	2.50
			2.52 ± 0.18	3.27 ± 0.20	3.11 ± 0.96	2.94 ± 0.18
MMC	1.25	1	8.00	1.50	5.10	1.90
		2	7.50	1.60	6.60	1.55
		3	6.40	1.50	7.20	2.05
		4	8.40	1.70	8.50	1.90
		5	8.20	1.55	8.60	1.65
		6	6.40	1.70	4.70	2.10
		7	3.90	3.00		
		8	7.20	1.70		
			7.00 ± 0.52**	1.78 ± 0.18**	6.78 ± 0.67*	1.86 ± 0.09**
BLM	2.5	1	7.50	2.00	6.90	2.15
		2	5.80	2.15	6.10	2.10
		3	4.40	4.90	5.90	2.25
		4	5.60	2.10	7.40	1.70
		5	5.10	2.45	5.40	1.95
		6	8.20	2.25	5.40	1.50
			6.10 ± 0.59**	2.64 ± 0.46	6.18 ± 0.33*	1.94 ± 0.12**
γ-irradiation	5 Gy	1	ND[a]	ND	7.50	1.40
		2			6.30	1.85
		3			5.00	2.25
		4			5.50	2.35
		5			4.50	2.85
		6			5.10	2.05
					5.65 ± 0.44*	2.13 ± 0.20*

[a]ND = not determined

* $p<0.05$

** $p<0.001$

Discussion

In the present paper, we investigated whether clastogenic properties of compounds can be detected in mice harbouring the *lacZ* gene. This was performed by exposure of these mice to three clastogens: MMC, BLM or γ-irradiation. To increase the sensitivity of mice towards clastogens, we used mice (also harbouring the *lacZ* gene) with a defect in the *Rad54* and *Rad54B* genes. These genes are involved in the HR repair. With a defect in the HR, there may be a potential shift in the repair of DNA double-strand breaks towards NHEJ repair. NHEJ is an error-prone mechanism causing an accumulation of chromosomal damage, which can lead to an increase in gene mutations.

None of the clastogens tested showed an absolute induction in the *lacZ* MF in the bone marrow, liver and spleen of the WT mice compared to the untreated control at 4 days and 28 days. The lack of *lacZ* MF induction in the WT mice could not be ascribed to the performance of the test because ENU, the positive control (15,18), showed a statistically significant *lacZ* MF induction in all organs tested. In contrast, exposure to BLM (both at 4 and 28 days after treatment) and γ-irradiation (only 28 days after treatment) showed a slight but statistically significant induction in the *lacZ* MF in the bone marrow of the *Rad54/Rad54B* mice. In the other tissues tested no statistically significant induction of *lacZ* MF was observed.

Surprisingly, there was a higher background *lacZ* MF found in the liver of the *Rad54/Rad54B* mice. This higher background is probably due to deficiencies in the *Rad54* and the *Rad54B* genes. *Rad54/Rad54B* mutants cause an accumulation of mutations and a higher *lacZ* MF baseline frequency in some, but certainly not all, organs. We propose that in fast proliferating tissues, like the spleen and bone marrow, there is a selection in cell division on cells having no DNA damage, leading to the suppression of the *lacZ* MF, even in a DNA repair-deficient background. This selection mechanism is absent in slow or non-proliferating (somatic) tissues like the liver.

The responses of *in vivo* assays are a reflection of different processes such as absorption, distribution, metabolism and elimination. The low *lacZ* MF induction upon clastogen exposure could be due to an inappropriate route of administration. This could result in insufficient exposure of the target cells to the different compounds, hence leading to low induction in *lacZ* MF. Lynch *et al.* (12) showed that bioavailability is important for the effect of compounds on different tissues/organs. In their study, BLM was found both clastogenic

and cytotoxic to the bone marrow when administered *i.p.*, but not when given orally. Thus bio-availability is an important factor in the genotoxicity testing of compounds in vivo. However, in our present study a decreased % of PCEs after treatment in the MN test indicated that the target organ (bone marrow) was clearly exposed to the various compounds.

Since we found only a slight induction of the *lacZ* MF in the *Rad54/Rad54B* mice, we wanted to investigate whether there was a difference in a "mutational fingerprint" between the two genotypes. This was performed by hybridizing the mutant colonies with totally labelled mouse DNA. A positive signal is indicative for large deletions (size-change mutations) or chromosomal rearrangements (5).

An increase was seen both in WT and *Rad54/Rad54B* mice. Interestingly, the response in the bone marrow of WT mice was most overt 4 days after treatment, whereas the response in *Rad54/Rad54B* mice was optimal 28 days after treatment with both MMC and BLM (see Fig. 3). An explanation for this finding could be that WT mice, having active DNA-repair, are able to repair DNA damage caused by MMC and BLM. Apparently, 4 days after treatment this repair is not complete yet, leading to size-change mutations at this point. In contrast, the *Rad54/Rad54B* mice have compromised HR repair, consequently, leading to a shift in the repair of DNA double strands through NHEJ repair, which is not error free. Apparently, size-change mutations accumulate under these conditions and are highest 28 days after treatment with the clastogens MMC and BLM. Based on these findings we propose that the pUR288 model is promising in detecting clastogens, but the optimal conditions are not established yet.

Unexpectedly, we observed that ENU induced MN in peripheral blood of WT mice (Table 1). However, it is known from the literature (7) that ENU is capable to form various types of DNA adducts. One of these, N7-ethylguanine, may easily lead to apurinic sites, which on their turn may result in chromosomal damage causing this compound to be positive in the MN test.

In conclusion, the aim of this study was to investigate whether the *lacZ* plasmid mouse model is capable of detecting clastogenic effects of compounds. Although there was no clear induction in the *lacZ* MF after treatment with the clastogens in WT mice and only a subtle induction in the *lacZ* MF in the bone marrow of *Rad54/Rad54B* mice, the hybridization studies demonstrate an induction of rearranged *lacZ* variants carrying mouse chromosomal DNA after treatment with MMC and BLM. These results indicate that the pUR288 model still may be promising enough for the detection of clastogenic properties of compounds. Apparently, the correct conditions are not established yet. Next, the present approach is quite laborious and therefore, certainly is not suitable for high-throughput-testing. It may be

recommendable to develop an in vitro system using cells obtained from the mice used in this study to find out whether the plasmid system is not only promising but indeed capable of efficiently recognizing clastogens. The latter approach, if successful, will have an additional advantage, in that far less animals will be required for testing.

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Chapter 5

LacZ mouse embryonic fibroblasts detect both clastogens and mutagens

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Abstract

The clastogenic effects of MMC and BLM and the mutagenic effects of B[a]P, N-ac-AAF and ENU were studied in mouse embryonic fibroblasts derived from wild-type (WT) and *Rad54/Rad54B*-deficient mice. Clastogens as well as mutagens showed a statistically significant induction of mutations in the *lacZ* reporter gene both in a WT and *Rad54/Rad54B*-deficient genetic background. *Rad54/Rad54B* MEFs appeared equally sensitive to the clastogens compared to WT MEFs, except for MMC. The type of mutations induced by the different compounds was investigated further by hybridizing the mutant colonies with total mouse DNA. An obvious increased number of mouse DNA positive clones was observed after BLM and MMC exposure, indicating that after these treatments genome rearrangements/translocations had occurred. In this hybridization assay, *Rad54/Rad54B* MEFs did not show more rearrangements/translocations than WT MEFs. As expected, the mutagens used showed no increase in chromosomal rearrangements or transloctions in MEFs derived from both genotypes. These results show that WT MEFs carrying the *lacZ* reporter gene on a plasmid are capable to detect both clastogenic as well as mutagenic effects of compounds *in vitro*. Deletion of the *Rad54* and *Rad54B* genes did not further enhance the sensitivity of MEFs towards clastogens.

Introduction

Humans are frequently exposed to different chemicals in food/feed additives, packaging material, drugs, cosmetics, pesticides etc. It is very important that these chemicals are properly evaluated for their potential adverse health effects. Some chemicals are genotoxic and cause DNA damage. Not or incorrectly repaired DNA damage may lead to gene mutations, changes in metabolic processes, cell death, accelerated ageing or carcinogenesis. Fortunately, cells are equiped with many DNA repair mechanisms which can remove DNA lesions efficiently and accurately (7,11).

Genotoxicity has two different endpoints: gene mutations and chromosomal aberrations. The latter can be either numerical (aneugenic) or structural (clastogenic). Consequently, to cover these endpoints, at least two tests have to be performed in safety assesment. Problems occur when a chemical induces gene mutations *in vitro*, since there are no comparable well validated and reliable endogenous gene mutation assays *in vivo*. The use of a gene mutation test with transgenic animals may be used as an alternative (8,10). The commercially available transgenic mouse models have a very low response to clastogens and, as a consequence, do not detect chromosomal rearrangements (12). Boerrigter *et al.* (1) have designed a *lacZ* plasmid-based transgenic mouse model, the pUR288 plasmid mouse model, which can overcome this restriction.

Previously, we have investigated whether the plasmid *lacZ* mouse model, is able to detect both mutagens and clastogens (8). These plasmid *lacZ* mice are theoretically capable of detecting small mutations as well as large deletions (>500 base pairs; see Refs. 3,9,12), this in contrast to the phage lambda-based models (6). These large deletions (or size mutations) are detected as inter- or intrachromosomal rearrangements (e.g. translocations) due to incorrect fusion or repair of chromosomal breaks. Consequently, the plasmid *lacZ* mouse model theoretically should be able to detect the genotoxic potency of clastogens.

There was no clear induction in the overall *lacZ* mutant frequency *in vivo* in pUR288 mice after treatment with clastogens. However, the model still appeared to be promising (8), since the induction of chromosomal rearrangements and translocations were indirectly measured through hybridization studies using total mouse DNA as a probe. Therefore, we decided to further study the model using mouse embryonic fibroblasts (MEFs) derived from the repair-proficient and *Rad54/Rad54B*-deficient mice, carrying the *lacZ* reporter gene.

Cells with a defect in the *Rad54* and *Rad54B* genes are deficient in homologous recombination (HR) repair. As such we assume that in these cells the repair of double-strand breaks will be primarily accomplished through non-homologous-end-joining (NHEJ) repair in stead of HR. NHEJ is an error-prone mechanism causing an accumulation of chromosomal damage, which may lead to an increase in mutations, especially after exposure to clastogens. Both WT and *Rad54/Rad54B* MEFs, carrying ~20 copies of the *lacZ* plasmid, were treated with clastogens (mitomycin C, bleomycin) and mutagens (benzo[a]pyrene, *N*-acetoxy-2-acetylaminofluorene, *N*-ethyl-*N*-nitrosourea). It was our hypothesis that the repair-deficient cells would be more sensitive than WT cells when exposed to clastogens in terms of cell survival, gene mutation fixation and micronuclei induction. The sensitivities towards mutagens were expected to be identical in both WT and repair-deficient MEFs. Surprisingly, however, the WT *lacZ* reporter cells were in general equally sensitive to clastogens as compared to the repair-deficient *Rad54/Rad54B* cells, with the exception of MMC.

Materials and methods

Isolation of mouse embryonic fibroblasts (MEFs)

Embryos of 13.5 days were harvested from C57BL/6 wild-type (WT) and *Rad54/Rad54B* repair-deficient (*Rad54/Rad54B*) mice harbouring the *lacZ* transgene. Mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Netherlands Vaccine Institute (NVI, Bilthoven, The Netherlands). The liver and head were discarded from the embryonic body (to avoid disturbance during fibroblast growth). The remainder of the embryonic body was trypsinised and cultured in a 75 cm^2 flask containing 15 ml culture medium (Dulbecco's Modified Eagle Medium, DMEM) supplemented with 1% Modified Eagles Medium Non-Essential Amino Acids (MEM NEAA), 1% Penicillin-Streptomycin (PS) and 10% Fetal Bovine Serum (FBS) at 37^oC in a humidified atmosphere containing 3% O_2 and 10% CO_2 for 3 days. These incubation conditions were the same for all experiments described in this paper.

After 3 days, the cells were trypsinised, equally divided over two 175 cm^2 culture flasks, and were grown for another 4 days. Thereafter, the cells were collected using trypsin and counted in a Bürker-Türk. After centrifugation at 1200 rpm and 4^oC for 5 min, the cell pellet was resuspended in freezing medium (DMEM supplemented with 20% FBS, 10% dimethylsulfoxide (DMSO), 1% MEM NEAA and 1% PS) at a concentration of $3x10^6$ cells per ml while keeping it on ice. One ml portions were kept at -80^oC for at least 24 hours and were then stored in liquid nitrogen. In all experiments as described below, exposure to mutagens and clastogens were concurrently performed in both WT and DNA-repair deficient MEFs.

Cell survival

A cell survival assay (Cell Proliferation Kit II (XTT), Roche, Germany) was used to determine the doses of the genotoxic compounds to be used for treatment of MEFs. For every dose of each compound 4 days before treatment, aliquots of $3x10^6$ MEFs were seeded and cultured in a 175 cm^2 flask containing 30 ml culture medium. Twenty-four hours before treatment, the cells were dissociated with trypsin and cultured in a 24-wells plate containing $0.3x10^6$ cells and a final volume of 1 ml culture medium.

On the day of treatment, the cells were washed once with D-PBS (Dulbecco's phosphate-buffered saline, containing KCl, KH_2PO_4, NaCl, Na_2HPO_4, without calcium and magnesium)

before treatment with the various compounds: mitomycin C (MMC, CAS no. 50-07-7), bleomycin (BLM, CAS no. 9041-93-4), benzo[a]pyrene (B[a]P, CAS no. 200-028-5), *N*-acetoxy-2-acetylaminofluorene (N-ac-AAF, CAS 6098-44-8) and *N*-ethyl-*N*-nitrosourea (ENU, CAS no. 759-73-9) (see Table 1).

The compounds were dissolved in the appropriate solvent (see Table 1) on the day of treatment. After treatment, the cells were washed once with 4 ml D-PBS and cultured in 0.5 ml culture medium for 5 days. The survival of the cells was determined using the XTT assay, according to the Manufacturer's instructions. Briefly, after removing of the culture medium, MEFs were incubated for 2 hours in 0.5 ml culture medium with 100 µl of XTT reagent (20 µl electon coupling reagens (PMS (N-methyldibenzopyrazine methyl sulfate) and 1.25 mM phosphate buffered saline (PBS)) in 1 ml XTT reagent (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate)) was added, incubation was for 2 hours. After incubation, 100 µl of the suspension was transferred into a 96-wells plate and analyzed using a Spectra Max 190 (Molecular Devices Corp.) ELISA reader and SoftMax Pro 5.0 software. The fluorescence measured with the ELISA reader was used to calculate the amount of living cells for each treatment.

***LacZ* gene mutation assay**

Treatment of MEFs

For every dose of each compound and for every time point, $1x10^6$ cells of both genotypes were seeded into Petri dishes containing 10 ml culture medium 24 hours before treatment. The cells were exposed to the compounds at doses indicated in Table 1 in a total volume of 4 ml culture medium for 3 h. After treatment, the cells were washed twice with 4 ml D-PBS before adding 10 ml culture medium and cultured for 1, 3 or 6 days. After incubation, the cells were collected in 2 x 1 ml D-PBS using a cell scraper. Finally, the cell suspension was centrifugated at 1200 rpm and 4°C for 5 minutes. The pellet was stored at -20°C until the *lacZ* rescue assay was performed and the mutant frequency determined.

Table 1. Concentrations of all compounds used for the survival assay.

Compound	Concentration	Solvent	Treatment time (*lacZ* MF)[b]	Treatment time (MN)[c]
MMC	0, 0.2, 0.4 and 0.8 μg/ml	D-PBS	3 hours	12 hours
BLM	0, 20, 40 and 80 μg/ml	D-PBS	3 hours	12 hours
B[a]P	0, 10, 25, 50 and 100 μM	DMSO + S9-medium[a]	3 hours	3 hours
N-ac-AAF	0, 4, 8, 12, 15 and 30 μM	DMSO	3 hours	12 hours
ENU	0, 2, 4, 6 and 8 mM	Culture medium	3 hours	12 hours

[a]S9-medium consisted of 82.5% culture medium (DMEM supplemented with 1% MEM NEAA and 1% PS, no FBS), 12.5% Cofactor solution (NADP, Glucose-6-phosphate, KCl, $MgCl_2.6H_2O$, 1 M Hepes pH 7.4 and phosphate buffer (containing NaCl, KCl, $Na_2HPO_4.2H_2O$ and KH_2PO_4)) and 5% S9 fraction from Aroclor 1254-induced rats

[b] *lacZ* MF, mutant frequency measured in the *LacZ* reporter gene

[c]MN, micronucleus test

Determination of the mutant frequency

The mutant frequency in MEFs was determined using the procedure described by Dollé *et al* (2). Briefly, total genomic DNA was isolated using a phenol extraction. The total genomic DNA (20–50 μg) was digested with the restriction enzyme *Hind*III to obtain either pUR288 plasmids (gene mutations) or parts of a pUR288 plasmid with murine DNA (size mutations) both containing (parts of) the *lacZ* gene. The *lacZ*-containing plasmids were rescued from the solution using magnetic beads coated with the *lacZ*/*lacI* fusion protein. After detachment from the beads, these plasmids were subsequently transfected into electrocompetent *E. coli* strain C (*lacZ*$^-$ *galE*$^-$). A fraction (2 μl of the 2 ml total) of the bacterial sample was plated on non-selective X-galactosidase plates to determine the rescue efficiency; the remainder on selective P-glycosidase plates to select for mutants. The *lacZ* mutant frequency was calculated by dividing the number of mutants by the total number of rescued colonies x dilution factor (1000).

Hybridization of mutant colonies with total mouse DNA

Individual mutant colonies were grafted on a Hybond-N^+ filter (Amersham). To obtain exclusively DNA bound to the filter, the cells were lysed and washed twice with a denaturation buffer and twice in a neutralization buffer to remove cell debris and unbound DNA. The filter was washed with 5xSSC (3 M NaCl and 0.3 M Na-citrate) and dried avoiding contact with other DNA sources.

Total mouse DNA (250 ng DNA in milliQ-UF) was digested with *Hind*III in digestion mix (100 mM Tris pH 7.6, 80 mM $MgCl_2$ and 10 mM DTT). After digestion, a maximum of 10 ng/µl total mouse DNA diluted in milliQ-UF was denaturated at 100°C and put on ice. The DNA was labelled and detected with the Amershan Gene Images AlkPhos Direct Labelling and Detection System kit (GE Healthcare, UK). After gentle centrifugation, the DNA was collected and a mixture of reaction buffer, labelling reagent (containing 0.1% (w/v) sodium azide) and cross-linker working solution (containing 4.7% (w/v) formaldehyde), was added to the denaturated DNA before incubation at 37°C for 30 minutes (see for further details the Manufacturer's instructions). The reaction was stopped by cooling on ice.

For the hybridization, the AlkPhos Direct hybridization buffer (containing 0.5 M NaCl and 4% (w/v) blocking reagent) was pre-warmed at 55°C. The dried filters were gently put into a tube and the pre-hybridization mix (1/100 volume) solution was added to the filters. The mixture was pre-hybridized at 55°C for 15 minutes, before the probe was added. The filters were hybridized overnight at 55°C while shaking carefully. After hybridization, the filters were first washed twice with a primary wash buffer (containing 1 M Tris base and 2 M NaCl) at 55°C while shaking carefully. The second step was performed with a freshly made secondary wash buffer (containing 1 M Tris base, 2 M NaCl and 1 M $MgCl_2$). The filters were washed twice at room temperature while shaking carefully. The wet filters were packed in Saran wrap foil and incubated for 5 minutes with an ECF (Enhanced Chemifluorescence) substrate. The filters were illuminated using a Phosphor imager (Storm 860, Molecular Dynamics, GE Healthcare Bio-Sciences). Mutant colonies that hybridized with total mouse DNA were counted.

***In vitro* micronucleus test (MNT)**

Cell proliferation assay

The cell division time of MEFs was determined using a cell proliferation assay (Cell Proliferation Kit II (XTT), Roche, Germany). Three x 10^6 WT MEFs were cultured in 30 ml culture medium in 175 cm^2 flask for 3 days. After incubation, the cells were washed once with D-PBS and cultured in low serum medium (DMEM supplemented with 1% MEM NEAA, 1% PS and 0.1% FBS) for 3 days. Subsequently, the cells were dissociated with trypsin and subcultured in a 24-wells plate at $0.3x10^6$ cells per 2 ml culture medium for 6 h. Cell division was determined using an XTT assay according to the manufactures instructions. Briefly, the culture medium was removed from the 24-wells plate and 0.5 ml culture medium and 100 µl of XTT reagent (20 µl electon coupling reagens in 1 ml XTT reagent) was added. The 24-wells plate was incubated for 4, 8, 12, 16, 20, 24, 28 and 32 hours, respectively. After each incubation time, 100 µl of the suspension was transferred into a 96-wells plate and analyzed using a Spectra Max 190 (Molecular Devices Corp.) ELISA reader and SoftMax Pro 5.0 software. The fluorescence measured with the ELISA reader was used to calculate the amount of living cells for each time point.

The in vitro MNT

For each compound at all doses tested $1x10^6$ cells from both genotypes were seeded in a 25 cm^2 flask contaning 5 ml culture medium 24 h before treatment. The cells were exposed to MMC, BLM, N-ac-AAF and ENU as indicated in Table 1 in a volume of 4 ml for 12 hours. At the start of the treatment, cytochalasin B was added to the cell suspension in a final concentration of 6 µg/ml. For the B[a]P treatment, cells were first incubated for 3 h with a S9 mix containing B[a]P and cytochalasin B. After treatment, the cells were washed twice with D-PBS and incubated for the remaining period of time (Table 1) with culture medium containing cytochalasin B.

Twelve hours after the start of the treatment, the cells were trypsinised and centrifuged at 1000 rpm and 4°C for 5 min. The pellet was dissolved in culture medium and 50 µl of an 8x dilution of the cell suspension was spotted onto a microscope slide using a Cytospin 3 (Thermo Shandon Limited, U.K.) at 500 rpm for 5 min. The slides were allowed to dry on air before they were fixated with methanol for 5 min and stained with a 6% Giemsa solution for approximately 5-10 min.

The percentage of micronucleated bi-nuclear cells was determined in 1000 bi-nuclear cells for each compound. The percentages bi-nuclear cells were determined by counting the amount of bi-nuclear cells in at least 2000 cells. This percentage is an indication for cell toxicity.

Statistical analysis

The differences in the cell survival test (XTT test) were analyzed by an one-way ANOVA with a Bonferroni correction. Differences in mutant frequencies were evaluated using a Mann-Whitney "*U*" test whereas differences in MN were determined with the Pearson's X^2-test. Significant differences were accepted at the level of 0.05 ($p<0.05$). All data in tables and figures are presented as the mean ± SEM (standard error of the mean).

Results

Cell survival assay

The sensitivity of the WT and *Rad54/Rad54B* MEFs, in terms of cell survival, was tested after treatment with 2 clastogens: MMC and BLM and 3 mutagens: B[a]P, N-ac-AAF and ENU.

The clastogenic compounds showed a dose dependent and statistically significant ($p<0.05$) decrease in the amount of living cells of both genotypes compared to the untreated controls (Fig. 1).

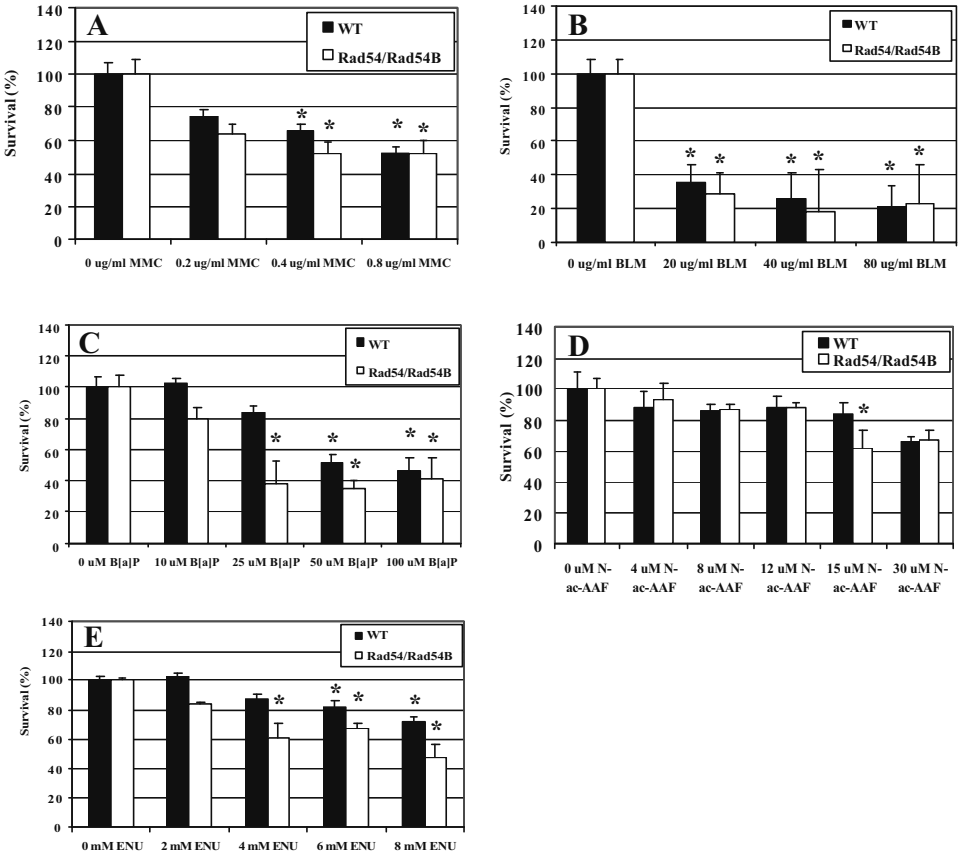

Figure 1: Survival of WT and *Rad54/Rad54B* MEFs after treatment with various compounds. Asterisks indicate a significant difference ($p<0.05$) between the percentages living cells in the treated groups compared to the untreated control.

A: MMC; **B**: BLM; **C**: B[a]P; **D**: N-ac-AAF and **E**: ENU.

There was no significant difference in sensitivity to clastogen exposure between the two genotypes. Surprisingly, the lowest concentration of BLM tested (20 µg/ml) already induced the maximal decrease in the percentage living cells, indicating that the lowest concentration tested is probably quite toxic to the cells.

Concerning the mutagens, a dose dependent and statistically significant decrease in the amount of living cells was found with ENU and B[a]P. However, such an effect was not observed with N-Ac-AAF (Fig. 1D). In contrast to the clastogens, the *Rad54/Rad54B* MEFs were more sensitive to the mutagens B[a]P and ENU resulting in decreased cell survival at lower doses in *Rad54/Rad54B* MEFs.

The doses selected for *lacZ* analysis and micronucleus induction, as shown in Table 1, were chosen on the basis of these results given that the observed cytotoxic effects are linked to mutagenic processes.

***LacZ* gene mutation assay**

The *lacZ* MF was determined in WT and *Rad54/Rad54B* MEFs at 1, 3 and 6 days after treatment with clastogens (Fig. 2) or mutagens (Fig. 3).

The clastogens MMC and BLM induced a dose dependent and statistically significant ($p<0.05$) increase in the *lacZ* MF in MEFs of both genotypes and for the 3 time points tested. An exception, however, was found for BLM exposure where the WT cells showed, in comparison to its untreated controls, a biological relevant but not statistically significant dose response at the 3-day sampling time in comparison to its untreated controls. In contrast to the XTT test, where no genotype effect was detected with the clastogens, a statistically significant genotype effect ($p<0.030$-0.017) at all three sampling times was observed for the highest dose MMC (*i.e.* 0.8 µg/ml) tested.

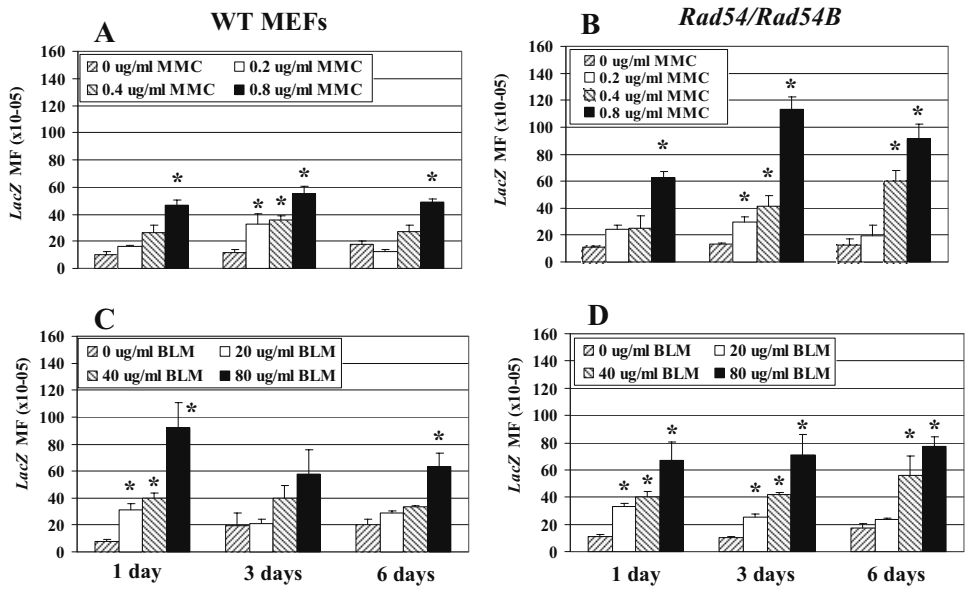

Figure 2: The *lacZ* MF in WT (A+C) and *Rad54/Rad54B* (B+D) MEFs, 1, 3 and 6 days after treatment with clastogens. Asterisks indicate a significant difference (p<0.05) between the *lacZ* MF in the treated groups compared to the untreated control.
A and **B**: MMC; **C** and **D**: BLM.

The mutagens also showed a dose-dependent and statistically significant (p<0.05) increase in the *lacZ* MF for most time points tested. The only exception was with B[a]P tested in *Rad54/Rad54B* MEFs at 1 day after treatment where although biologically relevant no statistically significant dose-response relationship was detected. A statistically significant genotype effect (p=0.016) was only found for N-ac-AAF (6 day sampling time at the highest concentration tested). The *lacZ* MF for mutagens was on average at least 1.5-fold higher than the *lacZ* MF observed for clastogens (p<0.05).

For all compounds tested (both clastogens and mutagens) a biologically relevant dose dependent increase in the *lacZ* MF was already detectable 1 day after treatment. No significant better results were obtained when the *lacZ* MF was determined 3 or 6 days after treatment.

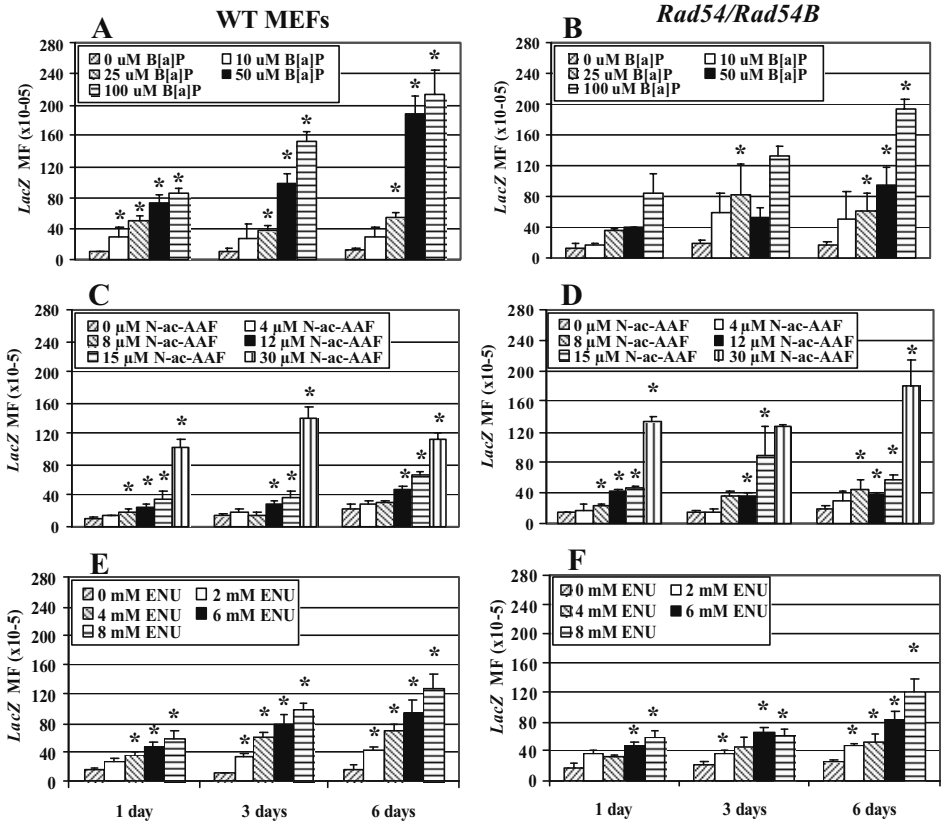

Figure 3: The *lacZ* MF in WT (A+C+E) and *Rad54/Rad54B* (B+D+F) MEFs, 1, 3 and 6 days after treatment with various mutagens. Asterisks indicate a significant difference (p<0.05) between the *lacZ* MF in the treated groups compared to the untreated control.
A and **B**: B[a]P; **C** and **D**: N-ac-AAF; **E** and **F**: ENU.

Hybridization of mutant colonies with total mouse DNA

The number of mutant colonies with possible chromosomal rearrangements or translocations (both referred to as size-change mutants) were subsequently determined by hybridization of the mutant colonies with total mouse DNA (3). Both clastogens (MMC and BLM) showed a 4-8-fold increase in the percentages of hybridization positive clones in WT MEFs as compared to the untreated control (Fig. 4A). Such an increase was absent in BLM-treated *Rad54/Rad54B* MEFs. However, we found a 3-fold increase in the number of size-change mutants in MMC-treated *Rad54/Rad54B* MEFs (Fig. 4A). As expected, treatment with

mutagens did not result in any significant accumulation of hybridization positive clones for both genotypes (Fig. 4B).

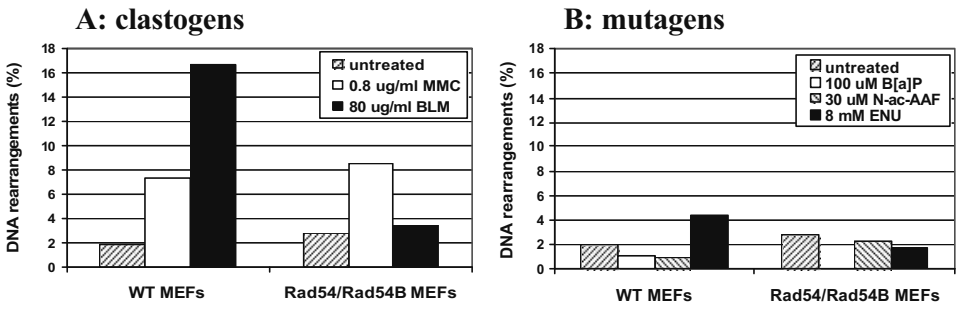

Figure 4: Hybridization of mutant colonies from wild-type and *Rad54/Rad54B* MEFs 6 days after treatment with various compounds.

A: MMC and BLM; **B**: B[a]P, N-ac-AAF and ENU

***In vitro* MNT**

Under conditions used in this study, it appeared that WT MEFs had a division time of approximately 8 h (results not shown). Based on our previous observations (not shown) we assumed that the division time for *Rad54/Rad54B* MEFs does not dramatically differ from that of WT MEFs. Therefore, the micronucleus test was performed with harvest times 8 and 12 h after treatment with the different genotoxins.

Exposure to clastogens was sufficient as demonstrated by the statistically significant ($p<0.05$) decrease in the number of binuclear cells (Table 2), except for MMC in *Rad54/Rad54B* MEFs at 0.2 and 0.4 μg/ml. Toxicity (% bi-nuclear cells) upon mutagen exposure varied, but was in general in the same range within the genotypes tested. The 12-h time point appeared more suitable for future MN experiments, as on average at this time point a higher amount of bi-nuclear cells was found as compared to the 8-h time point. Therefore, only the results with the 12-h time point are shown (Table 2) and discussed.

A dose dependent and statistically significant increase in the number of cells with micronuclei (MN) was observed for MMC and BLM in both genotypes. A genotype effect with MMC was found since the number of cells with MN was approximately 2-fold higher in *Rad54/Rad54B* MEFs. A dose dependent increase in the number of micronucleated MEFs was also found for B[a]P in both genotypes but these should be taken with care owing to high

cytotoxicity observed at the highest doses tested. A biologically relevant increase in micronucleated MEFs was generally not found after N-ac-AAF and ENU treatment.

Table 2. Micronucleus induction in WT and *Rad54/Rad54B* MEFs 12 hours after treatment.

		WT MEFS		*Rad54/Rad54B* MEFs	
Compound	**Dose**	**%bi-nuclear cells with a MN (mean ± SEM)**	**%bi-nuclear cells (mean ± SEM)**	**%bi-nuclear cells with a MN (mean ± SEM)**	**%bi-nuclear cells (mean ± SEM)**
MMC	0 µg/ml	0.50 ± 0.30	44.65 ± 1.25	2.98 ± 1.04	16.24 ± 0.70
	0.2 µg/ml	2.10 ± 0.10*	27.16 ± 0.34*	1.70 ± 0.13	21.04 ± 1.11
	0.4 µg/ml	2.90 ± 0.30*	26.35 ± 3.65*	5.77 ± 0.34*	17.97 ± 3.05
	0.8 µg/ml	2.78 ± 0.58*	22.15 ± 1.05*	4.6 ± 0.51*	38.36 ± 2.31*
BLM	0 µg/ml	0.91 ± 0.31	43.25 ± 0.85	1.62 ± 0.59	28.62 ± 0.32
	20 µg/ml	6.17 ± 0.53*	13.15 ± 1.65*	7.38 ± 0.51*[b]	9.30 ± 1.28*
	40 µg/ml	NC[a]	8.80 ± 0.10*	12.27 ± 1.39*[b]	8.19 ± 0.75*
	80 µg/ml	NC	8.00 ± 1.10*	11.92 ± 0.29*[b]	6.14 ± 0.02*
B[a]P	0 µM	0.41 ± 0.06	33.20 ± 1.10	0.8 ± 0.36	33.24 ± 0.26
	10 µM	0.48 ± 0.12	26.35 ± 1.25	2.84 ± 0.14*	24.19 ± 5.11
	25 µM	2.51 ± 0.82*[b]	7.35 ± 0.65*	3.76 ± 1.19*	12.71 ± 0.14
	50 µM	NC	4.40 ± 0.70*	4.49 ± 2.08*	3.71 ± 0.01[b]
	100 µM	NC	1.90 ± 0.20*	NC	NC
N-ac-AAF	0 µM	0.44 ± 0.25	46.00 ± 0.80	0.57 ± 0.08	44.65 ± 3.90
	4 µM	0.40 ± 0.06	38.55 ± 2.65	0.87 ± 0.12	39.38 ± 0.80
	8 µM	0.36 ± 0.09	38.05 ± 2.95	0.58 ± 0.28	23.61 ± 0.04*
	12 µM	0.40 ± 0.20	29.05 ± 0.95*	1.27 ± 0.27*	18.35 ± 1.84*
	15 µM	NC	22.95 ± 1.65*	1.78 ± 0.79*	13.80 ± 1.45*
	30 µM	NC	16.66 ± 0.45*	0.65[b]	9.20 ± 0.00*
ENU	0 mM	1.10 ± 0.16	52.29 ± 3.35	0.91 ± 0.28	29.43 ± 1.41
	2 mM	1.01 ± 0.03	44.26 ± 3.10*	1.19 ± 0.16	24.59 ± 4.20
	4 mM	1.08[b]	36.91*	1.32 ± 0.36	23.44 ± 2.21
	6 mM	1.01 ± 0.25	30.29 ± 2.51*	1.36 ± 0.20	19.97 ± 2.29
	8 mM	1.61 ± 1.02*	19.53 ± 0.36*	1.49 ± 0.37[b]	11.16 ± 0.88*

[a]NC = not counted due to a low amount of bi-nuclear cells

[b]Less than the required number of bi-nuclear cells analyzed

* $p<0.05$

Discussion

In the current paper, we investigated the possibility to detect clastogenic as well as mutagenic properties of compounds using an *in vitro* model system consisting of mouse embryonic fibroblasts (MEFs) derived from WT or *Rad54/Rad54B* mice carrying the *lacZ* reporter gene. Using a proof of principle approach, MEFs were treated with two clastogens, *i.e.* MMC and BLM, and three mutagens, *i.e.* B[a]P, N-ac-AAF and ENU, and mutant frequencies as well as specific mutation types were determined.

In MEFs of both genotypes a dose dependent and statistically significant increase in the *lacZ* MF was found after treatment with all three mutagens as well as the two clastogens (Figs. 2 and 3). As expected, no obvious differences in *lacZ* MF induction were observed between cells of the two different genotypes when treated with mutagens (Fig. 3).

The only consistent genotype effect on *lacZ* MF induction was observed after MMC treatment (highest dose tested, compare Figs. 2A and B). For unknown reasons this effect was absent after BLM treatment. Maybe in *Rad54/Rad54B* cells repair of MMC-induced lesions relies more on active HR repair than the lesions induced by BLM. A presumptive shift towards repair through the error-prone NHEJ machinery of MMC-induced lesions might be the underlying mechanism explaining the higher *lacZ* MF in these DNA repair-deficient cells. Apparently, repair in MEFs of the lesions induced by BLM do not heavily rely on active HR repair.

The lack of a genotype effect after treatment with the clastogen BLM could also be due to the type of cells used in our experiments. The formation of chromosomal breaks triggers both HR- as well as NHEJ-repair. Essers *et al.* (5) showed that the contribution of the HR and NHEJ repair pathways can be different depending on the mammalian developmental stage and also on the specific type of DNA damage. Using *mRad54*-deficient mice (5) and *mRad54*-deficient ES cells derived from these mice (4), they demonstrated that HR repair plays a role at the embryonic stage, but, unexpectedly, not at the adult stage. Here we used MEFs, derived from embryonic stages, in which HR repair is apparently not as essential as it is in ES cells. Obviously, this hypothesis needs further experimental support.

Our results clearly indicate that several classes of clastogens exist. BLM induce DNA lesions that can be repaired equally effective in cells without active HR repair compared to WT cells. The cross-linking agent MMC did, however, show a differential effect in repair-deficient cells compared to WT cells. Both the *lacZ* gene mutation induction in total (Fig. 2B), but also

number of the *lacZ* mutations carrying mouse DNA fragments (size-change mutants; Fig. 4A) were only elevated upon MMC treatment in *Rad54/Rad54B* cells. Apparently, HR repair is necessary to repair DNA cross-links and if not repaired efficiently, as possibly occurs in *Rad54/Rad54B* cells, this will lead to more *lacZ* mutations of which a substantial part are the results of large deletions and/or chromosomal rearrangements. Such an induction of 'size-change mutants' was virtually absent in DNA repair-deficient cells when treated with BLM and, as expected, upon treatment with the three mutagens. The remaining puzzling issue is that in WT cells both MMC and BLM induced significant levels of size-change mutations. Probably, BLM lead to double-strand DNA breaks in *Rad54/Rad54B* cells, which are quite toxic to these cells and therefore these DNA aberrations may lead to cell death rather than detectable size-change mutants.

An aberrant behaviour of MMC in DNA repair deficient cells was also seen in the classical MN assay. In WT MEFs MMC as well as BLM induced bi-nucleated cells carrying a MN. The test was, as expected, in WT cells restrictively positive to clastogens, since the mutagens did not induce significant levels of bi-nuclear cells carrying a MN. Only B[a]P appeared to induce some MN especially in *Rad54/Rad54B*-deficient cells. However, these results need to be interpreted with care, since very low levels of binucleated cells were left, indicating that under our conditions B[a]P is relatively toxic to MEFs. In contrast, and for unknown reasons, in the *Rad54/Rad54B* MEFs MMC showed relatively high levels of binucleated cells, indicating that MMC is far less toxic than BLM both in WT and DNA repair deficient cells. This finding is probably the underlying reason for the observed (significant) increase in *lacZ* mutants in DNA repair deficient cells as compared to their WT counterparts. Taken together we conclude that *Rad54/Rad54B* MEFs respond differently upon different clastogens. The DNA repair deficient cells appear to react more specifically to the cross-linking agent MMC as compared to the double-strand DNA break-inducing agent BLM.

The aim of this study was to test whether it is possible to measure clastogenic and mutagenic effects of compounds in one *in vitro* cellular system. We showed that, when taking the induction of *lacZ* gene mutants as a measure for genotoxic features of compounds, we are able to detect both their mutagenic or clastogenic features. With the exception of somewhat higher MMC-induced *lacZ* mutant levels in the repair-deficient cells, it appeared that WT MEFs and *Rad54/Rad54B* MEFs are equally sensitive in this assay. In conclusion, we propose that WT MEFs, carrying the *lacZ* reporter gene on a plasmid, are ideally suited for *in vitro* genotoxic screening of compounds. These cells eliminate the need to perform two separate tests for the different compounds, leading to a more appropriate and cost-effective

test. In addition, genotoxic properties of chemicals and pharmaceuticals can be analyzed *in vitro* and *in vivo* using the same target gene analysis. Further validation studies are needed, specifically with a broader group of clastogens before this model can routinely be used as an alternative high-throughput-screening test.

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Chapter 6

Comparison of clastogen-induced gene expression profiles in wild-type and DNA repair-deficient *Rad54/Rad54B* cells

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Abstract

Background: Previously we found that *Rad54/Rad54B* cells are more sensitive towards mitomycin C (MMC) as compared to wild-type (WT) cells. This difference in sensitivity was absent upon exposure to other clastogens like bleomycin (BLM) and γ-radiation. In order to get further insight into possible underlying mechanisms, gene expression changes in WT and *Rad54/Rad54B* MEFs (mouse embryonic fibroblasts) after exposure to the clastogens MMC and BLM were investigated. Exposures of these cells to mutagens (N-ac-AAF and ENU) and vehicle were taken as controls.

Results: Most exposures resulted in an induction of DNA damage signalling and apoptosis genes and a reduced expression of cell division genes in cells of both genotypes. As expected, responses to N-ac-AAF were very similar in both genotypes. ENU exposure did not lead to significant gene expression changes in cells of both genotypes, presumably due to its short half-life. Gene expression responses to clastogens, however, showed a genotype-dependent effect for BLM and MMC. MMC treated *Rad54/Rad54B* MEFs showed no induction of p53-signaling, DNA damage response and apoptosis as seen for all the other treatments.

Conclusion: These data support our finding that different types of clastogens exist and that responses to these types depend on the DNA repair status of the cells.

Background

DNA double-strand breaks (DSBs) have detrimental effects on the integrity of chromosomes and cell viability. Unrepaired or incorrectly repaired DSBs can lead to loss of chromosomes or cell cycle arrest which may lead to uncontrolled cell growth, cell death or carcinogenesis (1,2). DSBs mainly arise through exogenous DNA-damaging agents (clastogens) and endogenous sources. Clastogens can be divided into compounds that induce single/double-strand breaks, like bleomycin (BLM) and γ-radiation, and compounds that induce interstrand crosslinks (ICLs), like mitomycin C (MMC). The latter are extremely cytotoxic (3).

The clastogenic potential of chemicals can be tested with different types of genotoxicity assays. In previous studies we measured the *lacZ* mutant frequencies (*lacZ* MF) in both wild-type (WT) and DNA repair-deficient *Rad54/Rad54B* MEFs derived from mice carrying the *lacZ* gene in a plasmid vector. Cells were treated with both mutagenic (causing gene mutations) and clastogenic (causing chromosome aberrations) compounds (4). The *Rad54/Rad54B* MEFs have a defect in the *Rad54* and the *Rad54B* genes (both involved in Homologous Recombination (HR) repair), which we assume may cause a shift in the repair of single- or double-strand breaks from HR repair towards non-homologous end-joining (NHEJ) repair, which is an error-prone repair system**.** This presumed shift between repair systems might cause an accumulation of chromosomal damage induced by clastogens. Since *Rad54/Rad54B* cells have a defect in HR repair, it is to be expected that upon clastogen exposure these cells will accumulate higher *lacZ* mutant frequencies (MF) as compared to WT cells.

It was shown that MEFs isolated from both WT as well as *Rad54/Rad54B* MEFs were able to detect gene mutations and chromosomal aberrations. Surprisingly, of the clastogens used (BLM and MMC), only MMC showed a genotype-dependent effect; *Rad54/Rad54B* MEFs were more sensitive towards MMC treatment as compared to the WT MEFs (4). Bleomycin (BLM) induced DNA lesions which could be repaired equally effective in cells without active HR repair compared to WT cells, whereas mitomycin C (MMC) showed a differential effect in repair-deficient cells compared to WT cells. This confirms the difference in DNA damage caused by the clastogens, chromosomal breaks (BLM) versus cross linking (MMC).

As different types of clastogens result in genotype-dependent differences in genotoxic sensitivity, we hypothesized that these compounds also trigger separate pathways of (geno)toxicity in the two genotypes. Hence, we further investigated whether different

clastogens also led to specific different changes in gene expression patterns upon exposure, and thus if indeed different types of clastogens exist. To this end, we performed microarray analysis with WT and *Rad54/Rad54B* MEFs treated with the clastogens: MMC and BLM, and the mutagens: *N*-acetoxy-2-acetylaminofluorene (N-ac-AAF) and *N*-ethyl-*N*-nitrosourea (ENU). The two mutagens were included as controls aimed at distinguishing general genotoxicity responses as well as genotype-independent responses specific to either clastogens or mutagens. The outcome of our studies confirms our hypothesis that different clastogens lead to specific gene expression changes and moreover the responses are genotype specific.

Methods

Isolation of mouse embryonic fibroblasts (MEFs)

Embryos of 13.5 days were harvested from wild-type (WT) and *Rad54/Rad54B* repair-deficient (*Rad54/Rad54B*) mice. All mice were in a C57/BL6 genetic background and were bred and maintained under specific pathogen-free conditions at the animal facility of the Netherlands Vaccine Institute (NVI, Bilthoven, The Netherlands). All animal experiments were approved by the Institute's Animal Ethics Committee and were carried out in accordance with Dutch and international legislation. The liver and head were discarded from the embryonic body (to avoid disturbance during fibroblast growth). The remainder of the embryonic body was trypsinised and cultured in a 75 cm^2 flask containing 15 ml culture medium (Dulbecco's Modified Eagle Medium (DMEM) completed with 1% Modified Eagles Medium Non-Essential Amino Acids (MEM NEAA), 1% Penicillin-Streptomycin (PS) and 10% Fetal Bovine Serum (FBS)) at 37°C in a humidified atmosphere containing 3% O_2 and 10% CO_2 for 3 days.

After 3 days, the cells were trypsinised, equally divided over two 175 cm^2 culture flasks, and were grown for another 4 days. Thereafter, the cells were collected using trypsin and counted in a Bürker-Türk. After centrifugation at 1200 rpm and 4°C for 5 minutes, the cell pellet was resuspended in freezing medium (DMEM completed with 20% FBS. 10% dimethylsulfoxide (DMSO), 1% MEM NEAA and 1% PS) at a concentration of $3x10^6$ cells per ml while keeping it on ice. One ml portions were kept at -80°C for at least 24 hours and were then stored in liquid nitrogen.

Treatment of MEFs

For each compound and each assay, aliquots of $3x10^6$ cells of both genotypes were seeded and cultured in a 175 cm^2 flask containing 30 ml culture medium. Twenty-four hours before treatment, the cells were dissociated with trypsin and cultured into petridishes containing $1x10^6$ cells and a final volume of 10 ml culture medium.

On the day of treatment, the cells were washed once with D-PBS (Dulbecco's phosphate-buffered saline, containing KCl, KH_2PO_4, NaCl, Na_2HPO_4, without calcium and magnesium) before treatment with the various genotoxic compounds (see Table 1).

Table 1. Concentrations of all compounds used for the microarray experiment.

Compound	Concentration	Solvent	CAS number
MMC (Mitomycin C)	0.2 μg/ml	PBS	50-07-7
BLM (Bleomycin)	20 μg/ml	PBS	9041-93-4
N-ac-AAF (*N*-acetoxy-2-acetylaminofluorene)	30 μM	DMSO	6098-44-8
ENU (*N*-ethyl-*N*-nitrosourea)	4 Mm	Culture medium	759-73-9

Untreated MEFs were used as a control. The concentration of each compound was chosen from previously obtained survival data of both WT as well as *Rad54/Rad54B* MEFs treated with the different compounds (4). In the XTT test, these concentrations resulted in approximately 80% survival.

The compounds were dissolved in the appropriate solvent (see Table 1) on the day of treatment. The cells were treated with the different compounds in a final volume of 4 ml for 3 hours. After treatment, the cells were washed once with D-PBS and cultured in 10 ml culture medium for an additional 5 hours before collecting them for RNA isolation. Thereafter, the cells were washed once with D-PBS. The cells were dissociated with 750 μl RLT-buffer using a cell scraper and were collected in a 2 ml tube and stored overnight at -80°C until RNA isolation and microarray analysis was performed.

RNA isolation

Total RNA from each sample was extracted using the Rneasy Mini kit (Qiagen, Valencia, CA, USA), followed by a DNase treatment with RNase-Free DNase Set (Qiagen, Valencia, CA, USA). The RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and RNA quality was determined on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Measurements were performed according to the manufacturer's protocols. Total RNA samples with an RNA integrity number (RIN) > 7 were used for further analysis.

Microarray analysis

Mouse oligonucleotide libraries were obtained from Sigma-Compugen Incorporated. The libraries represent a total of 21,825 LEADS™ clusters plus 231 controls. The oligonucleotide libraries and additional control oligos from the Lucidea™ Microarray ScoreCard™ (GE Healthcare) were printed with a Lucidea Spotter (Amersham Pharmacia Biosciences, Piscataway, NJ, USA) on UltraGAPS slides (amino-silane-coated slides, Corning #40017, Corning Life Sciences, Lowell, MA, USA) and processed according to the manufacturer's instructions.

Mouse genome microarrays were used in the analysis of gene expression profiles of WT and *Rad54/Rad54B* MEFs exposed to mutagens and clastogens (see Table 1 in the previous section). For each exposure or control group, five RNA samples were analyzed, each of which was hybridized to an individual microarray slide. In short, Cy3 and Cy5 labelled cRNA samples were prepared as described in the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, Texas, USA) using 1 μg of purified total RNA and a 100 fold dilution of Lucidea Spike-ins (GE Healthcare) as template for the reaction. Test samples were labelled with Cy3 and the common reference was labelled with Cy5. The common reference was made by combining 1 μg of each test sample, labelling 1 μg portions of this pool with Cy5 and pooling the resulting Cy5 labelled material afterwards. 1 μg of Cy3 cRNA was combined with 1 μg Cy5 labelled cRNA and incubated for 30 minutes at 60°C in the presence of fragmentation buffer (Agilent). The fragmentized solution was mixed in a 1:1 ratio with a 2 x hybridization buffer (Agilent) and transferred to the microarray. A sandwich of the microarray with a backing slide was hybridized overnight in a Surehyb chamber. Hybridization was performed overnight according to the Agilent, Low NA input Linear Amplification procedure at 65°C. The Surehyb chamber was disassembled in GE wash buffer 1 and washed for 1 minute at room temperature and for another minute in GE wash buffer 2 at 37°C. Microarrays were quickly dried by dipping in isopropanol and a short spin at 230 rcf. Arrays were scanned at two wavelengths (Cy3 and Cy5, or 532 and 633 nm, respectively) on an Agilent G2565 microarray scanner.

Data analysis and statistics

Array Vision software (Imaging Research, St. Catherine's, Ontario, Canada) was used to determine median Cy3 and Cy5 signal intensities for each separate spot and background noise. Quality control was performed on raw data by means of visual inspection of the

scanned images, as well as a check on the scatter and MA (ratio-intensity) plots. The control spots present on the slide were used for quality control, but excluded from the further analysis. Raw signal data for oligonucleotide-containing spots were normalized with R software (www.r-project.org) by using a three-step approach (5) that consisted of (1) natural log transformation, (2) quantile normalization of all scans, (3) correction of the sample spot signal for the corresponding reference spot signal.

Differences in gene expression between experimental MEFs sample groups were determined by a one-way ANOVA. Genes with a False Discovery Rate (FDR) < 0.05 and a Fold Ratio (FR) > 1.5 (between treated and control group) were considered differently expressed. Correlations between sets of differently expressed genes were calculated for the union of the two sets. Enrichment for Gene Ontology and other functional terms was determined by DAVID / EASE (http://david.abcc.ncifcrf.gov) (6). Additional analyses were performed using GeneMaths (Applied Maths, St-Martens-Latem, Belgium). Principal Component Analysis (PCA) was performed on the average whole-genome gene expression profile of the experimental groups. For clustering analysis, the merged data of all genes regulated in at least one treatment-control comparison were combined into a table with the appropriate ln-ratio/control values, which was further analyzed using Euclidean distance and Ward linkage.

Groupwise regulation of Gene Ontology categories and gene sets were determined by Gene Set Enrichment Analysis (GSEA) (7) using the pre-ranked GSEA option under default analysis parameters. Gene set collections used were the c5 (Gene Ontology) and c2 (expert curated) gene sets provide by MsigDB (http://www.broad.mit.edu/gsea/msigdb/), and additional gene set collections developed in house. Gene sets were considered regulated if the GSEA *p*-value was < 0.05 and the FDR was < 0.25.

Results

Basal genotype gene expression differences.

After microarray data normalization, Principal Component Analysis was performed on the average gene expression profiles of the experimental groups (Figure 1). This indicates a separation between all exposed and control WT versus *Rad54/Rad54B* MEFs, respectively. However, in contrast to the relatively large genotype difference suggested by the PCA, only 12 genes were found to be differentially expressed between these groups (FDR < 5%, FR > 1.5). GSEA analysis showed a subtle but coordinate relative down-regulation of cell division related genes in the *Rad54/Rad54B* MEFs compared to the WT MEFs. Likewise, this genotype showed a similarly subtle but coordinate trend in up-regulation of immunological pathways and immune-cell associated genes as compared to the WT MEFs. This indicates that the genotype separation indicated by the PCA is due to small changes in the activity of the cell division machinery and a higher percentage of (precursors of) immune cells. It should be mentioned here that MEFs are derived from a large part of the embryo and consist of a mixed cell population, containing mostly fibroblast but also other cell types. Further GSEA analysis using cell type-specific gene sets could not ascribe the shift in immune cell expression to specific immunological cell lineages.

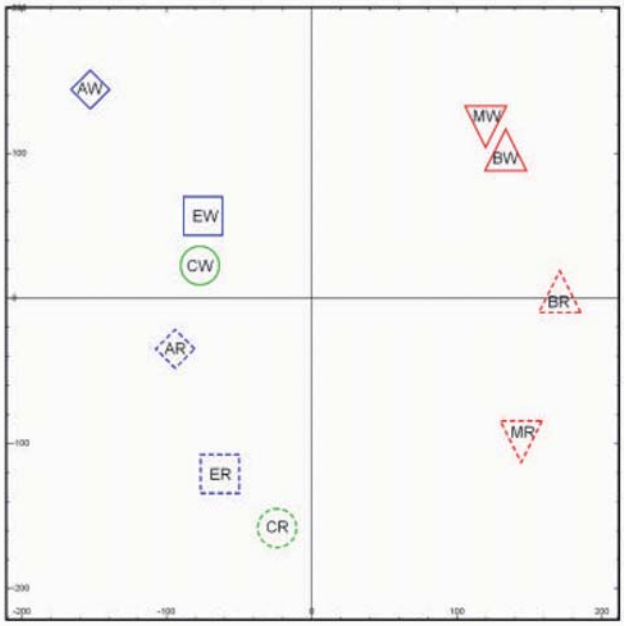

Figure 1. Principal component analysis on genome-wide expression profiles for the experimental groups.

CW: WT MEFs – untreated, **CR**: *Rad54/Rad54B* MEFs – untreated, (circle);

AW: WT MEFs - N-ac-AAF, **AR**: *Rad54/Rad54B* MEFs - N-ac-AAF, (diamond);

EW: WT MEFs – ENU, **ER**: *Rad54/Rad54B* MEFs –ENU, (square);

BW: WT MEFs – BLM, **BR**: *Rad54/Rad54B* MEFs –BLM, (triangle);

MW: WT MEFs – MMC, **MR**: *Rad54/Rad54B* MEFs –MMC (inverted triangle).

Gene expression response to mutagens.

Exposure to N-ac-AAF resulted in 150 and 143 differently expressed genes in WT and *Rad54/Rad54B* MEFs, respectively. The responses were highly comparable (R = 0.96) for both genotypes, as can also be seen from Figure 2.

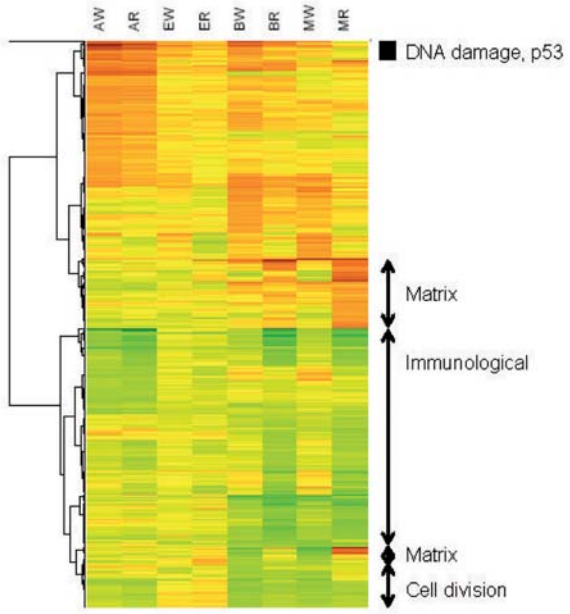

Figure 2. Hierarchical clustering of gene expression changes in WT and *Rad54/Rad54B* MEFs after mutagen or clastogen exposure. Expression changes are given relative to the corresponding control, where red represents up-regulation, green down-regulation and yellow no difference in gene expression levels. The black bar indicates the cluster showing the strongest genotype-dependent effect for the MMC-exposed MEFs.
Exposures are as follows: **AW**: WT MEFs - N-ac-AAF, **AR**: *Rad54/Rad54B* MEFs - N-ac-AAF, **EW**: WT MEFs – ENU, **ER**: *Rad54/Rad54B* MEFs – ENU, **BW**: WT MEFs – BLM, **BR**: *Rad54/Rad54B* MEFs – BLM, **MW**: WT MEFs – MMC, **MR**: *Rad54/Rad54B* MEFs – MMC.

Functional annotation showed that the strongest induced genes were involved in a number of connected pathways such as p53-signaling, DNA damage response, and apoptosis (*Pmaip1*, *Ccng1*, *Btg2*, *Mdm2*, *Cdkn1a*, *Rprm*, *Perp, Bax*). In addition, there was up-regulation for a number of oxidative stress genes (*Mt1*, *Gsta1*, *Gsta2*, *Gsta4*, *Gclm*). Down-regulation was mainly observed for cell division genes (e.g. *Ccnb1*, *Ccnb2*, *Ccna2*, *Aurka*).
ENU exposure did not result in any differently expressed genes in WT and only 2 in *Rad54/Rad54B* MEFs. Although hardly any genes were significantly regulated, we did observe that the DNA damage responsive genes regulated by N-ac-AAF showed a similar but weaker trend upon ENU exposure (Figure 2 and Table 2).

Table 2. Gene expression changes for DNA damage response and cell cycle genes in WT and *Rad54/Rad54B* MEFs after exposure to mutagens and clastogens.

Gene symbol	Alias	WT[1] - N-ac-AAF	Rad[2] - N-ac-AAF	WT – ENU	Rad - ENU	WT - BLM	Rad - BLM	WT - MMC	Rad - MMC
Pmaip1	Noxa	3.26*	2.40	1.39	1.29	4.12*	2.86*	2.26	-1.02
Ccng1		2.74*	2.00*	1.77	1.28	2.99*	2.13*	1.54	-1.11
Btg2		2.46*	1.68*	1.59	1.09	2.77*	1.75*	1.55	1.13
Mdm2		6.37*	3.40*	2.21	1.54	3.08*	1.95*	1.99	1.08
Cdkn1a	P21	4.20*	2.81*	2.09	1.54	3.06*	2.00*	1.67	1.35
Rprm		2.41*	2.37*	1.21	1.31	2.50*	1.97*	1.14	1.38
Perp		1.54*	1.40	1.07	1.08	1.48	1.46	1.05	1.08
Bax		1.55*	1.52*	1.14	1.29	1.50	1.40	1.25	-1.05
Bbc3	Puma	1.34	1.24	1.16	1.10	1.54*	1.33	1.41	1.11
Mgmt		1.01	1.06	1.15	1.16	1.72*	1.29	1.07	-1.08
Ccnb1		-1.63*	-1.78*	1.07	1.20	-1.6*	-1.43	-1.58	-1.61
Ccnb2		-1.64*	-1.56*	-1.08	1.16	-1.67*	-1.48	-1.79*	-1.42
Plk1		-1.55	-1.72*	1.07	1.19	-2.00*	-1.57*	-2.04*	-1.51
Ccna2		-1.24	-1.37	1.07	1.16	-2.01*	-1.43	-1.58*	-1.38
Ube2c		-1.25	-1.48	1.25	1.23	-1.44	-1.42	-1.70*	-1.58
Aurka		-1.28	-1.59*	1.29	1.22	-1.81*	-1.50*	-1.71*	-1.58*
Cdkn3		-1.44	-1.45	-1.01	1.16	-1.48	-1.22	-1.53*	-1.24
Cdca3		-1.31	-1.39	-1.00	1.17	-1.51*	-1.32	-1.44	-1.38
Cdc2a		-1.16	-1.22	1.18	1.27	-1.64*	-1.33	-1.56*	-1.44
Bub1b		-1.10	-1.31	1.10	1.07	-1.67*	-1.43	-1.49	-1.27
Cdca8		-1.12	-1.29	1.17	1.11	-1.57*	-1.32	-1.34	-1.35
Cdkn2d	P19	-1.24	-1.17	1.10	1.45	-1.47	-1.26	-1.65*	-1.04
Cdkn2c	P18	1.05	-1.05	1.19	1.42	-1.59*	-1.34	-1.60*	-1.09

[1]WT are wild-type MEFs treated with the different compounds

[2]Rad are *Rad54/Rad54B* MEFs treated with the different compounds

*An asterisk indicates a significant regulated gene (at FDR < 5% and ratio > ± 1.5)

Gene expression response to clastogens.

The numbers of differently expressed genes in WT and *Rad54/Rad54B* MEFs after BLM treatment were 166 and 178, respectively. Responses for both genotypes were comparable (R = 0.75), but more different than those observed for the mutagen exposures (see above). Functional annotation of differentially expressed genes showed the predominant effect was induction of genes involved in p53-signaling, DNA damage response, and apoptosis (*Pmaip1*, *Ccng1*, *Btg2*, *Mdm2*, *Cdkn1a*, *Rprm, Bbc3*). Down-regulation was observed for several cell

division genes (e.g. *Ccnb1*, *Ccnb2*, *Ccna2*, *Aurka*, *Cdca3*, *Cdc2a*, *Bub1b*, *Cdca8*). These changes were overall similar to those observed for the mutagen N-ac-AAF.

For MMC treatment, the numbers of differentially expressed genes were 76 and 156 for WT and *Rad54/Rad54B* MEFs, respectively. Here, the responses were different (R = 0.29) between the MEFs of the two genotypes. For the WT MEFs, functional annotation showed induction of p53-signaling and DNA damage response genes (*Pmaip1*, *Ccng1*, *Btg2*, *Mdm2*, *Cdkn1a)*, and down-regulation of cell division (*Ccnb1*, *Ccnb2*, *Ccna2*, *Ube2c*, *Aurka*, *Cdc2a*) genes, in a similar manner as seen for BLM and N-ac-AAF. For the *Rad54/Rad54B* MEFs, we observed down-regulation of cell division genes similar to that found in the corresponding WT exposure. However, induction of p53-signaling, DNA damage response, and apoptosis were not found in the MMC-treated *Rad54/Rad54B* MEFs. Comparing these responses among the clastogen-exposed groups showed that these were almost absent in the MMC-treated *Rad54/Rad54B* MEFs but present in the other groups (Figure 2 (indicated as a black block) and Table 2). This latter finding was confirmed in the GSEA results: the canonical p53-related pathways in the MsigDB C2 database all showed a significant pathway-level induction for the different treatment *vs* control comparisons; except for the MMC exposed *Rad54/Rad54B* MEFs where p values as well as the corresponding FDR were > 0.25.

Gene expression response comparison clastogens to mutagens.

Comparing the gene expression changes for mutagen and clastogen exposed MEFs showed a cluster of commonly regulated genes for all the exposures except for the MMC-treated *Rad54/Rad54B* MEFs. This cluster is indicated in Figure 2 with a black bar. Additionally, for both BLM and MMC exposed MEFS, gene expression down-regulations were found that were not (or less) present in mutagen exposed MEFs of both genotypes (Figure 2). Functional annotation showed that the majority of the genes involved are either involved in immunological pathways or known immune cell markers. Interestingly, these genes show a stronger down-regulation in the *Rad54/Rad54B* than in the WT MEFs, indicating that this phenomenon is linked to the higher proportion of immune cells in the *Rad54/Rad54B* MEFs. Clustering also revealed an increase in extracellular matrix gene expression in the clastogen treated MEFs, which was very pronounced in the *Rad54/Rad54B* MEFs but less induced or even down-regulated in WT MEFs (Figure 2). As extracellular matrix genes are highly expressed in fibroblast this indicates a relative enrichment for fibroblast mRNA when compared to the down-regulation of immune cell mRNA in the total mRNA fraction.

Discussion

We have previously demonstrated that WT and *Rad54/Rad54B* MEFs were able to detect both mutagen and clastogen activity using *lacZ* as a reporter gene. For the MMC-exposed, but not the BLM-exposed MEFs, we observed an increase in *lacZ* mutant frequency in the *Rad54/Rad54B* MEFs compared to the WT MEFs. This confirms that different types of clastogens exist, which causes genotype-dependent differences in genetic damage. To investigate whether these differences are reflected in triggering differences in genotoxicity response pathways, we examined gene expression changes in WT and *Rad54/Rad54B* MEFs upon exposure to two types of clastogens, two mutagens and unexposed controls.

PCA analysis shows that an overall genotype-dependent difference exists between all WT versus all *Rad54/Rad54B* MEFs. A combination of statistical analysis at the gene expression level and a threshold-free whole-genome analysis (GSEA) showed that these differences could be ascribed to a combination of lower activity of cell division genes and an increase in the proportion of immune cells in the *Rad54/Rad54B* MEFs compared to the WT MEFs. The presence of immune cells among mouse embryonic fibroblast cells can be explained because MEFs are not exclusively derived from fibroblast containing tissues. Instead, MEFs originate from a larger part of the embryo, excluding liver and head but including immunologically relevant tissues. By the choice of culturing conditions, further MEF culture selects for fibroblast cells. However, this selection will not be complete and some immune cells remained present in the MEFs used for the experiments, especially those described for *Rad54/Rad54B* MEFs.

PCA visualization of the whole-genome data (Figure 1) shows a similar shift in both direction and length for the gene expression profiles for each compound compared to their respective control. In the case of ENU exposed MEFs the overall effect is small and for these exposures hardly any significantly regulated genes were found in MEFs of both genotypes. Clastogen-exposed MEFs (both MMC and BLM) show an overall similar trend in the PCA and were different for the N-ac-AAF-exposed MEFs, indicating that there is a difference in gene expression response after mutagens and clastogens exposure.

For most of the exposures, a broad-scale DNA damage response was observed. This included genes that are involved in apoptosis (*Pmaip1*, *Mdm2, Cdkn1a*, *Perp*, *Bax*, *Bbc3*), cell cycle arrest (*Ccng1*, *Btg2*, *Mdm2, Cdkn1a*, *Rprm*), and DNA repair (*Mgmt*); with a role for p53 in their activation being a common factor. Responses in these genes lead to temporarily cell

cycle arrest and DNA repair or apoptosis. In line with this finding, we also observed a down-regulation of cell division genes for most of the exposures. Induction of oxidative stress genes was only found for the N-ac-AAF exposed cells by means of induction of several GST enzymes. Although oxidative stress plays a role in inducing genotoxicity, the response found here can also be part of a general protection mechanism against this compound. Exposure to clastogens, but not mutagens, resulted in a relative down-regulation of immune cell-associated genes and a relative up-regulation of fibroblast-associated genes. Both these effects were more pronounced in the *Rad54/Rad54B* MEFs as compared to the WT MEFs. The relative down-regulation of immune cell-associated genes suggests that immune cells and their precursors are more sensitive to clastogen exposure than to mutagen exposure. The higher proportion of immunological cells in the *Rad54/Rad54B* MEFs population provides an explanation for the enhanced effects observed for this genotype. The relative up-regulation of extracellular matrix genes can be explained in that a lower presence of immune cell-derived mRNA in the total mRNA fraction leads to a relative increase for mRNA highly expressed in other (e.g. fibroblast) cells. This is consistent with the finding that this effect is also more pronounced in the *Rad54/Rad54B* genotype than WT MEFs. It should be noted that the differences in cell composition, and therefore transcriptional changes in lineage specific genes upon treatment, are not necessarily related to genotype-dependent differences between WT and *Rad54/Rad54B* MEFs. As MEFs are not clonal in their origin, some variations in cell composition between isolations are inherent to the use of such cells. Transcriptional changes in cell lineage specific genes reflect the natural variation between MEF batches coinciding with different sensitivities between cell lineages to different (classes of) genotoxic compounds.

The concentrations used in the experiment were chosen to result in comparable effects on survival and indeed the WT response for N-ac-AAF, BLM, and MMC was found to give similar degrees of gene expression changes. In contrast, ENU gave much weaker gene expression changes in both MEF genotypes. A possible explanation for this could be the half life of ENU which is approximately 1 hour in culture medium (8). The gene expression changes were measured 8 hours after treatment with the different compounds. Since ENU only has a half-life of 1 hour, most of its reactivity will have disappeared within the first few hours after exposure. The remaining time gives the cells the ability to restore the DNA damage caused by ENU exposure. Therefore, there will be no or a very low effect measured of the ENU exposure 8 hours after treatment. This was seen as both ENU treated as well as

untreated samples showed quite similar gene expression changes in both WT as well as *Rad54/Rad54B* MEFs.

Effects for the N-ac-AAF treatments in WT and *Rad54/Rad54B* MEFs were similar in their response, and the response for ENU treatment was virtually absent in MEFs of both genotypes. Therefore, it can be said that mutagens give the same gene expression response in both WT and *Rad54/Rad54B* MEFs. For the clastogen treatments, however, this was not the case. For the BLM treatment, the response was moderately similar, albeit comparable ($R = 0.75$) for MEFs of both genotypes. An even stronger difference was found for after MMC treatment ($R = 0.29$) between MEFs of both genotypes. Thus, the response for *Rad54/Rad54B* MEFs is different from the WT response to clastogens, making it (at least partially) clastogen specific. Remarkably, in addition to differences in gene expression profiles after clastogen and mutagen treatment, there is also a difference between the two clastogens used (BLM and MMC). The major difference between the responses to BLM and MMC lies in a weaker response through the p53-signaling pathway upon MMC exposure of *Rad54/Rad54B* MEFs, whereas the reduction in cell division genes was not affected.

The reduction in p53-signaling pathway genes for MMC, though not for BLM, is in line with our previous finding that two types of clastogens exist (4). One including BLM and γ-radiation, acting mainly through single- and/or double-strand breaks, that can be repaired equally effective in cells with and without active HR repair. MMC on the other hand belongs to a class that causes DNA crosslinks which show a differential effect in WT versus HR-repair-deficient cells. All these findings indicate that MMC damage repair is HR dependent. In this study, the MMC response deviates between WT and *Rad54/Rad54B* MEFs compared to the BLM response. The reduced p53-signaling after MMC exposure in *Rad54/Rad54B* MEFs could provide a mechanistic explanation for the increased *lacZ* MF after MMC exposure (4), as an impaired DNA damage response will lead to a weaker DNA repair response and therefore a larger percentage of the cells will carry a *lacZ* mutation.

This study provides evidence that the difference in DNA damage response between BLM and MMC is caused by insufficient p53-signaling at the gene expression level, presumably due to lack of DNA crosslink damage recognition in MMC-exposed *Rad54/Rad54B* MEFs. This suggests that HR is not only necessary for crosslink repair, but that the *Rad54* and/or *Rad54B* genes are involved in DNA crosslink damage recognition.

Conclusion

In this study, we used WT and DNA repair deficient *Rad54/Rad54B* MEFs to study transcriptional responses to two different clastogens; bleomycin (BLM), which causes chromosomal breaks, and the crosslinking agent mitomycin C (MMC). The mutagens N-acetoxy-2-acetylaminofluorene (N-ac-AAF) and N-ethyl-N-nitrosourea (ENU) induced similar gene expression changes in MEFs of both genotypes, however, the two clastogens triggered different responses. In MMC-exposed *Rad54/Rad54B* MEFs we could not detect a p53-dependent response to genotoxic offence, which was as expected normally induced in MMC-exposed WT MEFs as well as in BLM-exposed MEFs being either repair deficient or proficient. This study is in line with our previous study (4) and lends further support to our hypothesis that different types of clastogens exist. This can have implications for compound hazard identification.

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Chapter 7

Summary and General Discussion

Summary and General Discussion

The aim of the studies described in this thesis was to develop an *in vitro* model consisting of mouse embryonic fibroblasts (MEFs) derived from the pUR288 mouse model, harbouring the *lacZ* gene, for the simultaneous detection of both mutagenic as well as clastogenic features of compounds. The effect of exposure to mutagens (chemical compounds causing predominantly gene mutations) and clastogens (chemical compounds causing predominantly chromosomal aberrations) on the *lacZ* mutant frequency (*lacZ* MF) was extensively studied by performing both *in vivo* and *in vitro* studies with wild-type (WT) pUR288 and DNA repair deficient *Rad54/Rad54B* mice, both carrying the *lacZ* gene, and MEFs isolated from these mice. The results are summarized, discussed and some future prospectives are given.

7.1. The pUR288 mice versus the Muta™Mouse model

The sensitivity of pUR288 mice was compared to that of the Muta™Mouse model. The Muta™Mouse model, a bacteriophage λ-based assay, is a commercially available model and detects gene mutation and small deletions, but not large deletions. This restriction is caused by the fact that Muta™Mouse is a bacteriophage model and thus the length of the insert as well as the presence of active *cos*-sites are essential (13,25,29).

It was hypothesized that while the Muta™Mouse model would only be able to detect the mutagens, the pUR288 mouse model would be able to detect both the mutagens as well as the clastogens (3). Previously, it has been shown in Muta™Mouse that clastogen treatment induced low or no increase in the *lacZ* MF in contrast to the MNT (26). It appeared that mitomycin C (MMC), a clastogen, was negative in the Muta™Mouse assay, but induced micronuclei in the bone marrow of the same animals. Suzuki *et al.* (20) have also shown that single treatment of Muta™Mouse with MMC induced a significant increase in the percentages cells containing a micronucleus, but no significant induction in the *lacZ* MF of the bone marrow or liver. Surprisingly, exposure of these mice for 5 consecutive days induced an approximately 2-fold increase in the *lacZ* MF in the bone marrow (20). However, this was not confirmed by another study of Cosentino and Heddle (6). In general, it appears that clastogens like MMC do not induce detectable levels of *lacZ* gene mutations in Muta™Mouse (20).

As expected in our studies both Muta™Mouse and the pUR288 mouse model were able to detect genotoxic activities of mutagens like 2-AAF, ENU and B[a]P. On the other hand there

was hardly any induction of the *lacZ* MF in both models after exposure to the used clastogens. Only BLM showed a small but statistical significant increase in the *lacZ* MF in bone marrow cells of the pUR288 mice only (**Chapter 2**). These findings only partially underline the above mentioned hypothesis (6,20,26). The pUR288 model appears, to some extent, to be able to detect clastogenic activities of compounds. Choosing more optimal conditions like different (higher) concentrations, routes of excposure and timepoints can possibly improve the low sensitivity towards clastogens.

Further analysis of hybridization studies, using total murine DNA as a probe (**Chapter 2** and **4**), revealed that, after treatment with clastogens, the number of pUR288 mutants, containing mouse DNA, was increased. This indicates that the pUR288 mouse model is, to some extent, able to detect clastogenic acivities of compounds, this in contrast to the Muta^{TM}Mouse model.

To further investigate the sensitivity of the Muta^{TM}Mouse model, several dose-range exposure studies with different clastogens were conducted**.** The studies described in **Chapter 3** showed that for all compounds the *i.p.* route of exposure gave higher results as compared to the oral route. Apparently, exposure procedures and thus the systemic bio-availability, are important factors for efficient monitoring of activities of genotoxic (clastogenic) compounds.

The studies performed in **Chapter 3** showed that BLM treatment led to a significant induction of the *lacZ* MF in the Muta^{TM}Mouse model. However, the dose used was 25-times higher as the one used for the studies described in **Chapter 2**. It is known from previous literature that BLM is a genotoxic agent (17) that can cause gene mutations due to oxidative damage next to single- and double-strand breaks (17,19). Probably the induction of the *lacZ* MF in the Muta^{TM}Mouse model is a result of gene mutations caused by oxidative damage, and is not due to the number of single- or double-strand breaks. This oxidative activity could also be the explanation for the positive results found with BLM, but not with the other clastogens, in the pUR288 mouse model (**Chapter 2**).

7.2 *Rad54/Rad54B* and homologous recombination repair

Only weak *lacZ* MF inductions were found after clastogen treatment in repair-proficient pUR288 mice (**Chapter 2** and **4**). Clastogens induce single- or double-strand breaks, which are repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ) repair (9,14,23,24,28). It was hypothesized that knocking down *Rad54* and *Rad54B*, both involved in HR repair, would create a model which becomes more sensitive towards clastogens (1,8,9,21,27). If HR repair is knocked-out, this will either give rise to an accumulation of DNA strand-breaks or to a shift into NHEJ repair. This repair system is

error-prone and therefore may lead to an accumulation of *lacZ* mutations. For this reason we additionally introduced a *Rad54/Rad54B* deficiency in pUR288 mice in order to more efficiently test the clastogenic properties of compounds.

The results described in **Chapter 2** and **4** show that BLM, but not MMC, treatment led to a moderately higher, but statistically significant, increase in the *lacZ* MF in *Rad54/Rad54B* mice compared to those observed in WT mice. Even the results obtained in the MNT showed that the *Rad54/Rad54B* mice were slightly more sensitive towards the used clastogens. This in contrast with previous literature where it was shown that the *Rad54/Rad54B* mice and ES cells derived from these mice were hypersensitive for MMC compared to WT mice and ES cells. However, the hypersensitivity towards MMC of the double-mutant model was similar as seen for the single-mutant (*Rad54* or *Rad54B*) model (27). Essers *et al.* (10) have also shown that MMC-treated *Rad54* and *Rad54B* mice were indeed more sensitive than WT mice (10).

A possible explanation for the differences between MMC and BLM in our experiments (in *lacZ* MF induction) might be the type of DNA damage induced by these compounds. BLM induces single- or double-strand breaks, while MMC induces crosslinks (2,7,15-17,19). Apparently, clastogens with different features, like inducing DNA breaks or crosslinks, are repaired through different mechanisms. Previous studies performed with *Rad54/Rad54B* ES cells showed that repair of MMC is HR dependent (10,27). Consequently, this may suggest that, in the absence of HR repair, the repair of DSB caused by BLM can be dealt with by NHEJ, whereas the repair of DNA crosslinks totally depends on active HR repair. This latter theory needs, however, further experimental support.

One may argue that the concentrations of the used clastogens (**Chapter 4**) were too high and thus toxic to the cells, hence leading to cell death. The HRR-deficient *Rad54/Rad54B* mice are probably more sensitive compared to the WT mice for the high toxicity of the clastogens, which resulted in more cell death in *Rad54/Rad54B* mice and therefore in equal induction in *lacZ* MF in mice of both genotypes.

It is clear that the protocol used in these experiments is not optimal and needs further improvement.

7.3 Development of an *in vitro* genotoxicity screening assay: combining different genotoxic endpoints

Fibroblasts were chosen because they are easy to culture, studies are fast and and are relatively inexpensive. Previous exposure studies conducted with MEFs, showed that UV causes a statistically significant increase in the *lacZ* MF in a dose-dependant manner (4,5). These findings indicated that the use of MEFs derived from the pUR288 plasmid mice are useful to detect *lacZ* mutations.

MEFs, derived from pUR288 (WT) and *Rad54/Rad54B* mice, were tested in order to investigate whether these cells were able to detect both clastogenic and mutagenic features of compounds (**Chapter 5**). For this purpose several exposure studies with different clastogens and mutagens were conducted. A clear dose-dependent induction in *lacZ* MF in the WT MEFs after treatment with mutagens as well as clastogens was observed. The hybridization studies, to support the *lacZ* MF induction of clastogenic properties of compounds, showed a clear increase in DNA rearrangements for clastogens but as expected not for mutagens. Also the classical control test for clastogens, MNT, was only positive for the clastogens and not for the mutagens.

For the same reason as in the *in vivo* experiments, the *lacZ* MF induction in the WT MEFs was compared to that in the *Rad54/Rad54B* deficient MEFs. Only MMC-treated *Rad54/Rad54B* MEFs showed a higher *lacZ* MF as compared to the WT MEFs. These results were further confirmed by hybridization data and results obtained from the MNT. As discussed before for the *in vivo* experiments (section 7.2), these results suggest that clastogens with different features, like inducing DNA breaks or crosslinks, are repaired through different mechanisms. Crosslinks not repaired correctly and efficiently in *Rad54/Rad54B* mice or MEFs caused more *lacZ* mutations of which a substantial part are large deletions and/or chromosomal rearrangements. However, the genotypic effect of BLM found in the *in vivo* studies (**Chapter 4**) could not be recapitulated by the *in vitro* studies (**Chapter 5**). Although, this finding is not easy to explain, we should bear in mind that the cells taken *in vivo* (i.e. from the bone marrow) are intrinsically not comparable to those used *in vitro*, the MEFs. Apparently, DNA damage responses are cell-type specific, differ *in vivo* compared to *in vitro* and possibly these differences are influenced by the status of repair capacities of the cells.

Although we cannot explain all DNA damage responses *in vivo* and *in vitro* upon genotoxic exposure, we can, however, conclude that the WT MEFs are sensitive enough to measure the

effect of both mutagens as well as clastogens and for future experiments it is not necessary to use the DNA repair-deficient *Rad54/Rad54B* MEFs for this purpose.

7.4 Different clastogens trigger different damage response pathways

In the studies described so far different results were obtained with BLM inducing single- or double-strand breaks and with MMC causing DNA crosslinks. Comparison of the transcriptional responses in MEFs upon exposure to BLM, MMC and 2 control mutagens revealed that differences in gene expression patterns occur between the 2 clastogens tested (**Chapter 6**). In BLM treated WT and *Rad54/Rad54B* MEFs a p53-dependent DNA damage repair was found. These observed transcriptional fingerprints suggest that, upon exposure to these compounds, the MEFs go into apoptosis and they down-regulate cell cycling. In contrast, the MMC-treated *Rad54/Rad54B* MEFs, but not the WT MEFs, showed almost no p53-dependent transcriptional responses These micro-array studies confirm the difference in DNA damage response between BLM and MMC, which is possibly caused by insufficient p53-signaling in MMC-treated *Rad54/Rad54B* MEFs. This finding possibly explains the genotypic (WT versus *Rad54/Rad54B*) effects on the *lacZ* MFs in MMC-treated MEFs.

What the effects of BLM and MMC treatment are on transcription *in vivo* (*e.g.* in the BM of treated mice) need further experiments. However, it is our hypothesis that transcriptional responses *in vivo* will not perfectly match those found *in vitro*, especially not in a *Rad54/Rad54B*-deficient genetic background.

7.5 Final conclusions and future prospectives

The studies described in this thesis have shown that the *in vivo lacZ* pUR288 mouse model was not sensitive enough to detect clastogenic features of compounds. The sensitivity towards clastogens was not substantially increased by using a HR-repair-deficient *Rad54/Rad54B* mouse model. Only a weak positive result was observed after BLM exposure. In mouse embryonic fibroblast (MEFs) derived from the pUR288 mouse model positive effects on the *lacZ* MF after treatment with both clastogens as well as mutagens were found. It appeared even possible to detect both double-strand breaks and cross-links.

The major advantage of *in vitro* screening with *lacZ* MEFs is the simultaneous detection of both clastogenic and mutagenic activities. Although there are several appropriate *in vitro* tests available for the detection of the genotoxic properties of compounds, none of these tests is able to detect simultaneously gene mutations and chromosomal aberrations. Using this *in vitro* test can be of great advantage, since this can lead to a reduction in the number of tests

needed. The reduction in time and costs and the increase in the sensitivity makes this *in vitro lacZ* assay a good alternative for existing tests for screening of genotoxic compounds.

However, before this test can be used as an alternative for existing screening genotoxic compounds further studies are needed. These should include testing of a wider range of genotoxic compounds, non-genotoxic compounds, non-carcinogens and most importantly also low potency genotoxic compounds. Then it can be assessed whether this assay is reproducible and whether the results obtained can replace existing genotoxicity assays (29).

For the *in vitro* studies mouse embryonic fibroblasts (MEFs) were used, because they are primary cells, easy to obtain and easy to store for future use. Tests with these cells are relatively inexpensive, can be performed fast and ultimately may lead to a reduction in animal use for genotoxicity testing. On the other hand, although these cells have primary features, they most likely lack sufficient metabolic activity. Therefore, our *in vitro* studies with benzo[a]pyrene (B[a]P) were performed in the presence of exogenous metabolic activation enzymes (S9) to warrant metabolism. Metabolism is important for the conversion of compounds into active metabolites; and the lack of metabolism will have an impact on the genotoxic effect of compounds (13). Moreover, MEFs are a heterogenic population of cells consisting of mainly fibroblasts, but also many other cell types like immune cells are present. The heterogeneity of MEFs is important to acknowledge in future experiments since they may interfere with the effect measured in MEFs **(see Chapter 6)**.

Because of the disadvantages of MEFs, the use of primary hepatocytes derived from pUR288 mice should also be considered for their use in genotoxicity testing. Primary hepatocytes obtained by perfusion are metabolically active and are a (more) homogenic and stable population (11,12,22). Furthermore, hepatocytes are one of the most widely used cell types for toxicological studies (Sacco *et al.* 2004). However, before hepatocytes can be used as attractive alternatives in genotoxicity testing, one need to show whether simultaneous detection of different endpoints are also possible, like in MEFs, in these cells.

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Nederlandse samenvatting

Curriculum Vitae

List of Publications

Dankwoord

Samenvatting

De mens wordt dagelijks blootgesteld aan verschillende chemicaliën en agentia die aanwezig kunnen zijn in bijvoorbeeld voedsel of voedingssupplementen, verpakkingsmateriaal, medicijnen, cosmetica en pesticiden. Het is belangrijk dat, voordat deze producten op de markt komen, de chemicaliën die aanwezig zijn in deze producten worden geëvalueerd op potentiële schadelijke effecten voor mens, dier en milieu. Chemicaliën kunnen genotoxisch zijn en DNA schade veroorzaken, die leiden tot versnelde veroudering, ontwikkeling van kanker of celdood (geprogrammeerde celdood, ook wel 'apoptose' genoemd). De genotoxische verbindingen zorgen voor schade aan de structuur van het DNA molecuul, waardoor er een genverandering kan optreden. Hierdoor kan de functie van de cel veranderen. Genotoxiciteit kent twee eindpunten: genmutaties (mutageen) en chromosoom afwijkingen (clastogeen). Voorbeelden van genmutaties zijn puntmutaties (het uitwisselen van de ene nucleotide (bouwsteen voor het DNA) voor een andere), inserties (toevoegen van één of enkele nucleotiden) en kleine deleties (het verwijderen van <500 nucleotiden). Een voorbeeld voor chromosoom afwijkingen zijn chromosoom breuken.

Door de jaren heen zijn er diverse genotoxiciteitstesten ontwikkeld. Geen van deze testen is echter in staat zowel genmutaties als chromosoom afwijkingen tegelijkertijd vast te stellen. Om deze reden worden voor de beoordeling van een stof op genotoxische eigenschappen verschillende testen ingezet, zowel *in vitro* als *in vivo*. Voorbeelden van *in vitro* genmutatie (GM) testen zijn de Ames test (in bacteriën), de muis lymphoma test en de *hprt* test (de laatste twee zijn testen in zoogdiercellen). De chromosoom aberratie test (CAT) en de micronucleus test (MNT) zijn de meest gebruikte *in vitro* testen voor chromosoom afwijkingen. Indien er aanwijzingen zijn dat chemicaliën *in vitro* genotoxisch zijn, wordt er meestal ook *in vivo* onderzoek uitgevoerd. Bij *in vivo* onderzoek spelen een aantal fysiologische processen een rol die niet na te bootsen zijn *in vitro*, zoals de opname van stoffen (ook wel 'absorptie' genoemd), transport van stoffen ('distributie'), metabolisme (omzetten van stoffen in actieve en niet-actieve bestanddelen) en de uitscheiding van stoffen ('excretie'). De meest gebruikte *in vivo* genmutatie modellen zijn het Muta™Mouse model en het Big Blue® muismodel, terwijl de *in vivo* CAT en de MNT de meest gebruikte testen voor chromosoom afwijkingen zijn.

Zoals hiervoor is aangegeven, kunnen genotoxische stoffen DNA schade veroorzaken die schadelijk kan zijn voor mens, dier of milieu. Gelukkig zijn er in een cel mechanismen aanwezig om verschillende vormen van DNA schade snel en efficiënt te herstellen of te verwijderen. De belangrijkste DNA herstel mechanismen betrokken bij herstel van DNA laesies zijn direct repair, base excision repair (BER), nucleotide excision repair (NER) en mismatch repair (MMR). Specifiek voor chromosoom afwijkingen zijn homologe recombinatie repair (HRR) en niet-homologe end-joining (NHEJ) de belangrijkste DNA herstel mechanismen.

Het doel van de onderzoeken uitgevoerd in dit proefschrift was het ontwikkelen van een model dat simultaan genmutaties en chromosoom afwijkingen kan meten. Voor de *in vivo* experimenten is er gebruik gemaakt van pUR288 muizen. Dit model bevat het *lacZ* indicator gen dat niet alleen kleine deleties en puntmutaties kan meten maar ook grote deleties en chromosoom re-arrangements als gevolg van de grote deleties. Aangezien het nog niet eerder was aangetoond dat het pUR288 muismodel inderdaad gevoelig genoeg is voor het meten van clastogenen, is er een tweede muismodel gebruikt, namelijk het *Rad54/Rad54B* muismodel. Dit muismodel heeft een defect in zowel het *Rad54* als *Rad54B* gen; die beide deel uitmaken van het HRR. In een dergelijke genetische achtergrond kunnen DNA breuken alleen nog via het NHEJ worden hersteld. Aangezien NHEJ repair niet zonder fouten gaat, zou dit moeten leiden tot een hogere *lacZ* mutant frequentie (*lacZ* MF) in het muismodel met een defect in de *Rad54/Rad54B* genen. De verwachting was dus dat het *Rad54/Rad54B* muismodel gevoeliger is voor clastogenen dan het pUR288 muismodel. Voor de *in vitro* experimenten zijn embryonale fibroblasten (MEFs) geïsoleerd uit beide muismodellen. Dit *in vitro* model kan uiteindelijk gebruikt worden als een alternatieve genotoxiciteits test en kan leiden tot een efficiënter gebruik van *in vitro* methoden, omdat dit kan leiden tot een vermindering in het aantal testen die nodig zijn voor het bepalen van de genotoxiciteit van stoffen en een vermindering in tijd en kosten.

In dit proefschrift werd de gevoeligheid van het pUR288 muismodel voor clastogene stoffen onderzocht. Als controle werd het Muta^{TM}Mouse model, een veel gebruikte genmutatie test, meegenomen. De verwachting was dat het Muta^{TM}Mouse model niet erg gevoelig zou zijn voor effecten van clastogene stoffen, dit in tegenstelling tot het pUR288 muismodel. Gebleken is (Hoofdstuk 2) dat van de gebruikte clastogenen, alleen bleomycine (BLM) een kleine inductie gaf in de *lacZ* MF van het pUR288 muismodel en, zoals verwacht, niet in het

Muta^{TM}Mouse model. Verdere analyse met hybridisatie studies, gebruikmakend van muis DNA, toonde aan dat blootstelling aan alle geteste clastogenen een stijging geeft in het percentage chromosoom afwijkingen van het pUR288 muismodel. Dit laat zien dat het pUR288 muismodel inderdaad een veelbelovend model is om de clastogene effecten van stoffen aan te tonen. De test dient nog geëvalueerd te worden met diverse andere genotoxische stoffen, zwak genotoxische-, niet-genotoxische en niet-carcinogene stoffen. Hierdoor kan er onderzocht worden of deze test reproduceerbaar is en routinematig gebruikt kan worden en of deze test de huidige genotoxiciteitstesten kan vervangen.

De lage gevoeligheid van het Muta^{TM}Mouse model voor clastogene stoffen is verder onderzocht (Hoofdstuk 3) door verschillende routes van toediening en hogere doseringen van clastogene stoffen te gebruiken. De clastogenen werden zowel oraal (via de mond) als *intraperitoneaal* (*i.p.*; injectie in de buikholte) toegediend. Met behulp van de micronucleus test, een klassieke clastogeniteitstest, is aangetoond dat *i.p.* toediening van stoffen een groter effect geeft vergeleken met de orale toediening. Ook de *lacZ* MF test heeft bewezen dat de route van toediening een belangrijke factor is bij het meten van het effect van genotoxische stoffen. Voor toekomstige experimenten is het daarom belangrijk om hiermee rekening te houden.

Aanvankelijk leek het pUR288 muismodel niet optimaal voor het routinematig testen van clastogene stoffen. De gevoeligheid kon ook niet verhoogd worden door kruising met *Rad54/Rad54B* muizen (Hoofdstuk 4), die een defect hebben in het HRR. De hypothese was dat in dit model herstel van dubbelstreng breuken alleen nog kan plaatsvinden door middel van het NHEJ repair dat (kleine) fouten maakt, die in theorie door het *lacZ* systeem wel te meten zijn. Echter de resultaten van het onderzoek (Hoofdstuk 4) lieten zien dat dit laatste niet het geval is. In vergelijking met het pUR288 muismodel bleek het *Rad54/Rad54B* muismodel slechts gering gevoeliger te zijn voor de clastogenen BLM en γ-straling, maar niet voor mitomycine C (MMC). De conclusie was dat (nog) geen van beide muismodellen gevoelig genoeg is voor het detecteren van clastogene stoffen.

Om clastogene eigenschappen van stoffen ook in *in vitro* studies te kunnen bepalen, werden muis embryonale fibroblasten (MEFs) geïsoleerd uit zowel pUR288 als *Rad54/Rad54B* muizen. De MEFs werden behandeld met clastogene en mutagene stoffen (Hoofdstuk 5). Interessant genoeg konden we aantonen dat zowel de mutagene als de clastogene stoffen een

dosisafhankelijke stijging veroorzaken in de *lacZ* MF in MEFs geïsoleerd uit beide muismodellen. Er werd een genotype effect waargenomen na behandeling met MMC. MMC induceerde een hogere *lacZ* MF in de *Rad54/Rad54B* MEFs vergeleken met de pUR288 MEFs. Dit werd tevens bevestigd door hybridisatie studies. Omdat dit effect alleen met MMC werd waargenomen, en niet met BLM, konden we concluderen dat verschillende clastogenen soms ook verschillende type DNA schades veroorzaken. Mogelijk leidt BLM behandeling voornamelijk tot enkel en/of dubbelstreng breuken, die hersteld kunnen worden door zowel HRR als NHEJ. Herstel van DNA interstrand crosslinks, geïnduceerd door stoffen als MMC, is (voor een deel) afhankelijk van HRR.

Om deze hypothese verder te ondersteunen werden microarray studies uitgevoerd (Hoofdstuk 6). In deze studies zijn de effecten op genexpressie niveau onderzocht na behandeling van *Rad54/Rad54B* en pUR288 MEFs met verschillende mutagene en clastogene stoffen. De resultaten lieten zien dat het genotype effect werd veroorzaakt door een zwakke activatie van de *p53* route in de MMC-behandelde *Rad54/Rad54B* MEFs. Dit kan een verklaring zijn voor de hogere *lacZ* MF gevonden in de *Rad54/Rad54B* MEFs vergeleken met de pUR288 MEFs na behandeling met MMC. Een zwakke DNA schade response in de MMC-behandelde *Rad54/Rad54B* MEFs kan leiden tot een hoger percentage cellen met een *lacZ* mutatie. Hierdoor zouden er hogere *lacZ* MF in de *Rad54/Rad54B* MEFs gevonden worden, dit ten opzichte van de herstel-proficiënte pUR288 MEFs. Deze resultaten geven aan dat zowel het *Rad54* als het *Rad54B* gen mogelijk betrokken zijn bij herkenning en herstel van DNA crosslinks, veroorzaakt door bijvoorbeeld MMC.

Ons uiteindelijke doel van het uitgevoerde werk was het ontwikkelen van een *in vitro* testsysteem voor het meten van zowel de mutagene als de clastogene effecten van stoffen. De studies beschreven in dit proefschrift hebben ons meer inzicht gegeven over genotoxiciteit en het effect van mutagene en clastogene stoffen op de *lacZ* mutant frequentie (MF) en de expressie van genen. We hebben kunnen aantonen dat in MEFs zowel de mutagene als de clastogene effecten van stoffen gemeten kunnen worden. Interessant was de ontdekking dat het uitschakelen van HRR een hogere gevoeligheid induceerde voor het clastogeen MMC maar niet voor BLM, vergeleken met het wildtype model. De resultaten geven aan dat zowel het *Rad54* als het *Rad54B* gen mogelijk betrokken zijn bij herkenning en herstel van DNA crosslinks, veroorzaakt door bijvoorbeeld MMC. Het *in vitro* MEF systeem dient nog gevalideerd te worden met verschillende genotoxische (mutagene en clastogene), carcinogene

en niet-genotoxische stoffen. Bovendien moet de test verder geoptimaliseerd worden en moet worden onderzocht hoe stoffen die eerst gemetaboliseerd moeten worden, getest kunnen worden. Als alternatief voor MEFs zouden levercellen ('hepatocyten') bruikbaar kunnen zijn, omdat deze een werkend metabolisme hebben en bovendien een veel homogenere celpopulatie vertegenwoordigen dan MEFs.

Mogelijk kan de *in vitro* genmutatie test met MEFs of in de toekomst met hepatocyten gebruikt worden als een alternatief voor bestaande testen en het mogelijk maken om zowel de mutagene als clastogene eigenschappen van stoffen (routinematig) te testen in één gecombineerd *in vitro* testmodel. Dit zal dan leiden tot een vermindering in het aantal te gebruiken proefdieren en het aantal testen die nodig zijn voor het bepalen van de genotoxiciteit van stoffen.

Curriculum Vitae

Op 14 maart 1979 werd Anuska Gautmiedevie Mahabir, dochter van Paresram en Agnes Chandra Mahabir, geboren te Paramaribo in Suriname. Na het behalen van haar VWO diploma in 1998 aan het Dr. Mr. J.C. De Miranda Lyceum in Paramaribo, Suriname, werd in september 1998 begonnen met de studie Bio-Farmaceutische Wetenschappen (BFW) aan de Rijksuniversiteit Leiden in Nederland. Tijdens deze studie werd een hoofdonderzoeksstage uitgevoerd bij de vakgroep Farmaceutische Technologie onder begeleiding van L. Honeywell-Ngyuen. In augustus 2004 begon ze aan haar promotieonderzoek bij de afdeling Gezondheidsbeschermingsonderzoek (GBO, voormalig TOX) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven in samenwerking met de afdeling Dier, Wetenschap en Maatschappij (DWM) van de Faculteit Diergeneeskunde aan de Universiteit Utrecht. Het promotieonderzoek, waarvan de resultaten in dit proefschrift beschreven staan, is uitgevoerd onder begeleiding van dr. J. van Benthem, prof. dr. H. van Steeg en prof. dr. C.F.M. Hendriksen.

List of Publications

Mahabir AG, van Benthem J, Korsten H, Lynch AM, Bailey L, de Vries A, Hendriksen CFM, van Steeg H. (2008). Detecting genotoxic effects of potential clastogens: an *in vivo* study using the transgenic *lacZ* plasmid and the Muta^{TM}Mouse model. Mutation Research 652(2):151-157

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