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Detecting genotoxic effects of potential clastogens: An *in vivo* study using the transgenic *lacZ* plasmid and the Muta^{TM}Mouse model

Anuska G. Mahabir[a,c], Jan van Benthem[a], Hanneke Korsten[a], Anthony M. Lynch[d], Lorna Bailey[d], Annemieke de Vries[a], Coenradus F.M. Hendriksen[b,c], Harry van Steeg[a,*]

[a] *National Institute of Public Health and the Environment (RIVM), Laboratory for Health Protection Research, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands*
[b] *Netherlands Vaccine Institute (NVI), Joint Centre for Laboratory Animal Studies, Bilthoven, The Netherlands*
[c] *Netherlands Centre Alternatives to Animal Use (NCA), Utrecht University, The Netherlands*
[d] *GlaxoSmithKline, Department of Genetic Toxicology, Ware, United Kingdom*

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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 with 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. The latter results indicate that the plasmid mouse assay may still be a promising model for the detection of clastogens.

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1. Introduction

Regulatory agencies are frequently confronted with the legislation of new chemicals, re-evaluation of existing chemicals and with risk estimation for 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 substitutions, frame shifts, deletions and insertions, and chromosome aberrations, which in turn can be structural (clastogenic effect) and/or numerical (aneugenic effect) [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 pairs) 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

* Corresponding author. Tel.: +31 30 274 2102; fax: +31 30 274 4446.
E-mail address: harry.van.steeg@rivm.nl (H. van Steeg).

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

Dose (mg/kg bw)	Animal number	Bone marrow			Liver		
		Total number of plasmids	Number of mutants	MF × 10^{-5}	Total number of plasmids	Number of mutants	MF × 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			3.4 ± 1.5	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
							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			3.7 ± 2.0	184	9	4.9
							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

* $p < 0.05$.
** $p < 0.001$.

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* [19,24] 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 have been shown to induce micronuclei in non-transgenic mice [Refs. 12, 13 and 28, respectively]. 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.

2. Materials and methods

2.1. 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

Table 1B
The *lacZ* MF in bone marrow and liver of the Muta™Mouse

Dose (mg/kg bw)	Animal number	Bone marrow			Liver		
		Total number of plasmids	Number of mutants	MF × 10^{-5}	Total number of plasmids	Number of mutants	MF × 10^{-5}
Methyl cellulose							
0	1	113,220	25	22.1	740,520	12	16.2
	2	832,830	19	22.8	279,480	9	32.2
	3	191,250	6	31.4	572,730	25	43.7
	4	256,530	8	31.2	549,780	26	47.3
	5	222,870	16	71.8	450,330	33	73.3
	6	270,810	7	25.9	775,200	25	32.3
	7	83,130	1	12.03	355,470	39	109.7
	8	293,250	31	105.7	315,333	14	44.4
	9	283,050	1	3.53	424,830	21	49.4
	10				188,700	18	95.4
				36.3 ± 32.2			54.4 ± 29.5
ENU							
50	1	204,000	294	1441.2	163,200	36	220.6
	2	339,150	428	1262.0	665,550	159	238.9
	3	267,750	300	1120.5	687,990	238	354.9
	4	371,790	449	1207.7	435,030	202	464.3
	5	175,440	305	1738.5	387,090	150	387.5
				1354.0 ± 244.9**			333.3 ± 102.8
Bleomycin							
2.5	1	427,890	15	35.1	249,900	3	12.0
	2	308,040	23	74.7	435,030	19	43.7
	3	397,290	6	15.1	366,690	23	35.7
	4	334,050	22	65.9	368,730	30	81.4
	5	314,670	9	28.6	364,650	35	96.0
				43.9 ± 25.4			53.7 ± 34.3
m-AMSA							
3.0	1	431,970	13	30.1	384,540	18	46.8
	2	313,242	35	111.7	302,940	11	36.3
	3	69,870	3	42.9	175,950	13	73.9
	4	759,900	17	22.4	138,210	9	65.1
	5				168,810	7	41.5
				51.8 ± 40.9			52.7 ± 16.1
o-AMSA							
3.0	1	409,020	21	51.3	1,483,080	16	10.8
	2	496,740	26	52.3	916,470	9	9.8
	3	177,480	11	62.0	415,650	15	36.1
	4	1,945,600	12	6.2	189,210	12	63.4
				43.0 ± 25.0			36.4 ± 26.8
Camptothecin							
0.5	1	290,700	3	10.3	341,230	8	33.2
	2	174,420	5	28.7	1,048,800	19	18.1
	3	201,450	19	94.3	554,370	21	37.9
	4	154,530	3	19.4	310,080	16	51.6
	5	230,010	19	82.6	316,200	22	69.6
				47.1 ± 38.6			42.1 ± 19.5

** $p < 0.001$.

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 function 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.

2.2. 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 × 1000.

In the Muta™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 deter-

mine 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.

2.3. 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 2× SSC (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 min 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 2× SSC, the filter was packed in Saran wrap foil, put against a Phosphor imager and illuminated. The clones positive for mouse DNA were quantified.

2.4. 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 microlitres of fetal calf serum (FCS) and 1 μl blood were 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™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.

2.5. Statistical analysis

The mean *lacZ* plasmid mutant frequency and the micronucleus frequency for the different treatment groups were compared with Student's *t*-test. The percentages micronucleated PCEs and the size-change mutations (chromosomal rearrangements) were tested for significance with the χ^2 test ($\alpha = 0.05$).

Data (unpaired mutant frequency, and paired micronucleus frequency) obtained with the Muta™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].

3. Results

3.1. Gene mutation assay with transgenic animals

The *lacZ* mutant frequency (MF) was determined in bone marrow and liver of the *lacZ* plasmid and Muta™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™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.

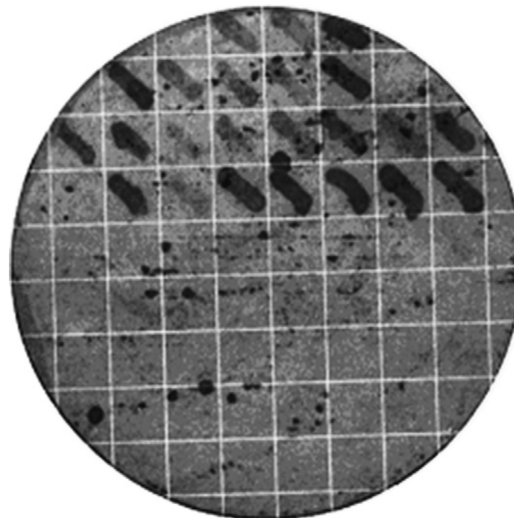

Fig. 1. Hybridisation of mutant colonies with total mouse DNA. *LacZ*-negative mutant clones were collected and grown overnight on a separate plate. Colonies were lifted and hybridised with a probe containing total mouse DNA (for details see Section 2). All clones containing (fractions of) mouse DNA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

3.2. 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). In the mutant colonies derived from bone marrow of clastogen-treated mice, an increase in the percentages of mouse DNA-positive clones as compared with 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-positive clones compared with the negative control.

3.3. Micronucleus test (MN test)

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

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

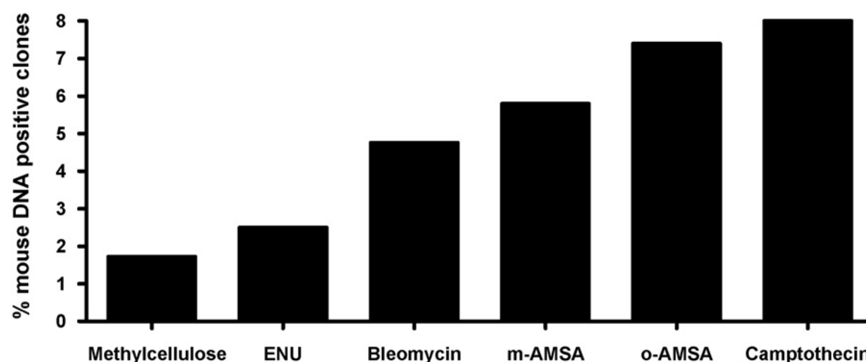

Fig. 2. Quantification of mouse DNA-positive *lacZ* mutant clones upon treatment with different clastogens. The percentages of mutant colonies hybridised with total mouse DNA in the bone marrow of the *lacZ* plasmid mouse after treatment with methylcellulose (negative control), ENU (positive control),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

Table 3
Micronucleus induction 24 h after the last treatment in the *lacZ* plasmid mouse and the Muta™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	0.42 ± 0.19	21.2 ± 17.1	0.37	2.12
		9			0.33	2.99
		10			0.32	10.97
					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

* $p < 0.05$.
** $p < 0.001$.

tically 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 the micronucleus test [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™Mouse, was decreased in the blood of

almost all treated mice and is indicative of sufficient exposure in the target cells (Table 3).

4. Discussion

In the present paper, we studied the capacities of the *lacZ* plasmid mouse model and the Muta^{TM}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^{TM}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^{TM}Mouse have different genetic backgrounds, which could have an effect on the responses to the different compounds. The plasmid mouse is derived from a B6 background (like the BigBlue mouse), whereas the Muta^{TM}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^{TM}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, confirm this expectation. The low induction of *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^{TM}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 does not meet those considered optimal in the current 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 to 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 ($0.42 \pm 0.19\%$) was comparable with that observed in the Muta^{TM}Mouse (0.35 ± 0.04) in the current study, and is consistent with published data (0.42%, see 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 adduct 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-positive clones by hybridisation with labelled total mouse DNA. In this hybridisation study, all clastogens showed an increase in mouse DNA-positive 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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