National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Estimating the carcinogenic potency of chemicals from the *in vivo* micronucleus test

RIVM report 340700007/2012

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Rapport in het kort

Schatting van de carcinogene potency van chemische stoffen uit de in vivo micronucleus test

Het RIVM heeft een methode ontwikkeld waarmee sneller en met minder proefdieren een schatting kan worden gemaakt van de mate waarin een chemische stof kankerverwekkend is.

Normaal gesproken wordt de mate waarin een stof kankerverwekkend is gebaseerd op het aantal tumoren dat in langdurige dierstudies wordt aangetroffen. Dergelijke langdurige studies zijn nodig omdat tumorvorming een langzaam proces is. Deze studies duren twee jaar en vergen veel proefdieren (rond de 400). Voordat tot een langetermijnstudie wordt overgegaan, wordt eerst met behulp van een kortetermijnstudie bekeken of een stof wel of geen DNA-schade veroorzaakt. Hiervoor zijn circa 50 proefdieren nodig. Als DNA-schade optreedt, is dit een indicatie dat de stof kankerverwekkend kan zijn. De langetermijnstudie wordt vervolgens ingezet om na te gaan of de stof inderdaad kankerverwekkend is, maar ook om een indicatie te krijgen van de mate waarin.

Uit het RIVM-onderzoek blijkt nu dat op basis van de kortetermijnstudie niet alleen duidelijk wordt of een stof DNA-schade veroorzaakt, maar ook een indicatie kan worden verkregen van de mate waarin de stof kankerverwekkend is. De langetermijnstudie met veel proefdieren kan dan in veel gevallen vermeden worden. Dit is van belang aangezien er internationaal naar wordt gestreefd het proefdiergebruik terug te dringen en het aantal langdurige studies te minimaliseren.

Voor de nieuwe methode is in kortetermijnstudies onderzocht bij welke concentratie (bijvoorbeeld in het voer van de dieren) een bepaalde mate van DNA-schade optreedt. Tevens is onderzocht bij welke concentratie een bepaald percentage van de proefdieren tumoren krijgt in de langetermijnstudies. Beide concentraties bleken aan elkaar gerelateerd.

Trefwoorden:
vervanging, voorspelbaarheid, *in vivo* genotoxiciteit, carcinogeniteit, benchmark dose, *in vivo* micronucleus test

Abstract

Estimating the carcinogenic potency of chemicals from the *in vivo* micronucleus test

The RIVM has developed a faster method for estimating the carcinogenic potency of compounds, using less animals than with existing methods.

Currently, the degree to which a substance is carcinogenic is estimated from the number of tumors found in animals in long-term studies. These long-term studies are necessary because the development of tumors is a slow process. Normally, these studies take two years and make use of many animals (around 400). Prior to deciding whether to perform a long-term study, short term-studies are always performed to examine if a compound causes DNA damage. The presence of DNA damage (a positive result in short-term test) is generally indicative that a compound might be carcinogenic. Long-term studies are therefore performed to confirm whether a compound is carcinogenic or not, and to assess how potent the substance is in inducing tumors (carcinogenic potency).

Research at the RIVM has shown that short-term studies can not only provide an indication as to whether a compound causes DNA damage, but also can provide an estimate of the carcinogenic potency of a chemical. With this new approach, long-term studies can be avoided. This is of interest given the international aim for reducing animal use and long-term studies.

In this new method, a comparison was made between the concentration that induced a selected degree of DNA damage in short-term studies and the concentration at which a selected percentage of animals developed tumors. Results demonstrated a relationship between concentrations in short- and long-term studies, thus providing the possibility to use short-term studies to obtain an indication of the carcinogenic potency of chemicals.

Keywords:
potency correlation, *in vivo* genotoxicity tests, carcinogenicity, benchmark dose approach, *in vivo* micronucleus test

Contents

Summary

The goal of this report was to investigate the use of short-term genotoxicity tests in predicting the carcinogenic potency of chemicals as measured in a chronic cancer bioassay. This bioassay has several practical drawbacks: it uses large numbers of animals, has a long duration (two years plus one year of analysis), and high costs. For this reason, alternative methods are desirable. *In vivo* genotoxicity assays might be useful as an alternative for estimating carcinogenic potency given the experience that genotoxicty tests measure mutations and/or chromosomal aberrations, which are associated with carcinogenesis.

In this study we focused on the murine *in vivo* micronucleus test as a potential predictor of the carcinogenic potency of compounds in the same species. We applied the benchmark dose (BMD) approach for estimating the genotoxic and the carcinogenic potency for a total of 51 compounds: 41 were studied in the National Toxicology Program and 10 were re-analyzed from the Carcinogenic Potency Database and public literature. Both genotoxicity and carcinogenicity studies showed large variability in estimated BMDs for the same compounds when data from studies with different study conditions were compared. In spite of this variability, the BMDs derived from the genotoxicity studies were clearly correlated to the BMDs from the carcinogenicity studies, in particular when the lowest BMD was selected for each compound, both for the micronucleus and the carcinogenicity studies. Results suggest that the lowest tumor BMD10 can be estimated from *in vivo* micronucleus BMDL05s (lower confidence bound of BMD05) by multiplying the latter by a scaling factor of 10. Thus, the uncertainty in the MN BMD05 is taken into account by using the BMDL05 as the starting point. This value would predict the tumor BMD10 within an uncertainty range of around two orders of magnitude (the true BMD10 might be a factor of 10 higher or lower with an overall uncertainty range of 100). Therefore, an uncertainty factor of only 10 would be sufficient to turn the value obtained into a conservative estimate of the tumor BMD10.

Our results challenge the way genotoxicity data are currently analyzed by showing that genotoxicity potencies only differ gradually among chemicals without any demarcation between genotoxic and non-genotoxic chemicals. Thus, the distinction between genotoxic or non-genotoxic can only be made by appointing a value of the potency parameter for use in practical decisions. Given that a BMD analysis of haematopoietic micronucleus (hMN) data from blood and bone marrow provides better and more useful information, it is worthwhile to re-think the current experimental designs and to consider using designs with more doses without increasing the total number of animals.

Overall, these results show that the clastogenic potency from short-term studies may be used as an estimate of the carcinogenic potency, at least for compounds that are positive in the *in vivo* micronucleus test. The next step would be to investigate the carcinogenic potency for chemicals that appear to be non-clastogenic.

1 Introduction

The 2-year cancer bioassay (OECD, 2008) plays an important role in cancer risk assessment. Unfortunately, this assay has several drawbacks which include the large number of animals utilized, the long time it takes to get the results (two years plus one year of analysis), and the high cost (up to several million euros depending on the route of exposure (Jacobson-Kram et al., 2004). Therefore, the decision to perform such a study is not easily made. In the European legislation REACH (Registration, Evaluation, Authorization, and restriction of Chemicals), carcinogenicity studies are considered at all annexes but testing is only allowed at the highest production volume (REACH Annex X) and under specific conditions: (i) the test substance is either genotoxic or induces hyperplasia and/or pre-neoplastic lesions in repeated dose studies and (ii) the substance has a widespread dispersive use or there is evidence of frequent or long-term human exposure. Both criteria have to be fulfilled for carcinogenicity studies to be performed. There are also a number of conditions that waive carcinogenicity studies such as classification as a category B or category C-mutagen. A test proposal needs to be submitted and approved by the European Chemical Agency (ECHA) before any carcinogenicity study is allowed to be performed. As a result, carcinogenicity assays in Europe will often be lacking and thus in many instances human risk assessment for a potential carcinogen cannot be completed, leaving the carcinogenic potency of the chemical unknown.

This raises the question of whether carcinogenic potency of substances can be estimated in the absence of carcinogenicity data from a chronic bioassay. One way to address this issue for genotoxic carcinogens is to use *in vivo* genotoxicity tests. Genotoxicity is generally a parameter used for hazard identification, but the applicability of using the magnitude of response from *in vivo* genotoxicity tests for hazard characterization has never been thoroughly investigated in a quantitative manner. Genotoxicity assays could be useful for the determination of cancer potency parameters given that genotoxicity tests measure mutations and/or chromosomal aberrations which are strongly associated with carcinogenesis. These *in vivo* tests are relevant candidates for predicting the carcinogenic potency of chemicals, as there is accountability for metabolic disposition of a xenobiotic including absorption, tissue distribution, metabolism and excretion; all these factors are important parameters in determining the carcinogenic potency of chemicals *in vivo*.

If genotoxic potency were found to be correlated with carcinogenic potency, then *in vivo* genotoxicity tests might be used to assess the carcinogenic risk of substances in the absence of carcinogenicity data. To the best of our knowledge, only one study has attempted to address the relationship between dose-response data from genotoxicity tests and carcinogenicity studies (Sanner and Dybing, 2005). They compared the Lowest Effective Dose (LED) for *in vivo* genotoxicity (micronuclei, sister chromatid exchange, DNA adducts, chromosomal aberrations and comet assay) to a dose that resulted in a 25% increase in tumor load (T_{25}) in mice and rats. Positive correlations were found both in mice and rats, and both for oral and inhalation exposure (Sanner and Dybing, 2005). A drawback of this study was that the LED and T_{25}, both being estimated by simple and imprecise methods, were used as estimates of equipotent doses. We performed dose-response analysis in an earlier study where equipotent doses were estimated as benchmark doses (BMDs) from *in*

vivo genotoxicity tests (micronucleus test and transgenic rodent mutation assay) as well as from carcinogenicity studies. In this study we found a positive correlation between genotoxicity tests and tissue-matched carcinogenicity, based on dose-response data from 18 compounds (Hernandez et al., 2011). This promising result stimulated us to proceed and try to validate this result based on a larger sample of chemicals. In the present study we investigated 51 compounds: 41 compounds which were evaluated by the U.S. National Toxicology Program (http://ntp.niehs.nih.gov/) in a *in vivo* micronucleus test, as well as in a 2-year cancer bioassay and 10 compounds from our previous analysis (Hernandez et al., 2011). Table 1 provides a list of compounds considered.

The *in vivo* erythrocyte micronucleus test is the most common *in vivo* genotoxicity test. It measures the ability of a test compound to induce micronuclei in progenitor red blood cells in bone marrow (Heddle et al., 1983). The incidence of micronucleated polychromatic erythrocytes (reticulocytes) from bone marrow (Heddle et al., 1983), and micronucleated reticulocytes from peripheral blood (Hamada et al., 2001) are considered a valid index for identifying chemicals that induce chromosomal damage or cause chromosomes to lag at anaphase.

We analyzed the *in vivo* micronucleus tests from hematopoietic cells (blood and bone marrow, hMN) and carcinogenicity studies for the 51 chemicals by re-analyzing the dose-response data and by quantifying their respective potencies in terms of equipotent doses, i.e. doses that are associated with the same change in response. Such equipotent doses can be effectively estimated using the BMD approach, where dose-response curves are fitted to the overall dose-response data resulting in confidence intervals for the BMDs. Confidence intervals quantify the precision of each estimated BMD in dependence of the quality of the specific dataset.

1.1 Aim

The aim of this report was to investigate whether the carcinogenic potency of a compound can be estimated from an *in vivo* hMN. This would be the case if the following conditions are fulfilled:

1. a relationship between the hMN and carcinogenic potency of chemicals must exist, and;
2. the dose-response data from hMN and carcinogenicity tests are of sufficient quality to establish that correlation with sufficient precision.

The first condition relates to the scientific question if the correlation exists at all. The second condition relates to the practical (risk assessment) question if current test protocols are of sufficient quality to make predictions about the carcinogenic potency of a chemical in the absence of a bioassay. If it can be established that condition 1 holds, it would be worthwhile to investigate how study protocols could be improved to make the prediction of a chemical's carcinogenic potency more precise.

2 Analysis

2.1 Data

A total of 222 technical reports in the National Toxicology Program were surveyed for carcinogens that had both hMN and carcinogenicity dose-response data and 44 compounds met this criterion (http://ntp.niehs.nih.gov/). For another 10 compounds, dose-response analysis was performed (BMD analysis) for carcinogenicity data obtained from the Carcinogenic Potency Database (CPD, http://potency.berkeley.edu/); for DMH, NDA, BAP, 2AAF, AZA and PHIP a literature search was performed to obtain *in vivo* micronucleus data (Asano and Hagiwara, 1992; Durling and Abramsson-Zetterberg, 2005; Meli and Seeberg, 1990; Shimada et al., 1992; Smith et al., 1999; Suzuki et al., 1996; Vrzoc and Petras, 1997). The basis for selection of these 10 compounds is the availability of both carcinogenicity and hMN data for BMD comparisons. The compounds selected were not exclusively compounds classified as clastogenic; many of them were also classified as mutagenic. Compounds in Table 1 that were selected from the NTP database were assigned a consistency score by Levy (2010), both for hMN and carcinogenicity, based on the consistency of the results of multiple tests of the same compound when tested in one or both sexes of mice and/or rats:
0 = all negative results wherever tested;
1 = some negative and some equivocal, never positive;
2 = never positive or negative; always equivocal;
3 = at least one positive and one negative; and
4 = at least one positive; no negative whenever tested.
Many compounds considered in this study had equivocal (score 2) or inconsistent (score 1 or 3) test results for the hMN test, while four of them were negative with a score 0 (i.e. classified as non-clastogenic). Similarly for carcinogenicity, where seven compounds were negative in both sexes of both mice and rats or had intermediate scores in at least one sex or species.

Table 1: List of compounds

Carcinogenicity					NTP Score		In vivo hMN	
Source	Cas #	IARC	Compound	AB	hMN	Cancer	M	F
1) TR-042	320-67-2	2A	5-Azacytidine	ACD	4	3	+	NT
2) CPD	306-37-6	2A	1,2-Dimethyl-hydrazine	DMH	-	-	+[1]	NT
3) CPD	305-03-3	1	Chlorambucil	CBC	-	-	+	NT
4) CPD	148-82-3	1	Melphalan	MEL	-	-	+	NT
5) CPD	62-75-9	2A	N-Nitrosodimethyl-amine	NDA	-	-	+[2]	NT
6) CPD	684-93-5	2A	N-Nitroso-N-methylurea	MNU	-	-	+	NT
7) TR-028	96-12-8	2B	1,2-Dibromo-3-chloropropane	DBCP	0	4	+[3]	NT
TR-206	96-12-8		1,2-Dibromo-3-chloropropane		0	4	+[3]	NT
8) CPD	50-32-8	1	Benzo(a)pyrene	BAP	-	-	+[4,5]	NT
9) CPD	50-18-0	1	Cyclo-phosphamide	CPA	-	-	+	NT
10) TR-086	106-93-4	2A	1,2-Dibromoethane	DBE	4	4	+	NT
11) CPD	53-96-3	-	2-acetylaminofluorene	2AAF	-	-	+[6]	NT
12) CPD	446-86-6	1	Azathioprine	AZA	-	-	+[7]	NT
13) TR-288	106-99-0	1	1,3-Butadiene	BUT	4	4	+	+
14) TR-316	513-37-1	2B	Dimethylvinyl chloride (DMVC)	DMVC	4	4	+	NT
15) TR-510	51-79-6	2A	Urethane	URE	3	4	+	NT

IARC, International Agency for Research on Cancer; CPD, carcinogenic potency database (http://potency.berkeley.edu/); hMN, hematopoietic micronucleus test; AB, abbreviation; M, male; F, female; NT, not tested; TR, National Toxicology Program technical report; +, positive; -, negative;

[1](Meli and Seeberg, 1990); [2](Suzuki et al., 1996);

[3] (Morita et al., 1997), [4](Shimada et al., 1992);

[5](Vrzoc and Petras, 1997); [6](Asano and Hagiwara, 1992);

[7](Smith et al., 1999); [8](Durling and Abramsson-Zetterberg, 2005).

hMN and carcinogenicity test results were scored as follows: 0 for all negative results wherever tested; 1 for some negative and some equivocal, never positive; 2 for never positive or negative; always equivocal; 3 for at least one positive and one negative; and 4 for at least one positive; no negative whenever tested in any strain or sex of both mice and rats (Levy, 2010).

Table 1: List of compounds cont…

Carcinogenicity					NTP Score		In vivo hMN	
Source	Cas #	IARC	Compound	AB	hMN	Cancer	M	F
16) CPD	105650-23-5	2B	PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine)	PHIP	-	-	+[8]	NT
17) TR-205	101-80-4	2B	4,4'-Oxydianiline	OXY	4	4	+	NT
18) TR-374	556-52-5	2A	Glycidol	GLY	3	4	+	NT
19) TR-027	79-34-5	3	1,1,2,2-Tetrachloro-ethane	TET	4	3	+	+
20) TR-194	7446-34-6	-	Selenium sulfide	SEL	0	0	-	NT
21) TR-289	71-43-2	1	Benzene	BEN	4	4	+	NT
22) TR-351	20265-96-7	-	p-Chloraniline hydrochloride	PCH	4	3	+	NT
23) TR-502	302-17-0	3	Chloral hydrate	CHL	4	2	+	NT
24) TR-67-66-3	67-66-3	2B	Chloroform	FOR	4	3	+	NT
25) TR-376	106-92-3	-	Allyl glycidyl ether	AGE	3	3	+	NT
26) TR-366	123-31-9	3	Hydroquinone	HYD	4	3	+	NT
27) TR-486	78-79-5	2B	Isoprene	ISO	4	4	+	NT
28) TR-469	30516-87-1	2B	3'-Azido-3'-deoxy-thymidine (AZT)	AZT	4	4	+	NT
29) TR-448	542-56-3	-	Isobutyl nitrite	ISN	4	4	+	+
30) TR-002	79-01-6	2A	Trichloro-ethylene	TCE	0	2	-	NT
31) TR-465	77-09-8	2B	Phenol-phthalein	PHP	4	4	+	+
32) TR-063	95-83-0	2B	4-Chloro-o-phenylene-diamine	COP	4	4	+	NT

IARC, International Agency for Research on Cancer; CPD, carcinogenic potency database (http://potency.berkeley.edu/); hMN, hematopoietic micronucleus test; AB, abbreviation; M, male; F, female; NT, not tested; TR, National Toxicology Program technical report; +, positive; -, negative. hMN and carcinogenicity test results were scored as follows:
0 for all negative results wherever tested; 1 for some negative and some equivocal, never positive; 2 for never positive or negative; always equivocal; 3 for at least one positive and one negative; and 4 for at least one positive; no negative whenever tested in any strain or sex of both mice and rats (Levy, 2010).

Table 1: List of compounds cont…

Carcinogenicity					NTP Score		In vivo hMN	
Source	Cas #	IARC	Compound	AB	hMN	Cancer	M	F
33) TR-515	57018-52-7	3	Propylene glycol mono-t-butyl ether	PGE	3	3	-	+
34) TR-266	150-68-5	3	Monuron	MON	4	3	+	NT
35) TR-287	868-85-9	3	Dimethyl hydrogen phosphite	DHP	4	3	+	NT
36) TR-309	1163-19-5	3	Decabromodiphenyl oxide	DPO	4	3	NT	NT
37) TR-203	108-95-2	3	Phenol	PHE	4	1	+	NT
38) TR-385	74-83-9	3	Methyl bromide	MBR	4	0	+	NT
39) TR-403	108-46-3	3	Resorcinol	RSC	4	0	+	NT
40) TR-473	58-55-9	3	Theophylline	TEO	3	0	+	-
41) TR-200	15481-70-6	-	2,6-Toluene-diamine dihydrochloride	TAC	4	0	+	NT
42) TR-247	50-81-7	-	L-Ascorbic acid	LAS	4	0	+	NT
43) TR-330	136-77-6	-	4-Hexylresorcinol	HRC	3	1	+	NT
44) TR-447	75-05-8	-	Acetonitrile	ACE	3	1	+	-
45) TR-493	518-82-1	-	Emodin	EMO	3	1	-	+
46) TR-501	80-07-9	-	p,p'-Dichloro-diphenyl sulfone	CPS	4	0	+	NT
47) TR-097	13463-67-7	2B	Titanium dioxide	TIO	4	1	+	NT
48) TR-237	630-20-6	3	1,1,1,2-Tetrachloro-ethane	ETH	4	3	+	NT
49) TR-513	91-17-8	-	Decalin	DCN	3	3	+	-
50) TR-527	129-73-7	-	Leucomalachite green	LEU	4	3	NT	+

IARC, International Agency for Research on Cancer; CPD, carcinogenic potency database (http://potency.berkeley.edu/); hMN, hematopoietic micronucleus test; AB, abbreviation; M, male; F, female; NT, not tested; TR, National Toxicology Program technical report; +, positive; -, negative. hMN and carcinogenicity test results were scored as follows: 0 for all negative results wherever tested; 1 for some negative and some equivocal, never positive; 2 for never positive or negative; always equivocal; 3 for at least one positive and one

negative; and 4 for at least one positive; no negative whenever tested in any strain or sex of both mice and rats (Levy, 2010).

Table 1: List of compounds cont…

Carcinogenicity					NTP Score		In vivo hMN	
Source	Cas #	IARC	Compound	AB	hMN	Cancer	M	F
51) TR-543	98-83-9	-	alpha-Methylstyrene	AMS	3	3	-	+
52) TR-546	7789-12-0	-	Sodium dichromate dihydrate (VI)	SCD	3	4	+	NT
53) TR-551	97-54-1	-	Isoeugenol	IEG	3	3	-	+
54) TR-479	68603-42-9	2B	Coconut oil acid diethanolamine condensate	COC	3	3	+	+

The *in vivo* micronucleus tests examined blood and/or bone marrow using a variable study design, with group sizes between 5 and 15, and number of doses ranging from three to eight (including controls). They further differed in the exposure regimen, varying from a single dose to treatment for 182 days or 26 weeks. Both sexes were used in some of the studies, but most studies used only males. While for some chemicals only a single dose-response dataset was available, other chemicals were tested more extensively by varying factors like sex, strain, or exposure regimen, resulting in various dose-response datasets for the same compound. Thus, individual datasets were defined by having the same levels for the following factors: compound, sex, strain, route, tissue observed, exposure regimen, exposure duration, and sampling time (24 or 48 hrs). Genotoxic potency parameters were derived in terms of BMDs for each of these individual datasets. Thus, there were many BMDs derived per compound and the lowest BMD was selected for final analysis.

The carcinogenicity dose-response data varied largely in group sizes (ranging between as few as 7 and as many as 999). Number of dose groups ranged from three to eight (including controls). Individual datasets were defined by the following factors: compound, sex, strain, route, exposure duration, study duration, and tissue with lesion, type of lesion, tissue specific lesion, and lesion category (see below). Carcinogenic potency parameters were derived in terms of BMDs for each individual dataset. The number of individual dose-response datasets available for a given compound varied between a single dose-response for some chemicals to a large number of them in others by using, i.e. different exposure durations or study durations (with 2AAF as the most prominent example). Similar to the analysis of the *in vivo* hMN, there were many BMDs derived per compound and the lowest BMD was selected for final analysis.

One of the complications of the dose-incidence data reported by carcinogenicity studies is that they may relate to quite different types of response with varying degrees of severity, such as hyperplasia, adenomas, carcinomas, or tumor bearing animals (tba). Clearly, a dose that causes a 10% increase in animals with hyperplasia is not equipotent to a dose that causes a 10% increase in tumor bearing animals. To keep track of such differences, we assigned 'lesion class' scores, roughly representing different descriptions of malignant or potentially pre-malignant lesions in each dose-response dataset. There was a

group of compounds which had no evidence of a dose-related trend in carcinogenicity and we assigned this group category 'A'. The remaining lesions were designated as follows:

B- hyperplasia, effects in hematopoetic system, neoplasms;
C- adenoma, benign tumors, cystioadenoma, leukemia;
D- carcinoma, papilloma, sarcoma, angiosarcoma, lymphoma, hamangioendothelioma, stomal tumor, granulosa cell tumor, pheochromocytoma;
E- tumors mixed, any mixed tumor (i.e. lung mixed, liver mixed);
F- combination of tumors: papilloma-carcinoma, carcinosarcoma, adenocarcinoma;
G- tba, tba mixed (more than one tumor type combined by NTP or by Berkeley for the CPD);
H- tba malignant tumor(s) (See Appendix 1 for a detailed description of all the tumor lesions in each category).

Three approaches were taken in correlating the hMN potency to the tumour potency:
1. by including all BMDs for all endpoints and study conditions in one graph, irregardless of tumor lesion category;
2. by selecting the lowest BMD for each compound, irregardless of the associated tumor lesion category, and;
3. by selecting the lowest BMD for each compound, for the same tumor lesion category. See Figures 1-3, respectively.

2.2 Deriving BMD confidence intervals

Equipotent doses are defined by BMDs at a given constant benchmark response (BMR). For the carcinogenicity studies we used a BMR of 10% extra risk, which is the most commonly used value of the BMR in dose-response characterization of quantal endpoints (EFSA, 2009). For the continuous dose-response data from the *in vivo* hMN we used a 5% increase in the mean count in the controls as the BMR, as being the recommended BMR for continuous response data by the European Food Safety Authority (EFSA, 2009).

Rather than deriving BMDs as single values, we derived the (two-sided) 90%-confidence intervals for them. In this way, the potency of chemicals can be quantified even if they do not show a significant trend in the dose-response. For our purposes this is particularly important because this makes it possible to include chemicals with 'negative' test outcomes in our sample of chemicals, and thus contribute to establishing the correlation of interest. For chemicals with a negative test outcome, the upper confidence limit for the BMD will be very large or even infinite. However, there will be a lower bound, implying that, if the chemical would at all induce a response (at the value of the BMR used), then it will occur at a higher dose than that lower bound. On the other side of the range, it may occur that the lower confidence limit results in 'zero', meaning that no lower bound can be assessed given the dose-response data available. Such may occur in datasets when even the lowest dose shows a response that is substantially higher than the chosen BMR. In these cases, the upper bound of the confidence interval provides some information on the potency of the chemical: the effect (at effect size = BMR) will occur at a dose lower than that upper bound.

Correlation plots of hMN against carcinogenic BMDs can be created by plotting the confidence intervals (in both the x- and y-direction) for each dataset, including the ones that resulted in (one-sided) infinite confidence intervals. Another option is to plot single points rather than confidence intervals, and in that case we used the (geometric) mean of the upper and lower confidence bound. For intervals with zero lower bound we used the minimum nonzero lower limit over all intervals assessed, and for intervals with infinite upper bound we used the maximum of the finite upper bounds of all intervals assessed.

Many of the dose-response data available did not contain much information on the shape of the dose-response by themselves; for instance due to a limited number of dose levels tested, or with responses in only one or a few dose groups. This would lead to highly imprecise estimates of equipotent doses, and a potential correlation would be concealed by the large variability in the data However, a recent re-analysis of a large number of toxicological datasets (Slob and Setzer, in prep.) showed that the dose-responses (from similar studies) of different chemicals tend to be parallel on log-dose scale. We used this result by fitting the model to combined clusters of datasets, where the common value of the steepness parameter is informed by all datasets in the cluster. This approach results in a considerable improvement of the precision of individual BMDs *(*i.e. smaller confidence intervals).

The *continuous* dose-response data from the micronucleus tests were analysed by fitting the exponential model, which is one of the recommended models for continuous data (EFSA, 2009), and known to be generally applicable to toxicity data:

$$y = a[c-(c-1)\exp\left(-bx^{d}\right)$$

where *y* is the response (number of cells with micronuclei) and *x* the dose. In fitting the model to the combined cluster of datasets, separate values for parameters *a* (reflecting the response at dose 0) and *b* (reflecting the potency of the chemical) are estimated for each individual dose-response dataset, while parameters *c* and *d* are kept constant over all datasets within the cluster analyzed. The within group variance was estimated separately for each individual dataset as well. See Slob (2002) for a more detailed discussion on this method.

For the *quantal* dose-response data from the carcinogenicity studies the log-logistic model was fitted.

$$y = \frac{a + (1-a)}{1+\exp[-c\log(x/b)]}$$

where *y* is the response (fraction of affected animals) and *x* the dose. Again, parameters *a* (reflecting the response at dose 0) and *b* (reflecting the potency of the chemical) are estimated for each individual dose-response dataset, while parameter *c* is kept constant over all datasets within the cluster analyzed.

A BMD analysis normally applies various models to take 'model uncertainty' into account. The overall confidence interval (CI) for the BMD is then obtained by integrating the results from the various models (for which various methods may be used (EFSA, 2009)). We only used one model in the present analysis. In general, experience has shown that the log-logistic model describes toxicological dose-responses data in nearly all cases. Further, systematic analyses of large numbers of historical datasets from all sorts of studies showed that the two

models just mentioned adequately described all datasets that were selected based on having sufficient doses and animals (Slob and Sezter, in prep.).

Dose-response modelling was performed with the software package PROAST. This package allows for dose-response analysis with covariates. This makes it possible to perform combined analyses of clustered sets of similar datasets related to different chemicals by assuming that the potencies of the chemicals differ, but not the shape parameters of dose-response model (see above). This assumption appeared to be satisfied by the data considered, at least approximately so.

2.3 Steps of analysis

To overall analysis consisted of the following steps.

- A large datasheet was created for the hMN data with columns dose (mg/kg/day), mean response, Standard Error of the Mean (SEM), group size, and a number of relevant factors (chemical tested, sex, strain, route of exposure, tissue (blood or bone marrow), exposure duration, sampling time (24 or 48 hours), or vehicle used in controls.
- Similarly, a large datasheet was created for the tumor data with columns dose (mg/kg/day), number of animals with response, group size, and a number of relevant factors (chemical tested, sex, strain, route of exposure, exposure duration, duration of study, tissue, type of lesion, combination of lesion and tissue, severity of the response.
- In both datasheets, a column with study number was added, such that dose-response data differing in any of the associated factors were labelled with a unique number.
- For each datasheet the total number of individual datasets was too large to be analysed as a single combined dataset. Each datasheet was split up in a number of manageable clusters, with around 60 datasets max. Each cluster combined similar datasets like same route of administration, and/or same sex, etc.
- A dose-response model was fitted to each cluster of datasets, with individual dataset as a covariate for parameter *a* (estimated response in controls) and for parameter *b* (potency of the dose-response), and, in the case of hMN data, for the within group variance. In this way, the steepness of the curves is estimated from all dose-response data in the cluster, since it is assumed to be constant among the individual studies.
- For each cluster of datasets, the confidence intervals were calculated for the BMD related to each individual dataset.
- The CIs for all hMN clusters were combined and the columns with the associated factors were added. The same was done for the tumor clusters.
- The hMN and tumor CIs were combined in a single table, such that for matching chemicals each genotoxic study with a CI matches each tumor study with a CI, and vice versa. The factors related to the hMN and to the tumor studies were maintained as additional columns.
- The CIs, or the geometric means of the intervals related to matching chemicals were plotted against each other. Any of the factors in the table could be used to mark the points. In this way, the impact of each factor could be further examined.

3 Results

From the 54 compounds considered, three were omitted: TIO as it resulted in (two-sided) infinite BMD CI for all micronucleus data considered, DPO as there were no micronucleus data available, and AZA because it was tested in a genetically modified strain (MM). Thus, 51 chemicals were left for further analysis.

All hMN and carcinogenic BMDs were plotted against each other in Figure 1. Even though the scatter is large, from this figure it can be concluded that a positive (and approximately linear) correlation between the hMN potency of a compound and its carcinogenic potency exists. The large scatter and low correlation coefficient (Pearson's r = 0.35) could hardly be better given the large variability in BMDs within the same compounds, in the X-direction (hMN), and even more so in the Y-direction (carcinogenicity). The latter could partly be due to the fact that the carcinogenic dose-response datasets varied in important factors like study duration (and exposure duration), and lesion class description of the endpoint considered. A first exploration did not reveal which factors contributed systematically to the variation in BMDs within compounds, apart from the factor 'lesion severity' in the carcinogenicity studies.

The next question is to what extent the carcinogenic potency (BMD) can be estimated from the hMN potency (BMD). Normally, the lowest carcinogenic BMD is selected as a point of departure (PoD) in risk assessment. Figure 2 shows the correlation between the lowest hMN and lowest carcinogenic BMDs (for any lesion category), each selected from all datasets available for a given compound. In this figure, the CI in both directions were plotted and from this figure, we noted that some of the CIs in the right upper corner have infinite upper bounds, but the lower bounds are in line with the overall correlation. This Figure contains 7 compounds in category A, 1 compound in category B, 3 compounds in category C, 11 compounds in category D, 3 compounds in category E, 21 compounds in category F, and 5 compounds in category G (See Appendix 2 for summary of the hMN and tumor BMDs).

As already noted, part of the scatter in the correlation is due to the different 'lesion classes' for the endpoint considered representing different descriptions of malignant or potentially pre-malignant lesions. Indeed, the scatter can be reduced by selecting one lesion category. Figure 3 shows the results for lesion category D (defined as tissue-specific tumor, see section 2.1 for lesion category definitions). The middle smooth line represents all points where the tumor BMD10 is ten times higher than the hMN BMD05. Hence, if all points would lie on this line, the carcinogenic BMD10 would be predicted by multiplying the hMN BMD05 by a scaling factor of 10. Note that this value of the scaling factor specifically holds for the value for the BMR chosen for the hMN BMD; if a higher value than 5% been chosen, this scaling factor would have been found to be lower (possibly lower than 1). Obviously, both the uncertainty in BMDs (represented by the confidence intervals) and the scatter among the points need to be taken into account. Most of the associated uncertainty/variability can be captured by the two dotted lines, representing a factor of 10 higher/lower than the middle smooth line. Taking a specific hMN BMDL05 (i.e. BMD05 lower bound) which lies exactly on the lower dotted line, multiplying that value by ten would result in a 'best' estimate of the tumor BMD. When that value is subsequently divided by ten so as to take the uncertainty into account, the

resulting value would be back on the dotted line, and the associated value on the y-axis would now represent a conservative estimate of the tumor BMD.

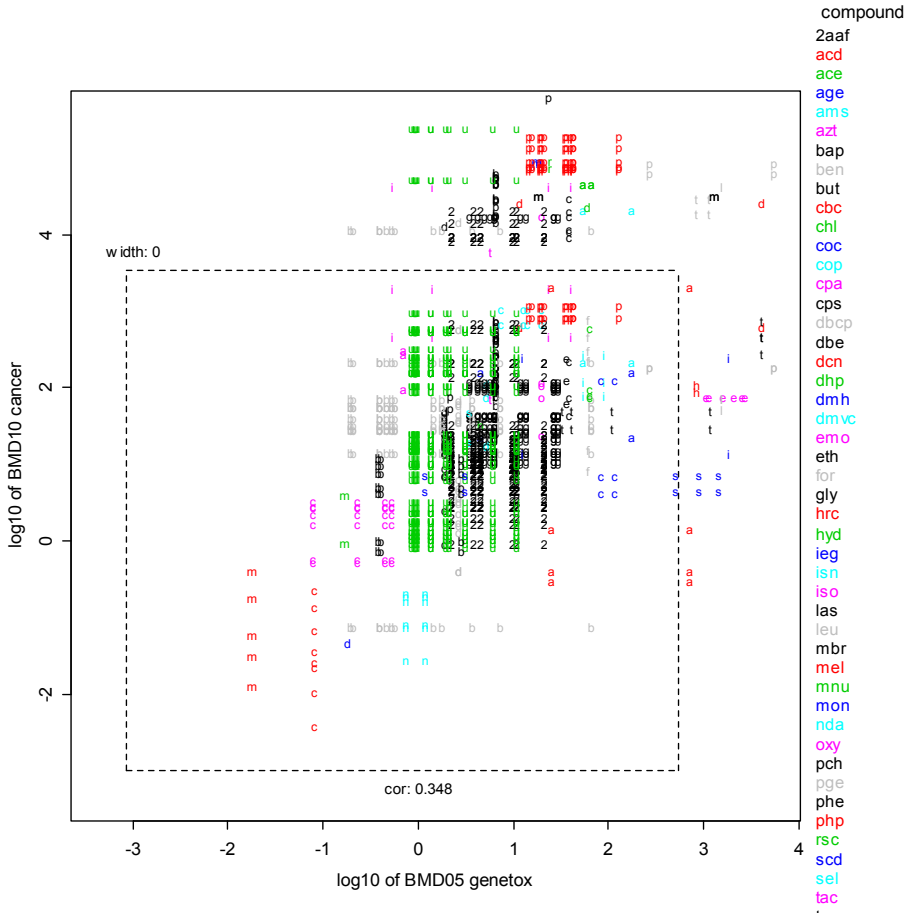

Figure 1. All hMN BMDs plotted against all carcinogenic BMDs (n = 1452 pairs of BMDs, related to 51 compounds). Note the large variation in BMDs within chemicals, both for the hMN and for the carcinogenic BMDs. The lower and left dashed lines of the inner block indicate the smallest nonzero lower confidence limit over all datasets; the right and upper dashed lines indicate the largest finite upper confidence limit over all datasets. So, outside these lines the uncertainty is infinite in the outer direction.

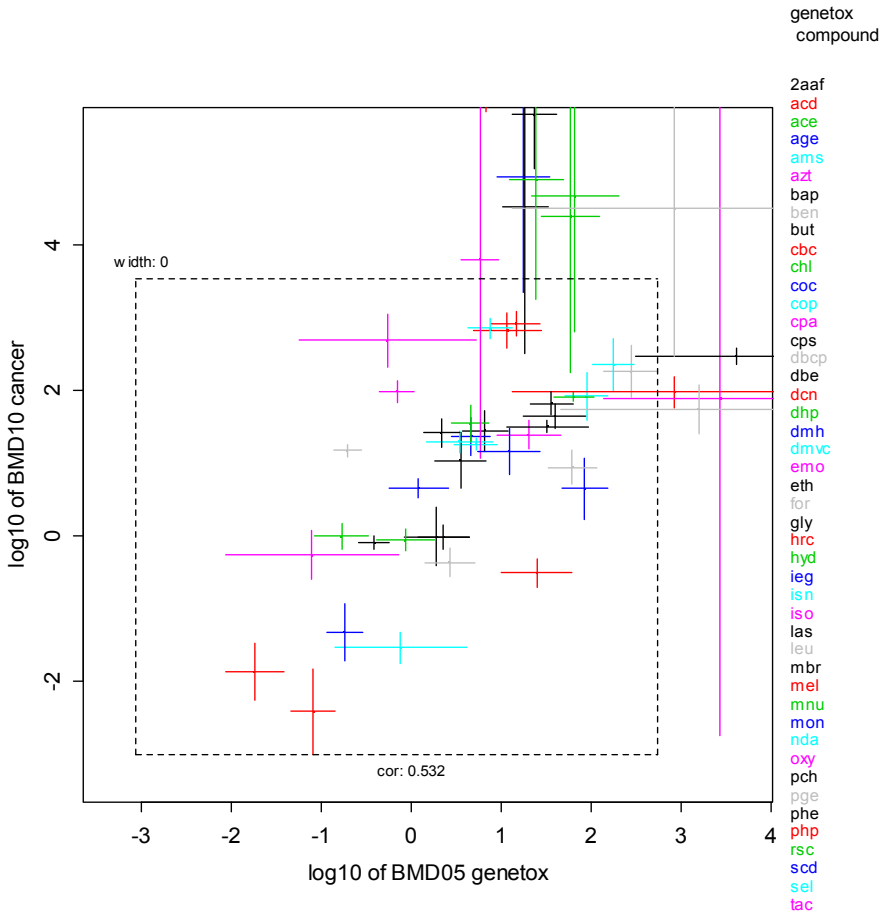

Figure 2. The 90% CIs for the lowest carcinogenic BMD were plotted against the CIs for the lowest hMN BMD for the 51 chemicals considered. Upper confidence bounds that hit the outer frame are in fact infinite (See Appendix 2 for hMN and tumor BMD values).

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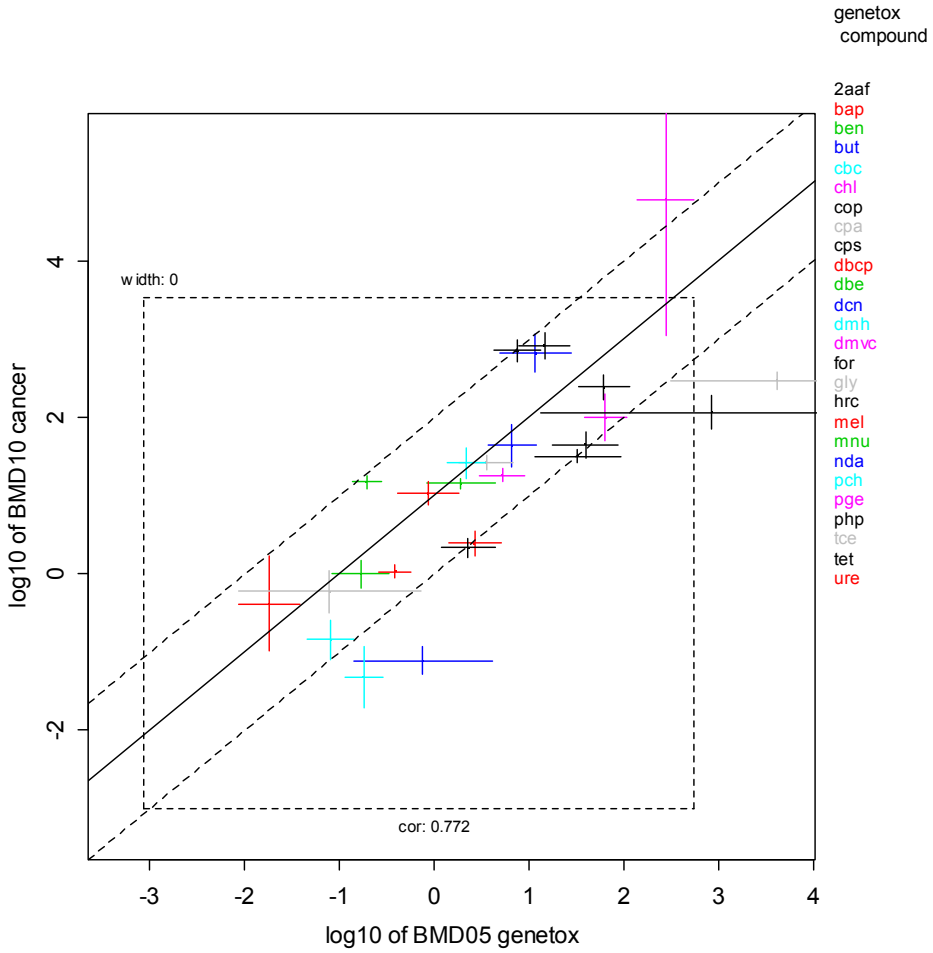

Figure 3. Lowest carcinogenic BMD for lesion category D against lowest hMN BMD (n = 26 compounds). The 90%-CIs are plotted in both directions. The middle solid line represents the case where the carcinogenic BMD10 is 10 fold higher than the hMN BMD05s. The upper and lower dotted lines are 10 times higher and lower than that (in both directions). So, the lower dotted line is the line where the hMN BMD05 is equal to the tumor BMD10. The confidence intervals for chemicals DMH and NDA are outside the lines, but their dose-response data were poor (e.g., only two nonzero doses) (see Appendix 3 for hMN and tumor BMD values).

4 Discussion

The aim of this report was to extend our initial study (Hernandez et al., 2011) which was based on only 18 compounds and to investigate whether BMDs from the *in vivo* hMN are correlated with carcinogenic BMDs. Increasing the number of compounds to 51 confirmed our earlier result (Hernandez et al., 2011) that the hMN potency of a chemical is correlated to its carcinogenic potency. There is remaining scatter in the correlation plots, but this could not have been otherwise given the observation that carcinogenic BMDs vary considerably within the same compounds. This variability can be partly explained by the fact that the individual dose-response datasets (for a given compound) often relate to endpoints of different type and lesion category. Further, the individual datasets for a given compound may vary regarding other factors, such as study duration, route, strain, and sex. The individual dose-response datasets for the various chemicals form a complex database, and more work would be needed to see if systematic patterns could be revealed between the various factors involved and the resulting BMDs.

This analysis differed in approach to our previous study (Hernandez et al., 2011) in several ways. First, we analyzed 51 compounds, 138 micronucleus and 388 cancer bioassay data sets, in comparison to 18 compounds, 54 micronucleus and 224 cancer bioassay data sets in our previous study. Second, data were analyzed separately in our previous study while the data were grouped into clusters for combined analyses in the present study. This was performed because combining datasets into clusters with chemical as a covariate generally leads to more precise BMD estimates, in particular for poor dose-response datasets that by themselves provide little information on the dose-response shape. Third, in the present study we only used hMN tests, while in the previous study we included various types of genotoxicity assays, including the transgenic rodent mutation assay and the comet assay.

Regarding the results, there were both similarities and differences between both studies. The correlation coefficients between lowest tumor BMD10 and lowest micronucleus BMD10 were similar in both studies: 0.54 in our previous analysis and 0.53 (Figure 2) in this study. Importantly, tumor lesion was taken into account in the present analysis, which was not the case in our earlier study, resulting in an improvement in the correlation to 0.77 (Figure 3). However, an important difference was that we found the tumor BMD to be proportional to the hMN BMD in the present study, while in the previous study the tumor BMD was proportional to (approximately) the square root of the genotoxic BMD. Further, in our present analysis we found an uncertainty factor of 10 would probably be sufficient, in comparison to an uncertainty factor of 100 in our earlier analysis (Hernandez et al., 2011).

The approach of producing correlation plots between hMN and carcinogenic potency of chemicals (Figures 1 to 3) challenges the way genotoxicity data are analyzed, in particularly the way we categorize chemicals as genotoxic or non-genotoxic, and the way we view false positive and false negative results in genotoxicity tests. As an illustration, we plotted the geometric means of the CIs in Figure 2, and indicated which chemicals were negative in the hMN and/or carcinogenicity test (Figure 4). Several examples from Figure 4 will be highlighted to illustrate the advantages of using correlation plots, in comparison to traditional yes/no analysis. The first example concerns a set of chemicals (ACE, CPS, EMO, HRC, MBR, PHE, TAC, TEO, TIO and RSC) which are positive for

hMN and negative for carcinogenicity (+,- in Figure 4), and would be considered a false positive in the traditional approach. As Figure 4 shows, this set of compounds does not deviate from the correlation plot. The fact that they are located in the right upper corner means that the hMN test correctly predicts them to have low carcinogenic potency (if any). The second set of examples are CHL (+/e) and TCE (-,e) where equivocal results were obtained in the carcinogenicity study. In this case, both the hMN and the tumor BMDs were found to be high, and the hMN test correctly predicts that CHL and TCE are weak carcinogens. These examples illustrate how informative the correlation plot (Figure 4) is in gaining insight on the carcinogenic potency of chemicals using the estimated potency (BMD) from the *in vivo* hMN test. Thus, all 'false positives' and 'false negatives' in the database considered comply with the overall correlation plot. As Figure 4 shows, negative tests only occur in the right upper quarter of the plot (above around 1 mg/kg), but this region also includes many positives (for both tests). Clearly, highly potent compounds (i.e. low BMD) will virtually always be found to be positive, while lower potency chemicals have an increasing probability to result in a negative test outcome. Given that our data set had very few negative hMN and non-carcinogens, further analysis is needed to investigate whether negative hMN compounds provide useful information in regards to carcinogenic potency. This is of particular importance as analysis of hMN generally show low sensitivity for prediction of carcinogenicity (Witt et al., 2000). It is clear from the aforementioned correlation plots that carcinogenic potency information can be derived from positive hMN compounds in this data set and our results are in line with the notion that a positive hMN is highly predictive of rodent carcinogenicity (Witt et al., 2000).

As illustrated by Figure 4, positive and negative test outcomes do not necessarily provide a discrete distinction between compounds with or without the hazard considered. Overall, Figures 2, 3 and 4 show that different chemicals gradually differ in potency without any clear demarcation between highly and less potent chemicals because there is no gap between low and high BMDs, neither for hMN nor for carcinogenicity. Therefore, it is not possible to objectively define the border between positive and negative chemicals (for hMN or for carcinogenicity). For purposes of classification and labelling, the boundary between positive and negative chemicals may be an appointed dose level that is based on consensus, using practical considerations such as maximally feasible or realistic doses.

Finally, results suggest that the lowest tumor BMD10 can be estimated from *in vivo* micronucleus BMDL05s (lower confidence bound of BMD05) by multiplying the latter by a scaling factor of 10. Here, the uncertainty in the MN BMD05 is taken into account by using the BMDL05 as the starting point. This value would predict the tumor BMD10 within an uncertainty range of around two orders of magnitude (the true BMD10 might be a factor of 10 higher or lower with an overall uncertainty range of 100). Therefore, an uncertainty factor of only 10 would be sufficient to obtain a conservative estimate of the tumor BMD10.

Altogether, these results demonstrate that the clastogenic potency from short-term studies may be used as an estimate of the carcinogenic potency, at least for compounds with a positive result in *in vivo* micronucleus studies.

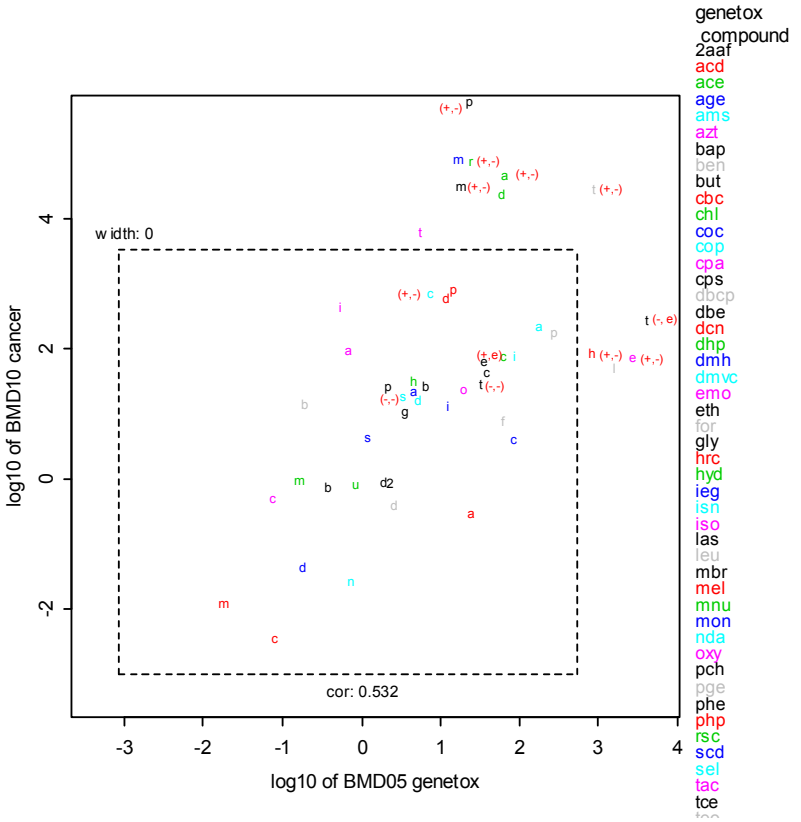

Figure 4. Geometric means of the confidence intervals shown in Fig. 2, related to lowest hMN BMD and lowest tumor BMD for each chemical. Between brackets the micronucleus and tumor test outcome are given, respectively (+ for positive, - for negative and e for equivocal result). So, (-,+) would represent a false positive. If omitted, both test outcomes were positive. Note that the points outside the dashed box are rough approximations only, as their confidence limits had infinite upper bounds (See Appendix 2 for hMN and tumor BMD values).

4.1 Impact for risk assessment

Our results have a significant impact for risk assessment, both regarding hazard identification and hazard characterization.

4.1.1 Hazard identification

Our results show that there are only gradual differences in genotoxic (hMN) (or carcinogenic) potencies and suggest that a clear demarcation between negative and positive compounds is not distinguishable. To inform risk management decisions that require a yes/no answer to the question whether a chemical may be considered to be genotoxic (or carcinogenic) or not, is currently based on the associated statistical test being significant or not. The outcome of significance tests is largely driven by coincidental experimental circumstances, and may be misleading, as illustrated in Figure 4. The approach of estimating the potency of the compound (in terms of BMD) is much more reliable. To translate this quantitative outcome into a yes/no answer, consensus would be needed as to where to draw the line on the potency (BMD) scale that distinguishes chemicals with the hazard from those without the hazard in a decision framework. For

instance, one might decide to demarcate chemicals with a BMDL05 below 1000 mg/kg body weight as genotoxic and above that level as non-genotoxic. This would be a better funded and more transparent way of classifying chemicals as compared to the current approach. Regulatory agencies should be responsible for determining their own demarcating dose values.

4.1.2 Hazard characterization

The practical impact of our results is that, in the absence of a carcinogenicity study, it may be possible to prepare a conservative estimate of the carcinogenic potency from a (short-term) *in vivo* micronucleus test. As Figure 3 showed, the carcinogenic BMD10 can, on average, be estimated by multiplying the micronucleus BMDL05 (note the lower bound) by a scaling factor of 10. An uncertainty factor of 10 can account for the uncertainty of the correlation between micronucleus and tumor potency. Put together, the rodent genotoxicity BMDL05 can be directly used as a conservative estimate of the rodent tumor BMD10. This relationship is based on the results for carcinogenic BMD10s associated with effects of lesion category D (see Figure 3). For lesion categories C, E and F the average relationship also resulted in a scaling factor of 10, although the scatter in the correlations was slightly larger. A further analysis of the data (with the extensions mentioned above) should be performed to better substantiate the overall uncertainty in the correlation plots.

Overall, our findings have a significant impact on the way risk assessment is currently performed by providing an option of using the *in vivo* MN test for both hazard identification and hazard characterization.

4.2 Impact for study design

One possibility to further reduce the observed scatter in the correlation between hMN and carcinogenicity BMDs resulting from current studies is to improve the study designs of both carcinogenicity and hMN studies. Since the trend is to avoid carcinogenicity studies wherever possible, focus should primarily be on changes to improve the genotoxicity studies. Although OECD guidelines are currently available for the *in vivo* hMN test, these guidelines should be updated to improve the interpretation and applicability of the results. Here, we briefly discuss general statistical aspects of an optimal study design for the purpose of effectively quantifying the genotoxic potency of chemicals.

One thing to note in thinking about experimental designs using the BMD approach is that it is not driven by statistical power, but rather by statistical precision in estimating the potency (BMD) of the chemical. The precision of the BMD is determined by the total number of animals in the study rather than by individual group sizes. For this reason, experimental designs using the BMD approach can have fewer animals per dose and more doses without increasing the total number of animals used. Indeed, for a given total number of animals in the study, the precision as well as the accuracy can be improved by distributing the animals over more dose groups (keeping the total number of animals the same (Slob et al., 2005). Therefore, experimental designs of genotoxicity studies may provide better information for BMD analysis when six or more dose groups are employed. Although less animals per dose are used, the same information on inter-animal variability is obtained, with a gain of better understanding the dose-response relationship. Group sizes are important for approaches that rely on pairwise comparison of dose groups using significance testing but not in a dose-response analysis.

5 Conclusions

Our current and previous results (Hernandez et al., 2011) support the existence of a quantitative relationship between *in vivo* hMN potency and the carcinogenic potency.

The gradual variation in hMN potencies among chemicals challenges the current approach of assessing the genotoxicity of chemicals, usually based on significance testing. The demarcation between genotoxic and non-genotoxic compounds is better set by appointing a value of the potency (BMD) based on biological or practical considerations.

The observation that chemicals show little variation in dose-response shapes supports our approach of analyzing datasets as combined clusters. In this way, BMDs could be better estimated for chemicals with limited dose-response data.

More chemicals with 'negative' outcomes in the hMN test should be added to this analysis to check whether the hMN test for these chemicals predicts cancer potency. If not, it should be investigated if these chemicals can be covered by another genotoxicity test, e.g. one that measures mutations.

Overall, these results support the notion that cancer potency can be estimated from a positive *in vivo* micronucleus test in the absence of a 2-year cancer bioassay.

6 Future research

The analysis of the database from the 51 compounds in this study could be further extended by examining the following:

- **Refine lesion categories**: For the purpose of this first exploration of the data, we devised a rough lesion categorization representing different descriptions of malignant or potentially pre-malignant lesions in each dose-response dataset. This should be revised by consulting with experts (cancer pathologists) and re-analyzing the database with the new categorization.

- **Include more compounds with 'negative' hMN results**: It is possible that some of these chemicals fall outside the correlation found in this study. If so, examination of other genotoxicity tests may fill this gap.

- **Other *in vivo* genotoxicity tests**: It would be useful to investigate to what extent other genotoxicity tests (*e.g.* transgenic rodent mutation assay and comet assay) could predict the carcinogenic potency of chemicals and to what extend this analysis can complement the findings in this report.

- **Variable study conditions**: Insight in study conditions involved in the various micronucleus tests and carcinogenicity studies might reduce the scatter in the correlation plots even further. Therefore, a further investigation of the impact of study conditions on the potency estimates would be very useful to improve the current OECD guidelines.

- **Extended dose-response analysis**: The present study used a 5% increase in micronucleus frequency as the BMR. However, this value may have been too low for obtaining a reasonable BMD confidence interval. The correlation should be re-assessed based on a larger effect size (BMR) than 5% (e.g. 10-50%). This may result in a different scaling factor, while the uncertainty factor is expected to remain the same. In addition, the assumption that dose-responses are parallel on log-dose scale (for a given cluster of datasets) should be further validated.

- ***In vitro* genotoxicity tests**: Explore potency correlations between *in vitro* genotoxicity tests and carcinogenicity. Even if a weaker correlation is observed, results can inform risk assessment, help improve testing strategies, and possibly further reduce the number of animals currently used in evaluating the carcinogenic potential of chemicals. This is of particular importance for cosmetics where *in vivo* testing is no longer allowed.

7 Acknowledgements

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Appendix 1: Description of tumor lesions per class

		Number of tumors per lesion category								
Tissue	**Tissue.lesion**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
adrenal.gland	adrenal.gland.fibrosarcoma				3					3
	adrenal.gland.squamus.cell.carcinoma				3					3
	adrenal.squamous.cell.carcinoma				6					6
	pheochromocytoma				6					6
	pheochromocytoma.lymphosarcoma				3					3
bladder	bladder.carcinoma				145					145
	bladder.mix					64				64
brain	forebrain.olf.neuroepithelioma				4					4
epididymis	epididymis.squamous.cell.carcinoma				3					3
esophagus	esophagus.mixed					4				4
	esophagus.squamous.cell.carcinoma				4					4
forestomach	foresquamous.cell.carcinoma				6					6
	foresquamous.cell.papilloma				8					8
	foresquamous.cell.papillomaorcarcinoma						3			3
	foresquamouscell.papilloma.carcinoma						4			4
	forstomach.mixed					4				4
	forstomach.squamous.cell.carcinoma				4					4
harderian.gland	adenoma			12						12
	adenoma.carcinoma				8		9			17
	adenomaoradenocarcinoma						6			6
	carcinoma.adenoma						24			24
heart	hemangiosarcoma				20					20

		Number of tumors per lesion category								
Tissue	**Tissue.lesion**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
hematopoietic.system	blood.vessel.angiosarcoma				4					4
	bone.marrow.depletion.cellular		12							12
	decrease.mononuclear.leukemia			4						4
	hematopietic.system.lymphoma.thymicorigin				8					8
	hematopietic.system.sarcoma				4					4
	hematopietic.system.sarcoma.lymphoma				4					4
	hematopoietic.system		4							4
	hematopoietic.system.lymphoma				11					11
	lymphnode.depletion.lymphoid		12							12
	lymphoma				9					9
histiocytic.sarcoma	allorgans.histiocytic.sarcoma					4				4
	histiocytic.sarcoma				5					5
kidney	adenoma.carcinoma						4			4
	nephropathy		12							12
	renal.tubule.adenoma.carcinoma						3			3
	renal.tubule.adenoma.carcinoma.std&extended						4			4
lagn	lymphocytic.and.granulocytic.neoplasm		3							3
larynx	larynx.mixed					4				4
liver	adenoma			3						3
	adenoma.carcinoma				6		69			75
	adenoma.carcinoma.hemangiosarcoma						16			16
	adenoma.carcinoma.hepatoblastoma						6			6
	carcinoma				16					16
	carcinoma.myelosarcoma						3			3
	carcinoma.sarcoma.hemangioma						3			3
	decrease.hepatocellular.adenoma.carcinoma						8			8
	hemangiosarcoma				12					12
	hepatoblastoma				8					8
	hepatocellular.adenoma.carcinoma						14			14

		Number of tumors per lesion category								
Tissue	**Tissue.lesion**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
liver	hepatocellular.carcinoma				4					4
	liv.mix					64				64
	liver.benign			4						4
	liver.carcinoma				133					133
	liver.malignant				4					4
	liver.squamous.cell.carcinoma				12					12
lung	adenoma			16						16
	adenoma.carcinoma						47			47
	lung.alveolar.bronchiolar.carcinoma				6					6
	lung.malignant				3					3
	lung.mix					9				9
	lung.mixed					6				6
	lung.tumor					3				3
lymphosarcoma	lymphosarcoma				6					6
	lymphsarcoma				3					3
mammary.gland	mammary.gland.adenoma			3						3
	mammary.gland.carcinoma.carcinosarcoma						4			4
	mammary.gland.mixed.tumors					15				15
mixed.tumor	multiple.organs					3				3
neoc	no.evidence.of.carcinogencity	29								29
ovary	ovary.cystadenoma			4						4
	ovary.degeneration		6							6
	ovary.granulosa.celltumor				4					4
	ovary.mixed.tumors.benign					4				4
	ovary.mixedtumors					8				8
	ovary.squamous.cell.carcinoma				3					3
	ovary.stromal.tumor				4					4
pancreas	pancreas.squamous.cell.carcinoma				9					9
pituitary.gland	adenoma			7						7
	gland.adenoma			9						9
preputial.gland	carcinoma				7					7
small intestine	adenoma.carcinoma						10			10

		Number of tumors per lesions category								
Tissue	**Tissue.lesion**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
skin	fibrosarcoma				3					3
	skin.aquamouscell.papilloma				3					3
	skin.hemangiosarcoma				8					8
	skin.sarcoma				4					4
	skin.squamouscell.papilloma.carcinoma						3			3
spleen	hemangiosarcoma				4					4
	spleen.depletion.lymphoid		12							12
	spleen.hemangioendothelial.sarcoma				4					4
	spleen.hemangiosarcoma				12					12
	spleen.sarcoma.lymphosarcoma				3					3
	spleen.squamous.cell.carcinoma				6					6
spleen	spleen.squamous.cell.carcinoma				6					6
stomach	papilloma.carcinoma						6			6
	stomach.glandular.adenocarcinoma						4			4
	stomach.squamous.cell.carcinoma				12					12
tumor bearing animal	animals.with.malignant.tumors								3	3
	tba.malignant								12	12
	tba.mix							9		9
	tba.mixed							16		16
	tumor.bearing.animal							3		3
testis	testis.degeneration		6							6
	testis.squamous.cell.carcinoma				3					3
thymus	thymus.depletion.lymphoid		12							12
thyroid.gland	.adenoma			4						4
	.adenoma.carcinoma						7			7
tonge	tonge.mixed					4				4
	tonge.squamous.cell.carcinoma				4					4
uterus	uterus.carcinoma.adenocarcinoma						3			3
	uterus.hemangioendothelioma				3					3
	uterus.hemangiosarcoma				4					4
vagina	squamouscell.carcinoma.papilloma						4			4
zymbal.gland	zymbal.gland.carcinoma				8					8
Grand Total		29	79	66	595	196	264	28	15	1272

Appendix 2: Data used to generate Figure 2

				hMN (Genotoxicity endpoint)								Carcinogenicity (Tumor Endpoint)								
compound	strain	sex	route	tissue	treatment regimen	duration exposure	vehicle control	sampling time	source	$BMDL_{05}$	$BMDU_{05}$	compound	strain	sex	route	exposure time	duration exper	severity	tissue	tissue.lesion	$BMDL_{10}$	$BMDU_{10}$
dbe	B6	m	inh	blood	125	175	air	24	ntp	0.83257	4.4119	dbe	b6c	f	inh	96	104	G	tba	tba.mix	0.38631	2.6025
but	B6	m	inh	brn	14	14	air	24	ntp	3.6422	11.807	but	b6c	m	inh	60	60	F	lung	adenocarc.	14.67	52.304
age	B6	m	ip	brn	3	3	PBS	24	ntp	2.8126	7.5742	age	b6c	m	inh	103	103	C	nasal cavity	nas.cav.ade	12.722	43.036
iso	B6	f	inh	brn	65	91	air	24	ntp	0.055659	5.184	iso	b6c	m	inh	72	72	F	liver	hepatocellular.ade.carc	211.24	1113.5
isn	B6	f	inh	blood	65	91	air	24	ntp	50.605	153.93	isn	b6c	f	inh	103	103	F	lung	ade.carc	39.116	178.51
pge	B6	f	inh	blood	65	91	air	24	ntp	139.45	538.29	pge	b6c	m	inh	104	104	F	liver	ade.carc	79.184	407.45
mbr	B6	m	inh	blood	10	14	air	24	ntp	10.346	33.151	mbr	b6c	m	inh	104	104	A	NA	NA	318.25	Inf
ace	B6	f	inh	blood	65	91	air	24	ntp	21.697	201.04	ace	b6c	m	inh	104	104	A	NA	NA	633.68	Inf
coc	B6	f	dermal	blood	92	66	ethanol	0	ntp	47.346	150.8	coc	b6c	f	dermal	104	104	F	liver	ade.carc.hepatoblas	1.6957	11.648
dcn	B6	m	inh	blood	65	91	air	24	ntp	4.8684	27.706	dcn	b6c	f	inh	105	105	D	uterus	stromal.polyp.scarcom	380.73	1147.6
ams	B6	f	inh	blood	65	91	air	24	ntp	104.29	302.55	ams	b6c	f	inh	105	105	F	liver	ade.carc	98.814	504.93
acd	B6	m	ip	blood	3	3	corn.oil	48	ntp	10.108	60.529	acd	b6c	f	ip	81	103	B	lymph	ytic.and.granulocytic.ni	0.19844	0.48384
dmvc	B6	m	ip	brn	3	3	corn.oil	24	ntp	3.0136	8.6822	dmvc	b6c	f	gav	103	103	D	forstomach	foresquam.carc	14.879	22.092
oxy	P16	m	gav	blood	195	273	water	24	ntp	8.8444	45.85	oxy	b6c	f	feed	104	104	F	liver	ade.carc	15.688	36.294
gly	P16	m	ip	brn	3	3	corn.oil	24	ntp	1.8469	6.7115	gly	b6c	m	gav	104	104	F	liver	ade.carc	4.4968	25.35
sel	B6	m	ip	brn	3	3	corn.oil	24	ntp	1.4662	8.0835	sel	b6c	f	gav	103	103	F	liver	ade.carc	14.004	27.619
ben	P16	m	gav	blood	32	42	corn.oil	24	ntp	0.13461	0.27361	ben	b6c	m	gav	103	103	D	preputial gland	carc	12.409	18.08
chl	B6	m	ip	brn	3	3	PBS	24	ntp	38.049	107.22	chl	b6c	f	gav	105	105	C	pituitary gland	gl.ade	69.98	96.298
for	B6	m	ip	brn	3	3	corn.oil	24	ntp	33.492	113.1	for	b6c	f	gav	93	93	F	liver	carc.myelosarc	5.1245	14.821
hyd	B6	m	ip	brn	3	3	PBS	24	ntp	2.7673	7.3699	hyd	b6c	f	gav	104	104	F	liver	ade.carc	19.406	63.061
cop	cd1	m	ip	brn	1	2	na	48	Morita	4.2448	13.213	cop	b6c	m	feed	96	96	D	liver	ade.carc	522.07	969.95
mon	B6	m	ip	brn	3	3	corn.oil	24	ntp	8.9375	34.913	mon	b6c	m	feed	104	104	F	liver	ade.carc	2160.8	Inf
dhp	B6	m	ip	brn	3	3	corn.oil	24	ntp	28.015	125.18	dhp	b6c	m	gav	103	103	A	NA	NA	178.1	Inf
tio	B6	m	ip	brn	3	3	PBS	24	ntp	0.00085	Inf	tio	b6c	m	feed	104	104	A	NA	NA	677.390	Inf
tac	B6	m	ip	brn	3	3	PBS	24	ntp	3.5391	9.3742	tac	b6c	f	feed	104	104	C	pituitary gland	ade	11.792	Inf
phe	B6	m	ip	brn	3	3	PBS	24	ntp	13.282	40.826	phe	b6c	m	water	103	103	A	NA	NA	108.690	Inf
las	B6	m	ip	brn	3	3	PBS	24	ntp	69.725	204.69	las	b6c	m	feed	103	103	A	NA	NA	815.520	Inf
hrc	B6	m	ip	brn	3	3	corn.oil	24	ntp	13.2	Inf	hrc	b6c	m	gav	104	104	F	harderian gland	ade.carc	57.689	156.45
rsc	B6	m	ip	brn	3	3	PBS	24	ntp	12.118	48.762	rsc	b6c	m	gav	104	104	A	NA	NA	1774.6	Inf
erno	B6	m	ip	brn	3	3	corn.oil	24	ntp	138.63	Inf	erno	b6c	m	feed	105	105	F	kidney	ren.tub.ade.carc	0.00178	Inf
cps	B6	m	ip	brn	3	3	corn.oil	24	ntp	17.834	85.395	cps	b6c	f	feed	104	104	D	skin	skin.sarc	30.396	66.02
eth	B6	m	ip	brn	3	3	corn.oil	24	ntp	20.989	62.853	eth	b6c	f	gav	103	103	F	liver	ade.carc	45.942	94.694
mel	cd1	m	ip	brn	1	1	na	24h	Morita	0.008496	0.037978	mel	swiss	f	ip	26	26	G	tba	tba.mix	0.005465	0.033153
cbc	bdf1	m	ip	brn	1	1	na	48hrs	Morita	0.046114	0.14097	cbc	swiss	f	ip	26	78	G	tba	tba.mix	0.001	0.014859
nda	0	m	ip	blood	1	1	na	48	suzuki	0.14075	4.0427	nda	cbl	f	gav	50	72	G	tba	tba.mix	0.017799	0.048266
cpa	balb	m	ip	blood	1	1	NaCl	48	Vrzoc	1.3964	5.0091	cpa	swiss	f	ip	79	79	G	tba	mix	0.27675	0.67592
2aaf	bdf1	m	ip	blood	1	1	na	48	Asano	1.1875	4.3712	2aaf	b6c	m	feed	82	82	E	tba	tba.mix	0.24923	1.1873
ure	B6	f	water	brn	90	91	water	24	ntp	0.40415	1.816	ure	b6c	m	water	104	104	F	harderian gland	carc ade	0.619	1.231
rnnu	balb	m	ip	blood	1	1	NaCl	48	Vrzoc	0.084731	0.33333	rnnu	c3h	m	water	30	54	D	spleen	spl.hemangioendoth.sar	0.65234	1.5042
bap	bdf1	m	gav	blood	1	1	na	48	Shimada	0.26131	0.57301	bap	b6c	f	feed	95.6	104	E	forstomach	forest.mix	0.64629	0.99282
tet	B6	f	feed	blood	92	92	feed	0	ntp	11.603	91.846	tet	b6c	f	gav	90	90	D	liver	carc	25.909	38.959
pch	B6	m	gav	brn	3	3	PBS	24	ntp	1.3507	3.4433	pch	b6c	m	gav	103	103	D	liver	hemangiosarc	16.274	41.28
tce	B6	m	gav	brn	3	3	corn.oil	24	ntp	305.23	Inf	tce	b6c	m	gav	104	104	D	liver	carc	230.45	377.83
php	P16	f	feed	blood	182	182	feed	24	ntp	7.7055	27.231	php	b6c	f	feed	104	104	D	haemartopo etic	hemat.sarc.lymph	567.42	1187.2
teo	B6	m	feed	blood	65	91	corn.oil	24	ntp	13.372	Inf	teo	b6c	m	gav	104	104	F	liver	decrease.hepc.ade.carc	296.6	Inf
scd	AM3	m	feed	blood	65	65	water	24	ntp	0.56674	2.5567	scd	b6c	f	water	104	104	F	small intestine	ade.carc	3.3753	6.133
ieg	B6	f	gav	blood	90	91	corn.oil	24	ntp	5.4738	27.191	ieg	b6c	m	gav	105	105	F	liver	ade.carc	7.0856	30.14
dmh	cd1	m	gav	brn	1	1	na	24	Meli	0.11373	0.28357	dmh	swa	m	water	52	52	D	haemartopo etic	blood.vessel.angiosarc	0.019671	0.11912
azt	B6	m	gav	blood	65x	90	na	24	ntp	0.44026	1.0861	azt	b6c	f	gav	105	105	F	vagina	squam.carc.papil	69.055	136.5
leu	B6	f	feed	blood	28	28	feed	24	ntp	45.562	Inf	leu	b6c	f	feed	104	104	F	liver]	ade.carc	25.616	121.02

Appendix 3: Data used to generate Figure 3

hMN (Genotoxicity endpoint)												Carcinogenicity (Tumor Endpoint)										
compound	strain	sex	route	tissue	treatment regimen	duration exposure	vehicle control	sampling time	source	$BMDL_{05}$	$BMDU_{05}$	compound	strain	sex	route	exposure time	duration exper	severity	tissue	tissue lesion	$BMDL_{10}$	$BMDU_{10}$
dbe	B6	m	inh	blood	125	175	air	24	ntp	0.83257	4.4119	dbe	b6c	f	gav	53	90	3	stom	stom.squam.carc	12.273	16.725
but	B6	m	inh	bm	14	14	air	24	ntp	3.6422	11.807	but	b6c	f	inh	36	36	3	forstomach	forepapil	23.309	80.514
pge	B6	f	inh	blood	65	91	air	24	ntp	139.45	538.29	pge	b6c	m	inh	104	104	3	liver	hepatoblas	1102.8	Inf
dcn	B6	m	inh	blood	65	91	air	24	ntp	4.8684	27.706	dcn	b6c	f	inh	105	105	3	uterus	ut.stromal.polyp scarcoma	380.73	1147.6
dmvc	B6	m	ip	bm	3	3	corn.oil	24	ntp	3.0136	8.8822	dmvc	b6c	f	gav	103	103	3	forest	foresquam.carc	14.879	22.092
gly	P16	m	gav	blood	195	273	water	24	ntp	1.8469	6.7115	gly	b6c	f	gav	104	104	3	skin	fibrosarc	21.477	32.515
ben	P16	m	gav	blood	32	42	corn.oil	24	ntp	0.13461	0.27361	ben	b6c	m	gav	103	103	3	preputial gland	carc	12.409	18.08
chl	B6	m	ip	bm	3	3	PBS	24	ntp	38.049	107.22	chl	b6c	m	gav	104	104	3	liver	hepc.carc	49.735	198.32
for	B6	m	ip	bm	3	3	corn.oil	24	ntp	33.492	113.1	for	b6c	m	gav	93	93	3	haematopoetic	hemat.lymph	168.89	347.3
cop	cd1	m	ip	bm	1	2	na	48	Morita	4.2448	13.213	cop	b6c	m	feed	96	96	3	liver	ade.carc	522.07	959.95
hrc	B6	m	ip	bm	3	3	corn.oil	24	ntp	13.2	Inf	hrc	b6c	m	gav	104	104	3	adrenal gland	pheochrom	71.112	187.08
cps	B6	m	ip	bm	3	3	corn.oil	24	ntp	17.834	85.395	cps	b6c	f	feed	104	104	3	skin	skin.sarc	30.396	66.02
mel	cd1	m	ip	bm	1	1	na	24h	Morita	0.008496	0.037978	mel	swiss	f	ip	26	79	3	adrenal gland	adr.gland.fibrosarc	0.10179	1.6978
cbc	bdf1	m	ip	bm	1	1	na	48hrs	Morita	0.046114	0.14097	cbc	swiss	m	ip	26	78	3	lymph	lymph	0.080981	0.25336
nda	na	m	ip	blood	1	1	na	48	suzuki	0.14075	4.0427	nda	cbl	m	gav	50	72	3	brain	forebrain.olf neuroepithelioma	0.050993	0.11527
dbcp	cd1	m	ip	bm	1	1	na	48hrs	Morita.1997	1.3964	5.0091	dbcp	b6c	f	inh	91.3	104	3	nasal cavity	nas.cav.carc	1.701	3.5211
cpa	balb	m	ip	blood	1	1	NaCl	48	Vrzoc	0.008473	0.73073	cpa	swiss	f	ip	26	79	3	lung	lung.malign	0.31888	1.1111
2aaf	bdf1	m	ip	blood	1	1	na	48	Asano	1.1875	4.3712	2aaf	b6c	m	feed	82	82	3	liver	liv.carc	1.6211	2.8581
ure	B6	f	water	bm	90	91	water	24	ntp	0.40415	1.816	ure	b6c	f	5.perc.EtOH	104	104	3	liver	hemangiosarc	7.5407	15.186
mnu	balb	m	ip	blood	1	1	NaCl	48	Vrzoc	0.084731	0.33333	mnu	c3h	m	water	30	54	3	spleen	spl.hemangioendoth sarc	0.65234	1.5042
bap	bdf1	m	gav	blood	1	1	na	48	Shimada	0.26131	0.57301	bap	b6c	f	feed	95.6	104	3	forstomach	forest.squam.carc	0.87406	1.284
tet	B6	f	feed	blood	92	92	feed	0	ntp	11.603	91.846	tet	b6c	f	gav	90	90	3	liver	carc	25.909	38.959
pch	B6	m	gav	bm	3	3	PBS	24	ntp	1.3507	3.4433	pch	b6c	m	gav	103	103	3	liver	hemangiosarc	16.274	41.28
tce	B6	m	gav	bm	3	3	corn.oil	24	ntp	305.23	Inf	tce	b6c	m	gav	104	104	3	liver	carc	230.45	377.83
php	P16	f	feed	blood	182	182	feed	24	ntp	7.7055	27.231	php	b6c	f	feed	104	104	3	haematopoetic	hemat.sarc.lymph	567.42	1187.2
dmh	cd1	m	gav	bm	1	1	na	24	Meli	0.11373	0.28357	dmh	swa	m	water	52	52	3	haematopoetic	blood.vessel.angiosarc	0.019671	0.11912

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