Critical Examination of Selected Aspects of the ToxTracker® *In Vitro* Genotoxicity Assay: Evaluation of S9 Metabolic Activation Protocols and Quantitative Interpretation of Dose-response Data

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Abstract

Genotoxic effects such as mutations and chromosome abnormalities can augment the risk of adverse health effects such as cancer and heritable genetic diseases; chemicals in commerce must be screened for genotoxic activity. To this end, Toxys B.V. developed the *in vitro* ToxTracker® assay, which detects (geno)toxicity by monitoring the activity of six reporter genes in cultured mES cells (murine embryonic stem cells), i.e., *Rtkn*, *Bscl2*, *Btg2*, *Srxn1*, *Blvrb* and *Ddit3*. The reporters respond to genotoxic stress, oxidative stress, and endoplasmic reticulum stress characterized by protein unfolding; reporter induction is monitored using flow cytometry. The ToxTracker® assay generates large amounts of multivariate concentration-response data; this study employed innovative quantitative methods to scrutinize ToxTracker® assay results. The work (i) defined a fold-change threshold for identification of a significant positive response, (ii) used two analytical approaches to define endpoint-specific Benchmark Response (BMR) values, (iii) used the BMD (Benchmark Dose) combined-covariate approach for potency ranking of assay validation compounds, and (iv) used PCA (Principal Component Analysis) to investigate functional and statistical relationships between the reporters. The results revealed fold-change cut-offs of 1.5 and 1.7 for identification of weak and strong positive responses, respectively. 1.5-fold is consistent with the value advocated by Toxys B.V.; 1.7-fold is more conservative than the Toxys-advocated 2-fold value. Potency ranking of the validation compounds permitted comparative identification of the most potent inducers of each reporter. The most potent compounds consistently included clastogens used for cancer chemotherapy. BMR values determined using the Zeller *et al.* (2017) approach ranged from 2.2% for *Blvrb* and *Rtkn*, to 7.0% for *Ddit3*, with an average of 3.9% across all the reporters. The Slob (2016) approach yielded values that ranged from 30% for *Ddit3*, to 52% for *Rtkn*, with an average of 43%. The PCA results indicated the *Rtkn*, *Bscl2* and *Btg2* reporters

are functionally redundant; collectively indicative of genotoxic stress. The *Blvrb* and *Ddit3* reporters are orthogonal indicators of oxidative stress and protein unfolding, respectively; they are essential for toxicological profiling using the ToxTracker® assay. PCA axis scores reflect the toxicological MOA (Mode of Action) of the tested compounds; hitherto unknown MOAs can be inferred using PCA axis-plot proximity to well-studied compounds. Like most *in vitro* (geno)toxicity assessment assays, ToxTracker® employs a material known as S9 to simulate mammalian hepatic metabolism. S9 is prepared from the livers of rats exposed to an inducer of microsomal CYP (Cytochrome P450) isozymes; the most common CYP inducer is the PCB (polychlorinated biphenyl) mixture known as Aroclor-1254. Due to restrictions in the availability of Aroclor-1254, this study also evaluated the utility of Phenobarbital (PB)/β-Naphthoflavone (BNF)-induced S9, a proposed substitute for Aroclor-induced S9. The results indicate that, despite differences in enzymatic profiles, a 24-hr protocol using 0.40% v/v PB/BNF-induced S9 yields results that are comparable to those obtained using 0.25% v/v Aroclor-induced S9. This study constitutes a significant step towards augmenting the utility of the ToxTracker® assay; it provides a foundation for eventual adoption of high-throughput reporter assays for routine regulatory screening of new and existing chemicals.

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List of Abbreviations

1,3-DPH	1,3-diphenyltriazine
17be	17*b*-estradiol
1-nap	1-naphthol
2AA	2-aminoanthracene
2AAF	2-acetylaminofluorene
2-mer	2-mercaptopurin
3MC	3-methylcholanthrene
4-a-3-n	4-amino-3-nitrophenol
4-NQO	4-nitroquinoline-1-oxide
4-Vinyl-1-cyclo	4-vinyl-1-cyclohexene diepoxide
5-azacytidine	5-azacytidine
8-oxo-Dg	*8-oxo*-2'-deoxyguanosine
A	Aroclor-1254 induced rat liver S9
Abac	abacavir
Acic	aciclovir
Adef	adefovir
AFB1	aflatoxin B1
Alach	alachlor
All. bro	allyl bromide
Alos	alosetron
Amiod	amiodarone
Amitrole	amitrole
Ant. A	antimycin A
ATM	ataxia telangiesctasia mutated
ATR	Rad3-related protein
Atraz	atrazine
Azat	azathioprine
Azin. met.	azinphos methyl
BAA	benz[*a*]anthracene
BaP	benzo[*a*]pyrene
Beno	benomyl
Benz. par.	benzyl paraben
Blvrb	biliverdin reductase B
BMD	Benchmark Dose
BMDL	lower confidence interval of the Benchmark Dose
BMDU	upper confidence interval of the Benchmark Dose
BMR	Benchmark Response
Bref. A	brefeldin A
Bscl2	Bernardinelli-Seip congenital lipodystrophy type 2
Btg2	B-cell translocation gene 2
C/EPB	CCAAT/enhancer-binding protein
Campto	camptothecin

Carbam	carbamazepine
Carben	carbendazim
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
$CdCl_2$	cadmium chloride
CED	Critical Effect Dose
CEDL	lower confidence interval of the Critical Effect Size
CEDU	upper confidence interval of the Critical Effect Dose
CEES	2-chloroethyl ethylsulfide
CES	Critical Effect Size
Chk	checkpoint kinase
Chloramb	chlorambucil
Chloramp	chloramphenicol
Chlorphe	chlorpheniramine maleate
Chlorpy	chlorpyrifos
Cicles	ciclesonide
Cispt	cisplatin
Climba	climbazole
Colce	colcemid
Colch	colchicine
CPA	cyclophosphamide
CYP	cytochrome P450
Daun	daunorubicin
Daz	dazomet
Ddit3	DNA damage inducible transcript-3
DEHP	di-(2-ethylhexyl)phthalate
Diclo	diclofenac
Dield	dieldrin
Diet. mal.	diethyl maleate
Diethyls	diethylstilbestrol
Diphenylh	diphenylhydantoin
DMBA	7,12-dimethylbenz[*a*]anthracene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Docet	docetaxel
Dode. ald.	dodecyl aldehyde
Econ	econazole
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Agency
Emod	emodin
EMS	ethyl methanesulfonate
Endos	endosulfan
ENU	*N*-Nitroso-*N*-ethylurea
ES	Effect size
Ethox. acid	ethoxyacetic acid
Ethyl par.	ethyl paraben

Ethylen	ethylenediaminetetraacetic acid
Etop	etoposide
Eug	eugenol
EURL-ECVAM	European Center for Validation of Alternative Methods
Famot	famotidine
Fludio	fludioxonil
Fluorou	5-fluorouracil
Flusi	flusilazole
GFP	Green Fluorescent Protein
Griseo	griseofulvin
H_2O_2	hydrogen peroxide
HBGV	Health-based Guidance Value
Hesp	hesperadin
HMOX1	heme oxygenase-1
HMPA	hexamethylphosphoramide
HOPO	2-pyridinol 1-oxide
Hydral	hydralazine
Hydrocor	hydrocortisone
Hydroq	hydroquinone
Hydroxybenz	hydroxybenzomorpholine
Hydroxyu	hydroxyurea
IL-1β	Interleukin 1 beta
IQ	2-amino-3-methyl-3H-imidazo[4,5-F]quinoline
KEAP1	Kelch-like ECH-associated protein 1
Lidocaine	lidocaine
Meben	mebendazole
MeIQ	2-amino-3,4-dimethylimidazo[4,5-F]quinoline
Menad	menadione
mES cells	mouse embryonic stem cells
Met. mer.	methyl mercury
MIT	methylisothiazolinone
Mit C	mitomycin C
Mitox	mitoxantrone
MMS	methyl methanesulfonate
MOA	mode of action
Monob. phtha.	monobutyl phthalate
Nali. Acid	nalidixic acid
NEMO	NF-kappa-B essential modulator
NF-κb	nuclear factor kappa-light-chain-enhancer of activated B
Nifed	nifedipine
Nitrof	nitrofurantoin
Nitrophe	nitrophenol
Nitropy	1-nitropyrene
NNK	4-[methyl(nitroso)amino]-1-(3-pyridinyl)-1-butanone
Nocod	nocodazole

NOGEL	No-Observed Genotoxic Effect Level
Norethy	norethynodrel
Noscap	noscapine
Novob	novobiocin
Nrf2	nuclear factor (erythroid-derived 2)-like 2
Nut-3	nutlin-3
OAT	*o*-aminoazotoluene
OECD	Organisation for Economic Cooperation and Development
Ofloxa	ofloxacin
PB/BNF	Phenobarbital/β-Naphthoflavone induced rat liver S9
PCA	Principal Component Analysis
p-Chloroa	p-chloroaniline
PFOA	perfluorooctanoic acid
PFOS	perfluorooctanesulfonic acid
Phen. par.	phenyl paraben
Phenanth	phenanthrene
PhIP	2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine
Phtha. anhyd.	phthalic anhydride
PoD	point of departure
Podophyl	podophyllotoxin
Pot. chrom.	potassium chromate
Predni	prednisolone
Pyrimeth	pyrimethamine
Querce	quercetin
ROS	Reactive Oxygen Species
RTKN	Rhotekin
Sabin. Hyd.	sabinene hydrate
Sod. ars.	sodium arsenite
Sod. az.	sodium azide
Sod. dode.	sodium dodecyl
Soft	softenon
Spirox	spiroxamine
Srxn1	Sulfiredoxin 1
Stavud	stavudine
Sulfuram	sulfuramid
Taxol	taxol
TDI	Tolerable Daily Intake
Temoz	temozolomide
Testos	testosterone
Tetraeth	disulfiram
Thapsi	thapsigargin
TNF-α	Tumor necrosis factor alpha
Topot	topotecan
Triadi	triadimenol
Triclos	triclosan

Troglit	troglitazone
Tuni	tunicamycin
Vinb	vinblastine sulphate
Vinclo	vinclozolin
Vincri	vincristine
Vindes	vindesine
Vinorel	vinorelbine
Vorico	voriconazole
Zafir	zafirlukast
Zido	zidovudine

Chapter 2 Statement of Contributions:

Title: Analysis and Interpretation of ToxTracker® Dose-response Data – Reporter Signal Interpretation, Compound Potency Ranking, and Determination of Endpoint-specific Benchmark Response (BMR) Values

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Chapter One

1.1 Chemical safety assessment and genotoxicity testing

Safety assessment of chemicals is essential in order to minimize the risks of adverse human and/or environmental health effects. In Canada, regulatory evaluations of substances in commerce is conducted in accordance with the Canadian Environmental Protection Act (CEPA, 1999) (1), the Pest Control Products Act (PCPA) (2), the Consumer Products Safety Act (3), and the Food and Drugs Act (4). In the United States, regulatory evaluations of new or existing chemicals is conducted under the Toxic Substances Control Act (TSCA) (5), and the Federal Insecticide, Fungicide and Rodenticide Act (FIRFA) (6). Similarly, in the European Union, the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations address the potential adverse impacts of chemicals on human and environmental health (7).

The regulatory initiatives mentioned above employ a variety of different *in vitro* and *in vivo* toxicity assessment tools. The ability to assess a substance's ability to adversely alter genetic material (i.e. genotoxicity) is a key component of all hazard assessments. Assessing genotoxicity is necessary in order to minimize the risk of adverse health effects associated with DNA damage, e.g., heritable genetic diseases, somatic mosaicism and cancer.

Organizations such as the ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) (8), the United States FDA (Food and Drug Administration) and the government of Canada (e.g., CEPA New Substances Notification Regulations) (9) define test strategies for genetic toxicity screening. Moreover, the OECD (Organisation for Economic Cooperation and Development) establishes internationally-standardized guidelines on how to properly perform the recommended assays, i.e., the OECD Test Guidelines program (10). *In vitro* tests constitute the cornerstone of regulatory frameworks used for genotoxicity assessment; these tests have been widely adopted, and are routinely employed

worldwide. For example, the aforementioned New Substances Notification Regulations (NSNR) outlined in CEPA requires use of OECD-recognized *in vitro* mutagenicity tests conducted in bacterial and/or mammalian cells (9). The required tests, such as the Salmonella Reverse Mutation Test (i.e., Ames test), mammalian cell gene mutation tests (e.g., *tk* or *hprt* locus mutagenicity assays), and mammalian cell chromosome damage assays (e.g., mammalian cell micronucleus assay) have been shown to reliably detect *in vivo* mutagens and genotoxic carcinogens. The global trend towards minimizing the use of animals for chemical safety assessments promotes the use of the aforementioned assays; indeed, all *in vitro* hazard assessment tools. The use of such tools is aligned with 3R principles, i.e., Replacement, Reduction, and Refinement of the use animals for scientific research and regulatory hazard assessments (11, 12).

Despite the utility of the established *in vitro* genotoxicity tests mentioned above, they are largely based on technologies available 30-50 years ago, and they are relatively laborious (13). Criticism of traditional *in vitro* tests also comments on their relatively high cost, low throughput, and lack of ability to provide detailed information about compound-specific mode of action (14). Moreover, with respect to assay performance, these tests often have either a high specificity or a high sensitivity, but not both. More specifically, tests with a high sensitivity and a low specificity can yield false-positive results, i.e., the test will incorrectly elicit a positive result in the absence of *in vivo* hazard (15).

1.2 *In vitro* metabolic activation

Some chemicals need to undergo metabolic transformation before they are able to adversely interact with cells and tissues, i.e., metabolic conversion of an otherwise benign substances to a (geno)toxic metabolite. With respect to genotoxicity specifically, chemicals that are not DNA-reactive can be metabolically-transformed into reactive metabolites (16). For example, polycyclic

aromatic hydrocarbons (PAHs) are generally benign until they are metabolically converted into DNA-reactive metabolites by cytochrome P450 (CYP) isozymes such as P4501A1 (Table 1.1) (17-19). In some cases, the organisms employed for *in vitro* genotoxicity assessment (e.g., *Salmonella enterica* serotype *typhimurium*, human TK6 lymphoblast cells are unable to carry out the conversion to DNA-reactive metabolites that commonly occurs in mammals *in vivo*, e.g., as a result of mammalian hepatic metabolism. Consequently, it is often necessary to simulate *in vivo* mammalian hepatic metabolism, and *in vitro* assays for genotoxicity generally employ an enzymatic preparation that is added to bacterial and mammalian cell cultures (20). The most common preparation used is the S9 fraction (i.e., 9,000g post-mitochondrial supernatant) obtained from homogenized livers of male Sprague-Dawley rats treated with an agent that upregulates production of microsomal enzymes such as the P450 isozymes. Aroclor 1254, phenobarbital, or a mixture of phenobarbital (PB) with β-Naphthoflavone (BNF), are commonly used forinduction of microsomal enzymes that confer metabolic activity of S9 (21). Aroclor 1254 is a mixture of polychlorinated biphenyls (PCBs) (20), including PCBs that are agonists of the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR), all of which are involved in the regulation of the cytochrome P450 (CYP) superfamily, i.e. isozymes that are involved in catalyzation of the aforementioned metabolic activation reactions (22). As for PB and BNF, they are ligands of CAR and AhR, respectively, and are often used together as microsomal enzyme inducing agents, i.e., for stimulation of CYP production and S9 metabolic activity (23). Because of recent restrictions put into place on the production and use of Aroclor 1254, there is a shift towards an alternative inducer of hepatic oxidative metabolism (24, 25). The combination of PB/BNF-induced rat liver S9 has been studied as a possible substitute for Aroclor 1254 induced-S9. These agents were selected because they are known to effectively

induce two major forms of cytochrome P450, i.e., CYP1A1 and CYP3A, as shown in Table 1.2. Although no two inducers of metabolic capacity are expected to have the same induction profile, it was considered that PB/BNF could be a suitable alternative to Aroclor 1254, i.e., the pattern of overall enzyme induction is sufficiently similar (23).

Table 1.1 Different classes of genotoxic chemicals and their metabolic requirements.

Chemical Class	**Example**	**Metabolic Requirements**	**References**
Polycyclic aromatic hydrocarbons	Benzo[*a*]pyrene (BaP)	CYP1A1 CYP1A2 CYP3A EH[a]	Jeffrey, 1985; Bauer et al., 1995; Kim et al., 1998 (17-19)
Aromatic amines	2-Aminoanthracene (2AA)	CYP1A1 CYP1A2 SULT[b] NAT[c]	Heflich and Neft, 1994 (26)
Heterocyclic amines	2-Amimo-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)	CYP1A1 CYP1A2 SULT NAT UGT[d]	Schut and Snyderwine, 1999; Cai et al., 2016 (27, 28)
Fungal metabolites	Aflatoxin B1 (AFB1)	CYP1A2 CYP3A	Gallagher et al., 1994 (29)

[a]Epoxide hydrolase
[b]Sulfotransferase
[c]*N*-acetyltransferase
[d]Uridine 5'-diphospho-glucuronosyltransferase

Table 1.2 Metabolic activity of Aroclor 1254 and Phenobarbital/β-Naphthoflavone-induced S9, expressed as mean specific activity and fold-change relative to uninduced control. Adapted from Cox *et al*., 2016 (30)

Enzyme	Mean activity in p/moles/min/mg protein (in fold change induction)		
	Uninduced rat liver S9	**Aroclor 1254-induced rat liver S9**	**Phenobarbital/β-Naphthoflavone-induced rat liver S9**
CYP1A1 CYP1A2	55.2	6580.9 (119.22)	5883.2 (106.58)
CYP2B1 CYP3A	80	3098.1 (38.73)	6525.2 (81.56)
CYP1A2	17.4	1856.1 (106.67)	833.2 (47.89)

1.3 Toxicity testing in the 21st Century: high-throughput reporter assays

In vitro (geno)toxicity assessment tools developed more recently often employ high-throughput (HT) reporter systems for rapid assessment of large numbers of compounds across numerous endpoints. Indeed, *in vitro* assays employed by the United States ToxCast and the Tox21 programs (31) can rapidly assess the toxicological activity of thousands of agents across hundreds of endpoints. With respect to genotoxicity specifically, assays such as the Multiflow™ and ToxTracker® genotoxicity assays (Table 1.3) are high-throughput reporter assays that employ readily-detected fluorescent or luminescent signals (e.g. Green Fluorescent Protein or GFP, luciferin) indicative of changes in gene expression or protein production. Immunocytochemical staining can also be used to rapidly assess compound-induced cellular stress via measurements of protein abundance, post-translational modification and/or cellular localization (32). High-throughput signal measurement techniques such as flow cytometry are often used to assess the magnitude of the stress signal relative to the control condition, i.e., cells exposed to carrier solvent. The test systems can rapidly assess single endpoints; or alternatively, multiple endpoints simultaneously (i.e., multiplexed assessment tools). The latter, by virtue of the ability to

simultaneously assess multiple cellular responses, can effectively provide information on the mode of action of the tested agent (i.e., type(s) of cellular changes underlying toxicological hazard). As can be seen in Table 1.3, there are multiple reporter assays that can be used to assess genotoxicity; moreover, gain insight on a chemical's mode of action (MOA). MOA information permits classification of chemicals into categories that provide information on mechanism of toxicity; this information can be used to support risk assessment (e.g., induction of chromosomal abnormalities via non-disjunction).

The assays listed in Table 1.3 are generally less costly and time-consuming compared to already established *in vitro* mutagenicity tests (Table 1.4). Although these novel HT tools have great potential, they have not been validated for regulatory application, and consequently, there are no OECD test guidelines (11). Moreover, the endpoints are not recognized for regulatory assessments conducted under, for example, CEPA's New Substances Notification Regulations (NSNR). Nevertheless, validation data are rapidly becoming available; to date they indicate that the performance of some reporter-based assays (e.g., ToxTracker®) exceeds that of the aforementioned conventional assays (Table 1.5). The performance of genotoxicity assays is often evaluated by its ability to determine whether a substance is carcinogenic; sensitivity and specificity are used to evaluate the assay's ability to correctly identify potential carcinogens and/or *in vivo* mutagens. These measures are calculated using the ratio of identified positive and negative carcinogens over expected positives and negatives, respectively. Importantly, the complements of sensitivity and specificity are indicative of false positive and false negative frequency, respectively (e.g., the ratio of substances not correctly classified with respect to carcinogenicity).

Table 1.3 Examples of high-throughput (HT) reporter-based (geno)toxicity assays. Gene and/or protein reporters are indicated.

Assay	Reporter(s)	Reference
GreenScreen®	*GADD45a*-GFP*	Cahill et al., 2004 (33)
BlueScreen™	*GADD45a*-GLuc**	Simpson et al., 2013 (34)
ToxTracker®	*Blvrb*-GFP; *Srxn1*-GFP; *Btg2*-GFP; *Ddit3*-GFP; *Bscl2*-GFP; *Rtkn*-GFP	Hendriks et al., 2016 (15)
Multiflow™	Nuclear p53; γH2AX foci, Phospho-Histone H3	Bryce et al., 2016 (35)
ATAD5 Assay	*ATAD5*-luciferase	Fox et al., 2012 (36)
Cytoprotex CellCiphr® Premier	Numerous including activated p53	Knight et al., 2009 (31)

*GFP, Green Fluorescence Protein
** GLuc, Gaussia luciferase

Table 1.4 Comparison of operational costs and turnaround time for genotoxicity assays. High-throughput reporter assays and traditional *in vitro* and *in vivo* assays are indicated.

	Cost per compound (k€)	Compound needed (mg)	Turnaround time (weeks)
Novel Screening Assays			
ToxTracker®	1.5-2	2-5	1-3
Ames MPF	1.2-1.8	20-150	1-3
GreenScreen HC™	1.5-2	10	1-3
Established Regulatory Assays			
Ames test	3.5-5.5	1000-2000	3-4
Micronucleus assay (*in vivo*)	12-22	2000-10000	10-14
Micronucleus assay (*in vitro*)	12-17	1000-2000	3-4
Chromosomal Aberration (*in vivo*)	14-22	1500-3000	10-15
Mammalian Mutation (*in vivo*)	12-18	1500-3000	8-11
Comet Assay (*in vivo*)	20-30	2000-10000	7-11

Table 1.5 Comparative performance of the ToxTracker® and conventional *in vitro* genotoxicity tests. Sensitivity indicates the ability to correctly identify known *in vivo* genotoxicants; specificity indicates the ability to correctly identify non-genotoxic substances. Adapted from Hendriks *et al*. (15, 37)

	Sensitivity (%)	**Specificity (%)**
Ames test[a]	46[b]	81
In vitro micronucleus test[a]	96	47
Chromosome aberration test[a]	94	50
ToxTracker® assay	95	94
Multiflow™ assay	92	96

[a]Data obtained from Kirkland *et al.*, 2008 (38).
[b]Sensitivity increases to 62% when microtubule disrupting compounds were omitted from the calculations.

1.4 The ToxTracker® assay

The ToxTracker® assay is an example of a multiplexed, high-throughput assay for the assessment of (geno)toxicological responses. The assay uses unique gene expression biomarkers to assess a variety of cellular responses related to toxicological stress; providing information to assess the magnitude of the detected hazard, as well as the substance's MOA. As such, it offers a high-throughput, multiplexed alternative to traditional *in vitro* genotoxicity assays (39). The biomarkers employed can detect activation of multiple stress response pathways associated with, for example, inhibition of DNA replication, DNA double strand breaks, generalized DNA damage activated as part of a p53-mediated stress response, and oxidative stress; these responses that have been empirically and mechanistically associated with increased likelihood of *in vivo* mutagenicity and carcinogenicity (39). The assay can also detect protein misfolding, which is indicative of more generalized cellular stress (39).

The ToxTracker® assay reporter system is mechanistically based on signaling proteins to detect toxicological responses induced by the tested agents. Signaling proteins, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), ataxia telangiesctasia mutated (ATM), and Rad3-related protein (ATR), are activated by cellular stressors; they in turn activate signaling pathways, such as p53-dependant pathways, which respond to DNA damage via ultimate activation of apoptosis and DNA repair, the heme oxygenase-1 (HMOX1) and nuclear factor (erythroid-derived 2)-like 2 (NRF2) antioxidant pathways, and the protein unfolding response pathways. Activation of these pathways triggers a signaling cascade that ultimately alters the expression of distinct genes associated with specific damage/repair responses and processes (e.g., *Bscl2*, *Rtkn*, *Srxn1*, *Blvrb*, *Ddit3* and *Btg2*). In the ToxTracker® assay system these genes have been genetically linked to green fluorescent protein (GFP), a fluorescent reporter, and changes in the expression can be readily detected using flow cytometry. The ToxTracker® assay therefore allows rapid *in vitro* detection of complementary cellular damage response pathways; providing insight into the magnitude and type(s) of toxicological effects induced by the test chemical (Figure 1.1) (39).

1.4.1 Development of the ToxTracker® reporter cell lines

The aforementioned toxicological-response reporter genes were selected using whole-genome transcriptional profiling of mouse embryonic stem (mES) cells exposed to a test set of chemical carcinogens. Following exposure to low, medium and high concentrations of test chemical, RNA was isolated from the mES cells, before being labeled and hybridized to Genechip Mouse Genome microarrays (40). Results from triplicate hybridizations were combined, and differential gene expression detected via statistical comparisons of treated cells and untreated controls. Genes with a false discovery rate ≤10% were considered as indicative of robust, chemically-induced changes in gene expression. The most significant changes in gene expression were identified using multiple

test-corrected p-values and fold changes (i.e., p-values < 0.01 and fold change >2). Genes with significant changes in gene expression, and large fold-changes in response to specific classes of carcinogenic compounds, were identified as potential biomarkers for different types of cellular damage, including DNA damage, generalized cellular stress, oxidative stress, and protein damage. These genes were selected as the basis for the construction of the ToxTracker® reporter cell lines (40).

The GFP-linked reporters were generated using bacterial artificial chromosomes (BAC). BACs containing the biomarker genes were modified with a C-terminal GFP marker. Successfully transfected mouse embryonic stem cells were selected based on the ability to induce GFP expression in response to a series of carcinogenic compounds, including genotoxins and pro-oxidants (39, 41). Six reporters were selected that collectively provide GFP signals for genes representing the aforementioned damage-associated pathways (Figure 1.1), i.e., DNA damage, oxidative stress, protein damage, and generalized cellular stress (40).

Although the results of the gene expression analyses were used to identify reporter genes that are independent (i.e., orthogonal), the functional relationships between the endpoints has hitherto not been investigated. In principle, a multivariate data analysis technique such as Principal Component Analysis (PCA) could be employed to investigate reporter signal redundancy.

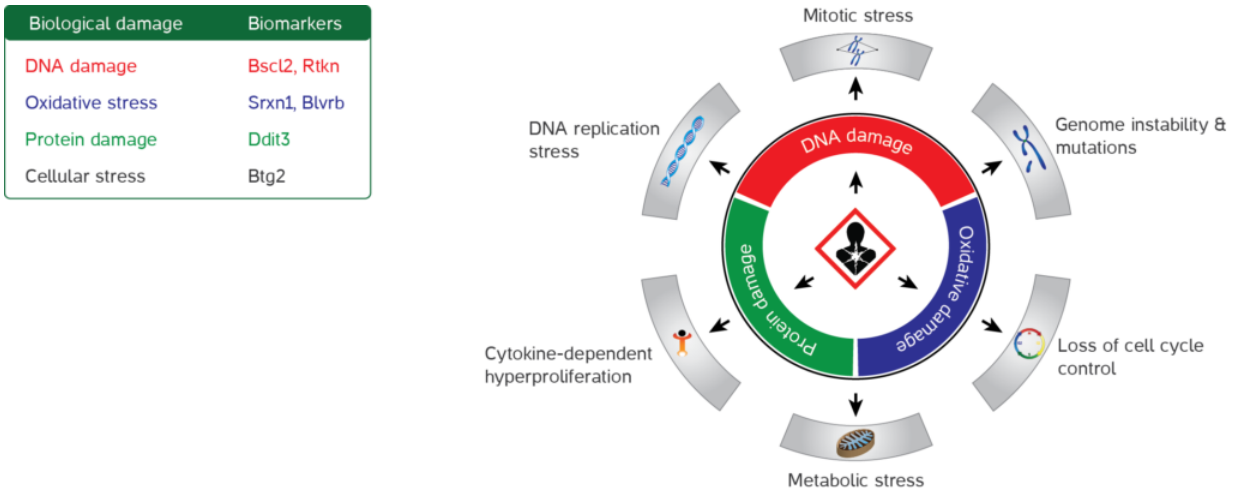


Figure 1.1 Cell damage pathways associated with ToxTracker® gene expression biomarkers. The graphic on the right summarizes the types of cellular responses that can be detected. The graphic on the left summarizes the corresponding biomarker genes monitored using flow cytometry. Figure from Toxys, 2018 (41). Used with permission.

1.4.2 The ToxTracker® reporters

Srxn1

The *Srxn1*-GFP reporter is activated by increased levels of oxidative stress, e.g., stress related to ROS (reactive oxygen species). *Srxn1* (i.e., *Sulfiredoxin 1*) is controlled by the NRF2 signaling pathway (39). NRF2 is a nuclear transcription factor that plays an important role in the cellular oxidative stress response (42). In its inactive state, under normal conditions, NRF2 interacts with the Kelch-like ECH-associated protein 1 (KEAP1), and is retained in the cytoplasm (43). KEAP1 acts as an adaptor for the Cullin 3 E3 ubiquitin ligase, which mediates the proteasomal degradation of NRF2 (43). Under stress conditions provoked by ROS increase, NRF2 allows expression of antioxidants to protect the cells against ROS-related damage (39). The positive control for this reporter is diethyl maleate, a substance known to generate cellular ROS (44).

Blvrb

The *Blvrb*-GFP reporter monitors activation of the *Hmox1* antioxidant response (41). *Hmox1* is a gene regulated by NRF2; its function is to remove toxic heme, in addition to regulating the production of proteins such as biliverdin reductase B (BLVRB), a reductase that catalyzes the final step in mammalian heme metabolism (45). *Hmox1* protects against oxidative stress, regulates apoptosis, modulates inflammation, and contributes to angiogenesis (45). Activation of the *Blvrb* reporter is thus indicative of oxidative stress; the positive control for this reporter is also diethyl maleate.

Rtkn

The *Rtkn*-GFP reporter is activated by DNA double stand breaks (41). The *Rtkn* gene encodes the Rhotekin protein (RTKN), an effector of the RHO GTPase; it has been implicated in neural stem cell differentiation and apoptosis regulation (46). Activation of *Rtkn* has been associated with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) cytokine signaling pathway, a protein complex that plays a major role in processes such as cytokine expression, stress regulation, cell division and transformation (47). Upon activation of NF-κB, the accelerated formation of replication protein A (RPA) and RAD51 foci stimulate the removal of DNA double strand breaks through homologous recombination (48). The positive control for this reporter is cisplatin, a clastogen known to cause chromosomal abnormalities via the induction of DNA double strand breaks (49).

Bscl2

The *Bscl2*-GFP reporter is activated by inhibition of replication. *Bscl2* (i.e., Bernardinelli-Seip Congenital Lipodystrophy Type 2) is controlled by ATM and ATR; elements of the checkpoint

kinase (*Chk*) 1 signaling pathway induced by DNA damaging agents. These serine/threonine protein kinases respond to genotoxic agents by stimulating cell cycle checkpoint activation and DNA repair (50). Importantly, unlike the *Btg2* reporter, *Bscl2*-GFP expression is not regulated by the p53 tumor suppressor (39). Thus, this reporter provides information on the genotoxic potential of the tested article and is not activated by other types of cellular stress.

ATR is a major signaling protein that, along with ATM, is known to be involved in the DNA damage response. ATR is activated by the stalling of DNA replication; it is recruited to stalled replication forks, and activation of ATR kinase results in the activation of *Chk1* (17). ATM is involved in repair of DNA double strand breaks; its activation phosphorylates *Chk2*. Both *Chk1* and *Chk2* are inhibitors of CDC25 phosphatase and the p53 tumor suppressor. Inhibition of these genes blocks the activation of the cyclin-dependent kinases and inhibits cell cycle progression, thus inducing apoptosis (51). The positive control for this reporter is also cisplatin.

Ddit3

The *Ddit3*-GFP reporter is activated by the protein unfolding response (41). The process is activated upon cellular stress, whereby changes in endoplasmic reticulum homeostasis are detected; the signal is transduced to the cytoplasm and nucleus, causing a compensating response that promotes apoptosis (52). The compensatory response increases expression of the DNA damage inducible transcript-3, (i.e., *Ddit3*), which is also implicated in adipogenesis and erythropoiesis (53). *Ddit3* encodes a leucine zipper transcription factor (53), which is a member of the CCAAT/enhancer-binding protein (C/EPB) family that is known to control responses to cellular stress. The DDIT3 protein forms heterodimers with other members of the C/EPB family, thereby preventing their DNA binding activity (54). The positive control for this reporter is

tunicamycin, a known endoplasmic reticulum stress inducer that causes activation of the unfolded protein response (55).

Btg2

The *Btg2*-GFP reporter is activated through a p53-dependant response to genotoxic agents (41). The p53 gene codes for a tumor suppressor that plays an important role in transcriptional regulation of proximate genes involved in the cellular response to DNA damage (56). It has been suggested that the *Btg2* gene (i.e., B-cell translocation gene 2) is stimulated by p53 functions, thereby playing a role in a variety of cellular processes. These include responses to DNA damage, with concomitant activation of apoptosis and DNA repair. It is likely that p53 mediates these responses via G1 growth arrest (56). Upon detection of DNA damage, eukaryotic cells prevent replication of damaged templates and segregation of damaged chromosomes by delaying cell cycle progression at both G1-to-S and G2-to-M transitions (56). The positive control for this reporter is cisplatin. Diethyl maleate and aflatoxin B1 can also activate the *Btg2* reporter.

1.4.3 The ToxTracker® assay procedure

Six mES (mouse embryonic stem cells lines), each containing one of the aforementioned GFP reporters, are used to quantitatively examine responses induced by *in vitro* exposure to the test substance. Briefly, cells are first seeded on to a gelatin-coated 96-wells microplate, and treated with various concentrations of the substances of interest. Following a 24-hour incubation, the induction of GFP is measured by flow cytometry (39). The reporter activity is determined as the mean fluorescence intensity of 5,000 intact cells. The activation of a reporter is considered to be a valid positive when the mean GFP level reaches >2-fold above the concurrent solvent control, and a valid negative when the magnitude of the response is <1.5-fold above the control. A chemical yielding a mean GFP level between 1.5 and 2-fold requires expert scrutiny to determine if the

response can be termed positive or negative (41). These thresholds represent a level that is at least 5 times higher than the standard deviation (SD) of background fluorescence in DMSO-exposed cells (39). ToxTracker® documentation notes that application of a 1.5-fold threshold cut-off permits identification of positive responses with a confidence level greater than 99.9% (39).

1.4.4 ToxTracker® assay validation

To evaluate the performance of the ToxTracker® assay, the sensitivity and specificity of the reporter cell lines (i.e., the ability to correctly identify carcinogens and non-carcinogens) was investigated. Each of the ToxTracker® mES cell lines were exposed to multiple concentrations of numerous genotoxic and non-genotoxic compounds, and GFP induction was measured by flow cytometry 24 hours after treatment (39). The compounds were selected from the list of chemicals recommended by the European Center for Validation of Alternative Methods (EURL-ECVAM) for validation of *in vitro* genotoxicity assays (see Appendix I) (38); more specifically, the assay's ability to correctly identify carcinogens and non-carcinogens. The assay validation compounds examined can be classified into three groups. ECVAM Class 1 compounds are *in vivo* genotoxins, most of them are mutagenic carcinogens that are expected to elicit a positive response in an *in vitro* genotoxicity assay. ECVAM Class 2 compounds consist of compounds that are non-carcinogenic and non-genotoxic. They should elicit a negative response in an *in vitro* genotoxicity assay. Finally, ECVAM Class 3 compounds are non-carcinogens and assumed to be non-mutagenic. They usually elicit negative responses in *in vivo* genotoxicity assays, but some of them have been reported to elicit positive responses in *in vitro* assays (i.e., *in vitro* false positives).

The tested concentrations of the ECVAM validation compounds were chosen according to the substances' cytotoxicity; the highest tested concentration reduced cellular survival by a maximum of 75%. A maximum concentration of 10 mM was used for compounds that did not affect cell

viability (39). The number of carcinogens and non-carcinogens that were correctly identified in the assay were used to determine the sensitivity and specificity of the assay, respectively, i.e., by dividing the amount of correctly identified compounds by the number of expected number of positives or negative.

Cross-assay comparative studies based on substances recommended by the EURL-ECVAM (38) allowed researchers (41) to show that responses elicited by ToxTracker® reporters for genotoxicity (i.e., *Rtkn* and *Bscl2*) correspond to responses elicited by traditional *in vitro* and *in vivo* regulatory assays for genotoxicity, e.g., those listed in Table 1.6 (41). More specifically, when comparing traditional assay responses with the response of the *Bscl2* reporter, which is associated with the induction of mutagenic DNA lesions, Hendriks *et al.* found that 93% of compounds that elicited a positive response in the Salmonella reverse mutation assay also elicited positive responses in *Bscl2*-GFP. Similarly, 95% of the compounds that failed to elicit a significant *Bscl2* reporter response also failed to elicit significant responses in the traditional regulatory assays (Figure 1.2). Thus, only 7% of known genotoxins failed to elicit a positive response in *Bscl2*-GFP, and only 5% of non-genotoxins elicited a positive response in the reporter. Those are representative of the false negatives and false positives, respectively (41).

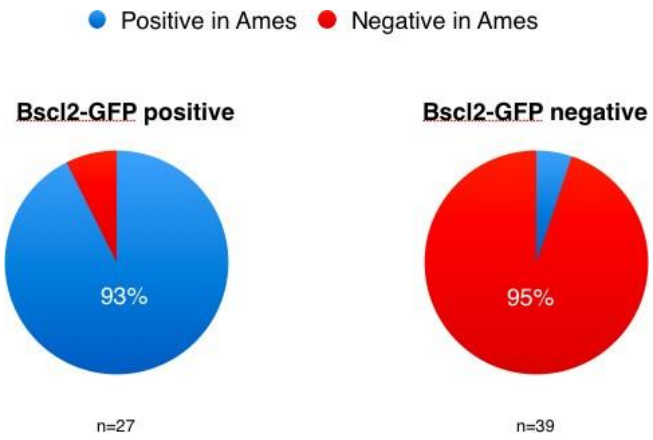


Figure 1.2 Correspondence between the ToxTracker® *Bscl2* reporter and the Salmonella reverse mutation assay (i.e., Ames assay). Percentage values indicate the proportion of compounds that elicited the same result for both the ToxTracker® and Ames assays. Figure taken from Toxys, 2018 (41). Used with permission.

As for the *Rtkn* reporter, which is activated by DNA double strand breaks, Hendriks *et al.* showed that 100% of the tested compounds that yielded a positive reporter response in the *in vitro* micronucleus or chromosomal aberration assays, i.e., traditional assays used to assess ability to induce chromosomal abnormalities, also elicited a positive response in *Rtkn*-GFP. However, 62% of the compounds that yielded a negative response on the traditional *in vitro* chromosomal damage assays also elicited a negative response in the *Rtkn* reporter. Thus, 38% of non-genotoxins elicited a positive response in the reporter, corresponding to the false positive rate. In addition, 92% of the compounds that elicited a positive response in the *in vivo* micronucleus and chromosomal aberrations assays also elicited a positive response in the *Rtkn* reporter. When testing compounds that elicited a negative response in the traditional *in vivo* micronucleus or chromosomal aberration assays, 93% of those compounds also elicited a negative response in *Rtkn*-GFP. Thus, the false positive and false negative rates are 8% and 7%, respectively (Figure 1.3) (41).

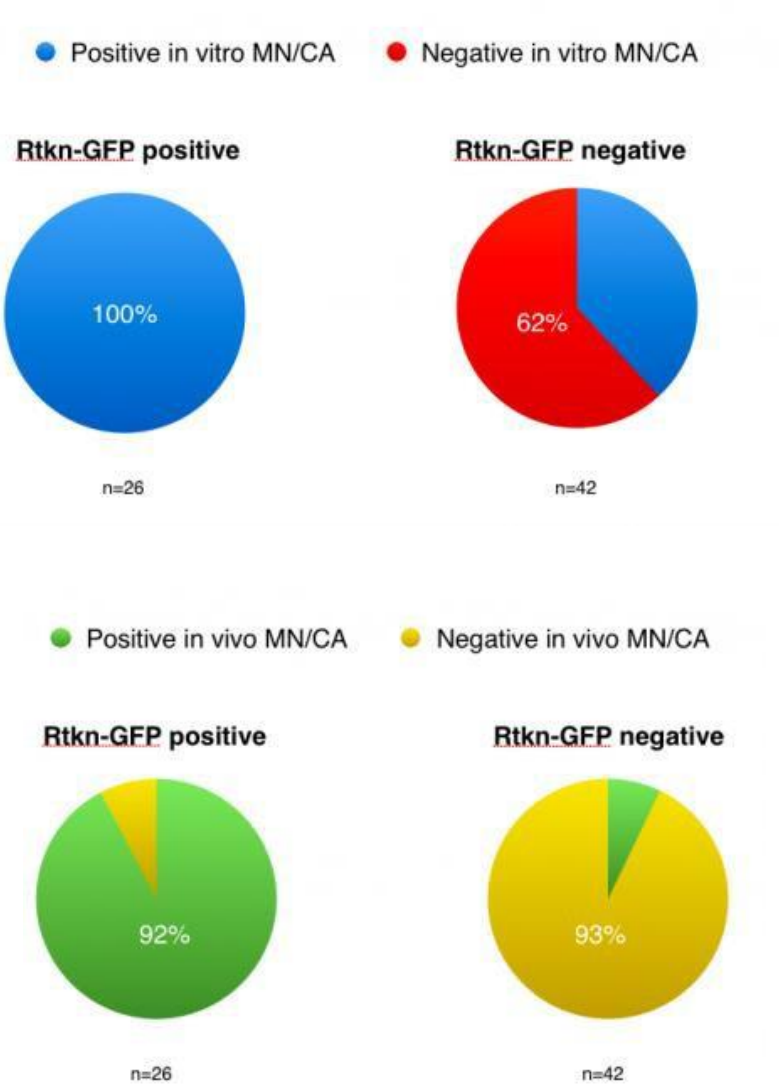


Figure 1.3 Correspondence between the ToxTracker® *Rtkn* reporter and the *in vitro* (top) and *in vivo* (bottom) micronucleus (MN) and chromosomal aberration (CA) assays. Percentage values indicate the proportion of compounds that elicited the same result for both the ToxTracker® and regulatory assays. Figure taken from Toxys, 2018 (41). Used with permission.

In contrast, the GreenScreen HC™ reporter assay has a sensitivity and specificity of 89% and 77%, respectively, when compared with the Ames assay. When compared with the *in vitro* and *in vivo* micronucleus and chromosomal aberration assay, the sensitivity corresponds to 71% and 81%,

respectively. Additionally, the specificity values are of 100% and 95% for the *in vitro* and *in vivo* assays, respectively (57).

1.5 Determination of a threshold for identification of a significant positive response

Qualitative interpretation of (geno)toxicity assay results requires the determination of a threshold beyond which a response can be deemed to be significantly different from background. In the case of the ToxTracker® assay, the variance of mean fluorescence values (i.e., GFP mean for 5,000 cells) for the solvent control can be used to determine a value that is significantly-elevated for a given level of statistical significance. This value can then be expressed as a fold-change increase over control. However, since any fold-change value is a ratio metric, determination of a statistically-robust fold-change threshold is unfortunately complex (58). There are several approaches that can be employed to effectively determine the variability of a ratio metric; one approach employs a technique commonly referred to as bootstrapping. More specifically, random sampling of numerator and denominator values and iterative ratio calculation. A large number of iterations (e.g., >1000) provides a ratio distribution; the 95^{th} percentile can be used to delineate a threshold for designation of a statistically significant fold-change.

1.6 Quantitative dose-response modelling and the BMD (Benchmark Dose) approach

Statistical methods are frequently used to determine if the magnitude of a bioassay response to a prioritized substance is significantly different from the solvent control response, i.e., determination if the observed response is positive or negative. However, it is frequently desirable to go beyond qualitative data evaluation (i.e., positive or negative), and quantitatively examine the dose-response relationship. This permits determination of a substance's toxicological potency, and metrics that denote potency can be used for comparative potency ranking and/or human health risk assessment. For example, comparative evaluation of the response at each dose (i.e., response at a given dose versus background response for the solvent control) can be used to determine a NOAEL

(No-observed Adverse Effect Level), the highest dose that fails to yield a response that is not statistically different from the solvent control. If all of the tested doses elicit response significantly different from the solvent control, then the dose-response data can be used to determine a LOAEL (Lowest Observed Adverse Effect Level). These metrics can subsequently be used to denote potency, and comparative potency analysis can be used to rank the toxicological hazards of tested substances. Moreover, via data extrapolation, NOAEL or LOAEL values from *in vivo* animal studies can be used to calculate an MOE (Margin of Exposure) or human exposure limit value. The former is simply the ratio of the NOAEL to the estimated or predicted human exposure whereby high values indicate low risk of human health effect, and conversely, low values indicate high risk of human health effects. The latter involves dividing NOAEL values by the product of a series of uncertainty factors to determine a human exposure limit value (e.g., Tolerable Daily Intake or TDI, Permitted Daily Exposure or PDE), collectively referred to as HBGVs (Health-based Guidance Values) (59). Uncertainty factors employed to calculate HBGVs account for uncertainties are related to, for example, animal-to-human data extrapolation and inter-individual human variability. A detailed discussion on the use of *in vivo* potency metrics for human health risk assessment is beyond the scope of this thesis, and the reader is referred to works such as White *et al*., 2020 (60) for a complete overview and discussion of the principles and methods. Dose-response data generated by *in vitro* assays, such as the GFP mean fluorescence for different chemical concentrations in the ToxTracker® assay, can readily be used for comparative potency analysis.

As an alternative to the NOAEL or LOAEL, quantitative dose-response modeling can be used to determine a robust potency metric such as the BMD (Benchmark Dose), i.e., the interpolated *in vitro* concentration or *in vivo* dose that yields a small, pre-defined increase in response over the

negative control (e.g., a 10% increase over background). The BMD is alternatively referred to as the critical effect dose (CED), and the pre-defined increase over background is referred to as the BMR (Benchmark Response) or CES (Critical Effect Size). More specifically, determination of BMD values analysis involves using freely-available software such as BMDS (https://www.epa.gov/bmds) or PROAST (https://www.rivm.nl/en/proast) to fit one of a series of mathematical functions to experimental dose/concentration-response data, and subsequent interpolation of the BMD/CED corresponding to a set BMR/CES[1] (61). In contrast to the quantal family of functions, where datasets analyzed have a quantal response metric that does not exceed 1.0 (i.e., 100% of the population infected), the exponential family of function has been deemed to be appropriate when analyzing data with a continuous response metric (i.e., values ranging from 0 to infinite) (61). In the case of ToxTracker® data, the response metric is the mean GFP fluorescence, which is considered continuous (39). The BMR is generally a percentage increase over background that represents a small, measurable, and toxicologically-relevant increase over control. Importantly, the BMD approach permits determination of lower and upper confidence limits, referred to as the BMDL and BMDU, respectively. The confidence interval provides information on the precision of the BMD value, and the regulatory utility of the underlying concentration-response data. More specifically, a wider confidence interval, as indicated by a large ratio of BMDL to BMDU, is indicative of low of BMD precision. Low BMD precision limits the ability to detect potency differences. A conceptually overview of BMD approach is illustrated in Figure 1.4 (62).

[1] For convenience and consistency, the terms BMD and BMR will be used hereafter. Moreover, since the thesis is dealing with *in vitro* bioassay data, the term concentration will be used instead of dose, a term for *in vivo* data.

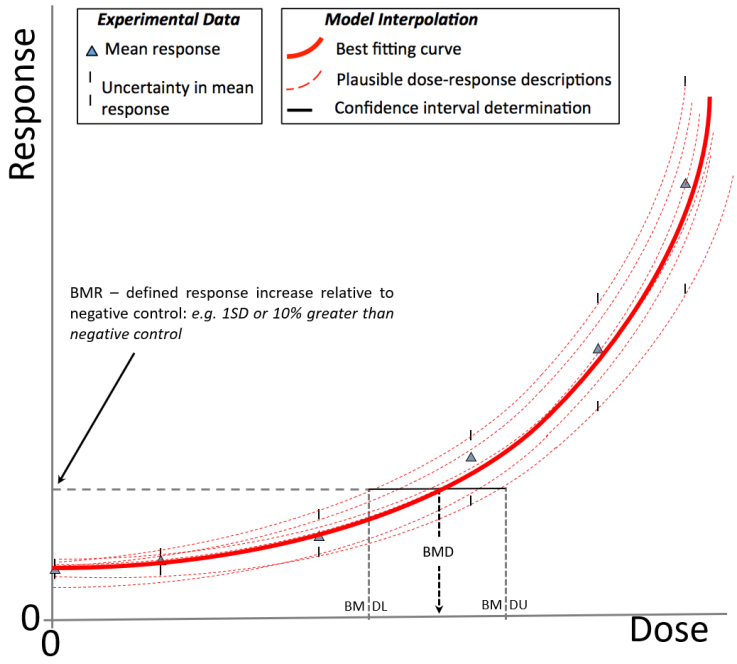


Figure 1.4 Schematic representation of the BMD approach for analyzing dose/concentration–response data. The solid red line indicates the best fitting curve; the dotted lines illustrate other plausible dose-response functions. Extrapolation is used to define the BMD that corresponds to a given BMR, and its lower and upper confidence limits, i.e., the BMDL and BMDU. The BMR, BMD, BMDL and BMDU are indicated. Taken from Wills *et al*., 2016 (62). Used with permission.

Using a method referred to as the BMD-combined covariate approach, BMD values can be simultaneously estimated for numerous dose-response datasets, and subsequently compared across a covariate of interest (e.g., compound). When compound is the specified covariate, the results can be used for compound potency ranking (Figure 1.5) (63). Overlapping BMD confidence intervals reflect compounds that are equipotent, and the results obtained often permit compound potency groupings. For example, Figure 1.5 illustrates the relatively potency of a series of aneugens

assessed using the *in vitro* micronucleus assay. The compounds were ranked by potency using the BMD covariate approach with a BMR of 10%.

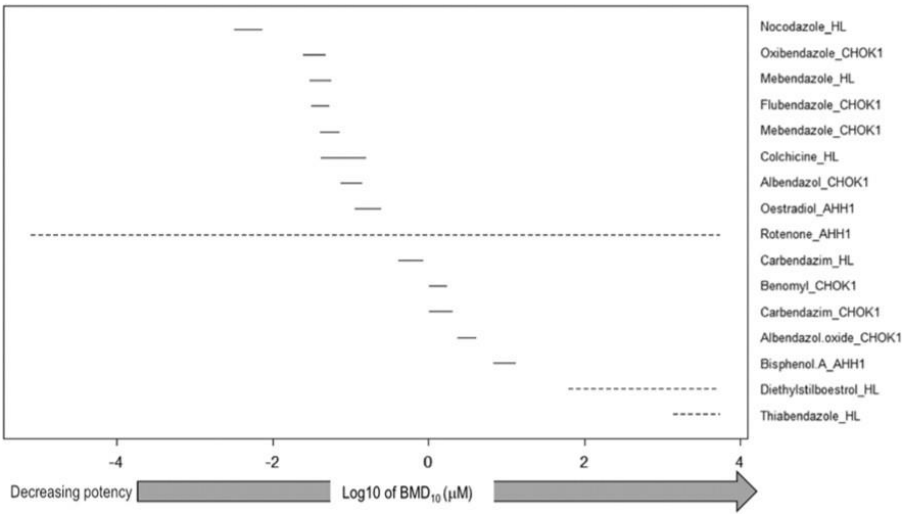


Figure 1.5 Illustration of BMD combined covariate results to rank the potency of selected aneugens. The panel shows the 90% confidence intervals for the BMD_{10} values associated with each substance, i.e., the concentration that yields a 10% increase over control. *In vitro* micronucleus frequency dose-response data collected from the literature was analyzed using an exponential model with BMD covariate analyses. Compounds were tested in human lymphocytes (HL) (64-66), Chinese hamster ovary (CHOK1) cells (67), or human lymphoblastoid (AHH-1) cells (68, 69). Lines represent the lower (left end of bar) and upper (right end of bar) confidence limits of each compound-specific BMD. Overlapping bars denote equipotent chemicals, and dotted lines represent compounds for which a finite interval could not be determined. An infinite upper bound indicate that there might not be any positive response for these chemicals. Taken from Wills *et al*., 2016 (62). Used with permission.

As previously mentioned, the BMR is the pre-defined increase in response over background. When carrying out BMD analyses on large datasets that include distinctive covariates (e.g., compound, sex, cell type, tissue, etc.), the choice of a BMR expressed as percentage increase above control has been shown to be fairly unimportant (i.e., for objectively comparing compound potencies) (70). However, when the purpose of the analysis is to compare potency values across distinct endpoints, or derive BMD values for risk assessment purposes, it becomes necessary to

define meaningful, endpoint-specific BMRs. These values can be used as the basis for robust cross-endpoint comparisons. A fair amount of literature (71, 72) has been devoted to determination of BMR values that can effectively be employed to generate BMD values that can be used for regulatory decision-making. For example, the EFSA recommends a BMR of 5% for determination of BMD values for continuous endpoints (71). In contrast, recommendations by the US Environmental Protection Agency (EPA) stipulate that "for continuous data, the preferred approach is to define a BMR based on the level of change in the endpoint at which the effect is considered to become biologically significant, as determined by expert judgment or relevant guidance documents" (72). Although multiple recommendations for setting the BMR have been published, no general consensus has been reached.

It is becoming increasingly clear that a single value of BMR (e.g. 10%) cannot be used to denote a uniformly meaningful effect size for all toxicological endpoints. For example, a 10% change in mutant frequency cannot be considered as toxicologically equivalent to a 10% change in red blood cell count (73). Alternatively, in the absence of any evidence for selecting a BMR representative of a biologically significant increase over background, a BMR of one control standard deviation change from the control mean (BMD_{1SD}) can be used. However, this approach has been criticized, notably because in the case of low variability of control responses, it is unlikely that a one standard deviation shift could be deemed adverse. In contrast, responses with high control variability will yield higher BMD values compared to the BMD_{10} approach. Table 1.6 summarizes the advantages and limitations associated with the BMD_{10} and BMD_{1SD} approaches, they are compared and contrasted with the NOAEL and LOAEL approaches

Table 1.6 Summary of advantages and disadvantages of different quantitative dose-response analysis methods. Based on information from Johnson *et al*., 2014 (74)

	Full name	Definition	Potential limitations	Advantages
NOAEL	No Observed Adverse Effect Level	The highest tested dose for which there is no statistically significant increase in adverse effect compared to the control	Statistical assumptions must be met, value dependent on study design	Easy to apply, doesn't require dose-response modelling, commonly used
LOAEL	Lowest Observed Adverse Effect Level	The lowest tested dose for which there is a statistically significant increase in adverse effect compared to the control	Statistical assumptions must be met, value dependent on study design	Easy to apply, doesn't require dose-response modelling, commonly used
BMD10	Benchmark Dose 10	A dose or concentration that elicits a 10% increase over background	MD approach requires consensus on biologically appropriate BMR, continuous and quantal data are modelled differently, often produces very low BMD metrics	Fits function to entire dose-response, currently used by many regulatory agencies
BMD1SD	Benchmark Dose 1 standard deviation	~ 10% excess risk for individuals below and above the 2nd and 98th percentiles, respectively	Continuous and quantal data are modelled differently, comparisons between endpoints and historical datasets influenced by variance of background response	Fits function to entire dose-response, currently used by many regulatory agencies

1.7 Determination of an effective, realistic BMR

As previously mentioned, the BMD combined covariate approach can be used to rank the potency of chemicals. For this purpose, the BMR employed does not have an effect on the relative ranking of compounds. That being said, the precision of the BMD values for comparative potency analysis will increase with an increase with a higher BMR. Although precise BMR values are not required for comparative potency analysis, comparison of BMD values across endpoints requires determination of endpoint-specific BMR values, e.g., BMR values for each of the ToxTracker® reporters.

As noted earlier, use of BMD values for risk assessment and regulatory decision-making (e.g., determination of HBGV values) requires specification of a toxicologically meaningful BMR. Unfortunately, there is no broad consensus regarding appropriate BMR values for various toxicological endpoints used for chemical safety assessments. As noted, common recommendations are 5% increase over background (71), and one standard deviation over background (72). Although these values are frequently employed, some authors and/or regulatory authorities indicate that BMR values can also be set using "expert judgement" (59); moreover, that a set value of 5% may be too low to reflect a toxicologically-meaningful response increase (72).

In addition to the lack of consensus for BMR values for toxicological endpoints in general, there is no consensus for BMR values used for analyses of genotoxicity dose-response data. In part this is due to the tradition to use qualitative data interpretation for simple of dichotomous compound classification (i.e., positive versus negative) (75, 60). However, a more recent shift from qualitative to quantitative genotoxicity data interpretation has led to discussion regarding the most appropriate BMR value(s) for genotoxicity endpoints (60). For example, although Johnson *et al.*, (2014) used 10% BMR to interpret 1-ethyl-1-nitrosourea (ENU) and 1-methyl-1-nitrosourea (MNU) dose–response data, Wills *et al.*, 2017, in their analysis of TGR (Transgenic Rodent) assay dose-response data, noted that BMR values should be in the range 18 to 66%. Table 1.8 provides a summary of BMR values recommended for regulatory interpretation of genotoxicity dose/concentration-response data.

With respect to the quantitative interpretation of ToxTracker® concentration-response data, there is a lack of overall consensus; thus, a noteworthy need to empirically determine a toxicologically meaningful BMRs for the ToxTracker® endpoints. The recent literature describes two approaches that can be employed to determine toxicologically-meaningful BMR values for

(geno)toxicity endpoints: One by Slob (76), and the other by Zeller *et al.* (73). The approach described by Slob, 2016, which is based on "effect size theory", scales BMR values to the dynamic range of each endpoint, i.e., the maximum different between background and maximum. The approach described by Zeller *et al*. (2017) determines meaningful BMR values by scrutinizing historical background values for each endpoint. Each of these approaches, and its application for interpretation of ToxTracker® dose-response data, are outlined in more detail below.

Although the Slob effect size theory scales BMR to maximum response (*M*), the *M* values can be very difficult to estimate. Consequently, Slob, 2016, established an approach based on within-group variance (i.e., average variability in dose-specific responses), which was shown to be empirically related to *M* (76). The Slob effect size theory predicts a general relationship between the maximum response (i.e., maximum fold change in response relative to control) and within-group variation (i.e., variability in dose-specific responses) for any toxicological endpoint. Slob demonstrates this relationship using dose-response data for over 25 different endpoints, the work thus provides a basis for setting BMRs appropriately in context of each endpoint's response maximum. One-eighth of log *M* is suggested as a meaningfully small effect size with the BMR being estimated as the eighth-root of *M*. Since, as noted by Slob, *M* can be difficult to estimate, an alternative approach can employ *s*, the average response variance across all doses. More generally, although the theory supports the BMD_{1SD} approach, the approach employs the geometric mean variance across all doses to establish a BMR, i.e., rather than the variance associated with the control group mean (76). This is rationalized by considerations of errors present in experimental measurements that are generally largest for the lowest concentrations (i.e., responses at very low doses cannot be precisely measured) (76). With respect to genotoxicity BMR values derived using the *s* values summarized by Slob, values range from 71-79% for the *in vivo* micronucleus assay,

58-70% for the *in vivo* peripheral blood *Pig-a* mutation frequency assay, and 46-74% for the transgene mutation frequency assay (i.e., mutation frequency in BigBlue® rat liver) (Table 1.7).

Table 1.7 Estimated BMR value ranges for a series of *in vivo* genotoxicity endpoints. The values were calculated using Slob ES theory (76). Data taken from White *et al*., 2020 (60).

Assay	BMR values (%)
In vivo peripheral blood micronucleus	71-79
In vivo peripheral blood Pig-a mutant frequency	58-70
In vivo transgenic rodent somatic and germ cell mutant frequency	46-74

An alternative approach is outlined in Zeller *et al*. (73); the authors recommend use of variability in the log-normal distributions of vehicle control values to define a small effect size that can be used to define minimal BMR values. More specifically, Zeller *et al*. (73) used a number of different metrics to scrutinize variability in study-specific or historical vehicle control responses, and employed the 95th percentile of the log-normal distribution in endpoint-specific responses to define endpoint-specific BMR values. The approach, which is conceptually similar to the aforementioned Slob (76) approach, focusses on variability of vehicle control to define meaningful, endpoint specific BMR values. Zeller *et al*. (73) noted that the most promising approach involves the use of "trimmed" historical controls, whereby highly deviant vehicle control responses, which are not representative of the true distribution of historical controls, are removed prior to the analysis. The final analysis uses the ratio of standard deviation over the mean of the trimmed historical control to determine endpoint-specific BMR values expressed as percentage increase about the mean.

1.8 Statement of purpose, thesis objectives and thesis hypotheses

Current regulatory strategies for the assessment of genotoxicity rely on the use of OECD-approved *in vitro* assays, with *in vivo* follow-up employed where necessary. These conventional *in vitro* assays are limited in the sense that they are costly and time consuming compared to novel DNA damage reporter assays (Table 1.4). The ToxTracker® reporter assay, which can simultaneously assess several cellular stress responses in mES cells, is well-suited for efficient, effective and affordable assessment of chemical hazard. High-throughput (geno)toxicity assays such as ToxTracker® generate vast amounts of complex, multivariate data; there is a concomitant need to develop and employ quantitative methods that improve and streamline interpretation of test results. Lastly, as technologies advance, it is necessary to periodically evaluate and modernise the protocols employed to effectively execute (geno)toxicity assays such as ToxTracker®. Hence, the objectives of this study are:

(1) examine background response levels for each ToxTracker® reporter, and define a fold-change threshold for identification of a significant positive response;

(2) employ the BMD combined-covariate approach to rank the potency of assay-validation substances examined to date;

(3) using the Zeller *et al.* (2017) and Slob (2016) approaches, determine reporter-specific BMRs for routine quantitative interpretation of dose-response data across ToxTracker® reporters;

(4) using PCA (principal component analysis), scrutinize the functional and statistical relationships between the multiplexed ToxTracker® endpoints;

(5) by comparing ToxTracker® responses elicited by reference genotoxicants, evaluate the suitability of PB/BNF-induced S9 as a substitute for Aroclor 1254-induced S9.

Regarding these aforementioned objectives, the following hypotheses will be tested:

(1) empirically-determined cut-off values for identification of a positive response will be consistent with the values currently used by Toxys;

(2) the use of the BMD combined-covariate approach will reveal that substances used as ToxTracker® positive controls are amongst the most potent substances tested to date. These will be followed by hitherto-recognized potent mutagens and oxidative stress-inducing substances;

(3) the Zeller *et al*. (2017) and Slob (2016) approaches will yield similar endpoint-specific BMR values;

(4) multivariate analyses of ToxTracker® responses to validation compounds will reveal functional redundancies in reporter-specific responses;

(5) a 24-hour continuous exposure protocol with 1% v/v PB/BNF S9 will yield results that are comparable to those generated using the current Aroclor S9-based assay protocol.

1.9 References

1. Government of Canada. Canadian Environmental Protection Act, 1999. Acts and Regulations. Ottawa, Canada, 2019. https://laws-lois.justice.gc.ca/eng/acts/c-15.31/page-1.html

2. Government of Canada. Pest Control Products Act. Acts and Regulations, 2002. Ottawa, Canada, 2020. https://laws-lois.justice.gc.ca/eng/acts/p-9.01/

3. Government of Canada. Canada Consumer Product Safety Act, 2010. Acts and Regulations. Ottawa, Canada, 2020. https://laws-lois.justice.gc.ca/eng/acts/c-1.68/

4. Government of Canada. Food and Drugs Act, 1985. Acts and Regulations. Ottawa, Canada, 2020. https://laws-lois.justice.gc.ca/eng/acts/f-27/

5. USEPA (United States Environmental Protection Agency). Summary of the Toxic Substances Control Act, 1976. Laws and Regulations. Washington, DC: USEPA. 2019. https://www.epa.gov/laws-regulations/summary-toxic-substances-control-act#:~:text=The%20Toxic%20Substances%20Control%20Act%20of%201976%20provides%20EPA%20with,%2C%20drugs%2C%20cosmetics%20and%20pesticides.

6. USEPA (United States Environmental Protection Agency). Summary of the Federal Insecticide, Fungicide, and Rodenticide Act, 1996. Laws and Regulations. Washington, DC: USEPA. 2020. https://www.epa.gov/laws-regulations/summary-federal-insecticide-fungicide-and-rodenticide-act

7. European Commission. Chemicals. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Brussels, Belgium, 2019. https://ec.europa.eu/environment/chemicals/reach/reach_en.htm

8. ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use). Safety Guidelines. Geneva, Switzerland: ICH. 1995.

9. Government of Canada. New Substances Notification Regulations (Chemicals and Polymers), 2005. Acts and Regulations. Ottawa, Canada, 2018. https://laws-lois.justice.gc.ca/eng/Regulations/SOR-2005-247/index.html

10. OECD (Organisation for Economic Co-operation and Development). Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Organisation for Economic Co-Operation and Development Publishing. Paris, 2005;14(34):96.

11. OECD (Organisation for Economic Co-operation and Development). Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines. Organisation for Economic Co-operation and Development. Paris, 2015:1-64.

12. MacArthur CJ. The 3Rs in research: A contemporary approach to replacement, reduction and refinement. The British Journal of Nutrition. 2018;120(S1):S1-7.

13. Li AP, Loretz LJ. Assays for Genotoxicity. In: Li AP, Heflich RH, editors. Genetic Toxicology. 1st ed. Boca Raton, Florida: CRC Press; 1946. p. 119-42.

14. MacGregor JT, Frötschlb R, White PA, Crump KS, Eastmonde DA, Fukushimaf S, Guérardg M, Hayashih M, Soeteman-Hernándezi LG, Kasamatsuj T, Levyk, DD, Morital T, Müllerm L, Schoenyn R, Schulero MJ, Thybaud V, Johnson GE. IWGT report on quantitative approaches to genotoxicity risk assessment I. Methods and metrics for defining exposure–response relationships and points of departure (PoDs). Mutation Research. 2015;783:55-65.

15. Hendriks G, Derr RS, Misovic B, Morolli B, Calleja, Fabienne M. G. R., Vrieling H. The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. Toxicological Sciences. 2016;150(1):190-203.

16. Glatt H. An overview of bioactivation of chemical carcinogens. Biochemical Society Transactions. 2000;28(2):1-6.

17. Jeffrey AM. DNA modification by chemical carcinogens. Pharmacology & Therapeutics. 1985;28:237-72.

18. Bauer E, Guo Z, Ueng Y, Bell LC, Zeldin D, Guengerich FP. Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. Chemical Research in Toxicology. 1995;8:136-42.

19. Kim J, Stansbury K, Walker N, Trush M, Strickland P, Sutter T. Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. Carcinogenesis. 1998;19:1847-53.

20. Ku WW, Bigger A, Brambilla G, Glatt H, Gocke E, Guzzie PJ, Hakura A, Honma M, Martus H, Obach RS, Roberts S. Strategy for genotoxicity testing-metabolic considerations. Mutation Research. 2007;627(1):59-77.

21. Organisation for Economic Co-operation and Development (OECD). Guidelines for Testing of Chemicals, Section 4, Test Guideline 471: Bacterial Reverse Mutation Test. Organisation for Economic Cooperation and Development. Paris, 1997. https://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en.

22. Waxman DJ. P450 Gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. Archives of Biochemistry and Biophysics. 1999;369(1):11-23.

23. Callander RD, Mackay JM, Clay P, Elcombe CR, Elliott BM. Evaluation of phenobarbital/beta-naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in in vitro genotoxicity assays. Mutagenesis. 1995;10(6):517-22.

24. Elliott BM, Combes RD, Elcombe CR, Gatehouse DG, Gibson GG, Mackay JM, Wolf RC. Alternatives to Aroclor 1254-induced S9 in in vitro genotoxicity assays. Mutagenesis. 1992;7(3):175-7.

25. Clare C. The state of play for PCBs. British Industrial Biological Research Association Bulletin.

1989;28:111-4.

26. Heflich R, Neft R. Genetic toxicity of 2-acetylaminofluorene, 2-aminofluorene and some of their metabolites and model metabolites. Mutation Research. 1994;318:73-174.

27. Cai T, Yao L, Turesky R. Bioactivation of heterocyclic aromatic amines by UDP glucuronosyltransferases. Chemical Research in Toxicology. 2016;29:879-91.

28. Schut H, Snyderwine E. DNA adducts of heterocyclic amine food mutagens: Implications for mutagenesis and carcinogenesis. Carcinogenesis. 1999;20:353-68.

29. Gallagher E, Wienkers L, Stapleton P, Kunze K, Eaton D. Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. Cancer Research. 1994;54:101-8.

30. Cox JA, Fellows MD, Hashizume T, White PA. The utility of metabolic activation mixtures containing human hepatic post-mitochondrial supernatant (S9) for *in vitro* genetic toxicity assessment. Mutagenesis. 2016;31(2):117-30.

31. Knight AW, Little S, Houck K, Dix D, Judson R, Richard A, McCarroll N, Akerman G, Yang C, Birrella L, Walmsley RM. Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast™ chemicals. Regulatory Toxicology and Pharmacology. 2009;55(2):188-99.

32. Curtis CD, Thorngren DL, Nardulli AM. Immunohistochemical analysis of oxidative stress and DNA repair proteins in normal mammary and breast cancer tissues. BMC Cancer. 2010;10(9).

33. Cahill P, Knight A, Billinton N, Barker M, Walsh L, Keenan P, Williams CV, Tweats DJ, Walmsley RM. The GreenScreen® genotoxicity assay: a screening validation programme. Mutagenesis. 2004;19(2):105-19.

34. Simpson K, Bevan N, Hastwell P, Eidam P, Shah P, Gogo E, Rees S, Brown A. The BlueScreen-384 assay as an indicator of genotoxic hazard potential in early-stage drug discovery. Journal of Biomolecular Screening. 2013;18(4):441-52.

35. Bryce S, Bernacki D, Bemis J, Dertinger S. Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach. Environmental and Molecular Mutagenesis. 2016;57:171-87.

36. Fox JT, Sakamuru S, Huang R, Teneva N, Simmons SO, Xia M, Tice RR, Austin CP, Myunga K. High-throughput genotoxicity assay identifies antioxidants as inducers of DNA damage response and cell death. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(14):5423-8.

37. Bryce S, Bernacki D, Bemis J, Spellman R, Engel M, Schuler M, Lorge E, Heikkinen PT, Hemmann U, Thybaud V, Wilde S, Queisser N, Sutter A, Zeller A, Guerard M, Kirkland D,

Dertinger SD. Interlaboratory evaluation of a multiplexed high information content in vitro genotoxicity assay. Environmental and Molecular Mutagenesis. 2017;58:146-61.

38. Kirkland D, Kasper P, Muller L, Corvi R, Speit G. Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: A follow-up to an ECVAM workshop. Mutation Research. 2008;1-2:99-108.

39. Hendriks G, Atallah M, Morolli B, Calleja, Fabienne M. G. R., Ras-Verloop N, Huijskens I, Raamsman M, van de Water B, Vrieling H. The ToxTracker Assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences. 2012;125(1):285-98.

40. Hendriks G, Attalah M, Raamsman M, Morolli B, Van der Putten H, Jaadar H, Tijdens I, Esveldt-van Lange R, Mullenders L, Van de Water B, Vrieling H. Sensitive DsRed fluorescence based reporter cell systems for genotoxicity and oxidative stress assessment. Mutation Research. 2011;709:49-59.

41. Toxys. ToxTracker. toxys.com. 2018.

42. Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radical Biology & Medicine. 2009;9:1304-9.

43. Niture SK, Kaspar JW, Shen J, Jaiswal AK. Nrf2 signaling and cell survival. Toxicology and Applied Pharmacology. 2010;1:37-42.

44. Boyland E, Chasseaud LF. Enzyme-catalysed conjugations of glutathione with unsaturated compounds. Biochemical Journal. 1967;104:95-102.

45. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism . Cellular Molecular Life Science. 2016;73:3221-47.

46. Reid T, Furuyashiki T, Ishizaki T, Watanabe G, Watanabe N, Fujisawa K, Morii N, Madaule P, Narumiya S. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rhobinding domain. Journal of Biological Chemistry. 1996;271:13556-60.

47. Collier FM, Loving A, Baker AJ, McLeod J, Walder K, Kirkland MA. RTKN2 induces NF-KappaB dependent resistance to intrinsic apoptosis in HEK cells and regulates BCL-2 genes in human CD4(þ) lymphocytes. Journal of Cell Death. 2009;2:9-23.

48. Volcic M, Karl S, Baumann B, Salles D, Daniel P, Fulda S, Wiesmuller L. NF-iB regulates DNA double-strand break repair in conjunction with BRCA1–CtIP complexes. Nucleic Acids Research. 2012;40(1):181-95.

49. Rezaee M, Sanche L, Hunting DJ. Cisplatin enhances the formation of DNA single- and double-strand breaks by hydrated electrons and hydroxyl radicals. 2013. Radiation

Research;179(3):323-31.

50. Reinhardt HC, Yaffe MB. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. Current Opinion in Cell Biology. 2009;2:245-55.

51. Meek DW. Tumour suppression by p53: A role for the DNA damage response? Nature Reviews Cancer. 2009;10:714-23.

52. Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E, Quest AFG, Lavandero S. Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. International Review of Cell and Molecular Biology. 2013;301:215-90.

53. National Center for Biotechnology Information. DDIT3 DNA damage inducible transcript 3. 2018.

54. Jauhiainen A, Thomsen C, Strombom L, Grundevik P, Andersson C, Danielsson A, Andersson MK, Nerman O, Rorkvist L, Stahlberg A, Aman P. Distinct cytoplasmic and nuclear functions of the stress induced protein DDIT3/CHOP/GADD153. Plos One. 2012;7(4):e33208.

55. Wang H, Wang X, Ke Z, Comer AL, Xu M, Frank JA, Zhang Z, Shi X, Luo J. Tunicamycin-induced unfolded protein response in the developing mouse brain. Toxicology and Applied Pharmacology. 2015;283(3):157-67.

56. Rouault J, Falette N, Guéhenneux F, Guillot C, Rimokh R, Wang Q, Berthet C, Moyret-Lalle C, Savatier P, Pain B, Shaw P, Berger R, Samarut J, Magaud JP, Ozturk M, Samarut C, Puisieux A. Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nature Genetics. 1996;14(4):482-6.

57. Hastwell P, Chai L, Roberts K, Webster T, Harvey J, Rees R, Brown A. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: Validation of the GreenScreen HC *GADD45a-GFP* genotoxicity assay. Mutation Research. 2006;607(2):160-75.

58. Welsh A, Peterson A, Altmann S. The fallacy of averages. The American Naturalist. 1998;132(2):277-88.

59. Haber LT, Dourson ML, Allen BC, Hertzberg RC, Parker A, Vincent MJ, Maier A, Boobis AR. Benchmark dose (BMD) modeling: current practice, issues, and challenges. Critical Reviews in Toxicology. 2018;48(5):387-415.

60. White PA, Long AS, Johnson GE. Quantitative interpretation of genetic toxicity dose-response data for risk assessment and regulatory decision-making: current status and emerging priorities. Environmental and Molecular Mutagenesis. 2020;61(1):66-83.

61. Slob W, Setzer RW. Shape and steepness of toxicological dose–response relationships of continuous endpoints. Critical Reviews in Toxicology. 2014;44(3):270-97.

62. Wills JW, Johnson GE, Doak SH, Soeteman-Hernández LG, Slob W, White PA. Empirical analysis of BMD metrics in genetic toxicology part I: in vitro analyses to provide robust potency rankings and support MOA determinations. Mutagenesis. 2016;31(3):255-63.

63. Wills JW, Alexandra S. Long, Alexandra S., Johnson GE, Bemis JC, Dertinger SD, Slob W, White PA. Empirical analysis of BMD metrics in genetic toxicology part II: in vivo potency comparisons to promote reductions in the use of experimental animals for genetic toxicity assessment. Mutagenesis. 2016;31(3):265-75.

64. Clare MG, Lorenzon G, Akhurst LC, Marzin D, van Delft J, Montero R, Botta A, Bertens A, Cinelli S, Thybaud V, Lorge E. SFTG international collaborative study on in vitro micronucleus test: II. Using human lymphocytes. Mutation Research. 2006;607:37-60.

65. Elhajouji A, Tibaldi F, Kirsch-Volders M. Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors in vitro in human lymphocytes. Mutagenesis. 1997;12:133-40.

66. Elhajouji A, Van Hummelen P, Kirsch-Volders M. Indications for a threshold of chemically-induced aneuploidy in vitro in human lymphocytes. Environmental and Molecular Mutagenesis. 1995;26:292-304.

67. Ermler S, Scholze M, Kortenkamp A. Seven benzimidazole pesticides combined at sub-threshold levels induce micronuclei in vitro. Mutagenesis. 2013;28(417):426.

68. Hernández LG, van Benthem J, Johnson GE. A modeof-action approach for the identification of genotoxic carcinogens. Plos One. 2013;8:e64532.

69. Johnson GE, Parry EM. Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-A and rotenone. Mutation Research. 2008;651:56-63.

70. Bemis J, Wills J, Bryce S, Torous D, Dertinger S, Slob W. Comparison of in vitro and in vivo clastogenic potency based on benchmark dose analysis of flow cytometric micronucleus data. Mutagenesis. 2016;31:277-85.

71. EFSA (European Food Safety Authority). Guidance of the Scientific Committee on a request from EFSA on the use of the benchmark dose approach in risk assessment. EFSA Journal. Parma, 2009;7(6):1-72.

72. USEPA (United States Environmental Protection Agency). Benchmark dose technical guidance. Report EPA/100/R-12/001. Washington, DC: USEPA. 2012.

73. Zeller A, Duran-Pacheco G, Guérard M. An appraisal of critical effect sizes for the benchmark dose approach to assess dose–response relationships in genetic toxicology. Archives of Toxicology. 2017;91(12):3799-807.

74. Johnson GE, Soeteman-Hernandez L, Gollapudi BB, Bodger OG, Dearfield KL, Heflich RH, Hixon JG, Lovell DP, MacGregor JT, Pottenger LH, Thompson CM, Abraham L,

Thybaud V, Tanir JY, Zeiger E, van Benthem J, White PA. Derivation of point of departure (PoD) estimates in genetic toxicology studies and their potential applications in risk assessment. Environmental and Molecular Mutagenesis. 2014;55(8):609-23.

75. Johnson G., White A., Wills J., White P., Slob W., Kenny J., Harvey J. Using standard in-vivo genotoxicity studies and benchmark dose modelling to determine acceptable daily intakes for mutagenic impurities. Mutagenesis. 2016;31(6):706.

76. Slob W. A general theory of effect size, and its consequences for defining the benchmark response (BMR) for continuous endpoints. Critical Reviews in Toxicology. 2016;47(4):342-51.

Appendix I

Table A1. Comparison of the response profiles for the current, conventional *in vitro* and *in vivo* genotoxicity tests, with ToxTracker® assay responses. Taken from Hendriks *et al*., 2012 (39). Used with permission.

Chemical	**CAS number**	***In vitro* genotoxicity**				***In vivo* genotoxicity**					**ToxTracker assay**					
ECVAM class 1		Ames test	MLA	*In vitro* MN	*In vitro* CA	*In vivo* MN	*In vivo* CA	UDS	Transgenic	Comet	Bscl2-GFP	Rtkn-GFP	Btg2-GFP	Srxn1-GFP	Blvrb-GFP	Ddit3-GFP
1. Ames-positive in vivo genotoxins.																
Cyclophosphamide	50-18-0	+	+	+	+	+	+	+	+	+	+	+++	++	-	+	-
ENU (N-Nitroso-N-ethylurea)	759-73-9	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
Methyl methanesulfonate (MMS)	66-27-3	+	+	+	+	+	+	+	E	+	-	+	+	++	+++	-
Benzo[a]pyrene	50-32-8	+	+	+	+	+	+	E	+	E	+	+++	++	+	+	+
7,12-dimethylbenz[a]anthracene	57-97-6	+	+	+	E	+	+	E	+	+	-	++	+	-	-	-
2-acetylaminofluorene (2-AAF)	53-96-3	+	+	+	+	+	+	+	+	+	I	I	I	I	I	I
2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)	105650-23-5	+		+	+	+	-	+	+	+	++	+++	++	+	+	-
Aflatoxin B1	1162-65-8	+	+	+	+	+	+	+	+	-	+	+++	++	+	-	-
Cadmium Chloride	10108-64-2	E	I	+	+	+	+			-	-	+	-	-	-	-
Cisplatin	15663-27-1	+	+	+	+	+	+	+	+	+	+	+++	++	+	+	-
p-chloroaniline	106-47-8	+	E		E	+		E		+	-	-	-	-	-	+

2. In vivo genotoxins, neg. or equivocal in Ames.																	
Etoposide	33419-42-0	+	+	+	+	+	+	+	-	+	+	+++	++	+	-	-	
Hydroquinone	123-31-9	-	+	+	+	+	+	+		-	+	+++	+	++	+++	-	
Zidovudine (Azidothymidine)	30516-87-1	+	+	+	+	+	-			+	+	-	+	+	-	++	
Sodium arsenite	7784-46-5	-	+	+	+	+	+		+	+	-	++	-	-	+	+	
Taxol / Paclitaxel	33069-62-4	I	+	+	+	+	+	+		+	+	-	++	+++	-	-	
Chloramphenicol	56-75-7	-	+	I	+	E	E	E			-	-	-	+	-	++	
ECVAM class 2																	
1. Non-carcinogens, neg. in in vivo genotoxicity tests.																	
Ampicillin	69-53-4	-	-	-	-	-	-			-	-	-	-	-	-	-	
D-mannitol	69-65-8	-	-	-	-	-	-			E	-	-	-	-	-	-	
2. Non-carcinogens, no in vivo genotoxicity data.																	
Phenformin HCl	834-28-6	-	-		-						-	-	-	-	-	-	
n-butyl chloride	109-69-3	-	E		-						-	-	-	-	-	+	
(2-chloroethyl)trimethylammonium chloride	999-81-5	-	-		-	-	-				-	-	-	-	-	+	
Cyclohexanone	108-94-1	-	- / + (+S9)		-	-	-				-	-	-	-	-	-	

N,N-dicyclohexyl thiourea	1212-29-9	-	E		-									-	-	-	-	-	+				
Ethylenediaminetetraacetic acid (EDTA)	60-00-4	-	E		E	+	+							-	-	+	-	-	-				
Erythromycin stearate	643-22-1	-	-		-									-	-	-	-	-	-				
Fluometuron	2164-17-2	-	-		-									-	-	-	-	-	+				
3. non-genotoxic carcinogens																							
D-limonene	5989-27-5	-	I	+	-								-	-	-	-	-	-	-				
Amitrole	61-82-5	-	-		E	-	-	-						-	-	-	-	-	-				
Tert-butyl alcohol	75-65-0	-	-		-	-								-	-	-	-	-	-				
Diethanolamine	111-42-2	-	-		-	-								-	-	-	-	-	-				
Methyl carbamate	598-55-0	-	-	-	-	-	E						-	-	-	-	-	-	-				
Progesterone	57-83-0	-	-		-			-						-	-	-	-	-	+				
Pyridine	110-86-1	-	-		-	-	-	-						-	-	-	-	-	-				
Tris(2-ethylhexyl)phosphate	78-42-2	-	-		-	-	-							-	-	-	-	-	++				
Hexachloroethane	67-72-1	-		-	-	-								-	-	-	-	-	-				
ECVAM class 3																							
1. non-carcinogens with negative or equivocal in vivo genotoxicity data																							

D,l-menthol	15356-70-4	-	E		+	-	-			-	-	-	-	-	-		
Phthalic anhydride	85-44-9	-	+		+					-	++	+	+++	-	-		
Tert-butylhydroquinone	1948-33-0	E			+	-	-			-	-	-	+++	++	+		
o-anthranilic acid	118-92-3	-	+	+	+	-	-			-	-	-	-	-	-		
1,3-dihydroxybenzene (Resorcinol)	108-46-3	-	+	+	+	-	-			-	-	-	-	-	-		
Sulfisoxazole	127-69-5	-	+		-					-	-	-	-	-	-		
2. non-carcinogens with no in vivo genotoxicity data																	
Ethionamide	536-33-4	-	+		+					-	-	-	-	-	+		
Curcumin	458-37-7	-		+						-	-	-	-	-	-		
Benzyl alcohol	100-51-6	-	E		+				+	-	-	-	-	-	-		
Urea	57-13-6	-	E		I				+	-	-	-	-	-	-		
3. non-genotoxic carcinogens or non-carcinogens																	
Sodium saccharin	128-44-9	-	-		+	-	-	-		-	-	-	-	-	-		
4. Supplementary list (less clear of in vitro genotoxicity prediction)																	
Propyl gallate	121-79-9	E	+	+	+	E	E			-	+	+	++	+	-		
p-Nitrophenol	100-02-7	-	E		E					-	-	-	-	-	+		

Ethyl acrylate	140-88-5	-	+	+	+	E	E					-	-	-	-	-	-
Eugenol	97-53-0	I	+	+	+	E		-			-	-	++	+	-	-	-
Isobutyraldehyde	78-84-2	E	+	+	+	-	+					-	-	-	-	-	-
2,4-dichlorophenol	120-83-2	E	I		E		+				+	-	-	-	-	-	+
Additional non-genotoxic carcinogens																	
1,4-bis(3,5-dichloro-2-pyridinyloxy)benzene (TCPOBOP)	76150-91-9	-										-	-	-	-	-	-
2,3,7,8-tetrachlorodibenzo-p-dioxin	1746-01-6	-	-	-	-							-	-	-	-	-	-
Aroclor-1254	27323-18-8	-										-	-	-	+	-	-
Beta-hexachlorocyclohexane	319-85-7	-	-		-							-	-	-	-	-	-
Carbon tetrachloride	56-23-5	E	I	+	-	-	-	-	-	-		-	-	-	-	-	-
Clofibrate	637-07-0	-	-	-	+	-	-					-	-	-	-	-	-
Cyclosporin A	59865-13-3	-	-			-	-					-	-	-	+	-	++
Heptachlor epoxide	1024-57-3	E										-	-	-	-	-	+
Lead acetate	301-04-2	-	I		E	E	E	-			+	-	-	-	+	-	-
Phenobarbital	50-06-6	+	I	-	+	E	-	-	E		-	-	-	-	-	-	++
Phorbol 12-myristate 13-acetate (PMA)	16561-29-8	-										-	-	-	++	-	+
Tacrolimus	109581-93-3											-	-	-	-	-	++

Wyeth-14643	50892-23-4	-			-					E			+	-	-	-	+	-	++				
Additional non-genotoxins																							
Copper sulfate	7758-98-7	I				+	+						+	-	-	-	+++	-	-				
Diethyl maleate	141-05-9	-	+											-	-	-	+++	++	-				
Quercetin	117-39-5	+	+		+	-	-	-						-	+	-	-	+	-				
Thapsigargin	67526-95-8													-	-	-	-	-	+++				
Tunicamycin	11089-65-9													-	-	-	-	-	+++				
Valproic acid	99-66-1	-												-	-	-	-	-	-				
Additional genotoxins																							
4-hydroxy-2-nonenal	75899-68-2													-	+	+	++	+	-				
4-nitroquinoline-1-oxide (of -N-oxide)	56-57-5	+	+	+	+	+	+	+	+					-	++	+	-	-	-				
Camptothecin	7689-03-4	-	+	+	+	+	+							+	+++	++	+	-	-				
Cytarabine	147-94-4	E	+		+	+	+							+	+++	++	-	+	-				
Doxorubicin (adriamycin)	23214-92-8	+	+	+	+	+	+	+						+	+++	+	++	+	-				
Flavopiridol	146426-40-6		E											-	-	-	-	-	-				

Hydrogen peroxide	7722-84-1	+	+	+	+	-	-	+				+	++	+	+	+	-
Hydroxyurea	127-07-1	+	+	+	+	+		+	+			+	+++	++	+	+	-
Mitomycin C	50-07-7	+	+	+	+	+	+		+	+		+	+++	++	+	-	-
Potassium bromate	7758-01-2	+		+	+							-	+++	++	+	+	-
Tert-butyl hydroperoxide	75-91-2	+			+							-	+	+	-	++	-
Microtubule-disrupting agents																	
Colcemid	477-30-5	-		+	+							++	+++	++	+	-	-
Colchicine	64-86-8	I	E	+	+	+	+					+++	+	+	++	+	-
Docetaxel	114977-28-5	-	-		+	+	+					-	+	-	-	-	-
Griseofulvin	126-07-8	-	+	+	+	E	+					-	-	-	-	-	+
Noscapine	912-60-7	-										-	++	-	-	-	-
Podophyllotoxin	518-28-5	-	-									+	+	+	+	-	-
Vinblastin	143-67-9	I	+	+	+	+	+					+	++	+	+++	+	+
Vincristine	57-22-7	-	+	+	+	+	+					+	+	++	+++	-	-
Vindesine	59917-39-4	-		+		+						-	-	-	+	-	-
Vinorelbine	71486-22-1	-	E	+	+	+						+	+	++	+++	-	-

2. In vivo genotoxins, neg. or equivocal in Ames.																	
Etoposide	33419-42-0	+	+	+	+	+	+	+	-	+	+	+++	++	+	-	-
Hydroquinone	123-31-9	-	+	+	+	+	+	+		-	+	+++	+	++	+++	-
Zidovudine (Azidothymidine)	30516-87-1	+	+	+	+	+	-			+	+	-	+	+	-	++
Sodium arsenite	7784-46-5	-	+	+	+	+	+		+	+	-	++	-	-	+	+
Taxol / Paclitaxel	33069-62-4	I	+	+	+	+	+	+		+	+	-	++	+++	-	-
Chloramphenicol	56-75-7	-	+	I	+	E	E	E			-	-	-	+	-	++

E=equivocal, I=(technically) inconclusive, +=positive, -=negative

Chapter Two

2.1 Introduction

Current regulatory strategies for the assessment of genotoxicity (i.e., ability to damage genetic material) rely on the use of OECD-approved *in vitro* assays, with *in vivo* follow-up where necessary (1). The required tests, such as the Salmonella Reverse Mutation Test (i.e., Ames test), mammalian cell gene mutation tests (e.g., *tk* or *hprt* locus mutagenicity assays), and mammalian cell chromosome damage assays (e.g., mammalian cell micronucleus assay) have been shown to reliably detect *in vivo* mutagens and carcinogens; thus permitting reduction in the use of *in vivo* methods, and unnecessary use of laboratory animals (2). Despite their utility, these conventional *in vitro* assays are limited in the sense that most are costly, time-consuming (i.e., low throughput), and laborious compared to novel DNA damage reporter assays (Chapter 1, Table 1.4). Standard mammalian-cell *in vitro* assays for the detection of mutation or chromosomal damage are considered low-throughput primarily because they require a post-exposure incubation that permits DNA damage to be "fixed" into observable mutations or chromosomal aberrations (3). The fixation requirements generally necessitate a post-exposure delay of 24-36 hours in order to cover at least one round of cellular replication. Thus, it could be asserted that conventional assays for the detection of mutation or chromosomal damage are poorly aligned with an expressed need for high-throughput, high-content chemical screening tools (4). With respect to high-content information, the older, traditional assays only monitor one endpoint at a time. In contrast, some novel reporter assays monitor numerous toxicological endpoints simultaneously (i.e., multiplexed assessment capability); because the resultant information covers a range of cellular processes, it can be used to unravel the MOA (mode of action) underlying observed genotoxic hazard (4).

In contrast to the aforementioned, conventional *in vitro* genotoxicity assays, the novel reporter-based, multiplexed assays permit high-throughput, high-content chemical safety assessment. Moreover, as noted, the multiplexed information provided can be used to determine MOA.

Therefore, novel reporter assays can efficiently and effectively provide detailed profiles for the assessment of toxicological hazards posed by chemicals in commerce. Notably, the ToxTracker® assay, which can simultaneously assess several cellular stress responses in mES (murine embryonic stem) cells, is well suited to efficient, effective and affordable assessment of chemical hazard (5). The assay detects (geno)toxicity by monitoring fluorescent biomarkers linked to six reporter genes. The reporters encode signal proteins that respond to chemically-induced stress responses such as DNA damage causing replication fork stalling (*Rtkn*), DNA strand breaks (*Bscl2*), generalized cellular toxicity related to P53 stabilization (*Btg2*), generalized oxidative stress (*Srxn1* and *Blvrb*), and protein damage (*Ddit3*).

High-throughput assays such as ToxTracker® generate vast amounts of complex, multivariate data, and comprehensive, rigorous interpretation of these data necessitates consideration of numerous quantitative issues. These include (1) considerations of variability in control responses, and definition of a threshold for identification of a significant positive response, (2) definition of a BMR (Benchmark Response) that delineates small, background responses from those deemed toxicologically meaningful (see below), (3) comparative interpretation of chemical-specific responses, and (4) determination of the functional and statistical relationships between multiplexed endpoints.

With respect to the variability of ToxTracker® control data (i.e., background fluorescence of unexposed cells), this can be evaluated by examining the distribution of fluorescence control values, and their associated distributional statistics (e.g., standard deviation). Alternatively, since toxicity assay responses are often reported as fold-changes relative to control, and 1.0 is associated with a lack of induced response, a robust approach involves determination of the variability in control fold-change (i.e., 1.0). However, since a fold-change value is a unitless ratio of two

variables, variance calculation presents unique challenges, and use of specialized methods are warranted (6, 7). Alternatively, the variance of fold-change values can be determined by bootstrapping, i.e., determination of ratio variance via iterative, random sampling of numerator and denominator values.

Numerous methods have been employed to assess the potency of (geno)toxic substances. Benchmark Dose (BMD) analysis involves fitting a mathematical function to dose/concentration-response data, and interpolating the dose or concentration required to elicit a predefined, fractional increase in response over control (e.g., ToxTracker® background reporter signals). The predefined increase, which is known as the BMR or Benchmark Response, can be rather subjective (8) with some regulatory authorities recommending 5 or 10% for a variety of toxicological endpoints. Alternatively, some regulatory authorities recommend a BMR equivalent to one standard deviation above mean background (i.e., 1SD) (9). In either case, if the BMR is indicative of an *in vivo* response deemed to be adverse, the BMD value can be used for human health risk assessment, i.e., determination of an HBGV (Health-based Guidance Value) such as a TDI (Tolerable Daily Intake). Slob, 2016 (10) discusses how analyses of maximum effect size for a given endpoint can be used to determine a percentage increase above background that can be considered minimally adverse. More specifically, the effect-size (ES) theory of Slob recommends calculation of BMR as the eighth root of the endpoint-specific maximum effect size ($M^{1/8}$). Since the maximum effect size is often difficult to estimate, Slob's ES theory provides the basis for BMR determination via estimation of the geometric mean variance of dose-specific responses. A somewhat similar approach for BMR determination was recently outlined by Zeller *et al*. (11), who used variability in the log-normal distributions of background (control) values to define a small effect size that can be termed the BMR. Briefly, Zeller *et al*. (11) used a number of different methods to scrutinize

variability in historical controls, and subsequently employed the 95th percentile of the log-normal distribution of control values to define endpoint-specific BMR values. Zeller *et al.* (11) noted that the most promising approach involves the use of trimmed historical controls, whereby highly deviant historical control response values, which are deemed not representative of the true distribution, are removed prior to the analysis. Importantly, Slob does not recommend BMR's based on analysis of control values alone. Rather, Slob recommends use of the geometric mean variance determined using concentration-response data across all concentration groups.

An extension of the BMD approach involves simultaneous analysis of numerous dose-response functions across a covariate (e.g., substance, sex, cell type, tissue, etc.); the approach is sometimes referred to as BMD combined-covariate analysis. For large datasets, such as those available for the ToxTracker® assay, the BMD combined-covariate approach can be used to rank the potency of a series of reference substances, and subsequent potency evaluation of hitherto untested chemicals. Conveniently, when the purpose of the analysis is to compare potency values for a given endpoint, the BMR employed is relatively unimportant. That being said, the precision of BMD values employed for comparative potency analysis will increase with increasing BMR, i.e., a BMD for a 100% increase will be more precise than a BMD for a small (e.g., 5%) increase.

As noted earlier, the ToxTracker® assay can simultaneously monitor responses across 6 reporters that are collectively indicative of a substance's toxicological properties. However, since the molecular signaling (i.e., ATM/ATR signaling) underlying some of the ToxTracker® reporter responses are functionally related, the reporters are likely redundant. For example, DNA-damaging substances that cause strand breakage and/or replication-fork stalling will likely elicit *Rtkn* and *Bscl2* responses, and perhaps a *Btg2* response as well. Moreover, substances that cause DNA damage via generation of ROS (reactive oxygen species) will likely elicit responses of the *Rtkn,*

Srxn1 and *Blvrb* reporters, and perhaps the *Btg2* reporter as well. Thus, a multivariate statistical technique such as PCA (Principal Component Analysis) can be used to investigate functional redundancy in ToxTracker® reporters. More specifically, if a dataset containing ToxTracker® reporter responses across a wide range of substances is available, then PCA, a variable reduction procedure, can be used be used to statistically delineate redundancy in the six ToxTracker® reporters. Correlations between the signals of the original 6 reporters and the Principal Components (i.e., the loadings) can subsequently be used to operationally interpret and classify the multivariate principal components (e.g., DNA damage stress, oxidative stress, etc.).

The work described herein employed a variety of data analysis strategies to address the aforementioned issues related to quantitative interpretation of ToxTracker® dose-response data. More specifically, using dose response data for over 90 reference substances, the work investigated (1) the fold-change response level required to identify a significant positive response, (2) substance potency ranking for each ToxTracker® reporter, (3) the BMR indicative of toxicologically-meaningful ToxTracker® responses, and (4) the functional redundancy of responses across the six ToxTracker® reporters.

2.2 Material and methods

2.2.1 Data compilation and formatting

Toxys B.V. (Leiden, The Netherlands) provided data files from over 100 studies; files include all non-confidential dose-response data, as well as solvent control response values associated with studies of confidential test articles (i.e., client projects). Each file contains the study date, the microplate and well number, the reporter studied, the replicate number, the substance tested, the presence or absence of an S9 metabolic activation mixture, the test article concentration in µM, the mean GFP response per cell, and the final cell concentration. Dose-specific reporter response values are GFP signals averaged over 5,000 scored cells. Collectively, the data files include over 86,000 dose-response observations across over 90 validation substances (i.e., over 13,000 observations per reporter), including the four positive control substances (i.e., tunicamycin, cisplatin, diethyl maleate, aflatoxin B1). Additionally, the files include extensive solvent control response values, i.e., over 20,000 negative control observations in total, with over 3,500 control values for each reporter. 106 text-only data files in tab-delimited format (tsv), containing the non-confidential data for validation substances, were imported into SAS v9.4; each file represents a study, each microplate on a given date represents a sub-study. The imported text files were combined into one master, permanent SAS dataset; the control values in the validation-substance master file were combined with a second data file containing control data associated with confidential client projects. Figure 2.1 illustrates the protocol for initial dataset handling and data compilation.

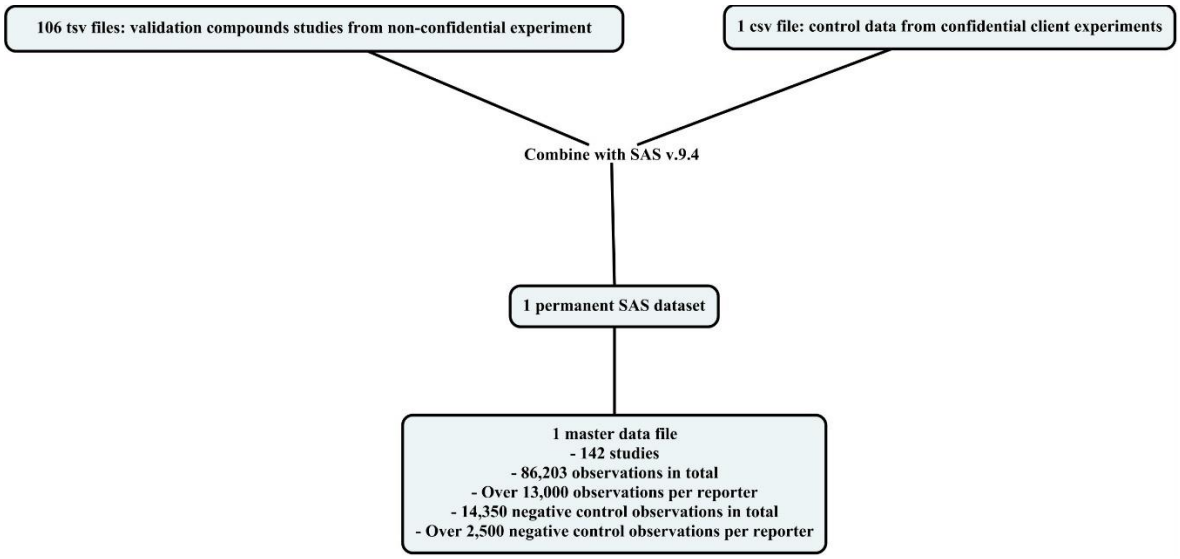


Figure 2.1 Flowchart illustrating initial dataset handling and data compilation. 106 tab-delimited (tsv), validation-substance files were combined with one comma delimited (csv) control-only file to create a single master, permanent SAS dataset, i.e., a dataset containing all ToxTracker® dose-response data for both non-confidential studies of validation substances, and all control data from confidential client projects. Where required, data from the master file were exported to Excel. Text outside boxes indicates processes, text in boxes indicates results.

2.2.2 Determination of fold-change cut-off values for identification of reporter-specific positive responses

All solvent control data were compiled into a single file containing all reporter-specific GFP values associated with dose=0 (i.e., unexposed control). The resulting control data, which included over 2,500 observations for each reporter, were then examined using a custom SAS macro. More specifically, a bootstrapping approach was used to iteratively calculate possible control GFP ratio values for each individual study (i.e., ratios of randomly selected numerator and denominator); this was done 5,000 times to generate a distribution of possible control ratios. The obtained distributions, which all have a mean of 1.0, were then scrutinized in order to determine the upper 95^{th} and 99^{th} percentiles of possible fold-change values for unexposed controls. These values are indicative of the minimum fold-change responses that denote a significant positive response. To ensure the normality of the distributions, all GFP control values were log-transformed prior to

calculation of control ratios. Visual inspection of box-plots and quantile-quantile plots was used to confirm the normality of the distributions. Using a bootstrapping approach, the 95th and 99th percentile values were determined for every study with a normal distribution. The 95th and 99th percentiles for every study with a normal distribution were averaged, and used to define a response-level threshold expressed as fold-change above control. The values were then compared with those recommended by Toxys B.V. Figure 2.2 illustrates the data handling and analysis protocol.

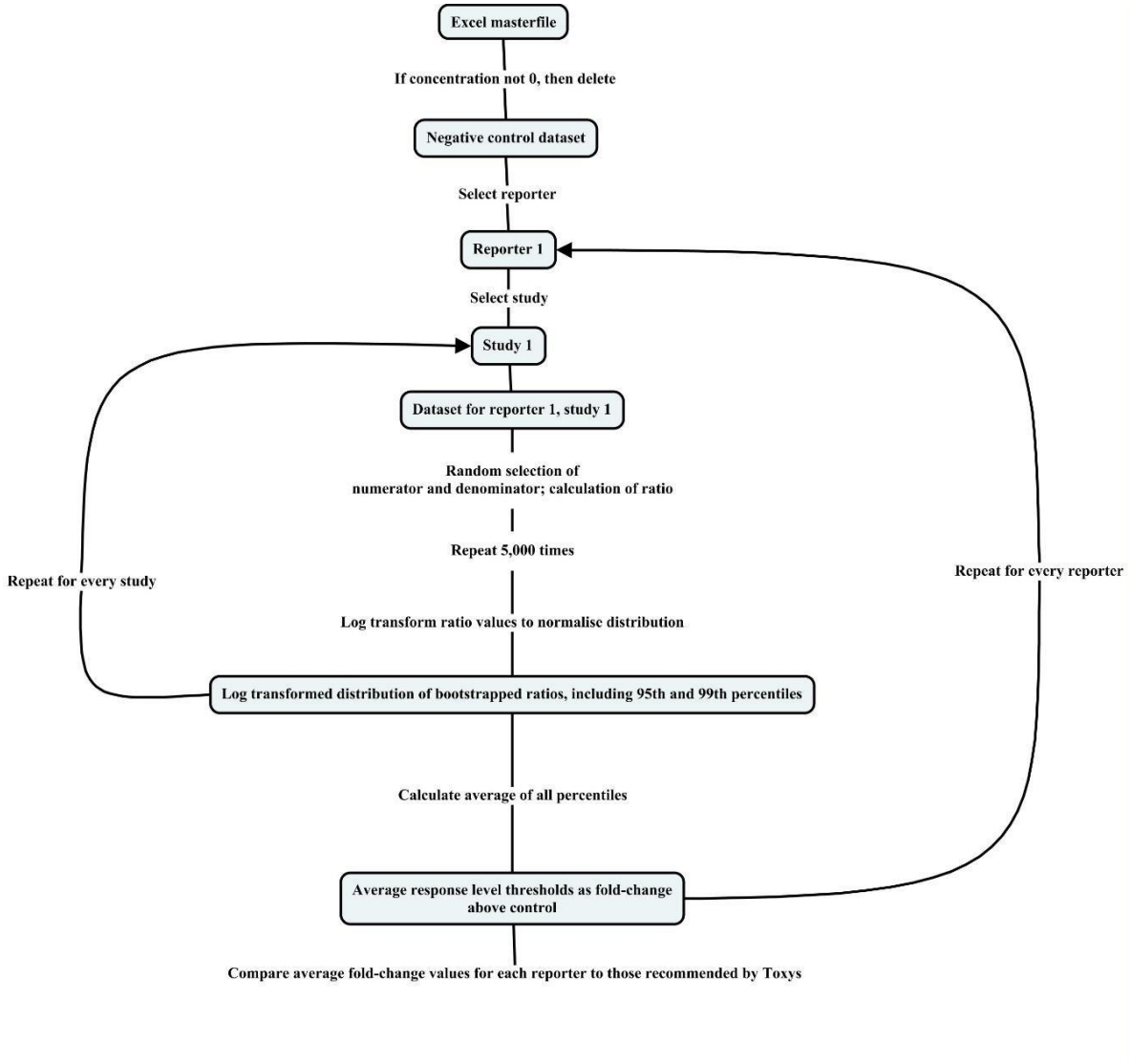


Figure 2.2 Flowchart illustrating the protocol employed to determine the fold-change cut-off for identification of reporter-specific positive responses. Text outside boxes indicate processes, text in boxes indicate results. See text for details.

2.2.3 Comparative potency analysis using the Benchmark Dose (BMD) combined-covariate approach

Prior to comparative potency analysis, the data were screened for cytotoxicity and dose-response pattern. Following recommendations of Toxys B.V., the cytotoxicity associated with each concentration of each chemical was calculated for each of the six reporters, i.e., the ratio of the post-exposure, dose-specific cell concentration to its corresponding control cell concentration. The cytotoxicity was used to exclude reporter response values deemed to be unreliable; more specifically, response values for concentrations wherein cytotoxicity was >75%. The GFP induction at tested concentrations was also examined; cases where the dose-response data showed a decrease in GFP induction with increasing concentration were excluded from dose-response analyses.

Cytotoxicity-screened dose-response data were used for BMD combined-covariate analysis conducted using PROAST v.66.14 in R (RIVM, Bilthoven, The Netherlands). More specifically, the approach, which has been described in more detail by Wills *et al.* (12, 13), was employed for simultaneous analysis of up to 10 dose-response relationships; the approach assumes that the maximum response (i.e., parameter *c* in PROAST), log steepness (i.e., parameter *d* in PROAST) and *var* (i.e., within group variation) are conserved across substance-specific dose-response relationships. This is consistent with conservation of dose-response shape parameters for continuous endpoints noted by Slob and Setzer (14). Parameters for background response (i.e., parameter *a* in PROAST) and substance potency (i.e., parameter *b* in PROAST) were assumed to be covariate (substance)-dependent (15). The PROAST output provided the BMD (i.e., potency) for a BMR of 100% (i.e., doubling dose), with BMDL and BMDU designating the lower and upper 90% confidence limits, respectively. The BMDL and BMDU were used for comparative potency evaluations of validation substances. The results obtained permitted robust ranking of chemical

potency for each reporter. BMD values were subsequently used to investigate the functional and statistical relationships between reporter responses (see Section 2.2.5). Some substances are benign until enzymatically converted into reactive metabolites (e.g., benzo[*a*]pyrene) (16). Consequently, significant positive responses for some substances were only elicited when tested in the presence of an exogenous metabolic activation mixture containing a rodent hepatic preparation known as S9 (i.e., 9000g supernatant). The S9 used was derived from Aroclor 1254-exposed male rat livers.

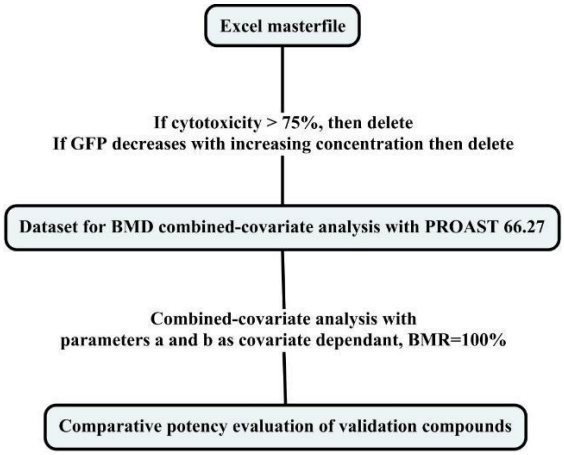


Figure 2.3 Flowchart illustrating the protocol employed for comparative potency ranking of validation substances. Text outside boxes indicates processes, text in boxes indicates results. See text for details.

2.2.4 Determination of endpoint-specific Benchmark Response (BMR) values

Determination of endpoint-specific BMR values employed two different computational approaches. More specifically, endpoint-specific BMR values were calculated using the aforementioned approaches outlined by Zeller *et al.* (11) and Slob (10). To facilitate comparisons to other BMR values (9), resultant endpoint-specific BMR values were expressed as percentage increases relative to the control-group mean.

Determination of reporter-specific BMR values using the Zeller *et al.* approach

With respect to the Zeller *et al.* (6) approach, the 95th percentile was used as a cut-off to create trimmed historical control distributions. More specifically, trimmed historical controls were defined as the set of control values remaining after removal of the upper-most 5% of values, which were considered significantly deviant, and therefore, not representative of typical deviations from the mean. The value obtained by calculating the standard deviation over the mean of the trimmed historical control distribution, expressed as a percentage relative to the mean control, was used to define the BMR. All analyses were conducted using SAS v9.4. Figure 2.4 provides an illustrative overview of the data analysis protocol employed.

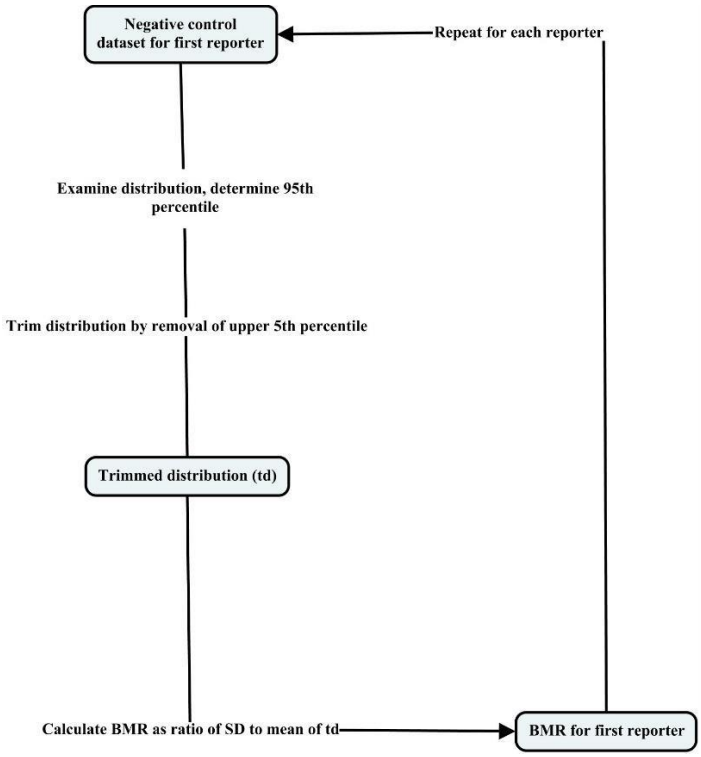


Figure 2.4 Flowchart illustrating the protocol employed for determination of reporter-specific BMR values based on the approach of Zeller *et al.* (2017). Text outside boxes indicates processes, text in boxes indicates results. See text for details.

Determination of reporter-specific BMR values using the Slob approach

With respect to the Slob (10) approach, the average dose-specific standard deviation was calculated via dose-response analysis in PROAST; average within-group standard deviation was calculated as the square root of the PROAST *var* estimate, i.e., the average within-group variance across all dose-groups and studies included in the dose-response analysis. More specifically, for each ToxTracker® reporter, validation substances eliciting a positive response in a specific reporter were analyzed in PROAST using the combined-covariate approach; parameters a and b (i.e., background response and potency) were defined as covariate-dependent, a single value for *var* was estimated across all data included in the analysis (i.e., not covariate dependent), and assumed to represent the average response variance across all doses and substances. The empirical relationship between *var* and *M* (i.e. maximum response parameter) described in the Slob ES theory was used to determine *M*, and the attendant small effect size termed the BMR (10).

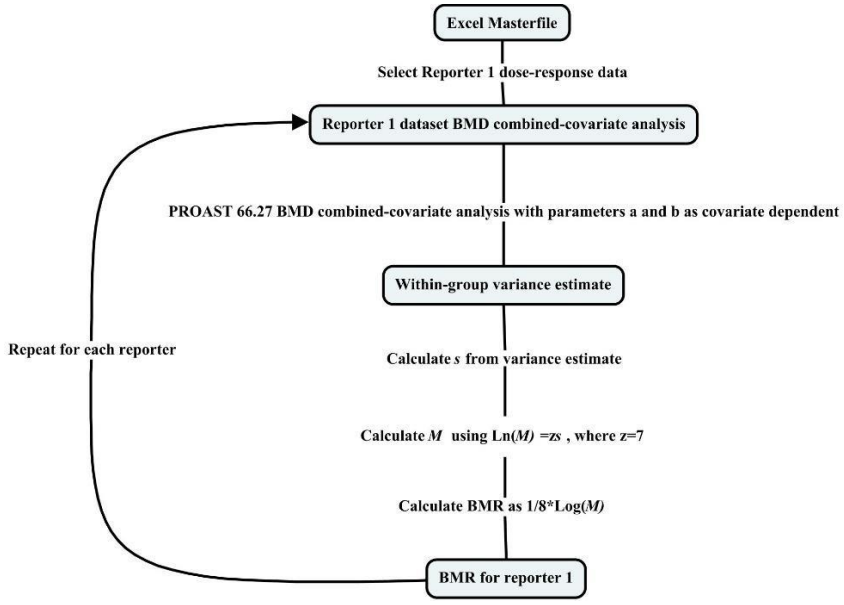


Figure 2.5 Flowchart illustrating the data analysis protocol employed to determine reporter-specific BMR values based on the Slob (2016) Effect-size (ES) theory. Text outside boxes indicates processes, text in boxes indicates results. See text for details.

2.2.5 Principal Component Analysis

SAS 9.4 was used for Principal Component Analysis of the dataset containing BMD values for all substance-reporter combinations. In order to base the PCA analyses on the most data possible, some BMD values were adjusted for inclusion in the dataset. More specifically, for cases where the BMD values were not significantly different from zero, an estimated lower limit of detection was used. The lower limit of detection was estimated as 0.01, and values that were not significantly different from 0 were replaced by 0.01/sqrt(2). This approach allowed the use of values that were otherwise unusable for statistical analyses. It is also used frequently in studies and recommended by the US EPA (17-19). This approach was used for 6 substances. Similarly, in cases where the BMD values were extremely large (e.g., $>10^4$), indicating a lack of response, an upper limit of 10,000 (10^4) was used. This value was used for 58 substances. Using the dataset containing BMD values for each substance-reporter combination, Principal Components were extracted that account for the largest amount of the variance in the multi-reporter response pattern, i.e., the six reporter response variables were optimally transformed into a smaller set of multivariate axes that are linear combinations of the variables in the observed dataset (20). The output provided substance-specific scores for each component, and the principal component factor pattern (i.e., the loadings of each reporter variable on each component). The loadings of each reporter variable on each component, which reflects the correlation between the variable and the component, were used to conceptually interpret the multivariate components. The Varimax rotation option was used to provide orthogonal factor solutions, i.e., maximally uncorrelated factors, or principal components, that account for the largest amount of the variation in the multivariate response data. By ensuring that each variable primarily loads on only one component, Varimax rotation simplifies interpretation of the component pattern. Indeed, it is recommended for exploratory data analysis that optimally facilitates interpretation of multivariate dimensions (20).

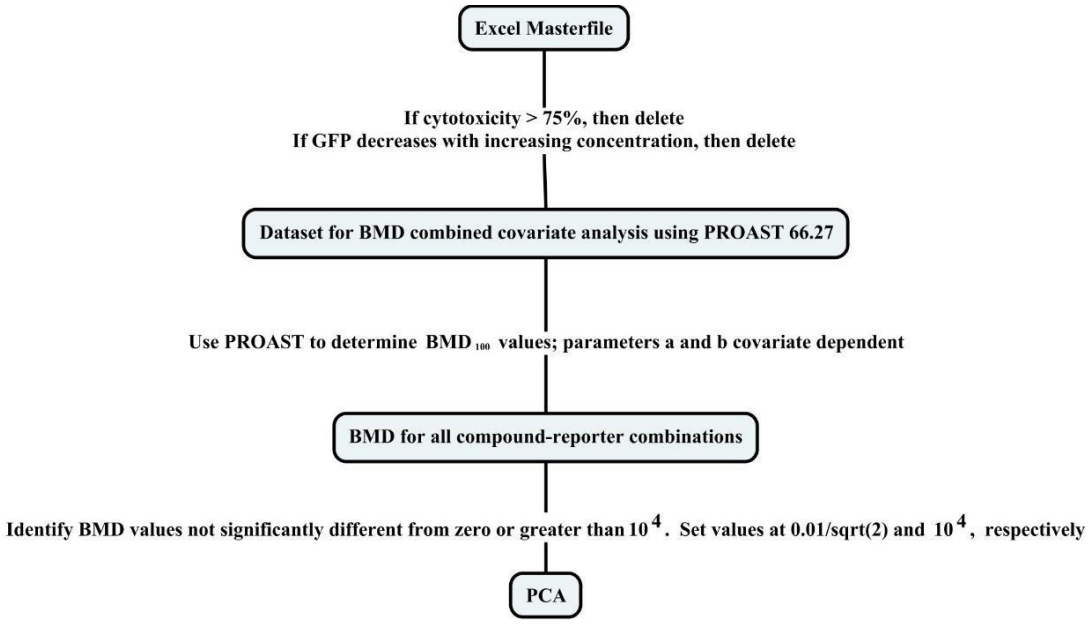
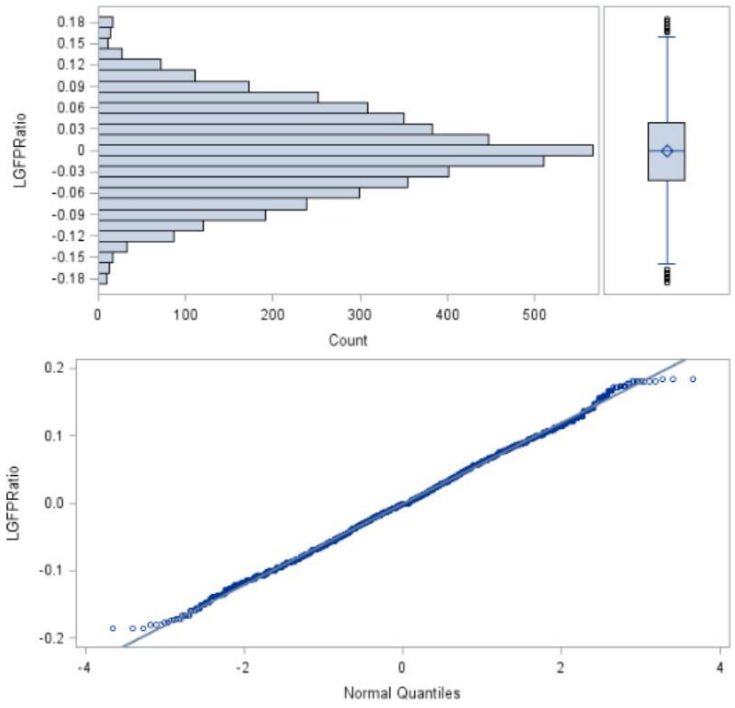


Figure 2.6 Flowchart illustrating the protocol for the PCA (Principal Component Analysis). Text outside boxes indicates processes, text in boxes indicates results. See text for details.

2.3 Results

2.3.1 Reporter-specific fold-change cut-off values for identification of a positive response

Bootstrapping analyses yielded distributions of possible fold-change values such as that shown in Figure 2.7. The distribution shown, which relates to control data for *Rtkn* generated on July 11, 2018, yielded 95th and 99th percentiles of 1.25 and 1.37, respectively. The resulting cut-off values for each reporter, which were determined as the geometric 95th percentile of the control ratio distribution, range from 1.46 to 1.62. The average values across all reporters is approximately 1.5, i.e., a 1.5-fold change indicates a significant positive response. In comparison, the cut-off values identified using the geometric 99th percentile of the control ratio distribution range from 1.64 to 1.88, with an average value of 1.70 for all the reporters (Table 2.1). Thus, the cut-off fold-change value for a weak positive response determined herein is identical to that already used by Toxys B.V., i.e., 1.5-fold increase over background. With respect to delineation of a strong positive response, the value currently used by Toxys (i.e., 2-fold increase) is 15% greater than that determined herein. This indicates that the Toxys approach for identification of strong positive responses is somewhat conservative, i.e., would be less than one percent probability that a GFP induction of 2-fold change above control could be obtained by chance alone.



Obs	N	Mean	StdDev	P99	P95	GMean	GMSD	GM95th	GM99th
1	5000	-.00100948	0.060129	0.13634	0.097457	0.99768	1.14850	1.25158	1.36881

Figure 2.7 Illustration of a SAS bootstrap output. Control ratios were calculated 5,000 times; the distribution of Log_{10} bootstrapped ratios was examined and compared to expectations for a normal distribution (e.g., histogram, box plot, and Q-Q plot shown at top left, top right and bottom). The geometric 95th and 99th percentiles were used to determine the cut-off values for determination of a positive response (bottom table). LGFPRatio = Log_{10} GFP Ratio, N = number of bootstrap iterations, GMean = Geometric Mean, GMSD = geometric standard deviation, GM95th and GM99th = geometric 95th and 99th percentiles, respectively.

Table 2.1 Summary of bootstrap analysis to determine fold-change cut-off values for delineation of significant positive responses. Values shown are geometric 95th (GM95th) and 99th percentiles (GM99th) of ratio distributions.

Reporter	GM95th	GM99th
Bscl2	1.46	1.66
Blvrb	1.62	1.88
Btg2	1.46	1.64
Ddit3	1.52	1.69
Rtkn	1.55	1.76
Srxn1	1.58	1.85
Average	1.51	1.74

2.3.2 Reporter-specific Benchmark Response (BMR) values

Values based on Slob ES (effect size) theory

The within-group standard deviation (*s*) for each reporter was obtained by calculating the square root of the variance, i.e., the parameter in the PROAST output designated as *var* (Figure 2.8). *s* was used to calculate *M* according to the aforementioned equation (Figure 2.5) Ln(*M*) = z*s, where z=7 (see Slob, 2016). According to the Slob ES theory, BMR was calculated as 1/8*Log(*M*). These analyses yielded values ranging from 0.30 for *Ddit3* (i.e., 30%), to 0.52 for *Rtkn*, with an average of 0.43. The calculated BMRs for each reporter are summarized in Table 2.2.

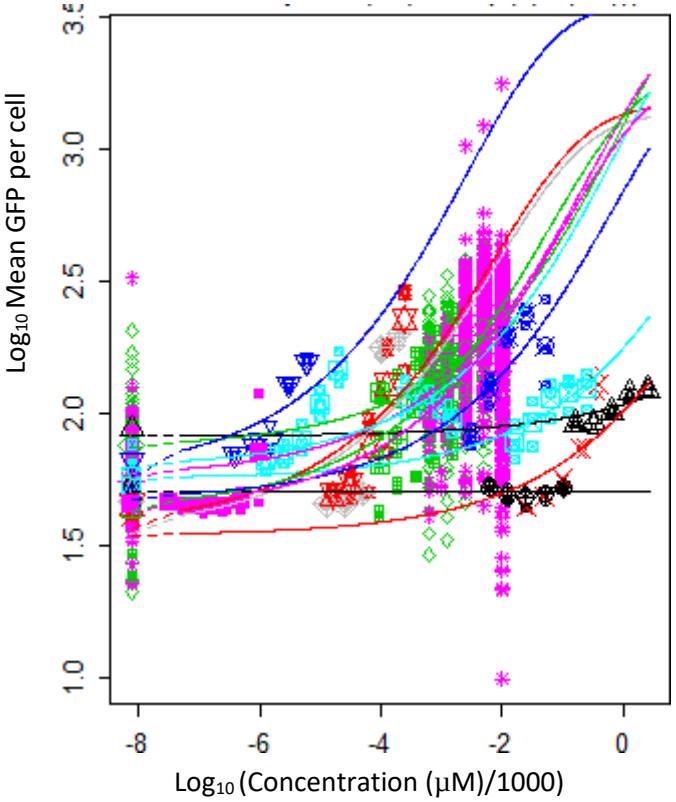


Figure 2.8 Concentration-response analysis used for determination of *var* for the *Bscl2* reporter. The analyses, which were conducted using PROAST 66.27 in R, included dose-response data for 13 substances. Substance was used as the covariate, and PROAST parameters a and b were covariate-dependent; a single variance (*var*) value was estimated. A scaling factor of 1,000 was used for the concentration variable. Large plotting symbols indicate concentration-specific mean values; smaller plotting symbols indicate response values for each individual replicate. Note that the pink and green symbols show the responses of cisplatin and aflatoxin B1, respectively, positive controls that were included in every assay plate. See section 2.2.4 for additional details.

Table 2.2 Reporter-specific Benchmark Response (BMR) values calculated by using the Slob ES theory. BMRs, expressed as percentage above control, were calculated as (100 * ($M^{1/8}$ – 1)); *M* was calculated according to the formula Ln(*M*) = z*s, where z is a constant equal to 7 (Slob, 2016). The within-group standard deviation (*s*) was calculated as the square root of the variance (i.e., $var^{1/2}$).

Reporter	Variance	*s*	*M*	BMR (%)
Bscl2	0.11	0.33	10.07	34
Blvrb	0.15	0.39	15.01	40
Btg2	0.15	0.39	14.91	40
Ddit3	0.09	0.30	8.00	30
Rtkn	0.28	0.52	39.17	58
Srxn1	0.24	0.49	31.09	54
Average	0.17	0.40	19.71	43

Values based on Zeller et al. control distribution method

BMR values for each reporter were also calculated according to the Zeller *et al*. (11) approach, i.e., by trimming the upper 95th percentile of the control distribution, and dividing the standard deviation of the trimmed distribution by its mean. This resulted in values ranging from 2.2% for *Blvrb* and *Rtkn*, to 7.0% for *Ddit3*, with an average of 3.9% for all the reporters (Table 2.3).

Table 2.3 Reporter-specific Benchmark Response (BMR) values calculated as the ratio of the standard deviation to the mean for the trimmed distribution of historical control values.

Reporter	BMR (%)
Bscl2	2.5
Blvrb	2.2
Btg2	4.1
Ddit3	7.0
Rtkn	2.2
Srxn1	5.3
Average	3.9

2.3.3 Benchmark Dose combined-covariate analysis for substance potency ranking

For each reporter, the BMD combined-covariate approach was used for comparative potency analysis and ranking of assay-validation compounds (Table 2.4). The substances selected for comparative potency analysis included only those that elicited a significant positive response (see Methods section 2.2.3). The combined-covariate approach determines BMD_{100} values for each compound included in the analysis (i.e., the dose required to elicit a doubling of the response); BMD_{100} confidence intervals are employed to compare potency values (i.e., BMD_{100} values). Figure 2.9 provides an example of comparative potency analysis of 5 substances for the DNA damage reporter *Bscl2*. The results clearly show that mitomycin C is significantly more potent than BaP and DMBA (Figure 2.9b, see Tables A1-6 for substance abbreviations). Where confidence limits (i.e., right and left extremes of horizontal lines) overlap, substances are termed equipotent. Where confidence intervals do not overlap, substance potencies can be ranked.

Table 2.4 Substances included in the BMD combined-covariate analysis for potency ranking. Only substances that elicited a positive response for multiple reporters were included in potency ranking analysis. x indicates that a defined confidence interval was determined; in other cases, the response was weak or erratic, and the BMD confidence interval could not be determined. The use of an S9 metabolic activation mixture is indicated as required.

Chemical	**CAS Number**	***Rtkn***	***Bscl2***	***Blvrb***	***Srxn1***	***Btg2***	***Ddit3***
17*b*-estradiol +S9	50-28-2	x	x	x	x	x	x
1-naphthol	1321-67-1	x	x	x	x	x	x
1-nitropyrene	5522-43-0	x	x	x	x	x	x
2-Chloroethyl ethylsulfide	693-07-2	x	x	x	x	x	
2-mercaptopurine	50-44-2	x	x	x	x	x	x
2-pyridinol 1-oxide	13161-30-3	x	x			x	x
2-pyridinol 1-oxide +S9	13161-30-3	x	x		x	x	x
4-amino-3-nitrophenol	610-81-1	x	x	x	x	x	
4-nitroquinoline-1-oxide	56-57-5	x	x	x		x	
4-vinyl-1-cyclohexene diepoxide	106-87-6	x	x	x	x	x	x
5-fluorouracil	51-21-8	x	x			x	
7,12-Dimethylbenz[*a*]anthracene +S9	57-97-6	x	x	x	x	x	x
Abacavir	136470-78-5	x	x	x	x	x	x
Aciclovir	59277-89-3	x	x	x	x	x	x
Adefovir	142340-99-6	x	x	x	x	x	x
Aflatoxin B1 +S9	1162-65-8	x	x	x	x	x	

Alachlor	15972-60-8			x			
Allyl bromide	106-95-6	x	x	x	x	x	x
Benzo[*a*]pyrene +S9	50-32-8	x	x	x	x	x	x
Camptothecin	7689-03-4	x	x	x	x	x	x
Carbamazepine	298-46-4	x	x		x	x	x
Chlorambucil	305-03-3	x	x	x	x	x	
Chloramphenicol	56-75-7				x		
Chlorpheniramine maleate	113-92-8	x	x	x		x	x
Cisplatin	15663-27-1	x	x	x	x	x	
Colcemid	477-30-5	x	x	x	x	x	x
Colchicine	64-86-8	x	x	x		x	x
Daunorubicin	20830-81-3	x	x	x	x	x	
Dazomet	533-74-4	x	x	x	x	x	x
Diclofenac +S9	15307-79-6	x	x		x	x	x
Diethylstilbestrol	56-53-1	x	x	x	x	x	x
Diethyl maleate	141-05-9	x	x	x	x	x	x
Diphenylhydantoin	57-41-0	x	x	x	x	x	x
Disulfiram	97-77-8	x	x	x	x	x	x
Docetaxel	114977-28-5	x	x		x	x	x
EDTA[a]	60-00-4	x	x	x	x	x	x
Emodin +S9	518-82-1	x	x	x	x	x	x
Ethyl paraben	120-47-8	x	x		x	x	x
Ethyl methanesulfonate	62-50-0	x	x	x	x	x	
Etoposide	33419-42-0	x	x	x	x	x	x
Eugenol	97-53-0	x	x	x	x	x	x
Fludioxonil	131341-86-1	x	x	x	x	x	
Hydralazine	304-20-1	x	x	x	x	x	
Hydrogen peroxide	7722-84-1	x	x	x	x	x	
Hydroquinone	123-31-9	x	x	x	x	x	x
Hydroxybenzomorpholine	26021-57-8	x	x	x	x	x	x
Hydroxyurea	127-07-1	x	x	x	x	x	x
Mebendazole	31431-39-7	x	x	x	x	x	x
Mebendazole +S9	31431-39-7	x	x	x	x	x	
Methylisothiazolinone	2682-20-4	x	x	x	x	x	x
Mitomycin C	50-07-7	x	x	x	x	x	
N-Nitroso-*N*-ethylurea	759-73-9	x	x	x	x	x	x
NNK[b]	64091-91-4	x	x		x	x	
Nocodazole	31430-18-9	x	x		x	x	x
Norethynodrel	68-23-5	x	x		x	x	x
Noscapine	912-60-7	x	x				
Novobiocin	303-81-1	x	x				
Nutlin-3	548472-68-0	x	x	x	x	x	x
PhIP[c] +S9	105650-23-5	x		x	x	x	x
Phthalic anhydride	85-44-9	x	x	x	x	x	x

Podophyllotoxin	518-28-5	x	x	x	x	x	x
Potassium chromate	7789-00-6	x	x	x	x	x	
Quercetin	117-39-5	x	x	x	x	x	x
Sodium azide	26628-22-8	x	x			x	x
Taxol	33069-62-4	x	x		x		
Testosterone	58-22-0	x	x	x	x	x	x
Temozolimide	85622-93-1	x	x		x	x	
Topotecan	123948-87-8	x	x	x	x	x	
Topotecan +S9	123948-87-8	x	x	x	x	x	
Tunicamycin	11089-65-9		x				x
Vinblastine sulphate	143-67-9	x	x	x	x	x	x

[a]Ethylenediaminetetraacetic acid
[b]4-(methylnitrosaino)-1-(3-pyridyl)-1-butanone
[c]2-Amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine

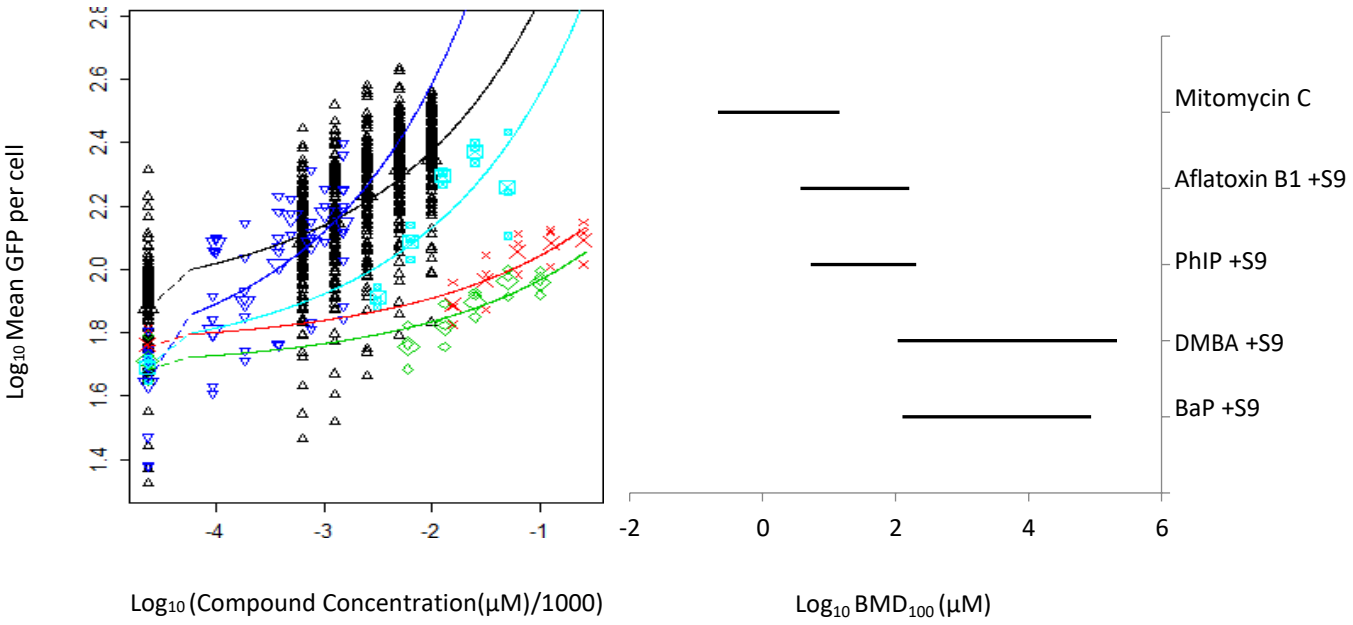
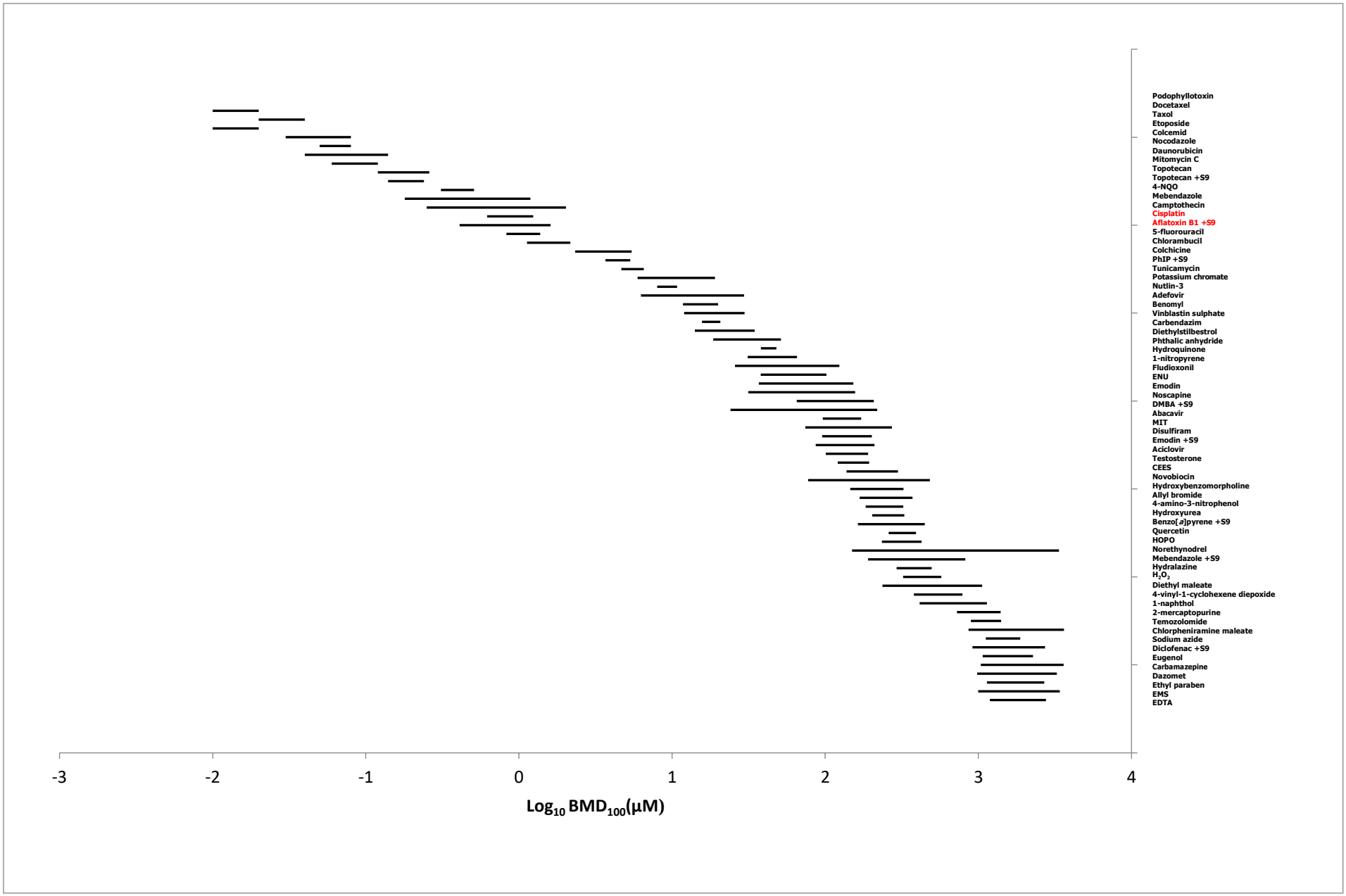


Figure 2.9 Example of BMD combined-covariate analysis for the *Bscl2* reporter. ToxTracker® dose-response data were analyzed using substance as a covariate; the fitted dose-response curves (A) illustrate the responses for 5 compounds. The colors represent the following substances: black, aflatoxin B1 +S9; red, benzo[*a*]pyrene +S9; green, DMBA +S9; dark blue, mitomycin C; light blue, PhIP +S9. A scaling factor of 1,000 was applied for the concentration axis. The BMD confidence intervals for each condition are shown in (B), the left-hand side of the horizontal lines are the BMDLs, the right-hand side of the horizontal lines are the BMDUs. Subset of analyzed substances shown for simplicity. Note that aflatoxin B1 (+S9) is a positive control that is routinely included in every assay plate.

The results of the comprehensive combined covariate analyses, which compared potency values across all positive substances for each reporter, are summarized and illustrated in Figures 2.10a through 2.10f, and Tables A1 through A6. For some substances, the PROAST software could not define confidence intervals, i.e., would indicate a BMDL of 0 and an infinite BMDU. The former indicates that the BMDL could not be differentiated from zero, i.e., lower confidence limit of BMD cannot be defined. The latter indicates that the upper BMDU cannot be defined, i.e., upper confidence limit of the BMD is infinite. These problems with BMDL and BMDU are indicated in the aforementioned tables; the substances for which the confidence interval could not be defined were excluded from the comparative potency figures (i.e., 2.10a through 2.10f). Where confidence limits (i.e., right and left extremes of horizontal lines) overlap, substances are termed equipotent. Where confidence interval calculated for each positive substance do not overlap, substance potencies can be ranked.

It is interesting to note that the positive controls for each reporter (i.e., potent substances routinely used to ensure assay performance) are not the most potent substances tested (see Figures 2.10a through 2.10f). Nevertheless, the potency rankings of cisplatin and tunicamycin, the positive controls for *Bscl2*, *Rtkn* and *Ddit3*, are always in the 3rd quartile (i.e. 75th percentile or higher). Interestingly, diethyl maleate, the positive control for oxidative damage, has an intermediate potency; the rank is about midway between most and least potent. Moreover, the potency rankings of the positive control substances for the reporters detecting genotoxicity (i.e., *Bscl2* and *Rtkn*) and oxidative stress (i.e., *Blvrb* and *Srxn1*) are similar, including the additional genotoxic stress positive control for metabolic activation, i.e., AFB1.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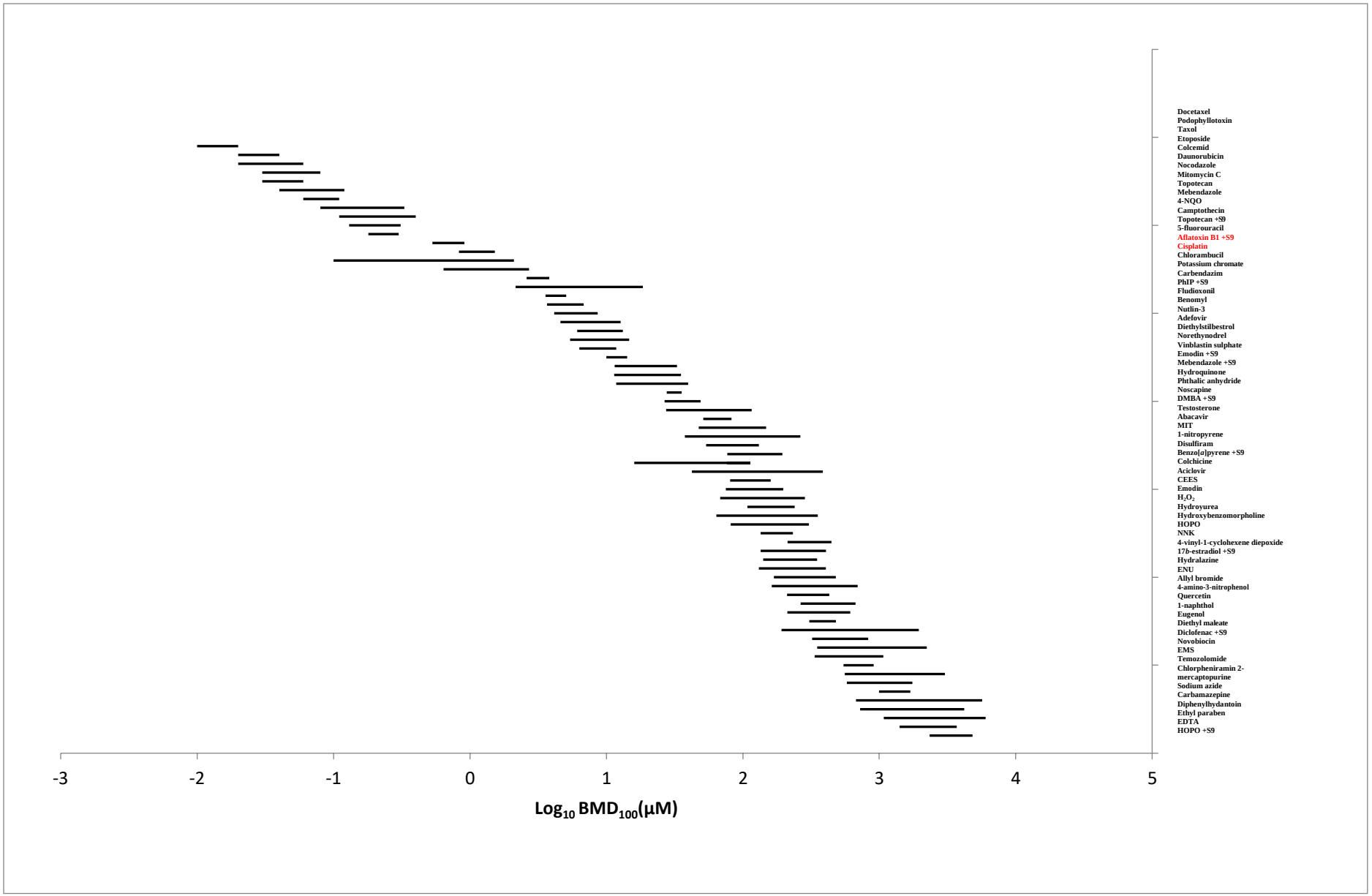
Mebendazole
Camptothecin
Cisplatin
Aflatoxin B1 +S9
5-fluorouracil
Chlorambucil
Colchicine
PhIP +S9
Tunicamycin
Potassium chromate
Nutlin-3
Adefovir
Benomyl
Vinblastin sulphate
Carbendazim
Diethylstilbestrol
Phthalic anhydride
Hydroquinone
1-nitropyrene
Fludioxonil
ENU
Emodin
Noscapine
DMBA +S9
Abacavir
MIT
Disulfiram
Emodin +S9
Aciclovir
Testosterone
CEES
Novobiocin
Hydroxybenzomorpholine
Allyl bromide
4-amino-3-nitrophenol
Hydroxyurea
Benzo[a]pyrene +S9
Quercetin
HOPO
Norethynodrel
Mebendazole +S9
Hydralazine
H2O2
Diethyl maleate
4-vinyl-1-cyclohexene diepoxide
1-naphthol
2-mercaptopurine
Temozolomide
Chlorpheniramine maleate
Sodium azide
Diclofenac +S9
Eugenol
Carbamazepine
Dazomet
Ethyl paraben
EMS
EDTA
-3
-2
-1
0
1
2
3
4
Log10 BMD100(µM)

Figure 2.10a Illustration of BMD combined-covariate results to rank the potency of ToxTracker® validation substances for the *Bscl2* reporter (i.e., mutagenic DNA lesions characterized by replication fork stalling). The BMR is 100%, and the positive controls (i.e., cisplatin and aflatoxin B1) are indicated in red. Use of an exogenous metabolic activation mixture indicated as "+S9". The left side of the horizontal bars indicates the BMDL, the right side indicates the BMDU. Substances that did not elicit a significant positive response, or that had undefined confidence intervals are not included, i.e., BMDL and BMDU are 0 and infinite, respectively. Doses for which cytotoxicity exceeded 75% (i.e., <25% survival) were not included in the analyses.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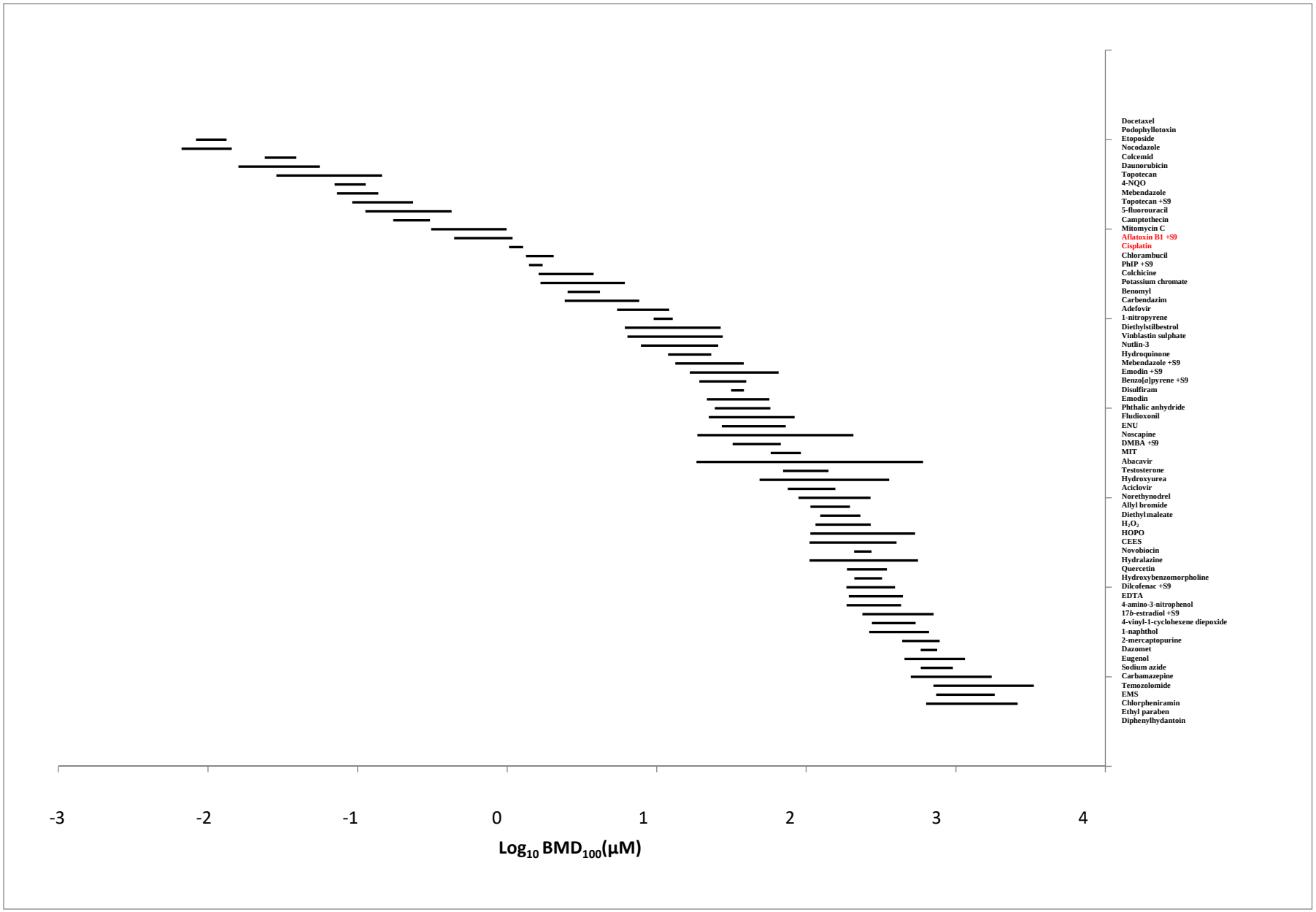
Topotecan +S9
5-fluorouracil
Aflatoxin B1 +S9
Cisplatin
Chlorambucil
Potassium chromate
Carbendazim
PhIP +S9
Fludioxonil
Benomyl
Nutlin-3
Adefovir
Diethylstilbestrol
Norethynodrel
Vinblastin sulphate
Emodin +S9
Mebendazole +S9
Hydroquinone
Phthalic anhydride
Noscapine
DMBA +S9
Testosterone
Abacavir
MIT
1-nitropyrene
Disulfiram
Benzo[a]pyrene +S9
Colchicine
Aciclovir
CEES
Emodin
H2O2
Hydroyurea
Hydroxybenzomorpholine
HOPO
NNK
4-vinyl-1-cyclohexene diepoxide
17b-estradiol +S9
Hydralazine
ENU
Allyl bromide
4-amino-3-nitrophenol
Quercetin
1-naphthol
Eugenol
Diethyl maleate
Diclofenac +S9
Novobiocin
EMS
Temozolomide
Chlorpheniramin 2-
mercaptopurine
Sodium azide
Carbamazepine
Diphenylhydantoin
Ethyl paraben
EDTA
HOPO +S9
-3
-2
-1
0
1
2
3
4
5
Log10 BMD100(μM)

Figure 2.10b Illustration of BMD combined-covariate results to rank the potency of ToxTracker® validation substances for the *Rtkn* reporter (i.e., DNA double strand breaks). The BMR is 100%, and the positive controls (i.e., cisplatin and aflatoxin B1) are indicated in red. Use of an exogenous metabolic activation mixture indicated as "+S9". The left side of the horizontal bars indicates the BMDL, the right side indicates the BMDU. Substances that did not elicit a significant positive response, or that had undefined confidence intervals are not included, i.e., BMDL and BMDU are 0 and infinite, respectively. Doses for which cytotoxicity exceeded 75% (i.e., <25% survival) were not included in the analyses.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4-NQO
Mebendazole
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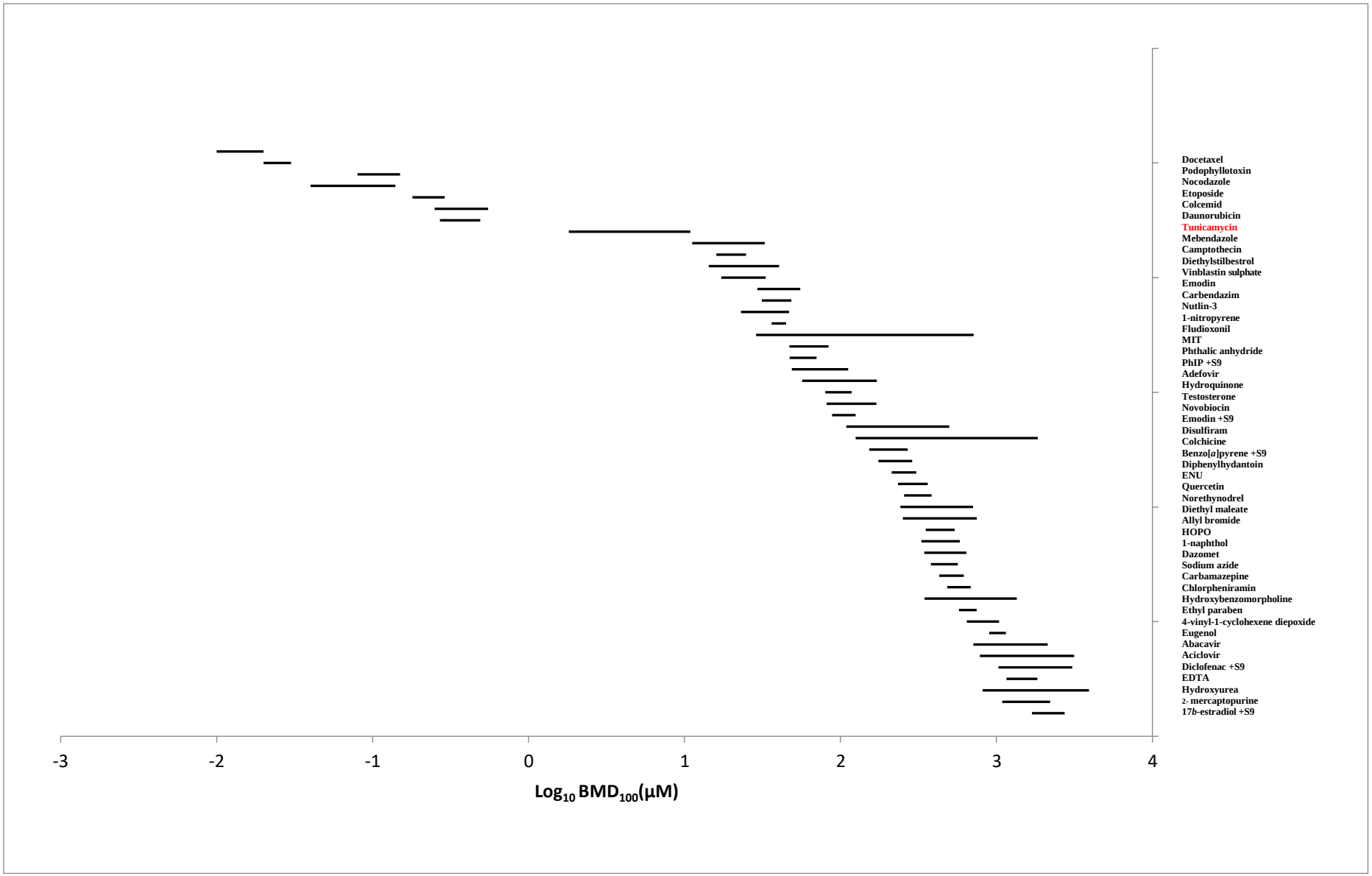
Camptothecin
Mitomycin C
Aflatoxin B1 +S9
Cisplatin
Chlorambucil
PhIP +S9
Colchicine
Potassium chromate
Benomyl
Carbendazim
Adefovir
1-nitropyrene
Diethylstilbestrol
Vinblastin sulphate
Nutlin-3
Hydroquinone
Mebendazole +S9
Emodin +S9
Benzo[a]pyrene +S9
Disulfiram
Emodin
Phthalic anhydride
Fludioxonil
ENU
Noscapine
DMBA +S9
MIT
Abacavir
Testosterone
Hydroxyurea
Aciclovir
Norethynodrel
Allyl bromide
Diethyl maleate
H_2O_2
HOPO
CEES
Novobiocin
Hydralazine
Quercetin
Hydroxybenzomorpholine
Dilcofenac +S9
EDTA
4-amino-3-nitrophenol
17b-estradiol +S9
4-vinyl-1-cyclohexene diepoxide
1-naphthol
2-mercaptopurine
Dazomet
Eugenol
Sodium azide
Carbamazepine
Temozolomide
EMS
Chlorpheniramin
Ethyl paraben
Diphenylhydantoin
-3
-2
-1
0
1
2
3
4
Log_{10} BMD_{100}(µM)

Figure 2.10c Illustration of BMD combined-covariate results to rank the potency of ToxTracker® validation substances for the *Btg2* reporter (i.e., p53-mediated stress response). The BMR is 100%, and the positive controls (i.e., diethyl maleate and aflatoxin B1) are indicated in red. Use of an exogenous metabolic activation mixture indicated as "+S9". The left side of the horizontal bars indicates the BMDL, the right side indicates the BMDU. Substances that did not elicit a significant positive response, or that had undefined confidence intervals are not included, i.e., BMDL and BMDU are 0 and infinite, respectively. Doses for which cytotoxicity exceeded 75% (i.e., <25% survival) were not included in the analyses.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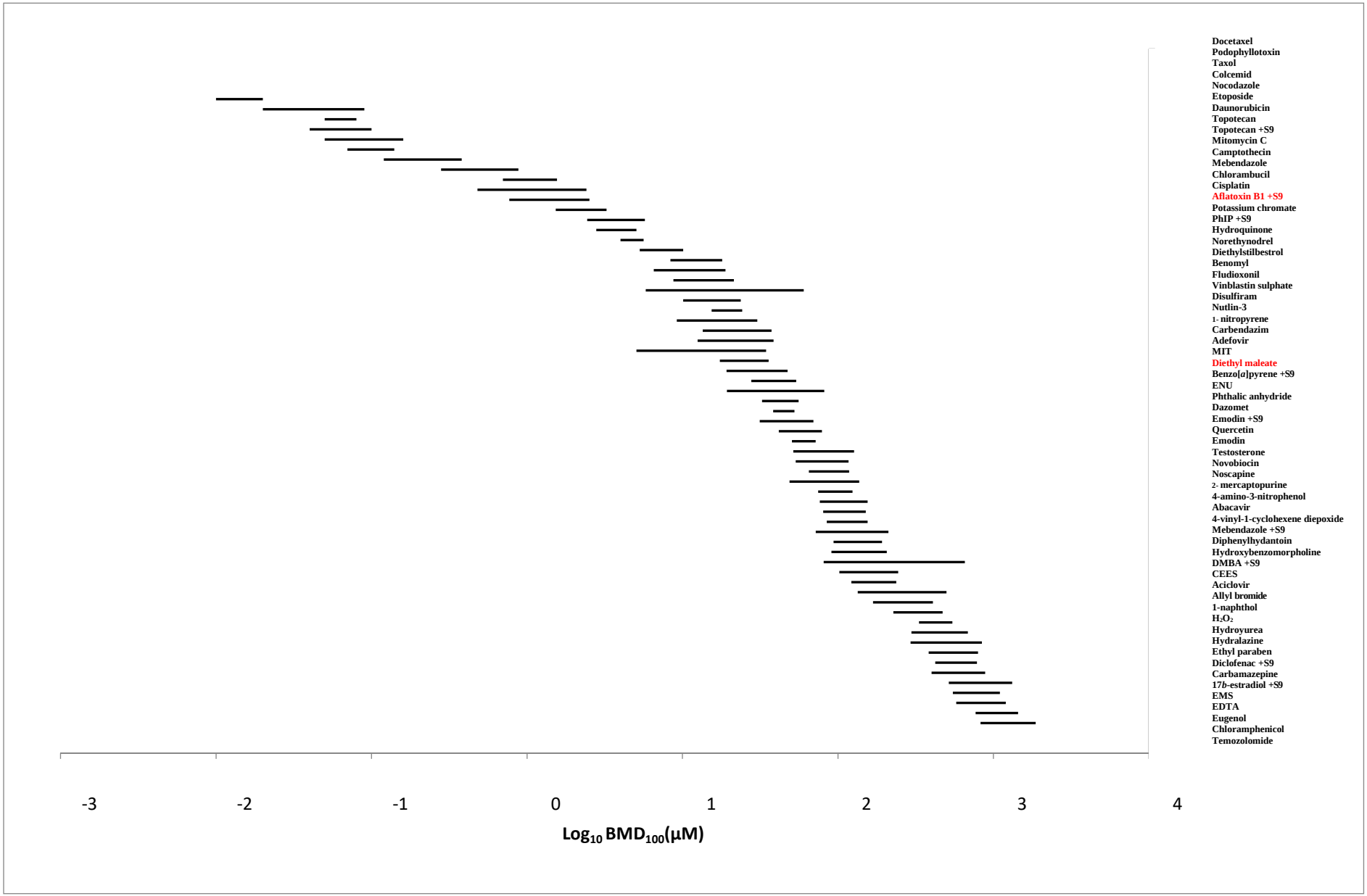
Emodin
Carbendazim
Nutlin-3
1-nitropyrene
Fludioxonil
MIT
Phthalic anhydride
PhIP +S9
Adefovir
Hydroquinone
Testosterone
Novobiocin
Emodin +S9
Disulfiram
Colchicine
Benzo[a]pyrene +S9
Diphenylhydantoin
ENU
Quercetin
Norethynodrel
Diethyl maleate
Allyl bromide
HOPO
1-naphthol
Dazomet
Sodium azide
Carbamazepine
Chlorpheniramin
Hydroxybenzomorpholine
Ethyl paraben
4-vinyl-1-cyclohexene diepoxide
Eugenol
Abacavir
Aciclovir
Diclofenac +S9
EDTA
Hydroxyurea
2- mercaptopurine
17b-estradiol +S9
-3
-2
-1
0
1
2
3
4
Log10 BMD100(µM)

Figure 2.10d Illustration of BMD combined-covariate results to rank the potency of ToxTracker® validation substances for the *Ddit3* reporter (i.e., unfolded protein response). The BMR is 100%, and the positive control (i.e., tunicamycin) is indicated in red. Use of an exogenous metabolic activation mixture indicated as "+S9". The left side of the horizontal bars indicates the BMDL, the right side indicates the BMDU. Substances that did not elicit a significant positive response, or that had undefined confidence intervals are not included, i.e., BMDL and BMDU are 0 and infinite, respectively. Doses for which cytotoxicity exceeded 75% (i.e., <25% survival) were not included in the analyses.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Podophyllotoxin
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1- nitropyrene
Carbendazim
Adefovir
MIT
Diethyl maleate
Benzo[a]pyrene +S9
ENU
Phthalic anhydride
Dazomet
Emodin +S9
Quercetin
Emodin
Testosterone
Novobiocin
Noscapine
2- mercaptopurine
4-amino-3-nitrophenol
Abacavir
4-vinyl-1-cyclohexene diepoxide
Mebendazole +S9
Diphenylhydantoin
Hydroxybenzomorpholine
DMBA +S9
CEES
Aciclovir
Allyl bromide
1-naphthol
H2O2
Hydroyurea
Hydralazine
Ethyl paraben
Diclofenac +S9
Carbamazepine
17b-estradiol +S9
EMS
EDTA
Eugenol
Chloramphenicol
Temozolomide
-3
-2
-1
0
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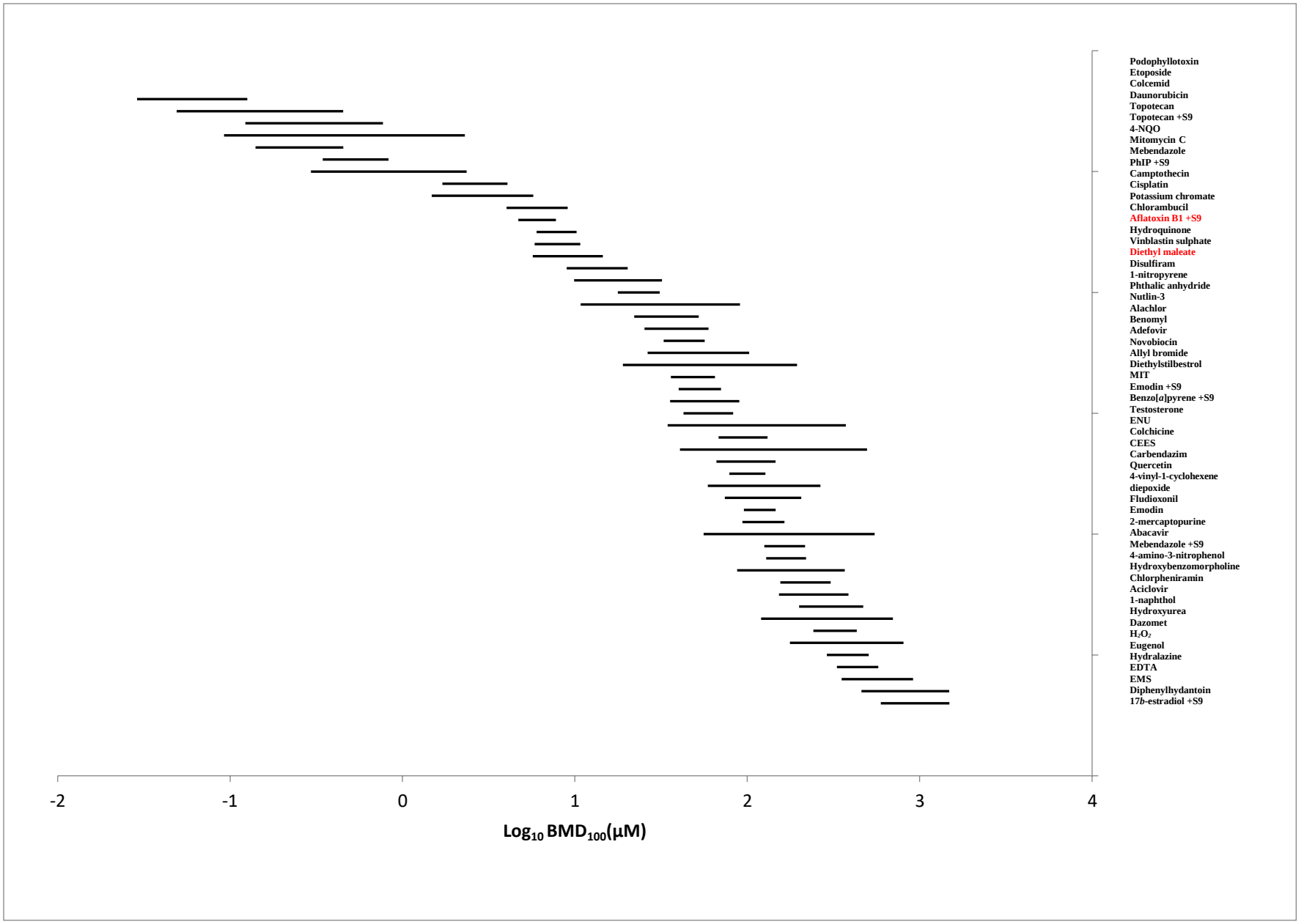
4
Log10 BMD100(µM)

Figure 2.10e Illustration of BMD combined-covariate results to rank the potency of ToxTracker® validation substances for the *Srxn1* reporter (i.e., oxidative damage). The BMR is 100%, and the positive controls (i.e., diethyl maleate and aflatoxin B1) are indicated in red. Use of an exogenous metabolic activation mixture indicated as "+S9". The left side of the horizontal bars indicates the BMDL, the right side indicates the BMDU. Substances that did not elicit a significant positive response, or that had undefined confidence intervals are not included, i.e., BMDL and BMDU are 0 and infinite, respectively. Doses for which cytotoxicity exceeded 75% (i.e., <25% survival) were not included in the analyses.


Podophyllotoxin
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Colcemid
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Topotecan +S9
4-NQO
Mitomycin C
Mebendazole
PhIP +S9
Camptothecin
Cisplatin
Potassium chromate
Chlorambucil
Aflatoxin B1 +S9
Hydroquinone
Vinblastin sulphate
Diethyl maleate
Disulfiram
1-nitropyrene
Phthalic anhydride
Nutlin-3
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Testosterone
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Emodin
2-mercaptopurine
Abacavir
Mebendazole +S9
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Hydroxybenzomorpholine
Chlorpheniramin
Aciclovir
1-naphthol
Hydroxyurea
Dazomet
H2O2
Eugenol
Hydralazine
EDTA
EMS
Diphenylhydantoin
17b-estradiol +S9
-2
-1
0
1
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3
4
Log10 BMD100(µM)

Figure 2.10f Illustration of BMD combined-covariate results to rank the potency of ToxTracker® validation substances for the *Blvrb* reporter (i.e., oxidative damage). The BMR is 100%, and the positive controls (i.e., diethyl maleate and aflatoxin B1) are indicated in red. Use of an exogenous metabolic activation mixture indicated as "+S9". The left side of the horizontal bars indicates the BMDL, the right side indicates the BMDU. Substances that did not elicit a significant positive response, or that had undefined confidence intervals are not included, i.e., BMDL and BMDU are 0 and infinite, respectively. Doses for which cytotoxicity exceeded 75% (i.e., <25% survival) were not included in the analyses.

2.3.4 Principal Component Analysis

BMD scores for each substance-reporter combination were used as the basis for Principal Component Analysis to assess reporter response redundancy, and investigate the MOA (mode of action) of the tested substances. The results show that the first 3 components explain over 93% of the variance, thus providing an adequate summary of the data (Table 2.5). The factor pattern, also referred to as the Principal Component loading matrix, reflects the correlation between the original variable and each Principal Component (i.e., axis). The reporter specific loadings are summarized in Table 2.6, and the loading pattern overlaid on orthogonal bi-plots (i.e., red arrows in Figures 2.11a-c); each plot also shows substance specific axis scores (Table A7). The plots, and respective loading values for each reporter, are indicative of the toxicological import of each multivariate axis. For example, since the reporters for genotoxicity, *Bscl2* and *Rtkn*, have strong positive loadings on the first component (i.e., 0.85 and 0.90), it can be termed a genotoxicity axis. Not surprisingly, the *Btg2* reporter, which reflects p53-driven effects, also has a strong positive axis 1 loading (i.e., 0.88). Loadings of *Blvrb* and *Ddit3* on axis 1 are both below 0.5, i.e., 0.40 and 0.29, respectively, which would generally be considered low (17). Axis 2 appears to be an oxidative stress axis, with a particularly high loading value for the *Blvrb* reporter (i.e., 0.82). With the exception of *Srxn1*, the loadings of the other reporters on axis 2 are low (i.e., <0.50). Interestingly, the results reveal moderate loadings of the *Srxn1* reporter on axes 1 and 2, i.e., 0.68 and 0.56, respectively. This likely indicates that the reporter response is indicative of both oxidative and genotoxic stress. As for the third axis, the loading values indicate that it can be considered a generalized cellular stress axis indicative of effects related to protein damage/unfolding, i.e., *Ddit3* induction. The *Ddit3* loading on axis 3 is very high (i.e., 0.92); loadings of all other response variables are well below 0.5. Inspection of the standardized scoring coefficients (not shown), which can be used to calculate an axis score for each substance, further illustrate the contrast

between the various reporters. More specifically, the standardized coefficients show that components 1 and 2 reflect the contrast between oxidative stress (i.e., primarily *Blvrb*) and genotoxic stress (i.e., *Btg2, Rtkn* and *Bscl2*); the coefficients for component 3 illustrate a strong contrast between protein damage (i.e., *Ddit3*) and all the other reporters.

The position of each substance score on each principal component bi-plot reflects its MOA (mode-of-action); indeed, its relative ability to induce the aforementioned stress responses (e.g., genotoxic stress, oxidative stress, generalized cellular stress). For example, keeping in mind that high potency is associated with low BMD, Figures 2.11a and 2.11c indicate that substances such as the positive control cisplatin (cispt) and aflatoxin B1 (AFB1 +S9), as well as mitomycin C (mit C), induce genotoxic stress almost exclusively, whereas substances such as vincristine (vincri) and 4-nitroquinoline-1-oxide (4-NQO) appear to equally induce genotoxic and oxidative stress. The aflatoxin B1 response is not surprising given that it has been used as an additional positive control for genotoxic stress whereby enzymatic activation is required to generate a reactive metabolite. Figure 2.11c indicates that 4-NQO and vincristine also induce a substantial protein damage response. With respect to the oxidative damage axis (i.e., axis 2), the positions of substances such as sodium arsenite (sod. Ars) and cadmium chloride (CdCl2) indicate that they induce oxidative stress almost exclusively. Interestingly, Figure 2.11b shows that both cadmium chloride and sodium arsenite are more potent inducers of oxidative stress than the positive control used for the oxidative stress reporters *Srxn1* and *Blvrb*. i.e., diethyl maleate. The data presented in Figure 2.11b also indicates that the positive control tunicamycin (tuni) induces a protein damage response almost exclusively; although, Figure 2.11c suggests that tunicamycin is also a weak inducer of genotoxic stress. Not surprisingly, substances with the lowest relative BMD values (e.g., the therapeutic products docetaxel, podophyllotoxin, taxol, etoposide and colcemid), which can be

considered the highest potency toxicants investigated (Figures 2.10a through 2.10f), all have exceptionally low scores on 2 or 3 of the multivariate axes. To facilitate examination of each substance's position on each principal component bi-plot, Figures A1, A2 and A3 provide an expanded view of Figures 2.11a through 2.11c (i.e., axes limits restricted to -2 and +2). These figures more clearly illustrate the positions of substances that are weaker inducers of the investigated toxicological stress responses, and the relative positions of the positive controls cisplatin, aflatoxin B1, diethyl maleate and tunicamycin.

Table 2.5 Proportion of variance explained by each principal component

	Proportion of variance (%)	**Cumulative (%)**
Component 1	78.14	78.14
Component 2	9.91	88.05
Component 3	5.23	93.29
Component 4	4.11	97.40
Component 5	1.50	98.90
Component 6	1.10	100.00

Table 2.6 Reporter specific loadings on the first three principal components. The loading for each reporter on each axis is illustrated in the PCA plots (Figures 2.11a-c)

	Component 1	**Component 2**	**Component 3**
Rtkn	0.90	0.29	0.22
Bscl2	0.85	0.36	0.32
Blvrb	0.40	0.82	0.35
Srxn1	0.68	0.56	0.21
Btg2	0.88	0.30	0.27
Ddit3	0.29	0.27	0.92

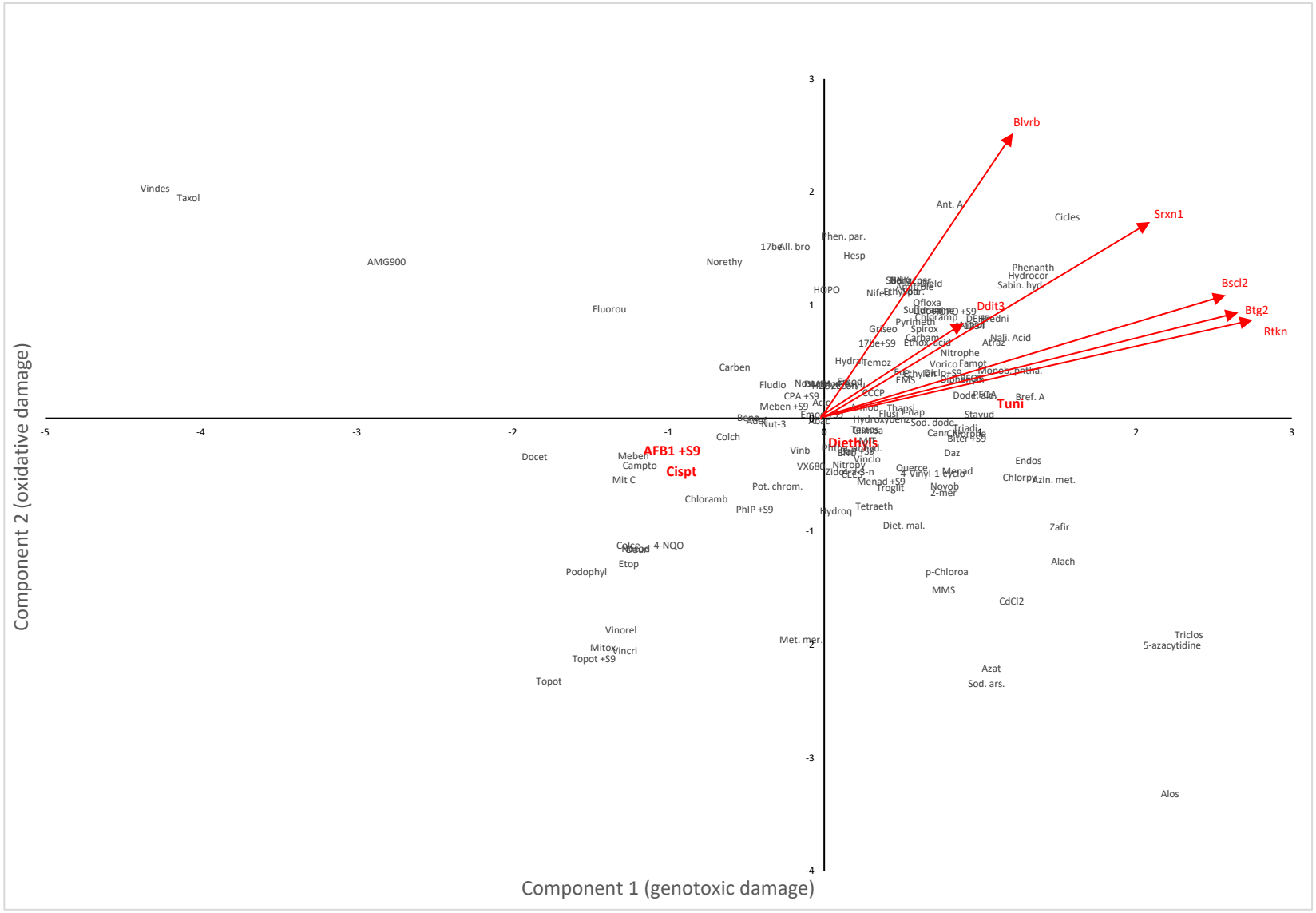

Figure 2.11a Principal Component Analysis results based on BMD values for each substance-reporter combination; Component 2 versus Component 1. Plot shows loadings of reporter responses on each component (i.e., axis) as red arrows. Arrow lengths and positions reflect loading values on each axis. The chemicals are represented according to the abbreviations in Table A7, with positive control substances shown in red. Analysis employed Varimax option to maximize percentage of variance explained by orthogonal axes.

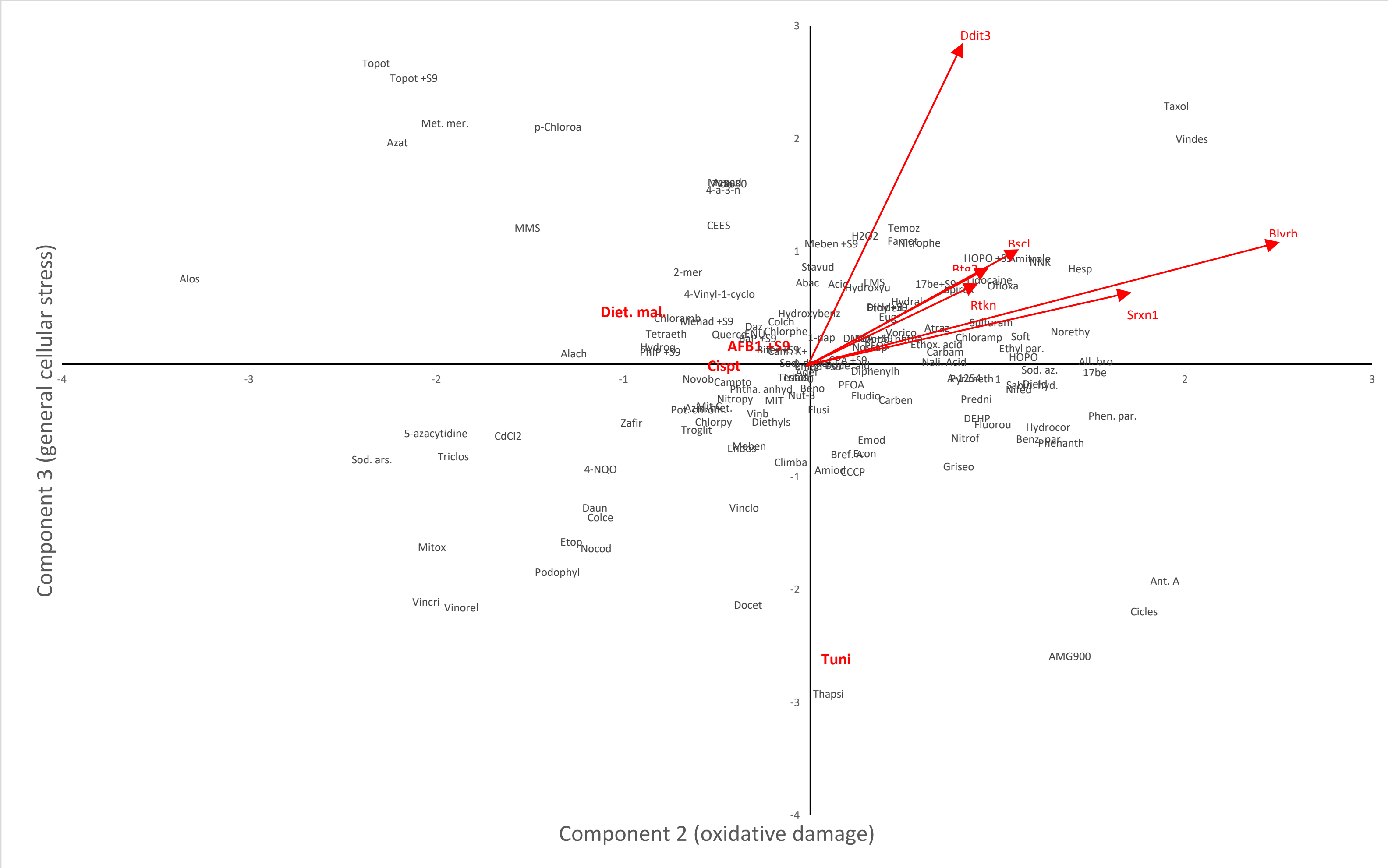

Figure 2.11b Principal Component Analysis results based on BMD values for each substance-reporter combination; Component 3 versus Component 2. Plot shows loadings of reporter responses on each component (i.e., axis) as red arrows. Arrow lengths and positions reflect loading values on each axis. The chemicals are represented according to the abbreviations in Table A7, with positive control substances shown in red. Analysis employed Varimax option to maximize percentage of variance explained by orthogonal axes.

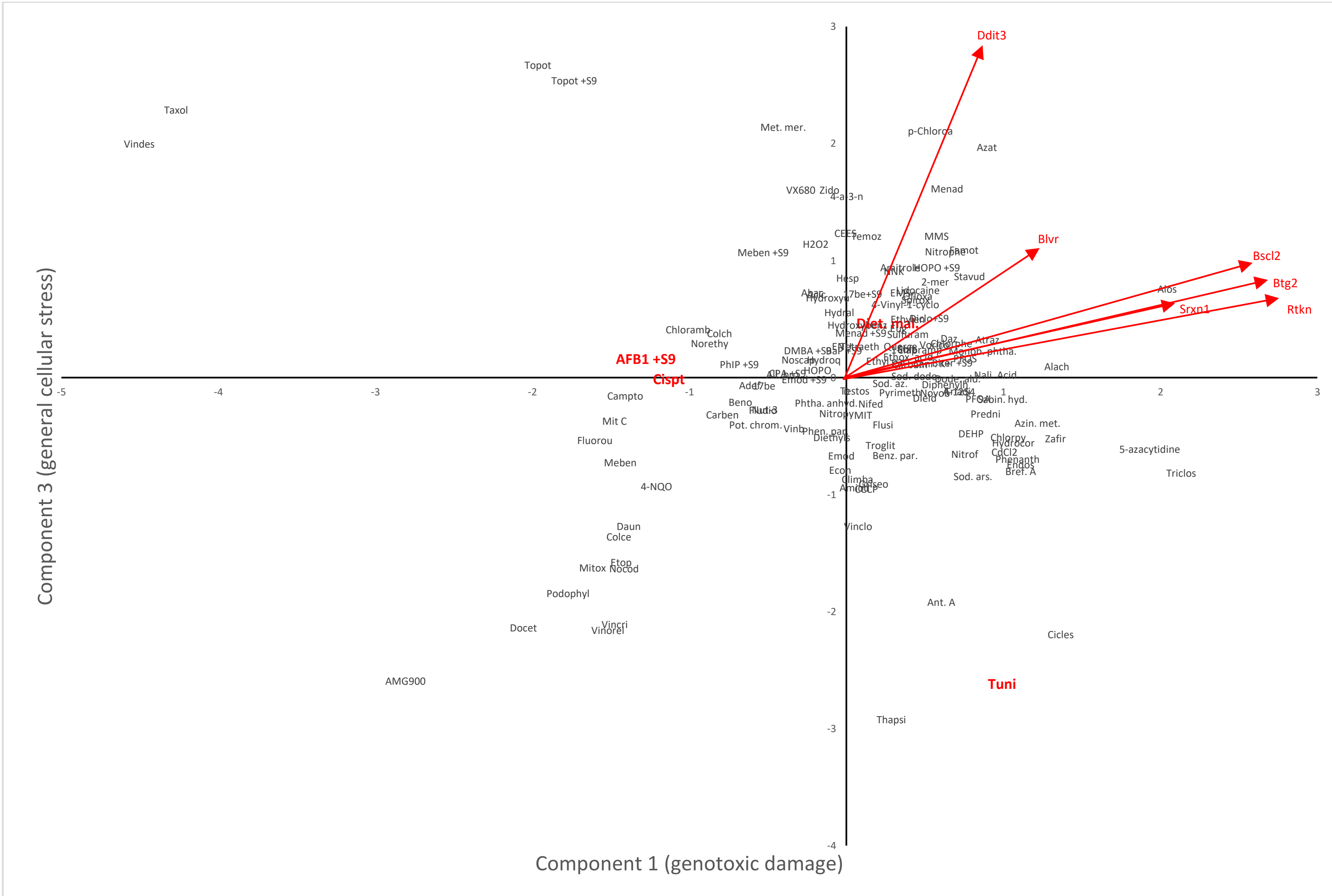

Figure 2.11c Principal Component Analysis results based on BMD values for each substance-reporter combination; Component 3 versus Component 1. Plot shows loadings of reporter responses on each component (i.e., axis) as red arrows. Arrow lengths and positions reflect loading values on each axis. The chemicals are represented according to the abbreviations in Table A7, with positive control substances shown in red. Analysis employed Varimax option to maximize percentage of variance explained by orthogonal axes.

2.4 Discussion

In vitro reporter-based assays for (geno)toxicity assessment afford the ability to rapidly and effectively assess chemical safety. The ToxTracker® assay employs six genetic reporters in engineered mES cells to assess a substance's ability to elicit a range of toxicological responses, e.g., DNA damage, oxidative stress, generalized toxicological stress, and protein damage. The format of the ToxTracker® assay, which can rapidly assess numerous substances in a single day, generates large amounts of dose-response data. This study analyzed historical ToxTracker® dose-response data, including data representing responses to over 90 substances used to evaluate assay performance (i.e., validation substances), as well as historical control data (i.e., background GFP signal). More specifically, the work presented investigated (1) the fold-change threshold to identify a significant positive response, (2) the BMRs (Benchmark Responses) representing small, adverse effects on ToxTracker® cells, (3) the relative potency of validation substances across all six reporters, and (4) the functional and statistical redundancy in the reporter responses.

2.4.1 Determination of fold-change thresholds for identification of reporter-specific positive responses

The distributions of control value ratios, i.e., range of fold-change values for unexposed controls, were used to determine the cut-offs for delineation of significant positive responses. Toxys B.V. previously stated that the activation of a ToxTracker® reporter is considered positive when the mean GFP level is >1.5-fold above the concurrent solvent control. According to Toxys, this threshold represents a signal level that is at least 5 times higher than the standard deviation of the background fluorescence of water/DMSO-exposed cells. The application of a 1.5-fold threshold cut-off allegedly permits identification of positive response with a confidence level greater than 99.9% (5). Toxys noted that a 1.5-fold increase should be used to denote a weak positive; 2-fold to denote a strong positive.

In comparison with the approach used by Toxys to determine cut-off values, the approach used herein is more appropriate for establishing the statistical limits of a fold-change value (i.e., a ratio). More specifically, Toxys examined the statistical limits of the fluorescence readings, and then calculated a *post-hoc* fold-change value; this study employed a bootstrapping approach to examine the statistical limits of actual florescence value ratios. The latter is more robust in light of the noted complexity of assessing the error associated with a ratio (6). As noted in Section 2.2.2, the cut-off values for a positive response determined in this study averaged 1.51-fold for the 95th percentile of the ratio distribution, and 1.74-fold for the 99th percentile. This corresponds to the values used by Toxys to delineate weak- and strong-positive ToxTracker® responses, respectively. Toxys noted that 1.5-fold is indicative of a weak positive response; 2-fold is indicative of a strong positive response. Thus, the 1.74-fold change value determined herein indicates that the 2-fold value advocated by Toxys is conservative, i.e., less than 1% chance that such an increase could be obtained by chance alone.

It is valuable to compare the fold-change threshold values noted above with those employed for interpretation of result for other *in vitro* reporter assays (Table 1.3), as well as traditional *in vitro* assays such as the *in vitro* mammalian cell gene mutation assays, and the *in vitro* mammalian cell micronucleus assay. Indeed, the GreenScreen® assay also uses a threshold of 1.5-fold change to differentiate between positive and negative results (21). Similarly, with respect to the MultiFlow™ multiplexed, reporter assay, threshold values used for identification of clastogens are also in the 1.5- to 2-fold range (22). In comparison, OECD test guidelines for the *in vitro* mammalian cell gene mutation assays and the *in vitro* mammalian cell micronucleus assay state that a test result is considered positive when there is a significant statistical difference from the control values at $p<0.05$ (23, 24). Threshold values used for the identification of genotoxicants in other reporter

assays (e.g., flow cytometric *in vitro* micronucleus assay and MultiFlow™) are in the range of 3-4.5-fold change (25, 26). The thresholds determined herein for the ToxTracker® assay are aligned with the recommendations of the OECD. More specifically, the 1.51-fold value for a weak positive is comparable to the statistical approach ($p<0.05$). Moreover, the 1.74-fold value for a strong positive is more conservative than the OECD recommendations.

2.4.2 Determination of reporter-specific BMR values

The choice of a BMR value is critically important when dose-response data are to be used for risk assessment. More specifically, as recently noted by White *et al.*, 2020, BMD values from *in vivo* animal studies, which are intended for use in the calculation of health based guidance values (HBGVs) such as the TDI (Tolerable Daily Intake) and PDE (Permitted Daily Exposure), must be based on a BMR reflective of adversity (8). In comparison, dose-response data generated using *in vitro* assays such as ToxTracker® assay, are primarily useful for qualitative identification of effects and determination of chemical MOA. Use of *in vitro* dose-response data for human health risk assessment is currently being explored; however, pharmacokinetic phenomena such as absorption, distribution, metabolism and excretion (ADME) necessitate the use of complex *in vitro*-to-*in vivo* (IVIVE) extrapolation models (27). Such models predict AED (administered equivalent *in vivo* dose) from *in vitro* concentration values, and the simulated dose-response relationship is used to calculate a PoD (point-of-departure) value such as the BMD. The PoD is then used as the basis for determination of a human exposure limit (i.e., HBGV). Although interesting, a detailed discussion on the use of *in vitro* BMD values, such as those presented herein, for human health risk assessment is beyond the scope of this thesis (27).

The choice of BMR is equally important if BMD values are to be used for meaningful cross-endpoint comparisons, e.g., comparisons of BMD values across the 6 ToxTracker® reporters. Thus,

with respect to current and future interpretations of ToxTracker® dose-response data, it is vitally important to determine reporter-specific BMRs; moreover, to compare and contrast the reporter-specific BMRs. The importance of endpoint-specific BMR values for interpretation of toxicological dose-response data was eloquently delineated in the aforementioned ES theory of Slob (Slob, 2016). That work noted that BMRs must be scaled relative to endpoint-specific maximum effect size (i.e., *M*). Moreover, that if the maximum effect size values (*M*) across the ToxTracker® reporters are similar, then BMRs aligned will also be similar, and cross-reporter BMD comparisons simplified accordingly.

In this study, two different approaches were used to determine reporter-specific BMR values, i.e., small, toxicologically-meaningful response increases. The first approach, which is based on the work of Zeller *et al*., employed the ratio of standard deviation to mean for the trimmed distribution of historical control values. Since it is based on analysis of historical control values, Zeller *et al.* noted that it should be considered as a BMR lower limit (11). The second approach, which is based on the Slob ES theory, employed endpoint-specific maximum effect size (*M*), or its surrogate, the within-group standard deviation (*s*), to calculate BMR. The first method resulted in BMR values from 2.2 to 7.0%, with an average of 3.9%; the second resulted in higher values, ranging from 33 to 58%, with an average of 43% (Table 2.2). Several factors need to be considered when comparing these ToxTracker® assay BMRs. The value determined using the Zeller *et al*. approach is dependent on the variability in the trimmed distribution of historical control values. Since the ToxTracker® dose-response data examined in this study were all generated by the same laboratory, and the experimental conditions for the solvent control is conserved, it is not surprising that the standard deviation of the trimmed distribution of historical control is only 2-7% of the mean. Thus, BMR values determined using the Zeller *et al.* approach could be termed the minimum

increase over background that is biologically significant (i.e., 4% above control). In contrast, BMR values based on the Slob ES theory are scaled according to the dynamic range of the endpoint, i.e., the difference between background and maximum possible effect (*M*). According to Slob, a small, toxicologically-meaningful response can be defined as 1/8 of Log(*M*), or the eight root of *M*. Thus, rather than reflecting the minimum significant response, they reflect the smallest response that is toxicologically-meaningful relative to the maximum possible response. Interestingly, the BMRs based on *M* are also more variable than those based on the Zeller *et al*. calculation. This is not surprisingly since the BMRs based on the Slob ES theory are calculated using the average standard deviation (*s*) across all tested doses, i.e., the surrogate for maximum response (*M*). Since substance preparation and cellular dosing are subject to experimental error, *s* can be expected to be more variable than the historical solvent control standard deviation used in the Zeller *et al.* approach. Interestingly, with respect to the other endpoints investigated by Slob, 2016, ToxTracker® BMRs based on maximum effect size (*M*) (Table 2.2) are relatively constant across the reporters, i.e., between 8.0 and 39.2. This is likely related to the functional similarity of the ToxTracker® endpoints. In contrast, *M* values reported by Slob, 2016, which span 27 distinct endpoints such as organ weight, tissue damage, and blood chemistry, range from a low of 1.4 to a high greater than 80,000. These latter values correspond to BMR values ranging from a low of 4.3% to a high of over 300%.

Taking into account the information above, and considering the low variability in historical control responses, it can be argued that the BMRs determined using Slob ES theory are more suitable for routine, cross-reporter interpretation of ToxTracker® dose-response data. These values correspond to a small, adverse effect, whereas the BMRs based on Zeller *et al*. approach only

indicate the minimum percentage increase over background that exceeds the historically-observed range.

It is interesting to compare the BMR values discussed above with the recommendation of different regulatory authorities, and/or the values discussed in the scientific literature. The Benchmark Dose technical guidance by the US EPA (United States Environmental Protection Agency) (9) states that ideally, the BMR for continuous data should be defined as a minimal level of change that is biologically significant for a given endpoint. In the event that such information is unavailable, other options can be used, such as using one standard deviation above the mean background levels. Moreover, the document also states that the selection of a BMR includes a judgement about the statistical and biological characteristics of the data, and the application of its resulting BMD. Guidance on how to make this judgement is not provided within the technical guidance document. The EFSA (European Food Safety Authority) guidance document on the use of the Benchmark Dose approach in risk assessment (28) outlines the weaknesses of using a BMR of one standard deviation, i.e., one standard deviation above the negative control. More specifically, it highlights that the resulting BMD would then be dependent on one particular study, and, due to study-specific factors (i.e., heterogeneity in experimental conditions), may not be reflective of the "true" BMD value. It also mentions that use of the 1 SD metric yields a BMD that cannot be translated into an equipotent dose in populations of cells or animals with larger within-group variation. Instead, the European Food Safety Agency (EFSA) recommends defining the BMD as a percent change in mean response as compared to background response. It highlights that although a BMR of 5% is generally recommended as default, it can be modified based on statistical or toxicological considerations.

White *et al.* also recommends against the use of the BMR_{1SD} method, since the use of historical control data with high variability will result in a higher standard deviation, which will lead to less restrictive BMD and BMDL values, i.e., higher values (8). White *et al.* also mention the need to determine endpoint-specific BMRs; moreover, that there is still controversy on how to determine a robust BMR for a toxicological endpoint. They also refer to the Slob approach, indicating that it can be used for robust determination of endpoint-specific BMR values. Unfortunately, it requires extensive collection and analysis of dose-response data from numerous studies covering a wide range of test articles (i.e., compounds).

Overall, the values presented herein, which employed both the Zeller *et al.* and Slob approaches, and were based on dose-response data from numerous ToxTracker® experiments, can be considered as robust endpoint-specific BMR values. Such values are required for (1) cross-endpoint (i.e., reporter) BMD comparisons, and (2) determination of BMD values that can be used for calculation of HBGVs. The latter requires the aforementioned *in vitro*-to-*in vivo* extrapolation, and determination of AEDs. As suggested earlier, this type of analysis is beyond the scope of the current study.

2.4.3 Benchmark Dose combined-covariate analysis for substance potency rankings

Using the Benchmark Dose combined-covariate approach, chemicals were ranked according to potency on each reporter. A BMR of 100% was specified, and the analyses yielded the doubling dose (i.e., BMD_{100}), plus upper and lower confidence limits. The BMD_{100} corresponds to an increase over control that is consistent with the Toxys fold-change cut-off for a strong positive response. In addition, a 100% increase is consistent with the 99^{th} percentile of the distribution of bootstrapped ratios discussed earlier. Moreover, although the choice of BMR is relatively

unimportant for comparative potency analyses, a higher BMR (i.e., 100%) will yield a more precise BMD, and an improved ability to discriminate potencies from one another.

Since this study primarily focused on the utility of the ToxTracker® reporter assay for genotoxicity assessment, the discussion of the relative potencies of the validation substances will focus on the *Rtkn* and *Bscl2* reporters. Nevertheless, since the *Srxn1*, *Blvrb* and *Btg2* reporters can also detect responses to substances that elicit genetic damage, relative ranking on those reporters will also be briefly discussed. Importantly, an extensive discussion of the relative ranking of all substances on all reporters, and their mechanistic interpretation, is beyond the scope of the thesis.

It is also important to note that only chemicals that were positive for multiple reporters were plotted on the potency figures (2.10a-f). Moreover, substances that had a BMD not significantly different from 0, or undefined confidence intervals (i.e., BMDL of 0 or BMDU of infinite) were also not included in the potency ranking. Details of the individual BMD values for each substance can be found in the Appendix II (Tables A1-6).

The *Rtkn* reporter is activated through DNA double strand breaks. It encodes the Rhotekin protein and has been associated with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) cytokine signaling pathway, a protein complex that plays a major role in processes such as cytokine expression, stress regulation, cell division and transformation. Its primary positive control is cisplatin, which is a known clastogen and mutagen (29). The additional positive control for metabolic activation, aflatoxin B1, is a known mutagen and carcinogen (30). Regarding the ranking of *Rtkn* potency values, the most potent substances are primarily strong clastogens (e.g., docetaxel, taxol, podophyllotoxin, etoposide, colcemid, nocodazole, daunorubicin). Interesting, all of these substances are cancer chemotherapy agents that cause cell death via severe chromosomal damage (31, 32). Although the primary positive control cisplatin is also a chemotherapeutic

clastogen (33), it is not among the most potent substances. Nevertheless, it still ranks in the upper 75th percentile. The positive control for metabolic activation, aflatoxin B1, also ranks in the upper 75th percentile. This substance can be enzymatically converted into a reactive metabolite (i.e., aflatoxin B1-8,9-epoxide), which subsequently forms guanine adducts (i.e., 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N^7-Guanine)). The adduct is unstable, and converts to formamidopyrimidine (FAPY)-base derivatives that can lead to an apurinic site (34). Studies report that the AFB1-N^7-guanine adduct can lead to G-to-T mutations (35, 36). The high potency of these substances on the *Rtkn* reporter is not surprising since clastogens cause chromosome breakage and/or mal-segregation. DNA damage by such chemicals will cause cell-cycle arrest that stimulates increase in the production of the RAD51 protein, which in turn stimulates the repair of DNA double strand breaks following the activation of NF-κB (37). This in turn will stimulate upregulation of repair genes and pathways, such as the *Chk1* signaling pathways controlled by ATM/ATR proteins.

Substances that yielded mid-range potency values for the *Rtkn* reporter include substances that can readily form bulky DNA adducts (e.g., AFB1, BaP, DMBA, PhIP). These substances can be enzymatically-converted into highly-unstable, reactive metabolites that readily form stable, bulky DNA adducts. For example, the electrophilic BaP metabolite BPDE (benzo[*a*]pyrene-diol-epoxide) yields N^2 guanine adducts that, when left unrepaired, can lead to mutations (38). Such agents can cause S-phase DNA replication stalling via increased production of the RAD51 repair protein (34, 39), which can in turn can lead to chromosomal breaks that necessitate upregulation of genes involved in DNA double-strand break repair (34), e.g., *ATM* and *ATR*. Thus, in response to the damage, production of repair proteins such as RAD51 are increased, which ultimately stimulates the activation of the *Rtkn* reporter (34). Another mid-range substance is vinblastine

sulphate. Like the top-ranking compounds, it is also a clastogenic cancer chemotherapeutic agent (40).

Substances that yielded low potency values for the *Rtkn* reporter include ENU, EMS, eugenol and sodium azide. Eugenol, which is a non-DNA-reactive antioxidant, has actually been reported to have a protective effect against DNA damage, and considered non carcinogenic and non-mutagenic by the USFDA (United States Food and Drug Administration) (41, 42). Nevertheless, it has been described as an *in vitro* positive that can indirectly elicit a genotoxic response due excessive cytotoxicity, and/or perturbations to the cell culture conditions (43). Interestingly, eugenol has been termed an *in vitro* false positive for *in vivo* genotoxicity and carcinogenicity (40). This would likely account for the fact that it is a less potent substance. Sodium azide is a positive control for the Ames assay, and a potent mutagen in many plants and bacteria. However, studies have shown that mammalian cells do not have the ability to convert the substance to a mutagenic intermediate (i.e., azidoalanine) (44). This likely account for the fact that the substance is a weak inducer of the *Rtkn* genotoxicity reporter (44). ENU is a weak-acting alkylating agent that transfers its ethyl group to nitrogen or oxygen in DNA, resulting in base mispairing that causes base-pair substitution mutations (45, 46). The ethyl group of EMS is transferred to guanine forming O^6-ethylguanine; the unrepaired adduct can lead to mutations, cell cycle arrest, or apoptosis. It can also lead to DNA double strand breaks in the S/G_2 transition phase of the cell cycle (47). This in turn would induce the *Rtkn* reporter via activation of the NF-κB cytokine signaling pathway.

With respect to substance potency rankings for *Bscl2*, the results show ranking that is similar to that described for *Rtkn.* Activation of the *Bscl2* reporter (i.e., Bernardinelli-Seip Congenital Lipodystrophy Type 2) is controlled by ATM and ATR; elements of the checkpoint kinase (*Chk*) 1 signaling pathway induced by DNA damaging agents (48). Since DNA double strand breaks

(i.e., *Bscl2* induction) will induce DNA replication stall (i.e., *Rtkn* induction), it is expected that the potency ranking on the two reporters will be similar (37). Like the *Rtkn* reporter, the top-ranking inducers of *Bscl2* are clastogens such as docetaxel, podophyllotoxin, taxol, etoposide, colcemid, nocodazole, and daunorubicin. Again, the positive controls, cisplatin and aflatoxin B1, rank in the upper 75th percentile. As previously mentioned, clastogenic substances cause DNA strand breaks and cell cycle arrest. In response to the damage, proteins involved in DNA repair are activated, e.g., activation of the ATM kinase that contributes to a signalling cascade contributing to activation of the *Bscl2* reporter (5, 49). Similar to what was observed for *Rtkn*, the mid-ranking substances (e.g., AFB1, PhIP, DMBA) include those that can be converted to highly-unstable electrophiles that readily form DNA adducts. The formation of stable adducts will cause S-phase cell cycle stalling and DNA strand breaks, which will in turn activate the ATR/ATM signaling pathways associated with *Bscl2* (5, 50).

As for the low-ranking substances, they also include EMS, eugenol and sodium azide. As indicated earlier, EMS can induce DNA double strand breaks and cell cycle arrest (47). This would also induce the *Bscl2* reporter through DNA-damage-associated recruitment of ATR/ATM (5, 50). In contrast, eugenol is considered non carcinogenic and non-mutagenic by the USFDA (United States Food and Drug Administration) (41, 42). As for sodium azide, as already indicated, it has a limited mutagenic potential in mammalian cells due to cellular inability to generate the substance's mutagenic intermediate azidoalanine (44).

As indicated above, rankings for selected substances on the two DNA damage reporters are similar; in fact, review of the ranking shown in Figures 2.10a-b does not show any major differences in substance rankings. This is consistent with the mechanistic overlap in the pathways responsible for inducing those reporters. Both ATR and ATM, which are part of the induction

pathway of the *Bscl2* reporter, interact with the NF-κB, which in turn is associated with upregulation of *Rtkn* via the NF-κB essential modulator (NEMO) (51). Some differences include the previously mentioned mid-range substances, i.e., AFB1, BaP, and DMBA, which rank higher for the *Rtkn* reporter relative to the *Bscl2* reporter. This could indicate that the DNA strand breaks created by those substances precede the cell cycle arrest that induces the *Bscl2* reporter. In any case, it is important to recognise that induction of the genotoxicity reporters is a dynamic process involving numerous cellular pathways; there is ample opportunity for pathway crossover that can result in activation of both reporters. Indeed, the PCA results, which are discussed in a subsequent section, are entirely consistent with the documented overlap in the signalling cascades that contribute to the activation of both of the genotoxicity reporters.

The same relative ranking can be applied to the *Btg2* reporter associated with general cellular stress mediated by P53 activation. More specifically, substances with a high potency include the aforementioned clastogens such as docetaxel, taxol, nocodazole, podophyllotoxin, etoposide, colcemid and daunorubicin; mid-ranking substances include AFB1, BaP and DMBA, and low-ranking substances include eugenol, EMS and sodium azide. The positive controls cisplatin and aflatoxin B1 again rank in the upper 75th percentile. It has been suggested that the *Btg2* gene (i.e., B-cell translocation gene 2) is stimulated by activated P53, thereby playing a role in responses to different types of DNA damage, and concomitant activation of apoptosis and DNA repair. It is likely that P53 activation facilitates these responses via G1 growth arrest (52). The high and mid-ranking substances mentioned earlier elicit DNA damage through several mechanisms; studies have shown that ATM, which induces the *Bscl2* reporter, is also implicated in response to DNA double strand breaks (49), and in the activation of the P53-mediated DNA damage response (53).

The overlap in the reporter pathways for DNA damage and general cellular stress likely accounts for the high-level ranking similarities.

The same relative conclusions can be made for the oxidative damage reporters, i.e., *Blvrb* and *Srxn1*. The *Blvrb* (biliverdin reductase B) reporter measures oxidative damage through the HMOX1 signaling pathway, which is regulated through NRF2. The rankings for this reporter compare to the other reporter for oxidative damage, *Srxn1*, which is controlled by the NRF2 signaling pathway. The highest-ranking substances also include colcemid, etoposide, daunorubicin and podophyllotoxin, which is similar to the rankings for the genotoxicity reporters. Studies have shown that these substances also induce oxidative damage (54, 55). Other known inducers of oxidative stress include potassium chromate, which contains the oxidant hexavalent chromium (56). Hydrogen peroxide is also a major redox metabolite that induces oxidative stress via the NRF2/Keap1 pathway (57). Interestingly, the former ranks in the upper 75th percentile, even higher than the positive control diethyl maleate, that is among the mid-ranking substances for the *Srxn1* reporter, and slightly below the upper 75th percentile for the *Blvrb* reporter. In comparison, the ranking of the latter substance is amongst the lowest.

Overall, although the definitive rankings of each substance on each reporter differ from each other, the relative positions of the chemicals are similar; there are no major differences between the substances' relative rankings. For both the DNA damage and the oxidative stress reporters, top ranking substances include potent clastogens, such as docetaxel, taxol, nocodazole, podophyllotoxin, colcemid, etoposide and daunorubicin. Mid-ranking substances include substances that can readily form DNA adducts, such as AFB1, BaP and DMBA. Finally, for the DNA damage reporters, low-ranking substances include eugenol, an *in vitro* false positive, as well as EMS and sodium azide. The rankings of these substances are also low for the oxidative stress

reporters *Blvrb* and *Srxn1*. The consistency in these rankings indicates functional overlap in the cellular pathways underlying induction of the genotoxicity and oxidative stress reporters. As mentioned, clastogens elicit DNA strand breaks, as well as cell cycle stalling. More specifically, proteins implicated in the DNA damage response include RAD51, which is modulated through NF-κB (5, 34). NF-κB, which is associated with the activation of the *Rtkn* reporter, can also be activated through oxidative stress, which induces the *Blvrb* and *Srxn1* reporters (5, 58). ATR and ATM, which are involved the cellular signalling cascade that stimulates the *Bscl2* reporter, are also involved in the DNA damage response. Those proteins are also implicated in the P53 cellular stress response, which induces *Btg2*. As for *Blvrb* and *Srxn1* reporters, substances that induce DNA damage, and thus the aforementioned reporters, can also elicit oxidative damage through a variety of mechanisms. For example, substances that elicit DNA adducts, such as BaP, can also create ROS through the production of *o*-quinones and redox cycling (19). Clastogenic substances can also induce the generation of ROS through the secretion and expression of proinflammatory cytokines, such as TNF-α and IL-1β (59). The PCA analysis, which is discussed in the succeeding section, provides empirical insight into the functional relationships between the ToxTracker® reporters.

2.4.4 Principal Component Analysis

BMD values for 144 treatments, across all six reporters, were used as the basis for PCA (Principal Component Analysis). The results revealed that the first three components provide an adequate summary of the data; accounting for over 93% of the variance in BMD values (Table 2.5). The bi-plots of orthogonal principal components 1, 2 and 3 show the loading patterns, as well as the axis-specific scores for each substance (Figures 2.11a-c). The loading values for each reporter (Table 2.6) on each orthogonal axis (i.e., Principal Component) reflect the relationship between the individual reporter variables and the multivariate axes. Given the very high loadings

of *Rtkn*, *Bscl2* and *Btg2* (i.e., >0.85), the results clearly indicate that axis 1 is a genotoxic stress axis. Thus, it can be argued that these three reporters are redundant, i.e., they are all indicative of genotoxicity. The very high loading of *Blvrb* (0.82) clearly indicates that axis 2 is an oxidative stress axis. Since it is the only reporter that is uniquely aligned with axis 2, it can be argued that this reporter is essential for toxicological profiling of tested substances. Interestingly, given the moderate loadings on axes 1 and 2 (i.e., 0.68 and 0.56), the *Srxn1* appears to reflect both oxidative and gentoxic stress. The very high loading of *Ddit3* (0.92) indicates that axis 3 is clearly a generalized toxicological stress axis reflective of effects related to protein damage.

These results are entirely consistent with the aforementioned functional inter-connectedness of the toxicological response pathways leading to upregulation of the six ToxTracker® reporters. For example, since the pathways leading to upregulation of *Bscl2*, *Rtkn* and *Btg2* are all influenced by ATM/ATR, coordination of reporter induction is expected. More specifically, *Rtkn* is activated by NF-κB, which stimulates the removal of DNA double strand breaks, and *Bslc2* is regulated by ATR and ATM, elements of the checkpoint kinase (*Chk*) 1 signaling pathway induced by DNA damaging agents (60). As noted earlier, both ATR and ATM interact with the NF-κB via its essential modulator (NEMO) (51). The *Btg2* reporter is activated by P53-mediated cellular stress; as previously mentioned, ATM is also involved in the P53-mediated cell response, indicating that the DNA damage and replication stall responsible for the induction of *Bscl2* should also play a role in the induction of *Btg2*.

Since the *Blvrb* reporter is regulated by HMOX1, which is known to respond to agents that contribute to intracellular ROS, it is not surprising that it is uniquely associated with a single axis reflective of oxidative stress. However, given the control of the *Srxn1* reporter by the NRF2-signalling pathway, which is also known to be induced by agents that contribute to intracellular

ROS, it is somewhat surprising that reporter response is only moderately aligned with axis 2. Relatedly, the alignment of the *Srxn1* reporter with axis 1 is also surprising since that axis clearly reflects genotoxic rather than oxidative stress. However, close inspection of the NRF2 signaling pathway, and the pathways induced by genotoxic stress, shows that they are indeed inter-connected. More specifically, there has been reports of multiple interactions and cross-talk between NRF2 and NF-κB. For example, absence of NRF2 is associated with increased oxidative stress, which increases the production of cytokines via expression of NF-κB (61). In addition, inhibition of NRF2 causes repression of ATM and ATR (62). Moreover, close inspection of the pathway that controls *Blvrb* induction (i.e., HMOX1-controlled pathway) indicates that while HMOX1 is regulated through NRF2, it is quite distinct from the pathways related to genotoxic stress.

Lastly, it is not surprising that the *Ddit3* reporter is uniquely associated with a single axis (i.e., axis 3). The reporter is induced by toxicological stress leading to protein unfolding (5), which is controlled by a signalling pathway that is distinct from those related to oxidative and genotoxic stress. More specifically, the *Ddit3* response is activated by endoplasmic reticulum stress. Changes in endoplasmic reticulum homeostasis induce a signal transduction to the cytoplasm and nucleus, causing a compensating response that promotes apoptosis. This increases expression of the DDIT3 protein, which forms heterodimers with other members of the CCAAT/enhancer-binding protein (C/EPB) family, thereby preventing their DNA binding activity. Proteins of this family are known to control responses to cellular stress (63, 64). Interestingly, *Ddit3* is also a target of P53, and it has been shown to be significantly induced in response to hypoxia (65). Nevertheless, *Ddit3* loading on axes 1 and 2 are both below 0.30.

It is interesting to note that few substances are located in the upper-left quadrant of the axis 2 versus axis 1 plot (i.e., high genotoxic stress, low oxidative stress). Indeed, most of the potent genotoxicants (e.g., docetaxel, podophyllotoxin, etoposide, mitomycin C, cisplatin, colcemid, nocodazole, vinblastine, etc.) are located in the lower-left quadrant, which would correspond to substances that elicit both strong genotoxic and oxidative stress responses. This is not unexpected since, as mentioned earlier, many genotoxic substances are known to elicit genotoxic effects via direct interactions with DNA or whole chromosomes, and/or the apparatus that ensures chromosomal integrity and replicative fidelity (e.g., centrosome), as well as via indirect, adverse interactions with the genome via ROS generation. These substances are all used as chemotherapeutic agents that cause cell death. For example, etoposide causes an accumulation of covalent enzyme-cleaved DNA complexes that lead to DNA strand breaks, eventually initiating death pathways (29). Docetaxel is a microtubule-stabilizing taxane that induces cell death via mitotic catastrophe (66). Colcemid, nocodazole, podophyllotoxin and vinblastine, inhibit cell division through their effect on mitotic assembly (28). Potassium chromate is an interesting example of a substance that would be expected to be in the lower-left quadrant of the axis 2 versus axis 1 orthogonal bi-plot. The substance, which contains a hexavalent chromium, is a well-known genotoxicants that causes genetic damage via generation of ROS and formation of oxidative DNA lesions, i.e., both genotoxic an oxidative stress (56). The oxidative lesions, such as 8-oxo-dG (8-oxo-2'-deoxyguanosine), are known to contribute to base-pair substitution mutations, i.e., G-to-T transversions (67).

In direct contrast to the upper-left quadrant, the lower-right quadrant of the axis 2 versus axis 1 bi-plot reflects potent induction of oxidative stress, and relatively weak induction of genotoxic stress. Examples include compounds such as sodium arsenite, cadmium chloride, alosetron,

alachlor, the positive control diethyl maleate, and methylmethane sulfonate. Methylmethane sulfonate is a monofunctional alkylating agent that causes mainly 7-methylguanine and 3-methyladenine adducts, resulting in GC-AT transitions (36). It also inhibits cellular respiration and rapidly increases ROS production (68). Sodium arsenite and cadmium chloride are examples of substances that would certainly be expected to be in the lower-right quadrant. Both substances are potent inducers of oxidative stress; generation of intracellular ROS is associated with aforementioned oxidative DNA lesions and associated mutations (69, 70). The results reveal that these substances are comparatively weak mutagens, i.e., relative to potassium chromate.

Substances located in the upper-right quadrant of the axis 2 versus axis 1 bi-plot would be considered relatively weak inducers of both genotoxic and oxidative stress. Examples include substances such as phenanthrene, ciclesonide, hydrocortisone and atrazine. Like BaP, phenanthrene is metabolized by CYP1A1/1A2 enzymes; however, in contrast, it cannot form a mutagenic diol-epoxide (71). Studies also show that it induces mild oxidative stress (72). Atrazine causes endocrine disruption; studies have shown that it is not genotoxic (73). The substance also can induce mild oxidative stress via modulation of NRF2 (74).

The additional bi-plots, which show the relationships between (1) axis 3 and axis 2, and (2) axis 3 and axis 1, i.e., Figures 2.11b and 2.11c, respectively, allow detection of substances that elicit (1) a strong protein damage response and/or oxidative stress response, and (2) a strong protein damage response and/or genotoxic stress response. For example, the lower-left quadrant of Figure 2.11b (i.e., axis 3 versus axis 2) includes substances that are potent inducers of the protein damage response, as well as the oxidative stress response. Examples include substances such as vincristine, mitoxanthrone, etoposide, podophyllotoxin, nocodazole, and daunomycin. This result is not surprising since these aforementioned cancer chemotherapy agents that are highly potent toxicants

capable of eliciting a wide range of adverse cellular effects (31, 32). In contrast, substances in the lower-right quadrant are potent inducers of the protein damage response, but relatively weak inducers of oxidative stress. Examples include tunicamycin, AMG900, ciclesonide, thapsigargin, and antimycin A. Tunicamycin, the positive control for the *Ddit3* reporter, as well as thapsigargin, are known for their ability to induce endoplasmic reticulum stress, resulting in the activation of the unfolded protein response (75, 76).

With respect to Figure 2.11c, substances in the lower-left quadrant would be those that are potent inducers of genotoxic and protein damage stress responses. Examples include the previously-highlighted substances docetaxel, podophyllotoxin, AMG900, etoposide, daunorubicin, nocodazole, and vincristine. As mentioned earlier, such cancer chemotherapy agents commonly elicit a wide range of adverse cellular effects (31, 32). Finally, substances in the lower-right quadrant would induce a protein damage response, but a relatively weak genotoxicity stress response. Examples include substances such as tunicamycin, thapsigargin, and antimycin A. As previously mentioned, both tunicamycin and thapsigargin are inducers of endoplasmic reticulum stress, and can also lead to accumulation of lipids, cell death, cytolysis, and inflammation (75, 76).

The absence of a discussion regarding substances in the upper-left and upper-right quadrants of Figures 2.11b and 2.11c is noteworthy. All substances on the top half of Figures 2.11b and 2.11c would be relatively weak inducers of a protein damage response; thus, their toxicological import with respect to this type of effect might be considered minimal. With respect to the upper-left of Figure 2.11b, the substances in this quadrant would be considered potent oxidative stressors, i.e., they have already been discussed. Similarly, with respect to the upper-left of Figure 2.11c, the substances in this quadrant would be considered potent genotoxic stressors; they have also been discussed.

Overall, the PCA results indicate that the ToxTracker® reporters are not entirely independent. The loadings of the various reporter variables on the first 3 Principal Components indicate that some of the reporters are functionally and statistically redundant. More specifically, the Principal Components indicate three types of orthogonal cellular stress, i.e., genotoxic, oxidative, and endoplasmic reticulum stress characterized by protein damage and unfolding. With respect to gentoxic stress, the axis 1 reporter loadings suggest that either one of *Rtkn*, *Bscl2* or *Btg2* could be employed to identify genotoxicants. Nevertheless, the functional redundancy of these three ToxTracker® reporters would ensure effective detection of genotoxicity, a toxicological hazard that is enshrined in chemical safety policies and legislation worldwide. Interestingly, the results show that the *Srxn1* reporter also supports detection of gentoxic stressors. With respect to oxidative stress, the axis 2 loading values indicate that the *Blvrb* reporter is essential; the *Srxn1* reporter also supports detection of substances that induce oxidative stress. With respect to endoplasmic reticulum stress characterized by protein damage and unfolding, the axis 3 loadings clearly indicate that the *Ddit3* reporter is orthogonal to the genotoxic and oxidative stress reporters, and therefore an essential element of the ToxTracker® assay system.

With respect to the MOA of each tested compound, the compound-specific scores on each PCA axis provides toxicologically-pertinent information. For example, axis scores for cancer chemotherapy agents such as docetaxel, taxol, nocodazole and daunorubicin, etoposide, and podophyllotoxin indicate that they are potent cellular toxicants that elicit genotoxic, oxidative, and endoplasmic reticulum stress. In contrast, substances such as sodium arsenite and cadmium chloride are strong inducers of oxidative stress, but weak inducers of genotoxic stress. Similarly, the positive control tunicamycin is a potent inducer of endoplasmic reticulum stress, but a weak inducer of genotoxic or oxidative stress. In principal, the standardised scoring coefficients (not

shown) associated with each principal component could be used to convert reporter-specific BMD_{100} values into axis scores that reflect MOA (i.e., genotoxic, oxidative, etc.). This MOA information could subsequently be used to generate hypotheses about cellular changes associated with selected toxicant exposures (e.g., oxidative DNA damage); moreover, to identify undesirable toxicological effects of therapeutic product candidates.

In summary, this work employed a variety of quantitative strategies to analyse and interpret large amounts of ToxTracker® dose-response data. First, a large historical database of control response values was used to determine robust cut-offs for the identification of significant positive responses for each reporter; values were determined by using a bootstrapping approach to determine the 95^{th} and 99^{th} percentiles of the distribution of possible fold-change control values. Second, endpoint-specific BMRs were defined using both the Zeller *et al*. method, and the Slob effect size theory. The differences in BMRs highlighted each method's assumptions and underlying principles; moreover, the lack of consensus in the literature regarding the optimal approach for determination of endpoint-specific BMR values. Theoretical considerations outlined by Slob (2016) indicate that the approach based on ES theory should provide more appropriate reporter-specific BMR values for the ToxTracker® assay, i.e., values scaled to the dynamic range rather than the statistical limits of the background response. Third, the BMD combined-covariate approach was used to rank chemical potency for each ToxTracker® reporter. The genotoxicity reporters (e.g., *Rtkn* and *Bscl2*) showed similar substance potency rankings, with clastogenic and aneugenic cancer chemotherapeutic agents being the most potent chemicals; the potency of the positive controls used to ensure assay performance ranked in the upper 75^{th} percentile. Responses to some chemicals on the oxidative stress reporters (e.g., *Srxn1*) revealed rankings similar to the genotoxicity reporters, suggesting that numerous substances elicit genotoxic and oxidative stress.

This is not surprising since some well-studied genotoxic substances (e.g., BaP) are known to form stable bulky DNA adducts and generate ROS through metabolite redox cycling (16). Overall, the rankings readily permit potency evaluations of assay validation substances for each of the six reporters; these ranking can be used by Toxys as a point of reference to objectively evaluate the potency of confidential test articles submitted for toxicological screening. Lastly, BMD values for all validation chemicals on all reporters were used in a PCA to investigate the statistical and functional relationships between the reporters. The results show that the ToxTracker® reporters can be divided into 3 toxicological components corresponding to DNA damage, oxidative stress and general cellular stress characterized by protein damage and unfolding. These results support the notion that cross-talk between cellular pathways likely accounts for simultaneous induction of multiple ToxTracker® reporters, e.g., genotoxic substances that also cause oxidative damage and/or generalized toxicological stress characterized by protein damage and unfolding. Overall, the results obtained contributed to the development of a comprehensive quantitative framework for efficient and effective interpretation of ToxTracker® assay dose-response data.

2.5 References

1. Organisation for Economic Co-operation and Development. Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines. Organisation for Economic Co-operation and Development Publishing. Paris, 2015:1-64. https://www.oecd.org/env/ehs/testing/Draft%20Guidance%20Document%20on%20OECD%20Genetic%20Toxicology%20Test%20Guidelines.pdf

2. Organisation for Economic Co-operation and Development. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Organisation for Economic Co-Operation and Development Publishing. Paris, 2005;14(34):96. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd-gd34.pdf

3. Li AP, Loretz LJ. Assays for Genotoxicity. In: Li AP, Heflich RH, editors. Genetic Toxicology. 1st ed. Boca Raton, Florida: CRC Press; 1946. p. 119-42.

4. MacGregor JT, Frötschlb R, White PA, Crump KS, Eastmonde DA, Fukushimaf S, Guérardg M, Hayashih M, Soeteman-Hernándezi LG, Kasamatsuj T, Levyk, DD, Morital T, Müllerm L, Schoenyn R, Schulero MJ, Thybaud V, Johnson GE. IWGT report on quantitative approaches to genotoxicity risk assessment I. Methods and metrics for defining exposure–response relationships and points of departure (PoDs). Mutation Research. 2015;783:55-65.

5. Hendriks G, Atallah M, Morolli B, Calleja, Fabienne M. G. R., Ras-Verloop N, Huijskens I, Raamsman M, van de Water B, Vrieling H. The ToxTracker assay: novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences. 2012;125(1):285-298.

6. Welsh A, Peterson A, Altmann S. The fallacy of averages. The American Naturalist. 1998;132(2):277-288.

7. Liermann M, Steel A, Rosing M, Guttorp P. Random denominators and the analysis of ratio data. Environmental and Ecological Statistics. 2004;11:55-71.

8. White PA, Long AS, Johnson GE. Quantitative Interpretation of genetic toxicity dose-response data for risk assessment and regulatory decision-making: current status and emerging priorities. Environmental and Molecular Mutagenesis. 2020;61(1):66-83.

9. United States Environmental Protection Agency (USEPA). Benchmark Dose Technical Guidance. https://www.epa.gov/risk/benchmark-dose-technical-guidance. 2012.

10. Slob W. A general theory of effect size, and its consequences for defining the benchmark response (BMR) for continuous endpoints. Critical Reviews in Toxicology. 2016;47(4):342-351.

11. Zeller A, Duran-Pacheco G, Guérard M. An appraisal of critical effect sizes for the benchmark dose approach to assess dose–response relationships in genetic toxicology. Archives of Toxicology. 2017;91(12):3799-3807.

12. Haber LT, Dourson ML, Allen BC, Hertzberg RC, Parker A, Vincent MJ, Maier A, Boobis AR. Benchmark dose (BMD) modeling: current practice, issues, and challenges. Critical Reviews in Toxicology. 2018;48(5):387-415.

13. Wills JW, Johnson GE, Doak SH, Soeteman-Hernández LG, Slob W, White PA. Empirical analysis of BMD metrics in genetic toxicology part I: *in vitro* analyses to provide robust potency rankings and support MOA determinations. Mutagenesis. 2016;31(3):255-263.

14. Slob W, Setzer RW. Shape and steepness of toxicological dose–response relationships of continuous endpoints. Critical Reviews in Toxicology. 2014;44(3):270-297.

15. Wills JW, Johnson GE, Battaion HL, Slob W, White PA. Comparing BMD-Derived genotoxic potency estimations across variants of the transgenic rodent gene mutation assay. Environmental and Molecular Mutagenesis. 2017;58(9):632-643.

16. Zhang L, Jin Y, Huang M, Penning TM. The role of human aldo-keto reductases in the metabolic activation and detoxication of polycyclic aromatic hydrocarbons: interconversion of PAH catechols and PAH o-quinones. Frontiers in Pharmacology. 2012;3:193.

17. Goldberg RJ. PROC FACTOR: How to interpret the output of a real-world example. http://www2.sas.com/proceedings/sugi22/STATS/PAPER268.PDF. 1997.

18. Hastwell P, Chai L, Roberts K, Webster T, Harvey J, Rees R, Brown A. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: Validation of the GreenScreen HC *GADD45a-GFP* genotoxicity assay. Mutation Research. Genetic toxicology and environmental mutagenesis. 2006;607(2):160-175.

19. Succop PA, Clark S, Chen M, Galke W. Imputation of data values that are less than a detection limit. Journal of Occupational and Environmental Hygiene. 2004;1(7):436-441.

20. Schisterman E, Hu J, Whitcomb B. Limit of detection bias and the correction of variance estimates. Epidemiology. 2004;15(4):S157.

21. United States Environmental Protection Agency (USEPA). Guidelines for Statistical Analysis of Occupational Exposure Data. https://www.epa.gov/sites/production/files/2015-09/documents/stat_guide_occ.pdf. 1994.

22. Bryce S, Bernacki D, Bemis J, Spellman R, Engel M, Schuler M, Lorge E, Heikkinen PT, Hemmann U, Thybaud V, Wilde S, Queisser N, Sutter A, Zeller A, Guerard M, Kirkland D, Dertinger SD. Interlaboratory evaluation of a multiplexed high information content in vitro genotoxicity assay. Environmental and Molecular Mutagenesis. 2017;58:146-161.

23. Organisation for Economic Co-operation and Development (OECD). Test No. 487: In Vitro Mammalian Cell Micronucleus Test. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, 1997. https://www.oecd.org/chemicalsafety/test-no-487-in-vitro-mammalian-cell-micronucleus-test-9789264264861-en.htm.

24. Organisation for Economic Co-operation and Development (OECD). Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, 1997. https://www.oecd.org/chemicalsafety/test-no-476-in-vitro-mammalian-cell-gene-mutation-tests-using-the-hprt-and-xprt-genes-9789264264809-en.htm.

25. Annemette V, Thougaard J, Christiansen TM, Hornberg JJ. Validation of a high-throughput flow cytometric in vitro micronucleus assay including assessment of metabolic activation in TK6 cells. Environmental and Molecular Mutagenesis. 2014;55:704-718.

26. Bryce SM, Avlasevich SL, Bemis JC, Dertinger SD. Miniaturized flow cytometry-based CHO-K1 micronucleus assay discriminates aneugenic and clastogenic modes of action. Environmental and Molecular Mutagenesis. 2011;52(4):280-286.

27. Wetmore BA, Wambaugh JF, Ferguson SS, Sochaski MA, Rotroff DM, Freeman K, Clewell HJ III, Dix DJ, Andersen ME, Houck KA, Allen B, Judson RS, Singh R, Kavlock RJ, Richard AM, Thomas RS. Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. Toxicological Sciences. 2012;125(1):157-174.

28. European Food Safety Authority (EFSA). Guidance of the Scientific Committee on a request from EFSA on the use of the benchmark dose approach in risk assessment. EFSA Journal. Parma, 2009;7(6):1-72.

29. Greene MH. Is cisplatin a human carcinogen? Journal of the National Cancer Institute. 1992;84(5):306-312.

30. Lin Y, Li L, Makarova AV, Burgers PM, Stone MP, Lloyd RS. Molecular basis of aflatoxin-induced mutagenesis—role of the aflatoxin B1-formamidopyrimidine adduct. Carcinogenesis. 2014;35(7):1461-1468.

31. Rieder CL, Palazzo RE. Colcemid and the mitotic cycle. Journal of Cell Science. 1992;102:387-392.

32. Baldwin EL, Osheroff N. Etoposide, topoisomerase II and cancer. Current Medicinal Chemistry Anti-Cancer Agents. 2005;5(4):363-372.

33. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. European Journal of Pharmacology. 2014:364-378.

34. Fasullo M, Chen Y, Bortcosh W, Sun M, Egner PA. Aflatoxin B1-associated DNA adducts stall S phase and stimulate Rad51 foci in *Saccharomyces cerevisiae*. Journal of Nucleic Acids. 2010;2010:456487.

35. Smela ME, Hamm ML, Henderson PT, Harris CM, Harris TM, Essigmann JM. The aflatoxin B1 formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(10):6655-6660.

36. Boysen G, Pachkowski BF, Nakamura J, Swenberg JA. The formation and biological significance of *N*7-Guanine adducts. Mutation Research. 2009;678(2):76-94.

37. Haaf T, Raderschall E, Reddy G, Ward DC, Radding CM, Golub EI. Sequestration of mammalian Rad51-recombination protein into micronuclei. Journal of Cell Biology. 1999;144(1):11-20.

38. Piberger AL, Krüger CT, Strauch BM, Schneider B, Hartwig A. BPDE-induced genotoxicity: relationship between DNA adducts, mutagenicity in the in vitro PIG-A assay, and the transcriptional response to DNA damage in TK6 cells. Archives of Toxicology. 2018;92(1):541-551.

39. Brown KL, Voehler MW, Magee SM, Harris CM, Harris TM, Stone MP. Structural perturbations induced by the α-anomer of the aflatoxin B1 formamidopyrimidine adduct in duplex and single-strand DNA. Journal of the American Chemical Society. 2009;131(44):16096-16107.

40. Tazi A, Lorillon G, Haroche J, Neel A, Dominique S, Aouba A, Bouaziz J, de Margerie-Melon C, Bugnet E, Cottin V, Comont T, Lavigne C, Kahn J, Donadieu J, Chevret S. Vinblastine chemotherapy in adult patients with langerhans cell histiocytosis: a multicenter retrospective study. Orphanet Journal of Rare Diseases. 2017;12(1):95.

41. Yogalakshmi B, Viswanathan P, Anuradha CV. Investigation of antioxidant, anti-inflammatory and DNA-protective properties of eugenol in thioacetamide-induced liver injury in rats. Toxicology. 2010;268:204-212.

42. Zhang L.L., Zhang L.F., Xu J.G., Hu Q.P. Comparison study on antioxidant, DNA damage protective and antibacterial activities of eugenol and isoeugenol against several foodborne pathogens. Food and Nutrition Research. 2017;61(1):1353356.

43. Maertens RM, Long AS, White PA. Performance of the in vitro transgene mutation assay in MutaMouse FE1 cells: Evaluation of nine misleading ("False") positive chemicals. Environmental and Molecular Mutagenesis. 2017;58(8):582-591.

44. Arenaz P, Hallberg L, Mancillas F, Gutierrez G, Garcia S. Sodium azide mutagenesis in mammals: inability of mammalian cells to convert azide to a mutagenic intermediate. Mutation Research Letters. 1989;227(1):63-67.

45. Olson E, Graham D. Chapter 5 - Animal Models in Pharmacogenomics. In: Handbook of Pharmacogenomics and Stratified Medicine. Elsevier; 2014. p.73-87.

46. Dobo KL, Fiedler RD, Gunther WC, Thiffeault CJ, Cammerer Z, Coffing SL, Shutsky T, Schuler M. Defining EMS and ENU dose-response relationships using the *Pig-a* mutation assay in rats. Mutation Research. 2011;725(1-2):13-21.

47. Quiros S, Roos PW, Kaina B. Processing of O6-methylguanine into DNA double-strand

breaks requires two rounds of replication whereas apoptosis is also induced in subsequent cell cycles. Cell Cycle. 2010;9(1):168-178.

48. Hendriks G, Derr RS, Misovic B, Morolli B, Calleja, Fabienne M. G. R., Vrieling H. The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. Toxicological Sciences. 2016;150(1):190-203.

49. Wakasugi M, Sasaki T, Matsumoto M, Nagaoka M, Inoue K, Inobe M, Horibata K, Tanaka K, Matsunaga T. Nucleotide excision repair-dependent DNA double-strand break formation and ATM signaling activation in mammalian quiescent cells. Journal of Biological Chemistry. 2014;289(41):28730-28737.

50. Jeffrey AM. DNA modification by chemical carcinogens. Pharmacology & Therapeutics. 1985;28:237-272.

51. Wu Z, Miyamoto S. Induction of a pro-apoptotic ATM–NF-κB pathway and its repression by ATR in response to replication stress. EMBO Journal. 2008;27(14):1963-1973.

52. Rouault J, Falette N, Guéhenneux F, Guillot C, Rimokh R, Wang Q, Berthet C, Moyret-Lalle C, Savatier P, Pain B, Shaw P, Berger R, Samarut J, Magaud JP, Ozturk M, Samarut C, Puisieux A. Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nature Genetics. 1996;14(4):482-486.

53. Sun B, Ross SM, Rowley S, Adeleye Y, Clewell RA. Contribution of ATM and ATR kinase pathways to p53-mediated response in etoposide and methyl methanesulfonate induced DNA damage. Environmental and Molecular Mutagenesis. 2017;58(2):72-83.

54. Shin H, Kwon H, Lee H, Anwar MA, Choi S. Etoposide induced cytotoxicity mediated by ROS and ERK in human kidney proximal tubule cells. Scientific Reports. 2016;6:34064.

55. Li Y, Xue Z, Dong X, Liu Q, Liu Z, Li H, Xing H, Xu Y, Tang K, Tian Z, Wang M, Rao Q, Wang J. Mitochondrial dysfunction and oxidative stress in bone marrow stromal cells induced by daunorubicin leads to DNA damage in hematopoietic cells. Free Radical Biology & Medicine. 2020;146:211-221.

56. Wise SS, Holmes AA, Wise Sr JP. Hexavalent chromium-induced DNA damage and repair mechanisms. Reviews on Environmental Health. 2008;23(1):39-57.

57. Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. Redox Biology. 2017;11:613-617.

58. Lingappan K. NF-κB in oxidative stress. Current Opinion in Toxicology. 2018;7:81-86.

59. So H, Kim H, Lee J, Park C, Kim Y, Kim E, Kim, J, Yun K, Lee K, Lee H, Moon S, Lim DJ, Park R. Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-κB. Journal of the Association for Research in Otolaryngology. 2007;8(3):338-355.

60. Reinhardt HC, Yaffe MB. Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. Current Opinion in Cell Biology. 2009;2:245-255.

61. Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. Biochemical Society Transactions. 2015;43(4):621-626.

62. Khalil HS, Deeni Y. NRF2 inhibition causes repression of ATM and ATR expression leading to aberrant DNA Damage Response. Biodiscovery. 2015;15:e8964.

63. Jauhiainen A, Thomsen C, Strombom L, Grundevik P, Andersson C, Danielsson A, Andersson MK, Nerman O, Rorkvist L, Stahlberg A, Aman P. Distinct cytoplasmic and nuclear functions of the stress induced protein DDIT3/CHOP/GADD153. Plos One. 2012;7(4):e33208.

64. Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E, Quest AFG, Lavandero S. Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. International Review of Cell and Molecular Biology. 2013;301:215-290.

65. Liu T, Laurell C, Selivanova G, Lundeberg J, Nilsson P, Wiman KG. Hypoxia induces p53-dependent transactivation and Fas/CD95-dependent apoptosis. Cell Death and Differenciation. 2007;14:411-421.

66. Morse DL, Gray H, Payne CM, Gillies RJ. Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. Molecular Cancer Therapeutics. 2005;4(10):1495-1504.

67. Nakabeppu Y. Cellular Levels of 8-Oxoguanine in either DNA or the nucleotide pool play pivotal roles in carcinogenesis and survival of cancer cells. International Journal of Molecular Sciences. 2014;14(7):12543-12557.

68. Kitanovic A, Walther T, Loret MO, Holzwarth J, Kitanovic I, Bonowski F, Van Bui N, Francois JM, Wölfl S. Metabolic response to MMS-mediated DNA damage in Saccharomyces Cerevisiae is dependent on the glucose concentration in the medium . FEMS Yeast Research. 2009;9(4):535-551.

69. Ramos RR, Lopez-Carrillo L, Rios-Perez AD, De Vizcaya-Ruíz A, Cebrian ME. Sodium Arsenite induces ROS generation, DNA oxidative damage, HO-1 and c-Myc proteins, NF-kappa B activation and cell proliferation in human breast cancer MCF-7 cells. Mutation Research. 2009;674(1-2):109-115.

70. Nemmiche S, Chabane-Sari D, Kadri M, Guiraud P. Cadmium chloride-induced oxidative stress and DNA damage in the human Jurkat T cell line is not linked to intracellular trace elements depletion. Toxicology in Vitro. 2011;25(1):191-198.

71. Sasaki JC, Arey J, Eastmond DA, Parks KK, Grosovsky AJ. Genotoxicity induced in human lymphoblasts by atmospheric reaction products of naphthalene and phenanthrene. Mutation

Research. 1997;393(1-2):23-35.

72. Burritt DJ. The polycyclic aromatic hydrocarbon phenanthrene causes oxidative stress and alters polyamine metabolism in the aquatic liverwort Riccia fluitans L. Plant, Cell & Environment. 2008;31(10):1416-1431.

73. A Pesticide Information Project of Cooperative Extension Offices of Cornell University, Michigan State University, Oregon State University, and University of California at Davis. Atrazine. Extension Toxicology Network. 1993.

74. Zhang C, Qin L, Dou D, Li X, Ge J, Li J. Atrazine induced oxidative stress and mitochondrial dysfunction in quail (Coturnix C. Coturnix) kidney via modulating Nrf2 signaling pathway. Chemosphere. 2018;212:974-982.

75. Wang H, Wang X, Ke Z, Comer AL, Xu M, Frank JA, Zhang Z, Shi X, Luo J. Tunicamycin-induced unfolded protein response in the developing mouse brain. Toxicology and Applied Pharmacology. 2015;283(3):157-167.

76. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods in Enzymology. 2011;490:71-92.

Appendix II

Table A1 Substance-specific Benchmark dose (BMD) values for the *Bscl2* reporter, and their lower (BMDL) and upper (BMDU) confidence limits. In some cases, the BMDL could not be differentiated from zero, in these cases the value is indicated as 0. In some cases, the BMDU values are infinite and listed as inf. The BMR value used for the analysis was 100%; thus, BMD values shown are doubling doses, i.e., the dose required to elicit a doubling of the response. BMD values >10,000 were assumed to be indicative of negative response and are not shown.

Chemical	BMD	BMDL	BMDU
AMG900	0	0	0.01
Vindesine	0	0	0
Podophyllotoxin	0.01	0.01	0.02
Mitoxantrone	0.01	0	0.01
Vincristine	0.01	0	0.01
Vinorelbine	0.01	0.01	0.01
Docetaxel	0.02	0.02	0.04
Taxol	0.02	0.01	0.02
Etoposide	0.05	0.03	0.08
Colcemid	0.07	0.05	0.08
Nocodazole	0.07	0.04	0.14
Daunorubicin	0.09	0.06	0.12
Mitomycin C	0.18	0.12	0.26
Topotecan	0.18	0.14	0.24
Topotecan +S9	0.39	0.31	0.51
4-nitroquinoline-1-oxide (4-NQO)	0.44	0.18	1.19
Mebendazole	0.45	0.25	2.03
Camptothecin	0.90	0.62	1.24
Cisplatin	1.02	0.41	1.61
Aflatoxin B1 +S9	1.07	0.83	1.38
5-fluorouracil	1.55	1.13	2.17
Sodium arsenite	2.83	2.48	3.33
Chlorambucil	3.60	2.33	5.45
Colchicine	3.68	0	5.34
PhIP[a]+S9	5.69	4.67	6.54
Tunicamycin	8.66	5.96	19.1
Potassium chromate	9.51	8.01	10.8
Nutlin-3	14.23	6.27	29.6
Adefovir	15.42	11.80	20.00
Benomyl	17.64	12.00	29.80
Vinblastine sulphate	18.06	15.70	20.70
Thapsigargin	19.01	10.40	Inf
Vinclozolin	21.06	14.90	Inf

Carbendazim	23.81	14.10	34.70
Diethylstilbestrol	29.84	18.60	51.50
Methyl mercury	38.53	20.00	Inf
Griseofulvin	39.56	26.50	59.40
Amiodarone	40.15	18.90	Inf
Phthalic anhydride	43.09	38.10	48.10
Cyclophosphamide +S9	45.35	26.10	83.40
Hydroquinone	45.69	31.20	65.60
1-nitropyrene	59.86	25.80	124.00
Fludioxonil	64.65	38.00	102.00
Troglitazone	64.97	50.20	94.70
N-nitroso-*N*-ethylurea (ENU)	74.20	36.90	153.00
Emodin	84.91	31.50	157.00
CCCP[b]	91.82	55.90	Inf
Econazole	92.73	45.50	456.00
Zafirlukast	106.45	82.80	Inf
Noscapine	108.06	65.30	208.00
7,12-Dimethylbenz[*a*]anthracene (DMBA) +S9	116.44	24.10	219.00
Abacavir	129.34	96.60	172.00
Methylisothiazolinone (MIT)	131.33	74.20	273.00
Disulfiram	132.45	95.70	202.00
Tozasertib (VX680)	133.59	113.00	181.00
Emodin +S9	134.41	86.80	210.00
Aciclovir	138.97	101.00	191.00
Testosterone	149.43	121.00	194.00
Cadmium Chloride ($CdCl_2$)	183.35	18.00	Inf
Climbazole	185.03	40.80	Inf
2-chloroethyl ethylsulfide (CEES)	204.27	138.00	299.00
Novobiocin	206.61	77.50	483.00
Hydroxybenzomorpholine	220.54	146.00	325.00
Allyl bromide	238.77	168.00	372.00
Flusilazole	240.11	155.00	509.00
Menadione +S9	243.16	114.00	Inf
4-amino-3-nitrophenol	245.35	184.00	324.00
Hydroxyurea	259.52	203.00	329.00
Benzo[*a*]pyrene +S9	269.72	164.00	448.00
Zidovudine	282.48	0	337.00
Lidocaine	283.60	0	548.00
Quercetin	302.56	260.00	392.00
2-pyridinol 1-oxide (HOPO)	313.09	235.00	427.00
Antimycin A	337.47	115.00	Inf
Norethynodrel	339.42	150.00	3.37E+03

Mebendazole +S9	355.59	191.00	824.00
Hydralazine	383.04	293.00	496.00
Phenyl paraben	419.71	312.00	Inf
Hydrogen peroxide (H_2O_2)	429.71	323.00	574.00
Diethyl maleate	441.82	237.00	1.06E+03
Sodium dodecyl	540.42	397.00	Inf
4-vinyl-1-cyclohexene diepoxide	553.63	380.00	788.00
1-naphthol	630.47	414.00	1.14E+03
Canrenoate K+	705.76	436.00	2.20E+03
Methyl methanesulfonate (MMS)	859.35	486.00	2.50E+03
Triadimenol	950.98	518.00	Inf
2-mercaptopurine	986.82	727.00	1.40E+03
Alosetron	998.37	433.00	Inf
Endosulfan	1.01E+03	220.00	Inf
Chlorpyrifos	1.08E+03	176.00	Inf
Temozolomide	1.11E+03	895.00	1.41E+03
Chlorpheniramine maleate	1.31E+03	864.00	3.63E+03
Sodium azide	1.33E+03	1.12E+03	1.88E+03
Perfluorooctanesulfonic acid (PFOS)	1.41E+03	1.15E+03	2.30E+03
Bitertanol +S9	1.43E+03	527.00	Inf
Benzyl paraben	1.43E+03	484.00	Inf
Diclofenac +S9	1.48E+03	917.00	2.73E+03
Eugenol	1.49E+03	1.07E+03	2.28E+03
Perfluorooctanoic acid (PFOA)	1.53E+03	558.00	Inf
Carbamazepine	1.54E+03	1.04E+03	3.61E+03
Nifedipine	1.63E+03	442.00	Inf
Dazomet	1.65E+03	984.00	3.26E+03
Chloramphenicol	1.66E+03	1.14E+03	3.70E+03
Ethyl paraben	1.66E+03	1.14E+03	2.70E+03
Ethyl methanesulfonate (EMS)	1.70E+03	1.00E+03	3.41E+03
Voriconazole	1.70E+03	1.32E+03	Inf
Ethylenediaminetetraacetic acid (EDTA)	1.71E+03	1.19E+03	2.77E+03
Azinphos methyl	1.73E+03	696.00	Inf
Dodecyl aldehyde	1.76E+03	695.00	Inf
Ethoxyacetic acid	1.94E+03	1.01E+03	Inf
Softenon	2.70E+03	1.41E+03	Inf
Monobutyl phthalate	2.87E+03	1.16E+03	Inf
Sulfluramid	3.08E+03	1.88E+03	Inf
Stavudine	3.23E+03	1.83E+03	Inf
Diphenylhydantoin	3.43E+03	1.68E+03	1.55E+04
Nitrofurantoin	3.46E+03	1.12E+03	Inf

Nitrophenol	4.31E+03	1.44E+03	Inf
17*b*-estradiol +S9	5.27E+03	3.21E+03	1.22E+04
Atrazine	5.43E+03	3.20E+03	1.50E+04
2-pyridinol 1-oxide (HOPO) +S9	7.65E+03	5.30E+03	1.20E+04
Spiroxamine	8.36E+03	2.55E+03	Inf

[a]2-Amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine
[b] Carbonyl cyanide 3-chlorophenylhydrazone

Table A2 Substance-specific Benchmark dose (BMD) values for the *Rtkn* reporter, and their lower (BMDL) and upper (BMDU) confidence limits. In some cases, the BMDL could not be differentiated from zero, in these cases the value is indicated as 0. In some cases, the BMDU values are infinite and listed as inf. The BMR value used for the analysis was 100%; thus, BMD values shown are doubling doses, i.e., the dose required to elicit a doubling of the response. BMD values >10,000 were assumed to be indicative of negative response and are not shown.

Chemical	BMD	BMDL	BMDU
AMG900	0	0	0
Mitoxantrone	0	0	0
Vincristine	0	0	NaN
Vindesine	0	0	0
Vinorelbine	0	0	NaN
Docetaxel	0.01	0	0.01
Podophyllotoxin	0.01	0.01	0.01
Taxol	0.02	0.01	0.02
Etoposide	0.03	0.02	0.04
Colcemid	0.05	0.02	0.06
Daunorubicin	0.05	0.03	0.08
Nocodazole	0.05	0.03	0.06
Mitomycin C	0.07	0.04	0.12
Topotecan	0.08	0.06	0.11
Mebendazole	0.16	0.08	0.33
4-nitroquinoline-1-oxide (4-NQO)	0.19	0.11	0.40
Camptothecin	0.21	0.13	0.31
Topotecan +S9	0.23	0.18	0.30
5-fluorouracil	0.70	0.53	0.91
Aflatoxin B1 +S9	1.13	0.83	1.52
Cisplatin	1.21	0	2.10
Chlorambucil	1.71	0.64	2.71
Potassium chromate	3.21	2.60	3.81
Carbendazim	3.60	2.16	18.50
PhIP[a] +S9	4.32	3.58	5.07
Fludioxonil	5.02	3.67	6.81
Sodium arsenite	5.25	3.33	Inf
Benomyl	5.99	4.14	8.63
Nutlin-3	7.53	4.60	12.70
Adefovir	8.96	6.11	13.20
Diethylstilbestrol	8.98	5.42	14.70
Cyclophosphamide	37.43	20.00	61.30
Norethynodrel	8.99	6.33	11.80
17*b*-estradiol	9.81	0	15.50
Thapsigargin	16.15	7.71	Inf
Vinblastine sulphate	12.09	10.00	14.20

Emodin +S9	19.24	11.50	32.90
Mebendazole +S9	20.50	11.40	35.20
Hydroquinone	22.40	11.80	39.70
Vinclozin	29.70	29.70	NaN
Phthalic anhydride	31.62	27.70	35.60
Noscapine	35.68	26.70	49.00
7,12-Dimethylbenz[*a*]anthracene (DMBA) +S9	59.85	27.40	116.00
Climbazole	49.34	21.90	Inf
Testosterone	66.08	51.30	82.60
Abacavir	80.78	47.50	148.00
Troglitazone	80.05	50.80	Inf
Methylisothiazolinone (MIT)	83.77	37.60	264.00
Ethoxyacetic acid	539.23	390.00	913.00
Nitropyrene	84.69	53.80	131.00
Econazole	105.99	37.20	Inf
Tozasertib (VX680)	108.32	108.00	NaN
Disulfiram	111.19	76.90	195.00
Benzo[*a*]pyrene +S9	113.46	76.60	16.00
Colchicine	113.91	42.30	386.00
Famotidine	13811.82	9310	27900
Menadione +S9	116.58	50.90	Inf
CCCP[b]	122.62	49.90	Inf
Aciclovir	114.93	80.70	160.00
2-chloroethyl ethylsulfide (CEES)	122.94	75.00	198.00
Emodin	127.59	68.20	285.00
Hydrogen peroxide (H_2O_2)	154.96	108.00	240.00
Amiodarone	164.12	24.10	Inf
Hydroyurea	157.76	64.00	354.00
Hydroxybenzomorpholine	164.46	81.50	305.00
2-pyridinol 1-oxide (HOPO)	177.98	135.00	233.00
Zidoudine	213.77	214.00	NaN
Flusilazole	214.56	122.00	Inf
NNK[c]	212.59	0	446.00
4-vinyl-1-cyclohexene diepoxide	223.79	135.00	407.00
17*b*-estradiol +S9	224.70	141.00	350.00
Hydralazine	238.04	131.00	406.00
Methyl mercury	19.30	8.94	2070
Brefeldin A	252.25	129.00	1230
N-nitroso-*N*-ethylurea (ENU)	253.44	169.00	480.00
Allyl bromide	273.82	163.00	695.00
4-amino-3-nitrophenol	306.42	211.00	430.00
Quercetin	339.58	265.00	670.00

1-naphthol	347.56	212.00	613.00
Phenyl paraben	397.68	288.00	Inf
Eugenol	386.31	307.00	481.00
Diethyl maleate	411.49	192.00	1.95E+03
Diclofenac +S9	512.50	322.00	830.00
Novobiocin	538.53	351.00	2.23E+03
Ethyl methanesulfonate (EMS)	599.23	336.00	1.07E+03
Canrenoate K+	646.16	312.00	Inf
Sodium dodecyl	659.17	406.00	Inf
Nifedipine	690.64	338.00	Inf
Temozolomide	717.26	547.00	910.00
Methyl methanesulfonate (MMS)	750.05	309.00	Inf
Chlorpheniramin	937.13	559.00	3.03E+03
Pyrimethamine	244.38	208.00	295.00
2-mercaptopurine	964.33	579.00	1.75E+03
Bitertanol +S9	1.17E+03	395.00	Inf
Amitrole	117E+03	806.00	1.61E+03
Spiroxamine	1.64E+03	1.14E+03	3.92E+03
Sulfluramid	1.51E+03	1.08E+03	2.99E+03
Sodium azide	1.21E+03	996.00	1.69E+03
Carbamazepine	1.24E+03	675.00	5.68E+03
Griseofulvin	1.58E+03	624.00	Inf
Diphenylhydantoin	1.42E+03	724.00	4.20E+03
Voriconazole	1.57E+03	1570	196.00
Benzyl paraben	1.76E+03	414.00	Inf
Triadimenol	1.78E+03	445.00	Inf
Prednisolone	1.93E+03	1.27E+03	Inf
Ethyl paraben	1.73E+03	1.08E+03	6.02E+03
Softenon	2.40E+03	1.35E+03	Inf
Ethylenediaminetetraacetic acid (EDTA)	1.98E+03	1.41E+03	3.70E+03
Azathioprine	2.87E+03	455.00	Inf
Chloramphenicol	3.29E+03	1.24E+03	Inf
Dazomet	2.49E+03	1.11E+03	2.99E+04
Ofloxacin	3.56E+03	2.15E+03	Inf
2-pyridinol 1-oxide (HOPO) +S9	3.40E+03	2.34E+03	4.83E+03

[a]2-Amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine
[b] Carbonyl cyanide 3-chlorophenylhydrazone
[c]4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Table A3 Substance-specific Benchmark dose (BMD) values for the *Srxn1* reporter, and their lower (BMDL) and upper (BMDU) confidence limits. In some cases, the BMDL could not be differentiated from zero, in these cases the value is indicated as 0. In some cases, the BMDU values are infinite and listed as inf. The BMR value used for the analysis was 100%; thus, BMD values shown are doubling doses, i.e., the dose required to elicit a doubling of the response. BMD values >10,000 were assumed to be indicative of negative response and are not shown.

Chemical	BMD	BMDL	BMDU
AMG900	0	0	0.01
p-chloroaniline	0	0	610.00
Vincristine	0	0	0.01
Vindesine	0	0	0
Docetaxel	0.01	0.01	0.01
Mitoxantrone	0.01	0	0.01
Podophyllotoxin	0.01	0.01	0.02
Vinorelbine	0.01	0	0.01
Taxol	0.03	0.02	0.09
Colcemid	0.07	0.05	0.08
Nocodazole	0.07	0.04	0.10
Etoposide	0.09	0.05	0.16
Daunorubicin	0.10	0.07	0.14
4-nitroquinoline-1-oxide (4-NQO)	0.11	0	0.28
Topotecan	0.20	0.12	0.38
Topotecan +S9	0.44	0.28	0.88
Sodium arsenite	0.59	0.51	0.69
5-Azacytidine	0.87	0.59	1.24
Mitomycin C	1.01	0.70	1.56
Camptothecin	1.12	0.48	2.41
Mebendazole	1.29	0.77	2.52
Azathioprine	1.80	0.53	4.14
Menadione	2.07	1.42	2.93
Chlorambucil	2.35	1.53	3.25
Methyl mercury	3.20	2.12	4.77
Cisplatin	3.68	2.44	5.73
Aflatoxin B1 +S9	3.76	2.79	5.06
Colchicine	3.92	0	5.37
Cadmium Chloride ($CdCl_2$)	4.29	3.10	6.19
Potassium chromate	4.70	3.99	5.62
PhIP[a] +S9	6.25	5.31	10.10
Climbazole	6.57	47.50	91.90
5-fluorouracil	11.95	6.66	Inf
Hydroquinone	12.43	8.36	18.00
Norethynodrel	13.53	6.54	18.90
Diethylstilbestrol	13.80	8.74	21.40

Vinclozin	14.05	10.30	31.20
Thapsigargin	14.16	7.26	Inf
Benomyl	14.21	5.80	60.20
Fludioxonil	15.11	10.10	23.70
Troglitazone	16.65	12.20	22.40
Econazole	17.56	12.10	27.70
Triclosan	17.84	13.40	23.20
Vinblastine sulphate	19.37	15.40	24.20
Disulfiram	20.59	9.19	30.30
Amiodarone	21.05	6.95	234.00
Nutlin-3	21.66	13.50	37.40
Griseofulvin	23.53	14.80	39.20
Nitropyrene	23.73	12.50	38.50
Carbendazim	24.18	5.06	34.50
Adefovir	24.26	17.40	35.80
Methylisothiazolinone (MIT)	30.08	19.20	47.40
Methyl methanesulfonate (MMS)	36.96	25.40	52.10
Diethyl maleate	39.37	27.70	53.90
Benzo[*a*]pyrene +S9	40.27	19.30	81.60
Alachlor	41.78	29.90	58.10
N-nitroso-*N*-ethylurea (ENU)	42.92	32.50	55.80
Phthalic anhydride	44.85	38.30	52.50
Menadione +S9	46.33	28.50	86.00
Dazomet	47.15	31.40	69.50
Endosulfan	47.46	32.20	78.30
Flusilazole	47.84	36.80	60.20
Nitrofurantoin	48.22	0	66.80
Zafirlukast	49.49	32.90	68.70
Chlorpyrifos	53.09	41.50	65.70
Emodin +S9	58.05	41.60	78.90
Quercetin	59.38	50.60	71.90
CCCP[b]	63.24	43.30	141.00
Zidoudine	68.38	0	321.00
Nifedipine	69.87	34.30	111.00
Emodin	79.20	51.60	127.00
Testosterone	81.10	53.50	117.00
Novobiocin	86.08	65.00	118.00
Azinphos methyl	88.50	44.60	185.00
Noscapine	95.99	48.80	137.00
Benzyl paraben	96.80	42.90	262.00
2-mercaptopurine	96.83	74.60	124.00
4-amino-3-nitrophenol	109.83	76.40	155.00
Abacavir	111.16	80.30	151.00

Cyclophosphamide	113.75	82.10	191.00
4-vinyl-1-cyclohexene diepoxide	115.64	84.70	155.00
Tozasertib (VX680)	116.42	83.60	195.00
Mebendazole +S9	118.43	72.00	211.00
Diphenylhydantoin	133.80	93.60	192.00
Hydroxybenzomorpholine	136.07	90.70	206.00
7,12-Dimethylbenz[*a*]anthracene (DMBA) +S9	142.56	80.90	655.00
Di-(2-ethylhexyl)phthalate (DEHP)	144.38	88.70	358.00
2-chloroethyl ethylsulfide (CEES)	153.64	102.00	244.00
Canrenoate K+	161.61	80.60	261.00
Aciclovir	169.72	122.00	237.00
Aroclor-1254	178.89	86.30	478.00
Dodecyl aldehyde	186.33	128.00	285.00
Triadimenol	196.35	138.00	270.00
Sodium dodecyl	201.90	171.00	236.00
Allyl bromide	225.23	134.00	499.00
Bitertanol +S9	241.45	102.00	955.00
Alosetron	242.54	100.00	989.00
1-naphthol	250.23	168.00	408.00
Perfluorooctanoic acid (PFOA)	302.72	213.00	412.00
Hydrogen peroxide (H_2O_2)	336.55	227.00	471.00
Phenyl paraben	379.74	275.00	Inf
Hydroyurea	423.53	332.00	544.00
Nalidixic acid	436.85	0	648.00
Hydralazine	441.54	297.00	684.00
Chlorpheniramin	454.24	330.00	609.00
Ethyl paraben	468.56	293.00	842.00
Brefeldin A	515.15	124.00	Inf
Diclofenac +S9	545.21	383.00	796.00
Carbamazepine	586.29	422.00	783.00
17*b*-estradiol +S9	600.87	400.00	884.00
17*b*-estradiol	617.10	292.00	3.71E+03
Pyrimethamine	654.38	311.00	Inf
2-pyridinol 1-oxide (HOPO)	657.79	394.00	Inf
Monobutyl phthalate	662.42	450.00	950.00
Stavudine	668.25	409.00	1.03E+03
Ethyl methanesulfonate (EMS)	776.47	516.00	1.32E+03
Ethylenediaminetetraacetic acid (EDTA)	782.89	548.00	1.10E+03
Perfluorooctanesulfonic acid (PFOS)	809.95	651.00	941.00
Eugenol	836.97	576.00	1.20E+03
Dieldrin	867.94	311.00	Inf

Famotidine	878.97	513.00	1.67E+03
Ethoxyacetic acid	972.04	566.00	Inf
Nitrophenol	990.66	553.00	1.77E+03
Sulfluramid	993.00	739.00	1.41E+03
Chloramphenicol	1.03E+03	768.00	1.44E+03
Spiroxamine	1.07E+03	786.00	1.58E+03
Prednisolone	1.08E+03	90.90	1.29E+03
Voriconazole	1.10E+03	761.00	1.71E+03
Softenon	1.11E+03	935.00	1.51E+03
Temozolomide	1.18E+03	826.00	1.87E+03
Sodium azide	1.45E+03	1.10E+03	Inf
Atrazine	1.59E+03	722.00	4.70E+03
Antimycin A	1.63E+03	110.00	Inf
Lidocaine	3.41E+03	2.16E+03	5.49E+03
Ofloxacin	3.79E+03	2.34E+03	Inf
Tunicamycin	6.25E+03	5.80	Inf
2-pyridinol 1-oxide (HOPO) +S9	6.69E+03	4.51E+03	1.24E+04
Hydrocortisone	8.11E+03	1.47E+03	Inf

[a]2-Amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine

[b] Carbonyl cyanide 3-chlorophenylhydrazone

Table A4 Substance-specific Benchmark dose (BMD) values for the *Blvrb* reporter, and their lower (BMDL) and upper (BMDU) confidence limits. In some cases, the BMDL could not be differentiated from zero, in these cases the value is indicated as 0. In some cases, the BMDU values are infinite and listed as inf. The BMR value used for the analysis was 100%; thus, BMD values shown are doubling doses, i.e., the dose required to elicit a doubling of the response. BMD values >10,000 were assumed to be indicative of negative response and are not shown.

Chemical	BMD	BMDL	BMDU
Vincristine	0	0	0.01
Mitoxantrone	0.01	0.01	0.04
Vinorelbine	0.01	0	0.01
Podophyllotoxin	0.06	0.03	0.20
Etoposide	0.09	0.06	0.15
Nocodazole	0.16	0.09	Inf
AMG900	0.21	0.04	Inf
Colcemid	0.25	0.12	0.95
Daunorubicin	0.25	0.11	97.00
Topotecan	0.25	0.13	0.45
Topotecan +S9	0.52	0.25	1.09
Sodium arsenite	0.66	0.47	0.90
4-nitroquinolone-1-oxide (4-NQO)	0.68	0.37	3.42
Docetaxel	0.80	0.20	Inf
Mitomycin C	2.55	1.25	7.85
Mebendazole	3.01	1.11	27.70
Methyl mercury	5.17	2.59	10.70
PhIP[a] +S9	5.79	3.25	10.50
Camptothecin	6.07	4.02	9.68
Tunicamycin	7.50	4.29	Inf
Cisplatin	7.60	3.29	26.30
Cadmium Chloride ($CdCl_2$)	7.79	5.02	13.20
Potassium chromate	8.10	5.41	12.90
Chlorambucil	9.29	4.56	17.70
Thapsigargin	10.75	6.36	Inf
Aflatoxin B1 +S9	13.58	9.42	21.30
Triclosan	14.05	5.98	24.90
Hydroquinone	15.65	12.10	20.00
Vinclozin	16.06	10.90	33.80
Methyl methanesulfonate (MMS)	20.60	10.60	36.40
Vinblastine sulphate	23.34	18.40	31.40
Diethyl maleate	23.44	16.90	31.10
Troglitazone	23.76	13.70	52.80
Disulfiram	30.38	0	91.80
1-nitropyrene	32.12	19.70	50.20
5-Azacytidine	37.22	22.40	74.10

Phthalic anhydride	39.85	26.30	59.40
Nutlin-3	42.87	27.10	87.70
Alachlor	45.80	34.80	60.90
Azathioprine	46.72	33.00	65.60
Benomyl	47.32	21.80	183.00
Adefovir	48.56	33.40	79.00
Novobiocin	53.20	33.80	79.50
Menadione +S9	54.14	24.50	169.00
Allyl Bromide	55.71	42.50	73.60
Diethylstilbestrol	58.98	34.00	132.00
Zafirlukast	84.26	55.60	160.00
Methylisothiazolinone (MIT)	91.73	36.80	377.00
Emodin +S9	93.55	69.40	125.00
Benzo[*a*]pyrene +S9	95.24	71.60	126.00
Testosterone	97.28	62.30	157.00
N-nitroso-*N*-ethylurea (ENU)	99.72	72.10	140.00
Climbazole	99.80	24.10	Inf
Colchicine	104.13	39.20	423.00
Cyclophosphamide + S9	109.90	46.00	457.00
5-fluorouracil	115.32	25.60	Inf
2-chloroethyl ethylsulfide (CEES)	116.32	84.70	163.00
Carbendazim	118.47	50.60	616.00
Amiodarole	120.33	11.50	Inf
CCCP[b]	122.32	60.00	389.00
Quercetin	123.48	84.00	185.00
4-vinyl-1-cyclohexene diepoxide	133.16	105.00	170.00
Tozasertib (VX680)	151.74	101.00	351.00
Chlorpyrifos	163.45	62.10	1190.00
Fludioxonil	163.83	92.80	418.00
Emodin	166.40	106.00	294.00
2-mercaptopurine	171.14	138.00	211.00
Abacavir	213.39	162.00	284.00
Flusilazole	214.07	136.00	409.00
Azinphos methyl	222.94	151.00	352.00
Mebendazole +S9	229.20	96.10	943.00
Econazole	247.43	84.90	4.93E+03
Noscapine	253.76	131.00	Inf
Sodium dodecyl	258.05	175.00	425.00
Zidovudine	259.12	0	416.00
4-amino-3-nitrophenol	259.85	199.00	343.00
Endosulfan	261.64	126.00	919.00
7,12-Dimethylbenz[*a*]anthracene (DMBA) +S9	268.13	147.00	658.00

Hydroxybenzomorpholine	270.22	209.00	356.00
Bitertanol	287.80	176.00	553.00
Canrenoate K+	313.48	187.00	591.00
Chlorpheniramine maleate	317.52	162.00	682.00
Aciclovir	336.34	245.00	480.00
Triadimenol	354.98	235.00	721.00
1-naphthol	375.23	250.00	632.00
Hydroxyurea	429.45	330.00	777.00
Brefeldin A	478.47	210.00	2.18E+03
Dazomet	536.91	239.00	1.39E+03
Hydrogen peroxide (H_2O_2)	758.38	577.00	1.03E+03
Eugenol	898.47	472.00	2.15E+03
Hydralazine	911.89	703.00	1.23E+03
Perfluorooctanoic acid (PFOA)	1.11E+03	528.00	Inf
Ethylenediaminetetraacetic acid (EDTA)	1.20E+03	927.00	1.61E+03
Ethyl methanesulfonate (EMS)	1.20E+03	790.00	2.05E+03
Dodecyl aldehyde	1.26E+03	602.00	4.95E+03
Voriconazole	1.54E+03	1.02E+03	3.68E+03
Perfluorooctanesulfonic acid (PFOS)	1.60E+03	923.00	1.15E+04
Stavudine	1.69E+03	969.00	3.98E+03
Diphenylhydantoin	1.73E+03	1.05E+03	3.39E+03
Ethoxyacetic acid	1.77E+03	952.00	4.65E+03
Pyrimethamine	2.05E+03	670.00	Inf
Diclofenac +S9	2.15E+03	739.00	2.48E+04
17*b*-estradiol +S9	2.20E+03	1.46E+03	3.65E+03
Temozolomide	2.26E+03	1.24E+03	5.96E+03
Griseofulvin	2.36E+03	1.08E+03	1.46E+04
2-pyridinol 1-oxide (HOPO)	2.50E+03	1.00E+03	5.30E+04
17*b*-estradiol	2.60E+03	810.00	Inf
Carbamazepine	2.61E+03	1.29E+03	8.05E+03
Monobutyl phthalate	3.33E+03	881.00	Inf
Chloramphenicol	5.74E+03	1.47E+03	3.85E+06
Prednisolone	5.86E+03	2.26E+03	8.83E+04
Spiroxamine	6.11E+03	2.32E+03	1.12E+05
Sodium azide	6.31E+03	2.35E+03	1.40E+05
Atrazine	6.69E+03	3.31E+03	2.10E+04
Sulfluramid	7.16E+03	2.51E+03	5.70E+05
Ofloxacin	8.59E+03	3.23E+03	Inf
Dieldrin	9.75E+03	638.00	Inf
Amitrole	9.81E+03	6.49E+03	1.68E+04

[a] 2-Amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine

[b] Carbonyl cyanide 3-chlorophenylhydrazone

Table A5 Substance-specific Benchmark dose (BMD) values for the *Btg2* reporter, and their lower (BMDL) and upper (BMDU) confidence limits. In some cases, the BMDL could not be differentiated from zero, in these cases the value is indicated as 0. In some cases, the BMDU values are infinite and listed as inf. The BMR value used for the analysis was 100%; thus, BMD values shown are doubling doses, i.e., the dose required to elicit a doubling of the response. BMD values >10,000 were assumed to be indicative of negative response and are not shown.

Chemical	BMD	BMDL	BMDU
AMG900	0	0	0.01
Mitoxantrone	0	0	0.01
Vincristine	0	0	0
Vindesine	0	0	0
Vinorelbine	0	0	0.10
Docetaxel	0.01	0.01	0.01
Podophyllotoxin	0.01	0.01	0.01
Taxol	0.01	0	0.01
Nocodazole	0.03	0.02	0.05
Etoposide	0.03	0.02	0.05
Colcemid	0.06	0.05	0.08
Daunorubicin	0.09	0.06	0.13
Topotecan	0.10	0.08	0.13
4-nitroquinoline-1-oxide (4-NQO)	0.15	0.08	0.28
Mebendazole	0.23	0.11	0.56
Topotecan +S9	0.23	0.18	0.29
5-fluorouracil	0.63	0.46	0.87
Camptothecin	0.70	0.43	1.10
Mitomycin C	1.14	0.56	2.11
Aflatoxin B1 +S9	1.34	1.01	1.78
Cisplatin	1.56	0.77	2.46
Chlorambucil	2.48	1.57	3.86
PhIP[a] +S9	3.08	2.79	3.46
Colchicine	3.26	0	4.99
Potassium chromate	4.62	4.15	5.13
Benomyl	7.91	5.19	12.10
Tunicamycin	10.98	6.48	Inf
Carbendazim	11.19	6.08	22.30
Adefovir	11.84	9.23	15.20
1-nitropyrene	13.64	7.18	22.60
Diethylstilbestrol	14.67	10.10	22.50
17*b*-estradiol	15.67	10.70	21.80
Thapsigargin	16.67	9.12	Inf
Vinblastine sulphate	16.75	14.30	19.20
Hesperadin	18.46	12.90	25.20

Nutlin-3	20.46	10.60	46.40
Methyl mercury	22.20	9.38	Inf
Cadmium Chloride ($CdCl_2$)	22.70	12.30	Inf
Cyclophosphamide +S9	24.29	14.10	43.00
Hydroquinone	27.56	12.90	56.00
Mebendazole +S9	27.82	14.90	49.00
Vinclozolin	28.16	17.70	Inf
Emodin +S9	34.95	24.90	48.40
Benzo[*a*]pyrene +S9	36.65	23.90	68.50
Disulfiram	37.51	22.70	89.10
Emodin	38.77	26.90	55.40
Phthalic anhydride	42.34	38.20	46.50
Fludioxonil	44.28	26.20	68.60
N-nitroso-*N*-ethylurea (ENU)	46.36	30.30	71.30
Amiodarone	49.55	18.50	Inf
Climbazole	50.10	27.20	Inf
Menadione +S9	55.00	25.50	133.00
Troglitazone	57.64	44.70	83.60
Econazole	58.43	28.70	274.00
CCCP[b]	60.09	38.00	116.00
Noscapine	73.62	42.40	159.00
7,12-Dimethylbenz[*a*]anthracene (DMBA) +S9	78.24	50.40	135.00
Tozasertib (VX680)	98.21	82.10	118.00
Methylisothiazolinone (MIT)	99.82	42.20	467.00
Abacavir	103.48	72.00	151.00
Testosterone	106.94	84.10	134.00
Methyl methanesulfonate (MMS)	136.57	62.70	265.00
Hydroxyurea	142.23	33.60	1.10E+03
Aciclovir	143.72	101.00	203.00
Flusilazole	163.31	108.00	305.00
Norethynodrel	168.79	79.60	586.00
Allyl bromide	177.33	125.00	260.00
Diethyl maleate	206.46	129.00	391.00
Hydrogen peroxide (H_2O_2)	208.58	155.00	285.00
Zidovudine	224.92	0	278.00
2-pyridinol 1-oxide (HOPO)	234.99	173.00	321.00
2-chloroethyl ethylsulfide (CEES)	242.80	158.00	370.00
Novobiocin	255.58	132.00	662.00
Hydralazine	259.56	131.00	500.00
Quercetin	271.70	242.00	316.00
Hydroxybenzomorpholine	285.85	124.00	659.00
Dilcofenac +S9	290.36	213.00	394.00

Sodium dodecyl	314.01	284.00	353.00
Chloryrifos	326.57	141.00	Inf
Phentyl paraben	331.79	281.00	485.00
Ethylenediaminetetraacetic acid (EDTA)	369.85	299.00	458.00
4-amino-3-nitrophenol	386.29	264.00	559.00
Pyrimethamine	390.93	311.00	Inf
Alachlor	392.88	227.00	1.29E+03
17*b*-estradiol +S9	397.71	268.00	617.00
Dodecyl aldehyde	454.68	254.00	1.20E+03
Azathioprine	541.29	208.00	Inf
Griseofulvin	571.36	383.00	981.00
4-vinyl-1-cyclohexene diepoxide	590.50	379.00	877.00
Antimycin A	592.18	118.00	Inf
1-naphthol	650.46	418.00	1.25E+03
Bitertanol +S9	674.82	332.00	1.07E+04
2-mercaptopurine	682.13	486.00	953.00
Benzyl paraben	690.87	300.00	Inf
Dazomet	738.33	490.00	1.23E+03
Triadimenol	752.64	437.00	Inf
Endosulfan	830.07	155.00	Inf
Eugenol	831.69	618.00	1.10E+03
Canrenoate K+	887.22	482.00	Inf
Nifedipine	894.50	260.00	Inf
Perfluorooctanoic acid (PFOA)	917.46	324.00	2.58E+04
Sodium azide	1.09E+03	978.00	1.26E+03
Carbamazepine	1.09E+03	729.00	1.85E+03
Temozolomide	1.10E+03	867.00	1.42E+03
Ethyl methanesulfonate (EMS)	1.18E+03	710.00	2.47E+03
Ofloxacin	1.20E+03	657.00	2.59E+03
Chlorpheniramin	1.24E+03	806.00	3.78E+03
Ethoxyacetic acid	1.28E+03	727.00	1.39E+04
Perfluorooctanesulfonic acid (PFOS)	1.31E+03	1.12E+03	1.75E+03
Aroclor-1254	1.32E+03	498.00	Inf
Ethyl paraben	1.34E+03	906.00	2.23E+03
Diphenylhydantoin	1.39E+03	802.00	3.28E+03
Di-(2-ethylhexyl)phthalate (DEHP)	1.46E+03	298.00	Inf
Alosetron	1.55E+03	483.00	Inf
Azinphos methyl	1.71E+03	611.00	Inf
Voriconazole	1.75E+03	1.23E+03	Inf
Spiroxamine	1.75E+03	1.30E+03	Inf
Softenon	1.92E+03	1.34E+03	Inf
Chloramphenicol	1.98E+03	1.22E+03	Inf

Nitrofurantoin	1.99E+03	655.00	1.51E+05
Sulfluramid	2.34E+03	1.54E+03	9.66E+03
Stavudin	2.47E+03	1.52E+03	Inf
Amitrole	2.53E+03	1.70E+03	3.64E+03
Monobutyl phthalate	4.89E+03	1.09E+03	Inf
2-pyridinol 1-oxide (HOPO) +S9	6.95E+03	4.67E+03	1.18E+04
Nitrophenol	7.32E+03	1.80E+03	Inf
Famotidine	9.59E+03	6.86E+03	1.38E+04

[a]2-Amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine
[b] Carbonyl cyanide 3-chlorophenylhydrazone

Table A6 Substance-specific Benchmark dose (BMD) values for the *Ddit3* reporter, and their lower (BMDL) and upper (BMDU) confidence limits. In some cases, the BMDL could not be differentiated from zero, in these cases the value is indicated as 0. In some cases, the BMDU values are infinite and listed as inf. The BMR value used for the analysis was 100%; thus, BMD values shown are doubling doses, i.e., the dose required to elicit a doubling of the response. BMD values >10,000 were assumed to be indicative of negative response and are not shown

Chemical	BMD	BMDL	BMDU
AMG900	0.01	0.01	Inf
Vincristine	0.01	0.01	0.01
Vinorelbine	0.01	0.01	0.02
Docetaxel	0.02	0.01	0.02
Podophyllotoxin	0.03	0.02	0.03
Thapsigargin	0.03	0	0.13
Mithoxantrone	0.04	0.03	Inf
Nocodazole	0.10	0.08	0.15
Etoposide	0.11	0.04	0.14
Colcemid	0.22	0.18	0.29
Daunorubicin	0.30	0.25	0.55
Tunicamycin	0.37	0.27	0.49
4-nitroquinolone-1-oxide (4-NQO)	0.91	0.53	Inf
Sodium arsenite	2.34	1.92	2.76
Vinclozin	2.42	1.95	2.97
Mebendazole	3.31	1.81	10.90
Ciclesonide	3.39	2.84	4.00
Antimycin A	4.64	2.47	6.58
Climbazole	7.54	6.36	8.94
Mitomycin C	7.60	2.69	Inf
Amiodarone	9.24	7.21	11.80
CCCP[a]	11.15	9.08	13.60
5-Azacytidine	11.43	9.01	14.20
Triclosan	11.50	9.31	14.60
Cadmium Chloride ($CdCl_2$)	11.90	9.37	17.10
Econazole	13.98	11.50	16.70
Potassium chromate	14.16	11.20	Inf
Camptothecin	15.94	11.20	32.70
5-fluorouracil	16.44	8.40	Inf
Troglitazone	16.91	13.80	20.70
Griseofulvin	18.69	16.40	21.00
Diethylstilbestrol	20.16	16.00	24.80
Vinblastine sulphate	22.67	14.30	40.40
Emodin	25.60	17.20	33.10
Endosulfan	26.25	23.10	30.70
Brefeldin A	33.94	27.50	40.80

Cisplatin	35.22	15.30	Inf
Carbendazim	37.52	29.30	55.20
Nutlin-3	37.62	31.30	48.40
1-nitropyrene	38.24	23.00	46.80
Benomyl	39.69	22.30	Inf
Fludioxonil	40.40	36.20	44.80
Zafirlukast	43.38	38.30	48.80
Chlorpyrifos	45.33	38.90	52.20
Methylisothiazolinone (MIT)	45.94	28.80	714.00
Flusilazole	49.03	44.00	54.10
Phthalic anhydride	55.15	47.00	83.90
Nitrofurantoin	55.18	45.20	69.20
PhIP[b] +S9	55.67	47.20	70.30
Benzyl paraben	56.75	43.20	75.80
Aflatoxin B1 +S9	59.99	26.40	Inf
Adefovir	67.33	48.80	112.00
Azinphos methyl	74.51	61.30	89.40
Hydroquinone	91.85	56.80	171.00
Testosterone	97.37	79.90	118.00
Chlorambucil	98.37	64.40	Inf
Novobiocin	99.11	81.40	170.00
Emodin +S9	105.68	88.30	125.00
Di-(2-ethylhexyl)phthalate (DEHP)	109.46	84.70	137.00
Phenyl paraben	122.34	101.00	147.00
Cyclophosphamide +S9	152.91	112.00	299.00
Nifedipine	156.56	128.00	214.00
Phenanthrene	165.22	142.00	204.00
Disulfiram	166.31	109.00	498.00
Alachlor	171.31	133.00	239.00
Triadimenol	173.31	145.00	207.00
Colchicine	185.06	125.00	1.84E+03
Benzo[*a*]pyrene +S9	205.08	153.00	270.00
Perfluorooctanoic acid (PFOA)	208.76	166.00	256.00
Sodium dodecyl	218.11	189.00	249.00
17*b*-estradiol	221.95	160.00	299.00
Noscapine	224.01	134.00	Inf
Diphenylhydantoin	224.90	175.00	288.00
Hydrocortisone	226.84	135.00	371.00
Prednisolone	231.53	180.00	273.00
Pyrimethamine	239.46	203.00	281.00
N-nitroso-*N*-ethylurea (ENU)	247.16	213.00	306.00
Quercetin	279.58	234.00	363.00
Canrenoate K+	285.14	217.00	360.00

Aroclor-1254	289.66	224.00	416.00
Menadione +S9	290.46	112.00	Inf
Dodecyl aldehyde	294.68	233.00	378.00
7,12-Dimethylbenz[*a*]anthracene (DMBA) +S9	310.00	136.00	Inf
Norethynodrel	317.15	256.00	384.00
Bitertanol +S9	321.41	244.00	501.00
Diethyl maleate	325.77	242.00	708.00
Dieldrin	349.98	261.00	1.07E+03
Allyl bromide	370.05	251.00	748.00
2-pyridinol 1-oxide (HOPO)	416.56	352.00	540.00
1-naphthol	422.83	330.00	583.00
Dazomet	435.99	345.00	642.00
Sodium azide	477.28	380.00	565.00
Carbamazepine	520.07	430.00	617.00
Nalidixic acid	531.07	429.00	655.00
Chlorpheniramin	574.39	484.00	684.00
Hydroxybenzomorpholine	585.21	346.00	1.35E+03
Ethoxyacetic acid	622.03	507.00	911.00
Sabinene hydrate	629.18	539.00	579.00
Ethyl paraben	660.06	575.00	746.00
Perfluorooctanesulfonic acid (PFOS)	679.93	591.00	768.00
Alosetron	686.61	360.00	Inf
4-vinyl-1-cyclohexene diepoxide	812.64	646.00	1.04E+03
Monobutyl phthalate	855.50	700.00	1.05E+03
Voriconazole	878.78	791.00	973.00
Chloramphenicol	978.95	816.00	1.22E+03
Eugenol	1.01E+03	899.00	1.15E+03
Abacavir	1.05E+03	711.00	2.13E+03
Softenon	1.07E+03	899.00	1.32E+03
Hydralazine	1.21E+03	733.00	2.41E+03
Aciclovir	1.22E+03	783.00	3.15E+03
Sulfluramid	1.34E+03	1.04E+03	1.99E+03
Diclofenac +S9	1.36E+03	1.03E+03	3.06E+03
Ethylenediaminetetraacetic acid (EDTA)	1.38E+03	1.16E+03	1.83E+03
Hydroxyurea	1.44E+03	814.00	3.92E+03
2-mercaptopurine	1.45E+03	1.09E+03	2.21E+03
Atrazine	1.48E+03	1.12E+03	1.88E+03
17*b*-estradiol +S9	2.12E+03	1.69E+03	2.74E+03
Methyl methanesulfonate (MMS)	2.30E+03	787.00	Inf
Mebendazole +S9	2.34E+03	489.00	Inf
Ethyl methanesulfonate (EMS)	2.35E+03	1.29E+03	Inf

Spiroxamine	2.82E+03	1.61E+03	Inf
Vindesine	3.22E+03	0	Inf
2-chloroethyl ethylsulfide (CEES)	3.99E+03	867.00	Inf
Stavudine	4.02E+03	1.73E+03	Inf
Ofloxacin	4.43E+03	2.68E+03	Inf
Hydrogen peroxide (H_2O_2)	4.89E+03	1.71E+03	Inf
Lidocaine	5.88E+03	3.74E+03	9.30E+03
Hesperadin	8.36E+03	5.04E+03	Inf
NNK[c]	8.53E+03	6.83E+03	1.10E+04

[a] Carbonyl cyanide 3-chlorophenylhydrazone
[b] 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine
[c] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Table A7 Substance-specific scores for each substance on components 1, 2 and 3. The scores indicate the influence of each multivariate axis. Each chemical was given an abbreviation to facilitate display on the PCA plots (Figures 2.11a-c).

Chemical	Abbreviation	Component 1	Component 2	Component 3
17*b*-estradiol	17be	-0.52	1.52	-0.07
17*b*-estradiol +S9	17be+S9	0.10	0.67	0.72
1-naphthol	1-nap	0.37	0.06	0.24
1-nitropyrene	Nitropy	-0.06	-0.40	-0.30
PhIP[a]+S9	PhIP +S9	-0.68	-0.80	0.11
2-chloroethyl ethylsulfide	CEES	-0.01	-0.49	1.24
2-mercaptopurin	2-mer	0.56	-0.65	0.82
2-pyridinol 1-oxide	HOPO	-0.18	1.14	0.07
2-pyridinol 1-oxide +S9	HOPO +S9	0.58	0.95	0.94
NNK[b]	NNK	0.30	1.23	0.91
4-amino-3-nitrophenol	4-a-3-n	0.00	-0.47	1.55
4-nitroquinoline-1-oxide	4-NQO	-1.21	-1.12	-0.93
4-vinyl-1-cyclohexene diepoxide	4-Vinyl-1-cyclo	0.38	-0.49	0.63
5-azacytidine	5-azacytidine	1.93	-2.00	-0.61
5-fluorouracil	Fluorou	-1.60	0.97	-0.53
7,12-Dimethylbenz[*a*]anthracene +S9	DMBA +S9	-0.25	0.31	0.23
Abacavir	Abac	-0.22	-0.02	0.73
Aciclovir	Acic	-0.19	0.15	0.71
Adefovir	Adef	-0.62	-0.02	-0.07
Aflatoxin B1 +S9	AFB1 +S9	-1.28	-0.28	0.17
Alachlor	Alach	1.34	-1.26	0.10
Allyl bromide	All. bro	-0.41	1.52	0.03
Alosetron	Alos	2.04	-3.32	0.76
AMG900	AMG900	-2.81	1.39	-2.59
Amiodarone	Amiod	0.05	0.11	-0.94
Amitrole	Amitrole	0.34	1.17	0.94
Antimycin A	Ant. A	0.60	1.89	-1.92
Aroclor-1254	A-1254	0.72	0.82	-0.12
Atrazine	Atraz	0.90	0.68	0.33
Azathioprine	Azat	0.89	-2.21	1.97
Azinphos methyl	Azin. met.	1.22	-0.54	-0.39
Benomyl	Beno	-0.68	0.01	-0.21
Benzo[*a*]pyrene +S9	BaP +S9	-0.01	-0.28	0.23
Benzyl paraben	Benz. par.	0.31	1.23	-0.66
Bitertanol +S9	Biter +S9	0.67	-0.17	0.13
Brefeldin A	Bref. A	1.11	0.19	-0.80
Cadmium chloride	CdCl2	1.01	-1.62	-0.63

Camptothecin	Campto	-1.41	-0.41	-0.15
Canrenoate K+	Canr. K+	0.55	-0.12	0.12
Carbamazepine	Carbam	0.41	0.72	0.11
Carbendazim	Carben	-0.79	0.46	-0.32
CCCP[c]	CCCP	0.13	0.23	-0.95
Chlorambucil	Chloramb	-1.01	-0.71	0.41
Chloramphenicol	Chloramp	0.47	0.90	0.24
Chlorpheniramine maleate	Chlorphe	0.67	-0.13	0.29
Chlorpyrifos	Chlorpy	1.03	-0.52	-0.51
Ciclesonide	Cicles	1.37	1.78	-2.19
Cisplatin	Cispt	-1.13	-0.46	-0.01
Climbazole	Climba	0.07	-0.11	-0.87
Colcemid	Colce	-1.45	-1.12	-1.36
Colchicine	Colch	-0.81	-0.16	0.38
Cyclophosphamide +S9	CPA +S9	-0.38	0.20	0.04
Daunorubicin	Daun	-1.39	-1.15	-1.27
Dazomet	Daz	0.65	-0.30	0.34
Diclofenac +S9	Diclo+S9	0.53	0.41	0.51
Dieldrin	Dield	0.50	1.20	-0.17
Diethyl maleate	Diet. mal.	0.26	-0.95	0.47
Diethylstilbestrol	Diethyls	-0.09	-0.21	-0.51
Diphenylhydantoin	Diphenylh	0.63	0.35	-0.06
Disulfiram	Tetraeth	0.08	-0.77	0.27
Di-(2-ethylhexyl)phthalate	DEHP	0.79	0.89	-0.48
Docetaxel	Docet	-2.06	-0.33	-2.13
Dodecyl aldehyde	Dode. ald.	0.71	0.20	-0.01
Econazole	Econ	-0.04	0.29	-0.79
Emodin	Emod	-0.03	0.33	-0.67
Emodin +S9	Emod +S9	-0.27	0.04	-0.01
Ethyl methanesulfonate	EMS	0.34	0.34	0.73
Endosulfan	Endos	1.11	-0.37	-0.74
Ethoxyacetic acid	Ethox. acid	0.39	0.67	0.18
Ethyl paraben	Ethyl par.	0.26	1.13	0.14
Ethylenediaminetetraacetic acid	Ethylen	0.39	0.40	0.50
Etoposide	Etop	-1.43	-1.28	-1.57
Eugenol	Eug	0.33	0.41	0.42
Famotidine	Famot	0.75	0.49	1.09
Fludioxonil	Fludio	-0.53	0.30	-0.27
Flusilazole	Flusi	0.23	0.04	-0.40
Griseofulvin	Griseo	0.17	0.79	-0.91
Hesperadin	Hesp	0.01	1.44	0.85
Hydralazine	Hydral	-0.05	0.51	0.56
Hydrocortisone	Hydrocor	1.06	1.27	-0.56
Hydrogen peroxide	H2O2	-0.20	0.29	1.14

Hydroquinone	Hydroq	-0.14	-0.81	0.15
Hydroxybenzomorpholine	Hydroxybenz	0.07	-0.01	0.45
Hydroxyurea	Hydroxyu	-0.12	0.30	0.68
Lidocaine	Lidocaine	0.46	0.96	0.75
Mebendazole	Meben	-1.44	-0.33	-0.72
Mebendazole +S9	Meben +S9	-0.53	0.11	1.07
Menadione	Menad	0.64	-0.46	1.62
Menadione +S9	Menad +S9	0.09	-0.55	0.38
Methyl methanesulfonate	MMS	0.57	-1.51	1.21
Methylisothiazolinone	MIT	0.11	-0.19	-0.32
Methyl mercury	Met. mer.	-0.40	-1.95	2.14
Mitomycin C	Mit C	-1.48	-0.54	-0.37
Mitoxantrone	Mitox	-1.62	-2.02	-1.62
Monobutyl phthalate	Monob. phtha.	0.87	0.43	0.23
Nalidixic acid	Nali. Acid	0.95	0.71	0.02
Nifedipine	Nifed	0.16	1.11	-0.22
Nitrofurantoin	Nitrof	0.75	0.83	-0.65
Nitrophenol	Nitrophe	0.63	0.58	1.08
Nocodazole	Nocod	-1.42	-1.15	-1.63
Norethynodrel	Norethy	-0.87	1.39	0.29
Noscapine	Noscap	-0.31	0.32	0.16
Novobiocin	Novob	0.56	-0.60	-0.13
Nutlin-3	Nut-3	-0.52	-0.05	-0.27
N-Nitroso-*N*-ethylurea	ENU	-0.03	-0.30	0.27
Ofloxacin	Ofloxa	0.45	1.03	0.70
Perfluorooctanoic acid	PFOA	0.84	0.22	-0.18
Perfluorooctanesulfonic acid	PFOS	0.76	0.35	0.17
Phenanthrene	Phenanth	1.09	1.34	-0.70
Phenyl paraben	Phen. par.	-0.13	1.61	-0.45
Phthalic anhydride	Phtha. anhyd.	-0.13	-0.26	-0.22
Podophyllotoxin	Podophyl	-1.77	-1.35	-1.84
Potassium chromate	Pot. chrom.	-0.58	-0.60	-0.40
Prednisolone	Predni	0.89	0.89	-0.31
Pyrimethamine	Pyrimeth	0.34	0.86	-0.13
p-chloroaniline	p-Chloroa	0.53	-1.35	2.11
Quercetin	Querce	0.34	-0.43	0.27
Sabinene hydrate	Sabin. Hyd.	1.00	1.19	-0.18
Sodium arsenite	Sod. ars.	0.81	-2.34	-0.84
Sodium azide	Sod. az.	0.28	1.22	-0.05
Sodium dodecyl	Sod. dode.	0.44	-0.03	0.01
Softenon	Soft	0.38	1.12	0.25
Spiroxamine	Spirox	0.44	0.79	0.67
Stavudine	Stavud	0.78	0.04	0.87

Sulfuramid	Sulfuram	0.39	0.96	0.37
Taxol	Taxol	-4.27	1.96	2.29
Temozolomide	Temoz	0.13	0.50	1.21
Testosterone	Testos	0.05	-0.09	-0.11
Thapsigargin	Thapsi	0.29	0.10	-2.92
Topotecan	Topot	-1.96	-2.32	2.67
Topotecan +S9	Topot +S9	-1.73	-2.12	2.54
Triadimenol	Triadi	0.71	-0.08	-0.11
Triclosan	Triclos	2.13	-1.91	-0.81
Troglitazone	Troglit	0.22	-0.61	-0.58
Tunicamycin	Tuni	0.99	0.14	-2.61
Vinblastine sulphate	Vinb	-0.33	-0.28	-0.43
Vinclozolin	Vinclo	0.07	-0.35	-1.27
Vincristine	Vincri	-1.47	-2.05	-2.11
Vindesine	Vindes	-4.50	2.04	2.00
Vinorelbine	Vinorel	-1.52	-1.86	-2.16
Voriconazole	Vorico	0.56	0.48	0.28
VX680	VX680	-0.29	-0.42	1.60
Zafirlukast	Zafir	1.33	-0.96	-0.52
Zidovudine	Zido	-0.11	-0.47	1.60

[a]2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine
[b]4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
[c] Carbonyl cyanide 3-chlorophenylhydrazone

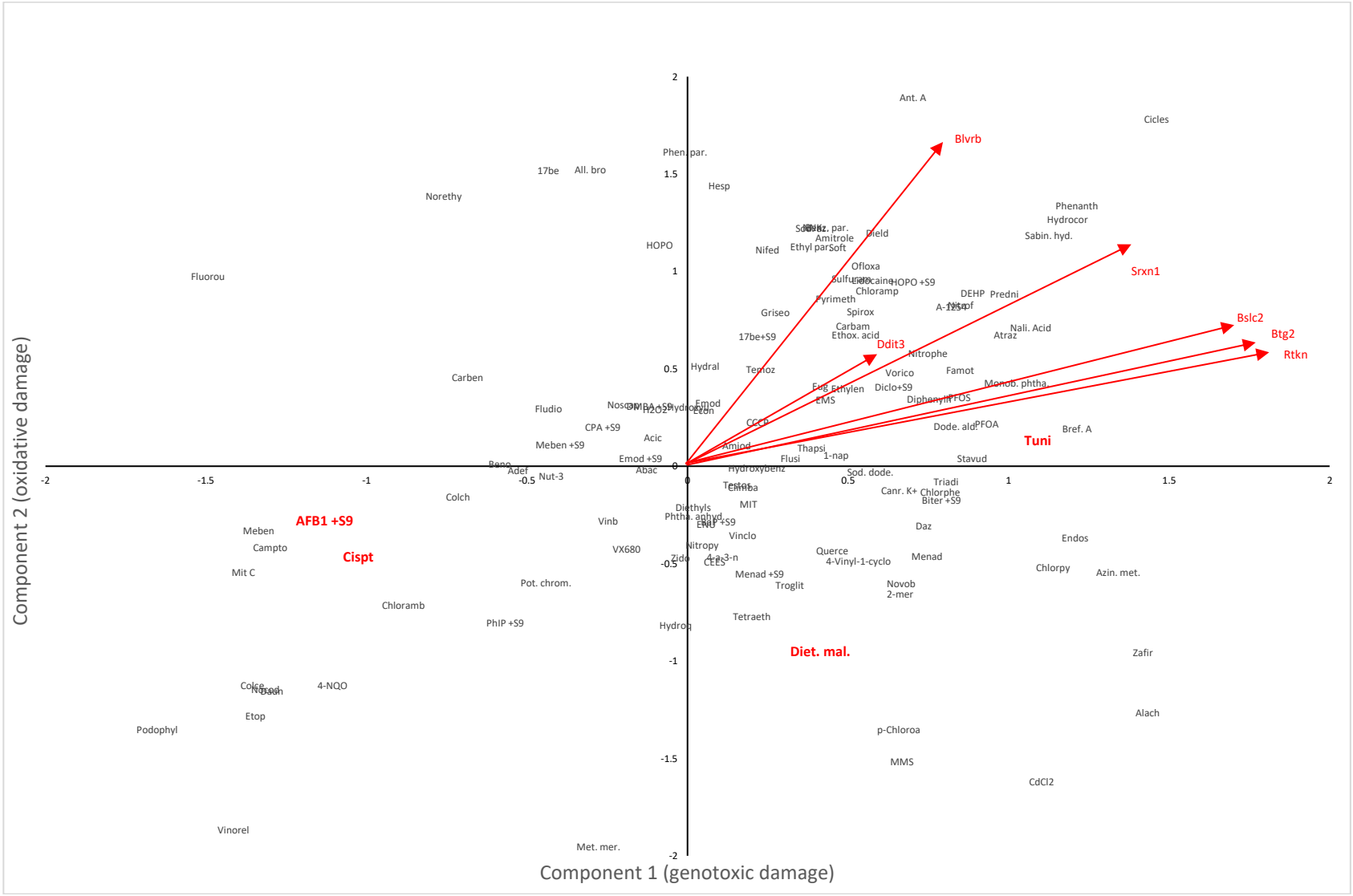

Figure A1 Zoomed Principal Component Analysis results for component 1 and 2 based on BMD values for each substance-reporter. Plot shows loadings of reporter responses on each component (i.e., axis) as arrows. Arrow length reflects loading magnitude. The chemicals are represented (Table A7) with positive controls in red. Analysis employed Varimax option to maximize percentage of variance explained by orthogonal axes.

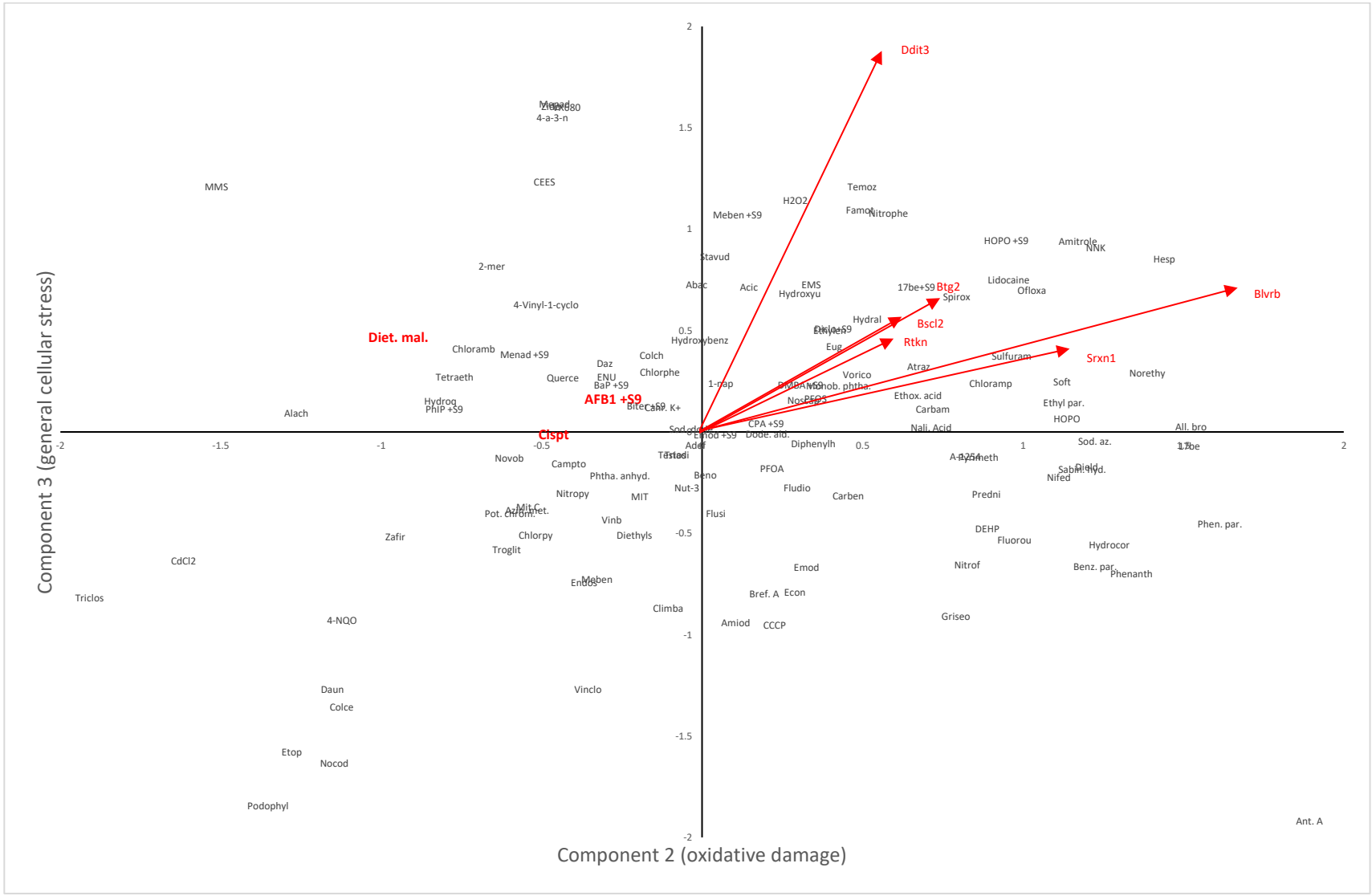

Figure A2 Zoomed Principal Component Analysis results for component 2 and 3 based on BMD values for each substance-reporter. Plot shows loadings of reporter responses on each component (i.e., axis) as arrows. Arrow length reflects loading magnitude. The chemicals are represented (Table A7) with positive controls in red. Analysis employed Varimax option to maximize percentage of variance explained by orthogonal axes.

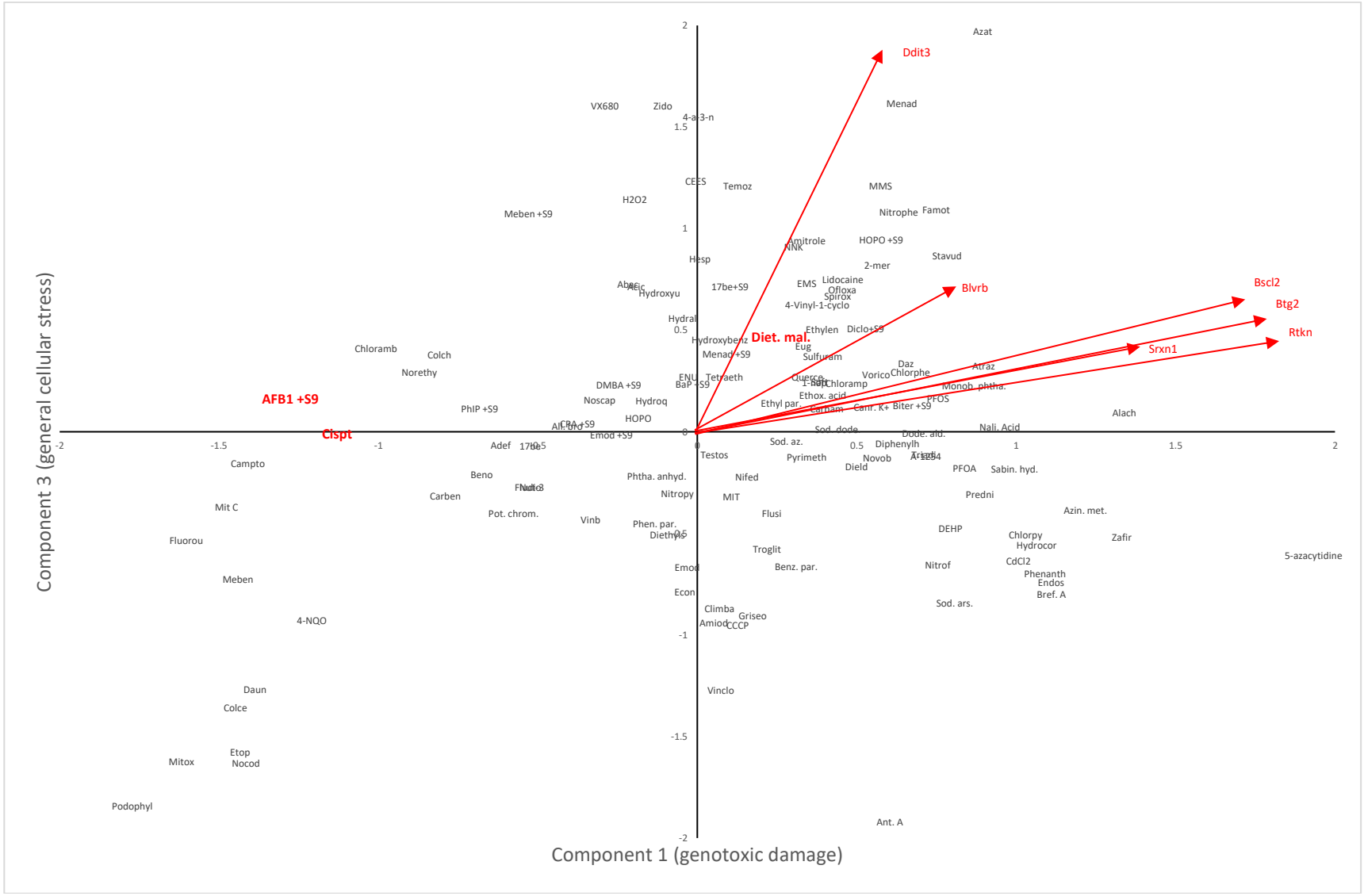

Figure A3 Zoomed Principal Component Analysis results for component 1 and 3 based on BMD values for each substance-reporter. Plot shows loadings of reporter responses on each component (i.e., axis) as arrows. Arrow length reflects loading magnitude. The chemicals are represented (Table A7) with positive controls in red. Analysis employed Varimax option to maximize percentage of variance explained by orthogonal axes.

Chapter Three

3.1 Introduction

To minimize the risk of adverse human health effects, chemical safety assessments require toxicity screening. Toxicity screening must include assessment of genotoxicity (i.e., ability to damage genetic material), which has been linked to heritable genetic diseases and cancer (1). A variety of different *in vitro* genotoxicity tests can be used for chemical hazard assessment. These tests, such as the Salmonella Reverse Mutation Test (i.e., Ames test), mammalian cell gene mutation tests (e.g., *tk* or *hprt* locus mutagenicity assays), and mammalian cell chromosome damage assays (e.g., mammalian cell micronucleus assay) constitute the cornerstone of regulatory frameworks used for genotoxicity assessment; they have been shown to reliably detect of *in vivo* mutagens and carcinogens (2). Moreover, the global push towards reduction of animal use in chemical safety assessment has contributed to the promotion of *in vitro* testing methods. However, some commonly-used *in vitro* tests have faced criticism, notably for their relatively high cost and low throughput, and because they are based on technologies developed 30-50 years ago (3, 4).

To permit efficient genotoxicity screening, a Dutch company Toxys B.V. recently developed a high-throughput assay system that comprises a suite of assessment tools collectively called the ToxTracker® assay (5). The system uses molecular reporters to detect toxicological responses induced by the tested agents. Sensor proteins, such as the NF-κB, ataxia telangiesctasia (ATM), and *Rad3*-related protein (ATR), detect cellular stressors and activate specific signaling pathways such as p53-dependant pathways (e.g., pathways related to DNA damage and concomitant activation of apoptosis and DNA repair), the HMOX1 and NRF2 antioxidant response pathways, and the protein unfolding response pathway. Activation of these signaling pathways results in signaling cascades that ultimately alters the expression of distinct genes associated with specific damage/repair processes (e.g., *Bscl2, Rtkn, Srxn1, Blvrb, Ddit3* and *Btg2*). In the ToxTracker® assay these genes are linked to green fluorescent protein (GFP), a fluorescent reporter, and changes

in gene expression are readily detected using flow cytometry. ToxTracker® therefore allows rapid *in vitro* detection of complementary cellular damage response pathways induced by exposures to toxicants; moreover, by virtue of its multiplexed capabilities, the assay provides insight into toxicant MOA (mode of action) (5-7).

Some genotoxic substances are relatively benign until they undergo enzymatic conversion to active metabolites that can, for example, react with DNA, e.g., PAHs (polycyclic aromatic hydrocarbons) such as benzo[*a*]pyrene. In mammals, enzymatic activation of substances such as PAHs is typically catalyzed by members of the cytochrome P450 super-family. More specifically, microsomal P450 isozymes (CYPs) such as 1A1, 1A2 and 3A (8-10). Unfortunately, the commonly used *in vitro* genotoxicity assessment systems mentioned earlier (e.g., Salmonella Reverse Mutation Test) generally employ cells that are metabolically deficient with respect to hepatic microsomal enzymes such as the cytochrome P450s (CYPs). To compensate for this metabolic deficiency, chemicals assessed using many *in vitro* genotoxicity assays are tested in the presence of a mammalian hepatic fraction called S9, i.e., the supernatant of homogenized rat liver centrifuged at 9,000g (11). Moreover, to enhance the activity of enzymes required for the activation of carcinogenic and mutagenic substances, the rodents from which S9 is derived are treated with a CYP-inducing agent, e.g., the PCB (polychlorinated biphenyl) mixture known as Aroclor-1254 (12).

The most commonly used, commercially-distributed S9 is derived from the livers of metabolically-stimulated, male rats. The aforementioned substance most-commonly employed for metabolic stimulation, i.e., Aroclor 1254, has the capacity to upregulate the expression of CYPs isozymes such as CYP 1A1, 1A2 and 3A (12). The S9 from Aroclor 1254-treated male rats is frequently used to assess the mutagenic potential of chemicals requiring metabolic activation, i.e.,

metabolic conversion to reactive metabolites (12). However, since PCB mixtures such as Aroclor 1254 are no longer commercially available, the commercial supply of Aroclor-induced rat liver S9 will eventually be depleted. Consequently, there is a need to find an alternative inducer of hepatic oxidative metabolism that confers a similar metabolic activation profile (13, 14). To this end, a combination of phenobarbital (PB) and β-naphthoflavone (BNF) has been investigated as a substitute for Aroclor 1254 (15). These agents were selected because they are known to effectively induce two major forms of CYPs, i.e., CYP1A1 and CYP3A (Chapter 1, Table 1.1). In light of the similar pattern of CYP isozyme induction, some researchers have considered S9 from PB/BNF-treated rats as a suitable alternative to S9 from Aroclor 1254-treated rats (15).

The ToxTracker® assay, which, as noted, assesses chemical induction of transgenic reporters, employs murine embryonic stem cells (mES) that also lack the endogenous metabolic capacity to enzymatically convert some genotoxic substances to their reactive metabolites. Consequently, S9 metabolic activation is routinely achieved by the addition of hepatic S9 derived from Aroclor 1254-induced male Sprague-Dawley rats. However, for the reasons outlined above, there is a need to shift to PB/BNF-induced S9, and an ensuing need to evaluate the performance of ToxTracker® assessments carried out using the replacement S9. More specifically, there is a critical need to comparatively examine the responses of the genotoxicity reporters (i.e., *Rtkn* and *Bscl2*) in the presence of Aroclor 1254-induced S9 or PB/BNF-induced S9. For the evaluation to be effective, the substances examined should require a diversity of CYP isozymes for conversion into reactive, genotoxic metabolite(s). More specifically, the evaluation should include substances such as PAHs (e.g., BaP), that require CYP1A1 to catalyze the production of the ultimate mutagen benzo[*a*]pyrene-diol-epoxide (BPDE), as well as substances like heterocyclic amines (e.g., 2-

Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine or PhIP) that require CYP1A2 to generate *N*-hydroxyarylamines (10, 16).

This work employed a series of carefully-selected test chemicals to evaluate the suitability of PB/BNF-induced rat liver S9 as a substitute for Aroclor 1254-induced rat liver S9. More specifically, the work examined the nature and magnitude of the responses of the ToxTracker® genotoxicity reporters (i.e., *Rtkn* and *Bscl2*) across a series of 20 substances, and a range of S9-based ToxTracker® protocols. As noted in Chapter 1, the *Rtkn* reporter is indicative of DNA double strand breaks; the *Bscl2* reporter is indicative of replication fork stalling that is commonly induced by agents that form stable, bulky DNA adducts. The initial work examined 20 substances; later, more focused work, examined a subset of 7 substances. With respect to protocols investigated, the work evaluated protocols designed to balance the cytotoxicity and metabolic activation capabilities of each S9 type. The effect of exposure time was initially investigated (i.e., 3 and 24 hr); the effect of S9-type was subsequently examined to evaluate the 24-hr protocol. Initial evaluations of PB/BNF-induced S9 were based on the correspondence between observed dichotomous responses (i.e., positive versus negative) and *a priori* expectations. The Benchmark Dose (BMD) approach was employed to quantitatively examine ToxTracker® dose-response data; the BMD combined-covariate approach was used to evaluate the effects of experimental conditions (i.e., S9 type, treatment time) on test substance potency.

3.2 Material and methods

3.2.1 List of chemicals and reagents[2]

Chemicals

- Benzo[*a*]pyrene (BaP)
- Benz[*a*]anthracene (BAA)
- Aflatoxin B1 (AFB1)
- 7,12-Dimethylbenz[*a*]anthracene (DMBA)
- Hexamethylphosphoramide (HMPA)
- 2-Amioanthracene (2AA)
- 2-Acetylaminofluorene (2AAF)
- 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ)[2]
- 2-Amino-1-methyl-6-phenylimidazo(4,5-*b*) pyridine (PhIP)[3]
- 2-Amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ)[3]
- Isoprene (ISP)
- Phenolphthalein (PHPT)
- Cyclophosphamide (CPA)
- *o*-Aminoazotoluene (OAT)
- Dimethyl yellow (DMYellow)
- Disperse Orange (DOrange)
- 3-Methylcholanthrene (3MC)
- 1,2-diphenylhydrazine (1,2-DPH)
- 1,3-diphenyltriazine (1,3-DPH)[3]
- Tryptophan-P2 (Trp-p-2)[2]

[2] Unless stated otherwise, all tested substances were purchased from Merck/Sigma Aldrich (Schnelldorf, Germany).
[2] Toronto Research Chemicals (Toronto, Canada)
[3] Apollo Scientific (Denton, Manchester, UK)

3.2.2 Culture of wild-type and ToxTracker® mES cells (mouse embryonic stem cells)

C57/Bl6 B4418 wild-type and ToxTracker® mES cells were cultured in Knockout D-MEM (Gibco, Paisley, Scotland) containing 10% fetal bovine serum, 2mM GlutaMAX (ThermoFisher Scientific, Paisley, Scotland), 1mM sodium pyruvate (ThermoFisher Scientific, Paisley, Scotland), 100μM β-mercaptoethanol (ThermoFisher Scientific, Paisley, Scotland), and leukemia inhibitory factor (Toxys, Leiden, Netherlands), as previously described (17). Wild-type mES cells were employed for initial cytotoxicity tests and dose range-finding. Subsequent toxicity assessment employed the transgenic ToxTracker® mES cells

3.2.3 Exposure of the wild-type mES cells and ToxTracker® reporter cell lines

mES cells were propagated on a feeder layer of irradiated primary mouse embryonic fibroblasts according to established protocols (5). Cells were seeded on gelatin-coated 96-wells plates in the absence of feeder cells 24-hr prior to chemical exposure.

For each chemical tested in the ToxTracker® assay, cytotoxicity in wild-type mES cells was first assessed in a dose range-finding experiment (Figure 3.1). In accordance with the recommendations for *in vitro* genotoxicity testing, the maximum concentration examined was 10 mM (18). In case of precipitation in the culture medium, the maximum soluble concentration was examined. 20 concentrations were tested in the dose range-finding experiment, starting at the maximum concentration followed by 19 consecutive 2-fold dilutions in DMSO or water. The 5 concentrations selected for the definitive ToxTracker® assay started with the highest concentration that elicited a cytotoxicity <75%, followed by the 4 consecutive, 2-fold serial dilutions.

0	0.02	0.04	0.08	0.15	0.31 Substance 1	0.61	1.22	2.44	4.88	9.77	0
0	19.53	39.06	78.13	156.25	312.5	625	1250	2500	5000	10000	0
0	0.02	0.04	0.08	0.15	0.31 Substance 2	0.61	1.22	2.44	4.88	9.77	0
0	19.53	39.06	78.13	156.25	312.5	625	1250	2500	5000	10000	0
0	0.02	0.04	0.08	0.15	0.31 Substance 3	0.61	1.22	2.44	4.88	9.77	0
0	19.53	39.06	78.13	156.25	312.5	625	1250	2500	5000	10000	0
0	0.01	0.02	0.04	0.08	0.16 CisPt	0.31	0.63	1.25	2.5	5	0
0	0.01	0.02	0.04	0.08	0.16 AFB1	0.31	0.63	1.25	2.5	5	0

Figure 3.1 Sample plate design for dose range-finding experiments with wild type mES cells. Twenty consecutive 2-fold dilutions were tested for each of the studied substances. Aflatoxin B1 (AFB1) and cisplatin (CisPt) were used as positive controls in the presence and absence of S9, respectively. Numbers indicate the substance concentrations in µM.

24-hr after seeding the cells in the 96-wells plates, the ToxTracker® mES cell lines were exposed to the selected concentrations of substances dissolved or suspended in Gibco D-MEM cell culture media (Figure 3.2). In the case of metabolic activation, two protocols were used. In the first, cells were exposed in the presence of 0.25% v/v Aroclor- or 1% PB/BNF-induced rat liver S9, and incubated for 24-hr at 37˚C, 5% CO_2 until GFP reporter analysis. In the second protocol, the same S9 concentrations were used; after 3-hr, the cells were washed with PBS, and cultured for 21-hr in cell culture medium without the tested substance. This was followed GFP reporter analysis. Each experiment was conducted in triplicate, i.e., with 3 separate batches of cultured mES cells.

Following the analyses of the data obtained from the 24- and 3-hr exposure experiments, additional experiments were conducted by Toxys B.V. to examine the 24-hr protocol in more detail. More specifically, Toxys B.V. compared the results obtained using PB/BNF-induced and Aroclor-induced S9; exposure time was held constant at 24-hr and S9 concentrations were fixed at 0.40% v/v for PB/BNF-induced S9 and 0.25% v/v for Aroclor-induced S9. The ToxTracker® assay was conducted on a subset of substances known to elicit strong responses on the genotoxicity reporters (i.e., *Rtkn* and *Bscl2*); the experiments were repeated twice.

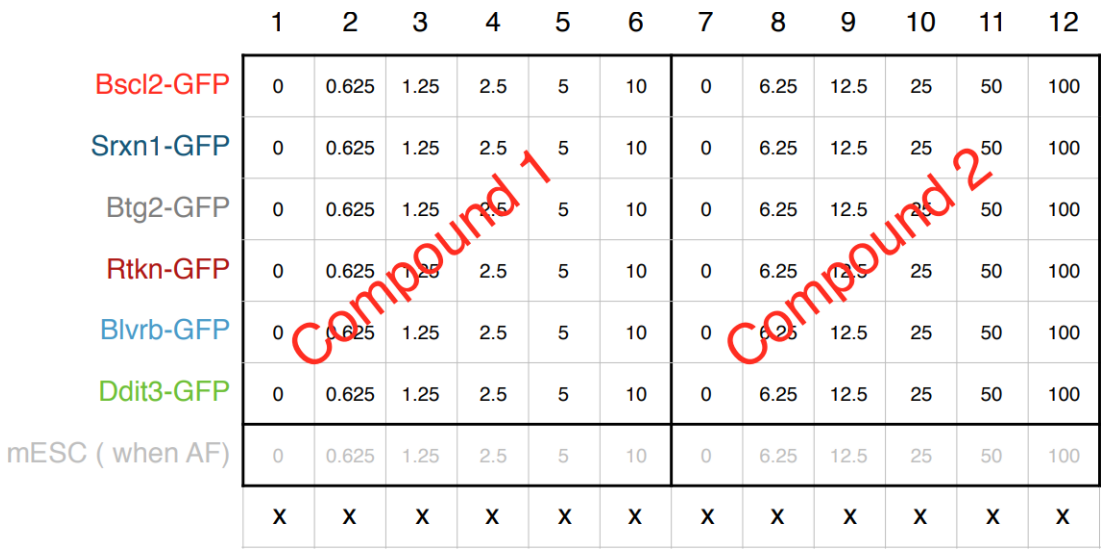

Figure 3.2 Sample plate design for substance testing in the ToxTracker® assay. Two substances are tested per plate, with 5 different concentrations, plus the negative solvent control. Each row is seeded with a different reporter cell line. Wild type cells are included when testing autofluorescent (AF) substances.

3.2.4 Analysis of ToxTracker® reporter induction

Detailed information about the ToxTracker® assay protocol is provided in Hendriks *et al.*, 2012 (5) and Hendriks *et al*., 2016 (6). Induction of the GFP reporters in the ToxTracker® cell lines was determined by flow cytometry. Briefly, after the selected treatment time (i.e., 24- or 3-hr), cells were washed with PBS, trypsinized, and resuspended in PBS with 2% serum; for the 24-hr treatment time, this was immediately followed by flow cytometry analysis. For the 3-hr treatment time, cells were incubated an additional 21-hr before being prepared for flow cytometry. GFP expression was measured only in intact single cells, and the mean GFP fluorescence was measured in each well. The cell number in each well was also determined for cytotoxicity assessment. The mean GFP fluorescence was determined by flow cytometry using a MACSQuant X flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). Reporter activity was determined by the mean fluorescence intensity (MFI) of 5,000 intact mES cells. Each dose-specific mean GFP per cell

value was compared to the concurrent control mean value; results were expressed as fold-change relative to the control. Cytotoxicity was expressed as the dose-specific percentage of dead cells relative to control. The positive controls used in the ToxTracker® assay are cisplatin for DNA damage (*Rtkn, Bscl2* and *Btg2*), diethyl maleate for oxidative stress (*Blvrb* and *Srxn1*), tunicamycin for protein unfolding (*Ddit3*), and AFB1 as an additional control when testing substances that require mammalian enzymatic conversion to reactive metabolites. 3 concentrations were tested for each positive control.

3.2.5 Selection of tested substances

Substances examined in this study, which evaluated the ToxTracker® reporter response in the presence of Aroclor 1254-induced S9 and PB/BNF-induced S9, were selected using a list of substances previously shown to be genotoxic, as specified by Jagger *et al.,* 2009 (19) (Table 3.1). Comparative data from *in vitro* and *in vivo* genotoxicity assays were largely retrieved from the National Toxicology Program's 11th Report on Carcinogens, which provides lists of substances both 'known' and 'reasonably anticipated' to be human carcinogens (20). The remaining substances were selected from the initial GreenScreen® (GADD45a–GFP) validation study conducted without metabolic activation (21).

Table 3.1 List of substances selected for the S9 protocol comparison. The corresponding CAS number and test results from the National Toxicology Program's 11th Report on Carcinogens (ROC) are presented. Adapted from Jagger *et al.*, 2009 (19).

Substance	CAS Number	Carc. Class[a]	Test Results[b]			
			Ames	Mammalian cell mutation	*in vitro* CA/MN	*in vivo* CA/MN
2AAF	53-96-3	B	+(22)	+(23)	+(23)	+(23)
AFB1	1162-65-8	A	+(23)	+(22)	+(20)	+(20, 24)
2AA	613-13-8	N/A	+(22, 23)	+(22)	Equiv. (23)	+(23)
OAT	97-56-3	B	+(22, 23)	+(22)	-(25)	-/+[c](25, 26)
BAA	56-55-3	B	+(22)	+(22, 23)	N/A	N/A
BaP	50-32-8	B	+(22, 23)	+(22, 23)	+(22, 23)	+(23)
CPA	6055-19-2	A	+(23)	+(22)	+(23)	+(22, 23)
DMBA	57-97-6	N/A	+(22)	+(22, 23)	+(22)	+(22, 23)
DMYellow	60-11-7	B	+(22, 23)	+(22, 23)	N/A	+(22)
1,2-DPH	122-66-7	B	+(23)	N/A	+(23)	-(23)
1,3-DPH	136-35-6	B	+(20, 23)	N/A	+(20)	+(20, 22)
DOrange	82-28-0	B	+(22, 23)	+(22)	+(23)	N/A
HMPA	680-31-9	B	-(23)	+(23)	-(23)	+(23)
IQ	77314-22-8	B	+(23)	+(22)	+(27)	+(24)
ISO	78-79-5	B	-(22, 23)	N/A	-(22)	-/+(22, 23)
MeIQ	77094-11-2	B	+(22)	+(22)	+(22)	+(24)
3MC	56-49-5	N/A	+(22, 23)	+(22)	-(23)	-(23)
Phenolphthalein	77-09-8	B	-(22, 23)	N/A	+(20, 23)	-/+(23)
PhIP	105650-23-5	B	+(22)	+(22)	+(22)	+(22, 24)
Trp-P-2	72254-58-1	N/A	+(22)	+(22, 28)	+(28)	+(29)

[a]Carcinogenicity classification according to the 11th RoC (20): A, known human carcinogen; B, reasonably anticipated to be a human carcinogen; N/A, not available and test result information shown not based on RoC documents.

[b]Summary results from genotoxicity tests: Salmonella reverse mutation test (Ames test); mammalian cell mutation tests (i.e., mouse lymphoma *tk* assay or *hprt* assay in CHO or V79 cells); CA, chromosome aberration; MN, micronucleus. Equiv indicates equivocal result.

[c]Positive result from *in vivo* MutaMouse transgenic rodent mutagenicity assay; multiple organs.

3.2.6 Comparative potency analysis using the BMD (Benchmark Dose) combined-covariate approach

The dose-response analysis package known as PROAST (v.66.14 in the R software environment) was used for dose–response analyses (https://www.rivm.nl/en/proast). For each analysis, combined data sets for ToxTracker® reporters were analyzed for each chemical using treatment, i.e., each combination of S9-type and incubation time, as a covariate. Following the approaches described by Hernández *et al*. (30) and Wills *et al*. (31, 32), the analyses were conducted using a 5-parameter exponential model. The dose-response analyses assumed that maximum response (parameter *c* in PROAST), log steepness (parameter *d* in PROAST) and *var* (i.e., within group variance) are conserved across dose-response functions; parameters for background response (parameter *a* in PROAST) and potency (parameter *b* in PROAST) are covariate dependent (33). The PROAST output provided the BMD (i.e., parameter *b* indicating potency) for a BMR (Benchmark Response) of 100%, i.e., the concentration that elicits a 2-fold increase in response over background, with BMDL and BMDU designating the lower and upper 90% confidence limits, respectively. The BMD confidence interval (i.e., BMDL to BMDU) was used for comparative potency comparisons across treatments.

Since the work described herein investigated the efficacy of different treatment protocols for detection of genotoxic substances, the analyses focused on ToxTracker® reporters that are indicative of genotoxic activity, i.e., *Rtkn* and *Bscl2*. Since some genotoxic substances induce oxidative stress and/or a p53-mediated response, the responses of *Srxn1, Blvrb* and *Btg2* reporters were also examined. The *Ddit3* response, which indicates protein damage, was not included in the analyses.

3.3 Results

3.3.1 Part I – The effects of S9-type and treatment time for 20 test substances

The initial experimental work examined the effect of treatment protocol (i.e., S9 type and treatment duration) on the magnitude of the response for several ToxTracker® reporters, i.e., chemical potency as indicated by the BMD_{100} (i.e., doubling dose). More specifically, the BMD combined-covariate approach was used to compare responses of ToxTracker® reporters *Rtkn*, *Bscl2*, *Btg2*, *Blvrb* and *Srxn1* across 20 substances (Appendix III, Tables A1-A5). Since the substances selected for the study include a preponderance of known genotoxicants, the results for *Rtkn* and *Bscl2* are highlighted. As noted, the other reporters are relevant to genotoxicant detection since oxidative stress and ROS (reactive oxygen species) can contribute to the formation of oxidative DNA lesions. Figure 3.3 shows an example of the results obtained; more specifically, the effect of treatment protocol on the *Rtkn* response of ToxTracker® cells exposed to the noteworthy mutagen BaP. The result show that the strongest response (i.e., lowest BMD_{100} or doubling dose) corresponds to a 3-hr treatment in the presence of Aroclor-induced S9. The weakest response (i.e., highest BMD_{100}) corresponds to 24-hr PB/BNF-induced S9. However, the BMD confidence limits shown in Figure 3.3B indicate that the two Aroclor conditions and the 3-hr PB/BNF condition are not significantly different; the BMD_{100} for the PB/BNF 24-hr condition is significantly higher than the others.

The following sections provide a brief summary of the results obtained for each of the ToxTracker® reporters investigated. Responses indicated as *preferable* reflect the experimental condition associated with the lowest BMD_{100} (i.e., lowest doubling dose). Table 3.2 provides a more detailed summary of the results obtained across all substances, all treatments, and all reporters. Appendix III provides a detailed overview of the results obtained, i.e., BMD_{100} values, and their upper and lower confidence limits, for each substance-treatment combination. It should

be noted that in some cases the PROAST software was unable to define a BMD and its confidence limits. This is generally due to the nature of the dose-response function, and software attempts to fit an exponential function to a flat or nearly flat response, i.e., a weak or negative response. In some circumstances, the BMDL and BMDU values were not significantly different from zero or infinite, respectively. The former is typically associated with very potent substances for which the lower confidence limit of the small BMD_{100} cannot be differentiated from zero (i.e., the BMD confidence limit encompasses zero). The latter typically occurs when there is no induction of the reporter, i.e., for the specified reporter and experimental conditions, the response is negative. In all cases where the BMD, BMDL and BMDU were defined, the BMDL-to-BMDU ratio reflects the precision of the BMD value. Ratio values greater than 100 are generally considered indicative of a BMD with notably low precision, i.e., a BMD for which comparative interpretation will be restricted (34).

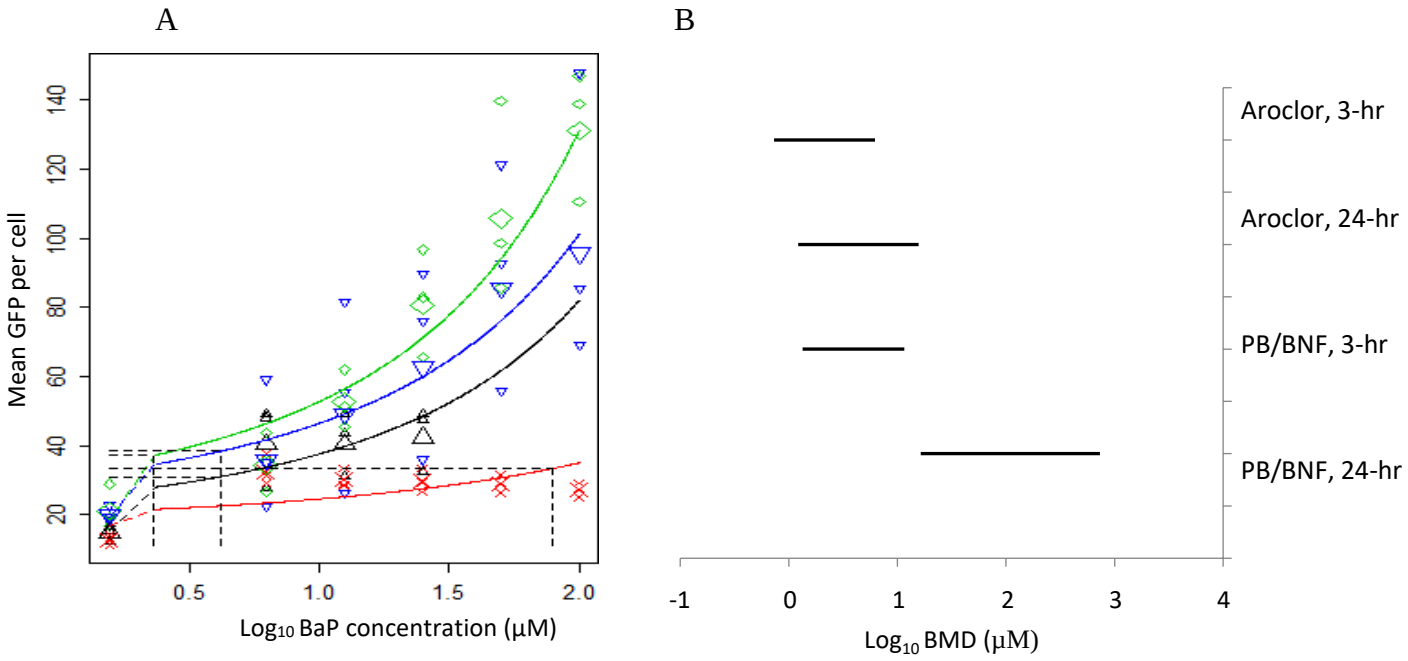

Figure 3.3 PROAST combined-covariate BMD analyses for different ToxTracker® assay treatment protocols – benzo[*a*]pyrene (BaP), *Rtkn* reporter. BaP dose-response data were analyzed using treatment as a covariate; exposure times of 3- and 24-hr for Aroclor- and PB/BNF-induced rat liver S9. The colors represent the following treatment conditions: green, 3-hr Aroclor; blue, 24-hr Aroclor; black, 3-hr PB/BNF; red, 24-hr PB/BNF. Small symbols represent values for individual biological replicates; large symbols represent mean values. A BMR of 100% was used to determine the BMD_{100} (µM) for the *Rtkn*-GFP reporter. Horizontal dotted lines indicate the BMD_{100}, i.e., the dose corresponding to a 100% increase in response over background (A). The BMD confidence intervals are shown in (B); the left-hand side of the horizontal lines indicates the BMDL, the right-hand side indicates the BMDU.

Bscl2

For the *Bscl2* DNA damage reporter, the lowest BMD_{100} values (i.e., doubling dose values) were associated with the PB/BNF-induced S9 for both CPA and AFB1. For the other substances, e.g., BAA, BaP, Trp-P-2, ISP, 2AA, 2AAF, IQ, MeIQ, PhIP, Phenolphthalein, 1,2-DPH, 1,3-DPH, DMBA, HMPA, DOrange, DMYellow, OAT and 3MC, the lowest BMD_{100} values are associated with Aroclor-induced S9. PB/BNF-induced S9 yielded significantly lower BMD_{100} values for CPA

only (i.e., significantly different at $p<0.05$). No significant differences in treatment-specific BMD_{100} values were observed for any of the other substances.

With respect to treatment time, the 24-hr treatment time yielded the lowest BMD_{100} values for BaP, 2AA, IQ, PhIP, 1,3-DPH and Phenolphthalein. In contrast, the lowest doubling dose was associated with the 3-hr exposure for the other substances investigated. The 24-hr treatment resulted in significantly lower BMD_{100} values for only two substances; 1,3-DPH and phenolphthalein. However, for DMBA and AFB1, the values were significantly higher for that exposure period, i.e., were significantly lower for the 3-hr treatment. There were no other significant differences in treatment-time BMD_{100} values.

Overall, although S9-type and/or treatment time altered the BMD_{100} for numerous test substances, in most cases the values were not significantly different. Nevertheless, there is some indication that BMD_{100} values are generally lower for Aroclor-induced S9 and 3-hr treatment.

Rtkn

For the *Rtkn* DNA damage reporter, the lowest BMD_{100} values were associated with the PB/BNF-induced S9 for CPA, AFB1, 1,3-DPH, 3MC and Phenolphthalein. For the other substances, e.g., BaP, BAA, 2AA, ISP, IQ, MeIQ, PhIP, 1,2-DPH, DMBA, HMPA, DOrange, DMYellow, Trp-P-2, OAT and 2AAF, the lowest BMD_{100} values were associated with Aroclor-induced S9. Aroclor-induced S9 yielded significantly lower BMD_{100} values only for BaP (Figure 3.3), and only for the 24-hr condition. No significant differences in S9-type BMD_{100} values were observed for any of the other substances investigated.

With respect to treatment time, the 24-hr condition yielded the lowest BMD_{100} values for 2AA, 2AAF, PhIP, Phenolphthalein, and 1,3-DPH. In contrast, the lowest BMD_{100} values for other

substances were associated with the 3-hr treatment. The 24-hr treatment yielded significantly lower BMD_{100} values for both PhIP and Phenolphthalein. Significantly lower BMD_{100} values were also observed for AFB1, CPA, and 3MC for the 3-hr treatment. No significant differences in treatment time-specific BMD_{100} values were observed for any other chemical.

Overall, there is some indication that BMD_{100} values are generally lower for Aroclor-induced S9 and 3-hr treatment.

Btg2

For the *Btg2* cellular stress reporter, the lowest BMD_{100} values were associated with PB/BNF-induced S9 for ISP, Trp-P-2, CPA and 3MC. For AFB1, the BMD_{100} values were very similar for both types of S9, although slightly lower for PB/BNF-induced S9. For the other substances investigated, e.g., BaP, BAA, 2AA, PhIP, MeIQ, IQ, Phenolphthalein, 1,2-DPH, 1,3-DPH, DMBA, HMPA, DOrange, DMYellow, OAT and 2AAF, the lowest BMD_{100} values were associated with Aroclor-induced S9. The S9 type associated with the lowest BMD_{100} values for BAA and HMPA was different for the 3-hr and 24-hr treatments, i.e., lower with Aroclor-induced S9 for the 24-hr treatment for BA, lower with PB/BNF-induced S9 for the 3-hr treatment for HMPA. The results did not reveal any significant S9-type effects.

With respect to treatment time, the 24-hr treatment yielded the lowest BMD_{100} values for BaP, 1,3-DPH, DMYellow, PhIP, and 2AA. In contrast, the lowest BMD values for other substances were associated with the 3-hr treatment. The 24-hr treatment yielded significantly lower BMD_{100} values for BaP only. In contrast, the 3-hr treatment yielded significantly lower BMD_{100} values for AFB1 and 3MC. The results did not reveal significant treatment-time effects for any of the other chemicals investigated.

Overall, there is some indication that BMD_{100} values are lower for Aroclor-induced S9 and 3-hr treatment.

Blvrb

For the *Blvrb* oxidative stress reporter, the lowest BMD_{100} values were associated with the PB/BNF-induced S9 for CPA, BAA and AFB1. For the other substances, e.g., DMBA, HMPA, ISP, MeIQ, IQ, PhIP, OAT, BaP, 2AA, 2AAF, Phenolphthalein, Trp-P-2, 1,2-DPH, 1,3-DPH, DOrange, DMYellow and 3MC, the lowest BMD_{100} values were associated with Aroclor-induced S9. No significant differences in S9-type BMD_{100} values were observed for any of the substances.

With respect to treatment time, the 24-hr treatment yielded the lowest BMD_{100} values for 2AA, 2AAF, 1,3-DPH, PhIP, Phenolphthalein and OAT. In contrast, the lowest BMD_{100} values for the other substances were associated with the 3-hr treatment. The 24-hr treatment yielded significantly lower BMD_{100} values for 2AA, BA, Phenolphthalein and 1,3-DPH. No significant differences in treatment-time BMD_{100} values were observed for the other substances.

Overall, there is some indication that BMD_{100} values are generally lower for Aroclor-induced S9 and 3-hr treatment.

Srxn1

For the *Srxn1* oxidative stress reporter, the lowest BMD_{100} values were associated with the PB/BNF-induced S9 for CPA, PhIP, DMYellow, DOrange, HMPA, AFB1 and 3MC. For the other substances, e.g., ISP, PHPT, Trp-P-2, BAA, DMBA, PhIP, MeIQ, IQ, OAT, 2AA, 2AAF, 1,2-DPH and 1,3-DPH, the lowest BMD_{100} values were associated with Aroclor-induced S9. No significant differences in S9-type BMD_{100} values were observed for any of the substances investigated.

With respect to treatment time, the 24-hr treatment yielded the lowest BMD_{100} values for 2AA, 2AAF, I,3-DPH, IQ, PhIP, OAT and HMPA. The lowest BMD_{100} value for other substances were associated with the 3-hr treatment. The 24-hr treatment yielded significantly lower BMD_{100} values for 2AA, 2AAF, PhIP, OAT and DMYellow. In contrast, the 3-hr treatment yielded a significantly lower BMD_{100} for AFB1. No significant treatment-time differences in BMD_{100} were observed for the other substances.

Overall, there is some indication that BMD_{100} values are lower for Aroclor-induced S9 and 3-hr treatment.

Unfortunately, no single treatment condition was uniformly associated with lowest BMD_{100}. Moreover, in most cases, the treatment variables did not have statistically significant effects on BMD_{100}. Nevertheless, the results were reviewed to determine the treatment condition most-commonly associated with the lowest BMD_{100} (i.e., lowest dose required to elicit a two-fold increase in reporter response). Table 3.2 provides an overall summary of the treatment condition associated with the lowest BMD for each substance-reporter combination.

For the genotoxicity reporters, i.e., *Bscl2* and *Rtkn*, the table reveals an overall preference for Aroclor-induced S9, i.e., lower BMD_{100} values. More specifically, nine of twelve assessments for *Bscl2*, and ten of fifteen assessments for *Rtkn*, and for the genotoxicity reporters overall, nineteen out of twenty-seven assessments (i.e., 70%). For Aroclor-induced S9 there does not seem to be any meaningful effect of treatment time on BMD_{100}. For PB/BNF-induced S9, most of the instances where the BMD_{100} value is lower than the corresponding Aroclor-induced S9 value are associated with 3-hr treatment duration.

For the oxidative stress reporters, i.e., *Blvrb* and *Srxn1,* the results reveal that overall, Aroclor-induced S9 is somewhat preferable, i.e., BMD_{100} values are lower. More specifically, ten out of thirteen assessments for *Blvrb,* and eight out of fourteen assessments for *Srxn1,* have lower BMD_{100} values with Aroclor, which corresponds to eighteen out of twenty-seven assessments overall (67%). The results also indicate that with regards to the treatment time, Aroclor-induced S9 is preferable when cells are exposed for 24-hr, and PB/BNF is preferable when the 3-hr treatment time is used (Table 3.2).

As for the p53 cellular stress reporter *Btg2,* the results also indicate that Aroclor-induced S9 is preferable overall, relative to the results associated with PB/BNF-induced S9. More specifically, nine out of thirteen assessments (70%) yielded lower BMD_{100} values when tested with Aroclor-induced S9. Additionally, one substance, i.e., AFB1, yielded equal BMD_{100} values for both types of S9. For Aroclor-induced S9, no meaningful effect regarding treatment time was observed. In contrast, for PB/BNF-induced S9, the condition associated with the lowest BMD_{100} values was 3-hr.

Table 3.2 Summary of the BMD comparative potency analysis. The condition (i.e., treatment time and S9 type) associated with the lowest BMD_{100} is indicated for each substance and each ToxTracker® reporter. Substances that had BMDLs and/or BMDUs of 0 and/or infinite, respectively, for all conditions are not included.

Reporter	Substance	Condition associated with lowest BMD_{100}	Comment
Bscl2	BaP	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values.
	CPA	PB/BNF, 3-hr	PB/BNF yielded significantly lower BMD_{100} values compared to Aroclor. No significant difference in treatment time BMD_{100} values.
	DMBA	Aroclor, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values in comparison with 24-hr. No significant differences in S9 type BMD_{100} values.
	2AA	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. BMDLs are undefined (i.e., equals 0) for the 24-hr conditions.
	1,2-DPH	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. BMDUs undefined (i.e., infinite) for the 3-hr condition.
	1,3-DPH	Aroclor, 24-hr	24-hr treatments yield significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
	MeIQ	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. 3-hr treatment BMDLs and/or BMDUs are undefined (i.e., equals 0 and infinite).
	IQ	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. Very large BMD_{100} confidence intervals (i.e., almost infinite), or undefined BMDLs or BMDUs (i.e., 0 or infinite).
	PhIP	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values.
	AFB1	PB/BNF, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values in comparison with 24-hr. No significant differences between S9 type BMD_{100} values.
	PHPT	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.

	3MC	PB/BNF, 3-hr	No significant differences between any of the BMD_{100} values. BMDLs are undefined (i.e., equals 0) for the 3-hr conditions.
Rtkn	BaP	Aroclor, 3-hr	For 24-hr, Aroclor yielded significantly lower BMD_{100} values. For PB/BNF, BMD_{100} values significantly lower for 3-hr. No significant differences in BMD_{100} values across other conditions.
	CPA	PB/BNF, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values in comparison with 24-hr. No significant differences between S9 type BMD_{100} values.
	DMBA	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
	2AA	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. BMDLs or BMDUs for the 24-hr condition undefined (i.e., 0 or infinite).
	DOrange	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
	1,3-DPH	PB/BNF, 24-hr	No significant differences between any of the BMD_{100} values.
	MeIQ	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. BMDLs not defined for the 3-hr condition (i.e., equals 0).
	IQ	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. BMDLs not defined (i.e., equals 0) for the 3-hr conditions and for 24-hr Aroclor.
	PhIP	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
	AFB1	PB/BNF, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values relative to 24-hr. No significant differences between S9 type BMD_{100} values.
	PHPT	PB/BNF, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
	3MC	PB/BNF, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values, although BMDLs not defined (i.e., equals 0) for this time point. No significant differences between S9 type BMD_{100} values.
	OAT	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
	2AAF	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values.

	1,2-DPH	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
Btg2	BaP	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values compared to 3-hr, although BMDLs undefined for this time point (i.e., equals 0). No significant differences between the S9 type BMD_{100} values.
	CPA	PB/BNF, 3-hr	PB/BNF treatments yield significant lower BMD_{100} values at 3-hr in comparison with 24-hr. No significant difference in S9 type.
	DMBA	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values
	2AAF	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. BMDUs not defined for 3-hr condition (i.e., infinite).
	DOrange	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. BMDUs not defined for 24-hr conditions (i.e., infinite). Very large confidence intervals (i.e., almost infinite) for 3-hr condition.
	1,3-DPH	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values
	DMYellow	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite) or undefined BMDUs (i.e., infinite).
	PhIP	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values.
	AFB1	PB/BNF, and Aroclor, 3-hr (equal values)	3-hr treatments yielded significantly lower BMD_{100} values. No significant differences between the S9 type BMD_{100} values.
	3MC	PB/BNF, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values, although BMDLs are not defined for this time point (i.e., equals 0). No significant differences between the S9 type BMD_{100} values.
	OAT	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite) or infinite BMDUs for all conditions.
	2AA	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite) or undefined BMDLs or BMDUs (i.e., 0 or infinite).
	1,2-DPH	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.

Blvrb	BaP	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
	CPA	PB/BNF, 3-hr	No significant differences between any of the BMD_{100} values.
	DMBA	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
	2AAF	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals or undefined BMDUs.
	1,3-DPH	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
	PhIP	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. BMDLs are undefined (i.e., equals 0) for the 24-hr conditions.
	AFB1	PB/BNF, 3-hr	No significant differences between any of the BMD_{100} values.
	PHPT	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
	3MC	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite).
	OAT	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. BMDLs or BMDUs are undefined (i.e., 0 or infinite) for the 3-hr conditions.
	2AA	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values, although BMDLs undefined for this time point (i.e., equals 0). No significant differences between S9 type BMD_{100} values.
	1,2-DPH	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
	BAA	PB/BNF, 3-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values. BMDUs undefined (i.e., equals 0) for the 24-hr conditions.
Srxn1	CPA	PB/BNF, 3-hr	No significant differences between any of the BMD_{100} values.
	DMBA	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.

2AA	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values, although BMDLs undefined for this time point (i.e., equals 0). No significant differences between S9 type BMD_{100} values.
DOrange	PB/BNF, 3-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite) or undefined BMDUs (i.e., infinite).
1,3-DPH	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values.
IQ	Aroclor, 24-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite) or undefined BMDLs or BMDUs (i.e., 0 or infinite).
PhIP	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
AFB1	PB/BNF, 3-hr	3-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values.
DMYellow	PB/BNF, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values. BMDUs undefined (i.e., infinite) for 3-hr conditions.
3MC	PB/BNF, 3-hr	No significant differences between any of the BMD_{100} values.
OAT	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values. BMDUs undefined (i.e., infinite) for 3-hr conditions.
2AAF	Aroclor, 24-hr	24-hr treatments yielded significantly lower BMD_{100} values. No significant differences between S9 type BMD_{100} values. BMDUs undefined (i.e., infinite) for 3-hr conditions.
1,2-DPH	Aroclor, 3-hr	No significant differences between any of the BMD_{100} values.
HMPA	PB/BNF, 24-hr	No significant differences between any of the BMD_{100} values. Very large confidence intervals (i.e., almost infinite) or undefined BMDUs (i.e., infinite).

3.3.2 Part II – Effect of S9-type for 24-hr treatment time and fixed S9 concentrations

Follow-up experiments evaluated the utility of two 24-hr metabolic activation protocols, i.e., 0.25% v/v Aroclor-induced S9 and 0.40% v/v PB/BNF-induced S9. The results obtained were compared using the BMD combined-covariate approach, i.e., statistical comparisons of the doubling doses (BMD_{100}) for seven genotoxicants (Table 3.1) across five ToxTracker® reporters. Figure 3.4 provides an example of the results obtained; a comparative illustration of the *Rtkn* reporter responses for MeIQ. The results revealed that the BMD_{100} is lower for the Aroclor protocol (Figure 3.4); from a statistical point of view, the difference is marginally significant.

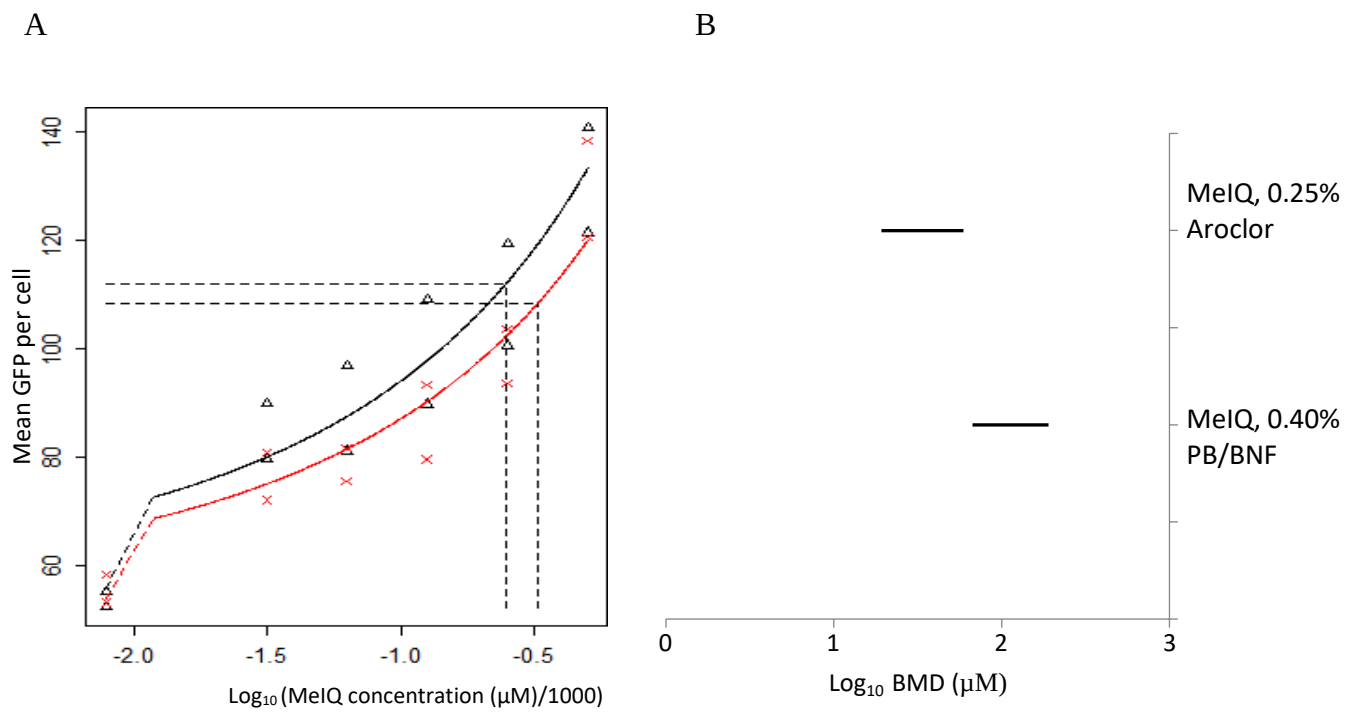

Figure 3.4 PROAST combined-covariate BMD results showing the effect of the different treatment protocols on the effect of MeIQ on the ToxTracker® *Rtkn* reporter. The fitted dose-response curves (A) show responses for the two different treatment protocols (i.e., 24-hr, 0.25% v/v Aroclor-induced S9 and 24-hr, 0.40% v/v PB/BNF-induced S9). The black symbols and fitted function show the Aroclor response; the red symbols and fitted function show the PB/BNF response. The BMR used is 100%, and the horizontal dotted lines indicate the BMD_{100} values (i.e., the doubling dose in µM). The plotting symbols show the results for duplicate experiments. The BMD confidence intervals for each condition are shown (B), the left-hand side of the horizontal lines are the BMDLs, the right-hand side of the horizontal lines are the BMDUs.

The results for both the *Bscl2* and the *Rtkn* genotoxicity reporters indicate that the BMD_{100} values for the Aroclor-induced S9 are lower for 5 of the 7 tested substances (i.e., 71%); the BMD_{100} values for the remaining 2 substances are lower for the PB/BNF-induced S9. That being said, the Aroclor-induced S9 BMD_{100} values are only significantly lower for the two heterocyclic amines, i.e., MeIQ and PhIP. The BMD_{100} value for 2AA with PB/BNF S9 is significantly lower than the corresponding Aroclor value. Overall, the results indicate that the Aroclor-induced S9 is preferable, i.e., BMD_{100} values are generally lower (Tables 3.3 and 3.4).

The results for the *Btg2* p53 cellular stress reporter indicate that the Aroclor-induced S9 treatment is slightly preferable, i.e., lower BMD_{100} values for 4 of the 7 tested substances (i.e., 57%), with the other 3 yielding lower BMD_{100} values with PB/BNF-induced S9. However, the values are only significantly different for BaP, which yielded a lower BMD_{100} when tested with Aroclor-induced S9. Overall, the results indicate that there is no noteworthy difference related to S9 type, i.e., condition for which BMD_{100} values are uniformly lower (Table 3.5).

With respect to the *Blvrb* reporter for oxidative stress, 5 of the 7 tested substances yielded lower BMD_{100} values when tested in the presence of Aroclor-induced S9 (i.e., 71%); the other 2 being lower when tested with PB/BNF-induced S9. However, there were no significant differences between any of the BMD_{100} values. Overall, the trend indicates that the Aroclor-induced S9 is preferable, i.e., BMD_{100} values are generally lower (Table 3.6).

With respect to the *Srxn1* reporter for oxidative stress, the results indicate that 4 of the 7 tested substances yielded lower BMD_{100} values when tested with PB/BNF-induced S9 (i.e., 57%), the other 3 being lower with Aroclor-induced S9. That being said, the BMD_{100} values are only significantly lower for 2AA, i.e., when tested in presence of PB/BNF-induced S9. Overall, the results indicate that there is no noteworthy preference regarding S9 type, i.e., S9 type for which the BMD_{100} values are uniformly lower (Table 3.7).

Table 3.3 Summary of ToxTracker® *Bscl2* BMD_{100} (µM) values employed to comparatively evaluate two metabolic activation protocols - 0.25% v/v Aroclor-induced S9 (A), and 0.40% v/v PB/BNF-induced S9. The *Bscl2* reporter results are shown; the treatment time was 24-hr for both S9 types. BMDLs of zero indicate that the value is not significantly different from 0. In those cases, BMDL/BMDU ratio was not calculated. Confidence intervals that could not be calculated are listed as NA (Not Available).

Chemical	Concentration (%)	S9 Type	BMD_{100} (µM)	BMDL	BMDU	BMDU/ BMDL
AFB1	0.25	A	4.07	2.53	6.68	2.64
	0.40	PB/BNF	2.85	1.84	4.54	2.47
BaP	0.25	A	21.90	0	918.00	N/A
	0.40	PB/BNF	107.09	21.00	1.82E+03	86.67
BAA	0.25	A	1.67E+06	4.05E+03	1.99E+07	4.91E+03
	0.40	PB/BNF	1.28E+06	3.12E+03	1.36E+07	4.36E+03
2AA	0.25	A	256.32	153.00	672.00	4.39
	0.40	PB/BNF	140.89	86.40	517.00	5.98
DMBA	0.25	A	24.31	12.60	49.80	3.95
	0.40	PB/BNF	70.89	33.10	189.00	5.71
MeIQ	0.25	A	246.76	129.00	399.00	3.09
	0.40	PB/BNF	323.15	201.00	565.00	2.81
PhIP	0.25	A	1.39	0.62	2.50	4.03
	0.40	PB/BNF	2.35	1.55	2.98	1.92

Table 3.4 Summary of ToxTracker® *Rtkn* BMD_{100} (µM) values employed to comparatively evaluate two metabolic activation protocols - 0.25% v/v Aroclor-induced S9 (A) and 0.40% v/v PB/BNF-induced S9. The *Rtkn* reporter results are shown; the treatment time was 24-hr for both S9 types. BMDLs of zero indicate that the value is not significantly different from 0. In those cases, BMDL/BMDU ratio was not calculated. Confidence intervals that could not be calculated are listed as NA (Not Available).

Chemical	Concentration (% v/v)	S9 Type	BMD_{100} (µM)	BMDL	BMDU	BMDU/ BMDL
AFB1	0.25	A	0.85	0.54	1.24	2.30
	0.40	PB/BNF	0.47	0.30	0.69	2.30
BaP	0.25	A	0.08	0	2.08	N/A
	0.40	PB/BNF	4.23	1.69	6.26	3.70
BAA	0.25	A	3.59E+04	1.30E+03	2.47E+05	190.00
	0.40	PB/BNF	1.51E+05	2.94E+03	1.03E+06	350.34
2AA	0.25	A	5.40E+03	287.00	5.46E+05	1.90E+03
	0.40	PB/BNF	3.01	0.30	64.50	215.00
DMBA	0.25	A	0.32	0.04	1.53	38.25
	0.40	PB/BNF	1.72	0.25	9.01	36.04
MeIQ	0.25	A	34.83	19.20	59.10	3.08
	0.40	PB/BNF	111.95	67.00	190.00	2.84
PhIP	0.25	A	0.48	0.34	0.63	1.85
	0.40	PB/BNF	1.00	0.75	1.25	1.67

Table 3.5 Summary of ToxTracker® *Btg2* BMD_{100} (µM) values employed to comparatively evaluate two metabolic activation protocols - 0.25% v/v Aroclor-induced S9 (A) and 0.40% v/v PB/BNF-induced S9. The *Btg2* reporter results are shown; the treatment time was 24-hr for both S9 types. BMDLs of zero indicate that the value is not significantly different from 0. In those cases, BMDL/BMDU ratio was not calculated. Confidence intervals that could not be calculated are listed as NA (Not Available).

Chemical	Concentration (% v/v)	S9 Type	BMD_{100} (µM)	BMDL	BMDU	BMDU/ BMDL
AFB1	0.25	A	1.00	0.73	1.33	1.82
	0.40	PB/BNF	0.67	0.49	0.88	1.80
BaP	0.25	A	0.51	0	2.58	N/A
	0.40	PB/BNF	11.49	5.49	23.10	4.21
BAA	0.25	A	1.51E+03	337.00	1.84E+05	545.99
	0.40	PB/BNF	241.42	73.30	3.04E+05	4.15E+03
2AA	0.25	A	6.64E+36	225.00	Infinite	N/A
	0.40	PB/BNF	0	NA	NA	N/A
DMBA	0.25	A	0.35	0.03	2.51	83.67
	0.40	PB/BNF	0.86	0.07	6.27	89.57
MeIQ	0.25	A	30.84	12.80	82.30	6.43
	0.40	PB/BNF	117.49	50.20	239.00	4.76
PhIP	0.25	A	0.98	0.57	1.39	2.44
	0.40	PB/BNF	1.22	0.76	1.68	2.21

Table 3.6 Summary of ToxTracker® *Blvrb* BMD_{100} (µM) values employed to comparatively evaluate two metabolic activation protocols - 0.25% v/v Aroclor 1254-induced S9 (A) and 0.40% v/v PB/BNF-induced S9. The *Blvrb* reporter results are shown; the treatment time was 24-hr for both S9 types. BMDLs of zero indicate that the value is not significantly different from 0. In those cases, BMDL/BMDU ratio was not calculated. Confidence intervals that could not be calculated are listed as NA (Not Available).

Chemical	Concentration (% v/v)	S9 Type	BMD_{100} (µM)	BMDL	BMDU	BMDU/ BMDL
AFB1	0.25	A	9.58E+03	22.30	2.79E+05	1.25E+04
	0.40	PB/BNF	2.57E+04	56.70	3.33E+06	5.87E+04
BaP	0.25	A	9.12	0	188.00	N/A
	0.40	PB/BNF	1.79	0	37.40	N/A
BAA	0.25	A	6.64	4.20	11.20	2.67
	0.40	PB/BNF	8.94	5.81	14.30	2.46
2AA	0.25	A	0.01	0	0.01	N/A
	0.40	PB/BNF	0	0	0.01	N/A
DMBA	0.25	A	7.26	2.97	17.50	5.89
	0.40	PB/BNF	7.63	3.10	20.50	6.61
MeIQ	0.25	A	1.19E+05	1.46E+03	2.93E+06	2.01E+03
	0.40	PB/BNF	5.24E+05	4.02E+03	2.50E+07	6.22E+03
PhIP	0.25	A	189.83	0	2.32E+03	N/A
	0.40	PB/BNF	1.30E+03	4.55	2.11E+04	4.64E+03

Table 3.7 Summary of ToxTracker® *Srxn1* BMD_{100} (µM) values employed to comparatively evaluate two metabolic activation protocols - 0.25% v/v Aroclor 1254-induced S9 (A) and 0.40% v/v PB/BNF-induced S9. The *Srxn1* reporter results are shown; the treatment time was 24-hr for both S9 types. BMDLs of zero indicate that the value is not significantly different from 0. In those cases, BMDL/BMDU ratio was not calculated. Confidence intervals that could not be calculated are listed as NA (Not Available).

Chemical	Concentration (% v/v)	S9 Type	BMD_{100} (µM)	BMDL	BMDU	BMDU/ BMDL
AFB1	0.25	A	4.44	1.91	11.50	6.02
	0.40	PB/BNF	1.42	0.57	4.00	7.02
BaP	0.25	A	0.76	0	5.39	N/A
	0.40	PB/BNF	1.23	0	5.80	N/A
BAA	0.25	A	20.27	6.59	111.00	16.84
	0.40	PB/BNF	2.25	0.61	6.99	11.46
2AA	0.25	A	0.04	0.01	0.22	22.00
	0.40	PB/BNF	0	0	0.03	N/A
DMBA	0.25	A	1.19	0.52	2.28	4.38
	0.40	PB/BNF	1.30	0.54	2.71	5.02
MeIQ	0.25	A	142.74	20.90	713.00	34.11
	0.40	PB/BNF	144.72	27.20	761.00	27.98
PhIP	0.25	A	2.63	0.98	2.86	2.92
	0.40	PB/BNF	2.55	1.01	12.78	12.65

Overall, the results indicate that for the genotoxicity reporters, i.e., *Rtkn* and *Bscl2*, Aroclor-induced S9 is marginally preferable, i.e., the BMD_{100} values are lower when substances are tested with this type of S9. In contrast, the results failed to indicate any S9-type preference for the other reporters. More specifically, for the *Btg2* p53 cellular stress reporter, only BaP yielded a significantly lower BMD_{100} when tested with Aroclor-induced S9. Additionally, although 2AA yielded a significantly lower *Srxn1* BMD_{100} when tested with PB/BNF-induced S9, with respect to both oxidative stress reporters (i.e., *Srxn1* and *Blvrb*), the other substances did not yield BMD_{100} values indicative of an S9-type preference.

3.4 Discussion

3.4.1 Part I – The effects of S9-type and treatment time for 20 test substances

As noted earlier, S9 is an essential reagent for effective *in vitro* genotoxicity assessment. S9, i.e., the post-mitochondrial supernatant from homogenized rodent liver, provides the basis for an exogenous metabolic activation mixture that permits *in vitro* simulation of mammalian hepatic metabolism, and *in vitro* conversion of some substances into DNA-reactive metabolites. More specifically, S9 supplements the metabolic deficiencies of cells commonly employed for *in vitro* assessment of genotoxicity, e.g., the ToxTracker® mES cells. To enhance the ability of an S9 metabolic activation mixture to support the enzymatic generation of DNA-reactive metabolites, rodents from which S9 is derived are commonly exposed to a CYP inducer. Traditionally, the S9-inducer employed is Aroclor 1254, a commercial mixture of PCBs. However, although Aroclor 1254 is a potent, effective inducer of CYP isozymes, especially CYP1A1 and CYP1A2, it is no longer commercially available. Thus, it is essential to delineate and evaluate an alternative. Although PB/BNF-induced S9 has been suggested as a suitable substitute (35), its utility and efficacy have not been sufficiently evaluated. The objective of the work presented herein was to compare Aroclor- and PB/BNF-induced rat liver S9 for genotoxicity assessment of selected chemicals. The first part of the work investigated the effect of S9-type and treatment time on induction of selected ToxTracker® assay reporters; testing involved 20 substances. This was followed by work that compared two metabolic activation protocols; the PB/BNF protocol investigated has been proposed for routine *in vitro* detection of genotoxic substances that require metabolic activation.

Effect of S9 type

The first part of the work examined the influence of treatment time and S9-type on the genotoxic potency of selected chemicals (i.e., BMD_{100} values). Several of the investigated substances, e.g., PAHs such as BaP and DMBA, are known to require metabolic conversion to DNA-reactive substances, and it was necessary to determine, for a given treatment time, if S9-type influences potency. Overall, for the reporters indicative of DNA-damage (i.e., *Bscl2* and *Rtkn*), oxidative stress (i.e., *Blvrb* and *Srxn1*), and p53-related cellular stress (*Btg2),* the results obtained for the tested substances showed a trend of lower BMD_{100} values (i.e., lower doubling dose) when Aroclor-induced S9 was used. However, in most instances the preference for Aroclor-induced S9 was not statistically significant. Nevertheless, this result is consistent with the requirement for CYP isozymes CYP1A1 and CYP1A2 to convert a substance such as BaP into a DNA-reactive electrophile capable of generating DNA (e.g., dG) adducts (8, 9) (Figure 3.1). The information presented in Chapter 1 (i.e., Table 1.2) indicates that Aroclor-induced S9 has higher CYP1A1 and CYP1A2 activity in comparison with PB/BNF-induced S9. As for heterocyclic and aromatic amines (e.g., PhIP, MeIQ, 2AA), Phase II enzymes such as NATs and SULT are also critically important for enzymatic conversion to reactive metabolites (36). The literature does not indicate that either inducer (i.e., Aroclor or PB/BNF) significantly elevates these Phase II enzymes, suggesting that neither inducer should have an influence on the magnitude or nature of the response. Nevertheless, since CYP1A2 is required to convert aromatic amines to hydroxyarylamines (37) (Chapter 1, Table 1.1), the preference for Aroclor-induced S9 was expected.

With respect to both the *Bscl2* and *Rtkn* DNA damage reporters, the results showed that most substances (e.g., PAHs) yielded lower, although not statistically-significant, BMD_{100} values with

Aroclor-induced S9, i.e., compared to PB/BNF-induced S9. Again, this is consistent with the metabolic profile of Aroclor-induced S9, i.e., higher activity of CYP1A1 and 1A2. In contrast, for *Bscl2* reporter, the lowest BMD_{100} for CPA, AFB1 and 3MC was associated with PB/BNF-induced S9. This is consistent with what is known about AFB1 and CPA. More specifically, they require CYP isozymes 3A and 2B for metabolic conversion to reactive metabolites, respectively; (38, 39) PB/BNF-induced S9 has higher CY3A and CY2B1 activities compared to Aroclor 1254-induced S9 (40) (Chapter 1, Table 1.2). As for 3MC, it was expected that the condition with the lowest BMD_{100} would be Aroclor-induced S9, since it is a PAH, and CYP1A1 and CYP1A2 are primarily required for metabolic conversion to a reactive metabolite (8, 9, 41). For the *Rtkn* reporter, BaP yielded a significantly lower BMD_{100} when tested in presence of Aroclor-induced S9 for the 24-hr condition. This was expected since, as already mentioned, PAHs require CYP1A1 and 1A2 isozymes for metabolic conversion to a reactive metabolite (8, 9). Substances such CPA, 1,3-DPH, PHPT, AFB1 and 3MC yielded lower BMD_{100} values when tested with PB/BNF-induced S9. As previously mentioned, the results are consistent with what is known about AFB1 and CPA (38, 39), although inconsistent with what is known about 3MC (41). Moreover, the results for PHPT and 1,3-DPH are also unexpected, since both substances require CYP1A1 and CYP1A2 for conversion to reactive metabolites (42, 43), and PB/BNF-induced S9 has lower 1A1 and 1A2 activities compared with Aroclor-induced S9.

It was expected that substances with CYP1A1 and CYP1A2 enzymatic requirements would yield significantly lower BMD_{100} values when tested with Aroclor-induced S9, i.e., across all reporters. Although BMD_{100} values were generally lower when tested with Aroclor-induced- S9, the results failed to show any significant differences between the two types of S9. Therefore, no firm conclusion can be made regarding the type of S9 that would be expected to uniformly yield

significantly lower BMD_{100} values. This suggests that, despite the fact that some of the required isozymes are more active in Aroclor-induced S9 (40) (Chapter 1, Table 1.2), PB/BNF-induced S9 could be used as an alternative to effectively detect the genotoxicity of substances such as PAHs, i.e., responses on the *Rtkn* and *Bscl2* reporters. That being said, it is important to note that the concentrations used for the S9 types were different, i.e., the concentration used for PB/BNF-induced S9 was four times higher than that used for Aroclor-induced S9. Given that the differences in induced enzymatic activity between the S9 types are only approximately 2-fold or less for CYP1A1, 1A2, 2B1 and 3A (Table 1.2), it seems that the higher concentration used for PB/BNF-induced S9 was able to compensate for the activity differences.

With respect to the reporters for oxidative stress, i.e. *Blvrb* and *Srxn1*, no significant differences in BMD_{100} values between the S9 types were observed. Interestingly, PAHs such as BaP can cause DNA damage via oxidative stress (44). More specifically, following P450 CYP1A1 and CYP1B1 metabolism, AKR (aldo-keto reductase) can generate *o*-quinones that generate ROS (reactive oxygen species) via redox cycling (44). However, since AKRs are phase II enzymes, and are mostly present in cytosol, Golgi apparatus and mitochondria (45) (46), an S9-source effect would not be expected. Indeed, there is no published information indicating that AKR activity in rat liver S9 would be affected by the chemical inducer of oxidative metabolism.

With respect to the reporter for p53-related cellular stress, i.e., *Btg2*, no significant difference in BMD_{100} values between the S9 types were observed. This was not expected since p53 translocation to the nucleus is influenced by ATR/ATM, which are affected by chemically-induced genetic damage (47). For example, CYP3A is required to generate DNA-reactive AFB1 metabolites (39) (Chapter 1, Table 1.1), and CYP3A activity is approximately 2-fold higher in PB/BNF-induced S9 compared to Aroclor-induced S9 (Chapter 1, Table 1.2). Thus, it would be

expected that the 1% v/v concentration of PB/BNF-induced S9 would result in a lower BMD_{100} value compared to 0.25% v/v of Aroclor-induced S9.

Overall, the results suggest that there is no meaningful difference between Aroclor-induced S9 and PB/BNF-induced S9, i.e., the results failed to yield statistically significant differences in BMD_{100} values. Although the results show some trends indicative of differences between Aroclor- and PB/BNF-induced S9, even some statistically significant differences, it is reasonable to assert that the differences are relatively inconsequential. This is certainly the case for the oxidative stress and p53 reporters (i.e., *Srxn1, Blvrb, Btg2*), and largely true for the genotoxicity reporters. There are exceptions, such as BaP for the *Rtkn* reporter, for which the BMD_{100} value is lower with Aroclor-induced S9, as well as a few substances (e.g., AFB1, CPA) for which the PB/BNF-induced S9 yielded lower BMD_{100} values. It is reasonable to assert that PB/BNF-induced S9 can be viewed as an effective substitute for Aroclor-induced S9. However, since S9 cytotoxicity has been observed (48, 49), it is possible that this has contributed to the utility of the results. Therefore, it would be important to take this factor into consideration when determining the concentration of PB/BNF-induced S9 that yields equivalent results compared to Aroclor-induced S9. Although the concentration of PB/BNF could theoretically be further increased (i.e., >1% v/v), the concentration of S9 in the exposure medium can only be increased until the 75% cytotoxicity cut-off is reached (i.e., the upper limit for an acceptable ToxTracker® assay). Therefore, future experiments should investigate the maximum concentration of PB/BNF-induced S9 that can be used without reaching the cytotoxicity cut-off.

Effect of exposure time

Since cultured animal cells, such as the ToxTracker® mES cells, are sensitive to rodent hepatic S9 (40), the metabolic activation conditions must balance S9 type, S9 concentration and treatment duration. Thus, the first phase of the work investigated both S9 type and treatment duration. With respect to treatment duration, the results indicate that, for a given S9-type, neither treatment time uniformly yielded lower BMD_{100} values (Table 3.2). For example, for the genotoxicity reporters *Rtkn* and *Bscl2*, AFB1 yielded lower BMD_{100} values for the 3-hr treatment; PHPT yielded lower BMD_{100} values for the 24-hr treatment. Moreover, for most treatments, there was no significant differences in the genotoxicity BMD_{100} values. Granted, the effect of treatment duration can be confounded with S9 type; thus, it is difficult to make definitive statements about the treatment-time effect.

In light of the results obtained, and the manageable cytotoxicity associated with a 24-hr treatment (data not shown), it should be possible to use the PB/BNF 24-hr protocol with 0.40% v/v as a substitute for Aroclor protocols. Indeed, the second part of the experimental work specifically compared the 24-hr protocol with 0.25% v/v Aroclor-induced S9 with the 24-hr protocol with 0.40% v/v PB/BNF-induced S9. This latter work focused on only a small number of known genotoxicants, and the responses of the *Rtkn* and *Bscl2* reporters. It is important to note that the 24-hr protocol is more practical for operational reasons, i.e., fewer sample manipulations and next day scoring. The 24-hr protocol involves continuous treatment in the presence of S9 before a single wash preceding scoring on the following day. In contrast, the 3-hr protocol necessitates two washes, i.e., one after 3-hr to stop the exposure, and one after 24-hr before the cells are prepared for flow cytometry.

3.4.2 Part II – Effect of S9-type for 24-hr treatment and defined S9 concentrations

In light of the results discussed above, Toxys B.V. endeavored to critically compare the responses of the genotoxicity reporters (i.e., *Rtkn* and *Bscl2*) across two alternate rat liver S9 treatment conditions and 7 known genotoxicants. More specifically, a continuous 24-hr treatment protocol in the presence of 0.25% v/v Aroclor-induced S9, or a 24-hr treatment in the presence of 0.40% v/v PB/BNF-induced S9. Toxys B.V. followed up on the earlier part of the study and generated the required data. The analyses were restricted to the following 7 known genotoxicants: AFB1, BAA, BaP, DMBA, 2AA, MeIQ and PhIP (Table 3.1) (19); importantly, all of these substances have been shown to have a noteworthy requirement for CYP isozymes 1A1, 1A2 or 3A (Chapter 1, Table 1.1). The significance and utility of the results obtained are discussed below.

The results obtained did not show any significant protocol differences for the *Bscl2* reporter, i.e., no difference in BMD_{100} values for all tested substances (i.e., AFB1, 2AA, BaP, BAA, MeIQ, PhIP and DMBA). Considering the metabolic requirements of these substances, it would be expected that Aroclor-induced S9 would yield lower BMD_{100} values; however, the lack of significant difference suggests that increasing the concentration of PB/BNF-induced S9 adequately compensated for the relative differences in the activity of CYP isozymes CYP1A1 and CYP1A2 (Chapter 1, Table 1.2). In contrast, for the *Rtkn* reporter, 2AA, MeIQ and PhIP had significantly lower BMD_{100} values when tested with Aroclor-induced S9. This was not surprising in light of the aforementioned difference in the relative activity of the CYP isozymes required for metabolic activation of these substances, i.e., the higher activity of CYP 1A1 and CYP1A2 for Aroclor-induced S9. More specifically, Table 1.2 shows that the differences in enzymatic activity for CYP1A1, 1A2, 2B1 and 3A is approximately 2-fold higher for Aroclor. Therefore, one might expect that the 1.6-fold difference in concentrations used in the ToxTracker® experiments would not be enough to compensate for the lower activity of isozymes in the PB/BNF-induced S9.

Although not significant, AFB1 had a lower BMD_{100} when tested with the PB/BNF-induced S9 protocol. This was also expected in light of the metabolic requirements of the fungal metabolite (Chapter 1, Table 1.1), and the enzymatic activity of PB/BNF-induced S9, i.e., increased relative activity of CYP3A (39, 40) (Chapter 1, Table 1.2).

For both oxidative stress reporters, i.e., *Blvrb* and *Srxn1*, there was no significant effect of S9 type. One exception is BAA with the *Srxn1* oxidative stress reporter; the BMD_{100} was lower when tested using the PB/BNF-induced S9 protocol. As previously mentioned, PAHs can cause oxidative stress via the formation of *o*-quinones, which are formed by AKRs, and can generate ROS via redox cycling. However, there is no published information regarding the differences in AKR activity between the S9 types, which suggests that S9-type should not have an influence on BAA potency. Thus, the lower BMD_{100} associated with PB/BNF-induced S9 cannot be explained.

Overall, the results suggest that, for a 24-hr treatment protocol, use of 0.40% v/v PB/BNF yields results that are aligned with those generated obtained using 0.25% v/v Aroclor-induced S9. Thus, it is reasonable to assert that, in the long term, PB/BNF-induced S9 can be used as a substitute for Aroclor-induced S9 for the ToxTracker® assay. The alternative S9 should permit effective and efficient identification of genotoxicants; moreover, determination of potency (i.e., BMD_{100}) for comparative potency evaluation and hazard assessment. That being said, to further refine the PB/BNF protocol, follow-up work should more comprehensively investigate the effect of S9 concentration on the response of the genotoxicity reporters, e.g., 0.25% v/v Aroclor versus 0.40, 0.50 and 0.60% PB/BNF. Additionally, the experimental design could be modified to increase the precision of the BMD_{100} values. Slob (2014) (50) showed that, instead of increasing the number of replicates per dose, BMD precision can be improved by increasing the number of concentrations/doses. The current experimental design included duplicates for 5 doses; it would

likely prove more beneficial to distribute the experimental units across 10 doses. Indeed, some researchers employing the BMD approach for analysis of genotoxicity reporter results are now opting for few replicates, if any, in favor of a large number of doses (51). This type of protocol also permits testing of a wider range of doses and/or finer dose spacing.

In conclusion, the results presented herein failed to reveal statistically-significant trends that can be used to definitely chose an effective protocol that avoids the use of Aroclor-induced S9; nevertheless, the results do provide data that can be used to support effective use of PB/BNF-induced S9 as a replacement for Aroclor-induced S9. That being said, the results also indicate that S9 type can influence genotoxic potency; thus, multiple factors need to be taken into consideration to ensure that the replacement protocol is effective. More specifically, the overall metabolic requirements for CYP isozymes in a metabolic activation mixture, the differences in the levels of the generally required isozymes in the two S9 types (i.e., CYPS 1A1, 1A2, 3A and 2B), the cytotoxicity of S9 preparations, and significant differences in the potency of noteworthy genotoxicants such as BaP and AFB1. Additionally, it will be necessary to ensure that a replacement protocol based on PB/BNF-induced S9 can effectively detect genotoxicants with a range of MOAs; moreover, effectively assess genotoxic potency for robust relative potency analysis. The former should probably include detailed performance assessment using metrics such as sensitivity, specificity and concordance, with *in vivo* genotoxicity as the *a priori* hazard point of reference (5, 7).

Going forward, it will be important to better understand the effect of S9 concentration on the response of each reporter, and to ultimately create an appropriate ToxTracker® protocol to permit use of PB/BNF-induced S9 as a routine substitute for Aroclor-induced S9. Most importantly, since the experimental designs employed herein frequently yielded BMD values with very low precision

(i.e., BMDU/BMDL>100), future work must examine far more, finely-spaced concentrations. This type of work would provide the dose-response data required to more definitively evaluate alternate protocols, thereby increasing confidence in the utility and efficacy a ToxTracker® protocol that employs PB/BNF-induced S9. Establishment and implementation of a reliable protocol based on PB/BNF-induced S9 will enable the high-throughput ToxTracker® assay to be used for routine genotoxicity assessment of substances that require safety evaluations prior to regulatory decision-making.

3.5 References

1. Organisation for Economic Co-operation and Development. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Organisation for Economic Co-Operation and Development Publishing. Paris, 2005;14(34):96. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd-gd34.pdf

2. Organisation for Economic Co-operation and Development. Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines. Organisation for Economic Co-operation and Development Publishing. Paris, 2015:1-64. https://www.oecd.org/env/ehs/testing/Draft%20Guidance%20Document%20on%20OECD%20Genetic%20Toxicology%20Test%20Guidelines.pdf

3. Li AP, Loretz LJ. Assays for Genotoxicity. In: Li AP, Heflich RH, editors. Genetic Toxicology. 1st ed. Boca Raton, Florida: CRC Press; 1946. p. 119-42.

4. MacGregor JT, Frötschlb R, White PA, Crump KS, Eastmonde DA, Fukushimaf S, Guérardg M, Hayashih M, Soeteman-Hernándezi LG, Kasamatsuj T, Levyk, DD, Morital T, Müllerm L, Schoenyn R, Schulero MJ, Thybaud V, Johnson GE. IWGT report on quantitative approaches to genotoxicity risk assessment I. Methods and metrics for defining exposure–response relationships and points of departure (PoDs). Mutation Research. 2015;783:55-65.

5. Hendriks G, Atallah M, Morolli B, Calleja, Fabienne M. G. R., Ras-Verloop N, Huijskens I, Raamsman M, van de Water B, Vrieling H. The ToxTracker Assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences. 2012;125(1):285-298.

6. Hendriks G, Derr RS, Misovic B, Morolli B, Calleja, Fabienne M. G. R., Vrieling H. The extended ToxTracker assay discriminates between induction of DNA damage, oxidative stress, and protein misfolding. Toxicological Sciences. 2016;150(1):190-203.

7. Toxys. ToxTracker. toxys.com. 2018.

8. Bauer E, Guo Z, Ueng Y, Bell LC, Zeldin D, Guengerich FP. Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. Chemical Research in Toxicology. 1995;8:136-42.

9. Jeffrey AM. DNA modification by chemical carcinogens. Pharmacology and Therapeutics. 1985;28:237-72.

10. Kim J, Stansbury K, Walker N, Trush M, Strickland P, Sutter T. Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. Carcinogenesis. 1998;19:1847-53.

11. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutation Research. 1983;113(3-4):173-215.

12. Ku WW, Bigger A, Brambilla G, Glatt H, Gocke E, Guzzie PJ, Hakura A, Honma M, Martus H, Obach RS, Roberts S. Strategy for genotoxicity testing-metabolic considerations. Mutation research. 2007;627(1):59-77.

13. Elliott BM, Combes RD, Elcombe CR, Gatehouse DG, Gibson GG, Mackay JM, Wolf RC. Alternatives to Aroclor 1254-induced S9 in in vitro genotoxicity assays. Mutagenesis. 1992;7(3):175-177.

14. Clare C. The state of play for PCBs. BIBRA Bulletin. 1989;28:111-4.

15. Callander RD, Mackay JM, Clay P, Elcombe CR, Elliott BM. Evaluation of phenobarbital/beta-naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in in vitro genotoxicity assays. Mutagenesis. 1995;10(6):517-22.

16. Schut H, Snyderwine E. DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. Carcinogenesis. 1999;20:353-68.

17. Hendriks G, Attalah M, Raamsman M, Morolli B, Van der Putten H, Jaadar H, Tijdens I, Esveldt-van Lange R, Mullenders L, Van de Water B, Vrieling H. Sensitive DsRed fluorescence based reporter cell systems for genotoxicity and oxidative stress assessment. Mutation Research. 2011;709:49-59.

18. Organisation for Economic Co-operation and Development (OECD). Document on Good In Vitro Method Practices. Organisation for Economic Co-Operation and Development Publishing. Paris, 2018. https://www.oecd-ilibrary.org/environment/guidance-document-on-good-in-vitro-method-practices-givimp_9789264304796-en.

19. Jagger C, Tate M, Cahill P, Hughes C, Knight AW, Billinton N, Walmsley RM. Assessment of the genotoxicity of S9-generated metabolites using the GreenScreen HC *GAAD45a-GFP* assay. Mutagenesis. 2009;24(1):35-50.

20. National Toxicology Program. 11th Report on Carcinogens. http://ntp.niehs.nih.gov/ntpweb/index.cfm?objectid5035E5806-F735-FE81-FF769DFE5509AF0A). 2005.

21. Hastwell P, Chai L, Roberts K, Webster T, Harvey J, Rees R, Brown A. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: Validation of the GreenScreen HC *GADD45a-GFP* genotoxicity assay. Mutation research. 2006;607(2):160-175.

22. United State National Library of Medicine. Toxicology Data Network (TOXNET), Chemical Carcinogenesis Research Information System (CCRIS). http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS. 2007.

23. National Toxicology Program. Data and resources. https://ntp.niehs.nih.gov/data/index.html. 2007.

24. International Agency for Research on Cancer (IARC). Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Lyon, 1992;56(165):245.

25. Ohsawa K, Hirano N, Sugiura M, Nakagawa S, Kimura M. Genotoxicity of o-aminoazoltoluene (AAT) determined by the Ames test, the in vitro chromosomal aberration test, and the transgenic mouse gene mutation assay. Mutation Research. 2000;471(113):126.

26. Wakata A, Miyamae Y, Sato S, Suzuki T, Morita T, Asano N, Awogi T, Kondo K, Hayashi M. Evaluation of the rat micronucleus test with bone marrow and peripheral blood: summary of the 9th collaborative study by CSGMT/JEMS.MMS. Environmental and Molecular Mutagenesis. 1998;32:84-100.

27. Loprieno N, Boncristiani G, Loprieno G. An experimental approach to identifying the genotoxic risk from cooked meat mutagens. Food and Chemical Toxicology. 1991;29:377-86.

28. Thompson LH, Carrano AV, Salazar E, Felton JS, Hatch FT. Comparative genotoxic effects of the cooked-food-related mutagens Trp-P-2 and IQ in bacteria and cultured mammalian cells. Mutation Research. 1983;117:243-57.

29. Minkler JL, Carrano AV. In vivo cytogenetic effects of the cooked-food-related mutagens Trp-P-2 and IQ in mouse bone marrow. Mutation Research. 1984;140:49-53.

30. Hernández L, Slob W, van Steeg H, van Benthem J. Can carcinogenic potency be predicted from in vivo genotoxicity data? a meta-analysis of historical data. Environmental and Molecular Mutagenesis. 2011;52(7):518-28.

31. Haber LT, Dourson ML, Allen BC, Hertzberg RC, Parker A, Vincent MJ, Maier A, Boobis AR. Benchmark dose (BMD) modeling: current practice, issues, and challenges. Critical Reviews in Toxicology. 2018;48(5):387-415.

32. Wills JW, Johnson GE, Doak SH, Soeteman-Hernández LG, Slob W, White PA. Empirical analysis of BMD metrics in genetic toxicology part I: *in vitro* analyses to provide robust potency rankings and support MOA determinations. Mutagenesis. 2016;31(3):255-63.

33. Wills JW, Johnson GE, Battaion HL, Slob W, White PA. Comparing BMD-Derived genotoxic potency estimations across variants of the transgenic rodent gene mutation assay. Environmental and Molecular Mutagenesis. 2017;58(9):632-43.

34. White PA, Zeller A, Pfuhler S, Johnson GE. In vivo positive mutagenicity of 1,4-dioxane and quantitative analysis of its mutagenicity and carcinogenicity in rats. Archives of Toxicology. 2019;93:211-2.

35. Organisation for Economic Co-operation and Development (OECD). Guidelines for Testing of Chemicals, Section 4, Test Guideline 471: Bacterial Reverse Mutation Test. Organisation for

Economic Cooperation and Development. Paris, 1997. https://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en

36. Gundert-Remy U, Bernauer U, Blömeke B, Döring B, Fabian E, Goebel C, Hessel S, Jäckh C, Lampen A, Oesch F, Petzinger E, Völkel W, Roos PH. Extrahepatic metabolism at the body's internal-external interfaces. Drug metabolism reviews. 2014;46(3):291-324.

37. Kim D, Guengerich FP. Cytochrome P450 activation of arylamines and heterocyclic amines. Annual review of pharmacology and toxicology. 2005;45:27-49.

38. Drug Bank. Cyclophosphamide. https://www.drugbank.ca/drugs/DB00531.2005.

39. Gallagher E, Wienkers L, Stapleton P, Kunze K, Eaton D. Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. Cancer Res. 1994;54:101-8.

40. Cox JA, Fellows MD, Hashizume T, White PA. The utility of metabolic activation mixtures containing human hepatic post-mitochondrial supernatant (S9) for *in vitro* genetic toxicity assessment. Mutagenesis. 2016;31(2):117-30.

41. Alfred LJ, Wojdani A, Nieto M, Perez R, Yoshida G. A chemical carcinogen, 3-methylcholanthrene, alters T-cell function and induces T-suppressor cells in a mouse model system. Immunology. 1983;50(2):207-13.

42. Bishop ME, Aidoo A, Domon OE, Morris SM, Casciano DA. Phenolphthalein induces micronuclei in transgenic human lymphoblastoid cells. Environmental and Molecular Mutagenesis. 1998;32(3):286-8.

43. Heflich R, Neft R. Genetic toxicity of 2-acetylaminofluorene, 2-aminofluorene and some of their metabolites and model metabolites. Mutation Research. 1994;318:73-174.

44. Zhang L, Jin Y, Huang M, Penning TM. The role of human aldo-keto reductases in the metabolic activation and detoxication of polycyclic aromatic hydrocarbons: interconversion of PAH catechols and PAH o-quinones. Frontiers in Pharmacology. 2012;3(193).

45. Barski OA, Tipparaju SM, Bhatnagar A. The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. Drug metabolism reviews. 2008;40(4):553-624.

46. Varma T, Liu SQ, West M, Thongboonkerd V, Ruvolo PP, May WS, Bhatnagar A. Protein kinase C-dependent phosphorylation and mitochondrial translocation of aldose reductase. FEBS Letters. 2003;534:175-179.47.

47. Meek DW. Tumour suppression by p53: A role for the DNA damage response? Nature Reviews Cancer. 2009;10:714-23.

48. Madle S. Evaluation of experimental parameters in an S9/human leukocyte sce test with cyclophosphamide. Mutation Research. 1981;85:347-56.

49. Kugler U, Bauchinger M, Schmid E, Goggelmann W. The effectiveness of S9 and microsomal mix on activation of cyclophosphamide to induce genotoxicity in human lymphocytes. Mutation Research. 1987;187:151-6.

50. Slob W. Benchmark dose and the three Rs. Part I. Getting more information from the same number of animals. Critical Reviews in Toxicology. 2014;44(7):557-67.

51. Bryce SM, Avlasevich SL, Bemis JC, Phonethepswath S, Dertinger SD. Miniaturized flow cytometric *in vitro* micronucleus assay represents an efficient tool for comprehensively characterizing genotoxicity dose-response relationships. Mutation Research. 2010;703(2):191-9.

Appendix III:

Table A1 Results of BMD (Benchmark Dose) analyses for the ToxTracker® *Bscl2* reporter; 20 substances and four treatment conditions. Results shown relate to Aroclor-induced (A) or PB/BNF-induced S9, and two different treatment times (i.e., 3-hr or 24-hr). The S9 concentrations for the 24-hr and 3-hr incubation times were 0.25% v/v and 1% v/v, respectively. Substances for which the lower and upper limit for the BMD is 0 and infinite, respectively, across all conditions, are excluded. For the specified reporter and experimental conditions, an infinite upper confidence limit (i.e., infinite BMDU) is indicative of a negative response (i.e., no significant reporter induction). The BMDU/BMDL ratio for substances with a lower or upper limit of 0 or infinite were not calculated (N/A). Full substance names and CAS numbers are provided in Table 3.1.

Chemical	Treatment Time (hr)	S9 Type	BMD	BMDL	BMDU	BMDU/ BMDL
1,2-DPH	24	A	2.32E+04	625.00	1.20E+09	1.92E+06
		PB/BNF	1.53E+04	471.00	1.90E+08	4.03E+05
	3	A	1.40E+05	373.00	Infinite	N/A
		PB/BNF	3.17E+05	803.00	Infinite	N/A
BaP	24	A	28.47	7.81	194.00	24.84
		PB/BNF	442.18	77.70	8.26E+03	106.31
	3	A	35.04	11.40	121.00	10.61
		PB/BNF	52.26	17.70	239.00	13.50
CPA	24	A	28.65	12.40	47.20	3.81
		PB/BNF	23.05	15.70	33.60	2.14
	3	A	11.44	7.77	17.30	2.23
		PB/BNF	10.39	7.09	15.40	2.17
MeIQ	24	A	404.18	22.50	7.58E+04	3.37E+03
		PB/BNF	2.00E+03	166.00	3.54E+03	21.33
	3	A	46.60	0	5.22E+03	N/A
		PB/BNF	7.61E+03	0	Infinite	N/A
PHPT	24	A	73.55	52.90	104.00	1.97
		PB/BNF	85.52	58.60	120.00	2.05
	3	A	918.67	542.00	2.38E+03	4.39
		PB/BNF	1.24E+03	608.00	Infinite	N/A
1,3-DPH	24	A	147.30	88.90	245.00	2.76
		PB/BNF	159.75	95.50	269.00	2.82
	3	A	951.11	476.00	2.30E+03	4.83
		PB/BNF	1.28E+03	600.00	3.45E+03	5.75
3MC	24	A	1.03E+03	27.60	7.63E+03	276.45
		PB/BNF	437.33	34.40	4.53E+03	131.69
	3	A	57.81	0	365.00	N/A
		PB/BNF	43.51	0	339.00	N/A
AFB1	24	A	2.87	1.47	5.65	3.84
		PB/BNF	3.27	1.65	6.55	3.97

	3	A	0.28	0.15	0.50	3.33
		PB/BNF	0.26	0.14	0.51	3.64
2AA	24	A	5.00	0	105.00	N/A
		PB/BNF	34.41	0	2.81E+03	N/A
	3	A	196.30	34.50	6.50E+03	188.41
		PB/BNF	97.31	17.80	4.39E+03	246.63
DMBA	24	A	81.24	26.20	1.06E+03	40.46
		PB/BNF	1.71E+03	76.70	Infinite	N/A
	3	A	4.15	2.29	7.06	3.08
		PB/BNF	8.96	2.22	17.50	7.88
IQ	24	A	1.70E+03	0	9.59E+06	N/A
		PB/BNF	2.59E+05	409.00	8.24E+08	2.01E+06
	3	A	9.88E+03	0	3.67E+07	N/A
		PB/BNF	2.12E+06	1.26E+03	Infinite	N/A
PhIP	24	A	1.90	0.91	3.69	4.05
		PB/BNF	1.96	0.94	5.06	5.38
	3	A	6.91	3.72	12.60	3.39
		PB/BNF	8.09	3.53	16.60	4.70

Table A2 Results of BMD (Benchmark Dose) analyses for the ToxTracker® *Rtkn* reporter; 20 substances and four treatment conditions. Results shown relate to Aroclor 1254-induced (A) or PB/BNF-induced S9, and two different treatment times (i.e., 3-hr or 24-hr). The S9 concentrations for the 24-hr and 3-hr incubation times were 0.25% v/v and 1% v/v, respectively. Substances for which the lower and upper limit for the BMD is 0 and infinite, respectively, across all conditions, are excluded. For the specified reporter and experimental conditions, an infinite upper confidence limit (i.e., infinite BMDU) is indicative of a negative response (i.e., no significant reporter induction). The BMDU/BMDL ratio for substances with a lower or upper limit of 0 or infinite were not calculated (N/A). Full substance names and CAS numbers are provided in Table 3.1.

Chemical	Treatment Time (hr)	S9 Type	BMD	BMDL	BMDU	BMDU/ BMDL
1,2-DPH	24	A	169.01	55.90	77.10	1.38
		PB/BNF	195.72	70.50	88.20	1.25
	3	A	85.34	0	767.00	N/A
		PB/BNF	212.09	77.00	954.00	12.39
BaP	24	A	4.19	1.22	15.70	12.87
		PB/BNF	79.17	16.40	734.00	44.76
	3	A	2.30	0.73	6.22	8.52
		PB/BNF	4.22	1.34	11.60	8.66
CPA	24	A	10.72	7.45	15.20	2.04
		PB/BNF	8.55	6.29	11.50	1.83
	3	A	3.08	2.21	4.22	1.91
		PB/BNF	2.66	1.90	3.65	1.92
MeIQ	24	A	49.16	3.48	294.00	84.48
		PB/BNF	170.41	21.80	1.97E+03	90.37
	3	A	4.55	0	36.10	N/A
		PB/BNF	411.35	0	9.18E+03	N/A
OAT	24	A	682.95	58.70	4.91E+05	8.36E+03
		PB/BNF	851.81	73.70	Infinite	N/A
	3	A	480.28	38.40	2.03E+04	528.65
		PB/BNF	1.29E+03	54.10	1.11E+05	2.05E+03
PHPT	24	A	38.65	26.40	53.70	2.03
		PB/BNF	37.05	24.40	51.80	2.12
	3	A	309.84	179.00	504.00	2.82
		PB/BNF	343.09	210.00	601.00	2.86
1,3-DPH	24	A	7.43	1.72	16.80	9.77
		PB/BNF	5.86	0	14.30	N/A
	3	A	22.50	9.50	41.70	4.39
		PB/BNF	28.09	12.80	52.50	4.10
2AAF	24	A	88.22	38.00	353.00	9.29
		PB/BNF	93.68	47.10	425.00	9.02
	3	A	128.02	36.90	472.00	12.79

		PB/BNF	184.25	62.90	Infinite	N/A
3MC	24	A	39.24	15.10	94.90	6.28
		PB/BNF	54.13	22.40	110.00	4.91
	3	A	3.83	0	9.06	N/A
		PB/BNF	3.24	0	8.32	N/A
AFB1	24	A	0.49	0.30	0.75	2.50
		PB/BNF	0.75	0.47	1.15	2.45
	3	A	0.06	0.04	0.08	2.00
		PB/BNF	0.04	0.02	0.05	2.50
2AA	24	A	8.80	0	462.00	N/A
		PB/BNF	283.79	29.40	Infinite	N/A
	3	A	22.29	5.96	90.80	15.23
		PB/BNF	17.04	3.90	76.10	19.51
DMBA	24	A	10.00	2.53	78.60	31.07
		PB/BNF	21.33	4.56	2.31E+03	506.58
	3	A	0.83	0.24	2.60	10.83
		PB/BNF	1.50	0.45	4.01	8.91
IQ	24	A	34.89	0	131.00	N/A
		PB/BNF	406.28	102.00	1.09E+03	10.69
	3	A	22.72	0	89.00	N/A
		PB/BNF	33.96	0	20.40	N/A
DOrange	24	A	297.98	71.20	1.14E+04	160.11
		PB/BNF	181.68	51.50	1.93E+03	37.48
	3	A	40.95	14.40	102.00	7.08
		PB/BNF	124.09	40.50	813.00	20.07
PhIP	24	A	0.75	0.41	1.17	2.85
		PB/BNF	0.93	0.53	1.66	3.13
	3	A	3.38	1.92	5.40	2.81
		PB/BNF	3.37	1.70	6.18	3.64

Table A3 Results of BMD (Benchmark Dose) analyses for the ToxTracker® *Btg2* reporter; 20 substances and four treatment conditions. Results shown relate to Aroclor 1254-induced (A) or PB/BNF-induced S9, and two different treatment times (i.e., 3-hr or 24-hr). The S9 concentrations for the 24-hr and 3-hr incubation times were 0.25% v/v and 1% v/v, respectively. Substances for which the lower and upper limit for the BMD is 0 and infinite, respectively, across all conditions, are excluded. For the specified reporter and experimental conditions, an infinite upper confidence limit (i.e., infinite BMDU) is indicative of a negative response (i.e., no significant reporter induction). The BMDU/BMDL ratio for substances with a lower or upper limit of 0 or infinite were not calculated (N/A). Full substance names and CAS numbers are provided in Table 3.1.

Chemical	Treatment Time (hr)	S9 Type	BMD	BMDL	BMDU	BMDU/ BMDL
1,2-DPH	24	A	118.33	45.30	497.00	10.97
		PB/BNF	138.67	54.60	540.00	9.89
	3	A	60.72	20.90	135.00	6.46
		PB/BNF	116.43	50.80	312.00	6.14
BaP	24	A	1.11	0	4.28	N/A
		PB/BNF	1.28	0	4.19	N/A
	3	A	12.50	7.20	21.90	3.04
		PB/BNF	18.75	11.40	30.50	2.68
CPA	24	A	6.16	4.26	8.76	2.06
		PB/BNF	5.46	5.20	10.60	2.04
	3	A	3.00	1.93	4.34	2.25
		PB/BNF	2.61	1.66	3.83	2.31
OAT	24	A	267.06	72.50	3.57E+07	4.92E+05
		PB/BNF	286.86	79.20	6.85E+07	8.65E+05
	3	A	704.26	41.00	1.56E+07	3.80E+05
		PB/BNF	1.24E+03	249.00	Infinite	N/A
1,3-DPH	24	A	33.03	17.30	56.90	3.29
		PB/BNF	33.05	17.40	56.80	3.26
	3	A	80.51	41.30	155.00	3.75
		PB/BNF	83.16	43.20	160.00	3.70
2AA	24	A	110.33	35.90	5.41E+03	150.70
		PB/BNF	116.59	39.00	6.33E+03	162.31
	3	A	192.62	30.10	Infinite	N/A
		PB/BNF	286.86	75.60	Infinite	N/A
3MC	24	A	167.44	27.10	1.31E+03	48.34
		PB/BNF	192.89	43.10	1.38E+03	32.02
	3	A	3.77	0	19.60	N/A
		PB/BNF	2.73	0	17.90	N/A
AFB1	24	A	0.58	0.23	1.29	5.61
		PB/BNF	0.90	0.36	2.11	5.86
	3	A	0.06	0.02	0.14	7.00

		PB/BNF	0.05	0.02	0.11	5.50
2AA	24	A	1.74	0	120.00	N/A
		PB/BNF	234.61	0	Infinite	N/A
	3	A	21.78	0.05	1.15E+04	2.30E+05
		PB/BNF	16.92	0	2.63E+05	N/A
DMBA	24	A	9.08	2.94	39.60	13.47
		PB/BNF	12.54	3.96	71.40	18.03
	3	A	1.59	0.58	3.61	6.22
		PB/BNF	2.78	1.03	6.90	6.70
DOrange	24	A	1.24E+10	695.00	Infinite	N/A
		PB/BNF	585.14	30.60	Infinite	N/A
	3	A	170.13	50.50	3.15E+05	6.24E+03
		PB/BNF	399.41	93.50	1.69E+06	1.81E+04
PhIP	24	A	0.69	0.14	1.71	12.21
		PB/BNF	21.36	0.41	392.00	956.10
	3	A	3.80	1.33	9.13	6.86
		PB/BNF	3.68	1.10	12.00	10.91
DMYellow	24	A	583.27	36.20	2.04E+05	5.64E+03
		PB/BNF	1.71E+03	72.30	Infinite	N/A
	3	A	1.86E+03	116.00	Infinite	N/A
		PB/BNF	3.82E+03	162.00	Infinite	N/A

Table A4 Results of BMD (Benchmark Dose) analyses for the ToxTracker® *Blvrb* reporter; 20 substances and four treatment conditions. Results shown relate to Aroclor 1254-induced (A) or PB/BNF-induced S9, and two different treatment times (i.e., 3-hr or 24-hr). The S9 concentrations for the 24-hr and 3-hr incubation times were 0.25% v/v and 1% v/v, respectively. Substances for which the lower and upper limit for the BMD is 0 and infinite, respectively, across all conditions, are excluded. For the specified reporter and experimental conditions, an infinite upper confidence limit (i.e., infinite BMDU) is indicative of a negative response (i.e., no significant reporter induction). The BMDU/BMDL ratio for substances with a lower or upper limit of 0 or infinite were not calculated (N/A). Full substance names and CAS numbers are provided in Table 3.1.

Chemical	Treatment Time (hr)	S9 Type	BMD	BMDL	BMDU	BMDU/ BMDL
1,2-DPH	24	A	137.07	20.70	480.00	23.19
		PB/BNF	269.37	70.80	1.36E+03	19.21
	3	A	17.95	4.60	71.10	15.46
		PB/BNF	77.76	21.30	290.00	13.62
BaP	24	A	23.05	8.14	92.20	11.33
		PB/BNF	206.25	58.90	1.19E+03	20.20
	3	A	17.59	7.40	39.40	5.32
		PB/BNF	30.63	12.70	77.80	6.13
CPA	24	A	37.55	20.30	66.30	3.27
		PB/BNF	62.19	32.50	116.00	3.57
	3	A	26.39	13.00	60.90	4.68
		PB/BNF	27.71	13.90	60.90	4.38
OAT	24	A	117.67	20.50	4.50E+04	2.20E+03
		PB/BNF	209.69	27.60	5.90E+04	2.14E+03
	3	A	467.12	0	6.40E+04	N/A
		PB/BNF	794.20	42.90	Infinite	N/A
PHPT	24	A	44.28	22.80	88.60	3.89
		PB/BNF	58.70	34.90	118.00	3.38
	3	A	341.30	215.00	650.00	3.02
		PB/BNF	391.68	233.00	727.00	3.12
1,3-DPH	24	A	42.81	28.00	62.40	2.23
		PB/BNF	58.17	38.10	85.10	2.23
	3	A	244.53	149.00	445.00	2.99
		PB/BNF	342.22	198.00	717.00	3.62
2AAF	24	A	249.20	10.70	1.32E+08	1.23E+07
		PB/BNF	537.80	35.70	Infinite	N/A
	3	A	946.87	59.00	Infinite	N/A
		PB/BNF	3.59E+03	120.00	Infinite	N/A
3MC	24	A	9.54E+04	1.28E+03	8.93E+05	697.66
		PB/BNF	5.18E+05	4.25E+03	8.37E+06	1.97E+03
	3	A	1.18E+04	259.00	8.62E+03	33.28

		PB/BNF	4.27E+04	754.00	3.73E+05	494.69
AFB1	24	A	59.26	10.50	421.00	40.10
		PB/BNF	129.00	21.50	1.27E+03	59.07
	3	A	5.99	1.07	33.40	31.21
		PB/BNF	3.75	0.62	26.00	41.94
2AA	24	A	0.02	0	0.42	N/A
		PB/BNF	0.09	0	1.04	N/A
	3	A	79.32	8.16	1.97E+03	241.42
		PB/BNF	128.61	11.30	5.12E+03	453.10
BA	24	A	83.40	18.10	Infinite	N/A
		PB/BNF	148.98	31.20	Infinite	N/A
	3	A	2.14	0.04	6.71	167.75
		PB/BNF	1.27	0.02	4.06	203.00
DMBA	24	A	34.61	16.20	96.00	5.93
		PB/BNF	28.65	14.20	73.80	5.20
	3	A	9.34	5.24	18.50	3.53
		PB/BNF	12.10	6.40	23.60	3.69
PhIP	24	A	6.39	0	137.00	N/A
		PB/BNF	8.58	0	1.36E+03	N/A
	3	A	30.58	6.31	450.00	71.32
		PB/BNF	37.19	3.84	720.00	187.50

Table A5 Results of BMD (Benchmark Dose) analyses for the ToxTracker® *Srxn1* reporter; 20 substances and four treatment conditions. Results shown relate to Aroclor 1254-induced (A) or PB/BNF-induced S9, and two different treatment times (i.e., 3-hr or 24-hr). The S9 concentrations for the 24-hr and 3-hr incubation times were 0.25% v/v and 1% v/v, respectively. Substances for which the lower and upper limit for the BMD is 0 and infinite, respectively, across all conditions, are excluded. For the specified reporter and experimental conditions, an infinite upper confidence limit (i.e., infinite BMDU) is indicative of a negative response (i.e., no significant reporter induction). The BMDU/BMDL ratio for substances with a lower or upper limit of 0 or infinite were not calculated (N/A). Full substance names and CAS numbers are provided in Table 3.1.

Chemical	Treatment Time (hr)	S9 Type	BMD	BMDL	BMDU	BMDU/ BMDL
1,2-DPH	24	A	39.20	16.60	86.40	5.20
		PB/BNF	72.05	30.90	149.00	4.82
	3	A	28.37	12.50	60.80	4.86
		PB/BNF	41.76	17.60	83.90	4.77
BaP	24	A	17.54	6.50	38.20	5.88
		PB/BNF	19.77	7.20	43.70	6.07
	3	A	74.96	30.00	203.00	6.77
		PB/BNF	89.05	35.20	269.00	7.64
CPA	24	A	9.71	1.45	24.70	17.03
		PB/BNF	14.14	3.03	41.60	13.73
	3	A	7.19	2.18	28.20	12.94
		PB/BNF	6.44	1.92	27.20	14.17
OAT	24	A	68.10	32.20	110.00	3.42
		PB/BNF	82.97	41.90	133.00	3.17
	3	A	803.71	291.00	Infinite	N/A
		PB/BNF	947.20	309.00	Infinite	N/A
1,3-DPH	24	A	17.54	6.50	38.20	5.88
		PB/BNF	19.77	7.20	43.70	6.07
	3	A	74.96	30.00	203.00	6.77
		PB/BNF	89.07	35.20	269.00	7.64
2AAF	24	A	33.47	5.99	96.80	16.16
		PB/BNF	61.09	17.70	321.00	18.14
	3	A	537.73	90.00	Infinite	N/A
		PB/BNF	1.76E+03	142.00	Infinite	N/A
3MC	24	A	33.49	11.00	92.90	8.45
		PB/BNF	30.80	11.40	82.30	7.22
	3	A	76.87	19.70	272.00	13.81
		PB/BNF	83.05	22.60	296.00	13.10
AFB1	24	A	2.13	0.83	5.22	6.29
		PB/BNF	2.83	1.11	7.66	6.90
	3	A	0.22	0.09	0.49	5.44

		PB/BNF	0.18	0.07	0.40	5.71
2AA	24	A	0.01	0	0.59	N/A
		PB/BNF	0.04	0	0.88	N/A
	3	A	22.84	1.63	492.00	301.84
		PB/BNF	38.43	2.34	154.00	65.81
DMBA	24	A	8.73	3.97	21.90	5.52
		PB/BNF	8.16	3.83	19.70	5.14
	3	A	2.74	1.37	4.99	3.64
		PB/BNF	4.00	2.02	7.63	3.78
HMPA	24	A	2.27E+04	1.02E+04	Infinite	N/A
		PB/BNF	2.14E+04	1.00E+04	8.59E+05	85.90
	3	A	8.39E+04	1.20E+04	Infinite	N/A
		PB/BNF	7.45E+04	1.20E+04	Infinite	N/A
IQ	24	A	0	0	975.00	N/A
		PB/BNF	516.19	23.40	1.65E+04	705.13
	3	A	177.29	0	7.96E+03	N/A
		PB/BNF	8.59E+03	479.00	Infinite	N/A
DOrange	24	A	5.41E+09	274.00	Infinite	N/A
		PB/BNF	2.30E+03	162.00	Infinite	N/A
	3	A	482.20	153.00	5.52E+08	3.61E+06
		PB/BNF	417.80	142.00	5.73E+07	4.04E+05
PhIP	24	A	1.15	0.63	1.98	3.14
		PB/BNF	1.22	0.66	2.30	3.48
	3	A	6.32	3.64	9.52	2.62
		PB/BNF	5.97	2.40	12.70	5.29
DMYellow	24	A	15.30	6.39	33.10	5.18
		PB/BNF	15.07	6.51	37.00	5.68
	3	A	403.91	152.00	Infinite	N/A
		PB/BNF	391.81	147.00	Infinite	N/A

Chapter Four

4.1 Summary of Study Outcomes

High-throughput, multiplexed, *in vitro* reporter assays such as the ToxTracker® assay rapidly generate large amount of dose response data. More specifically, the assay is conducted in a 96-well plate, and can assess up to 2 substances per plate with 5 concentrations for each substance, in addition to simultaneous positive controls. Including time for cell growth, the turnaround time is 1-3 weeks, compared to 3-15 weeks for other, more-established regulatory assays (see Chapter 1, Table 1.4). To evaluate assay performance, Toxys B.V. evaluated over 90 validation substances, generating over 86,000 observations. Moreover, with respect to response levels for solvent controls, Toxys has generated over 14,000 control observations. Thus, there is an opportunity to develop quantitative strategies to rigorously and comprehensively interpret available dose-response data. More specifically, to use the available data to (1) determine robust fold-change cut-off values for delineation of significant positive responses, (2) rigorously investigate BMR values to demarcate response levels that can be deemed toxicologically adverse, (3) using the BMD combined-covariate approach, rank the potency of validation substances tested to date, and (4) use multivariate analyses such as PCA to investigate functional and statistical redundancy in the six ToxTracker® reporters. With respect to the latter activity, individual substance axis scores can be used to evaluate MOA.

In addition to the necessity to employ quantitative methods to address the aforementioned issues, there is a necessity to modernize the assay protocol, specifically with respect to the hepatic S9 fraction used to prepare the requisite *in vitro* metabolic activation mixture. S9 is traditionally prepared from the livers of male rats exposed to an inducer of CYP isozymes; more specifically, the CYP isozymes involved in enzymatic conversion of some tested substances into toxicologically-active (i.e., reactive) metabolites, e.g., CYP isozymes 1A1, 1A2, 3A and 2B1.

Aroclor-1254 has traditionally been used as a CYP inducer. However, in light of the unavailability of Aroclor-1254, it is necessary to identify a substitute that can effectively induce production of the aforementioned hepatic CYPs. The substitute that is receiving the most attention world-wide is a mixture of PB (phenobarbital) and BNF (β-naphthoflavone) (i.e., PB/BNF-induced S9). Prior to the adoption of PB/BNF-induced S9 for routine use with *in vitro* (geno)toxicity assays such as ToxTracker®, it is necessary to evaluate assay protocols that employ PB/BNF-induced S9 in place of Aroclor-induced S9.

This project employed a variety of strategies and methods to quantitatively interpret the vast amounts of available ToxTracker® dose-response data. Moreover, in light of the operational shift towards the use of PB/BNF-induced S9 (i.e., instead of Aroclor 1254-induced S9), the project employed experimental methods to comparatively evaluate the utility of PB/BNF-induced S9.

4.1.1 Reporter-specific fold-change cut-off values for identification of a significant positive response

Objective: Using a bootstrapping approach, examine possible fold-change response values for the solvent control; define a fold-change threshold for identification of a significant positive response.

Outcome: This objective was fulfilled. The results show that the fold-change cut-off value for a weak positive response (i.e., 95th percentile) is 1.5, which is identical to that already used by Toxys B.V. This is consistent with the hypothesis outlined in Chapter 1, which specified that the determined cut-off for identification of a positive response will be aligned with values currently used by Toxys. The fold-change value indicative of a strong positive response (i.e., 99th percentile) is 1.7, which is lower than the 2-fold value currently used by Toxys. This indicates that the approach used by Toxys for the delineation of a strong positive response is conservative; indeed,

if the 2-fold cut-off is used, there would be less than a one percent probability that the observed GFP induction could be obtained by chance alone.

The fold-change cut-off values determined in this project are aligned with those used for other *in vitro* genotoxicity reporter assays, e.g., GreenScreen® and Multiflow™. The cut-offs for identification of significant responses for those assays are also in the 1.5- to 2-fold range. As for OECD-approved genotoxicity assays, test guidelines for the *in vitro* mammalian cell gene mutation assay and the *in vitro* mammalian cell micronucleus assay recommend the use of a statistical approach whereby a test result is considered positive when the response reaches a level that is statistically elevated relative to the concurrent control at $p<0.05$ (1, 2). The 1.5-fold cut-off value for identification of a weak ToxTracker® positive corresponds to the 95th percentile of the control fold-change distribution; it is thus comparable to the OECD statistical approach ($p<0.05$). The 1.7-fold cut-off value for identification of a strong ToxTracker® positive corresponds to the 99th percentile; thus, it is more conservative than the OECD recommendation, i.e., less than 1% probability that the response is obtained by chance alone.

4.1.2 Reporter-specific Benchmark Response (BMR) values

Objective: Using the Zeller *et al.* (2017) (3) approach, and the Slob (2016) (4) ES (effect size) theory, determine reporter-specific BMRs for routine quantitative interpretation of ToxTracker® dose-response data.

Outcome: This objective was fulfilled. Reporter-specific BMRs were defined using both the Zeller *et al*. method and the Slob ES theory. The values obtained differed between the approaches, which is not consistent with the hypothesis specified in Chapter 1. More specifically, the values for the Zeller *et al.* method ranged from 2.2% for *Blvrb* and *Rtkn*, to 7.0% for *Ddit3*, with an

average of 3.9% across all the reporters. In comparison, the Slob approach yielded values that ranged from 30% for *Ddit3* to 52% for *Rtkn*, with an average of 43%.

The differences in BMRs highlights each method's assumptions and underlying principles. Indeed, the Zeller *et al.* method is reflective of the variability in the trimmed distribution of historical control values. Since the data generated for this project all have the same provenance, i.e., the Toxys' laboratory, with conserved experimental conditions and sample handling, it was not surprising that the standard deviation of the trimmed distribution of historical controls is only 2-7% of the mean. Consequently, this value could be viewed as the minimum percentage increase above the controls that could be theoretically-associated with a toxicologically-adverse effect.

In contrast, the BMR values based on the Slob ES theory reflect the difference between background and the maximum possible effect, and are therefore scaled relative to the dynamic range of the endpoint. Therefore, they reflect the smallest response that is toxicologically-meaningful relative to the maximum possible response, e.g., the toxicological *ease* with which an adverse effect can be induced by the treatment. More specifically, if the maximum possible response is very large, a toxicologically-adverse response is only manifested when the response, expressed as a fold-change increase relative to the concurrent control, is substantial. Interestingly, the values obtained were also more variable than those obtained using the Zeller *et al.* method. Given that the Slob approach is based on the average variance across all tested doses, this was expected; substance preparation and cellular dosing can introduce more variability compared to what might be expected for the solvent control. Moreover, the higher variability across reporters is affected by cross-endpoint variability in maximum response; this was also expected since, to some extent, the pathways underlying induction of the ToxTracker® reporters are distinct.

With respect to the existing scientific literature, there is no consensus regarding BMR values that should be used for routine interpretation of *in vitro* dose-response data (5); moreover, methods for effective determination of endpoint-specific BMR values. However, the theoretical considerations outlined by Slob (2016) indicate that an approach based on ES theory would be expected to provide more toxicologically-appropriate, endpoint-specific BMR values for routine interpretation of ToxTracker® dose-response data, i.e., compared to the approach advocated by Zeller *et al*.

4.1.3 Benchmark Dose combined-covariate analysis for substance potency ranking

Objective: Employ the BMD combined-covariate approach to rank the potency of ToxTracker® assay validation substances.

Outcome: This objective was fulfilled. The BMD combined-covariate approach was used to rank the potency of ToxTracker® validation substances. The results showed that analysis of genotoxicity reporter dose-response data (i.e., *Rtkn* and *Bscl2* data) yielded similar rankings, with noteworthy clastogens being the most potent. The positive controls (i.e., cisplatin and aflatoxin B1) ranked in the upper 75th percentile. Rankings of some validation substances for the oxidative stress reporters (*Blvrb* and *Srxn1*) were similar to those observed for the genotoxicity reporters. This was not surprising, since some chemicals (e.g., BaP) can cause DNA damage via formation of bulky adducts, as well as via ROS production through metabolite redox cycling. Overall, the results obtained allowed effective potency ranking of validation substances for each of the reporters. The rankings obtained can serve as a point of reference for interpretation of potency values for confidential test articles submitted by Toxys clients for toxicological screening. The results are consistent with the hypothesis outlined in Chapter 1, i.e., that the use of the BMD combined-covariate approach should permit statistically-robust potency ranking of ToxTracker®

validation substances; moreover, comparative potency analysis that facilitates interpretation of ToxTracker® results for heretofore untested substances.

4.1.4 Principal Component Analysis

Objective: Utilize PCA (Principal Component Analysis) to scrutinise the functional and statistical relationships between the multiplexed ToxTracker® endpoints.

Outcome: This objective was fulfilled. The results showed that the reporter responses can be divided into 3 primary multivariate components that are linear combinations of the original reporter-response variables, i.e., components indicative of genotoxic stress, oxidative stress, and generalised cellular stress characterised by protein unfolding. The component loading values showed a high degree of response similarity for the genotoxicity-related reporters *Rtkn*, *Bscl2* and *Btg2*, thus, these reporters can be termed toxicologically redundant. Nevertheless, since genotoxicity assessment is a central prerequisite for prudent chemical safety assessment, some measure of redundancy is reasonable, if not critical, for precautious hazard identification and assessment. With respect to oxidative stress, the results revealed that the *Blvrb* reporter response is unique (i.e., orthogonal), and thus essential. Interestingly, the *Srxn1* reporter is aligned with both the genotoxicity and oxidative stress axes. Similar to the *Blvrb* response, and its unique utility as an oxidative stress indicator, the *Ddit3* reporter is orthogonal to the other axes. Thus, it is toxicologically-essential for test article assessment since it uniquely reflects endoplasmic reticulum stress manifested as undesirable protein unfolding. These results are consistent with the hypothesis outlined in Chapter 1, i.e., that multivariate analyses of ToxTracker® responses to validation substances will indicate functional redundancy in the reporter responses.

In addition to information about reporter response redundancy, which permitted evaluation of the hypothesis outlined in Chapter 1, the results obtained permit scrutiny of substance axis scores; moreover, enquiry regarding how these scores reflect toxicological MOA and substance similarities. For example, axis scores for cancer chemotherapy agents such as docetaxel, taxol, nocodazole, daunorubicin, etoposide, and podophyllotoxin indicate that they are potent cellular toxicants that elicit genotoxic, oxidative, and endoplasmic reticulum stress. In contrast, substances such as sodium arsenite and cadmium chloride are strong inducers of oxidative stress, but weak inducers of genotoxic stress. Similarly, the *Ddit3* positive control tunicamycin is a potent inducer of endoplasmic reticulum stress, but a weak inducer of genotoxic and oxidative stress.

4.1.5 Development of an alternative S9 protocol based on PB/BNF-induced rodent liver S9

Objective: By comparing ToxTracker® responses elicited by reference genotoxicants tested using different laboratory protocols, evaluate the suitability of PB/BNF-induced S9 as a substitute for Aroclor 1254-induced S9.

Outcome: This objective was partially fulfilled. The results showed that there were few significant, reporter-specific BMD_{100} differences values across the two different S9 types investigated. Although there were trends indicating some differences between Aroclor- and PB/BNF-induced S9, it is fair to state that they can be deemed inconsequential. There were some exceptions, whereby the BMD_{100} was significantly lower with Aroclor-induced S9 (e.g., BaP for *Rtkn*), or the BMD_{100} was significantly lower with PB/BNF-induced S9 (e.g., CPA for *Bscl2*). These results are reasonably consistent with the differences in the activity of CYP isozymes, such as 1A1, 1A2 and 3A, that have been described in the scientific literature. In light of these results,

it is reasonable to cautiously conclude that PB/BNF can be viewed as a suitable, alternative inducer of rodent hepatic metabolism, i.e., suitable alternative for routine production of rodent hepatic S9.

It is important to highlight that the experimental protocols employed herein were somewhat inconsistent, and consequently, the results obtained less than definitive. For example, with respect to the S9 concentrations examined, the first part of the experiment work was not well aligned with the second part (i.e., 1% v/v versus 0.40% v/v). In addition, the first phase of the work involved examination of two confounding variables, i.e., treatment time and S9 type. The type of S9 will likely affect the enzymatic conversion of the tested substances to reactive metabolites; however, treatment time might have coincidentally influenced S9 cytotoxicity, and cellular response capabilities. Additional work should more comprehensively investigate the effect of S9 concentration on the genotoxicity reporter responses, e.g., 0.25% v/v Aroclor-induced versus, for example, 0.40, 0.50 and 0.60% PB/BNF-induced. However, S9 cytotoxicity would need to be taken into account when considering the concentrations required to yield results aligned with those obtained using Aroclor-induced S9. The maximum S9 concentration cannot elicit cytotoxicity levels above 75%, the upper limit for an acceptable ToxTracker® assay.

With respect to treatment time, the results showed that it should be possible to judiciously use PB/BNF-induced S9 as alternative to Aroclor-induced if the test protocol employs 0.40% v/v S9 and a 24-hr treatment time. It should be noted that, as indicated in Chapter 3, a 24-hr protocol is far more practical compared with a 3-hr protocol. More specifically, the latter necessitates a 3-hr treatment time, followed by a wash, an additional 21-hr incubation period, and an additional wash prior to flow cytometry. In comparison, the 24-hr protocol involves continuous treatment in the presence of S9 before washing once prior to scoring.

Overall, the results suggest that a 24-hr protocol using 0.40% v/v PB/BNF-induced S9 yields results that are comparable to those obtained using 0.25% v/v Aroclor-induced S9. Thus, although not entirely definitive, the results presented herein indicate that PB/BNF-induced S9 is an appropriate substitute for Aroclor-induced S9. This is consistent with the hypothesis that was outlined in Chapter 1, i.e., that a protocol based on PB/BNF-induced S9 would yield results that are comparable to those obtained using the current protocol based on Aroclor-induced S9. This is consistent with a recent publication (i.e., Tian *et al*., 2020) that investigated alternative S9 protocols for the MultiFlow™ *in vitro* reporter assay. Although the Tian *et al*. work employed a lower concentration of S9 than that employed in the second part of the experimental work reported in Chapter 3 (i.e., 0.40% v/v), they confirmed the utility of an experimental protocol that employs a low S9 concentration, and an extended, continuous treatment for 24-hr (6).

4.2 Contribution to scientific knowledge

High-throughput assays such as ToxTracker® generate vast amounts of complex, multivariate data, and comprehensive, rigorous interpretation of these data necessitates quantitative consideration of numerous issues. To this end, this study employed a variety of strategies to analyse large amounts of ToxTracker® dose-response data, and the work presented in Chapter 2 constitutes a foundation for routine quantitative interpretation of ToxTracker® dose-response data. The work presented in Chapter 3 summarized experimental analyses that evaluated the suitability of PB/BNF-induced S9 as a suitable substitute for Aroclor-induced S9. That work provides a foundation for essential adoption of an alternative S9 type for routine toxicological screening using the ToxTracker® assay.

With respect to the results presented in Chapter 2, the thesis makes numerous contributions to original, scientific knowledge. By employing a robust bootstrapping approach, the thesis presents

original results that provide robust, fold-change cut-off values to delineate significant positive ToxTracker® responses. Additionally, the thesis presents the results of analyses that employed recently-published analytical frameworks (i.e., the Zeller *et al.* and Slob approaches) to determine and specify robust BMR values for routine interpretation of ToxTracker® dose-response data. To my knowledge, this is the first time that these analytical approaches have been employed to determine an *in vitro* assay response level, specified as percentage change above concurrent control, that can be deemed toxicologically-adverse.

Using the recently-developed and advocated BMD combined-covariate approach (e.g., 7, 8), the analyses presented herein permitted robust potency ranking of a large number of assay validation substances. Although other researchers have used the approach to rank substances tested *in vitro* (7, 9), to the best of my knowledge this work constitutes the first application of the approach for toxicological potency ranking of substances tested using a high-throughput, multiplexed reporter assay. These results permit potency ranking of hitherto untested substances; they also provide interesting and useful insight into the MOAs underlying the observed ToxTracker® reporter responses. For example, substances such as sodium arsenite and cadmium chloride induced an oxidative stress response (i.e., *Blvrb* and *Srxn1*) indicating that they are more potent than the positive control diethyl maleate. Further scrutiny of this potency pattern would likely yield hypotheses about a substance's ability to affect reporter-specific response pathways.

Lastly, PCA analysis of ToxTracker® reporter BMD_{100} values permitted novel insight into the functional and statistical relationships between the multiplexed endpoints. To my knowledge, this is the first time that a multivariate statistical technique has been used to investigate redundancy in reporters associated with a multiplexed, toxicological reporter assay; the results obtained constitute new scientific knowledge on several levels. The reporter variable loadings on each axis provide

insight into the functional redundancy of the reporters, and, perhaps more importantly, the interconnectedness of toxicological stress response pathways related to genetic damage, oxidative stress, and generalised toxicological stress characterized by protein unfolding. Follow-up work could scrutinize the pathways associated with the various stress reporters to investigate the mechanistic foundations underlying the presence or absence of hypothetical connectedness, e.g., *Srxn1* reporter relationship with the genotoxic and oxidative stress reporters. The PCA results further provide novel insight into the MOA of the tested substances; moreover, generation of related hypotheses. For example, substances used as anti-cancer therapeutics, such as vinorelbine, camptothecin, mitomycin C, and mitoxanthrone, elicited strong genotoxic and oxidative stress responses. Although it is well-known that oxidative stress in cancer patients is a side effect of chemotherapy, and that some cancer chemotherapy drugs are known to elicit a wide range of pathophysiologic effects (e.g., cyclophosphamide, doxorubicin and fluorouracil), the ability of the aforementioned substances to induce oxidative stress has not been well studied (10).

With respect to the results presented in Chapter 3, the thesis makes additional contributions to original, scientific knowledge. To develop a protocol that employs PB/BNF-induced S9 as an alternative to Aroclor-induced S9, the influence of treatment time and S9 type on ToxTracker® reporter responses were investigated. The effects of experimental conditions were evaluated using the BMD combined-covariate approach. Although multiple studies have investigated the use of PB/BNF-induced S9 as a substitute for Aroclor-induced S9 (11-13), the work presented in this thesis is the first study to examine the utility of PB/BNF-induced S9 for a high-throughput, multiplexed reporter assay. Overall, the work outlined in Chapter 3 constitutes a foundation for Aroclor substitution, and use of an alternative S9 type for multiplexed assays used for toxicological evaluations of substances with multiple MOAs.

4.3 Future steps and concluding remarks

The quantitative strategies used in Chapter 2 allowed the development of a data analysis and interpretation strategy to efficiently scrutinise large amounts of ToxTracker® dose-response data. With respect to the PCA work more specifically, although it provides some insight into the functional relationships between the pathways that influence the ToxTracker® reporters, future work could expand on the results by conducting a comparative analysis of the cellular pathways leading to augmentation of ToxTracker® reporter expression. This type of work could lead to a deeper understanding of the functional relationships between the ToxTracker® reporters, and, perhaps more importantly, lead to hypotheses regarding cross-talk between the cellular pathways controlling the nature and magnitude of toxicological responses. For example, future work could investigate overlap of the pathways controlling the *Srxn1* reporter and the DNA damage reporters. More specifically, future work could investigate the functional relationship between *Nrf2*, which activates the *Srxn1* reporter (14), and the ATM/ATR and NF-κB pathways for DNA damage (15, 16). Importantly, since the PCA results will be influenced by the toxicological profiles of the substances included in the dataset, follow-up work could repeat the PCA using a dataset with a more comprehensive range of substances. The analyses could even include results for confidential, coded substances previously examined by Toxys. Addition of more substances would certainly contribute to the robustness and utility of the PCA analyses. Lastly, substance specific PCA axis scores could be used to generate hypotheses about the MOA of hitherto poorly-understood toxicants, i.e., via the substance's bi-plot proximity to well-characterised substances.

Future work regarding the reporter specific BMRs could investigate whether the response levels designated as the "Benchmark" are really indicative of toxicologically-adverse outcomes. For example, follow up experiments could use cellular viability assays, such as the BrdU and EdU cell

proliferation assays (11), to examine the viability of cells exposed to the concentration required to elicit the BMR. This could be done for the BMR values determined using both the Zeller *et al.* and Slob ES approaches. It is realistic to hypothesize that concentrations which elicit the Zeller et al-determined BMR will not adversely affect cellular viability. In contrast, concentrations that elicit the Slob-determined BMR will be associated with a marked decline in cell viability. Indeed, this results would be entirely consistent with the acronym sometimes used instead of BMR, i.e., CES or 'Critical Effect Size' (4).

With respect to compound potency rankings, it would be interesting to further investigate the reasons underlying the low genotoxic potency (i.e., *Rtkn* and *Bscl2* responses) of noteworthy mutagens such as EMS and ENU. Follow-up experiments could examine the involvement of DNA repair pathways in controlling ToxTracker® mES cells' responses to these alkylating agents. Low potency ranking may be related to ToxTracker® mES cells' proficiency for effectively responding to base alkylation, i.e., relative to responses to, for example, substances involved in bulky adduct formation or chromosome breakage. Enzymes involved in alkyl removal (e.g., MGMT or methyl-guanine DNA methyltransferase) (17) or base-excision repair (e.g., MPG or methyl-purine DNA glycosylase) (18) could be knocked out or knocked down, and the magnitude of the response compared with that observed for unaltered ToxTracker® mES cells. The effect of MGMT and/or MPG knock-out/knock-down on EMS and ENU genotoxicity could then be iteratively compared to the effect of knocking out or knocking down pathways related to, for example, by-pass of bulky lesions (i.e., *Polh* for DNA polymerase η) (19). Similarly, elements of the ROS response pathways, e.g., *Nrf2* (20), could be knocked out or down, and the magnitude of the oxidative stress response (e.g., *Blvrb* and/or *Srxn1* responses) compared with that observed using unaltered ToxTracker® mES cells.

Establishment and implementation of a reliable PB/BNF-induced S9 protocol will allow the use of ToxTracker® assay for routine genotoxicity assessment of substances that require safety evaluations for regulatory decision-making. The results presented in thesis Chapter 3 suggests that, for the ToxTracker® assay, PB/BNF-induced S9 could be used as an alternative to Aroclor-induced S9. That being said, additional work is needed to ensure that an alternative protocol based on PB/BNF-induced S9 is sufficiently robust. For example, the alternate protocol would have to be able to detect a variety of genotoxicants with varied MOAs and metabolic requirements. Future work could test a wide range of substances with varied MOAs; test performance would subsequently be assessed using metrics such as sensitivity and specificity, with *a priori* information on *in vivo* genotoxicity used as the point of reference.

Since inducer-related differences in the levels of key CYP isozymes will almost certainly affect assay performance, additional work could carefully assess CYP isozyme activity in several commercially-available lots of Aroclor- and PB/BNF-induced S9. More specifically, ethoxy-, pentoxy-, methoxy- and benzyloxy-*O*-deethylase (i.e., EROD, PROD, BROD and MROD) activity could be measured, reflecting the activity of CYP isozymes 1A1/1A2, 2B1/3B2, 2B1/3A, and 1A2, respectively (21).The results of such analyses could be used to determine if an alternative protocol based on PB/BNF-induced S9 could be expected to compensate for any observed differences in isozyme specific activity levels, i.e., elevated concentration of PB/BNF-induced S9 compensates for lower specific activity relative to Aroclor-induced S9. Importantly, any alternate ToxTracker® assay protocol that employs PB/BNF-induced S9 would have to balance the activity of CYP isozymes with S9 cytotoxicity. Therefore, future work should also examine the comparative cytotoxicity of PB/BNF- and Aroclor-induced S9. This type of analysis would permit

determination of the concentration limit for PB/BNF-induced S9, i.e., the S9 concentration that elicits a response at the 75% cytotoxicity limit.

In order to further refine the PB/BNF-induced S9 protocol, the experimental design could be modified to increase the precision of BMD_{100} values. Slob (2014) (22) showed that, rather than increasing the number of replicates per dose, BMD precision can be improved by increasing the number of doses. The current ToxTracker® experimental setup includes duplicates for 5 doses; from a BMD precision point of view it would likely prove beneficial to distribute the experimental units across 10 doses. Indeed, some researchers employing the BMD approach for analysis of genotoxicity reporter results have opted for few, if any, replicates, in favour of a large number of experimental concentrations (23).

Another factor that could be considered is the concentration of co-factors in the S9 metabolic activation mixture, i.e., G6P or glucose-6-phosphate and NADP or nicotinamide adenine dinucleotide phosphate. The experiments outlined in Chapter 3 used standardised, commercial co-factor solutions purchased from Moltox (Boone, North Carolina, USA); future work could investigate the influence of S9 co-factor concentrations on ToxTracker® reporter responses. Although this type of work would be time-consuming and expensive, it could serve to optimise the performance of the ToxTracker® assay.

High-throughput assays such as ToxTracker® generate vast amounts of complex, multivariate data; Chapter 2 employed a variety of quantitative strategies to determine (1) the fold-change cut-off for identification of a significant positive response, (2) the relative potencies of a wide range of validation substances, (3) toxicologically-relevant, endpoint-specific BMR values, and (4) the functional relationships between reporter responses. Chapter 3 examined the effects of S9-type,

and treatment time on ToxTracker® reporter responses. Collectively, the work presented in the thesis constitutes a significant step towards improving the execution of the ToxTracker® assay, and subsequent interpretation of ToxTracker® concentration-response data. As such, the work provides a firm foundation for eventual adoption of high-throughput reporter assays such as ToxTracker® for routine regulatory evaluation and prioritization of chemicals in commerce. Future work could expand on the analyses presented herein, thereby further justifying adoption of the ToxTracker® assay for effective and efficient toxicological hazard assessment.

4.4 References

1. Organisation for Economic Co-operation and Development (OECD). Test No. 487: In Vitro Mammalian Cell Micronucleus Test. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, 1997. https://www.oecd.org/chemicalsafety/test-no-487-in-vitro-mammalian-cell-micronucleus-test-9789264264861-en.htm.

2. Organisation for Economic Co-operation and Development (OECD). Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes. OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, 1997. https://www.oecd.org/chemicalsafety/test-no-476-in-vitro-mammalian-cell-gene-mutation-tests-using-the-hprt-and-xprt-genes-9789264264809-en.htm.

3. Zeller A, Duran-Pacheco G, Guérard M. An appraisal of critical effect sizes for the benchmark dose approach to assess dose–response relationships in genetic toxicology. Archives of Toxicology. 2017;91(12):3799-807.

4. Slob W. A general theory of effect size, and its consequences for defining the benchmark response (BMR) for continuous endpoints. Critical Reviews in Toxicology. 2016;47(4):342-51.

5. White PA, Long AS, Johnson GE. Quantitative interpretation of genetic toxicity dose-response data for risk assessment and regulatory decision-making: current status and emerging priorities. Environmental and Molecular Mutagenesis. 2020;61(1):66-83.

6. Tian S, Cyr A, Zeise K, Bryce S, Hall M, Bemis J, Dertinger S.D. 3Rs-friendly approach to exogenous metabolic activation that supports high-throughput genetic toxicology testing. Environmental and Molecular Mutagenesis. 2020;61(4):408-32.

7. Allemang A, Mahony C, Lester C, Pfuhler S. Relative potency of fifteen pyrrolizidine alkaloids to induce DNA damage as measured by micronucleus induction in HepaRG human liver cells. Food and Chemical Toxicology. 2018;121(72):81.

8. Long AS, Wills JW, Krolak D, Guo M, Dertinger SD, Arlt VM, White PA. Benchmark dose analyses of multiple genetic toxicity endpoints permit robust, cross-tissue comparisons of MutaMouse responses to orally delivered benzo[a]pyrene. Archives of Toxicology. 2018;92(2):967-82.

9. Wills JW, Johnson GE, Doak SH, Soeteman-Hernández LG, Slob W, White PA. Empirical analysis of BMD metrics in genetic toxicology part I: in vitro analyses to provide robust potency rankings and support MOA determinations. Mutagenesis. 2016;31(3):255-63.

10. Chikako Y, Yuto S, Mika E, Yumi M, Kazuto T, Hisashi H. Induction of oxidative stress by anticancer drugs in the presence and absence of cells. Oncology Letters. 2017;14(5):6066-70.

11. Yauk CL, Buick JK, Williams A, Swartz CD, Recio L, Li H, Fornace Jr AJ, Thomson, EM, Aubrecht, J. Application of the TGx-28.65 transcriptomic biomarker to classify genotoxic and

non-genotoxic chemicals in human TK6 cells in the presence of rat liver S9. Environmental and Molecular Mutagenesis. 2016;57(4):243-60.

12. Escobar-Garcia D, Camacho-Carranza R, Pérez I, Dorado V, Arriaga-Alba M, Espinosa-Aguirre JJ. S9 Induction by the combined treatment with cyclohexanol and albendazole. Mutagenesis. 2001;16(6):523-8.

13. Callander RD, Mackay JM, Clay P, Elcombe CR, Elliott BM. Evaluation of phenobarbital/beta-naphthoflavone as an alternative S9-induction regime to Aroclor 1254 in the rat for use in in vitro genotoxicity assays. Mutagenesis. 1995;10(6):517-22.

14. Hendriks G, Atallah M, Morolli B, Calleja, Fabienne M. G. R., Ras-Verloop N, Huijskens I, Raamsman M, van de Water B, Vrieling H. The ToxTracker assay: novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences. 2012;125(1):285-298.

15. Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. Biochemical Society Transactions. 2015;43(4):621-6.

16. Khalil HS, Deeni Y. NRF2 inhibition causes repression of ATM and ATR expression leading to aberrant DNA damage response. Biodiscovery. 2015;15:e8964.

17. Christmann M, Verbeek B, Roos WP, Kaina B. O^6-Methylguanine-DNA Methyltransferase (MGMT) in normal tissues and tumors: enzyme activity, promoter methylation and immunohistochemistry. Biochimica et Biophysica Acta. 2011;1816(2):179-90.

18. Tang J, Svilar D, Trivedi RN, Wang X, Goellner EM, Moore B, Hamilton RL, Banze, LA, Brown AR, Sobol RW. N-methylpurine DNA glycosylase and DNA polymerase beta modulate BER inhibitor potentiation of glioma cells to temozolomide. Neuro-Oncology. 2011;13(5):471-83.

19. Cruet-Hennequart S, Gallagher K, Sokòl AM, Villalan S, Prendergast AM, Carty MP. DNA Polymerase eta, a key protein in translesion synthesis in human cells. Subcellular Biochemistry. 2010;50(189):209.

20. Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radical Biology & Medicine. 2009;9:1304-9.

21. Cox JA, Fellows MD, Hashizume T, White PA. The utility of metabolic activation mixtures containing human hepatic post-mitochondrial supernatant (S9) for *in vitro* genetic toxicity assessment. Mutagenesis. 2016;31(2):117-30.

22. Slob W. Benchmark dose and the three Rs. part I. Getting more information from the same number of animals. Critical Reviews in Toxicology. 2014;44(7):557-67.

23. Bryce SM, Avlasevich SL, Bemis JC, Phonethepswath S, Dertinger SD. Miniaturized flow cytometric *in vitro* micronucleus assay represents an efficient tool for comprehensively characterizing genotoxicity dose-response relationships. Mutation Research. 2010;703(2):191-9.