Optimisation of *in vitro* metabolism using S9 liver extract in ToxTracker®

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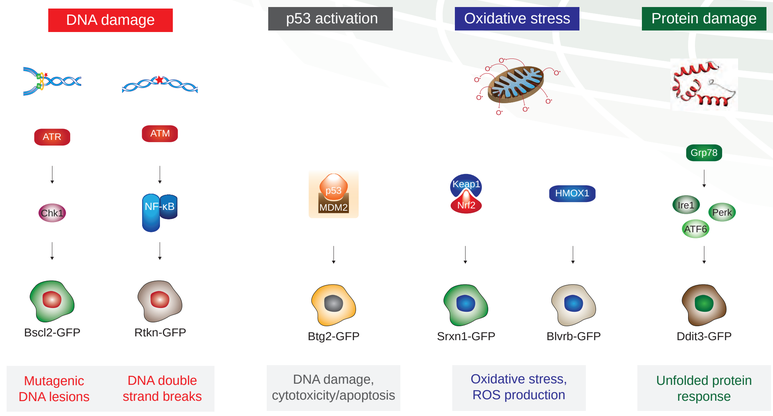
Introduction

Progenotoxic agents, such as Aflatoxin B1 and Benzo[a]pyrene, only become DNA reactive after metabolisation by detoxification enzymes. *In vivo* metabolism takes place in for example the liver, bone marrow or the lungs. In *in vitro* genotoxicity assays such as ToxTracker, metabolisation can be included by using S9 liver extract from rat or hamster. To induce the expression of detoxification enzymes, animals were treated with Aroclor-1254 or a mixture of phenobarbital and 5,6-benzoflavone.

In ToxTracker, we tested two different rat S9 liver extracts and compared their capacity to metabolise 20 progenotoxic compounds. Furthermore, we optimised the S9 concentration that was used in the assay to allow longer exposures in the presence of S9 without inducing cytotoxicity. The potency of the different S9 extracts and impact of the modifications of S9 concentration and exposure times were analysed by comparing the LOAEL.

The ToxTracker reporter assay

- ToxTracker is a stem cell-based reporter assay consisting of 6 GFP reporter cell lines.
- GFP-tagged biomarkers are activated upon specific cellular responses to DNA damage or other stress.
- Activation of the biomarkers is analysed using flow cytometry.



S9 toxicity

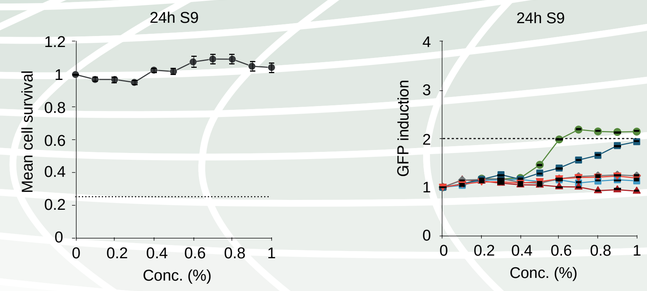


Figure 1: cytotoxicity testing of S9 treatment in ToxTracker. To test whether the S9 treatment could be extended from 3h to 24h, the ToxTracker reporters cell lines were exposed to increasing concentrations of S9 to assess reporter activation as well as cytotoxicity. An S9 concentration of more than 0.4% activated the Srxn1 oxidative stress and Ddit3 protein damage reporters, The dashed lines indicate the 0.25% cell survival minimum forToxTracker data acceptance and the 2-fold induction threshold for a positive ToxTracker result. A concentration of 0.25% S9 was selected for further S9 protocol optimisation experiments.

Optimisation of S9-mediated drug metabolisation

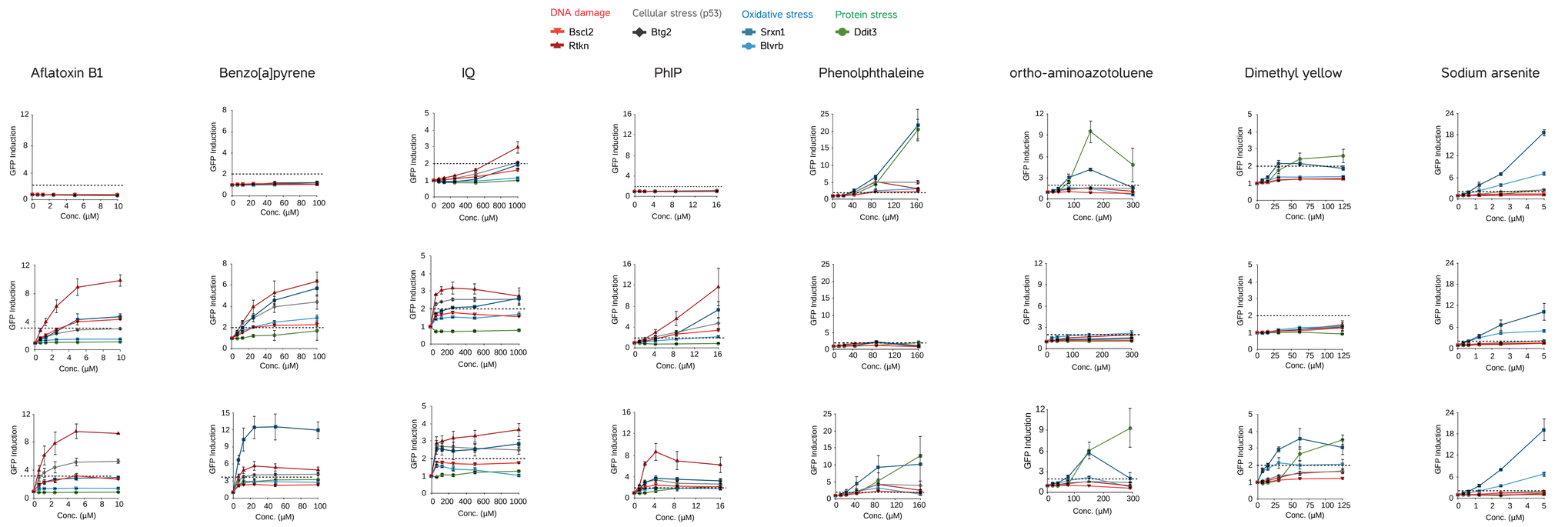


Figure 2: Differential activation of the ToxTracker reporters in absence and presence of an S9 metabolising system. The ToxTracker reporter cells were exposred to increasing concentrations of genotoxic compounds in absence of S9, in the presence of 1% S9 for 3h. or in presence of 0.25% S9 for 24h.. GFP induction levels in intact cells were determined by flow cytometry at 24 h. after initiation of the exposure. Cell survival was determined by flow cytometry after 24 h. exposure as the relative change in cell concentration compared to untreated controls.

Summary S9 metabolisation efficiency

Table 1: Lowest observed adverse effect concentration to induce genotoxicity or oxidative stress in the absence and presence of S9.

Compound	-S9		3h. 1% S9		24h. 0.25% S9	
	GTX	OX	GTX	OX	GTX	OX
Aflatoxin B1 (AFB1)	-	-	0.63	2.5	0.63	1.25
Benzo[a]pyrene (B[a]P)	-	-	12.5	12.5	6.25	6.25
Cyclophosphamide (CPA)	-	-	6.25	12.5	12.5	12.5
PhiP	-	-	3.91	7.81	0.98	1.95
Tryptophan-P-2	-	-	2.44	4.88	1.22	1.22
7,12-Dimethylbenz(a)anthracene (DMBA)	-	15.63	1.95	3.91	3.91	3.91
2-aminoanthracene (2-AA)	-	9.77	19.53	19.53	9.77	9.77
IQ	1000	-	62.5	250	62.5	62.5
3-methylcholanthrene (3-MC)	625	250	15.62	31.25	31.25	62.5
MeiQ	500	-	62.5	62.5	62.5	500
2-Acetylaminofluorene (2-AAF)	100	100	125	-	100	50
1,2-Diphenylhydrazine (1,2-DPH)	312.5	312.5	-	39.06	312.5	39.06
Disperse Orange	31.25	-	62.5	-	-	-
1,3-diphenyltriazine (1,3-DPT)	39.06	39.06	39.06	78.13	39.06	39.06
Phenolphthalein	39.06	39.06	312.5	312.5	39.06	19.53
Sodium arsenite	-	1.25	-	-	-	1.25
ortho-aminoazotoluene (OAT)	-	78.12	-	312.5	-	78.13
Dimethyl yellow	-	31.25	-	-	-	15.63
Isoprene	-	-	-	-	-	-
Hexamethylphosphoramide (HMPA)	-	-	-	-	-	-

All compounds were tested in the ToxTracker assay in the absence of S9, in the presence of 1% S9 for 3h. or 0.25% S9 for 24h. The LOAEL was determind based on ToxTracker data to compare the metabolic activation of the substances. For genotoxicity assessment, the LOAEL of Rtkn-GFP and Bscl2-GFP were determined. For the oxidative stress LOAEL, activation of the Srxn1-GFP and Blvrb-GFP reporters were assessed. The LOAEL was defined as the concentration at which a 2-fold induction of either reporter was observed.

Aroclor-1254 vs. Phenobarbital induced S9

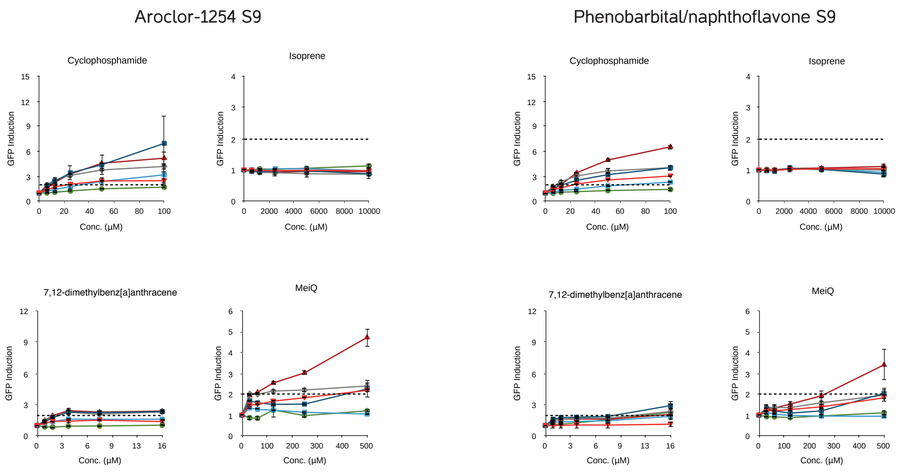


Figure 3: Differential activation of the ToxTracker reporters in presence of 0.25% aroclor-1254 of Phenobarbital induced S9 metabolising system.

Compound	Aroclor-1254 S9	Phenobarbital S9
Aflatoxin B1 (AFB1)	0.625	1.25
Benzo[a]pyrene (B[a]P)	6.25	6.25
Cyclophosphamide (CPA)	12.5	25
PhiP	0.98	1.95
Tryptophan-P-2	1.22	1.22
7,12-Dimethylbenz(a)anthracene (DMBA)	3.90	15.62
IQ	62.5	500
3-methylcholanthrene (3-MC)	31.25	62.5
MeiQ	62.5	500
2-Acetylaminofluorene (2-AAF)	100	100
1,2-Diphenylhydrazine (1,2-DPH)	312.5	312.5
1,3-diphenyltriazine (1,3-DPT)	39.06	39.06
Phenolphthalein	39.06	78.13
2-aminoanthracene (2-AA)	9.77	-
Benz[a]anthracene	62.5	-