CC/2017/06

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Input to OECD assessment of the ToxTracker Assay

Public Health England leads for the UK on human health discussions at the OECD meetings of the Working Group of National Coordinators of the Test Guidelines Programme (WNT). A submission has been made for a stem cell-based reporter assay for mechanistic genotoxicity and carcinogenicity hazard assessment, the ToxTracker assay. PHE have requested an opinion from the Committee on the use of the assay, in general and in particular for detection of non-genotoxic carcinogenicity. This will feed in to the UK's position during the discussions at the WNT meeting in April 2017.

The submission, in the Standard Project Submission Form (SPSF), outlines the validation process that is proposed for the assay, and which will contribute to a Detailed Review Paper on how the assay can be applied to support carcinogenicity hazard assessment. It is proposed that the ToxTracker assay would be a valuable component in an Adverse Outcome Pathway approach to identify carcinogenicity as it is able to assess both genotoxic modes of action and a number of non-genotoxic modes of action (oxidative stress and protein damage).

The submission is attached at Annex A, along with a number of annexed documents. In addition the comments received on the SPSF in early 2017 from Member States, and the responses to these comments are also in Annex A.

Attached at Annex B, is a consultation document and preliminary assessment report on the ToxTracker assay for the PARERE Network, which is an EU network of Member States to provide advice on the regulatory relevance and suitability of alternative approaches to animal testing proposed for validation. It is not proposed that the COC should attempt to address the questions in the consultation, however both these documents contain information on the assay, which Members may find useful.

The COM has previously considered the ToxTracker assay in 2014, as a paper in March 2014 and with a presentation in October 2014. The discussion paper and minutes of these meetings are provided in Annex C for Members' information.

Questions for the Committee

Members are invited to comment on the ToxTracker assay, which will feed in to the UK position on the assay, and in particular:

i. Members are requested to provide comments, based on the information provided, on the use of the assay to detect non-genotoxic carcinogens as part of hazard identification.

Secretariat
March 2017

CC/2017/06 – Annex A

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Input to OECD assessment of the ToxTracker Assay

This Annex contains the documentation provided via the OECD WNT:

- The Standard Project Submission Form and associated Annexes
  - including Hendriks et al. (2016) The Extended ToxTracker Assay Discriminates Between Induction of DNA Damage, Oxidative Stress, and Protein Misfolding. Toxicological Sciences, 150(1), 190-203.
- The comments from Member States on the SPSF with responses from the lead country.
- The draft project plan v1.3 for the Inter-laboratory validation of ToxTracker

These papers are attached. They are not being made publicly available for copyright reasons, but can be obtained from the OECD via the COC Secretariat in the first instance.

Secretariat
March 2017

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Input to OECD assessment of the ToxTracker Assay

This Annex contains the PARERE consultation:

- Consultation on the Regulatory Relevance of test submission ToxTracker® - TM2016-03
- Report on the Test Presubmission Assessment of the ToxTracker® Test Method

These papers are attached. They are not being made publicly available for copyright reasons, but can be obtained from the PARERE network via the COC Secretariat in the first instance.

Secretariat
March 2017

CC/2017/06 – Annex C

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Input to OECD assessment of the ToxTracker Assay

This Annex contains the considerations by COM of the ToxTracker Assay:

- MUT/2014/03 The ToxTracker assay for genotoxins
- MUT/MIN/2014/01 COM March 2014 minutes – see page 6
- MUT/MIN/2014/02 COM October 2014 minutes – see page 8

These papers are attached.

Secretariat
March 2017

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

The ToxTracker assay for genotoxins

Introduction

1. The Committee has previously evaluated assays for genotoxicity based on the response of a transgene fused to specific response elements for DNA damage signalling, in particular the GADD45a GreenScreen assay[1]. A similar principle is used in the CALUX assays, which are available coupled to the Ah receptor, and response elements for Nrf2, p53 and AP1, amongst others. Data using some of these test systems have been considered by sister committees, such as the COT[2].
2. The GADD45a assay lacks specificity, in that the response can be induced by a number of different signalling systems. Although responding primarily to p53, expression can also be modulated by ras, p73, JNK, NF-κB and Nrf2. Hence, a number of groups have been seeking more specific reporter systems not only to enable the identification of genotoxicants but also to determine the specific mechanism by which they act.
3. A significant disadvantage of many existing reporter systems of potential utility in testing for genotoxicity is that they are hosted in cells of tumorigenic origin and hence a number of signalling pathways, particular those reflecting DNA damage responses, will not be normal.

Development of ToxTracker system

4. In an effort to overcome this limitation, groups such as that of Hendriks *et al* (2011) have used undifferentiated mouse embryonic stem (mES) cells. These are non-transformed, yet are continuously renewable. The strategy adopted by Hendriks *et al* (2011, 2012, 2013) to develop suitable reporter cells for genotoxicity testing is illustrated in Fig. 1.
5. mES cells were exposed to model compounds to identify putative biomarker genes, specific to their mechanism of action, using transcriptional analysis. Preferentially responding genes coupled to the gene for green fluorescent protein (GFP), as a readily quantifiable expression marker, were transfected into mES cells, to produce pathway-specific reporter cells.
6. C57/Bl6 B4418 wild type embryonic stem cells were exposed in culture to a range of concentrations of a number of genotoxic and non-genotoxic compounds (Table 1). The concentrations used were those producing <10%, 10–30% and 30–50% apoptosis, respectively.

[1] Annual Report 2010 (http://cot.food.gov.uk/pdfs/cotcomcocreport2010.pdf)
[2] http://cot.food.gov.uk/pdfs/TOX-2006-09.pdf

Fig. 1. Strategy used by Hendriks *et al* (2011, 2012) to develop reporter cells for use in genotoxicity assays
Information from http://toxtracker.com/details/

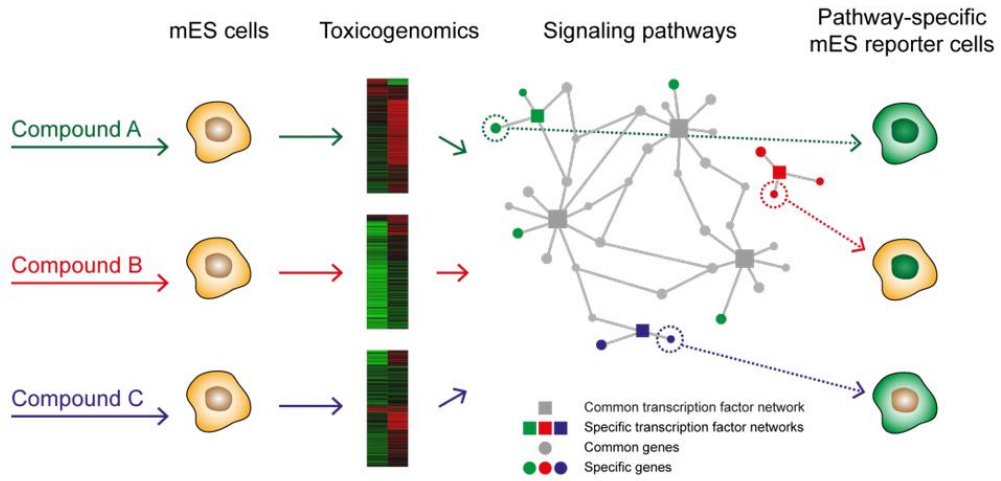


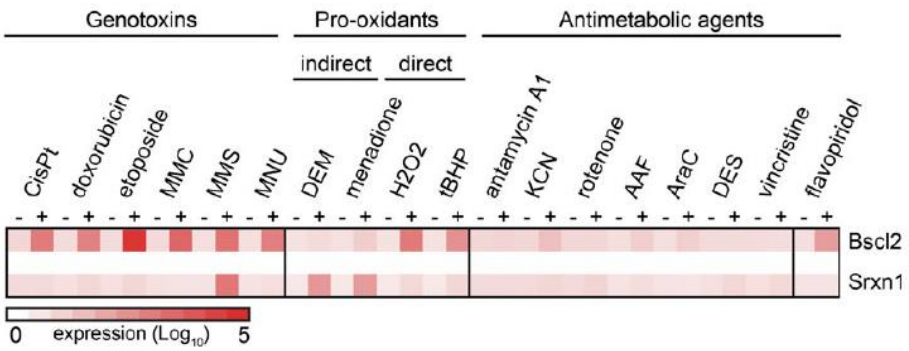
Table 1. Compounds* used for genome wide transcription profiling of mES cells by Hendriks *et al* (2011)

Cisplatin+	Menadione++	2-Acetylaminofluorene^
Doxorubicin+	Hydrogen peroxide++	Cytosine arabinoside^
Etoposide+	*Tert*-butyl hydroperoxide++	Vincristine^
Mitomycin-C+	Antimycin A1^	Flavopiridol^
Methyl methanesulfonate+	Potassium cyanide^	Cyclosporin A^
Diethyl malonate++	Rotenone^	Wyeth-14,643^

*Authors' classification: Red+ = genotoxin; Green++ = pro-oxidant; Blue^ = other

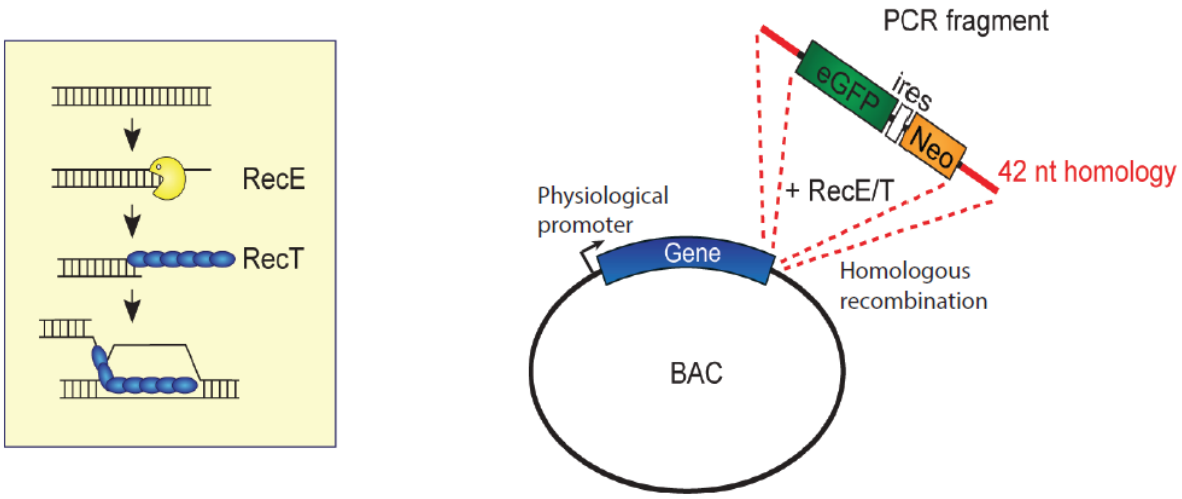
7. Where necessary, metabolic activation was achieved by including 1% S9 liver extract from rats treated with Arocolor 1254 in the cell culture system.
8. RNA isolated from treated cells was hybridised on Genechip Mouse Genome 430A arrays, comprising approx. 14,000 well-characterized genes, from Affymetrix.
9. In the published papers (Hendriks *et al*, 2011, 2012), the authors sought putative biomarker genes that were strongly and preferentially responsive to either DNA-damaging chemicals or oxidative stress. Through this strategy they identified *Bscl2* as preferentially responsive to genotoxins and *Srxn1* as preferentially responsive to pro-oxidants (Fig. 2).
10. The *Bscl2* gene is deficient in subjects with Berardinelli-Seip congenital lipodystrophy and encodes the protein Seipin. Previously, the *Bscl2* gene had not been implicated in a DNA damage response to genotoxins.
11. The *Srxn1* gene encodes the protein sulfiredoxin-1, which catalyses the reduction of oxidized cysteines in peroxiredoxins in peroxisomes. This is important in cellular defence against oxidative stress.

Fig. 2. Specificity of response of putative biomarker genes to genotoxins and pro-oxidants in mES cells (Hendriks *et al*, 2012)



12. Bacterial artificial chromosomes (BAC) were identified containing the biomarker genes and the gene encoding green fluorescent protein (GFP) was fused to the C-terminus of the biomarker gene. BAC transgenes were chosen to ensure the presence of most, if not all, of the regulatory elements of the respective biomarker gene. Following suitable manipulation and selection, modified BACs were transfected into mES cells. Monoclonal cells were isolated based on their response to representative genotoxins and pro-oxidants (cisplatin and DEM, respectively). The protocol is illustrated in Fig. 3.

Fig. 3. Protocol for preparation of reporter gene constructs for use in the ToxTracker system (Based on Hendriks *et al*, 2011, 2012)



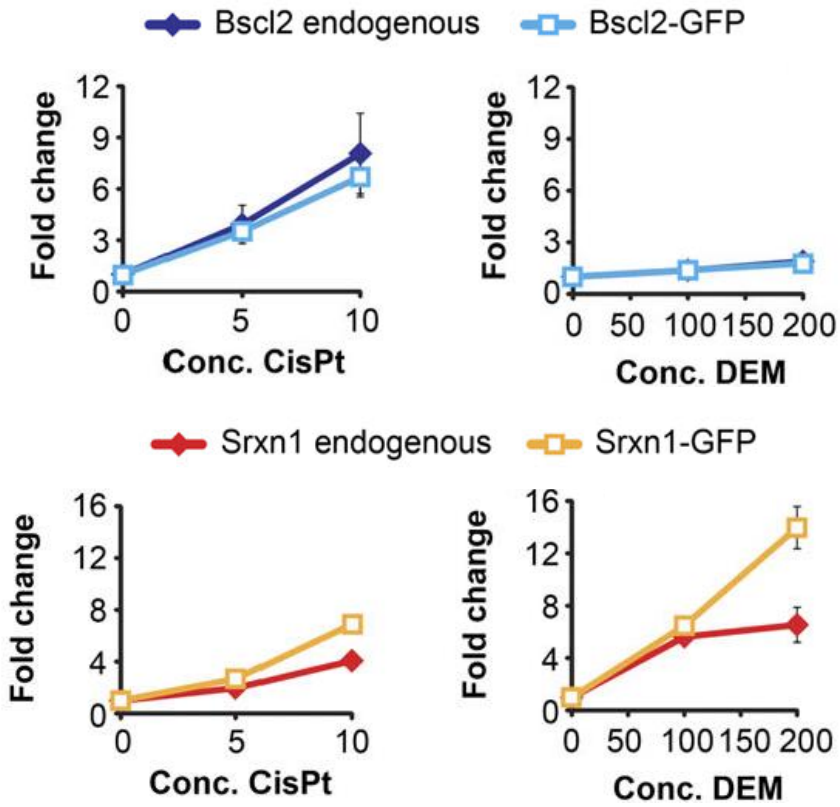
13. Assay read-out was expressed as the mean fluorescence intensity from GFP of 5000 viable cells, determined by flow cytometry. A response was considered positive when exposure to a compound resulted in > 1.5-fold increase in the GFP signal compared to the control. This is at least 5 times the SD of background fluorescence of mock transfected cells.

14. According to Hendriks *et al* (2012), use of a 1.5-fold cut-off provides >99.9% confidence in the reliability of a positive result and also provides comparability with the GreenScreen HC assay, in which the same cut-off is used.

15. *Bscl2* appears to be relatively specific to the genotoxic compound cisplatin, relative to the pro-oxidant DEM. *Srxn1* is less specific, responding to both compounds, though the response to DEM is more marked than to cisplatin, particularly of the trans-gene compared to the endogenous gene (Fig. 4).

16. Hendriks *et al* (2012) have termed the reporter cell lines derived using this approach as ToxTracker.

Fig.4. Specificity of transfected mES cell clones selected for assay development, to a representative genotoxin (cisplatin = CisPt) and pro-oxidant (diethyl maleate – DEM). Cells were exposed for 16 h (qualitatively very similar results were obtained after 8 h). Expression of GFP reporters was compared with that of the endogenous biomarker genes using qRT-PCR. Data from Hendriks *et al* (2012).



Preliminary evaluation of the ToxTracker system

17. In a preliminary study, Hendriks *et al* (2012) investigated the response of the two reporter cell lines to 8 representative genotoxins and pro-oxidants (listed in Table 2). Although methyl methanesulfonate is an alkylating agent, a number of groups have shown that the primary response of cells in culture to this compound is oxidative stress.

18. The results of this study confirmed the initial characterisation of the two cell lines. The *Bscl2* cells are relatively specific for genotoxins, with essentially no response to pro-oxidants. In contrast, the *Srxn1* cells respond more strongly to pro-oxidants, but they also respond positively to genotoxins (Table 2).

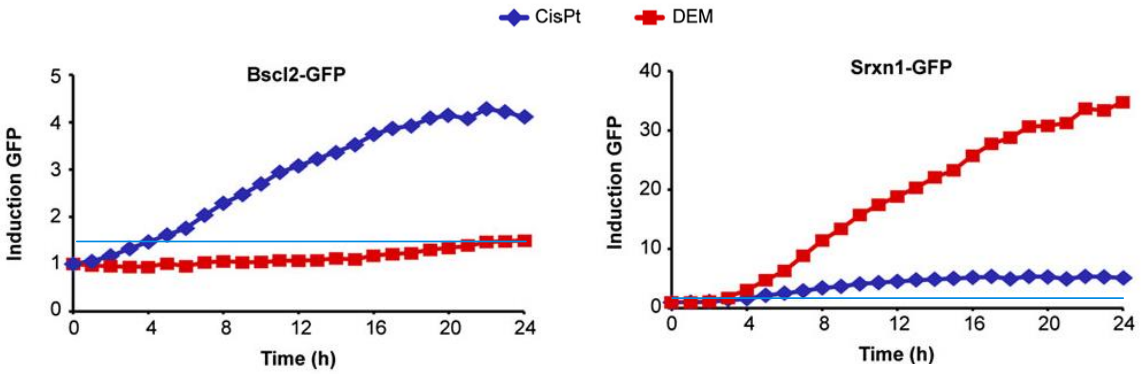
Table 2. Preliminary study of sensitivity and specificity of the ToxTracker reporter cell lines. Note that cytotoxicity was almost identical in the two cell lines. Data were abstracted from graphs provided in Hendriks *et al* (2012).

Compound	***Bscl2* cells Fold increase**	***Srxn1* cells Fold increase**	**Concentration***
Genotoxins			
Cisplatin	3.7	2.4	7 µM
Etopiside	4.6	3.2	1.5 µM
Doxorubicin	3.6	2.5	0.5 µM
Mitomycin C	4.3	2.4	1.5 µg/ml+
Pro-oxidants			
Diethyl maleate	1.3	14.1	250 µM+
Sodium arsenite	1.3	5.8	5 µM
Cadmium chloride	0.9	3.1	20 µM+
Methyl methanesulfonate	1.6	23.7	0.5 mM+

*Concentration at which fold-change was observed, which caused 75% cytotoxicity or was the +maximum concentration tested

19. A positive response is observed in the cells after only a few hours, for both a genotoxin (*Bscl2* cells) and a pro-oxidant (*Srxn1* cells), respectively (Fig. 5)

Fig. 5. Time course of response of ToxTracker cells to a representative genotoxin (cisplatin = CisPt) and pro-oxidant (diethyl maleate = DEM). Horizontal blue line = 1.5-fold induction. Modified from Hendriks *et al* (2012).



Validation of the ToxTracker system

20. The ToxTracker GFP reporter mES cells were subjected to a validation assessment using 50 compounds, a combination of genotoxins and non-genotoxins. Cells were exposed to a least five concentrations of the compounds, with at least three independent replicates. Choice of concentrations was based on cytotoxicity, the

highest concentration used causing a 75-90% reduction in viability after 24-h of treatment. Where viability was not affected, the maximum concentration used was 10 mM.

21. For those chemicals requiring metabolic activation, cells were exposed to the compounds for 3 h in the presence of 1% S9 liver extract (post-mitochondrial supernatant) from rats treated with Aroclor 1254. No details are provided as to which of the 50 chemicals were treated in this way.

22. Compounds were selected mainly from those recommended by ECVAM for validation of new *in vitro* genotoxicity assays (Kirkland *et al*, 2008). ECVAM Class 1 compounds comprise *in vivo* genotoxins, most which are known carcinogens with a mutagenic mode of action that should be positive in an *in vitro* genotoxicity assay. ECVAM Class 2 comprises compounds that are not genotoxic and are either not carcinogenic or are carcinogenic by a non-genotoxic mode of action. These compounds should not give any evidence of genotoxicity in an *in vitro* assay. ECVAM Class 3 also comprises compounds that are non-carcinogens and are not genotoxic in *in vivo* assays, but which have been reported positive in some *in vitro* genotoxicity assays.

23. All ECVAM class I compounds were positive in one or both of the reporter cell lines, other than *p*-chloroaniline. Although carcinogenic, this compound shows no evidence of genotoxicity in other *in vitro* assays (Hendriks *et al,* 2012*)*. None of the ECVAM class 2 compounds was positive with either of the reporter cell lines. Most non-carcinogenic ECVAM class 3 compounds were negative with the reporter cell lines (Table 3).

24. Amongst ECVAM class 3 compounds, *tert*-butylhydroquinone, resorcinol, sulfoxazole, *p*-nitrophenol, dichlorophenol and propyl gallate were positive with the reporter cell lines. These compounds were genotoxic in other *in vitro* assays. The response was generally selective for the Srxn1-GFP reporter, suggesting an oxidative stress mode of action. Resorcinol and propyl gallate were also positive with the Bscl2-GFP reporter, suggesting genotoxic potential, at least *in vitro*.

Table 3. Results of testing the ECVAM list of carcinogens and non-carcinogens in ToxTracker reporter cell lines (Hendriks *et al*, 2012).

Substance	**Ames test**[a]	**In vivo GTx**[a]	**In vitro GTx**[a]	**ToxTracker**	
				Response	**Pathway**[b]
Ames +ve, in vivo genotoxins (ECVAM 1a)					
Cisplatin	+	+	+	+	Gtx
MMS	+	+	+	+	Ox
$CdCl_2$	+	+	+	+	Ox
p-Chloroaniline	+	+	±	-	Ox
Ames –ve or ±, in vivo genotoxins (ECVAM 1b)					
Sodium arsenite	-	+	+	+	Ox
Taxol	-	+	+	+	Gtx

Substance	**Ames test**[a]	**In vivo GTx**[a]	**In vitro GTx**[a]	**ToxTracker**	
Non-carcinogens, -ve in vivo genotoxins (ECVAM 2a)					
n-Butyl chloride	-	No data	-	-	n/a
Phenformin HCl	-	No data	-	-	n/a
(2-Chloroethyl) trimethylammonium chloride	-	No data	-	-	n/a
N,*N*-Dicyclohexyl thiourea	-	No data	-	-	n/a
Cyclohexanone	-	No data	-	-	n/a
Erythromycin stearate	-	No data	-	-	n/a
Fluometron	-	No data	-	-	n/a
Non-genotoxic carcinogens (ECVAM 2b)					
D-Limonene	-	No data	-	-	n/a
Amitole	-	-	-	-	n/a
Tert-Butyl alcohol	-	-	-	-	n/a
Diethanolamine	-	-	-	-	n/a
Hexachloroethane	-	-	-	-	n/a
Methyl carbamate	-	-	-	-	n/a
Pyridine	-	-	-	-	n/a
Tris(2-ethylhexyl) phosphate (TEHP)	-	-	-	-	n/a
Non-carcinogens, -ve or ± in vivo genotoxins (ECVAM 3a)					
Tert-Butylhydroquinone	-	-	+	+	Ox
o-anthranilic acid	-	-	+	-	N/A
1,3-Dihydroxybenzene (resorcinol)	-	-	+	+	Equivocal
Sulfisoxazole	-	-	-	+	Ox
Non-carcinogens, no in vivo genotoxicity data (ECVAM 3b)					
Ethionamide	-	No data	+ (weak)	-	n/a
Curcumin	-	No data	+	-	n/a
Benzyl alcohol	-	No data	+ (weak)	-	n/a
Urea	-	No data	+	-	n/a
Rodent only non-genotoxic carcinogens (ECVAM 3c)					
Sodium saccharin	-	-	±	-	n/a
In vitro genotoxicity unclear (ECVAM 3d)					
p-Nitrophenol	-	No data	±	+	Ox
2,4-Dichlorophenol	-	+ (weak)	-	+	Ox
Eugenol	-	±	-	-	n/a
Ethyl acrylate	-	+ (weak)	-	-	n/a
Isobutyraldehyde	-	±/+	-	-	n/a

Substance	Ames test[a]	In vivo GTx[a]	In vitro GTx[a]	ToxTracker	
Propyl gallate	-	±	-	+	Equivocal

[a]From Kirkland *et al* (2008)
[b]Gtx = genotoxin; Ox = pro-oxidant; n/a = not applicable

25. A number of compounds in addition to those on the ECVAM list were also tested. All were correctly identified using the reporter cell lines as either genotoxic or pro-oxidant (Table 4).

Table 4. Results of testing of additional list of carcinogens and non-carcinogens in ToxTracker reporter cell lines (Hendriks *et al*, 2012).

Substance	Ames test[a]	In vivo GTx[a]	In vitro GTx[a]	ToxTracker	
				Response	Pathway[b]
Doxorubicin	+	+	+	+	Gtx
MMC	+	+	+	+	Gtx
Etoposide	-	+	+	+	Gtx
DEM	No data	No data	No data	+	Ox
Tert-Butyl hydroperoxide	+	-	-	+	Ox
Hydrogen peroxide	+	-	+	+	Ox
Flavopiridol	No data	No data	No data	-	n/a
Copper sulfate	No data	No data	No data	+	Ox
Potassium bromate	+	+	+	+	Ox
4-Nitroquinelone-1-oxide	+	+	+	+	Gtx
4-Hydroxy-2-nonenal	-	-	+	+	Ox
Cytarabine	-	No data	+	+	Gtx
Camptothecin	-	-	+	+	Gtx
Bleomycin	+	+	+	+	Gtx

[a]From Kirkland *et al* (2008)
[b]Gtx = genotoxin; Ox = pro-oxidant

Role of metabolic activation

26. The effects of metabolic activation were specifically investigated using four compounds known to depend on metabolism for their genotoxicity. These were aflatoxin B1 (AFB1), benzo[*a*]pyrene (B[*a*]P), cyclophosphamide and dimethylbenz[*a*]anthracene (DMBA). Cells were incubated for 3 h in the presence of hepatic S9 from Aroclor 1254 treated rats. The degree of cytotoxicity of S9 was unacceptable with longer incubation times.

27. All four compounds were positive with the Bscl2-GFP reporter in the presence of S9, but were negative in the absence of an activating system. There was a parallel increase in cytotoxicity with induction of Bscl2-GFP expression. There is no

information on the response of the Srxn1-GFP reporter with these compounds, or indeed whether they were tested with this system.

28. The response of the Bscl2-GFP reporter to cisplatin, which does not require metabolic activation for its genotoxicty, was unaffected by the addition of rat liver S9.

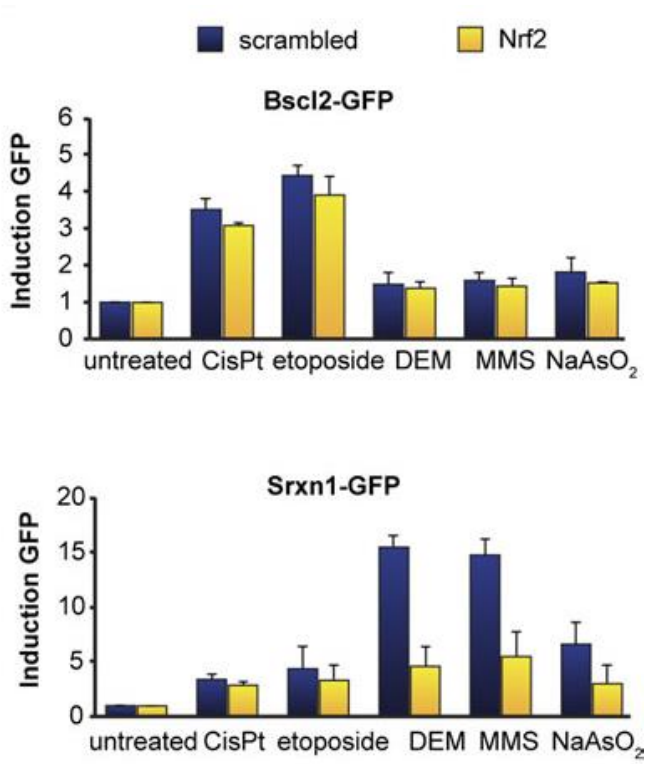
Mechanism of induction of ToxTracker reporter genes Srxn1 and Bscl2

29. Induction of the Srxn1-GFP reporter by the pro-oxidants diethyl maleate, $CuSO_4$, $NaAsO_2$, $CdCl_2$ and by methyl methanesulfonate was inhibited by N-actylcysteine, whilst the positive response to mitomycin C, a genotoxin, in neither the Srxn1-GFP nor the Srxn1-GFP reporter cell line was affected by N-acetylcysteine.

30. The response of the Srxn1-GFP reporter to the pro-oxidants diethyl maleate, $NaAsO_2$ and methyl methanesulfonate was markedly attenuated by Nrf2 knockdown using siRNA tranfection. In contrast, the response of the cells to the genotoxic compounds cisplatin and etoposide was unaffected by Nrf2 knockdown. Nrf2 knockdown had no effect on the response to any of the compounds on the response of Bscl2-GFP reporter cells (Fig. 6).

31. The authors (Hendriks *et al*, 2012) conclude that these data establish that Srxn1-GFP induction is via the formation of reactive oxygen species through the Nrf2 signalling pathway. However, it should be noted that N-acetylcysteine can inactivate electrophiles as well as scavenge reactive oxygen species, so the evidence implicating only ROS in the response could be stronger.

Fig. 6. Response of ToxTracker reporter cell lines to representative genotoxins (CisPt and etoposide) and pro-oxidants (DEM, MMS, $NaAsO_2$) before and after knockdown of Nrf2 by siRNA transfection. From Henriks *et al*, 2012



32. Genotoxins with quite different modes of action (cisplatin and mitomycin C by DNA cross-linking, etoposide and doxorubicin by causing double strand breaks by inhibiting topoisomerase II, N-nitroso-N-methylurea (MNU) via methylation of DNA and aflatoxin B1, benzo[a]pyrene and dimethylbenz[a]anthracene by arylation of DNA) all induced expression of the Bscl2-GFP reporter system. Interestingly, hydroxyurea, which depletes cellular free ribonucleotide levels by inhibiting ribonucleotide reductase, and aphidicolin, a direct inhibitor of DNA polymerase, also induced Bscl2-GFP reporter gene expression. This suggests that the response reflects inhibition of DNA replication rather than DNA damage *per se*. However, these two compounds also induced a response, albeit to a lesser extent, in Srxn1-GFP cells (maximum of 2-fold cf 4-fold with Bscl2-GFP cells).

33. Induction of Bscl2-GFP expression by cisplatin and aphidicolin was almost completely repressed by an inhibitor of ATR (schisandrin B) or of Chk1/Chk2 (UCN-01) but was unaffected by an inhibitor of ATM (ku55933). This provides evidence that Bscl2-GFP reporter expression on exposure to genotoxins reflects the response of the ATR-Chk1 signalling pathway to stalled DNA replication forks (Hendriks *et al*, 2012). Although the Srxn1-GFP reporter responded to these compounds (cisplatin and aphidicolin) this was independent of either ATM or ATR.

34. Knockdown of p53 by siRNA transfection had no effect on the response of the Bscl2-GFP (or the Srxn1-GFP) reporter cell line to genotoxic (cisplatin, etoposide) or pro-oxidant (diethyl maleate, methyl methanesulfonate, sodium arsenite) compounds. Hence, the response of the Bscl2-GFP reporter cell line is independent of p53 (Hendriks *et al*, 2012).

Btg2-GFP reporter cell line

35. Whilst most of the work reported by Hendriks *et al* (2012) on the ToxTracker system was on the Bscl2-GFP and Srxn1-GFP cell lines, limited information was also provided on a third member of the ToxTracker sytem, the Btg2-GFP cell line. Btg2 is a target of p53 that is transcriptionally activated by both genotoxins and pro-oxidants (Rouault *et al*, 1996).

36. Btg2-GFP expression was induced by genotoxic and pro-oxidant compounds tested in #32 above (cisplatin, etoposide, diethyl maleate, methyl methanesulfonate and sodium arsenite). Knockdown of p53 substantially reduced the response (Fig. 7).

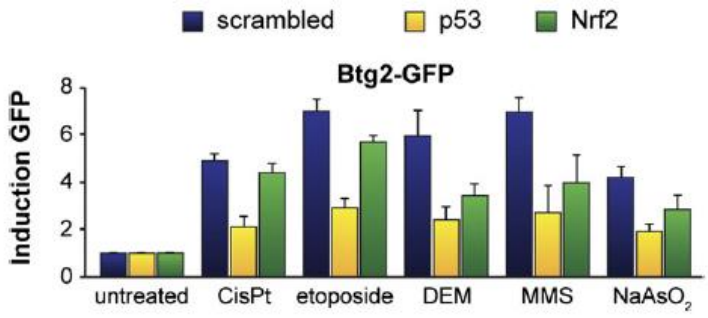
Recent developments in the ToxTracker system

37. Since publication of details of the ToxTracker system in the peer reviewed literature, the lead researchers involved have established a spin out company, called Toxsys, in part to commercialise the ToxTracker system (see http://toxtracker.com/, last accessed 08/01/14).

38. According to the ToxTracker website, the number of available reporter cell lines, all in mouse embryonic stem cells, has increased to 6. These are listed in Table 5. The

molecular signalling pathways involved in the response of these reporter systems are illustrated in Fig. 8.

Fig. 7. Response of Btg2-GFP reporter cell line to genotoxic (CisPt, etoposide) and pro-oxidant (DEM, MMS, Na AsO_2) compounds. Knockdown of p53 using siRNA transfection substantially attenuated the response. Knockdown of Nrf2 also reduced the response, particularly to the pro-oxidants (Hendriks *et al*, 2012).



39. The ToxTracker website (http://toxtracker.com/details/) reports that "A full interlaboratory cross-validation that was performed showed an excellent reproducibility and transferability of the ToxTracker assay." No further details are provided.

Fig. 8. Signalling pathways involved in response of current ToxTracker GFP reporter cell lines to genotoxic chemicals (from http://toxtracker.com/details/).

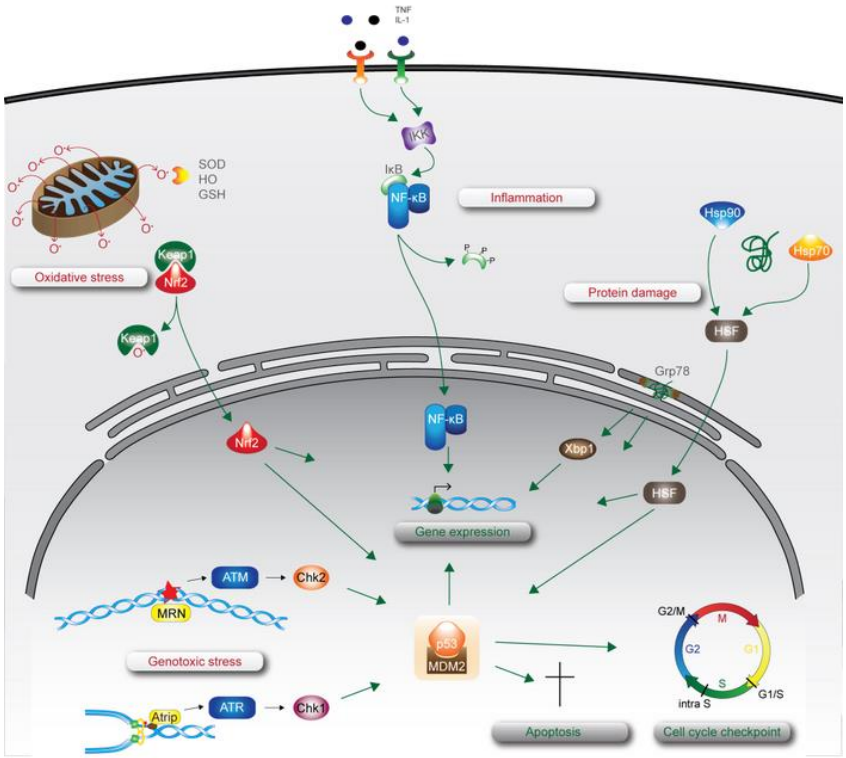
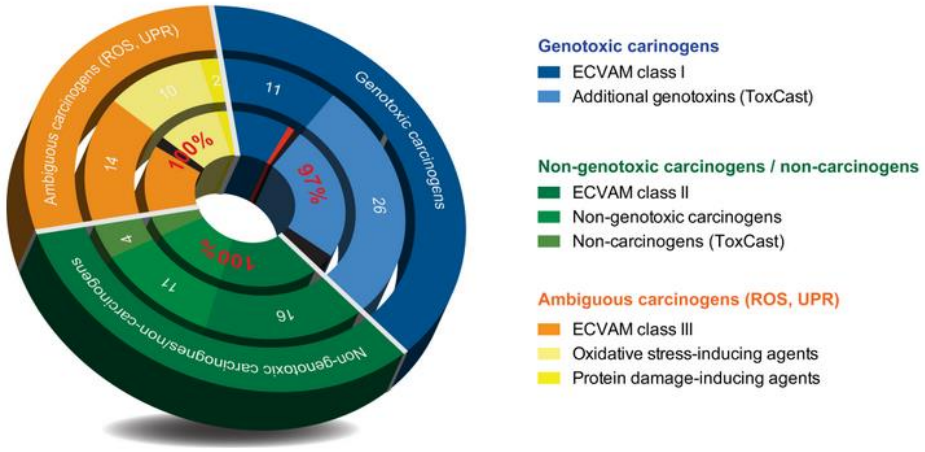


Table 5. Currently available GFP reporter mES cell lines in the ToxTracker system (information from http://toxtracker.com/details/)

Stressor	**Pathway**	**Molecular marker**
DNA damage	ATR/Chk1 DNA damage signalling NF-B signalling	Bscl2 Rtkn
Oxidative stress	Nrf2 antioxidant response Unknown	Srxn1 Blvrb
Protein damage	Unfolded protein response	Ddit3
Cellular stress	P53 signalling	Btg2

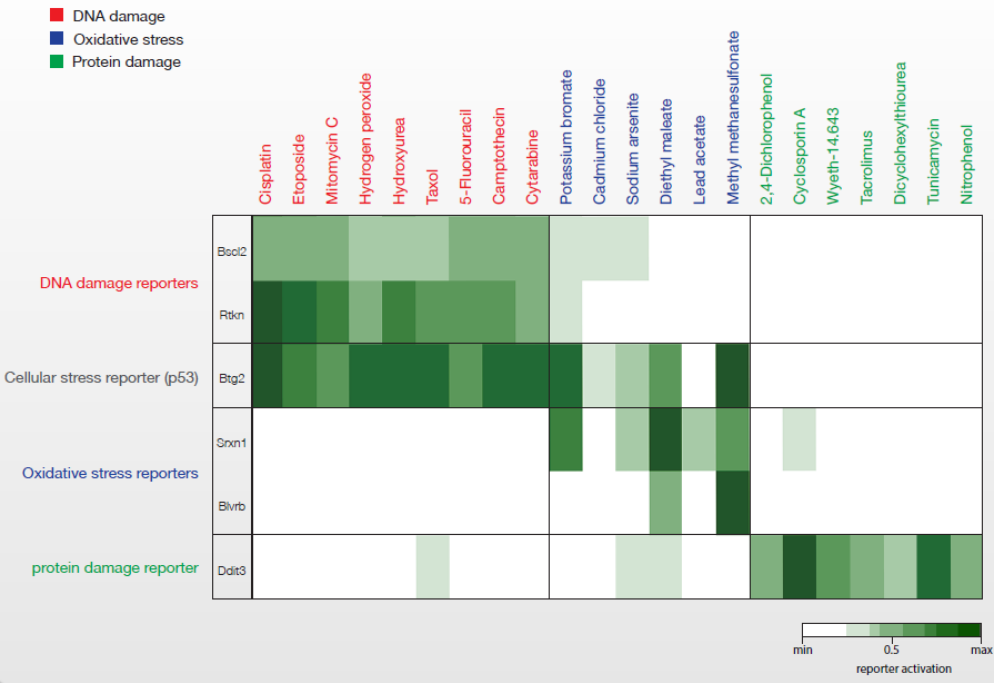
40. The ToxTracker website also indicates that the number of compounds tested has increased to 94, with a similarly high reliability in the accuracy of classification as genotoxic or not, to that reported by Hendriks *et al* (2012) (details as shown in Fig. 9). Over 97% of compounds were correctly classified according to their known biological reactivity using the ToxTracker system (http://toxtracker.com/details/).

Fig 9. Summary of performance of ToxTracker system in classifying 94 genotoxic and non-genotoxic compounds (from http://toxtracker.com/details/)



41. The identity of the additional 44 compounds included in the analysis on the ToxTracker website is not provided. Details of the response of the different reporter cell lines to representative compounds with different biological activities are provided in the form of a "heat map" in a FAQ sheet available from http://toxtracker.com/Toxtracker-Facts.pdf (Fig. 10).

Fig. 10. Specificity of the ToxTracker system according to the biological activity of the compound (from http://toxtracker.com/Toxtracker-Facts.pdf)



Conclusions

42. The approach taken to the development of the ToxTracker system is logical and overcomes some of the limitations of previous assays. The reporter genes are selectively responsive, having being identified in genome wide transcriptional analysis; they have been transfected in BACs (bacterial artificial chromosomes), which helps ensure the presence of most, it not all, appropriate regulatory gene sequences; and embryonic stem cells are used as hosts for the reporters, avoiding problems that can occur when tumour-derived cell lines as used, in which a number of signalling pathways, including those involved in DNA damage responses, are often abnormal.

43. Although six different cell lines are now available, reflecting different signalling pathways in response to genotoxic compounds, only two of these have been subjected to detailed investigation (or at least, data on only two of them have been reported). These are the Bscl2 reporter cell line, responding to genotoxins, and the Srxn1 reporter cell line, responding to pro-oxidants. Limited information has been provided on a third reporter cell line, Btg2, which responds to both genotoxins and pro-oxidants.

44. Like the cells used in most other *in vitro* genotoxicty assays, the ToxTracker reporter cell lines lack endogenous metabolic activity (they are derived from undifferentiated mouse embryonic stem cells). As in a number of such assays, this limitation can be overcome to some extent by the inclusion of post-mitochondrial supernatant (s9 extract) from the liver of rats treated with Aroclor 1254. The use of S9 with mES cells

was slightly problematic, in that exposure was possible for only 3 h, due to excessive cytotoxicity after this time.

45. The signalling pathways responsible for the responsiveness of the Bscl2-GFP and Srxn1-GFP reporter cell lines have largely been determined. The former appears to depend on the ATR-Chk1 signalling in response to stalled DNA replication forks. The latter appears to reflect the Nrf2 signalling pathway. There is evidence that the response of the Btg2-GFP reporter is p53-dependent.

46. The performance of the Bscl2-GFP and Srxn1-GFP reporter cell lines has been evaluated in detail with 50 compounds, including those recommended by ECVAM for the evaluation of *in vitro* genotoxicity assays. Less comprehensive Information was provided on an additional six compounds. Only one aneugen was tested. The number of Ames-positive mutagens was relatively low, particularly if pro-oxidants are excluded. The combination of the two reporter systems was very successful in classifying the compounds for their biological activity based on a weight of evidence consideration, in that a number of pro-oxidants were considered to be classified accurately if they were positive with the Srxn1-GFP reporter cell line, despite negative findings in other genotoxicity tests *in vitro* and *in vivo* (e.g. sulfisoxazole).

47. Compounds known to depend on metabolic activation for their genotoxicity could be successfully classified by the inclusion of S9 in the incubation. Specific information is provided on the role of S9 for five compounds, four requiring metabolism for activity and one that did not. It is implied that S9 was included in incubations with those other compounds where metabolism was necessary for their activity, but the basis for when this would be done, and the identity of the chemicals involved, are not provided. No strategy is proposed for how compounds, for which the role of metabolism is not known, should be tested with this system.

48. The ToxTracker system has been commercialised and additional information not yet available in the published literature on its performance is available on the website. This includes expansion of the number of available reporter cell lines to reflect additional signalling pathways, an increase in the number of compounds tested to almost double that reported in the published papers and an indication that an inter-laboratory comparison of the methodology has been performed, though no details are provided.

49. Hendriks *et al* (2011, 2012) make a distinction between chemicals they classify as “genotoxins” and those that are considered as pro-oxidants. Genotoxins include mutagens (alkylating and arylating agents), clastogens and aneugens. The pro-oxidants investigated were negative in the reporter system responsive to “genotoxins”. However, many studies suggest that pro-oxidants may be genotoxic though mutagenic or clastogenic effects (Klaunig *et al*, 2011; Lindholm *et al*, 2010). As the pro-oxidants tested had no effect in the Bscl2 reporter cell line, does this reflect a level of discrimination not available in other *in vitro* assays for genotoxicity, or does it reflect a deficiency in the test system? This will depend on the relevance of oxidant-induced genotoxicity as a mechanism in carcinogenesis.

Questions for Members

- What are members' views of the scientific merit of the approach adopted in the development of the ToxTracker system?
- Is sufficient information available to enable the validity of the assay system to be evaluated? If not, what information would be necessary?
- What are members' views of the role of such an assay system in a strategy for genotoxicity testing? For example should it be a first tier assay; for investigation of equivocal results in other assays; for mode of action studies?
- When, and should it be, necessary to identify pro-oxidants that are not genotoxic in any other genotoxicity assays, including the ToxTracker reporter system for DNA damage response (the Bscl2-GFP reporter system)? How would such information be used in the genotoxicity assessment of a novel chemical?
- The ToxTracker assay is available on purchase as a reagent kit under a non-exclusive user license. What are the implications, if any, of commercialisation of the assay system for its general use in genotoxicity testing?
- Would it be useful to members if further information could be obtained on the assay system and its performance, from the researchers responsible for its development?

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MUT/MIN/2014/1

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Minutes of the meeting held at 10.30 am on Thursday 6th March 2014 in Room 125A Skipton House, Elephant and Castle, London, SE1.

Present:

Chairman:	Dr D Lovell
Members:	Dr G Clare
	Professor M O'Donovan
	Dr B Elliot
	Ms P Hardwick
	Professor G Jenkins
	Professor D Kirkland
	Professor A Lynch
	Professor D Phillips
Secretariat:	Dr O Sepai (PHE Secretary)
	Dr D Gott (FSA Secretariat)
	Dr K Burnett (PHE Tox Unit)
	Mr S Robjohns (PHE Secretariat minutes)
Assessors:	Dr H Stemplewski (MHRA)
Observers:	Dr A Scott

ITEM 1: ANNOUNCEMENTS/APOLOGIES FOR ABSENCE

1. The Chair welcomed members, the secretariat and assessors. Dr D Gott was attending in place of Dr D Benford from the FSA. The Chair also welcomed Dr A Scott from Unilever who would be attending from 12.30 pm as a member of the OECD Expert Group for Cell Transformation Assays (CTA).

2. Apologies for absence were received from the members Dr S Dean, Dr S Doak, Professor F Martin, and Professor M Rennie. Apologies were also received from the assessors Dr C Ramsey, Mr S Fletcher (VMD) and Dr S Dutton (HSE).

3. The Chair congratulated the COM member Dr Shareen Doak on the birth of her baby boy Riley born in January. The committee was also informed that Professor Guy M. Poppy had been appointed as the new Chief Scientific adviser to the Food Standards Agency.

4. Members were reminded of the need to declare any interests before discussion of items.

ITEM 2: MINUTES OF MEETING ON 28th November 2013 (MUT/MIN/2013/3)

5. Members agreed the minutes subject to minor editorial changes.

ITEM 3: MATTERS ARISING

6. The committee was informed that the post for the secretary of the COM (previously the role of Jon Battershill) had been re-advertised. This post would also involve the evaluation of pesticides and biocides as a regulatory toxicologist. Members were requested to inform colleagues who may wish to apply. The COM expressed its concern over the difficulty and delay in recruiting for this post.

ITEM 4: ANNUAL REPORT FOR 2013 (MUT/2014/01)

7. The aim of the Committees on Toxicity, Mutagenicity, and Carcinogenicity of Chemicals in Food, Consumer Products and the Environment Annual Reports is to provide a brief toxicological background to the Committees' decisions.
Paper MUT/2014/01 provided draft summaries of the items and statements considered by the COM during 2013. This was intended to form the COM contribution to the joint COC/COM and COT 2013 Annual Report. The draft text had been summarised from the minutes and statements for 2013.

8. Insufficient time was available to consider this item at this meeting. However, members were asked to send any comments to the secretariat.

Members were also asked to send updated 'Declarations of Interest' statements to Gill Fisher the COM administrator.

ITEM 5: MUTATIONAL SPECTRA (MUT/2014/02)

9. The term 'mutation spectra' refers to the composite of the number, types and sites of all mutations observed in a given sequence. It is also more loosely used in referring to the number and types of mutation found or even the main type of mutation observed (e.g. GC to AT transversions).

10. The COM had previously advised on the significance of mutation spectra arising from a specific chemical exposure. In a 1999 statement, the COM reported on the high frequency of mutations at codon 61 of the K-ras gene in lung tumours from ozone exposed mice.

11. The topic of mutation spectra was also raised in the Horizon scanning exercise in 2006, when it was suggested that a review of studies examining mutational fingerprints and hotspots for mutation following carcinogen exposure could be conducted. The topic had also been raised at a subsequent horizon scanning exercise, but had not been undertaken due to other priorities.

12. Paper MUT/2014/02 presented an overview and summaries of a selection of studies retrieved from the literature, which analysed mutation spectra induced by different chemicals in different test systems. The paper was intended as an overview and summaries and findings of the reviewed papers were tabulated. A variety of test systems had been used. *In vitro* systems included bacterial, human, rodent and transgenic cell lines. *In vivo* systems identified were primarily transgenic models from which genes were more easily isolated and sequenced (i.e. Muta^{TM} mouse, Big Blue and gpt delta mice). A paper discussing the use of diagnostic mutations in establishing the mechanisms of carcinogenicity, presented to the Committee in 1999, was also appended at Annex 1.

13. Members considered that this was a very interesting area of research. Its main value lay primarily in evaluating a chemical's mode of carcinogenic action or in understanding cancer aetiology and types of adducts and mutation involved in cancer. Currently it was not suitable for regulatory purposes.

14. The committee advised that Ames tester Salmonella strains and the hprt locus are not suitable for use in mutation spectra analysis. Mutation spectra should be assessed in phenotypically neutral genes, which were not subject to selection. The *lacI or lacz* genes from transgenic rodents were considered to be examples of neutral genes that would not be selected for *in vivo*, however it was pointed out that the genes are selected for in the *ex vivo* part of the studies.

15. It was noted that the analysis of p53 across different models was of value in evaluating chemically-induced mutations as it has been shown that mutation patterns are conserved in different test systems (e.g. BaP induced GC →TA transversions). Mutations in p53 are seen following exposure to PAH's in animals and these are correlated with those seen in some human cancers, for example in smokers, as detailed in an IARC database.

16. The human p53 knock-in (Hupki) mouse model containing a human wild-type TP53 DNA sequence was considered to be useful for investigating experimentally induced mutations in the human TP53 gene. However, it was noted that not all clones will have the p53 mutation and that the acquired immortality could be due to a mutation in a gene other than p53. The nature of the transformed foci in *in vitro* Hupki cell lines are characteristic of the chemical tested and could be used for proof of principle evaluations. Important limitations in using *in vitro* systems include that DNA damage and mutation are more likely *in vitro* than *in vivo* due to the higher levels of oxygen and the greater potential of oxidative damage and results from cell lines may be unrepresentative of untransformed diploid cells. This could confound the results and interpretation.

17. Currently, there were only a few good examples of mutation spectra that could be associated with certain cancer causative agents e.g. UV light, aflatoxin B1, tobacco smoke and aristolochic acid. However, members agreed that the development of 'next generation sequencing' technologies, where the whole genome could be sequenced would provide a substantial amount of new data that could be very useful for evaluating and understanding the role of mutation patterns in cancer development. Current methods that looked at only a single reporter gene may only provide limited information. It would be important to distinguish between mutations in genes that drove the cancer process and mutations in genes that had no effect i.e. were only 'passengers' in the cancer process. Members agreed that an *in vitro* experimental test system (not Hupki) was needed in which the whole genome could be analysed in a non-selective model (representative of human cells), from which a mutation pattern seen in human tumours could be identified. Where possible it would be better to use human cells and a 3D model rather than a 2D model. It would also be important to identify key signal genes and pathways in the cancer process.

18. The committee also discussed the use of mutation spectra from transgenic animal models in interpreting the significance of a positive *in vitro* genotoxicity result and a negative *in vivo* genotoxicity test result where a chronic carcinogenicity assay was positive. It was suggested that in such cases, any differences in metabolism and target tissues exposure would be considered. Furthermore, the MHRA noted that mutation spectra had not been used in the regulation of pharmaceuticals and medicines. Rather, further tests would be conducted or a weight of evidence approach adopted and/or a risk/benefit analysis would be used.

19. Overall, the COM concluded that the identified and summarised papers provided a reasonable representation of the current methods used in

assessing mutation spectra. It was noted that 'next generation' sequencing and new technologies would soon provide substantial new data that would potentially be very useful. Members agreed that at present, mutation spectra could not be used for regulatory purposes, but would be useful in evaluating mode of action and understanding the link between mutation and cancer. The committee agreed that it would be useful to produce a statement on mutation spectra incorporating and building on the information from the 1999 COM paper on mutation spectra by Professor A Boobis. It was also agreed that the COM should maintain a watching brief on this topic and consider a joint meeting with the COC if there are important developments in this area.

ITEM 6: TOX TRACKER (MUT/2014/03)

20. At the previous November 2013 meeting, one member had informed the committee of development of a new genotoxicity test system called ToxTracker. This comprised a system of reporter cell lines where 6 identified genes reflecting key pathways had been cloned into mouse embryonic stem cells. It was suggested that this would be useful for the COM to review.

21. Paper MUT/2014/03 described the development of the test system and proof of concept exercises. Some validation data from the Bscl2-GFP and Srxn1-GFP reporter cell lines, as presented in two publications from Dr Giel Hendriks et al (2011, 2012) from Leiden University, was also included in the paper. These cell lines are considered to identify genotoxic and pro-oxidant chemicals respectively.

22. Members agreed that the assay appeared to be an interesting approach to identifying genotoxicants and would be potentially useful in evaluating mode of genotoxic action, although it was noted that the selection of the genes used in the test system could have been chosen on an empirical basis rather than on a mechanistic basis.

23. According to the Tox Tracker website the entire system comprising six cell lines would be required for the assay to be of sufficient value. Validation data from only two of the cell lines had been published namely the Bscl2 reporter cell line responding to genotoxins and the Srxn1 reporter cell line, responding to pro-oxidants. The COM was not aware of published validation data for the other cell lines, namely Rtkn, Blvrb, Ddit3 and Btg2. The small number of chemicals tested in the presence of S9; a lack of evaluation of the effects of S9 on the expressed genes; and the unexpected results for methyl methanesulphonate (i.e. did not indicate a predominantly genotoxic response); were all considered to be limitations of the data. Currently, the apparent high sensitivity of the test system indicated on the website could not be verified from the published data.

24. Members considered that pro-oxidants have genotoxic potential i.e. if the degree of oxidation is sufficient then genotoxicity may occur. It was noted that processes that lead to oxidative stress generated *in vitro* can be very

different to those generated *in vivo* (which may also be attributable to immune driven or inflammatory responses).

25. Members indicated an interest in a comparison between the response of pro-oxidants in the ToxTracker and the Green screen (GADD45 assay). However it was also considered that the ToxTracker may be able to identify non-genotoxic carcinogens which cause cellular stress independent of DNA damage and the system would also be useful to provide mode of action information. The committee considered that an inter-laboratory trial for the use of this assay would be useful, but queried how costly and resource demanding the assay would be to use.

26. Regarding the potential use of this assay within a genotoxicity testing strategy, it was considered that it would be more useful as a biomarker assay as it does not directly address one of the three mutagenic endpoints (i.e. aneuploidy). However, it may be potentially useful in a genotoxicity testing strategy where *in vivo* testing is not permitted, such as in the testing of cosmetics. Furthermore, the committee suggested that it would be very useful to invite the developers of this assay to provide a presentation at a future COM meeting where the unpublished validation data could also be presented.

ITEM 7: OECD UPDATES (MUT/2014/04)

27. The OECD Test Guidelines (TG) are a collection of the most relevant internationally agreed test methods used by government, industry and independent laboratories to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. Many of the OECD test guidelines for genotoxicity had not been revised since 1997 although one (for the *in vitro* micronucleus test) was more recent. Many are being reviewed and updated and there are additional TGs for new genotoxicity assays (cell transformation and *in vivo* comet). The committee was provided with draft updated OECD genotoxicity guidelines and members were asked to provide any relevant comments that could be presented at the next meeting of the OECD Working Group of National Coordinators to the Test Guidelines Programme (WNT).

7.1 Draft TG in vitro Syrian hamster embryo (SHE) cell transformation assay

28. The committee re-iterated its previous concerns over the cell transformation assay (CTA) i.e. it does not discriminate between genotoxic and non-genotoxic substances; that it was not ready for regulatory purposes; that there was a need for further validation; even with the development of a photo-catalogue to identify morphologically transformed cells there is still a need for peer review of morphologically transformed cells; and that the underlying mechanism of the CTA was not currently understood.

29. The COM considered that the endpoint detected and the applicability domain of the assay were not clearly defined. Members felt that it was not clear what criteria constitute a positive response and that there were

uncertainties over how to interpret a positive response. It was agreed that it should not be used as a core test, but may have some use as a supplementary test.

30. Some members also considered that not all colonies were derived from fully transformed cells therefore the assay detected 'morphological changes' rather than 'transformed cells'. There was also continued concern expressed over the use of two different pHs. It was suggested that the two pHs were not equivalent because of differing sensitivity and specificity. Ideally it would be better to have a TG for just one preferred option or a separate TG for each.

7.2 Draft TG 474 mammalian erythrocyte micronucleus test

31. The COM suggested that there should be editorial alignment across the TG's for the *in* vivo tests with regards to dosing and assessment of sufficient exposure of the target tissue. The description of how to achieve the top dose (extent of toxic signs at the MTD) is not consistent across different *in vivo* guidelines. It was also commented that the recommendation not to use the assay if the test chemical (or a metabolite) will not reach the target tissue is a strange recommendation because it requires use of animals to try to show that the target tissue is not exposed.

32. The importance of the use of plasma pharmacokinetics to establish exposure was emphasised by members. It was queried whether signs of toxicity were no longer sufficient to demonstrate exposure and whether measurement of exposure was required in every case. One member said that the TG comment on sampling time or treatment compared to the lifespan of erythrocytes was unclear.

7.3 Draft TG 475 mammalian bone marrow chromosomal aberration test

33 There were no substantial comments on this draft update other than to ensure editorial alignment across the TG's for the *in vivo* tests.

7.4 *TG 473: In vitro mammalian chromosome aberration test*

34. Establishing the rate of division of the target cells in any particular laboratory, maintenance of culture conditions to ensure a high proportion of dividing cells in the (negative control) cultures and knowing the background level of cytogenetic damage in the target cells is critical to ensuring valid outcomes of the *in vitro* cytogenetic assays (TG487 and 473).

35. The COM considered that for both TG 487 and 473, it is not acceptable to use single cultures with only 3 concentrations of test chemical unless there is a robust historical data base for a laboratory showing acceptable homogeneity between replicate control cultures. It is scientifically more reliable to use duplicate cultures.

7.5 TG 487 in vitro mammalian cell micronucleus test

36. There were no substantial comments (other than above as also applicable to TG473) on this draft update.

7.6 Draft TG in vivo mammalian alkaline comet assay

37. Some members considered that this draft TG was too prescriptive and was more like a protocol than a Guideline.

38. As above, the committee advised that there should be editorial alignment across the *in vivo* TGs regarding the requirements to demonstrate target tissue exposure i.e. the approach should be harmonised. Members added that signs of toxicity should be sufficient and there should be no need to go to lethality.

39. The COM agreed to accept the draft *in vivo* comet guidance, but to raise the issue for the potential for increased use of animals (see below).

40. Overall, for all of the *in vivo* guidelines (TG474, TG475 and the new comet assay guideline) the COM questioned the requirement to demonstrate lack of difference between males and females before deciding whether to test 5 males or 3 female with 3 male. It is rare that there are no differences between males and females, and even a small difference could be considered to represent sex differences. It was felt that this would lead to most laboratories erring on the side of caution and testing 6, or even 10 animals, which would be contrary to the 3Rs principles and animal welfare.

7.7 Draft TG genotoxicity testing for manufactured nanomaterials

41. It was noted that this document was currently not sufficient to be regarded as a TG as it was more of an introductory document with the main emphasis on the characterisation of nanomaterials to be tested. However, it was acknowledged as an important aspect of the guidance. Members considered that it will be important for this document to note that the Ames test is not suitable for the genotoxicity testing of nano-materials. It was suggested that individuals with expertise in this field not at the COM meeting (both internal and external to PHE) should be asked for comments on this document.

42. Members were asked to email any additional comments on the OECD test guidelines to the secretariat.

ITEM 8: ANY OTHER BUSINESS

43. There was no other business.

ITEM 9: DATE OF NEXT MEETING

44. 19th June 2014

Item	Actions	Responsibility
Item 5: Mutation spectra	Draft or update COM statement on mutation spectra	Secretariat
Item 6: ToxTracker	Invite speaker to provide a presentation to the Com on ToxTracker	Secretariat
Item 7: OECD TGs	Provide to the COM comments on OECD TGs to the WNT meeting.	Secretariat

MUT/MIN/2014/2

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Minutes of the meeting held at 10.30 am on Thursday 16th October 2014 in Room 125A Skipton House, Elephant and Castle, London, SE1.

Present:

Chairman:	Dr D Lovell
Members:	Dr G Clare Dr S Dean Professor M O'Donovan Ms P Hardwick Professor G Jenkins Professor D Kirkland Professor A Lynch Professor D Phillips
Secretariat:	Dr O Sepai (PHE Secretary) Dr D Gott (FSA Secretariat) Dr K Burnett (PHE Tox Unit) Mr S Robjohns (PHE Secretariat minutes)
Assessors:	Dr Lata Koshy (HSE)
Observers:	Dr M Cush (Delphic HSE Solutions Limited)
In attendance:	Dr G Hendriks (toxys - item 7) Mr K Okona-Mensah (PHE Tox Unit) Miss B Gadeberg (PHE) Ms F Pollitt (PHE)

ITEM 1: ANNOUNCEMENTS/APOLOGIES FOR ABSENCE

1. The Chair welcomed members, the secretariat and assessors. Dr D Gott was attending in place of Dr D Benford from the FSA. The Chair also welcomed Mr Ken Okona-Mensah (PHE Toxicology unit), Ms Frances Pollitt (PHE), Miss Britta Gadeberg (PHE), and Dr Meera Cush (observer – Delphic Limited). Dr Giel Hendriks (Toxys) would be attending later for item 7.

2. Apologies for absence were received from the members Dr S Doak, Dr B Elliot, Professor F Martin, and Professor M Rennie. Apologies were also received from the assessors Dr C Ramsey (Health Protection Scotland) and Dr H Stemplewski (MHRA).

3. Members were reminded of the need to declare any interests before discussion of items.

ITEM 2: MINUTES OF MEETING ON 6th March 2014 (MUT/MIN/2014/1)

4. Members agreed the minutes subject to minor editorial changes.

ITEM 3: MATTERS ARISING

5. The committee was updated on vacancies in the COM secretariat. The more senior post as the secretary to the COM had not been filled and would have to be re-advertised. This post may need to be amended to a more general toxicology role to make it easier to fill. The more junior secretariat post (previously Dr Lesley Hetherington) had been frozen. The COM administrative role was also vacant as Gill Fisher had recently left PHE. It was hoped that a replacement would be obtained.

6. The Chair would be having a meeting with Dr John Harrison the Director of CRCE Chilton to discuss matters relating to the COM and the support that the committee required.

7. Members were requested to update and send their declarations of interest to the secretariat and were informed that the COM 2013 annual report had been completed.

8. The committee was informed that the COM contact email address had changed to the more generic COM@phe.gov.uk.

ITEM 4: Update review of the mutagenicity of alcohol (MUT/2014/05)

9. Dr G Clare declared a personal, non-specific interest. It was agreed that Dr Clare would not participate in the discussion or conclusions of this topic.

10. This updated review of the mutagenicity of alcohol and its primary metabolite acetaldehyde was prepared following a request from COC to support its on-going review of alcohol induced carcinogenicity. This would help the COC regarding possible mechanisms of cancer causally associated with the consumption of alcoholic drinks.

11. A systematic review of the literature had been conducted to capture the available evidence on the genotoxic effects of alcohol and acetaldehyde. The COM previously evaluated alcohol in 1995 and 2000. The COM published a statement in 2000. Any revised advice from the COM would be fed back to the COC.

12. The chair suggested that the COM should consider the review in three sections for each chemical, focussing on the different genotoxic endpoints, followed by a general discussion at the end. There was also a need to consider whether any changes were required to the COM 2000 statement.

13. The committee noted that a substantial number of studies had been published since the COM's last consideration of the mutagenicity of alcohol. Members suggested that a number of potential confounding factors may also need to be considered in terms of potential cancer risk e.g. body mass index, type of alcoholic beverage, drugs, diet and protective mechanisms. It was also suggested that the concentration of alcohol in alcoholic drinks could be important, for example, for cancer of the upper aerodigestive tract. For alcoholics, it would be difficult to control for non-ethanol contribution to the total alcohol intake.

14. Regarding DNA adducts, the importance of cytochrome P450 2E1 (CYP 2E1) induction and its role in the generation of oxidative metabolites was emphasised (this was also considered in more detail in item 5). The COM also noted the potential importance of polymorphisms in the metabolism of alcohol and its effect on mutagenicity. The committee considered that there was some evidence suggesting an increased formation of DNA adducts and micronuclei in individuals deficient in acetaldehyde dehydrogenase.

15. Members noted how key papers on DNA adduct formation were difficult to compare. For example, two studies by Balbo et al (2008 & 2012) found an increase in DNA adducts in individuals following alcohol consumption, while another by Singh et al (2012) did not find an increase in DNA adducts. This discrepancy in the results could be due to differences in the sensitivity of the studies. The Balbo et al studies related to intermittent exposure and the Singh study related to acute exposure. However, after adjustment for the use of different units in the studies, the results appeared to be similar, despite the authors' differing interpretation over a positive or negative finding.

16. Members considered the adduct N2-ethyl-deoxyguanosine to be a good biomarker of acetaldehyde exposure, but noted that in general there were substantial differences in the way studies were conducted (e.g. sensitivity, duration of exposure, and understanding of background adduct

levels). The COM also agreed that there were not many good or informative studies on DNA adduct formation following alcohol exposure.

17. Regarding studies on micronuclei (MN) formation and alcohol consumption, the COM agreed that studies on alcoholics and drug users were difficult to interpret. It was also noted that MN formation in bi-nucleate cells occurs *ex vivo* and there may be a publication bias towards positive results. The committee was informed that the COM (in relation to a consideration of exposure to pesticides) had previously evaluated data on the background incidence of MN and chromosome aberrations when considering these endpoints as biomarkers of genotoxicity. A large variability in the background levels of these biomarkers was found, which complicated interpretation. The tabulated data (table 1.3 and 1.4) of studies on MN and chromosome aberrations in alcohol drinkers showed a mixture of effects with only about a ¼ of the studies reporting negative results. The *in vitro* data for acetaldehyde and induction of MN were more convincing than the *in vivo* data. The *in vivo* data were difficult to assess.

18. The COM considered potential modes of genotoxic mechanism in some highlighted papers. Kayani and Parry (2010) performed a cytokinesis-blocked MN assay with kinetochore staining *in vitro* which showed a dose dependent increase in kinetochore positive MN with ethanol treated cells, but not for MN in acetaldehyde treated cells. The authors contended that this indicated an aneugenic mode of action for ethanol and a clastogenic mode of action for acetaldehyde. Members considered that this was an interesting paper that could not be ignored. However, there were other possible explanations for a positive result (e.g. oxidation of spindle fibres or an artefact from the use of antibodies). Furthermore, it was only one study. Therefore, further investigation would be helpful before drawing conclusions. A study by Kotova et al (2013) investigated the mechanism of genotoxicity from sub-chronic ethanol exposure in rats. The study suggested that the genotoxicity (as detected by MN) was due to acetaldehyde induced DNA replication lesions in dividing cells. Again, the COM considered that this was an interesting paper, suggesting a plausible mechanism for genotoxicity, but further investigation would be required before conclusions could be drawn.

19. Members noted that in some studies, such as in gastric mucosa cells, exposure to relatively high concentrations of ethanol (e.g. 1M) could result in secondary or indirect DNA damage following irritation, inflammation or dehydration.

20. The committee also looked at a paper on ethyl sulfate by Mitchell et al (2014) that suggested that ethanol could also be metabolised to ethyl sulfate that can alkylate DNA potentially leading to mutation. However, sulfate compounds were difficult to test (e.g. sulphates do not pass through membranes very easily) and the COM considered that further data would be required before conclusions could be drawn on this hypothesis.

21. Overall the COM agreed that it was reasonable to assume that acetaldehyde was genotoxic from the available *in vitro* and *in vivo* data. The data for ethanol were not clear due to a number of other potential confounding factors, therefore it could not be concluded that ethanol is directly mutagenic in vivo.

22. The committee considered that the papers on genotoxic mechanism were interesting and plausible, but required further investigation before any conclusions could be drawn.

23. Members agreed that the alcohol metabolite acetaldehyde was the most concerning candidate for the observed genotoxicity arising from exposure to alcoholic beverages.

24. Regarding the recent paper by Mitchell et al (2014) suggesting that ethanol can also be metabolised to ethyl sulphate, which could alkylate DNA potentially leading to mutation, the committee considered that further investigation would be required to draw any conclusions on this proposed hypothesis.

25. The committee agreed that there were sufficient new data to suggest that mutagenicity following exposure to alcohol and its metabolites was biologically plausible, which would require a revision of the COM 2000 statement.

ITEM 5: ALCOHOL AND OXIDATIVE DNA DAMAGE – A PRELIMINARY OVERVIEW (MUT/2014/6)

26. Dr G Clare declared a personal, non-specific interest. It was agreed that Dr Clare would not participate in the discussion or conclusions of this topic.

27. MUT/2014/06 provided brief summaries of studies retrieved during the literature search for paper MUT/2014/05, which examined endpoints associated with alcohol-induced oxidative mechanisms (which may in turn contribute to its carcinogenic mode of action). The committee was asked to comment on oxidative damage to DNA as a potential mode of action for alcohol and whether it wished to see a detailed review of these papers on this topic.

28. Members noted that there was uncertainty over the use of the adduct 8-hydroxy-2'-deoxyguanosine (8-OH-dG) as a biomarker of effect. This is because it represents a pre-mutagenic lesion only. It is well repaired in normal circumstances, but it could lead to mutation under some circumstances. It was noted that a more accurate description or term was 'oxidative damage to DNA' rather than 'oxidative DNA damage'.

29. The impact of alcohol on DNA repair was a further factor to be considered, together with the oxidative pathways that would generate oxidative biomarkers i.e. following irritation or inflammatory responses to

alcohol. 8-OH-dG was considered to be a biomarker of oxidative stress in general and the most abundant DNA lesion.

30. Members agreed that it was too simplistic to consider that alcohol is just metabolised to acetaldehyde. Expression of CYP 2E1 could be induced, which is also involved in the metabolism of ethanol to acetaldehyde and the generation of reactive oxygen species. Therefore, there may be a need to separate papers that consider tissues where CYP2E1 is expressed or induced from others that don't i.e. there was a need to separate out circumstantial evidence of oxidative damage to DNA arising from secondary processes such as irritation etc. Studies using knockout mice may be useful in this regard.

31. It was agreed that acetaldehyde was likely to generate a plethora of effects, including oxidation, which could result in DNA damage. The papers on human subjects again reflected the difficulties in assessing results due to potential confounding factors – such as consumption of fruit and vegetables. There were a lot of studies where co-exposure to other substances in addition to ethanol would occur, which would make interpretation difficult.

32. It was agreed that a systematic or detailed evaluation of the literature in this area was not necessary, but the addition of an extra paragraph reflecting current hypotheses in the revised statement would be appropriate. The additional paragraph should focus on the importance of the induction of CYP2E1 in different tissues. This would also need to include the role of CYP2E1 in the metabolism of ethanol to acetaldehyde; the impact of oxidative damage induced by inflammatory or irritant responses; the relative contributions of alcohol and acetaldehyde on oxidation and carcinogenicity; and the influence of other dietary factors.

ITEM 6: STATEMENT ON THE USE OF MUTATION SPECTRA IN GENETIC TOXICOLOGY (MUT/2014/07)

33. A paper on chemically induced mutation spectra was reviewed by the committee at the previous meeting in March 2014. Members decided that it would be useful to produce a statement on mutation spectra following the discussion. A first draft statement had been produced and was presented to the COM for comment. The statement was drafted based on the previous committee discussion; a review of the literature; and on three specific chemical exposures with defined mutation spectra.

34. Members commented that overall the first draft statement was a fair evaluation of the topic. It was agreed that the paragraph describing why phenotypically neutral genes are most suitable for examining mutation spectra should be altered so as to not imply that the selectable genes used in mutagenicity tests are not useful indicators of mutagenicity.

35. The COM agreed that there are some examples where mutation spectra (MS) are conserved across test systems and species (e.g. for the food mutagen MeIQx.

36. With regard to the specific chemical examples – it was considered unnecessary to go into detail about which chemicals in tobacco smoke were responsible for the MS profile, because this was complicated and imprecise.

37. The statement should include the reasons why spectra in tumours differ in different sites, for example the role of repair locus effects. Furthermore it should be noted that the *Tp*53 mutation is a late mutation in colorectal cancers and therefore it may not be detected even when smoking contributed towards tumour progression. Members considered the MS of aflatoxin B1 to be clear and distinct in human liver tumours. It was noted that a number of factors could also affect mutation spectra for aflatoxin and other chemicals, such as the effect of viruses (e.g. hepatitis B for aflatoxin) and the time at which spectra are measured (i.e. mutation spectra may change over time after the initial chemical exposure).

38. Aristolochic acid was considered to generate a distinct MS and represent an example of an unusual tumour. It was considered to be the best example of a specific chemically induced MS. The picture may also be clear for smoking for example, when the sample or measurement was taken at the right time and in the right tissue.

39. Members requested that the statement should clarify how and when MS could be used. MS could be potentially useful as part of an overall tool box of non-standard methods, which could contribute towards the overall identification of genotoxic hazards. They could also contribute to a weight of evidence approach towards the generation of plausible, causative associations and in mode of action evaluation. However, it was also stated that lots of different mutagens could cause the same mutation and thus the applicability of MS for identifying mutagens would be very limited. A different change in sequence does not always mean a differing reactivity or potency of the mutagen.

40. The paragraph on next generation sequencing needed to be enlarged and be more specific to the evaluation of mutagenesis. Members offered to provide suitable references for this section. It was decided that the Table containing the references reviewed in the previous paper was not necessary for the current statement. The statement will be re-drafted to reflect Members' discussion and suggestions for the next meeting.

ITEM 7: TOXTRACKER – IN VITRO GENOTOXICITY TEST- DISCUSSION AND PRESENTATION BY DR GIEL HENDRIKS

41. The committee had considered a paper on the newly developed ToxTracker genotoxicity assay at its last meeting in March 2014. Members had expressed an interest in keeping up to date with the development of the assay when additional validation emerged.

42. Dr Giel Hendriks was invited to the COM and gave a presentation on ToxTracker. The ToxTracker genotoxicity assay comprised a system of reporter cell lines where 6 identified genes, reflecting key pathways, had been cloned into mouse embryonic stem cells. This assay could identify both genotoxic and pro-oxidant chemicals. Dr Hendriks said that one of the main advantages of this genotoxicity assay over standard in vitro test systems, was that it was able to provide some insight into the mechanism of genotoxicity e.g. oxidative damage to DNA or protein damage. Different types of genotoxicity could be detected by certain biomarker genes. These reporter genes could be related to certain cellular pathways and related biological damage (e.g. DNA damage detected by Bacl2 and Rtkn; oxidative stress detected by Srxn1 and Blvrb; and protein stress by Ddit3). Chemically induced genotoxicity, could be detected by the induction of Green fluorescent protein (GFP) determined by flow cytometry.

43. Following the presentation, there was a discussion and members had a number of questions. The COM heard that all compounds could be tested for the influence of metabolic activation by the addition of S9, which was found to be the most effective method for the inclusion of metabolic activation. The fold increase in GFP induction was used to determine an overall positive genotoxicity response. Using the designed software, GFP induction could be calculated for a certain degree of cytotoxicity (50% cytotoxicity was selected as the optimum value).

44. The sampling time was 24 hours after initial exposure. The exposure time was said to not markedly change the results. The time point for measurement had to be sufficiently long to detect aneugenic activity, which was a later event. The cut off point for a positive genotoxicity result was chosen as a 1.5 fold increase in the induction of GFP, which was 5 times the standard deviation.

45. For validation of the assay, the developers used the ECVAM suggested library for carcinogens and non-carcinogens and the USA Toxcast library.

46. The 6 chosen genes incorporated into mouse embryonic stem cells were the 6 best identified performers (i.e. Bscl2, Rtkn, Srxn1, Blvrb, Ddit3 and Btg2) for predicting genotoxicity. There were other reporter genes that could also be used. The results for methyl methanesulphonate did not predominantly indicate DNA damage, but had given a stronger signal for oxidative stress, which was unexpected. .

47. Regarding the role of this assay and where it might fit in a testing strategy, it was suggested that the current view was that it would be useful as an early screen before *in vivo* testing. There was a possibility that for a situation where there was a positive *in vitro* genotoxicity result, considered to be weak or a misleading positive, then results from ToxTracker may help with the overall interpretation.

48. Members suggested that there was a need to gain a better understanding of what the gene expression changes meant in terms of the mechanism of genotoxicity (i.e. what the genes were doing or reflecting). The COM were informed that this was on-going process and currently being examined e.g. the signalling pathways were being assessed and there was need to understand reporter gene activation and how this correlates with carcinogenicity.

49. It was pointed out, that if a 'heat map' (i.e. degree of GFP induction for each reporter gene following a chemical exposure) was examined for ECVAM model compounds then the profile for 'new' chemicals could be looked at to see what model chemicals they were closest to. For about two thirds of chemicals looked at so far, a primary activity could be identified.

50. The COM may consider this item further at the next meeting.

ITEM 8: OECD UPDATES

51. The COM were informed that there would no longer be an OECD test Guideline for the *in vitro* Syrian hamster embryo (SHE) cell transformation assay but a guidance document instead. This was due to the concerns that the COM and other countries had expressed over the development of a test guideline.

52. Members were asked to provide any comments to the secretariat that they might have on the draft update Dominant lethal test (TG 478) and the mammalian spermatagonial chromosomal aberration test (TG 483).

53. One member updated the committee on WNT meeting held in April 2014. Essentially everything that went to the WNT was approved. The *in vivo* tests would not have to provide a justification for sex differences. If there was no evidence for a difference then either sex could be used.

54. Regarding *in vitro* tests the request for wording to include duplicate cultures in preference to single replicates was agreed.

55. The latest draft revised guidelines would be circulated to members for comment i.e. mouse lymphoma assay and the mammalian cell gene mutation test (Thymidine Kinase and Hprt and xprt assays).

ITEM 9: ANY OTHER BUSINESS

56. Members were informed that there were some difficulties with the 'new' COM website now that its location had been moved to the .gov.uk site. Unfortunately, the secretariat had not been consulted over the changes. It was hoped that the minutes and statement etc. would be able to go on the new website in the future. Previous COM documents are no available from the main COM page, but could be assessed via links. The previous COM page

and documents were available from an archived site that cannot be changed over time. The secretariat was having on-going discussions with web publishing at PHE to try and improve the current site.

ITEM 10: DATE OF NEXT MEETING

57. 5th March 2015.