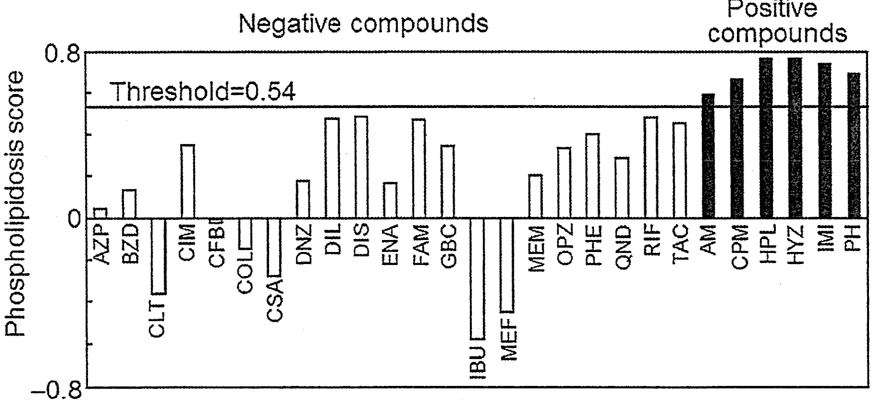


Fig. 1. Classification of the training set compounds. The phospholipidosis scores computed with the optimized biomarker set (the phospholipidosis signature in Table 3) correctly classified the full training set of compounds (white bars: negative compounds, black bars: positive compounds). For compound abbreviations, see Table 1.

11-compound external validation set, which was independent of the TGP database (Fig. 2a, Table 1). These eleven compounds were selected from lists of phospholipidosis-positive/negative compounds from published studies (Atienzar et al., 2007; Pelletier et al., 2007), purchased from available suppliers, and the corresponding 24 h single-dose microarray data measured in-house as described in Section 2.5. The phospholipidosis scores of all the negative validation compounds (CP, DF, DOX, OFL, and PROC) were correctly lower than the score threshold (0.54), and the scores of 4 out of 6 positive compounds were also correctly larger than the threshold (accuracy = 82%, sensitivity = 67%, and specificity = 100%). Whereas the two compounds (CQ and ZIM) became false negative, their scores were nonetheless close to the threshold. The area under the ROC curve (ROC-AUC) was 0.97 (Fig. 2b). These results suggest that the approach to phospholipidosis prediction as described in this paper can also be applied to data derived from outside of the TGP database.

4. Discussion

The earliest time point at which intracellular vacuolization has been observed among the data for the positive training set was the 7-day repeated administration of AM and IMI. We have hypothesized, however, that histopathologically visible, drug-induced phospholipidosis is preceded by a characteristic gene expression pattern, and that this pattern can be detected already at 24 h following administration of a compound with potential for inducing phospholipidosis. We therefore applied our genetic algorithm to gene expression data measured 24 h after single dose administration, and identified the set of 25 biomarker candidates (Fig. 1, Table 3). This set of biomarkers was assessed by the external validation set prepared in-house (Fig. 2).

The mechanisms underlying compound-induced phospholipidosis are not well understood, but several interesting explanations have been published (inhibition of phospholipases, decreased breakdown of phospholipids, increased synthesis of phospholipids) (Halliwell, 1997; Hruban, 1984). Different mechanisms may be also at play according to the characteristics of the individual compounds including in vivo half-life, compound structure, and dose level. We considered, therefore, that an extensive search for biomarkers based on genome-wide expression data would be the most effective approach in this complex situation, and that a multi-marker strategy could possibly improve prediction of compound-specific phospholipidosis-inducing potential. In order to identify such

Table 4
Ingenuity pathways analysis (IPA) of the set of 25 biomarker candidates.

Pathway name	*p*-Value
Metabolism of xenobiotics by cytochrome P450	**7.59E−08**
Linoleic acid metabolism	**3.02E−05**
Fatty acid metabolism	**8.32E−05**
Arachidonic acid metabolism	**2.69E−03**
Tryptophan metabolism	**2.69E−03**
LPS/IL-1 mediated inhibition of RXR function	**4.90E−03**
Glycolysis/gluconeogenesis	1.74E−02
14-3-3-mediated signaling	1.95E−02
Germ cell–Sertoli cell junction signaling	3.16E−02

Pathways outlined in bold font overlapped with the pathways represented in the biomarkers selected from gene expression data measured after repeated dosing in the Hirode et al.'s study.

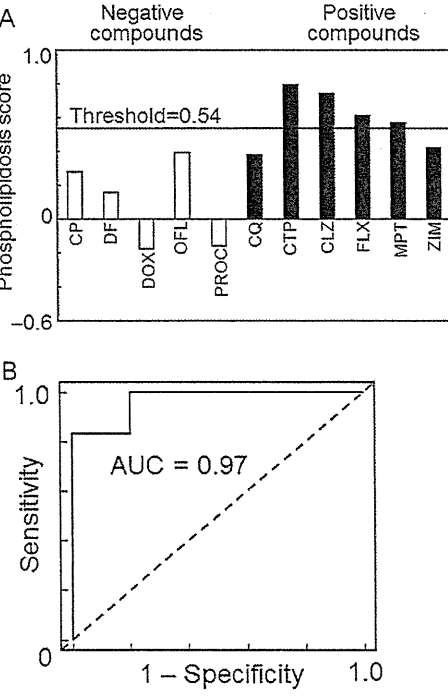


Fig. 2. Predictions for the independent validation set. The phospholipidosis-scores for the independent validation set (white bars: negative compounds, black bars: positive compounds). For compound abbreviation, see Table 1. The ROC curve from prediction of phospholipidosis-inducing potential. ROC, receiver operating characteristic; AUC, area under the curve.

markers, we initially searched for consistently regulated probe-sets in the training set, i.e., the probe-sets that were regulated across all compounds known to induce phospholipidosis, and that were not regulated in the negative compounds. Regulated probe-sets were defined as probe-sets with fold change values above the given threshold. It was, however, not possible to identify consistently regulated probe-sets for phospholipidosis prediction, even if it was possible to identify such markers across for example PPAR agonist compounds in the TGP database (unpublished data). Apparently, the difficulties in extracting marker candidates suggested that there might be different mechanisms, timings, or other factors involved for the selected training set compounds. Therefore, it was necessary to employ less strict criteria that did not require the probe-sets to be regulated in all phospholipidosis-inducing compounds in order to obtain probe-sets for further analysis. The results from the IPA pathway analysis and the 11-compound validation procedure suggest that it was possible in this way to pick up probe-sets for genes implicated in the mechanism of phospholipidosis, as the 25 markers contained in the phospholipidosis signature are related to lipid metabolism pathways (Table 4).

As mentioned in Section 1, alternative and publicly available sets of biomarkers exist for phospholipidosis prediction, notably the set from the Hirode et al.'s study (Hirode et al., 2008), which was also derived based on data from the TGP database. The study, however, employed microarray gene expression data measured after repeated dosing, i.e., at much longer time points. When we compared our set of biomarkers with the set of 78 biomarkers selected from the phenotypically anchored gene expression profiles in the Hirode et al. study, we made the remarkable observation that the top six of the nine pathways listed in Table 4 overlap with the pathways represented in the set of 78 biomarkers (Supplementary Table 1). This suggests that these two biomarker sets reflect to some extent similar biological processes underlying phospholipidosis. There is, however, only an overlap of 3 genes (Ugt2b17, Cyp2b2, and Slco1a4) when the two sets are directly compared. It is therefore thought that our novel set of biomarkers represents early gene expression changes before phospholipidosis is evident, and that we can predict phospholipidosis-inducing potential of compounds by our biomarkers using gene expression data at the 24 h time point following single dose administration.

Drugs may induce phospholipidosis on a more severe level in organs other than the liver, and phospholipidosis screening should preferably be able to correctly evaluate such compounds. In the present study, the compounds CPM and FLX are known to induce phospholipidosis mainly in the lung (Gonzalez-Rothi et al., 1995; Sgaragli et al., 1983), and it is promising that these compounds were correctly characterized by our set of biomarker candidates. Phospholipidosis may occur in multiple organs and tissues as stated above, but most compounds pass through liver and it would be therefore expected that phospholipidosis-inducing potential can be evaluated by analysis of the hepatic gene expression pattern even in the case that liver is not the main target organ for phospholipidosis.

In summary, a set of biomarker candidates have been optimized to predict phospholipidosis-inducing potential of compounds using rat liver microarray data measured 24 h following single dose administration. The set of biomarkers was capable of correctly classifying all training set compounds, and when applied to an external validation set comprising 11 compounds, the phospholipidosis-inducing potential for 9 compounds was correctly predicted. Whereas histopathological changes are only observed after repeated administration, we will be able to assess phospholipidosis-inducing potential of compounds using the set of early time-point biomarkers as described in the present study. We, therefore, conclude that the set of 25 biomarkers will be a valuable tool for phospholipidosis screening in the early phase of drug development and this should bring about distinct advantages in terms of cost and speed in drug discovery.

Conflict of interest

There is no conflict of interest in this study.

Acknowledgements

The authors would like to thank J. Deguchi, K. Kijima, and Y. Tominaga for valuable discussions. This work was supported in part by a grant from the Ministry of Health, Labour and Welfare (H14-Toxico-001 and H19-Toxico-001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2012.02.015.

References

Anderson, N., Borlak, J., 2006. Drug-induced phospholipidosis. FEBS Lett. 580, 5533–5540.

Atienzar, F., Gerets, H., Dufrane, S., Tilmant, K., Cornet, M., Dhalluin, S., Ruty, B., Rose, G., Canning, M., 2007. Determination of phospholipidosis potential based on gene expression analysis in HepG2 cells. Toxicol. Sci. 96, 101–114.

Baronas, E.T., Lee, J.W., Alden, C., Hsieh, F.Y., 2007. Biomarkers to monitor drug-induced phospholipidosis. Toxicol. Appl. Pharmacol. 218, 72–78.

Berridge, B.R., Chatman, L.A., Odin, M., Schultze, A.E., Losco, P.E., Meehan, J.T., Peters, T., Vonderfecht, S.L., 2007. Phospholipidosis in nonclinical toxicity studies. Toxicol. Pathol. 35, 325.

Blomme, E.A., Yang, Y., Waring, J.F., 2009. Use of toxicogenomics to understand mechanisms of drug-induced hepatotoxicity during drug discovery and development. Toxicol. Lett. 186, 22–31.

Carstensen, B., 2007. Age-period-cohort models for the Lexis diagram. Stat. Med. 26, 3018–3045.

Chiang, A.P., Butte, A.J., 2009. Data-driven methods to discover molecular determinants of serious adverse drug events. Clin. Pharmacol. Ther. 85, 259–268.

Delaney, J., Neville, W.A., Swain, A., Miles, A., Leonard, M.S., Waterfield, C.J., 2004. Phenylacetylglycine, a putative biomarker of phospholipidosis: its origins and relevance to phospholipid accumulation using amiodarone treated rats as a model. Biomarkers 9, 271–290.

DiMasi, J.A., Hansen, R.W., Grabowski, H.G., 2003. The price of innovation: new estimates of drug development costs. J. Health Econ. 22, 151–185.

Fujimura, H., Dekura, E., Kurabe, M., Shimazu, N., Koitabashi, M., Toriumi, W., 2007. Cell-based fluorescence assay for evaluation of new-drugs potential for phospholipidosis in an early stage of drug development. Exp. Toxicol. Pathol. 58, 375–382.

Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Lander, E.S., 1999. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286, 531–537.

Gonzalez-Rothi, R.J., Zander, D.S., Ros, P.R., 1995. Fluoxetine hydrochloride (Prozac)-induced pulmonary disease. Chest 107, 1763–1765.

Halliwell, W.H., 1997. Cationic amphiphilic drug-induced phospholipidosis. Toxicol. Pathol. 25, 53–60.

Hirode, M., Ono, A., Miyagishima, T., Nagao, T., Ohno, Y., Urushidani, T., 2008. Gene expression profiling in rat liver treated with compounds inducing phospholipidosis. Toxicol. Appl. Pharmacol. 229, 290–299.

Hruban, Z., 1984. Pulmonary and generalized lysosomal storage induced by amphiphilic drugs. Environ. Health Perspect. 55, 53–76.

Kasahara, T., Tomita, K., Murano, H., Harada, T., Tsubakimoto, K., Ogihara, T., Ohnishi, S., Kakinuma, C., 2006. Establishment of an in vitro high-throughput screening assay for detecting phospholipidosis-inducing potential. Toxicol. Sci. 90, 133–141.

Kruhlak, N.L., Choi, S.S., Contrera, J.F., Weaver, J.L., Willard, J.M., Hastings, K.L., Sancilio, L.F., 2008. Development of a phospholipidosis database and predictive quantitative structure–activity relationship (QSAR) models. Toxicol. Mech. Methods 18, 217–227.

Kuroda, Y., Saito, M., 2010. Prediction of phospholipidosis-inducing potential of drugs by in vitro biochemical and physicochemical assays followed by multivariate analysis. Toxicol. In Vitro 24, 661–668.

Nioi, P., Perry, B.K., Wang, E.J., Gu, Y.Z., Snyder, R.D., 2007. In vitro detection of drug-induced phospholipidosis using gene expression and fluorescent phospholipid based methodologies. Toxicol. Sci. 99, 162–173.

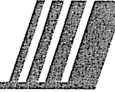
Pelletier, D.J., Gehlhaar, D., Tilloy-Ellul, A., Johnson, T.O., Greene, N., 2007. Evaluation of a published in silico model and construction of a novel Bayesian model for predicting phospholipidosis inducing potential. J. Chem. Inf. Model. 47, 1196–1205.

Ploemen, J.P., Kelder, J., Hafmans, T., van de Sandt, H., van Burgsteden, J.A., Saleminki, P.J., van Esch, E., 2004. Use of physicochemical calculation of pKa and CLogP to predict phospholipidosis-inducing potential: a case study with structurally related piperazines. Exp. Toxicol. Pathol. 55, 347–355.
Reasor, M.J., Hastings, K.L., Ulrich, R.G., 2006. Drug-induced phospholipidosis: issues and future directions. Expert Opin. Drug Saf. 5, 567–583.
Reasor, M.J., Kacew, S., 2001. Drug-induced phospholipidosis: are there functional consequences? Exp. Biol. Med. (Maywood) 226, 825–830.
Sawada, H., Takami, K., Asahi, S., 2005. A toxicogenomic approach to drug-induced phospholipidosis: analysis of its induction mechanism and establishment of a novel in vitro screening system. Toxicol. Sci. 83, 282–292.
Sgaragli, G.P., Della Corte, L., Gremigni, D., 1983. Chlorimipramine-induced phospholipidosis: biochemical and pharmacokinetic observations in the rat. Pharmacol. Res. Commun. 15, 231–246.
Taillardat-Bertschinger, A., Martinet, C.A., Carrupt, P.A., Reist, M., Caron, G., Fruttero, R., Testa, B., 2002. Molecular factors influencing retention on immobilized artificial membranes (IAM) compared to partitioning in liposomes and n-octanol. Pharm. Res. 19, 729–737.
Takashima, K., Mizukawa, Y., Morishita, K., Okuyama, M., Kasahara, T., Toritsuka, N., Miyagishima, T., Nagao, T., Urushidani, T., 2006. Effect of the difference in vehicles on gene expression in the rat liver—analysis of the control data in the Toxicogenomics Project Database. Life Sci. 78, 2787–2796.
Tengstrand, E.A., Miwa, G.T., Hsieh, F.Y., 2010. Bis(monoacylglycerol)phosphate as a non-invasive biomarker to monitor the onset and time-course of phospholipidosis with drug-induced toxicities. Expert Opin. Drug Metab. Toxicol. 6, 555–570.
Tomizawa, K., Sugano, K., Yamada, H., Horii, I., 2006. Physicochemical and cell-based approach for early screening of phospholipidosis-inducing potential. J. Toxicol. Sci. 31, 315–324.
Urushidani, T., Nagao, T., 2005. Toxicogenomics: the Japanese initiative. In: Borak, J. (Ed.), Handbook of Toxicogenomics—Strategies and Applications. Wiley-VCH, Weinheim, pp. 623–631.

特集

薬理学における動物実験代替法研究の重要性

大野　泰雄



要約：薬理学研究における薬物の作用標的の解析や医薬品候補物質の研究等に in vitro 試験法が広く利用されている．しかし，様々な理由で，in vitro で得られた結果が必ずしも in vivo で再現できないことが多く，in vivo 動物実験を欠かすことができない．平成23年3月に予定していた薬理学会年会においても，多数の動物実験結果が発表される予定であった．一方，動物実験については，市民による反対運動もあり，3Rs の原則に則り適正に実施することが法的に求められている．また，世界的に代替法の開発・評価を専門的に行う国立の機関も多く構築されている．薬理学が今後も社会のサポートを得，継続して発展していくためには，関連法規制を遵守し，動物実験を行う機関，施設，機材および人材を整備し，代替法について研究者教育を行うとともに，それらの適切性について第三者により評価を受けることが重要である．これは一研究者により対応できることではなく，研究機関が組織として対応して初めて達成することが可能である．

はじめに

近代における生物科学発展の多くは動物実験により支えられてきた．しかし，動物の福祉や権利の立場から動物実験への批判も多く，動物実験代替法が検討されてきた．

Russell と Burch（1959）(1) の定義によれば，動物実験代替法（代替法）とは科学研究や教育，毒性試験，生産等の目的のために動物を用いる方法を動物を用いない方法に置き換えること（Replacement）であり，動物使用数の削減（Reduction）や動物使用に伴う苦痛の削減（Refinement）を含むものである．この考えは日本も含め，国際的に広く受け入れられている．

代替法開発はもともと動物愛護の精神に根ざすものであるが，無駄な動物実験の廃止や多数の新規化学物質の経済的な安全性評価，極めて毒性の強い可能性のある化学物質の毒性を動物実験で調べることに伴う危険の回避，また，ヒトへの外挿のために必要な毒性発現機序に関する情報の確保のためにも有効である．新しい代替法の開発は独創性のある研究への道を開くのみならず，遺伝子突然変異を検出する Ames' 試験のように，発がん機構解明だけでなく，発がん物質スクリーニングのために行政的に広く使われ，発がん物質の管理に大きな貢献をしたものもある．

一方，新しい試験法，特に従来の動物実験に替わるものとして新規に開発された試験法を受け入れるためには，適切なバリデーションを行い使用目的に合致していることを確認する必要がある．特に，既存の毒性試験法を新しい試験法（代替法）に置き換えることにより，医薬品や農薬等，化学物質の安全性評価のレベルが低下することは許されない．従って，新規安全性試験法を公的な試験法として受け入れるためには，複数の機関により，GLP に準じて実施されたバリデーションにより，その利点と限界が明確にされ，既存の方法と比べ同等以上の有用性があることが，第三者により確認されなくてはならない．具体的なバリデーションの方法や行政的受入の基準については，小野（1994）(2)，大野（2004）(3)，OECD（2005）(4) などを参照されたい．

1. 動物実験代替法に関わる機関

代替法の研究は欧米では早くから行われており，イギリスでは医学分野における実験動物を他のものに置き換えるための基金（FRAME）が1969年に，米国では1981年にジョンズホプキンズ大学に代替法センターが開設された．日本では1982年に現在の動物実験

キーワード：動物実験代替法，薬理学
国立医薬品食品衛生研究所（〒158-8501 東京都世田谷区上用賀 1-18-1）
E-mail: ohno@nihs.go.jp　原稿受領日：2011年6月1日，依頼原稿
Title: Alternatives to animal experiments in pharmacology.　Author: Yasuo Ohno

代替法学会の前身となる研究会が設立された．これらの研究を通じて多くの方法が開発されてきた．一方，EU議会は動物愛護運動と動物実験に対する反対運動の高まりに対応して，代替法開発の拠点とし，代替法についてのデータベースを設置・維持するため，また，行政，産業，生物・医学分野の科学者，消費者，および動物愛護運動グループの対話を促進することを目的に1991年に代替法バリデーションセンター（European Center for the Validation of Alternative Methods: ECVAM）を設立した（1994年開所）．国レベルでも代替法研究のための機関として，ドイツはThe Centre for Documentation and Evaluation of Alternatives to Animal Experiments（ZEBET）を1989年に，オランダはNetherlands Centre Alternatives to Animal Use（NCA, 最近National Knowledge Centre on Alternatives（NKCA）に名称変更）を，イギリスはThe National Centre for the Replacement, Refinement and Reduction of Animals in Research（NC3Rs）を2004年に設立した．米国は，毒性試験法の開発，バリデーション，受入れ，および国内および国際レベルでのハーモナイゼーションに関する問題を連邦政府内で調整するためにNICEATM（NTP Interagency Center for the Evaluation of Alternative Toxicological Methods）の下にNIEHS（National Institute of Environmental Health Sciences）を含む14の行政機関および研究機関によりICCVAM（Interagency Coordinating Committee on the Validation of Alternative Methods）を1993年に設置した．このように，欧米の行政機関においては，新たに開発された動物実験代替法を科学的に評価し，可能なものについては取り入れていこうと，努力している．

わが国においても，動物福祉への関心の高まりに応じて，平成17年6月に交付された動物の愛護及び管理に関する法律の一部を改正する法律（改正動愛法，平成17年法律第68号）により，動物実験等に関する3Rの原則を尊重することが義務付けられるとともに，翌年，動物実験に関する基本指針が文部科学省(5)，厚生労働省(6)，環境省(7)，および農林水産省(8)から通知された．また，日本学術会議より動物実験に関する詳細指針が通知された．2008年には，主に動物実験代替法の評価を行う施設として，国立医薬品食品衛生研究所 安全性生物試験研究センター 薬理部内に新規試験法評価室（通称JaCVAM）が設立され，活動を始めた．その後，2010年には，その活動が認められ，ECVAM，ICCVAMおよびカナダ厚生省（Health Canada）とともに代替法に関する協力を目的にInternational Cooperation on Alternative Test Methods（ICATM）協定が結ばれ，代替法の評価が進んでいる．2011年にはこれに韓国FDAに属するKorean Center for the Validation of Alternative Methods（KoCVAM）も加わった．

2. 薬理学分野での動物実験代替法への対応について

薬理学の研究においては，医薬品や化学物質等の生体に及ぼす影響を検討するため，摘出臓器や組織，細胞，細胞下分画などを用いたインビトロ試験系が多用されてきたが，得られた結果が真に意義のあるものであることを証明するためには，インビボ実験が欠かせない．実際，表1に示したように，平成23年度に予定した第84回薬理学会における口頭発表の内，マウスやラット，ウサギ，およびモルモットなど哺乳動物を用いる研究は，139件で全体の約52%を占めていた（なお，第77回年会ではポスター発表のうち約70%が動物を用いた研究であった．）．このように，依然として，薬理学の研究において動物実験は欠かせないものである．

一方，動物実験に対する一般市民の反応は，その意義と説明の仕方により異なってくる．たとえば，イギリスで行われた調査では，白血病のような重篤な疾病の治療につながる研究では，80%以上の人がマウスによる実験を許容していたが，化粧品のための研究では，40%弱の人しか動物実験を許容しなかった．また，単に「科学者は動物実験を許容されるべきである」との質問では，64%の人が中程度以上の反対を示したが，「痛みを和らげる薬物の開発や生命を脅かす疾患の治療法を開発するための研究をしている科学者がいる」ことを説明すると，その反対が41%に減少した（New Scientist. 1999 May 22, p. 26-31）．

すなわち，動物実験を行う薬理学者は自己の研究目的に動物実験がやむを得ざるものであること，また，行う動物実験は動物実験倫理委員会により承認されたものであることを示す必要がある．また，動物実験を行っている施設は，指針に則り，当該施設が適切な施設で適切な動物実験を行っていることが第三者評価により認証されることが望ましい．

このようにして，動物実験に関する社会的要請に応えることが，継続的に薬理学研究を進める上で必須である．このことを広く会員に示し，適正な動物実験を進めるため，薬理学会では，改正動愛法の趣旨を尊重し，動物実験に関する日本薬理学会指針を改正し，「日本薬理学会は本指針に従った動物実験が行われることを期待するとともに，これに反する研究の成果は本会

表1 薬理学会での動物実験の調査（口頭発表のみ）

		初日	2〜3日	計	合計	%
動物を使用した実験	in vivo 実験*					
	マウス	39	51	90		
	GM マウス#	7	16	23		
	ラット	5	17	22		
	ウサギ	1	1	2		
	モルモット	1	1	2		
					139	51.87
	カエル	1	2	3		
	ゼブラフィッシュ	1	2	3		
					6	2.24
	不明##	6	8	14	14	5.22
	摘出組織・細胞実験**					
	マウス	7	5	12		
	ラット	2	12	14		
					26	9.70
	ヒト	1	8	9	9	3.36
	カエル	1	0	1		
	ゼブラフィッシュ	1	0	1		
					2	0.75
動物未使用***		10	38	48	48	17.91
不明		7	17	24	24	8.96
合計		90	178	268		

*：in vivo で動物に何らかの処置をしているもの．**：無処置の動物から摘出した組織や細胞等を利用しているもの．***：細胞株，微生物，線虫および屠殺場に由来する試料を用いているもの．#：GM と wild の両方を用いたものは重複して計算．##：動物を使用しているが，動物種が不明のもの．

の刊行する学術雑誌から排除する所存である」と宣言した（平成19年）(9)．薬理学研究者はこの指針に従い，研究を進めるよう留意すべきである．しかし，表2に示したように，改正動愛法が施行されて1年半が経過した平成19年2〜3月の時点でも，代替法に関する教育は必ずしも十分では無かった．現在は，改善されていることを望むものである．

医薬品開発のための国際的な指針「医薬品の臨床試験及び販売承認申請のための非臨床安全性試験の実施についてのガイダンス（ICH M3（R2））」(10) においても，3R の原則への配慮が多々なされており，本指針が「3R（使用動物数の削減／苦痛の軽減／代替法の利用）の原則に従って動物の使用を抑え，医薬品開発のための資源の有効利用に資する」よう期待している．「安全性評価のための新しいインビトロ代替法の利用について考慮すべきである．」とも述べている．これは今後開発されることが予想される iPS 細胞由来標本を用いた試験を配慮して記載されたものであるが，ICH で受け入れるためには，それらのバリデーションが完了し，全ての ICH 規制当局によって認められる必要があるとされた．

表2 動物実験倫理に関する薬学系大学の教育内容

教育内容*	教えている大学の割合
動愛法	80.80%
実験動物の生理，生態，習性	65.40%
実験動物の飼育，管理方法	84.60%
動物実験における 3R の原則	69.20%
動物の苦痛の評価	69.20%
苦痛の軽減方法	73.10%
安楽死の方法	80.80%
動物実験代替法	57.70%
その他	42.30%

*：文部科学省等の指針から教えることが望ましいと思われるもの．平成19年2〜3月にアンケート調査した結果の集計（回答数29，回答施設数24）．

効力を裏づけるためのインビボおよび／またはインビトロにおける薬力学的試験は，目的とする治療標的に対する被験物質の作用機序や効果を調べることを意図している．このような試験は，通常，医薬品開発の探索段階で実施され，通常は GLP に従っては，行われない．しかし，ICH M3（R2）指針では，これらの試験結果を非臨床試験および臨床試験の用量設定に役立てることを勧めている．逆に，有効に利用できるよう

にGLPに準じて明確な試験計画をたて，データの記録や保管に心がけるべきである．また，インビボで評価する場合には，使用動物を削減するため，可能な範囲内で，一般毒性試験や他の試験に組み込んで実施することを考慮すべきとした．乱用性試験についても，ヒト以外の霊長類を使用するケースは，霊長類がヒトでの乱用性を予測し得ると考える明確な根拠があり，しかも，げっ歯類のモデルは不適切である場合に限るべきであるとした．なお，実験の際の動物の苦痛の評価については，国立大学法人動物実験施設協議会の作成した「動物実験処置の苦痛分類に関する解説」が参考となる．

おわりに

動物実験を行う者はそれが本当に必要な実験であるか，動物を用いない他の試験法に置き換えることができないか，動物に不必要な苦痛を与えていないかを，施設毎の動物実験委員会で審議することが求められている．薬理学を含む生物科学を教える立場にある者は，動物実験代替法について適正な教育を行う必要がある．また，レギュラトリーサイエンスに携わる者には，ガイドライン等で定められた毒性試験法のように，行政的に要求される試験の妥当性について過去の経験，新しい技術・知見，現時点での社会情勢などに基づき常に見直さなくてはならない．EUで一時大きな問題となった動物の権利を標榜する団体による研究機関の攻撃も，最近は鎮静化している模様であるが，これらは行政機関や大学や企業などの研究機関，および動物実験実施者等による動物福祉への配慮や違法行為に対する毅然たる態度による成果であると考えている．

文　献

1) Russell WMS, et al. The principles of human experimental technique. Methuen; 1959.
2) 小野宏，大野忠夫編著．動物実験代替法マニュアル．共立出版; 1994. p. 123-133.
3) 大野泰雄．国立衛研報. 2004;122:1-10.
4) OECD: Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment, OECD series on testing and assessment 2005; No 34 (18 August 2005).
5) 文部科学省．研究機関等における動物実験等の実施に関する基本指針．文部科学省告示 第71号, 2006.6.1.
6) 厚生労働省．厚生労働省の所管する動物実験等の実施に関する基本指針．厚生労働省通知 科発0601002号, 2006.6.1.
7) 環境省．実験動物の飼育及び保管並びに苦痛の軽減に関する基準．環境省告示第88号, 2006.6.1.
8) 農林水産省．農林水産省の所管する研究機関等における動物実験等の実施に関する基本指針．2006.6.1.
8) 日本学術会議．動物実験の適正な実施に向けたガイドライン．2006.6.1.
9) 大野泰雄．日薬理誌. 2007;129:5-9.
10) 厚生労働省．医薬品の臨床試験及び販売承認申請のための非臨床安全性試験の実施についてのガイダンス ICH Consensus Guideline. 薬食審査発0219第4号, 2010.2.19.

著者プロフィール

大野　泰雄（おおの　やすお）

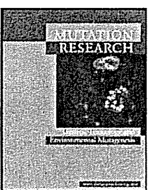
国立医薬品食品衛生研究所所長．
◇1976年東京大学薬学系大学院博士課程修了．同年現在の研究所に採用され，'11年より現職．肝・腎における代謝と毒性研究に従事．カロリンスカ研究所に留学後in vitro毒性および動物実験代替法研究に関与．日本動物実験代替法学会長および第6回国際動物実験代替法学会長を歴任．

Mutation Research 741 (2012) 32–56

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres



Effect of reducing the top concentration used in the *in vitro* chromosomal aberration test in CHL cells on the evaluation of industrial chemical genotoxicity

Takeshi Morita[a,*], Masamitsu Honma[b], Kaoru Morikawa[a]

[a] National Institute of Health Sciences, Division of Safety Information on Drug, Food and Chemicals, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] National Institute of Health Sciences, Division of Mutagenesis and Genetics, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

ARTICLE INFO

Article history:
Received 3 June 2011
Received in revised form
22 September 2011
Accepted 6 October 2011
Available online 17 October 2011

Keywords:
In vitro tests, Chromosomal aberration test, CHL cells, Test concentration limit, Industrial chemicals

ABSTRACT

A current concern with *in vitro* mammalian cell genotoxicity testing is the high frequency of false or misleading positive results caused in part by the past use of excessively high test concentrations. A dataset of 249 industrial chemicals used in Japan and tested for genotoxicity was analyzed. Of these, 116 (46.6%) were positive in the *in vitro* chromosomal aberration (CA) test, including 6 that were positive only at test concentrations >10 mM. There were 59 CA-positive chemicals at test concentrations ≤1 mM. At >1 mM, 51 chemicals were CA-positive, including 13 Ames-positive chemicals, which were therefore not "missed" by the test battery. Thus, 38 potentially positive chemicals would not have been detected in the test battery if the top test concentration was limited to 1 mM in CA test. Analysis of the relevance of CA results on the 38 missed chemicals was conducted based on a weight of evidence approach, including evaluations of effects of extreme culture conditions (low pH, high toxicity, or precipitation), *in silico* structural alert analysis, *in vivo* genotoxicity and carcinogenicity test data (where available), mode of action, or information from closely related chemicals. After an exhaustive review, there were four chemicals with some concern for human health risk assessment, nine with minimal concern, and the remaining 25 with negligible concern. We apply different top concentrations to the 38 missed chemicals to identify the most accurate approach for predicting the genotoxicity of industrial chemicals. Of these 2 mM or 1 mg/mL, whichever is higher, was the most effective in detecting these chemicals, *i.e.*, relatively higher (8/13) or lower (17/25) detection among 13 chemicals with some or minimal concern, or 25 with negligible concern, respectively. Lower top concentration limits, 1 mM or 0.5 mg/mL, whichever is higher, are not as effective (2/13) for detecting these chemicals with concern. Therefore, we conclude 2 mM or 1 mg/mL, whichever is higher, would be an appropriate top concentration limit for testing industrial chemicals for chromosome damage.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

The top concentrations used in *in vitro* mammalian cell genotoxicity tests are currently being re-examined with the goal of reducing the frequency of false or misleading positive results [1–6]. In the standard test battery, the mouse lymphoma assay and the *in vitro* micronucleus (MN) or chromosomal aberration (CA) test have low specificity for predicting carcinogenicity (e.g., <45%) [7–9], and the false positive results they generate lead to additional testing, often requiring the unnecessary use of animals [4]. The current top concentration limit specified in regulatory guidelines is 10 mM or 5 mg/mL, whichever is lower, when not limited by solubility or cytotoxicity [10,11]. The possible reasons for misleading or non-relevant results caused by testing at very high concentrations *in vitro* are: (1) un-physiological culture conditions including low pH, high osmolality and/or precipitation; (2) excessive cellular metabolic turn over, activation and defense/stress processes; and (3) results obtained at high concentrations that could not be reached *in vivo* and therefore not confirmed in *in vivo* genotoxicity or carcinogenicity tests. Therefore, more biologically relevant experimental conditions are needed. One way to lessen false positive results is to reduce the top test concentration [1]. The proposed revised International Conference on Harmonisation (ICH) test guidelines for pharmaceuticals recommends as the top concentration 1 mM or 0.5 mg/mL, whichever is lower [12], and almost same conclusion, *i.e.*,1 mM or 0.5 mg/mL, whichever is higher, was reached following an analysis of 384 genotoxic rodent carcinogens [3] and the retesting of selected chemicals [4]. A consensus for reducing the top concentration for testing in mammalian genotoxicity tests *in vitro* from 10 mM (but no agreement to what concentration should be) was reached in 2009 at the 5th International Workshop on Genotoxicity Testing, where the results from our preliminary analysis of 249 chemicals were presented [5]. Earlier in 2006, participants in a European Centre for the Validation of Alternative Methods workshop proposed that the published and industry data should

* Corresponding author. Tel.: +81 3 3700 1141; fax: +81 3 3700 1483.
E-mail address: morita-tk@nihs.go.jp (T. Morita).

1383-5718/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2011.10.005

be reviewed to determine whether the top test concentration should be lowered [1]. Therefore, our analysis will provide useful information for this debate.

As a member of the Organization for Economic Co-operation and Development (OECD) since 1991, Japan has been performing safety tests of high production volume (HPV) chemicals. The results of the 249 chemicals analyzed in this paper as part of that effort were published as hard copies [13–26], while recent results are available only online (http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp). The dataset includes results of the *in vitro* CA test and the Ames test, both conducted in accordance with OECD or Japanese test guideline and according to Good Laboratory Practice (GLP) regulations. In this paper, we report our analysis of "missed" chemicals—that is, those that were positive in the *in vitro* CA test at the 10 mM top concentration but not at 1 mM and were negative in the Ames test. We discuss the significance and relevance of the induction of CAs by these chemicals based on the weight of evidence approach including *in silico* analysis, review of literature on *in vivo* genotoxicity and carcinogenicity, or effects in structurally related chemicals. The levels of concern for human health risk assessment are defined for each chemical in this paper. Finally, we apply different top concentrations to missed chemicals to identify the most accurate approach for predicting the genotoxicity of industrial chemicals.

2. Materials and methods

2.1. Source of published in vitro CA test results

We obtained *in vitro* CA test data from reports published from 1994–2006 by the Ministry of Health, Labor, and Welfare of Japan [13–26].

2.2. In vitro CA test protocol

In vitro CA tests were conducted in Chinese hamster lung (CHL) cells in accordance with OECD test guideline no. 473 [11] or the Japanese test guideline for new chemicals [27] under GLP conditions. The outline of the protocol under the former Japanese test guideline were as follows: Treatment length was 6 h (with 18 h recovery), 24 h (continuous without recovery time), or 48 h (continuous without recovery time) without S9 mix, a rat liver homogenate microsomal fraction with co-factors for metabolic activation, and 6 h (with 18 h recovery) with S9 mix. The top concentration was 5 mg/mL (or equivalent of 10 mM) when no cytotoxicity was observed. In the presence of cytotoxicity, the top concentration selected was one that caused 50% or greater inhibition of cell growth compared to the negative control. Methods for measuring cytotoxicity, as relative cell growth, were an estimation of monolayer confluence using a monocellater or other equipment, or survival cell counts. Structural aberrations and polyploidy were evaluated independently in 100 or 200 metaphases per concentration. In some cases, 800 metaphases per concentration were analyzed for polyploidy. Pre-1997 studies did not follow the current OECD guideline that was published that year. Major differences from the current OECD guideline are use of long exposure time (48-h continuous treatment), use of concentrations which shows much greater than 50% relative cell growth as the top concentration when cytotoxicity was observed. More preferably 5 mg/mL was used, rather than 10 mM as the top concentration when cytotoxicity was not observed. In general, there was no consideration of physiological culture conditions (pH, osmolality, or precipitation), no concurrent cytotoxicity measurement, no ensuring that at least 200 metaphases were analyzed per concentration, and no statisticalanalyses were carried out [27,28].

2.3. Reporting of results

Each experiment was classified as (a) positive (+): ≥10% cells with CAs; (b) equivocal (?): ≥5–10% cells with CAs, or (c) negative (−): less than 5% cells with CAs. Then only chemicals showing at least one positive or equivocal experiment were considered positive or equivocal compound, respectively, in the original reports [13–26]. Statistical significance, reproducibility, culture conditions, or concentration relationship of the response were taken into consideration in some cases. Basically, calls of the classification of chemicals were based on the original calls. However, there are some exceptions to this analysis. If a chemical was classified equivocal in the original "call" and showed a reproducibility and/or concentration-related response with statistical significance, the chemical was considered positive. For example, two chemicals (Identifications (IDs) 61, 82) assigned equivocal in the original call were considered positive, because the effect was reproducible or there was a CA-induction of equal to or more than 10%. Three chemicals (IDs 81, 83, 84) assigned negative in the original call were also considered positive, because CA-induction of them was equal to or more than 10%; the original reports judged the CA-induction was due to low pH (see Section 3.1). Chemicals that show negative and/or equivocal (without reproducibility or concentration-related response) results were also considered as negative compounds.

Note that the percentages of cells with CAs refer to structural aberrations and do not include polyploidy. The percentages of polyploidy cells are presented in two chemicals (IDs 95, 96) in this analysis.

2.4. Analysis of the data

The different steps of the analysis (weight of evidence approach) used in this paper for the 249 chemicals on which *in vitro* CA tests were conducted, are shown in Fig. 1: (1) analysis of *in vitro* CA data excluding 48 h results; (2) classification of chemicals into positive and negative compounds; (3) for positive chemicals exclusion of those with the lowest effective concentration (LEC) of ≤1 mM or >10 mM; (4) for chemicals with LEC of >1–10 mM, review of Ames test data; if positive, chemicals would not be missed; (5) if Ames-negative (*i.e.*, possible "missed" chemicals in the test battery), further evaluation of the relevance of CA results, including evaluation of effects of extreme culture conditions (low pH, high toxicity and precipitation), *in silico* analyses using Deductive Estimation of Risk from Existing Knowledge (DEREK) for Windows and/or the Optimized Approach Based on Structural Indices Set (OASIS) tissue metabolic simulator (TIMES), and review of the literature to see if more *in vivo* genotoxicity and carcinogenicity data including on structurally related compounds can be found; and (6) conclusion on level of concern for human health risk assessment on missed chemicals.

2.5. In silico structure alert analysis

We used DEREK for Windows (version 12) for structure alerts for mutagenicity, clastogenicity, and carcinogenicity [29] and TIMES (version 2.26.3) for structural alerts for clastogenicity [30]. TIMES can predict CAs induced by metabolically activated chemicals that do not elicit activity in the parent form, in addition to alerting for mutagenic structures [30–33]. Basically, DEREK was applied to all "missed" chemicals. TIMES was also applied missed chemicals with the exception of chemicals which were considered positive due to possible effects of extreme culture conditions.

2.6. Literature search

For the literature search, we used *PubMed* and *TOXNET* and searched for "CAS number", "carcinogenicity", "genotoxicity", "mutagenicity", "micronucleus" and "chromosomal aberration". Data from structurally related chemicals were also taken into account. We also searched Screening Information Data Set (SIDS) documents in the United Nations Environment Programme (UNEP, http://www.chem.unep.ch/irptc/sids/OECDSIDS/sidspub.html) or the OECD chemical database (http://webnet.oecd.org/hpv/ui/Search.aspx) [34–53].

2.7. Level of concern

The level of concern for human health risk assessment on "missed" chemicals was defined based on the analysis by weight of evidence approach. General criteria are as follows: (1) negligible concern, negative result(s) in *in vivo* genotoxicity or carcinogenicity test, clear evidence(s) of irrelevancy (e.g., extreme culture condition) for CA-induction, and/or mode of action of non-DNA target; (2) minimal concern, some evidence(s) of irrelevancy of CA-induction or of increasing level of negligible concern, or negative result(s) in *in vivo* genotoxicity tests with some limitations; (3) some concern, positive result(s) in *in vivo* genotoxicity or carcinogenicity test, or no supporting evidence(s) for reducing the level of concern.

2.8. Application of different top concentrations to the "missed" chemicals

Several top concentration limits were applied to the "missed" chemicals in order to investigate their effectiveness for predicting the genotoxicity of industrial chemicals, which include 1 mM or 0.5 mg/mL, whichever is higher; 2 mM or 1 mg/mL, whichever is higher; 4 mM or 2 mg/mL, whichever is lower; and 10 mM or 2 mg/mL, whichever is lower. These top concentrations except for 2 mM or 1 mg/mL, whichever is higher, are under discussion by an OECD expert group (unpublished). The number of chemicals detected among missed chemicals with minimal or some concern, or with negligible concern was calculated.

3. Results

3.1. Analysis of in vitro CA and Ames test data

At first, we regarded two chemicals, benzyltrimethylammonium chloride (ID61, Table 2) and glycerol triacetate (ID82, Table 4), as positive in this analysis; their original "call" were equivocal [16,18], but the effects observed by benzyltrimethylammonium chloride or glycerol triacetate were reproducible or induced more than 10%

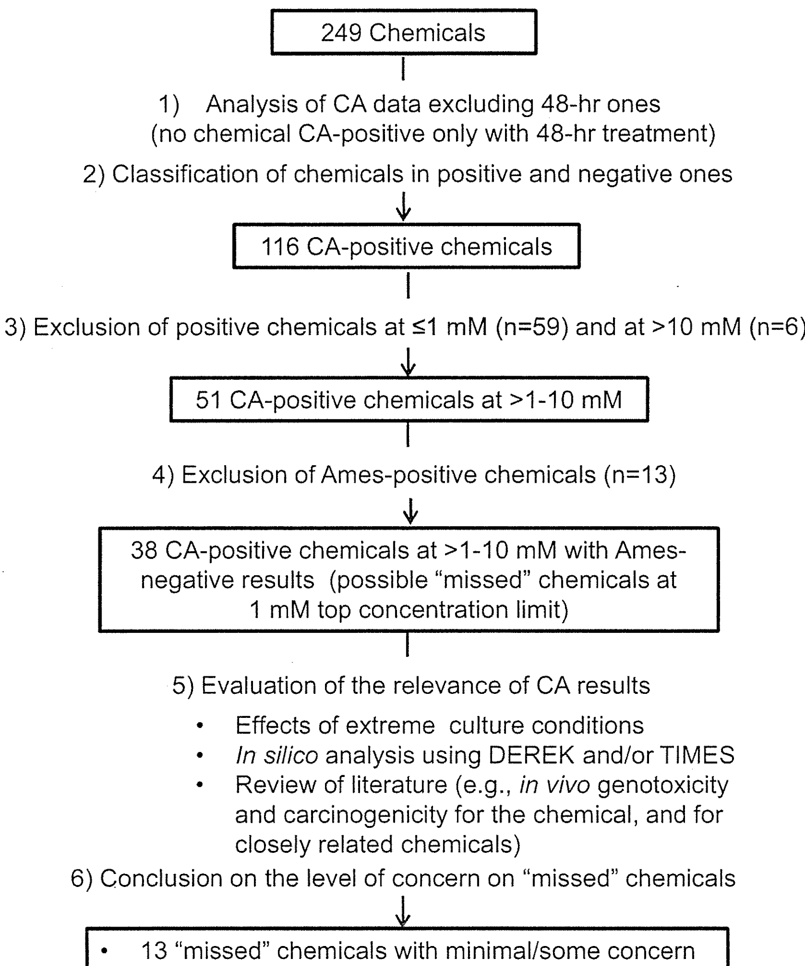


Fig. 1. Flow chart of the different steps of the analysis (weight of evidence approach).

cells with CAs, respectively. CA-induction by the latter chemical was considered to be due to low pH, so equivocal was assigned in the original report [18]. In addition, we regarded 3 chemicals, 2-amino-5-methylbenzenesulfonic acid (ID81), 4-hydroxybenzoic acid (ID83), and methyl acetoacetate (ID84) (Table 4), as positive in this analysis; their original "call" were negative, but the frequencies of CAs observed by the 3 chemicals were more than 10%. Clastogenicity induced by them was considered to be due to low pH, and neutralization of culture medium gave negative results. Thus a negative call was assigned for these chemicals in the original reports [16–18,54].

Among the 249 chemicals tested that had been subjected to the *in vitro* CA test, 116 (46.6%) were positive (Tables 1–4), and 133 (53.4%) were negative (Table 5). No chemicals were CA-positive only with a 48-h treatment. Almost all chemicals were also Ames tested, although we took some Ames data from other literature sources including US National Toxicology Program (NTP, http://ntp-apps.niehs.nih.gov/ntp_tox/) [55–57]. The Ames data are included in Tables 1–5.

Based on the steps of the analysis (Fig. 1), the 249 chemicals in the *in vitro* CA test were divided into five groups; (1) 59 chemicals positive at ≤1 mM (Table 1); (2) six chemicals positive at >10 mM which are considered negative following the criteria given in the current guidelines (Table 2); (3) 13 chemicals positive at >1–10 mM with Ames-positive results which were not "missed" by the test battery (Table 3); (4) 38 chemicals positive at >1–10 mM with Ames-negative results which would be missed in top concentration of 1 mM (possible "missed" chemicals, Table 4); and (5) 133 chemicals negative in the *in vitro* CA test (Table 5).

3.2. Evaluation of the relevance of *in vitro* CA results

Thirty eight chemicals were chemicals that would be missed if 1 mM was employed as the top concentration limit (*i.e., in vitro* CA negative at >1–10 mM and Ames-negative). The relevance of *in vitro* CA results was evaluated based on a weight of evidence approach including analysis of effects of extreme culture conditions (low pH, high toxicity, and precipitation), *in silico* structural alert analysis using DEREK and/or TIMES, and review of literature for *in vivo* genotoxicity and carcinogenicity tests and the genotoxicity/carcinogenicity of closely related chemicals (Table 4).

On measuring cytotoxicity, there were cases where even though there was apparently, for example 50% relative cell growth, measured by cell counts or confluence, there were insufficient mitotic cells to score.

3.2.1. Possible effects of extreme culture conditions (15 chemicals)

3.2.1.1. Low pH (seven chemicals). A low pH effect was defined as responsible for CA induction when the medium pH was 6.0 or below at the beginning of or just after treatment. Morita et al. reported that initial pH 6.2 or below in 6-h treatment with S9 mix, and initial pH 5.5 or below in 24-h treatment without S9 mix, were clastogenic

Table 1
59 Japanese high production volume chemicals positive at ≤1 mM[a]) in the *in vitro* chromosomal aberration test with CHL cells at (1994–2006, *n* = 249).

ID no.	Chemical name	CAS	MW	LEC (mM)	LEC (mg/mL)	Ames test	Reference
1	Acenaphthene	83-32-9	154.2	1.0	0.2	–	[17]
2	3-Aminophenol	591-27-5	109.1	0.3	0.03	–	[21]
3	4-Aminophenol	123-30-8	109.1	0.02	0.003	–	[17]
4	4,4′-Biphenyldiol	92-88-6	186.2	0.2	0.03	–	[25]
5	1,2-Bis(2-chloroethoxy)ethane	112-26-5	187.1	0.3	0.06	+	[24]
6	Bis(1-methylethyl)naphthalene	38640-62-9	212.3	0.7	0.14	–	[20]
7	N-tert-Butyl-2-benzothiazolesulfenamide	95-31-8	238.4	0.8	0.2	–	[17]
8	*o-sec*-Butylphenol	89-72-5	150.2	0.1	0.02	–	[19]
9	6-*tert*-Butyl-*m*-cresol	88-60-8	164.3	0.05	0.01	–	[19]
10	2-*tert*-Butylphenol	88-18-6	150.2	0.05	0.01	–	[20]
11	*p*-*tert*-Butylphenol	98-54-4	150.2	0.2	0.03	–	[16]
12	Cadmium nitrate tetrahydrate	10022-68-1	308.5	0.02	0.01	–	[22]
13	1-Chloro-2-(chloromethyl)benzene	611-19-8	161.0	0.6	0.1	+	[19]
14	4-Chloro-*o*-cresol	1570-64-5	142.6	0.6	0.1	–	[16]
15	Chloropentabromocyclohexane	87-84-3	513.1	0.06	0.03	–	[16]
16	4-Chlorophenol	106-48-9	128.6	0.4	0.05	–	[20]
17	Chromic acid disodium salt dihydrate	7789-12-0	297.8	0.002	0.001	+	[23]
18	2,4-Diamino-6-phenyl-*s*-triazine	91-76-9	187.2	0.4	0.08	–	[19]
19	1,3-Dibromopropane	109-64-8	201.9	0.3	0.06	+	[23]
20	2,4-Di-*tert*-butylphenol	96-76-4	206.3	0.04	0.01	–	[20]
21	3,4-Dichloro-1-butene	760-23-6	125.0	0.1	0.01	+	[16]
22	1,2-Dichloro-3-nitrobenzene	3209-22-1	192.0	0.6	0.1	–	[13,34]
23	1,4-Dichloro-2-nitrobenzene	89-61-2	192.0	0.8	0.15	+	[15]
24	*N,N*-Dicyclohexyl-2-benzothiazolesulfenamide	4979-32-2	346.6	0.6	0.2	–	[15]
25	*O,O*′-Diethyl dithiophosphate	298-06-6	186.2	0.6	0.12	+	[25]
26	Diethyl fumarate	623-91-6	172.2	0.1	0.01	–	[14]
27	2-(Dimethylamino)ethyl acrylate	2439-35-2	143.2	0.4	0.05	+	[17]
28	*N*-(1,3-Dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine	793-24-8	268.4	0.02	0.005	–	[19]
29	Diphenyl cresyl phosphate	26444-49-5	340.3	0.1	0.04	–	[14]
30	Disperse Yellow 42	5124-25-4	369.4	0.2	0.08	–	[22]
31	2,3-Epoxypropyl methacrylate	106-91-2	142.2	0.2	0.02	+	[17,35]
32	4-Ethoxybenzeneamine (*p*-Phenetidin)	156-43-4	137.2	0.4	0.05	–	[13]
33	2-Ethylanthraquinone	84-51-5	236.3	0.6	0.16	–	[20]
34	3-Ethylphenol	620-17-7	122.2	0.4	0.05	–	[21]
35	4-Ethylphenol	123-07-9	122.2	0.3	0.04	–	[20]
36	Hydrazine monohydrate	7803-57-8	50.1	1.0	0.06	+	[23]
37	2-Hydroxybenzaldehyde	90-02-8	122.1	0.8	0.1	–	[16]
38	Methacrylonitrile (methyl acrylonitrile)	126-98-7	67.1	1.0	0.07	–	[20]
39	Methoxymethanol	4461-52-3	62.1	0.3	0.02	+	[14]
40	1-Methoxynaphthalene	2216-69-5	158.2	0.1	0.02	–	[16]
41	4,4′-Methylenebis(2-chloroaniline)	101-14-4	267.2	0.1	0.04	+	[25]
42	Methylenediphenol	1333-16-0	200.2	0.05	0.01	–	[21]
43	4,4′-Methylenediphenol	620-92-8	200.2	1.0	0.2	–	[25]
44	4-(1-Methylethenyl)phenol	4286-23-1	134.2	0.5	0.06	–	[23]
45	Methyl isothiocyanate	556-61-6	73.1	0.03	0.003	–	[25]
46	3-Methyl-4-nitrophenol	2581-34-2	153.2	0.3	0.04	–	[14]
47	3-Methylphenol (*m*-Cresol)	108-39-4	108.1	0.2	0.03	–	[20]
48	2-(4-Morpholinyldithio)benzothiazole	95-32-9	284.4	0.3	0.1	–	[24]
49	4-Nitro-*o*-anisidine	97-52-9	168.2	0.5	0.08	+	[17]
50	2-Pentylanthraquinone	13936-21-5	278.4	0.2	0.06	+	[26]
51	*N*-Phenylmaleimide	941-69-5	173.2	0.02	0.01	+	[21]
52	*N*-Phenyl-*N*′-isopropyl-*p*-phenylenediamine	101-72-4	226.3	0.01	0.01	–	[23]
53	Phosphoric acid, dodecyl ester, sodium salt	50957-96-5	288.3	0.16	0.05	–	[26]
54	3a,4,7,7a-Tetrahydro-1*H*-indene	3048-65-5	120.2	0.8	0.004	–	[17]
55	Thymol	89-83-8	150.2	0.5	0.002	–	[16]
56	2,4,6-Tribromophenol	118-79-6	330.8	0.2	0.05	–	[19]
57	1,3,5-Trihydroxybenezene	108-73-6	126.1	1.0	0.1	–	[22]
58	2,3,6-Trimethylphenol	2416-94-6	136.2	0.4	0.05	–	[19]
59	2-Vinylpyridine	100-69-6	105.2	0.1	0.01	+	[17]

(–): Negative; (+): positive; MW: molecular weight; LEC: lowest effective concentration.
[a] As lowest effective concentration.

Table 2
6 Japanese high production volume chemicals positive at >10 mM[a]) in the *in vitro* chromosomal aberration test with CHL cells at (1994–2006, *n* = 249).

ID no.	Chemical name	CAS	MW	LEC (mM)	LEC (mg/mL)	Ames test	Reference
60	o-Acetoacetotoluidine	93-68-5	191.2	13.1	2.5	–	[19]
61	Benzyltrimethylammonium chloride[b]	56-93-9	185.7	10.2	1.9	–	[16]
62	3-Methylzenzoic acid	99-04-7	136.2	11.0	1.5	–	[19]
63	3-Nitrobenzenamine	99-09-2	138.1	11.6	1.6	+	[13]
64	Phthalimide	85-41-6	147.1	17.0	2.5	–	[19]
65	2,2,6,6-Tetramethyl-4-hydroxypiperidine	2403-88-5	157.3	12.7	2.0	–	[18]

(–): Negative; (+): positive; MW: molecular weight; LEC: lowest effective concentration.
[a] As lowest effective concentration.
[b] The original "call" was equivocal [16]. However, it regarded as positive in this analysis because the effect was reproducible.

Table 3
13 Japanese high production volume chemicals positive at >1–10 mM[a] in the *in vitro* chromosomal aberration test with CHL cells with Ames-positive results (1994–2006, n = 249).

ID no.	Chemical name	CAS	MW	LEC (mM)	LEC (mg/mL)	Ames test	Reference
66	*N*-(Aminoethyl)ethanolamine	111-41-1	104.2	9.6	1.0	+	[16,55]
67	2-Amino-1-naphthalenesulfonic acid	81-16-3	223.3	4.9	1.1	+	[15]
68	1-Bromo-3-chloropropane	109-70-6	157.4	1.6	0.3	+	[20]
69	2-(Dimethylamino)ethyl methacrylate	2867-47-2	157.2	4.0	0.6	+/−	[18,53]
70	2,3-Dimethylaniline	87-59-2	121.2	5.0	0.6	+	[17]
71	2,6-Dimethylaniline (2,6-Xylidine)	87-62-7	121.2	2.5	0.3	+	[25]
72	3,5-Dimethylaniline (3,5-Xylidine)	108-69-0	121.2	7.4	0.9	+	[17,56]
73	Disperse Red 206	26630-87-5	580.1	4.3	2.5	+	[23]
74	3-Methoxybenzeneamine	536-90-3	123.2	6.1	0.8	+	[13]
75	4,4′-Oxybis(benzenesulfonylhydrazide)	80-51-3	358.4	1.6	0.6	+	[24]
76	Thiourea dioxide	4189-44-0	108.1	5.5	0.6	+	[19]
77	Tolylene diisocyanate (Toluene diisocyanate)	26471-62-5	174.2	1.8	0.3	+	[21]
78	2,4,6-Trinitrophenol (Picric acid)	88-89-1	229.1	7.0	1.6	+	[20]

(−): Negative; (+): positive; MW: molecular weight; LEC: lowest effective concentration.
[a] As lowest effective concentration.

to CHO-K1 cells [58,59], and stable pH 6.5 or below for 24 h, or stable pH 5.8 or below for 6 h without S9 mix were clastogenic to CHL cells [60]. Therefore, pH 6.0 or below, regardless of fluctuation or stability, in the culture medium might cause CAs both with and without S9 mix.

ID79. 3-Aminobenzenesulfonic acid (CAS no. 121-47-1) [molecular weight (MW) = 173]: 3-Aminobenzenesulfonic acid induced CAs with S9 mix (5.0 and 16.5% at 2.4 and 4.8 mM (0.83 mg/mL), respectively) [14]. The pH of the medium was 5.8 or 6.3 at the beginning of the 6-h treatment and 6.2 or 6.5 just after it at 4.8 or 2.4 mM, respectively. Relative cell growth, as measured by monolayer confluence, was about 100% or 90% at 4.8 or 9.5 mM, respectively. However, there were no metaphases at 9.5 mM. Without S9 mix, no CA induction was observed after 6- or 24-h treatment. The reason for this will be due to the short duration in low pH culture condition; the pH of the medium without S9 mix was 5.8 or 6.5 at the beginning of the 6-h treatment and 6.6 or 6.9 just after it at 4.8 or 2.4 mM, respectively. Initial pHs of the medium were similar, but the pHs after the treatment without S9 mix were higher than that with S9 mix. The window of the induction of CAs by low pH is narrower without S9 mix than that with S9 mix, generally; the same effect was observed for hydrochloric acid and sulfuric acid [58]. 3-Aminobenzenesulfonic acid does not possess any DEREK structural alerts. In addition, an *in vivo* MN test was negative for a related structural analogue, 2-amino-5-methylbenzenesulfonic acid (CAS no. 88-44-8, ID81) [51]. The CAs observed are considered as irrelevant as they were only seen at low pH, and it is supported by all other available data. Thus the level of concern is negligible.

ID80. 2-Amino-5-chloro-4-methylbenzenesulfonic acid (CAS no. 88-53-9) [MW = 222]: 2-Amino-5-chloro-4-methylbenzenesufonic acid induced CAs (7.5% and 50.0%) at 9 mM (2 mg/mL) after 24-h treatment without S9 mix, and at 10 mM (2.2 mg/mL) after 6-h treatment with S9 mix, respectively..The pH of the medium was 6.4–6.6 without S9 mix and 5.5–5.8 with S9 mix in the beginning of the treatment [13]. Relative cell growth, as measured by monolayer confluence, was about 100% at 10 mM with S9 mix. Continuous low pH (6.5 or below) condition for 24 h without S9 mix was known to induce structural CAs in CHL cells [60]. No structural alerts were identified by DEREK. An *in vivo* MN test was negative for a related structural analogue, 2-amino-5-methylbenzenesulfonic acid (CAS no. 88-44-8, ID81) [51]. The CAs observed are considered as irrelevant due to low pH, and it is supported by all other available data. Thus the level of concern is negligible.

ID81. 2-Amino-5-methylbenzenesulfonic acid (CAS no. 88-44-8) [MW = 187]: 2-Amino-5-methylbenzenesulfonic acid induced CAs (7.0%) at 5.1 mM (1.0 mg/mL) after 6-h treatment with S9 mix, and relative cell growth, as measured by monolayer confluence, was about 40%. Only 5 metaphases were analyzed at 10 mM (1.9 mg/mL) due to severe cytotoxicity. The pH of the medium was 5.8 at the beginning of the treatment and 6.3 at the end [16]. No CAs were observed up to 10 mM when the pH of the culture medium was adjusted to about pH 7 by adding 1 N NaOH [16,54]. No structural alerts were identified by DEREK. Furthermore, an *in vivo* mouse bone marrow MN test was negative after oral administration up to 5000 mg/kg [51]. The CAs observed are considered as irrelevant due to low pH, and it is supported by all other available data. Thus the level of concern is negligible.

ID82. Glycerol triacetate (CAS no. 102-76-1) [MW = 218]: Glycerol triacetate induced CAs (42.0%) at 10 mM (2.2 mg/mL) with S9 mix, in which relative cell growth, as measured by monolayer confluence, was about 30%. The color of the medium became yellow only in the treatment with S9 mix. A confirmation study was conducted at neutral pH culture condition (adjusted to pH 6.9 by adding 1 N NaOH at the beginning of the treatment, but the pH value just after the addition of test chemicals was not described) [18]. However, the pH of the medium decreased to 4.9 after 6-h treatment with S9 mix [18,40]. Acidic metabolite(s) might be generated at high concentration with S9 mix, resulting in the induction CAs. No CA induction was observed at the lower concentration of 2.5 mM or 5 mM; relative cell growth was about 100% or 90%, respectively. The effect of the generation of acidic metabolite(s) will be *in vitro* specific at high concentration in the presence of S9 mix. No structural alerts were identified by DEREK. The CAs observed are considered as irrelevant due to low pH, and thus the level of concern is negligible.

ID83. 4-Hydroxybenzoic acid (CAS no. 99-96-7) [MW = 138]: 4-Hydroxybenzoic acid induced CAs (27.5% or 26.5%) at 5.1 mM (0.7 mg/mL) after 24-h treatment without S9 mix or 6-h treatment with S9 mix (181 cells analyzed); relative cell growth, as measured by monolayer confluence, was 58% or 28%, respectively. Severe cytotoxicity was observed at 10 mM (1.4 mg/mL) in all treatments. The pH of the medium was 6.1 or 5.8 at the beginning of the treatment and 6.6 or 6.2 at the end after 24-h treatment without S9 mix or 6-h treatment with S9 mix, respectively [17]. Culture medium with continuous low pH (6.5 or below) was known to induce CAs in CHL cells with S9 mix [60]. No CA induction was observed up to 10 mM when the pH of the culture medium was adjusted to about pH 7.5 by adding 1 N NaOH [17,52,54]. No structural alerts were identified by DEREK. The CAs observed are considered as irrelevant due to low pH, and thus the level of concern is negligible.

ID84. Methyl acetoacetate (CAS no. 105-45-3) [MW = 116]: Methyl acetoacetate induced CAs (11.0%) at 10 mM (1.2 mg/mL) after 6-h treatment with S9 mix; relative cell growth, as measured by monolayer confluence, was 88%. Though the color of the medium became yellow, no pH measurement was conducted [18]. No CA induction

Table 4
Analysis for the relevance of CA results in 38 "missed" chemicals which are CA-positive at >1–10 mM with Ames-negative results.

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
1. Possible effects of extreme culture conditions ($n = 15$)											
1.1. Low pH (7 chemicals)											
79	3-Aminobenzenesulfonic acid[e]	121-47-1		173.2	2.4	0.4	No alert	ND	– [14]	<–> [51]	Irrelevant due to low pH; negative in *in vivo* MN test for a related structural analogue, 2-mino-5-methylbenzenesulfonic acid (ID81) [51]; negligible concern.
80	2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9		221.5	9.0	2.0	No alert	ND	– [13]	<–> [51]	Irrelevant due to low pH; negative in *in vivo* MN test for a related structural analogue, 2-mino-5-methylbenzenesulfonic acid (ID81) [51]; negligible concern.
81	2-Amino-5-methylbenzenesulfonic acid[c,e]	88-44-8		187.2	5.1	1.0	No alert	ND	– [16]	– [51]	Irrelevant due to low pH; no increase in CAs by neutralization of culture medium; negative in *in vivo* MN test; negligible concern.
82	Glycerol triacetate[d,e]	102-76-1		218.2	10.0	2.2	No alert	ND	– [25]		Irrelevant due to low pH; possible generation of acidic metabolite(s) in *in vitro* specific condition with S9 mix; negligible concern.

333

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
83	4-Hydroxybenzoic acid[c]	99-96-7		138.1	5.1	0.7	No alert	ND	– [17,52]		Irrelevant due to low pH; no increase in CAs by neutralization of culture medium; negligible concern.
84	Methyl acetoacetate[c,e]	105-45-3		116.1	10.0	1.2	No alert	ND	– [18]		Not concluded irrelevant due to low pH; lack of information on pH in the initial phase and after the treatment; no increase in CAs by neutralization of culture medium; minimal concern.
85	1-Naphthylacetic acid	86-87-3		186.2	9.1	1.7	No alert	ND	– [14]	– [61]	Irrelevant due to low pH; negative in *in vivo* MN test; negligible concern.
1.2. High toxicity (6 chemicals)											
86	1,3-Bis(2-methylphenyl)guanidine[e]	97-39-2		239.3	2.5	0.6	No alert	ND	– [24]	– [42]	Not concluded irrelevant due to high toxicity; insufficient of negative in bone marrow MN test because *in vitro* CA-positive only with S9 mix; minimal concern.

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
87	*tert*-Butyl-methacrylate	585-07-9		142.2	2.8	0.4	CA-induction due to alpha, beta-unsaturated ester or thioester	ND	– [16]	<–> [44,53,62]	Maybe associated with cytotoxicity; negative in *in vivo* MN test for other methacrylates, 2-(dimethylamino)ethyl methacrylate (ID69) [53], 2-hydroxyethyl methacrylate (ID91) [44], and methyl methacrylate [62]; no evidence of carcinogenic potential for methyl methacrylate in rats and mice [62]; negligible concern.
88	*o*-Dichlorobenzene[e]	95-50-1		147.0	1.6	0.2	Carcinogenicity due to polyhalogenated aromatic	ND	– [20]	–, (–) [41]	Irrelevant due to high toxicity; negative in *in vivo* MN and CA tests, and in *in vivo* carcinogenicity tests; negligible concern.
89	Dicyclohexylamine	101-83-7		181.3	3.3	0.6	No alert	ND	– [18]	<(–)> [43]	Not considered to be irrelevant due to high toxicity; negative in *in vivo* CA test for a closely related structural analogue, *N*-methyl dicyclohexylamine [43]; negligible concern.
90	*N*-Ethylaniline	103-69-5		121.2	9.1	1.1	No alert	ND	– [15]		Maybe irrelevant due to high toxicity; no supporting evidence to reduce the level of concern; minimal concern.

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
91	2-Hydroxyethyl methacrylate	868-77-9		130.2	5.0	0.7	CA-induction due to alpha, beta-unsaturated ester or thioester	ND	– [17]	– [44]	Maybe associated with cytotoxicity; negative in *in vivo* MN test; no evidence of carcinogenic potential for methyl methacrylate in rats and mice [62]; negligible concern.
1.3. Precipitation coupled with high toxicity (2 chemicals)											
92	4-Methylbenzoic acid	99-94-5		136.2	8.8	1.2	No alert	ND	– [24]	– [45]	Irrelevant due to precipitation and following high toxicity; precipitation at all concentrations tested; negative in *in vivo* MN test; negligible concern.
93	Triphosphoric acid aluminium salt[e]	13939-25-8		317.9	6.3	2.0	No alert	ND	– [23]		Irrelevant due to precipitation and following high toxicity; precipitation at all concentrations tested; negligible concern.
2. Weak evidence for a positive ($n = 2$)											
94	4,4′-Sulfonyldiphenol	80-09-1		250.3	1.6	0.4	No alert	ND	– [19]		Low biological significance; statistically significant, but not tested for confirmation of reproducibility; negligible concern.

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
95	1,3,5-Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)isocyanuric acid	27676-62-6		784.1	3.2	2.5	No alert	ND	– [24]		Maybe low biological significance; statistically significant increase only in polyploidy based on the analysis of 800 cells per group, and outside of the historical range for negative controls; minimal concern.
3. Possible other factors ($n = 21$)											
3.1. Induction of polyploidy only (1 chemical)											
96	1,2-Dicyanobenzene	91-15-6		128.1	2.5	0.3	No alert	ND	– [20]	– [46]	Induction of polyploidy only; negative in *in vivo* MN test; mode of action by non-DNA target; negligible concern.
3.2. Selected chemical class with DNA reactivity (4 chemicals)											
97	2-(Diethylamino)ethyl methacrylate[e]	105-16-8		185.3	3.2	0.6	CA-induction due to alpha, beta-unsaturated ester or thioester	CA-induction due to possible metabolite(s): unsaturated aldehydes can interact with topoiso-merases/proteins.	– [18]	<–> [44,53,62]	Maybe associated with the DNA reactivity and/or cytotoxicity; negative in *in vivo* MN test for other methacrylates, 2-(dimethylamino)ethyl methacrylate (ID69) [53], 2-hydroxyethyl methacrylate (ID91) [44], and methyl methacrylate [62]; no evidence of carcinogenic potential for methyl methacrylate in rats and mice [62]; negligible concern.

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
98	Methacrylic acid, monoester with propane-1,2-diol	27813-02-1	$H_2C=C(CH_3)-C(=O)-O-CH(CH_2-OH)-CH_3$	144.2	5.0	0.7	CA-induction due to alpha,beta-unsaturated ester or thioester	CA-induction due to parent chemical and possible metabolite(s): acrylates can interact with DNA and topoiso-merases/proteins; unsaturated aldehydes can interact with topoiso-merases/proteins.	– [16]	<–> [44,53,62]	Maybe associated with the DNA reactivity and/or cytotoxicity; negative in *in vivo* MN test for other methacrylates, 2-(dimethylamino)ethyl methacrylate (ID69) [53], 2-hydroxyethyl methacrylate (ID91) [44], and methyl methacrylate [62]; no evidence of carcinogenic potential for methyl methacrylate in rats and mice [62]; negligible concern.
99	(Methacryloyloxyethyl) trimethylammonium chloride	5039-78-1	$CH_2=C(CH_3)-C(=O)-OCH_2CH_2-N^+(CH_3)_3$ Cl^-	207.7	10.0	2.1	CA-induction due to alpha,beta-unsaturated ester or thioester	CA-induction due to parent chemical: alkylamido betaines, theoretically interact with DNA or topoiso-merases/proteins.	– [21]	<–> [44,53,62]	Maybe associated with the DNA reactivity; negative in *in vivo* MN test for other methacrylates, 2-(dimethylamino)ethyl methacrylate (ID69) [53], 2-hydroxyethyl methacrylate (ID91) [44], and methyl methacrylate [62]; no evidence of carcinogenic potential for methyl methacrylate in rats and mice [62]; negligible concern.
100	Ethenyltrimethoxysilane[e]	2768-02-7	$CH_2=CH-Si(OCH_3)_3$	148.2	5.0	0.8	No alert	CA-induction due to possible metabolite(s): epoxides aziridines can interact with topoiso-merases/proteins.	– [26]	<–> [65]	Maybe associated with the DNA reactivity of metabolite(s); negative in *in vivo* MN test for an alkoxy silane, [3-(methacryloxy)propyl] trimethoxysilane [65], but insufficient of negative in *in vivo* MN test because *in vitro* CA-positive only with S9 mix; minimal concern.

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
3.3. Others (16 chemicals)											
101	2-Chlorophenol	95-57-8		128.6	2.0	0.3	CA-induction due to halophenol	No alert	– [20]	– [67]	Negative in *in vivo* MN test; not found to be carcinogenic in rats [68]. Negligible concern.
102	C.I. Fluorescent brightner 271	41267-43-0		1347.1	3.7	5.0	No alert	Not applied due to too large molecule	– [26]		Negative in *in vitro* CA test for other 3 structural related compounds (C.I. fluorescent brightner 24, 225 and 260) [64]; no *in vivo* supporting evidence of a reduced level of concern; minimal concern.
103	1,4-Dibromobenzene[e]	106-37-6		235.9	2.3	0.6	No alert	CA-induction due to possible metabolite(s): epoxides and aziridines can interact with topoiso-merases/proteins.	– [14]	<–> [69]	Negative in *in vivo* MN test for a closely related structural analogue, 1,4-dichlorobenzenen (non-genotoxic carcinogen) [69]; negligible concern.
104	Dibutyl adipate[e]	105-99-7		258.4	2.5	0.7	No alert	No alert	– [15]		No supporting evidence of a reduced level of concern; some concern.

Table 4 (*Continued*)

ID no.	Chemical name	CAS	Molecular structure	MW	LEC (mM)	LEC (mg/mL)	DEREK[a]	TIMES[b]	Ames test [Ref.]	*In vivo* MN (CA) [Ref.]	Note
105	2-(Di-*n*-butylamino)ethanol	102-81-8		173.3	1.9	0.3	No alert	No alert	– [25]	<–> [70,71]	Negative in *in vivo* MN test for related structural analogues, 2-aminoethanol [70] and 2-diethylaminoethanol [71]; negligible concern.
106	N,N-Dimethylbenzylamine[e]	103-83-3		135.2	2.8	0.4	No alert	CA-induction due to possible metabolite(s): benzylamines theoretically can interact with DNA.	– [17]	– [72]	Insufficient of negative in *in vivo* MN test because *in vitro* CA-positive only with S9 mix; minimal concern.
107	2,4-Dinitrophenol	51-28-5		184.1	6.5	1.2	Carcinogenicity due to aromatic nitro; CA-induction due to polynitrophenol or precursor.	CA-induction due to parent chemical and possible metabolite(s): nitro compounds can interact with DNA; amines, aminophenols and phenyle-neamines, aminophenols, or hydroxylamines can interact with DNA and topoiso-merases/proteins.	– [20]		Metabolic poison (uncouples oxidative phosphorylation, the mechanism with a threshold); Positive *in vitro* CA at cytotoxic levels in CHO and TK cells [73]; clastogenicity by indirect mechanism (energy depletion) [74]; negligible concern
108	2-Ethylbutyric acid	88-09-5		116.2	3.4	0.4	No alert	ND	– [22]	– [25,49]	Negative in *in vivo* MN test; negligible concern