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Genotoxic Impurities: From Structural Alerts to Qualification

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Abstract:

The presence of an *in cerebro* structural alert in a potential or actual impurity, most likely arising as a byproduct or carried-over reagent or starting material, in a drug substance or drug product is merely an indication that the compound *may* be a DNA-reactive genotoxin. The correlation between structural alerts for direct or indirect electrophilic characteristics and relevant biological activity is highly imperfect. For virtually all actual or potential impurities that are structurally alerting there is likely to be a variety of possibilities for clarifying their genotoxicity status based on published data, *in silico* assessments or *de novo* testing in bacterial reverse mutation assays. Even for compounds that test positive a number of options are available to enable a compound-specific qualification to be made involving the use of pre-existing toxicological data (particularly from lifetime rodent bioassays) and/or using information from appropriate additional studies. A review of representative compounds from several classes of structurally alerting substances (epoxides, hydrazines, aromatic amines, halides and aldehydes) provides examples of different types of qualification strategies Overall, it seems prudent to obtain maximum "leverage" from toxicological approaches, which are likely to be relatively low cost, before making any significant process-related changes to an active pharmaceutical ingredient (API) synthesis.

Introduction

Although the concept of using structural alerts to predict potential genotoxic activity for identified impurities is now well established, the concordance between such alerts and biologically relevant genotoxic potential (in the context of genotoxic impurities) can be highly imperfect. Assuming that any impurity with a structural alert is potentially DNA-reactive and thus subject to the default threshold of toxicological concern (TTC) limit of 1.5 μg/day may often lead to a drug substance specification limit that is unnecessarily restrictive. This article is intended to provide an overview of the key elements of an integrated chemical−toxicological approach to the optimal qualification of genotoxic impurities (GIs) especially when compound-specific biological data are available.

Structural Alerts for Genotoxicity

The concept of structural alerts for genotoxic activity was first elucidated in the 1980s/1990s particularly by Ashby and Tennant[1] who based their conclusions on correlations between electrophilicity and DNA reactivity (as assessed by Ames-testing data) for around 300 chemicals. Since then many more compounds have been tested (>8000) and sophisticated QSAR software-based systems have been developed such as DEREK, TOPKAT, MCASE and Toxtree. A compound containing a structural alert for the presence of an electrophilic moiety (or a substituent such as an aromatic primary amino group that can be metabolically activated) may or may not be Ames-positive. Similar alerts apply in terms of predicting chromosomal aberrations, but clastogenic compounds that are not DNA-reactive and nonmutagenic (in bacterial reverse mutation assays) are effectively excluded from consideration as genotoxic impurities under the EU guidance[2] (see below).

- alkyl esters of phosphonic or sulfonic acids
- aromatic nitro groups
- aromatic azo groups (reduction to amine)
- aromatic ring N-oxides
- aromatic mono- and dialkyl amino groups
- alkyl hydrazines
- alkyl aldehydes
- N-methylol derivatives
- monohaloalkanes
- N- and S-mustards, β-haloethyl-
- N-chloramines
- propiolactones and propiosulfones
- aromatic and aliphatic aziridinyl derivatives
- aromatic and aliphatic substituted primary alkyl halides
- urethane derivatives (carbamates)
- alkyl N-nitrosamines
- aromatic amines and N-hydroxy derivatives
- aliphatic epoxides and aromatic oxides
- centres of Michael reactivity
- halogenated methanes ($CX_{1-4}H_{3-0}$)
- aliphatic nitro groups.

Figure 1. **Conventional structural alerts for genotoxicity (based on bacterial reverse mutation assay data).**

A conventional list of structural alerts for genotoxicity[3] (as detected by bacterial reverse mutation assays) is shown in Figure 1. Other sources provide a more comprehensive list; for example the European Chemicals Bureau (ECB) Toxtree project[4] is based on 31 main structural alerts (plus some subcategories), although three main categories comprise nongenotoxic carcinogens.

Using a database of >4000 compounds, Sawatari et al[5] determined correlations between 44 substructures and bacterial mutagenicity data. A high proportion of genotoxic compounds was found for electrophilic reagents such as epoxides (63%), aromatic nitro compounds (49%) and primary alkyl monoha-

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(1) Ashby, J.; Tennant, R. W. Definitive Relationships among Chemical Structure, Carcinogenicity and Mutagenicity for 301 Chemicals Tested by the U.S. NTP. *Mutat. Res.* **1991**, *257*, 229–306.

(2) EU Guideline of Limits of Genotoxic Impurities, http://www.ema.europa.eu/pdfs/human/swp/519902en.pdf. Q&A supplement, http://www.ema.europa.eu/pdfs/human/swp/43199407en.pdf.

(3) Safety Evaluation of Certain Food Additives and Contaminants, WHO Food Additives Series 40; http://www.inchem.org/documents/jecfa/jecmono/v040je17.htm.

(4) Bossa, C.; Worth, A.; Netzeva, T.; Jeliazkova-Nikolova, N.; Benigni, R. A New Model for Predicting Mutagens/Carcinogens Implemented in Toxtree v1.4; http://www.scarlet-project.eu/posters/Bossa_C-scarlet.pdf.

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Table 1. **Bacterial reverse mutation assay data7 (*Salmonella typhimurium*) on representative sulfonamides, carboxylic acid amides, nitriles, halobenzenes, and primary aliphatic amines**

compound	CASRN	result	*S. typhimurium* strains
2-toluenesulfonamide	88-19-7	negative	TA98, 100, 1535, 1537, 1538
sulfanilamide	63-74-1	negative	TA98, 100, 1535, 1537, 1538
benzamide	55-21-0	negative	TA97, 98, 100, 1535,
stearic acid amide	124-26-5	negative	TA98, 100, 1535, 1537
acetonitrile	75-05-08	negative	TA98, 100, 1535, 1537
benzonitrile	100-47-0	negative	TA97, 98, 100, 1535, 1537, 1538
chlorobenzene	108-90-7	negative	TA98, 100, 1535, 1537
1,2-dichlorobenzene	95-50-1	negative	TA98, 100, 1535, 1537, UTH8413
bromobenzene	108-86-1	negative	TA98, 100, 1535, 1537
methylamine	74-89-5	negative	TA98, 100, 1535, 1537
ethylamine	75-04-7	negative	TA98, 100, 1535, 1537
1-butylamine	109-73-9	negative	TA98, 100, 1535, 1537
ethanolamine	141-43-5	negative	TA98, 100, 1535, 1537, 1538

lides (46%). Data on chloroalkanes and bromoalkanes are not reported separately. The publication contains several substructures (including sulfonamides, carboxylic acid amides, nitriles, halobenzenes and primary aliphatic amines) not normally associated with genotoxic activity; these structural features are not included in the ECB list and data shown in Table 1 indicate that representative compounds are all Ames-negative. The two simplest carboxylic acid amides (formamide and acetamide), although Ames-negative, show carcinogenic activity in rodents which, in the case of acetamide, may be due to the formation of hydroxylamine as an *in vivo* metabolite.[6] For carboxylic acid amides therefore, it is misleading to characterise the structural class as "alerting" based on what are essentially special circumstances in the cases of the simplest members of the class (typically containing 1−3 carbon atoms). Similar considerations applying to carbamate esters are discussed below.

It is not uncommon for some regulatory assessors to misdesignate particular structural features as an alert. For example queries are often raised on potential residues of methanesulfonic acid and *p*-toluenesulfonic acid, both of which have no structural alert and are Ames-negative.[7]

In a retrospective analysis of starting materials and intermediates involved in active pharmaceutical ingredient (API) synthesis,[8] the most common structurally alerting groups were found to be aromatic amines, aromatic nitros, alkylating agents and Michael acceptors. Other alerting groups occurring less frequently included acid halides, epoxides, hydrazines and oximes.

Testing for Genotoxicity and Carcinogenicity

The standard Ames bacterial reverse mutation assay is typically undertaken in up to 5 strains of *Salmonella typhimurium* (TA 98, 100, 1535, 1537, 1538) and possibly in *Escherichia coli* WP2 uvrA. Maximum plate loadings are 5−10 mg, and a minimum loading of 250 μg/plate is recommended for testing of potential genotoxic impurities (PGIs).[2] The microsomal fraction (S9 mix) of Aroclor 1254-induced rat liver homogenate is used as the default metabolic activating system, tests being conducted in the presence and absence of S9 (±S9). The assay is normally undertaken using plate incorporation of the test substance; a preincubation technique (in which there is an initial incubation period before plating onto minimal agar) can also be used. Closely similar results are normally obtained using either plate incorporation or preincubation assays although differences can occur in some cases. For example, crotonaldehyde (2-butenal) is Ames-positive only in preincubation assays,[9] suggesting that detoxification mechanisms need to be eliminated/saturated in order to obtain a positive response. The relevant OECD testing guideline (OECD 471)[10] indicates that both approaches should be used in cases where unexpected or equivocal results are obtained.

Mutagens compared to nonmutagens are more likely to be carcinogenic, more likely to induce tumours at multiple target sites and more likely to be carcinogenic in two species.[11] Genotoxic carcinogens are expected to exhibit a dose−response in terms of tumour incidence, whereas nongenotoxic carcinogens have to be administered at greater than a threshold dose in order to generate a tumorigenic response. This categorisation can break down since some so-called "genotoxic carcinogens", particularly reactive site-of-contact carcinogens such as vinyl acetate,[12] show clear evidence for a threshold.

In vivo assays (normally in rodents) for genotoxicity or carcinogenicity are carried out at the maximum practicable dose or the maximum tolerated dose (MTD) in order to minimise the likelihood of obtaining a false-negative result. The use of such high doses is considered to compensate to some extent for the relatively small numbers of animals used in carcinogenicity studies (normally 50 rodents/sex/group). For nongenotoxic compounds, or for genotoxic compounds subject to rapid detoxifying metabolism, two of the main potential consequences of administering such "heroic" doses are:

- Cytotoxicity (producing regenerative tissue repair with enhanced cell proliferation and turnover)
- Metabolic overload (leading to saturation of normal clearance mechanisms and/or aberrant high-dose metabolism producing toxic entities such as free radicals).

Since the majority of chemicals, many of which are nongenotoxic, tested adequately at the MTD demonstrate

(5) Sawatari, K.; Nakanishi, Y.; Matsushima, T. Relationships between Chemical Structures and Mutagenicity: A Preliminary Survey for a Database of Mutagenicity Test Results of New Work Place Chemicals. *Ind. Health* **2001**, *39*, 341–345.

(6) Sakano, K.; Oikawa, S.; Hiraku, Y.; Kawanishi, S. Mechanism of Metal-Mediated DNA Damage Induced by a Metabolite of Carcinogenic Acetamide. *Chem. Biol. Interact.* **2004**, *149*, 52–59.

(7) Chemical Carcinogenesis Research Information System (CCRIS); http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS.

(8) Dobo, K. L.; Greene, N.; Cyr, M. O.; Caron, S.; Ku, W. W. The Application of Structure-Based Assessment to Support Safety and Chemistry Diligence to Manage Genotoxic Impurities in Active Pharmaceutical Ingredients during Drug Development. *Regul. Toxicol. Pharmacol.* **2006**, *44*, 282–293.

(9) Concise International Chemical Assessment Document 74: 2-Butenal: http://www.inchem.org/documents/cicads/cicads/cicad74.pdf.

(10) Organisation for Economic Co-operation and Development (OECD) Guideline for Testing of Chemicals, No 471, Bacterial Reverse Mutation Test: http://www.oecd.org/dataoecd/18/31/1948418.pdf.

(11) Gold, L. S.; Slone, T. H.; Stern, B. R.; Bernstein, L. Comparison of Target Organs of Carcinogenicity for Mutagenic and Non-mutagenic Chemicals. *Mutat. Res.* **1993**, *286*, 75–100.

(12) Hengstler, J. G.; Bogdanffy, M. S.; Bolt, H. M.; Oesch, F. Challenging Dogma: Thresholds for Genotoxic Carcinogens? The Case of Vinyl Acetate. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 485–520.

carcinogenicity, the response may be due in large part to one or more nearly universal modes of action, such as regenerative cell replication at the MTD rather than due to some unique carcinogenic property of a chemical.[13] It is well established that dose can have a profound effect on mechanism, and so a carcinogenic effect observed at a high dose is not necessarily expected to occur at lower doses.[14] Bioassay results can exhibit major discordancies across rodent strains, species and genders, and most of this variability remains unexplained.[15] For example, aniline produced no positive response in an oral bioassay in male rats at doses up to 60 mg/kg/day,[16] whereas oral aniline hydrochloride at doses from 115 to 286 mg/kg/day tested positive in the rat, particularly in males (TD_{50} 269 mg/kg/day), and was noncarcinogenic in mice at doses up to 1500 mg/kg/day.[17]

Employing alternative species such as fish for carcinogenicity testing can make the use of larger group sizes and lower doses much more practicable. A study in over 40,000 rainbow trout using ultralow doses of dibenzo[a,l]pyrene (DBP) resulted in estimates of the doses producing a cancer incidence of 1 in a million (the normal regulatory threshold in the USA for concerns over carcinogenicity) that were 500−1500-fold higher than those predicted by linear extrapolation from much higher doses administered to rodents.[18]

In summary, the current rodent carcinogenicity testing paradigm seems heavily biased towards producing (false) positive results, caused mainly by the application of the MTD concept, and high-to-low-dose linear extrapolation techniques commonly employed in regulatory toxicology seem likely to overestimate human carcinogenic risk by several orders of magnitude.

Carcinogenic Potency

By analogy with the well-known LD50 ('lethal dose 50'), the concept of a 'tumorigenic dose 50' (TD_{50}) was first proposed by Sawyer et al[19] as an index of carcinogenic potency. A low value of TD_{50} indicates a potent carcinogen, whereas a high value indicates a weak one. The TD_{50} can be defined (in the absence both of tumours in the control group and of intercurrent deaths) as that (daily) dose of chemical which gives 50% of the test animals tumours by some fixed age. Since the tumour(s) of interest often does occur in control animals, TD_{50} is more precisely defined as: that dose-rate in mg/kg/day which, if administered chronically for the standard lifespan of the species, will halve the probability of remaining tumourless throughout that period. The use of correction factors is necessary when an experiment is terminated before the standard lifespan of the test species. TD_{50} values are available in the Carcinogenic Potency Database (CPDB);[20] headline values shown for each species in a summary table are averages calculated by taking the harmonic mean of the most potent TD_{50} values from among target sites in each positive experiment. Thus, the harmonic mean provides a "weight of evidence" TD_{50} value for a particular compound. In contrast, use of the most potent site (lowest statistically significant TD_{50} value in any experiment) would reflect only results from a single experiment. Use of a single-point value can also significantly distort the distinction between carcinogens and noncarcinogens. For example, whilst endosulfan, rotenone and 3-nitropropionic acid are considered to be noncarcinogenic in the CPDB, use of the lowest TD_{50} values effectively redesignates these compounds as high-potency "carcinogens" (TD_{50}s being <2.5 mg/kg/day).

TTC (Threshold of Toxicological Concern) Concept

In the context of EU guidance on genotoxic impurities the TTC is considered to be a pragmatic risk management tool using a probabilistic methodology. There is a high probability that a 10^{-5} lifetime cancer risk will not be exceeded if the daily intake of a genotoxic impurity with unknown carcinogenic potential/potency is below the TTC value.[2] The default TTC for genotoxic impurities in pharmaceuticals is 1.5 μg/day (based on a cancer risk at a probability of 10^{-5}), although a lower value (possibly 10-fold lower) is recommended for high-potency carcinogens (such as *N*-nitrosamines, aflatoxins and azoxy compounds) and higher values (based for example on the "staged TTC" concept) may be acceptable in some circumstances such as established dietary exposure, life-threatening indication such as cancer (often treated with highly toxic/genotoxic drugs), limited life expectancy and/or duration of exposure.

The derivation of the default TTC for genotoxic impurities is critically dependent on one publication[21] that claims to be based on TD_{50} data for 730 "carcinogens" in relation to risk assessment of food mutagens/carcinogens using a cancer probability value of 10^{-6} (compared to 10^{-5} for pharmaceutical impurities). A process of linear extrapolation (i.e. dividing TD_{50} values by 500,000) was used to estimate cancer risk at a probability of 10^{-6} for different groups of substances. However, the data set and the methodology involved are nontransparent particularly in respect of:

- The precise compounds evaluated;
- The representativeness of the data set;
- The TD_{50} values employed.

(13) Gaylor, D. W. Are Tumor Incidence Rates from Chronic Bioassays Telling Us What We Need to Know about Carcinogens? *Regul. Toxicol. Pharmacol.* **2005**, *41*, 128–133.
(14) Counts, J. L.; Goodman, J. I. Principles Underlying Dose Selection for, and Extrapolation from, the Carcinogen Bioassay: Dose Influences Mechanism. *Regul. Toxicol. Pharmacol.* **1995**, *21*, 418–421.
(15) Knight, A.; Bailey, J.; Balcombe, J. Animal Carcinogenicity Studies: 2. Obstacles to Extrapolation of Data to Humans. *ATLA, Altern. Lab Anim.* **2006**, *34*, 29–38.
(16) Carcinogenic Potency Database: Aniline; http://potency.berkeley.edu/chempages/aniline.html.
(17) Carcinogenic Potency Database: Aniline Hydrochloride; http://potency.berkeley.edu/chempages/aniline.HCl.html.
(18) Bailey, G. S.; Reddy, A. P.; Pereira, C. B.; Harttig, U.; Baird, W.; Spitsbergen, J. M.; Hendricks, J. D.; Orner, G. A.; Williams, D. E.; Swenberg, J. A. Nonlinear Cancer Response at Ultralow Dose: a 40800-Animal ED(001) Tumor and Biomarker Study. *Chem. Res. Toxicol.* **2009**, *22*, 1264–1276.
(19) Sawyer, C.; Peto, R.; Bernstein, L.; Pike, M. C. Calculation of Carcinogenic Potency from Long-Term Animal Carcinogenesis Experiments. *Biometrics* **1984**, *40*, 27–40.
(20) Carcinogenic Potency Database; http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CPDB.htm.
(21) Kroes, R.; Renwick, A. G.; Cheeseman, M.; Kleiner, J.; Mangelsdorf, I.; Piersma, A.; Schilter, B.; Schlatter, J.; van Schothorst, F.; Vos, J. G.; Würtzen, G. Structure-Based Thresholds of Toxicological Concern (TTC):Guidance for Application to Substances Present at Low Levels in the Diet. *Food Chem. Toxicol.* **2004**, *42*, 65–83.

Furthermore, based on information contained in an earlier similar publication[22] it seems highly likely that the lowest statistically significant point estimates for TD_{50}s were employed, which is particularly ironic in view of the fact that the EU guideline on limits of genotoxic impurities[2] emphasises the use of weight-of-evidence approaches in the interpretation of *in vivo* genotoxicity data.

Additional concerns relate to overconservatism introduced by use of linear extrapolation and the skewed nature of the CPDB data set which contains >50% of carcinogens. Moreover, there has been no attempt to incorporate any element of "calibration" based on a highly mutagenic compound such as ethyl methanesulfonate (EMS) which exhibits a threshold dose for unrepaired DNA alkylation in mice of 25 mg/kg/day, equivalent to a human dose of 2 mg/kg/day[23] (approximately 70,000 times the default TTC).

In spite of the serious criticisms that can be made regarding the validity and integrity of the current default numerical TTC value applied to genotoxic impurities, changes seem highly unlikely in the short term, although the creation of an ICH guideline on genotoxic impurities could present an opportunity for the major concerns to be raised. For the foreseeable future therefore, it seems prudent to assume a default TTC of 1.5 μg/day when dealing with genotoxic impurities.

Genotoxic Impurity: Definition

In the EU guidance[2] there is no comprehensive definition of "genotoxic impurity". Instead a statement is provided on what compounds might be classified as genotoxic:

"In the current context the classification of a compound (impurity) as genotoxic in general means that there are positive findings in established *in vitro* or *in vivo* genotoxicity tests with the main focus on DNA reactive substances that have a potential for direct DNA damage. Isolated *in vitro* findings may be assessed for *in vivo* relevance in adequate follow-up testing. In the absence of such information *in vitro* genotoxicants are usually considered as presumptive *in vivo* mutagens and carcinogens."

The Q&A supplement to the EU guideline contains additional clarification:

"...a negative Ames test (conducted to regulatory acceptable standards) will overrule a structural alert and no further studies would be required providing the level remains below ICH Q3A/B limits."

Thus, any Ames-positive impurity will be potentially DNA-reactive and so would always be considered as a "genotoxic impurity". Conversely, an Ames-negative compound should (almost) always be disqualified from being categorized as a "genotoxic impurity" within the context of the EU guidance. Issues of classification and perception can arise however with Ames-negative compounds that give positive results in other mutagenicity tests such as the mouse lymphoma assay (MLA), *in vitro* chromosomal aberration assay and *in vivo* micronucleus assay. At the very least Ames-negative compounds that are solely *in vitro* clastogens should be eliminated from the definition of "genotoxic impurity" since such substances lack DNA reactivity. Moreover, numerous Ames-negative drug substances are *in vitro* clastogens; in a recent publication[24] the genotoxicity status of small-molecule drug substances listed in the *Physicians' Desk Reference* (excluding drugs that exhibit class-specific genotoxicity such as anticancers, antivirals, nucleoside analogues and steroids) has been evaluated. Out of a total of 545 marketed drug substances the proportion (percentage) of positive responses was as follows: *in vitro* chromosome aberrations: 88/380 (26.1%); MLAs: 32/163 (19.1%), *in vivo* cytogenetics: 49/438 (11.1%); bacterial reverse mutation assays: 38/525 (7.1%). It is clear that a significant proportion of Ames-negative drug substances show *in vitro* clastogenicity. Furthermore, the MLA has, like all mammalian cell assays, extremely low specificity[25] and produces a high rate of false positives (in terms of predicting carcinogenic potential).[26] Overall, the result of Ames testing is overwhelmingly the most important criterion of in terms of "genotoxic impurity" classification; for an Ames-negative compound, positive *in vitro* clastogenicity results can probably be discounted, although any other positive test results should be thoroughly evaluated (for example in relation to dose−response, cytotoxicity and evidence for a threshold) before deciding on the classification of a specific impurity.

If an Ames-negative compound shows some (high-dose) carcinogenic activity in rodent bioassays, in no way does this mean that the substance should be categorized as a "genotoxic impurity". It should be evaluated as a conventional impurity using ICH Q3A/B/C criteria as appropriate. For example, five of the solvents listed in ICH Q3C (R2) (1,4-dioxane, ethanol, hexane, methyl *t*-butyl ether and toluene) produce tumours at high doses in rodents,[20] but are nevertheless Ames-negative.

Terminology

Two similar terms, both with the same "PGI" abbreviation, have been used to in relation to impurities with a potential for genotoxic activity and also with potential for being present in an API:

• Potentially genotoxic impurity: this term, used by the European Pharmacopeia (PhEur),[27] indicates a structurally alerting impurity (related-substance impurity in the context of the PhEur) known or highly likely to be present in an API. This type of PGI is expected to be a named impurity in the relevant PhEur monograph.

• Potential genotoxic impurity: most commonly this term is employed to describe a structurally alerting or confirmed

(22) Cheeseman, M. A.; Machuga, E. J.; Bailey, A. B. A Tiered Approach to Threshold of Regulation. *Food Chem. Toxicol.* **1999**, *37*, 387–412.

(23) Müller, L.; Gocke, E.; Lavé, T.; Pfister, T. Ethyl Methanesulfonate Toxicity in Viracept: A Comprehensive Human Risk Assessment Based on Threshold Data for Genotoxicity. *Toxicol. Lett.* **2009**, *190*, 317–29.

(24) Snyder, R. D. An Update on the Genotoxicity and Carcinogenicity of Marketed Pharmaceuticals with Reference to in Silico Predictivity. *Environ. Mol. Mutagen.* **2009**, *50*, 435–450.

(25) Kirkland, D.; Aardema, M.; Henderson, L.; Müller, L. Evaluation of the Ability of a Battery of Three in Vitro Genotoxicity Tests to Discriminate Rodent Carcinogens and Non-Carcinogens I. Sensitivity, Specificity and Relative Predictivity. *Mutat. Res.* **2005**, *584*, 1–256.

(26) Caldwell, J. Perspective on the Usefulness of the Mouse Lymphoma Assay As an Indicator of a Genotoxic Carcinogen: Ten Compounds Which Are Positive in the Mouse Lymphoma Assay but Are Not Genotoxic Carcinogens. *Teratog. Carcinog. Mutagen.* **1993**, *13*, 185–190.

(27) Potentially Genotoxic Impurities and European Pharmacopoeia Monographs on Substances for Human Use; http://www.edqm.eu/medias/fichiers/NEW_Potentialy_genotoxic_impurities_PhEur_monographs.pdf.

genotoxic reagent or intermediate used in an API synthesis but with no clear information on whether there is any carry-over to the drug substance. Such PGIs are normally controlled by showing essential absence in the drug substance using a limit of detection below the TTC or other justified concentration limit.

There is however an element of overlap for the two related terms; in this article the principal use of PGI is in relation to potential genotoxic impurities.

Approaches to Qualification and Specification Setting for GIs and PGIs

Identified Impurity in Drug Substance or Drug Product. If the impurity is a known compound and has a structural alert for genotoxicity, Ames-test data may be available in the public domain. Alternatively, if no published data are available and/or the compound is not known (by searching on PubChem for example), it is acceptable to assume that the compound is genotoxic in the Ames assay and control accordingly (in most cases using the default TTC limit of 1.5 μg/day), or an Ames test could be undertaken. Options for a compound testing positive in the Ames assay are:

- Control at TTC level
- Check for availability of additional relevant data in the public domain (particularly rodent bioassay data)
- Undertake additional genotoxicity studies (compound-specific programme).

If the impurity is Ames-negative (based on published or experimental results) then it can be controlled at the appropriate ICH Q3A/B (R2) qualification threshold, for example at 0.15% for drug substances used at up to 667 mg/day. If a substantial amount of toxicological data is available it may be possible to determine a PDE (Permitted Daily Exposure) based on published independent expert assessments (for example by the U.S. Environmental Protection Agency) or based on methodology described in the ICH Q3C (R3) guideline on residual solvents. It is also possible to calculate a PDE from TD_{50} data (if available) as shown below.

Options for dealing with impurities without a structural alert for genotoxicity include:

- Reading across from an Ames-negative drug-substance structure; if the impurity shows no structural alerts and contains no additional structural features compared to those present in the drug substance, it is highly likely that the impurity is nongenotoxic.
- Commissioning an *in silico* evaluation (using DEREK for example); this is now an acceptable approach[2] to confirm an *in cerebro* determination of the absence of structural alerts.
- Use of published or in-house confirmatory genotoxicity data (if available); this may be necessary in some cases to convince agencies that have a degree of scepticism over the predictivity of *in silico* evaluations.

The output of an *in silico* assessment should be interpreted with care in order to distinguish between "hits" representing potential DNA reactivity and those associated with possible clastogenic activity.

As shown in a previous section, the TTC concept is based on linear extrapolation of TD_{50} values (in mg/kg/day) for groups of compounds, and so based on this precedent it should be justifiable to determine PDEs for *individual* compounds using a similar process. Assuming a patient body weight of 50 kg and a cancer risk at a probability of 10^{-5}.

$$\mathrm{PDE}(\mu\mathrm{g/day}) = (\mathrm{TD50} \times 50)/50{,}000 = \mathrm{TD50} \times 10^{-3}$$

In other words, the PDE for a compound for which a TD_{50} value is available has the same numerical value as the TD_{50} but in units of μg/day. In view of the overestimation of risk built into the linear extrapolation process, PDEs obtained in this manner should be considered to be highly conservative estimates of safe human doses. For example, Müller and Gocke[28] cite a TD_{50} value for EMS of 11 mg/kg/day (implying a PDE of 11 μg/day) whereas use of compound-specific toxicokinetic data in conjunction with the human-equivalent threshold dose for DNA alkylation of 2 mg/kg/day suggests a PDE in the region of 10−100 mg/day. Use of compound-specific data on substances that might be considered to be of high potency, could produce a PDE much higher than the (implied) default value of 0.15 μg/day, 1-nitrosohydantoin (TD_{50} 43.8 mg/kg/day[29]) being one example.

Potential Carryover of Reagents/Intermediates with Structural Alerts. Since the introduction of the EU guideline on genotoxic impurities an increasing number of regulatory reviewers have focused their attention on reagents/intermediates used in API synthesis. A typical synthesis could use various reactive reagents such as acid chlorides and alkylating agents and starting materials/intermediates might contain aromatic amine or epoxide groups. For some synthetic routes there could be up to a dozen or more PGIs that could, in theory, be present as impurities in the API. Control of PGIs normally requires appropriate qualification and/or specification setting in order to meet regulatory requirements. Structurally alerting compounds, particularly highly reactive reagents, employed in the early stages of a complex synthesis are unlikely to be carried over to the API. Pierson et al[30] argue that most genotoxic reagents/intermediates present at four or more stages in the synthesis prior to API isolation/purification are likely to be deactivated owing mainly to reaction with other reagents and/or dissolution in solvents, or removed by vacuum distillation/purging procedures. However, reviewers generally show little sympathy with such arguments[31] and quantitative analytical information on the API and/or intermediates possibly combined with the results of spiking experiments (impurity fate analysis) could well be required in order to demonstrate the absence of carryover of PGIs at a suitably low (TTC) level. Such investigations can be highly resource-intensive and challenging, particularly in respect of developing validated analytical meth-

(28) Müller, L.; Gocke, E. Considerations Regarding a Permitted Daily Exposure Calculation for Ethyl Methanesulfonate. *Toxicol. Lett.* **2009**, *190*, 330–2.

(29) Carcinogenic Potency Database: 1-Nitrosohydantoin; http://potency.berkeley.edu/chempages/1-nitrosohydantoin.html.

(30) Pierson, D. A.; Olsen, B. A.; Robbins, D. K.; DeVries, K. M.; Varie, D. L. Approaches to Assessment, Testing Decisions, and Analytical Determination of Genotoxic Impurities in Drug Substances. *Org. Process Res. Dev.* **2009**, *13*, 285–291.

(31) Ganapthy, M. Control of Genotoxic Impurities and the Regulatory Impact of This: A Case Study; AAPS Annual Meeting, 12th November 2009; http://mediaserver.aapspharmaceutica.com/meetings/09AM/Slides/11.12.09_Thu/409%20AB/1330/Ganapathy%20Mohan.pdf.

ods for low levels of multiple PGIs.[32−34] Moreover, limits for certain PGIs may become part of the API specification and so become an ongoing quality-control commitment.

PGIs feature in a recent publication[35] reviewing quality concerns raised during the assessment of applications made via the EU Centralised Procedure: "Issues on potential genotoxic impurities have been identified by the CHMP as Major Objections due to confirmed "alerting structures" present in the starting materials and intermediates of drug substances". A review of EU withdrawal assessment reports suggests that the major objections based on PGIs relate to potential residues of mesityl oxide (4-methyl-3-penten-2-one) in a drug substance crystallised from acetone[36] and to possible traces of alkyl mesylates (methyl, ethyl and isopropyl mesylates) in a mesylate salt drug substance.[37]

Modification of the API synthesis in a way that minimises PGI levels in the drug substance has been undertaken in some cases; recent published examples have featured formaldehyde,[30] chloroalkanes[38] and acetamide.[39] Since acetamide is Ames-negative it is a moot point as to whether it should be considered as a "genotoxic impurity"; it is however a modest-potency carcinogen with a TD_{50} value of 180 mg/kg/day.[40]

Before committing extensive resources to the development of analytical methods and/or synthesis modification, it is highly advisible to evaluate whether a compound-specific toxicological risk assessment can be made for each PGI. Many PGIs are likely to be commonly employed synthetic reagents for which Ames-test results and other data may be available in the public domain.[41] Basing PGI limits on TTC or staged-TTC criteria without the benefit of a comprehensive toxicological assessment could be unnecessarily constraining in terms of the implied specification level (rather than using the appropriate ICH Q3A/B (R2) qualification threshold value for example). In turn this can affect the development cost/sophistication of appropriate analytical methods; use of mass spectrometry (MS) single-ion monitoring may be required to detect impurity levels determined using the standard TTC limit of 1.5 μg/day whereas conventional techniques using HPLC or gas chromatography may be perfectly adequate if a compound-specific limit of several hundred micrograms/day can be justified.

In view of these considerations, the following ranking of activities is recommended when dealing with PGIs associated with a particular API synthesis:

- Comprehensive toxicological risk assessment based on published information (if available) or on *in silico* evaluations
- Development of validated assays of appropriate sensitivity based on (tentative) safe limits determined as above in combination with maximum daily dose of API
- Impurity fate analysis, as appropriate
- Modifications to synthetic route, as appropriate and if necessary.

Dealing with highly reactive PGIs, including commonly used reagents such as thionyl chloride, acetyl chloride, chloroacetyl chloride, methanesulfonyl chloride and ethyl chloroformate, can be problematic. The results of Ames testing can be dependent on the solvent employed and, unfortunately, information on genotoxicity shown in Material Safety Data Sheets (MSDSs) may not specify the solvent that was used. DMSO has been shown to react with carboxylic and sulfonic acid chlorides[42] to form genotoxic chlorodimethylsulfide. On the other hand, using a much more physiologically relevant solvent such as water gave negative results for acid chlorides (Table 2). Since acid chlorides are likely to be hydrolysed to the corresponding carboxylic or sulfonic acid in the presence of water, some might argue that water is also an inappropriate solvent. One approach to this issue might be to use a nonhydroxylic, nonreactive solvent such as hexane in order to evaluate the intrinsic genotoxicity of acid chlorides. A second test using water as

Table 2. **Ames assay data on some highly reactive acid chlorides used as synthetic reagents[a]**

compound	DMSO solvent	water solvent	other solvents	reference
acetyl chloride	+	−	−(acetone)	42, 128
octanyoyl chloride	+	−	NA	42
benzoyl chloride	+	−	−(acetone)	42, 129
phenylacetyl chloride	+	−	NA	42
chloroacetyl chloride	NA	NA	−(solvent not specified)	130
methanesulfonyl chloride	+	−	NA	42
isopropylsulfonyl chloride	+	−	NA	42
benzenesulfonyl chloride	+	−	NA	42
4-chlorobenzenesulfonyl chloride	+	NA	−(ethanol)	42
ethyl chloroformate	NA	NA	−(solvent not specified)	131

[a] + positive assay; − negative assay; NA = not available; majority of tests carried out in *S. tyhimurium* strains TA98 and TAmix (±S9).[42]

(32) Liu, D. Q.; Sun, M.; Kord, A. S. Recent Advances in Trace Analysis of Pharmaceutical Genotoxic Impurities. *J. Pharm. Biomed. Anal.* **2010**, *51*, 999–1014.

(33) Sun, M.; Bai, L.; Terfloth, G. J.; Liu, D. Q.; Kord, A. S. Matrix Deactivation: a General Approach to Improve Stability of Unstable and Reactive Pharmaceutical Genotoxic Impurities for Trace Analysis. *J. Pharm. Biomed. Anal.* **2010**, *52*, 30–36.

(34) Bai, L.; Sun, M.; An, J.; Liu, D. Q.; Chen, T. K.; Kord, A. S. Enhancing the Detection Sensitivity of Trace Analysis of Pharmaceutical Genotoxic Impurities by Chemical Derivatization and Coordination Ion Spray-Mass Spectrometry. *J. Chromatogr., A* **2010**, *1217*, 302–306.

(35) Borg, J. J.; Robert, J.-L.; Wade, G.; Aislaitner, G.; Pirozynski, M.; Abadie, E.; Salmonson, T.; Vella Bonanno, P. Where is industry getting it wrong? A Review of Quality Concerns Raised at Day 120 by the Committee For Medicinal Products for Human Use during European Centralised Marketing Authorisation Submissions for Chemical Entity Medicinal Products. *J. Pharm. Pharm. Sci.* **2009**, *12*, 181–198.

(36) Arxxant withdrawal assessment report; http://www.ema.europa.eu/humandocs/PDFs/EPAR/arxxant/15096407en.pdf.

(37) Factive withdrawal assessment report; http://www.ema.europa.eu/humandocs/PDFs/EPAR/factive/Factive-WEPAR.pdf.

(38) Yang, Q.; Haney, B. P.; Vaux, A.; Riley, D. A.; Heidrich, L.; He, P.; Mason, P.; Tehim, A.; Fisher, L. E.; Maag, H.; Anderson, N. G. Controlling the Genotoxins Ethyl Chloride and Methyl Chloride Formed During the Preparation of Amine Hydrochloride Salts from Solutions of Ethanol and Methanol. *Org. Process Res. Dev.* **2009**, *13*, 786–791.

(39) Schülé A.; Ates, C.; Palacio, M.; Stofferis, J.; Delatinne, J.-P.; Martin, B.; Lloyd, S. Monitoring and Control of Genotoxic Impurity Acetamide in the Synthesis of Zaurategrast Sulfate. *Org. Process Res. Dev.* **2010**. DOI: 10.1021/op900330e.

(40) Carcinogenic Potency Database: Acetamide; http://potency.berkeley.edu/chempages/ACETAMIDE.html.

(41) Friscia, O.; Pulci, R.; Fassio, F.; Comelli, R. Chemical Reagents As Potential Impurities of Pharmaceutical Products: Investigations on Their Genotoxic Activity. *J. Environ. Pathol. Toxicol. Oncol.* **1994**, *13*, 89–110.

(42) Amberg, A.; Braun, K.; Czich, A.; Kauffmann, H.-M.; Spirkl H.-P.; Stammberger, I.; Troschau, G. Positive Genotoxicity Results Due to the Formation of Genotoxic Reaction-Products of the Solvent DMSO with Carboxylic/Sulfonic Acid Halides. *Toxicological Sciences, The Toxicologist Supplement* **2007**, Abstract No 1661.

solvent would evaluate physiologically relevant genotoxic effects, and, if appropriate, a third test in the presence of a relevant solvent (such as ethanol) used in later stages of the API synthesis would evaluate whether residues of the acid chloride might survive downstream processes such as recrystallisation. This type of approach seems distinctly preferable to analytical determination of residues of highly reactive reagents in APIs or intermediates since the instability of the analyte can lead to significant variability with such assays.

Procedures for Setting Specification Limits and Need for Routine Tests. EU guidance on specification setting is available for some types of impurities including metal catalysts and reagents[43] and residual solvents.[44] For residual solvents concentration data need to be made available on at least six pilot batches or three production batches. Routine tests are not required if a solvent is present at <30% or <10% of the specified limit for Class 1 and Class 2 solvents[45] respectively. Since Class 1 solvents are associated with significant toxicity (e.g., carcinogenicity), their treatment in terms of specification requirements appears to be a clear precedent for omitting routine tests for genotoxic impurities in drug substances if batch analysis data indicate that the concentration is <30% of the appropriate limit.

Use of Decision Trees. A number of decision trees on dealing with GIs and PGIs have been published including those in the EU guideline[2] and the FDA draft guideline[46] and the schemes recommended by Pierson et al[30] and Müller et al.[47] On the one hand such decision trees can be helpful, but on the other hand it is not possible to anticipate all possibilities for all compounds in such schemes; for example, for some compounds the duration of exposure, the patient population/indication and life expectancy may be important, whereas for others (such as crotonaldehyde[9]) dietary exposure may be a critical consideration. Overall, the most reliable approach is likely to involve assembly and collation of all relevant data followed by implementation of a compound-specific qualification strategy.

Genotoxicity and Carcinogenicity Data on Compounds with Common Structural Alerts. Data have been compiled on representative compounds containing some of the structural alerts shown in Figure 1. The principal sources of information are the CPDB[20] and CCRIS.[7]

Epoxides. Epoxide-containing substances from both natural and man-made sources are ubiquitous in the environment and food supply, as well as being generated endogenously by the action of cytochrome P450 epoxygenases on aromatic and olefinic compounds to produce arene and alkene oxides respectively. Epoxides as such are lipophilic and electrophilic, the latter property being related to the chemical reactivity of the strained epoxide ring. Alkene oxides tend to be more reactive than arene oxides and symmetrically substituted epoxides are less reactive than those with an asymmetric substitution pattern (as noted with the epoxides of chlorinated ethylenes).[48]

Table 3. **Genotoxicity and carcinogenic potency data on epoxides listed in the CPDB[a]**

compound	CAS number	Ames *Salmonella*	TD_{50} (mg/kg/day) mouse	TD_{50} (mg/kg/day) rat	route
glycidaldehyde	765-34-4	+	nda	NP	oral gavage
allyl glycidyl ether	106-92-3	+	182	NP	inhalation
D,L-diepoxybutane	298-18-0	+	nda	NP	oral gavage
dicyclopentadiene dioxide	81-21-0	nda	NP	NP	diet
1,2-epoxybutane	106-88-7	+	NP	220	inhalation
ethylene oxide	75-21-8	+	63.7	21.3	inhalation; oral gavage
glycidol	556-52-5	+	34.7	4.28	oral gavage
1,2-propylene oxide	75-56-9	+	912	74.4	inhalation; oral gavage
styrene oxide	96-09-3	+	118	55.4	oral gavage
epichlorohydrin	106-89-8	+	NP	2.96	inhalation; oral gavage

[a] nda = no data available; NP = no positive test.

Many epoxides are genotoxic in bacterial reverse mutation assays, although this is by no means a universal situation. Von der Hude et al (1990)[49] reported that 12/51 epoxides were nongenotoxic in the Ames *Salmonella* assay, Wade et al (1978)[50] reported that highly substituted aliphatic epoxides were Ames-negative and in 1983 Glatt et al[51] reported that the majority of epoxides derived from drugs, steroids and pesticides were Ames-negative. Examples of nongenotoxic epoxides include:

- 1,2-epoxyoctane, 1,2-epoxydecane, epoxycyclooctane, epoxycyclododecane, (+)-limoneoxide, α-pinaneoxide, *trans*-stilbeneoxide, and *cis*-2,3-epoxysuccinic acid.[49]
- caryophyllene oxide, ethylmethyphenylglycidate, *cis*-methylepoxycinnamate.[52]
- carbamazepine-10,11-oxide.[51]

A number of epoxides have produced positive responses in conventional oral rodent bioassays; summary data on bacterial genotoxicity and carcinogenicity for epoxides listed in the CPDB are shown in Table 3. Carcinogenic potencies span a 100-fold range and 3 of the 10 epoxides listed in Table 3 appear to be noncarcinogenic. Although carcinogenicity studies have

(43) Guideline on the Specification Limits for Residues of Metal Catalysts or Metal Reagents; http://www.ema.europa.eu/pdfs/human/swp/444600enfin.pdf.
(44) Specifications for Class 1 and Class 2 Residual Solvents in Active Substances; http://www.ema.europa.eu/pdfs/human/qwp/045003en.pdf.
(45) Impurities. Residual Solvents. ICH Topic Q3C (R3); http://www.emea.europa.eu/pdfs/human/ich/028395en.pdf.
(46) Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079235.pdf.
(47) Müller, L.; Mauthe, R. J.; Riley, C. M.; Andino, M. M.; Beels, C.; De Antonis, D.; DeGeorge, J.; De Knaep, A. G.; Ellison, D.; Fagerland, J. A.; Frank, R.; Fritschel, B.; Galloway, S.; Harpur, E.; Humfrey, C. D. N.; Jacks, A. S.; Jagota, N.; Mackinnon, J.; Mohan, G.; Ness, D. K.; O'Donovan, M. R.; Smith, M. D.; Vudathala, G.; Yotti, L. A Rationale for Determining, Testing, and Controlling Specific Impurities in Pharmaceuticals That Possess Potential for Genotoxicity. *Regul. Toxicol. Pharmacol.* **2006**, *44*, 198–211.
(48) Manson, M. M. Epoxides--Is There a Human Health Problem. *Br. J. Ind. Med.* **1980**, *37*, 317–336.
(49) von der Hude, W.; Seelbach, A.; Basler, A. Epoxides: Comparison of the Induction of SOS Repair in Escherichia coli PQ37 and the Bacterial Mutagenicity in the Ames Test. *Mutat. Res.* **1990**, *231*, 205–218.
(50) Wade, D. R.; Airy, S. C.; Sinsheimer, J. E. Mutagenicity of Aliphatic Epoxides. *Mutat. Res.* **1978**, *58*, 217–223.
(51) Glatt, H.; Jung, R.; Oesch, F. Bacterial Mutagenicity Investigation of Epoxides: Drugs, Drug Metabolites, Steroids and Pesticides. *Mutat. Res.* **1983**, *111*, 99–118.
(52) Epoxides (as flavouring agents); http://www.inchem.org/documents/jecfa/jecmono/v56je12.pdf.

been reported on various other epoxides,[48] the majority are pre-GLP involving topical or subcutaneous administration, and it is difficult to assess the reliability of the results.

Several epoxides are carcinogenic only at the point of administration. For example, when given by oral gavage, both ethylene oxide and propylene oxide caused late-onset tumours only in the rat forestomach.[53] When administered by inhalation, propylene oxide is a nasal carcinogen at doses causing glutathione depletion in the affected tissue, suggesting that a threshold mechanism could well apply[54] in a similar manner to that established for vinyl acetate.[55]

A number of epoxides are used as food flavourings; a recent review by the Joint Expert Committee on Food Additives (JECFA) on nine epoxide flavourings[52] (five of which occur naturally in foodstuffs) concluded that they do not present a safety concern at current estimated human intakes (approximately 15 mg/day in total; up to 90% of this is accounted for by ethyl methylphenylglycidate which has a JECFA ADI of 0−0.5 mg/kg/day). The three food-flavouring epoxides that have been evaluated in the Ames *Salmonella* assay (including ethyl methylphenylglycidate) produced negative results.

With a few exceptions, for example in respect of skin sensitisation, epoxides that are formed *in vivo* tend to have a greater potential to cause adverse effects than preformed epoxides present in food and the environment. Highly reactive epoxides are likely to interact with nucleophilic molecules, such as proteins in food, and not be absorbed in their active form. In addition they are readily degraded *in vivo* and would not be expected to reach target organs remote from the site of administration. Epoxides formed *in vivo*, such as those generated by epoxidation of alkenes and arenes, are often produced at close proximity to their site of action and so can reach their target quite readily (by diffusion for example). The principal routes of detoxification of preformed epoxides and those produced by (hepatic) metabolism involve:

- hydration by epoxide hydrolase (EH, EC 3.3.2.3)
- reaction with glutathione catalysed by glutathione *S*-transferase (GST, EC 2.5.1.18).

EH comprises a large and heterogeneous group of enzymes which effectively adds a molecule of water to an epoxide to produce a *trans* vicinal diol. EHs are located in the endoplasmic reticulum (microsomal epoxide hydrolase, mEH) and as soluble cytosolic enzymes (soluble epoxide hydrolase, sEH) in most mammalian cells.[56,57] In general, epoxide hydration leads to more stable and less reactive intermediates. In addition to their role in detoxification of genotoxic compounds, EHs are involved in the control of physiological signaling molecules.[58] mEHs act on their epoxide substrates by creating covalent enzyme−substrate complexes that are generated much faster, by several orders of magnitude, than the rate of subsequent hydrolysis leading to formation of terminal diols.[59] In normal circumstances epoxide levels *in vivo* would be expected to be extremely low and toxicologically insignificant, but at high epoxide concentrations mEH can be titrated out increasing the potential for genotoxic effects. Thus, a threshold is likely to apply for most epoxides in relation to *in vivo* genotoxicity and carcinogenicity, and data from high-dose rodent bioassays should be extrapolated with caution when applied to the risk assessment of low doses of exogenous epoxides. Furthermore EH activity varies across species being higher in the rat than the mouse, and higher still in primates, which has led to the conclusion that 1,3-butadiene is likely to be much less toxic in humans than in mice.[60] Similar observations regarding the much higher rate of epoxide hydrolysis in human compared to rat liver and its relevance to risk assessment have been made regarding dietary allylbenzene analogues.[61]

GSTs (human) are a multigene family of enzymes that are involved in the metabolism of a wide range of compounds of both exogenous and endogenous origin. They act principally as detoxifying enzymes by catalyzing the conjugation of electrophilic compounds, such as epoxides, with glutathione. The mammalian GSTs have been categorised in four classes of cytosolic enzymes and two membrane-bound enzymes. The cytosolic GSTs are the major forms involved in xenobiotic metabolism comprising 95% of total cellular GSTs and are capable of conjugating relatively hydrophilic electrophilic molecules.[62] Various types of reactions with glutathione can occur at electrophilic carbon including displacement reactions (e.g., halides, sulfonates, phosphates), opening of strained-ring structures (e.g., epoxides, β-lactones) and Michael addition to activated double bonds (e.g., α,β-unsaturated ketones). In addition, glutathione can interact directly with electrophilic nitrogen, sulfur and oxygen in specific cases.[63] In the case of epoxides however, glutathione conjugation tends to be a less important detoxification mechanism than EH-catalysed hydration since mEH has a higher affinity for many lipophilic epoxide substrates than GST. The balance between EH- and GST-catalysed detoxification might well change at higher epoxide doses when EH activity may be significantly depleted.

Several chemical examples of *in vivo* epoxygenase-catalysed formation of epoxides have been mentioned above (e.g.,

(53) Dunkelberg, H. Carcinogenicity of Ethylene Oxide and 1,2-Propylene Oxide upon Intragastric Administration to Rats. *Br. J. Cancer* **1982**, *46*, 924–933.

(54) Albertini, R. J.; Sweeney, L. M. Propylene Oxide: Genotoxicity Profile of a Rodent Nasal Carcinogen. *Crit. Rev. Toxicol.* **2007**, *37*, 489–520.

(55) Hengstler, J. G.; Bogdanffy, M. S.; Bolt, H. M.; Oesch, F. Challenging Dogma: Thresholds for Genotoxic Carcinogens? The Case of Vinyl Acetate. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 485–520.

(56) Morisseau, C.; Hammock, B. D. Epoxide Hydrolases: Mechanisms, Inhibitor Designs, and Biological Roles. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 311–333.

(57) Newman, J. W.; Morisseau, C.; Hammock, B. D. Epoxide Hydrolases: Their Roles and Interactions with Lipid Metabolism. *Prog. Lipid Res.* **2005**, *44*, 1–51.

(58) Arand, M.; Cronin, A.; Adamska, M.; Oesch, F. Epoxide Hydrolases: Structure, Function, Mechanism, and Assay. *Methods Enzymol.* **2005**, *400*, 569–588.

(59) Oesch, F.; Herrero, M. E.; Hengstler, J. G.; Lohmann, M.; Arand, M. Metabolic Detoxification: Implications for Thresholds. *Toxicol Pathol.* **2000**, *28*, 382–387.

(60) Henderson, R. F. Species Differences in the Metabolism of Olefins: Implications for Risk Assessment. *Chem. Biol. Interact.* **2001**, *135−136*, 53–64.

(61) Guenthner, T. M.; Luo, G. Investigation of the Role of the 2′,3′-Epoxidation Pathway in the Bioactivation and Genotoxicity of Dietary Allylbenzene Analogs. *Toxicology* **2001**, *160*, 47–58.

(62) Seidegård, J.; Ekström, G. The Role of Human Glutathione Transferases and Epoxide Hydrolases in the Metabolism of Xenobiotics. *Environ. Health Perspect.* **1997**, *105* (Suppl. 4), 791–799.

(63) Testa, B.; Krämer, S. D. The Biochemistry of Drug Metabolism: An Introduction: Part 4. Reactions of Conjugation and Their Enzymes. *Chem. Biodivers.* **2008**, *5*, 2171–2336.

allylbenzenes and 1,3-butadiene), and many other olefins and arenes are known to be metabolised through epoxide intermediates including cyclohexene, styrene, stilbene and benzo(a)pyrene.[48] The latter compound undergoes a double epoxidation and monohydration to form the 7,8-dihydrodiol-8,10-oxide which appears to be too hydrophilic to be effectively hydrated by EH.[64] A number of pharmaceutical actives have been shown to form relatively stable epoxide metabolites that may be excreted in urine; these include carbamazepine, cyproheptadine and protriptyline. On the other hand, based on *in vivo* studies in the rat, carbamazepine-10,11-epoxide, although Ames-negative, is reported to have the potential to initiate cellular damage if not adequately detoxified via conjugation with glutathione.[65] Other drugs for which there is indirect evidence (based on metabolite profiles) for formation of reactive arene oxide metabolic intermediates include phenytoin,[66] lamotrigine,[67] amitryptiline[68] and diclofenac.[69]

Although α,β-unsaturated ketones generally produce negative results in bacterial reverse mutation assays,[70] ethylvinyl ketone (1-penten-3-one) is an exception. The latter compound caused reverse mutation in an assay with preincubation in a single strain of *S. typhimurium* (TA100); in the presence of metabolic activation at a concentration of 84 μg/plate, it induced a 3-fold increase in the rate of reversions. In order to evaluate the effect of epoxidation of the double-bond on potential mutagenicity, 1-penten-3-one was further evaluated in the presence of SKF 525A, an inhibitor of microsomal monooxygenases, and 1,1,1-trichloropropene-2,3-oxide, an inhibitor of epoxide hydrolase. No mutagenic activity was observed in the presence of 100 μg/mL of SKF 525A, but addition of 1,1,1-trichloropropene-2,3-oxide resulted in an increase in the frequency of reverse mutations in a concentration-dependent manner.[71]

The standard metabolic activating system (Aroclor 1254-induced rat liver S9) used in *in vitro* genotoxicity assays contains greatly enhanced activity of P450 oxidative enzymes that are often capable of bioactivating compounds to electrophilic intermediates, epoxygenation of alkenes and arenes being just one example. On the other hand, since S9 is not supplemented with cofactors for conjugative enzymes, reactive electrophiles that would be rapidly quenched by conjugation *in vivo* before being able to cause mutation can be generated in these *in vitro* assays.[72] *In vitro* genotoxicity testing of ethylvinyl ketone is thus considered to produce false positive results since sufficient metabolic processes are available for detoxification at low levels of intake. These include known conjugation of α,β-unsaturated ketones with glutathione and biotransformation of metabolically formed epoxides to dihydrodiols by epoxide hydrolase.[70]

In conclusion, although many, but not all, epoxides are genotoxic in the Ames test, such assay systems are often deficient in enzyme systems providing critical defense mechanisms involved in detoxification. *In vivo* rodent bioassays on epoxides are by no means uniformly positive and in many cases tumours occur only at the point of administration, reflecting the long-term consequences of tissue damage by highly reactive compounds. At small-to-modest *in vivo* doses most epoxides are likely to show a threshold dose for toxic effects (including carcinogenicity) owing to the presence of two highly effective defense mechanisms, hydration by EH and epoxide-ring-opening by glutathione.

Hydrazines. The parent compound hydrazine, and some of its *N*-alkyl, *N*-aryl and *N*-acyl analogues, have been subjected to extensive toxicological evaluations. Independent expert reviews have been conducted by the International Programme on Chemical Safety (IPCS),[73] Agency for Toxic Substances and Disease Registry (ATSDR),[74] International Agency for Research on Cancer (IARC),[75] Environmental Protection Agency (EPA),[76] Office of Environmental Health Hazard Assessment (OEHHA),[77] and most recently in 2007 by the Chemicals Evaluation and Research Institute Japan (CERIJ).[78] Hydrazine is well absorbed following oral, topical or inhalation administration. Quite high concentrations have been noted in the liver and kidney shortly after dosing in rodents, although there is no convincing evidence for tissue accumulation. Around 20−30% of the dose is converted to nitrogen gas; nonvolatile metabolites are formed mainly by acetylation and CYP450-linked oxidative reactions producing radical species. The principal urinary component is the parent compound along with a variable amount of monoacetylhydrazine and a smaller contribution by

(64) Shimada, T. Xenobiotic-Metabolizing Enzymes Involved in Activation and Detoxification of Carcinogenic Polycyclic Aromatic Hydrocarbons. *Drug Metab. Pharmacokinet.* **2006**, *21*, 257–276.

(65) Madden, S.; Maggs, J. L.; Park, B. K. Bioactivation of Carbamazepine in the Rat in Vivo. Evidence for the Formation of Reactive Arene Oxide(s). *Drug Metab. Dispos.* **1996**, *24*, 469–479.

(66) Roy, D.; Snodgrass, W. R. Phenytoin Metabolic Activation: Role of Cytochrome P-450, Glutathione, Age, and Sex in Rats and Mice. *Res. Commun. Chem. Pathol. Pharmacol.* **1988**, *59*, 173–190.

(67) Maggs, J. L.; Naisbitt, D. J.; Tettey, J. N. A.; Pirmohamed, M.; Park, B. K. Metabolism of Lamotrigine to a Reactive Arene Oxide Intermediate. *Chem. Res. Toxicol.* **2000**, *13*, 1075–1081.

(68) Wen, B.; Ma, L.; Zhu, M. Bioactivation of the Tricyclic Antidepressant Amitriptyline and Its Metabolite Nortriptyline to Arene Oxide Intermediates in Human Liver Microsomes and Recombinant P450s. *Chem. Biol. Interact.* **2008**, *173*, 59–67.

(69) Yan, Z.; Li, J.; Huebert, N.; Caldwell, G. W.; Du, Y.; Zhong, H. Detection of a Novel Reactive Metabolite of Diclofenac: Evidence for CYP2C9-Mediated Bioactivation via Arene Oxides. *Drug Metab. Dispos.* **2005**, *33*, 706–713.

(70) WHO Food Additive Series 50, Aliphatic Secondary Alcohols, Ketones and Related Esters; http://www.inchem.org/documents/jecfa/jecmono/v50je15.htm.

(71) Deininger, C.; Eder, E.; Neudecker, T.; Hoffman, C. Mutagenicity and Genotoxicity of Ethylvinyl Ketone in Bacterial Tests. *J. Appl. Toxicol.* **1990**, *10*, 167–171.

(72) Obach, R. S.; Dobo, K. L. Comparison of Metabolite Profiles Generated in Aroclor-Induced Rat Liver and Human Liver Subcellular Fractions: Considerations for in Vitro Genotoxicity Hazard Assessment. *Environ. Mol. Mutagen.* **2008**, *49*, 631–641.

(73) International Programme on Chemical Safety, Environmental Health Criteria 68, Hydrazine; http://www.inchem.org/documents/ehc/ehc/ehc68.htm.

(74) Agency for Toxic Substances and Disease Registry, Toxicological Profile for Hydrazines; http://www.atsdr.cdc.gov/toxprofiles/tp100.pdf.

(75) International Agency for Research on Cancer, Hydrazine; http://www.inchem.org/documents/iarc/vol71/037-hydraz.html; http://monographs.iarc.fr/ENG/Monographs/vol71/mono71-43.pdf.

(76) Environmental Protection Agency, Integrated Risk Information System, Hydrazine/Hydrazine Sulfate; http://www.epa.gov/iris/subst/0352.htm.

(77) Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Hydrazine/Hydrazine Sulfate; http://oehha.ca.gov/prop65/pdf/2009FebruaryStat.pdf.

(78) Chemicals Evaluation and Research Institute Japan, Hazard Assessment Report - Hydrazine; http://www.cerij.or.jp/ceri_en/hazard_assessment_report/pdf/en_302_01_2.pdf.

Table 4. **Oral TD_{50} and genotoxicity data on hydrazines, hydrazides and hydrazones listed in the CPDB[a]**

compound	CAS number	Ames *Salmonella*	TD_{50} (mg/kg/day) mouse	TD_{50} (mg/kg/day) rat
		Hydrazines		
hydrazine	302-01-2	+	2.93	0.613
hydrazine sulfate	10034-93-2	+	7.59	40.8
methylhydrazine	60-34-4	+	7.55	nda
methylhydrazine sulfate	302-15-8	nda	2.72	nda
1,1-dimethylhydrazine	57-14-7	+	3.96	NP
1,2-dimethylhydrazine·2HCl	306-37-6	+	0.114	nda
ethylhydrazine·HCl	18413-14-4	nda	6.56	nda
n-pentylhydrazine·HCl	1119-68-2	nda	5.87	nda
benzylhydrazine·2HCl	20570-96-1	nda	85.3	nda
2-hydroxyethylhydrazine	109-84-2	+	0.397	nda
		Hydrazides		
acetylhydrazine	1068-57-1	+	9.85	nda
1,2-diacetylhydrazine	318-73-0	−	NP	nda
1-acetyl-2-phenyl-hydrazine	114-83-0	+	51.2	nda
formylhydrazine	624-84-0	nda	36.4	nda
1,2-diformylhydrazine	628-36-4	nda	668	nda
1-methyl-1-formylhydrazine	758-17-8	−	1.37	nda
1-ethyl-1-formylhydrazine	74920-78-8	nda	2.8	nda
benzoylhydrazine	613-94-5	nda	9.59	nda
carbamylhydrazine·HCl	563-41-7	±	223	nda
		Hydrazones		
acetaldehyde methylformylhydrazone	16568-02-8	−	2.51	nda
pentanal methylformylhydrazone	57590-20-2	nda	3.42	nda
3-methylbutanal methylformylhydrazone	57590-21-3	nda	2.03	nda
hexanal methylformylhydrazone	57590-22-4	Nda	3.42	nda

[a] nda = no data available; NP = no positive test.

diacetylhydrazine.[79] Other minor metabolites are formed by conjugation with 2-oxoglutarate (1,4,5,6-tetrahydro-6-oxo-3-pyridazine carboxylic acid) and pyruvate (pyruvate hydrazone) and reaction with ammonia to form urea. *In vivo* 1,2-dimethylhydrazine is metabolized to form azomethane, azoxymethane, methylazoxymethanol, ethane, and carbon dioxide; ethane formation is considered to arise by dimerization of methyl radicals. Overall, the data indicate that that oxidation can occur at both the nitrogen and the carbon of 1,2-dimethylhydrazine *in vivo* and strongly suggest that free radical formation occurs.

Hydrazines, hydrazides and hydrazones all show conventional structural alerts for genotoxic potential.[5] Hydrazine and its analogues generally produce positive results in bacterial and mammalian cell test systems, with a trend to increased activity in the presence of a metabolic activating system (such as rat liver homogenate S9 fraction), possibly reflecting the importance of radical active metabolites. Data from *in vivo* tests are equivocal or positive. The mechanism of DNA alkylation for hydrazine is thought to occur by an initial condensation reaction with endogenous formaldehyde to produce formaldehyde hydrazone, followed by other reactions leading ultimately to diazomethane or similar reactive alkylating compounds as the genotoxic moiety.[75]

Hydrazine, methyl hydrazine, 1,1- and 1,2-dimethylhydrazine and other analogues are carcinogenic in rodents and may be human carcinogens.[80] Human epidemiological studies on hydrazine-exposed plant operatives failed to show any association with carcinogenicity however, possibly indicating a threshold at low levels of exposure.[75] TD_{50} values and Ames *Salmonella* results for representative hydrazines, hydrazides and hydrazones are shown in Table 4. The presence of substituents that reduce the basicity of one or both nitrogen atoms clearly reduces the carcinogenic (and genotoxic) potency; for example, 1,2-diacetylhydrazine is Ames-negative and noncarcinogenic in the mouse. Hydralazine (1-hydrazinylphthalazine) and its hydrochloride are both Ames-positive and show evidence of tumorigenicity in rodent bioassays.[7] However, the hydrochloride salt is used in the treatment of hypertension at doses up to 200 mg/day - justified by the absence of any evidence for carcinogenicity over many years of clinical use.[81]

Aromatic Amines. Primary and secondary aromatic amines are generally not inherently genotoxic but require metabolic activation in order to generate an electophilic species. In other words, the presence of S9 mix is normally required in the Ames assay to produce a positive result (particularly in strains TA98 and TA100); 2,4-diaminotoluene, 2,4-diaminoethylbenzene, and a few amines containing a nitro-group are direct mutagens.[82] The main pathways of aromatic amine metabolism include ring oxidation, *N*-acetylation and *N*-oxidation;[83,84] the latter trans-

(79) Huq, F. Molecular modelling analysis of the metabolism of hydrazine. *J. Pharmacol. Toxicol.* **2006**, *1*, 485–489.

(80) *N*-Methylhydrazine, Evaluation of the Carcinogenicity and Genotoxicity, Nr 2002/07OSH; Dutch Expert Committee on Occupational Standards: The Hague, 2002; http://www.gezondheidsraad.nl/sites/default/files/02@07OSH.PDF.

(81) Summary of Product Characteristics, Apresoline Tablets 25 mg; http://www.medicines.org.uk/EMC/medicine/10820/SPC/Apresoline+Tablets+25+mg/.

(82) Chung, K.-T.; Kirkovsky, L.; Kirkovsky, A.; Purcell, W. P. Review of Mutagenicity of Monocyclic Aromatic Amines: Quantitative Structure-Activity Relationships. *Mutat. Res.* **1997**, *387*, 1–16.

(83) Bartsch, H. Metabolic Activation of Aromatic Amines and Azo Dyes. *IARC Sci. Publ.* **1981**, 13–30.

formation produces a *N*-hydroxy compound that is conjugated as an acetate, sulphate or glucuronide. Deconjugation then produces a nitrenium ion, $ArN^{+}H$, which is considered to be the proximate mutagen/carcinogen that binds to DNA.[85,86] The action of nitroreductase (present in the *Salmonella* strains used in reverse bacterial mutation assays) on aromatic nitro compounds also produces an *N*-hydroxylamine intermediate, thus explaining the genotoxic properties of many, but not all, aromatic nitro compounds; for example, nitrobenzene is Ames-negative,[87] whereas 2,4-dinitrotoluene is Ames-positive.[88] For an Ames-positive aromatic nitro compound (negative in nitroreductase-deficient strains TA98NR and TA100NR) under development as an anticonvulsant, it was possible to show with the use of a comprehensive battery of additional assays that the compound was unlikely to be genotoxic *in vivo*.[89] It seems unlikely, however, that such extensive testing would be undertaken on an Ames-positive aromatic nitro compound impurity.

Data on the Ames genotoxicity and carcinogenic potency (TD_{50}) of a number of representative aromatic amines are shown in Table 5. It can be seen that a significant proportion are Ames-positive, although the presence of a hydroxyl or carboxy substituent eliminates the genotoxic response. Secondary and tertiary amines are generally Ames-negative whilst chloro compounds appear to be both positive and negative. Most Ames-positive compounds, if tested for carcinogenicity, produce positive results, although *p*-anisidine and *p*-chloroaniline are noncarcinogenic in rodent bioassays. Somewhat confusingly, Ames-negative *N*,*N*-dimethylaniline is reported to be of modest carcinogenic potency with a rat TD_{50} of 125 mg/kg/day. Numerous attempts have been made to construct structure−activity relationships, based for example on the nature and positioning of benzene-ring substituents, in order to predict aromatic amine genotoxicity/carcinogenicity, all with limited success owing to the complex inter-relationships between chemical and biological factors.[82,90] Ames genotoxic potency, which is often poorly correlated with carcinogenic potency, seems to be related to the predicted stability of the appropriate nitrenium ion.[91−93] More recent publications suggest that separate quantitative structure−activity relationships may apply to genotoxicity and carcinogenicity[94,95] and that even when end points from multiple *in silico* and *in vitro* evaluations are available the goal of accurately predicting quantitative genotoxic and carcinogenic potency remains elusive.[96]

Table 5. **Oral TD_{50} and genotoxicity data on aromatic amines**[a]

compound	CAS number	Ames *Salmonella*	TD_{50} (mg/kg/day) mouse	rat
	Primary Monoamines			
aniline	62-53-3	−	nda	NP
aniline·HCl	142-04-1	−	NP	269
o-anisidine·HCl	134-29-2	+	966	29.7
p-anisidine·HCl	20265-97-8	+	NP	NP
p-chloroaniline	106-47-8	+	NP	NP
p-chloroaniline·HCl	20265-96-7	+	89.5	7.62
3,4-dichloroaniline	95-76-1	−	nda	nda
3-chloro-*p*-toluidine	95-74-9	−	NP	NP
4-chloro-*o*-toluidine·HCl	3165-93-3	−	25.8	NP
5-chloro-*o*-toluidine	95-79-4	−	195	NP
4-nitroaniline	100-01-6	+	NP	NP
2-amino-4-nitrophenol	99-57-0	+	NP	839
2-amino-5-nitrophenol	121-88-0	+	NP	111
4-amino-2-nitrophenol	119-34-6	+	NP	309
o-toluidine·HCl	636-21-5	+	840	43.6
m-toluidine·HCl	638-03-9	−	1440	NP
p-toluidine·HCl	540-23-8	+	83.5	NP
o-aminophenol	95-55-6	−	nda	nda
m-aminophenol	591-27-5	−	nda	nda
p-aminophenol	123-30-8	−	nda	nda
2,4-xylidine·HCl	21436-96-4	+	12.4	NP
2,5-xylidine·HCl	51786-53-9	+	626	152
2-aminobenzoic acid	118-92-3	−	NP	NP
4-aminobenzoic acid	150-13-0	−	nda	nda
	Primary Diamines			
2,4-diaminotoluene	95-80-7	+	26.7	2.47
2,4-diaminotoluene·HCl	636-23-7	+	203	4.42
2,6-diaminotoluene·HCl	15481-70-6	+	NP	NP
2,5-diaminotoluene·HCl	6369-59-1	+	NP	NP
	Secondary Monoamines			
N-methylaniline	100-61-8	−	nda	nda
N-phenylaniline	122-39-4	−	nda	nda
3-hydroxy-*N*-phenylaniline	101-18-8	−	nda	nda
acetanilide	103-84-4	−	nda	nda
	Tertiary Monoamines			
N,*N*-dimethylaniline	121-69-7	−	NP	125

[a] nda = no data available; NP = no positive test.

Halo Compounds. Numerous halogen-containing compounds have been evaluated for genotoxicity/carcinogenicity, Given the difficulties in obtaining reliable predictions, particularly for aromatic amines, it may be prudent to undertake an Ames assay on any PGI containing an aromatic amine structural alert if data are not already available in the public domain.

(84) Gorrod, J. W.; Manson, D. The Metabolism of Aromatic Amines. *Xenobiotica* **1986**, *16*, 933–955.
(85) Williams, G. M. DNA Reactive and Epigenetic Carcinogens. *Exp. Toxicol. Pathol.* **1992**, *44*, 457–463.
(86) Kerdar, R. S.; Dehner, D.; Wild, D. Reactivity and Genotoxicity of Arylnitrenium Ions in Bacterial and Mammalian Cells. *Toxicol. Lett.* **1993**, *67*, 73–85.
(87) Environmental Health Criteria 230: Nitrobenzene: http://www.inchem.org/documents/ehc/ehc/ehc230.htm.
(88) Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS); 2,4-dinitrotoluene: http://www.inchem.org/documents/sids/sids/121142.html.
(89) Suter, W.; Hartmann, A.; Poetter, F.; Sagelsdorff, P.; Hoffmann, P.; Martus, H.-J. Genotoxicity Assessment of the Antiepileptic Drug AMP397, an Ames-Positive Aromatic Nitro Compound. *Mutat. Res.* **2002**, *518*, 181–194.
(90) Colvin, M. E.; Hatch, F. T.; Felton, J. S. Chemical and Biological Factors Affecting Mutagen Potency. *Mutat. Res.* **1998**, *400*, 479–492.
(91) Ford, G. P.; Herman, P. S. Relative Stabilities of Nitrenium Ions Derived from Polycyclic Aromatic Amines. Relationship to Mutagenicity. *Chem. Biol. Interact.* **1992**, *81*, 1–18.
(92) Hatch, F. T.; Colvin, M. E. Quantitative Structure-Activity (QSAR) Relationships of Mutagenic Aromatic and Heterocyclic Amines. *Mutat. Res.* **1997**, *376*, 87–96.
(93) Bentzien, J.; Hickey, E. R.; Kemper, R. A.; Brewer, M. L.; Dyekjaer, J. D.; East, S. P.; Whittaker, M. An in Silico Method for Predicting Ames Activities of Primary Aromatic Amines by Calculating the Stabilities of Nitrenium Ions. *J. Chem. Inf. Model.* **2010**, *50*, 274–297.
(94) Benigni, R.; Passerini, L. Carcinogenicity of the Aromatic Amines: From Structure-Activity Relationships to Mechanisms of Action and Risk Assessment. *Mutat. Res.* **2002**, *511*, 191–206.
(95) Benigni, R.; Bossa, C.; Netzeva, T.; Rodomonte, A.; Tsakovska, I. Mechanistic QSAR of Aromatic Amines: New Models for Discriminating between Homocyclic Mutagens and Nonmutagens, and Validation of Models for Carcinogens. *Environ. Mol. Mutagen.* **2007**, *48*, 754–71.
(96) Benfenati, E.; Benigni, R.; Demarini, D. M.; Helma, C.; Kirkland, D.; Martin, T. M.; Mazzatorta, P.; Ouédraogo-Arras, G.; Richard, A. M.; Schilter, B.; Schoonen, W. G. E. J.; Snyder, R. D.; Yang, C. Predictive Models for Carcinogenicity and Mutagenicity: Frameworks, State-of-the-Art, and Perspectives. *J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotoxicol. Rev.* **2009**, *27*, 57–90.

ranging from simple, volatile halomethanes to stable and persistent compounds such as dioxin, DDT and PCBs. This section is focused mainly on simple chloro and bromo compounds that may be used as synthetic reagents or could, at least in theory, be present in APIs as side-reaction products, for example as chloroalkanes formed during hydrochloride salt synthesis in the presence of one or more short-chain alcohols. Polyhalo compounds such as chlorobromopropanes, fluorinated propellants, halogen-containing anaesthetics and chloroparaffins will not be discussed in detail.

The reactivity of halo compounds in biological systems can be predicted to a significant extent on the basis of their relevant chemical properties such as alkylating potential and susceptibility to hydrolysis. Thus, bromo compounds are expected to be more reactive than chloro compounds; S_N1 and S_N2 characteristics will determine the nature and the extent of reactivity towards nucleophiles, and steric factors may also play a part in some cases. In the NBP [4-(*p*-nitrobenzyl)pyridine] alkylation assay alkyl halides generally show negligible activity,[97] MMS being at least 40 times more active than ethyl, propyl or butyl bromide. Allyl bromide appears to be more active[98] (around one-eighth of the activity of MMS) although allyl chloride shows minimal activity. Benzyl chloride, quite active for a chloro compound, is around 20-fold less active than allyl bromide.[98] Owing to their volatility and/or hydrophobicity many alkyl halides show negative results in conventional Ames *Salmonella* assays, and it is often necessary to employ vapour-phase exposure in a closed system (using a desiccator for example) in order to obtain positive results.[99,100] As shown in Table 6 most alkyl halides, especially bromides, are Ames-positive (using a closed test system if necessary), although 1-chloropropane, 1-chlorobutane and neopentyl bromide are all Ames-negative. As expected, based on their lack of alkylating activity, both chloro- and bromobenzene are Ames-negative.

Some unsaturated halo compounds have the potential to be metabolised to form quite active mutagenic molecular species. For example, evidence suggests that oxidative biotransformation of vinyl chloride produces chloroethylene oxide and 2-chloroacetaldehyde as active metabolites.[101,102] Binding of bromobenzene 3,4-oxide to liver proteins is thought to account for the hepatotoxicity of bromobenzene.[103] The predominant metabolic pathway for simple alkyl halides is halide displacement by GSH, although some C-hydroxylation reactions may occur as, for example, chloroethane leading to the production of acetaldehyde and chloroethanol.[104]

Rodent bioassay data on alkyl halides (Table 6) strongly suggest that these compounds are either noncarcinogens (1-chlorobutane, bromomethane) or low-potency carcinogens (chloroethane, bromoethane). Both chloroethane and bromoethane produced an increased incidence of a rare type of endometrial tumour in female mice[105] and it seems highly plausible that the carcinogenic effect is caused by a species-/gender-specific stress-related adrenal overstimulation and exces-

Table 6. **Oral TD_{50} and genotoxicity data on various halo compounds**[a]

compound	CAS number	Ames *Salmonella*	TD_{50} (mg/kg/day) mouse	rat
Haloalkanes				
chloromethane	74-87-3	+	nda	nda
chloroethane	75-00-3	+	1810	NP
1-chloropropane	540-54-5	−	nda	nda
2-chloropropane	75-29-6	+	nda	nda
1-chlorobutane	109-69-3	−	NP	NP
2-chlorobutane	78-86-4	+	nda	nda
tert-butyl chloride	507-20-0	±	nda	nda
bromomethane	74-83-9	+	NP	NP
bromoethane	74-96-4	+	535	149
1-bromopropane	106-94-5	±	nda	nda
2-bromopropane	75-26-3	+	nda	nda
1-bromobutane	109-65-9	+	nda	nda
2-bromobutane	78-76-2	+	nda	nda
tert-butyl bromide	507-19-7	+	nda	nda
neopentyl bromide	630-17-1	−	nda	nda
Haloalkenes				
vinyl chloride	75-01-4	+	21.8	6.11
allyl chloride	107-05-1	+	NP	inadequate
1-chloropropene	590-21-6	+	5.05	nda
3-chloro-2-methylpropene	563-47-3	−	1000	nda
vinyl bromide	593-60-2	+	nda	18.5
allyl bromide	106-95-6	+	nda	nda
Hydroxyls, Carbonyls, Ethers				
1-chloro-2-propanol	127-00-4	+	NP	NP
2-chloropropenol	78-89-7	+	nda	nda
2-chloropropenal	683-50-1	nda	12.9	nda
chloroacetaldehyde	107-20-0	+	36.1	nda
chloromethylmethylether	107-30-2	−	nda	5.5
bromoethanaol	540-51-2	+	76.1	nda
bromoacetaldehyde	17157-48-1	−	NP	nda
Aromatics				
benzyl chloride	100-44-7	+	61.5	nda
chlorobenzene	109-90-7	−	nda	247
1-chloro-2-nitrobenzene	88-73-3	+	157	NP
1-chloro-4-nitrobenzene	100-00-5	+	473	NP
1-chloro-2,4-dinitrobenzene	97-00-7	+	NP	NP
2-chloroacetophenone	532-27-4	−	NP	NP
2-(chloromethypyridine)·HCl	6959-47-3	+	NP	NP
3-(chloromethypyridine)·HCl	6959-48-4	+	229	433
benzyl bromide	100-39-0	+	nda	nda
bromobenzene	100-86-1	−	nda	nda
Polyhalogen Compounds				
chloroform	67-66-3	−	111	262
1,1-dichloroethane	75-34-3	−	NP	NP
1,2-dichloroethane	107-06-2	+	138	14.6
1,2-dichloropropane	78-87-5	+	276	NP
dichloroacetic acid	79-43-6	+	119	161
1,2-dichlorobenzene	95-50-1	−	NP	NP
1,4-dichlorobenzene	106-46-7	−	323	644
chlorofluoromethane	593-70-4	+	nda	27.5
chlorodibromomethane	124-48-1	−	139	NP
bromodichloromethane	75-27-4	+	47.7	72.5
1,2-dibromoethane	106-93-4	+	7.45	1.52

[a] nda = no data available; NP = no positive test.

(97) Sobol, Z.; Engel, M. E.; Rubitski, E.; Ku, W. W.; Aubrecht, J.; Schiestl, R. H. Genotoxicity Profiles of Common Alkyl Halides and Esters with Alkylating Activity. *Mutat. Res.* **2007**, *633*, 80–94.

(98) Eder, E.; Neudecker, T.; Lutz, D.; Henschler, D. Correlation of Alkylating and Mutagenic Activities of Allyl and Allylic Compounds: Standard Alkylation Test vs. Kinetic Investigation. *Chem. Biol. Interact.* **1982**, *38*, 303–315.

(99) Ashby, J. The Evaluation of Volatile Chemicals for Mutagenicity. *Mutagenesis* **1989**, *4*, 160–162.

(100) Simmon, V. F. Applications of the Salmonella/Microsome Assay. In *Short-Term Tests for Chemical Carcinogens*; Stich, H. F., San, R. H. C., Eds.; Springer-Verlag: New York, 1981; pp 120−126.

(101) Vinyl chloride (WHO Food Additives Series 19); http://www.inchem.org/documents/jecfa/jecmono/v19je16.htm.

(102) Bolt, H. M. Metabolism of Genotoxic Agents: Halogenated Compounds. *IARC Sci. Publ.* **1984**, 63–71.

(103) Rombach, E. M.; Hanzlik, R. P. Detection of Adducts of Bromobenzene 3,4-Oxide with Rat Liver Microsomal Protein Sulfhydryl Groups Using Specific Antibodies. *Chem. Res. Toxicol.* **1999**, *12*, 159–163.

(104) ATSDR Toxicological Profile for Chloroethane; http://www.atsdr.cdc.gov/toxprofiles/tp105.pdf.

sive corticosteroid production. The rodent carcinogenicity profile for chloroethane (increased incidence of a rare tumour type at an extremely high concentration of 15,000 ppm[106] in one species/gender) is thus much closer to that for a nongenotoxic carcinogen than for a genotoxic carcinogen. Thus, the (feeble) alkylating activity of chloroethane seems largely incidental to its carcinogenic activity, a scenario likely to apply to many other similar alkyl halides. This prediction is strongly supported by the fact that benzyl, ethyl, isopropyl, and trityl bromides were inactive as carcinogens at doses up to 0.83, 12.5, 8.3, and 0.25 mmol/kg, respectively, when administered by single subcutaneous injection to female rats.[107]

A number of independent expert assessments are available on halo compounds, some of which are summarised in Table 7. Acceptable/tolerable exposures are expressed in various ways, for example as minimal risk levels (MRLs) by the Agency for Toxic Substances and Disease Registry, or as reference concentrations/doses (RfCs/RfDs) by the U.S. Environmental Protection Agency. There is a clear consensus that chloroethane is less hazardous than the more reactive chloromethane, although recommended safe exposures for the former range from the highly conservative OEHHA value of 150 μg/day to 200 mg/day (10 mg/m^3 at an average air intake of 20 m^3/day[43]) based on the EPA IRIS assessment. Acceptable exposures in the context of genotoxic impurities can also be calculated on the basis of the TD_{50} values as described above, resulting in PDEs of 1810 and 149 μg/day for chloroethane and bromoethane respectively. A PDE for noncarcinogenic 1-chlorobutane could be determined using ICH Q3C (R3) methodology on the basis of the most appropriate NOAEL in lifetime studies.

Aldehydes. Human exposure to aldehydes is significant: preformed aldehydes are ubiquitous in the environment and in our food supply, and some aldehydes such as formaldehyde, acetaldehyde, crotonaldehde and malondialdehyde are formed endogenously as metabolic intermediates.[108] In biological systems aldehyde reactivity is governed principally by Schiff base formation, which has the potential to affect proteins, DNA bases and other macromolecules. Prolonged exposure of rats to high concentrations of inhaled formaldehyde has been shown to significantly decrease the density of structural proteins of the junctional complex in the nasoepithelium.[109] On the other hand, a number of highly effective detoxification reactions based mainly on aldehyde dehydrogenase activity and glutathione conjugation (for unsaturated aldehydes) are capable of offsetting

Table 7. **Independent expert summaries/assessments on some simple halo compounds[a]**

compound	IPCS EHC and NTP	CICAD	OECD SIDS	EPA IRIS	OEHHA	ATSDR	screening assessment
chloromethane	–	X (guidance value for indirect exposure 0.018 mg/m^3)[132]	X[133]	X (inhalation RfC 0.09 mg/m^3)[134]	–	X (chronic inhalation MRL 0.1 mg/m^3)[135]	X[136]
chloroethane	–	–	–	X (inhalation RfC 10 mg/m^3)[137]	X (NSRL 150 μg/day)[138]	X (inhalation MRL 40 mg/m^3)[104]	–
1-chlorobutane	–	–	X[139]	–	–	–	–
benzyl chloride	–	–	X[140]	–	X (NSRL 4 μg/day)[141]	–	X[142]
bromomethane	X (ADI 1 mg/kg/day) [EHC][143]	–	X[144]	X (oral RfD 0.0014 mg/kg/day)[145]	–	X (oral MRL 3 μg/kg/day)[146]	–
bromoethane	–	X[147]	–	–	–	–	–
1-bromopropane	X [NTP][148]	–	–	–	–	–	–

[a] IPCS EHC = International Program on Chemical Safety Environmental Health Criteria; NTP = National Toxicology Program; CICAD = Concise International Chemical Assessment Document; OECD SIDS = Organization for Economic Co-operation and Development Screening Information Data Sets; EPA IRIS = Environmental Protection Agency Integrated Risk Information System; OEHHA = Office of Environmental Health Hazard Assessment; ATSDR = Agency for Toxic Substances and Disease Registry; Screening Assessment = Environment Canada Screening Assessment Report; ADI = Acceptable Daily Intake; RfC = Reference Concentration; RfD = Reference Dose; NSRL = No Significant Risk Level; MRL = Minimal Risk Level; X = Review available.

(105) Holder, J. W. Analysis of Chloroethane Toxicity and Carcinogenicity Including a Comparison with Bromoethane. *Toxicol. Ind. Health* **2008**, *24*, 655–675.

(106) Gargas, M. L.; Sweeney, L. M.; Himmelstein, M. W.; Pottenger, L. H.; Bus, J. S.; Holder, J. W. Physiologically Based Pharmacokinetic Modeling of Chloroethane Disposition in Mice, Rats, and Women. *Toxicol. Sci.* **2008**, *104*, 54–66.

(107) Dipple, A.; Levy, L. S.; Lawley, P. D. Comparative Carcinogenicity of Alkylating Agents: Comparisons of a Series of Alkyl and Aralkyl Bromides of Differing Chemical Reactivities As Inducers of Sarcoma at the Site of a Single Injection in the Rat. *Carcinogenesis* **1981**, *2*, 103–107.

(108) O'Brien, P. J.; Siraki, A. G.; Shangari, N. Aldehyde Sources, Metabolism, Molecular Toxicity Mechanisms, and Possible Effects on Human Health. *Crit. Rev. Toxicol.* **2005**, *35*, 609–662.

(109) Arican, R. Y.; Sahin, Z.; Ustunel, I.; Sarikcioglu, L.; Ozdem, S.; Oguz, N. Effects of Formaldehyde Inhalation on the Junctional Proteins of Nasal Respiratory Mucosa of Rats. *Exp. Toxicol. Pathol.* **2009**, *61*, 297–305.

Table 8. **Toxicological properties of some representative aldehydes[a]**

compound (CAS no.)	Ames *Salmonella*	*in vitro* clastogenicity	carcinogenicity	possible limits
formaldehyde (50-00-0)	+	+	oral: -inhalation: + (nasal tumours); rat/mouse TD_{50} = 1.35/43.9 mg/kg/day[149]	oral RfD: 10 mg/day[150]
				oral: tolerable concentration 2.6 ppm[151]
				inhalation RfD: 16 μg/day[5]
acetaldehyde (75-07-0)	−	+	oral: - (suboptimal rat bioassay)[152]	oral: no safety concern when used as a food flavourings.[7]
			inhalation: + (nasal tumours); rat/hamster TD_{50} 153/565 mg/kg/day[153]	occurs naturally in wine/fruit juice up to 300 ppm
				inhalation RfD: 100 μg/day[154]
propionaldehyde (123-38-6)	−	±	NT	ICH Q3A/B qualification threshold
benzaldehyde (100-52-7)	−	−	oral: no positive in rat; high-dose forestomach tumours in mouse. mouse TD_{50} 1490 mg/kg/day[155]	oral RfD: 5 mg/day[156]
acrolein (107-02-8)	+	+	oral: no positive in rat or mouse[157,158]	oral RfD: 25 μg/day.[159] inhalation RfD: 0.4 μg/day
crotonaldehyde (4170-30-3)	+ (preincubation only)	+	oral: rat TD_{50} 4.2 mg/kg/day[160]	oral: ≥ 150 μg/day from food[161]
cinnamaldehyde (*trans*) (104-55-2)	−	−	oral: no positive in rat or mouse (*trans* isomer)[162]	ICH Q3A/B qualification threshold

[a] Data from CCRIS unless otherwise stated; limits based on cancer risk of 10^{-5}; typical patient assumed to weigh 50 kg and breathe 20 m^3 air/day; NT = not tested; RfD = reference dose (equivalent to PDE).

potential toxic effects to a greater or lesser extent, depending on the particular biological system.[110] Thus, aldehyde toxicology is characterized by the presence of thresholds, marked differences between *in vitro* and *in vivo* evaluations and differential effects depending on the route of administration.

Highly reactive low-molecular-weight aldehydes tend to be Ames-positive, whilst less volatile compounds such as benzaldehyde and cinnamaldehyde test negative as bacterial mutagens. α,β-Unsaturated aldehydes such as acrolein and crotonaldehyde are considered as bifunctional in that they can react by Michael addition or directly via the aldehyde group, thus adding further potential complexity in their interactions with biological matrices. Table 1 summarizes relevant toxicological properties of some representative aldehydes.

Formaldehyde is present in the atmosphere, particularly from automobile exhaust fumes, in the home, in occupational settings and finally as a naturally occurring component of meats, fish, vegetables and fruit. Formaldehyde exposure from dietary sources alone is estimated at between 1.5 to 14.0 mg/day. Formaldehyde undergoes rapid oxidation to formic acid, a reaction catalysed by formaldehyde dehydrogenase. The daily endogenous turnover of formaldehyde is estimated to be 31−59 g at a rate of around 41 mg/min.[111] By contrast the body's exogenous exposure is at least 2 orders of magnitude lower. Thus, whilst formaldehyde is a well-documented local irritant of the skin, lungs, nose, and GI tract[112,113] rapid metabolic clearance linked to the short half-life, makes it unlikely that exogenous oral exposure will be associated with toxicity.[114] Formaldehyde is a metabolic intermediate in the oxidative biotransformation of methanol to formic acid, both of which are permitted solvents with PDEs of 30 and 50 mg/day, respectively.[45] Formaldehyde is also a primary metabolite of methyl ethers such as the Class 3 permitted solvent methyl tert-butyl ether.[115] Moreover, formaldehyde is reported to be negative in terms of oral rodent carcinogenicity (Table 8).

The toxicological profile of acetaldehyde is quite similar to that of formaldehyde except that it is overall less reactive (Ames-negative and with a higher inhalation TD_{50}). Although acetaldehyde is formed as the primary metabolite of ethanol and so would not be expected, in moderate amounts, to constitute a carcinogenic hazard, no good quality oral rodent bioassay data are available. Nevertheless, an oral intake of several tens of milligrams/day should be considered acceptable given that ethanol is categorized as a Class 3 solvent in pharmaceuticals with an acceptable exposure of up to 50 mg/day.[45]

As can be seen in Table 8 several aldehydes have been subjected to reasonably adequate toxicological testing. Therefore, it is possible, instead of relying on the default generic TTC limit of 1.5 μg/day, to apply compound-specific limits based on negative Ames assays or derived from prior regulatory assessments (for example by the U.S. Environmental Protection Agency).

European Pharmacopoeia and Structurally Alerting Related-Substance Impurities in Generic Drug Substances

As indicated above, the PhEur has released a detailed policy statement on potentially genotoxic impurities,[27] particularly those listed as named impurities in PhEur monographs. The PhEur policy is cross-referenced in the Q&A supplement to the EU guideline.[2] Drug substance monographs constitute the

(110) Morris, J. B. Dosimetry, toxicity and carcinogenicity of inspired acetaldehyde in the rat. *Mutat. Res.* **1997**, *380*, 113–124.

(111) Dhareshwar, S. S.; Stella, V. J. Your Prodrug Releases Formaldehyde: Should You Be Concerned? No! *J. Pharm. Sci.* **2008**, *97*, 4184–4193.

(112) Collins, J. J.; Acquavella, J. F.; Esmen, N. A. An Updated Meta-Analysis of Formaldehyde Exposure and Upper Respiratory Tract Cancers. *J. Occup. Environ. Med.* **1997**, *39*, 639–651.

(113) Arts, J. H.; Rennen, M. A.; de Heer, C. Inhaled Formaldehyde: Evaluation of Sensory Irritation in Relation to Carcinogenicity. *Regul. Toxicol. Pharmacol.* **2006**, *44*, 144–160.

(114) Restani, P.; Galli, C. L. Oral Toxicity of Formaldehyde and Its Derivatives. *Crit. Rev. Toxicol.* **1991**, *21*, 315–328.

(115) Amberg, A.; Rosner, E.; Dekant, W. Toxicokinetics of methyl tert-butyl ether and its metabolites in humans after oral exposure. *Toxicol Sci.* **2001**, *61*, 62–67.

most visible part of the output of the PhEur, and such monographs are drawn up typically when a drug has been marketed for 10 years or more. API impurity profile data are submitted to the EDQM (European Directorate for the Quality of Medicines, which provides the secretariat for PhEur) particularly by the originator company but also by generic manufacturers. Structurally alerting related-substance impurities are normally considered to have been qualified (toxicologically) by the Originator Company and/or "qualification by use" will probably apply (only up to typical concentrations in the originator product). This highly pragmatic approach by PhEur can and does lead to situations where specification limits for structurally alerting impurities may be significantly in excess of TTC levels. A similar approach is acceptable for generic versions of originator products authorised prior to first January 2007 (implementation date for EU guidance on genotoxic impurities[2]) although it is necessary for generic company applicants to provide confirmatory analytical data on various lots of the originator product.

Thus, the key issues for structurally alerting related-substance impurities in a generic API are:

- The originator product must have been authorised prior to January 2007.
- Study data (rather than a structural alert) must be available demonstrating the genotoxic potential of an impurity in order to trigger any follow-up measures unless the alert relates to a structure of high concern (such as for *N*-nitroso or azoxy compounds).
- This policy applies equally to drug substances that are controlled by a PhEur monograph and those that are not.[2]

As part of an EU Marketing Authorisation Application (MAA) review in 2007, a suitable limit for epoxide photodegradation impurities[116,117] (Figure 2) in a generic version of atorvastatin calcium was considered to be 0.15% (for each impurity) since levels of the two impurities were similar in both the originator and the generic drug products.[118] To support the safety of the two epoxide impurities the applicant for the MAA showed in a 4-week bridging oral rat repeated-dose toxicity study that there were no toxicologically significant differences between atorvastatin calcium with impurities and atorvastatin calcium without impurities. Although comparative toxicity studies using spiked and unspiked drug substance are recommended in ICH Q3A (R2)[119] as a means of qualifying impurities, such studies have extremely low sensitivity. Suppose for example a drug substance with a rat NOAEL of 100 mg/kg/day were spiked at up to the 5% level, the maximum dose of impurity (5 mg/kg/day) would, unless extremely toxic, be most unlikely to cause any adverse effects. By way of

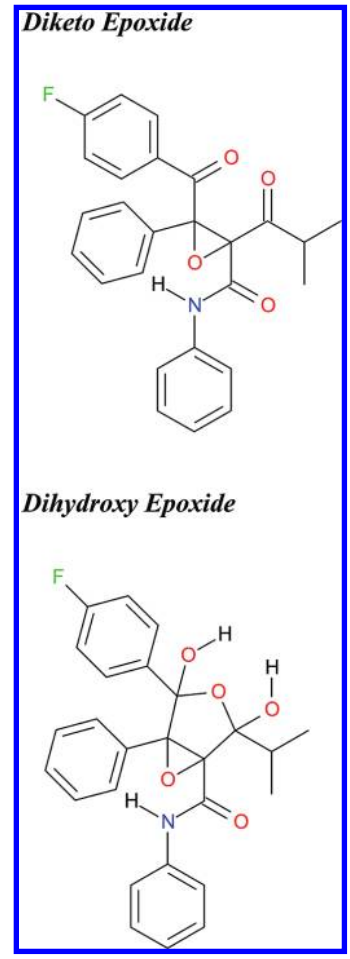

Figure 2. **Atorvastatin epoxide photodegradation impurities.**

comparison glycidol, which is highly reactive and genotoxic, has an oral rat NOAEL of 25 mg/kg/day[120] and so only impurities significantly more toxic than glycidol would produce a signal in this kind of comparative toxicity study. In view of the fact that highly substituted epoxides are, in general, too sterically hindered to be genotoxic,[50] both of the atorvastatin photodegradation impurities are likely to be nongenotoxic; however, obtaining negative and unequivocal results from Ames assays is by no means completely certain, thus applicants might be best advised to rely on regulatory and analytical approaches when dealing with structurally alerting impurities present in specific generic and originator products.

Regulatory Assessments

Achieving robust and credible evaluations of PGIs often requires an integrated approach taking into account chemical, toxicological and regulatory considerations. Regulatory agency assessors tend to specialise in a range of quite narrow disciplines; in most cases Module 3 data (Quality) are reviewed by pharmacists, Module 4 data (Nonclinical Safety) are reviewed by toxicologists, Module 5 data (Clinical Safety and Efficacy) are reviewed by physicians and statisticians and Drug Master Files (DMFs) by chemists. A significant number of

(116) Petkovska, R.; Cornett, C.; Dimitrovska, A. Development and Validation of Rapid Resolution RP-HPLC Method for Simultaneous Determination of Atorvastatin and Related Compounds by Use of Chemometrics. *Anal. Lett.* **2008**, *41*, 992–1009.

(117) Hurley, T. R.; Colson, C. E.; Clipper, S. A.; Uhlendorf, S. E.; Reily, M. D. Photodecomposition of CI-981, an HMG-CoA Reductase Inhibitor. *Tetrahedron* **1993**, *49*, 1979–1984.

(118) Mutual Recognition Procedure IS/H/100-102/01-03/MR; http://www.hma.eu/fileadmin/dateien/Human_Medicines/CMD_h_/cmdh_pressreleases/2007_03.pdf.

(119) Impurities in new Drug Substances. ICH Topic Q3A (R2): http://www.ema.europa.eu/pdfs/human/ich/273799en.pdf.

(120) National Toxicology Program (NTP) Report on the Toxicology and Carcinogenesis Study of Glycidol (CAS No. 556-52-5) in Genetically Modified Haploinsufficient $p16^{Ink4a}/p19^{Arf}$ Mice; http://ntp.niehs.nih.gov/files/gmm13_web1.pdf, 2007.

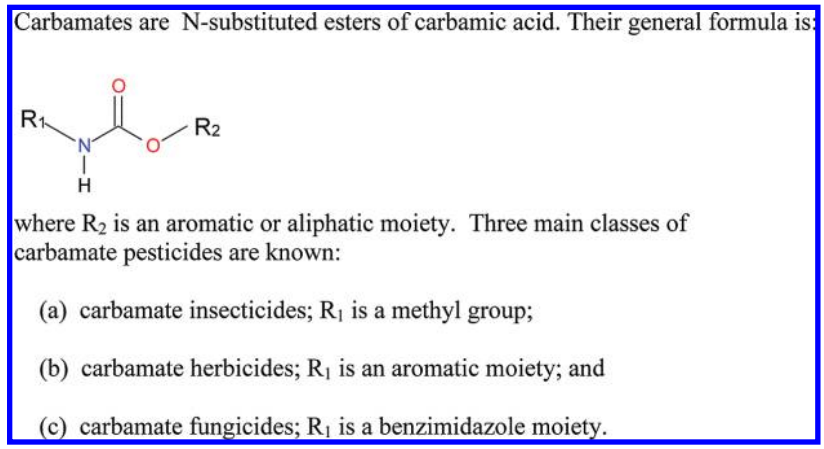

Figure 3. **Carbamate pesticides.**

questions on PGIs, most of which appear to be raised by Quality and DMF assessors, can exhibit a variety of misperceptions, possibly reflecting an incomplete understanding of the regulatory guidance and a lack of knowledge of toxicology. Examples include: incorrect attribution of structural alerts (in compounds such as sulfonic acids, *tert*-butanol and aliphatic amines); application of the guidance to Ames-negative clastogens; confusion over the role and utility of carcinogenicity data for both Ames-positive and Ames-negative compounds; use of structural alerts alone as a basis for application of the TTC limit (Ames-negative and mouse micronucleus-negative mesityl oxide[121] being a constantly recurring example). In some cases assessors may attempt to hamper the ability of applicants to respond through the use of published data by stipulating that only original reports containing raw data will be taken into consideration. Such a stance is highly unreasonable, disproportionate and untenable since assessors should be well aware of the fact that full reports containing all raw data are rarely available in the public domain. If this policy were to be applied across the board it would render virtually all published reports unusable to support both guidelines and applications; in particular, all MAAs made under Directive 2001/83 Article 10a (well-established use) would be nonviable.

Discussion and Conclusions

The following sequence of evaluations, in terms of escalating cost and complexity, could be applied to the safety assessment of a new PGI:

- *in cerebro* structural alerts
- *in silico* structural alerts
- bacterial reverse mutation assay
- additional *in vitro*/*in vivo* genotoxicity assays (with agency consultation on the most appropriate case-specific package)
- rodent carcinogenicity bioassay.

For an assumed or confirmed Ames-positive PGI or GI the standard TTC limit (1.5 μg/day) would normally apply; most companies would halt toxicological evaluations at this stage and attempt to employ one or more process-related measures such as increasing their understanding of the API synthesis, adjusting process parameters or altering the synthesis to avoid formation of the GI.[122] Some of these process-related approaches may turn out to be highly resource-intensive and in some circumstances a PGI may arise by degradation (as is the case with the epoxide photodegradation impurities in atorvastatin), a situation that may not be readily addressed by changing the process conditions. The option of undertaking additional toxicological studies should not necessarily be discounted since it is likely that a limit considerably higher than the generic TTC value could be determined on the basis of compound-specific data. For example, as noted above, a human threshold dose of 2 mg/kg/day was established for EMS as a means of resolving an accidental contamination issue for an anti-HIV drug.[123] In virtually all cases there will most likely be toxicological options in terms of undertaking additional qualification studies, which may or may not be preferable in individual cases to applying process-related measures.

There are numerous cases where structural alerts overpredict DNA reactivity, particularly for some aromatic amines and alkyl halides as shown in Tables 5 and 6. On the other hand most epoxides and hydrazines (Tables 3 and 4, respectively) are expected to be Ames-positive when tested up to the maximum feasible concentration. Saturated and α,β-unsaturated aldehydes (Table 8) at low doses/concentrations are not generally DNA-reactive, but they may cause clastogenic effects *in vitro*.[124,125] The structural alert for carbamates seems to be based on a few well-known esters that are genotoxic/carcinogenic (such as methyl, ethyl and vinyl carbamate), whereas N-substituted carbamates used as insecticides, herbicides or fungicides (Figure 3) are, in general, nongenotoxic and noncarcinogenic.[126]

The EU guidance describes two categories of genotoxic impurities: those with a threshold-related mechanism and those without. Only compounds in the latter category are controlled

(121) Mesityl Oxide. Hazard Assessment by U.S. Environmental Protection Agency. http://www.epa.gov/opprd001/inerts/mesityl.pdf.
(122) Robinson D. I. Control of Genotoxic Impurities in Active Pharmaceutical Ingredients: A Review and Perspective. *Org Process Res Dev*. **2010**. DOI: 10.1021/op900341a.
(123) Gocke, E.; Müller, L. In Vivo Studies in the Mouse to Define a Threshold for the Genotoxicity of EMS and ENU. *Mutat. Res.* **2009**, *678*, 101–107.
(124) Adams, T. B.; Gavin, C. L.; Taylor, S. V.; Waddell, W. J.; Cohen, S. M.; Feron, V. J.; Goodman, J.; Rietjens, I. M. C. M.; Marnett, L. J.; Portoghese, P. S.; Smith, R. L. The FEMA GRAS Assessment of α,β-Unsaturated Aldehydes and Related Substances Used As Flavor Ingredients. *Food Chem. Toxicol*. **2008**, *46*, 2935–2967.
(125) IPCS, WHO Food Additive Series 40; Saturated aliphatic acyclic linear primary alcohols, aldehydes and acids. http://www.inchem.org/documents/jecfa/jecmono/v040je10.htm.
(126) IPCS, EHC 64; Carbamate pesticides: a general introduction. http://www.inchem.org/documents/ehc/ehc/ehc64.htm.

using the TTC threshold approach (in the absence of rodent bioassay data or other extenuating circumstances). Mechanisms leading to a threshold in terms of genotoxic potential are described in the EU guidance[2] as follows:

Examples of mechanisms of genotoxicity that may be demonstrated to lead to nonlinear or thresholded dose−response relationships include interaction with the spindle apparatus of cell division leading to aneuploidy, topoisomerase inhibition, inhibition of DNA synthesis, overloading of defense mechanisms, metabolic overload and physiological perturbations (e.g., induction of erythropoeisis, hyper- or hypothermia).

Most if not all genotoxins show some evidence, at least *in vitro*, for the existence of a threshold in relation to overloading of defense mechanisms; this is particularly the case for epoxides based on *in vitro* data and for aldehydes based on both *in vitro* and *in vivo* data. However, determination of a threshold is likely to require *in vivo* studies in order to gain regulatory acceptance. Standard designs for such studies are lacking although the approach taken on EMS[123] might be appropriate.

In conclusion, it is emphasised that the presence of a genuine *in cerebro* structural alert in a potential or actual impurity is an indication that the compound *may* be a DNA-reactive genotoxin. Application of a TTC-based PGI specification limit is feasible at this point, although in many cases it is likely to be unnecessarily constraining and may stimulate quite costly process modifications to the API synthesis. A recent suggested approach to remove residues of reactive GIs from an API involves treatment with a nucleophilic resin.[127] This might be useful in removing traces of alkyating or acylating agents, although many alkyl halides would probably be insufficiently reactive to enable removal, and GIs such as hydrazines and aromatic amines that require metabolic activation would be unaffected. For virtually all PGIs there are likely to be multiple possibilities for clarifying the genotoxicity status based on published data, *in silico* assessments or *de novo* Ames testing. For an Ames-positive PGI or GI a variety of options are available to enable a compound-specific qualification to be made involving the use of existing toxicological data (if available) and/or using information from appropriate additional studies. Overall, it seems prudent to obtain maximum "leverage" from toxicological approaches, which are likely to be relatively low cost, before making any significant process-related changes to the API synthesis.

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(127) Lee, C.; Helmy, R. ; Strulson, C.; Plewa, J.; Kolodziej, E.; Antonucci, V.; Mao, B.; Welch, C. J.; Ge, Z. ; Al-Sayah, M. A. Removal of Electrophilic Potential Genotoxic Impurities Using Nucleophilic Reactive Resins. *Org. Process Res. Dev.* **2010**. DOI: 10.1021/op1000397.
(128) Zeiger, E.; Anderson, B.; Haworth, S.; Lawlor, T.; Mortelmans, K. Salmonella Mutagenicity Tests: IV. Results from the Testing of 300 Chemicals. *Environ. Mol. Mutagen*. **1988**, *11* (Suppl. 12), 1–157.
(129) IUCLID Data Set for Benzoyl Chloride; http://www.epa.gov/chemrtk/pubs/summaries/benzchlr/c14969.pdf.
(130) Acute exposure guideline level for chloroacetyl chloride; http://www.epa.gov/oppt/aegl/pubs/chloroacetylchloride_and_dichloroacetylchloride_tsd_interimversion1_8_28_07.pdf.
(131) BASF Safety Data Sheet for Ethyl Chloroformate; http://worldaccount.basf.com/wa/PublicMSDS/PDF/09007af88009b79d.pdf.
(132) Chloromethane, CICAD 28; http://www.inchem.org/documents/cicads/cicads/cicad28.htm.
(133) Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS); Chloromethane. http://www.chem.unep.ch/irptc/sids/OECDSIDS/CLMETHANE.pdf.
(134) Chloromethane, EPA IRIS; http://www.epa.gov/iris/subst/1003.htm.
(135) ATSDR Toxicological Profile for Chloromethane; http://www.atsdr.cdc.gov/toxprofiles/tp106.html.
(136) Environment Canada Screening Assessment Report for Chloromethane; http://www.ec.gc.ca/substances/ese/eng/challenge/batch6/batch6_74-87-3.cfm.
(137) Chloroethane, EPA IRIS; http://www.epa.gov/iris/subst/0523.htm.
(138) Chloroethane: Proposition 65 Safe Harbor Levels Development; Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency: Sacramento, CA, 2009; p 3; http://oehha.ca.gov/prop65/pdf/2009FebruaryStat.pdf.
(139) 1-Chlorobutane: Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS); http://www.inchem.org/documents/sids/sids/109693.pdf.
(140) Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS); Benzyl chloride. http://www.chem.unep.ch/irptc/sids/OECDSIDS/100447.pdf.
(141) Benzyl chloride: Proposition 65 Safe Harbor Levels Development; Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency: Sacramento, CA, 2009; p 3; http://oehha.ca.gov/prop65/pdf/2009FebruaryStat.pdf.
(142) Environment Canada Screening Assessment Report for Benzyl Chloride; http://www.ec.gc.ca/substances/ese/eng/challenge/batch6/batch6_100-44-7_en.pdf.
(143) Bromomethane, IPCS EHC166; http://www.inchem.org/documents/ehc/ehc/ehc166.htm.
(144) Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS); Bromomethane. http://www.chem.unep.ch/irptc/sids/OECDSIDS/Methyl%20bromide.pdf.
(145) Bromomethane, EPA IRIS; http://www.epa.gov/iris/subst/0015.htm.
(146) ATSDR Toxicological Profile for Bromomethane: http://www.atsdr.cdc.gov/toxprofiles/tp27.pdf.
(147) Bromoethane CICAD 42; http://whqlibdoc.who.int/HQ/2002/a75356.pdf.
(148) 1-Bromopropane, NTP Review; http://ntp.niehs.nih.gov/index.cfm?objectid=4E0C03A9-F1F6-975E-79F1E370B9027815.
(149) Carcinogenic Potency Database: Formaldehyde; http://potency.berkeley.edu/chempages/formaldehyde.html.
(150) EPA IRIS Reference Dose for Formaldehyde; http://www.epa.gov/iris/subst/0419.htm.
(151) Concise International Chemical Assessment Document 40: Formaldehyde; http://www.inchem.org/documents/cicads/cicads/cicad40.htm.
(152) Evaluation Report of Food Additives: Acetaldehyde; http://www.fsc.go.jp/english/evaluationreports/foodadditive/acetaldehyde_report.pdf.
(153) Carcinogenic Potency Database: Acetaldehyde; http://potency.berkeley.edu/chempages/acetaldehyde.html.
(154) EPA IRIS Reference Dose for Formaldehyde; http://www.epa.gov/IRIS/subst/0290.htm.
(155) Carcinogenic Potency Database: Benzaldehyde; http://potency.berkeley.edu/chempages/benzaldehyde.html.
(156) EPA IRIS Reference Dose for Benzaldehyde; http://www.epa.gov/iris/subst/0332.htm.
(157) Carcinogenic Potency Database: Acrolein ; http://potency.berkeley.edu/chempages/acrolein.html.
(158) Concise International Chemical Assessment Document 43: Acrolein; http://www.inchem.org/documents/cicads/cicads/cicad43.htm.
(159) EPA IRIS Reference Dose for Acrolein; http://www.epa.gov/ncea/iris/subst/0364.htm.
(160) Carcinogenic Potency Database: Crotonaldehyde; http://potency.berkeley.edu/chempages/crotonaldehyde.html.
(161) Concise International Chemical Assessment Document 74: 2-Butenal; http://libdoc.who.int/publications/2008/9789241530743_eng.pdf.
(162) Carcinogenic Potency Database: Cinnamaldehyde; http://potency.berkeley.edu/chempages/trans-cinnamaldehyde.html.