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Genotoxic Impurities: A Regulatory Toxicology Commentary on Recent Articles in *Organic Process Research & Development*

David J Snodin*

Xiphora Biopharma Consulting, 9 Richmond Apartments, Redland Court Road, Bristol, BS6 7BG U.K.

ABSTRACT: Although it has now been over four years since the introduction of the EU guideline on limits of genotoxic impurities, even after various amendments the regulatory guidance is still associated with ambiguity and differing interpretations. In spite of this, it is believed that a critical toxicological analysis can shed light on a number of key issues ranging from the definition of a genotoxic impurity to the determination of appropriate limits and performing compound-specific risk assessments on the basis of public-domain data.

INTRODUCTION

Genotoxic impurities (GIs) continue to be a "hot" topic across a number of disciplines including process development, chemical analysis, toxicology, and regulatory affairs. Several articles on GIs were published in *Organic Process Research & Development* in 2010, and a number of themes emerge when viewed from a regulatory toxicology perspective. These are discussed below.

DEFINITION OF GENOTOXIC IMPURITY (GI) AND SCOPE OF EU REGULATORY GUIDANCE

In the EU guideline,[1] genotoxic impurities are defined as: "DNA-reactive substances that have a potential for direct DNA damage". In the context of EU guidance, "genotoxic" is focused on a particular type of mutagenicity, essentially that detected by the Ames test. It can be argued that in vitro chromosomal aberration data have essentially no role in the characterization of potential genotoxic impurities (PGIs) since clastogenic events reflect effects at the chromosomal level rather than direct DNA damage and are generally considered to be thresholded. This distinction is emphasized by the Q&A supplement[2] to the EU guideline and by the draft FDA guidance[3] which indicate that that no further testing is required (in terms of genotoxicity) if a structurally alerting compound is shown to be Ames-negative. For example, if a compound is tested only in the Ames assay and is found to be negative, it will not be considered as a GI. The compound could well show clastogenic effects if evaluated in vitro; however, the Q&A document does not call for such testing, and so it follows that in vitro clastogenic activity is not relevant under the terms of reference of the EU guideline. According to a remark made at a recent presentation by the rapporteur for the EU guideline,[4] results from mammalian-cell in vitro tests, such as the mouse lymphoma assay, can also be discounted provided that a compound is clearly Ames-negative. Fumaric acid, *trans*-butenedioic acid, a naturally occurring compound in many plants and a permitted food additive (E297), used as a counterion for tenofovir disoproxil (Viread) leading to a daily patient exposure of 55 mg,[5] is both Ames-negative and noncarcinogenic;[6] however, it produces a positive response in the mouse lymphoma assay in both the absence and the presence of metabolic activation.[6]

Chromosomal effects are detected in vitro, often only at high/cytotoxic concentrations, for many Ames-negative compounds including a high proportion (>25%) of pharmaceutical APIs.[7] For example, benzaldehyde and many other aldehydes give sporadic positive results in terms of in vitro chromosomal aberrations (see case study below) but are considered to be nongenotoxic and suitable for use in foods.[8] Moreover, positive in vitro chromosomal aberration assay results on Ames-negative compounds are extremely poorly correlated with carcinogenic potential, the false-positive rate (in terms of the correlation between in vitro genotoxicity data and in vivo rodent bioassay results) being estimated to be at least 75%. Kirkland et al.[9] identified 183 chemicals that were noncarcinogenic after testing in both male and female rats and mice. There were genotoxicity data on 177 of these. The specificity of the Ames test was reasonable (73.9%); however, all mammalian-cell tests had very low specificity (i.e., below 45%), and this declined to extremely low levels in combinations of two and three test systems. When all three tests were performed, 75−95% of noncarcinogens gave positive (i.e., false positive) results in at least one test in the battery. In a joint report of several EU expert committees released in 2009,[10] a similar opinion is expressed concerning the low predictivity of in vitro chromosomal mammalian cell assays for carcinogenic activity: "...the predictivity of positive results from in vitro assays responding to the clastogenic activity of chemicals in mammalian cells, i.e., the test for chromosome aberrations, the micronucleus assay and the mouse lymphoma assay, is very limited". ICH guidance on genotoxic impurities (ICH M7—in preparation[11]) already emphasizes the focus on DNA reactivity particularly in relation to potential carcinogenicity in its working title: "M7 Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk".

On the basis of the foregoing arguments, it would seem reasonable to conlude that any Ames-negative impurity should

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not be classified or evaluated as a GI irrespective of any evidence from in vitro mammalian-cell assays (for chromosomal aberrations for example); this is not a completely universal view however[12] since the issue is not presented with complete clarity in the current guidance. For example, in the draft FDA guidance,[3] on the one hand impurities that give "positive results in one or more genotoxicity assays" are a cause for concern, but on the other hand, if the initial evaluation (involving a bacterial reverse mutation assay) of the genotoxic potential of an impurity with an identified structural alert is negative, no further genotoxicity studies are recommended. In terms of applying quantitative limits, compounds for which carcinogenicity data are available, if Ames-positive, should be subjected to a compound-specific risk assessment. Ames-negative compounds for which carcinogenicity bioassay data are available should be considered to be outside of the scope of the EU guidance and evaluated on a compound-specific basis. This is not a universal experience in regulatory assessments however, and it may require forceful arguments to make the case.

Case Study on Linear Aliphatic Aldehydes. Genotoxicity data available from two sources (Toxnet[13] and a JECFA flavouring assessment[14]) on C1−C11 linear aliphatic aldehydes have been evaluated. Except for formaldehyde (Ames-positive but negative in terms of oral carcinogenicity), all other compounds in the series are Ames-negative. Data from additional in vitro assays (mainly forward mutation in mammalian cells and mammalian-cell cytogenetics) are available on seven of the series, and for six of these seven (C2, C3, C4, C5, C6, and C9 compounds) positive results are reported in at least one of these assays. For the remaining four compounds, only Ames data are available on the C8, C10, and C11, whereas for heptanal (C7), negative results in both Ames and mouse lymphoma assays are reported. In the impurity context, it appears wholly illogical to categorize heptanal, octanal, decanal, and undecanal any differently from the other compounds in the C2−C11 series; performing additional cytogenetic and forward mutation assays would undoubtedly give at least one positive result on each compound. Determination of GI or non-GI status should not rely on the absence or presence in the literature of data from ancillary in vitro assays (in non-Ames assays) at a particular point in time. The absence of any evidence of DNA reactivity for the entire series is believed to be sufficient to conclude that all compounds in the C2−C11 linear aliphatic aldehyde series should be classified as non-GIs.

GENOTOXIC IMPURITY LIMITS

If an impurity meets the criteria discussed above in terms of being classed as a GI (Ames-positive or, in the absence of published or in-house data, *assumed* to be Ames-positive based on the presence of a structural alert), it can be controlled at the default TTC (threshold of toxicological concern) limit of 1.5 μg/day. However, there are many exceptions to this general rule including:

- Compounds with evidence for a threshold,e.g., topoisomerase II inhibitors and classical intercalating agents;
- Compounds with carcinogenicity bioassay data, e.g., formaldehyde and allyl chloride;
- "Severe" indications, such as cancer treatment (see ICH S9 guidance);
- Indications where life expectancy is <5 years;
- Limited duration of exposure, ≤1 year (applies to both clinical trials and marketing in the EU and apparently only to clinical trials according to the draft US guidance);
- GI formed as a "significant metabolite" (examples of which are pretty rare);
- Exposure via food, e.g., acetaldehyde and crotonaldehyde.

A limit less than the default TTC is recommended in the draft FDA guidance[8] for juveniles and infants, by applying additional safety factors of 3 and 10, respectively. However, no such provisions are made in the EU guideline owing to the fact that the current TTC limit is considered exceptionally conservative.[4] Given this explanation, it is hard to understand why the EU guideline (and the draft FDA guideline) apply a group limit for GIs that are structurally similar (effectively using an additional safety factor of 2 or 3 in the case of most structurally similar GIs). Many other arguments refuting the multiple GI approach are presented by Elder and Harvey.[15]

STRUCTURAL ALERTS AND IN-SILICO EVALUATIONS

It should be emphasized that in-silico systems such as DEREK are not essential for determination of structural alerts; as indicated in the draft FDA guidance,[8] an in-cerebro assessment, combined with published information on representative compounds in the same structural class, can be just as effective in many cases. Agencies that have internal access to such in-silico systems will most likely undertake their own evaluation which can throw up concerns, whereas that undertaken by the applicant did not. For a significant number of chemical classes, structural alerts massively overpredict mutagenicity (Ames-positivity) owing to numerous modulating factors including high molecular weight, hydrophilicity, high reactivity, steric hindrance, molecular symmetry, and ready metabolism. Such overpredictions were clearly demonstrated by Raillard et al.[16] for a number of structural classes, including aldehydes, α,β-unsaturated carbonyls, and aromatic amines, that can be associated with drug substance degradation pathways. It may be possible to "read across" with confidence for some tightly defined structural classes, but it might be necessary to provide compound-specific data to convince some sceptical regulators.

RISK ASSESSMENT OF PGIS AND DEVELOPMENT OF METHODS OF ANALYSIS

Although several articles[5,17,18] stress the major challenges faced in developing methods of analysis for determination of parts per million and subparts per million levels of GIs controlled at a TTC-based limit, it is not clear in all cases that the decision to apply such a limit has been preceded by a thorough risk assessment. Sun et al.[7] describe the enormous effort required to develop a robust, validated assay for just one GI (dimethyl sulfate) at the TTC level. Dimethyl sulfate (DMS) is Ames-positive and considered to be carcinogenic in rodents, although no data on oral carcinogenicity appear to be available.[19] Since DMS has the same Swain−Scott s constant (an index of nucleophilic selectivity) of 0.86 as methyl methanesulfonate (MMS; TD_{50} mouse 31.8 mg/kg/day), in theory, it may be reasonable to assume that the two compounds possess similar moderate carcinogenic potency (based on the established approximate correlation of TD_{50} and Swain−Scott s).[20] In addition, it can be argued that DMS probably exhibits a threshold for carcinogenicity since the compound is hydrolysed extremely

rapidly in aqueous environments.[21] In spite of such considerations, it may be prudent to assume a worst-case TTC limit for DMS since most regulatory agency assessors may be reluctant to accept evaluations based on "read across" approaches. On the other hand, for other structurally alerting potential impurities such as 4-chlorobutyryl chloride in levetiracetam,[22] application of the TTC limit is not justified (since the PGI is reported in TOXNET to be Ames-negative[23]).

DISCUSSION OF SOME INDIVIDUAL IMPURITIES

Acetamide. Describing and controlling acetamide as a "genotoxic impurity"[24] would seem inappropriate since the compound is clearly Ames-negative.[25] Several positive oral rodent carcinogenicity bioassays have been reported on acetamide. A detailed assessment of the carcinogenicity data reveals that the studies are quite old and involved the use of "heroic" doses (often just one or two dose levels) of ≥1 g/kg/day. Although, in principle, a threshold dose can be determined for a nongenotoxic carcinogen such as acetamide, it is not possible since in this case all doses that have been tested were associated with an increased tumour incidence. Nevertheless, a risk assessment for acetamide can be made on the basis of the cancer slope factor,[8] which assumes a linear dose response, or alternatively it is possible to determine a threshold dose based on data for the closely related analogue *N*, *N*-dimethylacetamide (DMAC; a Class 3 solvent). The latter compound is metabolized by sequential N-demethylation, ultimately to acetamide. On the basis of a NOAEL for DMAC from an oral, 24-month rat chronic toxicity study, and a worst-case estimate of metabolic conversion to acetamide,[26] a "metabolic" NOAEL and, in turn, a PDE of 15 mg/day can be determined [using ICH Q3C (R5) criteria].

Benzene and Thiourea. Two further examples of compounds inappropriately tagged as "genotoxic" impurities are mentioned by Robinson:[27] benzene and thiourea. The former is a nongenotoxic carcinogen that is a Class 1 solvent restricted to a maximum concentration of 2 ppm in a drug substance or drug product based on the provisions of ICH Q3C (R5).[28] Unfortunately, this limit is expressed only as a concentration and so tends to be applied somewhat inflexibly. It is based on an estimated exposure of 20 μg/day associated with a risk level of 1 in 10^5 in terms of increased tumour incidence and a maximum daily dose of 10 g of drug substance. This is a highly conservative limit especially when viewed in the context of the normal human exposure to benzene from environmental and dietary sources of 100−200 μg/day for nonsmokers,[29] much higher for smokers (60 μg/cigarette). Thiourea is also nongenotoxic but has tested positive for carcinogenicity, particularly in terms of thyroid tumour formation, in several rodent bioassays thought to be unacceptable by modern standards. A further consideration is that rodents are more susceptible than humans to thyroid tumour induction.[30] On the basis of human data from the use of thiourea as a medicament, a dose of 15 mg/day is normally considered to be without effect on thyroid function and essentially without risk.[24]

STRUCTURE−ACTIVITY RELATIONSHIPS

Callis et al.[8] have determined short-term limits for a range of PGIs based on carcinogenicity slope factors or the default TTC. In such an exercise, it is striking that the TTC approach acts as a "straightjacket" that lumps together under one limit compounds of strikingly different reactivities, isopropyl chloride and isopropyl mesylate for example. Isopropyl chloride is Ames-negative in assays using standard conditions; testing has to be carried out in a desiccator to obtain a (feebly) positive result; on the other hand, isopropyl mesylate (Swain−Scott $s = 0.29$) is a potent mutagen in the standard Ames test and gives positive results in several in vivo assays.[9,31] Hydrolysis half-lives at pH 7.0 and 25 °C are 38 days (the same as for chloroethane; TD50 1810 mg/kg/day) and 4.5 h, respectively.[20] Unfortunately, no rodent bioassay data appear to be available for either compound, although 1,2-dichloropropane is Ames-positive in standard assays and has a mouse TD50 of 276 mg/kg/day (negative in the rat).[32] A Risk Specific Dose (RSD) for isopropyl chloride of approximately 37 μg/day (for a 50 kg patient) has been determined by Bercu et al.[33] and Contrera[34] based on QSAR (quantitative structure−activity relationship) techniques using regression analysis of "training sets" of TD50 data. [The choice of compounds in the isopropyl chloride training set could be questioned in that it contained several nongenotoxic polychloro compounds but not 1,2-dichloropropane or chloroethane.] Use of QSAR models to predict genotoxic/carcinogenic potency relies on rule-based techniques (such as DEREK) or statistical techniques (using regression analysis of training data sets), and particularly in relation to carcinogenicity, the latter approach is considered to provide more reliable results.[35]

Since halo compounds in particular and other compounds containing "Ashby alerts" are often identified as PGIs, it is interesting to note that in the determination of the TTC limit Kroes et al.[36] classified only two such compounds (5% of the total in the data set) in the lowest potency category (equivalent to 1.5 μg/day), but since the data set is nontransparent it is not possible to identify these two key compounds. [In a prior publication[37] using essentially the same data set, the TD50 for MMS is listed as 0.178 mg/kg/day, 179 times lower than the true value, and it is unclear whether this mistranscription was carried forward to the data set used by Kroes et al. in the more "definitive" publication.] In the Kroes publication,[34] no carbamates were classified in the lowest potency category, suggesting that the standard TTC of 1.5 μg/day may be unnecessarily conservative for this structural class.

Issues mentioned in the foregoing discussion may, hopefully, lead to the consideration of introducing structural class-based limits, for halo compounds and carbamates for example. The current TTC is considered to be highly conservative in general,[4] and so it is clearly the case for structural classes associated with low carcinogenic potency.

CONCLUDING REMARKS

Genotoxic impurities are associated with considerable scientific complexity and regulatory ambiguity, and so it is not surprising that published articles contain a number of conflicting interpretations. Misapplication of a TTC-based limit to compounds that are structurally nonalerting or alerting but Ames-negative appears commonplace. Sufficient data are often available in the public domain to support a robust, compound-specific risk assessment for many genotoxic and nongenotoxic carcinogens and the occasional genotoxic noncarcinogen. Basing a programme of process development and/or analytical method development on an inappropriate TTC-based limit for a PGI could lead to the unnecessary expenditure of key resources, and consequently an up-front, thoroughly researched toxicological

assessment based on data already available is considered to be a sine qua non when dealing with PGIs. Various lines of evidence suggest that simple halo compounds (and other compounds with Ashby alerts) are of relatively low carcinogenic potency, and the use of limits based on RSDs rather than the TTC seems to be scientifically justified, although regulatory acceptance of such approaches is far from certain.

AUTHOR INFORMATION

Corresponding Author

*E-mail: snodind@xiphora.com.

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