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Is there a Real Case for Cumulative Control of Structurally Related Genotoxic Impurities?

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Abstract:

Recent guidance from U.S. and E.U. regulators is that "structurally similar" impurities should be evaluated as a class and not on an individual basis. However, the perceived additive risks do not appear to have been particularly well articulated by regulators. Recent modelling studies have indicated that there is a slight increased cancer risk from multiple (≤3) genotoxic impurities, but this is insignificant compared to the very conservative assumptions incorporated into the TTC. There are serious difficulties associated with defining both "common mechanisms of toxicity" and "concurrent exposure", and this together with the limited data and associated methodologies required to conduct cumulative risk assessment suggests that these procedures are not well established. Methyl and ethyl chloride could be considered structurally similar; however, the spectrum of tumours induced by each compound is quite distinct. McGovern and Jacobsen-Kram recognized that the TTCs that are being proposed by regulators could not be divorced from their corresponding analytical challenges. They indicated that when multiple structurally related impurities were involved, that the control of individual impurities may be more difficult. Industry has extensively invested in genotoxic risk assessment and demonstrated that downstream chemistries can effectively purge these impurities from the final API. This approach reaffirms the regulators' proposition that an understanding of the underpinning science and risk assessment, which are the foundations of Quality by Design (QbD), should eliminate the need/dependency on end product testing for genotoxic impurities (Quality by Testing).

Introduction

The recent guidance from the U.S. Food and Drug Administration[1] (FDA) followed the lead of the earlier European guidance[2] in suggesting that structurally similar impurities should be evaluated as a class and not on an individual basis. FDA summarised their position as: "However, in cases where a class or family of structurally similar impurities is identified and is expected to have similar mechanisms resulting in their genotoxic and carcinogenic potential, the total daily exposure to the related compounds should be evaluated relative to the recommended threshold exposure."

Earlier, the EMEA in their Question and Answer[3] responses indicated that: "In the case of structural similarity it can be assumed that the impurities act by the same mode of action and have the same molecular target, and thus might exert effects in an additive manner. In such a situation, the limit of the sum of the genotoxic impurities at 1.5 μg/day is recommended. This might not be practically achievable with reasonable efforts, in particular when the maximum daily dose is very high and thus may demand application of lower group limits. Justifications should be made on a case by case basis, taking into consideration issues such as the following:

- Maximum daily dose of the active substance
- Therapeutic indication
- Step of the synthesis at which the genotoxic impurity(ies) arise
- Capability of the manufacturing process (purification steps) to eliminate these impurities
- Capability of the analytical procedure to control these impurities.

In cases where routine use of more powerful detection methods would be difficult, one could consider using such methods in development or testing of the first commercial batches, in order to demonstrate that the actual values are sufficiently below the Threshold of Toxicological Concern (TTC). In such a case skip testing could be considered instead of routine testing, providing that the Competent Authorities, based on a risk assessment, consider the approach as acceptable."

As with all guidance documents the "devil is often in the detail", and Industry has struggled over how best to interpret these particular requirements. This short paper looks at the above issues and other related complexities that can arise and that will undoubtedly cause confusion and implementational difficulties and will not materially affect the safety of patients in either the United States or the European Union.

Inherent Conservatism of the TTC for Medicinal Products

The current TTC is based on the pioneering work of Cheeseman et al.[4] and Kroes et al.[5] These research groups compiled extensive databases of known mutagens and carcino-

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(1) Guidance for Industry. Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER). December 2008.

(2) Guideline on the Limits of Genotoxic Impurities, Committee for Medicinal Products (CHMP), European Medicines Agency, London. 28 June 2006. (CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006).

(3) EMEA. Question and Answers on the CHMP Guideline on Genotoxic Impurities. Committee for Medicinal Products for Human Use (CHMP), European Medicines Agency, London. 9 January 2008, CHMP/SWP/431994/2007.

(4) Cheeseman, M. A.; Machuga, E. J.; Bailey, A. B. *Food Chem. Toxicol.* **1999**, *37*, 387–412.

(5) Kroes, R.; Renwick, A. G.; Cheeseman, M.; Kleiner, J.; Mangelsdorf, I.; Piersma, A.; Schilter, B.; Schlatter, J.; van Scothorst, F.; Vos, J. G.; Würtzen, G. *Food Chem. Toxicol. Lett.* **2004**, *42*, 65–83.

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gens (700+) obtained from the Carcinogenic Potency Database[6] and then used this data to extrapolate the risk inherent from exposure to novel chemicals. On the basis of these underpinning studies a probability distribution of carcinogenic potencies was used to assess the risk inherent from a daily exposure of these carcinogens that would result in less than 1×10^{-6} increased risk in contracting cancer over a whole lifetime's exposure (deemed to be 70 years). This was deemed to be a virtually safe dose and equated to a daily exposure of 0.15 μg/day for potentially genotoxic compounds. However, when applied to pharmaceuticals the overall risk was lowered to a 1×10^{-5} increased risk in contracting cancer over a whole lifetime's exposure with an associated increase in calculated TTC to 1.5 μg/day. It is worth reiterating that the CHMP committee indicated that: "It should be recognized in this context that the methods on which the original TTC value is based are generally considered very conservative since they involved a linear extrapolation from the dose giving a 50% tumour incidence (TD_{50}) to a 1 in 10^6 incidence, using TD_{50} data from the most sensitive species and most sensitive site (several "worst-case" assumptions)." It has been estimated that these worst-case assumptions may in fact exaggerate the predicted lifetime cancer risk associated with the TTC by as much as 2 orders of magnitude.[4]

Additionally, these authors identified and *excluded* from the TTC approach those compounds with structural motifs that were highly alerting and considered that there would be a significantly increased probability of cancer risk if the public were exposed to these compounds, e.g. aflatoxin-like, *N*-nitroso and azoxy compounds. The Cheeseman et al[4] cohort of 703 compounds contained 101 *N*-nitroso compounds (14.4%), i.e. one-seventh of the total database. Similarly, Kroes et al[5] in their database of 730 compounds included 105 *N*-nitroso compounds, 5 aflatoxin-like compounds, 5-azoxy compounds, 11 steroids, and 5 tetrahalogenated dibenzodioxins and dibenzofurans, which equate to 131 excluded compounds (17.9%); or about one-sixth of their total database.

Therefore, despite the fact that these databases included significant percentages of these highly potent compounds which will inevitably have biased the whole data set towards lower TTC values, these compounds were then excluded from the resultant pharmaceutical TTC concept.

Cheeseman et al[4] reported that the MALV (median adjusted log value) of their full data set was 5.47 but that if they removed those compounds containing highly alerting structures from their database, (those exhibiting carcinogenicity), that the MALV of remaining compounds within the database would fall to 4.85. This is the same value as substances testing negative in the Ames assay. The authors indicated that there was a 24-fold decrease in potency between those compound with structural alerting motifs compared with those that did not contain these moieties. Delaney[7] indicated that the majority of compounds containing structural alerting motifs would never be used by synthetic chemists as active pharmaceutical intermediate (API) reactive intermediates. Therefore, the inevitable impact of exclusion of these highly potent compounds from the pharmaceutical TTC is that these "very conservative assessments" are undoubtedly even more conservative than fully appreciated.

The main conclusion of an International Life Science (ILSI) Expert group[8] tasked with reviewing the TTC for chemical substances present in the diet reiterated that a "TTC of 1.5 μg/day/person provides adequate safety assurance and that chemicals present in the diet that are consumed below this threshold level pose no appreciable risk". This Expert group looked at the holistic risk inherent in exposure from the ***many*** reactive chemicals in the diet and did not feel the need to specify any additional additive risks inherent from the presence of multiple structurally related chemicals. Similarly during the adoption of the "Threshold of Regulation" concept for food contact materials by the FDA in the U.S. there was no discussion of potential additional risks associated with multiple structurally related chemicals.[9] The ILSI Expert group also considered that the impact of minor variations around the TTC value of 1.5 μg/day on the predicted lifetime cancer risk was in itself considered to be negligible. Concluding that, if one makes the reasonable assumption that approximately 5−10% of unstudied chemicals will be carcinogenic, then the probability of not exceeding a 1×10^{-6} lifetime risk is effectively the same for a TTC of 1.5 μg/day or 6 μg/day (96% and 95%, respectively).[10,11] This would indicate that variations around the TTC of 1.5 μg/day for a single compound are not associated with an increased lifetime cancer risk, and hence, the same rationale would apply to multiple (<4) structurally related compounds considered to possess a similar genotoxic mechanism of action.

Additional Risks Inherent in Exposure from Several Structurally Related Impurities

There is limited information in the public domain to support the argument that there are indeed additional risks inherent from inadvertent exposure to more than one structurally related impurity or, for that matter, several structurally unrelated impurities at intrinsically low levels (<TTC). Our diet exposes us to significant quantities of similar and dissimilar genotoxins and carcinogens on a daily basis, and the body has evolved sophisticated defense mechanisms to address these repeated insults.

First, as the numbers of naturally occurring toxic chemicals are significant and have been estimated at between 5,000−10,000 different natural pesticides and their breakdown products,[12] animals have of necessity developed broad-based defense mechanisms. These include a continuous shedding of external cells exposed to exogenous and endogenous toxins. The surface layers of mouth, oesophagus, and the remainder of the gastrointestinal (GI) tract as well as the skin are shed every few days.[13]

Second, there is the mobilization of the so-called 'electrophile response attack' involving the induction of phase II enzymes

(6) Berkeley Carcinogenic Potency Database (http://potency.berkel.edu/pdfs/herp.pdf).
(7) Delaney, E. J. *Regul. Toxicol. Pharmacol.* **2007**, *49*, 107–124.
(8) Barlow, S. M.; Kozianowski, G.; Wûrtzen, G.; Sclatter, J. *Food Chem. Toxicol.* **2001**, *39*, 893–905.
(9) *Federal Register* **1995**, *60*(136), 36582−36595.
(10) Munro, I. C.; Kennepohl, E.; Kroes, R. *Food Chem. Toxicol.* **1999**, *37*, 207–232.
(11) Munro, I. C. *Regul. Toxicol. Pharmacol.* **1990**, *12*, 2–12.
(12) Maarse, H. Visscher, C. A., Eds. *Volatile Compounds in Foods, CIVO-TNO*; Zeist: The Netherlands, 1989.
(13) Ames, B. N.; Profet, M.; Gold, L. S. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7782–7786.

such as glutathione-*S*-transferases (GST), NAD(P)H-quinone acceptor oxidoreductases (QRs), UDP glucosonyltransferase (UGT), and epoxide hydrolyase (EH) as well as the elevation of intracellular levels of reduced glutathione in peripheral tissues in response to a variety of electrophiles and antioxidants.[14]

Third is the active efflux via P glycoprotein (PgP)-mediated mechanisms (or similar) of planar hydrophobic molecules, both natural and synthetic, out of the GI tract and liver cells. Fourthly, the body uses effective and inducible DNA repair mechanisms against electrophile-induced DNA−adduct formation, and hydrolysis and oxidation of DNA via reactive oxygen species (ROS). Finally, the body has effective olfactory and gustatory senses which have the ability to discern bitter, pungent, astringent, or acidic chemicals, often at low concentrations, and the ability to consciously or unconsciously (the latter via vomiting and/or diarhorrea) remove these offending foodstuffs and beverages.[12]

A good example of the body's exposure to multiple structurally related genotoxins and carcinogens is provided by lipid metabolism. Volatile aldehydes are the major byproducts of lipid oxidative degradation. They are also extremely biologically reactive, forming adducts with DNA, proteins, and phospholipids.[15] Amongst the myriad of volatile aldehydes formed by lipid metabolism are formaldehyde, acetaldehyde, 4-hydroxy-2-nonenal, malonaldehyde, acrolein, β-methylacrolein (crotonaldehyde), glyoxal, and methyl glyoxal.

High concentrations of aldehyde-metabolizing enzymes are found in both cytosols and endoplasmic reticulum, and these enzymes have overlapping substrate specificity; as a result, most cells appear to be relatively resistant to aldehyde-induced toxicity. The most electrophilic aldehydes, for example, acrolein, 4-hydroxy-2-nonenal, and methylglyoxal are the most readily metabolized (<3 min) and hence detoxified. For 4-hydroxy-2-nonenal this short half-life was attributed to a combination of GST, aldehyde dehydrogenase-2 (ALD-2), and to a limited extent, alcohol dehydrogenase (ADH), the latter case being a prime example of the broad-based, overlapping substrate specificity of many of these enzyme systems. In contrast, aldehydes do appear to have a role in cell growth and proliferation and may indeed have a role in actually preventing carcinogenesis by inducing phase II metabolizing enzymes, such as aldehyde dehydrogenase and aldehyde reductase.[16]

Bercu et al.[17] recently published an evaluation of the impact of combining multiple impurities that were either in similar or dissimilar structural classes and concluded that estimates of risk were not greatly impacted. They performed simulations to assess the additive risk (if any) inherent in exposure to structurally related genotoxic compounds compared to the overall risk inherent in exposure to structurally unrelated genotoxic compounds and demonstrated similar findings. The authors indicated that there is a slight increase in cancer risk for 2−3 impurities, but concluded that this was insignificant compared to the very conservative assumptions that were incorporated into the TTC. Importantly, they indicated that toxicological synergies were unlikely at these extremely low doses (<TTC).

In addition, Ames et al.[18] have concluded that the cancer hazards from natural carcinogens are higher than from their synthetic cousins based on the HERP Index (Human Exposure/Rodent Potency). These findings were corroborated by the UK National Research Council[19] who indicated that they had greater concerns regarding natural, rather than synthetic carcinogens. However, several researchers commented on the logic disconnect whereby the lay public appears to believe naturally occurring substances to be safe, and industrially derived materials to be dangerous.[20]

The regulatory authorities also seem to view the perceived risk of genotoxic impurities from medicinal and herbal products from very different perspectives. The EMEA's Herbal Medicinal Products Committee (HPMC) has issued a draft guideline[21] on the assessment of genotoxic constituents of herbal medicines. However, in contrast to the CHMP[2,3] guidance, HPMC have highlighted that the growth in the use of herbal medicines for self-treatment is unlikely to be impacted by this guidance and cautioned that regulatory authorities should not be overzealous in banning such products based on "extrapolated suspicions". HPMC stressed the need to develop robust risk-benefit assessments for herbal products. They conceded that the complex and variable (season to season, geographical origin, or mode of preparation) nature of herbal products presents additional challenges compared to standard medicinal products. The committee accept that herbal medicines are complex mixtures with large numbers of components which can have highly variable composition. The HPMC further indicated that "the complete composition is very difficult to unravel, so one can argue that there are always many unknown constituents and thus there may be hidden dangers".

The committee[21] further cautioned that, even for well-established genotoxins with known safety profiles, the complexity of the herbal medicine may make it difficult, if not impossible, to establish a TTC. Thus, multiple genotoxic components in herbal products appear to be viewed very differently from multiple genotoxic impurities in medicinal products.

The body's daily exposure to natural pesticides (many of which either contain structural alerts for potential genotoxicity or have been demonstrated to be positive in recognised *in vitro* or *in vivo* genotoxicity assays), based on the quantity (5,000−10,000 different natural pesticides and their breakdown products),[13] diversity (from simple volatile aldehydes such as formaldehyde to complex phytochemicals such as the potato

(14) Prestera, T.; Zhang, Y.; Spencer, S. R.; Wilczak, C. A.; Talalay, P. *Adv. Enzyme Reg.* **1993**, *33*, 281–296.
(15) Shibamoto, T. *J. Pharm. Biomed. Anal.* **2006**, *41*, 12–25.
(16) O'Brien, J.; Renwick, A. G.; Constable, A.; Dybing, E.; Müller, D. J. G.; Schlatter, J.; Slob, W.; Tueting, W.; van Benthem, J.; Williams, G. M.; Wolfreys, A. *Food Chem. Toxicol.* **2006**, *44*, 1613–1635.
(17) Bercu, J.; Hoffman, W. P.; Lee, C.; Ness, D. K. *Regul. Toxicol. Pharmacol.* **2008**, *51*, 270–277.
(18) Ames, B. N.; Profet, M.; Gold, L. S. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7777–7781.
(19) National Research Council *Carcinogens and Anticarcinogens In the Human Diet: a Comparison of Naturally Occurring and Synthetic Substances*; National Academies Press: Washington, D.C., 1996; http://wwww.nap.edu/readingroom/books/diet/index.html.
(20) Sugimura, T. *Carcinogenesis* **2000**, *21*, 387–395.
(21) Concept paper on the Development of a Guideline on the Assessment of Genotoxic Constituents in Herbal Substances/Preparations; Committee for Medicinal Products (CHMP), European Medicines Agency: London, 25 October 2006; EMEA/HPPC/413271/2006.

glycoalkaloids, α-chaconine and α-solanine) and absolute exposure (about 1.5 g/day of naturally occurring pesticides[18] is staggering.

However, the body is well adapted to cope with this onslaught and has a well-developed protection mechanism that includes inducible metabolic responses. Walker et al.[22] indicated that animal cells successfully resolve an estimated 10,000 chromosomal lesions/day. Given that there is an intrinsically low background level of persistent DNA mutations, which is in the range of 1−8 mutations per million cells, this stands testimony to the damage-resolution capacity of genomic cellular maintenance processes. On the basis of this background it seems illogical that regulators should believe that exposure to low levels (<TTC) of multiple GIs should in anyway constitute a greater threat to public safety.

Relative Toxicity of Structurally Similar GIs and Setting of Logical Specification Limits

The underlying assumption behind this aspect of the guidance[3] appears to be that both parties, Industry and Regulators, would have a similar understanding of the concept of Structural Similarity. This is probably true for well-documented classes of genotoxic compounds, for example, alkyl chlorides (e.g., methyl, ethyl, propyl, isopropyl chloride, etc.), sulfonate esters (e.g., methyl, ethyl, propyl, isopropyl mesylate). However, even in these well-documented cases the relative toxicity of the different members of that class is often different. McGovern and Jacobsen-Kram[23] stated, "However, it cannot be an *a priori* assumption that all members of a related series of compounds possess the same degree of genotoxic potency and/or analytical response factor (e.g. the polyaromatic hydrocarbons). Such cases should be studied on a case-by-case basis."

For example, the simple alkyl chlorides methyl and ethyl chloride could be considered structurally similar and indeed share very similar positive Ames profiles in the bacterial strains TA100, TA1535, and WP2UvrA,[24] and furthermore both compounds are carcinogenic in rodents. However, the spectrum or tumours induced by each compounds are quite distinct, with methyl chloride inducing renal tumours in male mice[25] and ethyl chloride inducing uterine tumours in female mice.[26]

This view was also articulated by Wilkinson et al.[27] The authors stated that in order for a compound to be considered to be acting through a common mechanism of toxicity, the chemicals should exert an identical toxic effect in the same target organ via an identical biological mechanism. The authors went on to indicate that there are serious difficulties associated with defining both "common mechanisms of toxicity" and "concurrent exposure" and that this together with the limited data and associated methodologies required to conduct cumulative risk assessment led them to suggest that these procedures could not by applied to pesticide regulation in food. It is pertinent to ask that, if this is the case in the well-studied field of pesticide regulation, will it not also be the case in genotoxic impurity regulation in medicinal products?

The recent Viracept withdrawal issue, which was attributed to elevated levels of ethyl methanesulfonate (EMS) in the drug product,[28] highlights the problems of trying to set rational combined specifications. After a thorough review by the company and regulatory agencies, there was also deemed to be a related risk of methyl methanesulfonate (MMS) formation due to possible methanesulfonyl chloride contamination in input batches of methane sulfonic acid. Initially a specification level of 0.6 ppm EMS was proposed, which was based on the TTC limit of 1.5 μg/day and doses of 2.5 g/day drug substance. However, after discussions with CHMP, a slightly lower combined limit for both EMS and MMS was set (0.5 ppm), equivalent to ≤1.25 μg/day total genotoxic impurity (<TTC). This combined limit is somewhat conservative when one considers that a compound-specific assessment for MMS using the existing carcinogenicity data (i.e. linear extrapolation from the TD_{50} of 31.8 mg/kg/day[6]) would result in a proposed Acceptable Daily Intake of MMS in excess of the TTC (∼31.8 μg/day based on a 50 kg human). In addition, after additional *in vitro* and *in vivo* genotoxicity studies on EMS, the proposed Acceptable Daily Intake for this particular compound was set at 104 μg/day which is also well in excess of the TTC.[29]

The volatile aldehydes also exemplify the different toxicities that are possible within the same structural class of compounds. Acrolein is the most cytotoxic volatile aldehyde, followed by 4-hydroxy-2-nonenal, whereas methylyglyoxal is the most mutagenic and the most significant of the endogenously encountered volatile aldehydes (which is attributed to its origins in glycolysis) followed by formaldehyde. Cinnamaldehyde is clastogenic and may be weakly carcinogenic. Glyoxal and methylglyoxal both have tumour-promoting potential.[16] The carcinogenic classifications of some of these volatile aldehydes are provided in Table 1.

On the basis of these variable and often confounding factors, how does Industry set realistic specifications for structurally related genotoxins, which may exhibit differing toxicities? ICH Q3C[30] provides some precedence, inasmuch as there are no requirements within this guideline for imposing group limits on class I solvents, e.g., halogenated alkanes.

The Impact of Metabolic Activation on the Concept of Structural Similarity

One area of potential interpretation (or misinterpretation) of this part of the guidance that appears not to have been fully considered by the regulators concerns the impact of metabolic activation on the toxicity of genotoxic impurities. Only a small

(22) Walker, V. E.; Casciano, D. A.; Tweats, D. J. *Toxicol. Lett.* **2009**, DOI: 10.1016/j.toxlet.2009.03.027.
(23) McGovern, T.; Jacobsen-Kram, D. *Trends Anal Chem.* **2006**, *25*, 790–795.
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(25) International program on chemical safety (http://www.inchem.org/documents/sids/sids/CLMETHANE.pdf).
(26) Picut, C. A.; Aoyama, H.; Holder, J. W.; Gold, L. S.; Maronpot, R. R.; Dixon, D. *Exp. Toxicol. Pathol.* **2003**, *55*, 1–9.
(27) Wilkinson, C. F.; Christoph, G. R.; Julien, E.; Kelley, J. M.; Kronenburg, J.; McCarthy, J.; Reiss, R. *Regul. Toxicol. Pharmacol.* **2000**, *31*, 30–43.
(28) Press Release: European Medicines Agency Agrees on Action Plan Following the Recall of Viracept and Recommends Suspension of the Marketing Authorization, London, 2007. EMEA/275367/2007.
(29) Müller, L.; Gocke, E. *Toxicol. Lett.* **2009**, *190*, 30–332.
(30) ICH Expert Working Group. Impurities: Guidelines for Residual Solvents, ICH Q3C (R4), **2009**.

Table 1. **Carcinogenic classifications of volatile aldehydes (derived from O'Brien et al., 2007[16])**

volatile aldehyde	IARC[a] classification	IARC definition	IRIS[b] classification	IRIS definition
formaldehyde	I	carcinogenic to humans	B1	probable human carcinogen (limited evidence)
acetaldehyde	IIB	possibly carcinogenic in humans	B2	probable human carcinogen (inadequate evidence)
acrolein	III	not classifiable as to human carcinogenicity	D	not classifiable
β-methylacrolein (crotonaldehyde)	III	not classifiable as to human carcinogenicity	C	possible carcinogen

[a] IARC (International Agency for Research on Cancer). [b] IRIS (Integrated Risk Information System).

Table 2. **Compound classes showing structural alerts leading to high risks of carcinogenicity and their route of metabolic activation (if any)[a]**

number	structural motif alerting for carcinogenicity	metabolic activation (if any)
1	primary alkyl halides[b]	directly acting electrophile
2	aryl amines and alkylated aryl amines	*in situ* formation of nitrenium ion
3	aromatic nitro (and some aliphatic nitro)	*in situ* formation of nitrenium ion
4	azo compounds[c]	*via* reduction and *in situ* formation of diazonium ion
5	unsymmetrical hydrazines[c]	*in situ* formation of carbocation or diazonium species
6	disubstituted hydrazines[c]	*via* reduction and *in situ* formation of diazonium ion
7	alkyl aldehydes	directly acting electrophile
8	epoxides (both alkyl and aryl)[e]	formation of α,β-unsaturated carbonyl compounds
9	aziridines (both alkyl and aryl)[e]	nucleophilic attack by cellular nucleophiles
10	*N*-nitroso	*via* formation of carbocation or diazonium species
11	esters of sulfonic and phosphonic acids (both alkyl and aryl)[b]	directly acting electrophile
12	alkyl *N*-oxides, aryl *N*-oxides	unknown
13	*N*-chloro amines[b]	accumulation possible
14	Michael reagents (amides e.g. acrylamide, nitriles, α,β-unsaturated esters)[c]	intrinsic electrophilicity
15	carbamate derivatives (urethanes)	*in situ* formation of epoxide
16	*N*-methylol derivatives	*in situ* formation of formaldehyde
17	*N*- and *S*-mustards (β-haloethyl)	*in situ* aziridine formation
18	propiolactones (and their thiolated derivatives, propiosultones)	nucleophilic attack by cellular nucleophiles
19	monohaloalkenes	*in situ* formation of epoxide
20	heavy metal compounds[e]	not included in pharmaceutical TTC
21	polycyclic amines	*in situ* formation of nitrenium ion
22	organophosphorous compounds[e]	not included in pharmaceutical TTC
23	aflatoxin-like compounds[e]	*in situ* formation of epoxide
24	azoxy compounds[c]	*via* reduction and *in situ* formation of diazonium ion
25	benzidine compounds[f]	*in situ* formation of nitrenium ion
26	steroid-like compounds[g]	not included in pharmaceutical TTC
27	tetrahalogenated dibenzodioxins and dibenzofurans[g]	accumulation possible
28	vinyl containing compounds	*in situ* formation of epoxide
29	*N*-hydroxy aminoaryls	*in situ* formation of nitrenium ion
30	*N*-acetylated aminoaryls	*in situ* formation of nitrenium ion

[a] Based on Cheeseman et al., 1999, and Kroes et al., 2004. [b] Part of miscellaneous Ashby alerts (Kroes et al., 2004). [c] Part of Cheeseman's original hydrazine grouping (Cheeseman et al., 1999). [e] Part of Cheeseman's original strained ring grouping (Cheeseman et al., 1999). [e] Often viewed separately under neurotoxic classification (Kroes, 2004). [f] Benzidine is often included in polycyclic amine grouping (Kroes, 2004). [g] Part of Cheeseman's original endocrine disrupter grouping (Cheeseman et al., 1999).

number of GIs are directly acting mutagens, which is based on their electrophilic nature, e.g. alkyl esters of sulfonic acids, alkyl halides, *N*-chloro compounds, and alkyl aldehydes. In contrast, the vast majority of GIs display little or no intrinsic genotoxic potential prior to undergoing metabolic activation to an electrophilic metabolite[31] (see Table 2).

Aromatic amines and aromatic nitro compounds both form the same highly reactive nitrenium ion, the former via an *N*-hydroxylation pathway whereas the latter is via reduction through the intermediate nitroso compound.

Polyaromatic hydrocarbons are activated to the corresponding diol epoxides. Alkyl *N*-nitroso compounds and unsymmetrical alkyl hydrazines are bioactivated to carbocations and diazonium compounds, whereas dialkyl hydrazines, azo, and azoxy compounds are metabolized to the corresponding diazonium compound via the *C*-hydroxylated intermediates.

It is often difficult for synthetic chemists to fully recognize structural similarity within GIs with complex, multifunctional reactive intermediates; however, if they were also required to incorporate metabolic activation and include structural motifs that share a common metabolic end-point, then it would be a very difficult task indeed. For example, 2,4-diaminotoluene and 2,6-diaminotoluene could be considered structurally similar, and furthermore they are both positive in the Ames assay in the presence of metabolic activation in the bacterial strains TA98

(31) Testa, B.; Krämer, S. D. *Chem. Biodiversity* **2009**, *6*, 591–684.

and TA100. However, the carcinogenicity of these compounds is quite distinct. Whereas 2,4-diaminotoluene induces tumours in the liver of rats and mice, 2,6-diaminotoluene is not carcinogenic in rodents.[32] The relationship between aromatic amines and aromatic nitro compounds is also difficult to interpret. For example 2,6-dinitrotoluene and 2,6-diaminotoluene are both Ames positive[28] (the former in the presence and absence of metabolic activation, the latter in the presence of metabolic activation only). However, while 2,6-dinitrotoluene induces liver tumours in rats,[6] 2,6-diaminotoluene is not carcinogenic in rodents.

In short, even within the known carcinogens belonging to the various chemical classes used to derive the original "threshold of regulation" the potency of compounds in a related class varied by up to several orders of magnitude.[4] This data alone demonstrate that members of a related chemical class may not necessarily possess the same degree of genotoxic and carcinogenic potency. Clearly, the default assumption concerning the combined control of structurally related impurities requires further examination.

Dose

The European guidance[3] indicates that the maximal daily dose of the API is an important factor as this may demand application of lower group limits, which might not be practically achievable. Although this is an important factor, especially from an analytical consideration, it is not the only consideration. For low-dose compounds, e.g. inhaled drugs where doses of 100 μg are not atypical, the challenge here is that the TTC (1.5 μg/day) will be significantly higher than the ICH Q3A limit,[33] and Industry applies the standard ICH criteria (which in this case are more stringent), and group limits for structurally similar GIs are therefore not applicable.

However, consideration of the API dose should not be the only deliberation. It is a well-accepted tenant of toxicology that it is the dose that makes the poison. There is ample literature precedent for naturally occurring antioxidants that, whilst having genuine antimutagenic activity at low concentrations, are often themselves mutagenic at higher concentrations.[34] Hence, the lower the dose the less toxic the compound, and even genotoxic alkylating agents have been shown to exhibit thresholded mechanisms of toxicity.[29] Hence, at very low doses (<TTC) genotoxic substances are deemed to be present at virtually safe levels (VSL) and there is little evidence for additive effects.

Therapeutic Indication

It has been reported that half of the drugs cited in the *Physician's Desk Reference* are carcinogenic in rodent studies.[35] Similarly, nearly half of the new drug submissions to U.S. Food and Drug Administration (FDA) are also carcinogenic.[36]

A recent ICH guidance document (ICH S9[37]) has indicated that, as the clinical dose levels in oncology studies are often closely aligned with the adverse effect doses, there is scope for flexibility in these studies. Whilst recognizing that genotoxic impurities in oncology products are unlikely to be beneficial, the guidance contends that the existing guidance based on negligible lifetime risk (based on 1 in 10^5 or 10^6 increased risk) might be inappropriate for an oncology drug where the intended patient population is suffering from a life-threatening disorder and where the life expectancy could be measured in months, rather than the 70 years used in the determination of the TTC limit of 1.5 μg/day. In addition, the patient will often be treated with a cocktail of cytotoxic drugs to help alleviate the condition. Against this background it would be illogical to be overly concerned about multiple genotoxic impurities in a potentially genotoxic or carcinogenic API.

In addition, many drugs have often been taken to market for nonlife-threatening therapies because the observed genotoxicity was not found to present hazard to humans. These include acyclovir (over the counter antiviral), citalopram (antidepressant), claritin (over the counter antiallergy), griseofulvin (antifungal), theophylline (bronchodilator), and zolmitriptan (antimigraine). There are currently efforts from many international agencies to address the perceived 'false positive' rate associated with *in vitro* genotoxicity tests in terms of their correlation with rodent carcinogenicity.[38]

Capability of the Analytical Procedure To Control These Impurities

While stating that impurities should always be reduced to the lowest levels that are reasonably practical, Jacobson-Kram and McGovern[39] at the Center for Drug Evaluation and Research, FDA, acknowledged that impurities cannot be reduced to zero and meaningful specifications for impurities need to be established. The authors advised that the presence of genotoxic impurities should be avoided; however, they also recognized that complete removal is often not possible. In these cases, the amounts of genotoxic impurity present should be limited to a level that represents an insignificant risk to clinical trial subjects or patients, for example, the TTC.

Thus, there is a requirement for stringent analytical control measures for reactive and often volatile genotoxic impurities. These measures, which often necessitate control of these analytes at the low ppm level relative to the API, pose very real analytical challenges. This in turn necessitates the application of sensitive, sophisticated, and often hyphenated analytical techniques e.g. GC-MS, HPLC-MS. However, this analytical control strategy needs to be based on a sound appreciation of process understanding aligned with quality by design (QbD) approaches.

As with all types of trace analysis the most significant challenge is often posed by the sample matrix (in the intermedi-

(32) http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr200.pdf.
(33) ICH Expert Working Group. Impurities in New Drug Substances. ICH Q3A(R2), **2006**.
(34) Cao, J.; Li-Ping, J.; Liu, Y.; Yang, G.; Xiao-Feng, Y.; Lai-Fu, Z. *Toxicon* **2007**, *49*, 1219–1222.
(35) Davies, T. S.; Monro, A. *J. Am. Coll. Toxicol.* **1995**, *14*, 90–107.
(36) Contrera, J.; Jacobs, A.; DeGeorge, J. *Regul. Toxicol. Pharmacol.* **1997**, *25*, 130–145.
(37) ICH Expert Working Group. Non-Clinical Evaluation for AntiCancer Pharmaceuticals, Step 3. ICH S9. EMEA/CHMP/ICH/646107/2008, December **2008**
(38) ICH Expert Working Group. Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use, ICH S2 (R1); http://www.ich.org/LOB/media/MEDIA3304.pdf.
(39) Jacobson-Kram, D.; McGovern, T. *Adv. Drug Delivery Rev.* **2007**, *59*, 38–42.

ate, API, and particularly, the drug product), and both resolution and sensitivity can be impacted by substrate solubility and analytical interference from both the main component of the substrate (e.g., API), but also minor components of the substrate, e.g., other impurities, degradation products, contaminants, and excipients in drug products.[40]

This testing strategy must be phase appropriate, and ironically more rigour and analytical sophistication, e.g., HPLC-MS or GC-MS, is typically required during the early stages of clinical development, where there is limited knowledge and experience of both the chemistry and any relevant control strategies, than at the commercial stage where there is a full understanding of both, supplemented by extensive testing of key intermediates and if required, the API itself.

Limit tests are often employed, with an absolute pass/fail criteria based on allowable exposure, e.g. TTC or staged TTCs.[41] These methods are fit for purpose and validated, but the validation requirements are less demanding than expected for a fully quantifiable method. Limit tests are validated for specificity (especially absence of interference), sensitivity (often just LOD), and accuracy. In the latter case, the demands of trace analysis often necessitate broader limits for recoveries of the analytes; indeed, it is possible to accept some level of bias as long as the method 'fails safe'. An obvious disadvantage of the limit test approach is that trending is not possible, and lowering of the toxicology aligned limit based on new information would necessitate retesting of all affected batches.

There are many different approaches to either increasing selectivity and/or improving sensitivity. Reduction of the matrix effect can be facilitated by extraction techniques, e.g., solid/solid, liquid/liquid, liquid/solid extraction, and the volatility of the analyte can be utilised to minimise matrix interference. The latter example has seen the renaissance of both GC and the related headspace GC techniques. Indeed, headspace GC can be used with nonvolatile analytes by introducing a volatile derivative, e.g. *in situ* derivatization of EMS with pentafluorothiophenol and headspace GC-MS.[42]

McGovern and Jacobson-Kram[23] recognized that the acceptable daily intakes (ADI) that are being proposed by regulators could not be divorced from their corresponding analytical challenges. However, they contended that the current state of analytical science was adequately advanced to meet the challenges inherent in low-level analyte(s) determination. They asserted that GC or HPLC, coupled with MS detection, should provide an adequate starting point for the development and validation of appropriately sensitive methods.

However, they indicated that, when multiple structurally related impurities were involved, for example, polymeric oligonucleotides or polyaromatic hydrocarbons, that the control of individual impurities may be more difficult. In these cases they proposed that control of all of the related impurities within that group could be countenanced, provided that the toxicology within the group was common and that relative potency was similar. They stated that: "it may be proposed that the group collectively meets the exposure limits as if it were a single compound."

Step of the Synthesis at Which the Genotoxic Impurity(ies) Arise and Capability of the Manufacturing Process (Purification Steps) to Eliminate These Impurities

The European guidance[3] indicates that the capability of the synthetic process to remove these impurities is an important factor. Although general guidance in this critical area is somewhat limited, the Production Statements of the *Ph. Eur.*, covering the potential formation of GIs, e.g. sulfonate esters in salts of sulfonic acids, do address the philosophy of this approach, whereby the production process must be evaluated to determine the likelihood of alkyl sulfonate formation and control measures that are applicable (including fully validated analytical methodologies), demonstrating that these impurities would be adequately controlled and would not be carried forward into the resultant API.[43]

Several Industry groups have reviewed control strategies aimed at eliminating or at least controlling genotoxic impurities within synthetic processes.

Argentine et al.[44] reported on the analytical control strategy for the control of residual formaldehyde in a novel API. The authors showed via process assessments that control of formaldehyde in either intermediate 1 or 2 at 10 ppm levels would ensure acceptable levels in the final API, even with no downstream purging of this volatile analyte. They developed a derivative-based approach allowing a suitably validated HPLC-UV method to be employed with an LOD of 1 ppm. Batches of intermediate 1 all showed levels well below the 10 ppm limit. They then assessed the capability of the downstream processes to effectively purge this analyte from the process using spiking, purging, and impurity fate mapping strategies. Intermediate 1 was spiked with formaldehyde at 1000 and 5000 ppm levels, and subsequent analysis of the resultant intermediate 2 showed up to 500-fold reduction in the signal analyte down to levels below the 10 ppm alerting limit. The authors considered the combination of these impurity fate mapping experiments coupled with low levels of formaldehyde typically seen in intermediate 1 (<10 ppm) and final stage purification of API were adequate to support the contention that specifications for residual formaldehyde in the final API were not justified.

Pierson et al[40] reported on a generic approach to the assessment, testing strategies and analytical assessments of genotoxic impurities in API. Their approach was predicated on where in the synthetic process the potential genotoxic impurity was introduced. Introduction in the final stage of the API was the worst-case scenario and would necessitate the introduction of specifications (dependent on the parallel toxicology assessment), with the caveat that demonstration of absence through purging strategies might be supportive of omission. They

(40) Pierson, D. A.; Olsen, B. A.; Robbins, D. K.; DeVries, K. M.; Varie, D. L. *Org. Process Res. Dev.* **2009**, *13*, 285–291.

(41) Mûller, L.; Mauthe, R. J.; Riley, C. M.; Andino, M. M.; de Antonis, D.; Beels, C.; DeGeorge, J.; De Knaep, A. G. M.; 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. *Regul. Toxicol. Pharmacol.* **2006**, *44*, 198–211.

(42) Alzaga, R.; Ryan, R. W.; Taylor/Worth, K.; Lipczynski, A. M.; Szucs, R.; Sandra, P. *J. Pharm. Biomed. Anal.* **2007**, *45*, 472–479.

(43) Amlodipine Besylate monograph, Ph.Eur. monograph 1491. *European Pharmacopoeia* , 6th ed. (as amended by Supplements 6.1 and 6.2, Council of Europe, Strasbourg), **2009**.

(44) Argentine, M. D.; Owens, P. K.; Olsen, B. A. *Adv. Drug Delivery Rev.* **2007**, *59*, 12–28.

exemplified this with the strategies that can be applied for the control of genotoxic sulfonate esters that can be formed from sulfonate salts crystallised from alcoholic media.

Pierson et al[40] exemplified the process by reporting on the impurity fate mapping of residual formaldehyde used in the manufacture of phenylmethylaminopropanol (PMAP), a starting material for fluoxetine and atomexetine. The proposed limit for residual formaldehyde in API was 16 ppm. The purging investigations were complicated by the fact that PMAP can further react with residual formaldehyde to form the cyclic phenyloxazine, and methods were developed to determine formaldehyde or formaldehyde equivalents (paraformaldehyde or phenyloxazine). There were no reportable levels of free formaldehyde in multiple batches of PMAP, and spiking experiments using both formaldehyde and phenyloxazine at levels as high as 5000 ppm showed residual levels of less than 10 ppm (i.e., 500-fold reduction). These data were used to justify the omission of a specification for residual formaldehyde in the API.

The recent withdrawal and subsequent reapproval of Viracept from the European markets focussed regulatory attention on sulfonate esters.[28] However, it is worth noting that the ethyl methanesulfonate (EMS) was preformed and found to originate from contamination of a reagent (methane sulfonic acid) and was not formed during mesylate salt synthesis. Implementation of a range of remedial actions led to a rapid reintroduction of the product onto the EU market.

European regulators have advocated subsequently a variety of risk mitigation strategies to ensure that alkyl sulfonate residues are controlled to appropriate, safety-based limits. The preventative measures together with a specification limit of 0.5 ppm for residual methyl methane sulfonate (MMS) and EMS in nelfinavir mesylate API, was deemed to be acceptable by EMEA.

There appears to be an underlying assumption on behalf of the regulators that in those cases where there are multiple structurally related genotoxic impurities within a synthetic process that they all will carry a similar risk in terms of likely carryover into the final API. However, even those structurally related genotoxic impurities that are formed at the same stage of the synthetic process often show different reactivities and will be purged to **different** extents by the common downstream chemistries. A recent good example of this is the control strategies for the structurally related sulfonate esters: MMS, EMS, and IMS (isopropyl methane sulfonic acid) in a novel API. Cimarosti et al[45] reported that when the API was crystallised with MsOH (methane sulfonic acid) containing elevated levels (up to 5× the specification limits) of the three sulfonate esters (MMS, EMS, and IMS) and, in addition, extra quantities of the three esters were added to the mother liquors just prior to filtration, that levels after deliquoring were inversely proportional to their Swain−Scott[46] *s* values,[47] i.e. the most reactive ester had the lowest levels of residual ester present in the API (MMS < EMS < IMS).

Companies often need to assess the issue of purging of genotoxic impurities in drug product processes and developing and appropriately validating supporting sensitive analytical methodologies. Gerber and Toelle[48] reported that between 60−70% of EMS contained within API was hydrolysed during tablet manufacture, storage, and distribution. They reported that EMS hydrolysis rate in Viracept tablets (250 mg) was approximately 0.3%/day at 25 °C and 0.2%/day at 20 °C, corresponding to rates of 9%/month and 5%/month, respectively.

Liu et al[49] reported on the analytical control strategy for five potential genotoxic impurities in a novel oncology product, pazopanib hydrochloride. They focussed on upstream control in starting materials or intermediates, thereby circumventing the need for control in the final drug substance. The attractiveness of this approach is that it allows for control limits to be set at higher levels, with the full knowledge that subsequent downstream purging will reduce the levels of analytes to acceptable levels and allow the control strategy to be based on less sophisticated and sensitive analytical methods, which are much more aligned to a routine, quality control environment.

Industry has extensively invested in genotoxic risk assessment, demonstrating that downstream chemistries can effectively purge these impurities from the final API. This approach reaffirms the regulators' proposition that an understanding of the underpinning science and risk assessment, which are the foundations of Quality by Design (QbD), should eliminate the need/dependency on end-product testing for genotoxic impurities (Quality by Testing).

Conclusion

The TTC concept was originally derived from a group of about 700+ genotoxic compounds. Interestingly, when applied to pharmaceutical assessments the most toxic compounds, e.g., *N*-nitroso, aflatoxins, etc., were specifically excluded from any further considerations. This inevitably biases the database towards a lower TTC value than supported by the original data. Indeed, Cheeseman et al.[4] showed that if the highly alerting compounds were removed from their database that the Median Adjusted Log Value (MALV) of the remaining compounds fell to 4.85, a value similar to those of compounds which were negative in the Ames test. Whereas, the MALV of the high concern subset rose to 6.18. This was corroborated by Barlow et al.,[7] who indicated that a "TTC of 1.5 μg/day/person provides adequate safety assurance and that chemicals present in the diet that are consumed below this threshold level pose no appreciable risk". The additive risk inherent in exposure from the ***many*** reactive chemicals in the diet was felt to be acceptable. They further stated that "the inclusion of large numbers of chemicals does not radically alter the distributions for noncarcinogenic end-points".

Indeed, the perceived additive risks inherent in the exposure to several, or indeed many, structurally related impurities do

(45) Cimarosti, Z.; Bravo, F.: Stonestreet, P.; Tinnazi, F.; Vecchi, O.; Camurri, G. *Org. Process Res. Dev.* **2009**, DOI: 10.1021/op900242x.
(46) Swain, C. G.; Scott, C. B. *J. Am. Chem. Soc.* **1953**, *75* (1), 141–147.
(47) Some data are available in the preceeding reference to assess relative chemical reactivity, in particular, the Swain−Scott *s* constants, which assess the sensitivity of an electrophilic substrate to nucleophilic attack. The sulfonate esters (MMS, EMS, and IMS) have very different *s* values: MMS (0.83) > EMS (0.67) > IMS (0.29).
(48) Gerber, C.; Toelle, H.-G. *Toxicol. Lett.* **2009**, DOI: 10.1016/j.toxlet.2009.02.020.
(49) Liu, D. Q.; Chen, T. K.; McGuire, M. A.; Kord, A. S. *J. Pharm. Biomed. Anal.* **2009**, *50*, 144–150.

not appear to have been particularly well articulated by regulators. From a physiological perspective the body appears to be well adapted to cope with repeated insults from the many structurally related genotoxic compounds in the diet. A particularly compelling example of this is the volatile aldehydes e.g., formaldehyde, acetaldehyde, acrolein, etc., a group of structurally related naturally occurring aldehydes, which are produced by the oxidation of lipids. All members of this class are extremely biologically reactive, forming adducts with DNA, proteins, and phospholipids. However, mammalian cells appear to be resistant to aldehyde-induced toxicity. This is reassuring as Ames[18] has reflected that the cancer hazard from natural carcinogens in the diet is higher than from the corresponding synthetic compounds based on Human Exposure/Rodent Potency (HERP) Index assessments.

Recently the EMEAs Herbal Medicinal Products Committee (HPMC)[21] cautioned that, even for well-established genotoxins with known safety profiles, the complexity of herbal medicines may make it difficult, if not impossible, to establish a TTC, thus appearing to reflect that the multiple components in herbal products (which are often genotoxic due to their plant origins) are viewed very differently from multiple impurities in medicinal compounds, even though the former are likely to be more toxic than the latter, based on HERP assessments.

Bercu et al.[17] recently showed that there was little enhanced risk from exposure to either multiple related or unrelated GIs. They concluded that any synergistic effects were unlikely at low doses ($\leq$TTC) and that the inherently conservative nature of the TTC adequately covered any minor increased risk.

The issue of metabolic activation of many GIs has not being considered when assessing 'structural similarity'. This will undoubtedly cause further confusion during the implantation of this particular part of the guidance.

The introduction of ICH S9[37] appears to offer greater flexibility in the need to control PGIs, and particularly structurally related PGIs in oncology products. This is to be welcomed.

Industry has extensively invested in genotoxic risk assessment, demonstrating that downstream chemistries can effectively purge these impurities from the final API. This approach reaffirms the regulators' proposition that an understanding of the underpinning science and risk assessment, which are the foundations of Quality by Design (QbD), should eliminate the need/dependency on end-product testing for genotoxic impurities (Quality by Testing).

In concluding, it seems to be a legitimate question to ask - if the body can cope with repeated insults of very low levels (<TTC) of structurally related (and nonstructurally related) genotoxins and carcinogens from the diet that pose a greater hazard than corresponding synthetic genotoxins, why is there a need to introduce further controls into the TTC approach, based on structural similarity concerns, when the TTC is already acknowledged to be conservative in nature? This is especially true as the supporting science required to assess the cumulative risk of exposure (in particular "common mechanisms of toxicity" and "concurrent exposure") to multiple genotoxins is still in its infancy. The introduction of additional requirements for structurally similar GIs appears not to be based on a good understanding of the underpinning science and is unlikely to materially affect the safety of patients in either the United States or the European Union.

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