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Control of Genotoxic Impurities in Active Pharmaceutical Ingredients: A Review and Perspective

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Abstract:

Recent guidelines from drug regulatory authorities in Europe and the Untited States of America (USA) require the control of genotoxic and potentially genotoxic impurities at parts per million levels in drug substances. This review will discuss the background to the guidelines and the various strategies drug substance manufacturers have employed to comply with the very tight constraints. These strategies include (a) redesigning the drug substance synthesis to avoid introducing problematic impurities, (b) altering relevant process parameters to remove or reduce such impurities to insignificant levels, (c) deploying process understanding to prove that a particular genotoxic impurity either cannot be formed or will be efficiently removed, (d) conducting toxicity studies to demonstrate that a suspect impurity is not harmful at the low levels envisaged for it. Examples of each approach are given.

1. Introduction

Ensuring the safety of pharmaceutical products is a primary responsibility of the chemists, engineers and formulators involved in their manufacture - regardless of whether the products are intended for commercial purposes or for clinical investigation. When focusing on safety, particular attention is paid to the quality and purity of the raw materials used in the formulation, especially of the active pharmaceutical ingredient(s) (API(s)). A drug substance will typically contain a range of low-level impurity compounds, for example arising as residues of starting materials, reagents, intermediates, or as side-products generated by the synthetic processes or degradation reactions; these need to be understood and controlled within tight limits. The drug substance itself is unlikely to be entirely safe, but a certain level of risk to the patient can be tolerated when weighed against the anticipated health benefits. This balance between risk and benefit has to be finely judged by pharmaceutical manufacturers and regulatory authorities on a case-by-case basis. Impurities, however, are expected to bring no benefits - only risk. Manufacturers must therefore eliminate them (or at least mitigate the risk associated with them) to the greatest extent possible.

The International Conference on Harmonization (ICH) began to publish definitive guidelines on impurities in drug substances and drug products in the late 1990s.[1] These guidelines have been adopted by the regulatory bodies of, among others, the USA, the European Union, and Japan, and have enjoyed wide support within the pharmaceutical industry over the intervening decade. Under this guidance, the normal qualification threshold for impurities is 0.15% (1500 parts per million(ppm)) or 1 mg/day, whichever is lower, for drug substances whose intake is up to 2 g/day. Impurities which exceed this threshold must have their toxicity specifically investigated. Below the qualification threshold, no investigation is required, although impurities at levels above 1000 ppm (or 1 mg/day) are expected, at the least, to be identified.[2]

While these thresholds are considered adequate for the general run of process-related impurities, the guideline also recognises that impurities which are "unusually toxic" are of increased concern and deserving of a qualification threshold significantly lower than the default values. Subsequent guidance from the European Medicines Agency (EMEA)[9] and the U.S. Food and Drug Administration (USFDA)[17] confirmed that the ICH thresholds may not be acceptable for genotoxic or carcinogenic impurities. This, however, has proved to be more controversial within the industry - initially because of the lack of clear regulatory guidance, and subsequently because of the increased stringency imposed when more detailed guidance finally did emerge. Several commentators[3−5] have critically reviewed the history of the evolving guidance on genotoxic impurities.

Genotoxic compounds are those which cause damage to DNA, for example by alkylation or intercalation, which could lead to mutation of the genetic code. The terms "genotoxic" and "mutagenic" are usually employed synonymously by chemists, although there is a subtle distinction.[6] The property, however named, can be easily demonstrated by subjecting the chemical to standard *in vitro* tests,[7] the best known being the Ames mutagenicity test. Whether a given genotoxic compound is also carcinogenic (the real worry from the safety viewpoint) is more difficult to determine; it relies on longer-term *in vivo*

(1) *ICH Guideline: Impurities in New Drug Substances Q3A*, (R2); International Conference on Harmonisation, **2006**. *ICH Guideline: Impurities in New Drug Products Q3B*, (R2); International Conference on Harmonisation, **2006**.

(2) A recent FDA guideline (July 2009) on impurities in generic drugs would effectively reduce the qualification threshold to 1000 ppm or 1 mg/day for new impurities which were not present in the reference listed drugs.

(3) Delaney, E. J. An impact analysis of the application of the threshold of toxicological concern concept to pharmaceuticals. *Regul. Toxicol. Pharmacol.* **2007**, *49* (2), 107–124.

(4) Humfrey, C. D. N. Recent Developments in the Risk Assessment of Potentially Genotoxic Impurities in Pharmaceutical Drug Substances. *Toxicol. Sci.* **2007**, *100* (1), 24–28.

(5) Snodin, D. EU Guideline on Genotoxic Impurities Needs Updating. *RAJ Pharma* **2008**, 593–598 and 663−670.

(6) "Genotoxicity" is a broader term in that it covers all types of DNA interactions, whereas "mutagenicity" covers only DNA-impacting events transmissible from cell to cell or generation to generation.

(7) *ICH Guideline: A Standard Battery for Genotoxicity Testing of Pharmaceuticals S2B*; International Conference on Harmonisation, **1997**.

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studies using animal models, and there is always a question of the extent to which the results of such studies can be extrapolated to humans. The conservative approach is to assess known genotoxic compounds as potential carcinogens unless there is experimental evidence to the contrary.

2. The Regulatory Viewpoint

In December 2002 the Safety Working Party (SWP) of the European Committee for Proprietary Medicinal Products (CPMP) published a "Position paper on the limits of genotoxic impurities", signaling an intention to fill the gap in ICH guidelines. Genotoxic impurities was a topic selected for a joint meeting of the Drug Information Association (DIA) and the EMEA in October 2003, where scientific and regulatory updates were presented and discussed in the light of case studies. The outcome of these discussions, along with other industry and regulatory comment, was subsequently published in a special edition of the *International Journal of Pharmaceutical Medicine*.[8] The EMEA published a guideline on the subject in June 2006 that finally came into effect in January 2007.[9]

The EMEA guideline recommends that any potentially genotoxic impurities (PGIs) in the drug substance should be identified, either from existing genotoxicity data or through the presence of "structural alerts". PGIs should then be dichotomized into those for which there is "sufficient (experimental) evidence for a threshold-related mechanism" and those "without sufficient (experimental) evidence for a threshold-related mechanism." The former category would include *inter alia* compounds that induce aneuploidy by interfering with the mitotic spindle, compounds that interfere with the activity of topoisomerase, and/or compounds that inhibit DNA synthesis. The limits for impurities with clear evidence for a threshold mechanism can be addressed using methods similar to those recommended by ICH for setting limits on Class 2 solvents.[10] This approach calculates a "permitted daily exposure," which is derived using the "no observed effect level" or, alternatively, the "lowest observed effect level" from the most relevant animal study and incorporating a variety of uncertainty factors.

Where there is no, or insufficient, evidence that the genotoxic impurity acts via a threshold-related mechanism, establishing a safe limit is much more problematic. The EMEA approach has been to adopt a concept originally applied by the USFDA to contaminants leaching from food packaging materials and known as the "threshold of regulatory concern".[11] This principle states that regulators ought not to be concerned with extremely low levels of contamination where the risk of harm is negligible. Subsequent research aimed to quantify such a regulatory threshold, which became redesignated as the "threshold of toxicological concern" (TTC).[12] On the basis of an analysis of the carcinogenic potency in rodents of over 700 carcinogens,[13,14] it was estimated that exposures less than 0.15 μg/day of these substances are unlikely to increase a lifetime cancer risk by more than 1 in one million; it is therefore reasonable to regard 0.15 μg/day as a "virtually safe dose" for all but the most potent carcinogens. The actual limit recommended by the EMEA is 1.5 μg/day, representing a 1 in 10^5 excess lifetime cancer risk, the extra latitude being justified by the presumed benefit to the patient of taking the medicine. It is recognized that certain classes of compounds, specifically aflatoxin-like, *N*-nitroso, and azoxy compounds, will require even tighter control. This type of compound is unlikely to feature in a typical drug substance synthesis. However, the general TTC limit, being several orders of magnitude lower than the normal qualification threshold suggested by ICH guidelines, still presents manufacturers with a host of technical and analytical challenges.

The EMEA guideline summarises their recommendations in the form of a decision tree (Figure 1). Their preferred option is, if possible, to eliminate the opportunities for the genotoxic impurity(ies) to appear at all. The second preference is for manufacturers to reduce the level(s) to as low as is reasonably practicable (ALARP principle). Application of TTC concepts is - according to this decision tree - only third favorite. (This view has subsequently been modified, as discussed later in this section.[16])

One issue which the EMEA guideline originally failed to address was appropriate limits for investigational drugs. At early stages of development the process understanding which is required to control trace-level impurities would likely be limited. On the other hand, clinical subjects are typically exposed to investigational drugs only for limited periods - certainly not the "lifetime" which is assumed in the TTC derivation. The industry group Pharmaceutical Research and Manufacturers of America (PhRMA) have therefore proposed a "staged TTC" approach for the intake of genotoxic impurities over various periods of exposure during clinical trials.[15] Extrapolating from the established acceptable intake over a lifetime, and resetting the acceptable risk to 1 in 10^6 (since clinical trial subjects may be volunteers who derive no specific benefit from the drug), they proposed limits of up to 120 μg/day for exposures lasting up to one month, decreasing to 10 μg/day for >6−12 month

(8) *Int. J. Pharm. Med.* **2004**, *18* (4). Special edition.
(9) *Guideline on the Limits of Genotoxic Impurities*, CPMP/SWP/5199/02; European Medicines Evaluation Agency, Committee for Medicinal Products for Human Use (CHMP): London, 28 June 2006.
(10) *ICH Guideline: Impurities - Guideline for Residual Solvents Q3C*, (R4); International Conference on Harmonisation, **2009**.
(11) Rulis, A. M. De minimis and the threshold of regulation. In *Food Protection Technology*; Felix, C. W., Ed.; Lewis Publishers: Chelsea, Michigan, USA, 1986; pp 29−37.
(12) Munro, I. C.; Renwick, A. G.; Danielewska-Nikiei, B. The Threshold of Toxicological Concern (TTC) in Risk Assessment. *Toxicol. Lett.* **2008**, *180* (2), 151–156.
(13) Cheeseman, M. A.; Machuga, E. J.; Bailey, A. B. A Tiered Approach to Threshold of Regulation. *Food Chem. Toxicol.* **1999**, *37* (4), 387–412.
(14) 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 at Low Levels in the Diet. *Food Chem. Toxicol.* **2004**, *42* (1), 65–83.
(15) Müller, 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. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Regul. Toxicol. Pharmacol.* **2006**, *44* (3), 198–211.
(16) *Questions & Answers on the CHMP Guideline on the Limits of Genotoxic Impurities*, Revision 1; EMEA/CHMP/SWP/43 1994/2007; CHMP Safety Working Party: London, 26 June 2008. (A second revision was published on 17 December 2009, with yet more clarification.)
(17) Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches. FDA Center for Drug Evaluation and Research. Guidance for Industry (Draft); 03 December 2008.

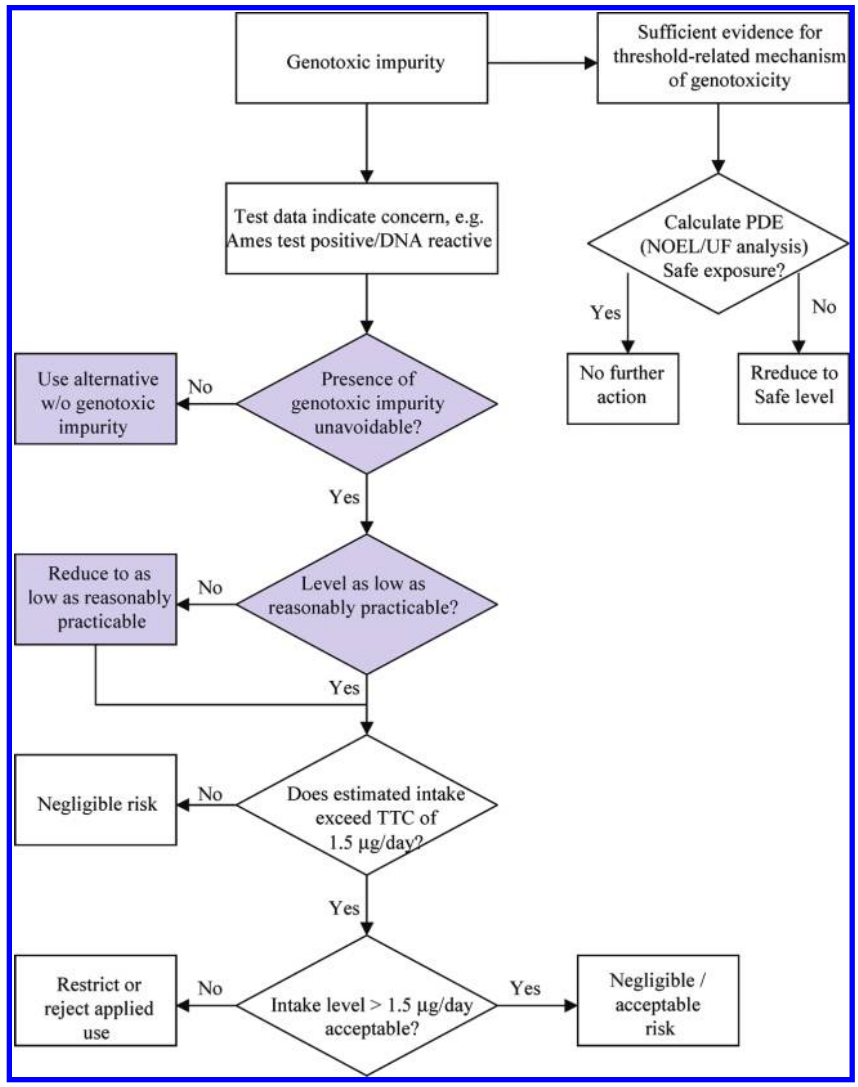


Figure 1. **EMEA decision tree for assessment of acceptability of genotoxic impurities.**

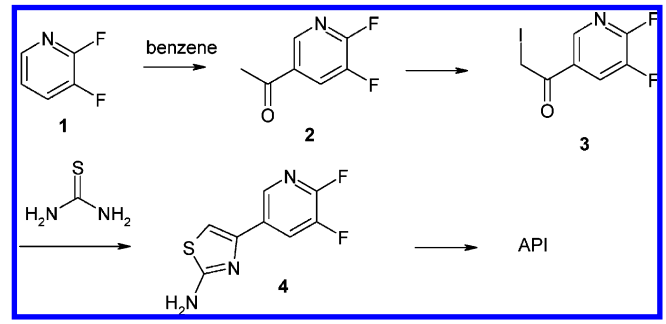
Table 1. **Proposed allowable daily intake (μg) for genotoxic impurities of unknown carcinogenic potential during clinical development**

	duration of exposure	single dose	NMT 14 days	14 days to 1 month	1−3 months	3−6 months	6−12 months	>12 months
PhRMA recommendations	ref 15	120	120	120	40	20	10	1.5
EMEA limits	ref 16	120	60	60	20	10	5	1.5
draft USFDA limits	ref 17	120	120	60	20	10	5	1.5

exposures. (In all cases a maximum limit of 0.5% would be observed on quality grounds.) This staged approach was subsequently endorsed by the EMEA in a 2008 "Question and Answer" document clarifying their original guidance,[16] albeit with reduced limits for each duration of exposure. Similar limits were proposed by the USFDA in their draft guideline published in December 2008.[17] A summary of the current recommendations from both authorities, as well as PhRMA, is provided in Table 1.

An example of this "staged TTC" approach has been presented by Syntagon in a recent web newsletter.[18] They evaluated the synthetic route to an investigational API (Scheme 1) intended for a phase I study with a duration of 20 days and a daily dose of approximately 100 mg. They identified two reagents, benzene and thiourea, as known genotoxins, and the iodo- intermediate **3** as potentially genotoxic. For the initial clinical study they set specifications of NMT 0.06% w/w (equivalent to 60 μg/day) for all three. It could be argued that the available compound-specific data for benzene and thiourea should have been consulted in preference to applying a staged

Scheme 1. **Four last chemical steps in the manufacture of an API (from ref 18)**



TTC. In the case of benzene, this would have justified a higher limit of 800 μg/day (0.80% w/w) for the short study, but they defaulted to the lower level as it was easily controlled for using standard analytical techniques (HPLC-UV and GC-FID).

It is possible to argue that the "staged" levels could be equally valid for approved drugs which are intended to be administered only for short periods.[19] This suggestion, however, has been specifically dismissed by the USFDA on the grounds that a drug may be used multiple times by the same individual, or may be used outside of its approved indication. However, applicants may provide the agency with a detailed rationale to support higher limits on a case-by-case basis - for example if the patient is likely to be exposed to higher levels of the potential genotoxic impurity (PGI) from other sources or if the drug is intended to treat a life-threatening condition.

The EMEA's 2008 Question and Answer document made a number of other welcome clarifications.

- The guideline need not be applied retrospectively to existing authorised products so long as the manufacturing procedure remains essentially unchanged, unless newly acquired knowledge flags a potential problem.
- It is not necessary to apply ALARP principles to genotoxic impurities which are controlled below the TTC, unless they have structures of very high concern. (This, as pointed out by Snodin,[5] actually contradicts the original guideline.)
- Absence of a "structural alert" based on a well-performed assessment would allow the impurity concerned to be classified as nongenotoxic, without the requirement for specific testing.
- A negative Ames test (properly conducted) would be sufficient to overrule any structural alerts and allow an impurity to be classified as nongenotoxic.
- It is acceptable to assume that an identified PGI is in fact genotoxic without specifically testing it.
- Unidentified impurities which occur below the ICH identification threshold (0.10% or 1 mg/day intake) need not be considered further.
- Identified impurities, even those below the identification threshold, should always be screened for structural alerts.
- When more than one genotoxic impurity is present, the TTC value of 1.5 μg/day can be applied to each, provided the impurities are not structurally related. Where structurally related PGIs are present, the 1.5 μg/day limit should be applied to the whole group.

3. Scientific Assessment of the Regulatory Proposals

While the guidelines appear to have been broadly accepted by the pharmaceutical industry at large, the basis of the regulatory approach has been challenged from a variety of viewpoints. Bouder[20] has contended that the risk-reduction model introduced by the EMEA is not optimal from a risk-management point of view. Instead, he advocates adopting the "Tolerability of Risk" approach, which has been used by the UK Health and Safety Executive to manage acute risks such as accidents in the nuclear and off-shore industries. In this context "tolerable" does not mean "acceptable", but refers rather to a "willingness by society as a whole to live with a risk so as to secure certain benefits, in confidence that the risk is one worth taking and that it is being properly controlled".

Delaney[3] notes that the TTC concept was originally employed by the USFDA in the context of food contaminants in 1995[21] as a device to enable them to comply with legislation dating from 1958[22] - a time when analytical chemistry was not advanced enough to enable routine detection of impurities at such low levels. This particular legislative provision does not exist for drug products, and the general thrust of USFDA drug regulation in more recent times has been to take a more balanced approach between risk and benefit.[23] Delaney also believes the assumptions upon which the 1.5 μg/day limit rests are overly conservative. Cheeseman et al.[13] had tabulated rodent TD_{50} values for a variety of carcinogens and derived a lower statistical limit for this measure. This was then linearly extrapolated from the "1 in 2" risk to estimate the lower limit for a "1 in 10^6" risk as 0.15 μg/day. However, many of the substances considered in this analysis are of historical concern and have structures which are unlikely to occur in a typical drug substance synthesis. The types of compounds which are likely to occur, for example alkylating agents, tend to fall at the weaker end of the potency spectrum, and if the analysis were restricted to those compounds, the TTC could be raised by approximately 2 orders of magnitude. Snodin[5] goes further and claims that many of the TD_{50} values used by Cheeseman et al. are significantly lower than those found in the Cancer Potency Database (CPDB),[24] sometimes by a factor of 180, and complains of a lack of transparency in the selection and use of data.

(18) *Syntagon Newsletter. A Pragmatic Approach to Controlling Potentially Genotoxic Impurities for Phase 1*; June **2009**; Issue 2 www.syntagon.com/PGI.aspx.

(19) Kirkland, D.; Snodin, D. Setting Limits for Genotoxic Impurities in Drug Substances: Threshold-Based and Pragmatic Approaches. *Int. J. Pharm. Med.* **2004**, *18* (4), 197–207.

(20) Bouder, F. Regulating impurities in pharmaceutical products: a tolerability of risk approach? *Expert Rev. Clin. Pharmacol.* **2008**, *1*, 241–250. Bouder, F.; Löfstedt, R. Tolerability of risk approach and the management of pharmaceutical risks. *Expert Rev. Clin. Pharmacol.* **2008**, *1*, 187–190.

(21) Federal Register Environmental Documents, 1995. Food additives: threshold of regulation for substances used in food-contact articles, final rule. 60 (136), 36581−36596 and 21 CFR Parts 5, 25, 170, 171 and 174, 2000; Vol. 65, No. 182.

(22) In 1958 and 1960, legislative amendments to the Food, Drug and Cosmetic Act of 1938 were passed by the U.S. Congress requiring manufacturers to establish the safety of additives in foods, drugs and cosmetics. A controversial provision included in each was the "Delaney Clause" which specified that no additive could be deemed safe (or given FDA approval) if found to cause cancer in man, or experimentally in animals. This provision was initially opposed by the FDA and by scientists, who agreed that an additive present in food at very low levels should not necessarily be forbidden solely on the basis of its capability to cause cancer when tested in animals at very high levels. However, since the amendment was passed, the FDA is required by law to apply a "zero risk tolerance" standard for potential carcinogens in food.

(23) (a) *Pharmaceutical CGMP for the 21st Century−A Risk-Based Approach*, US Food and Drug Administration, **2003**. (b) *Guidance for Industry: Q9 Quality Risk Management*, US Food and Drug Administration, June **2006**.

(24) Gold, L. S.; Slone, T. H.; Bernstein, L. Summary of Carcinogenic Potency and Positivity for 492 Rodent Carcinogens in the Carcinogenic Potency Database. *Environ. Health Perspect.* **1989**, *79*, 259–272.

Humfrey[4] points out additional sources of conservatism, such as the use of linear extrapolation, and questions the "no threshold" assumption that "one molecule can cause cancer", which takes no account of biological homeostatic or repair mechanisms.[25] The impact of compounding numerous sources of conservatism in this way can be to overestimate actual risk by a factor of 400, as illustrated by a case study of perchloroethylene contamination of water supplies.[26]

The specific EMEA requirement for structurally related PGIs to be controlled as a group below the TTC has also been challenged. Bercu et al.[27] used statistical simulations to assess the impact of a mixture of genotoxic compounds at TTC levels. While some increase in cancer risk was observed, it was thought to be modest in the light of the conservative assumptions referred to above, regardless of structural similarity. However, since the analysis indicates some increase in excess cancer risk with increasing number of genotoxic impurities, applying the TTC separately for four or more genotoxic impurities would warrant further discussion.

From a quality perspective, it is certainly desirable that manufacturers strive to attain the low impurity content envisioned by the new guidelines, but it must also be recognised that this can require the deployment of significant resources - in process development and in quality control - which in many cases achieves little by way of increased patient safety. The detection and quantitation of trace levels of an impurity typically requires cutting edge analytical techniques such as HPLC/MS or GC/MS. Such methods have been reported for controlling traces of organohalides[28] and of alkyl sulfonates.[29−32] However, this technology may not be readily available to all API manufacturers, particularly generic manufacturers.

The alternative to controlling a PGI at very low levels is to test it and hopefully demonstrate that it is not in fact genotoxic. This will usually require the PGI to be isolated or synthesized for testing. ICH guidelines allow an ordinary process-related impurity to be qualified on the basis of testing API lots which contain the impurity at representative levels. However, this approach is usually inadequate for controlling substances at the ppm level, because the normally employed Ames assay would not be sufficiently sensitive. USFDA scientists,[33] for example, have pointed out that "powerful mutagens such as 9-aminoacridine and methyl methanesulfonate which are used as positive controls would not be detected in the assay." Kenyon et al.[34] conducted a literature survey of approximately 450 mutagens, and estimated that 85% of these are detected at concentrations of 250 μg/plate or less. The EMEA[15] and PhRMA[14] therefore recommend this as the lower limit for a drug substance matrix test to be acceptable. The USFDA[17] continues to be suspicious of that approach entirely. It is, in any case, of limited practicality; if the API is tested at a concentration of 5 mg/plate, an impurity would need to be present at 5% or greater concentration for its genotoxicity to have a good chance of detection.

4. Industry Approaches

The pharmaceutical industry has responded to the PGI challenge in a variety of ways. The PhRMA group[15] proposed a model in which all impurities in the drug substance would be classified into one of five classes, using a combination of experimental data and comparative structural analysis.

- Class 1 impurities known to be both genotoxic (mutagenic) and carcinogenic
- Class 2 impurities known to be genotoxic (mutagenic), but with unknown carcinogenic potential
- Class 3 impurities containing alerting structures, unrelated to the structure of the API, and of unknown genotoxic (mutagenic) potential
- Class 4 impurities containing alerting structures, which are related to the API
- Class 5 impurities with no alerting structures, or where sufficient evidence exists that genotoxicity is absent

Figure 2 provides some examples of "alerting" functional groups that are known to be involved in reactions with DNA. The list is not exhaustive. A more thorough analysis of particular structures may be performed using proprietary computer software.[35] These programs have been demonstrated to be highly predictive for genotoxicity.[36,37] USFDA scientists have discussed considerations when using such computational toxicology to support regulatory decisions.[38]

(25) Calabrese, E. J.; Baldwin, L. A. Toxicology rethinks its central belief. *Nature* **2003**, *421*, 691–692.
(26) McCone, T. E.; Bogen, K. Y. Predicting the uncertainties in risk assessment. *Environ. Sci. Technol.* **1991**, *25* (10), 1674–1681.
(27) Bercu, J. P.; Hoffman, W. P.; Lee, C.; Ness, D. K. Quantitative assessment of cumulative carcinogenic risk for multiple genotoxic impurities in a new drug substance. *Regul. Toxicol. Pharmacol.* **2008**, *51* (3), 270–277.
(28) Elder, D. P.; Lipczynski, A. M.; Teasdale, A. Control and analysis of alkyl and benzyl halides and other related reactive organohalides as potential genotoxic impurities in active pharmaceutical ingredients (APIs). *J. Pharm. Biomed. Anal.* **2008**, *48*, 497–507.
(29) Alzaga, R.; Ryan, R. W.; Taylor-Worth, K.; Lipczynski, A. M.; Szucs, R.; Sandra, P. A generic approach for the determination of residues of alkylating agents in active pharmaceutical ingredients by *in situ* derivatization-headspace-gas-chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *45* (3), 472–479.
(30) An, J.; Sun, M.; Bai, L.; Chen, T.; Liu, D. Q.; Kord, A. A practical derivatization LC/MS approach for determination of trace level alkyl sulfonates and dialkyl sulfates genotoxic impurities in drug substances. *J. Pharm. Biomed. Anal.* **2008**, *48* (3), 1006–1010.
(31) Elder, D. P.; Teasdale, A.; Lipczynski, A. M. Control and analysis of alkyl esters of alkyl and aryl sulfonic acids in novel active pharmaceutical ingredients (APIs). *J. Pharm. Biomed. Anal.* **2008**, *46*, 1–8.
(32) Jacq, K.; Delaney, E.; Teasdale, A.; Eyley, S.; Taylor-Worth, K.; Lipczynski, A.; Reif, V. D.; Elder, D. P.; Facchine, K. L.; Golec, S.; Oestrich, R. S.; Sandra, P.; David, F. Development and validation of an automated static headspace gas chromatography-mass spectrometry (SHS-GC/MS) method for monitoring the formation of ethyl methane sulfonate from ethanol and sulfonic acid. *J. Pharm. Biomed. Anal.* **2008**, *48* (5), 1339–1344.
(33) Jacobson-Kram, D.; McGovern, T. Toxicological overview of impurities in pharmaceutical products. *Adv. Drug Delivery Rev.* **2007**, *59*, 38–42.
(34) Kenyon, M. O.; Cheung, J. R.; Dobo, K. L.; Ku, W. W. An evaluation of the sensitivity of the Ames assay to discern low-level mutagenic impurities. *Regul. Toxicol. Pharmacol.* **2007**, *48* (1), 75–86.
(35) Examples include DEREK (www.chem.leeds.ac.uk/luk/derek), Mcase (www.multicase.com/products/prod01.htm), and TOPKAT (http://accelrys.com/products/discovery-studio/predictive-toxicology.html).
(36) 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* (3), 282–293.
(37) Mayer, J.; Cheeseman, M. A.; Twaroski, M. L. Structure-activity relationship analysis tools: Validation and applicability in predicting carcinogens. *Regul. Toxicol. Pharmacol.* **2008**, *50* (1), 50–58.
(38) Kruhlak, N. L.; Contrera, J. F.; Benz, R. D.; Matthews, E. J. Progress in QSAR toxicity screening of pharmaceutical impurities and other FDA regulated products. *Adv. Drug Delivery Rev.* **2007**, *59*, 43–55.

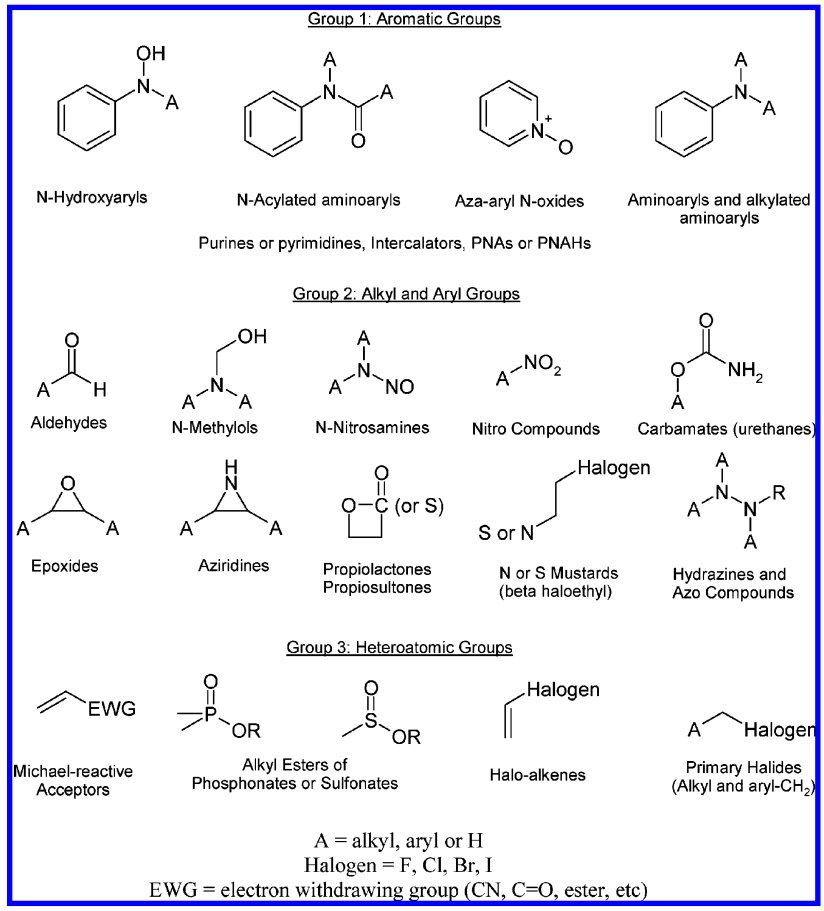


Figure 2. **Some examples of structurally alerting functional groups (from ref 15).**

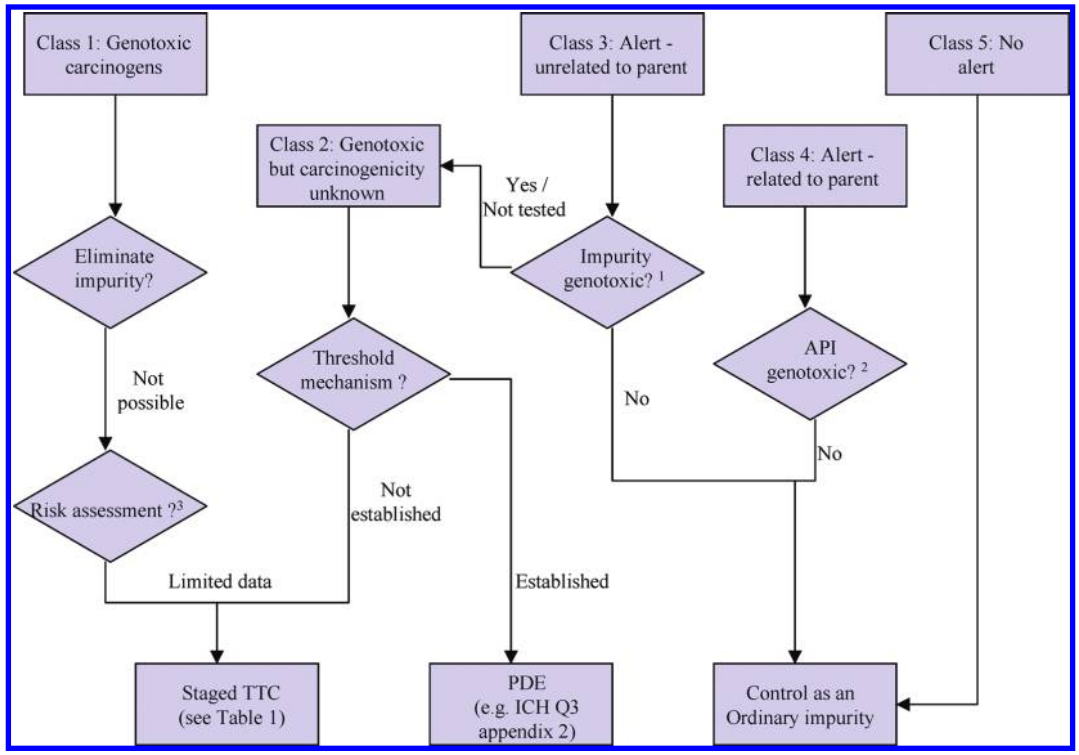
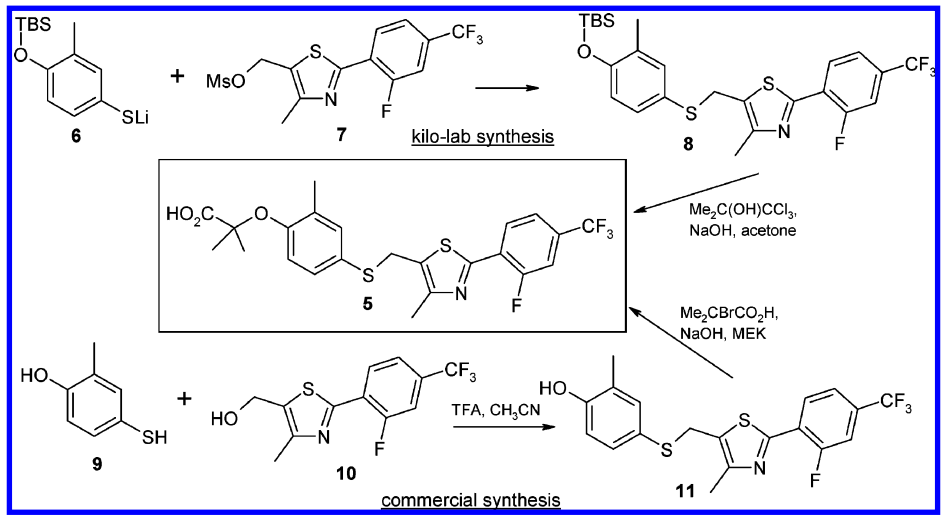


Figure 3. **PhRMA decision tree for action on impurities depending on classification (from ref 15). (1) Either tested neat or spiked into the API and tested up to ≤250 μg/plate. (2) If the API is positive, then a risk-benefit analysis is required. (3) Quantitative risk assessment to determine ADI.**

Figure 3 presents PhRMA's suggested decision tree detailing what action to take according to the assigned classifications. Class 5 compounds are treated as normal impurities and controlled according to the long-established ICH guidelines -

Scheme 2. **Kilo-lab and commercial syntheses of sodelglitazar (5) (from ref 40)**



as are those Class 3 compounds which are subsequently shown to be nongenotoxic, and Class 4 compounds where the API is shown to be nongenotoxic. Where Class 3 or Class 4 compounds are genotoxic, or if they are not tested, they are moved into Class 2. Compounds in this class are controlled using the staged TTC principles - unless evidence exists for a threshold-related genotoxicity mechanism (in which case a permitted daily exposure (PDE) is derived). Class 1 compounds represent the most serious risk; here the default preference is to eliminate the impurity by modifying the process. If this is not practical or realistic, a compound-specific calculation of risk should be conducted. Only as a last resort should the TTC concept be employed in these cases.

Pierson et al. (Eli Lilly & Co.)[39] have described an approach taken in their own company, with particular focus on the clinical development stages, illustrated with some examples. Once an impurity is assigned PGI status, a key feature of their strategy is to determine at what point in the synthesis it is introduced. Compounds introduced four or more synthetic steps before the final API are of lesser concern; here it should be sufficient to provide a chemical rationale that the impurity will be removed during the subsequent steps, in which case a specification for it would not be necessary. For PGIs occurring later in the synthesis, some experimental evidence of consistent removal should be provided to justify the absence of a specification. If the PGI is introduced in the penultimate step, and found in the penultimate at a level of concern, then a specification limit should be set in the final API. Where the PGI is introduced in the final step itself, then a specification should be applied in most cases at the API stage, on the basis of a toxicological assessment. The "levels of concern" are revised as the clinical project develops, in line with the staged TTC principles.

Once a PGI has been identified as an actual or possible contaminant, there are, broadly speaking, four courses of action open to the process development chemists: (1) alter the route of synthesis so as to remove the PGI entirely; (2) alter relevant process parameters to reduce the PGI to below a level of concern; (3) deploy chemical and mechanistic arguments, ideally backed with experimental evidence, to demonstrate that the PGI will not be present at significant levels; (4) conduct testing to demonstrate that the PGI is not actually harmful at its typical level in the API.

4.1. Altering the Synthesis To Avoid PGIs. According to the EMEA decision tree (Figure 1), avoidance of PGIs entirely is the number one preferred option. It may not always be practical to do this, but the potential to generate genotoxic impurities is one of several considerations that development chemists do employ when assessing the merits of competing syntheses, and indeed is frequently cited as a reason for changing synthetic route during development, particularly as processes are scaled up. A few recent examples are given below.

In describing the process development of sodelglitazar (**5**, Scheme 2), a potential type-2 diabetes drug, Brown et al. (GlaxoSmithKline [GSK])[40] identified the mesylate intermediate **7** in their kilo-lab route as having a potential for genotoxicity. In the commercial route, the problem was avoided by employing an alternative strategy, involving the nongenotoxic alcohol **10**, for formation of the thioether linkage.

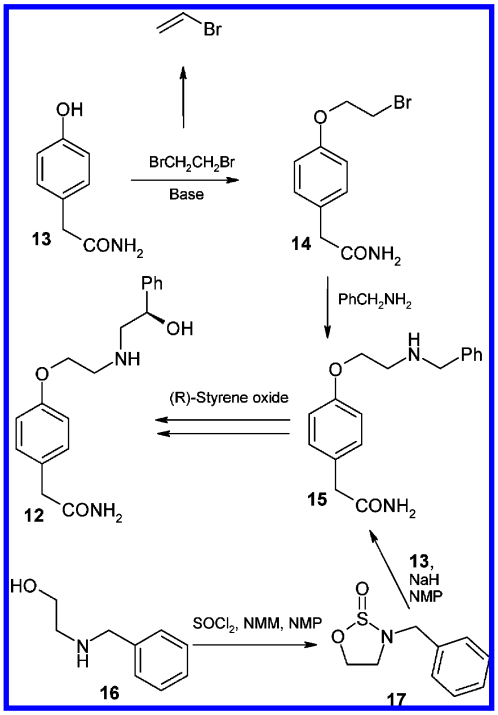
Vinyl bromide was identified as a potential genotoxic hazard in the medicinal chemistry route to an earlier diabetes drug candidate ZD-2079 (**12**, Scheme 3).[41] It arises as a result of a side reaction between dibromoethane and a base and was considered an unavoidable feature of this process. In this case the concern was more for worker safety than for product quality. Thus, for scale-up an alternative strategy for providing the two-carbon unit was developed - using the commercially available *N*-benzylethanolamine (**16**) and activating it as the oxathiazolidine-*S*-oxide (**17**), which then combines with the original starting material (**13**). (The article does not discuss how residues of styrene oxide, another PGI, are controlled.)

(39) 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.

(40) Brown, A. D.; Davis, R. D.; Fitzgerald, R. N.; Glover, B. N.; Harvey, K. A.; Jones, L. A.; Liu, B.; Patterson, D. E.; Sharp, M. J. Process Development for Sodelglitazar: A PPAR Panagonist. *Org. Process Res. Dev.* **2009**, *13* (2), 297–302.

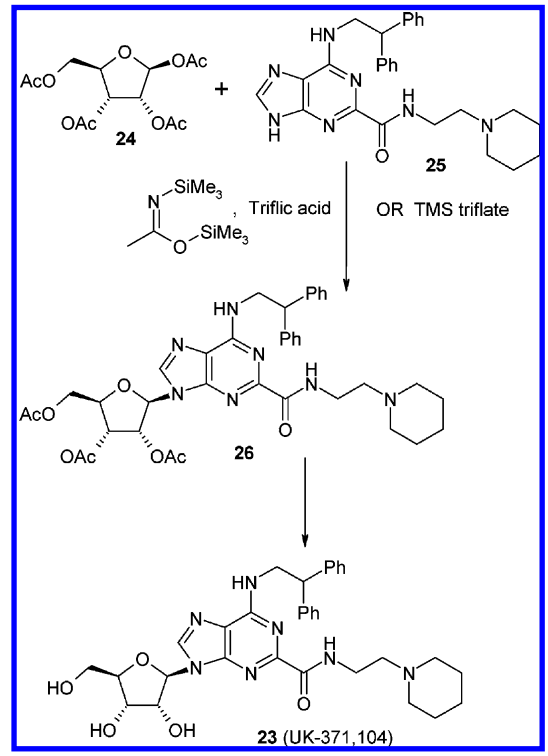
(41) Butters, M.; Catterick, D.; Craig, A.; Curzons, A.; Dale, D.; Gillmore, A.; Green, S. P.; Marziano, I.; Sherlock, J.-P.; White, W. Critical Assessment of Pharmaceutical Processes: A Rationale for Changing the Synthetic Route. *Chem. Rev.* **2006**, *106* (7), 3002–3027.

Scheme 3. **Routes to ZD-2079 (12) (from ref 41)**



For the large-scale preparation of denagliptin (**18**, Scheme 4), Patterson et al. (GSK)[42] employed a late-stage dehydration of amide **21** to nitrile **22**. With the majority of dehydrating agents studied, the reaction either did not go to completion or generated impurities. The most promising reagents were *p*-toluenesulfonic anhydride, which proved to be too expensive, and methanesulfonic anhydride, which gave a very clean conversion. Being introduced at such a late stage, the potential for this reagent to react with IPA to give a potentially genotoxic mesylate ester was a concern. This was overcome by a fortuitous discovery. Previous work on a preceding step of the synthesis had identified *n*-propanephosphoric acid cyclic anhydride (T3P) as the optimum reagent to effect the coupling of pyrrolidine **19** and acid **20** to generate **21**. They observed that, when the coupling was performed at elevated temperature, some dehydration occurred. They therefore introduced a second equivalent of the T3P to drive this to completion, thus avoiding the PGI and providing a more efficient manufacturing process overall.

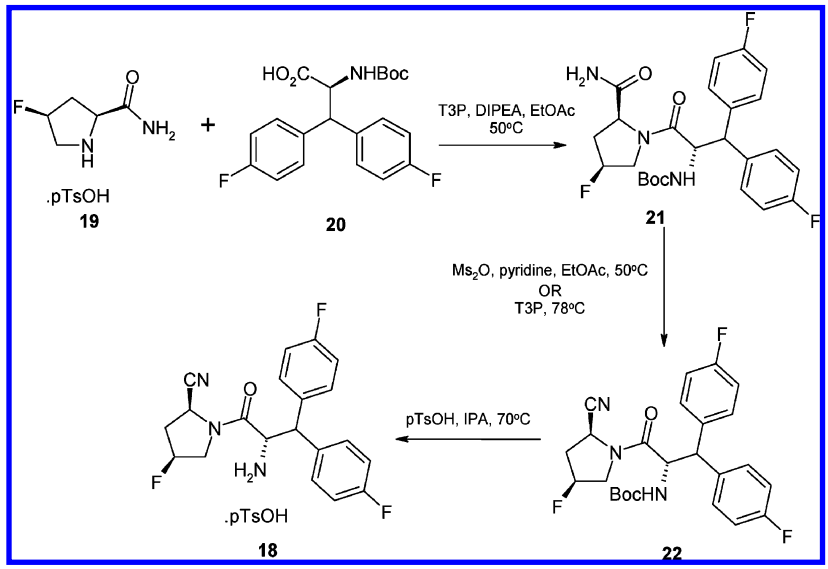
Scheme 5. **Final steps in synthesis of UK-371,104 (23) (from ref 43)**



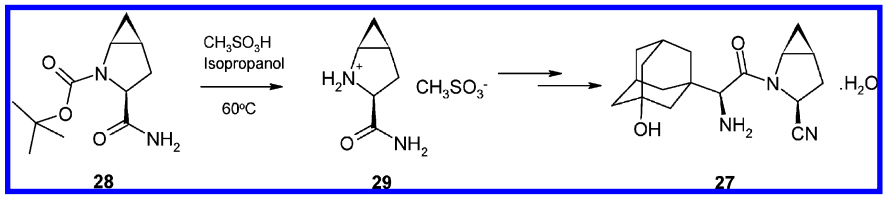
For the glycosidation reaction between furanose **24** and purine **25** (Scheme 5), Challenger et al. (Pfizer)[43] identified the combination of *N*,*O*-bis(trimethylsilyl)acetamide and triflic acid as giving a clean reaction leading to desired nucleoside **26**, the penultimate intermediate in the synthesis of API **23**. However, since workup of such reactions would generate stoichiometric amounts of acetamide (a category 2B carcinogen) close to the end of the synthesis, they elected to use trimethylsilyl triflate to effect the coupling instead.

In these cases, the potential to form genotoxic impurities is rarely the most critical factor affecting the decision to alter a synthetic route or to replace a reagent. If the existing process

Scheme 4. **Synthesis of denagliptin tosylate (18) (from ref 42)**



Scheme 6. **Synthesis of saxagliptin (27) (from ref 44)**

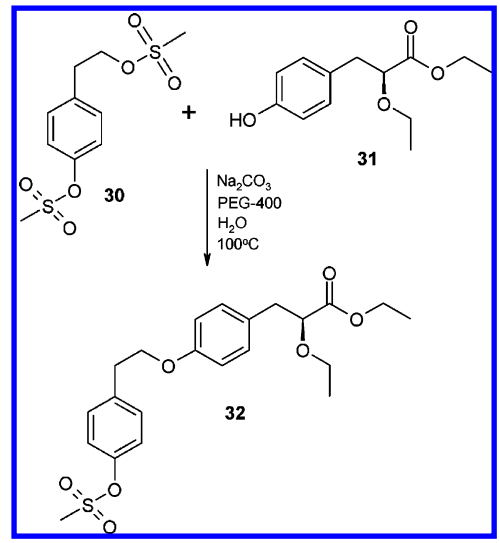


was otherwise advantageous, the PGI problem would usually be worked around in other ways. Savage et al. (Bristol-Myers Squibb)[44] identified methanesulfonic acid (MSA) as an ideal agent for the deprotection of pyrrolidine **28** (Scheme 6) to give a precursor of saxagliptin (**27**). Their preference would have been to avoid alcoholic solvents in combination with MSA, to prevent the formation of genotoxic methanesulfonate esters. However, the purification and yield provided by isopropanol outweighed the liabilities of using this combination, especially as extremely low levels of isopropyl methanesulfonate (IPMS) were in fact observed in the product. Despite the regulatory preference cited at the beginning of this section, it is unrealistic to avoid all procedures and reagents which might generate PGIs. The use of highly reactive electrophilic species is an indispensable strategy for the synthesis of complex organic molecules; the properties which render these species useful to chemists are precisely those associated with (suspected) genotoxicity.

4.2. Adjustment of Process Parameters. In many cases, PGIs have been successfully reduced below the TTC simply by altering appropriate conditions in either the reaction or workup stages. This can often be achieved without significant loss of yield.

For the synthesis of ether **32** (Scheme 7), chemists at AstraZeneca[45] were required to reflux the precursor **31** with an excess (1.6 equiv) of the potentially genotoxic bismesylate reagent **30** in the presence of sodium carbonate and a phase-transfer catalyst PEG-400. The conversion proceeded efficiently in 4−5 h, but it was necessary to continue refluxing for an additional 3−4 h in order to hydrolyse the excess of reagent. A low level of <0.03 area % was required in order to meet later-stage product specifications. In the original process, the extended reflux time led to increased hydrolysis of the carboxylate ester group in **32**, the product being completely hydrolysed after approximately 16 h exposure to the reaction conditions. To overcome this difficulty, the effect of pH on the rate of hydrolysis was studied. While the carboxylate hydrolysis was found to be strongly dependent on hydroxide ion catalysis, the sulfonate hydrolysis was characterized by a high uncatalysed rate. Thus, by adjusting the pH after the coupling is complete from 10 to around 7, now the bismesylate reagent **30** could be removed just as effectively, with no observable loss of the

Scheme 7. **Synthesis of an intermediate with carboxylate and sulfonate ester groups (from ref 45)**



product **32**. The conclusions of this study should be applicable to other processes where sulfonate ester reagents are used in excess.

Yang et al. (AMRI)[46] have recently reported the investigation of another problem which may be of more general relevance - the formation of small amounts of genotoxic alkyl chlorides when preparing amine hydrochloride salts in lower alcohol solvents. For one (unspecified) tertiary amine API, the hydrochloride salt was chosen for development because of its high solubility, and crystallization from methanol was preferred for producing the desired polymorph. Hydrochloric acid (37% aq) was employed as the salt-forming reagent, rather than anhydrous HCl, to minimize the potential for MeCl formation. However, an initial scale-up batch (3 kg) contained 11−12 ppm of this impurity. The batch was rescued by dissolving in a small amount of water and adding MTBE to crystallize; the salt was recovered in 92% yield with <1 ppm of MeCl. In order to avoid this reprocessing step with future batches, the influence of five crystallization parameters was studied, and using a lower temperature (10 °C) for the salt formation was identified as the primary key for producing the salt with lower MeCl levels (<1 ppm). A second tertiary amine hydrochloride API was crystallized from ethanol and found to contain around 10 ppm of EtCl impurity. However, this was of lesser concern, presumably because of a reduced dosage for that drug.

Vilsmeier reactions are often a cause for concern because of their potential to generate dimethylcarbamoyl chloride

(42) Patterson, D. E.; Powers, J. D.; LeBlanc, M.; Sharkey, T.; Boehler, E.; Irdam, E.; Osterhout, M. H. Development of a Practical Large-Scale Synthesis of Denagliptin Tosylate. *Org. Process Res. Dev.* **2009**, *13* (5), 900–906.

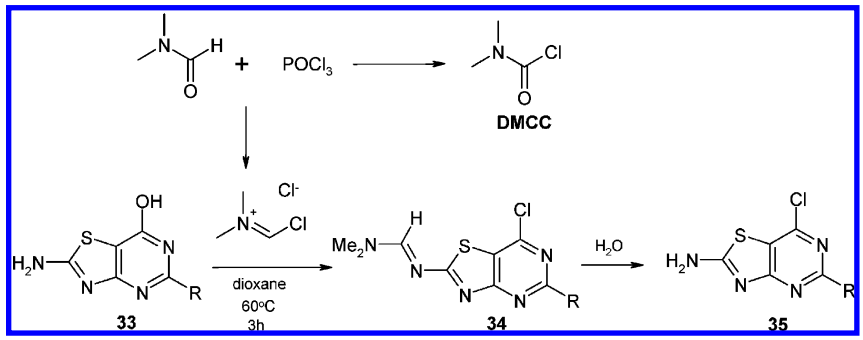
(43) Challenger, S.; Dessi, Y.; Fox, D. E.; Hesmondhalgh, L. C.; Pascal, P.; Pettman, A. J.; Smith, J. D. Development of a Scaleable Process for the Synthesis of the A2a Agonist, UK-371,104. *Org. Process Res. Dev.* **2008**, *12*, 575–583.

(44) Savage, S. A.; Jones, G. S.; Kolotuchin, S.; Ramrattan, S. A.; Vu, T.; Waltermire, R. E. Preparation of Saxagliptin, a Novel DPP-IV Inhibitor. *Org. Process Res. Dev.* **2009**, *13* (6), 1169–1176.

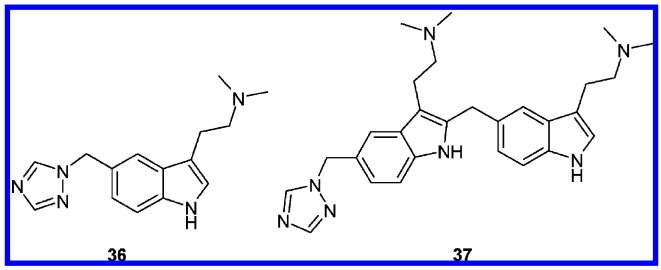
(45) Chan, L. C.; Cox, B. G.; Sinclair, R. S. Selective Hydrolysis of Methanesulfonate Esters. *Org. Process Res. Dev.* **2008**, *12*, 213–217.

(46) 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.

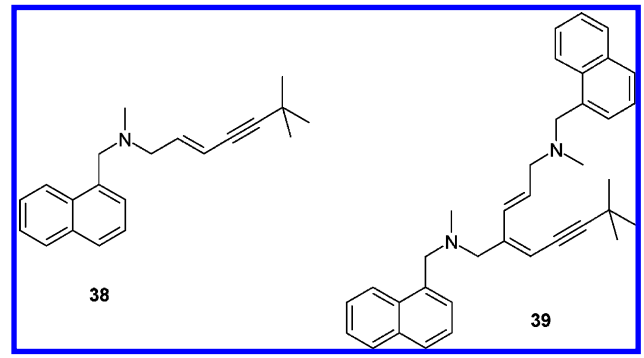
Scheme 8. **Generation of Vilsmeier reagent and its use in a chlorination procedure (from ref 47)**



Scheme 9. **Rizatriptan (36) and the dimeric impurity (37) (from ref 48)**



Scheme 10. **Structures of terbinafine (38) and a process-related genotoxic impurity (39) (from ref 49)**



(DMCC), which is known to be an animal carcinogen although its human carcinogenicity is still uncertain. Stare et al. (AstraZeneca)[47] employed Vilsmeier conditions for chlorination in the penultimate step of an API synthesis (Scheme 8) and conducted extensive studies on the formation and hydrolysis of DMCC. It was found that the choice of chlorinating agent was particularly critical, with $POCl_3$ producing far less of this side product than either thionyl chloride or oxalyl chloride. The use of a solvent, specifically dioxane, significantly suppressed the formation of DMCC, but the presence of bases, such as the substrate **33**, led to substantial enhancement. In a typical reaction mixture, after 3 h at 60 °C, 87 ppm of DMCC was detected using a GC/MS-SIM method. However, although DMCC is formed under the reaction conditions, a subsequent aqueous workup will rapidly hydrolyse it. A kinetic study showed a reduction in concentration from 11.4 mM to 0.03 mM over a one hour period, equivalent to a half-life of 4.13 min at 80 °C. Levels less than 3 ppm were found in batches of the isolated intermediate **35**.

In an extreme case, "end-of-pipe" solutions may have to be applied to the final API. Maddula et al. (Dr. Reddy's Laboratories)[48] have described the development of a negative hydrophobic interaction chromatographic process for removal of a genotoxic dimer impurity (**37**, Scheme 9) from the anti-migraine drug substance rizatriptan (**36**). Conventional processes such as fractional crystallization and recrystallization were unable to reduce the level below the acceptable limit of 0.01 wt %. By understanding the physicochemical properties of API, impurity, and adsorbent material, they were able to achieve a throughput of 33 g/L/h, reducing the impurity to below 0.008%, with 95% recovery of the API. However, it is not clear whether the company operates this process commercially.

Mathad[49] presented an example where a genotoxic impurity (**39**, Scheme 10) was found at levels around 200 ppm in batches of terbinafine (**38**). In order to isolate it, the reaction conditions were changed to increase its level from 0.1% to 10% in the reaction mix, followed by further enrichment to 97% by preparative chromatography. A purification process was established to reduce the impurity to below 6 ppm in affected batches. Subsequently, changes in the early stages of the synthesis resulted in impurity levels <2 ppm.

In some cases, conditions which actually promote the formation of potential genotoxins have been chosen in order to overcome other problems. In working up the cyclization reaction to form triazolone **41** (Scheme 11), the final intermediate in the synthesis of PPARα agonist LY518674, Argentine et al. (Eli Lilly & Co.)[50] elected to reflux the crude reaction mixture with sulfuric acid and ethanol. This converts various low-level reaction side products, particularly the hydrazide **42**, into diethyl ester **43** that unlike **42** was readily rejected by crystallization. The use of ethanol in the presence of sulfuric acid gave rise to three potentially genotoxic impurities, **44**, **45**, and **46**, but these

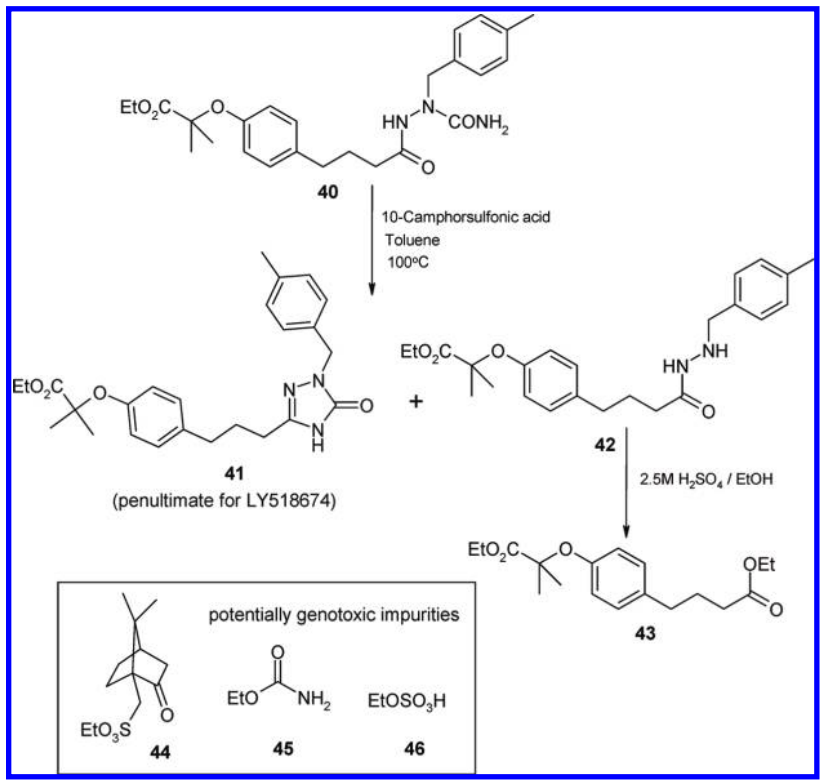
(47) Stare, M.; Laniewski, K.; Westermark, A.; Sjögren, M.; Tian, W. Investigation on the Formation and Hydrolysis of *N,N′*-Dimethylcarbamoyl Chloride (DMCC) in Vilsmeier Reactions Using GC/MS as the Analytical Detection Method. *Org. Process Res. Dev.* **2009**, *13* (5), 857–862.

(48) Maddula, S. R.; Kharkar, M.; Manudhane, K.; Kale, S.; Bhori, A.; Lali, A.; Dubey, P. K.; Sarma, K. R. J.; Bhattacharya, A.; Bandichhor, R. Preparative Chromatography Technique in the Removal of Isostructural Genotoxic Impurity in Rizatriptan: Use of Physicochemical Descriptors of Solute and Adsorbent. *Org. Process Res. Dev.* **2009**, *13* (4), 683–689.

(49) Mathad, V. T. Genotoxic Impurities in Pharmaceutical Products. USP-IPC 8th Annual Scientific Meeting, Hyderabad, India, February 2009 (http://www.usp.org/pdf/EN/meetings/asMeetingIndia2009/session3Track1bMathad.pdf).

(50) Argentine, M. D.; Braden, T. M.; Czarnik, J.; Conder, E. W.; Dunlap, S. E.; Fennell, J. W.; LaPack, M. A.; Rothhaar, R. R.; Scherer, R. B.; Schmid, C. R.; Vicenzi, J. T.; Wei, J. G.; Werner, J. A.; Roginski, R. T. The Role of New Technologies in Defining a Manufacturing Process for PPARα Agonist LY518674. *Org. Process Res. Dev.* **2009**, *13* (2), 131–143.

Scheme 11. **Synthesis and purification of LY518674 penultimate (from ref 50)**



were shown to be readily removed during workup operations such as extractions and crystallization.

4.3. Deployment of Process Understanding. Over the past decade, pharmaceutical manufacturers have been encouraged to employ Quality by Design (QbD) principles in the development of their processes, and thus rely less on routine testing.[51] As regards to genotoxic impurities, regulatory authorities increasingly require applicants to give detailed consideration to any potential for their formation, even when this appears highly unlikely. Following the recent well-publicized case of mesylate ester contamination of the antiviral drug Viracept (see section 4.4), the EMEA wrote to all marketing authorization holders for medicinal products[52] requesting them to undertake a risk assessment on the occurrence of similar impurities in their preparations. In one case in 2007 an application was rejected by the EMEA,[53] in part because the drug substance was recrystallized from acetone, and the applicant had failed to consider potential contamination with mesityl oxide arising from this.

The issue of sulfonate esters has probably generated more discussion recently than any other type of PGI. The development of APIs in the form of mesylate, tosylate or besylate salts has become more prevalent in recent years, in response to increasingly lower solubilities, as well as pK_a's, of basic drugs.[54] In particular, the practice of crystallizing or recrystallizing such salts from lower alcohols has come under suspicion as a potential source of sulfonate esters, which can cause alkylation of DNA. Snodin[55] used mechanistic considerations to argue that the reaction of an alcohol with methanesulfonic acid (MSA) to generate a mesylate ester ought not to occur to any significant extent under normal API processing conditions in the absence of impurities in the MSA such as the chloride or anhydride. The nucleophilicity of the mesylate anion would be too low for the reaction to proceed to any extent during a typical salt-formation time frame. Also, any mesylate ester that was formed would be hydrolysed or otherwise degraded more rapidly than it was formed. He proposed several precautions which could be applied, either separately or in combination, to provide further assurance: control of pH to maintain its value above 7, the use of high-purity MSA, and the use of alternative solvents.

Members of the Product Quality Research Institute (PQRI) have subsequently carried out detailed mechanistic studies of the reaction between MSA and methanol.[56] Their experiments with $H^{18}OCH_3$ clearly demonstrated that the esterification reaction proceeds entirely via initial protonation of methanol

(51) *FDA Guidance for Industry: Q8 Pharmaceutical Development*, (R2); International Conference on Harmonisation, November **2009**.

(52) *Request to Assess the Risk of Occurrence of Contamination with Mesilate Esters and Other Related Compounds in Pharmaceuticals*, EMEA/CMDh/98694/2008; Coordination Group for Mutual Recognition and Decentralised Procedures - Human Committee (CMDh): London, 27 February 2008.

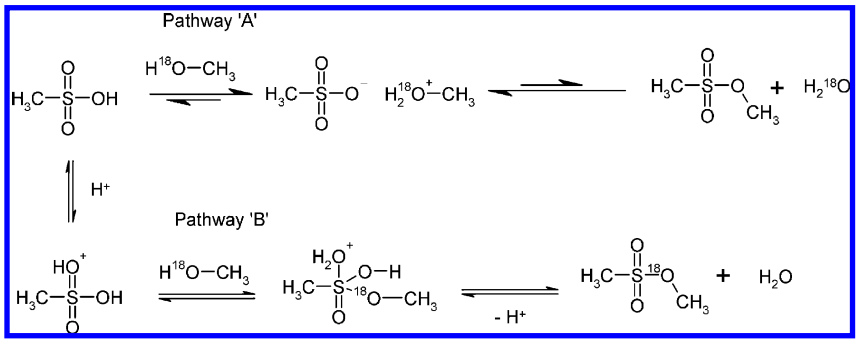
(53) *Withdrawal Assessment Report for Arxxant (Ruboxystaurin mesilate monohydrate)*, EMEA/150964/2007; European Medicines Agency: London, 24 May 2007.

(54) Elder, D. P.; Snodin, D. J. Drug substances presented as sulfonic acid salts: overview of utility, safety and regulation. *J. Pharm. Pharmacol.* **2009**, *61*, 269–278.

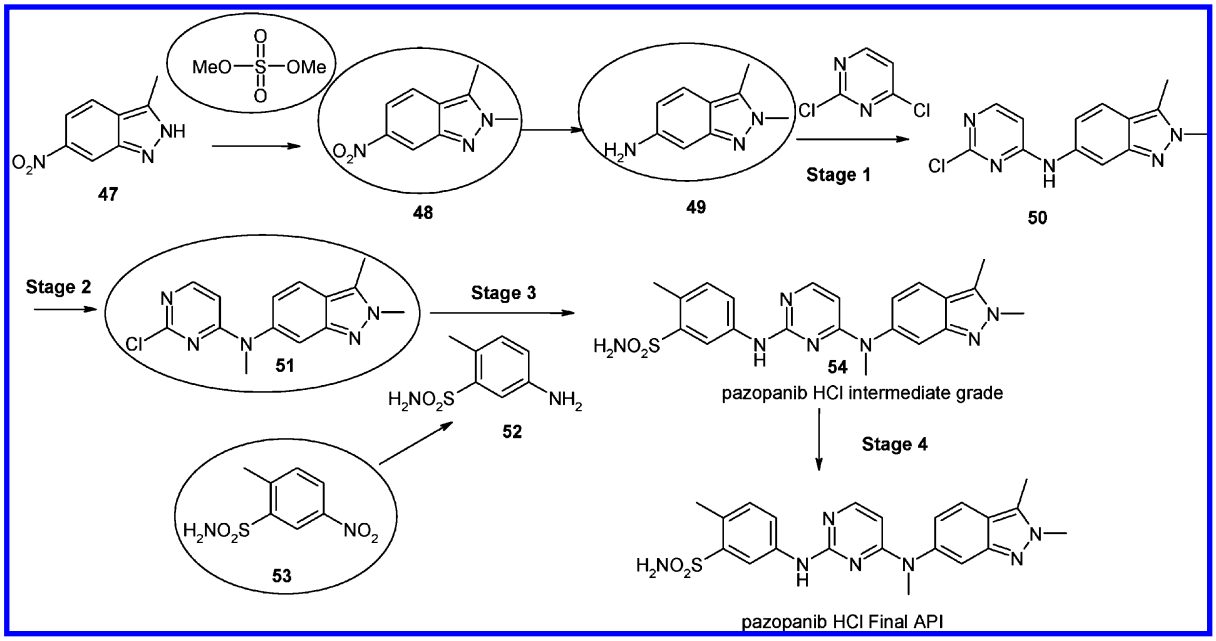
(55) Snodin, D. J. Residues of genotoxic alkyl mesylates in mesylate salt drug substances: Real or imaginary problems. *Regul. Toxicol. Pharmacol.* **2006**, *45*, 79–90.

(56) Teasdale, A.; Eyley, S. C.; Delaney, E.; Jacq, K.; Taylor-Worth, K.; Lipczynski, A.; Reif, V.; Elder, D. P.; Facchine, K. L.; Golec, S.; Oestrich, R. S.; Sandra, P.; David, F. Mechanism and Processing Parameters Affecting the Formation of Methyl Methanesulfonate from Methanol and Methanesulfonic Acid: An Illustrative Example for Sulfonate Ester Impurity Formation. *Org. Process Res. Dev.* **2009**, *13*, 429–433.

Scheme 12. **Alternative pathways for reaction of $H^{18}OCH_3$ with MSA (from ref 56)**



Scheme 13. **Manufacturing process of pazopanib HCl showing the five genotoxic impurities (circled) (from ref 57)**



and subsequent displacement of water (Scheme 12, Pathway A), rather than via pathway B, which is analogous to carboxylate ester formation. The conversion would thus require the presence of a strong acid in excess. Weaker acids such as phosphoric acid failed to catalyze the reaction, and a slight excess of base in the system inhibited it entirely. Thus, the formation of sulfonate esters in reaction mixtures relevant to API salt formation could be minimized by measures such as reducing time−temperature envelopes for exposure of the sulfonic acid to alcohols, incorporating water into the process, and reducing or eliminating excesses of sulfonic acid used in API salt formation. More detailed kinetic experiments have also been performed by this group, and further reports are anticipated.

Process understanding can also be demonstrated by means of spiking studies. Liu et al. (GSK)[57] identified five PGIs in the synthesis of pazopanib hydrochloride, a phase III anticancer candidate (Scheme 13). API dosage was around 800 mg/day; therefore, in view of the long-term nature of this advanced study, each PGI required to be controlled to <1.7 ppm in the final API. Trace analysis LC/MS methods were developed for this purpose, but it would be costly to use these for routine quality control purposes. Instead, spiking studies were used to demonstrate the capability of the processes to remove all five PGIs at earlier points in the synthesis. By shifting the analytical controls upstream, it was possible to set specifications at percent levels, and to control the impurities in the intermediates using standard LC-UV methods.

For example, compound **51** is possibly the most serious worry, as this intermediate goes into the final bond-forming step. This compound, although genotoxic, had been shown to be non-DNA reactive; therefore, a higher TTC limit of NMT 115 ppm in the API could be justified. All released clinical batches were tested and found to be below this limit. In these campaigns, the levels of **51** in intermediate grade pazopanib (**54**) had ranged from 0.1% to 0.6%. In order to examine process tolerability, 2% of **51** was spiked into **54**, which was then processed through the Stage 4 workup. It was established that concentrations of **51** in the final API were well below 115 ppm. Therefore, a limit of 0.6% (w/w) of **51** in **54** was proposed and discussed with the USFDA.

A similar example of using spiking studies, combined with statistical Design of Experiments, to control levels of mesylate esters in a fluoroaryl-amine salt, is reported elsewhere in this issue.[58]

(57) Liu, D. Q.; Chen, T. K.; McGuire, M. A.; Kord, A. S. Analytical control of genotoxic impurities in the pazopanib hydrochloride manufacturing process. *J. Pharm. Biomed. Anal.* **2009**, *50* (2), 144–150.

4.4. Toxicological Investigations. The experimental investigation of the toxicology of impurities, with a view to determining an acceptable intake specific for each compound, is likely to be a very expensive and time-consuming exercise - hence something which would only be contemplated in extreme circumstances. The EMEA guideline insists on this approach for the most potent classes of carcinogen (the so-called "cohort of concern") - namely, *N*-nitroso compounds, azoxy compounds, and aflatoxin-like compounds. Additionally, PhRMA recommend this for their Class 1 impurities - i.e. those which are known to be carcinogenic. It may be necessary for other impurities also, however, if it proves impractical to reduce their concentration below the threshold of toxicological concern (appropriately staged).

An example of this latter situation occurred recently after batches of the antiviral drug Viracept (nelfinavir mesylate) were found to be contaminated with the supposed genotoxic compound ethyl methanesulfonate (EMS). In this case the EMS was introduced as a result of a manufacturing error rather than from any of the standard chemical processes employed.[59] However, since the problem was only discovered after the batches had been released, many patients would have been exposed to levels of EMS approaching 1000 ppm over several months. The manufacturer, Hoffmann-La-Roche, therefore undertook a thorough investigation of the toxicology of EMS, the results of which have been published in a special 12-paper edition of Toxicology Letters.[60] The conclusions were somewhat surprising. Alkyl esters of sulfonic acids have been shown to exert genotoxic effects in bacterial and mammalian cells[61] and have for some time been a particular focus of industry and regulatory concern - as witnessed by some of the examples above. Roche's *in vivo* rodent study, however, demonstrated that there is in fact a threshold level of 2 mg/kg below which EMS does not have a harmful effect on DNA. This threshold level, fully 5 orders of magnitude higher than the recommended TTC level, has now been fully accepted by the EMEA's Safety Working Party.[62] The implications of this finding on the perception of risk arising from other sulfonate esters remains an open question.

Many other impurities which have at first given rise to concern over their genotoxic potential have turned out, on close examination, to pose less risk than originally supposed. In many cases, sufficient toxicology information may already be available in the open literature, especially for commonly used reagents. For example, Bercu and Callis[63] have recently summarized data on ethyl chloride (see section 4.2 above) which suggests that an acceptable daily intake of this compound could be 100-fold higher than the standard TTC of 1.5 μg.

Another example is formaldehyde. This simple compound has been the subject of considerable public health concerns over the decades. The International Agency for Research on Cancer (IARC) has classified formaldehyde as a known human carcinogen,[64] although the concern is mainly confined to inhalation exposure, and even then the research seems inconclusive.[65] It was found to be noncarcinogenic when administered to rats by the oral route in a lifetime bioassay;[66] nonetheless, its control to very low levels has been discussed by several process chemistry groups.[39,67] Dhareshwar and Stella[65] have pointed out that formaldehyde is ubiquitous in the environment and that humans are continuously exposed to it, both through inhalation and oral ingestion. For example, it is a natural component of many foods such as fruits, meat, vegetables, or fumigated grains. It also arises endogenously from the metabolism of ingested methanol. Several approved prodrugs, such as phosphenytoin, become activated *in vivo* with concomitant release of stoichiometric amounts of formaldehyde. Its toxicity is efficiently mitigated by rapid metabolism to formate by a variety of mechanisms. Its steady state concentration in tissues, cells and body fluids has been estimated as around 0.1 mM, regardless of exposure, and turnover is 30−60 g/day.[66] Thus, it seems unnecessary to limit daily intake from an orally administered pharmaceutical to just 1.5 μg. (Inhalation and parenteral products may be different.)

Acrylates represent another class of reagent often supposed to be genotoxic. Johannsen et al.[68] evaluated results of over 200 short-term *in vitro* and *in vivo* mutagenicity studies of acrylates available in the open literature. Acrylic acid and the entire acrylate and methacrylate chemical class produced a consistently positive response when tested in a mouse lymphoma assay and other *in vitro* mammalian cell assays designed to detect clastogenicity (causing breakage or disruption of DNA strands). However, no evidence of mutagenic or clastogenic effects was seen when the compounds were tested in whole animal studies.

In a similar vein, Eichenbaum et al.[69] assessed the genotoxic risks of *p*-nitrophenol as an impurity. Existing study results

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indicated it should be considered genotoxic, having demonstrated positive *in vitro* clastogenicity in mammalian cells. They therefore conducted *in vivo* mouse micronucleus and dermal pharmacokinetic bridging studies and found no evidence of clastogenicity or carcinogenicity. Following the procedures of ICH Q3C, a threshold limit of 4 mg/day could be set - though this would likely exceed any level tolerable on quality grounds.

The discrepancy between *in vitro* and *in vivo* studies may be explained by the existence of cellular DNA repair mechanisms, which can take care of low-level damage and thus effectively create a threshold which must be exceeded before any lasting damage ensues. It has been suggested[54,70] that chemical reactivity parameters such as the Swain−Scott *s* constant may be useful in predicting genetic activity profiles of monofunctional alkylating agents. A high *s* constant indicates that the reagent will react selectively with the more nucleophilic N-atoms, rather than O-atoms, on DNA bases, causing damage which can be routinely repaired. Reagents with lower *s* constants are less discriminating and are likely to have significantly higher carcinogenic potential. For example, EMS has a high *s* constant (0.67), and - as discussed above - there is now evidence that it carries minimal risk when present at trace levels.[62] *N*-ethyl-*N*-nitrosourea (ENU), in contrast, has a low *s* constant (<0.5). Gocke et al.[71] examined the literature on dose−response relations of both these reagents; whereas ENU appeared to induce genotoxic effects with a linear dose relationship, the response to EMS was sublinear. This suggests there should be a practical threshold dose for the carcinogenicity of EMS, but not for ENU. Thus, a knowledge of *s* constants could indicate whether it may be worthwhile conducting the *in vivo* toxicity studies, as an alternative to controlling the PGI at the very low TTC levels.

5. Conclusion

Genotoxic and potentially genotoxic impurities have been the subject of increasing regulatory and industry attention since the beginning of the 21st century. The EMEA was the first authority to implement detailed guidelines on how such impurities should be controlled. The USFDA have subsequently released their own draft guideline, but at the time of writing this has not been finalized. Although the avoidance of such substances in the API syntheses is the preferred option, it is understood that this will be impractical in many cases, given the need to synthesize the drug substance efficiently. Both authorities have set a limit of 1.5 μg/day - the threshold of toxicological concern - for most known and all suspect carcinogens, unless experimental evidence can justify higher limits. For APIs undergoing clinical testing, a staged TTC approach has been accepted, where higher levels can be set to support shorter-term studies. The scientific underpinning of this low level (3 orders of magnitude lower than the normal impurity qualification threshold set by ICH guidelines) has been challenged in many quarters. Additionally, many of the most notorious suspects have, when tested, turned out to pose far less risk than supposed. Although a degree of conservatism is not unreasonable, given the context, the extreme conservatism inherent in the derivation of TTC levels has resulted in significant expenditure of development time and resources by the industry. Nonetheless, it is clear that the control of PGIs at levels well below those of other impurities is here to stay. Indeed, industry has risen to the challenge of meeting the ppm levels with a variety of control strategies, which in many cases have avoided undue increases in manufacturing costs. The key, though, is to put more effort into process understanding during the development stages - and this should reap rewards in terms of improved process efficiency as well as in better product quality.

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