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Genotoxic impurities Evaluation in Active Pharmaceutical Ingredients (API)/ Drug Substance

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ABSTRACT

The main areas discussed in this review are evaluation of control strategies of genotoxic impurities at different levels in the manufacturing of active pharmaceutical ingredients. The acceptable levels and control strategies of various guidelines are evaluated in detail.

INTRODUCTION

A substance or a compound that is intended to be used in the manufacture of a pharmaceutical product as a therapeutically active compound, is called as active pharmaceutical ingredient (API). The manufacturing process of active pharmaceutical ingredients (APIs) often involve use of reactive materials (e.g.: starting materials, intermediates, catalysts and reagents). These reactive materials along with certain reactive byproducts could remain at trace levels in final pharmaceutical products. Based on their structure and reactivity some of these compounds have been classified as genotoxic impurities (GTIs). Genotoxic impurities at trace levels, are of increasing concern to both pharmaceutical industry and regulatory agencies as potential human carcinogenic substance [1-6].

The potential consequences of genetic damage that a cell can incur is called genotoxicity. Genotoxicity is the tendency of genotoxic compounds to attack electron rich centers in DNA generating chemically altered bases [7]. Genotoxic compounds can cause genomic insult by chromosomal alterations or DNA damage by various mechanisms such as intercalation, alkylation and other mechanisms that can lead to mutation of the genetic code.

There are many guidelines for genotoxic compounds, but the regulations are inconsistent and provide unprecise recommendations. Comprehensive and clear guidance for genotoxic impurities M7 was introduced by EDQM which has provided adequate understating up to certain levels.

Genotoxicity assays have become an integral component of regulatory requirements.
Sources of impurities in drug substances consist of the following:

- Starting materials and their contaminants
- Reagents and catalysts
- Solvents
- Intermediates
- Excipients and their contaminants

- Leachable
- Degradation products
- Salt and by products which lead to genotoxic compounds.

Figure 1: Flowchart defining starting materials

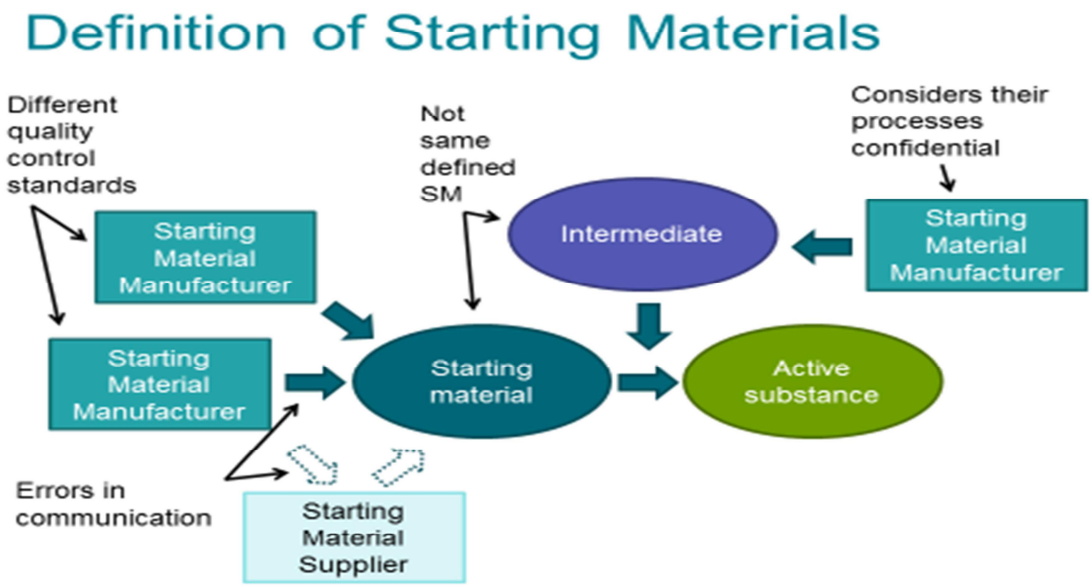


Figure 2: Starting material scientific definition

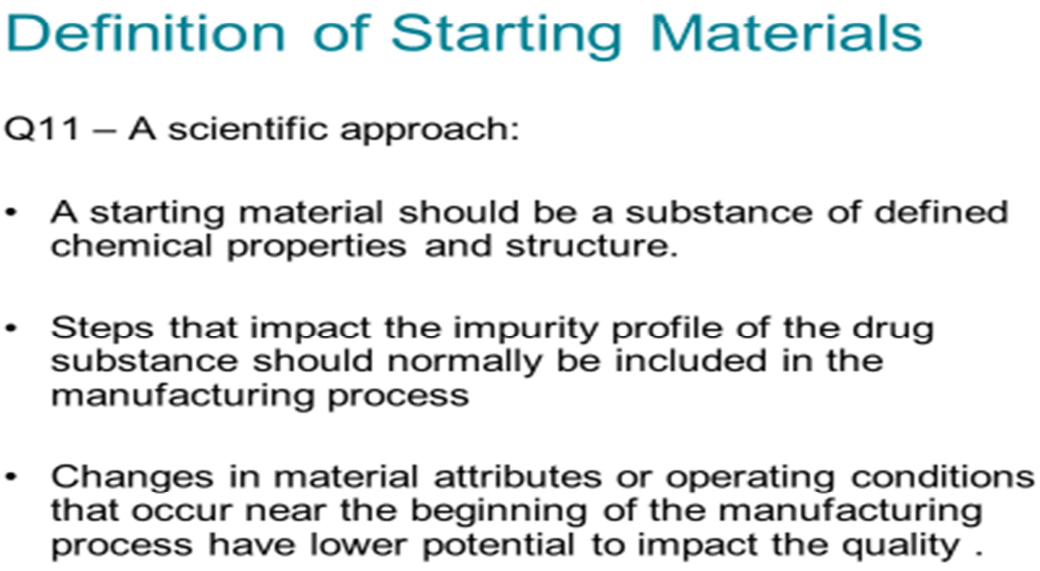
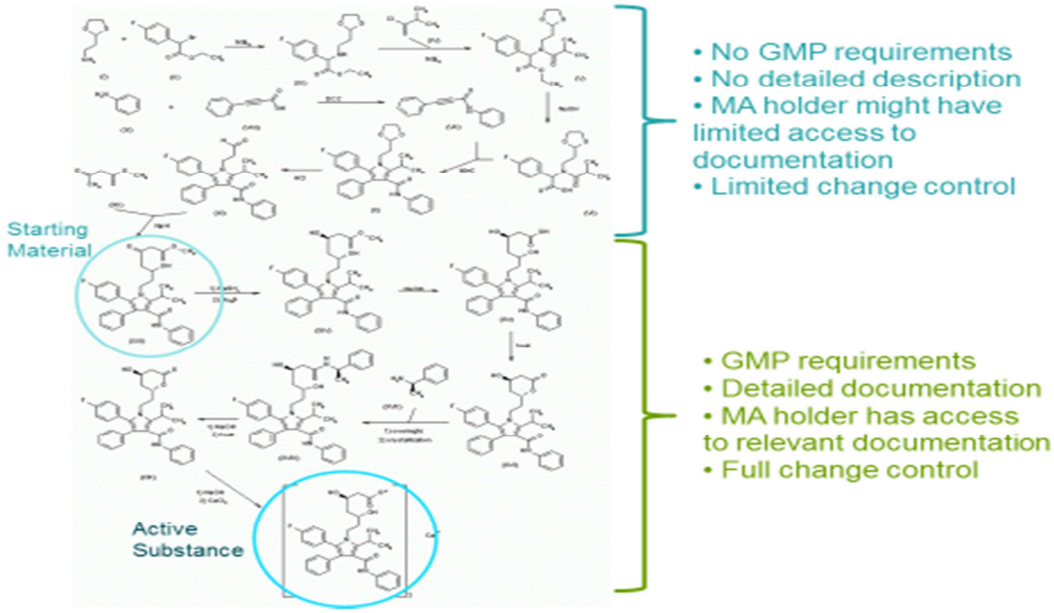


Figure 3: Various impurities sprouting from the starting material



Impurities related to drug substances can be classified into three main categories: organic impurities, inorganic (elemental) impurities, and residual solvents. Within these categories, genotoxic impurities form a special case that poses a significant safety risk, even at low concentrations, because they may be mutagenic and are therefore potentially damaging to DNA. As a result they can lead to mutations or cause cancer.

The guidance documents which discuss GTIs are as follows:
1. Guideline on the limits of genotoxic impurities (European medicines agency [EMA], 2006).
2. Genotoxic and carcinogenic impurities in rus substances and products: Recommended approaches (Food and Drug Administration [FDA] draft guidance, 2008).
3. Questions and answers on the "Guideline on the limits of Genotoxic Impurities" (EMA, 2010).
4. M7: Guideline for Genotoxicity

The scope of ICH Q3A, Q3B and Q3C is limited to marketed drugs rather than drugs in clinical development.

Pharmaceuticals are considered to be the most highly regulated industry, worldwide. There are international and country specific regulatory bodies that ensures compliance in various legal and regulatory aspects of a drug like drug development process, licensing, registration, manufacturing, marketing and labeling of pharmaceutical products. There are international regulatory bodies like International conference on harmonization (ICH), World health organization (WHO), world intellectual property organization (WIPO) which also play essential role in all aspects of pharmaceutical regulations.

The international conference on harmonization (ICH) brings together the regulatory authorities of Europe, Japan and USA.

Genotoxic impurity classification:
Compounds are classified according to their risk potential. Muller et al. proposed a five class system for categorizing genotoxic impurities [8].

Class 1
Impurities known to be genotoxic (mutagenic) and carcinogenic. This group includes known animal carcinogens with reliable data for a genotoxic mechanism, and human carcinogens. The genotoxic nature of the impurity is demonstrated using published data on the chemical structure.

Class 2
Impurities known to be genotoxic (mutagenic), but with unknown carcinogenic potential. This group includes impurities with demonstrated mutagenicity based on testing of the impurity in conventional genotoxicity tests.

Class 3
Impurities that have an alerting structure unrelated to the structure of the API, and of unknown genotoxic (mutagenic) potential. This group includes impurities with functional moieties that can be linked to genotoxicity based on structure. However, these moieties have not been tested as isolated compounds and are identified based on chemistry and using knowledge-based expert systems for structure activity relationships (SAR).

Class 4
Impurities with an alerting structure related to the API and impurities that contain an alerting functional moiety that is shared with the structure of the API.

Class 5
No alerting structure or indication of genotoxic potential.

Compounds in class 5 yield negative result in structure alert assessment and hence no additional action beyond normal impurity monitoring is required. Compounds in class 3 and 4 which show positive results are further submitted for mutagenicity testing, with Ames and mini mutagenicity test [9-10].

Figure 4: Potential Genotoxic Structures Characterization and Qualification

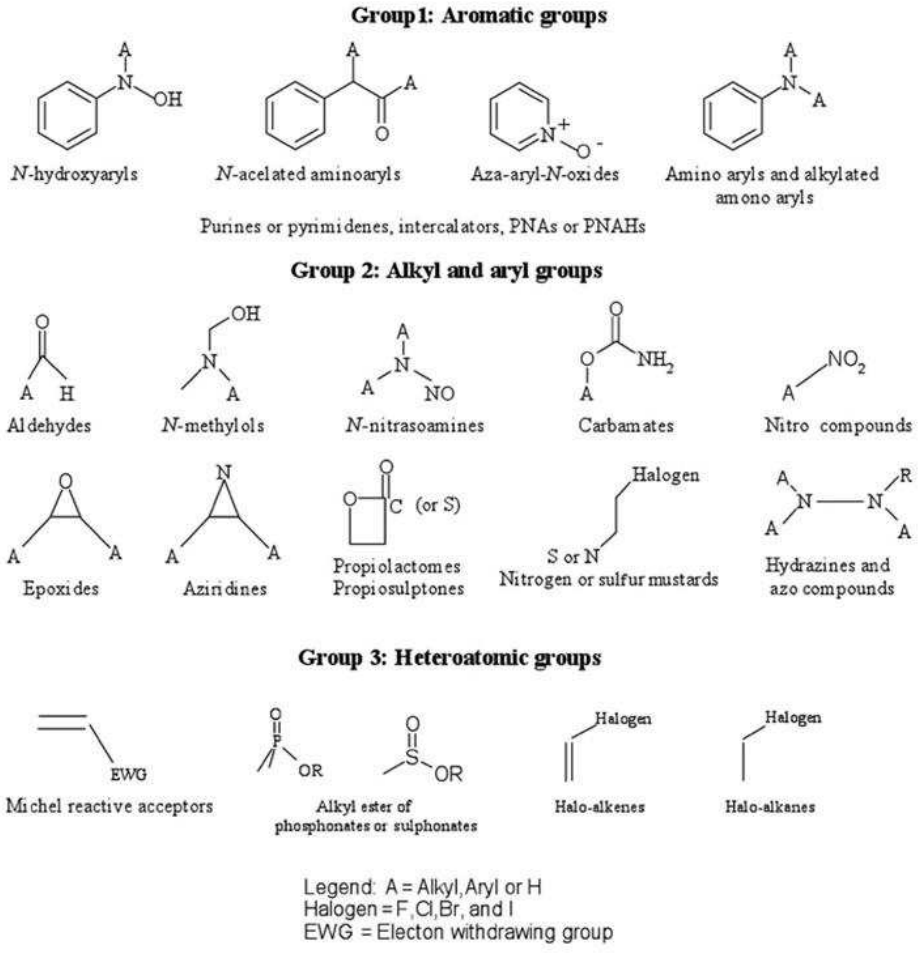


Figure 5: Classification of genotoxic impurities

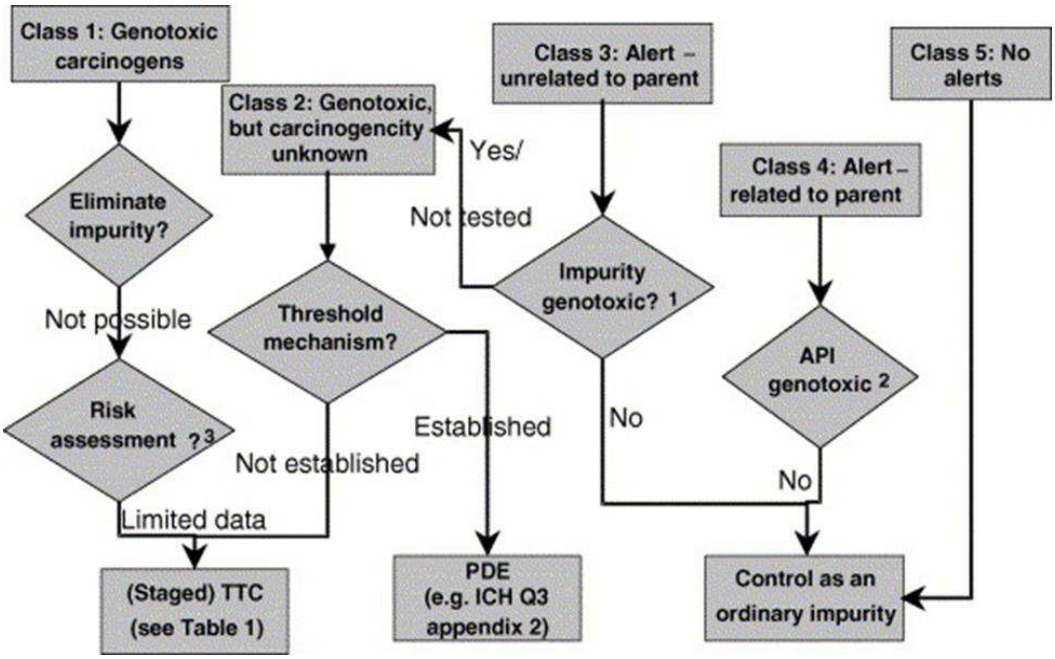
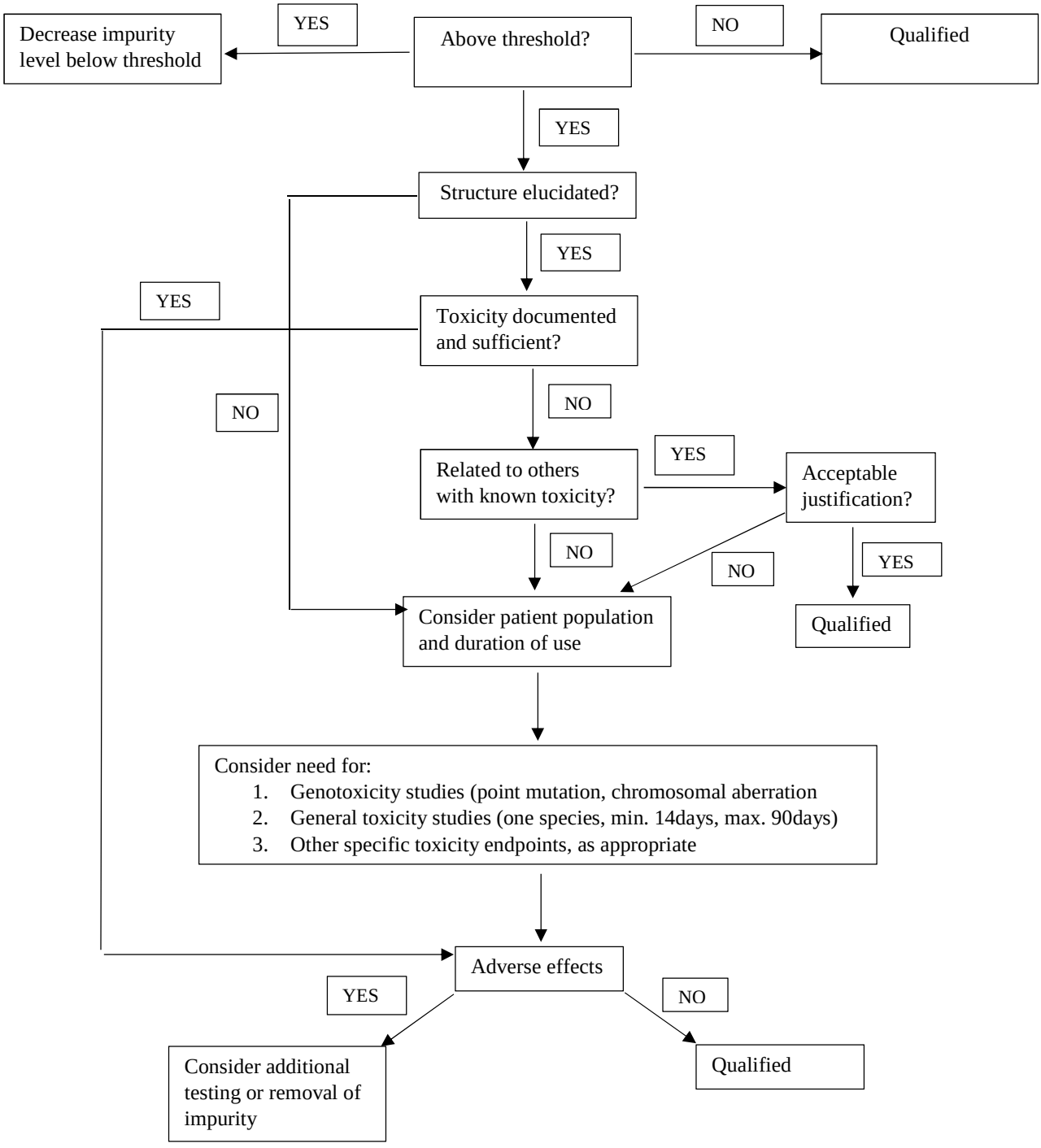


Figure 6: Flowchart describing identification of genotoxic impurities and its solution.



Genotoxicity assay methods:

Genotoxicity of a compound can be tested both in-vitro and in-vivo. One single test cannot provide all information of genetic damage caused by a compound hence a battery of tests are recommended. Genotoxicity assay tests as per the guidelines of FDA are as follows: a) test for gene mutation in bacteria. b) In vitro test with cytogenic evaluation of chromosomal damage with mammalian or rodent cells. c) An in vivo test for chromosomal damage [11].

As mentioned before, bacterial mutagenic assay (Ames test) is used in detection of point mutation or frame shift mutation using bacteria. Various modification of Ames test are also in practice. Most of the genotoxic carcinogens can be detected by Ames test [12-13]. Micronucleus test which uses Rodent bone marrow or peripheral blood is a reliable in vivo genotoxicity assay method [14]. Micronucleus are cytoplasmic bodies having a portion of acentric chromosome or whole chromosome which are not carried to the opposite pole during anaphase. Micronuclei reflects chromosomal damage. Comet assay or rat comet assay is another method commonly employed for genotoxicity assessment. The technique involves cell lysis and electrophoresis of the released DNA in agarose gel. DNA with more double strand break migrates quicker to the anode. This technique offers the advantage of having the ability to detect low levels of DNA damage, requires very low number of cells, is cheaper and displays results quicker. A credible degree of consonance have been shown between micronucleus test and comet assay by Hartmann et al. [15]. Comet assay however suffers the drawback of not being able to identify the exact chemical component causing the breaks (16). Rat liver unscheduled DNA synthesis (UDS) assay is an in vivo test method for investigating genotoxic effects of chemicals in the liver. Liver is a major site of metabolism of absorbed compounds and hence used in measuring DNA damage in vivo (17). ^{32}P post labeling is a DNA binding assay for the detection and quantification of DNA adducts and is used in the detection of genotoxic properties of chemical compounds [18-19]. Another genotoxicity assay called, Vitotox test is a high throughput bacterial genotoxicity test. Shigeharu muto et al. showed 94% concordance between Ames and vitotox test [20].

Mutagenic potential at the levels of gene, chromosome and genome are essential to determine the mutagenic potential of a drug [21]. Genotoxicity tests are not able to detect more than one end point in a single assay system [22].

Structural alerts/SAR evaluation and their drawbacks:
Prior knowledge of genotoxicity of a chemical/compound is very useful for drug manufacturers. In vivo testing in animals requires huge amount of resources whereas in vitro genotoxic assays in bacteria are comparatively quick and relatively less expensive. But even in-vitro testing can be cumbersome while testing huge number of chemicals. Hence, in-silico methods such as quantitative structure activity relationship (QSAR) which are based on existing data and knowledge have come more into use. The concept of structural alerts was first elucidated by Ashby and Tennant [23]. Genotoxic impurities can be identified by studying the functional groups. Several structural alerts were proposed by Ashby et al. The presence and absence of a structural alert can be evaluated using commercially available software packages. If an impurity contains a structural alert then the evaluation of its genotoxic potential is carried out using Ames test.

The need of anticipating clinical safety based on chemical structural alert is becoming an increasingly important part of regulatory decision making. Structure activity relationship (SAR) is used in predicting drug efficacy.

Some of the structural alert databases, are listed below:
1. National toxicology program database
(http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm)
2. TOXNET database (http://toxnet.nlm.nih.gov)
3. Univ California Berkeley Carcinogenic potency database (http://potency.berkeley.edu)
4. DEREK software (https://www.lhasalimited.org/)
5. MultiCASE software (http://www.multicase.com/)
6. Leadscope software (http://www.leadscope.com/)
7. Toxtree (http://ecb.jrc.it/QSAR)

Many pharma companies today, use a combination of *in vitro* screening and *in silico* analysis towards genetox screening. The genotoxicity prediction programs however suffer some drawbacks. As discussed by Synder and Smith in their review, the computer based programs have poor sensitivity for detecting Ames positives, and poorer sensitivity towards non-bacterial genotoxic assays along with their inability to identify non-alerting structures which are of greater interest [24]. Prediction software systems have their weaknesses along with their strengths [25]. There are some indirect mechanisms of genotoxicity as well, whereby the mutagen interacts with non-DNA targets [26]. Considering the alternative mechanisms of genotoxicity the most commonly used computational databases like MCASE, DEREK, and TOPKAT do not have sufficient sensitivity at predicting genotoxicity [27].

Genotoxic impurities: Sulfonate esters, Alkyl halides and chloroformates, epoxides and hydro peroxides, hydrazines and hydrazides, N-Nitroso, aromatic amines, aldehydes

Analytical techniques for detection of genotoxic impurities and associated challenges:

Different analytical techniques are employed towards detection of impurities like LCMS/MS NMR, HPLC-UV, and GC-MS/MS. Each technique has a different level of sensitivity capable of sensing impurities at various concentrations. Analytical approaches are based on physicochemical properties of the compounds. Volatility, polarity and reactivity of impurities pose a major challenge in the analysis of genotoxic impurities.

Figure 7: Limit of impurity vs daily dose of drug graphed

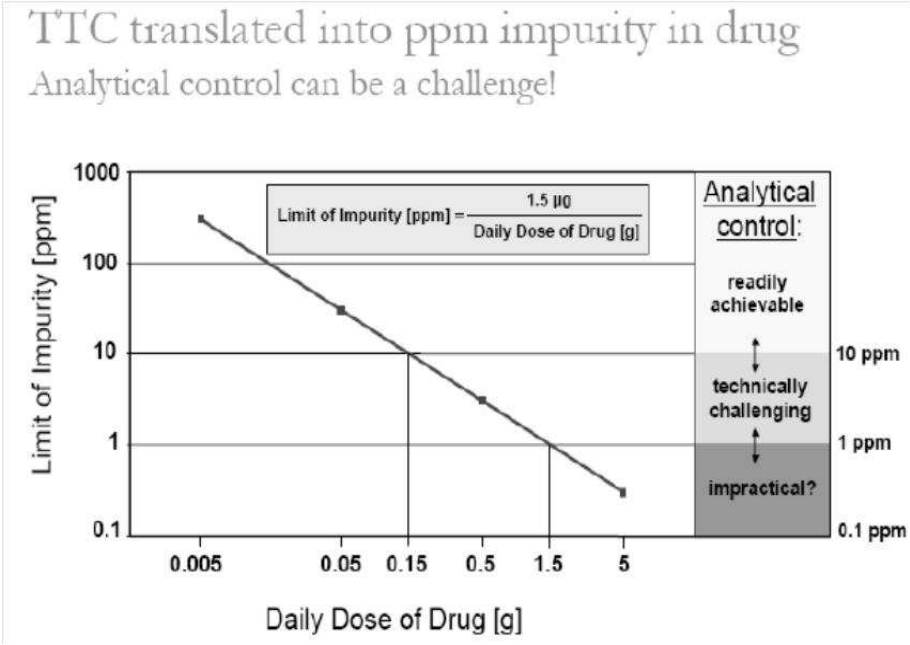


Figure 8: Relationship between Staged TTC, Drug Dose and Impurity Concentration Limit

Duration of exposure	Allowable daily intake[2]	Impurity Concentration Limit (ppm) based on drug daily dose[1]								
		1 mg	5 mg	10 mg	50 mg	0.1 g	0.2 g	0.5 g	1.0 g	2.0 g
>12 months	1.5 µg/day	1500	300	150	30	15	7.5	3	1.5	0.75
≤ 12 months	5 µg/day	5000	1000	500	100	50	25	10	5	2.5
≤ 6 months	10 µg/day	5000	2000	1000	200	100	50	20	10	5
≤ 3 months	20 µg/day	5000	4000	2000	400	200	100	40	20	10
≤ 1 month	60 µg/day	5000	5000	5000	1200	600	300	120	60	30
Single Dose	120 µg/day	5000	5000	5000	2400	1200	600	240	120	60

Application of genotoxic compound:
Genotoxic compounds find application in cancer therapy. Resveratrol, genistein and baicalcein which have genotoxic effects are shown to have chemotherapeutic properties [28].

Drugs containing genotoxic impurities:
Omeprazole is a proton pump inhibitor used in the treatment of gastric acid disorders and ulcers. The in vitro and in vivo assays have shown omeprazole to be a potential genotoxic compound [29-31]. Member of Statin drug family, Rosuvastatin is a HMG-CoA reductase inhibitor used in lowering cholesterol. Berber using comet assay has shown Rosuvastatin to cause chromosomal aberration, micronucleus induction and DNA damage [32]. Etanercept treats autoimmune diseases by inhibiting/interfering with TNF (tumor necrosis factor) [33]. It has FDA approval to treat rheumatoid arthritis and juvenile arthritis [34, 35] although safety concerns have been raised for Etanercept [36]. It also shows genotoxicity in juvenile idiopathic arthritis patients [37]. Co-vasotec is an antihypertensive drugs, which is a combination of angiotensin II receptor antagonist with hydrochlorothiazide (HCTZ). HCTZ was found to cause increase in micronucleus frequencies by the mechanism of chromosome delay and chromosome breakage [38]. Imatinib marketed as Gleevec or Glivec is a tyrosine kinase inhibitor used in the treatment of multiple cancers. 2-methyl-5-aminophenyl)-4-(3-pyridyl)-2-pyrimidine (Imp-A) is used during manufacture of imatinib mesylate (API), as an intermediate or raw material which is a well known carcinogen [39]. Celecoxib is a NSAID used for the treatment of osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis. Reddy et al. have shown the presence of (4-sulfamoylphenyl) hydrazine hydrochloride (SHH) and (4-methyl-acetophenone) para-sulfoamide phenylhydrazine hydrochloride (MAP) genotoxins using LC-MS method [40]. Atenolol is a drug belonging to the group of beta blockers used primarily in cardiovascular diseases, contains allyl chloride, 1, 3-dichloro-2-propanol and 2, 3-dichloro-1-propanol which are known genotoxins [41]. Emtricitabine (trade name – Emtriva) is used for the treatment of HIV infection, has been shown to contain genotoxic impurities, methyl methanesulfonate and ethyl methanesulfonate [42]. Amlodipine mesylate is used to lower blood pressure. It acts by relaxing muscles of blood vessels in the body. Amlodipine has also been shown to contain genotoxic impurity alkyl benzenesulfonate [43]. Cloperastine which is a cough suppressor contains genotoxic impurities of alkyl halide, methyl p-toluenesulfonate and 2-chloroethyl p-toluenesulfonate [44]. Metronidazole marketed as Flagyl is an antibiotic used for anaerobic bacteria and protozoa. Abrevaya et al showed metronidazole to be genotoxic using micronucleus test. [45].

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REFERENCES

[1] Humfrey CD, *Toxicol. Sci*. **2007**,100 (1), 24-28.
[2] Giordani A, Kobel W, Gally HU, *Eur J Pharm Sci*. **2011**, 43, 1-15.
[3] Jacobson-Kram D, McGovern T, *Adv. Drug. Deliv. Re*. **2007**, 59 (1), 38-42.
[4] Committee for medicinal products for human use, Guideline on the limits of genotoxic impurities. European medicines agency: London UK, June **2006**; Vol. CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006.
[5] Committee for medicinal products for human use, Questions and answers on CHMP guideline on the limits of genotoxic impurities. European medicines agency: London, UK, June **2008**; Vol. EMEA/CHMP/SWP/431994/2007.
[6] US department of health and human services, Guidance for industry, genotoxic and carcinogenic impurities in drug substances and products: recommended approaches. Food and drug administration, Centre for drug evaluation and research: Silver spring, MD, December **2008**; vol. http://www.fda.gov/cder/guidance/7834dft.pdf.
[7] Miller E C and Miller JA, *Cancer*, **1981**, 47, 1055 – 1064.
[8] Muller L, Mauthe RJ, Riley CM, Andino MM, Antonis DD, Beels C, DeGeorge J et al., *Regul. Toxicol. Pharmacol*. **2006**, 44(3), 198 – 211.
[9] Mortelmans K and Zeiger E., *Mut Res/Fund mol mech of mutagenesis*. **2000**, 455 (1-2), 29-60.
[10] Flamand N, Meunier JR, Meunier, Agapakis-Causse C., *Tox in Vitro* **2001**, 15 (2), 105 – 114.
[11] Guidance for industry S2B genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals; **2007**.www.fda.gov

[12] McCann J, Choi E, Yamasaki E, Ames BN., *Proc. Natl. Acad. Sci*. **1975**, 72, 5135 – 9.
[13] Zeiger E, Anderson B, Howorth S, Lawlor T, Mortlemans K., *Environ. Mol. Mutagen*. **1992**, 19, 2-141.
[14] http://www.oecd.org/dataoecd/18/34/1948442.pdf
[15] Hartmann A, Elhajouji A, Kiskinis E, Poetter F, H-J Martus, Fjallam A, Frieauff W and Suter W., *Food Chem. Toxicol*., **2001**, 39 (8), 843 – 858.
[16] Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamare Y, RojasE, Ryu JC and Sasaki YF., *Environ. Mol. Mutagen* **2000**, 35, 206- 221.
[17] OECD guideline for the testing of chemicals. Unscheduled DNA synthesis (USD) test with mammalian liver cells In vivo, **1997**.
[18] Schmeiser HH, Stiborova M, Arlt VM. *Methods Mol. Biol*. **2013**, 1044, 389 – 401.
[19] Whysner J, Redyy MV, Ross PM, Mohan M, Lax EA., *Mutat Res*. **2004**, 566 (2), 99 – 130.
[20] Shigeharu M, Hiroshi B and Yoshifumi U., *Environ Mutagen Res*., **2003**, 25, 69 – 75
[21] Shelby M, *Mutat. Res*., **1988**; 204, 3 – 15.
[22] Anderson D, Sorsa M, Waters MD., *Mutat. Res*. **1994**, 313, 101-15
[23] Ashby J.; Tennant, R. W., *Mutat. Res*., **1991**, 257, 229–306.
[24] Snyder RD and Smith MD., *Drug. Discov. Today*, **2005**, 10 (16), 1119-1124.
[25] Custer LL and Seder KS., *Cur. Drug. Metabol*., **2008**, 9(9), 978 – 985.
[26] Volders MK, Vanhauwaert A, Ritter UE, **2003**, *Toxicol letter*, 140-141, 63-74.
[27] Snyder RD, Pearl GS, Mandakas G, Choy WN, Goodsaid F and Rosenblum IY., Env. Mol. Mutagenesis., **2004**, 43 (3), 143 – 158.
[28] Myung K et al., *PNAS*, **2012**,109(14), 5423 – 5428
[29] Martelli A, Mattiolli F, Mereto E, Campart GB, Sini D, Bergamaschi G and Brambilla G. **1998**, 130 (1), 29-41.
[30] Rosenkranz HS and Klopman G., *Mutagenesis*, **1991**, 6(5), 381-384.
[31] Furihata C, Hirose K, Matsushima T., *Mut. Res. Letters*., **1991**, 262 (1), 73 – 76.
[32] Berber AA, Celik M, Aksoy H., *Drug Chem Toxicol*., **2014**, 37 (3), 316 – 21
[33] Fledmann M and Maini RN., *Nat Med*., **2003**, 9, 1245 – 1250.
[34] Siegel Jay P., Letter to Sally Gould, FDA, **1998**.
[35] Weiss K D., Letter to Sally Gould, FDA, **1999**.
[36] Safety update on TNF-α antagonists: Infliximab and Etanercept. FDA, 13 – 14.
[37] Demirkaya E, Cok I, Durmaz E, Ulutas OK, Ayaz NA, Besbas N, Ozen S., *Arthritis Care Res* **2010**, 62 (1), 73-7.
[38] Andrianopoulos C, Stephanou G, Demopoulos NA., *Environ Mol Mutagen*, **2006**, 47(3), 169 – 78.
[39] Bhatt V, Prasad G, Bhatt H, Sharma A., *Acta Chim. Pharm. Indica*, **2013**,3(2), 182 – 191.
[40] Reddy A V B, Venugopal N, Madhavi G., *J Ana Sci Tech*., **2014**, 5, 18.
[41] Mamilla Y R, Chetyala KR, Venna R, Gajjela R, Katragadda K, Govindasamy S, Mulukutla S, Datta D., *Chromatographia*; 71, 733 – 736.
[42] Kakadiya PR, Chandrashekhar TG, Ganguly S, Singh DK, Singh V., *Anal chem Insights* **2011**, 6, 21 – 28.
[43] Raman NVVSS, Reddy KR, Prasad AVSS, Ramakrishna K., *J phar biomed ana*., **2008**, 48(1), 227 – 230.
[44] Gracia A, Ruperez FJ, Ceppa F, Pellati F, Barbas C., *J Pharma biomed ana*, 61, 230 – 236.
[45] Abrevaya XC, Carballo MA, Mudry MD., *Genent. Mol. Biol*. **2007**, 30 (4), 1139 – 1143.