

**GENOTOXIC IMPURITIES IN ACTIVE PHARMACEUTICAL  
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Author****Debabrata Sanyal**Pacific University, Udaipur,  
Rajasthan, India – 313003.**ABSTRACT**

Impurities in pharmaceuticals are redundant chemical entities that remain with active pharmaceutical ingredients (APIs), or develop during formulation, or upon aging of both API and formulations. Genotoxic impurities are distinguished class of impurities that can induce genetic mutations and/or chromosomal rearrangements at very

low levels. Confirming to this threat towards safety of APIs, global regulations are evolved for their control. The article primarily discusses all aspects related with generation, identification, monitoring and control of genotoxic impurities at suitable level for safe intake of pharmaceutical products.

**KEYWORDS:** Genotoxic impurities.**INTRODUCTION**

Impurities in pharmaceuticals are redundant chemicals that remain with API or form during formulation or upon storage of both API and formulation. Presence of these redundant chemicals even in trace amount may influence efficacy and safety of pharmaceutical product. Control of impurities is currently a critical issue for pharmaceutical industry. These are classified into following categories as per guideline ICH Q3A (R2).<sup>[1]</sup>

Organic impurities can arise during the manufacturing process and/or storage of the API. They can be identified or unidentified, volatile or non-volatile, and include: starting materials, by-products, intermediates, degradation products, reagents, ligands and catalysts. Inorganic impurities can result from the manufacturing process. They are normally known

and identified and include: reagents, ligands and catalysts, heavy metals or other residual metals, inorganic salts, other materials.

Residual Solvents are remnant of inorganic or organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of API. Since these are generally of known toxicity, the selection of appropriate controls is easily accomplished.

Within these categories, genotoxic impurities form a special class that poses a significant safety risk of damaging the DNA; even at very low concentrations. As a result they can lead to mutations and / or cause cancer. Drug substances / APIs and their relative compounds such as impurities constitute an important group of genotoxic compounds. Thus, these compounds pose a serious concern to clinical subjects and patients. The present article will describe the significant aspects related to genotoxic impurities like genetic materials in human body, genetic materials in affected by genotoxic impurities, evolution of genotoxic impurities as a global concern, its significance towards adequate monitoring and control, recent regulations, control mechanisms depicted literatures and guidance documents. To confirm a pragmatic approach, further efforts have been made to discuss simplified mechanisms for identifying, monitoring and control of genotoxic impurities at suitable level for safe intake of pharmaceutical products.

### **Elaboration of the word Genotoxic**

GENOTOXIC Merriam-Webster Medical Dictionary.<sup>[2]</sup>

Main Entry: ge·no·tox·ic

Pronunciation: j -n -tāk-sik

Function: adjective: damaging to genetic material.<sup>[3]</sup>

-ge·no·tox·ic·i·ty/-tāk-sis-t-/noun, plural–ties

Genotoxic compounds induce genetic mutations and/or chromosomal rearrangements and can therefore act as carcinogenic compounds (McGovern and Jacobson-Kram, 2006).<sup>[4]</sup> These compounds cause damage to DNA by different mechanisms such as alkylation or other interactions that can lead to mutation of the genetic codes. In general, chemists employ the terms "genotoxic" and "mutagenic" synonymously; however, there is a subtle distinction. The majority of chemical carcinogens are capable of causing DNA damage, i.e., "genotoxic" (Ashby, 1990).<sup>[5]</sup> Moreover, a genotoxic compound also carries with it the carcinogenic effect which causes additional concern from the safety viewpoint. Term "genotoxic" is applied to

agents that interact with DNA and/or its associated cellular components (e.g. the spindle apparatus) or enzymes (e.g. topoisomerases) (Robinson, 2010).<sup>[6]</sup> Irrespective of the mechanism by which cancer is induced, it is now well agreed that it involves a change in the integrity or expression of genomic DNA.

### **Genetic materials in human body**

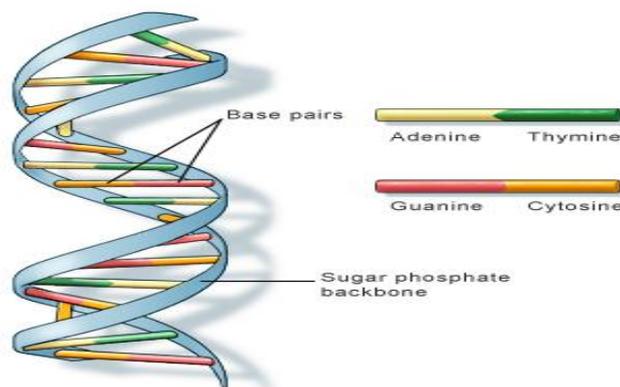
Genotoxic impurities (GTIs) are the chemical compounds that may be mutagenic and could potentially damage DNA with an accompanying risk of cancer. To understand the mechanism for control of genotoxic impurities, discussion has been made about the genetic material in human body,<sup>[7]</sup> and types of damages.

### **DNA**

DNA, or deoxyribonucleic acid, is the hereditary material in humans and almost all other organisms. Nearly every cell in a human body has same DNA. DNA is primarily located in the cell nucleus (where it is called nuclear DNA), but a small amount of DNA can also be found in the mitochondria (where it is called mitochondrial DNA).

The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). The sequence of these bases determines the information available for building and maintaining an organism. DNA bases pair up with each other, A with T and C with G, to form units called base pairs. Each base is also attached to a sugar molecule and a phosphate molecule. Together, a base, sugar, and phosphate are called a nucleotide.

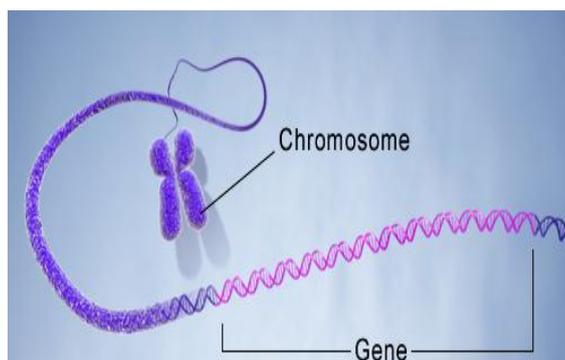
Nucleotides are arranged in two long strands that form a spiral called a double helix. The structure of the double helix is somewhat like a ladder, with the base pairs forming the ladder's rungs and the sugar and phosphate molecules forming the vertical sidepieces of the ladder. An important property of DNA is that it can replicate, or make copies of itself. Each strand of DNA in the double helix can serve as a pattern for duplicating the sequence of bases. This is critical when cells divide because each new cell needs to have an exact copy of the DNA present in the old cell.



DNA is a double helix formed by base pairs attached to a sugar-phosphate backbone.

### Gene

A gene is the basic physical and functional unit of heredity. Genes that are made up of DNA, act as instructions to make molecules called proteins. In humans, genes vary in size from a few hundred DNA bases to more than 2 million bases. The Human Genome Project has estimated that humans have between 20,000 and 25,000 genes.



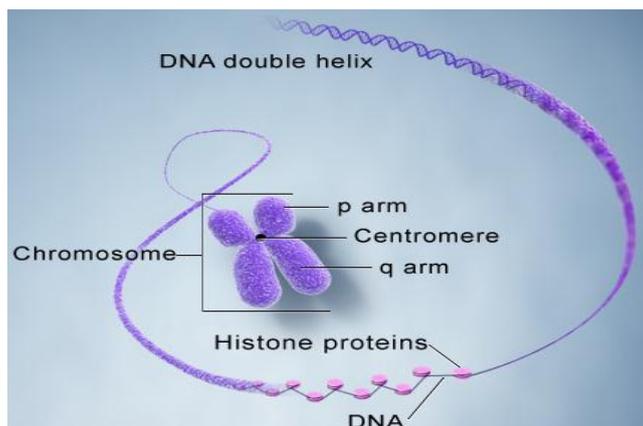
Genes are made up of DNA. Each chromosome contains many genes.

Every person has two copies of each gene, one inherited from each parent. Most genes are the same in all people, but a small number of genes (less than 1 percent of the total) are slightly different between people. Alleles are forms of the same gene with small differences in their sequence of DNA bases. These small differences contribute to each person's unique physical features.

### Chromosomes

In the nucleus of each cell, the DNA molecule is packaged into thread-like structures called chromosomes. Each chromosome is made up of DNA tightly coiled many times around proteins called histones that support its structure. Chromosomes are not visible in the cell's nucleus—not even under a microscope—when the cell is not dividing. However, the DNA

that makes up chromosomes becomes more tightly packed during cell division and is then visible under a microscope. Most of what researchers know about chromosomes was learned by observing chromosomes during cell division. Each chromosome has a constriction point called the centromere, which divides the chromosome into two sections, or “arms.” The short arm of the chromosome is labeled the “p arm.” The long arm of the chromosome is labeled the “q arm.” The location of the centromere on each chromosome gives the chromosome its characteristic shape, and can be used to help describe the location of specific genes.



Type of DNA damage induced through genotoxic impurities

Genotoxicity describes property of chemical agents that damages genetic information within a cell causing mutations, which may lead to cancer. While genotoxicity is often confused with mutagenicity, all mutagens are genotoxic; however, not all genotoxic substances are mutagenic. The alteration can have direct or indirect effects on the DNA: the induction of mutations, mistimed event activation, and direct DNA damage leading to mutations. The permanent, heritable changes can affect either somatic cells of the organism or germ cells to be passed on to future generations. Cells prevent expression of the genotoxic mutation by either DNA repair or apoptosis; however, the damage may not always be fixed leading to mutagenesis.<sup>[8]</sup>

The DNA sequence of a gene can be altered in a number of ways. Gene mutations have varying effects on health, depending on where they occur and whether they alter the function of essential proteins,<sup>[9]</sup> The types of mutations include:

Missense mutation - This type of mutation is a change in one DNA base pair that results in substitution of one amino acid for another in the protein made by a gene.

Nonsense mutation - A nonsense mutation is also a change in one DNA base pair. Instead of substituting one amino acid for another, however, the altered DNA sequence prematurely signals the cell to stop building a protein. This type of mutation results in a shortened protein that may function improperly or not at all.

Insertion - An insertion changes the number of DNA bases in a gene by adding a piece of DNA. As a result, the protein made by the gene may not function properly.

Deletion - A deletion changes the number of DNA bases by removing a piece of DNA. Small deletions may remove one or a few base pairs within a gene, while larger deletions can remove an entire gene or several neighboring genes. The deleted DNA may alter the function of the resulting protein(s).

Duplication - A duplication consists of a piece of DNA that is abnormally copied one or more times. This type of mutation may alter the function of the resulting protein.

Frameshift mutation - This type of mutation occurs when the addition or loss of DNA bases changes a gene's reading frame. A reading frame consists of groups of 3 bases that each code for one amino acid. A frameshift mutation shifts the grouping of these bases and changes the code for amino acids. The resulting protein is usually nonfunctional. Insertions, deletions, and duplications can all be frameshift mutations.

Repeat expansion - Nucleotide repeats are short DNA sequences that are repeated a number of times in a row. For example, a trinucleotide repeat is made up of 3-base-pair sequences, and a tetranucleotide repeat is made up of 4-base-pair sequences. A repeat expansion is a mutation that increases the number of times that the short DNA sequence is repeated. This type of mutation can cause the resulting protein to function improperly.

Genotoxic effects such as deletions, breaks and/or rearrangements can lead to cancer if the damage does not immediately lead to cell death. Regions sensitive to breakage, called fragile sites, may result from genotoxic agents. Some chemicals have the ability to induce fragile sites in regions of the chromosome where oncogenes are present, which could lead to carcinogenic effects.

### **Wakening of Regulatory Action for the concern Genotoxic Impurities**

Two incidents had triggered change in regulatory authorities stand point and realized the significance of Genotoxic Impurities [GTIs]. First one is the well-publicized case of Roche's Antiviral molecule, Nelfinavir, marketed under the brand name of Viracept.

Batches of the Nelfinavir manufactured at Roche's plant in Switzerland were apparently contaminated with traces of ethyl methanesulfonate [EMS] arising from reactor cleaning procedures, wherein trace levels of methanol is reacted with Methane sulphonic acid to yield EMS. In terms of assessing the risk to patients, Roche has investigated based on the toxicology of EMS and patient exposure limits and finally, Roche had recalled all nelfinavir (Viracept) manufactured at its Swiss plant with immediate effect in June 2007. EMS is a well-established genotoxic agent that has been used extensively as model compound in experimental work to establish the responsiveness of the test system under investigation and found that EMS induces DNA damage by a direct mechanism, acting as a mono functional ethylating agent.

In another case, where-in the API was re-crystallized from acetone and the applicant had failed to consider potential contamination of mesityl oxide arising from this. The application was rejected by the European Medicines Agency's (EMA) during 2007.

### **Regulatory Progressions for the control of Genotoxic Impurities**

Observers have critically reviewed the history of the evolving guidance on genotoxic impurities. There have been many discussions about the definition of genotoxins and genotoxicity. 'International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use' project represents the main group of guidelines with topics such as "Quality" topics and "Safety" topics.

Quality topics relate to chemical and pharmaceutical quality assurance (stability testing, impurity testing, etc.) and safety topics deal with in vitro and in vivo pre-clinical studies (carcinogenicity testing, genotoxicity testing, etc.) ICH Guideline on safety topics defined genotoxicity as "a broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced." [ICH guideline S2 (R1)].<sup>[10]</sup>

Table below illustrates a series of thresholds described in ICH Q3A (R2)<sup>[11]</sup> that trigger reporting, identification and qualification requirements in API.

Thresholds	Maximum daily dose	
	≤2 g/day	>2 g/day
Reporting threshold	0.05%	0.03%
Identification threshold	0.10% or 1.0 mg per day intake (whichever is lower)	0.05%
Qualification threshold	0.15% or 1.0 mg per day intake (whichever is lower)	0.05%

The below table depicts the thresholds for reporting, identification, and qualification of impurities in drug products ICH Q3B (R2).<sup>[12]</sup>

Reporting Thresholds	
Maximum Daily Dose <sup>i</sup>	Threshold <sup>ii,iii</sup>
≤ 1 g	0.1%

Identification Thresholds	
Maximum Daily Dose <sup>i</sup>	Threshold <sup>ii,iii</sup>
< 1 mg	1.0% or 5 µg TDI, whichever is lower
1 mg - 10 mg	0.5% or 20 µg TDI, whichever is lower
>10 mg - 2 g	0.2% or 2 mg TDI, whichever is lower
> 2 g	0.10%
Qualification Thresholds	
Maximum Daily Dose <sup>i</sup>	Threshold <sup>ii,iii</sup>
< 10 mg	1.0% or 50 µg TDI, whichever is lower
10 mg - 100 mg	0.5% or 200 µg TDI, whichever is lower
>100 mg - 2 g	0.2% or 3 mg TDI, whichever is lower
<i>i. Amount of API administered per day</i>	
<i>ii. Thresholds for degradation products are expressed either as a percentage of the API or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation product is unusually toxic.</i>	
<i>iii. Higher thresholds should be scientifically justified.</i>	

ICH safety guideline has presented specific guidance on ‘Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use’. Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds that induce genetic damage by various mechanisms as discussed under earlier section of this article. Compounds that are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens.

The guidance confirms a test series [battery] approach because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis.

The following two options for the standard battery are considered equally suitable:

Option 1 – i. A test for gene mutation in bacteria; ii. A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test), or an in vitro mouse lymphoma Tk gene mutation assay; iii. An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

Option 2 – i. A test for gene mutation in bacteria; ii. An in vivo assessment of genotoxicity with two tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay.

The reasoning behind considering Options 1 and 2 equally acceptable is as follows: When a positive result occurs in an in vitro mammalian cell assay, clearly negative results in two well conducted in vivo assays, in appropriate tissues and with demonstrated adequate exposure, are considered sufficient evidence for lack of genotoxic potential in vivo. Thus a test strategy in which two in vivo assays are conducted is same strategy that would be used to follow up a positive result in vitro.

The European Medicines Agency's [EMA] was the pioneering regulatory body to impose detailed guidelines to handle GTIs which came into operation at the beginning of 2007. The European Medicines Agency (EMA) guideline describes a general framework and practical approaches on how to deal with genotoxic impurities in new API. The USFDA subsequently released a draft guideline in December, 2008.

Essentially both of these guidelines mention the recommended approaches to deal with GTIs, especially its control limits in the form of Threshold of Toxicological concern (TTC), wherein 1.5 microgram per day daily intake of impurity is considered as virtually safe dosage, while low and high limits are case specific based on the toxic potential of a given compound. Therefore, GTIs have to be controlled below the TTC limit.

Based on the importance of the mechanism of action and the dose-response relationship in the assessment of genotoxic compounds, the EMA guideline,<sup>[13]</sup> presents two classes of genotoxic compounds: 1. Genotoxic compounds with sufficient (experimental) evidence for a threshold-related mechanism, 2. Genotoxic compounds without sufficient (experimental) evidence for a threshold related mechanism.

Those genotoxic compounds with sufficient evidence would be regulated by evaluating the permissible daily exposure or acceptable daily intake. For genotoxic compounds without sufficient evidence for a threshold related mechanism, the guideline proposes a policy of controlling levels to “as low as reasonably practicable” (ALARP) principle, where avoiding is not possible.

The Pharmaceutical Research and Manufacturing Association (PhRMA) has established a procedure for the testing, classification, qualification, toxicological risk assessment, and control of impurities processing genotoxic potential in pharmaceutical products. As most medicines are given for a limited period of time, this procedure proposes a staged TTC to adjust the limits for shorter exposure time during clinical trials. Thus, the staged TTC can be used for genotoxic compounds having genotoxicity data that are normally not suitable for a quantitative risk assessment (Müller et al., 2006.<sup>[14]</sup>). Apart from this various scientific authors have designed their proposals for the assessment and control of potential genotoxic impurities based on the various proceedings, findings and observations at various institutional and organization levels.

### Major breakthrough in dealing with genotoxic impurities

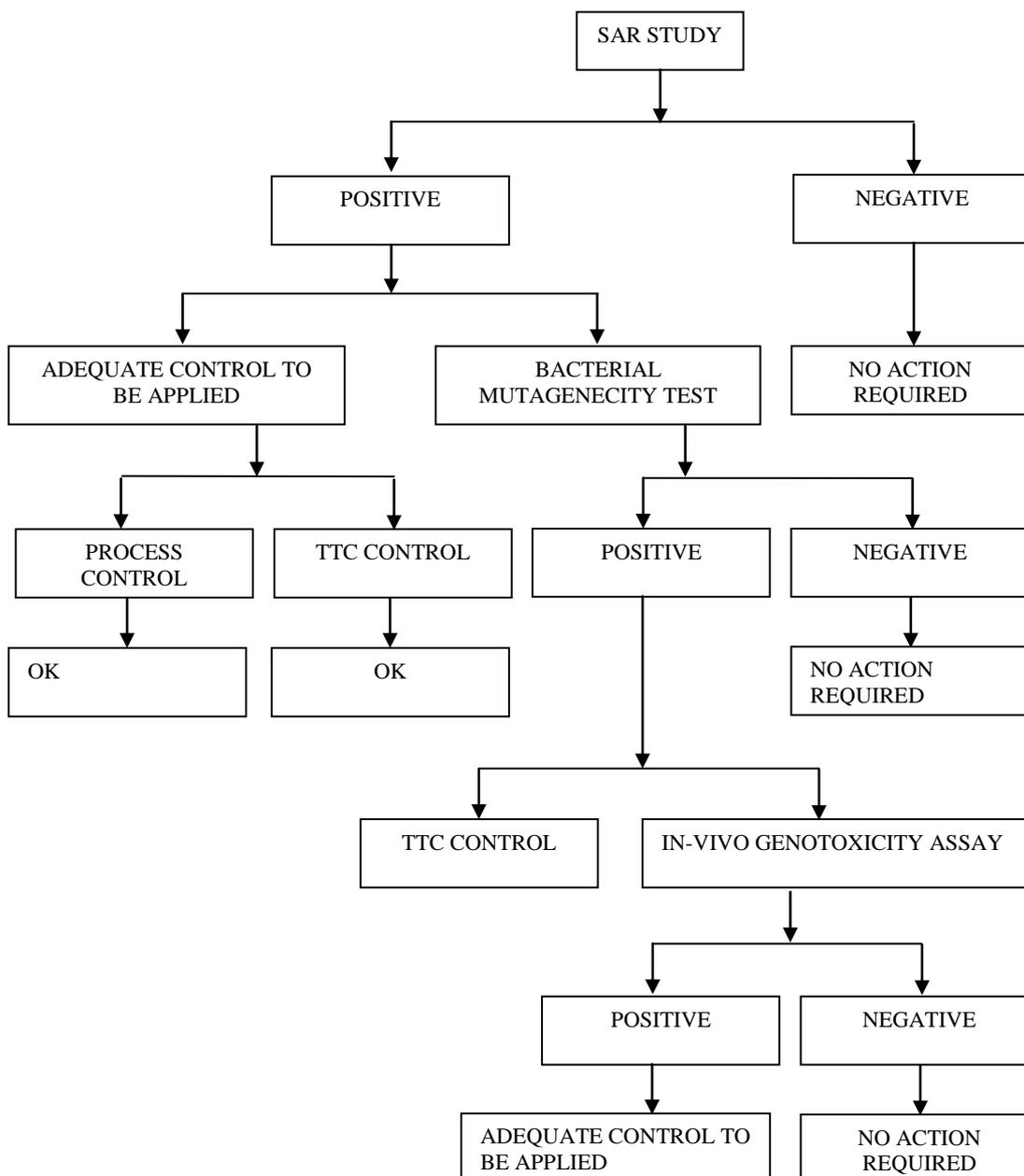
As an update of ICH M7,<sup>[15]</sup> a guideline for DNA-reactive impurities in pharmaceuticals developed by an ICH expert working group is major breakthrough in recent times for dealing with genotoxic impurities. The prime focus of the guidance is towards hazard assessment relating an initial analysis of actual and potential impurities by conducting database and literature searches for carcinogenicity and bacterial mutagenicity data. This assessment leads for classification of these impurities under Class 1, 2, 3, 4 or 5 according to following table.

Class	Definition	Proposed action for control
1	Known mutagenic carcinogens	Control at or below compound specific acceptable limit
2	Known mutagens with unknown carcinogenic potential (bacterial mutagenicity positive*, no rodent carcinogenicity data)	Control at or below acceptable limits (generic or adjusted TTC)
3	Alerting structure, unrelated to the structure of the API; no mutagenicity data.	Control at or below acceptable limits (generic or adjusted TTC) or do bacterial mutagenicity assay; If non-mutagenic= Class 5 If mutagenic=Class 2
4	Alerting structure, same alert in API which has been tested and is non-mutagenic	Treat as non-mutagenic impurity
5	No structural alerts, or alerting structure with	Treat as non-mutagenic

sufficient data to demonstrate lack of mutagenicity	impurity
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*\*Or other relevant positive mutagenicity data indicative of DNA-reactivity related induction of gene mutations (e.g., positive findings in in vivo gene mutation studies)*

If data for such a classification are not available, an assessment of Structure-Activity Relationships (SAR) that focuses on bacterial mutagenicity predictions is ideally performed. Brief sketch presented in the following confirms the logical progression using the above mechanism for identification, verification, monitoring and control.



### Methodologies employed for specific results

In absence of defined guideline for handling of Genotoxic impurities during synthesis of API, a chemist should demonstrate a unique skill set by harmonizing the chemistry, toxicology,

regulatory and analytical aspects related to minute level impurities. In this article efforts have been highlighted for devising a pragmatic framework for addressing genotoxic impurities in API.

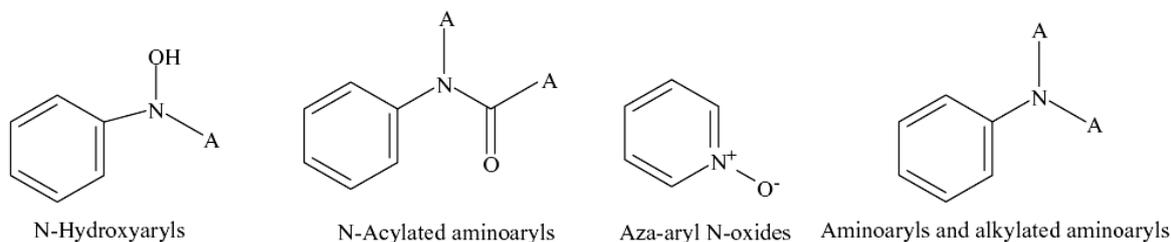
Amongst the latest publications, *Genotoxic Impurities: Strategies for Identification and Control*, Edited by Andrew Teasdale gives adequate discussion for the mechanism to deal with potential genotoxic impurities based on structural assessment. The structural assessment is based on the evaluation of the structural alert having susceptibility to possess the risk for genotoxicity. Structural alerts are defined as molecular functionalities (structural features) that are known to cause toxicity, and their presence in a molecular structure alerts the investigator to the potential toxicities of the test chemical. Nevertheless, the assumption that any impurity with a structural alert is potentially DNA-reactive and thus subject to the default TTC limit may often lead to unnecessary restrictive limits. From a resource and time table viewpoint of a new drug production, the experimental determination of genotoxicity is not feasible for millions of drug candidates in the pharmaceutical industry. Thus, compounds identified as potential hazards by *in silico* methods would be high priority candidates for confirmatory laboratory testing (Kruhlak et al., 2007<sup>[16]</sup>; Snodin, 2010<sup>[17]</sup>).

*In silico* toxicology is the application of computer technologies to analyze existing data, model, and predict the toxicological activity of a substance. In sequence, toxicologically based QSARs are mathematical equations used as a predictive technique to estimate the toxicity of new chemicals based upon a model of a training set of chemicals with known activity and a defined chemical space (Valerio, 2009<sup>[18]</sup>). Ashby and Tennant (1991) reported some correlations of electrophilicity with DNA reactivity (assessed by Ames-testing data) for about 300 chemicals and elucidated the concept of structural alerts for genotoxic activity in the 1980s/1990s.

The structural alerts identified to be associated with the risk for genotoxicity has been illustrated in subsequence.

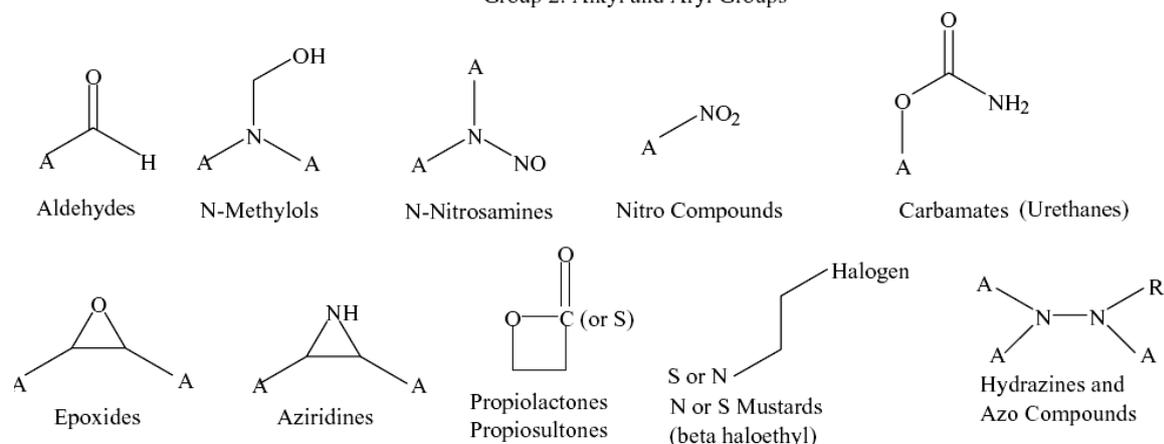
## Structural Alerts for Mutagenicity

## Group 1: Aromatic Groups

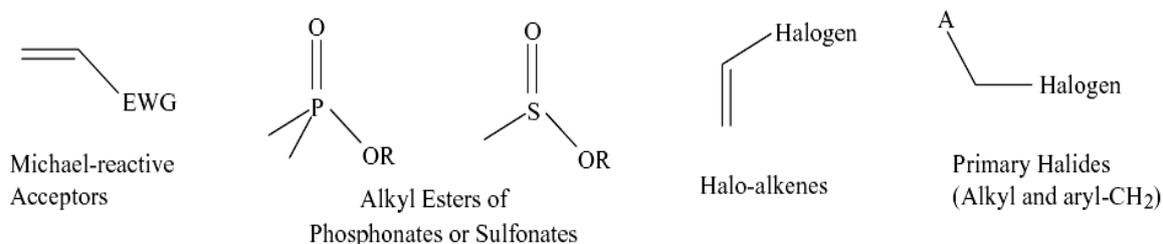


## Purines or Pyrimidines, Intercalators, PNAs or PNAHs

## Group 2: Alkyl and Aryl Groups



## Group 3: Heteroatomic Groups



Legend: A = Alkyl, Aryl, or H

Halogen = F, Cl, Br, I

EWG = Electron withdrawing group (CN, C=O, ester, etc)

**A simplified approach - dealing with genotoxic impurities**

The designing of the pathway is based on identification of the actual and potential impurities most likely to arise during synthesis, purification and storage of the API, based on sound scientific appraisal of chemical reactions involved in the synthesis.

Evaluation to be extended for impurities associated with raw materials that can contribute to the impurity profile of the API and possible degradation products. This discussion is limited with impurities that might reasonably be expected based on knowledge of the chemical reactions and conditions involved. Guided by existing genotoxicity data or the presence of structural alerts, potential genotoxic impurities are identified.

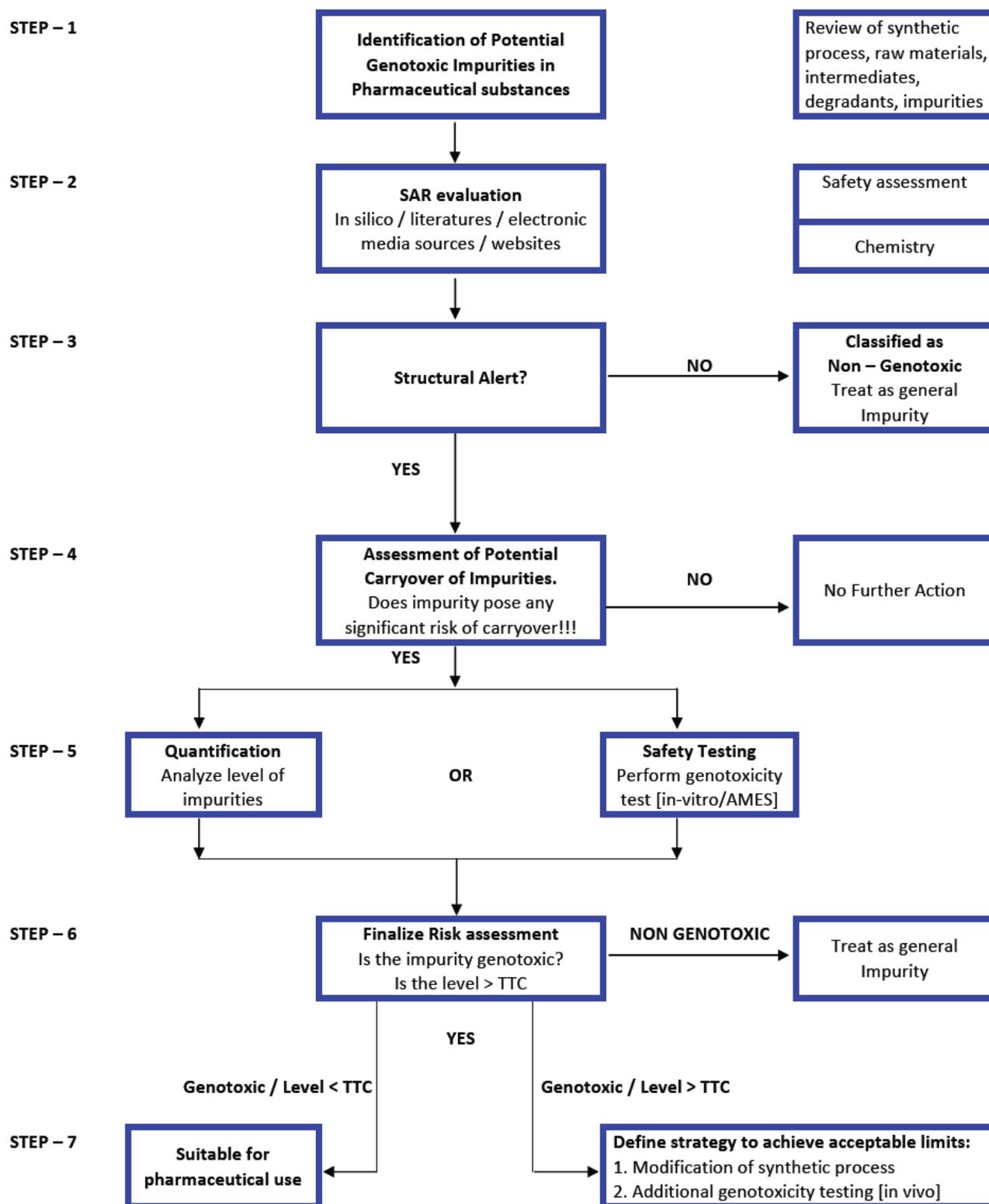
The genotoxic impurities with adequate experimental evidence for a threshold-related mechanism, is regulated using methods based on evaluation of Permissible Daily Intake. For genotoxic impurities with inadequate experimental evidence for a threshold-related mechanism, the existing research studies propose a limit called as "threshold of toxicological concern (TTC)." A TTC value of 1.5 g/day intake of a genotoxic impurity is considered to be associated with an acceptable risk. The concentration limit in parts per million (ppm) of genotoxic impurity permitted in API is the ratio of TTC ( $\mu\text{g}/\text{day}$ ) and daily dose (g/day).

When a potential impurity contains structural alerts, safety testing of the impurity, typically in a bacterial reverse mutation assay, can additionally be considered (Dobo et al. 2006,<sup>[19]</sup> Müller et al. 2006.<sup>[20]</sup>). The safety studies are essentially approached to ensure the confirmatory interpretation for the suspected genotoxic impurities.

Risk assessment for identified potential genotoxic impurity in API is concluded with risk characterization through any one of the approach or combination thereof for reducing potential cancer risk with patient exposed to genotoxic impurities.<sup>[21]</sup>

1. Modify synthesis or purification to minimize formation or removal of impurity.
2. Allowing maximum daily exposure target of 1.5  $\mu\text{g}$  per day of relevant impurity.
3. Characterize genotoxic and carcinogenic risk to support appropriate impurity specifications, either for higher or lower values.

The simplified pathway for assessment of genotoxic risk has been illustrated as flow chart in subsequence.



## CONCLUSION

The above pathway is designed taking into consideration the available published guidance to deal with genotoxic impurities. Efforts have been made to incorporate simplicity in the

approach for devising a pragmatic framework to address genotoxic impurities in API without compromising the output for the studies.

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