Journal of the Iranian Chemical Society, Vol. 2, No. 4, December 2005, pp. 244-267.

JOURNAL OF THE
Iranian
Chemical Society

The Identification of Toxicophores for the Prediction of Mutagenicity, Hepatotoxicity and Cardiotoxicity

G.H. Hakimelahi*[,a,b,c] and G.A. Khodarahmi[c]

[a]*TaiGen Biotechnology, 7F, 138 Shin Ming Rd., Neihu Dist., Taipei 114, Taiwan, ROC*

[b]*Institutes of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*

[c]*Department of Medicinal Chemistry, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran*

(Received 26 September2005, Accepted 24 October 2005)

The toxic properties of compounds can be related to chemical structures, and more specifically, to particular substructures, called toxicophores. Reliability and accuracy of mutagenicity, hepatotoxicity, or cardiotoxicity predictions may be achieved by identifying toxicophores. These predictions can guide the design of chemical libraries for hit and lead optimization. As such, a thorough molecular knowledge in drug-induced toxicity is required to aid the development of new therapeutic agents and prevent the release of potentially toxic drugs onto the market. The incorporation of these potentially reactive chemical moieties within new therapeutic agents should be limited. This, however, can not always be prevented, particularly when the structural feature responsible for toxicity is also responsible for the pharmacological efficacy.

In recent years, there has been strong pressure from society in general, and from government agencies in particular, to develop "general" prediction models in order to cope with the thousands of chemicals present in the environment for which experimental data are not available and likely will never exist. Therefore, one of the objectives of this work is to introduce methodologies capable of identifying the potential environmental health hazards of chemicals. This review also summarizes the evidence for reactive metabolite formation from chemical carcinogens, hepatotoxic drugs, and also describes how and where molecules bind and inhibit hERG K^+ channels, causing cardiotoxicity by QT prolongation. Such information should dramatically improve our understanding of drug-induced toxic reactions. Indeed, pharmaceutical companies are striving to improve the drug discovery and development process to identify, as early as possible, the risk of novel agents, or their metabolites, causing mutagenicity, hepatocellular toxicity, or QT interval prolongation and to make appropriate go/no-go decisions or modify their development programs accordingly.

Keywords: Toxicophore, Mutagenicity, Carcinogenicity, Hepatotoxicity, Cardiotoxicity, Metabolism

INTRODUCTION

Adverse drug reactions (ADRs) are significant health problems that contribute to the morbidity and mortality of patients. There are many different types of ADRs, affecting every organ system in the body. Drug-induced liver injury, carcinogenicity, and/or cardiac liability associated with the blockade of hERG (human ether a-go-go) are the most frequent reasons for the withdrawal of approved drugs from market [1,2].

The identification of chemical functionalities, and the knowledge of the conditions under which these functionalities could be changed constituted the basis of modern chemistry

*Corresponding author. E-mail: hosein@chem.sinica.edu.tw

and encompass the art of chemical synthesis as well as that of designing theoretical molecules that may possess desirable properties. Once these functionalities (*i.e.*, toxicophores) were identified by chemists, a further problem had to be resolved: identifying how the main structural backbone or other groups, including detoxifiers, affected the magnitude of the effect of the functionality. Toxicophores are substrates that indicate an increased potential for mutagenicity, whether this is caused by DNA reactivity or not. A toxicophore can represent a reactive substructure or a substructure that is prone to either metabolic activation or intercalation. Selection/validation criteria and the available knowledge of the chemistry and the metabolism of substructures combined with a statistical analysis based on sensitivity and *p*-values can lead to identification of novel toxicophores. Toxicophores can be applied to different phases of the drug optimization process, from supporting early risk and hazard assessments to guiding the design, synthesis, and ranking of chemicals.

The screening of drug candidates for mutagenicity is essential for drug approval [1,2] since mutagenic compounds (*i.e.*, aromatic amines, amides, hydroxylamines, nitroso and azo compounds) pose a toxic risk to humans [3]. Mutagenicity is the ability of a compound to cause DNA mutation. This effect can take place *via* several different mechanisms [2-4]. A compound's reactivity toward DNA can result in the creation of DNA adducts or base deletions [4]. Conversion of non-reactive compounds into DNA-reactive metabolites through enzyme-catalyzed metabolic activation, DNA distortion *via* intercalation, and reversible non-covalent fixation of DNA through π stacking interactions can disrupt enzymatic DNA repair and replication [4], increasing the chance of erroneous base replacements or deletions or insertion of base pairs.

In designing novel drugs, if the presence of a toxicophore for efficacy is a must, the corresponding detoxifying substructure should be identified [2c]. Detoxifying substructures can make toxicophore-containing compounds nonmutagenic because of their inhibition of mechanisms such as metabolic activation, DNA reactivity, or intercalation. This effect may be caused by steric hindrance or by a disruption of the required electronic charge distribution near the toxicophore. The aromatic nitro and aromatic amine toxicophores are specific examples of how toxicophore accuracy could be improved by the introduction of electron-withdrawing detoxifying substructures such as sulfonamide or trifluoromethyl groups [2].

The Chemical Carcinogenicity Research Information System (CCRIS) database [2d] contains scientifically evaluated toxicity test data for approximately 7000 compounds and mixtures, each identified with a CAS registry number and/or chemical name. Additional toxicity data are also available from other public toxicity databases [2e].

MUTAGENICITY

Ames *in vitro* Assay for Prediction of Mutagenicity

The Ames test is a simple *in vitro* assay designed to detect genetic damage caused by chemicals [5-7]. It uses a histidine-free medium with an engineered strain of bacteria that can proliferate into colonies after certain mutations restore their ability to synthesize histidine. A chemical is considered mutagenic (Ames test positive) when its addition to the assay causes a significant increase in the number of bacterial colonies compared to a control experiment. To mimic *in vivo* metabolism, a metabolic activation mixture containing liver microsomes (S9) can be added to this test. The reproducibility of Ames test data from the National Toxicology Program (NTP) was determined to be 85% (intra-assay agreement) [8]. Although the ability of *in vitro* genotoxicity and mutagenicity tests to predict *in vivo* toxicity has limits, Ames test results [5] as predictors for rodent carcinogenicity have been well established. As such, no other *in vitro* assay has been reported to better predict carcinogenicity [7,9-11].

Definition and Use of Toxicophores

Generally, we can recognize well-defined toxicophores from the literature [2] (aromatic nitro, aromatic amine, nitrosoamine, epoxide, aziridine, aromatic azo, nitrogen or sulfur mustard, α,β-unsaturated aldehydes, β-propiolactone, aliphatic halides and heteroatom bonded heteroatom derivatives) [9,10]. Toxicophores that are not defined or are ill defined in the literature may be judged by common knowledge, or one may suspect that new substructures are mutagenic. The α,β-unsaturated alkoxy toxicophore, detoxifying substructures and polycyclic planar system belong to a novel set of compounds, which deserve further study [10b]. Heteroatom-bonded heteroatom toxicophores, such as

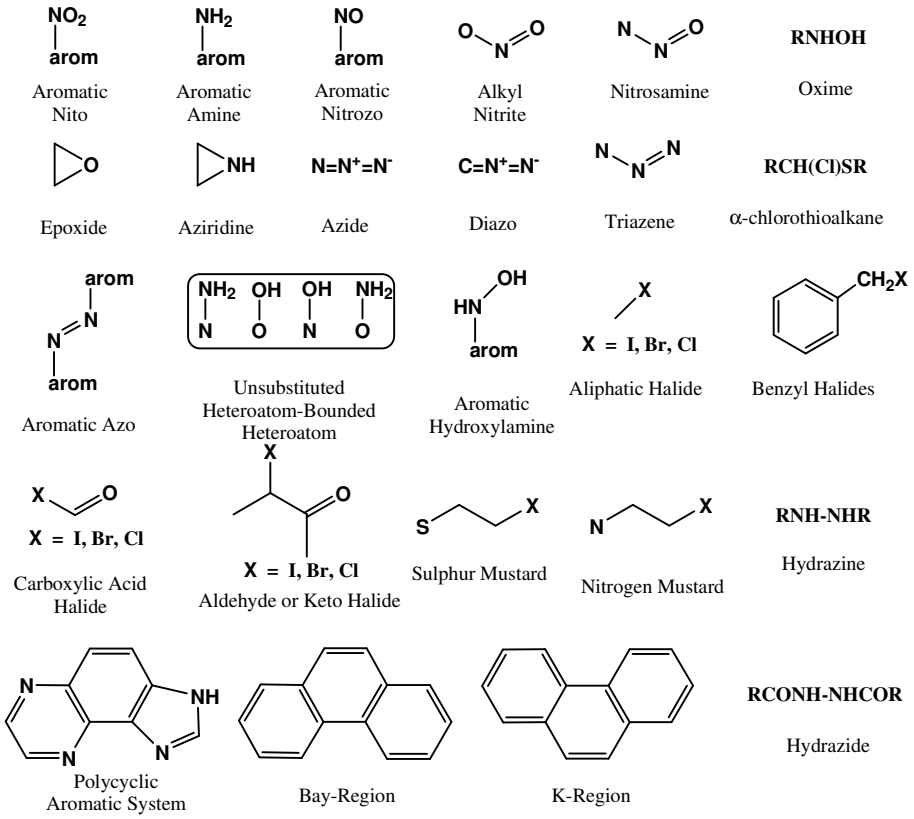


Fig. 1. Substructure representations and example compounds of approved and specific toxicophores.

hydroperoxide, oxime, diazohydroxyl, hydrazine, hydrazide, and hydroxylamine on aliphatic chains of chemicals may also be metabolized, *via* one electron oxidation or reduction step, into unstable radicals or more stable reactive substrates, which can react with DNA [2c,10]. Furan rings may also form epoxides as the entire furosemide molecule bonds to hepatic macromolecules and induce a hepatotoxic response [10b]. With aliphatic halide toxicophores, a variety of mutagens were detected. They possess chloride, bromide, or iodide substructures [2c]. No detoxifying substructures were identified for aliphatic halide toxicophores. Epoxides and aziridines are electrophilic, alkylating substructures that possess significant intrinsic reactivity [2]. The polycyclic aromatic toxicophores consist of a system of three or more fused aromatic rings [4]. Many compounds with polycyclic aromatic systems have been reported to intercalate into DNA. The sulfonate-bonded carbon atom, aromatic hydroxylamine substructure, azide, diazo, and triazene groups as well as an azo or an azoxy group that are located between two aromatic rings were also recognized as general toxicophores [2]. The mechanism of mutagenicity of compounds containing the aromatic amine, nitro, nitroso, or hydroxylamine moieties can be explained by partially overlapping metabolic activation pathways [1-4]. It is obvious that the approved toxicophores can aid in the prediction of mutagenicity in early risk assessment as well as in the design of chemical libraries for hit and lead optimization.

Figure 1 shows substructure representation of approved and specific toxicophores.

Approved additional toxicophores and their mode of action are summarized in Fig. 2. The reactivity of some of these compounds possessing an electrophilic carbon atom explains their DNA reactivity, and thus their mutagenicity. Compounds having polycyclic planar systems are, generally, mutagenic because they are capable of acting as intercalating agents. However, the mechanisms of their metabolic activation cannot

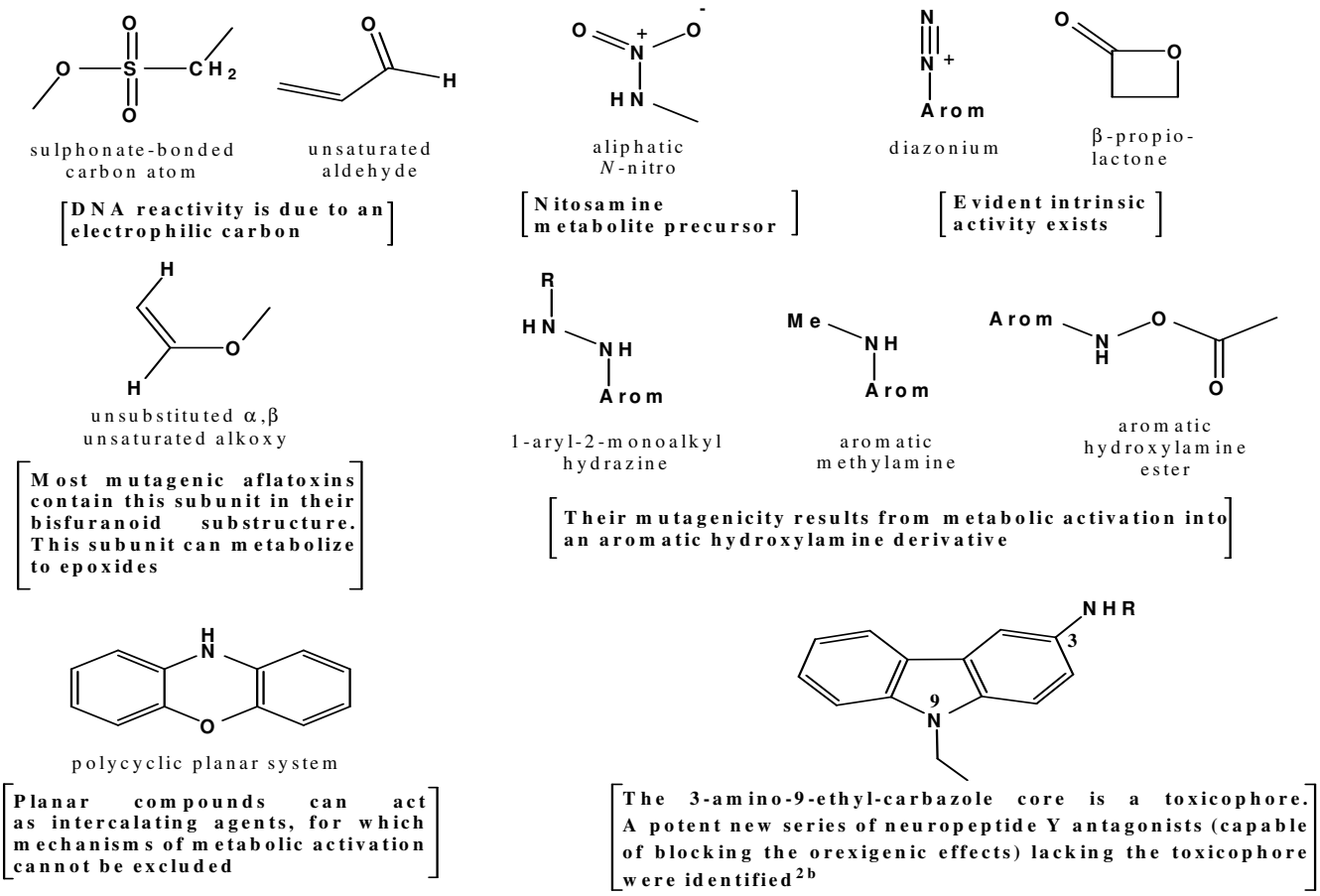


Fig. 2. Approved additional toxicophores and their modes of action in biological systems.

be excluded.

Mechanisms of Mutagenicity of Compounds Containing Aromatic Amine, Nitro, Nitroso or Hydroxylamine by Overlapping Metabolic Activation Pathways

An aromatic nitro group requires enzymatic reduction, catalyzed by both cytosolic and microsomal enzymes, to form an aromatic hydroxylamine intermediate. However, the reduction of an aromatic nitroso group is probably non-enzymatic (in general Fig. 3a; more specifically Fig. 3b) [12-14]. On the other hand, an aromatic amine requires enzymatic oxidation by liver enzymes, such as cytochrome P450, to form the same aromatic hydroxylamine intermediate [9,13]. Then, aromatic hydroxylamine forms electrophilic intermediates by *O*-acylation, *O*-sulfation, or *O*-protonation (*i.e.*, nitronium ion intermediate) that covalently bind to DNA and as a result causes mutation [14].

In general, chemicals can undergo enzyme-catalyzed bioactivation reactions within cellular systems with the formation of reactive chemical species. These active metabolites can either react with DNA or lead to thiol depletion [15a], known as reversible protein modification or glutathionylation, respectively [15,16]. They can also cause irreversible protein adduct formation and subsequent protein damage [17].

Detoxifying Substructures

The toxicity of the aromatic nitro substructure can be confirmed by the identification of detoxifying substructures in

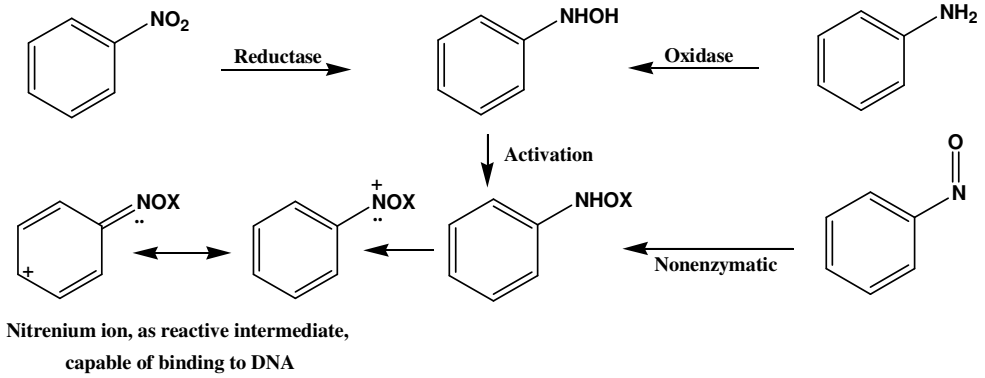


Fig. 3a. Biotransformations of aromatic nitro, aromatic amine and aromatic nitroso compounds to their reactive metabolite, nitrenium ion intermediate.

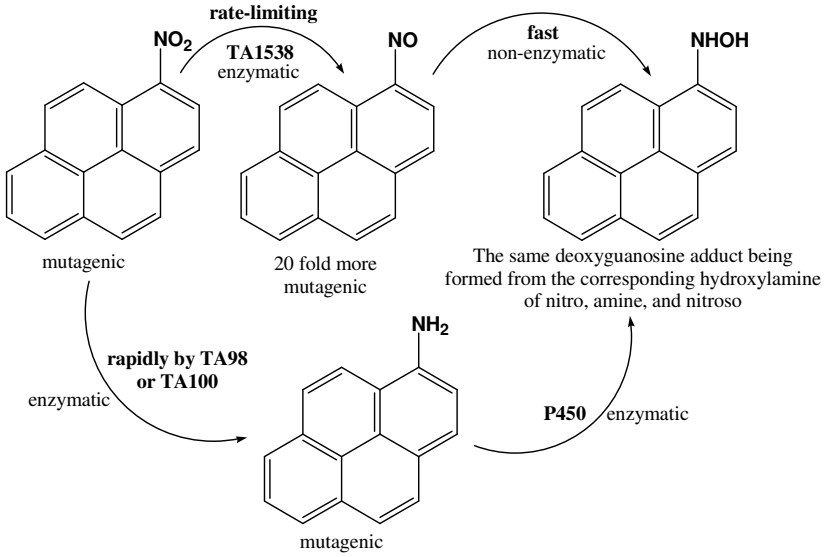


Fig. 3b. Mutagenicity of 1-nitropyrene and 1-aminopyrene and their metabolites.

ortho, *meta*, and/or *para* position(s) with respect to this toxicophore. Detoxifying substructures include CF_3, SO_2NH, SO_2OH, and the arylsulfonyl derivatives (strong electron withdrawing groups) that are incorporated into the specific aromatic nitro toxicophore [2].

As illustrated in Fig. 4, detoxifying substructures can render toxicophore-containing compounds nonmutagenic because of their inhibition of mechanisms such as metabolic activation, DNA reactivity, or intercalation. This effect may be caused by steric hindrance [12] or by a disruption of the required electronic charge distribution near the toxicophore [2]. Likewise, a sulfonic acid group at the appropriate position in a molecule-containing aromatic amine can also inhibit its undesirable metabolic activation [13]. Detoxification is due to the lack of availability of the amine's lone pair of electrons. This inhibits oxidation of aromatic amines by P450 liver enzymes, consequently lowering both mutagenicity and liver toxicity, as expected.

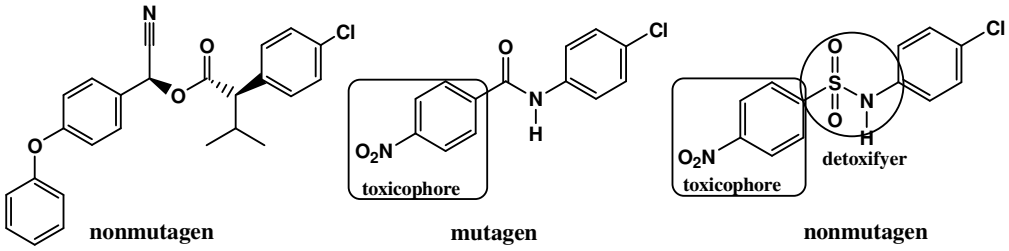
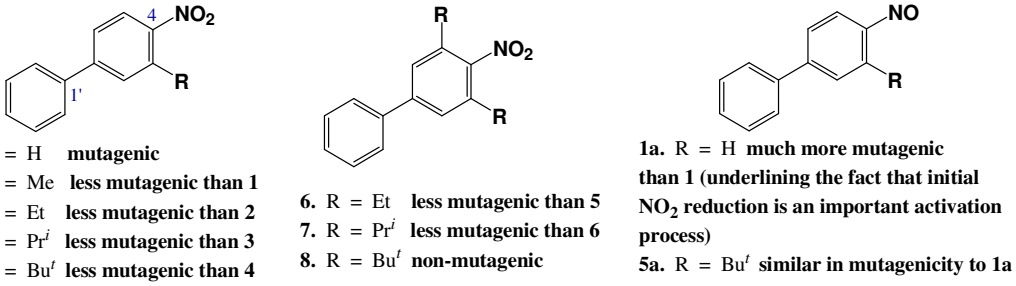


Fig. 4. Detoxifiers disturb the electronic charge distribution near the toxicophore.



1. R = H **mutagenic**
2. R = Me **less mutagenic than 1**
3. R = Et **less mutagenic than 2**
4. R = Pr^i **less mutagenic than 3**
5. R = Bu^t **less mutagenic than 4**

6. R = Et **less mutagenic than 5**
7. R = Pr^i **less mutagenic than 6**
8. R = Bu^t **non-mutagenic**

1a. R = H **much more mutagenic than 1 (underlining the fact that initial NO_2 reduction is an important activation process)**
5a. R = Bu^t **similar in mutagenicity to 1a**

Fig. 5. Effect of bulky alkyl substituents on the mutagenic activity of 4-nitrobiphenyl in *Salmonella typhimurium*.

The classification of mutagenicity is as follows: if a compound does not contain any toxicophore, it is classified as a nonmutagen; if a compound contains a toxicophore, like the aromatic nitro group, it is classified as a mutagen; if a compound contains both a toxicophore (*i.e.*, aromatic nitro group) and a detoxifying group (*i.e.*, CF_3, sulfonamide, sulfonic acid, arylsulfonyl, and possibly COOH), it is classified as a nonmutagen (Fig. 4).

Effect of Steric Hindrance on Mutagenicity

Besides causing acute and chronic toxicity, nitro-aromatics are mutagenic and many of them are carcinogenic. Metabolic activation of NO_2 to NO to NHOH to N^+OR (nitrenium electrophilic species) by mammalian or bacterial enzymes renders it capable of reacting with bionucleophiles such as proteins (*i.e.*, hemoglobin) or DNA, thereby disturbing replication and inducing mutation [12]. The mutagenic potential of nitro-aromatics through adduct formation depends on their structural features, such as the position and orientation of NO_2 with respect to the ring, the ability of the aromatic ring (planarity) to stabilize the ultimate N^+OR, and the influence of additional substituents. As such, the steric influence of alkyl substituents on the reduction of mutagenicity of NO_2-substituted aromatics is important (Fig. 5). *O*-Alkyl nitro-aromatics are less mutagenic, yet *O*-alkyl aromatic NH_2, NO, and NHOH are often more mutagenic [9,13,14]. This could be due to steric inhibition of the nitroreductase system. The initial reduction of NO_2 is a rate-limiting step in nitro-arene activation [15,16].

As illustrated in Fig. 5, alkyl substituents *ortho* to the nitro group of 4-nitrobiphenyl reduce or eliminate the mutagenicity (*i.e.*, NO_2 has no planarity in **8**). This effect was not observed for the nitroso compounds (compare **1a** with **5a**). Thus, the mutagenicity of this class of compounds increases if compounds are more easily reduced.

From Mutagenic to Nonmutagenic Nitroarenes. Effect of Bulky Alkyl Substituents Far Away from the Nitro Group

Since regioselective introduction of bulky alkyl substituents *ortho* to a nitro group is often difficult, and the chemical properties of the nitro functionality are changed,

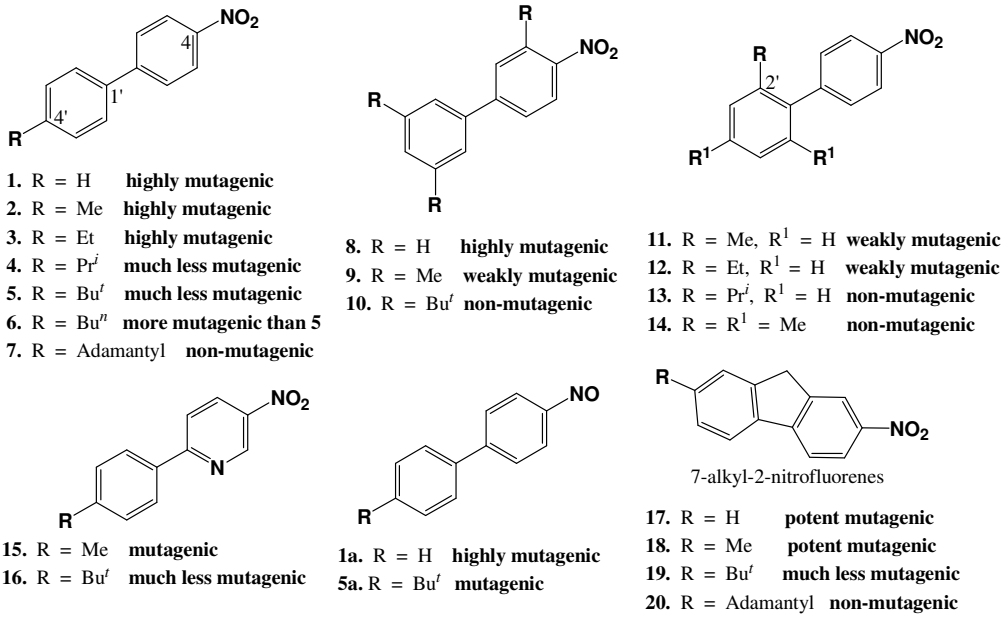


Fig. 6. Effect of bulky alkyl substituents far away from the nitro group.

electronically and/or sterically, by a bulky neighbor, this strategy to reduce mutagenicity may be of limited applicability. As such, bulky groups are introduced far away from NO_2 to examine the effect of the alkyl groups in the positions that neither directly affect the NO_2 nor the respective π-system, but change the shape of the molecule from flat to three-dimensional [16].

Research has shown [12,16] that alkyl groups far away from NO_2 exhibit a size-dependent effect on the mutagenicity of nitro-aromatics and nitroso-aromatics (Fig. 6). Small groups do not change the mutagenicity in the absence of the metabolic activator S9, but rather enhance it in the presence of S9 due to an electronic effect. Mutagenic responses, however, are reduced or even inhibited by the introduction of bulky groups, such as isopropyl, *tert*-butyl, or adamantyl, due to the steric effect. As the result of mutation, mutagenic compounds, at any dose level, could cause histidine-deficient bacteria to produce histidine, at least twice that of the solvent control. The mutagenicity (log revertant/nanomoles) was calculated by the linear slope of the dose-response curve using linear regression analysis [5-7,12,16].

In contrast to the alkyl-substitution at the *ortho* or 2'-position of the 4-nitrobiphenyl unit, the *para* alkyl groups at the 4'-position are far away from the nitro group and neither affect the charge delocalization nor the nitro group orientation. The binding interference of bulky *para* alkyls with the nitroreductase enzyme, however, cannot be excluded; yet it is unlikely that this effect alone is responsible for the strong reduction in mutagenicity.

Further evidence of the involvement of other effects arises from the related nitroso compounds, in which a more difficult first reduction step of the mutagenic activation is bypassed. It should be noted that the ability of metabolization of the toxicophores, either by oxidation or reduction is dependent on the size of the aromatic rings. The ease of metabolization can also be due to substituents on the aromatic rings.

It has been demonstrated that a flat aromatic system, capable of intercalating DNA, reacts as a frame-shift mutagen [15c]. Nitrated planar fluorenes **17** and **18** are much more mutagenic than the less planar nitrobiphenyls **1**-**3** (with dihedral angles of 40 degrees). By the insertion of the bulky groups, molecular planarity is lost (see compounds **4**-**7**, **19**, and **20**). Thus, they cannot intercalate well into the DNA, which results in much less mutagenicity. Therefore, reduction in mutagenicity is due only to a modification of the molecular shape while the chemical properties of the nitro group and the

degree of aromaticity remain essentially unaffected. Therefore, all factors must be taken into account, rather than a single factor alone; in fact, a single factor may originate from other factors.

Predicted and Experimental Mutagenicities

Although nitro-aromatics and related compounds such as aromatic amines, hydroxylamines, and nitroso compounds are important products and intermediates in industry and research, currently their use is restricted because of their well known mutagenic and carcinogenic properties [3]. The same is true for azo compounds that are widely used as dyes, since these compounds can be metabolically cleaved into the corresponding amines [16]. The problem of toxicity in drug development becomes of ever greater importance as more sophisticated methods of epidemiology uncover more subtle forms of toxicity. Concerns have shifted from acute toxicity to that resulting from long-term exposure to drugs and/or their metabolic products. Avoiding such potential toxicity, when identified early in drug development, can avert needless expense and loss of time.

Indeed, it is time to move from "descriptive toxicology" to "predictive toxicology" [15]. As such, in this section, a rather general Quantitative Structure Activity Relationship (QSAR) analysis for correlating the mutagenicity of aromatic and heteroaromatic nitro compounds is described. By use of the following equations [15], considering only hydrophobicity and electronic factors, the mutagenic activities of nitro-aromatics in Salmonella strains TA98 and TA100 in the absence of the S9 metabolic activator were determined:

$$\log TA98 = 0.65 \log P - 2.90 \log (\beta 10^{\log p + 1}) - 1.38 E_{LUMO} - 4.15; (\log \beta = -5.48)$$

$$\log TA100 = 1.20 \log P - 3.40 \log(\beta 10^{\log p + 1)} - 2.05 E_{LUMO} - 6.39; (\log \beta = -5.70)$$

Mutagenicity increases with hydrophobicity until ($\log P_{max} < 5$) and then drops rapidly for more hydrophobic compounds ($\log P_{max} > 6$). According to the electronic factors, a linear dependence between mutagenicity and E_{LUMO} was found. This means that the ease of the initial reduction of the NO_2 rate-limiting step in nitroarene activation plays the main role in mutagenicity [9,12-14]. Steric effects, however, have not been considered in their equations. The log*P* increases with the number and size of the alkyl substituents; the lowest-unoccupied molecular orbital (LUMO) energies are raised (+I effect) with alkyl substituents. Thus, based on the prediction, mutagenicity is expected to increase with alkyl substituents; yet, experimentally, mutagenicity decreases with the number of alkyl groups (Figs. 5 and 6). These deviations point to a steric effect, which is not incorporated in the equations. We believe that hydrophobicity increases mutagenicity; yet the steric factor decreases mutagenicity because it interferes with the planarity of the system and, in turn, the +I effect of alkyls cannot enhance the ease of the initial reduction of the NO_2. Therefore, we observe the adverse hydrophobic and steric effects of alkyls. Hydrophilic sulfonic acid substituents were shown to suppress the mutagenic properties. Decreased hydrophobicity may slow down cellular penetration and may weaken the binding of the mutagen to the activating enzyme. If NO_2 is oriented perpendicular or nearly perpendicular to the aromatic plane, the polycyclic nitro-aromatics exhibit weak or no mutagenicity. This may be due to their inability to fit into the active site of the nitroreductase. In contrast to the different orientation of the NO_2 group, the NO group is oriented coplanar to the aromatic plane, and the NO reduction is not inhibited (Figs. 5 and 6). Consequently, compounds with nearly orthogonal phenyl rings are not mutagenic. The weaker π-overlapping destabilizes the intermediate nitrenium ions (N^+OR), reducing their lifetime.

Compounds with adamantyl and *tert*-butyl ($\log P > 7$) are most lipophilic. They show no mutagenicity (Fig. 6). This is consistent with the calculation. The impact of the alkyl group (+I effect) on LUMO energies is strong (ease of NO_2 reduction causes mutagenicity, Fig. 3), but the compounds are non-mutagenic (Fig. 6). As expected, the conversion of phenyl to pyridine lowers the LUMO energies. Therefore, the compounds are expected to be less mutagenic, but little change in mutagenicity of the related analogs was observed (compare compound **2** with **15** in Fig. 6). In contrast to predictions, the overall experimental trend exhibits a decrease in mutagenicity with a decrease in the number and size of the substituents. Good agreement between predicted and experimental results exists for non-substituted compounds.

The number of deviations increases with the steric

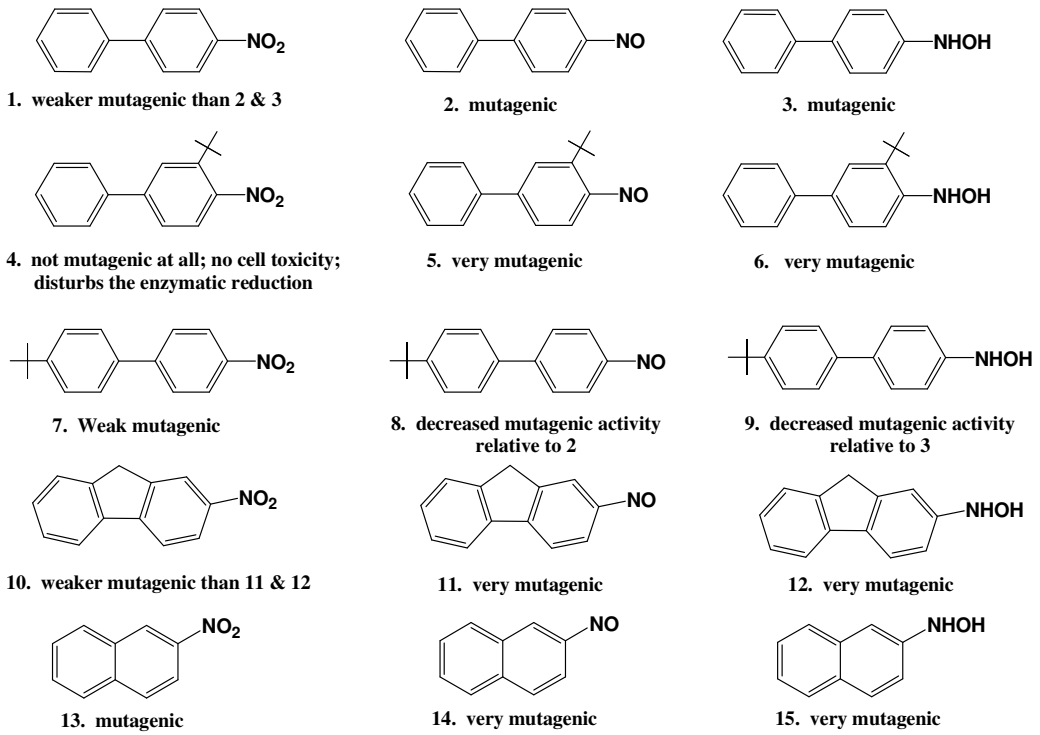


Fig. 7. Some mutagenic compounds and their metabolites.

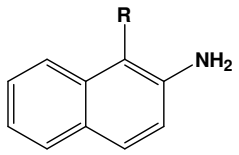
demands of the substituents. As such, in addition to lipophilicity and electronic factors, the steric factors must also be considered in a new mathematical formula for the prediction of mutagenicity. Steric effects and other structural factors, which have not been built into the equations, must be responsible for some observed variation in activity. The order of lipophilicity is Me < Et < Pr^i < Bu^t < Bu^n; yet the order of mutagenicity is Bu^t << Pr^i < Bu^n < Et = Me. No correlation between lipid solubility and mutagenicity is observed experimentally (Fig. 6). The chemical structures of some mutagenic compounds and their metabolites are shown in Fig. 7.

Aromatic amines are also widespread chemicals with considerable industrial and environmental importance. For example, aromatic amine-derived dyes are synthetic organic colorants widely used in the textile, paper, leather, plastics, cosmetics, drugs, and food industries. Moreover, several types of aromatic amines are generated during cooking [9]. Empirical correlations were developed for aromatic amines and tested in Salmonella strains TA98 and TA100 with metabolic activation:

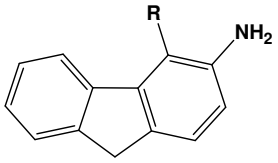
$$\log TA98 = 1.08 \log P + 1.28\, E_{HOMO} - 0.73\, E_{LUMO} + 1.46\, I_L + 7.20$$

$$\log TA100 = 0.92 \log P + 1.17\, E_{HOMO} - 1.18\, E_{LUMO} + 7.35$$

Accordingly, hydrophobicity (log$P$) was found to be related to mutagenicity [9a]. Electronic factors (E_{HOMO} and E_{LUMO}) have a smaller influence on mutagenicity (17% for TA100 and 4% for TA98). Nonetheless, a positive correlation exists between mutagenic activity and E_{HOMO} because of the ease of oxidation to the ultimate mutagen [9-11]. Indeed, from the above empirical equations for the prediction of mutagenicity, it is known that, for the determination of the mutagenicity of aromatic amines and nitro compounds, the Ames test is generally correlated with electronic energies and especially hydrophobicity. The introduction of very

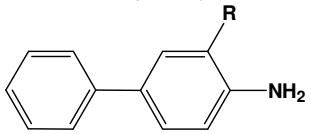


1. R = H **TA100, mutate; TA98, no mutation (+S9)**
2. R = Et **slightly more mutation in both strains**
3. R = Pr^i **TA98, weak mutation; TA100, mutation**
4. R = Bu^n **mutagenic in both strains**
5. R = Bu^t **no mutagenic in both strains**

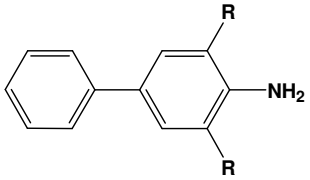


6. R = H **mutagenic (+S9)**
7. R = Et **more mutagenic**
8. R = Pr^i **even more mutagenic than 6 & 7**
9. R = Bu^n **20 times more mutagenic than 6**
10. R = Bu^t **lowest mutagenicity**

As expected, mutagenicity is not observed without metabolic activation (without S9), but observed in the presence of metabolic activator S9 (+S9). With increasing log *P*-values mutagenicity in general increases. In the case of the *tert*-butyl substituted compounds, however, the sterically crowded substituent leads to a reduction or elimination of mutagenicity.



11. R = H **mutagenic (+S9)**
12. R = Et **mutagenic**
13. R = Pr^i **slightly less mutagenic**
14. R = Bu^n **lower mutagenicity**
15. R = Bu^t **lowest mutagenicity**



16. R = Me **9 times lower mutagenic than 11 (+S9)**
17. R = Et **no mutagenicity**
18. R = Pr^i **a non-mutagenic compound**

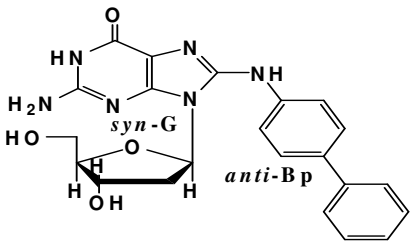
Double-alkylation at *ortho*-positions of the amino function with bulky alkyl groups causes an enhanced decrease in mutagenicity. We can also keep cytotoxicity; yet remove mutagenicity as observed in **10**. Compound **8** is very mutagenic with cytotoxicity at 500 ug/plate. Compound **10** is not mutagenic with the same value of cytotoxicity. As a result, we may be able to design and synthesize non-mutagenic anticancer compounds.

Fig. 8. Mutagenicity of some aromatic amines in the presence of a metabolic activator.

hydrophobic sulfonic acid substituents, for example, was shown to suppress the carcinogenic and mutagenic properties of benzidine, aminobiphenyl, and *N,N*-dimethylaminobenzene [16]. The decreased hydrophobicity, however, may slow down the penetration of the reactive intermediates through the cell wall or may weaken the binding of the mutagen to the activating enzyme.

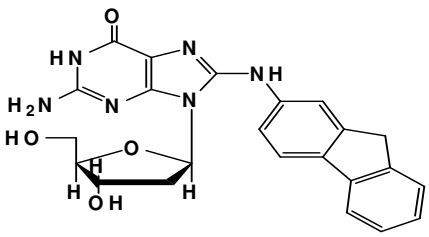
In some cases, strong hydrophobicity may dominate the steric demand, as observed with the higher mutagenicity of 1-*n*-butyl-2-aminofluorene relative to its ethyl or isopropyl analogues (Fig. 8). The reason for the high mutagenicity of methyl- and ethyl-substituted arylamines may be the increase of log*P* values relative to the steric demand, the stabilization of the ultimate nitrenium ion by the +I effect, and the probable oxidation of methyl and ethyl to the corresponding benzyl alcohols, which are converted by sulfation or phosphation into the ultimate mutagens that react with DNA [15]. Aromatic amines bearing alkyl groups far away from the amino group are also expected to exhibit mutagenicity similar to that of aniline, if their substituted alkyls distort the ring. Finally, the dialkylated NH_2 of aromatic amines reduces the mutagenicity drastically because of the inability to produce nitrenium ions *in vivo* (see compounds **16**-**18** in Fig. 8).

Aromatic amines will first be oxidized by cytochrome P450 to hydroxylamine, and then further *O*-acylation may generate a nitrenium ion to react with bionucleophiles, with DNA as the main target. The Ames test [5] has been performed with *Salmonella typhimurium* TA98 and TA100 in the presence and absence of the S9 mix [6], and calculations using the QSAR equations of Debnath *et al.* [21] are in



(*syn*)dG-C(8)-Aminobiphenyl

Normal *anti*-conformation (major) and abnormal *syn-conformation* (minor) were evidenced by NMR. Arylamine moiety in *anti* was predicted in the major groove of the relatively undistorted helix. Carcinogenicity may be due to the lack of recognition of the biphenyl in the *anti*-position by mammalian repair enzymes. In the minor conformer *syn*, on the other hand, aryl-amine may be stacked with neighboring bases or situated in the minor groove, as observed at physiological temperature.



(*syn*)dG-C(8)-Aminofluorene

In the case of fluorene, the situation was found t similar. The 9-mer, with a higher G:C content, both conformations. The 15-mer has a hi population of the mutagenic minor *syn*. As a re the sequence, nature of the carcinogen, and lengt the DNA duplex will affect the distribution *syn:anti* conformers in duplexes with these type modifications.

Fig. 9. DNA-adducts of aminobiphenyl and aminofluorene as metabolic intermediates.

agreement for unsubstituted compounds, and are nearly consistent for small alkyl functionalities (*i.e.*, 1-alkyl-2-aminofluorenes and 1-alkyl-2-aminonaphthalenes).

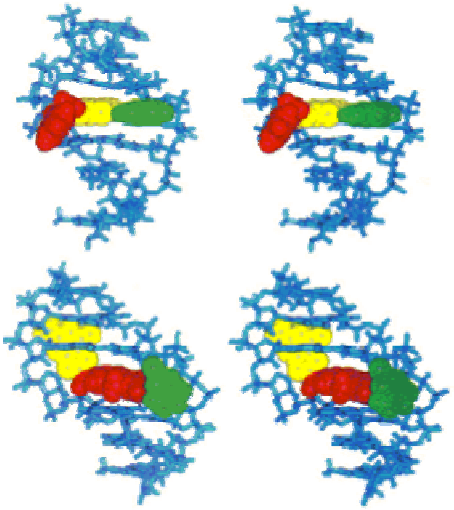
The largest difference between theory and experiment was found for bulky group-containing molecules such as *tert*-butyl substituted compounds. Unlike the theoretical prediction, it was found experimentally that the bulky substitution reduces or eliminates mutagenicity. Therefore, it is essential to introduce steric parameters to mathematical equations for the correct prediction of mutagenicity of proposed compounds.

To date, most efforts are directed toward the development of carcinogenicity prediction models [9]. Although some rule-based expert approaches, such as the OncoLogic system [9e], have attempted semiquantitative estimations of carcinogenic potency based on mechanistic considerations, these efforts have not attempted to incorporate any type of quantitative modeling or QSAR analysis [9e].

The Role of Planarity of Molecules in Mutation

Chemical carcinogens that covalently bind to DNA may result in base substitution, frame-shift mutations, deletions or gene rearrangements within the cell. Adducts to nuclear DNA are biomarkers for evaluating the biologically effective dose, and more realistically indicate an enhanced risk of developing a mutation-related disease than the external exposure dose method. The localization and accumulation of these promutagenic lesions in different organs are the composite result of several factors, including toxicokinetics, local and distant metabolism, efficiency and fidelity of DNA repair, and cell proliferation rate [19a]. The last factor will affect not only the dilution of DNA adducts but also the possible evolution towards either destructive processes, such as emphysema or cardiomyopathies, or proliferative processes, such as benign or malignant tumors at various sites. They also include heart tumors affecting fetal myocytes after transplacental exposure to DNA-binding agents, blood vessel tumors and atherosclerotic plaques. Mutations in mitochondrial DNA increase the number of oxidative phosphorylation-defective cells, especially in energy-requiring tissues such as brain, heart, and skeletal muscle, thereby playing an important role in aging and a variety of chronic degenerative diseases [19a]. A decreased formation of DNA adducts is an indicator of a reduced risk of developing the associated disease. Therefore, avoiding exposure to adduct-forming agents (*i.e.*, smoke, chemicals containing toxicophores) is important.

2-Aminofluorene, an aromatic amine, and the related *N*-acetyl-2-aminofluorene have been extensively studied as model chemical carcinogens since the discovery of their ability to induce a variety of tumors in experimental animals during toxicity testing as potential insecticides [19b]. C(8)-Deoxyguanosine adducts, as metabolic intermediates, were detected when unsubstituted 4-amino- or 4-nitro-biphenyl and 2-amino- or 2-nitro-fluorene were used *in vivo* and *in vitro* (Fig. 9) [17-19]. C8-Substitution can change the ratio of *syn*



External aminofluorene (AF, red) conformation (*Anti*). Fluorene ring (red) in the major roove with modified G (yellow) and complementary C (green). In (AF-G6-C15) Watson-Crick pair is consistent with the NMR data.

Inserted aminofluorene (AF, red) conformation (*Syn*). Fluorene ring (red) stacks between adjacent base pair; (G: yellow in minor groove, C: green) with denaturation of (AF-G6-C15). Watson-Crick pair is consistent with the NMR data.

Fig. 10. The energy-minimized conformation of the aminofluorene (AF)-modified seven bases of the duplex: d(A3-C4-A5-{AF-G6}-G7-A8-A9)d(T12-T13-C14-[C15]-T16-G17-G18).

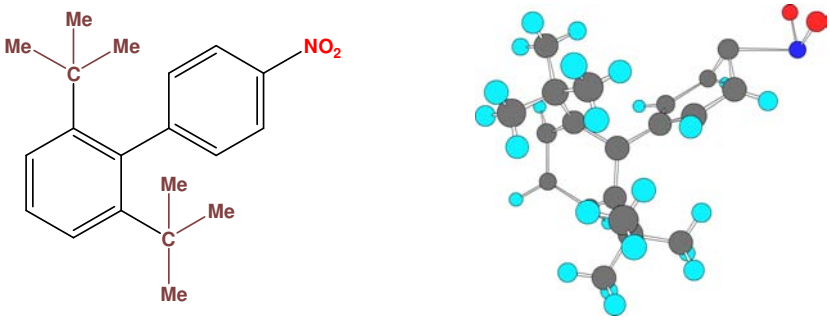


Fig. 11. Three-dimensional structure exhibits steric crowding, which inhibits the approach towards DNA.

and *anti* conformers [18], resulting in DNA conformational heterogenicity. The adduct conformation within the DNA plays an important role in determining mutation rates. In the "B-type" conformer, the aromatic moiety of the mutagen is located in the major or minor groove of the DNA helix and the overall structure is relatively undisturbed, but in the "stacked" conformer, the adduct moiety is inserted within the helix and is associated with an increased mutation frequency per adduct [19,20]. These DNA adducts probably destabilize the mutagenic stacked conformer by interfering with the intercalation into the helix (changing the ratio of "B-type" and "stacked"). Note that the mutagenic amino- or nitro-aromatics can be rendered nonmutagenic simply by the introduction of bulky groups without changing the chemical properties significantly [16].

Since significant local perturbation, observed in the minor conformer (*syn*), could provide a mechanism for mutations, the structural differences in the toxicophore fragments have a direct influence on the conformational heterogeneity, which may play a significant role in carcinogenesis (Fig. 10).

Alkyl substituted 4-nitrobiphenyls (Fig. 11) as well as 4-aminobiphenyls or 2-aminofluorenes are expected to have different steric behaviors in the presence of the double-stranded DNA. Reduction in mutagenicity of bulky-substituted analogues could be due to their incapability to react with DNA or intercalate into DNA.

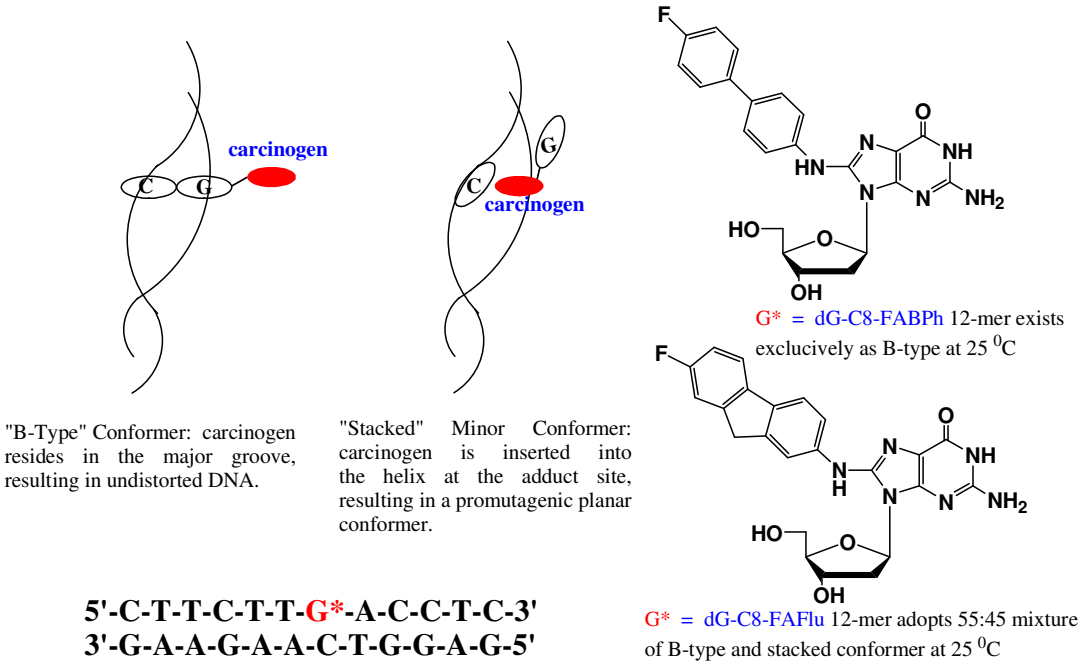


Fig. 12. Carcinogen-modified DNA duplex. ^{19}F NMR evidence of Conformational heterogeneity.

One- and two-dimensional ^{19}F NMR spectroscopy was also used to investigate the conformational heterogeneity of arylamine-modified DNA duplexes [20]. The ^{19}F NMR spectrum of *N*-(deoxyguanosin-8-yl)-4'-fluoro-4-aminobiphenyl (dG-C8-FABPh) showed a single peak, while that of *N*-(deoxyguanosin-8-yl)-7-fluoro-2-aminofluorene (dG-C8-FAFlu) revealed two prominent signals with a 55:45 ratio. The results are illustrated in Fig. 12.

In the "B-type" conformer, the overall structure is relatively undisturbed, but in the "stacked" conformer, the adduct moiety is inserted into the helix. This is associated with an increased frequency of mutation, as expected [19,20].

HEPATOTOXICITY

Idiosyncratic Toxicity: The Role of Toxicophores and Bioactivation

Drug toxicity is a major complication of drug therapy and drug development. Common ADRs include hepatotoxicity, severe cutaneous reactions, anaphylaxis, tissue damage, cellular stress, cardiotoxicity, and blood dyscrasias [22-32]. The pharmaceutical industry spends more than US$ 20 billion on drug discovery and development per year, with about one fifth of this total put into screening assays and toxicity testing. Therefore, a thorough molecular knowledge concerning the early events in drug-induced toxicity is required to aid in the decision-making processes during the development of new therapeutic agents. One of the main problems involves reactive metabolites. Electrophilic compounds resulting from the parent drug or an increment in the cellular production of reactive oxygen or nitrogen species (*i.e.*, HO-radicals, superoxide or peroxynitrite) can cause tissue damage, modify cellular proteins, or the oxidation of redox-sensitive thiols or amines within proteins. These can cause apoptosis, necrosis, perturbation of the host's immune system (*i.e.*, drug allergy), or modification of DNA, leading to carcinogenicity or teratogenicity [27].

The biotransformation of lipophilic compounds into water-soluble derivatives that are more readily excreted is a physiological role of the liver. Cytochrome P450 enzymes play a primary role in the metabolism of an incredibly diverse range of foreign compounds, including therapeutic agents [33-

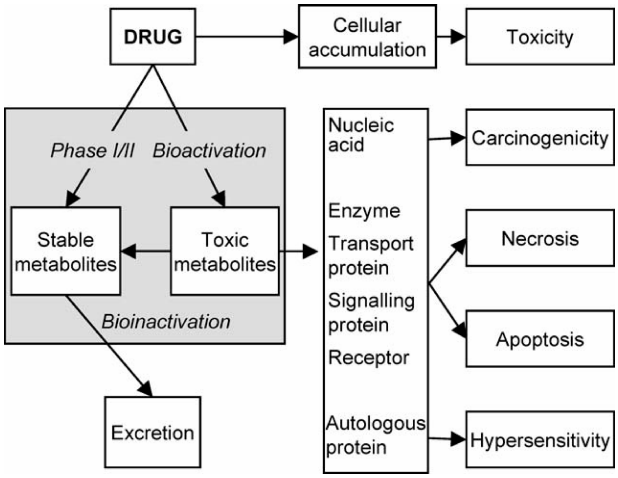


Fig. 13. Relationship between drug metabolism and toxicity. Toxicity may occur through accumulation of a parent drug or, *via* metabolic activation, formation of a chemically reactive metabolite, which, if not detoxified, can affect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequences of its modification will dictate the resulting toxicological response.

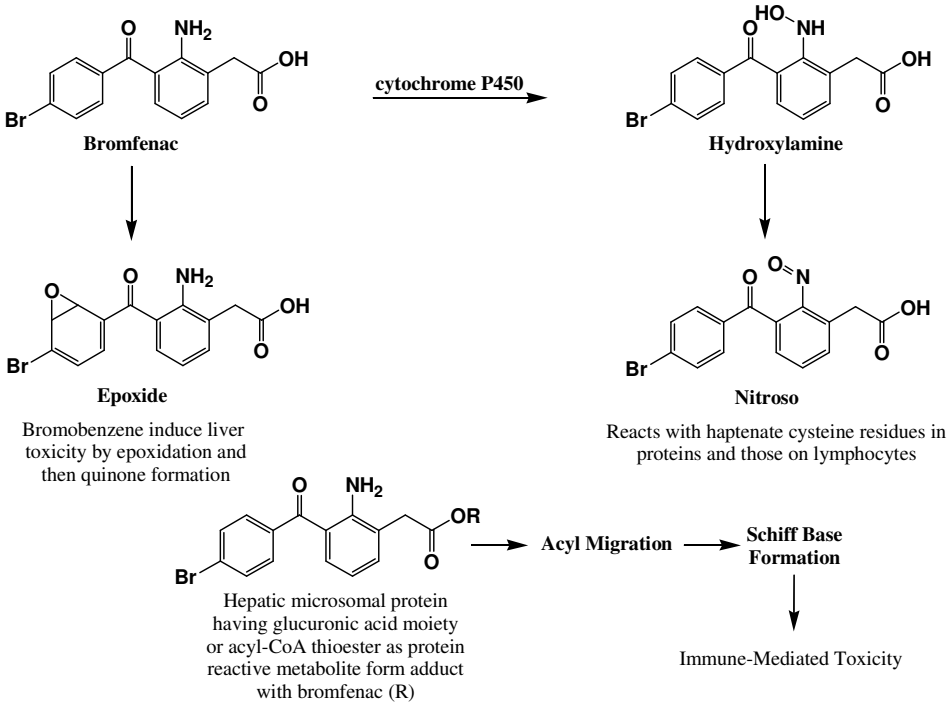


Fig. 14. Reactive metabolites from bromfenac (NSAID) that was removed from the market in 1998, due to idiosyncratic hepatotoxicity.

50]. Although the major role of drug metabolism is detoxication, it can also act as an "intoxication" process. Thus, foreign compounds can undergo biotransformation to metabolites that have intrinsic chemical reactivity toward cellular macromolecules. The propensity of a molecule to form such chemically reactive metabolites is a function of its chemistry, and structural alerts are well defined [33-50]. The versatility of cytochrome P450 together with the reactivity of its oxygen intermediates enables it to functionalize even relatively inert substrates, leading to the formation of chemically reactive species. Figure 13 illustrates relationship between drug metabolism and toxicity.

Formation of Hepatotoxins from Drugs

Bromfenac. Further chemical nowledge of the toxicophores contained within the molecule of the anti-inflammatory drug, bromfenac, before its release onto the market could have saved lives (Fig. 14) [49].

Acetaminophen. Acetaminophen is a major cause of drug-related morbidity and mortality in humans, producing massive hepatic necrosis after a single toxic dose (Fig. 15) [39,44,45].

The massive chemical stress mediated by an acetaminophen overdose leads to an immediate adaptive defense response in the hepatocyte [33]. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors, such as Nrf-2 (enhances GSH synthesis), that "sense" chemical danger and orchestrate cell defense by activation of the antioxidant response element (ARE) and transcription of a gene battery encoding antioxidant proteins and drug metabolizing enzymes (Fig.16).

Halothane. Halothane is the best-studied drug with respect to immunoallergic hepatitis (see Fig. 17) [46,47].

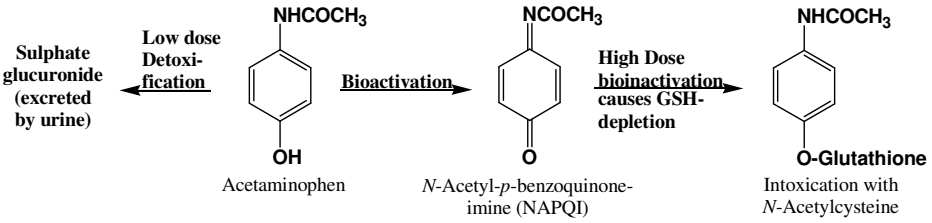


Fig. 15. Bioactivation and bioinactivation of acetaminophen.

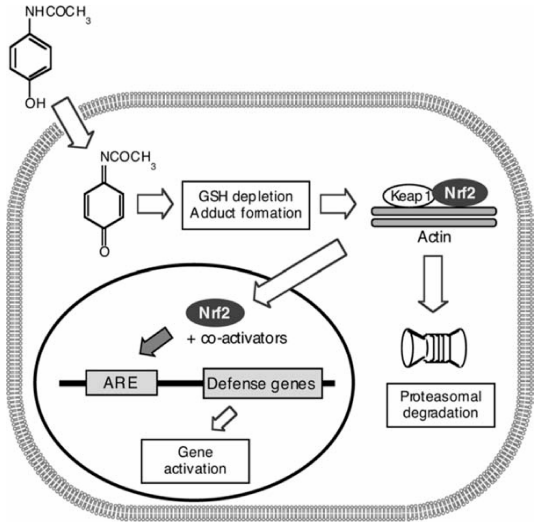


Fig. 16. Release of Nrf2 from its cytoplasmic inhibitor, keap 1, toward the nucleus.

The drug undergoes bioactivation in hepatocytes leading to drug-protein conjugate formation, through the modification of lysine residues in target proteins such as CYP 2E1 (P450), in the liver, increasing transaminase levels. The resulting modified protein is internalized by Kupffer cells and presented to cognate T cells that recognize the modified and native peptide. This in turn can lead to the generation of cytotoxic T cells and B-lymphocytes producing antibody (anti-TFA antibodies, anti-tissue antibodies, anti-CYP2E1). In theory, such an unregulated response could explain the severe idiosyncratic hepatotoxicity associated with halothane.

Isoniazid. Isoniazid (INH) is still the most widely used drug in the treatment of tuberculosis (TB) [48,50,51]. The combination of INH with rifampicin and/or pyrazinamide reduces the chance of inducing resistant strains of *Mycobacterium tuberculosis*. INH causes hepatitis and peripheral neuropathy. The pharmacology and toxicology of INH are due to reactive metabolites, including isonicotinoyl radical, which leads to adduct formation with NADP, and inhibition of enoyl-acyl carrier protein reductase (InhA) that is involved in the biosynthesis of mycolic acids present in the *Mycobacterium* cell wall (Fig. 18).

Diclofenac. The nonsteroidal *anti*-inflammatory drugs (NSAIDs) have a strong association with hepatotoxicity.

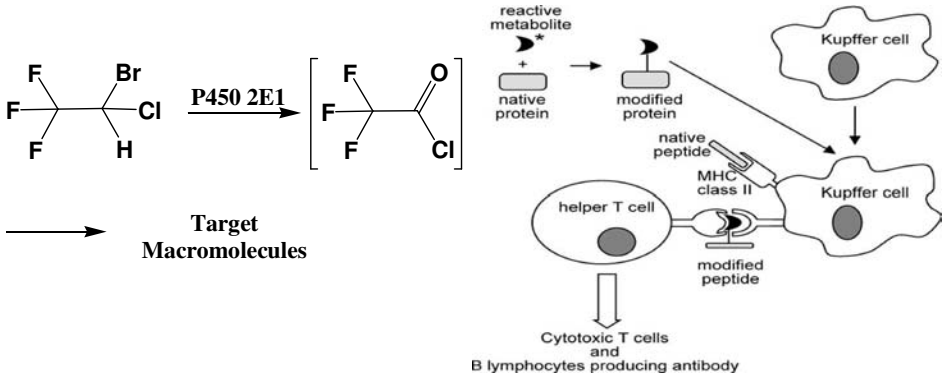


Fig. 17. Mechanism for the role of reactive metabolite in allergic hepatitis.

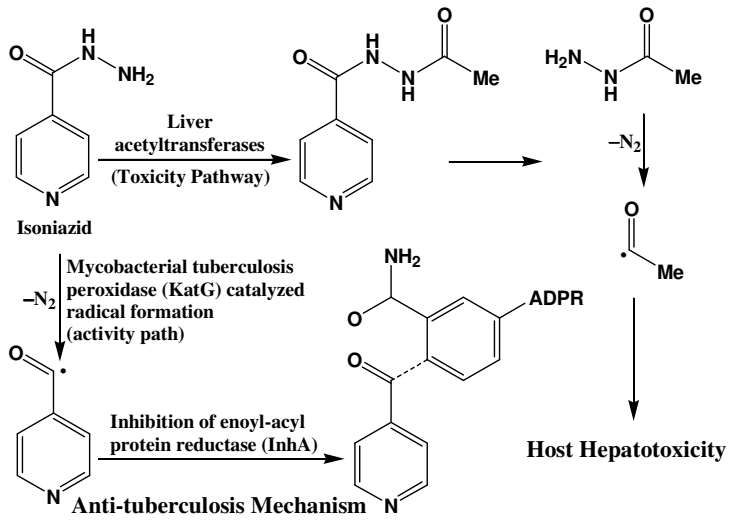


Fig. 18. Bioactivation and hepatocarcinogenesis of INH with high anti-TB activity.

Several NSAIDs have been withdrawn after obtaining approval for a license, including diclofenac [52-58]. Inhibition of the COX enzymes may lead to a reduction in the cell's protective agents, prostaglandins. Bioactivation, however, may occur both by oxidation (cytotoxic) in the presence of P450 enzymes and conjugation through acyl glucuronylation, which drives the binding of circulating proteins and hepatic proteins, known as acylthiolation. Diclofenac-protein adduct formation, especially on the cell surface, might be causally relevant to the expression of immune-mediated hepatitis. The mechanism of cytotoxicity and/or immune response to diclofenac is illustrated in Fig. 19.

Thiazolidinedione Antidiabetics. Troglitazone, a 2,4-thiazolidinedione, is the first of a new class of drugs for type 2 diabetes [59,61-63]. It was associated with a significant frequency of reversible increases in serum transaminases. Reports of severe and fatal liver injury finally led to the withdrawal of this important drug [59]. This drug, possessing a *p*-dimethylphenyl (toxicophore) moiety, capable of *in vivo* oxidation to a quinone moiety, could be replaced by the safer 2,4-thiazolidinediones, namely pioglitazone and rosiglitazone.

Its thiazolidinedione ring, however, forms several reactive metabolites, which could be eliminated as thioester and thioether conjugates of glutathione [56] as seen in Fig. 20 [59b,c].

Like troglitazone, the less hepatotoxic and cytotoxic pioglitazone and rosiglitazone, however, undergo NADPH-dependent covalent binding to human microsomal proteins [63a]. At present, the toxicological significance of troglitazone's metabolic activation remains an open question; even the relative extent of bioactivation of this class of compounds *in vitro* is unqualified. Finally, it is important to note that the heterogeneous clinical picture of troglitazone hepatotoxicity has prompted the suggestion that this may be a reflection of inter-individual variation in the balance of different mechanisms of drug toxicity as well as varying patient characteristics [63b].

Hard-Soft Chemistry and Toxicological Insult

Hard electrophiles generally react with hard nucleophiles, such as functional groups in DNA and lysine residues in proteins [64a]. Soft electrophiles react with soft nucleophiles, for example, cysteine residues in proteins and glutathione in the liver [64a]. Free radicals can also react with lipids and initiate lipid peroxidative chain reactions. Noncovalent interactions may also play a role in toxicity. On the other

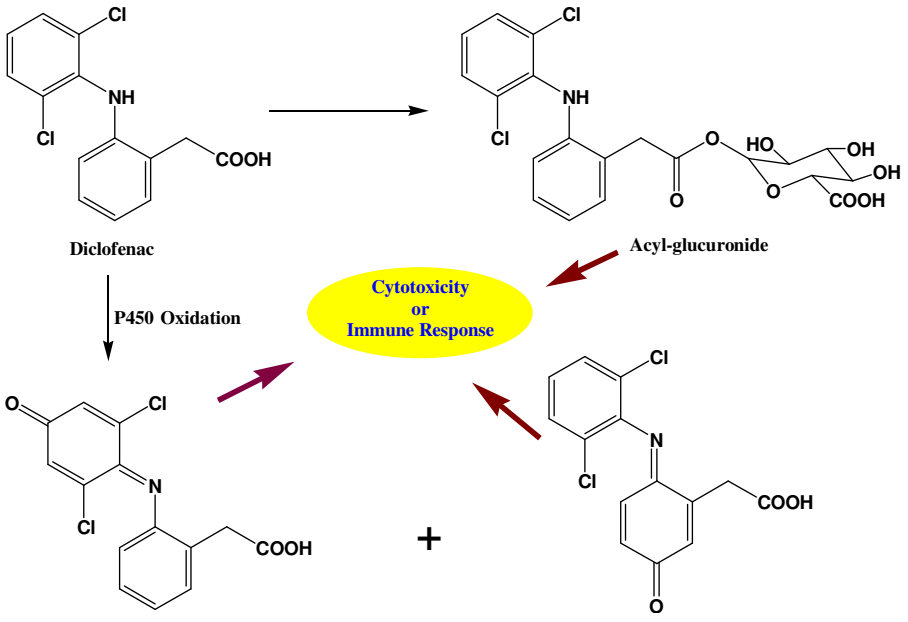


Fig. 19. Metabolic bioactivation of diclofenac.

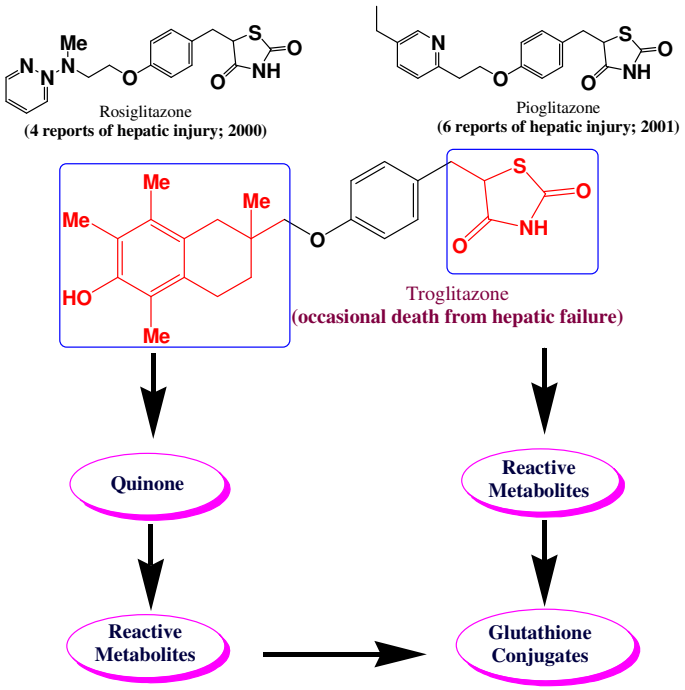


Fig. 20. Metabolism and toxicity of glitazones (troglitazone, rosiglitazone, and pioglitazone).

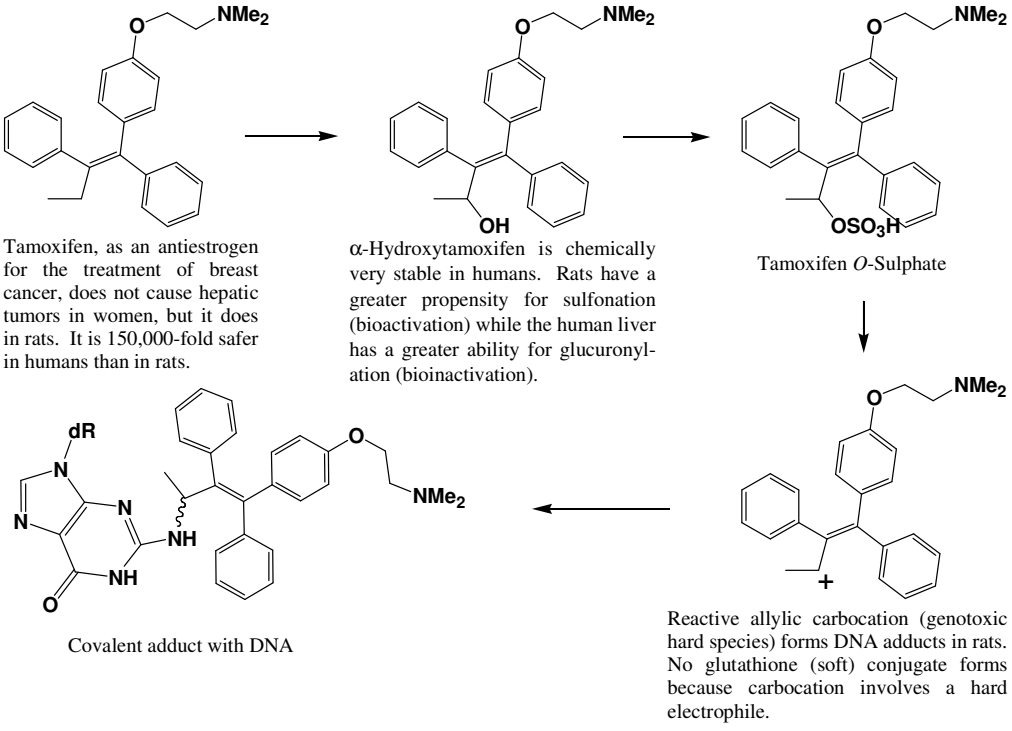


Fig. 21. Lack of detoxication of tamoxifen's hard carbocation metabolite by soft glutathione in rats.

hand, a number of drugs, such as penicillin, aspirin and omeprazole, rely on covalent binding to proteins for their efficacy; thus, prevention of covalent binding *via* chemical modification of the compounds may also lead to loss of efficacy. Therefore, the essential task is to differentiate between those protein modifications that are critical for drug toxicity and those necessary for drug efficacy. As a result, recognition of the extent of hardness-softness functionality of the enzymes responsible for toxicity and those essential for efficacy, and then modification of the important drug functionalities according to the hard-soft theory may result in safer medications. Figure 21 illustrates bioactivation of tamoxifen [33] and, on the basis of the hard-soft theory, exhibits its hepatocarcinogenesis.

CARDIOTOXICITY

Compounds with Human Ether-A-Go-Go Related Gene Channel (hERG) Liability

Avoiding cardiac liability caused by the QT prolongation associated with the blockade of hERG is another major problem in drug development [64-68].

An understanding of how and where a compound is binding to hERG can help a medicinal chemist to design out hERG blockade for a compound. Many compounds have been removed from the market due to cardiotoxicity caused by QT prolongation caused by hERG K^+ blockage [66].

Neurion Pharmaceuticals designed a kit (hERG-Mutant Activity Panel; hERG-MAP) [67] to allow a scientist to discover how and where molecules bind to hERG. By creating pharmacophores, one can determine if a compound or a series of molecules can be redesigned to avoid hERG interactions [67]. Mutation of the receptor with unnatural amino acids was performed to gain a better understanding of the H-bonding, cation–π and π–π interactions, and hydrophobic interactions involved. Using hERG-MAP, pharmacophores for hERG were derived (Fig. 22). Finally, one can design out hERG liability using hERG pharmacophores and target molecule pharmacophores.

Figure 23 displays hERG-MAP, which is a plot of the difference in the binding energy of a given compound with the mutant receptor relative to the wild type (WT) receptor.

Figure 24 shows the steps involved in the incorporation of unnatural amino acid mutants into the hERG channel [67,68]. Mutations that prohibit hydrogen bonding involve the following replacements: threonine 623 to hydroxy-threonine or serine and serine 624 to threonine or alanine. Mutations for cation–π/π–π interactions were replacement of tyrosine 652 to phenylalanine, 4-fluorophenylalanine, 3-fluorophenylalanine, or 3,5-difluorophenylalanine as well as the replacement of phenylalanine 656 to 4-fluoro-phenylalanine, 3,5-difluoro-phenylalanine, or cyclohexylalanine. The mutant channels are expressed in *Xenopus oocytes* and K^+ currents are measured using a two-electrode voltage clamp.

Building homology models and docking in screened compounds are the future objective of Neurion Pharmaceuticals to design out hERG liability using hERG pharmacophores derived by hERG-MAP.

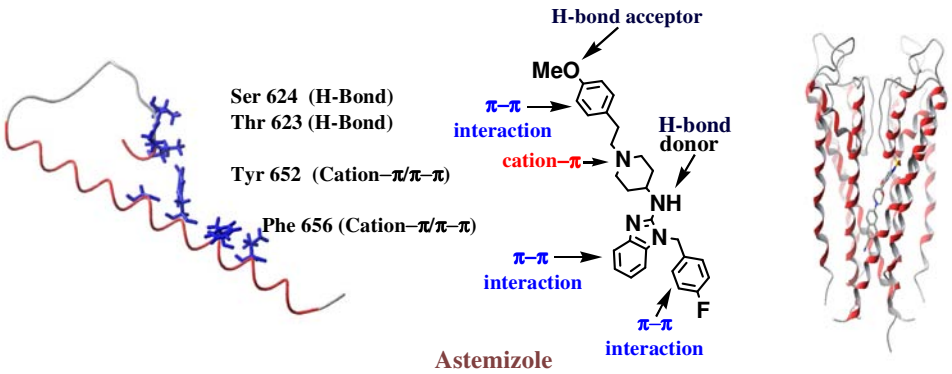


Fig. 22. hERG S6-P-Loop. Four amino acids involved in the binding of hERG blockers.

THE SOLUTION TO THE POTENTIAL TOXICITY PROBLEM

Prevention of the Release of Potentially Toxic Drugs onto the Market

To prevent the release of potential toxic drugs onto the market, first identify potential toxicophores within the proposed candidate compound as well as the potential metabolites or anabolites [22]. Then, identify the specific disposition, efficacy, and dose of the compound in question. For example, the antipsychotic drug, clozapine, at 900 mg/day, is bioactivated in the liver and in neutrophils transforming it into a toxic protein-reactive nitrenium metabolite [69-73]. Olanzepine, a neuroleptic with a similar structure to clozapine,

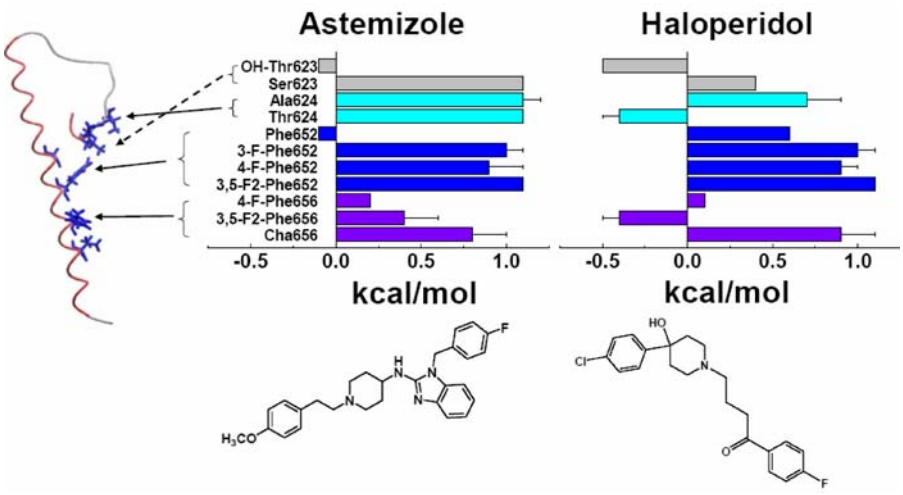


Fig. 23. Examples of distinct binding energy profiles for two common drugs. Y-axis: mutant receptor; X-axis: $\Delta\Delta G = -RT \ln(IC_{50}(MUT)/IC_{50}(WT))$.

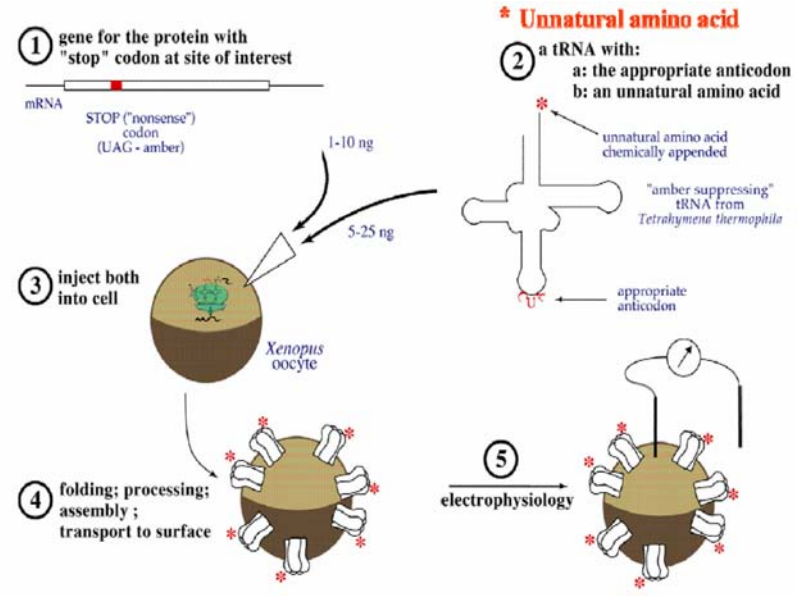


Fig. 24. Incorporation of unnatural amino acid probes into the hERG channel.

also undergoes nitrenium ion formation, but so far has not been associated with severe toxicity because of its low maximum daily dose of 10 mg/day. A dose-dependent relationship exists between reactive metabolite formation and development of idiosyncratic toxicity [71]. Unrelated to the administered drug, however, viral infections or inflammatory conditions could alter the drug metabolism, initiating a dose-dependent toxicity that is not idiosyncratic. Concurrent drug administration may also result in idiosyncratic reactions, including hepatic cholestasis, neuroleptic malignant syndrome and increased serum creatinine kinase activity (rhabdomyolysis) [22].

Downstream molecular pathways, such as transcription factor activation, and gene and protein expression or degradation, will give greater insight into the mechanism of toxicity. In the short term, the drug metabolist can determine the propensity of a novel chemical entity to undergo bioactivation in model systems ranging from expressed enzymes, through genetically engineered cells, to animals. Bioactivation can be assessed by trapping experiments with model nucleophiles [72a] through the measurement of uncharacterized covalent binding to endogenous proteins *in vitro*and *in vivo* [72b]. Then, the medicinal chemist can address the issue by seeking a stable pharmacophore to replace the potential toxicophore. In the long term, we need to know the ultimate toxin interference with signaling, and the sequence of molecular events that impair cell defense, ultimately leading to cellular destruction. When such a mechanistic framework is established, we will be in position to understand the time-course of toxicity, the nature of toxicity, and the direction that the toxicity takes in a particular patient [73].

Toxicity may also be estimated for molecules that have not been synthesized. With the help of MULTICASE, an artificial intelligence program capable of uncovering the relationship between the presence of specific substructures in a molecule and its toxicity, and TOX II, a program capable of identifying the existence of such substructures in new molecules, it is possible to predict whether a new molecule will be toxic. TOX II will uncover any functionality that was previously found to be associated with toxicity in any organic molecule [74-80]. The evaluation can also include automatically generated metabolites by use of the program META [74,75].

The program can select its modulators from a variety of structural ensembles as well as from a number of physical properties such as the logarithm of the octanol/water partition coefficient, water solubility, HOMO/LUMO energies, charge densities located in the various atoms of the toxicophore, and

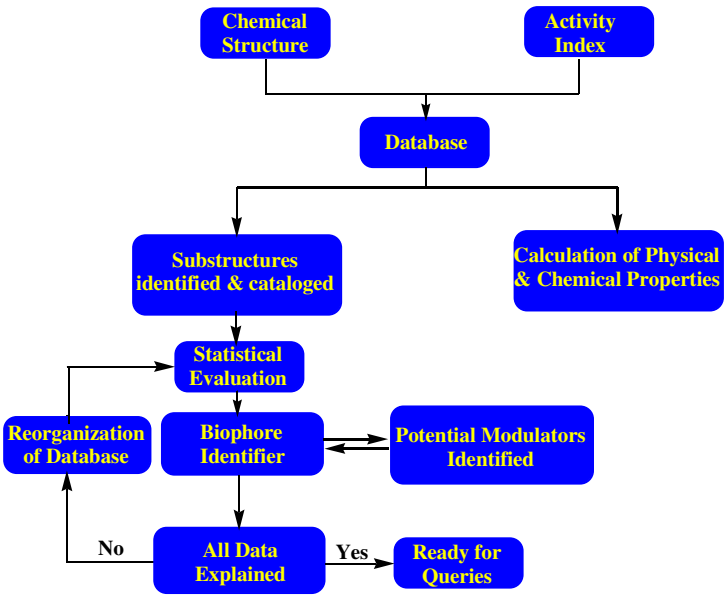


Fig. 25. The MULTICASE algorithm.

the location of hydrogen donors, hydrogen acceptors and lipophilic centers.

Once the information contained in the learning set has been digested by MULTICASE (Fig. 25), and transformed into the appropriate dictionaries, the TOX II program can be used to query the dictionaries and to generate predictions of activity for new molecules. Numerous specialized dictionaries capable of addressing questions about a number of different toxic endpoints have been created over the last ten years [74]. Predictions, however, should be taken as a guide to rank and prioritize chemicals for evaluation rather than as a crystal ball for the prediction of toxicity. Last but not least, predictions are functions of the nature, origin, diversity, and size of the databases.

CONCLUSIONS

Researches have shown that to avoid toxicity, it is better to avoid using potential toxicophores for the development of novel marketable drugs. The use of Ames test results to predict the mutagenicity of toxicophores has been described. Approved toxicophores can aid in the prediction of mutagenicity early in the risk assessment as well as in the design of chemical libraries for hit and lead optimization.

Chemically reactive metabolites derived from simple organic molecules, including therapeutic agents, were shown to cause a wide range of hepatic injuries. Short- and long-term solutions to the problem have been described. Metabolic transformation of drugs are catalyzed by numerous enzymes present in the body; the more important being cytochrome P450s. The actual knowledge of such enzymes enables us to postulate the main pathways of biotransformation of a given drug.

Avoiding the blockade of K^+ ion channels is costly and time consuming for industry and health care. By subtle modifications to residue side chains in the channel-binding pockets and measuring how these mutations affect a drug's ability to block hERG, specific interactions responsible for drug binding have been found. It has been shown that a hERG-MAP data set describes a compound's unique electrophysiological signature using a proprietary panel of hERG mutants. This information may be used to design drugs that do not undergo problematic interactions.

The TOX II program, capable of identifying the potential environmental health hazards of chemicals and predicting the toxicity of newly designed drugs, was also described.

A desirable drug, however, should be nontoxic, well absorbed, reach its target, undergo limited metabolism, and be excreted. Drug candidates should not readily inhibit the metabolism of marketed drugs, and it is desirable to market orally delivered drugs that have a once-daily dose.

REFERENCES

[1] International Conference on Harmonization: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Fed. Regist. 61 (1996) 18197.

[2] a) J. Kazius, R. McGurie, R. Bursi, J. Med. Chem. 48 (2005) 312; b) M. Hammond, R.L. Elliot, M.L. Gillaspy, D.C. Hager, R.F. Hank, J.A. LaFlamme, R. M. Oliver, P.A. DaSilva-Jardine, R.W. Stevenson, C.M. Mack, J.V. Cassella, Bioorg. Med. Chem. Lett. 13 (2003) 1989; c) J. Ashby, R.W. Tennant, Mutat. Res. 204 (1988) 17; d) Chemical Carcinogenesis Research Information System is available through TOXNET at http://toxnet.nlm.nih.gov. (2e) National Toxicity Program at http://ntp-server.niehs.nih.gov.; http://www.epa.gov.; Beilstein CrossFire Commander, v 4.0, MDL Information Systems, Inc., San Leandro, CA; and http://potency.berkeley.edu/CPDB-tab.html.

[3] C.S. Cooper, P.L. Grover (Eds.), Handbook of Experimental Pharmacology, Vol. 94/1, Springer-Verlag: Heidelberg, 1990, pp. 267-325.

[4] L.H. Garret, C.M. Grisham, Biochemistry, Saunders College Publishing: Orlando, FL, 1995, pp. 929-932.

[5] B.N. Ames, H. McCann, E.Yamasaki, Mutat. Res. 31 (1975) 347.

[6] D.M. Maron, B.N. Ames, Mutat. Res. 113 (1983) 173.

[7] K. Mortelmans, E. Zeiger, Mutat. Res. 455 (2000) 29.

[8] W.W. Piegorsch, E. Zeiger, Measuring intra-assay agreement for Ames Salmonella assay. Lecture notes in Medicinal Informatics, Springer-Verlag: Heidelberg, 1991, pp. 35-41.

[9] a) R. Benigni, A. Giuliani, R. Franke, A. Gruska, Chem. Rev. 100 (2000) 3697; b) R. Lahana, Drug Discovery Today 4 (1999) 447; c) C.G. Newton, in:

P.M. Dean, R.A. Lewis (Eds.), In Molecular Diversity in Drug Design, Kluwer Academic Publishers, Dordrecht, 1999; d) A.P. Watt, D. Morrrison, D.C. Evans, Drug Discovery Today 5 (2000) 17; e) Y.T. Woo, D.Y. Lai, J.C. Arcos, M.F. Argus, M.C. Cimino, S. DeVito, L. Keifer, Environ. Carcinog. Ecotoxicol. Rev. C15 (1997) 139.

[10] a) B.S. Kim, B.H. Margolin, Environ. Mol. Mutagen. 34 (1999) 297-304; b) F.P. Guengerich, M.S. Kim, M. Muller, L.G. Lowe, Recent results Cancer Res. 143 (1997) 449.

[11] E. Zeiger, J.K. Haseman, M.D. Shelby, B.H. Margolin, R.W. Tennant, Environ. Mol. Mutagen. 16 (1990) 1.

[12] M. Klein, U. Voigtmann, T. Haack, L. Erdinger, G. Boche, Mutat. Res. 467 (2000) 55.

[13] a) C. Glende, H. Schmitt, L. Erdinger, G. Engelhardt, G. Boche, Mutat. Res. 498 (2001) 19; b) S. Srivastava, P.H. Ruane, J.P. Toscano, M.B. Sullivan, C.J. Cramer, D. Chiapperino, E.C. Reed, D.E. Falvey, J. Am. Chem. Soc. 122 (2000) 8271.

[14] T. Haack, L. Erdinger, G. Boche, Mutat. Res. 491 (2001) 183.

[15] a) A.K. Debnath, R.L. Lopez de Compadre, G. Debnath, A.J. Shusterman, C. Hansch, J. Med. Chem. 34 (1991) 786 and references therein; b) G.G. Cash, Mutat. Res. 491 (2001) 31; c) G. Streiinger, Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, M. Inouye, Cold Springer Harbor Symp. Quant. Biol. 31 (1966) 77.

[16] a) M. Klein, L. Erdinger, G. Boche, Mutat. Res. 467 (2000) 69; b) H.A. Nieper, Naturwissenschaften 43 (1956) 500.

[17] B.P. Cho, F.A. Beland, M.M. Marques, Biochem. 31 (1992) 9587.

[18] B.P. Cho, F.A. Beland, M.M. Marques, Biochem. 33 (1994) 1373.

[19] a) S. De Flora, A. Izzotti, K. Randerath, E. Randerath, H. Bartsch, J. Nair, R. Balansky, F. van Schooten, P. Degan, G. Fronza, D. Walsh, J. Lewtas, Mutat. Res. 366 (1996) 197; b) L.M. Eckel, T.R. Krugh, Biochem. 33 (1994) 13611.

[20] L. Zhou, M. Rajabzadeh, D.D. Traficante, B.P. Cho, J. Am. Chem. Soc. 119 (1997) 5384.

[21] A.K. Debnath, R.L. Lopez de Compadre, A.J. Shusterman, C. Hansch, Environ. Mol. Mutagen 19 (1992) 53.

[22] D.P. Williams, B.K. Park, DDT 8 (2003) 1044 (www.drugdiscoverytoday.com) and references cited therein.

[23] M. Meadows, Serious liver injury. FDA Consum. 35 (2001) 8.

[24] S. Michelson, K. Joho, Curr. Opin. Mol. Ther. 2 (2000) 651.

[25] S.D. Nelson, Adv. Exp. Med. Biol. 500 (2001) 33.

[26] D.E. Slaughter, R.P. Hanzlik, Chem. Res. Toxicol. 4 (1991) 349.

[27] U.A. Boelsterli, Curr. Drug. Metab. 3 (2002) 439.

[28] U.A. Boelsterli, Curr. Opin. Drug Discov. Devel. 6 (2003) 81.

[29] B.K. Park, Toxicology 153 (2000) 39.

[30] Uetrecht, J.P. Chem. Res. Toxicol. 12 (1999) 887.

[31] M. McMahon, J. Biol. Chem. 278 (2003) 21592.

[32] J. Lazarou, J. Am. Med. Assoc. 279 (1998) 1200.

[33] B.K. Park, N.R. Kitteringham, J.L. Maggs, M. Pirmohamad, D.P. Williams, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 177.

[34] W.M. Lee, New. Engl. J. Med. 349 (2003) 474.

[35] E. Miller, J. Miller, Cancer Res. 7 (1947) 468.

[36] E. Miller, J. Miller, Cancer Res. 12 (1952) 547.

[37] B.B. Brodie, W.D. Reid, A.K. Cho, G. Sipes, G. Krishna, J.R. Gillette, Natl. Acad. Sci. USA, 68 (1971) 160.

[38] J.R. Gillette, J.R. Mitchell, B.B. Brodie, Annu. Rev. Pharmacol. 14 (1974) 271.

[39] M.A. Tirmenstein, S.D. Nelson, J. Biol. Chem. 264 (1989) 9814.

[40] H.J. Gonzalez, S. Kimura, Cancer Lett. 143 (1999) 199.

[41] C.J. Henderson, A.G. Smith, J. Ure, K. Brown, E.J. Bacon, C.R. Wolf, Proc. Natl., Acad, Sci. USA, 95 (1998) 5275.

[42] P. Carthew, K.J. Rich, E.A. Martin, F. De Matteis, C. K. Lim, Carcinogenesis 16 (1995) 1299.

[43] D.J. Boocock, J.L. Maggs, K. Brown, I.N.H. White, B. K. Park, Carcinogenesis 21 (2000) 1851.

[44] J.L. Raucy, J.M. Lasker, C.S. Lieber, M. Black, Arch.

Biochem. Biophys. 271 (1989) 170.
[45] C. Goldring, N. Kitteringham, R. Elsby, L. Randle, Y. Clement, Hepatology 39 (2004) 1267.
[46] J.G. Kenna, J. Hepatol. 26 (1997), (Suppl. 1) 5.
[47] N.R. Kitteringham, J.G. Kenna, B.K. Park, Br. J. Clin. Pharmacol. 40 (1995) 379.
[48] J.R. Mitchell, H.J. Zimmerman, K.G. Ishak, U.P. Thorgeirsson, J.A. Timbrell, Ann. Intern. Med. 84 (1976) 181.
[49] H.J. Zimmerman, Semin. Liver Dis. 10 (1990) 322.
[50] T.C. Sarich, T. Zhou, S.P. Adams, A.I. Bain, R.A. Wall, J.M.A. Wright, J. Pharmacol. Toxicol. Methods. 34 (1995) 109.
[51] D.A. Rozwarski, G.A. Grant, D.H. Barton, W.R.Jr. Jacobs, J.C. Sacchettini, Science 279 (1998) 89.
[52] J.R. Kenny, J.L. Maggs, X. Meng, D. Sinnott, S.E. Clarke, J. Med. Chem. 47 (2004) 2816.
[53] M.P. Grillo, F. Hua, C.G. Knutson, J.A. Ware, C. Li, Chem. Res. Toxicol. 16 (2003) 1410.
[54] S. Shen, M.R. Marchick, M.R. Davis, G.A. Doss, L.R. Pohl, Chem. Res. Toxicol. 12 (1999) 214.
[55] W. Tang, R.A. Stearns, S.M. Bandiera, Y. Zhang, C. Raab, Drug Metab. Dispos. 27 (1999) 365.
[56] G.H. Hakimelahi, G. Sh Gassanov, M.-H. Hsu, J.R. Hwu, S. Hakimelahi, Bioorg. Med. Chem. 10 (2002) 1321.
[57] Y. Masubuchi, A. Ose, T. Horie, Drug Metab. Dispos. 30 (2002) 1143.
[58] U.A. Boelsterli, Mechanistic Toxicology; Lonndon: Taylor & Francis, 2003, Chapter 9, p. 202.
[59] a) K.G. Tolman, J. Chandramouli, Clin. Liver Dis. 7 (2003) 369; b) K. Kassahun, P.G. Pearson, W. Tang, I. McIntoch, K. Leung, Chem. Res. Toxicol. 14 (2001) 62; c) J.N. Tettey, J.L. Maggs, W.G. Rapeport, M. Pirmohamed, B.K. Park, Chem. Res. Toxicol. 14 (2001) 65.
[60] U.A. Boelsterli, Toxicol. Appl. Pharmacol. 192 (2003) 307.
[61] A.J. Scheen, Drug Saf. 24 (2001) 873.
[62] D.J. Graham, L. Green, J.R. Senior, P. Nourjah, Am. J. Med. 114 (2003) 299.
[63] a) Z. Jayyosi, M. Muc, M. Gallagher, H. Toutain, J. Stevens, M. Kelley, Proc. N. Am. ISSX Meet., 9th., Nashville, 2004, Oct., 24, p. 200; b) M.T. Smith, Chem. Res. Toxicol. 16 (2003) 679.
[64] a) B. Fermini, A.A. Fossa, Nature Rev. Drug Discov. 2 (2003) 439; b) M. Roy, R. Dumaine, A.M. Brown, Circulation 94 (1996) 817.
[65] S.-H. Jo, J.B. Youm, C.O. Lee, Y.E. Earm, W.-K. Ho, Br. J. Pharm. 129 (2000) 1474.
[66] H.R. Alderton, Can. J. Psychiatry 40 (1995) 325.
[67] M.W. Nowak, N.M. Zacharias, A.A. Kulkarni, J.B. Nicholas, H.P. Lesso, S.J. Reyes, S. dee Sahba, B. Lally, E.D. Mackey, N. Shiva, P.B. Bennett, Neurion Pharmaceuticals Inc., 2004, www.neurionpharma.com.
[68] S. Ekins W.J. Crumb, R.D. Sarazan, J.H. Wikel, S.A. Wrighton, J. Pharmacol. Exp. Thera. 301 (2002) 427.
[69] a) R.G. Pearson, Hard and Soft Acids and Bases. Hutchinson & Ross: Canada, 1973; b) D.P. Williams, Mol. Pharmacol. 58 (2000) 207.
[70] M. Pirmohamed, B.K. Park, CNS Drugs 7 (1997) 139.
[71] J.P. Uetrecht, Curr. Opin. Drug Didcov. Devel. 4 (2001) 55.
[72] a) G.H. Hakimelahi, T. Sambaiah, J.-L. Zhu, K.S. Ethiraj, M. Pasdar, S. Hakimelahi, Eur. J. Med. Chem. 37 (2002) 207; b) D.C. Evans, A.P. watt, D.A. Nicoll-Griffith, T.A. Baillie, Chem. Res. Toxicol. 17 (2004) 3.
[73] J.W. Drake, R.H. Baltz, Annu. Rev. Biochem. 45 (1976) 11 (see: http://www.annualreviews.org/aronline; http://pharmtox.annualreviews.org).
[74] G. Klopman, H.S. Rosenkranz, Tox. Lett. 79 (1995) 145.
[75] H.S. Rosenkranz, G. Klopman, in: A.M. Goldberg, M. L. Principe (Eds.), Expert System Approach to the Prediction and Elucidation of the Structural Basis of Toxicological Activities. Alternative Methods in Toxicology, Mary Ann. Liebert, 1991, pp 145-162.
[76] G. Klopman, H.S. Rosenkranz, Environ. Health Perspect. 96 (1991) 67.
[77] G. Klopman, I. Kolossvary, J. Math. Chem. 5 (1990) 389.
[78] D.G. Bailey, Can. J. Clin. Pharmacol. 2 (1995) 10.
[79] M. Delaforge, Nucl. Med. Biol. 25 (1998) 705.
[80] O. Llorenz, J.J. Perez, H.O. Villar, J. Med. Chem. 44 (2001) 2793.