

PREDICTION OF HEPATOCARCINOGENIC EFFECT OF STRUCTURAL DIVERSE CHEMICALS BY COMPUTATIONAL TECHNOLOGIES

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Abstract

The primary testing strategy to identify chemical (hepato)carcinogens largely relies on the 2-year rodent bioassay, which is time-consuming and labor-intensive. There is an increasing effort to develop alternative approaches to prioritize the chemicals for, supplement, or even replace the cancer bioassay. In silico approaches based on quantitative structure-activity relationships (QSAR) are rapid and inexpensive and thus have been investigated for such purposes. Since the correlation between carcinogenicity and Ames mutagenicity test results was found to be significant enough it is expected that models based on Ames data could be used successfully for identification of chemical carcinogens. In the current study the implemented profiler for DNA binding prediction in non-commercial software tool was used to predict the hepatocarcinogenic effect of 55 representative chemicals. The obtained results show that 73% of the hepatocarcinogens can be successfully identified as genotoxic carcinogens. The role of nongenotoxic mechanisms has been assessed by application of profiling scheme for identification of nongenotoxic chemical carcinogens. As a result of combined application of both profilers 87% of hepatocarcinogens have been correctly identified.

Key words: liver, hepatocarcinogenicity, QSAR, genotoxicity, metabolic activation

1. INTRODUCTION

To assess the potential risk of human carcinogens, long-term animal bioassays for carcinogenicity are regularly used to resolve whether chemical agents are proficient of inducing cancer in humans. According to the regulatory authorities of Europe, USA and Japan, carcinogenicity studies should be performed before the application for marketing approval of pharmaceuticals and chemicals (Müller et al., 1999). Guidelines for carcinogenicity testing of pharmaceuticals and chemicals specify that long-term carcinogenicity studies in rodents should be carried out to establish chemicals as a carcinogen. Rodent carcinogenicity studies have been used for many years to assess carcinogenic potential of chemicals with an ultimate goal of assessing human carcinogenic risk (Ward 2010).

The liver is one of the main target organs of toxicity caused by various type of chemicals [Chang and Schiano 2007). The effect is known as hepatotoxicity and implies chemical-driven liver damage. A large number of chemicals are known to induce liver cancer (hepatocarcinogenicity) in rodents. Based on the predominant mechanism of action, liver carcinogens may be classified as genotoxic and epigenetic/nongenotoxic. Genotoxic carcinogens, also known as DNA - reactive carcinogens, generally are chemicals that directly interact with DNA (causing mutations) either as parent chemicals or as reactive metabolites to form DNA adducts or lesions which, if unrepaired, may initiate carcinogenesis. Epigenetic carcinogens are agents that act through secondary mechanisms that do not involve direct DNA damage.

The recognition that chemical carcinogenicity usually represents a mutation led to the study of in vitro models that permit demonstration of the mutating ability of chemical agents. In this respect the Salmonella reverse mutation test known as Ames test (Ames et al., 1975) seemed particularly successful in demonstrating mutation when it is applied for chemicals that produce carcinomas. Since the large part of carcinogens are procarcinogens, the Ames test is also able to assess the activating phase of the metabolism responsible for formation of highly reactive mutating metabolites. The correlation between mutagenicity in bacteria and carcinogenicity had been a subject in large number of

investigations (Mortelmans and Zeiger 2000). In general it may be concluded that the Ames test detects most of the known organic chemicals that are rodent carcinogens.

Expert analysis of large chemical collections with mutagenicity data allows definition of structural alerts which may serve as a basis for the development of computer-assisted (in silico) prediction technologies such as Quantitative Structure-Activity Relationships (QSAR). Currently, several commercial and non-commercial programs with integrated models for mutagenicity could be used for identification of mutagenic compounds (Cronin et al. 2003). The predictions can be further related to carcinogenicity, however additional information in terms of organ specificity is usually not provided. Such information is very important due to the surmise that the Ames test is probably not equally applicable for identification of carcinogens without taking into account the target organ. Furthermore, such investigation is expected to help in reducing the number of false positively predicted carcinogens which is a problem often associated with practical limit of the Ames test.

The aim of this study is to investigate the possibility to predict organ specific carcinogenicity in terms of hepatocarcinogenicity by application of profiling scheme for mutagenicity integrated in non-commercial tool for chemical risk assesement.

2. MATERIALS AND METHODS

2.1. Ames test for mutagenicity

One of the most important types of toxic response a chemical can produce is mutation. Mutations arise when the DNA in a cell is damaged in such a way that the information contained in the genetic code is altered. The Salmonella reverse mutation test – known as the Ames test (Ames et al., 1975) has been used for several decades as a useful tool for detection of potentially mutagenic chemicals. Genetically different strains of *S. typhimurium* are used for testing. They all carry some type of defective (mutant) gene that prevents them from synthesizing the amino acid histidine. In the presence of mutagenic chemicals, the defective gene may be mutated back to the functional state, allowing the bacterium to grow on the minimal medium.

Many chemicals are not mutagenic by themselves, but may be metabolized to mutagenic forms. Bacteria and most cultured mammalian cells cannot perform most of the metabolic conversions found in mammals and humans because they do not contain the necessary metabolizing enzyme systems. This limitation has been partially overcome by the development of exogenous metabolic activation systems that can be added to the test procedure. These systems usually consist of homogenates of liver fractions (S9) of rodents, usually rats that had been pretreated with substances to enhance the levels of the preferred metabolic enzymes (Wright 1980). A complete in vitro testing protocol includes tests with and without S9 fractions.

2.2. Validation chemical set

The validation dataset comprises 55 molecules (Massarelli et al. 2009). The hepatocarcinogenic effect was assessed in terms of TD₅₀ values for female mouse only. The authors stated that in order to prevent the influence of the molecular weight of the compounds, the TD₅₀ values, originally expressed as mg/kg, were transformed into the corresponding $\mu\text{M/Kg}$ values and submitted to the $-\log$ function, thus the final data correspond to pTD₅₀ values. It should be noted that high values of pTD₅₀ means high hepatocarcinogenicity.

2.3. OECD QSAR Toolbox

This is a software tool especially designated for chemical risk assessment (QSAR Tollbox). A key part of the system is so called categorization of chemicals. The categorization allows grouping of chemical substances into chemical categories. The chemical category is such a group of substances possessing similar physicochemical, toxicological and ecotoxicological properties or their fate in environmental and occupational surrounding or they behave using the common pattern as a result of chemical similarity.

An important advantage of the system is the large number of built-in profilers for different biological/toxic endpoints. Each profile consist a set of rules related to specific or general criteria associated to the respective endpoint.

In the following two sections details will be given for current versions of both profile associated with DNA damages and in vitro metabolic simulator incorporated in version 3.3 of the Toolbox.

2.4. Profiling scheme for DNA damages

The profiler is based on Ames mutagenicity model part of OASIS TIMES system (Mekenyan et al. 2012). The profiler contains exact definitions of 78 structural alerts responsible for interaction of chemicals with DNA. The scope of this profiler is to investigate the presence of alerts within the target molecules responsible for interaction with DNA, especially related to Ames mutagenicity.

2.5. Metabolism simulator

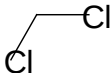
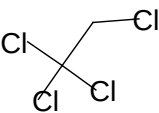
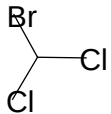
The current in vitro rat liver metabolic simulator represents electronically designed set of 509 structurally generalized, hierarchically arranged biotransformation reactions, which are characteristic for the metabolism for in vitro experimental systems such as rodent (mostly rat) liver microsomes and S9 fraction. A training set of 647 xenobiotic chemicals of a wide structural diversity, with experimentally observed metabolic reactions and pathways has been built, using published data on their metabolism in rodent liver microsomes and S9 fraction. On the whole, the simulator contains 450 – 470 enzymatic phase I transformations, such as aliphatic C-oxidation, aromatic C-hydroxylation, oxidative N- and O-dealkylation, epoxidation, ester and amide hydrolysis, carbonyl group reduction, nitro and azo group reduction, N-hydroxylation, etc. Additionally, 15 – 20 enzymatic phase II transformations, such as glucuronidation, sulfation, glutathione conjugation, N-acetylation, etc. are included with significantly lower priority than phase I ones.

The principal applicability of this simulator is associated with the reproduction as well as the prediction of the metabolic activation reactions and pathways of xenobiotic chemicals, which may elicit diverse array of in vitro biological effects.

3. RESULTS AND DISCUSSION

The validation set was transferred into the QSARToolbox v3.3 and the profiling scheme for DNA damages (DNA alerts for AMES, MN and CA by OASIS v.1.3) was applied on parent structures. Structural alerts have been identified in 23 out of all 55 chemical structures. It was found that almost half of the chemicals were classified as Geminal Polyhaloalkane Derivatives or Single-Ring Substituted Primary Aromatic Amines (Table 1 and 2 respectively).

Table 1. Chemicals classified as *Geminal Polyhaloalkane Derivatives*.

#	CAS	Chemical name	2D representation	pTD50	Ames test
1	75-09-2	Dichloromethane		1.58	positive
2	630-20-6	1,1,1,2-tetrachloroethane		2.42	positive
3	75-27-4	Bromodichloromethane		3.06	positive

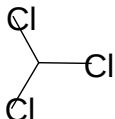
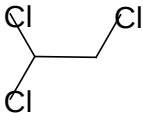
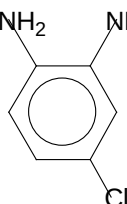
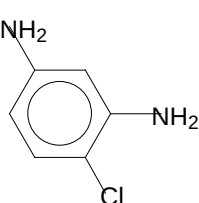
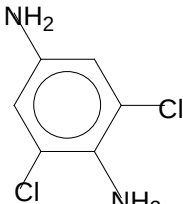
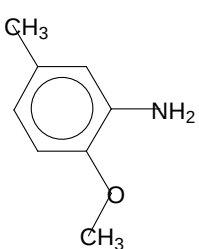
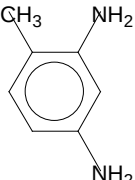
4	67-66-3	Chloroform		3.36	positive
5	79-00-5	1,1,2-trichloroethane		3.38	positive

Table 2. Chemicals classified as *Single-Ring Substituted Primary Aromatic Amines*.

#	CAS	Chemical name	2D representation	pTD ₅₀	Ames test
1	95-83-0	4-chloro-o-phenylenediamine		1.73	positive
2	5131-60-2	4-chloro-m-phenylenediamine		1.90	positive
3	609-20-1	2,6-dichloro-1,4-benzenediamine		1.94	positive
4	120-71-8	P-cresidine		2.64	positive
5	95-80-7	2,4-diaminotoluene		3.66	positive

While the structural alerts are part of the parent structures it should be noted that the associated interaction mechanisms with DNA for both groups implicit requires metabolic activation. For example it is considered that chloroform (representative for Geminal Polyhaloalkane Derivatives) undergoes oxidative biotransformation (catalyzed by cytochrome P450) leading to formation of trichloromethanol. Further loss of hydrogen chloride from trichloromethanol produces phosgene

which is considered to be responsible for DNA damages (details for interaction mechanism were taken from OASIS 1.3 profiler supporting materials). Similarly, bromodichloromethane and dibromochloromethane are also metabolized to brominated analogues of phosgene.

The group of Single-Ring Substituted Primary Aromatic Amines requires also metabolic activation to exert DNA damages. The obligatory step in bioactivation of all aniline derivatives involves enzymatic N-hydroxylation on the primary amine nitrogen, leading to the formation of N-hydroxylamine intermediate. These reactive N-hydroxylamine derivatives can undergo phase II conjugation, to generate the more reactive N-O sulfate and/or N-O acetyl conjugates. The excellent leaving group capability of sulfonyloxy- and acetoxy-functionalities in these conjugates is believed to lead to a highly reactive nitrenium ion. As a result nitrenium ion electrophilic species may readily bind covalently to cellular DNA and RNA (Kalgutkar et al. 2005).

Besides both groups discussed above the rest of the chemicals were found to be related to several classes including quinoneimines, nitroaniline derivatives, monohaloalkanes, nitrobiphenyls and bridged nitrobiphenyls, polynitroarenes, haloalkenes with Electron-Withdrawing Groups, N-Nitroso compounds and Anthraquinones.

As a conclusion at this point of the study one may state that the percentage of identified hepatocarcinogens based on presence of structural alert in the parent structure is 42% (23/55 chemicals) only. In respect to hepatocarcinogenic potency it was found that strong hepatocarcinogens with pTD50 values above 5 are not presented among them. As a logical consequence the next step of the analysis was directed in a way to account the influence of the metabolism as important factor for hepatocarcinogenicity.

The rest of 32 chemicals without identified DNA alert were screened again in combination with in vitro metabolism simulator. The result presented in Table 3 shows that DNA alert were found in metabolites for 17 chemicals.

Table 3. Chemicals classified as mutagens after metabolic activation.

#	Number of chemicals	Interaction mechanism of metabolites
1	8	N-hydroxylamine formation
2	5	Geminal Polyhaloalkane Derivatives
3	4	Epoxides and aziridines

In order to assess the reliability of the simulated metabolites and respective prediction results a comparison study with documented metabolism was performed. It is based on available data for observed metabolism for large number of chemicals included in the QSAR Toolbox. It was found that there are documented metabolic maps for six out of all seventeen chemicals. An example is provided for Trichloroethene (CAS# 79-01-6). Elfarra et al. (1998) reported three observed metabolites for this chemical illustrated in Figure 1. The positive mutagenic prediction is as results of identified epoxide structural moiety in metabolite #2 highlighted in Figure 1.

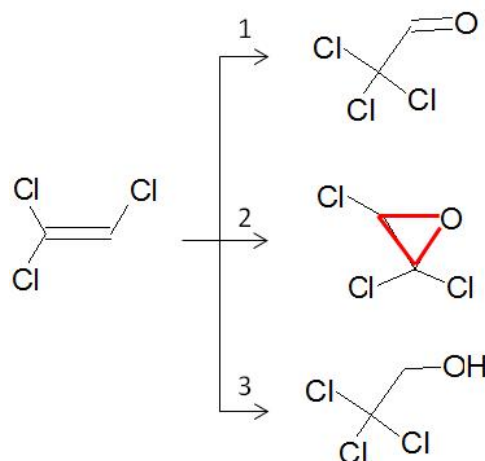


Fig1. Documented metabolism for *Trichloroethene* (CAS# 79-01-6, Elfarrar et al. (1998)).

Strobel et al. (1987) confirmed that *Trichloroethene* requires metabolic activation in order to elicit mutagenic effect. In this case it is evident that the ultimate hepatocarcinogenic effect is a result of metabolic conversion which leads to formation of highly reactive metabolite.

Taken together predictions with and without metabolic activation leads to 73% (40/55) correct identified hepatocarcinogens considered to act via genotoxic mechanism. Regarding the group of the chemicals which do not contain any alert (15 chemicals) with and without metabolic activation it is expected that the hepatocarcinogenicity is a result of nongenotoxic mechanism. For example the most potent hepatocarcinogens in this study TCDD (pTD50=8.8) and Kepone (pTD50=5.7) are supposed to act exactly via nongenotoxic mechanism. TCDD is a liver tumor promoter in female rodents (Bock 1993). The lack of genotoxicity and the sex difference in tumor promotion suggests that the mechanism is possibly related to hormonal factors (Williams 1996).

The possibility to predict the carcinogenic potential of the chemicals considered to act via nongenotoxic mechanisms was analyzed by making use of the profiler “*Carcinogenicity (genotox and nongenotox) alerts by ISS*” (built-in Toolbox). Eight out of all fifteen putative nongenotoxic carcinogens were found to have structural alert related to nongenotoxic mechanism. However, due to the limited number of the investigated chemicals it was not possible to set the focus on specific alerts which predominantly contribute to hepatocarcinogenicity. Nevertheless, for the sake of future analysis one should point out that the identified alerts represents a set of halogenated (PAHs, cycloalkanes, dibenzodioxins) and thiocarbonyl type of chemicals.

The ultimate prediction results assessed by the performance of both profilers correctly identifies 87% (48/55) of all investigated hepatocarcinogenic chemicals. The result seems promising, however, again due to the limited number of the analyzed chemicals it is expected that only part of the possible carcinogenic mechanisms have been highlighted. For example there is no even a single chemical which is thought to exert the effect through interaction with specific receptors. A step further in this way could be construction of a new extended set of validation chemicals for which the built-in profilers related to receptor mediated effects in the Toolbox can be applied.

4. CONCLUSIONS

In general, the mechanisms associated to chemical-induced (hepato)carcinogenicity can be classified as (a) *direct*, which are often triggered by parent molecule or after bioactivation as a result of formation of highly reactive metabolite(s) capable to bind covalently DNA and (b) *indirect* (nongenotoxic) typically complex interactions which affects DNA expression without modifying or directly damaging DNA structure. In the present study it has been shown that *in silico* tools in terms of built-in profilers in the QSAR Toolbox can be successfully applied for identification of chemical

hepatocarcinogens by accounting both mechanisms associated with carcinogenesis. A larger part – 73% of the investigated hepatocarcinogens were found to contain structural alert(s) either as parent structure or after metabolic activation able to cause direct damage on DNA. Concerning the second type - nongenotoxic mechanisms it was found that only limited number of alerts can be identified. In conclusion, the obtained result of total 87% correctly identified hepatocarcinogens suggests that combination of both profilers could be used successfully for screening of potential chemical carcinogens.

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