JRC Scientific and Technical Reports

Development of structural alerts for the *in vivo* micronucleus assay in rodents

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EUR 23844 EN - 2009

JRC EUROPEAN COMMISSION

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http:// http://ecb.jrc.ec.europa.eu/qsar/
http://ec.europa.eu/dgs/jrc/

JRC 52274

EUR 23844 EN

ISSN 1018-5593

Luxembourg: Office for Official Publications of the European Communities

Printed in Italy

ABSTRACT

In vivo mutagenicity and carcinogenicity studies are posing a high demand for test-related resources. Among these studies, the micronucleus test in rodents is the most widely used, as follow up to positive *in vitro* mutagenicity results. A recent survey of the (Q)SAR models for mutagenicity and carcinogenicity has indicated that no (Q)SAR models for *in vivo* micronucleus are available in the public domain. Therefore, the development and extensive use of estimation techniques such as (Q)SARs, read-across and grouping of chemicals, promises to have a huge animal saving potential for this endpoint. In this report, we describe the identification of structural alerts for the *in vivo* micronucleus assay, and provide the list of underlying chemical structures. These structural alerts provide a coarse-grain filter for the preliminary screening of potential *in vivo* mutagens.

LIST OF ABBREVIATIONS

EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
HOMO	Highest Occupied Molecular Orbital
ISS	Istituto Superiore di Sanita'
JRC	Joint Research Centre
LUMO	Lowest Unccupied Molecular Orbital
OECD	Organisation for Economic Cooperation and Development
(Q)SAR	(Quantitative) Structure-Activity Relationship
REACH	Registration Evaluation and Authorisation of CHemicals
ROC	Receiver Operating Curve
SA	Structural Alert
SA_BB	Benigni-Bossa structural alerts for mutagnicity / carcinogenicity in Toxtree
SA_Mic	Structural alerts refers for the *in vivo* micronucleus assay in Toxtree
SA_Prot	Structural alerts for protein binding in the OECD QSAR Toolbox

CONTENTS

1. Introduction

Mutagenicity testing is an important part of the regulatory hazard assessment of chemicals. It is undertaken for two main reasons: a) to detect chemicals that might cause genetic damage in germ cells, and thus increase the burden of heritable (genetic) disease in the human population; and b) to detect chemicals that might be carcinogenic (based on the assumption that mutagenesis, for example in somatic cells, is a key event in the process of carcinogenesis). Since no method is able alone to detect all possible genotoxic events, a wide array of test systems has been developed and accepted internationally in regulatory schemes.

Most often, these methods are used within a 2-tiered integrated testing approach: Tier 1 includes *in vivo* assays, and Tier 2 includes *in vivo* assays. As a matter of fact, mutagenicity testing was the first toxicity endpoint for which *in vivo* assays were accepted for regulatory testing, some 25 years ago. The latter usually comprise bacterial mutagenicity and cytogenetics tests, although gene mutation testing in cultured mammalian cells is sometimes also undertaken.

Tier 2 of the testing strategy involves the use of short-term *in vivo* studies (usually a bone-marrow cytogenetics assay) to assess whether any potential for genotoxicity detected at the Tier 1 *in vivo* stage is actually expressed in the whole animal. Thus, negative results *in vivo* are usually considered sufficient to indicate lack of mutagenicity, whereas a positive result is not considered sufficient to indicate that the chemical represents a mutagenic hazard (i.e. it could be a false positive). The above approach to genotoxicity testing has been adopted throughout the EU[1], and has been recommended internationally as part of the strategy for predicting and quantifying mutagenic and carcinogenic hazard (Ashby et al., 1996; Combes et al., 2007; Kirkland and Speit, 2008; Lilienblum et al., 2008).

[1] http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r7a_en.pdf?vers=20_08_08

According to an assessment carried out by the former European Chemicals Bureau (ECB), the *in vivo* mutagenicity studies, shortly followed by carcinogenicity, are posing high demand for test-related recourses (Pedersen et al., 2003; Van der Jagt et al., 2004). Among those, the micronucleus test in rodents is the most widely used, as follow up to positive *in vivo* mutagenicity results. A recent survey of the (Q)SAR models for mutagenicity and carcinogenicity (performed jointly by ISS and the JRC) has indicated that no (Q)SAR models for *in vivo* micronucleus are available in the public domain (Benigni et al., 2007): therefore, the development and extensive use of estimation techniques such as (Q)SARs, read-across and grouping of chemicals, might have a huge saving potential for this endpoint.

In this report, we describe: a) the collection of data on chemicals tested with the *in vivo* micronucleus assay; b) preliminary analyses of the data; c) the identification of Structural Alerts (SA) proper to this toxicological endpoint. First, some background information on the concept of SA is provided.

2. Structural alerts

The SAs for a toxicological endpoint are molecular functional groups or substructures known to be linked to that type of toxicity. The SAs are a coarse-grained approach to the use of Structure-Activity Relationships (SAR) to understand the toxicity mechanisms and to predict the toxic activity of chemicals. Because of their nature, the SAs have the role of pointing to chemicals potentially toxic, whereas no conclusions or indications about nontoxic chemicals are possible (except by exclusion) (Benigni and Bossa, 2006; Benigni and Bossa, 2008).

A set of chemicals characterized by the same SA constitute a family (class) of compounds that share the same mechanism of action. The reactivity of a SA can be modulated or abolished by the remaining part of the molecule in which the SA is embedded. At a coarse-grain level, such modulating effects can be represented by other molecular substructures (e.g., bulky groups ortho to an aromatic amine group) that are known to have an influence on the reactivity of the SA. Usually, the knowledge on the modulating substructures is quite limited for most of the SAs, thus it provides limited help in deciding which chemicals in a class will actually be toxic and *viceversa*. A powerful generalization of the Structure-Activity Relationships is provided by the Quantitative Structure-Activity Relationship (QSAR) analysis, which produces a mathematical model that links the biological activity to a limited number of physical chemical or other molecular properties (descriptors) with general relevance. Since most of the descriptors have continuous values, the QSARs provide fine-tuned models of the biological activity, and can give account of subtle differences. General introductions on QSAR are given elsewhere (Hansch and Leo, 1995, Hansch et al., 2002). Thus the SAs are not a discriminant model on the same ground of the QSAR models: the latter produce estimates for both positive and negative chemicals, as well as for the gradation of toxic potency.

The main role of the SAs is that of preliminary, or large-scale screenings. They are excellent tools for coarse-grain characterization of chemicals, including: description of sets of chemicals, preliminary hazard characterization, category formation and priority setting (enrichment). Since fine-tuned QSARs do not exist for many types of chemicals, the models based on SAs hold a special place in predictive toxicology. The

knowledge on the action mechanisms as exemplified by the SAs is routinely used in SAR assessment in the regulatory context (see, for example, the mechanistically-based reasoning as presented in Woo et al. (2002). In addition, the SAs are at the basis of popular commercial (e.g., DEREK, by Lhasa Ltd.[2]) and non-commercial software systems (e.g., Oncologic, by US Environmental Protection Agency [EPA][3]).

Recently, as follow-up of the collaboration between ISS and JRC, a rulebase for mutagens and carcinogens has been designed and implemented in the software Toxtree 1.51. It uses a structure-based approach consisting of a new compilation of SAs for carcinogenicity and mutagenicity. It also offers three mechanistically based QSARs for congeneric classes (aromatic amines and aldehydes) (Benigni et al., 2008a). Toxtree 1.51 is freely available from the JRC website.[4]

[2] http://www.lhasalimited.org/
[3] http://www.epa.gov/oppt/newchems/tools/oncologic.htm
[4] http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE

3. Development of structural alerts for the *in vivo* micronucleus assay

3.1 Data

The compilation of SAs for the *in vivo* micronucleus assay in rodents provided here, is based on both the existing knowledge on the mechanisms of toxic action and a structural analysis of the chemicals tested in the assay.

The *in vivo* micronucleus data in the public domain is quite limited. A search of the Chemical Carcinogenesis Research Information System (CCRIS) at the Toxnet website with the query: "in vivo micronucleus" points only to 240 chemicals.[5]

For this work, the remarkably larger commercial database by Leadscope Inc., called "FDA SAR Genetox Database" was used.[6] This database contains more than 700 chemicals tested in *in vivo* micronucleus with rodents, and includes data from both the public domain and the US Food and Drug Administration (FDA) files. A large majority of data were based on the analysis of micronuclei in bone marrow cells; for details on the technique, see for example, Krishna and Hayashi (2000).

3.1 Preliminary analyses

Since the main role of the *in vivo* micronucleus assay in regulatory schemes is that of confirming (or disproving) the positive *in vitro* results, it is of interest to check how the *in vivo* micronucleus results relate to the rodent carcinogenicity data and to the primary *in vitro* prediction test, i.e., the *Salmonella typhimurium* (Ames) test.

Tables I and II display the relationships between the *in vivo* micronucleus ad the two reference tests. The results for rodent carcinogenicity and the Ames test were retrieved from the freely available ISSCAN v3a database,[7] which is characterized by:

[5] http://toxnet.nlm.nih.gov/cgi-bin/sis/search
[6] http://www.leadscope.com/product_info.php?products_id=77
[7] http://www.iss.it/ampp/dati/cont.php?id=233&lang=1&tipo=7

a) the high quality of both chemical and biological information; b) the QSAR-ready format (Benigni et al., 2008b). Obviously, the total numbers of chemicals in the two tables are relative only to those chemicals tested in both systems.

Table I. Contingency table comparing the results of the rodent carcinogenicity test with the micronucleus test

Carcinogenicity test	**Micronucleus test**		
	Negative	Positive	Total
Negative	30	10	40
Positive	86	57	143
Total	116	67	183

Table II: Contingency table comparing the results of the *Salmonella typhimurium* assay with the micronucleus test

Salmonella assay	**Micronucleus test**		
	Negative	Positive	Total
Negative	74	36	110
Positive	41	34	75
Total	115	70	185

Table I shows that is the *in vivo* micronucleus assay is poorly sensitive to the rodent carcinogens: about 60% of the rodent carcinogens are not detected by the micronucleus. The poor sensitivity of the micronucleus assay to potential genotoxins is also apparent from Table II.

It should be emphasized that the present results obtained with the large Leadscope micronucleus database are in agreement with previous analyses based on smaller datasets in the public domain (Benigni, 1995).

In a second round of analyses, the extent to which the micronucleus data are related to well established indicators of DNA and protein binding was checked. This in view of the plethora of the reported mechanisms of micronucleus induction. As a matter of fact, micronuclei are markers of both aneugenic (change in the chromosomes number, usually by loss) and clastogenic (chromosome breakage) effects. It is generally assumed that such effects are generated through a range of different pathways. Evidence (mainly gathered from *in vitro* studies) indicates that micronuclei can be induced e.g., by typical DNA-attacking agents (e.g., alkylating agents like methylmethane sulfonate), by mitotic spindle poisons (e.g., colcemide, vincristine), or by inhibitors of cytokinesis (e.g., cytochalasin B). The latter effects are probably due to interference with proteins. Other chemicals are thought to be clastogenic through aspecific disturbance of cytokinesis due to lipophilicity (Dorn et al., 2007).

The relative influence of DNA and protein binding on micronucleus generation was checked by recording the distribution of structural alerts for the two effects in the Leadscope *in vivo* micronucleus database. As probes for DNA binding, we used the structural alerts for carcinogenicity / mutagenicity implemented in Toxtree 1.51. As a matter of fact, the large majority of these alerts refer to genotoxic carcinogenicity, which is assumed to be caused through direct interaction with DNA (Benigni and Bossa, 2008). As probes for protein binding, we used the alerts implemented in the Organisation for Economic Cooperation and Development (OECD) QSAR Toolbox.[8] These alerts were mainly developed from the mechanistic knowledge on skin sensitization, and model the covalent binding to proteins.

The results of the above analysis is displayed in Figure 1 as a ROC graph. It appears that the structural alerts for carcinogenicity / mutagenicity correlate to some extent with the induction of micronuclei, whereas those for protein covalent binding show no correlation (in the graph, they are on the diagonal line which represents random results).

[8] http://www.oecd.org/document/23/0,3343,en_2649_34379_33957015_1_1_1_1,00.html

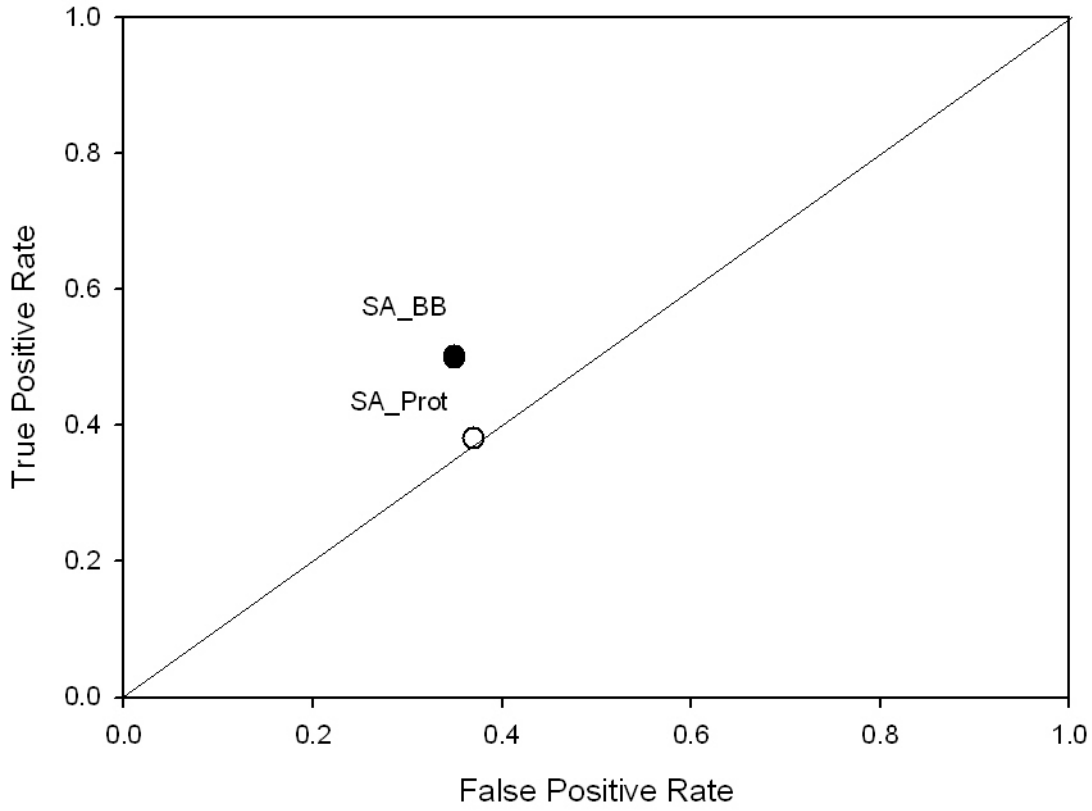

Figure 1. Receiver Operating Curve showing the concordance of two sets of structural alerts with the results of the *in vivo* micronucleus assay

(SA_BB refers to the Benigni-Bossa alerts in Toxtree; SA_Prot refers to the alerts for protein binding in the OECD QSAR Toolbox)

3.3 Structural Alerts for *in vivo* micronucleus assay

Since the above analyses pointed to genotoxic effects as an important determinant of micronuclei induction, we developed the list of Structural alerts for *in vivo* micronucleus using the carcinogenicity / mutagenicity alerts in Toxtree as a core , and then searching for additional substructures specific to the micronucleus-positive chemicals. From the Toxtree alerts for carcinogenicity / mutagenicity, we excluded four alerts specific for non-genotoxic mechanisms of carcinogenicity.

Using linear discriminant analysis as an analytical tool and ROC plots as a graphical tool, a series of additional substructures were added / removed to / from the Toxtree alerts in order to increase sensitivity and specificity. In these exploratory analyses, we

screened the very large collection of substructural patterns and functional groups (more than 27,000) contained in the software Leadscope Enteprise 2.4.15-6. We also re-checked the Toolbox protein binding alerts for individual substructures related with micronucleus induction.

The result is the optimized list of alerts in Appendix 1. Together with the Toxtree alerts, it contains five additional substructures identified in the course of this research. For the sake of clarity, the codes of the alerts in Toxtree are maintained, whereas the five additional alerts have new codes.

Figure 2 displays the agreement between the alerts for *in vivo* micronucleus, and the experimental results for this endpoint. Out of 547 negatives, the specificity of the SAs is 0.57. The sensitivity is 0.65 out of 182 positives. The overall accuracy is 0.59. For a comparison, the ROC graph shows the newly developed alerts for micronucleus together with those for DNA and protein binding. It appears that the performance of the final list of alerts is considerably higher than that of the DNA binding and Protein binding alerts.

Table III gives the true positive rate for the individual alerts.

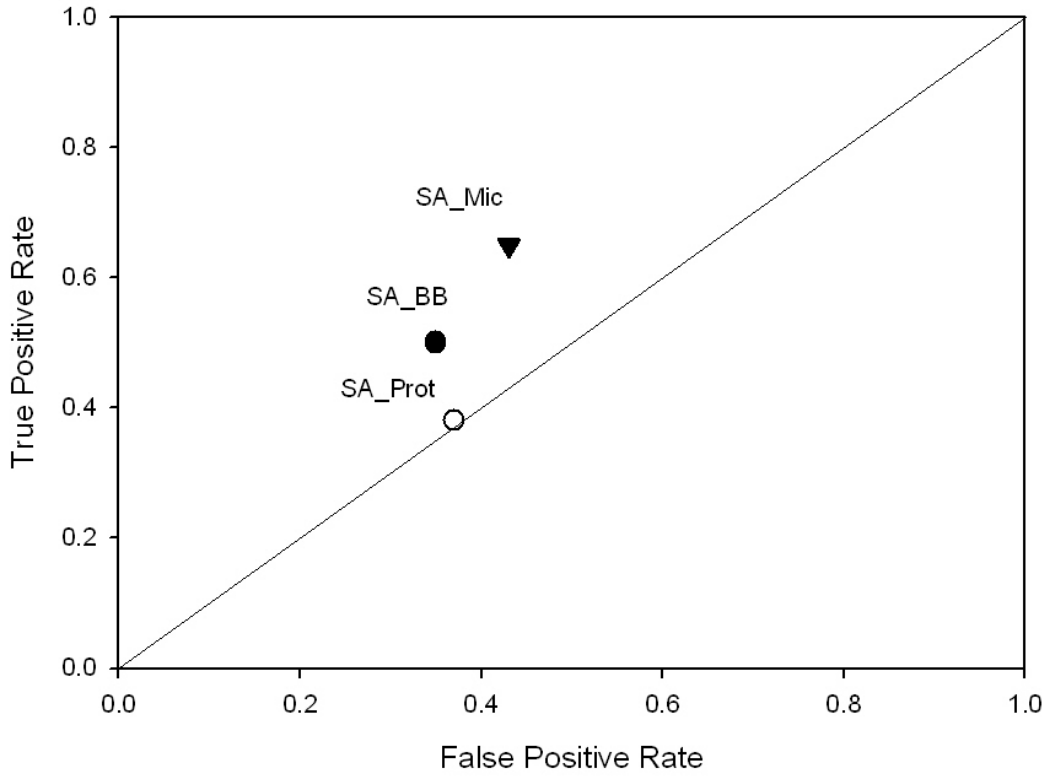

Figure 2 Receiver Operating Curve showing the concordance of structural alerts for the *in vivo* micronucleus assay with the experiemtnal results for this assay *(SA_Mic refers to the in vivo micronucleus alerts in Toxtree)*

Table III: Characterisation of Structural Alerts.

STRUCTURAL ALERT		No. Substances fired	No. of positive substances	True Positives (%)
SA_1:	acyl halides	0	0	
SA_2:	alkyl (C<5) or benzyl ester of sulphonic or phosphonic acid	4	3	75
SA_3:	N-methylol derivatives	1	0	0
SA_4:	monohaloalkene	3	3	100
SA_5:	S or N mustard	4	4	100
SA_6 :	propiolactones or propiosultones	0	0	
SA_7:	epoxides and aziridines	20	12	60
SA_8:	aliphatic halogens	35	9	26
SA_9:	alkyl nitrite	1	1	100
SA_10:	α, β unsaturated carbonyls	58	16	28
SA_11:	simple aldehyde	9	2	22
SA_12:	quinones	9	4	44
SA_13:	hydrazine	6	0	0
SA_14:	aliphatic azo and azoxy	0	0	
SA_15:	isocyanate and isothiocyanate groups	2	0	0
SA_16:	alkyl carbamate and thiocarbamate	9	2	22
SA_18:	Polycyclic Aromatic Hydrocarbons	1	1	100
SA_19:	heterocyclic Polycyclic Aromatic Hydrocarbons	7	0	0
SA_21:	alkyl and aryl N-nitroso groups	6	5	83
SA_22:	azide and triazene groups	2	2	100
SA_23:	aliphatic N-nitro group	2	1	50

SA_24: α, β unsaturated aliphatic alkoxy group	1	1	100
SA_25: aromatic nitroso group	0	0	
SA_26: aromatic ring N-oxide	0	0	
SA_27: nitro-aromatic	17	2	12
SA_28: primary aromatic amine, hydroxyl amine and its derived esters	50	19	38
SA_28bis: aromatic mono- and dialkylamine	5	2	40
SA_28tris: aromatic N-acyl amine	2	0	0
SA_29: aromatic diazo	8	4	50
SA_30: coumarins and Furocoumarins	3	0	0
SA_32: 1,3-dialkoxy-benzene	6	5	83
SA_33: 1-phenoxy-benzene	5	4	80
SA_34: hacceptor-path3-hacceptor	163	55	34
SA_35: cxolane	21	9	43
SA_36: carbodiimides	2	2	100

3.4 Further analyses on the alerts for micronucleus

A striking evidence in Table III is the relatively low percentage of true positives identified by many SAs. In other words, often the toxic potential of the alerts is not translated into actual toxicity in the experimental system. For a comparison, the True Positive Rate of the various alerts for mutagenicity / carcinogenicity in Toxtree is remarkably higher, ranging from 70 to 100% (Benigni and Bossa, 2008).

The above result contributes to better understand the evidence in Tables I and II, where it appears that the micronucleus assay has many more negatives than the carcinogenicity bioassay and the Salmonella mutagenicity test. Table III indicates that the low sensitivity of the micronucleus assay is largely due to the fact that often,

chemical functionalities and substructures which are supposed to be reactive do not exert their potential reactivity in this experimental system.

The issue of the low sensitivity of the micronucleus assay has been recognized by scientists involved in research aimed at improving the available short-term mutagenicity assays; as a matter of fact, validation of further, more sensitive *in vivo* assays (e.g., *in vivo* Comet assay) is presently in progress (Kirkland and Speit, 2008).

In the context of this research, we investigated if a general effect of bioavailability on the limited sensitivity of micronucleus was apparent. To this aim, we considered two chemical descriptors well known as to be linked to bioavailability: logP (hydrophobicity) and Molar Refractivity (MR) (Hansch and Leo, 1995). The two descriptors were calculated with the C-QSAR software (Daylight, Inc.)[9] for all the chemicals in the micronucleus database. For the two parameters, Table IV reports the ranges of values for positive and negative micronucleus results.

Table IV: Ranges of C-logP and C-MR in chemicals assayed with the micronucleus test

	C-logP	C-MR (x 10-1)
Micronucleus Negatives	-18.64 – 20.43	0.10 – 33.73
Micronucleus Positives	-9.58 – 15.23	0.15 – 32.91

It appears that the micronucleus positives cover a more limited range of logP values than the micronucleus negatives; however, the consideration of exclusion values for logP in combination with the SAs did not improve the overall performance (results not shown).

Whereas no general effect of logP (or MR) was found, analyses on the individual chemical classes showed that logP cut-offs can be identified for the classes of Nitroaromatics (Negatives at logP > 0.0), Aromatic Diazo (Negatives at logP < 3.7),

[9] http://www.daylight.com/about/index.html

and Oxolanes (Negatives at logP > 1.5). The consideration of these cut-offs increases the specificity of the SAs from 0.57 to 0.60.

The above result suggests a possible strategy to understand and modeling the many negative results observed with the micronucleus. Since the bone marrow (main target of the test) is an organ easily accessible by the blood stream, it can be hypothesized that the lack of effect shown by several chemicals with SAs (hence potentially reactive) is due to the many possible targets for reaction encountered in the *in vivo* situation; this diminishes the probability for the chemicals of reaching, and interacting with the molecular target(s) of the micronucleus test. For example, highly reactive chemicals will probably react with any target encountered in their way (e.g., proteins, water) before reaching the bone marrow. Thus it can be envisaged that QSARs for individual chemical classes should be developed, and that they should consider parameters linked to chemical reactivity (such as HOMO and LUMO energies). It can be hypothesized that the models derived from these QSARs will contribute to modulate the individual SAs.

4. Final considerations

Structural alerts point to classes of chemicals with the potential to cause toxic effects (here, *in vivo* micronucleus). Since this potential is modulated in each molecule by the rest of the structure (e.g., other functional groups, electronic structure, bulky groups), not all chemicals in a class are equally toxic. In the case of the SAs identified in the present study for the *in vivo* micronucleus test, the percentage of chemicals that have SAs but are not active in the test system is particularly high. This evidence agrees with, and rationalizes the notion that this test system is sensitive to genotoxins to a limited extent, and does not respond to a large number of recognized carcinogens and mutagens. For this reason, a positive *in vivo* micronucleus result adds a strong weight to an *in vivo* positive mutagenicity result, whereas a negative *in vivo* micronucleus result has a much lower relevance. The availability of a wider range of *in vivo* mutagenicity assays is a priority for the present regulatory strategies.

Within the above perspective, the SAs identified in this study provide a coarse-grain filter for a preliminary screening of potentially *in vivo* mutagens. In a risk assessment process, further information (e.g., QSARs for individual classes, experiments) is necessary to complete this initial screening step.

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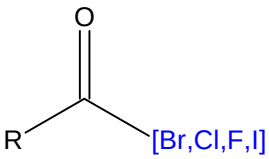
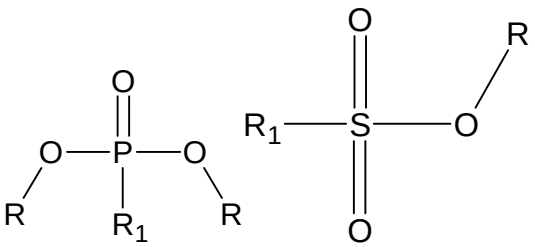
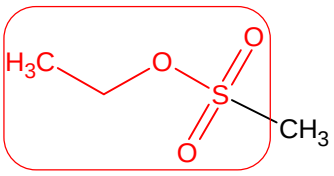
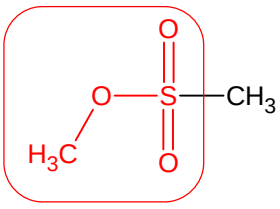
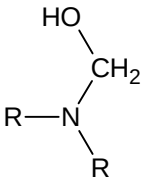
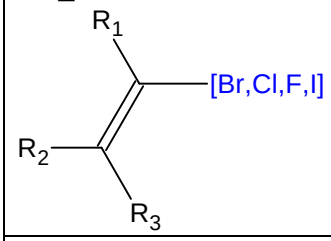
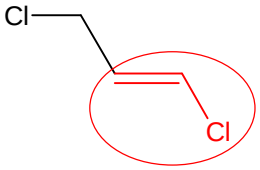
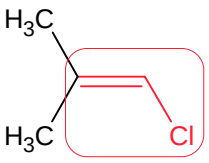
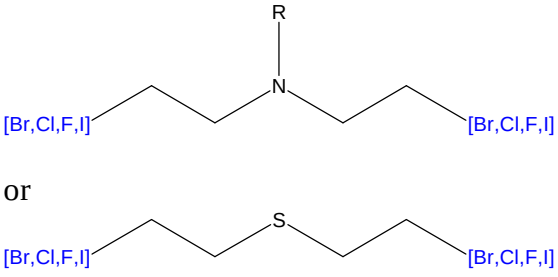
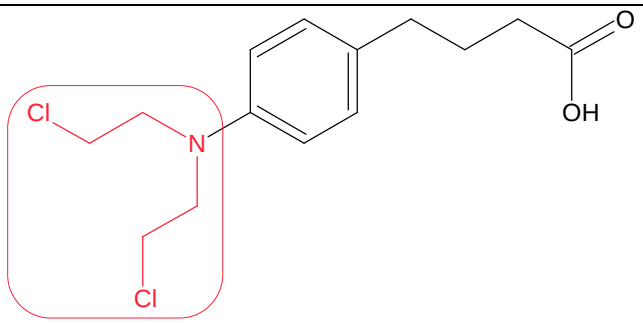
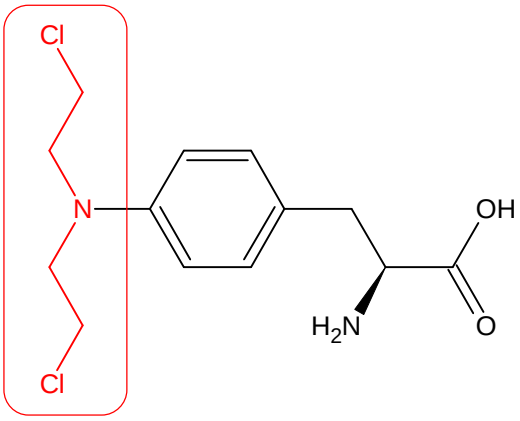
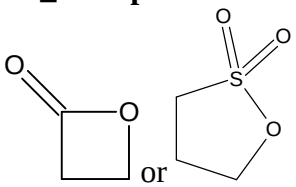
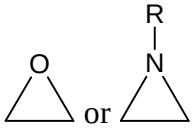
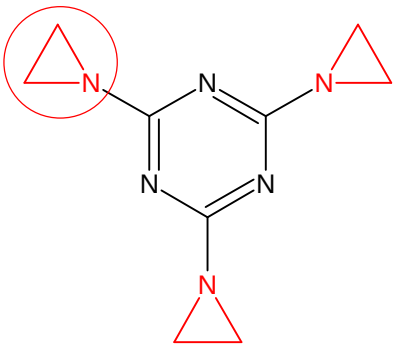
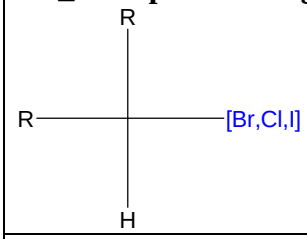
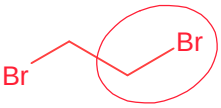
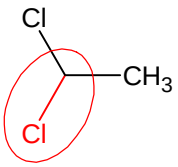
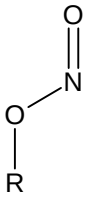
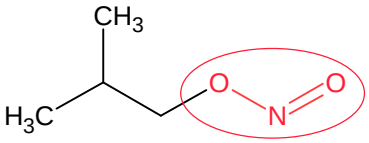
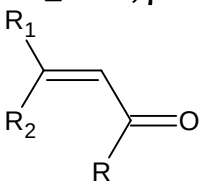
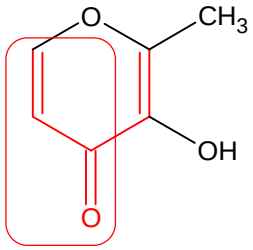
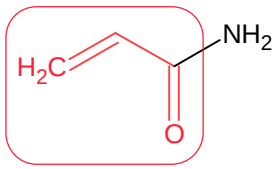
Appendix 1

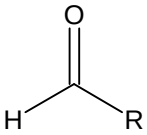
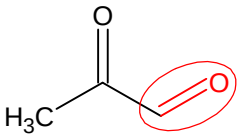
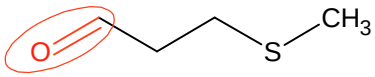
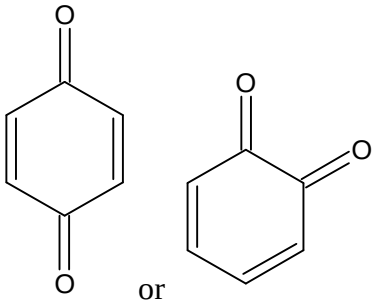
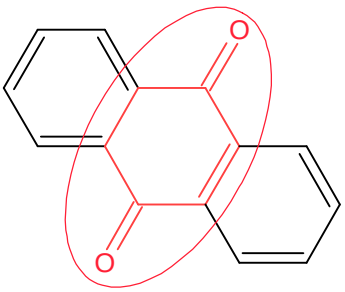
STRUCTURAL ALERT	DETAILS AND EXAMPLES
SA_1: Acyl halides 	R = any atom/group, except OH, SH
	No representatives
SA_2: alkyl (C<5) or benzyl ester of sulphonic or phosphonic acid 	R= Alkyl with C<5 (potentially substituted by halogens), or benzyl R1= any atom/group except OH, SH, O^-, S^-
	 Name: Ethyl Methanesulfonate CAS: 62-50-0 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Methyl Methanesulfonate CAS: 66-27-3 In vivo Micronucleus (Rodent): Positive Reference: CCRIS

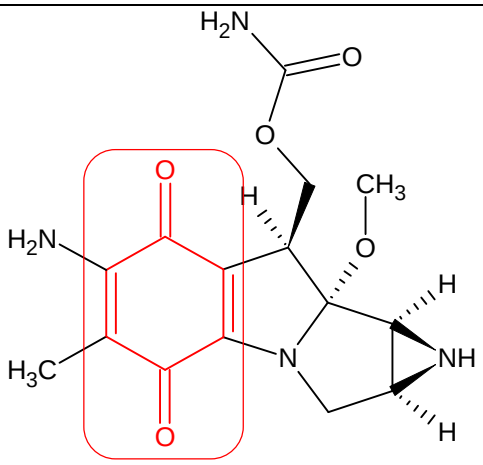
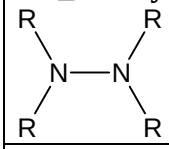
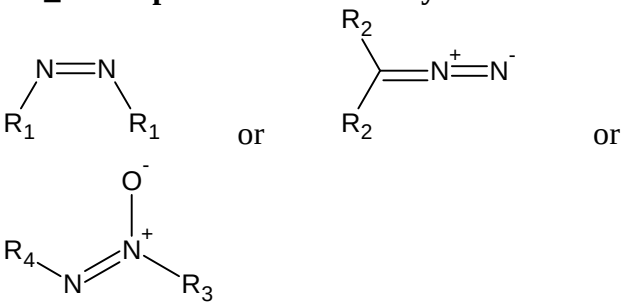
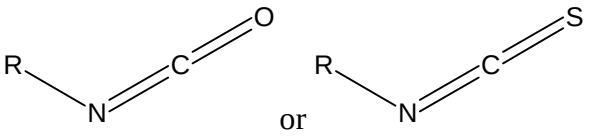
SA_3: N-methylol derivatives 	R = any atom/group
	No positive representative
SA_4: Monohaloalkene 	R_1, R_2 (or R_3) = H or Alkyl R_3 (or R_2) = any atom/group except halogens
	 Name: 1,3-dichloropropene CAS: 542-75-6 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Dimethylvinyl Chloride CAS: 513-37-1 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_5: S or N mustard 	R = any atom/group

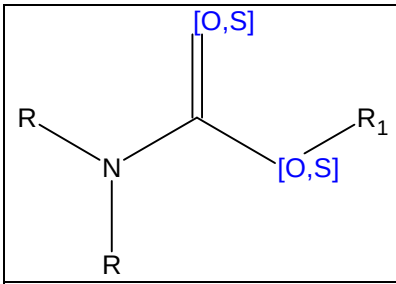
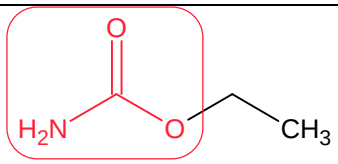
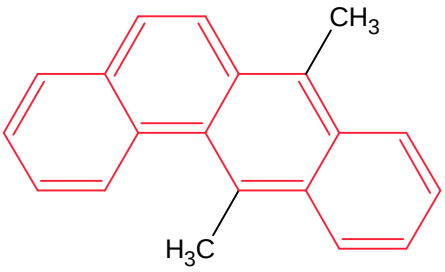
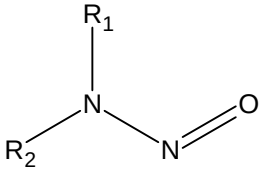
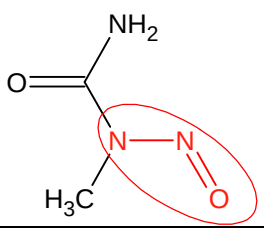
	 Name: Chloroambucil CAS: 305-03-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Melphalan CAS: 148-82-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_6 Propiolactones or propiosultones 	Any substance with the displayed substructures
	No representatives
SA_7:Epoxides and aziridines 	R = any atom/group
	

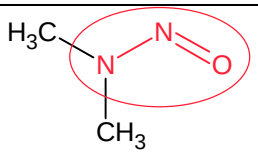
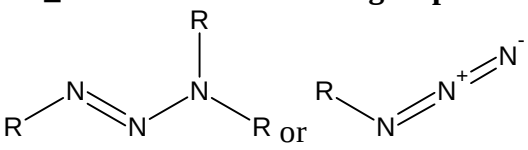
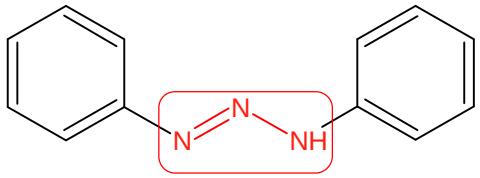
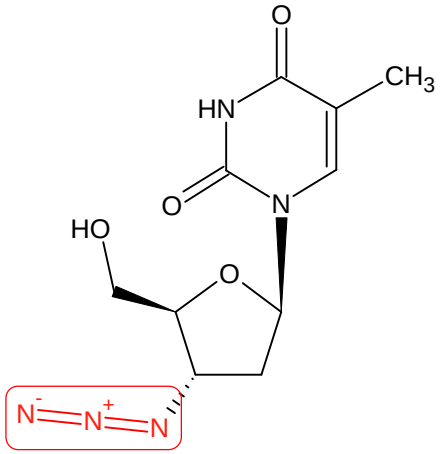
	Name: Ethylene Oxide CAS: 75-21-8 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
	 Name: Triethylenemelamine CAS: 51-18-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_8: Aliphatic halogens 	R = any atom/group
	 Name: 1,2-dibromoethane CAS: 106-93-4 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: 1,1-dichloroethane CAS: 75-34-3 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_9: Alkyl nitrite	R= any alkyl group

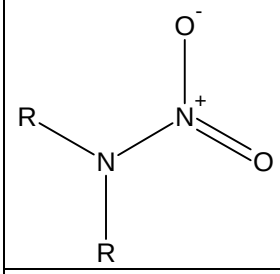
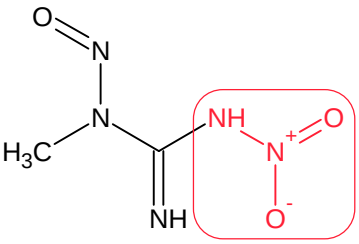
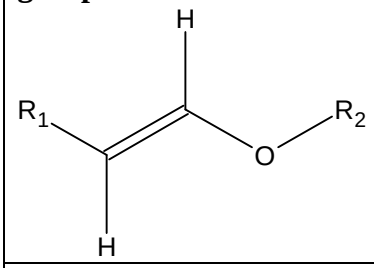
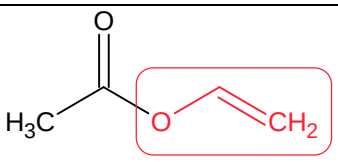
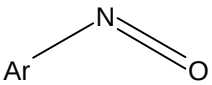
	
	 Name: Isobutyl Nitrite CAS: 542-56-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_10: α, β unsaturated carbonyls 	R1 and R2 = any atom/group, except alkyl chains with C>5 or aromatic rings. R= any atom/group, except OH, O^-
	 Name: Maltol CAS: 118-71-8 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
	 Name: Acrylamide CAS: 79-06-1 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_11: Simple aldehyde	R= aliphatic or aromatic carbon α,β unsaturated aldehydes are excluded

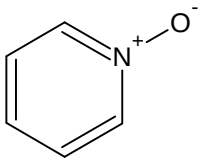
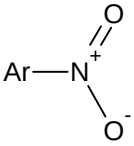
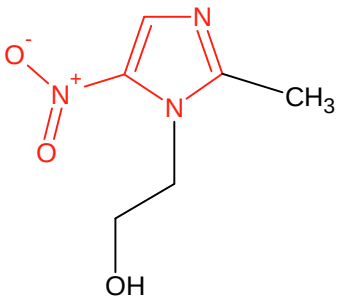
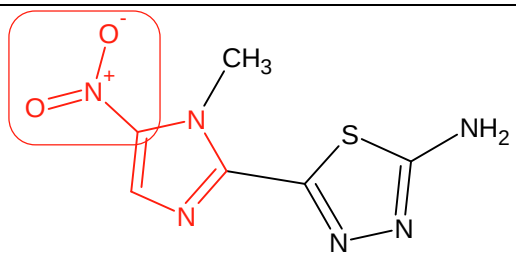
	
	 Name: Pyruvaldehyde CAS: 78-98-8 In vivo Micronucleus (Rodent): Positive Reference: Leadscope
	 Name: 3-(methylthio)propionaldehyde CAS: 3268-49-3 In vivo Micronucleus (Rodent): Positive Reference: Leadscope
SA_12: Quinones 	Any substance with the displayed substructures
	 Name: 9,10-Anthraquinone CAS: 84-65-1 In vivo Micronucleus (Rodent): Positive Reference: NTP

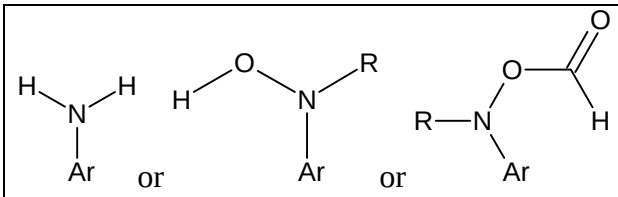
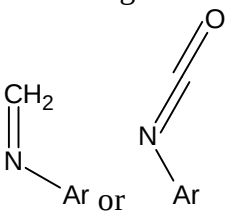
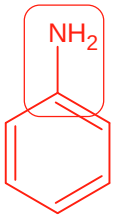
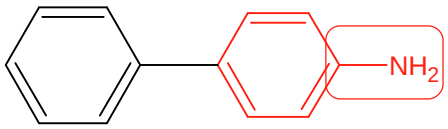
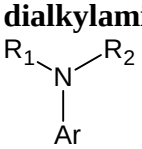
	 Name: Mitomycin C CAS: 50-07-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
SA_13: Hydrazine 	R= any atom/group
	No positive representative
SA_14: Aliphatic azo and azoxy 	R1= Aliphatic carbon or hydrogen R2, R3 = Any atom/group R4 = Aliphatic carbon
	No representatives
SA_15: isocyanate and isothiocyanate groups 	R= any atom/group
	No positive representative
SA_16: alkyl carbamate and thiocarbamate	R = Aliphatic carbon or hydrogen R1 = Aliphatic carbon

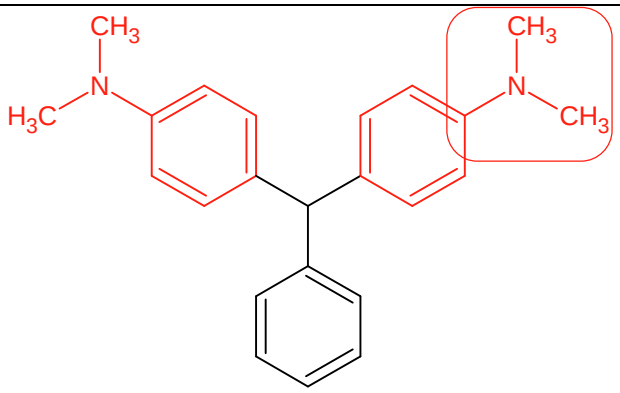
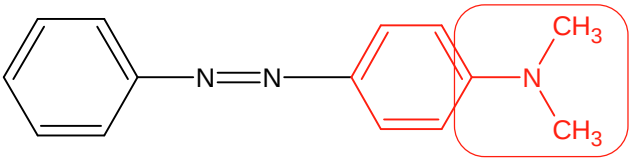
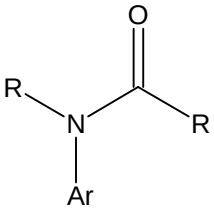
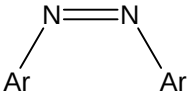
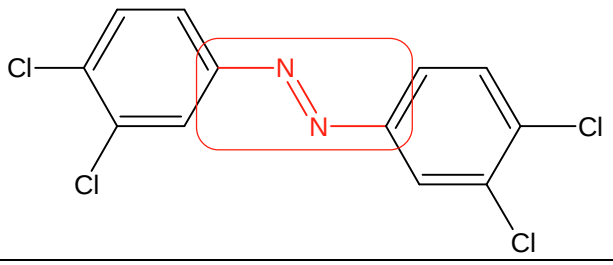
	
	 Name: Urethane CAS: 51-79-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
SA_18: Polycyclic Aromatic Hydrocarbons	Three or more fused rings, not heteroaromatic
	 Name: 7,12-Dimethylbenz(a)anthracene CAS: 57-97-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
SA_19: Heterocyclic Polycyclic Aromatic Hydrocarbons	Three or more fused rings, heteroaromatic
	No positive representative
SA_21: alkyl and aryl N-nitroso groups 	R1= Aliphatic or aromatic carbon, R2= Any atom/group
	

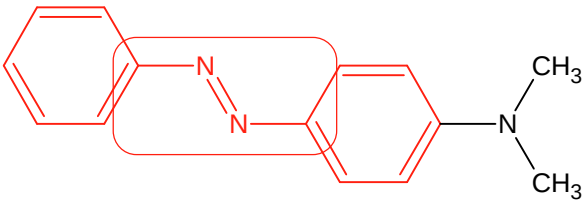
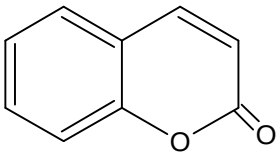
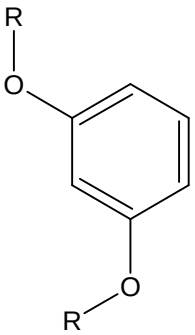
	Name: N-methyl-N-nitrosourea CAS: 684-93-5 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: N-nitrosodimethylamine CAS: 62-75-9 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_22: azide and triazene groups 	R= Any atom/group
	 Name: Diazoaminobenzene CAS: 136-35-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
	 Name: Zidovudine CAS: 30516_87-1

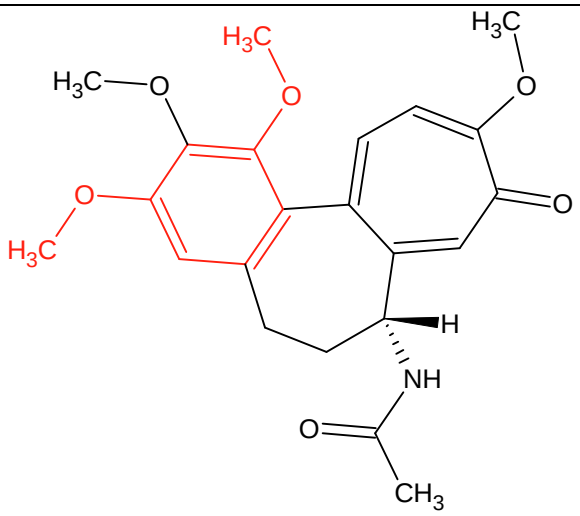
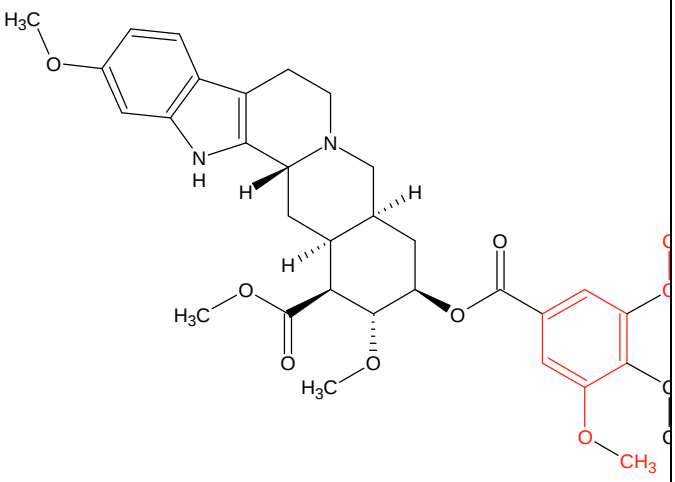
	In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_23: aliphatic N-nitro group 	R= Aliphatic carbon or hydrogen
	 Name: N-methyl-N'-nitro-N-nitrosoguanidine CAS: 70-25-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_24: α, β unsaturated aliphatic alkoxy group 	R1= Any aliphatic Carbon R2 = Aliphatic or aromatic carbon
	 Name: Vinyl Acetate CAS: 108-05-4 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_25: aromatic nitroso group 	Ar = Any aromatic/heteroaromatic ring
	No positive representative

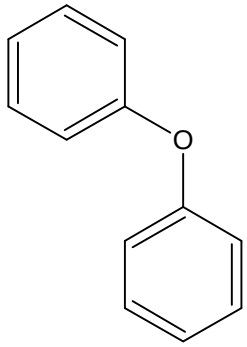
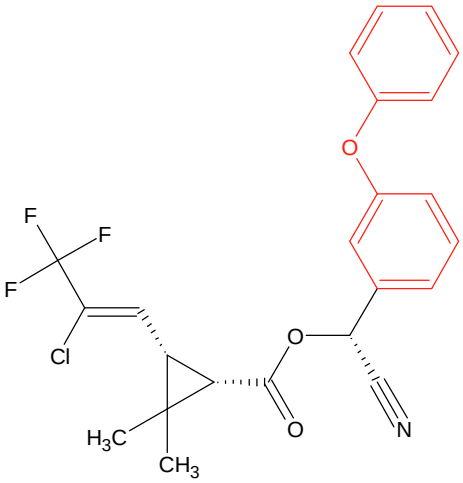
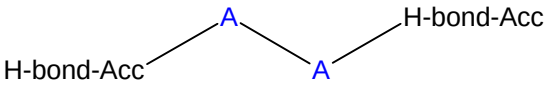
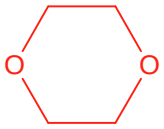
SA_26: aromatic ring N-oxide 	Any aromatic or heteroaromatic ring
	No positive representative
SA_27: Nitro-aromatic 	Ar = Any aromatic/heteroaromatic ring • Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded. • Chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded.
	 Name: Metronidazole CAS: 443-48-1 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
	 Name: CL 64855 CAS: 19622-55-0 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_28: primary aromatic amine, hydroxyl amine and its derived esters	Ar = Any aromatic/heteroaromatic ring R= Any atom/group • Chemicals with ortho-disubstitution, or

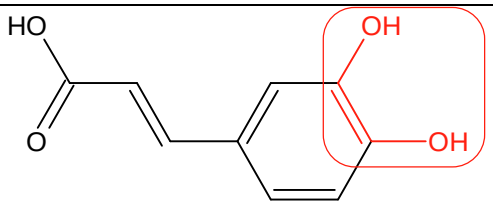
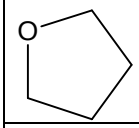
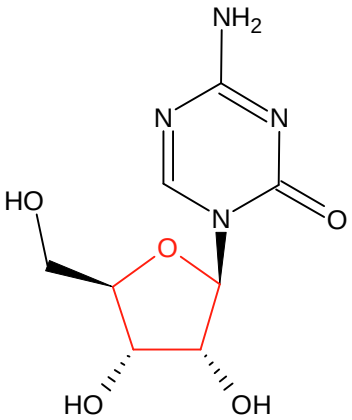
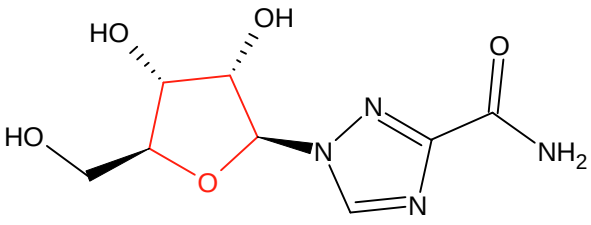
 or or or amine generating group:  or	with an ortho carboxylic acid substituent are excluded. • Chemicals with a sulfonic acid group (-SO3H) on the same ring of the amino group are excluded.
	 Name: Aniline CAS: 62-53-3 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
	 Name: 4-Biphenylamine CAS: 92-67-1 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_28bis: Aromatic mono- and dialkylamine 	Ar = Any aromatic/heteroaromatic ring R1 = Hydrogen, methyl, ethyl R2 = Methyl, ethyl • Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded. • Chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded.

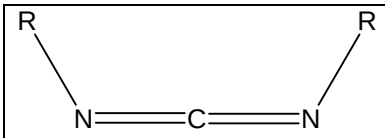
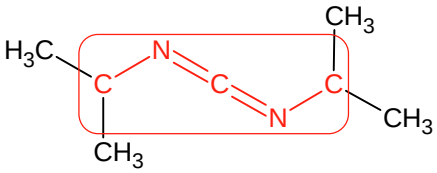
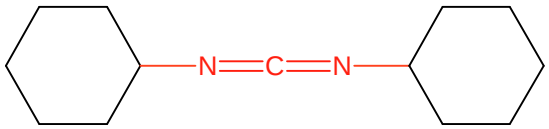
	 Name: Leucomalachite Green CAS: 129-73-7 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: 4-Dimethylaminoazobenzene CAS: 60-11-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_28tris: aromatic N-acyl amine 	Ar = Any aromatic/heteroaromatic ring R = Hydrogen, methyl • Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded. • Chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded.
	No positive representative
SA_29: Aromatic diazo 	Ar = Any aromatic/heteroaromatic ring • Chemicals with a sulfonic acid group (-SO3H) on both rings that contain linked to the diazo group are excluded.
	

	Name: 3,3',4,4'-Tetrachloroazobenzene CAS: 14047-09-7 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: 4-Dimethylaminoazobenzene CAS: 60-11-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_30: Coumarins and Furocoumarins 	Any substance with the displayed substructure
	No positive representative
SA_32: 1,3-dialkoxy-benzene 	R= any alkyl group

	 Name: Colchicine CAS: 64-86-8 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
	 Name: Reserpine CAS: 50-55-5 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_33: 1-phenoxy-benzene	Any substance with the displayed substructure.

	
	 Name: Lambda-cyhalothryn CAS: 91465-08-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_34: hacceptor-path3-hacceptor 	A= Any atom, except Hydrogen H-bond-Acc= Any atom that is a potential Hydrogen bond acceptor
	 Name: p-Dioxane CAS: 123-91-1 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP

	 Name: 3,4-Dihydroxycinnamic acid CAS: 331-39-5 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_35: Oxolane 	Any substance with the displayed substructure.
	 Name: 5-Azacytidine CAS: 320-67-2 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Ribavirin CAS: 36791-04-5 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_36: Carbodiimides	R= any alkyl group

	
	 Name: Diisopropylcarbodiimide CAS: 693-13-0 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Dicyclohexylcarbodiimide CAS: 538-75-0 In vivo Micronucleus (Rodent): Positive Reference: NTP

European Commission

EUR 23844 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: Development of Structural alerts for the *in vivo* micronucleus assay in rodents

Author(s): Benigni R, Bossa C, Tcheremenskaia O and Worth A

Luxembourg: Office for Official Publications of the European Communities

2009 – 42 pp. – 21 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593

Abstract

In vivo mutagenicity and carcinogenicity studies are posing a high demand for test-related resources. Among these studies, the micronucleus test in rodents is the most widely used, as follow up to positive in vitro mutagenicity results. A recent survey of the (Q)SAR models for mutagenicity and carcinogenicity has indicated that no (Q)SAR models for *in vivo* micronucleus are available in the public domain. Therefore, the development and extensive use of estimation techniques such as (Q)SARs, read-across and grouping of chemicals, promises to have a huge animal saving potential for this endpoint. In this report, we describe the identification of structural alerts for the *in vivo* micronucleus assay, and provide the list of underlying chemical structures. These structural alerts provide a coarse-grain filter for the preliminary screening of potential *in vivo* mutagens.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.