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Hazard assessment of nitrosamine and nitramine by-products of amine-based CCS: Alternative approaches

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ABSTRACT

Carbon capture and storage (CCS) technologies are considered vital and economic elements for achieving global CO_2 reduction targets, and is currently introduced worldwide (for more information on CCS, consult for example the websites of the International Energy Agency (http://www.iea.org/topics/ccs/) and the Global CCS Institute (http://www.globalccsinstitute.com/)). One prominent CCS technology, the amine-based post-combustion process, may generate nitrosamines and their related nitramines as by-products, the former well known for their potential mutagenic and carcinogenic properties. In order to efficiently assess the carcinogenic potency of any of these by-products this paper reviews and discusses novel prediction approaches consuming less time, money and animals than the traditionally applied 2-year rodent assay. For this, available animal carcinogenicity studies with N-nitroso compounds and nitramines have been used to derive carcinogenic potency values, that were subsequently used to assess the predictive performance of alternative prediction approaches for these chemicals. Promising cancer prediction models are the QSARs developed by the Helguera group, *in vitro* transformation assays, and the *in vivo* initiation-promotion, and transgenic animal assays. All these models, however, have not been adequately explored for this purpose, as the number of N-nitroso compounds investigated is yet too limited, and therefore further testing with relevant N-nitroso compounds is needed.

1. Introduction

Carbon capture and storage (CCS; alternatively referred to as carbon capture and sequestration), is a means of mitigating the contribution of fossil fuel emissions to climate change. The increase of the CO_2 concentration in our atmosphere leads to climate change and ocean acidification. CCS is based on capturing CO_2 from large emissions sources such as fossil fuel power plants, and storing it subsurface, from where it will not enter the atmosphere.

A leading CCS technology is the amine-based post-combustion process, specifically for retrofit options for existing large scale stationary emitters (IPCC, 2005). Post-combustion capture involves separating the CO_2 from other exhaust gases after combustion of the fossil fuel. A CO_2 rich gas stream, such as a power plant's flue gas, is brought into contact with a "lean" amine solution in an absorber. The amine solution binds CO_2 as it passes through, while other gases remain in the flue gas. The CO_2 in the resulting CO_2-saturated amine solution is then removed from the amines, dried and conditioned, after which it is ready for carbon storage. The amines themselves are continuously recycled and re-used. While post-combustion CO_2 capture is technically available for fossil fuel power plants, it has not yet been commercially used for large-scale CO_2 removal (IPCC, 2005).

One of the drawbacks associated with post-combustion amine based CCS technology is, however, the formation of potentially harmful by-products: i.e. degradation products from reactions during the capturing process that are subsequently emitted into the atmosphere. Of particular concern among these by-products are nitrosamines and their related nitramines (de Koeijer et al., 2013), both by-products of CCS, of which the former are well known for their potential mutagenic as well as carcinogenic properties (IARC, 2012). So far the nitrosamines dimethylnitrosamine (NDMA), diethylnitrosamine (NDEA), diethanolnitrosamine (NDELA), nitrosomorpholine (NMOR), nitrosopiperidine (NPIP) and nitrosopiperazine (NPIPz), and the nitramines methylnitramine (MA-NO_2), dimethylnitramine (DMA-NO_2) and methylethylnitramine (MEA-NO_2) have been identified as by-products formed in post-combustion amine based CCS technology (Bråten et al., 2008). Structurally, nitrosamines and nitramines are related, the difference being that the former possesses a N-nitroso-group and the latter a N-nitro-group (see Fig. 1). Nitrosamines are N-nitroso compounds of which the radical groups are alkyl or aryl groups

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0273-2300/

Table 1
Basic information on the N-nitroso compounds and nitramines discussed in this paper (sorted in alphabetic order on abbreviation).

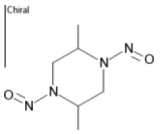
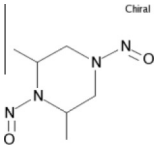
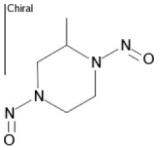
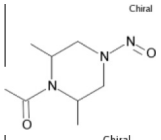
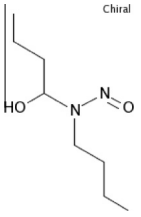
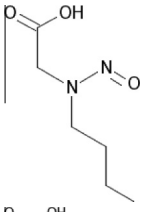
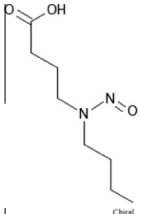
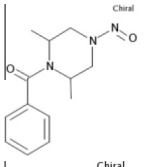
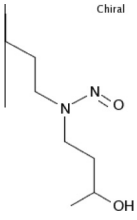
Name	Abbreviation	CAS-RN	MW	Structure
2,5-Dimethyl-1,4-dinitrosopiperazine	2,5-DMDNPIPz	55556-88-2	172.19	
2,6-Dimethyl-1,4-dinitrosopiperazine	2,6-DMDNPIPz	55380-34-2	172.19	
2-Methyl-1,4-dinitrosopiperazine	2-MDNPIPz	55556-94-0	158.16	
1-Nitroso-4-acetyl-3,5-dimethylpiperazine	AcNM2PIPz	75881-17-3	185.22	
Butylhydroxybutylnitrosamine	BBN	78619-31-5	174.24	
N-butyl-N-(carboxymethyl)nitrosamine	BCMN	61864-02-6	160.17	
Butyl(3-carboxypropyl)nitrosamine	BCPN	38252-74-3	188.12	
1-Nitroso-4-benzoyl-3,5-dimethylpiperazine	BeNM2PIPz	61034-40-0	247.29	
N-Butyl-N-(3-hydroxybutyl)nitrosamine	BHBN-3	40911-07-7	174.24	

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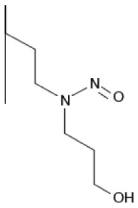
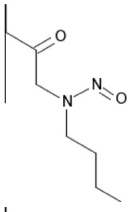
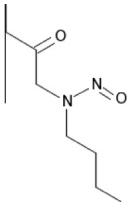
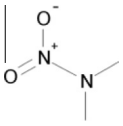
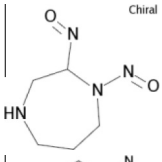
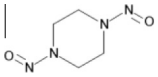
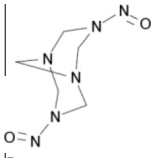
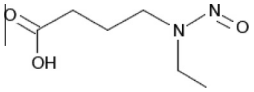
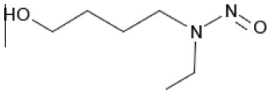
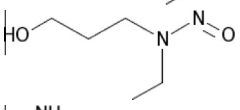
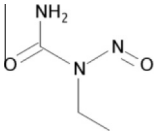
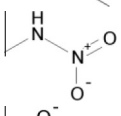
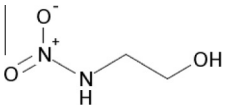
Table 1 (*continued*)

Name	Abbreviation	CAS-RN	MW	Structure
N-Butyl-N-(3-hydroxypropyl)nitrosamine	BHPN	51938-13-7	160.12	
N-Butyl-N-(2-oxobutyl)nitrosamine	BOBN-2	61734-90-5	172.22	
N-Butyl-N-(3-oxobutyl)nitrosamine	BOBN-3	61734-89-2	172.22	
N-methyl-N-nitro-methanamine	DMA-NO_2	4164-28-7	90.08	
1,4-Dinitroso-1,4-diazepane	DNhPIPz	55557-00-1	158.16	
1,4-Dinitrosopiperazine	DNPIPz	140-79-4	144.13	
N,N-dinitrosopentamethylenetetramine	DNPT	101-25-7	185.18	
N-Ethyl-N-(3-carboxypropyl)nitrosamine	ECPN	54897-63-1	160.17	
N-Ethyl-N-(4-hydroxybutyl)nitrosamine	EHBN	54897-62-0	146.19	
N-Ethyl-N-(3-hydroxypropyl)nitrosamine	EHPN	61734-88-1	132.16	
N-nitroso-N-ethylurea	ENU	759-73-9	117.10	
N-nitro-methanamine	MA-NO_2	598-57-2	76.05	
Ethanolnitramine	MEA-NO_2	74386-82-6	106.04	

(*continued on next page*)

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Table 1 (*continued*)

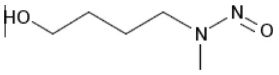
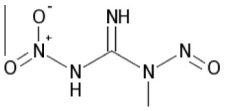
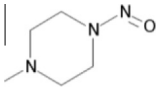
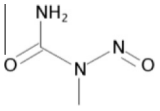
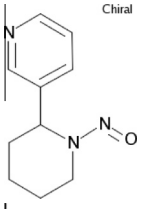
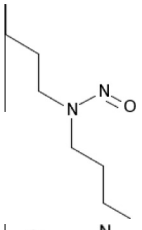
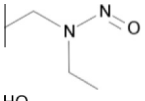
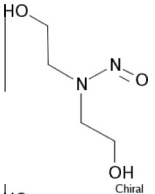
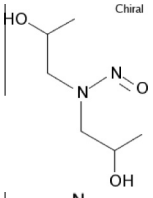
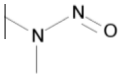
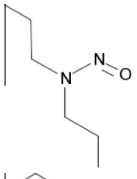
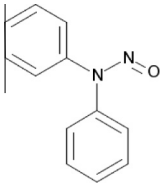
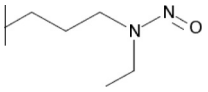
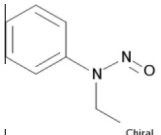
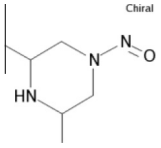
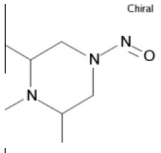
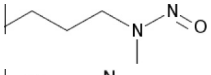
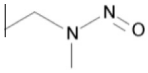
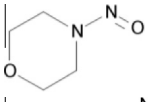
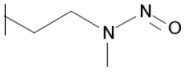
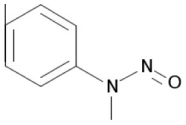
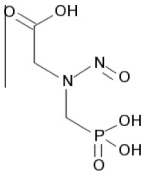
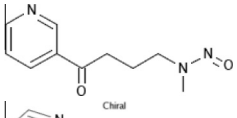
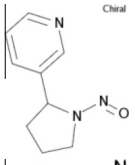
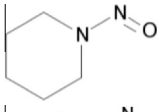
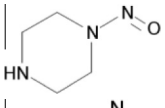
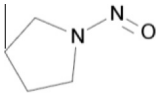
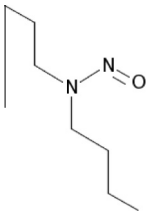
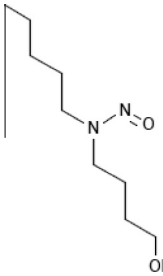
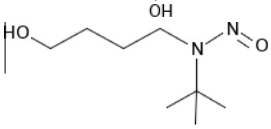
Name	Abbreviation	CAS-RN	MW	Structure
N-Methyl-N-(4-hydroxybutyl)nitrosamine	MHBN	51938-16-0	132.16	
N-methyl-N-nitro-N-nitrosoguanidine	MNNG	70-25-7	147.10	
N-Nitroso-N′-methylpiperazine	NMPIPz	16339-07-4	129.16	
N-nitroso-N-methylurea	MNU	684-93-5	103.08	
N′-Nitrosoanabasine	NAB	1133-64-8	191.23	
N-nitrosodi-n-butylamine	NDBA	924-16-3	158.24	
N-nitrosodiethylamine	NDEA	55-18-5	102.14	
N-nitrosodiethanolamine	NDELA	1116-54-7	131.14	
N-nitrosodiisopropanolamine	NDiPLA	53609-64-6	162.19	
N-nitrosodimethylamine	NDMA	62-75-9	74.08	
N-nitrosodi-n-propylamine	NDPA	621-64-7	130.19	
N-nitrosodiphenylamine	NDPhA	86-30-6	198.08	

Table 1 (*continued*)

Name	Abbreviation	CAS-RN	MW	Structure
N-nitrosoethylbutylamine	NEBA	4549-44-4	130.19	
N-nitrosoethylphenylamine	NEPhA	612-64-6	150.18	
1-Nitroso-3,5-dimethylpiperazine	NM2PIPz	67774-31-6	143.19	
3,4,5-Trimethyl-nitrosopiperazine	NM3PIPz	75881-18-4	157.21	
N-nitrosomethylbutylamine	NMBA	7068-83-9	116.16	
N-nitrosomethylethylamine	NMEA	10595-95-6	88.11	
N-nitrosomorpholine	NMOR	59-89-2	16.12	
N-nitrosomethylpropylamine	NMPA	924-46-9	102.14	
N-nitrosomethylphenylamine	NMPhA	614-00-6	136.15	
N-nitrosoglyphosphate	NNG	56516-72-4	198.07	
4-(methylnitrosamino)-1-(3-pyridyl)-1-Butanone	NNK	64091-91-4	207.23	
N′-nitrosonornicotine	NNN	64162-58-9	177.09	
N-nitrosopiperidine	NPIP	100-75-4	114.15	
N-nitrosopiperazine	NPIPz	5632-47-3	115.13	
N-nitrosopyrrolidine	NPYR	930-55-2	100.12	

(*continued on next page*)

Table 1 (*continued*)

Name	Abbreviation	CAS-RN	MW	Structure
N-Propyl-N-butylnitrosamine	PBN	25413-64-3	144.21	
N-Pentyl-N-(4-hydroxybutyl)nitrosamine	PeHBN	61734-86-9	188.27	
N-tert-butyl-N-(4-hydroxybutyl)nitrosamine	t-BBN	61734-87-0	174.24	

(WHO, 1978). In principle, every nitramine has a nitrosamine counterpart and vice versa: e.g. dimethylnitramine (DMA-NO_2) and NDMA.

Considering their potential mutagenic as well as carcinogenic properties, it is important that the issue of potentially harmful nitrosamine and nitramine by-products of this new CO_2-emission reducing technology is well addressed. The potential human exposure as well as the mutagenic properties and carcinogenic potencies of the specific nitrosamines and nitramines generated need to be adequately assessed to enable conclusions about the risk to human health. As the amine technology will continue to develop within the coming decades, new nitrosamine and nitramine structures with unknown mutagenic and carcinogenic properties may be generated as by-products. In this paper we will exclusively focus on alternative approaches to predict the carcinogenic properties of these compounds, i.e. to search for alternatives to the time-consuming, costly and impractical 2-year rodent assay traditionally applied in carcinogenicity assessment. First, we will shortly describe the human health profile of nitrosamines and other N-nitroso compounds, and nitramines, followed by reviewing available data on mutagenic and carcinogenic properties as their most critical effects. Table 1 presents all N-nitroso compounds and nitramines discussed in this paper and their structures. Subsequently, we will review and discuss alternative methodologies that were used to predict their carcinogenic properties, and explore the potential of these methodologies to predict the carcinogenic potency of nitrosamine or nitramine structures, and their potential use in place of the classical *in vivo* animal bioassay. Supplemental material is provided in two Appendices (A and B), accessible via the online version, at http://dx.doi.org/10.1016/j.yrtph.2014.01.017.

2. Human health toxicity profiles of N-nitroso compounds and CCS nitramines

2.1. Short term effects

2.1.1. Genotoxicity

Regarding short term toxicity, most data available concern the genotoxic properties of N-nitroso compounds and nitramines. Appendix A of the supplemental material presents a summary table of all the study results and references. In Table 2 the genotoxicity profiles of those N-nitroso compounds and nitramines are presented. From this inventory it is clear that all the listed N-nitroso compounds are positive in one or the other genotoxicity test. However, although NPIPz is positive when tested in the presence of metabolic activation in an Ames test with *Salmonella typhimurium* strain 1535 (Zeiger and Sheldon, 1978), this positive result is canceled by the negative result obtained with the *in vitro* HPRT gene mutation test with Chinese Hamster Ovary cells in the presence of metabolic activation (Jones et al., 1981). In a comparable test with Chinese Hamster Lung cells, the CCS nitrosamines NMOR (Robichová et al., 2004) and NDELA (Liu and Russell, 2008) did test positive. Still NPIPz is carcinogenic (see Table 3), and it might be a (very) weak mutagen. The fact that it is the least potent carcinogen of the CCS nitrosamines supports this last notion, while also the main target organ of NPIPz (the nasal cavity, same as for the structurally related DNPIPz) supports, or at least does not contradict, a comparable mechanism of carcinogenicity as its genotoxic congener. Except for ENU and MNU, all N-nitroso compounds completely tested in cytogenic tests or indicator tests for chromosome damage needed metabolic activation to be positive.

The nitramines DMA-NO_2, MA-NO_2 and MEA-NO_2 appear to be negative in indicator tests for chromosome damage. For the gene mutation tests the picture is more varied, some N-nitroso compounds only score positive in the presence of metabolic activation, others also in its absence. The nitramines DMA-NO_2, MA-NO_2 and MEA-NO_2 are mutagenic, even without metabolic activation. Unfortunately, for those nitrosamines scoring negative in carcinogenicity assays (EHPN, PeHBN, t-BBN, and BHBN-3) no genotoxicity data could be found in pubic literature.

2.1.2. Effects other than genotoxicity

Acute oral toxicity data retrieved from public literature are listed in Table 4. Oral LD_{50}'s range from 0.3 to 60 mmol/kg bw, with an average of 12 mmol/kg bw. The three nitramines included in the list all have a LD_{50} of around 10 mmol/kg bw and are in the mid-range of N-nitroso compound acute toxicity.

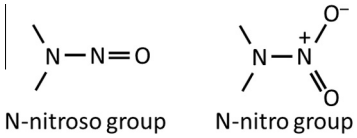


Fig. 1. Structure of N-nitroso (nitrosamine) and N-nitro (nitramine) groups.

Only for a few nitramines data on skin irritation and corrosion, eye irritation, and skin sensitization are available, all generated by Fjellsbø et al. (2011, 2013). The first three endpoints were tested using OECD guidelines under GLP conditions, while the last was assessed using the VITOSENS assay as described by Hooyberghs et al. (2008), which has no regulatory status. The nitramines, MA-NO and MEA-NO_2 tested negative for skin irritation and corrosion, positive for ocular corrosion and were classified as non-sensitizers in the VITOSENS assay. DMA-NO_2 also tested negative for skin irritation and positive for eye corrosion, but could not or was not tested in the other two assays.

2.2. Long term effects

2.2.1. Carcinogenicity

Human and animal studies on the carcinogenicity of N-nitroso compounds and CCS nitramines were collected in order to derive carcinogenic potency values for nitroso substances emitted to the air in the CCS process. However, human data on CCS nitramines could not be retrieved from public literature and none of the epidemiological dose–response studies we retrieved from public literature could link observed carcinogenic effects clearly to a single nitrosamine; only one study seemed to evaluate exposure to one specific nitrosamine, NDMA, but unfortunately, it is not suitable to derive a carcinogenic potency value since the investigated German cohort was not only exposed to NDMA but also to NMOR as well as to a number of nitrosamines which have not been measured (Straif et al., 1998, 2000; Weiland et al., 1996). Therefore the carcinogenic potency values derived for all N-nitroso compounds and CCS nitramines are based on animal data only (see Appendix B of the supplemental material).

In order to assess the relative carcinogenic potency of nitr(os)amines emitted to the air in the CCS process, preferably inhalation studies, performed with the same strain of animals, with guideline-conform protocols under identical experimental conditions should be used. However, in most studies available the oral route of administration has been investigated. In that case one needs to extrapolate the carcinogenic potency value obtained via the oral route to the respiratory route. Differences in toxicokinetic behavior between the oral and respiratory route (first pass effect, differences in biotransformation enzyme make up between the portals of entry, differences in uptake) may complicate this extrapolation. In particular when local tumors occur (i.e. tumors occurring at sites that are exposed even when there is no systemic uptake), oral and respiratory doses are difficult to compare. Among toxicological risk assessors there is consensus that only systemic effects may be extrapolated from one route to the other (see a.o. ECHA, 2012b). Consequently, no respiratory potency values were derived for the few N-nitroso compounds for which only oral studies were available that just showed local effects (e.g. esophagus tumors).

In principle, in absence of suitable human data, data from the most sensitive animal species should be used to derive human carcinogenic potency values, unless the tumors observed are irrelevant for humans. The vast majority of the studies encountered investigated carcinogenicity in rats. In case other species had been studied as well, the rat often was the most sensitive species (e.g. for NDELA, NMEA, NMPA, NDPA, NDBA, NPYR and NPIP: see Appendix B of the supplemental material). To avoid species bias when evaluating the predictive value of tests, the rat was chosen as the species to derive carcinogenic potency values from. As a matter of fact, in the few cases that other species were more sensitive than the rat, the values derived from them were not very different from those derived from rats (see Table 3).

The incidence per unit dose/concentration ($I_{dose/conc}$) was chosen as potency value, because it shows a direct proportional relationship with carcinogenic potency, making it easier to use than alternative measures like the T_{25}[1] or the TD_{50},[2] which show an inversely proportional relation with carcinogenic potency. For the calculation of I_{dose} (for oral studies) and I_{conc} (for respiratory studies), it was assumed that the general population would be exposed daily for lifetime. The incidence of tumor-bearing animals per mmol/kg bw/day for oral studies was calculated as follows[3]:

$$I_{dose} = [I_e - I_c]/[(D/AS \times (X_{po}/L) \times (X_{pe}/L) \times (\text{hours per day}/24) \times (\text{days per week}/7)]$$

For inhalation studies a similar formula was used[4]:

$$I_{conc} = [I_e - I_c]/[(C \times (X_{po}/L) \times (X_{pe}/L) \times (\text{hours per day}/24) \times (\text{days per week}/7)]$$

To estimate the extra lifetime risk of cancer in humans under lifespan conditions on the basis of results in animal experiments, it was assumed that no difference exists between experimental animals and man with respect to toxicokinetics, mechanism of tumor induction, target susceptibility, etc., unless specific information is available which justifies a different approach. Furthermore, it was assumed that the average human being lives 75 years, inhales 20 m^3 per day, is exposed 24 h per day, 7 days per week, 52 weeks per year, for lifetime. These proceedings are very similar to those used by e.g. the EU Scientific Committee on Occupational Exposure Limits (SCOEL) (Bolt and Huici-Montagud, 2008), the European Chemicals Agency (ECHA, 2012b) and the Dutch Health Council (Gezondheidsraad, 2012).

Since inhalation will be the main route of exposure for nitrosamines associated with CCS, and often only oral studies are available, I_{dose} for *systemic* tumors is extrapolated to I_{conc} using the following formula[5]:

$$I_{conc} = I_{dose} \times RV/BW \times RAbs/OAbs$$

[1] The chronic dose rate which will give 25% of the animals tumors at a specific tissue site, after correction for spontaneous incidence, within the standard life-time of that species.

[2] Daily dose-rate in mg/kg/body weight/day for life to induce tumors in half of test animals that would have remained tumor-free at zero dose.

[3] I is estimated tumor incidence; I_e and I_c are tumor incidences in exposed and control animals, respectively; AS is the allometric scaling factor, D is the daily dose (mmol/kg bw/day) in the lowest dose group showing a statistically significant increase in number of animals with a specific tumor, consistent with the dose–response; X_{po} and X_{pe} are observation and exposure period, respectively; L is the standard lifespan for the animal species in question.

[4] C is the exposure concentration (mmol/m^3) in the lowest dose group showing a statistically significant increase in number of animals with a specific tumor, consistent with the dose–response, and allometric scaling is not applied because it is proportional to the respiratory rate and thus implicitly taken into account.

[5] RV is respiratory volume, 20 m^3/day by default for the general population; BW is body weight, 70 kg by default for the general population; RAbs is percentage absorption of the nitrosamine via the respiratory route and OAbs is percentage absorption of the nitrosamine via the oral route, if no measured values are available, by default a ratio of 2 is used for RAbs/OAbs. It should be noted the I_{conc} for the general population con be simply converted into an occupational one by correcting for differences in exposure duration (assumed to be continuous throughout life time (75 years) for the general population and intermittent (e.g. 8 h/day, 5 days/week, 48 weeks/year for 40 years) and respiratory volume (10 m^3/8 h for workers) (see e.g. ECHA, 2012b).

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Table 2
Overall genotoxicity test results for the N-nitroso compounds and nitramines discussed in this paper.

Nitr(os)amine	Cytogenic tests			Gene mutation tests			Overall genotoxicity		
	No MA	MA	Overall	No MA	MA	Overall	No MA	MA	Overall
Alkyl-nitr(os)amines									
MA-NO_2	eq.	neg	eq.	pos	pos	pos	pos	pos	pos
NDMA	neg	pos	pos	neg	pos	pos	neg	pos	pos
DMA-NO_2	neg	neg	neg	pos	pos	pos	pos	pos	pos
NDEA	neg	pos	pos	neg	pos	pos	pos	pos	pos
NMEA					pos	pos		pos	pos
NMPA					pos	pos		pos	pos
NMBA					pos	pos		pos	pos
NEBA					pos	pos		pos	pos
NDPA	neg	neg	neg		pos	pos	neg	pos	pos
NDBA					pos	pos		pos	pos
Alkohol-nitr(os)amines									
MEA-NO_2	neg	neg	neg	pos	pos	pos	pos	pos	pos
NDELA	neg	pos	pos		pos	pos	neg	pos	pos
Heterocyclic nitrosamines									
NPIP	pos	pos	pos		pos	pos	pos	pos	pos
NPYR		pos	pos	pos	pos	pos	pos	pos	pos
NPIPz	neg	neg	neg		neg			neg	neg
DNPIPz	neg	pos	pos		pos	pos	neg	pos	pos
NMPIPz		pos	pos	neg	pos	pos	neg	pos	pos
NM_2PIPz				neg	pos	pos	neg	pos	pos
NBeM_2PIPz				neg	neg	neg	neg	neg	neg
NMOR	neg	pos	pos	neg	pos	pos	neg	pos	pos
Phenyl-nitrosamines									
NMPhA					pos	pos		pos	pos
NDPhA		pos	pos	eq.	eq.	eq.	eq.	pos	pos
Nitroso-ureas									
MNU	pos		pos	pos		pos	pos		pos
ENU	pos		pos	pos		pos	pos		pos

MA = metabolic activation.
Also *in vitro* tests executed with cells that clearly have xenobiotic metabolizing capacity (e.g. hepatocytes) were considered to have been executed in presence of metabolizing activity. Positive tests are shaded. Nitr(os)amines demonstrated to be produced in the CCS process are bold-faced.
The following test were considered to be "*cytogenic tests*": CA *in vitro*, Comet *in vitro*, Comet *in vivo*, MN *in vitro* and MN *in vivo*. "*Gene mutation tests*" were considered to be GM Bacteria, Germ line *in vivo*, GM *in vitro*, GM *in vivo* and UDS *in vivo*. In one category, a result was considered positive if at least one test in the category tested positive (pos); if no test was positive and one or more test were equivocal, the category was labeled "equivocal" (eq.); if no test was positive or equivocal and one or more tests were negative, the category was labeled "negative" (neg), else the category was left blank (no data).
The data and the corresponding literature references on which this table is based are summarized in Appendix A of the supplemental material.

In view of the uncertainties inherent to the procedure followed to derive limit values, all calculations were rounded off to two significant figures only. Whenever specific values needed for the calculations were not available from the original articles, default values were used. These values and their sources are listed in Table 1 of Appendix B of the supplemental material.

The studies available for the derivation of cancer potency values as well as the selection of the most suitable study to derive them and the exact calculation are described in Appendix B of the supplemental material. The potency values are listed in Table 3.

2.2.2. Effects other than carcinogenicity

No general studies on repeated dose toxicity of N-nitroso compounds or nitramines were encountered in public literature. Likewise data on reproductive and developmental toxicity are very scarce, however, since the vast majority of these N-nitroso compounds are genotoxic carcinogens, carcinogenicity is most likely the leading health effect for risk assessment purposes: For that reason e.g. the EU requires no data on reproductive and developmental toxicity for genotoxic carcinogens (ECHA, 2012a).

Only the nitrosourea ENU has been amply investigated for reproductive and developmental toxicity (see a.o. Akiyama et al., 2008; Katayama et al., 2001, 2000a, 2000b, 2002; Kennedy and O'Bryan, 2006; Lee et al., 2009; Sussman et al., 2001). It was proven to be a developmental toxicant and toxic for reproduction. For other N-nitroso compounds and the CCS nitramines hardly any data are available for these endpoints. An *ex vivo* study with

Table 3
Respiratory carcinogenic potencies of N-nitroso compounds and nitramines based on rat data. If data on other more sensitive species were available, the potency derived from the most sensitive species is listed as well.

Compound	MW	I_{conc} based on				Rank (oral studies)[a]
		Oral route (rat)		Respiratory route (rat)		
		$(mmol/m^3)^{-1}$	$(mg/m^3)^{-1}$	$(mmol/m^3)^{-1}$	$(mg/m^3)^{-1}$	
Alkyl-nitr(os)amines						
DMA-NO_2	90.08	7.4×10^1	8.2×10^{-1}	–	–	30
MA-NO_2	76.05	1.2×10^1	1.6×10^{-1}	–	–	36
NDBA	158.24	1.2×10^3	7.3×10^0	–	–	4
NDEA	102.14	1.1×10^3	1.1×10^1	–	–	5
NDMA	74.08	3.0×10^2	4.0×10^0	4.2×10^3	5.7×10^1	18
NDPA	130.19	4.1×10^2	3.1×10^0	–	–	13
NEBA	130.19	3.0×10^1	2.3×10^{-1}	–	–	33
NMBA	116.16	–	–	–	–	–
		Hamster: 5.5×10^2	4.8×10^0	–	–	–
NMEA	88.11	4.5×10^2	5.1×10^0	–	–	12
NMPA	102.14	–	–	–	–	–
		Hamster: 6.0×10^2	5.9×10^0	–	–	–
PBN	144.21	1.3×10^2	9.1×10^{-1}	–	–	23
Alcohol-, aldehyd- and carboxylnitr(os)amines						
BBN	174.24	9.4×10^2	5.4×10^0	–	–	8
BHBN-3	174.24	neg	neg	–	–	–
BHPN	160.12	neg	neg	–	–	–
EHBN	146.19	1.1×10^2	7.7×10^{-1}	–	–	26
		Mouse: 1.3×10^2	9.2×10^{-1}			
EHPN	132.16	neg	neg	–	–	–
MHBN	132.16	8.7×10^1	6.6×10^{-1}	–	–	29
NDELA	131.14	4.0×10^1	3.0×10^{-1}	–	–	32
PeHBN	188.27	neg	neg	–	–	–
t-BBN	174.24	neg	neg	–	–	–
NDiPLA	162.19	1.6×10^2	1.0×10^0	–	–	22
BOBN-2	172.22	1.1×10^2	6.5×10^{-1}	–	–	25
BOBN-3	172.22	2.2×10^1	1.3×10^{-1}	–	–	34
BCMN	160.17	neg	neg	–	–	–
BCPN	188.12	2.4×10^2	1.3×10^0	–	–	21
ECPN	160.17	1.1×10^2	7.1×10^{-1}	–	–	25
Phenyl-nitrosamines						
NDPhA	198.08	1.2×10^0	5.8×10^{-3}	–	–	38
NMPhA	136.15	1.10×10^3	7.7×10^0	–	–	5
NEPhA	150.18	–	–	–	–	–
Heterocyclic nitrosamines						
2,5-DMDNPIPz	172.19	3.8×10^2	2.2×10^0	–	–	14
2,6-DMDNPIPz	172.19	9.8×10^2	5.7×10^0	–	–	7
2-MDNPIPz	158.16	7.2×10^2	4.5×10^0	–	–	10
AcNM2PIPz	185.22	–	–	–	–	–
BeNM2PIPz	247.29	1.5×10^1	6.3×10^{-2}	–	–	35
DNhPIPz	158.16	9.2×10^2	5.8×10^0	–	–	9
DNPIPz	144.13	3.1×10^2	2.2×10^0	–	–	17
		mouse: 4.6×10^2	3.2×10^0	–	–	–
DNPT	185.18	neg	neg	–	–	–
NMPIPz	129.16	4.3×10^1	3.3×10^{-1}	4.6×10^2	3.5×10^0	31
NAB	191.23	–	–	–	–	–
NM2PIPz	143.19	2.5×10^2	1.8×10^0	–	–	20
NM3PIPz	157.21	3.0×10^2	1.9×10^0	–	–	18
NMOR	116.12	2.1×10^3	1.8×10^1	4.1×10^2	3.5×10^0	3
NNK	207.23	3.0×10^3	1.5×10^1	–	–	2
NNN	177.09	1.2×10^2	7.0×10^{-1}	–	–	24
NPIP	114.15	6.9×10^2	6.0×10^0	–	–	11
NPIPz	115.13	6.6×10^0	5.7×10^{-2}	–	–	37
NPYR	100.12	1.1×10^2	1.1×10^0	–	–	25
Nitroso-ureas and -guanidines						
ENU	117.10	3.9×10^3	3.3×10^1	–	–	1
MNU	103.08	3.3×10^2	3.2×10^0	–	–	16
MNNG	147.10	3.5×10^2	2.4×10^0	–	–	15
NNG	198.07	–	–	–	–	–

I_{conc} = incidence of tumor bearing animals per unit of concentration (lifetime continuous exposure).
The derivation of these carcinogenic potency values and the studies on which they are based are described and referenced in Appendix B of the supplemental material.
[a] Based on the values displayed in the third column.

NDMA conducted by Annola et al. (2009) demonstrated that the human fetus can be exposed to NDMA from the maternal circulation and that NDMA is not metabolized in full-term human placenta from healthy non-smoking, non-drinking mothers. This suggests that NDMA may cause developmental toxicity by direct interaction with the fetus.

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Table 4
Oral LD_{50} for selected N-nitroso compounds and nitramines in rats.

Nitr(os)amine	LD_{50}		References
	mmol/kg bw	mg/kg/bw	
Alkyl-nitr(os)amines			
DMA-NO_2	8.50	770	Fjellsbø et al. (2011)
MA-NO_2	11.00	840	Fjellsbø et al. (2011)
NDBA	7.60	1200	Druckrey et al. (1967)
NDEA	2.80	290	Druckrey et al. (1967)
NDMA	0.54	40	Druckrey et al. (1967)
NDPA	3.70	480	Druckrey et al. (1967)
NMBA	2.90	340	Druckrey et al. (1967)
NMEA	1.00	88	Druckrey et al. (1967)
NMPA	9.50	970	Druckrey et al. (1967)
Alcohol-nitr(os)amines			
MEA-NO_2	9.10	970	Fjellsbø et al. (2011)
BBN	10.00	1740	Druckrey et al. (1967)
NDELA	60.00	7870	Druckrey et al. (1967)
Phenyl-nitrosamines			
NDPhA	15.00	2970	Druckrey et al. (1967)
NEPhA	0.30	45	Druckrey et al. (1967)
NMPhA	2.10	290	Druckrey et al. (1967)
Heterocyclic nitrosamines			
DNPIPz	1.10	160	Druckrey et al. (1967)
NMPIPz	7.80	1010	Druckrey et al. (1967)
NMOR	2.80	330	Druckrey et al. (1967)
NPIP	1.70	190	Druckrey et al. (1967)
NPYR	9.00	900	Druckrey et al. (1967)
Nitroso-ureas			
ENU	2.00	230	Druckrey et al. (1967)
MNU	1.10	110	Druckrey et al. (1967)

2.3. Target-organs of tumor formation

Based on the studies described in Appendix B of the supplemental material, an inventory was made of the principle target organs of tumor formation in the carcinogenicity studies with CCS nitrosamines, other N-nitroso compounds and nitramines. Table 5 displays the results of this effort for rat carcinogenicity studies, which constitute the majority of the available studies.

When comparing target-organs for carcinogenicity for these compounds, the data clearly show that many organs are potentially at risk. It is not evident from the data what determines the specific targeting.

Many CCS nitrosamines and also the nitramine DMA-NO_2 have the liver as main target organ in oral studies. Also for other carcinogens the liver is often the main target organ after oral administration, which is probably related to the relatively high dose this organ will face and its high concentration of a broad spectrum of xenobiotic metabolizing enzymes, including many CYPs.

The second most frequent target organs are the esophagus and the nasal cavity. There are indications that CYP2A3 may play a role in this organ preference: CYP2A3 is reported to be present in rat nasal and lung tissue but not in the liver (Honkakoski and Negishi, 1997; Robottom-Ferreira et al., 2003), and it is also present in the esophagus (Ribeiro Pinto et al., 2001; Robottom-Ferreira et al., 2003). Unfortunately no data on the interaction between the CCS nitrosamines mainly targeting the nasal mucosa, DNPIPz and NPIPz, and CYPs are available.

From the overview one remarkable observation is the absence of tumors of the upper part of the digestive tract for the group of alcohol-, keto-, and carboxynitrosamines, as compared to the other groups. Also, a relatively high number of these nitrosamines have the bladder as their main target for cancer induction. Another remarkable observation is that piperazine derivatives as a group clearly do not target the liver.

Both DMA-NO_2 and MA-NO_2 target the Central Nervous System (CNS) (Hassel and Frei, 1987; Mirvish et al., 1980; Scherf et al., 1989), which is the only and main target organ of the latter compound (Hassel and Frei, 1987; Scherf et al., 1989), while for the former the liver is the most sensitive for tumor development (Druckrey et al., 1967, 1961; Goodall and Kennedy, 1976; Mirvish et al., 1980).

There seem to be some species differences in N-nitroso compound carcinogenicity as can be seen from the data on hamster and mouse compared to those on rats, although the number of hamster and mouse studies is too small to draw firm conclusions. For example, via the oral route the main target organ of NMPA and NMBA is the esophagus in rats (Lijinsky et al., 1983) while it is the liver in the hamster (Lijinsky and Kovatch, 1988). Furthermore, the main target organ of NDELA via the oral route in the rat is the liver, while in the mouse it is the lung (Hecht et al., 1989).

Based on the few available respiratory rat carcinogenicity studies, also some route differences in organ specificity are apparent: e.g. for NDMA the main target via the respiratory route is the nasal cavity and via the oral route the liver. This issue, and its importance for the extrapolation to humans, is touched upon in more detail in the discussion (Section 4).

Besides route and species, also animal strain and even dose-regime appear to be of influence: e.g. a single high oral dose of NDMA led to kidney tumors, while prolonged exposure mainly induced liver tumors (Magee and Barnes, 1967).

Fairly small differences in chemical structure may change organ specificity. For example, NDMA and NDEA induce tumors at many sites, e.g. in liver, kidney and upper digestive tract, but not in the bladder (Peto et al., 1991a,b), which is the major target organ of NDBA (Druckrey et al., 1967). Also the piperazine derivatives show a clear target-organ shift with relatively small structural changes: replacing a 4-methylgroup in NM_3PIPz with a 4-acetylgroup ($AcNM_2PIPz$) results in the complete disappearance of lymphomas and leukemia, and the appearance of esophagus and tongue tumors (Singer et al., 1981).

Overall, a great variety of organs may be targeted by carcinogenic CCS nitrosamines and nitramines, of which the liver is the most frequent one, followed by the esophagus and the nasal cavity. Although some generalizations appear possible, the nature of the target organs is determined by many different factors and the present database only shows some tendencies that do not allow firm conclusions.

3. Alternative methodologies for predicting carcinogenicity of N-nitroso compounds and nitramines

3.1. Approaches using chemical structure

3.1.1. Structural classes and carcinogenic potency

From Table 3 one can observe that estimated potencies of N-nitroso compounds and nitramines span over 3 orders of magnitude. Potency values of the alkylnitrosamines (based on oral studies) have a smaller potency range of only 50-fold (comparing NDBA and NEBA), of which NDBA appears the most potent carcinogen on a mmol/m^3 basis. Actually, all these nitrosamines cluster within one order of magnitude potency difference, only NEBA being about one order less potent than the second least potent one of this group. There is no other evident trend, e.g. with alkyl chain length.

None of the alkanolnitrosamines has a higher potency than NDEA, and the potency range of nitrosamines listed here is somewhat similar: about 10-fold. It should be recognized though that all 'negative' nitrosamines in this group may in fact be relatively weak carcinogens, as the experimental design used for these nitrosamines is not sensitive enough to conclude that they are not carcinogenic. Thus, should these negative nitrosamines have been tested

Table 5
Overview of target organs of carcinogenesis of N-nitroso compounds.

	Oral																				Respiratory			
Compound	**Oc**	**Oe**	**GSt**	**Fst**	**Di**	**Na**	**PEx**	**Tr**	**Lu**	**Pe**	**Li**	**He**	**Bl**	**Ki**	**Ov**	**Ty**	**Ma**	**Te**	**Ut**	**CNS**	**HPS**	**Na**	**Li**	**Th**	**CNS**
Alkyl-nitr(os)amines																									
MA-NO_2																				X		--	--	--	--
DMA-NO_2		X				X					X			X						X		--	--	--	--
NDMA									X		X			X								X			
NDEA		X									X											--	--	--	--
NMEA		X				X			X		X			X								--	--	--	--
NMPA	X	X		X																		--	--	--	--
NMBA	X	X		X		X																--	--	--	--
NEBA	X	X									X											--	--	--	--
NDPA	X	X		X		X					X											--	--	--	--
NPBA (PBN)		X									X											--	--	--	--
NDBA	X	X		X							X		X									--	--	--	--
Alcohol- keto- and carboxy- nitrosamines																									
NDELA						X					X			X				X				--	--	--	--
ND*i*PLA									X		X											--	--	--	--
MHBN													X									--	--	--	--
EHBN													X									--	--	--	--
EHPN																						--	--	--	--
BBN													X									--	--	--	--
PeHBN																						--	--	--	--
t-BBN																						--	--	--	--
BHBN-3																						--	--	--	--
BCPN													X									--	--	--	--
ECPN													X									--	--	--	--
BCMN																						--	--	--	--
	Oral																				Respiratory				
Compound	**Oc**	**Oe**	**GSt**	**Fst**	**Di**	**Na**	**PEx**	**Tr**	**Lu**	**Pe**	**Li**	**He**	**Bl**	**Ki**	**Ov**	**Ty**	**Ma**	**Te**	**Ut**	**CNS**	**HPS**	**Na**	**Li**	**Th**	**CNS**
BHPN																						--	--	--	--
BOBN-3											X											--	--	--	--
BOBN-2											X											--	--	--	--
Heterocyclic nitrosamines																									
NPIP		X		X				X			X											--	--	--	--
NMOR		X									X											X	X	X	X
NPYR											X							X				--	--	--	--
NPIPz						X																--	--	--	--
DNPIPz						X		X			X									X		--	--	--	--
MNPIPz						X																X			
M_3NPIPz						X										X						--	--	--	--
M_2NPIPz						X										X						--	--	--	--
AcM_2NPIPz	X	X																				--	--	--	--
BeM_2NPIPz				X																		--	--	--	--
2,5-DMDNPIPz		X				X														X		--	--	--	--
DNhPIPz		X				X														X		--	--	--	--
2-MDNPIPz		X				X																--	--	--	--
2,6-DMDNPIPz		X				X																--	--	--	--
NNK							X			X		X										--	--	--	--
NNN		X	X			X				X												--	--	--	--
Phenylnitrosamines																									
NMPhA		X		X						X												--	--	--	--
NDPhA												X										--	--	--	--
Nitrosoureas and -guanidines																									
ENU					X			X	X						X		X		X	X	X	--	--	--	--
	Oral																				Respiratory				
Compound	**Oc**	**Oe**	**GSt**	**Fst**	**Di**	**Na**	**PEx**	**Tr**	**Lu**	**Pe**	**Li**	**He**	**Bl**	**Ki**	**Ov**	**Ty**	**Ma**	**Te**	**Ut**	**CNS**	**HPS**	**Na**	**Li**	**Th**	**CNS**
MNU	X			X	X						X			X						X		--	--	--	--
MNNG			X	X	X					X												--	--	--	--
																						--	--	--	--

– = no data; Bl = bladder; CNS = Central Nervous System, Di = digestive tract (after stomach), Fst = forestomach, He = heart, HPS = haematopoietic system, Ki = kidneys, Li = liver, Lu = lungs, Ma = mammary gland, Na = nasal cavity, Oc = oral cavity, Oe = oesophagus, Ov = ovary, PEx: pancreas, exocrine, Te = testis, Th = thyroid, Tr = trachea, Ty = thymus, Ut = uterus. Empty cells indicate no effects were observed on the organ mentioned in the column heading. The most sensitive organ is indicated by a shaded cell. The CCS nitrosamines and nitramines are printed in bold face. The studies on which these data are based are described and referenced in Appendix B of the supplemental material.

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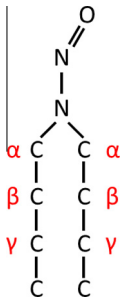

Fig. 2. The α-, β- and γ-positions in the N-nitroso compound structure.

in a study design with a higher resolution, i.e. closer to OECD guidelines, the potency range of this group may well expand into the lower potency region. From direct comparison of potencies of the respective pairs NMBA/MHBN, NEBA/EHBN, and NDBA/BBN, there is no clear effect of alkyl group hydroxylation on carcinogenic potency discernible; this also holds for the NDEA/NDELA and NDPA/NDiPLA pairs.

Also, all cyclic nitrosamines have lower potency estimates than NDEA, except NMOR that shows a comparable carcinogenic potency. Potencies of NPIP, and NPYR are within about one order of magnitude of NMOR. The structural changes in the NPIPz analogues show substantial impact on the carcinogenic potency set off against NPIPz itself: compare e.g. NPIPz with NM_2PIPz or NM_3PIPz.

The most potent N-nitroso compounds, e.g. NMOR, NDEA, NPIP and BBN do not show a clear structural similarity, the same is valid for the least potent N-nitroso compounds NPIPz, NEBA, and NDELA. Thus, from this data a clear relationship between structure and carcinogenic potency is not evident.

3.1.2. SARs and QSARs

In recent years a limited number of studies have been performed on predicting the carcinogenic potency of N-nitroso compounds using chemical structures as a basis. In a mechanistic study by Zhao et al. (2005) the carcinogenic activity of nitroso compounds was correlated with the reactivity of these compounds at two sites in their molecular structure. Next to bioactivation by α-hydroxylation or hydrolysis to form alkyldiazonium ions – the commonly accepted route for carcinogenesis – the formation of β- and γ-positioned esters as a key determinant for toxification was postulated (see Fig. 2). Based on the calculated lower activation energy of β-metabolites of methylethylnitrosamine as a model compound, it was concluded that they display a higher reactivity than γ-metabolites. The ability of N-nitroso compounds to form reactive intermediates from β- or γ-metabolites may potentially provide a means to rank the carcinogenic potency of this compound class by means of their activation energies. However, the accuracy of such a correlation has not yet been described. Therefore the practical use of this study for the prediction of N-nitroso compound carcinogenic potency is unclear.

Other studies on the carcinogenic potency of N-nitroso compounds focus less on mechanistic aspects, and instead involve various methods to derive Quantitative Structure Activity Relationships (QSAR) and classification models. In QSAR studies, the *in vivo* carcinogenic potencies of N-nitroso compounds are correlated with their molecular properties (descriptors). This allows predictions regarding the carcinogenic potency of new structures. Classification models, on the other hand, merely provide a categorization of compounds into carcinogenic and non-carcinogenic classes. The Carcinogenic Potency Database (CPDB)[6] is used as major data source for most studies. This database contains TD_{50} values as a potency measure.

Support vector machines (SVM) and linear discriminant analysis (LDA) were used by Luan et al. (2005) to classify 148 N-nitroso compounds as carcinogenic or non-carcinogenic.[7] The dataset used in this study, with about 80% of compounds classified as carcinogenic, was randomly divided in a training (118) and test set (30). Equal percentages of non-carcinogenic entities were maintained in both sets. A wide range of molecular descriptors was calculated and used to generate both LDA and SVM prediction models. The most optimal LDA model with a total accuracy of 89.8% (test and training set) was obtained using seven descriptors. Using identical descriptors, a nonlinear SVM model was built providing an overall accuracy of 95.2%. Thus both methods gave satisfactory results in overall predictive power, with a slightly better performance by SVM. However, when zooming in on the non-carcinogenic compounds of the test set the predictive power was less: 71.4% and 57.1% for LDA and SVM respectively. For the test and training set combined, the predictivity of the LDA model for non-carcinogenic compounds (68.6%) was lower than that of SVM (84.4%).

In the most recent studies on building a classification model, Yuan and colleagues linked molecular descriptors to the carcinogenicity of N-nitroso compounds. The studies, focusing on overall (Yuan et al., 2011) and liver-specific (Yuan et al., 2012) tumorigenic potential in rats, follow a similar approach to Helguera et al. (2007, 2008a,b, 2010; see below) by incorporating molecular TOPS-MODE[8] and Abraham solute descriptors. Neither of the studies was restricted to a specific route of administration or gender. Thus, a larger dataset could be used. In the 2011 study, 95 carcinogenic and 16 non-carcinogenic compounds were used, whereas the liver-specific study entailed 32 hepatocarcinogenic, 60 carcinogenic non-hepatocarcinogenic and 16 non-carcinogenic nitroso compounds. Non-carcinogenic and non-hepatocarcinogenic compounds were combined into one class of "non-hepatocarcinogens", the other class being the hepatocarcinogens. Both models were assessed by means of a test set (37 and 27 compounds, respectively) and by leave-one-out cross validation. Overall prediction accuracies of

[6] http://potency.berkeley.edu/cpdb.

[7] SVM is a method that constructs hyperplanes in a multidimensional space such that samples belonging to two different classes are separated in space. Any new sample is mapped in that space, and depending on which side of the plane it falls, belongs to one of the classes. LDA is a method to find a linear combination of features (descriptors) which characterizes or separates two or more classes of samples. The resulting combination may be used as a linear classifier to categorize new samples, in this case nitroso compounds.

[8] TOPS-MODE (TOPological Substructural MOlecular Design) measures the concentration of structural or physicochemical properties in regions of different sizes in the molecule.

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respectively 90% (overall carcinogenicity) and 94% (hepatocarcinogenicity) were reported, with acceptable statistical quality. However, the predictive value for the non-(hepato)carcinogenic group was significantly lower. Reported values for the test and training set were 87.5% and 65% in the 2011 study, and 71.4% and 76% in the 2012 study.

Helguera et al. (2007) reported a QSAR study using the TOPS-MODE approach. Several multilinear regression models were built based on TD_{50} values of 35 nitroso compounds.[9] Only compounds orally administered to male rats via the gavage route were taken into account, as species differences and administration routes may impact the measured TD_{50}. The best model was obtained using 5 parameters, yielding a model able to explain 84% of the experimental variance (1 outlier: 1-(2-hydroxyethyl)-1-nitrosourea) and showed good statistical quality. An analogous model was subsequently generated for female rats, applying a dataset of 26 compounds, which accounted for more than 86% of the variance in the experimental activity (Helguera et al., 2008b). In two follow-up studies, the TOPS-MODE approach was further explored. Abraham solute descriptors were incorporated in the model to reflect the effect of solvent on the carcinogenicity of nitroso-compounds orally administered to male and female rats via drinking water (Helguera et al., 2008a, 2010). The resulting QSAR models (dataset of resp. 39 and 56 nitroso compounds) displayed a performance and quality similar to that of the first study reported. A nice add-on of the TOPS-MODE approach is the ability to identify quantitative contributions of substructures to the studied endpoint. Thus, properties such as the length of the alkyl chain and substitution patterns can be used as alerts for the carcinogenic potential of nitroso compounds.

The TD_{50} data from the 2007 Helguera study were extracted and extended with 12 other compounds with identical endpoint by Harju et al. (2010). Using 33 compounds as training set and the remaining 7 as test set, a QSAR model was built with ADMEWORKS™ using interactive multi-linear regression and the most relevant descriptors as assessed by a genetic algorithm.[10] The final model had a satisfactory overall performance of 82%. Further analysis of the dataset, however, showed an underrepresentation of low carcinogenic nitroso compounds, i.e. the TD_{50} values did not fulfill the requirements for a normal distribution of data. Recommendations were therefore made to rebuild the model once additional data become available to confirm the validity of the prediction model and used descriptors.

The subsequent report by this group (Harju et al., 2011) describes this effort. The structures (ChemIDplus) and TD_{50} data (CPDB) of 92 N-nitroso compounds were collected and curated. The harmonic mean of the most potent TD_{50}value of each available study was used as quantitative measure as opposed to the earlier used lowest TD_{50} value obtained from rats. The remaining set of 58 N-nitroso compounds all contained alkyl or aryl N-nitroso groups[11] as the only structural alert for mutagenicity and carcinogenicity, and are – as far as known – bioactivated by CYPs. For this data set a large number of physicochemical and structural descriptors was calculated with ADMEWORKS™. By using self-organizing maps, a neural network based on unsupervised learning, a training (41 nitrosamines) and test set (17 nitrosamines) were defined. The best descriptors for model building were derived by a variable selection procedure and subsequent Multiple Linear Regression. A maximum of 6 descriptors was allowed to prevent overfitting. The best model had an R^2 of 0.72 for the training set which falls within the acceptable limit. However, the model completely failed to predict the test set ($Q^2 = -0.69$). This may be due to the heterogeneous nature of the historical TD_{50} data of the CPDB: non-standardized test protocols, different laboratories, and different application methods. Furthermore, it should be emphasized that the harmonic mean is based on all available positive studies, while in human risk assessment it is customary to use the valid study with the highest carcinogenic potency (which in this case would correspond to the lowest TD_{50}).

In summary, the availability of a large, structurally diverse dataset with consistently measured biological data is essential for generating a prediction model with a broad ('global') applicability domain. Often such data are lacking. This is also true for N-nitroso compounds. Apart from a small, diverse dataset this compound class additionally suffers from a misbalance between carcinogenic and non-carcinogenic entities, and the fact that TD_{50} values derived from differing experimental setups are used. The latter includes differences in the organ-specificity of tumors but also the potential dependency of a measured endpoint on differences in species, sex and administration route. Hence, the approach taken by Helguera to generate separate prediction models. One can thus conclude that despite the apparent good quality of the generated models, it is difficult to assess how these models will perform for new compounds – especially non-carcinogenic ones. Regardless, it is clear that new predictions rely heavily on the application domain of the model. An approach other than striving for a global prediction model would be the development of a local model. This would entail inclusion of a single mechanism of action in model building as described by the Zhao study and the 2011 NILU report. None of the other QSAR studies described have explicitly taken the mechanism of action into account. The studied compounds do, however, have different molecular pathways to become carcinogenic. *N*-nitrosoureas undergo non-enzymatic decomposition: breakage of the amide bond generates unstable carbamic acid and carbonium ions, resulting in alkylation and carbomoylation reactions of various biological macromolecules including DNA (Faustino et al., 2005; Golding et al., 1997). *N*-nitrosamines on the other hand require biotransformation by cytochromes P450 (CYP) to exert their full carcinogenic effect. Thus, predictive model building may benefit from taking activation routes into account. However, as *N*-nitrosamine bioactivation involves a large battery of CYP isozymes, including CYPs 1A1, 1A2, 1B1, 2A6, 2C8, 2C19, 2E1, 3A4 and 3A5 (see e.g. Cooper and Porter, 2000; Duarte et al., 2005; Emmert et al., 2006; Fujita and Kamataki, 2001a,b; Kushida et al., 2000), a CYP-specific 3D-modeling approach, pursued for individual compounds by DeVore and Scott (2012), will probably not be feasible, also taking into account that some CYPs may be deactivating and that other xenobiotic metabolizing enzymes may be involved as well.

3.2. Approaches using mutagenicity or alternative carcinogenicity tests

3.2.1. Introduction

Public literature was screened for papers on genotoxicity of N-nitroso compounds for which carcinogenicity data were acquired and for alternative carcinogenicity test results involving N-nitroso compounds. The papers retrieved were screened for their predictive potential with respect to N-nitroso compound carcinogenic potency.

3.2.2. Bacterial mutation tests

One of the main aims of making this inventory, was to investigate whether it would be possible to predict carcinogenic potency of N-nitroso compounds based on mutagenic potency. Since our objective was to find a quantitative prediction of potency,

[9] Taken from the published peer-reviewed papers from public literature included in the CPDB, excluding the NTP studies also contained in the CPDB. The authors stated they aimed to test the developed models on these additional data. Sofar no such efforts have been reported in public literature.

[10] Genetic algorithms encompass a variety of search paradigms inspired by nature: selection and recombination operators are applied to evolve a solution (best combination of descriptors) to a problem (variance in TD_{50} value).

[11] SA_21 from the Benigni-Bossa rulebase for mutagenicity and carcinogenicity.

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the results of the available tests were expressed as much as possible in a quantitative measure of mutagenicity: number of revertants per μmol of N-nitroso compound exposure. When the papers publishing the results of these tests did not mention potency figures, it was attempted to calculate them from the published data. Most of the bacterial mutagenicity studies were executed with *S. typhimurium* strains, some with *Escherichia coli* strains. To avoid species based bias, only the *Salmonella* results were considered.

In Table 6 the N-nitroso compounds are ranked for their mutagenic potency. Unfortunately, only for a few N-nitroso compounds the required data are available, covering only a small range of mutagenic potencies. Most of the tests have been executed with some sort of metabolic activation (221 of the 239 tests). Conspicuous is the wide range of mutagenic potencies found for most N-nitroso compounds. This is a reflection of the variety of tests with respect to strain, and metabolic activation source (S9 mixes of rats induced with different drugs, bacteria transfected with various human CYPs and CYP reductase). Upon analysis of the combined data, no apparent relationship exists between mutagenic and carcinogenic potency, not even when contemplating broad potency classes (see Fig. 3). This may be in part due to the heterogeneous nature of the dataset.

Guttenplan (1987) has studied the relation between mutagenic potency of a series of nitrosamines tested with the *S. typhimurium* strain TA100 in the presence of S9 and carcinogenic potency classes attributed by Lijinsky (1984), based on a subjective judgment of relative potency. He did not find any relationship when the mutagenic potency was expressed as number of revertants per μmol nitrosamine. However, he did find a positive correlation between carcinogenic potency classes and mutagenic efficiency, defined by him as the number of revertants per μmol nitrogen produced in an assay designed to measure α-hydroxylation of nitrosamines. However, neither measures showed any relationship with the carcinogenic potencies listed in Table 3.

Fujita and Kamataki (2001a, 2001b) have studied the relation between metabolic activation by various CYP isozymes and nitrosamine mutagenicity by transfecting *S. typhimurium* strain YG7108 with different human CYPs and CYP reductase. They have expressed mutagenic potency as the number of revertants per nmol nitrosamine per pmol CYP around the Minimum Effect Concentration of the nitrosamine. The nitrosamines investigated were NNK, NDEA, NPYR, NPIP, NMOR, NNN, NABS, NATB, NDBA, NDMA, NDPA, NEBA, NMBA, NMEA and NMPA. Nitrosamine mutagenic potency in the presence of human CYPs did not clearly correlate positively with their carcinogenic potency except for CYP3A4, with Pearson (r) and Spearman (ρ) correlation coefficients >0.6. However, even for this CYP, no clear separation between carcinogenic potency classes could be made based on the mutagenic potency of the nitrosamines in the YG7108 strain expressing it (see Fig. 4). Overall mutagenic potency parameters in this study did not correlate or even correlated negatively with carcinogenic potency (see Table 7).

Also using strain YG7108, the mutagenic potencies determined by Hakura et al. (2005) for the nitrosamines NDBA, NDEA, NDMA, NDPA and NMOR in the presence of human or rat S9 mix did not show a positive correlation with their carcinogenic potency ($r = -0.59$ to -0.12, $\rho = -0.9$ to -0.3). Also the mutagenic potencies derived by Cooper and Porter, 2000 for NDBA, NDEA, NDMA and NDPA using strains YG7104 and YG7108 and human CYP2E1 showed a negative correlation with carcinogenic potency ($r = -0.49$ and -0.39, respectively, $\rho = -0.8$ and -0.4, respectively). To conclude, Wagner et al. (2012) determined mutagenic potencies for NDMA, NMOR, NPIP and NPYR using strain YG7108 and rat S9 and did not find a correlation with carcinogenic potency ($r = -0.16$, $\rho = 0.2$).

Summarizing, mutagenic potencies derived from Ames tests do not seem to correlate well with N-nitroso compound carcinogenic potency. However, the present test set of N-nitroso compounds shows a narrow range of carcinogenic potencies (all within one order of magnitude), while mutagenic potencies vary up to three orders of magnitude. Therefore, testing with a good number of N-nitroso compounds representing a broader range of carcinogenic potencies could yield better results when using a well-defined and consistent AMES testing protocol.

3.2.3. In vitro mammalian mutation tests

Only one systematic exploration of *in vitro* mammalian cell mutagenicity systems for the prediction of carcinogenic potency of N-nitroso compounds has been traced. Jones et al. (1981) described the relationship between carcinogenicity and mutagenicity measured in Chinese hamster V79 cells co-cultivated with primary rat hepatocytes. They found a good correlation between the carcinogenic potency index[12] and the mutagenic potency index[13] for the 6-thioguanine marker ($r = 0.85$). They used a dataset of 26 N-nitroso compounds, most of which have a potency deviating at most a factor 2.7 from the linear regression line determined, with the notable exception of NDMA, which had circa 7 times higher carcinogenic potency index. The mutagenic potency derived by Jones et al. (1981) was plotted against the more commonly used carcinogenic potency we have calculated from oral rat studies and expressed as I_{conc} in $(mmol/m^3)^{-1}$. No correlation between the two parameters was observed (see Fig. 5, $r = -0.06$ and $\rho = 0.17$).

Another dataset was published by Bradley et al. (1981) and also contained a number of N-nitroso compounds. Various study designs were used, with different selective agents (8-azaguanine, 6-thioguanine, ouabain) or none and different activation systems (microsomes/S9, hepatocytes) or none. The mutagenic potency ($1/D_{10}$ in nM^{-1}) of the most frequently used marker, 8-azaguanine was plotted against the carcinogenic potency we have calculated from oral rat studies. As with the Jones study, no apparent relationship was observed (see Fig. 6).

The results of neither study could be used in an obvious way to discriminate between carcinogenic potency classes. This lack of correlation may be due to interlaboratory variation, since potencies for the same mutagen may vary between 30 and 60-fold (based on $n = 2$) (Bradley et al., 1981). However, N-nitroso compounds with approximately the same carcinogenic potency showed a variation in mutagenic potency over 3 orders of magnitude. Therefore, interlaboratory variation cannot be the only explanation. Concluding, in the form as reported by Bradley et al. (1981) and Jones et al. (1981), this test appears not to be able to predict carcinogenic potency of N-nitroso compounds.

3.2.4. In vitro transformation assays

Dunkel et al. (1981) compared the neoplastic transformation responses to chemical carcinogens of BALB/c 3T3 cells, Syrian Hamster Embryo Cells (SHE) and Rauscher Murine Leukemia Virus infected Fischer 344 Rat Embryo cells (RMLV-FRE). The authors included a number of N-nitroso compounds in their evaluation: MNNG, NDEA, NDMA, NDPhA and NEPhA. The compounds were tested without bioactivation. The test with RMLV-FRE only gives qualitative results, and can therefore not be used for quantitative prediction. The results with the other two tests are shown in Table 8. Without bioactivation even the qualitative performance of the two transformation tests for the selected N-nitroso compounds

[12] Defined as $\ln(1 + \frac{100}{D \times T^3})$, in which D is the total dose in mol/rat and T is the time (weeks) for death of 50% of the animals from tumours.

[13] Defined as $1/D_{10}$, in which D_{10} was defined as the concentration in nM of the compound that yielded mutant frequencies 10 times higher than the spontaneous mutant frequency.

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Table 6
Ames tests with nitrosamines of known carcinogenic potency for which mutagenic potency could be expressed in revertants/μmol.

Nitrosamine	Number of tests	Mutagenic potency (rev/μmol)			Carcinogenic potency	
		Range	Geometric mean	Ranking	([mmol/m^3]$^{-1}$)	Ranking
NDEA	53	330–110,000	2200	1	1100	3
NEBA	11	140–38,000	1700	2	30	9
NMEA	11	79–29,000	1400	3	452	5
NPIP	17	3900–9600	760	4	687	4
NDPA	21	220–96,000	620	5	409	6
NDMA	26	43–22,000	490	6	297	7
NPYR	17	110–30,000	400	7	108	8
NMOR	16	240–6700	370	8	2118	1
NDBA	17	97–8200	250	9	1150	2

The CCS nitrosamines and nitramines are printed in bold face.
The studies on which the mutagenic potencies are based, are summarized and referenced in Appendix A of the supplemental material. The derivation of the carcinogenic potency values and the studies on which they are based are described and referenced in Appendix B of the supplemental material.

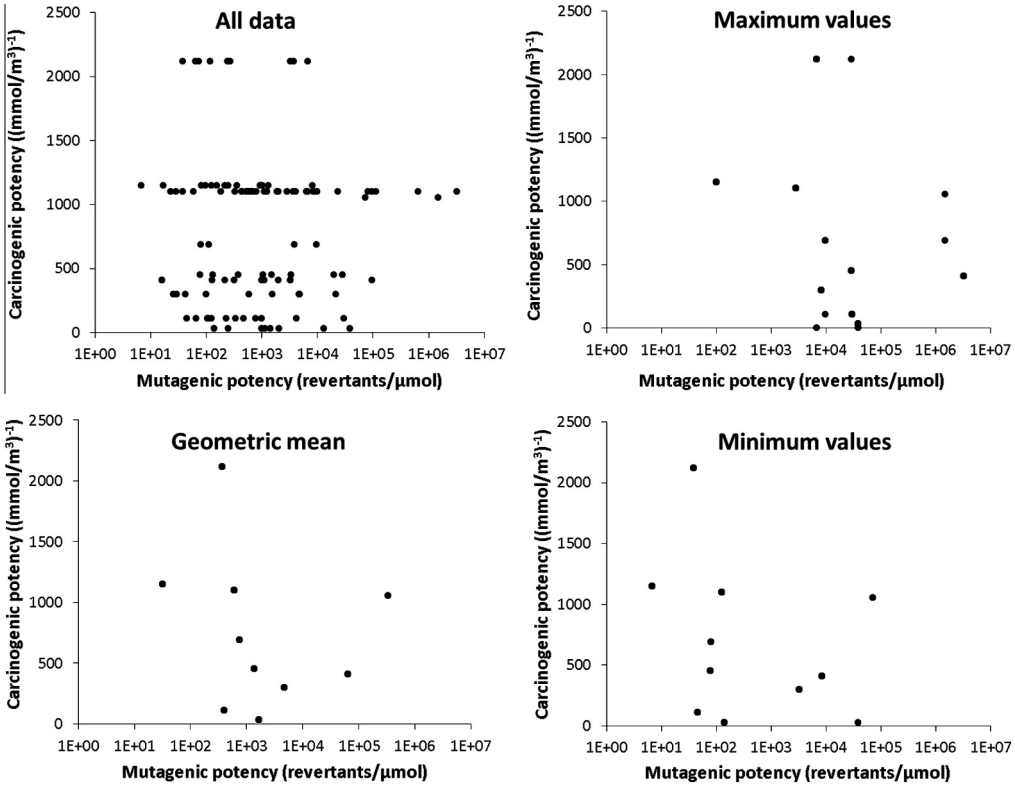

Fig. 3. Plot of mutagenic potency of N-nitroso compounds in bacterial reverse mutation tests against their carcinogenic potency. All data represent the mutagenic potency data of every Ames test of each N-nitroso compound we retrieved from public literature. The references to these studies are given in Table 9 of Appendix A of the supplemental material. Maximum and minimum values and geometric mean refer to the mutagenic potencies of the individual N-nitroso compounds. Carcinogenic potency data are the oral values listed in Table 3 of this paper.

is poor, so they certainly are not useful in a quantitative prediction. NDEA was also tested in the presence of hepatocytes and found to be positive (Dunkel et al., 1981). However, in this form the test will probably not be useful in quantitative predications because of its bad resolution: the maximum response was 4 transformed colonies in 664 surviving colonies for a concentration of 2.5 mg/mL of this potent carcinogen (at the next concentration, 5 mg/mL, survival dropped from approximately 100% to approximately 10%).

The Detailed Review Paper (DRP) number 31 of the Organization for Economic Cooperation and Development (OECD, 2007) analysed the performance of three models used in Cell Transformation Assays (CTAs) to screen the carcinogenic potential of chemicals: the Syrian hamster embryo (SHE) cells, and the mouse cell lines BALB/c 3T3 and C3H/10T$^1/_2$ (OECD, 2007; Vasseur and Lasne, 2012). The report compared CTA results collected from public literature to results from recent genotoxicity tests using mammalian and non-mammalian cell systems. The performance of the CTAs in predicting carcinogenic potential has been established for several hundreds of chemicals, comprising organic and inorganic substances. In this report (OECD, 2007) several N-nitroso compounds are listed. Unfortunately the report only published the categorical conclusion for each test result taken from public literature. Therefore, the source data of the N-nitroso compound results published in the OECD report were analyzed. Most of the original publications retrieved reported either the number of foci induced per culture dish or flask or the percentage of transformed cells or both. For each concentration reported the number of foci/dish (flask) or % cells transformed per mM concentration

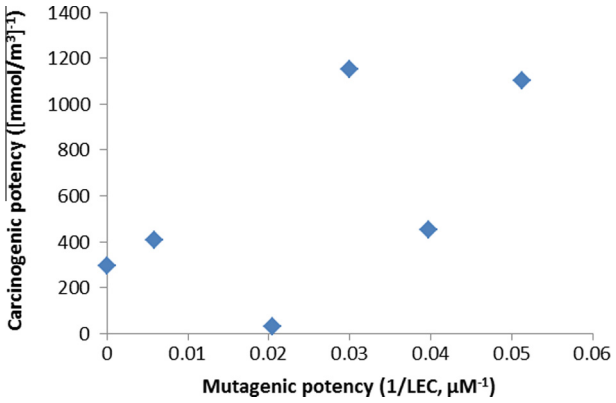

Fig. 4. Relationship between mutagenic potency in the *S. typhimurium* strain YG7108 hCYP3A4 and carcinogenic potency of N-nitroso compounds. The mutagenic potency is expressed as 1 divided by the Lowest Effect Concentration (LEC), and the data are derived from Fujita and Kamataki (2001a, 2001b). The carcinogenic potency data are the oral values listed in Table 3 of this paper.

were calculated. The maximum of these values calculated for each tested N-nitroso compound per cell line in one publication was taken as a measure of its transformation potency as measured in that publication. When more than one set of testing conditions were used, only the "standard conditions" were considered. The values thus calculated are listed in Table 11 of Appendix B.

Most of the transformation assays were executed without metabolic activation, a few assays did include metabolic activation in some form. For one publication hepatocytes had been added to the exposed cells, for another S9 fraction, while two publications used a cell line clone expressing human CYP2A6. On average, there is a very good correlation between the transformation potency of the various N-nitroso compounds in the three assays ($r = 0.98$–1.00). Furthermore, it is apparent that MNNG shows the highest transformation potency in the three transformation assays, much higher than would be expected based on its carcinogenic potency: it is number four in carcinogenic potency of the 8 N-nitroso compounds tested, with a potency ten times lower than that of number 1 (ENU), while in transformation potency it is number 1 with a potency 10 times higher than number 2 (NDMA). MNNG is the most tested N-nitroso compound in the three CTA assays, since it is often used as a positive control. Therefore, MNNG was used as a yard stick of transformation potency in order to analyze the results of the tested N-nitroso compounds across the three assays, expressing their transformation potency in each assay relative to the average potency of MNNG in that assay (see Table 9).

The mean relative potency of the tested N-nitroso compounds over all three assays executed without metabolic activation does not correlate at all with their carcinogenic potency ($r = -0.23$ and $\rho = -0.10$). However, in spite of the diverse nature of the metabolic activation employed, a good association was observed for the relative potencies determined in the presence of metabolic activation ($r = 0.95$ and $\rho = 0.8$; see Fig. 7). Since the number of N-nitroso compounds is rather low ($n = 4$) and the degree of association is mainly determined by one data point (NNK), more N-nitroso compounds need to be tested in a cell transformation assay (CTA) in the presence of metabolic activation in order to pass a definitive judgment.

Kowalski et al. (2000) evaluated an *in vitro* transformation test for predicting the rodent carcinogenicity TD_{50} (taken from the CPDB) against a testing database of 64 chemicals including both genotoxic and non-genotoxic carcinogens and carcinogens that normally require addition of a S-9 microsomal fraction for detection in the bacterial mutagenicity assay. The assay uses focus formation in a stable, bovine papillomavirus type 1 (BPV-1) DNA carrying C3H/10T½-mouse embryo fibroblast cell line (T1). Ninety-two percent of the compounds were correctly predicted as carcinogens, promoters, or non-carcinogens (NC). The authors listed the lowest transforming dose in the assay for the positive substances. Two N-nitroso compounds were mentioned: NDEA and MNNG, with lowest transforming doses of 0.05 mg/mL and 0.01 µg/mL, respectively. However, this clearly is not in line with the observed carcinogenic potencies of the CPDB: i.e. the TD_{50} in the rat for MNNG is 0.803 mg/kg bw/day, while for NDEA it is 0.0265 mg/kg bw/day. It confirms the remarkable potency of MNNG in cell transformation assays already noted in the assays with cell lines not transfected with viral genes (see above).

3.2.5. In vivo initiation assay

Only few studies were identified investigating the relation between *in vivo* mutagenic potency and carcinogenic potency.

Sakai et al. (2002) investigated the initiating activities of 26 chemicals by single intragastric administration in an *in vivo* 5 week initiation model in male Fischer 344 rats by evaluation of the induction of glutathione S-transferase placental form (GST-P)

Table 7
Correlation between mutagenic potency and carcinogenic potency of selected nitrosamines.

Parameter	Corr. coeff.	
	r	ρ
CYP1A1	−0.29	+0.09
CYP1A2	−0.46	+0.14
CYP1B1	−0.39	−0.20
CYP2A6	−0.10	+0.26
CYP2C8	+0.11	+0.26
CYP2C9	−0.10	+0.59
CYP2C19	+0.10	+0.45
CYP2D6	−0.13	+0.13
CYP2E1	+0.04	−0.09
CYP3A4	+0.62	+0.66
CYP3A5	−0.13	+0.13
Maximum mutagenic potency[a]	−0.01	+0.09
Average mutagenic potency[a]	−0.12	−0.14
Geometric mean mutagenic potency[a]	−0.44	−0.37

The mutagenic potencies are derived from Fujita and Kamataki (2001a, 2001b), the derivation of the carcinogenic potency values and the studies on which they are based, are described and referenced in Appendix B of the supplemental material.

[a] Calculated as 1/Lowest Effect Concentration (negatives set to 0).

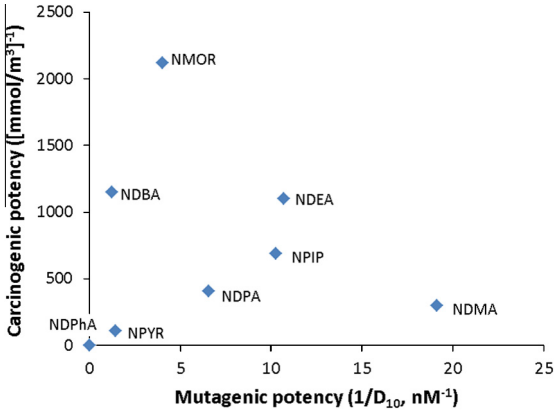

Fig. 5. Relationship between mutagenic potency of N-nitroso compounds in Chinese hamster V79 cells using thioguanine as marker gene and their carcinogenic potency. The mutagenic potency is expressed as 1 divided by the concentration in nM that yields mutant frequencies 10 times higher than the spontaneous mutant frequency (D_{10}). These mutagenic potency data are derived from Jones et al. (1981). The carcinogenic potency data are the oral values listed in Table 3 of this paper.

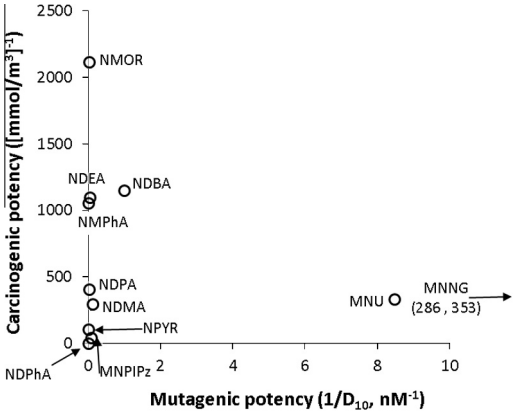

Fig. 6. Relationship between mutagenic potency of N-nitroso compounds in Chinese hamster V79 cells using 8-azaguanine as marker gene and their carcinogenic potency. The mutagenic potency is expressed as 1 divided by the concentration in nM that yields mutant frequencies 10 times higher than the spontaneous mutant frequency (D_{10}). These mutagenic potency data are derived from Bradley et al. (1981). The carcinogenic potency data are the oral values listed in Table 3 of this paper.

positive foci in the liver as end-point marker for hepatocarcinogenesis. Among the substances tested were NDEA, NDMA, NDiPLA, BBN, MNNG and MNU. The test was able to correctly identify genotoxic and non-genotoxic carcinogens as well as mutagenic non-carcinogens. Some association was found between the number of induced foci and the carcinogenic potency of these N-nitroso compounds ($r = 0.68$, $\rho = 0.20$), but which appears to strongly depend on one data point (see Fig. 8 and Table 10). However, when only considering those N-nitroso compounds which principally target the liver (all nitrosamines), a very good correlation is observed. The number of data points ($n = 3$) is too small to draw definitive conclusions, but this test does seem to hold promise for a quantitative prediction, especially for nitrosamines targeting the liver.

Also Fukushima et al. (2005) studied induction of liver lesions in male F344 rats by NDMA provided in a wide range of dose levels in drinking water, i.e. from 0.001 to 10 ppm (c. 0.142–1420 μg/kg bw/day), for a period of 16 weeks. A dose related and statistically significant increase of GST-P-positive foci, in the 1 and 10 ppm dose groups (c. 142 and 1420 μg/kg bw/day, respectively) was observed, corresponding to a relative increase of 3460 at the highest dose. This relative increase is circa 10 times higher than observed for NDMA by Sakai et al. (2002), which is probably related to the difference in dosing route and regime (single gavage of a relatively high dose (5 mg/kg bw) versus 16 week repeated administration via the drinking water). No increment in foci could be detected with the lower doses (0.001, 0.01, and 0.1 ppm (c. 0.142, 1.42 and 14.2 μg/kg bw/day, respectively)).

3.2.6. In vivo mutation assays

Sanner and Dybing (2005) have compared the lowest effective dose (LED) giving a response in an *in vivo* genotoxic test after oral or inhalation exposure with the carcinogenic dose descriptor T_{25}. The authors used a variety of *in vivo* genotoxicity assays: micronucleus, sister chromatid exchange, DNA adducts, chromosomal aberrations and Comet. The 34 genotoxic carcinogens in this analysis represented different classes of carcinogens and different genotoxic endpoints, exhibiting carcinogenic and mutagenic potencies covering four orders of magnitude, including one nitrosamine: NDELA. A linear correlation was found between the log of the lowest effective dose (LED) for *in vivo* genotoxicity after oral administration or inhalation exposure and the log of the lowest dose descriptor T_{25} for tumor formation ($r = 0.84$). Including 8 non-genotoxic substances in the analyses did not substantially change the degree of correlation ($r = 0.82$). The median of the ratio LED/T_{25} was 1.05% and for 90% of the substances this ratio fell in the range of 0.21–9.2, which shows that the numerical value of the LED is similar to the numerical value of the T_{25} within 1.5 orders of magnitude.

Table 8
Carcinogencity prediction by two transformation assays (data from (Dunkel et al., 1981).

N-nitroso compound	Balb/3T3	SHE	Carcinogenic potency ([mmol/m³]⁻¹)[a]
NDEA	±	–	1100
MNNG	+	+	350
NDMA	+	–	300
NDPhA	–	±	1

[a] Based on oral rat carcinogenicity studies (see Table 5).

Hernandez et al. (2011) performed a literature search for dose–response data in various *in vivo* genotoxicity and carcinogenicity studies. They applied the benchmark dose (BMD) approach using the dose–response modeling program PROAST on dose–response data from 18 compounds in the micronucleus assay (MN) and the *in vivo* transgenic rodent mutation assay (TGM). They compared their BMD_{10} values to the BMD_{10} from carcinogenicity studies in mice. The dataset contained 18 mostly genotoxic[14] carcinogenic compounds with potencies in the mouse spanning 4 orders of magnitude, including two N-nitroso compounds: MNU and NDMA. The authors observed a positive correlation between the logarithm of the lowest BMD_{10} from the genotoxicity tests (MN and TGM) and the logarithm of the tissue-matched[15] carcinogenicity BMD_{10} ($r = 0.54$ and 0.59, respectively). However, the correlation between the lowest overall genotoxicity BMD_{10} and the lowest tumor BMD_{10} was poor ($r = 0.43$) and the correlation between the lowest TGM BMD_{10} and the lowest tumor BMD_{10} was even poorer ($r = 0.22$). Since in risk assessment carcinogenic risk values are usually based on the tumors that appear in the highest frequency per unit dose, this means the evaluated tests cannot be used to predict carcinogenic potency thus derived, based on the available data. The authors blame this on the lack of proper dose–response studies due to the focus on qualitative hazard identification. Another cause could be the variety in species/strains treated, route of administration, exposure duration, tissues examined and genes used.

Of all the *in vivo* mutagenicity data for N-nitroso compounds that could be collected, only the *in vivo* transgenic rodent mutation assay (TGM) yielded relatively sufficient data for a quantitative analysis.

When plotting the maximum mutagenic potency value against the oral carcinogenic potency of the N-nitroso compounds a reasonable correlation is observed ($r = 0.74$ and $\rho = 0.43$, see Fig. 9). However, it all depends on one data point for ENU and no obvious carcinogenic potency classes could be discriminated using mutagenic potency determined in lacZ transgenic mice. On the other hand, the tobacco-specific nitrosamine NNK seems to be an outlier. When this data point is removed, the correlation improves dramatically, but still is dominated by one data point. However, without a good mechanistic reason for the exclusion of NNK, its removal remains arbitrary.

Concluding, potentially *in vivo* mutagenic potency *may* predict the oral carcinogenic potency of N-nitroso compounds. However, the database needs to be increased considerably using a uniform study protocol in order to verify this.

[14] Except perhaps benzene and dichloroacetic acid.

[15] Meaning genotoxic potency and carcinogenic potency were compared for identical tissues only.

Table 9
Transformation potencies of N-nitroso compounds.

Met Act	Compound	Cell lines						Primary cells		
		Balb/c 3T3			C3H/10T½			SHE		
		n	Mean transformation potency[a]	Relative transformation potency	*n*	Mean transformation potency[a]	Relative transformation potency	*n*	Mean transformation potency[b]	Relative transformation potency[c]
No	ENU	2	1.4×10^{0}	3.4×10^{-4}	1	3.5×10^{0}	4.7×10^{-2}	1	2.9×10^{-1}	4.0×10^{-4}
	MNNG	6	4.2×10^{3}	1.0×10^{0}	3	7.5×10^{1}	1.0×10^{0}	4	7.2×10^{2}	$1.0E \times 10^{0}$
	MNU	2	8.1×10^{0}	1.9×10^{-3}	1	1.9×10^{-2}	2.5×10^{-4}	1	2.4×10^{1}	3.3×10^{-2}
	NDEA	2	6.5×10^{0}	1.5×10^{-3}	2	7.7×10^{-2}	1.0×10^{-3}	2	1.1×10^{-1}	1.5×10^{-4}
	NDELA	1	4.8×10^{-2}	1.1×10^{-5}						
	NDMA	3	5.3×10^{1}	1.3×10^{-2}				1	1.4×10^{2}	2.0×10^{-1}
	NDPhA	1	2.0×10^{2}	4.7×10^{-2}	1	1.1×10^{0}	1.7×10^{-2}	1	$0.0 \times 10^{\circ}$	0.0×10^{0}
	NEPhA	1	9.4×10^{1}	2.2×10^{-2}				1	1.1×10^{2}	1.5×10^{-1}
	NNK				2	1.2×10^{-1}	1.7×10^{-3}			
Total		18			11			11		
Yes	MNU	1	1.6×10^{0}	3.8×10^{-4}						
	NDEA				1	1.8×10^{-1}	2.4×10^{-3}			
	NDMA	1	8.4×10^{-1}	2.0×10^{-4}	1	3.3×10^{-1}	4.5×10^{-3}			
	NNK				2	5.6×10^{-1}	7.5×10^{-3}			
Total		2			4					

Source data listed in Table 11 of Appendix A of the supplemental material. The transformation potency in a study is the maximum of the values calculated for each concentration tested. The mean is the average of the transformation potencies of all studies available for a particular cell line and nitrosamine.

[a] Number of foci/dish (flask)/mM.

[b] % Transformation/mM.

[c] The mean transformation potency in a cell line divided by the mean transformation potency of MNNG in that cell line; n = number of studies.

3.2.7. Summary of predictive performance of mutagenicity or alternative carcinogenicity tests

The results of some of the mutagenicity and alternative carcinogenicity tests discussed in the previous sections show a reasonable correlation with carcinogenic potency based on oral rat carcinogenicity studies. These tests are the combination of three *in vitro* cell transformation assays with the BALB/c 3T3, the SHE or the C3H/10T½ cell line executed in the presence of metabolic activation system, the *in vivo* GST-P assay and the *in vivo* lacZ mutation assay in transgenic mice. However, as shown in Table 11, the available N-nitroso compound datasets for these tests are too small to draw definitive conclusions.

We have also tested the predictive performance of the tests discussed in the previous sections against N-nitroso compounds potency data from the CPDB (inverse harmonic minimum TD_{50}'s for oral rat carcinogenicity studies), but this did not lead to significantly better or worse predictions (data not shown).

4. Discussion

Based on the available evidence, genotoxic carcinogenicity is the leading health effect for both the CCS nitrosamines as well as the CCS nitramines. All CCS nitrosamines and nitramines, except NPIPz, are genotoxic. Every CCS nitrosamines and nitramines that was tested for carcinogenicity is carcinogenic, even NPIPz. Most N-nitroso compounds and nitramines have a carcinogenic potency within two orders of magnitude of each other in oral studies. Notable exceptions are NDPhA, which is more than 4 orders of magnitude less potent than the most potent N-nitroso compound NNK, and NPIPZ which is more than 3 orders of magnitude less potent.

Many nitrosamines and also the nitramine DMA-NO_2 have the liver as main target organ for carcinogenicity in oral studies. Among the CCS substances the exceptions are the piperazines DNPIPz and NPIPz and the nitramine MA-NO_2, which is most potent in the CNS, although it does target the liver as well. The tested piperazines do not target the liver, and most of them induce nasal cavity tumors. For some other nitrosamines the esophagus is the main target: NMPA, NMBA, and NEBA. In general, tissues at the portals of entry, such as the oral cavity, esophagus, forestomach (after oral exposure), and the nasal cavity, trachea, and lung (after inhalation) appear at risk as well. However, the only more or less clear trends discernible from these carcinogenicity data are the tendency to induce nasal cavity tumors for nitrosopiperazines and bladder tumors for those (hydroxy)butyl nitrosamines that proved carcinogenic under the test conditions.

Based on the process chemistry involved in the CCS systems, the general population is potentially exposed via the environment through inhalation (de Koeijer et al., 2013). Although no data on workplace exposure are available, also occupational exposure to nitramines and nitrosamines as by-products of the CCS process may occur via this route (Gentry et al., 2013). For most CCS nitrosamines, as well as for most other N-nitroso compounds, only oral studies are available. That raises the question of how reliable extrapolation from oral to respiratory cancer values is. At present only for two CCS nitrosamines and one other nitrosamine oral as well as respiratory studies are available: NDMA, NMOR and NMPIPz.

The respiratory cancer risk value of NDMA derived from oral studies is circa 14× lower than the one directly derived from respiratory studies, and also the critical target organ is different: the liver after oral exposure and the olfactory mucosa after respiratory exposure. The epidemiological evidence by Straif et al. (2000) suggests that the cancer risk value derived from oral rat studies is closer to the real human respiratory cancer risk of NDMA than the one derived from the respiratory rat studies: Carrier et al. (2011) reanalyzed the data presented by Straif et al. (2000) and found that the threshold inducing an excess risk of mortality from cancers associated with exposure to nitrosamines for an average exposure period of approximately 10 years in a German cohort, corresponds to an average concentration between 2.5 μg/m^3 and 15 μg/m^3 of total nitrosamines (NDMA + NMOR). Overall, exposure to NDMA and NMOR was about equally high. Based on the NDMA carcinogenic potency value of 57 $(mg/m^3)^{-1}$ derived from the respiratory rat carcinogenicity study, one would expect an excess cancer mortality of $57/1000 \times 2.5/2 \times 10/40 \times 100 = 1.8\%$ cancer deaths already at the lower threshold concentration of 2.5 μg/m due to NDMA exposure alone, while based on the NDMA potency of 4 $(mg/m^3)^{-1}$ derived from the oral rat study one would expect an excess

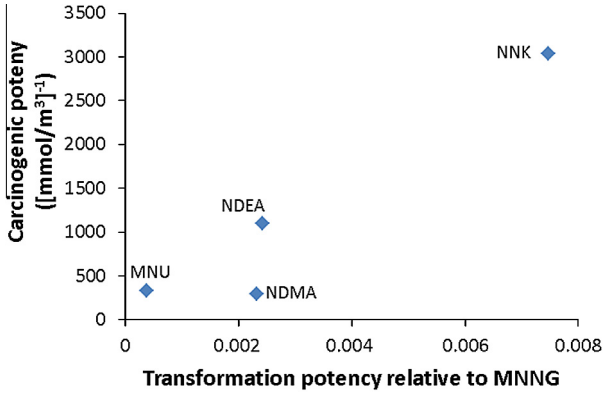

Fig. 7. Correlation between relative transformation potency in the presence of metabolic activation and carcinogenic potency of some N-nitroso compounds. The relative transformation potency data are taken from Table 9 and the carcinogenic potency data are the oral values listed in Table 3.

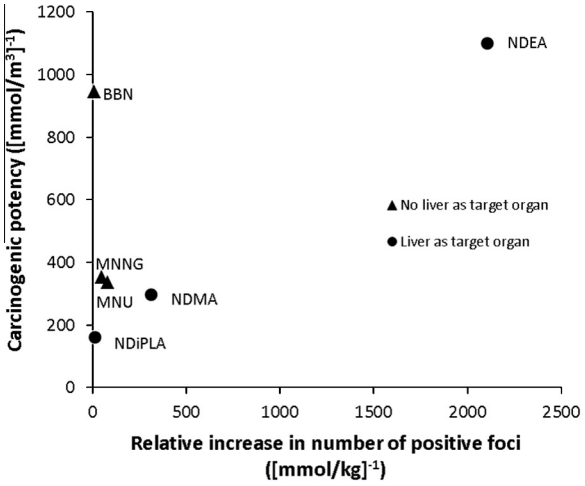

Fig. 8. Relation between carcinogenic potency and increase in GST-P positive foci. The GST-P data are derived from Sakai et al. (2002) and are listed in Table 10 and the carcinogenic potency data are the oral values listed in Table 3.

mortality of 0.15%. Furthermore, in this epidemiological study the most common nitrosamine induced cancers are upper gastrointestinal tract (oesophageal and oral) cancers. Therefore, NDMA-induced local tumors in the nose observed in respiratory carcinogenicity studies in rats may not be relevant for humans.

For NMOR both routes of exposure are more in line with each other: the critical target organ is the same, the liver, and the predicted human respiratory cancer risk values are in the same order of magnitude, the one derived from the oral studies being 5× higher than the one derived from the rat respiratory study.

NMPIPz targets the nasal cavity in the rat, both after oral and respiratory administration: via the latter route, where this target site constitutes the portal of entry, it is ten times as potent as the extrapolation from the oral route suggests (see Table 3).

Concluding, for one nitrosamine the oral rat study seems to be more predictive of the human respiratory cancer potency that the respiratory study (NDMA), for another there is reasonable agreement between the oral and respiratory rat study with respect to carcinogenic potency (NMOR) while for the third the oral rat study underestimates carcinogenic potency via the respiratory route (NMPIPz). Overall, based on this limited and somewhat contradictory evidence for these three nitrosamines, there is not sufficient evidence to depart from the simple absorption based oral to respiratory route extrapolation for systemic toxic effects.

N-nitroso compounds may differ quite substantially in carcinogenic potency (see Fig. 10): the potency range for the group described here stretches more than 3 orders of magnitude. Some of the presently negatively tested nitrosamines may turn out to be very low-potent carcinogens after all (even weaker than the weakest one identified here), when tested in regulatorily acceptable testing protocols. Also apparent from the data is that relatively small changes in molecular structure may result in quite different target organs for carcinogenicity, or in substantial potency changes.

Most of the available carcinogenicity data come from studies in which one or just a few of the substances presently investigated were tested. The majority of these studies were not designed for specifically estimating cancer potency of the investigated compound, but merely to identify its carcinogenic properties. Therefore, only few of these studies meet guideline-standards for estimating potency, and many suffer from shortcomings in this respect, such as an insufficient number of animals used, the absence of concurrent controls, a too short period of exposure and/or observation, testing (far) beyond the maximum tolerable dose, and incomplete toxicological examinations (including histopathological detail) and/or reporting (for references and more details see Appendix B of the supplemental material). The following substances have rat oral cancer risk values based on appropriate studies: NDMA (Peto et al., 1991a,b), NDEA (Peto et al., 1991a,b), NMEA (Lijinsky and Reuber, 1980), DNPIPz (taking into account the study by Lijinsky and Taylor (1975) and the study by Takano et al. (1982), when combining its different exposure/observation time groups), NDPhA (Cardy et al., 1979) and ENU (Maekawa et al., 1984). NDELA has an appropriate oral study on male rats only (Berger et al., 1987), NMOR has an appropriate oral study only including female rats (Lijinsky et al., 1988). All the other studies were less appropriate. Also the few available inhalation studies by Klein et al. (for NDMA (1991), NMOR (1990) and NMPIPz (1999)) were less fit for potency estimation, especially with respect to the number of doses and the number of animals tested. The few available dermal studies (NDEA: Iversen (1980) and Hoffmann and Graffi (1964); NDELA: Hoffman et al. (1983); NDPhA: Iversen (1980)) are not suitable for deriving cancer risk values. Clearly, one should recognize that most of the available studies are less appropriate for potency estimation, and that this may hamper the establishment of firm conclusions.

There are only two nitramines for which we could allocate carcinogenicity data, MA-NO_2, and DMA-NO_2, whose potency was in the same order of magnitude. On this basis it is not possible to validate any prediction model for this class of compounds. For N-nitroso compounds, on the other hand, carcinogenicity data are available for a substantial number of substances, covering a potency range of well over 3 orders of magnitude, including some very weak, if not non-carcinogenic compounds. Therefore, predictive models for this chemical group could be evaluated, as summarized below.

With regard to *in silico* prediction models, both classification models as well as QSARs have been developed, all using the CPDB as the major source of *in vivo* carcinogenicity data. The reported sensitivity of the classification models is rather good, but their specificity is quite poor. This latter result was to be expected given the limited number of non-carcinogenic N-nitroso compounds. Additionally these 'non-carcinogenic' N-nitroso compounds may in fact be very low-potent carcinogens as the performed tests are not adequate to definitively classify compounds as 'non-carcinogenic'. The QSAR models developed by the groups of Helguera et al. (2007, 2008a, 2008b, 2010) and Harju et al. (2010), respectively, showed a good predictive performance when the lowest TD_{50} values in the CPDB were selected as measure of carcinogenic potency. They have attempted to reduce bias by

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Table 10
Results initiation assay with N-nitroso compounds.

Nitrosamine	Dose (mg/kg)	Carcinogenicity target organ(s)	Test group		Control group		Potency (relative increase in no. of foci/(mmol/kg))
			n	No. of foci (Mean ± SD)	*n*	No. of foci Mean ± SD	
BBN	450	Bladder	6	29.5 ± 5.7	18	1.8 ± 1.5	6.3
NDEA	10	Liver	12	123.6 ± 30.8	9	0.6 ± 0.7	2104.1
NDMA	5	Liver	10	46.1 ± 21.1	19	2.2 ± 2.3	310.5
NDiPLA	500	Liver, kidney	16	75.5 ± 26.8	18	1.8 ± 1.5	13.6
MNU	35	Stomach	12	43.0 ± 22.6	20	1.6 ± 2.0	79.2
MNNG	80	Stomach	12	41.1 ± 16.4	20	1.6 ± 2.0	47.2

n = number of animals in group.
The potency values are calculated by the authors of this paper by dividing the number of foci observed by Sakai et al. by the administered doses (converted to mmol/kg).

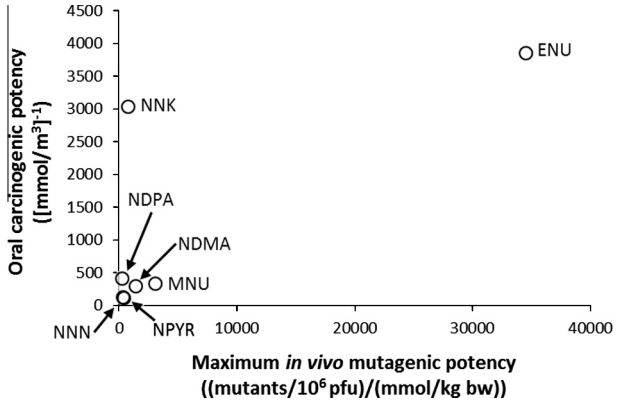

Fig. 9. Correlation between *in vivo* gene mutation in the transgenic rodent mutation assay and carcinogenic potency of N-nitroso compounds. pfu = plaque forming unit. The transgenic rodent mutation assay (TGM) data are listed in Table 8 of Appendix A of the supplemental material. For each N-nitroso compound the maximum of the potency values over all available studies is used. The carcinogenic potency data are the oral values listed in Table 3.

limiting the carcinogenicity data to specific study designs and protocols, such as oral gavage carcinogenicity studies with male rats. As their models were not validated with an external validation set, predictions for non-tested N-nitroso compounds are still to be interpreted with caution. In a subsequently developed QSAR model by NILU (Harju et al., 2011), the harmonic mean of the most potent TD_{50} values in rats was taken as measure of carcinogenic potency, and just N-nitroso compounds with only their trade-mark structural alert of the N-nitroso group were selected. This model satisfactory predicted the potency of the training set compounds, but was non-predictive for a predefined test set. Thus, the confined QSAR model of Helguera et al. (2008a) appears most suitable for generating a first estimate of the TD_{50} of an untested nitrosamine. The training sets used to develop these models, though, consist of rather diverse chemical structures with just a few nitrosamine structures similar to those identified in the CCS process, making predictions for new untested compounds that structurally relate to these compounds more uncertain. Therefore, a way forward with respect to making better predictive QSAR models essentially entails adequately addressing the above complicating factors. Thus, N-nitroso compounds should preferably be modeled as separate classes characterized by a common bioactivation reaction underlying their toxicity induction. At the same time, all potency values should be derived from a single species, exposed via the same route. However, should this result in a limited training set, the criteria may need to be less strict in order to obtain a sufficient number of chemicals in the set.

Potentially, biological models may produce better predictions than purely chemical models. There are quite a number of *in vitro* and *in vivo* biological models that could provide adequate

Table 11
Datasets of tests with a reasonable correlation with carcinogenic potency.

Compound	Tests			Total
	CTA + MA	lacZ mouse	GST-P assay	
NDMA	X	X	X	3
NDEA	X		X	2
MNU	X	X	X	3
NNK	X	X		2
NDPA		X		1
NNN		X		1
NPYR		X		1
ENU		X		1
MNNG			X	1
NDiPLA			X	1
BBN			X	1
Total	4	7	6	

Based on the data described in Section 3.2.

predictions of the carcinogenic potency of N-nitroso compounds. Firstly, of course, the many tests that assess mutagenic or genotoxic potential and potency. They could well reflect the chemical's carcinogenic potency, especially when focusing on a specific group of structurally related, genotoxic carcinogens. This especially holds for short-term *in vivo* models in which in principle all potential target organs may be investigated for mutations or pre-neoplastic lesions, indicative of the carcinogenic potency of the respective N-nitroso compound in that specific organ. But before stepping into *in vivo* studies, which actually should be seen as a last resort in this respect, first *in vitro* options should be explored.

Clearly, bacterial mutagenicity data are most abundantly available for this exploration. However, attempts to correlate mutagenic potency in any bacterial model to carcinogenic potency of N-nitroso compounds have not been recovered from public literature, and our own attempts to correlate mutagenic potency of N-nitroso compounds in all available Ames tests with carcinogenic potency were not successful. Though this may in part be due to the heterogeneity of the available data, also standardized mutagenicity data from Fujita and Kamataki, 2001a did not give any satisfying correlation with carcinogenic potency values. From this it must be concluded that this bacterial assay cannot serve as predictive model.

With regard to *in vitro* mammalian mutagenicity tests specifically focusing on N-nitroso compounds, only one attempt by Jones et al. (1981) with Chinese hamster V79 cells could be identified. They generally found a good correlation between carcinogenicity potency values and mutagenicity measured in V79 cells co-cultivated with primary rat hepatocytes for 26 N-nitroso compounds. However, they formulated a rather unconventional potency value, defined as $\ln(1+\frac{100}{D\times T^3})$,[16] which is difficult to interpret biologically.

[16] In which *D* is the total dose in mol/rat and *T* is the time (weeks) to death of 50% of the animals from tumors.

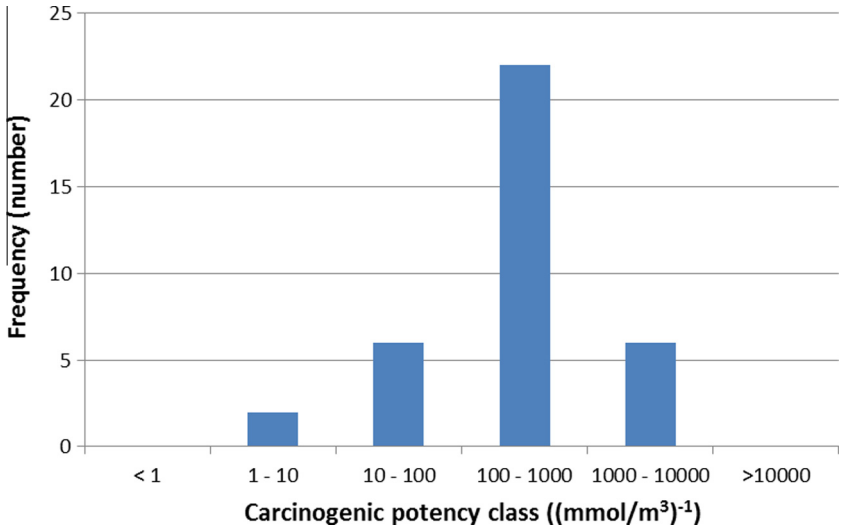

Fig. 10. Frequency distribution of respiratory carcinogenic potencies of the N-nitroso compounds described in this paper, derived from oral rat studies. Based on the data listed in Table 3.

When using the underlying original mutagenicity data together with our potency values, we were not able to find any correlation though. The absence of any other attempts in this field suggests that many options are still waiting to be explored. Quite a number of *in vitro* assays with mammalian cells potentially are suitable for this purpose, given their high true prediction rate of *in vivo* carcinogenicity potential (Kirkland, 2011).

We have explored the dose–response data for *in vitro* cell transformation assays (CTAs) and found a promising association between carcinogenic potency and transformation potency relative to the positive control MNNG, but only when metabolic activation was applied. As the dataset only comprises four N-nitroso compounds, this work needs to be further extended before a proper judgment about the predictive value of these assays can be made.

Toxicogenomic studies on N-nitroso compounds and on other well-known carcinogens that included a few N-nitroso compounds show that toxicogenomic read outs in *in vitro* or *in vivo* models apparently have the potential to distinguish genotoxic carcinogens, non-genotoxic carcinogens, and non-carcinogens (see e.g. Magkoufopoulou et al., 2012; Ellinger-Ziegelbauer et al., 2008), but attempts to predict carcinogenic potency on the basis of toxicogenomic data were not encountered, leaving this an option for future research.

Also for *in vivo* models detecting mutagenic potency we could not find any attempt to specifically predict N-nitroso compound carcinogenic potency. In all the studies retrieved, only a few N-nitroso compounds were included in a group of structurally diverse carcinogens. In the only initiation-promotion model study that involved more than two N-nitroso compounds, performed by Sakai et al. (2002), who used six N-nitroso compounds, indeed a promising association was found between the number of induced GST-P positive foci and the carcinogenic potency of the hepatocarcinogenic nitrosamines (NDEA, NDMA, and NDiPLA). It should be noted that only a weak association was found when including the non-hepatocarcinogens (BBN, MNU and MNNG).

Furthermore, an association was found between mutation induction in transgenic mice using the Lac Z gene and the carcinogenic potencies of the tested N-nitroso compounds. Thus, also this *in vivo* model may hold promise as prediction model for carcinogenic potency. However, the number of N-nitroso compounds tested was limited, there was an unexplained outlier and the association depended very strongly on one data point (leverage point).

5. Conclusions

Overall, no single model was identified that resulted in adequate and correct predictions of the carcinogenic potency of N-nitroso compounds as a group. Some models seem to hold promise in this respect, such as the QSARs developed by the Helguera group, the *in vitro* transformation assays, and the *in vivo* initiation-promotion, and transgenic animal assays. Most of the discussed models, however, have not been adequately explored for this purpose, as the number of N-nitroso compounds investigated is yet too limited. Therefore, further systematic data generation is needed to definitely assess their predictive power. As far as biological models are concerned, first preference should be given to *in vitro* models, for pragmatic as well as ethical reasons. Therefore, creating an extended dataset for one of the CTAs executed in the presence of a bioactivating system (e.g. rat S9), including a wide variety of N-nitroso compounds with respect to potency and chemical class and all CCS nitramines so far identified, would be a good first step in developing a model for N-nitroso compound and nitramine carcinogenic potency prediction. Based on the available data, none of the three evaluated CTAs is clearly preferred, as they show, at least for N-nitroso compounds, a strong correlation with each other. The SHE assay and the BALB/c 3T3 assay are currently in the process of being included in an OECD testing guideline, while the C3H/10T½-assay is not (Vasseur and Lasne, 2012), so one of the first two (or both) would be the first option to be followed up. Should the *in vitro* CTA model not provide the desired certainty and accuracy, one could embark on extending the datasets for one of the *in vivo* tests. In this case, the transgenic animal assay would be preferred, since it is already useable for the evaluation of various potential target organs. Once the cancer potency prediction of individual models has been established, combining these models should be explored to see whether this will raise the predictive capacity even further.

Though the carcinogenicity data on nitramines are too few to validate any prediction model, it may be anticipated that any prediction model (or combination of models) identified as suitable for nitrosamines might also predict carcinogenic potency for untested nitramines, noting their potential interconversion *in vivo* and the comparable carcinogenic potency and target-organs of DMA-NO_2 and NDMA when tested via the oral administration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2014.01.017.

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