Do all nitrosamines pose a significant level of genotoxic risk?

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Agenda

- Mechanism
- Metabolism
- Ames assay variabilities
- Is mutagenicity predictive of carcinogenicity?
- SAR for mutagenicity
- Carcinogenic potency
- SAR for carcinogenicity
- Expert review
Mechanism of action

Diazomethane formation

Carbocation formation

→ DNA Alkylation
Metabolic activation

- Metabolised by cytochrome P450 enzymes
  - CYP2A6 and CYP2E1 isozymes
- Mutagenic activity appears to be greater with hamster > mouse > rat S9
  - Shown for N-nitrosodimethylamine (DMN), N-nitrosodiethylamine and N-nitrosodimethylbutylamine
- Aroclor 1254 induction may repress activity of DMN demethylase activity in rat and mouse, but not hamster S9
- S9 from liver fractions more active than kidney and lung tissues
- Microsome and cytosol fractions required for activity of hamster S9
  - Microsome fraction of mouse and rat liver S9 inhibits the mutagenicity of DMN mediated by hamster liver S9
- S9 concentration important

Ames assay variabilities

- Base pair substitution strains (TA1535, TA100 and TA1530) most sensitive

- Pre-incubation/liquid suspension protocols may increase sensitivity compared to the standard Salmonella plate incorporation assay
  - DMN positive in pre-incubation assay in TA1530, but not plate incorporation assay, with rat liver S9
  - Likely due to higher component concentrations

- pH dependence
  - Activity is reported to be greater at pH 5 than at pH 7
  - The α-hydroxydialkynitrosamine active intermediate has greater stability in weakly acidic media

Effectively SARs cover all dialkylnitrosamines*

- Can Derek alert be refined?
- Can additional data be added to Sarah?

*nb well-aligned with cohort of concern
“It is therefore prudent to consider all N-nitrosamines containing a $\alpha$-hydrogen that can be metabolically activated as potentially mutagenic and carcinogenic to humans, however with different potencies depending on nature of the functional group, specifics of metabolic activation and repair efficiency and capacity” – EMA, 25th June 2020

Assessment report: Procedure under Article 5(3) of Regulation EC (No) 726/2004, Nitrosamine impurities in human medicinal products
Data gathering

• Searched for mutagenicity and carcinogenicity data in the public domain
  - also asked members for any data
• Identified 252 papers with (potentially new) data
• Updated the coverage in Vitic database
  - now contains 518 nitrosamines; 411 with Ames test data and 234 with rodent carcinogenicity data
• Updated the coverage in Lhasa Carcinogenicity Database
• Conducted targeted searching, to identify whether a change to SAR is feasible
  - searching for compounds (i) with a-steric hindrance and (ii) Ames data

SOT poster – Feb 2020
Mutagenicity and Carcinogenicity Study

• Analysed data

• Identified pivotal compounds

• Reviewed Ames and carcinogenicity data
  • Ames data → modify SAR
    • Co-ordinated with industry members to ensure repeat testing where data is incomplete or of insufficient quality
    • Acceptable purity, tested in modern 5-strain test, under most appropriate conditions
  • Carcinogenicity data
    • Does carcinogenicity data fully support SAR identified from Ames studies?
    • Are carcinogenicity data for pivotal compounds consistent with corresponding Ames studies? If not,
      • Can it be explained based on MoA (tumours unrelated by genotoxic MoA)?
      • Can class-specific AI/PDEs be generated for specific subclasses of nitrosamines?
Does Ames predict carcinogenicity?

Concordance between Ames/Carc for nitrosamines is generally very good in comparison to the wider performance

- Performance is highest for potent carcinogens (TD$_{50} < 1.5$)
  - 2/51 compounds incorrectly predicted negative but have poor/incomplete data
  - Both compounds are being re-tested to modern standards
  - One compound is a complex structure with other possible MoA

<table>
<thead>
<tr>
<th>Statistic</th>
<th>$R_2N-NO$</th>
<th>All data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced Accuracy</td>
<td>71.4%</td>
<td>66.2%</td>
</tr>
<tr>
<td>F-Score</td>
<td>90.2%</td>
<td>64.7%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92.8%</td>
<td>56.7%</td>
</tr>
<tr>
<td>Specificity</td>
<td>50.0%</td>
<td>75.7%</td>
</tr>
<tr>
<td>PPV</td>
<td>87.8%</td>
<td>75.3%</td>
</tr>
<tr>
<td>NPV</td>
<td>64.3%</td>
<td>57.2%</td>
</tr>
</tbody>
</table>
Exploring the SAR

**Piperazines**
- Metabolism possible
- Metabolism partially blocked

**Piperidines**
- Metabolism possible
- Metabolism partially blocked

**Dialkyl nitrosamines**
- Metabolism possible
- Metabolism partially blocked

Derek alert refinement

007: N-Nitro or N-nitroso compound

Alert Matches

Description Image

Comments


This alert describes the mutagenic and clastogenic potential of N-nitro or N-nitrosamine compounds.

N-nitro- and N-Nitrosamines are believed to require metabolic activation to exhibit genotoxic activity [Maga and Tu, Montesano and Hall], alpha-Hydroxylation and subsequent elimination of a carbonyl compound may lead to the formation of an alkylidiazohydroxide, which, on loss of a hydroxide, decomposes to give a carbonyl ion capable of alkylating DNA. The key hydroxylation step is primarily mediated by cytochrome P450, particularly isozymes 2A6 and 2E1.
Derek alert refinement

Click above to view the original structure.
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- Mechanism
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Carcinogenicity of N-Nitrosamines

• As mutagenic genotoxicants, N-nitrosamines are also typically carcinogens, and often potent ones
  • There are exceptions, however
    • Alternate mechanisms of carcinogenicity
    • Variation in available experimental data
  • Included in the ICH M7 Cohort of Concern since they are often more potent than the TTC \(^1\)
• Recent EMA guidance \(^2\) suggests a limit of 18 ng/day for nitrosamines with insufficient data, however also leaves the door open to SAR considerations
  • “If a MAH \(^3\) intends using a higher limit than 18 ng/day, an approach based on structure-activity-relationship (SAR) considerations is acceptable. The approach taken needs to be duly justified by the applicant/MAH.”
  • “The TD50 of the structurally closest related N-nitrosamine for which robust data are available to calculate a reliable TD50 should be applied”

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1 Threshold of Toxicological Concern
3 Marketing Authorisation Holder
Carcinogenicity of N-Nitrosamines

- The question therefore becomes “how do you define ‘structurally closest’”?
  - Nitrosamine TD50s span four orders of magnitude!
  - Choice of the correct analogue is critical
- NDEA and NDMA, the most studied, are amongst the most potent
  - Suggests they may not be good analogues for the majority of nitrosamines
Carcinogenicity of N-Nitrosamines

• Rough analysis of the SAR suggests that nitrosamines with methyl/ethyl groups are statistically more potent than any other category
  • Figure shows estimated 5\textsuperscript{th} percentiles for a variety of rough SAR classes
  • Methyl/ethyl are lower, and compounds with bulky groups are higher, than the bulk dataset
tert-Butylamine derivatives

- N-Nitroso-N-methyl-tert-butylamine and ethyl analogue are non-carcinogenic
  - Despite containing methyl/ethyl groups
  - No mutagenicity data available
  - Hypothesis is that the tert-butyl carbonium ion is unable to alkylate DNA
    - Shown in vivo with labelled N-nitroso-N-methyl-[2,2'-14C2]tert-butylamine
      
      ![Diagram](image)
      
      \( R = \text{Me, Et} \)

- Some Ames data is available for N-nitroso N-\( n \)-butyl tert-butylamine – TA1535 +/- S9 negative

1 Magee and Lee (1964), Biochem J, 91, 35-42
N-Nitrosodiphenylamine

- N-Nitrosodiphenylamine is a weak carcinogen and non-mutagenic in standard Ames strains
  - Expect that alpha-hydroxylation cannot occur
  - Expect the phenyl carbonium ion to be fairly unreactive
- But it is still carcinogenic!
  - Potential alternative mechanism – trans-nitrosation to the aryl nitroso\textsuperscript{1,2}
- Mutagenicity is in TA104 and TA2638 (+ S9)
  - Strains sensitive to oxidative damage
  - Oxidative damage is a mechanism proposed for aryl nitroso compounds

\textsuperscript{2} Challis and Osborne (1973), *J Chem Soc Perkin Trans II*, 1526-1533
\(\alpha\)-Substitution

- N-Nitrosodiisopropylamine is weakly carcinogenic
  - Non-mutagenic in a recent OECD-compliant Ames test
- N-Nitrosodisebutylamine cannot be confirmed to be non-carcinogenic
  - Increase in tumour rate but not statistically significant (few animals)
  - Non-mutagenic in incomplete Ames assay
- N-nitroso-2,6-dimethylpiperidine is non-carcinogenic and non-mutagenic
- Principal mechanism requires CH2 group \(\alpha\) to the nitrosamine
  - One removed mechanistically, one for steric reasons
- Possible (weaker) alternative carcinogenicity mechanisms:
  - Singlet oxygen formation via \(\alpha\)-hydroperoxidation\(^1\)
  - \(\beta\)-Oxidation and subsequent formation of alternate carcinogens\(^2\)

Cyclic nitrosamines

- N-Nitrosoazetidine, pyrrolidine, piperidine and piperazine are all mutagenic, but weak carcinogens
- N-Nitrosoazepane, azocane, morpholine and 1,2,3,6-tetrahydropyridine are all strong carcinogens
  - N-nitrosomorpholine is significantly more metabolically labile\(^1\)
  - Expect the same to apply to 1,2,3,6-tetrahydropyridine
  - Unclear what is occurring with the azepane/azocane
    - Propose that larger rings have less impact

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\(^1\) Hecht et al (1981), ACS Symposium Series, 174, 49-75
Electron-withdrawing groups

- N-Nitroso-(2,2,2-trifluoroethyl)ethylamine is a weak carcinogen, N-nitroso-bis(2,2,2-trifluoroethyl)amine is non-carcinogenic and non-mutagenic in TA1535
  - The same trend is seen with cyano groups
  - Electron-withdrawing groups reduce rate of metabolism and thus potency
  - Need to have strong EWG on both sides to remove carcinogenic liability
Carboxylic acids

- Carboxyl substituents at the α-position, analogous to amino acids, e.g. N-nitrosoprolpine, tend to reduce potency as electron-withdrawing groups (discussed above).
- N-nitroso-3-carboxypiperidine and N-nitroso-4-carboxypiperidine also negative – note they are cyclic.
- Hypothesis for these is polarity.
  - N-Nitrosomethyl-3-carboxypropylamine is carcinogenic but only a bladder carcinogen, supporting this.
  - No overall correlation between logP and TD50.

1 Hasegawa et al (1998), Cancer Lett, 123, 185-191
Updated Derek alert

070 N-Nitro or N-nitroso compound

Description Image

This alert describes the carcinogenicity of N-nitro- and N-nitroso compounds.

The mechanism of action of both N-nitro and N-nitroso compounds requires metabolic activation. [Maga and Tu, Scherf et al.]. Alpha-Hydroxylation and subsequent elimination of a carbonic compound may lead to the formation of an alkyldiazoalkylate which, on loss of a hydride, decomposes to give a carbocation capable of alkylating DNA. The key hydroxylation step is primarily mediated by cytochrome P450, particularly isozymes 2A6 and 2C1, but this may vary between compounds [Kamataki et al., Camus et al.]. The alpha-hydroxylation mechanism is supported by reduced potency for compounds with branched alkyl chains as discussed below and for deactivation at the alpha positions, indicating a kinetic isotope effect [Lijinsky et al. 1978, Lijinsky 1977]. N-Nitrosamines and N-nitrosoureas are chemically unstable at physiological pH and decompose to the alkyldiazoalkylate directly [Maga and Tu].
Updated Derek alert
ICH M7 allows the use of **two in silico** predictions in lieu of an Ames test

Sarah Nexus has a strongly positive hypothesis for nitrosamines

- Those compounds with known experimental data will return an exact match
- Many unknown nitrosamines will result in a positive call

Negative hypotheses exist for e.g. $\alpha$-substituted nitrosamines

- Negative nearest neighbours in these hypotheses can be used as supporting evidence
- Even in the general, positive, hypothesis, local areas of negative chemical space may exist

Expert review of the prediction is required in these cases

- And should be performed for all compounds
Expert review

Both positive

Carc +ve, Mut -ve

Both negative

“Nothing to report” for carcinogenicity

Carc –ve, Mut unknown

Unknown compounds

“Nothing to report” for carcinogenicity

“Nothing to report” for carcinogenicity
Expert review

Both positive, Mut positive

Both negative

“Nothing to report” for carcinogenicity

Carc -ve, Mut unknown

“Nothing to report” for carcinogenicity

“Nothing to report” for carcinogenicity
Acknowledgements

- Richard Williams
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Any questions?