N-Nitrosamine SAR Modeling of Carcinogenic Potency

current status and future needs

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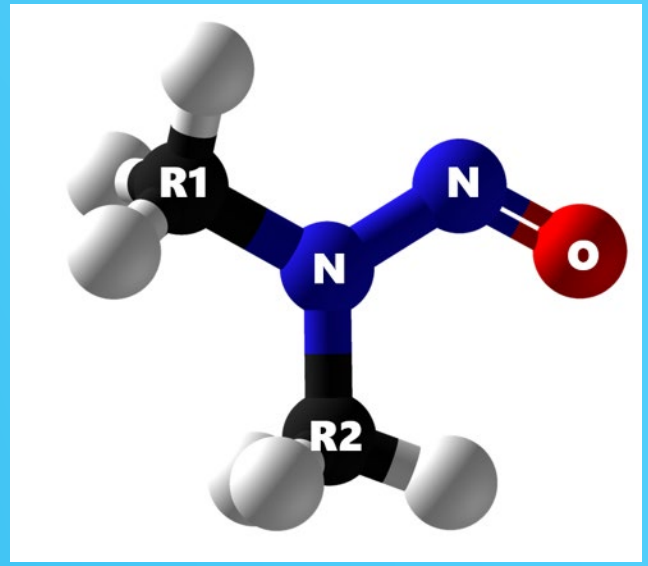
N-nitrosamine impurities in drugs

- Many N-nitrosamines (NAs) are known carcinogens and are cohorts of concern impurities (in the ICH M7 guidance on assessment of impurities in pharmaceuticals)
- N-nitrosamine impurities have occurred in marketed drugs due to process and synthesis impurities as well as compound degradation (starting in 2018)
- N-Nitrosamine impurities may be small molecules or nitrosamine drug substance-related impurities (NDSRIs), which are large and may be drug-like
- Sensitive mutagenicity assays are needed to detect nitrosamine levels
- Acceptable Intake regulatory limits must be established
  - Regulatory guidelines suggest using data and SAR information[1,2,,3,4]



[1]U.S. FDA, August 2023. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance Related Impurities (NDSRIs) https://www.fda.gov/media/170794/download

[2]European Medicines Agency (EMA), January 2024. Questions and answers for marketing authorisation holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products. EMA/409815/2020 Rev 20. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders/applicants-chmp-opinion-article-53-regulation-ec-no-726/2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf

[3]European Medicines Agency (EMA), 2019. Assessment Report: Referral under Article 31 of Directive 2001/83/EC: Angiotensin-II-receptor antagonists (sartans) containing a tetrazole group. EMA/217823/2019 https://www.ema.europa.eu/en/documents/variation-report/angiotensin-ii-receptor-antagonists-sartans-article-31-referral-chmp-assessment-report_en.pdf

[4]Swissmedic, 2019. Potential nitrosamine contamination: Request to perform a risk evaluation. https://www.swissmedic.ch/swissmedic/en/home/news/mitteilungen/aufforderung-zlinhaberinnen-ham.html.

Acceptable Intake limits for N-nitrosamines

- Available TD_{50} (i.e., the daily dose causing tumors in 50% of animals in a lifetime bioassay) data are used to obtain acceptable intake (AI) limits
- For N-nitrosamines without available toxicological data, a default class-specific limit has been set by regulators - 18 ng/day (EMA) or 26.5 ng/day (FDA)[1,3]
- Deviation from the default approach can be justified on a case-by-case basis applying Structure-Activity-Relationship (SAR) considerations and a read-across approach or the new Carcinogenic Potency Categorization Approach (CPCA)[2,3]

[1]European Medicines Agency (EMA), 2019c. Temporary interim limits for NMBA, DIPNA, EIPNA, impurities in sartan blood pressure medicines. EMA/351053/2019 rev 1. https://www.ema.europa.eu/en/documents/other/temporary-interim-limits-nmba-dipna-eipna-impurities-sartan-blood-pressure-medicines_en.pdf

[2]European Medicines Agency (EMA), January 2024. Questions and answers for marketing authorisation holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products. EMA/409815/2020 Rev 20. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders/applicants-chmp-opinion-article-53-regulation-ec-no-726/2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf

[3]U.S. FDA, August 2023. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance Related Impurities (NDSRIs) https://www.fda.gov/media/170794/download

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Health authority approach for assessing N-nitrosamine risk (U.S.FDA, EMA, MHRA, Health Canada)

1. Enhanced Ames test protocol (EAT)[1,2]
   - An Ames protocol more sensitive to N-nitrosamines, hamster S9, 30% S9, etc.
2. **The Carcinogenic Potency Categorization Approach (CPCA)[1,2]**
   - **A Structure-Activity Relationship (SAR) approach to establishing AI categories**
   - **A 2-dimensional, non-stereo chemical structure as input**
3. Read-across to surrogates with robust data[1,2,3,4]
   - Analogs with TD_{50} data in Carcinogenicity Potency Database[5]
4. *In vivo* mutagenicity testing
   - Follow-up testing when other approaches are inadequate

[1]European Medicines Agency (EMA), June 2020. Assessment report, Procedure under Article 5(3) of Regulation EC (No) 726/2004 Nitrosamine impurities in human medicinal products, Procedure number: EMEA/H/A-5(3)/1490 https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf

[2]U.S. FDA, August 2023. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance Related Impurities (NDSRIs) https://www.fda.gov/media/170794/download

[3]Australian Government Dept of Health and Aged Carc, January 2024, Established acceptable intake for nitrosamines in medicines https://www.tga.gov.au/how-we-regulate/monitoring-safety-and-shortages/industry-information-about-specific-safety-alerts-recalls-and-shortages/nitrosamine-impurities-medicines/appendix-1-established-acceptable-intake-nitrosamines-medicines

[4]Health Canada October 2023, Guidance on nitrosamine impurities in medications https://www.canada.ca/content/dam/hc-sc/documents/services/drugs-health-products/compliance-enforcement/information-health-product/drugs/nitrosamine-impurities/medications-guidance/guidance-nitrosamine%20impurities-medications.pdf

[5]Gold, L.S., Manley, N.B., Slone, T.H., Rohrbach, L., Garfinkel, G.B., 2005. Supplement to the Carcinogenic Potency Database (CPDB): Results of animal bioassays published in the general literature through 1997 and by the National Toxicology Program in 1997–1998. Toxicol. Sci. 85, 747–808. https://doi.org/10.1093/toxsci/kfi161

Why use a SAR approach to assess N-nitrosamines?

- Adequate data for many Nitrosamine Drug Substance-Related Impurities (NDSRIs) are not available. Historically, small molecule nitrosamines have primarily been studied.
- Adequate analogs for many nitrosamines for read-cross are not available
- Read-across arguments are hard to justify to regulators
- *In vivo* mutagenicity testing is costly and time-consuming

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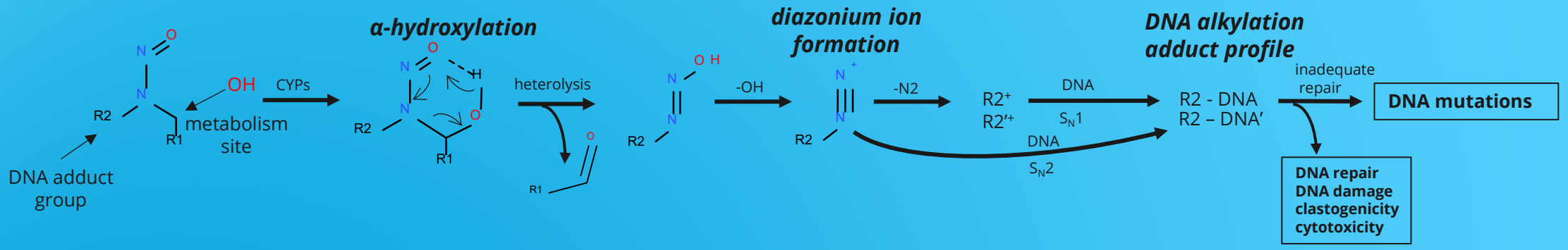
What can SAR approaches provide?

- NDSRI data gap filling (support from carc studies and enhanced Ames studies)
- Identification of structural features and physiochemical properties increasing and decreasing potency
- Mutagenicity and carcinogenicity potency estimations for NDSRIs

Principal mechanism for N-nitrosamines

α-carbon hydroxylation via CYPs (2E1, 2A6, 2C9, 2C19, 3A4) [1]



- Metabolic activation is the principal SAR driver of high potency
  - steric considerations (number of hydrogens, etc.)
  - electronic considerations (electron withdrawing groups)

- Formation of DNA adducts also depends on physicochemical properties
  - Compound solubility
  - ion stability

[1]Cross, K.P., Ponting, D.J., 2021. Developing structure-activity relationships for N-nitrosamine activity. Comput. Toxicol. 20, 100186. https://doi.org/10.1016/j.comtox.2021.100186

SAR considerations for N-nitrosamines

- **Aim:** identifying the structural features that most affect carcinogenic potency
- Different events may affect the carcinogenic potency:
  1. **Metabolic activation** (alpha-hydroxylation)
  2. **Diazonium ion stability**
  3. **DNA-alkylation formation and stability** (different adducts have different levels of mutagenicity)
  4. **Repair of potential DNA adducts**
- These events are also dependent on **physicochemical properties** of the nitrosamines
- While these events could potentially result in different SARs, **metabolic activation mechanism** is understood to be of principal concern for the overall SAR

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The Carcinogenic Potency Categorization Approach (CPCA)[1] – FDA potency categories

Similar potency categories are recommended by different Health Authorities but with an 26.5 ng/day lowest recommended AI limit[1,2]

Potency Category	Recommended AI (ng/day)	Comments
1	26.5	The recommended AI limit of 26.5 ng/day* is equal to the class-specific limit for nitrosamine impurities based on the most potent, robustly tested nitrosamine, *N*-nitrosodiethylamine (NDEA).** NDSRIs assigned to Category 1 are predicted to have carcinogenic potency no higher than the class-specific limit for nitrosamine impurities.
2	100	The recommended AI limit of 100 ng/day is representative of two potent, robustly tested nitrosamines, *N*-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-(butanone) (NNK), which have recommended AI limits of 96 ng/day and 100 ng/day, respectively. NDSRIs assigned to Category 2 are predicted to have carcinogenic potency no higher than NDMA and NNK.
3	400	Compared to Potency Category 2, NDSRIs in this category have lower carcinogenic potency due to, for example, the presence of a weakly deactivating structural feature. The recommended AI limit was set to reflect a 4-fold decrease in carcinogenic potency from Category 2.
4	1500	NDSRIs assigned to Category 4 may be metabolically activated through an alpha-hydroxylation pathway but are predicted to be of low carcinogenic potency, for example, because the pathway is disfavored due to steric or electronic influences, or because clearance pathways are favored. The recommended AI limit of 1500 ng/day is set at the TTC per ICH M7(R2).***
5	1500	NDSRIs assigned to Category 5 are not predicted to be metabolically activated via an α-hydroxylation pathway due to steric hindrance or the absence of α-hydrogens, or are predicted to form unstable species that will not react with DNA. The recommended AI limit of 1500 ng/day is set at the TTC per ICH M7(R2).***

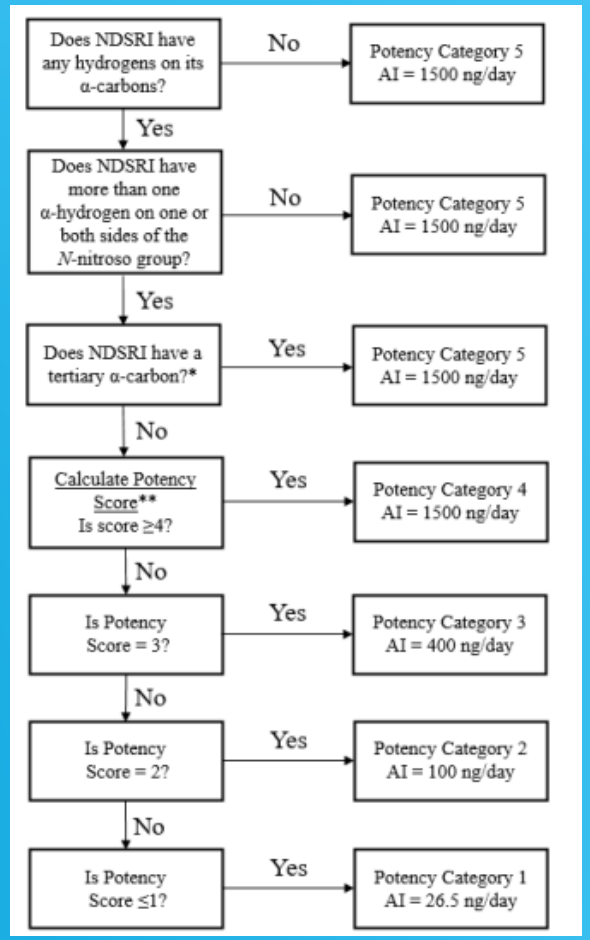
[1]U.S. FDA, August 2023. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance Related Impurities (NDSRIs) https://www.fda.gov/media/170794/download

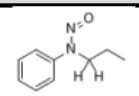
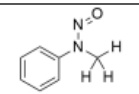
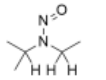
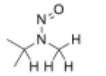
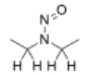
[2]European Medicines Agency (EMA), January 2024. Questions and answers for marketing authorisation holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products. EMA/409815/2020 Rev 20. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders/applicants-chmp-opinion-article-53-regulation-ec-no-726/2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf

The Carcinogenic Potency Categorization Approach (CPCA)[1] – algorithm flowchart

1. Predict the Carcinogenic Potency Category of an NDSRI
2. Identify an Associated Recommended AI Limit

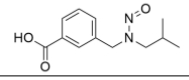
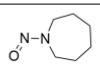
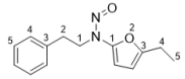
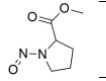
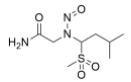
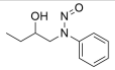
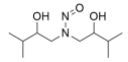
Calculation:
Potency Score = **α-Hydrogen Score** + **Deactivating Feature Score** (sum all scores for features present in NDSRI) + **Activating Feature Score** (sum all scores for features present in NDSRI)

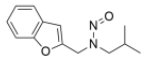
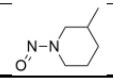


Count of Hydrogen Atoms on Each α-Carbon, Lowest First	Example	α-Hydrogen Score
0,2		3[$]
0,3		2
1,2		3
1,3		3
2,2		1

[1]U.S. FDA, August 2023. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance Related Impurities (NDSRIs) https://www.fda.gov/media/170794/download

The Carcinogenic Potency Categorization Approach (CPCA)[1] – algorithm features

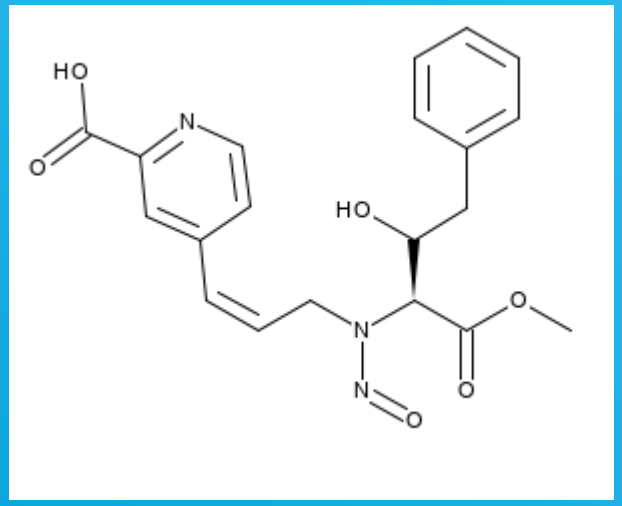
Deactivating Feature	Example	Individual Deactivating Feature Score
Carboxylic acid group anywhere on molecule		+3
N-nitroso group in a pyrrolidine ring		+3
N-nitroso group in a 6-membered ring containing at least one sulfur atom		+3
N-nitroso group in a 5- or 6-membered ring*		+2
N-nitroso group in a morpholine ring		+1
N-nitroso group in a 7-membered ring		+1
Chains of ≥5 consecutive non-hydrogen atoms (cyclic or acyclic) on both sides of acyclic *N*-nitroso group. Not more than 4 atoms in each chain may be in the same ring.		+1
Electron-withdrawing group** bonded to α-carbon on only one side of *N*-nitroso group (cyclic or acyclic) [2]		+1
Electron-withdrawing groups** bonded to α-carbons on both sides of *N*-nitroso group (cyclic or acyclic) [2]		+2
Hydroxyl group bonded to β-carbon*** on only one side of *N*-nitroso group (cyclic or acyclic)		+1
Hydroxyl group bonded to β-carbons*** on both sides of *N*-nitroso group (cyclic or acyclic)		+2

Activating Feature	Example	Individual Activating Feature Score
Aryl group bonded to α-carbon (i.e., benzylic or pseudo-benzylic substituent on *N*-nitroso group)		-1
Methyl group bonded to β-carbon (cyclic or acyclic)		-1

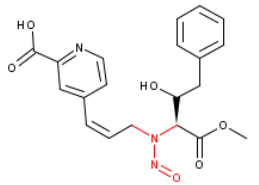
- Most features reduce metabolism (electron withdrawing)
- One feature increases metabolism
- One feature reflects increased solubility
- One feature increases ion stability

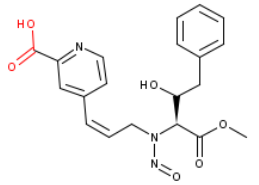
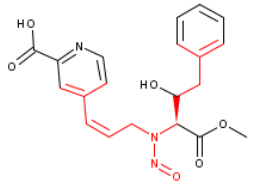
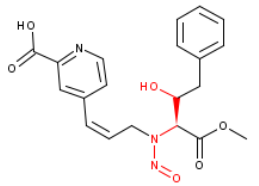
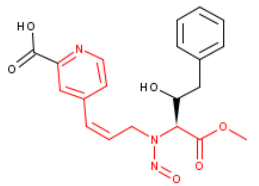
[1]U.S. FDA, August 2023. Recommended Acceptable Intake Limits for Nitrosamine Drug Substance Related Impurities (NDSRIs) https://www.fda.gov/media/170794/download
[2]Cross, K.P., Ponting, D.J., 2021. Developing structure-activity relationships for N-nitrosamine activity. Comput. Toxicol. 20, 100186. https://doi.org/10.1016/j.comtox.2021.100186

The Carcinogenic Potency Categorization Approach (CPCA) – example

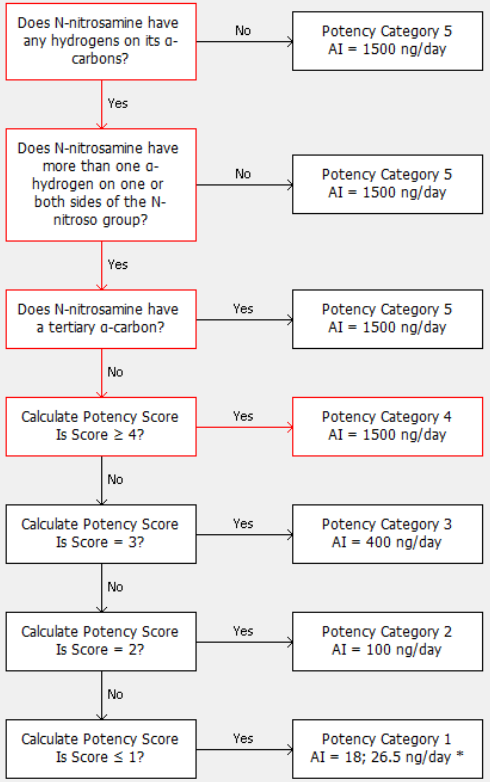


N-Nitrosamine Potency Assessment - test

Count of α-Hydrogens	Score	Feature Highlighted in Red
More than one alpha-carbon hydrogens present on either substituent (count 1,2).	3	 test

Deactivating Features	Score	Feature Highlighted in Red
Carboxylic acid group anywhere on molecule	+ 3	 test
Chains of >= 5 consecutive non-hydrogen atoms on both sides of acyclic N-nitroso group. Not more than 4 atoms in each chain may be in the same ring.	+ 1	 test
Hydroxyl group bonded to beta-carbon on only one side of N-nitroso group	+ 1	 test
Electron-withdrawing groups bonded to alpha-carbons on both sides of the N-nitroso group: Michael acceptor aryl EWG (A) : Carbonyl/carboxyl derivative EWG (1B4)	+ 2	 test

No Activating Features Present		
Potency Score = 3 + 3 + 1 + 1 + 2 = 10	Potency Category 4	AI = 1500 ng/day



* According to FDA the AI limit for Potency Category 1 = 26.5 ng/day
For EMA, the AI limit for Potency Category 1 = 18.0 ng/day

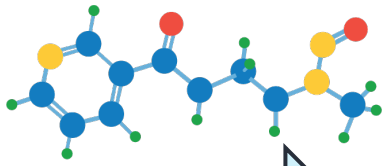
Ready

The Carcinogenic Potency Categorization Approach (CPCA) – considerations for the future

- The list of deactivating electron-withdrawing groups may be expanded to include similar ***deactivating*** features
  - Large rings, non-carbon aromatic systems, mixed di- and tri-halo features, phosphate/phosphites
  - Bulky groups near nitroso amines - used in drug synthesis
- Additional chemical features (in addition to beta-methyl groups) may stabilize diazonium and carbenium cations and be ***activating*** features
  - Dimethyl, tertiary methyl and secondary propyl groups, etc.
- The role of other common drug features should be clarified
  - Ketones, alcohols, etc.
  - Michael Acceptors vs benzylic features
- The limited ability of bulky carbenium ions to form ***DNA adducts*** should be considered
- Use of QM methods for **knowledge gap filling** – computing resources required

Summary

- Regulatory risk assessment now includes enhanced *in vitro* testing (EAT), evaluation using SAR knowledge (CPCA), and *in vivo* follow-up.
- Future directions for updating the SAR of *N*-nitrosamines includes:
  - Additional nitrosamine test data (TGR and *in vivo* Duplex Sequencing assays)
  - Results from applying theoretical Quantum Mechanical methods.
  - CPCA updates:
    - refinement and addition of structural features and physicochemical properties
    - improved accuracy of predictions
    - expansion of applicability domain to predict additional nitroso classes
- Formation of the HESI QSAR Nitrosamine team (including regulators)



Nitrosamines Research Program

Develop an Ames protocol predictive for carcinogenic potential of Nitrosamines

Goal 2: In vitrotest strategy

Identification and verification of in vitro assays with alternative cell models supporting Nitrosamines risk identification

Goal 3 In vivo Ames follow-up

Develop invivo Strategy to verify Ames Data within the frame of ICH M7

Goal 4: (Q)SAR/QM

Refine and extend the CPCA using (Q)SAR and QM for more predictive and accurate predictions

Nitrosamine (Q)SAR Team

Aim: Refine and extend the CPCA using (Q)SAR and QM for improved predictive performance.

Interrogate and validate **existing** CPCA features by (Q)SAR and QM

Interrogate and validate **new** CPCA features by (Q)SAR and QM

Assess the accuracy, confidence of each feature

Quantify the domain of applicability

Ranges of physicochemical properties;
New models to extend the current domain

To learn more about the full (Q)SAR-QM initiative or participate

- Validate electron conjugating and withdrawing groups and steric hinderance effects in the CPCA
- Refine ion stability assessment in the CPCA
- Refine ring size effects in the CPCA using electronic and experimental data

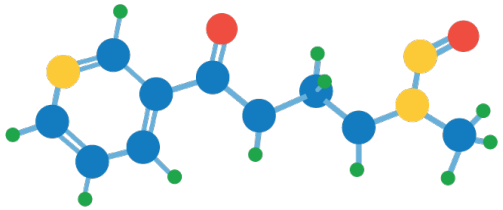
New global structure considerations

- Determine whether there is data to support features that encode pharmacokinetics/metabolism; focus on things that act like carboxylic acids, bioisosteres; Alpha, beta, gamma positions; Use of molecular weight and other descriptors

New activating/deactivating (local) structure features

- Mechanism of carbonyls and inclusion of this feature; Can additional data on allylic & propargylic groups can be generated; Address apparent discrepancy between mechanistic consideration; Explore ethers where O is in beta position and if this mimics effect of beta OH group

Instem Information Solutions For Life



Nitrosamines Research Program

10 Government/ Regulatory

- Agence nationale de sécurité du médicament et des produits de santé (ANSM)
- Danish Medicines Agency
- **European Medicines Agency**
- Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, (BfArM)
- Health Canada
- Institut National de lar recharge agronomique (INRA)
- National Institute of Health Sciences (NIHS), Japan
- National Institute for Public Health and the Environment (RIVM)
- SwissMedic
- **US Food and Drug Administration**

26 Private Industry

- AbbVie
- Amgen
- AstraZeneca
- BASF
- Bayer
- Boehringer Ingelheim
- Bristol Myers Squibb
- Charles River Laboratories
- Corteva Agriscience
- Eli Lilly
- Gilead
- GSK
- Inotiv
- Janssen
- Labcorp
- Leadscope/Instem
- Litron Laboratories
- Merck
- Merck kGA
- MultiCASE, Inc.
- Novartis
- PepsiCo
- Pfizer
- Proctor & Gamble
- Roche
- Sanofi
- Teva Pharmaceuticals
- Xenometrix

7 Academic, NGO, Consultants

- FSTox Consulting
- Lhasa Limited
- George Washington University
- New York Medical College
- St. George's Medical School, University of London
- Swansea University
- University of Colorado
- University of Minnesota

THANK YOU

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