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European Federation of Pharmaceutical
Industries and Associations

20 February 2023

Nitroso-Derivatives of ACE Inhibitors

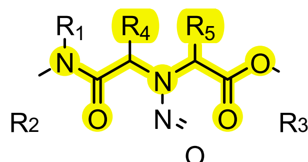
INTRODUCTION

Angiotensin-converting enzyme (ACE) inhibitors are identified by the suffix "-pril". Many of these ACE inhibitors have secondary amines including Benazepril, Enalapril, Lisinopril, Moexipril, Perindopril, Quinapril, Ramipril, and Trandolapril. Other ACE inhibitors do not contain secondary amines, such as Captopril and Fosinopril which have amide bonds but no amine functionality. In the presence of nitrosating conditions, the ACE inhibitors containing secondary amines can theoretically be nitrosated during the synthetic process of the drug substance or during formulation of the drug product. This document will focus only on the nitrosamines of ACE inhibitors that contain secondary amines ("nitroso-ACE inhibitors" henceforth).

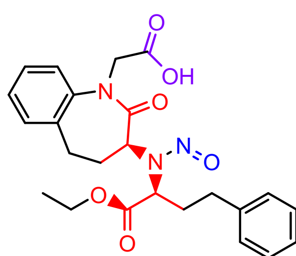
ASSESSMENT

Structure analysis

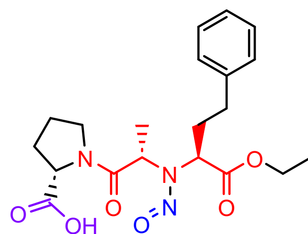
The nitroso-ACE inhibitors all have a very similar structural motif (Fig. 1), consisting of a common scaffold (highlighted in yellow below and in red in Fig. 1) where both carbons at the α-position to the nitrosamine are tertiary carbons and both β-positions are carboxy groups, one being a carboxamide and the other a carboxylate (in most cases an ethyl ester and only in Lisinopril it is a carboxylic acid).



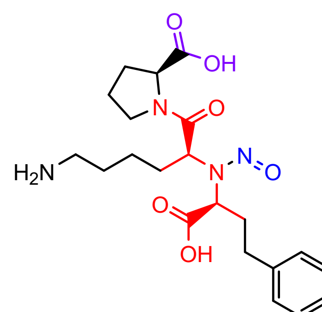
Furthermore, all the nitrosamines of the ACE inhibitors have a free carboxylic acid on the α-position to the amide (purple in Fig. 1), and the nitrogen of the amide is part of a rigid cyclic system (in many cases a bicyclic system).



Nitroso-Benazepril



Nitroso-Enalapril



Nitroso-Lisinopril

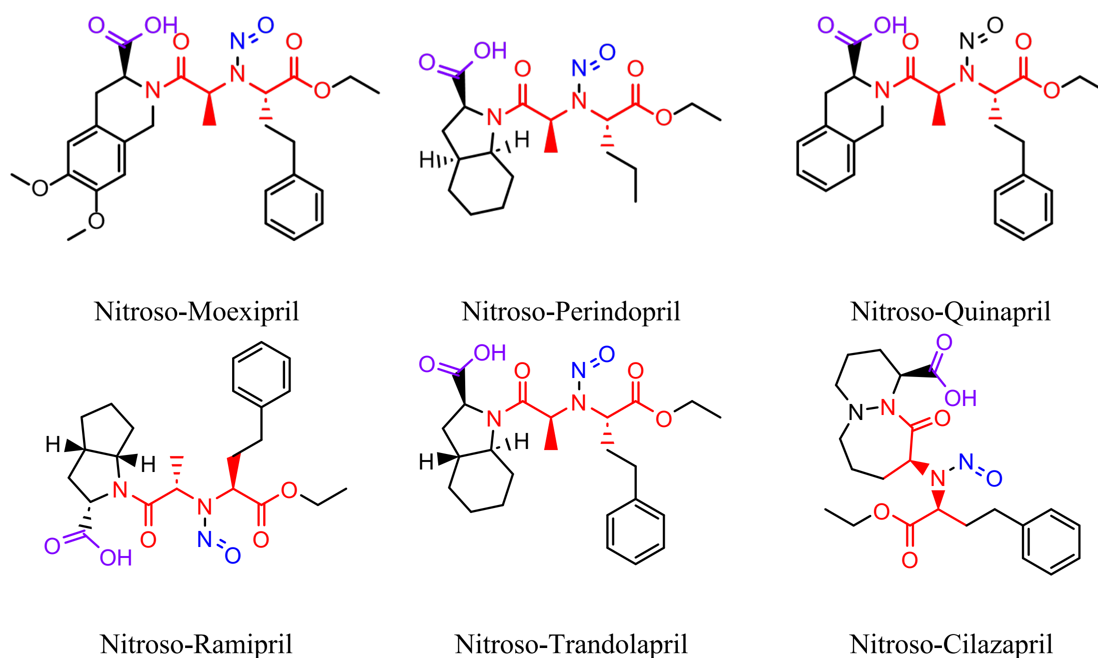
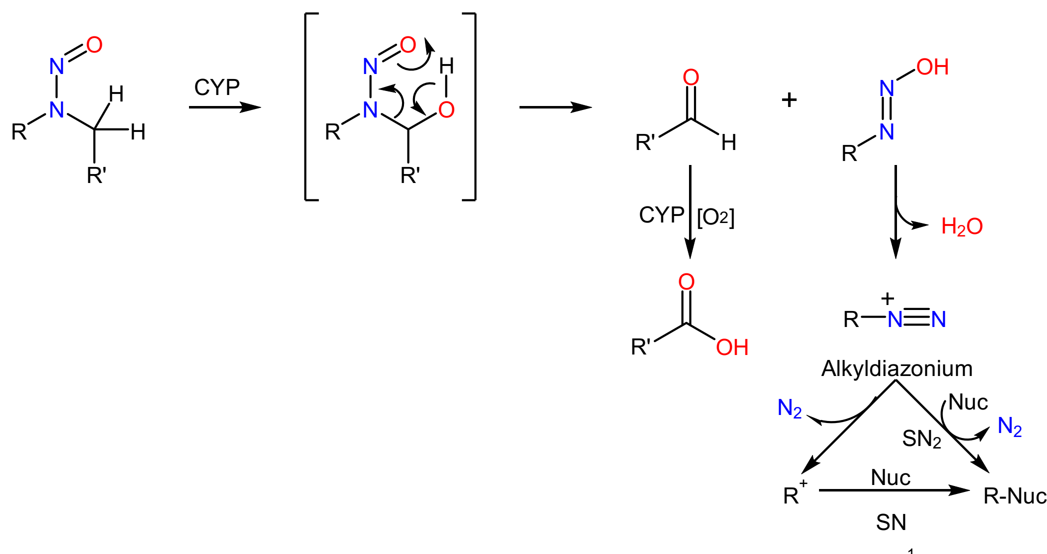


Figure 1. Structures of potential *N*-nitrosamines derived from ACE inhibitors that contain a secondary amine

Steric Hindrance

The importance of the α-position and its involvement in the bioactivation of nitrosamines has been discussed in various reviews.[1,2,3,4] The mutagenic mechanism of action of potent nitrosamines follows a cascade of events, starting from cytochrome P450 (CYP) mediated hydroxylation at the α-position to the nitrosamine. Subsequent elimination of a carbonyl compound leads to the formation of an alkyldiazohydroxide, which, on loss of a hydroxide, gives an alkyldiazonium or decomposes further to a carbonium ion. Both diazonium salt, or derived carbonium ion, are potentially capable of alkylating DNA (Scheme 1).



Scheme 1. Mechanism of CYP-mediated metabolism of nitrosamines causing DNA alkylation.

The initial α-hydroxylation is critical from a mechanistic perspective and interference or inhibition of this step has a major influence on the mutagenic potential of such compounds.

Both α-positions in the nitrosamines of the ACE inhibitors are very sterically hindered. Each α-carbon has only one hydrogen and the substitution pattern on these α-carbons is large and bulky. A simple 3-D model (MM2 energy minimized) of nitroso-enalapril, as a representative nitroso-ACE inhibitor, visualizes the steric hindrance around the two α-carbons (yellow arrows in Fig. 2).

In addition, if in theory a diazonium ion would be formed, the steric hindrance around the diazonium ion would prohibit DNA binding, as demonstrated for *N*-nitroso-ramipril in Fig. 3.

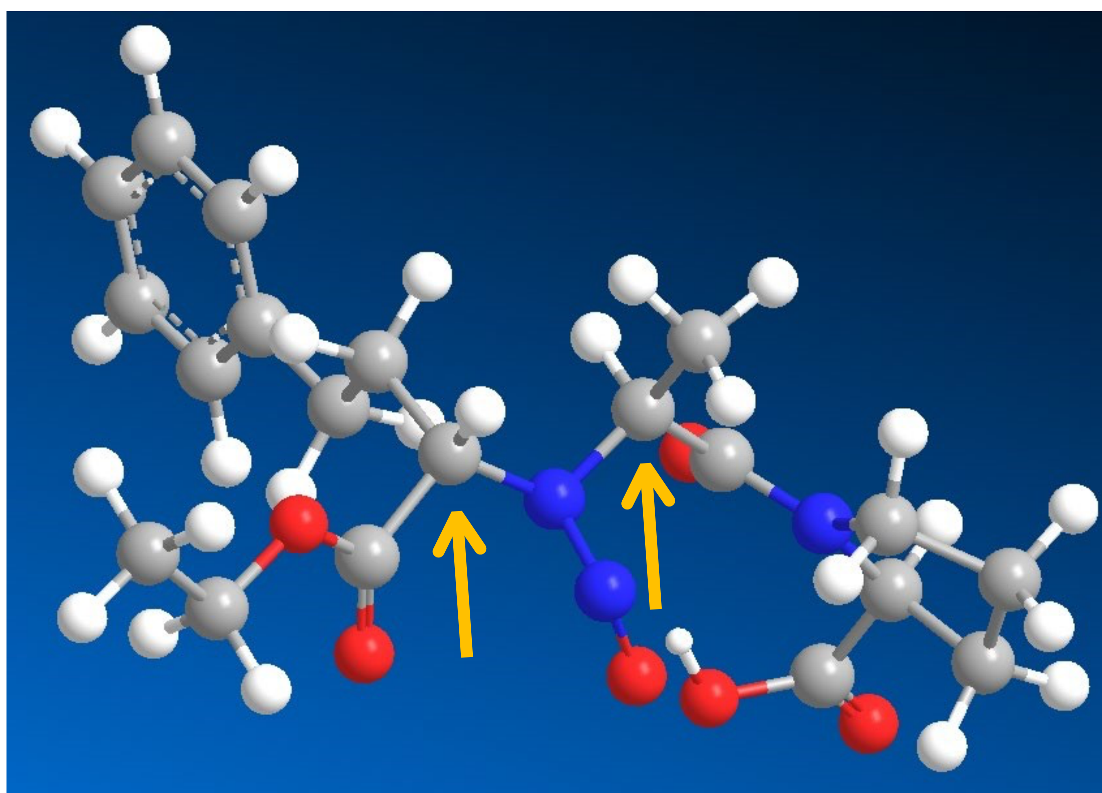


Figure 2. 3-D structure of nitroso-enalapril. Yellow arrows indicate α-carbon positions.

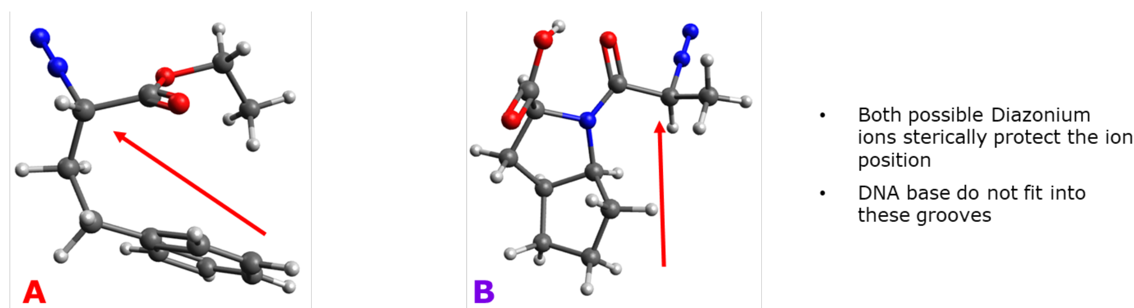


Figure 3. 3-D structure of diazonium derivative of ramipril that would theoretically derive from nitroso-ramipril, demonstrating steric hindrance of potential DNA binding

Simple branching at the α-position has been shown to strongly reduce carcinogenic potential, where even simple small alkyl substitutions, such as isopropyl or isobutyl groups can render small dialkyl nitrosamines as being weakly positive or negative in carcinogenicity assays.[5,6,7]

Cross and Ponting (2021)[5] show "that substitution of an isopropyl group (or longer) at one of the α-carbon positions reduces the prevalence of positive carcinogenicity compounds while "Two iPr groups" illustrates that isopropyl (or longer) substitutions at both positions reduces positive prevalence even more so".

Thomas et al. (2022)[6] shows that one of the features that show an association with a large decrease in potency is for "compounds with at least one isopropyl-like group (i.e., the *α*-carbon has two carbon substituents). The presence of even one isopropyl group leading to a reduction in potency may be an extension of the observation that a *tert*butyl group leads to an elimination of the potency and the reasons for it while less sterically hindered than a *tert*-butyl, the isopropyl is less likely to be a site of metabolism than a CH_2 group and, should metabolism occur on the other side of the nitrosamine, the formed diazonium or cation will be less reactive with DNA than a CH_2 group".

Ponting et al (2022)[7] bring evidence that "reaction of DNA with the reactive species generated via the bioactivation of an *N*-nitrosamine could be perturbed due to steric hindrance posed by neighboring substituents (e.g., isopropyl or *tert*-butyl groups)". They further elaborate on the reactivity of branched alkyl side chains and state that "The introduction of steric hindrance at the carbon *α* to the *N*-nitrosamine moiety has a dramatic effect on carcinogenic potency in animals. Branching in the form of a single methyl (or larger alkyl) group adjacent to the *N*-nitrosamine motif significantly reduces carcinogenicity and also the likelihood of genotoxicity. The presence of two such groups results in *N*-nitrosamines with minimal carcinogenic properties and mostly negative genotoxicity results. A potential reason for these observations is that the steric hindrance posed by the isopropyl-like *α* substituent (even a mere methyl) perturbs *α*carbon hydrogen abstraction in the active site of CYP2E1 or CYP2A6 considerably, particularly for low-molecular-weight *N*-nitrosamines".

The reduction in prevalence of positive carcinogenicity outcome has been shown for small nitrosamines where the branching at the α-positions is by small alkyl substituents, such as isopropyl or *tert*-butyl groups. For larger substituents that cause considerably more steric hindrance, the reduction of carcinogenic potency is expected to be much more significant. For the nitroso-ACE inhibitors (Fig 1.) the substituents on both sides of the nitrosamine are large and bulky; on one α-carbon of the nitrosamine there is a phenethyl group (all except for nitroso-perindopril that has an n-propyl substituent) and an ethyl ester moiety (except for nitroso-lisinopril that has a carboxylic acid); and the other α-carbon is substituted by various bulky groups, all containing an amide at the βposition, a cyclic moiety (often bicyclic) and a free carboxylate branched off of the ring system. All this bulkiness renders this class of nitrosamines as being extremely sterically hindered, which would make the binding to the CYP responsible for αhydroxylation of nitrosamines extremely difficult.

Carboxylic Acid Substituents

Ponting et al. (2022)[7] explain that "Incorporation of a carboxylic acid substituent(s) anywhere in the *N*-nitrosamine scaffold results in a dramatic reduction in both the carcinogenic potency and the positivity rate in genotoxicity assays. Potential reasons for this trend include the following: (a) Increased polarity (and therefore hydrophilicity) of low-molecular-weight *N*-nitrosamines, which potentially disfavors CYP (e.g., CYP2E1)-mediated oxidations in general and (b) preponderance for non-metabolic elimination routes (e.g., renal clearance as unchanged parent) and/or metabolic elimination via phase 2 conjugation pathways such as glucuronidation."

All the nitroso-ACE inhibitors have a carboxylic acid, and nitroso-lisinopril even has two free carboxylates. Furthermore, The majority of ACE inhibitors are prodrugs converted by hepatic esterolysis to a major active diacid metabolite,[8] which makes them more polar and even more disfavors CYP-mediated metabolism.

Metabolism

Metabolic activation at the *α*-position and formation of a diazonium ion is the key mechanistic driver for the highly potent Cohort of Concern nitrosamines. As an example, investigations on the metabolism of *N*-nitroso-ramipril were conducted *in vitro* and *in silico*. The *in vitro* investigations using human aroclor induced rat and araclor induced hamster S9, observed hydroxylation(s) located on the toluene moiety of the *N*-nitroso-ramipril, and/or on the octahydrocyclopenta-pyrrole, and/or the formation of *N*-nitrosoramiprilat, but no hydroxylation in *α*- or β-position of the nitroso group.

Quantum chemical calculations were performed at the B3LYP / 6-31+G* level of theory in order to study the ratio of possible metabolites, which support the findings of the metabolism studies (Fig. 4). Free energy calculations were used to estimate metabolic ratios at thermodynamic equilibrium. In accordance with the experimental study, the

level of expected α-hydroxylated metabolite at the concerning sites adjacent to the N-nitrosamine were estimated to be negligible (1 : < 3.0 $*10^{-5}$ compared to main metabolites). Considering the steric hindrance and free energy calculations, the opportunity for N-nitrosoramipril to be α-hydroxylated is strongly reduced. The highly probable lack of the formation of the diazonium ion is providing strong evidence that N-nitrosoramipril at least does not belong to the class of Cohort of Concern.

Nitrosoramipril: α-hydroxylation vs predicted SOM positions

Metabolite*	Gibbs free energy [hartree]**	Boltzmann distribution [%]
A	-1585.4737	0.0
B	-1585.4930	0.003
M1	-1585.4874	0.0
M2	**-1585.5020**	**97.3**
M3	-1585.4864	0.0
M4	-1585.4993	2.6
M5	-1585.4945	0.03

*Hydroxylation at respective position
**Level of theory: B3LPY / 6-31+G* / no solvent

- **M2 position most likely** based on Metasite SOM predictions and thermodynamically based on QM computations
- Both α-hydroxylation positions (A & B) very unlikely compared to the other positions considered above

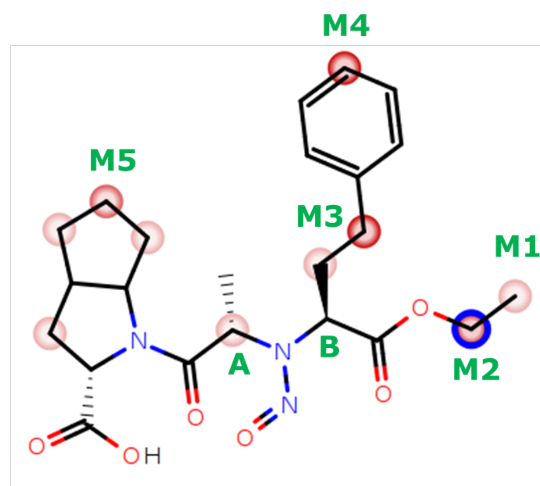


Figure 4. Quantum chemical calculations at the B3LYP / 6-31+G* level of theory in order to study the ratio of possible metabolites of *N*-nitroso-ramipril.

In vitro Test Results

Several nitroso-ACE inhibitors have been tested in the Ames test by various companies and the data was shared (anonymously) with the Lhasa Complex Nitrosamines Data Sharing Initiative (Vitic Complex Nitrosamines Database version 2022.2.0).The detailed data of these studies is restricted to the members of the data sharing initiative, however, from the 4 nitroso-ACE inhibitors currently listed in the Vitic database, three are negative and one is positive. Typically, nitrosamines that elicit a positive mutagenic response in the Ames test require metabolic activation to generate the diazonium metabolite that is responsible for the alkylation of DNA. The most sensitive strain to nitrosamines is TA1535, but also other strains react to nitrosamines. On occasion the use of hamster S9 is a more sensitive metabolic system than rat S9 when testing nitrosamines. The one positive Ames test result mentioned above was found to be positive in strain 1535 with hamster S9 but also in strain TA98 without S9 metabolic activation, indicating that it is probably positive due to a different mechanism of mutagenicity and not by the known mechanism that requires enzymatic α-hydroxylation. Several additional nitroso-ACE inhibitors have been tested in the Ames test by members of the data sharing initiative and all have shown to be negative.

Comparison with small dialkyl nitrosamines

For nitrosamines that do not have robust carcinogenicity data available the current regulatory guidances advise to use a SAR approach to read-across from a structurally similar nitrosamine to derive a permissible AI.[9,10,11,12,13] The use of a SAR approach to set AIs for newly found nitrosamines, including nitrosamine drug substance related impurities (NDSRI) must be scientifically justified and properly documented. NDSRIs are typically in a different chemical space than the simple alkyl nitrosamines (that form the basis of the current EMA NDSRI limits of 18 and 178 ng/day[8]) which are reported to be highly potent rodent carcinogens and consequently global structural similarity "read-across" approaches to assign specific AIs for NDSRIs can be problematic. The carcinogenicity of nitrosamines in rodents is known to range across several orders of magnitude of potency,[6] and all highly potent nitrosamines that have robust carcinogenicity data curated in the LCDB[14], are small molecular weight dialkyl nitrosamines. Therefore, it is not scientifically justified to use the established AIs of potent nitrosamines as surrogates for NDSRIs, and particularly for nitroso-ACE inhibitors that have multiple potency-reducing structural elements.
Using a weight of evidence approach, we are proposing the use of other measures such as SAR and metabolism, which should lead to a more appropriate AI, as discussed above.

SUMMARY AND CONCLUSION

Negative bacterial mutation assay (Ames test) under specific experimental conditions conducted (metabolic activation with hamster and rat S9) with additional supporting evidence by SAR and metabolism data could be used to set an AI for nitroso-ACE inhibitors.

Based on the evaluation and discussion of data from the Ames Test, SAR, metabolism and quantum chemical calculations, there is strong evidence that nitroso-ACE inhibitors are not mutagenic or at least not strong mutagens or carcinogens, therefore not belonging to the class of compounds within the Cohort of Concern.
Therefore, we are proposing as a precautionary and conservative measure an interim Acceptable Intake of 1.5 µg/day for the nitroso-ACE Inhibitors for discussion with the authorities, taking into account that risk/benefit may change this interim AI. This recommended interim limit will be revisited when more *in vivo* mutagenicity studies (e.g. TGR or duplex sequencing) will become available.

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Acknowledgments

The authors thank Susanne Glowienke (Novartis AG), Jim Harvey (GSK), and Richard Weaver (Servier) for their considered input and advice in the preparation of this paper.

This position paper was reviewed and agreed by the EFPIA Nitrosamine Team.

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