



Impact of ramipril nitroso-metabolites on cancer incidence — in silico and in vitro safety evaluation

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ABSTRACT

Background: Angiotensin-converting enzyme inhibitors (ACE-I) and their pharmacologically related sartans have been associated with an increased cancer incidence in several clinical observations. In 2018, sartans were revealed as being significantly contaminated with nitrosamines. Nitrosamines are potent human mutagens that can be formed ex vivo and, more concerningly, also in vivo from nitrosatable drug precursors. Their formation in sartans may justify the reported cancer risk and, by analogy, this may also apply to ACE-Is.

Materials and methods: We investigated a commonly used ACE-I, ramipril (RAM). We checked its susceptibility to in vivo interaction with nitrite, potentially resulting in the generation of mutagenic N-nitrosamines. To that end, in silico simulation of mutagenicity of RAM nitroso-derivatives was performed using VEGA-GUI software. Then, the Nitrosation Assay Procedure was conducted which served as a model of endogenous reaction. The resulting post-nitrosation mixtures were subjected to a bacterial reverse mutation test employing *Salmonella typhimurium* strains TA98 and TA100 with and without metabolic activation.

Results: Our results showed that studied samples did not induce point mutations in the test bacteria, regardless of the catalytic cytochrome activity.

Conclusion: We concluded that RAM endogenous nitrosation is not the reason for increased cancer incidence. However, other ACE-Is must be verified in a similar manner.

Key words: carcinogen; nitrosamines; Ames test; QSAR; mutagenicity

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Introduction

Angiotensin-converting enzyme inhibitors (ACE-Is) are a valuable class of drugs with well-established use as a first-line option in cardiovascu-

lar and renal system related diseases. Their pharmacological background translates into their high prevalence and wide-spread use [1], with ramipril (RAM) holding one of the highest market shares in Europe [2]. Despite the unquestionable on-target

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efficiency, there are accumulating reports suggesting serious off-target toxicity of these drugs. Safety concerns, in particular, relate to their pro-carcinogenic potential, evidenced by increased incidence of malignancy among ACE-Is users [3, 4]. Specifically, the risk regards common neoplastic conditions such as: endometrial [5, 6], melanoma, kidney and female reproductive cancers [7–11]. Furthermore, their capacity to stimulate lung cancer, which is the most common human neoplastic condition [12–14], was implied. Here, at chronic use exceeding 5 years, or at high exposures of more than 540 defined daily doses per year, a moderately increased hazard occurred. At short-term treatment, in turn, ACE-Is were relatively safe [15–17]. Hence, given the broad clinical use of ACE-Is, even the moderately increased cancer incidence would translate into a significant absolute number of individuals at risk. Thus, the above safety concerns cannot be ignored.

In pharmaceutical toxicology, cancer-inducing capacity is characteristic of mutagenic drug components. They are able to cause genomic damage by forming DNA adducts. This may lead to malignant cell transformation [18]. Well-known and potent human mutagens that can contaminate drug products or are formed endogenously from nitrosatable drug precursors are N-nitrosamines. Their presence in pharmaceutical formulations of sartans has recently become the reason for a global recall of over 1800 contaminated drug batches [19]. Noteworthy, sartans are pharmacologically allied to ACE-Is, and the link between their use and an increased cancer risk has also been reported previously. Potentially, the susceptibility of both drug families to form nitrosamines may be the reason here. Notably, the above high profile nitrosamine crisis prompted an immediate regulatory response to enhance vigilance over the potential nitrosamine contamination for all pharmaceuticals on market. Mandatory risk assessments and process changes are still underway with a final deadline of Oct. 1st, 2023. Their aim is to mitigate the risk and ensure that the contents of nitrosamines in dosage forms are below acceptable limits of 18 ng. Hence, adequate control strategies involving identification, purification or stabilization procedures need to be developed and implemented by Marketing Authorization Holders [20, 21].

The risk of exogenous exposure to nitrosamines has gained a great understanding of authorities, including the European Medicines Agency (EMA) and, more recently, also the European Food Safety Authority (EFSA) [22]. However, more problematic than drug and food nitroso-impurities are mutagenic N-nitroso derivatives produced *in vivo* from N-nitrosatable drug precursors, interacting with dietary nitrite in the acidic environment of gastric juice. Unfortunately, their levels cannot be controlled by conventional methods, while their real yield is unpredictable [23]. By extension, the resulting intermediates can either undergo enzymatic bioactivation to electrophilic reactive species or directly alkylate DNA causing mutations, even at nano-level exposures. Of further concern, a great proportion of existing drugs is theoretically vulnerable to endogenous N-nitrosation owing to the prevalent manifestation of the following structural motifs: amine, amide, cyanamide, guanidine, hydroxylamine, amidine, hydrazine, hydrazide, piperazine and diketopiperazine [19, 24]. Indeed, a real significance of this problem was evidenced by Schmidtsdorff et al. [25], who reported that a total of 33 out of 67 pharmaceuticals formed nitrosamine derivatives in a Nitrosation Assay Procedure (NAP test), described in detail in the Materials and Methods section. Similarly, Ozahn et al. [23] found 22 drugs, out of 28 examined, which produced mutagenic nitroso-metabolites. However, absolute human exposure to endogenous drug nitroso-derivatives for the majority of pharmaceuticals on market still remains unverified since here, the appropriate regulatory requirements for the industry have not been implemented by the authorities [19, 20]. Of note, since food is commonly enriched with nitrate and nitrite additives, it provides a significant source of substrates to endogenous nitrosation [22]. Hence, we emphasize that not independent, but joint actions by EMA and EFSA are necessary to control this problem.

For all these reasons, in the present pilot study, we intended to verify whether ACE-Is could form mutagenic nitroso-metabolites following drug administration. We selected RAM as a model ACE-I based on its prevalence of use and its chemical properties [2]. In fact, RAM is a 2-methylpropionyl-L-proline derivative with an unsubstituted nitrogen atom available for interaction with nitrite [26]. Firstly, we performed *in silico*

analysis to screen for potential mutagenicity of RAM nitroso-derivatives. Then, the NAP test was done in order to induce RAM nitrosation under the conditions resembling in vivo environment. The obtained nitrosation mixture was subjected to the in vitro bacterial reverse mutations test (Ames test), which is the method of choice for screening of potential mutagens, recommended by ICH M7 (R1) guideline for the industry. According to this guideline, Ames test is also sufficient to confirm or exclude the mutagenicity of a test compound without the need for confirmatory testing [20]. Our intention was to support the idea of implementing an additional drugs' safety assessment aimed at verification of their susceptibility to in vivo nitrosation. Hence, a model procedure was demonstrated herewith.

Materials and methods

In silico mutagenicity evaluation

We assumed that the process of RAM nitrosation in vivo can yield a variety of products of enzymatic catalysis. Thus, in order to pre-screen their potential mutagenicity, we considered all possible molecules that could be formed. For this purpose, we relied on RAM's fragmentation pattern [27]. The investigated structures were provided in Figure 1. For their identification by QSAR software, the corresponding SMILES (Simplified Molecular Input Line Entry System) were obtained from Online SMILES Translator and Structure File Generator (available at <https://cactus.nci.nih.gov/translate/>). They were used as input for the (Q) SAR simulation.

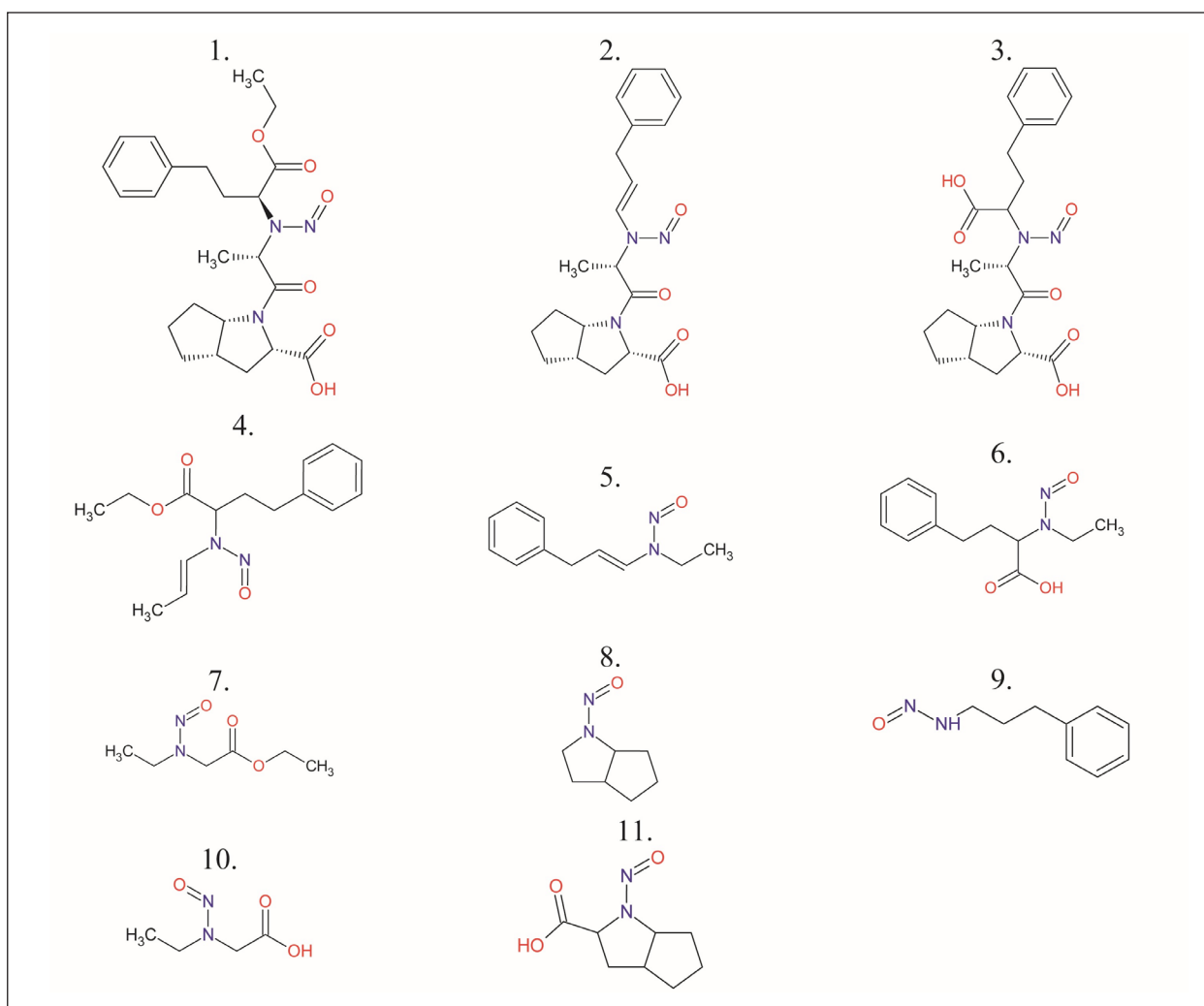


Figure 1. Structures of probable N-nitroso derivatives of ramipril (RAM)

An open-access software VEGA-GUI, version 1.2.0, from www.vegahub.eu was used for mutagenicity screening of possible RAM nitroso-derivatives [28]. Their SMILES identifiers were inserted and the predictions were computed based on five different mutagenicity models: CONSENSUS, CAESAR, ISS, SarPy and KNN. We also made a prediction for Octanol/Water partition coefficient (logP) using ALogP v.1.0.1 model. The output was provided in wording format 'mutagenic' or 'non-mutagenic'. The reliability of the simulation was expressed as Applicability Domain Index (ADI) in the following manner: for $1 \geq \text{ADI} > 0.85$ the reliability was high, for $0.85 \geq \text{ADI} > 0.7$ it was moderate, for $\text{ADI} \leq 0.7$ it was low. Then, we translated the predictions from the CAESAR, ISS, SarPy and KNN models into numeric values (scores), as follows: mutagenic with good reliability — 0.9, mutagenic with moderate reliability — 0.7, mutagenic with low reliability — 0.5, non-mutagenic with low reliability — 0.5, non-mutagenic with moderate reliability — 0.3, non-mutagenic with good reliability — 0.1. The CONSENSUS model (which displays a consensus result of CAESAR, ISS, SarPy and KNN) was treated independently, without assignment of numerical scores. Here, probability of being active (P_a) and probability of being inactive (P_i) was provided. If $P_a > P_i$, the compound was considered as mutagenic. For LogP prediction the result was provided as a numerical value.

For comparative analysis, further molecular parameters of the tested compounds, including molecular weight (MW) and volume were also obtained from Molinspiration cheminformatics, accessed at: <https://molinspiration.com> [29].

Nitrosation Assay Procedure (NAP test)

In order to verify the vulnerability of pharmaceuticals to form mutagenic N-nitroso compounds in vivo, a simple screening method has been proposed by the World Health Organization. It is referred to as the Nitrosation Assay Procedure (NAP test) and it involves treatment of tested compounds with a fourfold excess of nitrite in an acidic solution, which mimics conditions of the stomach [19, 24]. This method was applied in our study. For this purpose, the following chemicals were used: pepsin (No. 9001-75-6) from Pol-Aura (Poland), analytical grade hydrochloric acid 1.0 mol/L, sodium nitrite (NaNO_2) and ammonium sulfamate

($\text{NH}_4\text{SO}_3\text{NH}_2$) from Merck (Darmstadt, Germany). Firstly, simulated gastric juice (SGJ) (pH 1.2) was prepared as per the Polish Pharmacopoeia (ed. XII, 2020) by dissolving 2 g of sodium chloride and 3.2 g of pepsin in 80 mL HCl (1 mol/L) and a sufficient amount of ultrapure water to make 1000 mL (30). RAM (batch No. 11.PT24.01.02) was purchased from Rolabo (Zaragoza, Spain).

Subsequently, the sample of RAM (125.0 mg) was dissolved in DMSO, adjusted to pH 1.2 with SGJ and treated with sodium nitrite at a molar ratio of 10:40 mM. Then, pH was readjusted to 1.2. The resulting nitrosation mixture was incubated at 37°C for 60 minutes in the dark on a shaker. DMSO with nitrite was incubated under the same conditions to serve as a negative control for mutagenicity assay. The reaction was stopped by the addition of ammonium sulfamate at a molar ratio 4:4 of NaNO_2 vs. $\text{NH}_4\text{SO}_3\text{NO}_2$. The so prepared post-nitrosation mixture was used for mutagenicity evaluation. The concentrations of the tested nitrosation product were expressed as the concentrations of the parent compound prior nitrosation as follows: 4.5, 2.25, 1.125, 0.56, 0.28, 0.14 mg/dL. This allowed evidencing the level of exposure that may pose a safety concern.

Mutagenicity assay — bacterial reverse mutation test

The Ames test was performed to check the mutagenic activity of N-nitroso metabolites of RAM. By principle, this assay involves the exposition of histidine-auxotrophic bacteria to test compound in histidine-rich medium. Following exposure, test bacteria are transferred to a histidine-deficient medium. Reversion to histidine prototrophy and bacterial growth is a sign of mutation [31]. In this study, the commercial Ames MPF 98/100 microplate format mutagenicity assay kit from Xenometrix (Switzerland) was used. It contained *Salmonella typhimurium* strains TA98 (with frameshifts mutation hisD3052, rfa uvrB, pKM101) and TA100 (with base-pair substitution mutation hisG46, rfa uvrB, pKM101). Positive controls were: 2-nitrofluorene, 4-nitroquinolone-N-oxide and 2-aminoanthracene. The metabolic activation system involved Aroclor 1254-induced rat liver fraction S9. The kit also provided a ready-to-use growth medium, exposure medium and indicator medium. The S9 mix was prepared using a ready-to-use kit from Xenometrix (Switzer-

land), containing Buffer Salts solution (phosphate buffer, MgCl₂, KCl, NADP solution, G-6-P solution). Dilutions of the tested samples were made by sterile DMSO from Merck (Darmstadt, Germany).

The procedure provided by Ames MPF Instruction for use was followed [19, 24]. It was in agreement with OECD guideline 471: Bacterial Reverse Mutation Test [31]. Firstly, Salmonella typhimurium strains TA98 and TA100 were cultured overnight (15 h) in a histidine-rich growth medium at 37°C on a shaker set at 250 rpm. Exposure concentrations were pre-screened for cytotoxicity and solubility using TA98 strain, as per the kit instructions. Both Salmonella typhimurium strains TA98 and TA100 were then exposed to the tested concentrations of RAM post-nitrosation mixture in the presence and absence of the rat liver S9 fraction. S9 was to reveal potential mutagens that need enzymatic activation. In experiments without S9 fraction, 2-nitrofluorene (2.0 µg/mL) for TA98 and 4-nitroquinolone-N-oxide (0.1 µg/mL) for TA100 were used as positive controls. In experiments with S9 fraction, 2-aminoanthracene served as a positive control at 1.0 µg/mL for TA98

and at 2.5 µg/mL for TA100. The baseline concentration of the parent compound was 112.5 mg/mL. Then, a serial ½-log dilution was performed on a 96-well plate to obtain six stock solutions. They were diluted with a histidine-rich exposure medium, and with the metabolic activation S9 fraction mix (30%) if necessary. A bacterial culture containing approximately 10⁷ cells for each strain was added and the following test concentrations were obtained in triplicate on a 24-well plate: 4.5 mg/mL, 2.25 mg/mL, 1.125 mg/mL, 0.56 mg/mL, 0.28 mg/mL, 0.14 mg/mL. They were in agreement with OECD recommendations [31]. They were incubated at 37°C on a shaker (250 rpm) for 90 min. Afterwards, a pH indicator medium without histidine was added to the exposure cultures. They were distributed to 48 wells of a 384-well plate (50 µl per well) and incubated for 48 h at 37°C to allow the revertant bacteria to grow. If a mutation occurred, the pH indicator dye changed colour from purple to yellow secondary to bacterial metabolism, giving a positive result. Scoring of positive wells was performed visually. The procedure was graphically demonstrated in Figure 2. Mutagenicity was con-

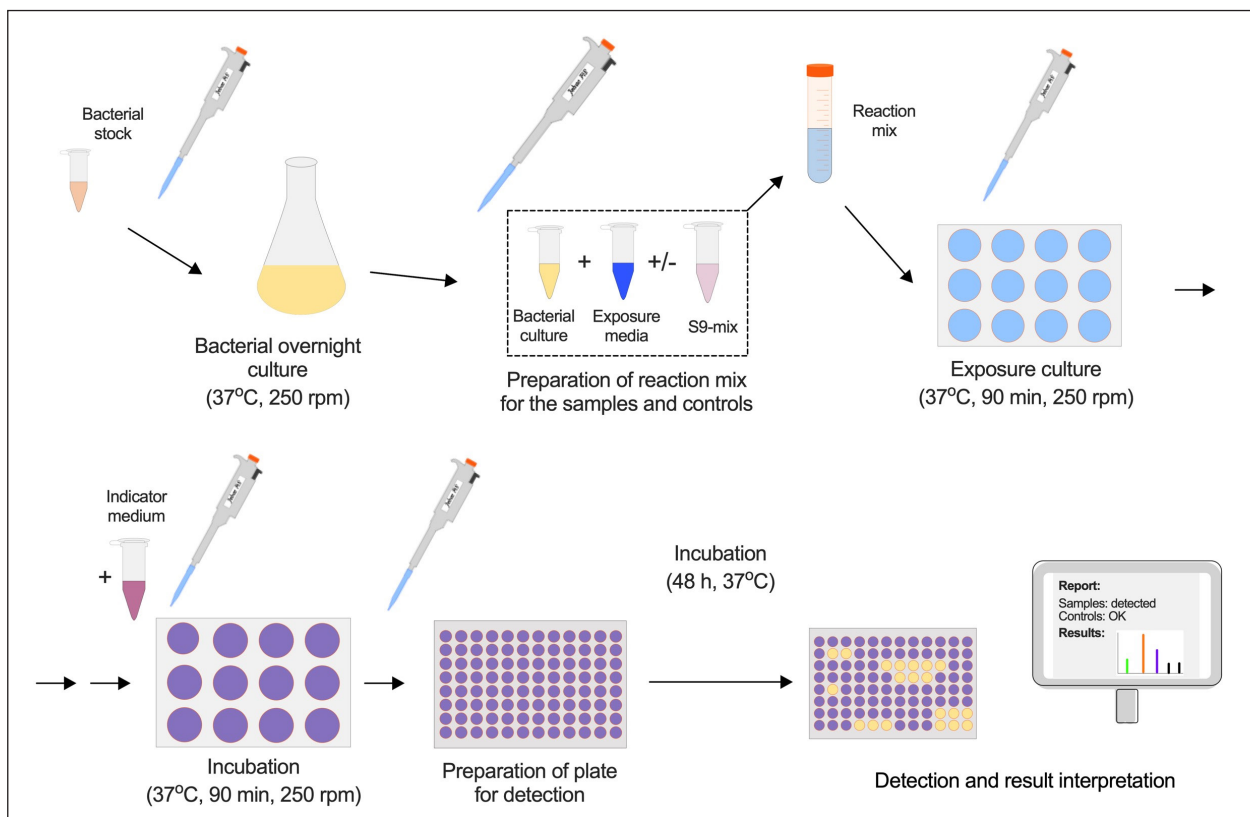


Figure 2. Ames test procedure

firmed if there was at least two-fold increase over the baseline ($FIB > 2$). The baseline equaled to the mean number of positive wells in the negative control plus one standard deviation (SD).

$$FIB = \frac{\text{mean number of positive wells}}{\text{zero-dose baseline}}$$

$$\text{Baseline} = \text{mean zero-dose control} + 1 \text{ SD}$$

The cumulative binominal test was employed for statistical analysis and the binominal B-value was calculated, as recommended by Xenometrix (Switzerland).

Results

In silico mutagenicity by VEGA

The results of in silico mutagenicity prediction and calculations of molecular properties obtained from employed (Q)SAR tools were provided in Table 1. It was concluded that the in vivo formation of mutagenic nitroso-derivatives of RAM is possible but not inevitable. In fact, the predictions for the least fragmented compounds, i.e.: No.1 (N-nitroso-ramipri), No.2 (N-nitroso-decarboxy-ramiprilat) and No.3 (N-nitroso-ramiprilat), were inconclusive ($P_a = P_i$) or non-mutagenic ($P_i > P_a$). The experimental verification of this claim was necessary.

Mutagenicity by Ames test

Mutagenicity was evaluated for RAM post-nitrosation mixtures at different concentrations of

the parent compound. Based on visual assessment, it was determined that none of them was cytotoxic against the TA98 strain. Then, the number of revertants in the treated cultures was achieved and the results of plate scoring were provided in Figure 3 and Table 2. FIB and B-value were adopted as criteria for determining mutagenicity. The primary criterion was FIB and the secondary criterion was B-value. A sample plate for scoring is demonstrated in Figure 4.

Discussion

This pilot study was designed to verify a probable mechanism of carcinogenicity of ACE-Is, the use of which has been associated with an increased cancer incidence in several clinical observations. The model compound selected for our research was RAM due to its wide-spread use and a potentially significant impact on human health. The scope of our investigation covered aspects of RAM indirect mutagenicity secondary to its in vivo nitrosation in the presence of dietary nitrite. Hence, we intended to address the problem of nitrosatable drug — food nitrite interaction, which, to our mind, requires combined and coordinated actions by relevant regulatory institutions. For this reason, we firstly performed in silico simulation of mutagenicity for RAM nitroso-derivatives. The structures selected for the analysis were based on RAM's fragmentation pattern. We assumed that under the conditions of in vivo catalysis the nitrosation yield could be

Table 1. Scores for mutagenicity prediction by VEGA* and molecular properties by Molinspiration cheminformatics

Comp. No.	CAESAR	ISS	SarPy	KNN	CONSENSUS		ALogP	Volume	MW*
1	0.5	0.5	0.5	0.3	$P_a = 0.15$	$P_i = 0.15$	3.61	411.58	445.52
2	0.5	0.5	0.5	0.1	$P_a = 0.15$	$P_i = 0.225$	3.31	352.79	369.46
3	0.5	0.5	0.5	0.1	$P_a = 0.15$	$P_i = 0.225$	3.01	377.25	417.46
4	0.7	0.5	0.7	0.7	$P_a = 0.5$	$P_i = 0.0$	2.78	250.59	262.31
5	0.7	0.5	0.7	0.7	$P_a = 0.5$	$P_i = 0.0$	2.41	161.68	164.21
6	0.7	0.5	0.7	0.7	$P_a = 0.35$	$P_i = 0.15$	2.50	236.27	222.45
7	0.9	0.9	0.9	0.9	$P_a = 0.9$	$P_i = 0.0$	0.71	151.74	160.17
8	0.9	0.9	0.9	0.9	$P_a = 0.9$	$P_i = 0.0$	1.40	136.23	140.19
9	0.7	0.9	0.7	0.7	$P_a = 0.675$	$P_i = 0.0$	2.50	189.24	190.25
10	0.9	0.9	0.9	0.9	$P_a = 0.9$	$P_i = 0.0$	0.71	117.41	132.12
11	0.7	0.9	0.7	0.9	$P_a = 0.75$	$P_i = 0.0$	1.10	163.25	184.19

*Red boxes correspond to active-mutagenic predictions, green boxes represent non-mutagenic predictions. Comp. No. — compound number, MW — molecular weight

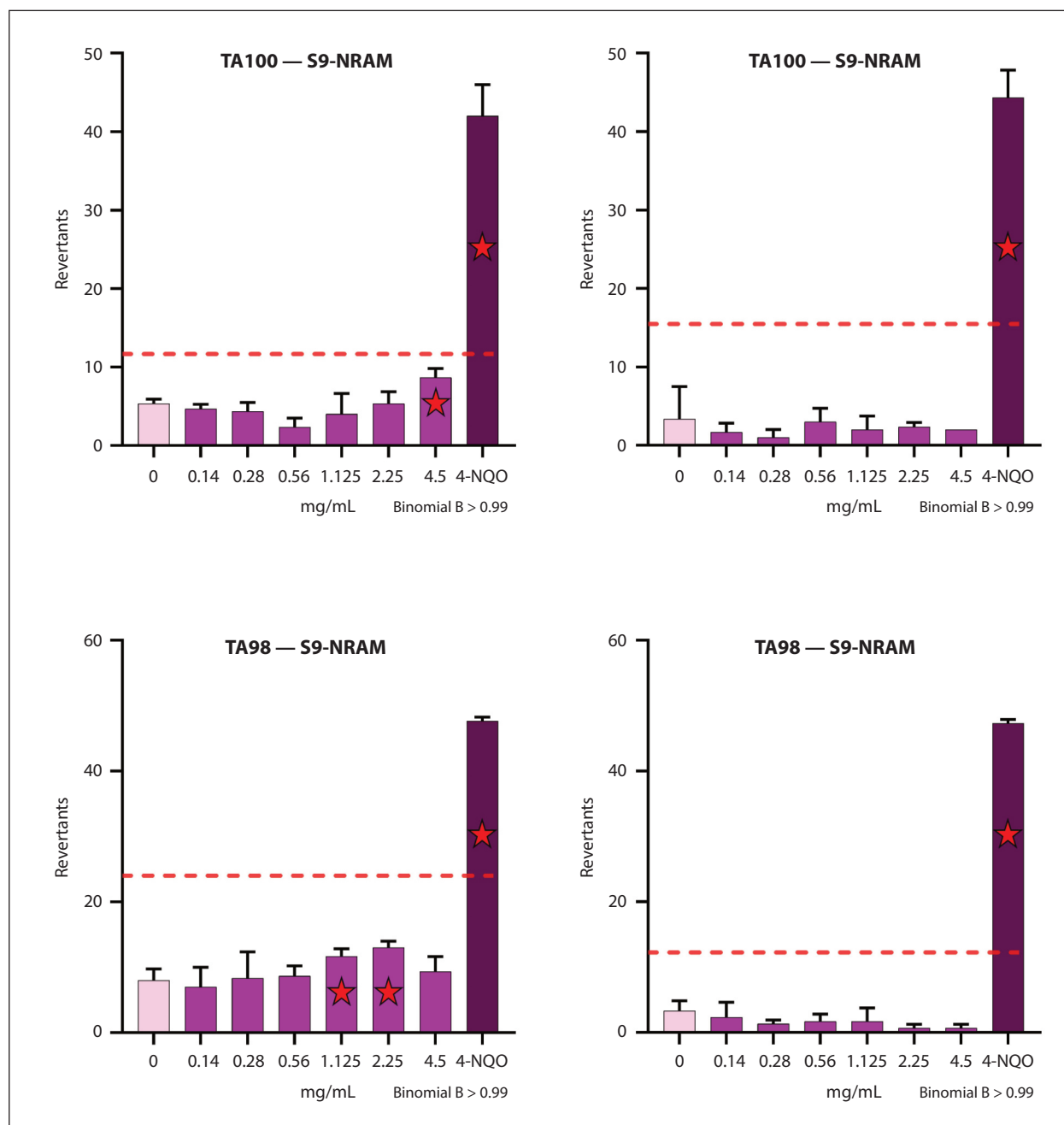


Figure 3. Results of plate scoring. Dashed line represents a two-fold increase over baseline, over which mutagenic activity was evidenced. Red asterisks indicate concentrations for which the B-value was above the critical one at 0.99

extensive and multidirectional. As demonstrated in Table 1, the mutagenicity of RAM nitroso-derivatives was heterogeneous. Less fragmented and more lipophilic molecules with higher molecular weight and volume (No. 1, 2, 3) seem to pose no mutagenic risk. However, the reliability of these predictions was low, as suggested by the assigned scores equalling 0.5. Thus, they need experimental verification. With increasing fragmenta-

tion and decreasing lipophilicity and particle size, the reliability of the predictions and the probability of being active increased. Small-molecule compounds with LogP below 2, volume below 155 and MW below 160, exhibited the highest P_a which equalled 0.9. For moderately lipophilic derivatives ($2.0 > \text{LogP} > 2.9$, MV and volume above 160), the prediction was 'mutagenic' with moderate reliability, while large lipophilic molecules

Table 2. The results of bacterial reverse mutation test for RAM post-nitrosation mixture (NRAM)

NRAM	TA98 -S9		TA98 +S9		TA100 -S9		TA100 +S9	
c [mg/mL]	FIB	B-value	FIB	B-value	FIB	B-value	FIB	B-value
Baseline	9.73		4.86		5.91		7.50	
0.14	0.72	0.2941	0.48	0.2105	0.79	0.3564	0.22	0.0605
0.28	0.86	0.6396	0.27	0.0254	0.73	0.2601	0.13	0.0086
0.56	0.89	0.7177	0.34	0.0605	0.39	0.0072	0.40	0.4534
1.125	1.20	0.9930	0.34	0.0605	0.68	0.1777	0.27	0.1212
2.25	1.34	0.9994	0.14	0.0022	0.90	0.5661	0.31	0.2105
4.5	0.96	0.8430	0.14	0.0022	1.47	0.9954	0.27	0.1212
K+	4.90	1.0000	9.74	1.0000	7.11	1.0000	5.91	1.0000

K+ — positive control; FIB — Fold increase over baseline; B-value — indicates the probability of spontaneous mutations events alone

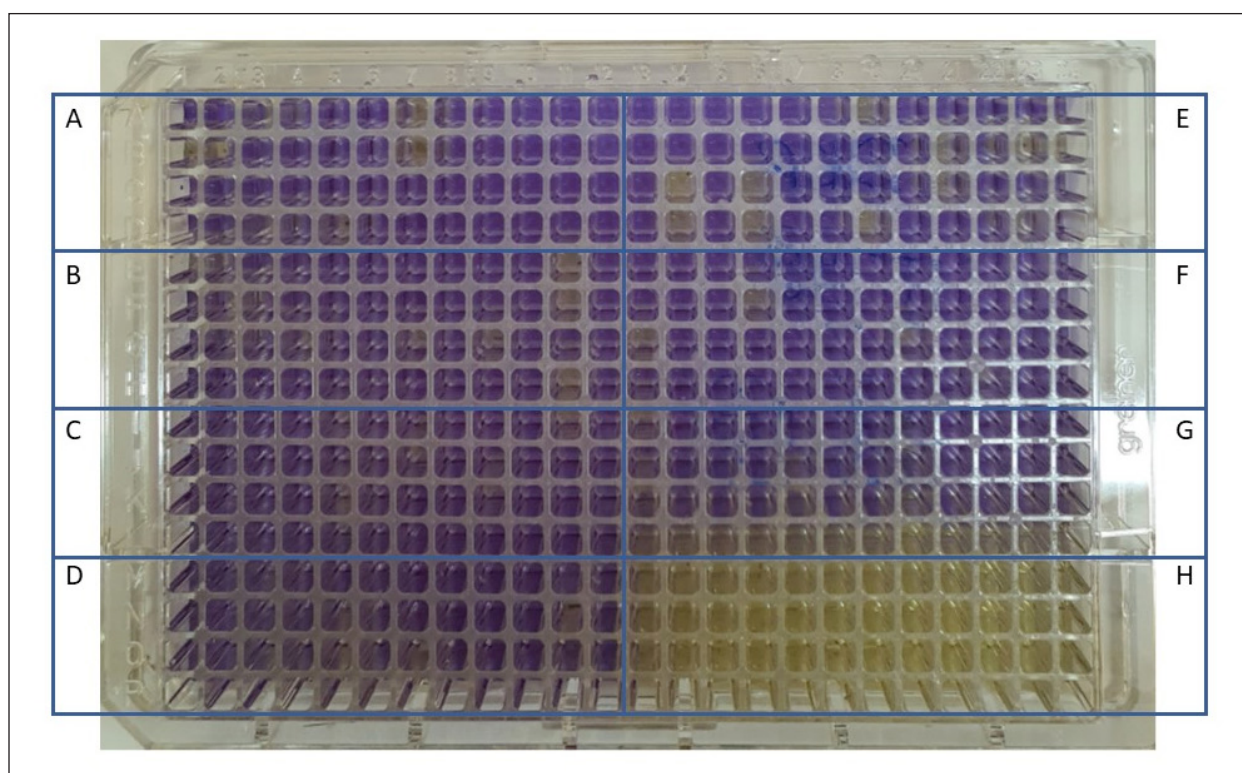


Figure 4. A sample plate for revertants scoring in mutagenicity assay. **A.** Negative control; **B.–G.** Concentrations tested; **H.** Positive control

(LogP > 3, MV and volume > 350) were predicted to be non-mutagenic or inconclusive.

In order to verify these results, the nitrosation procedure for RAM was conducted. Then, bacterial reverse mutation test was applied. As evidenced in Figure 3, none of the concentrations tested induced two-fold increase of revertants over the baseline. Therefore, the nitrosation of RAM was not associated with the induction of point mutations in neither

TA98 nor TA100 *Salmonella typhimurium* strain irrespective of metabolic activation. Consequently, the direct mutagenic effect of RAM nitrosation products by frameshift mutations or base-pair substitution was excluded. Probably, N-nitrosoramiprilat (No. 2) or N-nitroso-deoxy-ramiprilat (No. 3) or their mixture was formed, as suggested by in silico analysis of mutagenicity discussed above. Also, pure RAM is not mutagenic, as stated by

the available regulatory safety information [32]. However, our recent toxicological evaluation of the degradation impurity of RAM — its diketopiperazine derivative — provided a clear positive result for its post-nitrosation mixture in the experiment in the *Salmonella typhimurium* TA100 system after metabolic activation [19]. This indicates that it is not pure RAM, but its degradation impurity that could pose a toxicological concern. Therefore, its formation in dosage forms must be prevented by appropriate stabilization methods, for example by redox-based inhibitors [21].

The available literature dealing with the problem of mutagenic drug nitroso-metabolites notes that the vulnerability of ACE-Is to endogenous nitrosation is non-uniform. Indeed, there are two reports for a structural analogue of RAM, enalapril, which turned out to be susceptible to nitrosation in NAP test. Moreover, the resulting N-nitrosoenalapril was mutagenic in *Salmonella typhimurium* TA 1535/pSK1002 in vitro assay [23, 25]. On the contrary, imidapril and its DKP impurity were inactive in the Ames test after nitrosation, irrespective of metabolic activation [24]. This heterogeneity can be explained by the fact that the mechanisms underlying drug — nitrite interactions in vivo in the presence of metabolic activation system, catalysts and inhibitors — are extremely complex. It is also likely that more than one nitrosamine product is formed. Meanwhile, not all nitrosamines are mutagenic, as evidenced by Elder et al. [33]. Consequently, their endogenous formation would pose no mutagenic risk, with the concurrent negative result of the dedicated in vitro assays. Consistently with this, Ozhan et al. [23] reported that there was no structure-activity relationship in the group of 28 drugs subjected to NAP test and bacterial mutation assay. This means that the effect observed for a single compound after nitrosation cannot be extrapolated to its structural analogue. Therefore, in the group of ACE-Is, the tendency to form mutagenic nitrosamines in vivo should be verified on a case-by-case basis before general conclusions are made. Without these data for ACE-Is, as well as for other potentially nitrosatable drugs, the public health problem will persist.

In summary, based on the currently available literature, it can be speculated that the increased cancer incidence among ACE-I users might have been caused by in vivo formation of N-nitrosoe-

nalapril [23] or production of nitroso-derivatives from RAM DKP [19], but not by RAM or imidapril [24] endogenous nitrosation. For the ACE-Is of concern, we suggest implementing adjuvant therapy with antioxidants such as α -tocopherol or ascorbic acid [19, 21]. This would mitigate the risk of in vivo formation of mutagenic nitroso-metabolites and allow safer continuation of therapy with valuable drugs of proven clinical efficacy [34–36].

Conclusions

There are toxicological concerns about ACE-Is, as accumulating observational studies suggest their pro-carcinogenic potential. RAM is a model ACE-I with significant prevalence among patients. Our studies have clearly shown that this drug is not vulnerable to form mutagenic nitrosamines in vivo. Therefore, the pro-carcinogenic effect of RAM, if any, is not related to its endogenous nitrosation. Furthermore, with RAM as an example, the easy and inexpensive procedure for additional safety assessment of potentially nitrosatable drugs was proposed. It involves in silico simulation of mutagenicity, NAP test and Ames test. In case of positive results, adjuvant treatment should be recommended. The remaining ACE-Is must also be examined in this regard.

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Authors' contributions

Authors' individual contributions: conceptualization: K.R.; methodology: K.R.; investigation: K.R.; writing — original draft preparation: K.R.; writing — review and editing: K.R., M.M.; visualisation: T.K., K.R.; supervision: B.S.; funding acquisition: K.R. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Consent for publication

All authors read and approved the final manuscript.

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