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The mutagenicity analysis of imidapril hydrochloride and its degradant, diketopiperazine derivative, nitrosation mixtures by *in vitro* Ames test with two strains of *Salmonella typhimurium*

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ABSTRACT

Aim: The evaluation of mutagenic properties of imidapril hydrochloride (IMD) and its degradation impurity, diketopiperazine derivative (DKP), nitrosation mixtures was conducted in order to analyze the carcinogenic risk of IMD long-term treatment in patients. In this study an *in vitro* Ames test with *Salmonella enterica serovar Typhimurium* TA 98 and TA 100 strains was used.

Background: IMD and DKP contain nitrogen atoms, which makes them theoretically vulnerable to *in vivo* nitrosation with the production of N-nitroso compounds (NOC). NOC, in turn, are known animal mutagens indicating that their endogenous production from nitrosable drugs constitutes a carcinogenic hazard.

Materials and methods: Pure IMD sample was exposed to forced degradation conditions of increased temperature and dry air in order to achieve a DKP sample. Both samples were then treated with a nitrosating agent and the obtained nitrosation mixtures were subjected to mutagenicity analysis by the Ames test with *S. typhimurium* TA 98 and TA 100 strains in the presence and absence of metabolic activation system (S9 mix) using a commercial Ames MPF 98/100 microplate format mutagenicity assay kit.

Results: None of the six concentrations of the investigated nitrosation mixtures exhibited any mutagenic potential in both *S. typhimurium* strains. The addition of S9 mix did not alter the non-mutagenic properties of the studied compounds.

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Conclusions: The nitrite treatment of both studied compounds has no impact on their mutagenic properties under the conditions of the present studies. Hence, IMD and DKP nitrosation mixtures are classified as non-mutagens in this test.

1. Background

N-nitroso compounds (NOC) are the group of chemicals that contain the —N=O functional attached to a nitrogen atom of an organic moiety.[1] They are commonly present in food treated with sodium nitrite, cosmetics, pharmaceutical products, tobacco smoke, drinking water and air.[2] In multiple preclinical experiments, they have been shown to induce carcinogenic and tumor-promoting effects in as much as 39 species of laboratory animals, including higher primates. Furthermore, there is a supporting evidence confirming the participation of various NOC in the development of several human neoplasms including: gastric, esophageal, nasopharyngeal, pancreatic, colon and brain cancer.[3–6] The occurrence of NOC-induced malignancies results generally from a large-scale human exposure to these compounds which can be both, exogenous (as listed above) and endogenous after their *in vivo* formation from N-nitrosable precursors and nitrosating agents in the strongly acidic environment of gastric juice.[4] In fact, NOC precursors can be easily recognized by the presence of several functional groups, such as: dialkyl-, alkylaryl-, diaryl-, cyclic secondary amine, tertiary amine,[7,8] N-alkylurea, N-alkylcarbamate, N-alkylamide, cyanamide, guanidine, amidine, hydroxylamine, hydrazine, hydrazone, hydrazide, piperazine[4] and probably diketopiperazine.[9,10] These chemical structures, in turn, are commonly present in a large fraction of the available drug molecules making them a possible source of NOC *in vivo*. Indeed, a wide array of drugs is known to produce NOC after treatment with nitrosating agent and many of these nitrosation products have been proven to exert mutagenic actions.[4] Thus, the mutagenicity assessment for N-nitrosable drugs seems to be a reasonable means of cancer prevention aimed at the reduction of the risk associated with patient exposure to mutagenic and carcinogenic drugs.

The induction of genome mutations (especially point mutations in human oncogenes or tumor suppressor genes) is the most common mechanism by which drugs could contribute to cancer development.[11] A simple method that determines the patterns of missense mutations, commonly used for mutagen screening and recommended by regulatory agencies, is the *in vitro* bacterial reverse mutation assay (Ames test). This test is designed to detect a point mutation-inducing activity of investigated compounds, evidenced by the alternation of growth requirements of tester organisms.[12] Unfortunately, the analysis of the possible *in vivo* NOC production from nitrosable drugs and the assessment of their mutagenicity still remains outside the regulatory recommendations for standard carcinogenicity tests prior to drug registration.[4]

Another important aspect that needs consideration with respect to carcinogenic risk associated with the use of N-nitrosable drugs is their constant chemical degradation that leads to the formation of degradation impurities present in final dosage forms. Drug decay is, in fact, an unavoidable process resulting from a multi-component and multi-phase character of each formulation, causing the gradual loss of drug's potency and the accumulation of its degradation products.[13] This, in turn, indicates that the administration of rapidly-degrading pharmaceuticals is associated with patient exposure to their degradants which similarly to active ingredients could present the potential for N-nitrosation. For this reason, the appropriate stability tests, identification of drug degradation impurities, evaluation of their N-nitrosating tendency and assessment of their ability to induce genome mutations seem essential for safety assurance especially with respect to cancer initiation.

The subject of our studies, imidapril hydrochloride (IMD), is an angiotensin-converting enzyme inhibitor (ACE-I) indicated for the long-term treatment of essential hypertension.[14] The presence of nitrogen atoms in IMD molecule makes it theoretically N-nitrosable (Fig. 1). What is more, the previously-performed stability assessment of this drug proved that IMD undergoes the process of degradation, accelerated by heat and moisture, following different pathways dependent on the environmental conditions.[15] Under high moisture conditions, IMD decay results in the production of two degradation impurities, *i.e.*: a diacidic derivative (imidaprilat), which is an active metabolite, and a diketopiperazine derivative (DKP)[16]; however, under dry air conditions, IMD forms only one degradation impurity identified as DKP.[17] The structural analysis of the formed degradation impurities indicates that these compounds, like IMD, demonstrate the vulnerability to N-nitrosation, rising some justified safety concerns. It should, however, be emphasized that the non-mutagenic character of pure IMD and its active metabolite has been confirmed in the course of its legal registration process.[18] Furthermore, our previous mutagenicity studies of pure DKP with *Salmonella typhimurium* TA 98 and TA 100 strains, both with and without metabolic activation system, showed a negative result, confirming no impact of pure degradation impurity on cancer initiation by the mechanism of mutagenesis.[17] However, some available literature data demonstrate that other structurally-related ACE-Is, such as enalapril, do exhibit a mutagenic potential in *S. typhimurium* TA 1535 strain after N-nitrosation (1.12 mg/mL).[19] Moreover, there is evidence that the chemicals containing diketopiperazine motif could be carcinogenic to humans after exposure to nitrite in the environment of gastric juice.[9,10]

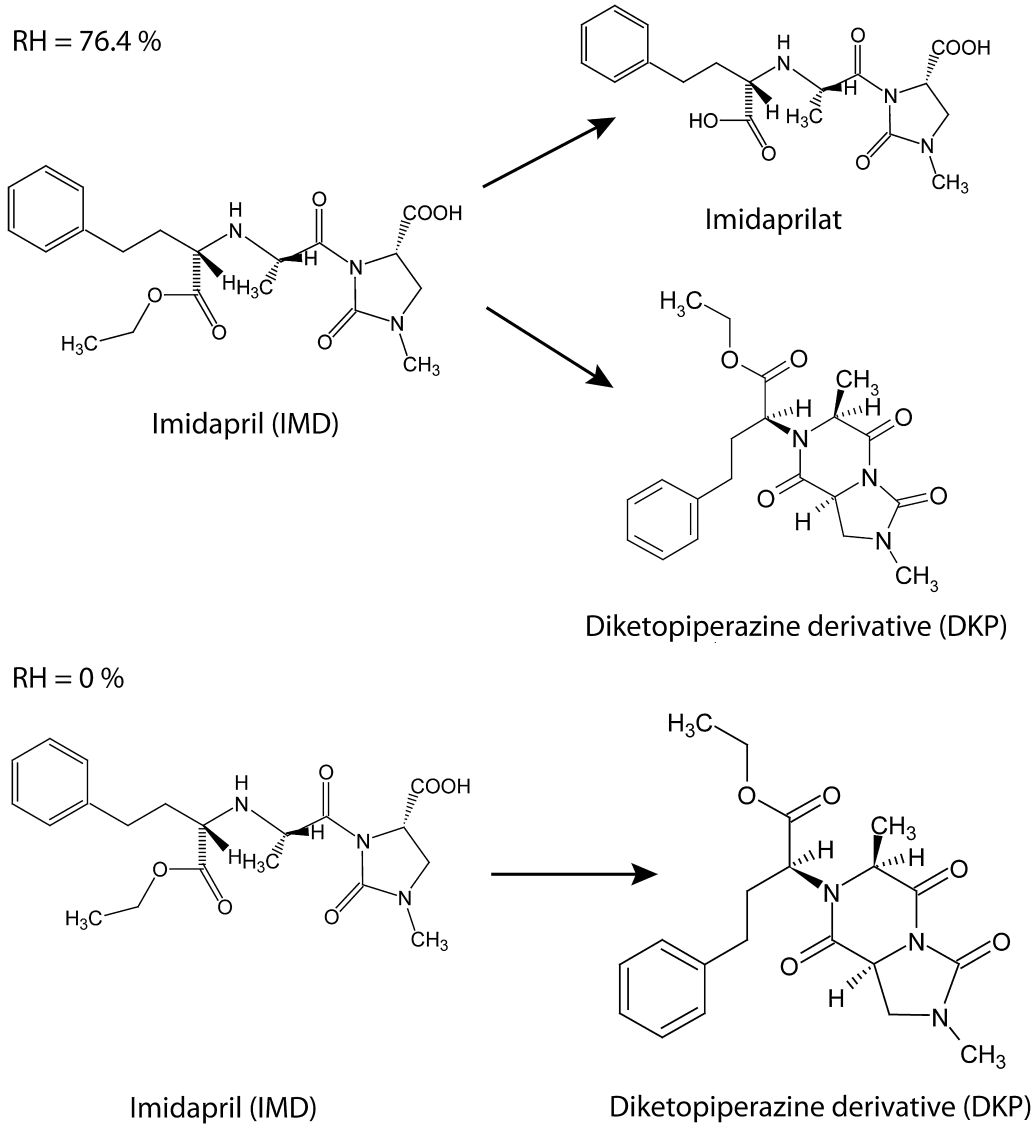

Fig. 1 – Pathways of IMD degradation under different *RH* conditions.

2. Aim

The aim of our study was to assess the mutagenic hazard of IMD and DKP nitrosation mixtures in order to investigate their impact on cancer initiation and confirm safety of IMD-based therapy. We did not aim at the mutagenicity evaluation of pure imidaprilat because of its close structural similarity to IMD (Fig. 1) suggesting the analogy of their chemical properties.

Our adopted analytical approach involved the exposure of pure IMD sample to stress conditions of increased temperature and dry air according to the previously-established procedure in order to achieve a DKP sample.[17] For the identification of the obtained degradation impurity a RP-HPLC method was applied. Pure IMD and DKP were subsequently subjected to N-nitrosation according to NAP test criteria[4,20] and the obtained nitrosation mixtures were finally analyzed for their cytotoxicity and mutagenicity using a commercial Ames MPF 98/100 microplate format mutagenicity assay kit with *S. typhimurium* TA 98 and TA 100 strains exposed to six concentrations of tested compounds in the presence and absence of a metabolic activation system.[12]

3. Materials and methods

3.1. *Chemicals*

3.1.1. *IMD degradation procedure*

Imidapril hydrochloride was supplied by Jeleniogorskie Zaklady Farmaceutyczne "JELFA" (Jelenia Gora, Poland). Analytical grade potassium phosphate monobasic and *ortho*-phosphoric acid were obtained from POCh S.A. (Gliwice, Poland) and HPLC grade methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled water was used.

3.1.2. *Nitrosation procedure*

Analytical grade hydrochloric acid 1.0 mol/L, sodium nitrite ($NaNO_2$) and ammonium sulfamate ($NH_4SO_3NH_2$) were obtained from Sigma–Aldrich. Ultrapure water was used.

3.1.3. *Mutagenicity assay*

A commercial Ames MPF 98/100 microplate format mutagenicity assay kit (Xenometrix, Switzerland) with *S. typhimurium* strains TA 98 (*hisD3052*, *rfa uvrB*, *pKM101*) and TA 100 (*hisG46*, *rfa uvrB*, *pKM101*) was used. It contained the following chemicals: 2-nitrofluorene, 4-nitroquinolone-N-oxide, 2-aminoanthracene as positive controls, phenobarbital/β-naphtoflavone-induced rat liver fraction S9 as the activation system, sterile ampicillin (50 mg/mL), growth medium, exposure medium (histidine-rich) and indicator medium (lacking histidine). The S9 mix was prepared by the use of a commercial kit from Xenometrix containing phosphate buffer pH 7.4, $MgCl_2$, KCl, G-6-P and NADP. The tested samples were diluted in sterile DMSO (Sigma–Aldrich).

3.2. *Apparatus*

3.2.1. *RP-HPLC method*

A Shimadzu liquid chromatograph comprising a Rheodyne 7125, 100 μL fixed loop injector, UV-VIS SPO-6AV detector, LC-6A pump and C-RGA chromatopac integrator was used. The chromatographic separation was performed under the following conditions: LiChrospher 100 RP-18 (size 5 μm) 250 mm × 4 mm I.D. column as a stationary phase and acetonitrile–methanol–aqueous phosphate buffer, pH 2.0; 0.035 mol/L (60:10:30, v/v/v) as a mobile phase. Aqueous phosphate buffer was prepared by dissolving 68.1 mg of potassium dihydrogen phosphate (KH_2PO_4) in 450 mL of bidistilled water. The obtained solution was further adjusted to desired pH 2.0 with 1.0 mL of phosphoric (V) acid (85%) and then it was completed to the volume of 500.0 mL with bidistilled water. The mobile phase had been filtered (filter 0.22 μm), degassed by ultrasound and pumped isocratically at a flow rate of 1.2 mL/min at ambient temperature. The detector wavelength was 218 nm and the injection volume was 20 μL.[21]

3.2.2. *Bacterial reverse mutation assay*

A Dry Incubator KBC-125W (WAMED) set at 37 °C and microplate shaker DTS-4 were used for the incubation of the tester strains. The optical density (OD_{600}) of the overnight culture was measured by UV-VIS spectrophotometer, Lambda-6 UV WinLab Version 2.70.01, Perkin Elmer. The analytical operations were performed under safety cabinet so that no contamination of the cultures could occur.

3.3. *Procedures*

3.3.1. *Obtainment of DKP sample*

Pure IMD was analytically weighted (200.0 mg for a mutagenicity study and 10.0 mg as a reference) into glass vial and placed in a sand bath that ensured the appropriate conditions of relative air humidity (*RH* 0%). The so prepared samples were heated to 100 °C for 15 days so as to induce the process of accelerated drug degradation. Afterwards, the degraded IMD sample (10.0 mg) was cooled to room temperature, dissolved in bidistilled water, quantitatively transferred into volumetric flask, made up to a total volume of 25.0 mL with methanol, filtered and chromatographed by RP-HPLC method in order to confirm its qualitative and quantitative composition by comparing the obtained chromatogram with the reference.[17]

3.3.2. *Nitrosation procedure*

The nitrosation of IMD and DKP was performed according to the 'nitrosation assay procedure' (NAP test) recommended by a WHO Expert group.[4,20] The samples of the investigated chemicals (125.0 mg) were dissolved in 1.0 mL of sterile DMSO, adjusted to pH 3 with 1 M hydrochloric acid and treated with sodium nitrite ($NaNO_2$) in the molar ratio of 1:4. Then pH was again adjusted to 3. The so obtained nitrosation mixtures were incubated for 1 h at 37 °C in the dark on a shaker. The solvent (sterile DMSO) with nitrite treatment was incubated under similar conditions as a negative control for the mutagenicity assay. The reaction was subsequently stopped by the addition of ammonium sulfamate (molar ratio $NaNO_2$ *vs.* $NH_4SO_3NO_2$ was 4:4). The obtained reaction mixtures were subjected to a cytotoxicity and mutagenicity assay. The concentrations of the tested nitrosation products were expressed as a concentration of the parent compounds prior nitrosation.

3.3.3. *Preparation of test bacterial strains*

The *S. typhimurium* strains TA 98 and TA 100 were cultured overnight (16 h) in a histidine-rich medium at 37 °C on a shaker in the presence of ampicillin and only cultures with optical density above 2.0, measured at λ = 600 nm (OD_{600}), were used.

3.3.4. *Cytotoxicity assay*

Pre-screen determination of dose range was performed prior to mutagenicity analysis by the evaluation of cytotoxicity of the tested compounds. With this aim, TA 98 strain was used. The nitrosation mixtures were sequentially diluted on a 96-well plate so that the following primary concentrations were obtained: 113.2 mg/mL, 35.95 mg/mL, 11.44 mg/mL, 3.64 mg/mL, 1.16 mg/mL, 0.37 mg/mL, 0.118 mg/mL. Aliquots of 10.0 μL of each primary concentration were transferred to 24-well plate and diluted with 240.0 μL of the mixture prepared beforehand by mixing 2.7 mL of an exposure medium with 0.3 mL of the overnight culture. The following exposure concentrations were thus obtained: 4.5 mg/mL, 1.44 mg/mL, 0.46 mg/mL, 0.15 mg/mL, 0.046 mg/mL, 0.015 mg/mL, 0.0047 mg/mL. The analysis was performed in parallel with a negative control (30.0 μL) obtained as described in Section 3.3.2. The so prepared solutions were incubated on a shaker at 37 °C for 90 min. The lowest concentration for which the lysis of bacteria was observed was accepted as the highest concentration for the mutagenicity assay.

3.3.5. *Ames test*

The mutagenicity assay was performed according to manufacturer instructions. The selected *S. typhimurium* strains were exposed to six concentrations of the tested nitrosation mixtures, both with and without rat liver S9 fraction. The stock solutions of positive controls in DMSO necessary for analysis were prepared as demonstrated in Table 1. The nitrosation mixtures were sequentially diluted with sterile DMSO, transferred to 24-microwell plates and mixed with 240 μL of the previously prepared mixture containing an exposure medium (histidine-rich), overnight culture and 4.5% microsomal S9

Table 1 – Positive controls applied for the mutagenicity assay.

S. typhimurium strain	Positive control	Stock solution	Final concentration
Without metabolic activation			
TA 98	2-Nitrofluorene	50.0 μg/mL	2.0 μg/mL
TA 100	4-Nitroquinolone-N-oxide	2.5 μg/mL	0.1 μg/mL
With metabolic activation induced with phenobarbital/β-naphtoflavone			
TA 98	2-Aminoanthracene	12.5 μg/mL	0.5 μg/mL
TA 100	2-Aminoanthracene	31.25 μg/mL	1.25 μg/mL

fraction (if necessary) so that the following final test concentrations in triplicate were produced: 0.14 mg/mL, 0.28 mg/mL, 0.56 mg/mL, 1.125 mg/mL, 2.25 mg/mL, 4.5 mg/mL. They were incubated for 90 min at 37 °C in parallel with positive and negative controls. Afterwards, the exposed cultures were diluted in a specially formulated medium containing a pH indicator and lacking histidine, and the contents of each 24-well culture were distributed into 48 wells of a 384-well plate (50 μL per well) to be incubated for 48 h. Mutagenicity was measured by a color change from purple to yellow caused by pH drop due to bacterial metabolism. The scoring of colonies that had undergone the reversion to histidine prototrophy was performed manually. A sample was considered positive when there was at least a twofold increase over the baseline (*FIB* > 2) and a positive dose–response tendency occurred. The baseline was defined as the mean number of positive wells in the negative controls plus one standard deviation (SD). The dose–response relationship was described by the increase in at least three consecutive doses maintaining statistical significance expressed as *p*-value below 0.05. The statistical criteria of the *t*-test were evaluated as a supplementary means of results interpretation.

FIB = mean number of positive wells/zero-dose baseline
Baseline = mean zero-dose control + 1 SD

4. Results

The 15-dayed stressing of IMD sample caused the complete degradation of the drug (IMD content in the sample ~0%) and the formation of DKP (content in the sample ~100%) as evidenced by the recorded RP-HPLC chromatogram (Fig. 2) which was compared with the reference.[17]

The pre-screen determination of dose range evidenced that none of the tested concentrations of the analyzed nitrosation mixtures exhibited any cytotoxicity signs of increased bacterial lysis. A bacteria growth was observed in each well indicated by the liquid turbidity comparable to zero-dose. Thus, according to *Organization for Economic Co-operation and Development Recommendations* – OECD guideline 471 adopted at 21 July 1997, the concentration of 4.5 mg/mL was selected as the highest concentration for mutagenicity assay.

The revertant frequencies obtained in the mutagenicity assay of the analyzed nitrosation mixtures for both *S. typhimurium* strains with and without metabolic activation are demonstrated in Figs. 3 and 4.

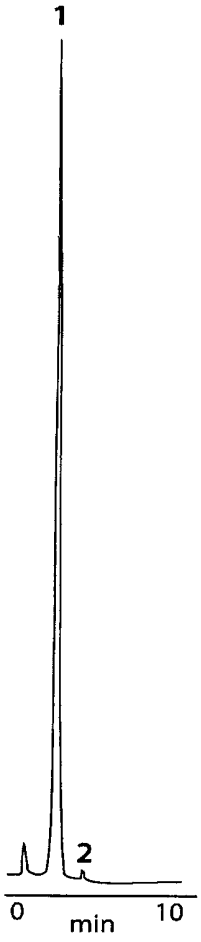

Fig. 2 – RP-HPLC chromatogram of degraded IMD sample after 15-dayed stressing under *T* = 100 °C and *RH* = 0%. 2 – IMD (t_R = 5 min); 1 – DKP (t_R = 3 min).

5. Discussion

The performed studies were aimed at the evaluation of carcinogenic hazard associated with the administration of the theoretically-nitrosable drug, IMD and its impurity, DKP which is formed in the course of IMD degradation and, thus, is present in the final dosage form. As indicated by the pathways of IMD decay (Fig. 1), its exposure to stress conditions of increased temperature and dry air leads to the formation of pure DKP.[17] Hence, by applying the previously-established and validated procedure of accelerated IMD degradation for 15 days we achieved the desired compound and its presence in the studied sample was confirmed by comparing the

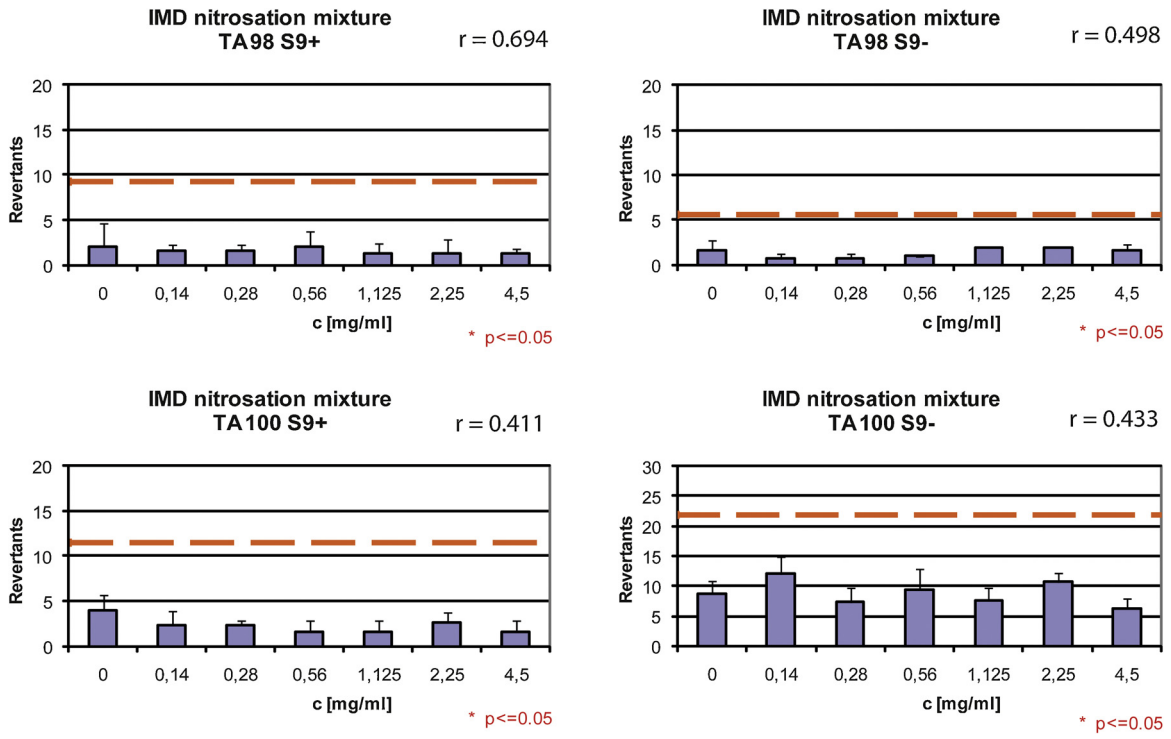

Fig. 3 – The results of bacterial reverse mutation test with TA 98 and TA 100 strain in the presence and absence of metabolic activation for IMD nitrosation mixture. Dashed line corresponds to *FIB* = 2.

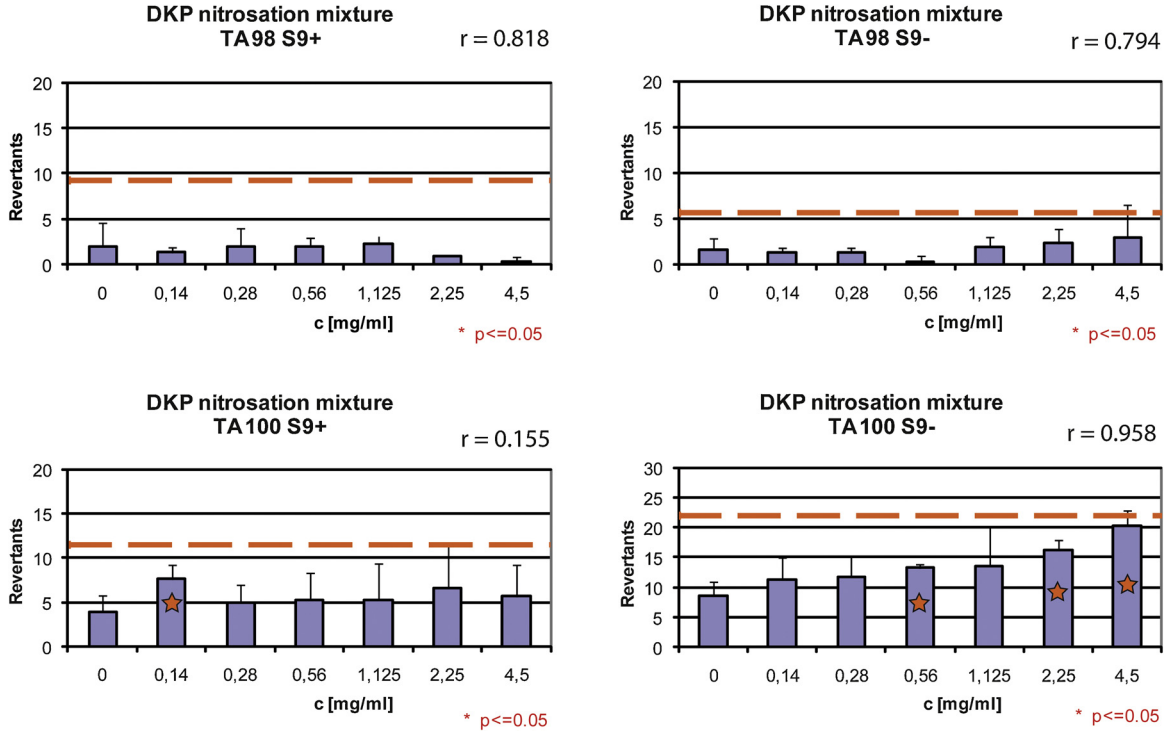

Fig. 4 – The results of bacterial reverse mutation test with TA 98 and TA 100 strain in the presence and absence of metabolic activation for DKP nitrosation mixture. Dashed line corresponds to *FIB* = 2.

obtained RP-HPLC chromatogram with the reference one.[17] Since both chromatograms represented a high level of analogy – the retention times of the peaks for IMD (t_R = 5 min) and DKP (t_R = 3 min) were comparable, and the DKP content was ~100% (Fig. 2), we accepted the sample for further studies.

Both IMD and DKP exhibit the theoretical capacity of forming NOC *in vivo*, thus, in our research they were subjected to nitrosation under the standard conditions established by NAP test guidelines. The applied nitrosation procedure provided an excess of nitrite to promote the reaction and ensured the optimal temperature and pH for the highest reaction efficiency so that the sensitivity of the analysis could be maximized. It should, however, be emphasized that the so obtained product yields exceed by far the clinically reachable NOC levels *in vivo* and thus the employed scientific approach was used only as a reference test to screen and discriminate between compounds that represent a potential risk and drugs with low N-nitrosation potential.

Cytotoxicity assay for both nitrosation mixtures was further performed as a pre-screen determination of a dose range in order to ensure the highest sensitivity of the Ames test. This procedure was in agreement with OECD guideline 471. In fact, all the tested concentrations were found to be non-cytotoxic indicating that the highest one can be applied to the mutagenicity assay.

The mutagenicity of both nitrosation mixtures was finally investigated. For this purpose the bacterial reverse mutations assay was selected. This test is recommended by ICH guideline *CMPH/ICH/174/95* which approves it as a standard method for pharmaceutical genotoxicity testing. It utilizes the specially engineered *S. typhimurium* strains that detect either frameshift (TA 98 with *hisD3052*) or base-pair substitution (TA 100 with *hisG46*) mutations so that mutagens acting *via* different mechanisms may be identified. Thus, by the application of the Ames test we evaluated the capability of the investigated nitrosation mixtures (expressed as *FIB* value) to reverse the histidine-dependent, auxotrophic mutants of *S. typhimurium* to the protrophic forms that can grow on a growth factor-deficient medium. The critical *FIB* value for the classification of positive results was accepted as 2. We found that none of the tested concentrations for both *S. typhimurium* strains exhibited a twofold increase of revertant number over the baseline (*FIB* below 2). What is more, the addition of S9 mix did not affect the mutagenicity of the tested compounds in both strains. Also, no dose–response tendency was observed for IMD nitrosation mixture in both strains, for DKP nitrosation mixture in TA 98 strain and for DKP nitrosation mixture in TA 100 with S9 mix, indicated by a low value of the correlation coefficient ($r < 0.818$). However, DKP nitrosation mixture with TA 100 strain without metabolic activation (S9-) gave a dose-related increase over the tested range (correlation between concentration and revertants number $r = 0.960$), while the result for the highest concentration was close (but below) to the adopted criteria of reproducible increase in revertants number. Since three consecutive concentrations with *p*-value below 0.05 were assumed as minimum to determine the dose–response as not random, the obtained result was interpreted as negative. Therefore, since none of the tested compounds met the criteria for positive result classification, it was concluded that the exposure to nitrosating agent does not influence the non-mutagenic properties of tested chemicals under the conditions of this study, indicating that the tested chemicals have no impact on cancer initiation by this mechanism. Basing on the obtained results, also some real-life implications can be made. According to literature data, the maximum blood concentration of IMD after 28-dayed administration equals 34.7 ng/mL[22] which is as much as 10^6 less than the concentration range tested. This indicates that the clinically obtainable level of its nitrosation and degradation product is even lower, advocating our no-effect argument. It should, however, be emphasized that *CPMP/ICH/141/95* and *CPMP/ICH/174/95* guidelines issued by ICH recommend the performance of additional studies, such as: *in vitro* assay with mammalian cells and *in vivo* experiments with laboratory animals as a corroborative evidence prior to final classification of a drug as a non-mutagen.

6. Conclusions

Exposure to nitrosating agent has no impact on IMD and DKP mutagenic properties under the conditions of the present test. Just like pure compounds, IMD and DKP after nitrite treatment give a negative result in the Ames test with *S. typhimurium* TA 98 and TA 100 strains, both with and without metabolic activation system (S9 mix). These results indicate that the *in vivo* nitrosation of IMD and its degradation impurity probably has no impact on cancer initiation by the mechanism of mutagenesis studied in our research. However, an appropriate clinical trial is necessary in order to conclusively confirm our results.

Conflict of interest

None declared.

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