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3-NOP: Mutagenicity and genotoxicity assessment

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ABSTRACT

3-NOP (3-nitroxy-propanol) is a new development compound which reduces methane emission from ruminating animals. For registration purposes with emphasis on EU and North America data requirements, mutagenic and genotoxic potential was assessed following OECD protocols and respective guidance documents. 3-NOP mutagenicity and genotoxicity testing raised no flags with regard to these endpoints. *In silico* assessment of 3-NOP and its major plasma metabolite NOPA (3-nitroxy-propionic acid) were predicted negative with regard to the bacterial reverse mutation (Ames) test. Ames test, mouse lymphoma assay, *in vitro* micronucleus test, and the oral *in vivo* micronucleus test using rat bone marrow were all negative. Exposure of the rat bone marrow was verified by the presence of 3-NOP and its metabolites NOPA and HPA (3-hydroxy-propionic acid) a naturally occurring substance in mammals) in plasma following oral dosing. It is therefore concluded that 3-NOP and its metabolites pose no mutagenic and genotoxic potential.

1. Introduction

3-nitroxy-propanol (3-NOP, CAS 100502-66-7) has been reported to reduce methane emission from ruminants (Hristov et al., 2015; Reynolds et al., 2014; Romero-Perez et al., 2014; Romero-Perez et al., 2015) by targeting the nickel enzyme methyl CoM reductase in rumen methanogenic archaea that catalyzes the methane forming reaction (Duin et al., 2016).

3-NOP was selected out of a series of compounds. For early toxicological assessment, mutagenicity and genotoxicity screening studies were conducted followed by regulatory compliant studies. For regulatory safety assessment purposes, the evaluation of the mutagenic and genotoxic potential of an active ingredient and its metabolites is essential to enable safe use of the compound in animal feed (EFSA, 2017a). In this context, the number of studies and endpoints to be investigated varies depending on the nature of the compound but also on the regulatory requirements. Table 1 compares the requirements of the European Union as compared to the USA for substances added into the feed of food-producing animals.

An important aspect for *in vitro* mutagenicity and genotoxicity testing is information on metabolism. 3-NOP (see Fig. 1) is a unique compound: It has two functional groups (i) primary alcohol, (ii) organic nitrate ester group and is metabolized into endogenous compounds (Thiel et al., 2018). The two major metabolic steps *in vivo* are oxidation of the primary alcohol function to the corresponding carboxylic acid (yielding 3-nitroxy-propionic acid, NOPA) followed by nitrate ester cleavage (resulting in the formation of 3-hydroxy-propionic acid, HPA, see Fig. 1).

Oxidation of short-chain primary alcohols like 3-NOP is catalyzed by a number of enzymes including alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), or Cytochrome P450 enyzmes (CYPs). ADH and ALDH are quantitatively dominant to oxidize short-chain primary alcohols, are located in the cytosol and in the cytosol/mitochondria, respectively, and usually require NAD as cofactor. CYPs are more located in the endoplasmic reticulum and to a lesser extent in mitochondria and require NADPH (Parkinson et al., 2013). CYP may be quantitatively less important for the oxidation reaction of primary alcohols than ADH/ALDH.

The standard OECD *in vitro* studies on this endpoint are designed to incorporate metabolism by adding liver homogenate S9-mix which contains both cytosolic and microsomal enzymes including ADH/ALDH. In a standard genotoxicity setting, NADPH regenerating system is added and the metabolism of the preparation is driven by enzymes requiring NADPH such as CYPs and take little account for phase II or ADH/ALDH catalyzed reactions (Gatehouse, 2012).

Thus, a concern using standard genotoxicity and mutagenicity testing *in vitro* could be that 3-NOP cannot be oxidized to NOPA or is oxidized to a limited extent only. To fully account for the oxidation of 3-NOP to NOPA and to fulfill data requirements of the US, we added two additional investigations: an *in vivo* study and *in silico* assessment of 3-

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Abbreviations

3-NOP	3-nitroxy-propanol
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
CNS	Central nervous system
CYP	Cytochrome P450 monooxygenase
HPA	3-hydroxy-propionic acid
HPLC-UV	High performance liquid chromatography with ultraviolet detector
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS/MS	Liquid chromatography with triple-quadrupole mass spectrometry detection
LLQ	Lower limit of quantification
MLA	Mouse lymphoma assay
MNT	Micronucleus test
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCE	Normochromatic erythrocytes
NOPA	3-nitroxy-propionic acid
PCE	Polychromatic erythrocytes
WoE	Weight of evidence

Table 1
Comparison of recommended *in vitro* and *in vivo* mutagenicity and genotoxicity studies.

	EFSA (2017a)	Redbook (2007)	VICH GL 23(R)
Bacterial reverse mutation test (OECD 471)	X	X	X
Forward mutation in mammalian cells	–	X[a]	X[e]
Clastogenicity testing *in vitro*	X[b]	X[a]	X[e]
In vivo test	X[c]	X[d]	X[f]

X = study required or to be considered.

[a] Either an *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells or an *in vitro* mouse lymphoma thymidine kinase gene mutation assay are to be conducted. The mouse lymphoma assay is preferred (OECD 490, former OECD 476).

[b] *In vitro* MNT (OECD 487).

[c] Consider specific features of the test substance: In case of positive results from *in vitro* battery, appropriate *in vivo* study(ies) should be conducted.

[d] Not required for cumulative estimated intake of the additive equal or less than 50 ppb but greater than 0.5 ppb; an acceptable *in vivo* test to study for chromosomal damage using mammalian hematopoietic cells is the *in vivo* MNT (OECD 474).

[e] VICH GL23(R) indicates that one of the following three tests can be used: *in vitro* chromosomal aberration test, *in vitro* micronucleus test or the mouse lymphoma test.

[f] Either an *in vivo* micronucleus test or and *in vivo* cytogenicity test.

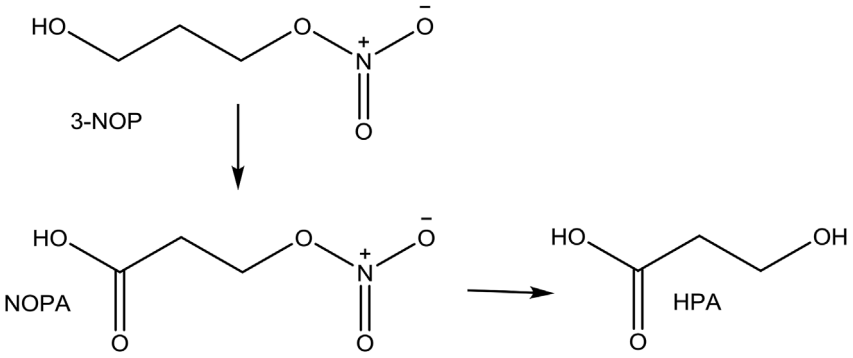

Fig. 1. Chemical structure of 3-NOP and its plasma metabolites NOPA and HPA.

NOP and its metabolite NOPA. Derek Nexus (Lhasa Limited) and Leadscope Model Applier (Leadscope Inc.) were used for the *in silico* evaluation.

2. Materials and methods

2.1. Test and control items

3-NOP a clear, pale yellow liquid with a purity of 99.4% was used. It was supplied by DSM Nutritional Products AG.

3-hydroxy-propionic acid sodium salt (purity 98%) was obtained from Toronto Research Chemical; NOPA (97.9%), ^{13}C-NOPA (99.4%), Sodium 3-hydroxypropanoate-2,2-d2 (=d2-HPA; 82.5%) were obtained from DSM Nutritional Products AG.

Sodium Azide (Sigma), ICR-191 (Acros Organics), 2-nitrofluorene (Merck), methylmethane sulfonate (Sigma), 4-nitroquinoline (Sigma), 2-aminoantracene (Sigma) and mitomycin C (Calbiochem, Sigma), colchicine (Acros Organics), Griseofulvin (Sigma), cyclophosphamide (Baxter) were used as positive controls.

Water, physiological saline, or DMSO were used as vehicle as appropriate.

2.2. Other materials and chemicals

Salmonella typhimurium tester strains TA1535, TA1537, TA98, TA100, TA102, and *Escherichia coli* WP2uvrA, rat liver S9 (obtained from male Sprague-Dawley rats induced with Aroclor 1254 or with phenobarbital/beta-naphthoflavone) were obtained from Trinova Biochem. L5178Y/TK$^{+/-}$-3.7.2C mouse lymphoma cells were obtained from ATCC. Chinese hamster V79 cells were obtained from Technical University of Darmstadt.

Acetonitrile, methanol, 2-propanol, formic acid and acetic acid were obtained from Merck. Ammonium formate, methansulfonic acid and zinc sulfate monohydrate were supplied by Fluka. Bovine serum albumin and sodium chloride were obtained from Sigma.

2.3. Guidelines, ethical approval, and animal housing

All regulatory mutagenicity/genotoxicity studies described were conducted in compliance with OECD GLP requirements. The protocols of the *in vivo* studies were approved by the Laboratory Animal Welfare Officer and the Ethical Committee.

The animals were housed in groups of up to 5 animals of the same dose group in polycarbonate cages (Makrolon type IV) containing appropriate bedding equipped with water bottles. Animals received pelleted rodent diet (SM R/M-Z from SSNIFF®) and had ad libitum access to water and feed unless indicated otherwise. The animal room had a temperature of 18–24 °C, a humidity of 40–70%, a 12 h light cycle and at least 10 air changes per hour with some minor fluctuations during cleaning procedures.

2.4. In vitro studies

2.4.1. Ames test

In the screening test *Salmonella typhimurium* tester strains were incubated in the presence and absence of S9-mix at test substance concentrations from 1.6 to 500 μg/plate using the pre-incubation method. This study is based on OECD 471 (1997) with some modifications: the smaller highest test concentration is justified by increased incubation time (60 min), higher bacteria and compound concentration (Kado et al., 1983; Muster et al., 2000). Negative controls were tested concurrently. In the absence of S9-mix, the following positive controls were test concurrently: 2-nitrofluorene (0.4 μg/plate, TA98), sodium azide (1.0 μg/plate TA100, 0.5 μg/plate TA1535), mitomycin c (0.01 μg/plate TA102), ICR 191 (0.1 μg/plate TA1537). In the presence of S9 2-aminoanthracene (0.4 μg/plate TA98, TA100, TA1535, 1.0 μg/plate TA1537, or 10 μg/plate TA102) was used.

The regulatory Ames test was conducted in compliance with OECD 471 (1997). 3-NOP was tested either in the presence or absence of metabolic activation (S9-mix) in *Salmonella typhimurium* and *E. coli* tester strains. Two independent experiments were performed using the plate-incorporation method with either 5% (v/v) S9 homogenate or 10% (v/v) S9 homogenate. 52, 164, 512, 1600, and 5000 μg 3-NOP/plate were used. As positive controls sodium azide (TA1535, 5 μg/plate), ICR-191 (TA1537, 2.5 μg/plate), 2-nitrofluorene (TA98, 10 μg/plate), methylmethane sulfonate (TA100, 650 μg/plate), or 4-nitroquinoline N-oxide (WP2uvrA, 10 μg/plate) were used in the absence of S9. 2-aminoanthracene was used in the presence of S9 for all strains (TA1535 and TA1537 2.5 μg/plate, TA98 and TA100 1 μg/plate, WP2uvrA 15 μg/plate). The study was considered acceptable when the number of revertants in the negative and positive control were within the historical control range. A test substance was considered negative when the total number of revertants is not greater than two times (TA100) or three times (TA1535, TA1537, TA98 or WP2uvrA) the concurrent vehicle control and is reproduced in a second independent experiment. No formal hypothesis testing was done.

2.4.2. In vitro mammalian cell gene mutation test

3-NOP was tested for forward mutation at the thymidine-kinase locus in L5178Y mouse lymphoma cells in compliance with OECD 476 (1997) in two independent experiments. The first experiment was performed in the absence and presence of S9 with a 3 h treatment period. The second mutation experiment was performed in the absence of S9 with a 24 h treatment period. The concentration range tested was 0.55, 1.7, 5.4, 17, 52, 164, 512 and 1211 μg/mL exposure medium. The highest concentration 1211 μg/mL is equivalent to 0.01 M. For expression of the mutant phenotype, the remaining cells were cultured for 2 days and plated for determination of the cloning efficiency and the mutation frequency. The plates for the cloning efficiency and mutation frequency were scored with the naked eye or with the microscope. The colonies were divided into small and large colonies. Methylmethane sulfonate and cyclophosphamide were used as positive controls. The test substance was considered negative (not mutagenic) in the test when none of the tested concentrations reached a mutation frequency (MF) of 126 above the control and such a response was repeated in a second independent experiment. The negative and the positive controls should produce the expected responses.

2.4.3. In vitro micronucleus test

In the screening *in vitro* MNT, 3-NOP was tested using V79 cells in the absence of S9-mix (24 h exposure) at concentrations of 310.8, 621.6, and 1243.2 μg/mL or presence (4 h exposure) of S9-mix at concentrations of 77.7, 155.4, 310.8 μg/mL. Two duplicate cultures per concentration were set up. At least 1000 cells per culture were scored. In the absence of S9 no clear cytotoxicity was observed. Clear cytotoxicity would be a reduced proliferation index to 50% of the control cells. In the presence of S9, higher concentrations were not evaluable because there were only few or no cells left.

The regulatory *in vitro* MNT was performed in compliance with OECD 487 (2010). Briefly, cultured peripheral human lymphocytes were collected from healthy adult, non-smoking, male volunteers (aged 18–35 years). The lymphocytes in the presence of phytohaemagglutinin were cultured for 46 ± 2 h and thereafter exposed to 3-NOP at 0, 164, 512, 1211 μg 3-NOP/mL. 3-NOP was tested based on solubility, pH and osmolarity as well as a dose-range-finding test. In the 1st cytogenetic experiment, cells were exposed for 3 h in the absence and presence of S9 in duplicate. 3-NOP was removed by centrifugation and cells were exposed to cytochalasin B. In the 2nd cytogenetic experiments, cells were exposed in duplicate for 24 h in the absence of S9 followed by cytochalasin B treatment. Afterwards cells were harvested, fixed, and mounted on slides. After Giemsa staining, slides were coded and examined for micronuclei. At least 1000 binucleated cells per culture were examined by light microscopy for micronuclei. In addition, at least 1000 mononucleated cells per culture were scored for micronuclei separately. For cytotoxicity assessment, a minimum of 500 cells per culture was counted, scoring cells with one, two or more nuclei (multinucleated cells). The cytostasis/cytotoxicity was determined by calculating the Cytokinesis-Block Proliferation Index. Mitomycin C and colchicine were used as positive controls in the absence of metabolic activation. Cyclophosphamide was used as positive control in the presence of metabolic activation. The incidence of micronucleated cells for each exposure group was compared to that of the solvent control using Chi-square test (one-sided).

2.5. In vivo micronucleus test

In a screening *in vivo* MNT, 6-week old male NMRI mice were treated intraperitoneally with 3-NOP at doses of 250, 500, or 1000 mg/kg. Dose levels were selected based on a range-finding study. Five animals were used per treatment group. The remaining procedures are the same as described for the regulatory test below except that 2000 Polychromatic erythrocytes (PCE) instead of 4000 PCE were examined for micronuclei.

The *in vivo* MNT was conducted in compliance with OECD 474 (1997) and draft OECD 474 (2013). Crl:WI(Han) rats (outbred, SPF-Quality) being 6–7 weeks old at the start of treatment. Based on the results of a dose-range-finding experiment, where sex differences were observed, at least 5 overnight fasted animals/sex/group/sampling time point received a single gavage dose of 0, 375, 750, or 1500 mg 3-NOP/kg bw. The dose levels were based on a dose-range-finding experiment. The positive control animals were treated with 20 mg cyclophosphamide/kg bw. Bone marrow of the groups treated with 3-NOP was sampled 24 h (all treatment groups) and 48 (highest dose only) hours after dosing. Bone marrow of the negative control group was isolated 24 h after dosing and bone marrow of the positive control group was isolated 48 h after dosing. The bone marrow cells were mounted on microscopic slides and stained with Giemsa. For each animal 4 slides were prepared. To prevent bias, all slides were randomly coded before

examination. The number of micronucleated PCE was counted in at least 4000 PCE. The ratio of polychromatic to NCE was determined by counting and differentiating at least the first 1000 erythrocytes at the same time. Micronuclei were only counted in PCE. The frequency of micronucleated PCE (at any dose, at any sampling time or any sex) was compared to the respective controls using the Wilcoxon Rank Sum Test, one sided).

2.6. Toxicokinetic studies for confirmation of systemic exposure upon oral dosing

Single and a repeat dose oral toxicokinetic experiments were performed. In the single dose test, 3-NOP was dosed by gavage to 1 male Crl: WI(Han) rat at 1000 mg/kg. Blood was drawn, 5, 15, and 30 min, as well as after 1, 2, and 3 h. In the repeat dose test, 3-NOP was dosed via gavage to 5 male Crl: WI(Han) rats initially at 1000 mg/kg/d once daily. From day 2 onwards, the dose was reduced to 800 mg/kg/day due to severe clinical signs. Animals had an age of at least 12–13 weeks. Animals were observed for clinical signs daily. Body weights and food consumption were measured at 5-day intervals. On day 10, blood was drawn from the jugular vein in K_3-EDTA tubes at 5 min, 15 min, 30 min, and 1, 2, 3, 6, and 24 h after dosing. In the obtained plasma samples, the concentrations of 3-NOP and its metabolites 3-nitroxypropionic acid (NOPA) and 3-hydroxy-propionic acid (HPA) were determined by chemical analysis.

2.7. Analytical methods

2.7.1. Dose formulation verification by analytical means

Dose formulations in the oral *in vivo* MNT study were verified by analytical means. Duplicate samples of all dose groups and the negative control were analyzed for 3-NOP concentration thereby determining accuracy and homogeneity using an HPLC-UV method. Samples were analyzed on a Symmetry Shield RP-18 column (100 mm × 4.6 mm, 3.5 µm) from Waters as stationary phase and 0.1% methanesulfonic acid in 15/85 (v/v) acetonitrile/water as mobile phase. UV-detection was at 210 nm.

2.7.2. Determination of 3-NOP, NOPA, and HPA concentration in rat plasma

3-NOP was extracted from plasma samples by addition of a zinc sulfate solution ($ZnSO_4$ 0.5 M in H_2O, pH 2.0), to remove proteins. After centrifugation, the supernatant was injected into an HPLC-UV system. Separation was achieved on a Hypersil Gold aQ column (150 × 2.1 mm, 1.9 µm) from ThermoFischer Scientific as stationary phase and a gradient with water 0.1% methanesulfonic acid and methanol + 0.1% methanesulfonic acid as mobile phase. The detection was done with a UV detector at 210 nm. The analytical range of the method (1.00 (= LLQ) to 250 µg/mL) was sufficient to detect 3-NOP in all samples obtained from animals exposed to 3-NOP.

For the analysis of NOPA or HPA, internal standard (^{13}C-NOPA or d2-HPA) was added to a sample aliquot of plasma and proteins were removed by addition of an organic solvent. The supernatant was analyzed by liquid chromatography with triple-quadrupole mass spectrometry detection (LC-MS/MS). Separation was achieved on a Zic®-Hilic column (100 × 2.1 mm, 3.5 µm) from Merck as stationary phase and a gradient with water and acetonitrile, both containing 50 mM of ammonium formate and 0.1% of formic acid, as mobile phase. The detection of specific fragment ions was performed by using multiple reaction monitoring (MRM) in negative mode. The analytical range of the method for NOPA (5.00 ng/mL (=LLQ) to 5000 ng/mL) or for HPA (100 ng/mL (=LLQ) to 20 000 ng/mL), was sufficient to detect NOPA or HPA, respectively, in all samples obtained from exposed animals.

2.8. In silico assessment

Two complementary *in silico* tools were used to predict the outcome of NOPA and 3-NOP regarding mutagenicity in bacteria.

Derek Nexus v5.0.1 (Lhasa Limited, www.lhasalimited.org, Marchant et al., 2008) with Derek Knowledge Base 2015 1.0 was used with the following settings: selected species: bacterium, mammal; endpoint: Mutagenicity *in vitro*; perceive tautomers: yes; perceive mixtures: yes, Match alerts without rules: no; show open likelihood: no; show rapid prototypes: yes. Alerts with reasoning level equivocal or above were considered positive and negative predictions were accepted.

Leadscope Model Applier Version 2.1.2 (Leadscope Inc. www.leadscope.com), was used as follows: Models E Coli - Sal 102 A-T Mut v1 and Salmonella Mut v3 from the Genotox Statistical suite were run with domain analysis and equivocal threshold of 0.4–0.6. Thus, predictions with probability < 0.4 were assigned negative, and with probability > 0.6 positive.

3. Results

3.1. In vitro studies

3-NOP was tested negative in the screening and the regulatory Ames Tests. In both regulatory Ames experiments, 3-NOP did not increase the number of revertants at all concentrations tested. In the 1st experiment, cytotoxicity was observed in TA1537 in the presence of S9 as indicated by a reduction in the number of revertant colonies at 5000 µg/plate. 3-NOP did not precipitate on the plates. The negative controls revertant rates were within the laboratory historical control ranges, except the response for TA98 in the presence of S9 in the 1st experiment only. Due to the low magnitude, this was not considered to influence the validity of the study. The positive controls produced the expected increases in revertant colonies and were within the laboratories historical control data ranges. This indicates that the test conditions were adequate and that the metabolic activation system functioned properly. To conclude, 3-NOP was not mutagenic in the Ames test.

Mouse Lymphoma Assay: 3-NOP did not precipitate in the exposure medium up to and including the concentration of 1211 µg/mL (= 0.01 M). Since testing up to 0.01 M is recommended in the guidelines, this concentration was used as the highest test substance concentration in the mutation experiments. The pH and osmolarity at a concentration of 1211 µg/mL were 7.25 and 0.300 Osm/kg respectively (compared to 7.24 and 0.291 Osm/kg in the solvent control). No toxicity was observed and all dose levels were evaluated in the absence and presence of S9-mix in both experiments. No significant increase in the mutation frequency at the TK locus was observed after treatment with 3-NOP either in the absence or in the presence of S9-mix. The numbers of small and large colonies in the 3-NOP treated cultures were comparable to the numbers of small and large colonies of the solvent controls. The spontaneous mutation frequencies in the solvent-treated control cultures were between the minimum and maximum value of the historical control data range. Mutation frequencies in cultures treated with positive control chemicals were increased by 11- and 14-fold for methylmethane sulfonate in the absence of S9-mix, and by 32-fold for cyclophosphamide in the presence of S9. In the absence of S9-mix, 3-NOP did not induce a significant increase in the mutation frequency in both experiments. In the presence of S9-mix, 3-NOP did not induce a significant increase in the mutation frequency. Thus, 3-NOP is not mutagenic in the TK mutation test system.

In the screening *in vitro* MNT, no increase in micronuclei frequency was observed in the absence of S9-mix. In the presence of metabolic activation, statistically significant increases in the percentage of micronucleated cells were noted (see Table 2).

Regulatory *in vitro* MNT: At a concentration of 1211 µg/mL (equivalent to 10 mM), 3-NOP showed no precipitation in the culture

Table 2
Results screening *in vitro* MNT using V79 cells in the presence of S9-mix.

Treatment Group	Concentration (μg/mL)	Exposure period (h)	Micronuclei per 2000 examined cells	
			Number of cells	% of examined cells
DMSO	0	4	19	0.95
3-NOP	77.7	4	19	0.95
	155.4	4	37	1.85*
	310.8	4	53	2.65*
Cyclophosphamide	15	4	329	16.45*

Historical negative control range 0.05–1.6%, mean 0.81%.
* statistically significantly different from solvent control using Chi-square test, $p < 0.05$.

medium. The pH and the osmolarity at this concentration were 7.65 and 0.306 Osm/kg, respectively (compared to 7.57 and 0.276 Osm/kg in the solvent control). Cytotoxicity was not noted up to and including 1211 μg/mL. Both in the absence and presence of S9-mix, 3-NOP did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei in both experiments. The number of mono- and binucleated cells with micronuclei found in the solvent control was within the historical control data range. The positive control chemicals, mitomycin C and cyclophosphamide both produced a statistically significant increase in the number of binucleated cells with micronuclei. The positive control chemical colchicine produced a statistically significant increase in the number of mononucleated cells with micronuclei. In addition, colchicine also showed a statistically significant increase in the number of binucleated cells with micronuclei in the first cytogenetic assay. It was therefore concluded that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly. Thus, 3-NOP was not clastogenic or aneugenic in human lymphocytes.

3.2. In vivo MNT

Due to the positive response obtained in the screening *in vitro* MNT, a screening *in vivo* MNT was performed using intraperitoneal route. Clinical signs were noted in all 3-NOP treatment groups (250, 500 or 1000 mg/kg): all animals were lethargic and had rough coat, two low dosed males and all males of the mid and high dose showed hunched posture, and all animals of the high dose showed ataxia. Two animals had their eyes closed. Group mean and individual micronucleated PCE of the 3-NOP treated animals were comparable with the concurrent vehicle control and the historical negative control data. The negative and the positive control showed the expected responses and confirmed the sensitivity of the test system. The PCE to NCE ratio in the 3-NOP treated animals remained unchanged.

Regulatory *in vivo* MNT: No test substance was detected in the negative control formulation. The concentrations analyzed in the low, mid, and high dose group formulations were in agreement with target concentration and were homogenous (i.e. mean accuracies were between 90 and 110% and the coefficient of variation was equal or below 10%).

In the dose-range-finder, 3 males and 3 females were treated with 2000 mg/kg. Within 1–3 h after dosing clinical signs consisted of lethargy, hunched posture, ventral recumbency, ataxia, and rough coat. One female died after showing slow breathing and rales. The survivors had recovered from treatment the next morning. Since severe symptoms were observed and one female animal died the dose level of 2000 mg/kg was considered to be above the maximum tolerated dose. Therefore, 1500 mg/kg was tested and showed comparable clinical signs but produced no deaths. Thus, 1500 mg/kg was selected as the highest dose. Males and females were tested due to sex differences observed.

The animals of the groups treated with 375 mg/kg body weight and the animals of the negative and positive control groups showed no treatment related clinical signs of toxicity or mortality. The following clinical observations were made in the groups treated with 1500 mg 3-NOP/kg body weight within 1.5 h after dosing: lethargy, ataxia (10

Table 3
Mean number of micronucleated PCE and PCE/NCE ratio.

Treatment	Dose	Sampling time	Number of micronucleated PCEs (1,2)	% micronucleated PCE (1,2)	Ratio PCE/NCE (1,3)
	(mg/kg)	(h)		(mean ± SD)	
MALES					
Vehicle control	0	24	5.2 ± 3.1	0.13 ± 0.08	0.88 ± 0.39
3-NOP	1500	24	9.0 ± 1.4(5)	0.23 ± 0.04	0.71 ± 0.22
3-NOP	1500	48	4.4 ± 5.6	0.11 ± 0.14	1.38 ± 0.27
3-NOP	750	24	2.2 ± 2.8	0.06 ± 0.07	0.90 ± 0.53
3-NOP	375	24	2.4 ± 1.7	0.06 ± 0.04	1.25 ± 0.49
Cyclosphosphamide	20	48	55.0 ± 17.1(4)	1.38 ± 0.43	0.49 ± 0.12
FEMALES					
Vehicle control	0	24	3.6 ± 2.1	0.09 ± 0.05	0.66 ± 0.22
3-NOP	1500	24	7.2 ± 5.0	0.18 ± 0.13	0.69 ± 0.30
3-NOP	1500	48	1.1 ± 0.5	0.03 ± 0.01	1.12 ± 0.46
3-NOP	750	24	5.4 ± 4.6	0.14 ± 0.12	0.55 ± 0.24
3-NOP	375	24	2.0 ± 2.0	0.05 ± 0.05	0.86 ± 0.29
Cyclosphosphamide	20	48	85.8 ± 17.8(4)	2.15 ± 0.45	0.15 ± 0.06

Vehicle control = Milli-Q.
(1) Five animals per treatment group.
(2) At least 4000 PCE were evaluated with a maximum deviation of 5%.
(3) The ratio was determined from at least the first 1000 erythrocytes counted.
(4) Significantly different from corresponding control group (Wilcoxon Rank Sum Test, P = 0.01).
(5) Significantly different from corresponding control group (Wilcoxon Rank Sum Test, P = 0.05).

Historical Controls: % micronucleated PCE.

	Males	Females
Range	0–0.25	0–0.20
Mean	0.10	0.09
SD	0.06	0.06
Number	80	45

males, 2 females), rough coat (2 males, 2 females), ventral recumbency (3 males, 11 females). Animals dosed with 750 mg/kg body weight showed the following toxic signs within 1.5 h after dosing: lethargy (2 males, 5 females), ataxia (3 females) and a rough coat (2 females). Three male animals dosed with 750 mg/kg body weight showed no treatment related clinical signs of toxicity or mortality. Within 19 h after dosing all animals had recovered from the treatment.

No biologically relevant increase in the mean frequency of micronucleated PCE was observed in the bone marrow of 3-NOP treated animals compared to the vehicle treated animals. Although a statistically significant increase in the mean number of micronucleated PCE was observed at a dose of 1500 mg/kg body weight in male animals (24 h sampling time), the mean number of micronucleated PCE was within the historical control data range (Table 3) and the increase was considered not biologically relevant.

The incidence of micronucleated PCE in the bone marrow of all negative control animals was within the historical solvent control data range.

Cyclophosphamide, the positive control substance, induced a statistically significant increase in the number of micronucleated PCE in both sexes.

The animals of the groups, which were treated with 3-NOP showed no decrease in the ratio of PCE/NCE. The animals of the groups treated with cyclophosphamide showed an expected decrease in the ratio of PCE/NCE, demonstrating toxic effects on erythropoiesis. It is concluded that 3-NOP is not clastogenic or aneugenic in the bone marrow micronucleus test when sampled at 24 and 48 h post dosing of male and female rats up to a dose of 1500 mg/kg (the maximum tolerated dose).

3.3. Systemic exposure

After a single dose of 1000 mg/kg, clinical signs consisted of lethargy, piloerection, and uncoordinated movements up to a few hours after dosing. After repeated dose administration, similar signs were present, necessitating to reduce the dose from day 2 onwards to 800 mg/kg to allow completion of the 10-day dosing period. A slight decrease in mean body weight was seen on day 5, followed by slight weight gain. Food intake was slightly decreased during the 10-day study period.

The kinetics after single and multiple oral doses were comparable. The systemic exposure analysis showed that 3-NOP plasma concentration increased rapidly with the peak concentration being observed already after 5–15 min (see Table 4, Fig. 2). Thereafter 3-NOP declined and an increase in its metabolite NOPA was observed with a peak concentration after 1 h. Beyond 1 h after dosing, 3-NOP was no longer detectable in plasma. After 24 h, NOPA concentrations had also declined considerably indicating quick elimination from plasma. NOPA is metabolized into HPA which had a peak after 2 h and a rapid decline afterwards.

3.4. In silico predictions

Both 3-NOP and its metabolite NOPA were predicted negative in the Ames test by two complementary models. In one of the models, though, 3-NOP was out of domain and no prediction was made. Overall, the predictions for 3-NOP are in line with the experimental results.

The results of the predictions for Ames test results from Derek Nexus and Leadscope are tabulated in Table 5.

There is only a small structural difference between 3-NOP and NOPA i.e. primary alcohol-function versus its oxidation product a carboxylic acid, respectively (See Fig. 1 for structures). Based on the negative *in silico* predictions for both compounds, and the negative *in vitro* testing of 3-NOP which is structurally comparable to NOPA, further *in vitro* testing of NOPA regarding mutagenicity was not considered necessary.

Table 4
Individual and Mean plasma concentrations of 3-NOP, NOPA and HPA after repeated dose, Tmax and Cmax values are highlighted.

Analyte	Route	Animal number	Day 10 - Time (h)							
			0.083	0.25	0.5	1	2	3	6	24
			Concentration (µg/mL)							
3-NOP	Oral	6	131	**348**	57.4	23.5	< LLQ	< LLQ	< LLQ	< LLQ
		7	**137**	**137**	85.7	20.2	< LLQ	< LLQ	< LLQ	< LLQ
		8	**161**	159	126	45.6	< LLQ	< LLQ	< LLQ	< LLQ
		9	**178**	140	79.9	27.2	< LLQ	< LLQ	< LLQ	< LLQ
		10	69.5	**283**	196	132	< LLQ	< LLQ	< LLQ	< LLQ
		Mean	135	213	109	49.7	n/a	n/a	n/a	n/a
		SD	41.5	96.3	54.4	47.1	n/a	n/a	n/a	n/a
		CV%	31	45	50	95	n/a	n/a	n/a	n/a
NOPA	n/a	6	130	223	266	**273**	17.0	3.12	2.65	0.158
		7	89.6	237	322	**366**	152	13.4	4.22	0.049
		8	78.6	197	248	**332**	85.3	0.86	0.283	0.178
		9	71.3	173	251	**290**	57.0	0.33	0.328	0.245
		10	90.2	193	279	**387**	212	2.83	0.641	0.059
		Mean	91.9	205	273	330	105	4.11	1.62	0.138
		SD	22.7	25.4	30.0	48.5	77.6	5.33	1.75	0.083
		CV%	25	12	11	15	74	130	108	60
HPA	n/a	6	30.1	59.8	110	180	**225**	95.0	24.3	1.60
		7	16.1	34.3	61.4	111	**147**	79.2	22.5	0.45
		8	27.8	65.4	108	211	**314**	187	18.1	2.59
		9	29.6	62.1	110	224	**294**	97.7	14.1	2.17
		10	22.8	49.0	90.6	163	**262**	160	15.2	0.51
		Mean	25.3	54.1	96.0	178	248	124	18.8	1.46
		SD	5.89	12.7	21.0	44.5	65.9	46.9	4.46	0.97
		CV%	23	23	22	25	27	38	24	66

n/a: not applicable.
< LLQ: below lower limit of quantification, 1 µg 3-NOP/mL.

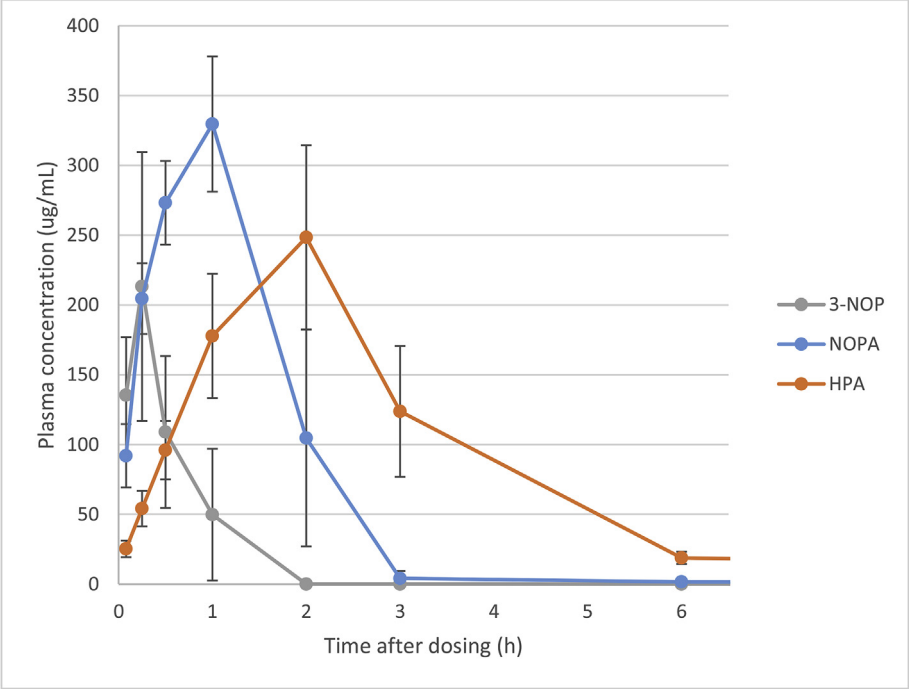

Fig. 2. Plasma kinetics of 3-NOP and its metabolite NOPA and HPA up to 6 h after dosing after 10 daily oral doses of 800 mg 3-NOP/kg bw/d. Data are expressed as group means ± standard deviation.

Table 5
Summary of *in silico* predictions of 3-NOP and its metabolite NOPA.

	Leadscope Model Applier Version 2.1.2		Derek Nexus v5.0.1
	Salmonella Mut v3	E coli-Sal 102 A-T Mut v1	Mutagenicity *in vitro*
3-NOP	No prediction (Out of domain)	Negative	Inactive
NOPA	Negative	Negative	Inactive

Inactive: Negative prediction based on no matching alerts or example compounds. Both compounds had no misclassified or unclassified features.

4. Discussion and conclusion

For candidate selection, screening assays for mutagenicity and genotoxicity were used as they require only limited amount of test item. While the screening Ames test showed negative responses, the screening *in vitro* MNT was positive in the presence of S9-mix at 3-NOP concentrations of 155.4 and 310.8 µg/mL. To investigate this further at a time where no information on metabolism and bioavailability of 3-NOP via the oral route was available, an *in vivo* MNT study was conducted using intraperitoneal dosing. In this study, 3-NOP was tested negative up to 1000 mg/kg and was therefore developed further. In the later, regulatory compliant *in vitro* MNT, 3-NOP was confirmed not inducing micronuclei *in vitro* even up to much higher concentrations (1211 µg/mL). Likewise, the regulatory *in vivo* MNT via the oral route was negative. At the dose levels employed (up to 1500 mg/kg) in this study, high 3-NOP plasma levels were achieved (see Table 4, Fig. 2) being in the area of the concentration tested positive in the screening *in vitro* MNT. Thus, the overall WoE suggests that 3-NOP is not clastogenic. The positive response in the screening *in vitro* MNT may be due to the characteristics of the cell line V79 used which renders the cells oversensitive. These characteristics were extensively discussed elsewhere and include impaired p53 status, karyotype instability, and DNA repair deficiencies which contributes to the known high false positive rate (Kirkland et al., 2007; Fowler et al., 2012).

The selection of the regulatory *in vitro* and *in vivo* genotoxicity studies was designed to fulfill data requirements for various regions with emphasis on the EU and the US. The standard OECD *in vitro* test utilizes S9-mix and addition of NADP as cofactor to account for phase I metabolism only with special emphasis on CYP-mediated metabolism. 3-NOP is special in terms of metabolism aspects for such *in vitro* approaches in genotoxicity testing because its metabolism is not necessarily CYP-mediated. The dominant metabolism pathway is the oxidation of primary short-chain alcohols yielding carboxylic acids. This step is mediated preferentially via the ADH and ALDH systems requiring NAD as cofactor in combination with specific CYPs being able to catalyze the oxidation of short-chain primary alcohols (Parkinson et al., 2013). The latter is active in the standard setting of S9-mix with NADP as cofactor and the formation of NOPA and thus testing of NOPA in the *in vitro* tests can be assumed. The second functional group - the organic nitrate ester – is cleaved yielding HPA from NOPA. This metabolism step was reported to be mediated via glutathione transferase enzymes (Govoni et al., 2006) for which the cofactor GSH is required but not added to standard S9-mix used for *in vitro* genotoxicity/mutagenicity testing.

For the sake of completeness, additional/alternative approaches were used to cover both mutagenicity and genotoxicity endpoints instead of testing NOPA per se. HPA is a natural occurring metabolite in mammalian cells being a degradation product of e.g. amino acids and DNA-bases and being further transformed into acetyl-CoA and/or propanoyl-CoA (KEGG pathway map00640 and map 00410). This made additional investigations unnecessary for HPA.

In silico predictions for mutagenicity using an expert alert based system (Derek Nexus) and statistical models (Leadscope) were employed for both NOPA and 3-NOP. The application of two systems is recommended by ICH M7 (2014). Derek Nexus made a negative prediction for mutagenicity in bacteria without any constraints. Likewise, Leadscope predictions did not raise a concern of bacterial mutagenicity: NOPA was predicted negative by both Leadscope models, and the negative experimental Ames result of 3-NOP confirms the negative prediction and fills in the gap for the “out of domain” result for this molecule.

The genotoxicity endpoint with regard to metabolites was addressed by an *in vivo* MNT because an *in vivo* study was an explicit data requirement in certain regions/certain legislations (see Table 1). The *in vivo* MNT in the bone marrow via oral dosing showed no relevant increase in the frequencies of micronuclei in the PCE. The frequencies were within the historical control background range.

To conclude firmly on a negative outcome of the *in vivo* MNT bone marrow study, evidence was required that the test item and/or its metabolites reached the bone marrow. The proof of bone marrow exposure is possible by different lines of evidence: toxicity to the bone marrow, presence of the test substance/metabolite in the bone marrow, systemic toxicity, or systemic detection of the test substance/its metabolites (EFSA, 2017b). In the data set presented here, data showing direct exposure of the bone marrow or toxicity to the bone marrow were not available. Systemic toxicity was noted in the *in vivo* MNT study being related to the CNS giving one line of evidence of systemic bioavailability and indication for bone marrow exposure. A second line of evidence is available from the plasma concentration measurements of 3-NOP as well as its metabolites NOPA and HPA from a set of separate dedicated toxicokinetic studies. The 3-NOP dose levels in the toxicokinetic studies were 800–1000 mg/kg and in the range of the dose levels used in the *in vivo* MNT with single exposure (375–1500 mg/kg).

The kinetics nicely show that 3-NOP oxidation to NOPA is rapid as evident by the difference in t_{max} being 5–15 min for 3-NOP and 1 h after dosing for NOPA. HPA is then formed by cleavage of the nitrate ester group as indicated by a later t_{max} of 2 h post-dose. The analytical method uses organic solvent or zinc sulfate solution to remove the protein by precipitation and thus measures both free and potentially non-covalently plasma protein bound 3-NOP and metabolites. However, the small size of the molecules and high-water solubility (e.g. 367 g 3-NOP/L) make protein binding unlikely. Thus, it can be concluded that the bone marrow was exposed to 3-NOP and its main metabolites NOPA and HPA and that both the 24 h and 48 h sampling time points were adequate. As the oxidation of 3-NOP to NOPA and further degradation to HPA is extensive *in vivo*, NOPA and HPA can be considered to be tested in the study with 3-NOP and a separate *in vitro* MNT study with NOPA or HPA was considered inappropriate.

In the present paper, we described our approach on mutagenicity and genotoxicity testing of 3-NOP from an early screening phase up to regulatory development phase. *In silico* predictions for mutagenicity, the Ames test and the mouse lymphoma test were all negative indicating that 3-NOP and its metabolites have no mutagenic potential. The overall evidence from *in vitro* and the *in vivo* MNT studies suggest that 3-NOP is neither clastogenic nor aneugenic. Thus, 3-NOP and its metabolites can be considered non-mutagenic and non-genotoxic.

Conflicts of interest

AT, EC, SE, and PB declare no conflict of interest other than employment. AS and IV declare that the studies were sponsored by DSM Nutritional Products AG.

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Transparency document

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