Outcome of assessment of 3-Nitrooxypropanol “3-NOP” - Appendix 1 List of toxicological studies

Tolerance and residue studies

Study	Year	OECD	Animals	Doses tested
Pilot tolerance study, 90 days	2018	N/A	16 (4 x4 groups) dairy cows	0, 1.6, 8, 16g 3-NOP/cow/day = 100, 500 and 1000mg/kg feed DM
Pivotal tolerance study, 56 days	109	N/A	80 (20 x 4 groups) dairy cows	0, 80, 100, 200 mg 3-NOP/kg DM
Milk analysis for NOPA from University of Reading efficacy study	2019	N/A	5 dairy cows	Milk samples from 5 cows receiving 3-NOP at approx 60 mg/kg, during 3 days in week 1, 6 and 15.

ADME

Study	Year	OECD	Animals	Doses tested
Stability of 3-NOP under Different Conditions	2015	N/A	N/A	202 µmol/L
Stability of 3-NOP under Different Conditions II – Plasma Protein Binding and Chemical Oxidation	2017	N/A	Wistar rat plasma	34 µmol/L
Plasma Protein Binding of 14C-NOPA	2019	N/A	Wistar rat plasma	31.3 µmol/L and 6.26 µmol/L at 37c for up to 24 hours
Stability of 3-NOP under Different Conditions III – In-vitro Incubations Leading to the Major Metabolite NOPA	2017	N/A	Rat (Wistar and Sprague Dawley), Dog (Beagle) and Human Liver Function	34 to 36 µmol/L
Metabolite Profiles and Kinetics of 3-NOP after In-vitro Incubation	2014	N/A	Cow rumen fluid	2.2 and 23 mg/L at 38 degrees for 24 hours
Metabolite Profiles of 3-NOP after In-vitro Incubation	2016	N/A	Sheep, Goat and Cow Rumen Fluid	1 mg/L at 39 degrees for 16 hours
ADME tissue distribution and plasma kinetics	2013	417	Wistar rats	505 mg/kg bw
ADME in the Rat Following Single and Multiple Oral Administration	2018	N/A	4M/4F Wistar rats	2 exps each with 50 and 500 mg/kg bw (exps in total). 50 given as a single dose and as a 50 x 5 daily doses. 500 just as single doses.
ADE with volatiles	2015	417	Wistar rats	506 mg/kg bw
Metabolites in plasma, liver and GIT	2014	417	Wistar rats	505 mg/kg bw
Nitrate/ nitrite in plasma	2014	417	Wistar rats	100 and 500 mg/kg bw

Study	Year	OECD	Animals	Doses tested
3-NOP in lactating goats	2015	503	2 goats	7 daily doses of 4.34 and 3.28 mg/kg bw being equiv to 112, 102 mg / kg DM (feed)
ADME in Dairy Cattle Following Multiple Oral Administration	2018	N/A	4 dairy cows	Every 12 hours for 7 days at dose level of 3.6 mg / kg bw / d (1.8 g / animal / d) being equiv to 150-160 mg / kg DM (feed)
ADME in Dairy Cattle Following Multiple Oral Administration (part 2)	2021	N/A	10 dairy cows	Every 12 hours for 5 days at dose level of 3.6 mg / kg bw / d (2.1 g/animal/day) being approximately equivalent to 150 mg/kg dry feed
NOPA and nitrate analysis of plasma	2016	N/A	4 Beef cattle and 4 controls	29 days of 3 mg/kg bw (2g / animal) being equiv to 284 mg/kg (feed)
NOPA and nitrate analysis of plasma	2016	N/A	28 beef cattle per dosing group	0,100,200 mg/kg feed for 238 days

Toxicity

Study	Year	OECD	Animals	Doses tested
In-vitro Ames Microsuspension Test	2010	471	N/A	0, 1.6, 5, 15.8, 50, 158, 500 ?g / plate, with and without S9 mix
In-vitro Salmonella typhimurium and Escherichia coli reverse mutation assay	2014	471	N/A	52, 164, 512, 1600 and 5000 ? g/plate, with and without S9 mix
In-vitro Salmonella typhimurium and Escherichia coli reverse mutation assay II	2015	471	N/A	52, 164, 512, 1600, 5000 µg/plate (experiment I), 492, 878, 1568, 2800, 5000 µg/plate (experiment II) with and without S9 mix
Screening in-vitro Micronucleus Test in Chinese Hamster V79 Cells	2010	487	N/A	0, 310.8, 621.6, 1243.2 ?g/mL (without S9-mix), 0, 77.7, 155.4, 310.8 ?g/mL (with S9-mix)
In-Vitro V79 Micronucleus Assay	2020	487	N/A	0, 300, 480, 540, 570, 600 ? g/mL (with S9-mix)
In-vitro Micronucleus assay in cultured peripheral human lymphocytes	2014	487	N/A	164, 512, 1211 ?g/mL, with and without S9 mix
In-vitro mammalian cell gene mutation test (Mouse lymphoma assay)	2015	476	N/A	0, 0.55, 1.7, 5.4, 17, 52, 164, 512 and 1211 ?g/mL, with and without S9 mix
Cell transformation (SHE) assay	2013	N/A (followed OECD draft proposal)	N/A	0, 500, 1000, 1500, 2000, 2250, 2500 ?g/mL
In-Vitro TK6 Micronucleus Assay	2021	487	N/A	0, 750, 1000, 1220 µg/ml with and without S9 mix
Salmonella typhimurium and Escherichia coli reverse mutation assay (NOPA)	2020	471	N/A	NOPA: 0, 3,10,33,100, 333, 1000, 2500 and 5000 ?g/plate with and without S9 mix
Micronucleus Test in Human Lymphocytes In vitro (NOPA)	2020	487	N/A	NOPA: 10.4,18.2,31.8,55.7,97.5,171,299,525,915,1372 µg/ml with and without S9 mix
Acute Oral Toxicity Test	2014	423	Wistar rats	300 - 2000 mg/kg bw
Assessment of acute inhalation toxicity	2017	436	Wistar rats	1 and 5 mg/L
Micronucleus test in bone marrow cells of the mouse (screening)	2011	474	NMRI Male mice (intraperitoneal)	0, 250, 500, 1000 mg/kg bw
Micronucleus test in bone marrow cells of the rat	2014	474	Wistar rats	0, 375, 750, 1500 mg/kg bw
10-day dose range finding study	2012	N/A	Wistar rats (n= 3 per group per sex)	0, 100, 300, 1000 mg/kg bw
Combined 28-day repeated dose toxicity study and reproduction / developmental toxicity screening test	2013	422, 407	Wistar rats	0, 10, 20, 100, 500 mg/kg bw

Study	Year	OECD	Animals	Doses tested
90-day oral gavage toxicity study	2015	408	Wistar rats	0, 50, 100, 300 mg/kg bw
Dose range finding study and the maximum Tolerated Dose (MTD study)	2014	N/A	Beagle dogs, n = 2 (1 xM, 1x F) DRF, n = 2 per sex per dose MTD	25, 125 and 500 mg/kg bw (DRF) 0,30,100,200 (MTD study) mg/kg bw
14-day oral gavage toxicity study	2016	N/A	Beagle dogs 2 x M and 2 x F per dose	0, 150, 300 mg/kg bw (300 given as a split dose of 150 x 2, each 6 hours apart)
3-months oral gavage toxicity study	2016	409	Beagle dogs	0, 10, 30, 100, 300 mg/kg bw
1 year oral gavage toxicity study	2016	452	Wistar rats	Males: 0, 25, 50, 100, 300 mg/kg bw Female: 0, 50, 100, 600 mg/kg bw
2-year carcinogenicity study	2019	451	Wistar rats	Males: 0, 25, 50, 100 mg/kg bw Female: 0, 50, 100, 300 mg/kg bw
6-day DRF in mice	2018	451 and 417	CbyB6F1 hybrid mouse	0, 124, 372, 742, 1224 mg/kg bw
28-day study in mice	2019	451	CbyB6F1 hybrid mouse	0, 100, 300, 700 mg/kg bw
NOPA In-Vivo 14-Day Dose Range Finder Assay in Rats	2021	N/A	Fischer rats	NOPA: 0, 112, 335, 558 and 892 mg/kg bw/d (n=6, male), 0, 335, 670 and 1000 mg/kg bw/day (n=6, female)
NOPA In-Vivo Mutation Assay at the cII Locus and In-Vivo Micronucleus Assay in Male and Female Big Blue® Transgenic F344 Rats	2021	488, 474	Fischer rats	NOPA: 0, 150, 300 and 600 mg/kg/day (n=6_ male), 0, 250, 500 and 1000 mg/kg/day (n=6_female)

Reprotoxicity

Study	Year	OECD	Animals	Doses tested
28-day oral gavage mechanistic study	2014	Based on 407	Wistar rats	0, 100, 300, 500 mg / kg bw
Prenatal developmental toxicity study	2015	414	Wistar rats	0, 100, 300, 1000 mg/kg bw
Prenatal developmental toxicity study	2016	414	NZW Rabbits	0, 50, 150, 450 mg/kg bw
Two-generation reproduction study	2016	416	Wistar rats	0, 25, 50, 100 (Male and Female), extra satellite group of females dosed at 600 mg/kg bw
6-10-day preliminary mechanistic study	2017	N/A	Wistar rats (n=9 across the two dosing levels)	800 and 1000 mg/kg bw
Dose range finding (mechanistic)	2018	N/A	Wistar rats (n=1 per dosing group)	3-NOP: 1000 mg/kg bw (Oral) NOPA (metabolite): 75,250,600 mg/kg bw (IV) HPA (metabolite): 75, 250, 400 mg/kg bw (IV) HPA: 75,250,350 mg/kg bw (SC)
Influence of metabolites on testicular toxicity in male rats, 10-day study	2018	N/A	Wistar rats (n=5 per dosing group)	3-NOP: 800 mg/kg bw (Oral) NOPA: 425 mg/kg bw (IV) HPA: 350 then 250 (day 3 onwards) mg/kg bw (IV) HPA: 350 mg/kg bw (SC)
Single dose transcriptomics study	2017	N/A	Wistar rats (n=8 per dosing group)	0, 100, 1000 mg/kg bw
Benchmark-Dose-Modelling	2019	N/A	N/A	N/A
In-vitro Steroidogenesis	2015	N/A	Human adrenal cells	0, 0.00001, 0.001, 0.01, 0.1, 1, 10 mM (3-NOP, NOPA and HPA)
Ex-vivo model testicular toxicity evaluation (3-NOP, NOPA, HPA, inorganic nitrate)	2015	N/A	Sprague Dawley rat	0, 0.002, 0.02, 0.5, 2 mM (all compounds)
Ex-vivo model testicular toxicity evaluation of NOPA	2016	N/A	Sprague Dawley rat	0, 0.02, 0.5, 2 mM (NOPA)

Study	Year	OECD	Animals	Doses tested
In-vitro / ex-vivo species comparison study using NOPA	2019	N/A	Testicular tissue from Wistar rats, Beagle dog, and Cynomolgus monkey (n=34 tissue samples for each species)	0,1,20,500,1200,2500 µM (NOPA)