Mutation induction in Muta™Mouse following exposure to N-Nitrosodimethylamine (NDMA) with evidence for sub-linear mutation accumulation following repeat dosing

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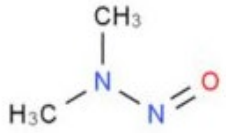
GSK

Introduction

N-Nitrosodimethylamine (NDMA)



- NDMA is the simplest dialkylnitrosamine belonging to the class of chemicals, N-nitrosamines.
- NDMA is an important natural & anthropomorphic environmental mutagen.
- NDMA genotoxicity has been demonstrated in multiple assays, both in vitro and in vivo (NTP).
- NDMA is a rodent carcinogen and induces tumours at multiple organ sites (Li et al, 2021).
  - In rats, the liver, lung, and nasal cavity are the most frequently occurring tumour sites.
  - In mice, the liver, lung and kidney are major tumour sites
- NDMA was recently identified as a contamination impurity in some commonly used marketed drugs; this has resulted in several product recalls.
- NDMA mutagenicity has been tested in 24+ TGR assays of varying quality
- In this study, NDMA mutagenicity was evaluated in an OECD compliant Muta™Mouse study (28-day oral dosing) across 7 doses (0.02-4 mg/kg/day) using an integrated design to better characterise the low doses that are more commensurate with impurity exposures.
- Acute treatments were included to investigate the accumulation and/or additivity of individual dose effects on mutation induction in liver (the most sensitive tissue for rodent mutagenicity and carcinogenicity).

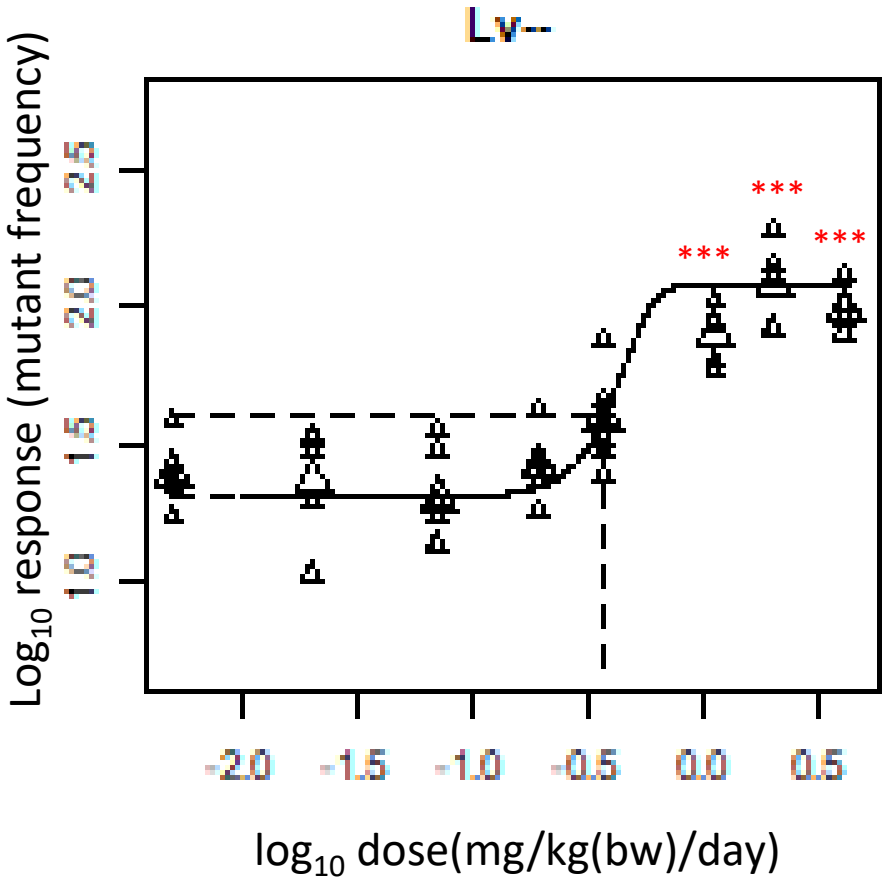
Results-1a

NDMA-induced mutant frequency @ the lacZ locus in male Muta™Mouse **liver**

Treatment Group	NDMA Dose (mg/kg(bw)/day)[1]	n	Group MF ($x10^{-6}$) Mean ± SD	Fold-change	Probability[2]
1	0 (Vehicle)	6	25.39 ± 6.97	-	-
2	0.02	5	25.08 ± 9.86	0.99	0.940
3	0.07	6	21.35 ± 8.88	0.84	0.994
4	0.19	6	27.01 ± 7.83	1.06	0.789
5	0.36	6	40.35 ± 17.78	1.59	0.092
6	1.1	5	80.52 ± 18.9	3.17	0.000 (***)
7	2.0	5	124.20 ± 43.7	4.89	0.000 (***)
8	4.0	5	98.94 ± 20.7	3.90	0.000 (***)

[1]Doses expressed in terms of the parent compound & dosed once daily for 28 days. Tissue sampled on day 31

[2]Pairwise comparison vs vehicle control (Dunnett's one-sided multiple comparison test) (* P < 0.05, ** P < 0.01, *** P < 0.001)



Results-1b

NDMA-induced mutant frequency @ the lacZ locus in male Muta™Mouse **liver**

Split dose v acute dose

Treatment Group (n = group size)	[1] NDMA daily dose (mg/kg((bw)/day)	[2] NDMA cumulative dose	Group MF (x10^{-6}) Mean ± SD	Fold-change	Probability[4]
1 (6)	0 (Vehicle)	0	25.39 ± 6.97	-	-
4 (6)	0.19 x 28	5.32	27.01 ± 7.83	1.06	0.824
9 (5)	5 x 1	5	31.15 ± 7.1	1.23	0.486
5 (6)	0.36 x 28	10.08	40.35 ± 17.78	1.59	0.086
10 (4)[3]	10 x 1	10	74.05 ± 17.0	2.92	0.000 (***)

Two repeat-dose groups (0.19 and 0.36 mg/kg(bw)day) were designed to cumulatively add up to the single-dose groups (5 and 10 mg/kg, respectively)

[1]Doses expressed in terms of the parent compound & dosed either on day 1 only or daily for 28days.

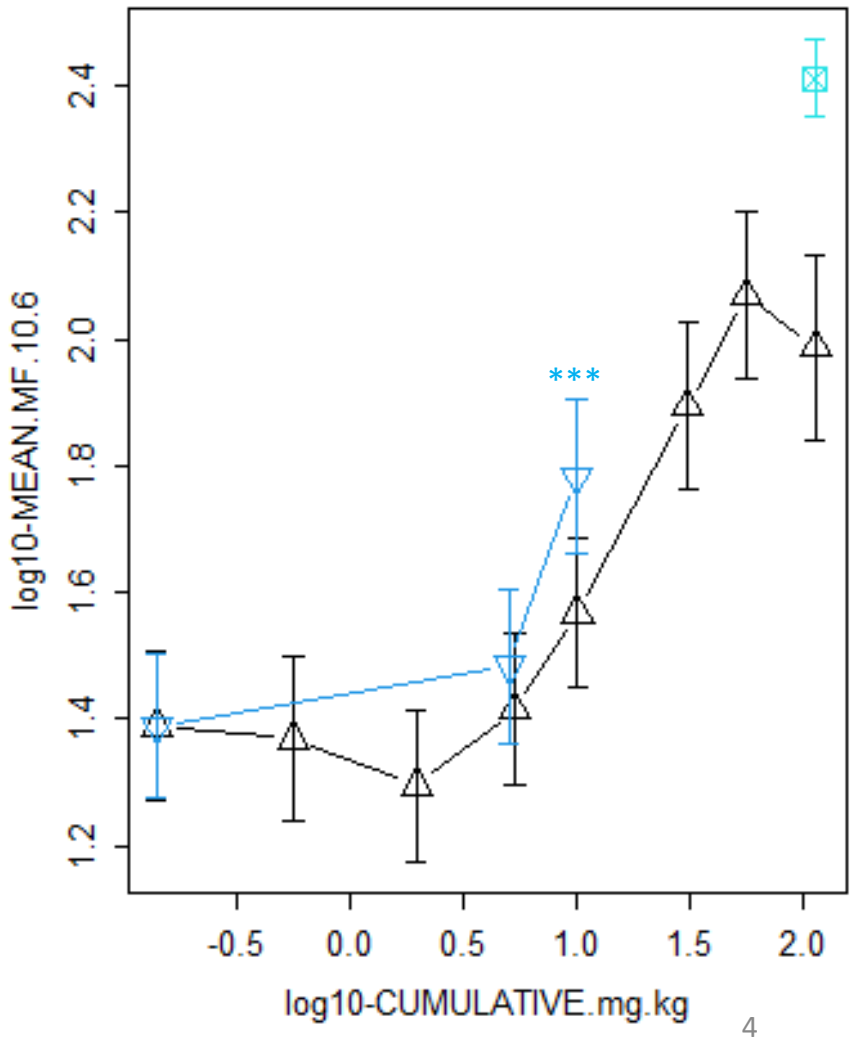
[2]Doses expressed as cumulative dose expressed in terms of the parent

[3]One animal died prior to scheduled termination; no sample taken

[4]Pairwise comparison vs vehicle control (Dunnett's one-sided multiple comparison test)
(* P < 0.05, ** P < 0.01, *** P < 0.001)

Single and **repeat** dosing-regimens

Positive control



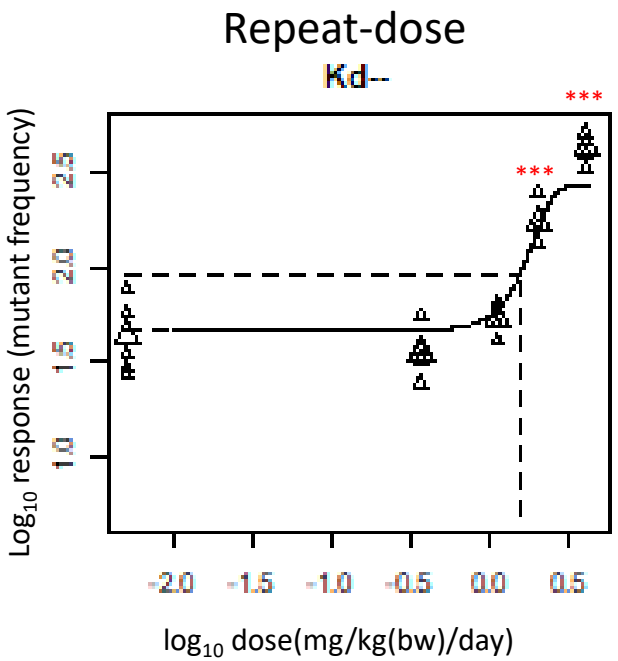
Results-1c

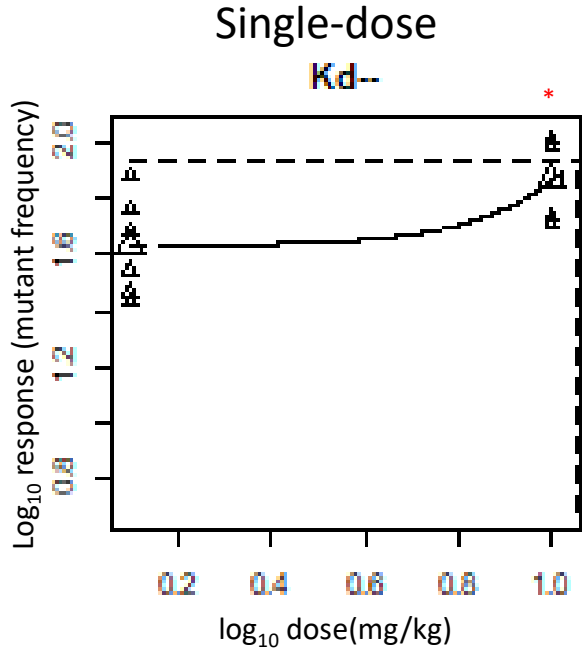
NDMA-induced mutant frequency @ the lacZ locus in male Muta™Mouse **Kidney**

Treatment Group	NDMA Dose (mg/kg(bw)/day)[1]	n	Group MF (x10^{-6}) Mean ± SD	Fold-change	Probability[2]
1	0 (Vehicle)	6	45.79 ± 19.3	-	-
5	0.36	4	37.05 ± 12.8	0.81	0.986
6	1.1	4	54.00 ± 10.8		0.438
7	2.0	3	181.32 ± 59.1	4.0	0.000 (***)
8	4.0	4	432.45 ± 82.0	9.44	0.000 (***)
10	10	4	76.44 ± 27.2	1.67	0.042 (*)

[1]Doses expressed in terms of the parent compound & dosed once daily for 28 days. Tissue sampled on day 31

[2]Pairwise comparison vs vehicle control (Dunnett's one-sided multiple comparison test) (* P < 0.05, ** P < 0.01, *** P < 0.001)

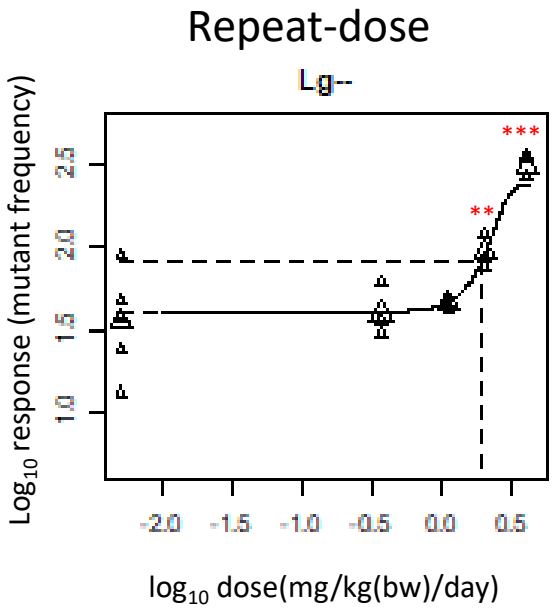


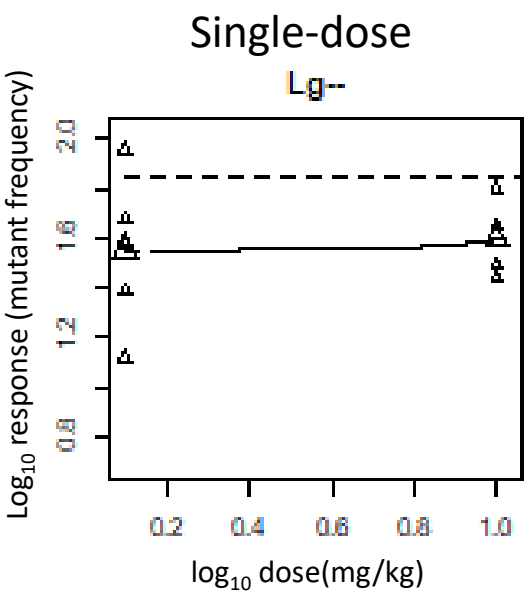


Results-1d

NDMA-induced mutant frequency @ the lacZ locus in male Muta™Mouse **Lung**

Treatment Group	NDMA Dose (mg/kg(bw)/day)[1]	n	Group MF (x10^{-6}) Mean ± SD	Fold-change	Probability[2]
1	0 (Vehicle)	6	42.25 ± 26.9	-	-
5	0.36	4	40.90 ± 14.4	0.97	0.842
6	1.1	4	45.46 ± 2.8	1.08	0.665
7	2.0	3	94.42 ± 21.4	2.2	0.005 (**)
8	4.0	4	310.15 ± 49.2	7.34	0.000 (***)
10	10	4	41.60 ± 16.3	0.98	0.831





[1]Doses expressed in terms of the parent compound & dosed once daily for 28 days.
Tissue sampled on day 31

[2]Pairwise comparison vs vehicle control (Dunnett's one-sided multiple comparison test)
(* P < 0.05, ** P < 0.01, *** P < 0.001)

Results-2

NDMA treatment was negative in bone marrow, spleen, bladder and stomach

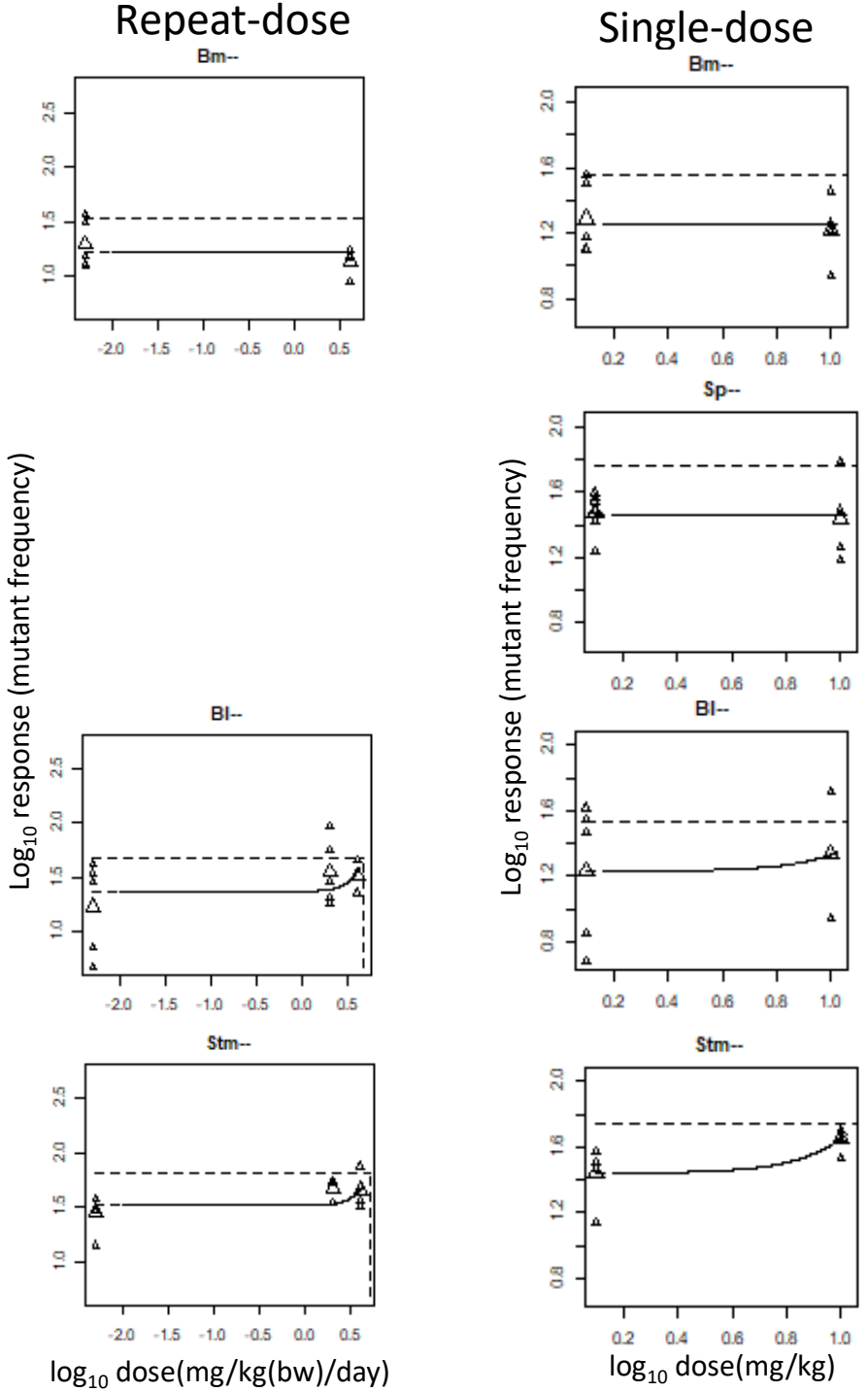
Mutant frequency @ the lacZ locus

Tissueddd	Treatment Group	NDMA Dose (mg/kg(bw)/day)[1]	n	Group MF ($x10^{-6}$) ± SD	Fold-change	Probability[2]
Bone marrow	1	0 (Vehicle)	5	21.63 ± 11.37	-	-
	8	4 x 28	4	14.07 ± 3.73	0.650	0.866
	10	10 x 1	4	18.01 ± 8.25	0.833	0.675
Spleen	1	0 (Vehicle)	6	30.99 ± 8.20	-	-
	-	-	-	-	-	-
	10	10 x 1	4	31.44 ± 20.9	1.015	0.634
Bladder	1	0 (Vehicle)	5	23.49 ± 16.60	-	-
	7	2 x 28	5	43.32 ± 31.50	1.844	0.248
	8	4 x 28	2	34.76 ± 16.80	1.480	0.413
	10	10 x 1	2	30.71 ± 30.90	1.307	0.665
Stomach	1	0 (Vehicle)	5	29.13 ± 9.00	-	-
	7	2 x 28	3	47.49 ± 11.5	1.630	0.069
	8	4 x 28	4	47.92 ± 19.6	1.645	0.055
	10	10 x 1	4	45.40 ± 8.0	1.559	0.071

(n) animal group size

[1]Doses expressed in terms of the parent compound & dosed either 1xdayx1days or 1xdayx28days.

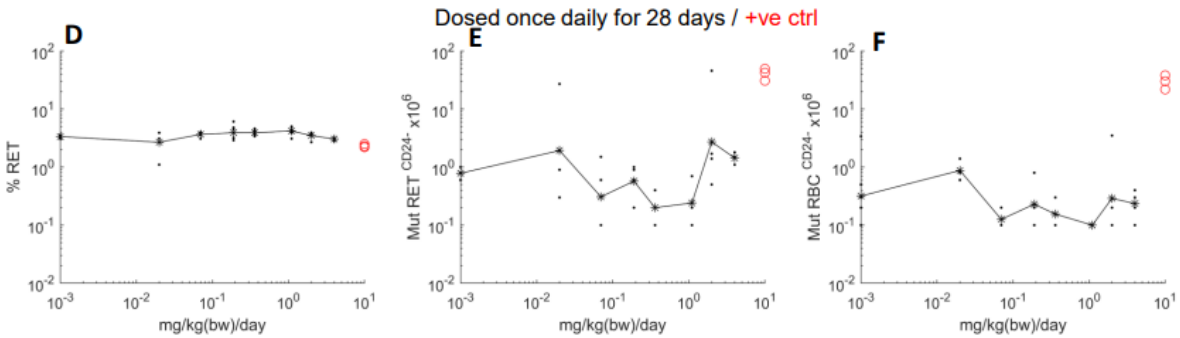
[2]Pairwise comparison vs vehicle control (Dunnett's one-sided multiple comparison test)
(* P < 0.05)



Results-3a

28-day MutaFlow assay results for NDMA (Pig-a mutation)

Group[1,2]	Dose (mg/kg)	n[4]	Group Mean %RET^{CD24+} (SD)	Group Mean mutant %RET^{CD24-} (SD)	Group Mean mutant %RBC^{CD24-} (SD)
1	0 (Vehicle)	6	3.37 (0.19)	0.77 (1.30)	0.40 (0.46)
2	0.02	5	3.00 (1.32)	0.70 (0.58)	7.55 (13.05)
3	0.07	6	3.68 (0.37	0.08 (0.08)	0.46 (0.63)
4	0.19	6	4.07 (1.29)	0.25 (0.28)	0.45 (0.45)
5	0.36	6	3.93 (0.45)	0.15 (0.12)	0.08 (0.16)
6	1.1	5	4.26 (0.78)	0.06 (0.05)	0.20 (0.29)
7	2.0	5	3.53 (0.56)	0.98 (1.68)	12.35 (22.31)
8	4.0	5	3.06 (0.19)	0.26 (0.11)	0.88 (0.84)
9	4.0	5	3.96 (0.93)	0.16 (0.13)	0.48 (0.97)
10	10.0	4[5,6]	3.40 (0.08)	0.43 (0.46)	0.43 (0.85)
ENU[3]	40.0	3	2.33 (0.15)	30.07 (8.20)[7]	40.80 (9.44)[7]



1. Groups 2-8 dosed once daily for 28 days and blood sampled on day 31
2. Groups 9 and 10 dosed on day 1 only and sampled on day 31
3. Positive control ENU (ethylnitrosourea) given once daily for 3 consecutive days (on Days 1, 2 and 3) and sampled on Day 31
4. Treatment group size (n) animals
5. One animal died prior to scheduled termination; no sample taken
6. Treatment group contained 1 animal with suspected jackpot mutation which was excluded from analysis
7. Statistically significant increases ($P < 0.05$)

RET = Reticulocyte; SD = standard deviation of mean; RBC = Red blood cell

Data analysed by Dunnett's t-test (one sided, upper), comparing treatment groups with the control.
A positive control standard was included in the study analysis as there was no concurrent MN positive control treatment group for the Muta™Mouse study.

There were no significant increases in % RET, mutant RET or mutant RBC at any NDMA dose relative to vehicle control

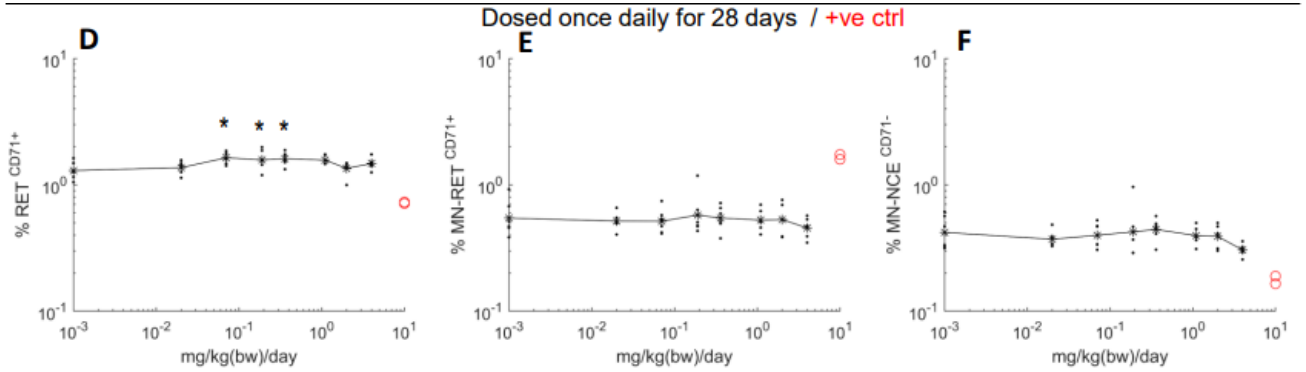
Results-3b

Micronucleus (MicroFlow) Assay for NDMA in Muta™mouse mice

Group	Dose[1] (mg/kg/day)	n[5]	Group Mean %RET^{CD71+} (SD)	Group Mean %MN-RET^{CD71+} (SD)	Group Mean %MN-RBC^{CD71-} (SD)
1[2]	0 (Vehicle)	6	1.31 (0.21)	0.57 (0.20)	0.44 (0.13)
2[2]	0.02	5	1.38 (0.18)	0.52 (0.09)	0.37 (0.07)
3[2]	0.07	6	1.65 (0.18)	0.53 (0.12)	0.41 (0.09)
4[2]	0.19	6	1.60 (0.29)	0.62 (0.29)	0.47 (0.25)
5[2]	0.36	6	1.62 (0.20)	0.56 (0.12)	0.45 (0.09)
6[2]	1.1	5	1.57 (0.14)	0.54 (0.12)	0.40 (0.07)
7[2]	2.0	5	1.36 (0.21)	0.55 (0.17)	0.40 (0.09)
8[2]	4.0	5	1.48 (0.18)	0.46 (0.09)	0.31 (0/04)
9[3]	5.0	5	1.86 (0.12)	0.66 (0.08)	0.50 (0.05)
10[3]	10.0	4[6]	1.67 (0.09)	0.67 (0.07)	0.51 (0.04)
Internal control[4]			0.72	1.66	0.18

1. Expressed in terms of the parent compound
2. Dosed once daily for 28 days and sampled on day 28
3. Single dose given on day 1 and sampled on day 28
4. MMS (methane methyl sulphonate) positive control standard provided as part of Litron MicroFlow^{BASIC} Kit (male CD1 mice dosed 50 mg/kg ip days 1-3 and sampled day 4).
5. (n) animal group size
6. One animal died prior to scheduled termination; no sample taken

RET = Reticulocyte; SD = standard deviation of mean; MN = Micronucleated



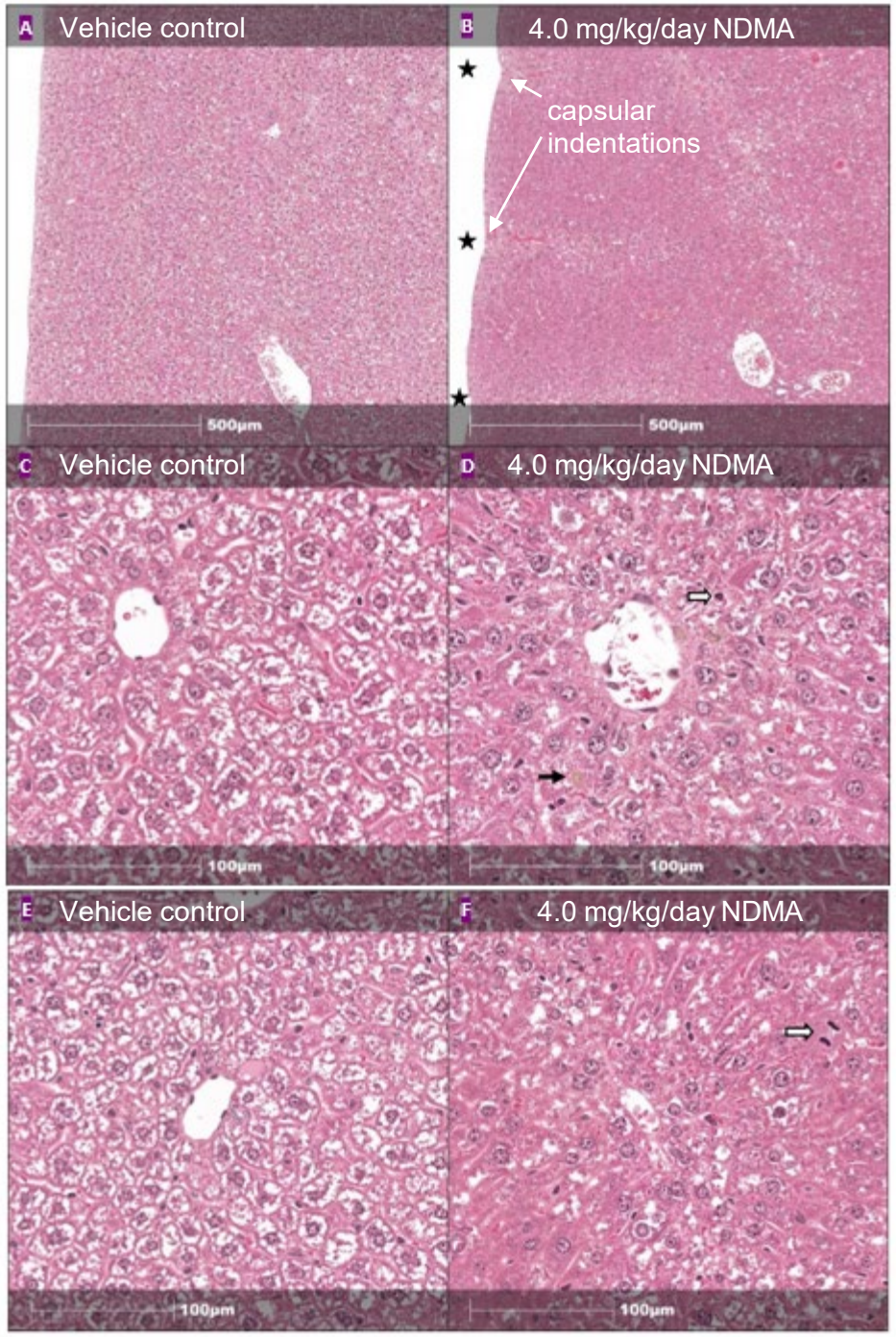
Data analysed by Dunnett's t-test (one sided, upper), comparing treatment groups with the control.
A positive control standard was included in the study analysis as there was no concurrent MN positive control treatment group for the Muta™Mouse study.

There were no significant increases in % MN-RET or % MN-NCE at any NDMA dose relative to vehicle control

Results-4a

Liver Pathology

- No remarkable liver microscopic pathology seen in any treatment groups dosed with 0.36 mg/kg/day NDMA or less.
- Microscopic observations were observed in animals dosed at 1.1 mg/kg/day NDMA and above. These included
  - Capsular indentations (B: indicated by stars)
  - Reduction in hepatocellular vacuolation (representing glycogen): see B compared to A
  - Pale golden pigment (D: black arrow) = hemosiderin (Schmorl negative, Perls prussian blue positive).
  - Single cell necrosis (D: white arrow) in the centrilobular area
  - Mitotic figures (F: white arrow)
- These findings were variably associated with centrilobular haemorrhage and congestion at the highest doses.
- An increase in hepatocellular mitotic figures was observed in mice given ≥ 2.0mg/kg/day and in mice given a single dose of ≥ 5.0mg/kg NDMA.

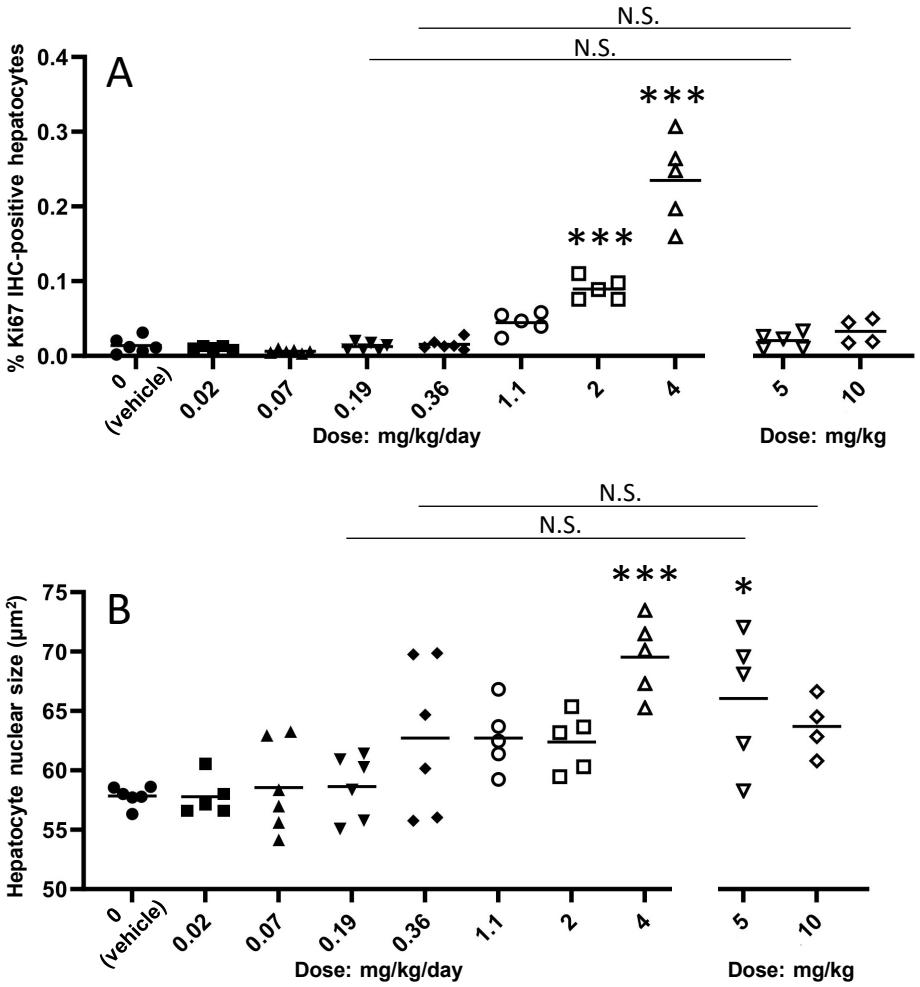


Conclusion: Evidence of hepatocellular toxicity at higher doses (1.1 mg.kg/day and above).

Confirms published observations (e.g. Souliotis et al (1998) PMID: 9635857 and references therein)

Results-4b

Liver Pathology Image Analysis



Data are for individual animals according to treatment group (NDMA dose in mg/kg/day). The NDMA 5 and 10 represent single dose treatments whereas all other treatments were 28 day daily doses. **Bars represent group means.**

Mice given repeat-doses ≥ 2mg/kg/day NDMA had a significantly higher percentage of hepatocyte nuclei which were Ki67 positive relative to the vehicle control group ($p<0.0001$)

Mice given repeat-doses of 4mg/kg/day NDMA or a single dose of 5.0mg/kg NDMA had significantly larger hepatocyte mean nuclear area relative to the vehicle control group ($p<0.0005$ and $p<0.05$, respectively).

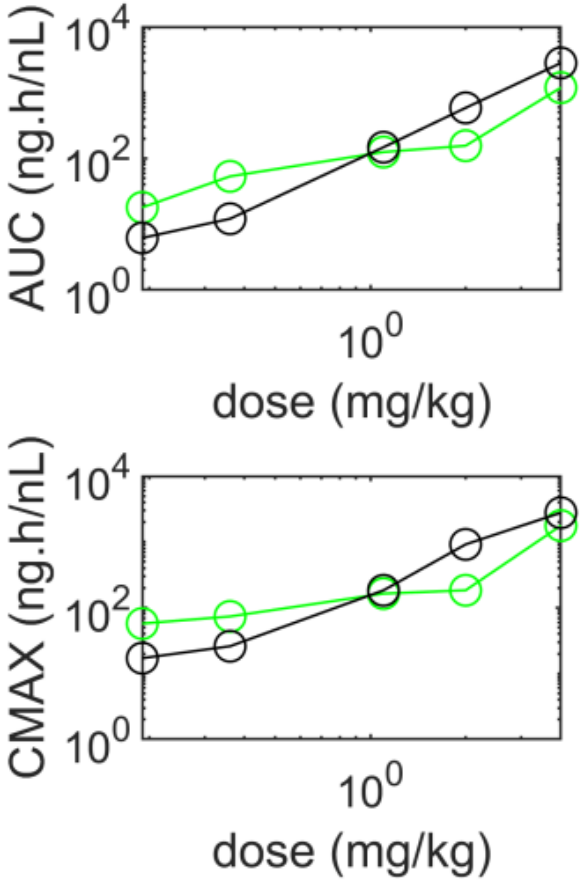
Conclusion: Further evidence of hepatocellular toxicity @ higher doses of NDMA

Results-5a
NDMA Disposition in Muta™Mouse

Composite Toxicokinetic Parameters (**blood**) Following Oral Administration

Parameter	Period	Dose of NDMA (mg/kg/day)						
		0.19	0.36	1.1	2	4	5	10
AUC_{0-t} (ng.h/mL)	Day 1	18.0	53.4	125	156	1190	401	2250
	Day 28	6.18	12.1	148	587	2820	NA	NA
C_{max} (ng/mL)	Day 1	57.5	73.7	164	184	1770	1340	3310
	Day 28	17.1	26.0	184	918	2780	NA	NA
T_{max} (h)	Day 1	0.083	1.00	1.00	1.00	1.00	0.083	0.083
	Day 28	0.083	0.083	0.083	0.083	0.25	NA	NA

5 and 10 mg/kg dose groups, animals given a single dose.



Repeat-dose, measured day 1

Repeat-dose, measured day 28

- **In blood, NDMA clearance increases on repeat dosing at doses of 0.36 mg/kg/day or less.**
- **In contrast, NDMA exposure accumulates at higher doses ≥ 2 mg/kg/day**

Results-5b
NDMA Disposition in Muta™Mouse

Liver tissue concentrations

Group	Total Dose (mg/kg)	Day	Hour	Average Conc (ng/g)	SD
2 TK	10.08	1	IAD	2.5	NC
Liver		1	1	NC	NC
0.36 mg/kg/day		1	4	NC	NC
		28	IAD	NC	NC
		28	1	NC	NC
		28	4	NC	NC
4 TK	56	1	IAD	2.4	1.2
Liver		1	1	1.0	0.8
2 mg/kg/day		1	4	NC	NC
		28	IAD	2.5	NC
		28	1	1.7	1.0
		28	4	NC	NC
5 TK	112	1	IAD	2.3	1.9
Liver		1	1	24.4	20.1
4 mg/kg/day		1	4	1.2	NC
		28	IAD	97.5	102.6
		28	1	28.4	25.1
		28	4	1.1	NC

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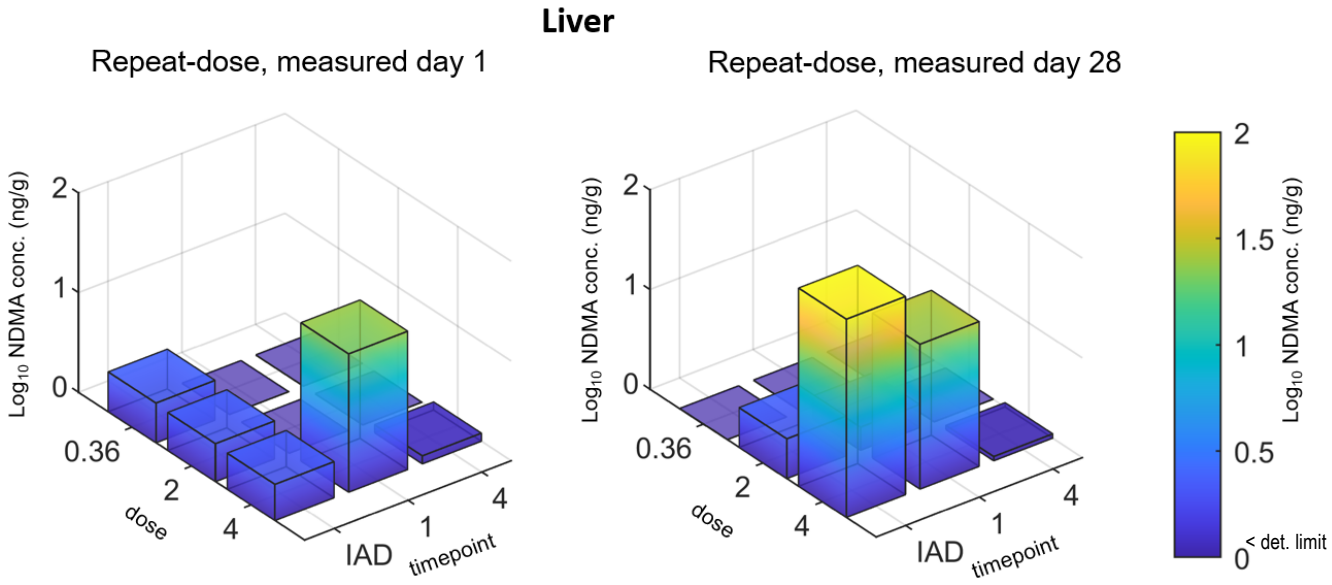
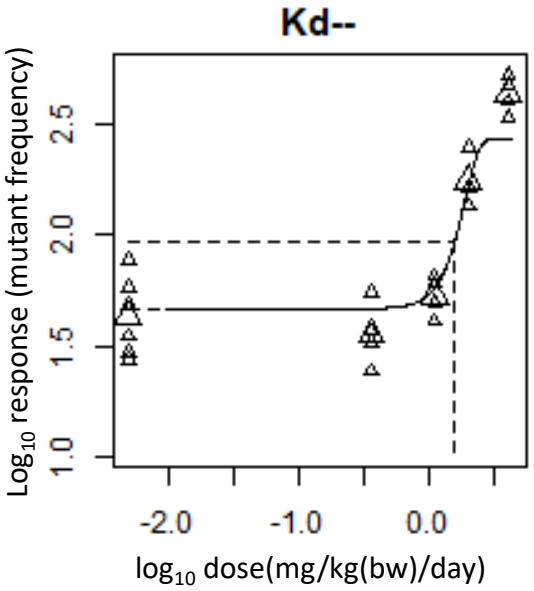
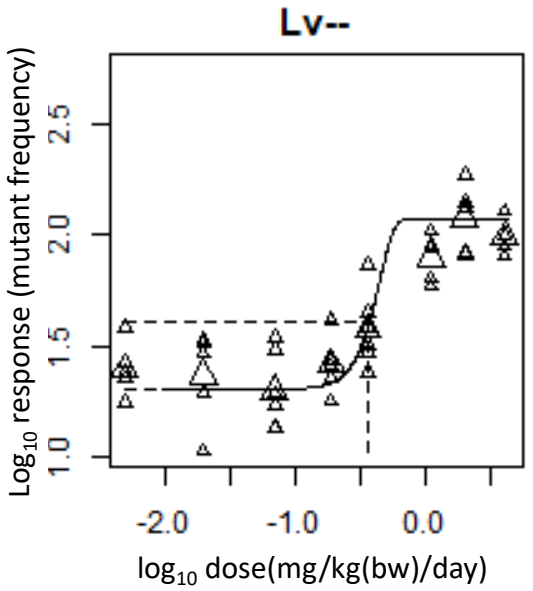


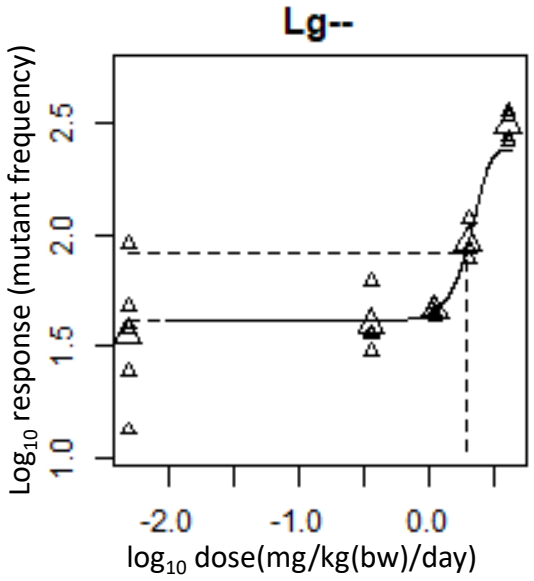
- In liver, after 28 days of dosing, NDMA is rapidly cleared at 0.36 mg/kg/day and 2 mg/kg/day
- At 4 mg/kg/day, the liver NDMA tissue concentration appears to persist for longer after dosing and there is accumulation after 28 days' repeat dosing.

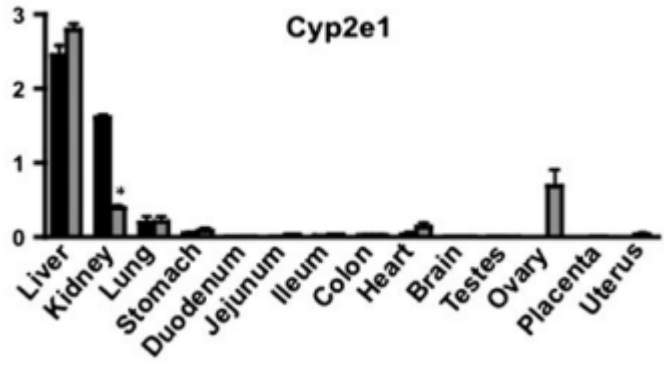
BMD-derived tissue sensitivity ranking (TGR endpoint)

In the mouse, Liver is ~ 4 - 5 times more sensitive than kidney or lung

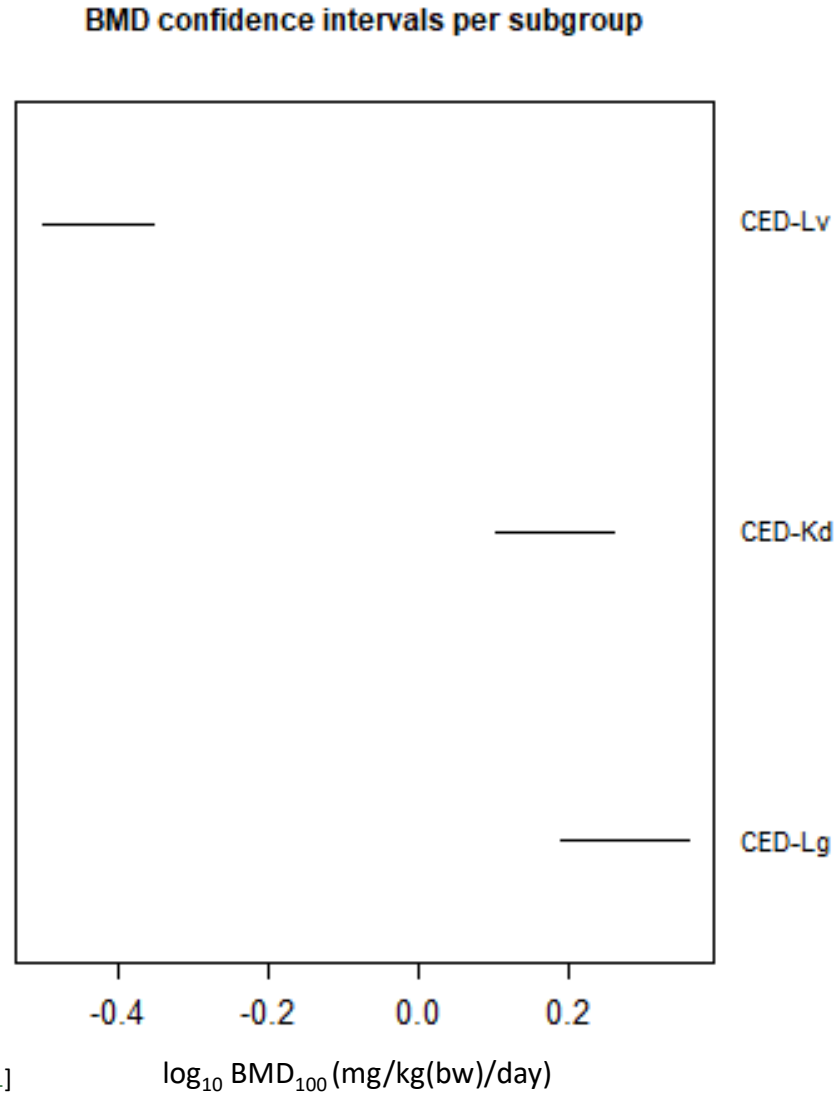




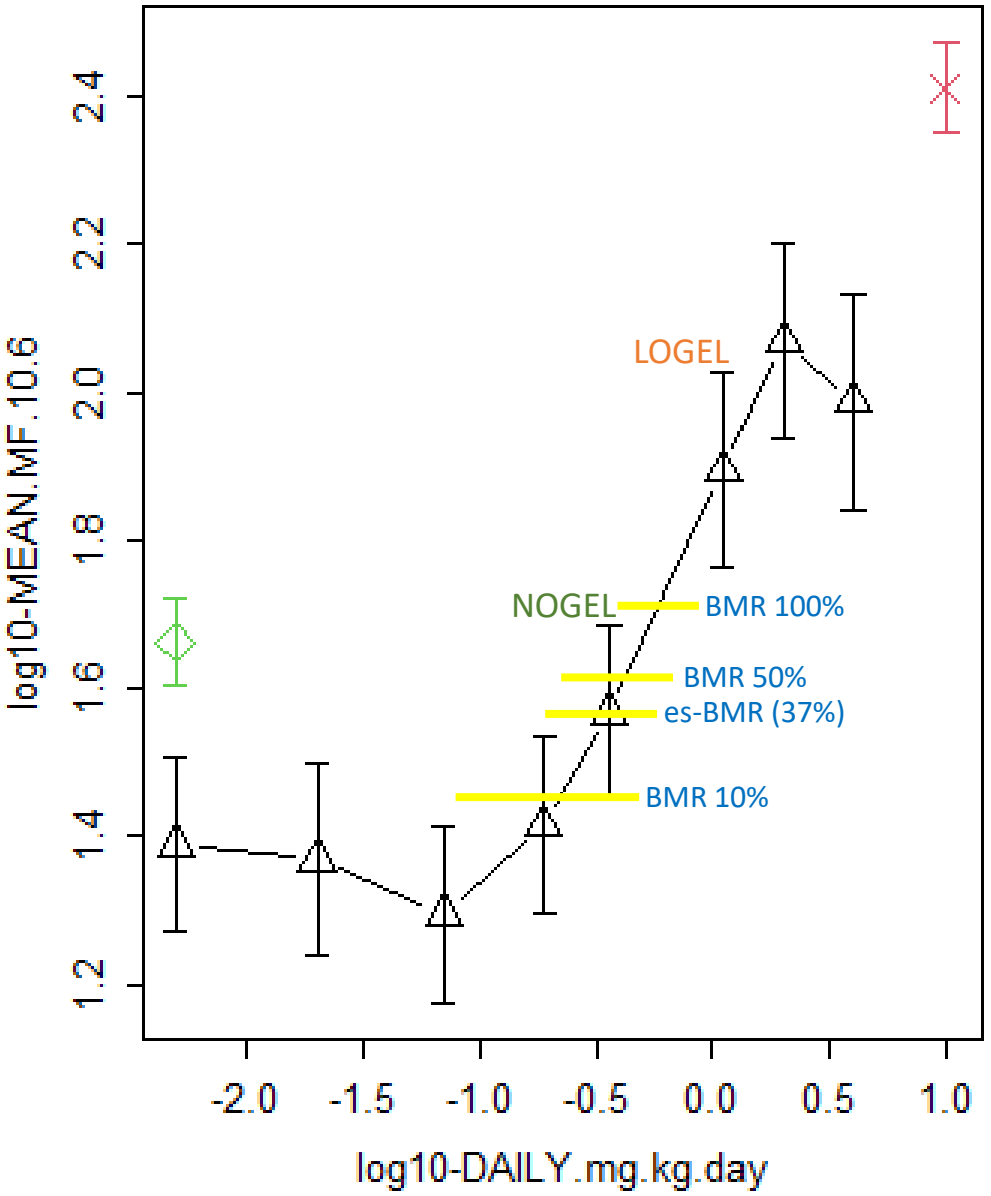




Expression (RNA) of Cyp2e1 in C57BL6 mice
Black: male; grey: female [Renaud et al, 2011 PMID: 21920951]



Point-of-departure determination: Liver MF

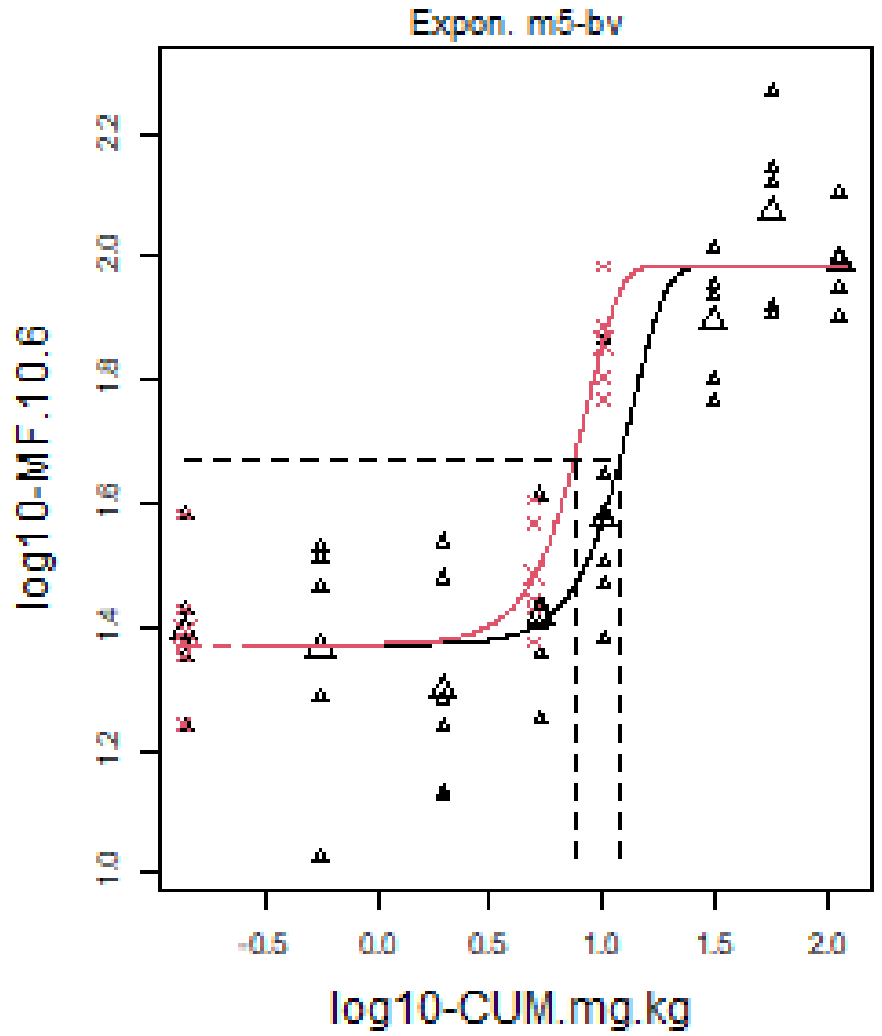


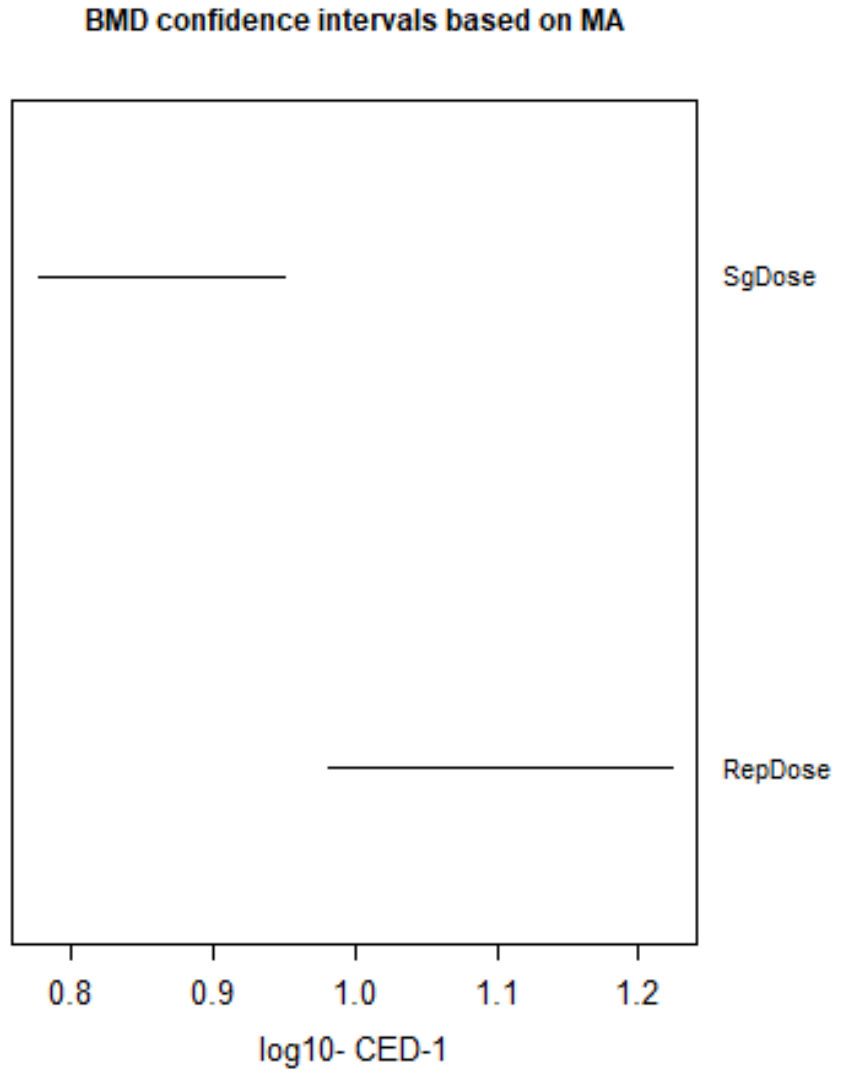
Liver repeat-dose NDMA BMDs in mg/kg(bw)/day				
Exp				
BMR	10%	50%	100%	esCES (37%)
BMD	0.12	0.33	0.51	0.29
BMDL	0.05	0.21	0.36	0.17
BMDU	0.24	0.51	0.72	0.46
Hill				
BMR	10%	50%	100%	esCES (37%)
BMD	0.15	0.32	0.46	0.29
BMDL	0.07	0.22	0.34	0.19
BMDU	0.25	0.46	0.65	0.42
Inv.Exp				
BMR	10%	50%	100%	esCES (37%)
BMD	0.19	0.31	0.44	0.28
BMDL	0.11	0.22	0.33	0.20
BMDU	0.29	0.42	0.60	0.40
Log Normal				
BMR	10%	50%	100%	esCES (37%)
BMD	0.16	0.32	0.46	0.29
BMDL	0.09	0.22	0.34	0.20
BMDU	0.26	0.45	0.64	0.41
MODEL AVERAGE	**0.07** **0.27**	**0.21** **0.46**	**0.34** **0.68**	**0.17** **0.42**

Liver, cumulative dose comparison

Acute vs chronic dosing regimen

Single-dose is ~1.6x (1.57x – 2.42x) more potent than fractionated, **repeat-dose** regimen

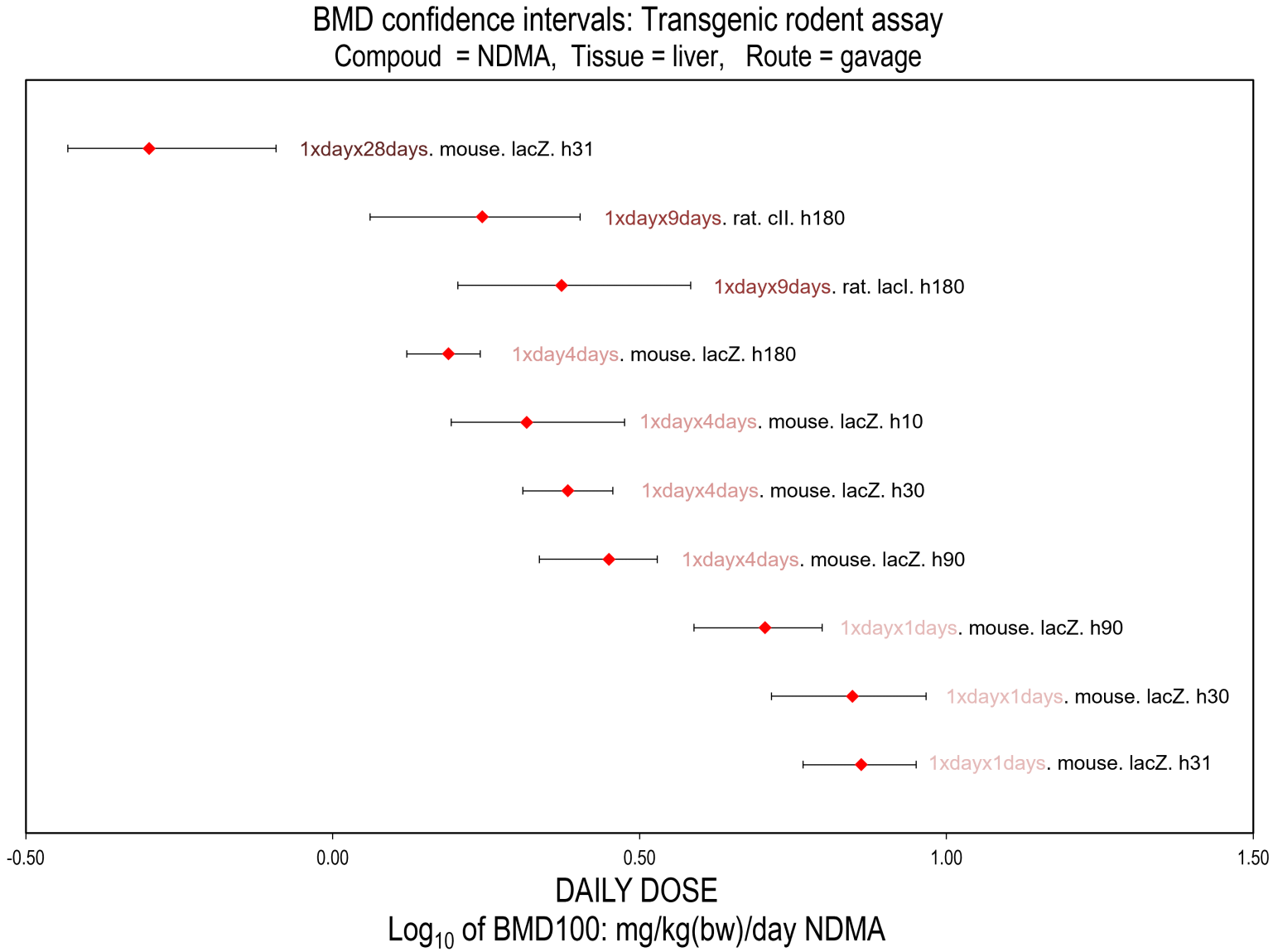




BMD analysis to quantitate NDMA mutagenic potency

Integrating published work alongside the new 28d TGR study

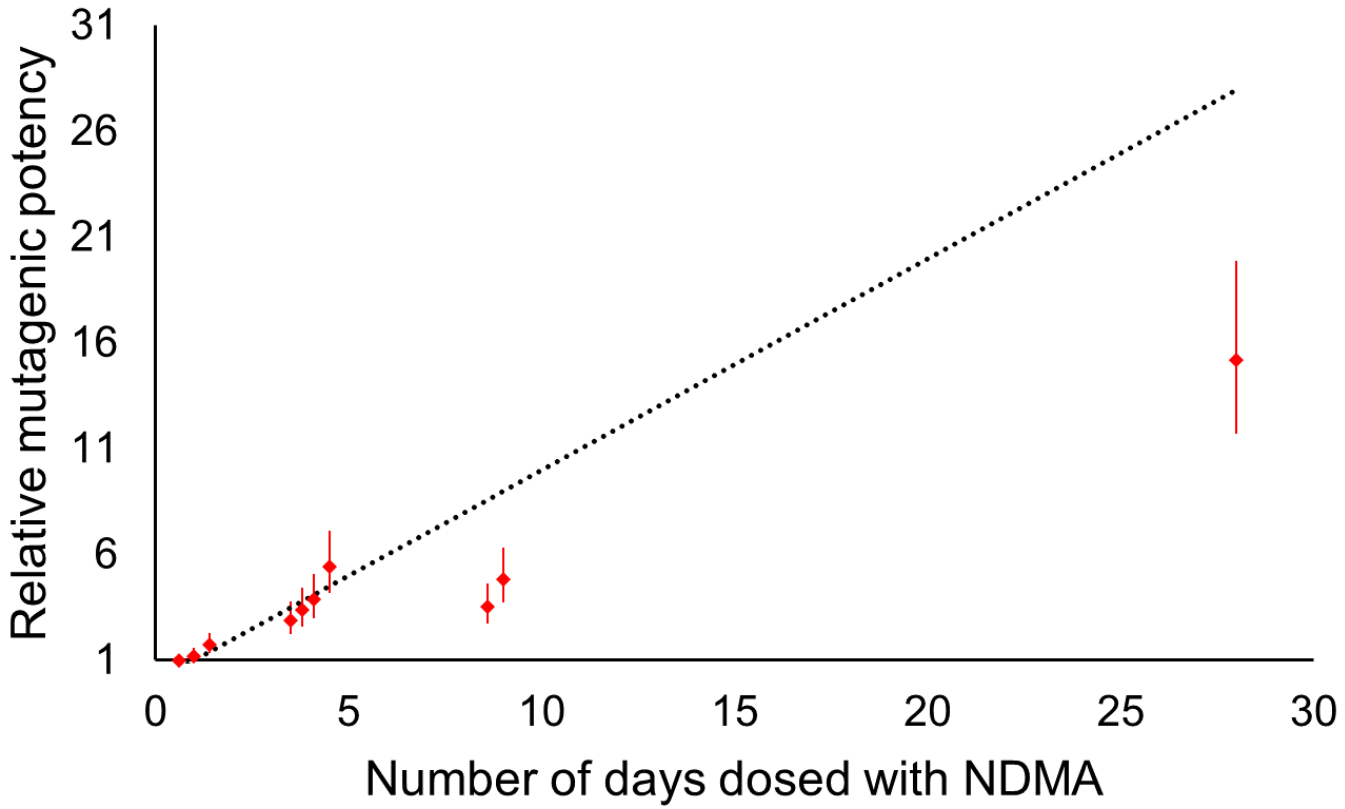
BMD analysis of the effect of exposure duration on mutant frequency dose-response relationships for NDMA



- Quantitative analysis shows that mutagenic potency increases with the number of days of exposure
- Mutation induction by short-term treatments is not the same as for a lifetime of exposure
- As such, this analysis is consistent with the concept of ‘less than lifetime’ risk assessment

BMD-derived relative potency comparison

TGR studies for NDMA
1xdayx1day / 1xdayx4days / 1xdayx9days / 1xdayx28days



Conclusions

NDMA 28d Muta™mouse study in the low dose region

1. NDMA-induced mutation was investigated in an OECD 488 compliant TGR assay.
   - Endpoints: lacZ mutation frequency analysis in various tissues, analysis of peripheral blood (Pig-a mutation & MN-Ret), histophathology and TK.
2. NDMA did not significantly elevate mutant frequency in bone marrow (lacZ) or peripheral blood (Pig-a) or micronucleus frequency in peripheral blood.
3. NDMA induced significant increases in mutant frequency @ the lacZ locus in liver following 28 day repeat dose treatments @ 1.1 mg/kg/day po and above, or following a single dose of 10 mg/kg po.
4. In Liver, the NOEL for NDMA was 0.36 mg/kg/day for 28 days or 5 mg/kg single dose po.
5. For a BMR of 50% using a 4-model average, the BMD for NDMA was 0.3 mg/kg(bw)/day (CI 0.21-0.46).
6. There was evidence of liver toxicity in mice given ≥ 2mg/kg/day NDMA or a single dose ≥ 5mg/kg.
7. At lower doses, NDMA was cleared quickly, whereas at higher doses there was evidence for accumulation in the liver and in blood.
8. Both mutation and liver toxicity appeared to correlate with NDMA exposure.
9. These data provide evidence to differentiate the risk of NDMA induced genotoxicity in the low dose region more commensurate with impurity exposures.

Next Steps

Quantitative potency ranking using benchmark dose analysis using all available TGR dose-response data for NDMA

- IP / gavage / drinking water routes-of-administration
- lacI / lacZ / cII transgenes in mice and rats
- Tissues: liver, bone marrow, spleen, kidney, lung
- Effect of different dosing regimens

Model data to determine less-than-lifetime risk assessment

Generate error corrected next-generation sequencing data for liver and bone marrow in collaboration with TwinStrand and AstraZeneca (data imminent)

Publish studies with full data sets

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