No Genotoxic Consequences of Daily Doses of EMS Inducing up to 380'000 DNA-Alkylations/Cell/Day

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Pharmaceuticals

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Why did we study EMS?

- June 2007: High impurity levels of EMS (ethyl methanesulfonate) were found in batches of an anti-AIDS medication (nelfinavir mesylate) produced in early 2007
- Information of health authorities (EMEA, Swissmedic); all batches on the market were recalled, marketing authorisation was suspended,
- request for patient registry was issued

Reason for accident:

- Residual cleaning fluid (ethanol) had not been removed from storage tank before filling with methanesulfonic acid, time of storage was 77 days until next campaign

Patient exposure:

→ Maximal content of EMS in tablets: ca 1000 ppm (TTC level: 0.6 ppm)

→ Maximal dose of EMS to patients: 0.055 mg/kg

→ Maximal duration of exposure: 3 months

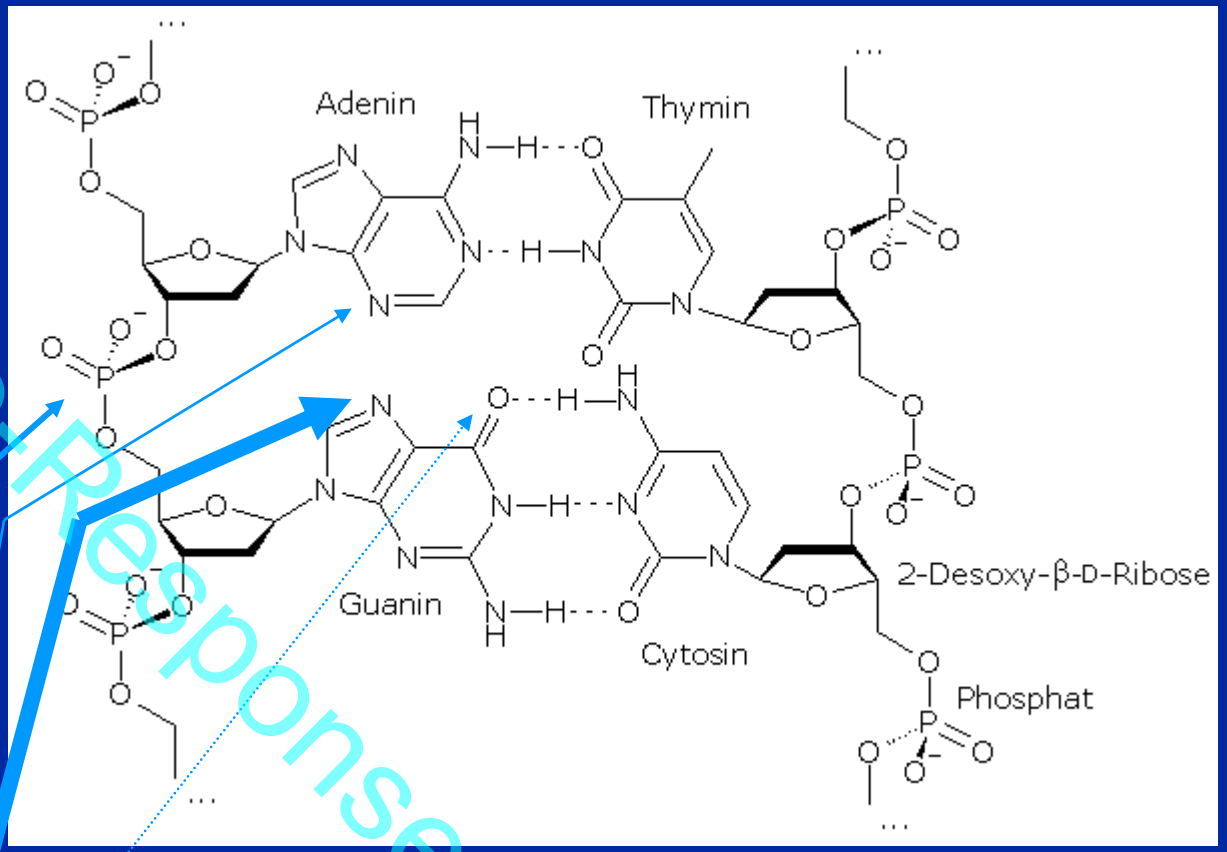
→ Number of patients: ≤ 40 000

What risk for adverse effects?

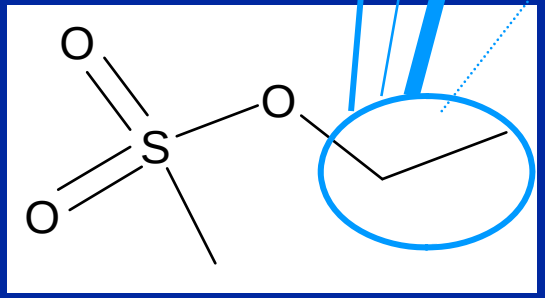
EMS is an exemplary alkylating genotoxin

→ so it shows linear dose response relations!!

or does it...?



DNA damage by EMS



Ethylmethanesulfonate

EMS ethylates DNA predominantly at N^7-Guanine

RISK ASSESSMENT APPROACHES

1) Linear back extrapolation (based on cancer data with MMS):

→ less than 1 addtl. cancer in 10 000 exposed patients

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2) Alternative approach:

Working hypothesis of thresholded dose response (based on Doak et al paper)

- If there exists a threshold for EMS-induced mutations also in vivo …
- If all adverse events due to EMS are secondary to DNA damage …
- If the exposure to EMS in patients was below this threshold …

↓

No additional risk for patients

EMS shows a threshold for genotoxicity in human cell line in vitro!

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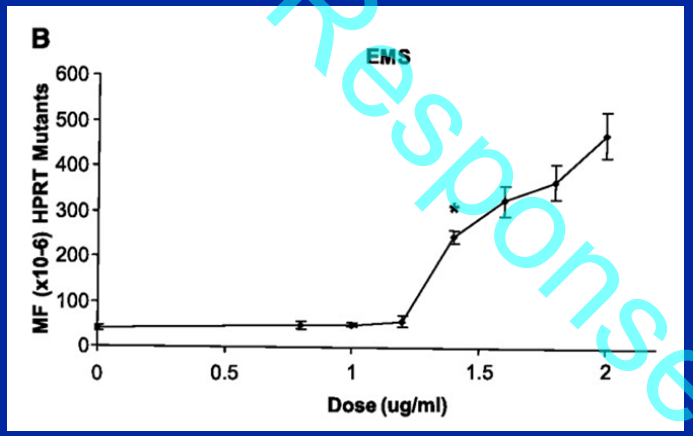
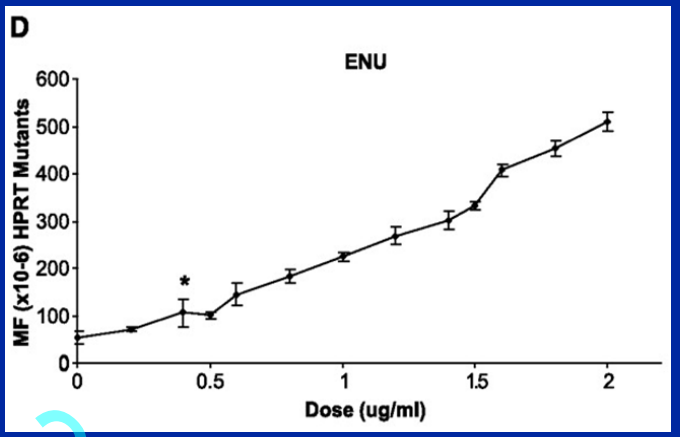
Dose-Response 2010

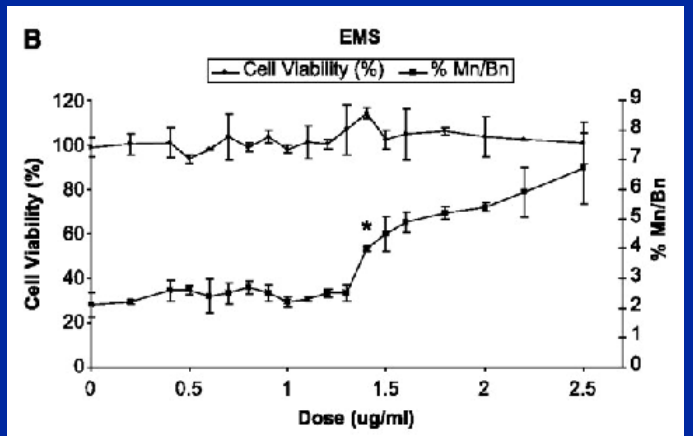
EMS Doak et al. (2007) **versus** **ENU**

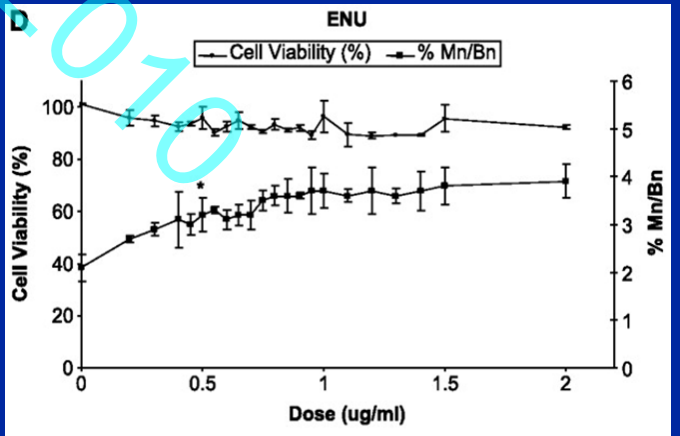
gene mutation induction





chromosomal damage induction





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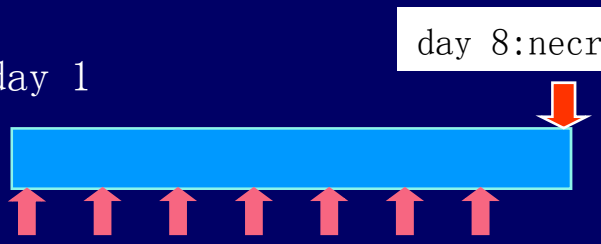
Decision:

Attempt to obtain solid in vivo evidence for thresholded dose response

to assure the patients that they do not carry an increased risk for mutations (and by inference for cancer, birth defects)

and with the hope that request for patient registry would be withdrawn

Induction of chromosomal damage in bone marrow of mice



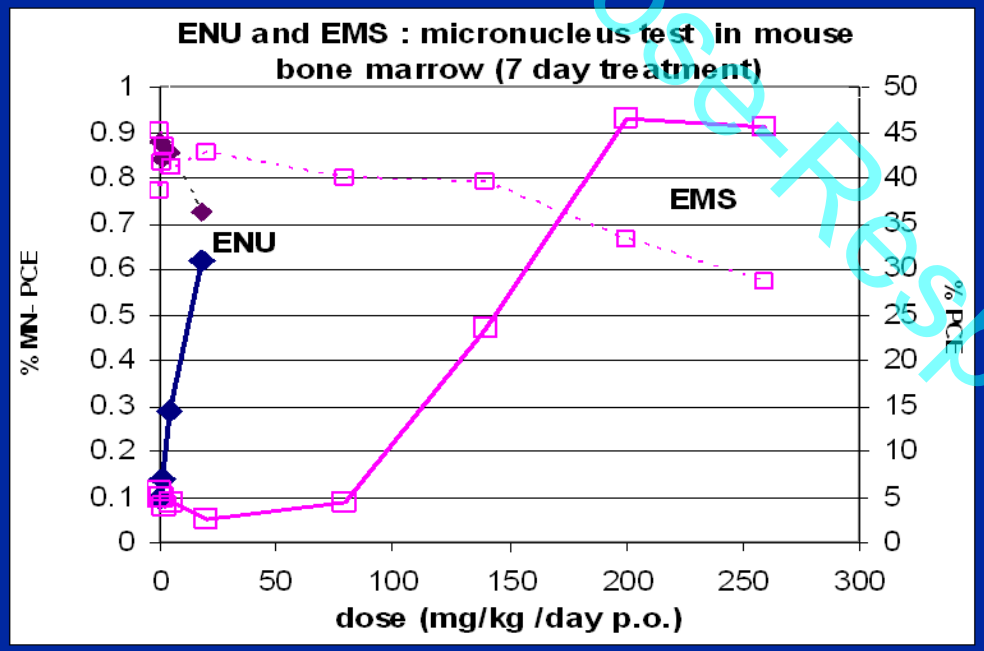
7 daily treatments

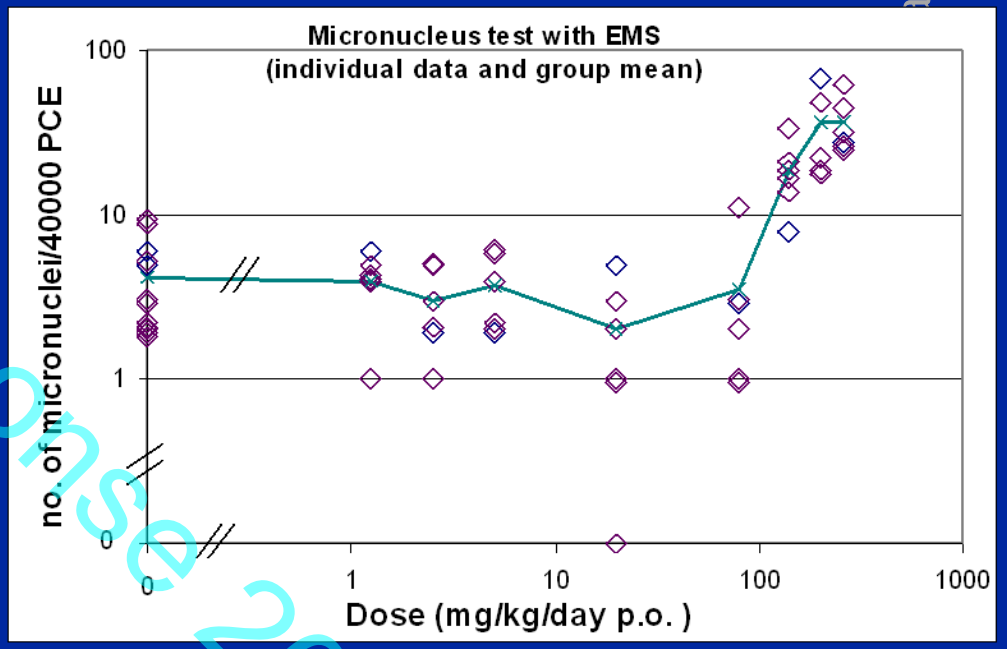
EMS: 0, 1.25, 2.5, 5, 20, 80, 140, 200, 260 mg/kg/day

ENU: 0, 1.11, 4.45, 17.8 mg/kg/day

- Erythrocytes prepared from bone marrow for assessment of micronuclei
- Assessment of exposure via ethyl- adducts in terminal valine of hemoglobin

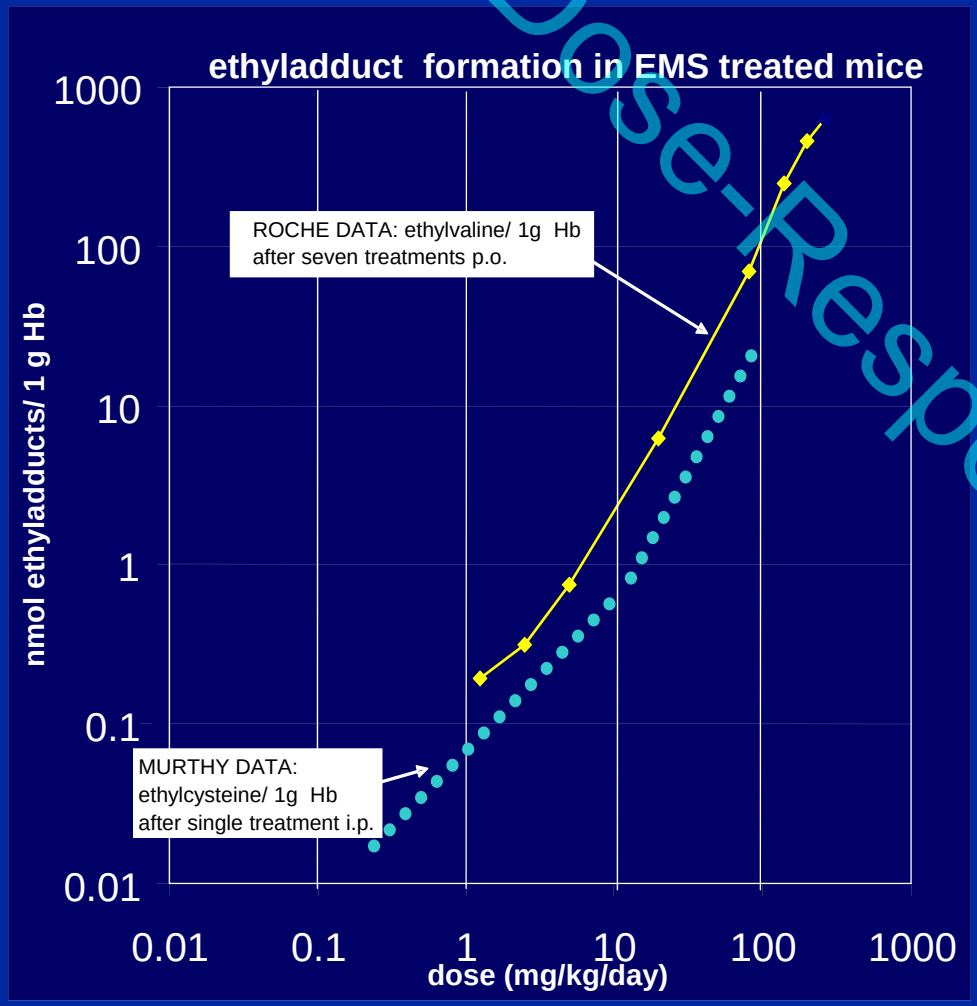
Micronuclei as a function of dose





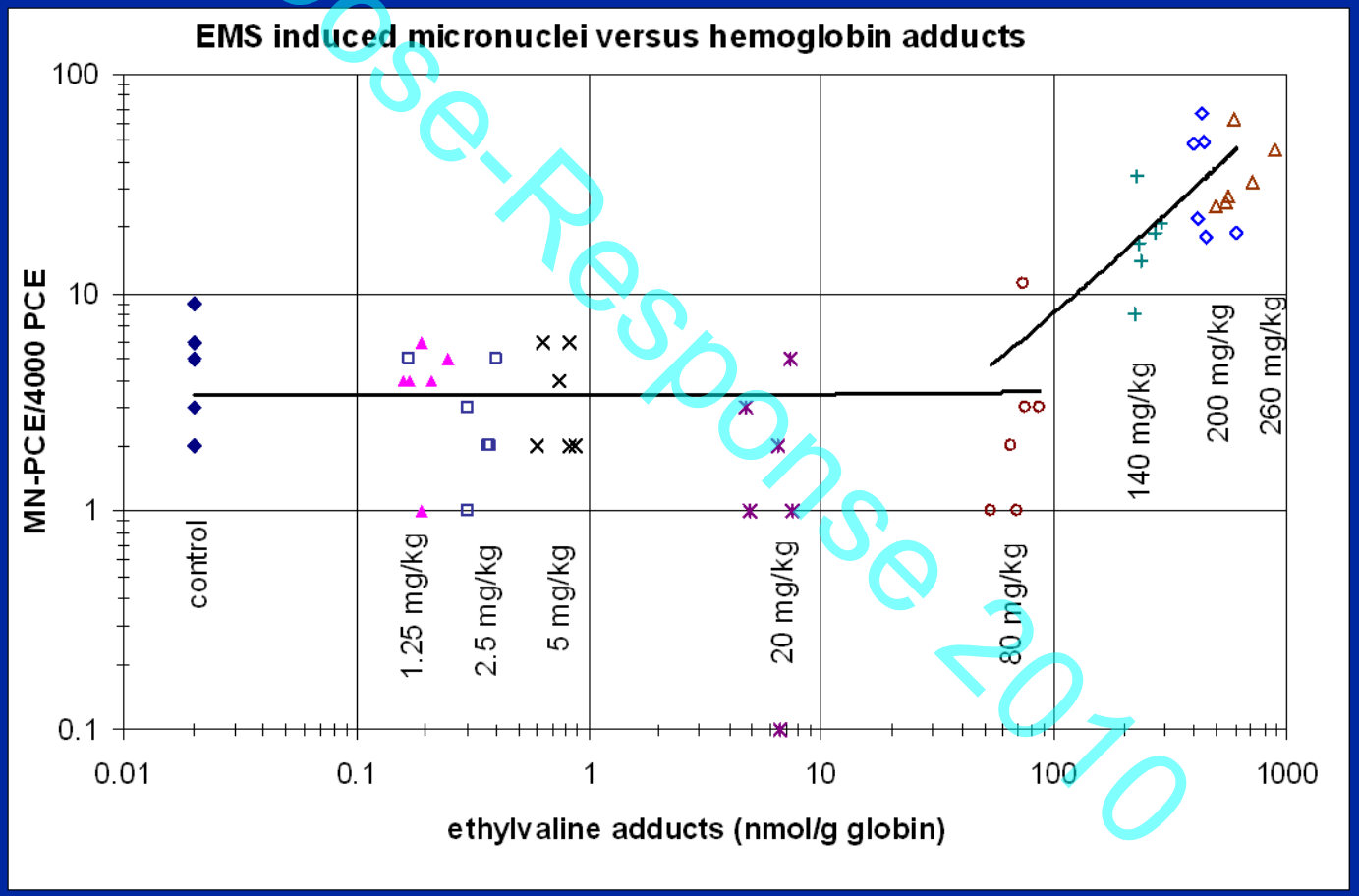
- EMS induces a thresholded dose response (80 mg/kg/day)
- ENU induces a linear response

Ethylvaline formation in globin as a biomarker of exposure



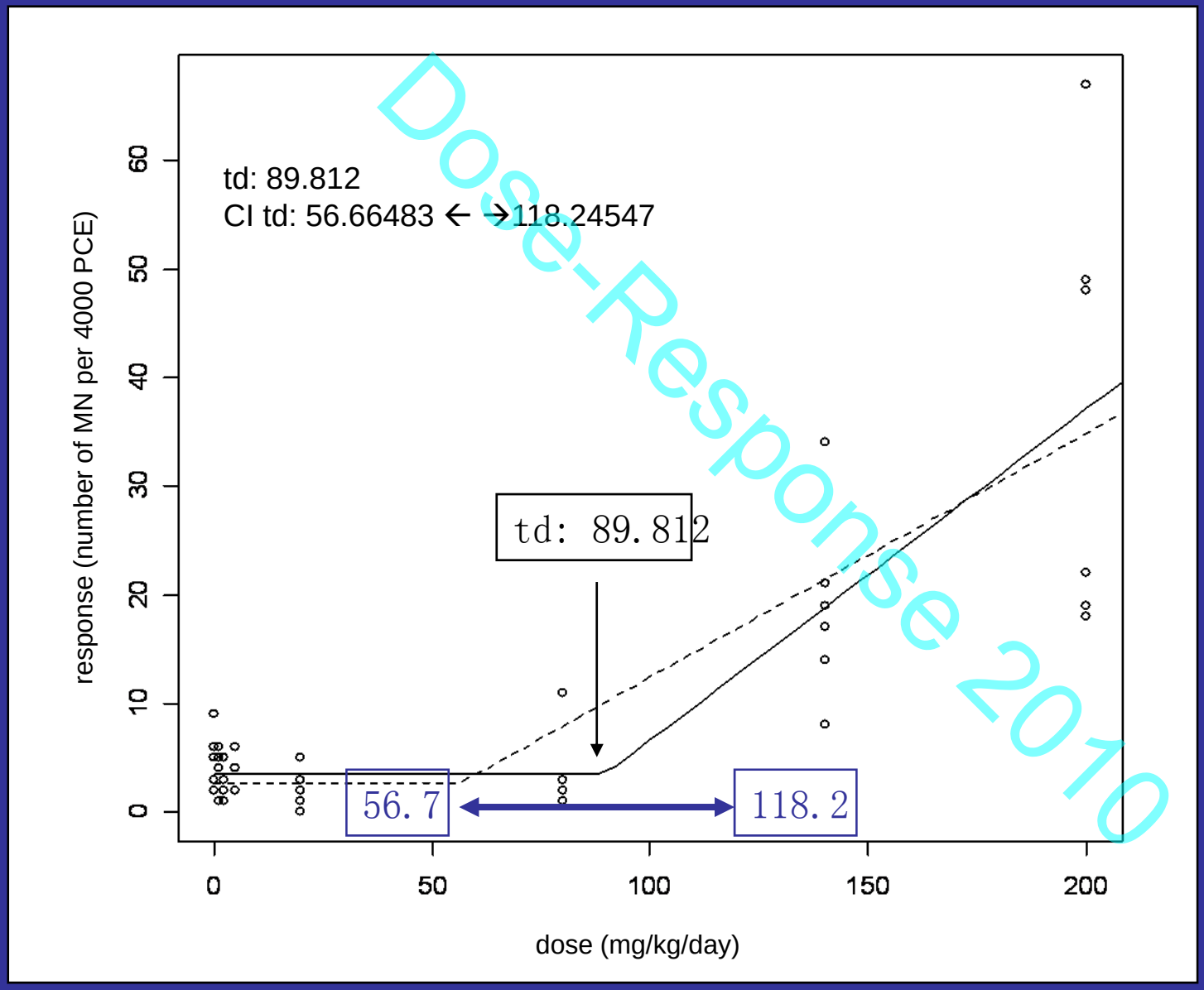
- Roughly linear increase at low doses, above linear increase at higher doses
- Data are fully in line with previous study by Murthy et al (1984)

Micronuclei as a function of exposure (ethylvaline adduct levels)

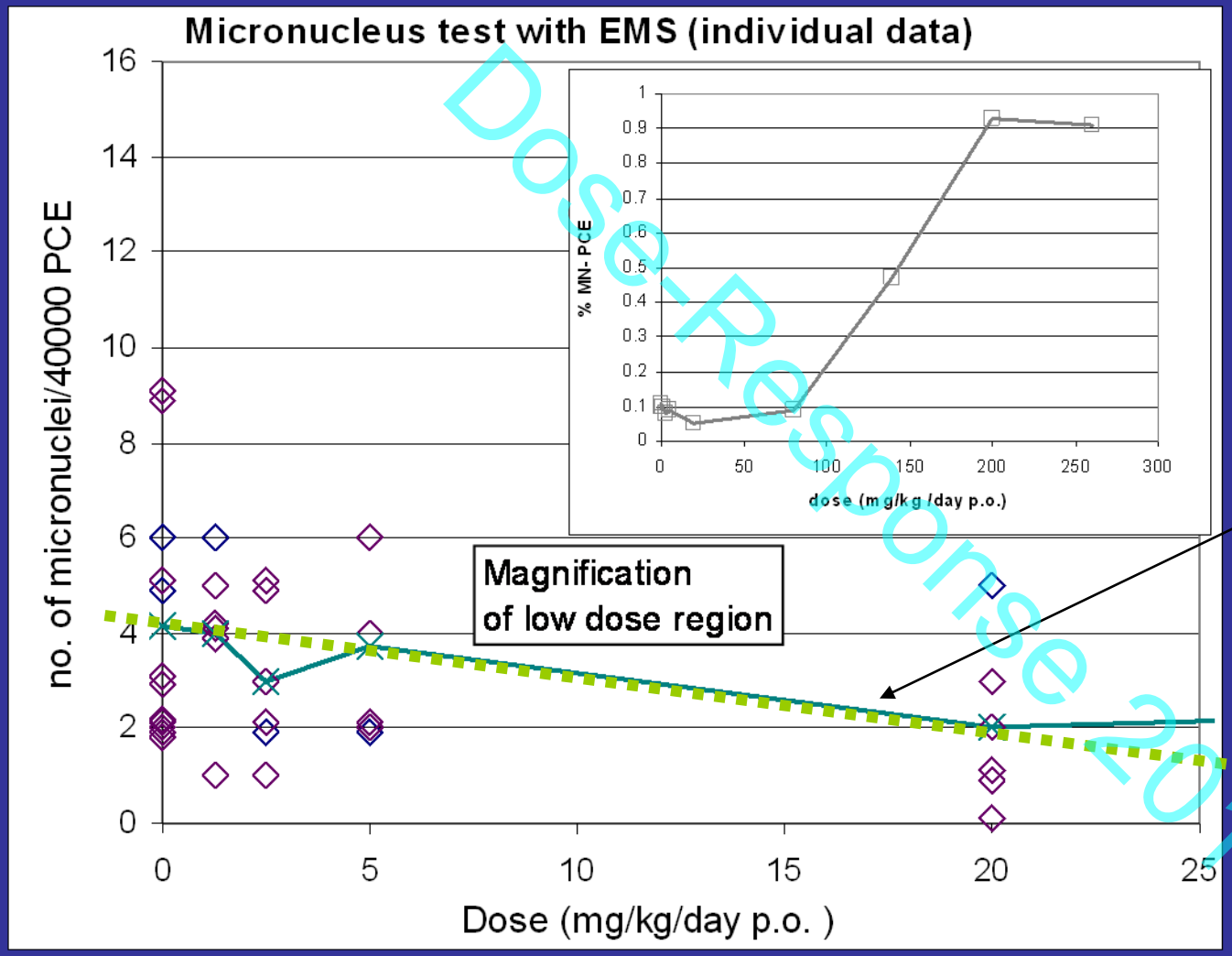


- No increase of micronuclei below ~100 nmol/g globin
  (more than 1000 fold higher than background level of < 0.1 nmol/g globin)

Statistics MNT bone marrow (assessed with stat. program developed by Lutz and Lutz, 2009)

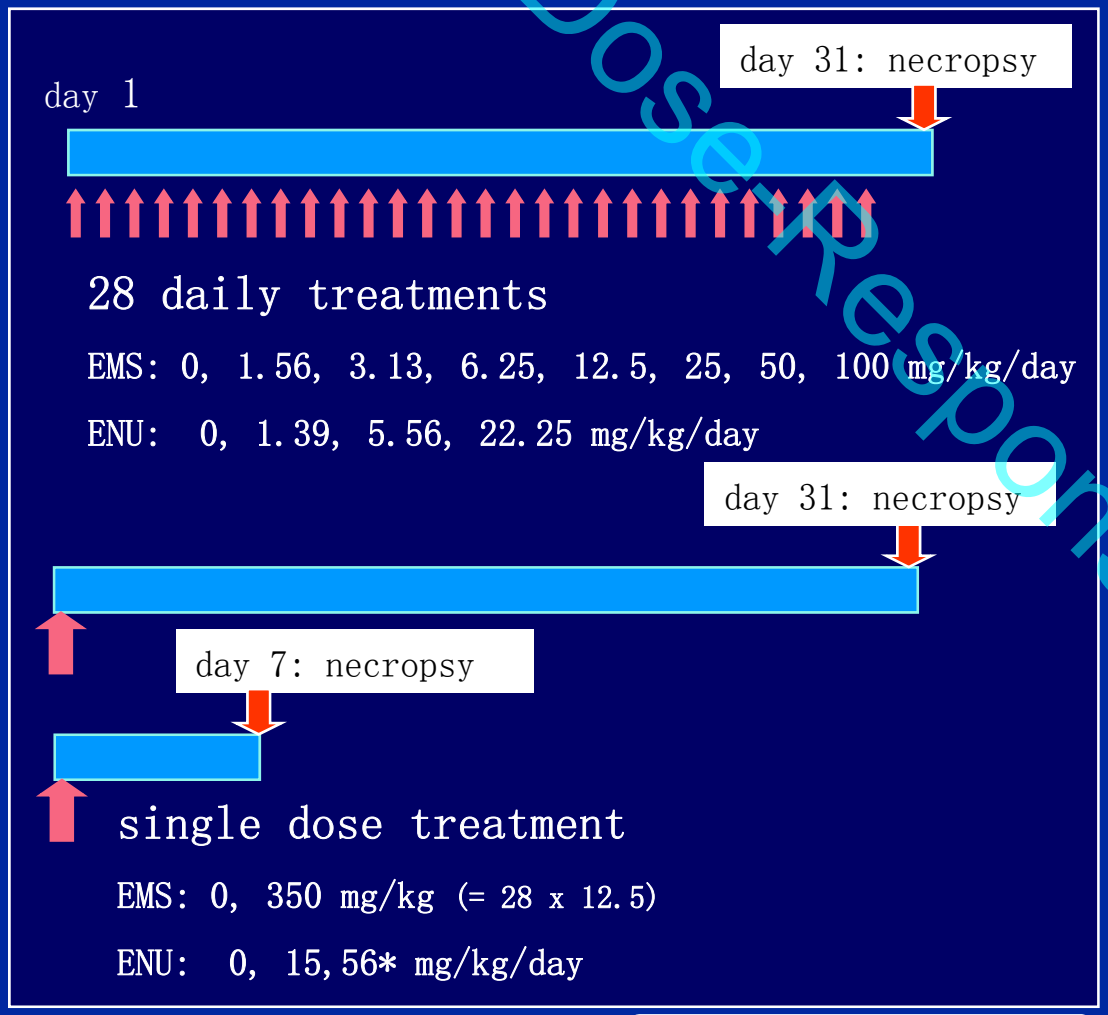


Dose response at low doses:



The slope at doses below 20 mg/kg/day is significantly negative ($p \leq 0.05$)

Induction of LacZ gene mutations in MutaMouse (transgenic) model



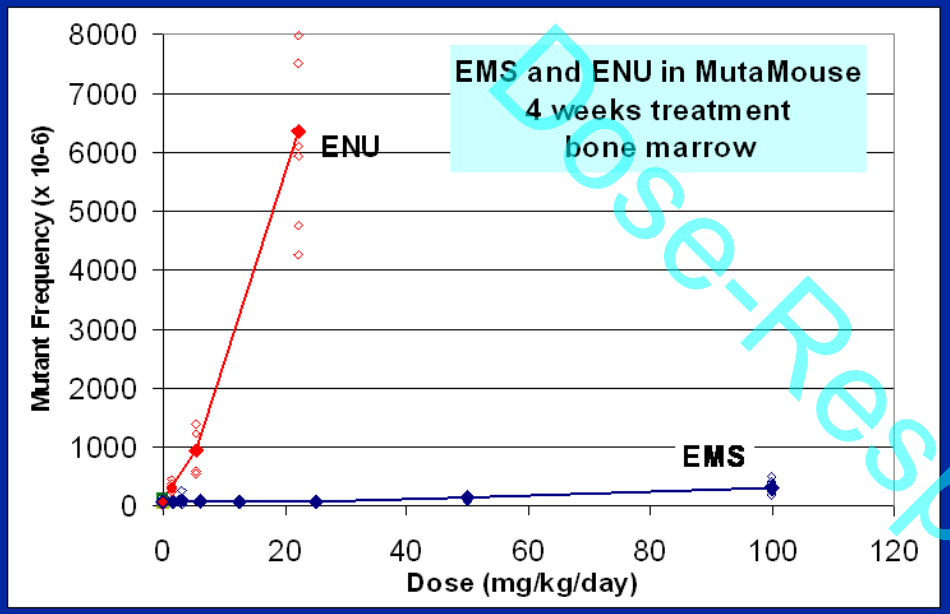
*planned was a tenfold higher dose (28 x 5.56 = 156 mg/kg)

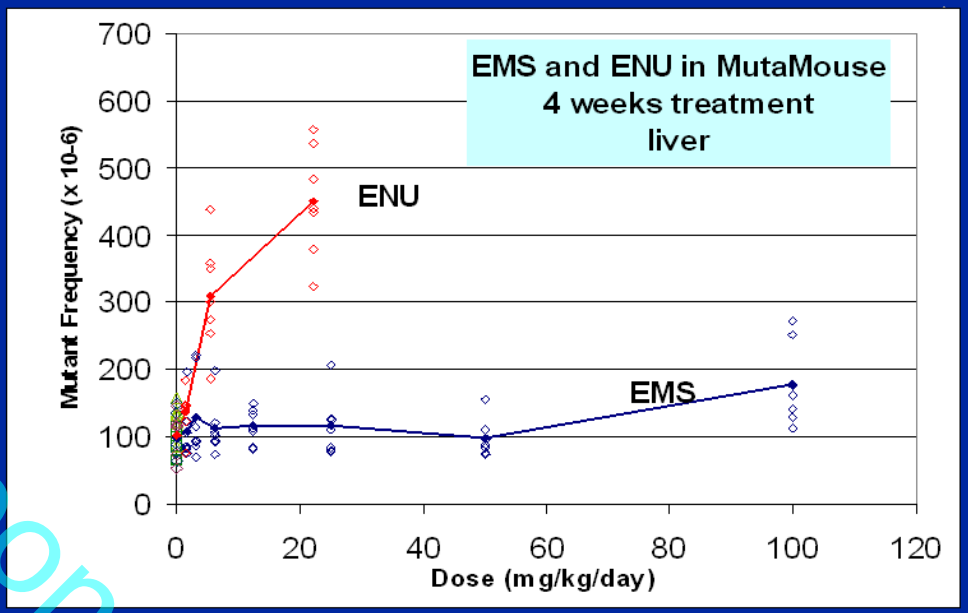
- DNA extracted from
  - bone marrow
  - liver
  - GI tract

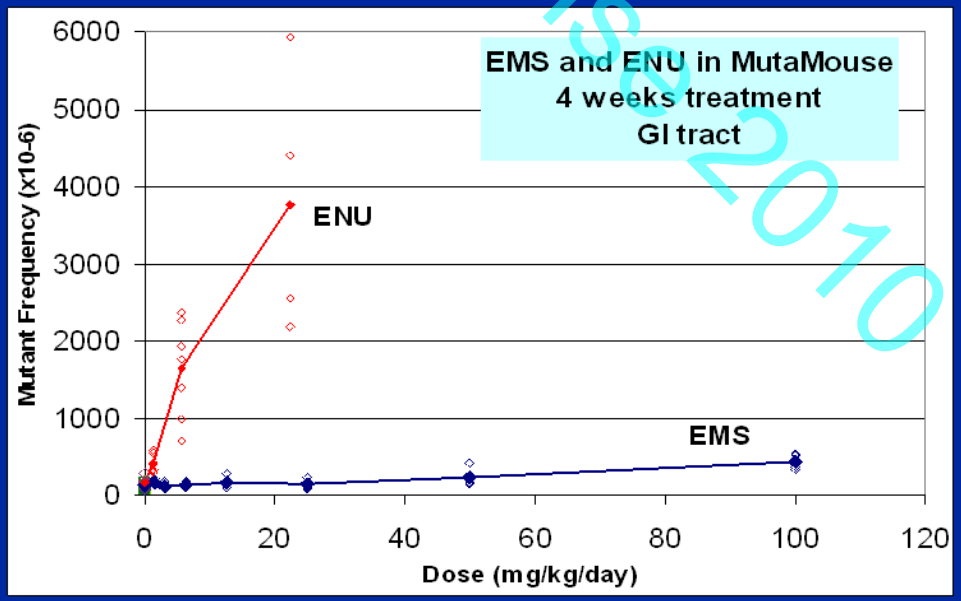
  for assessment of gene mutations at LacZ gene

- Assessment of exposure via ethyl- adducts in terminal valine of hemoglobin

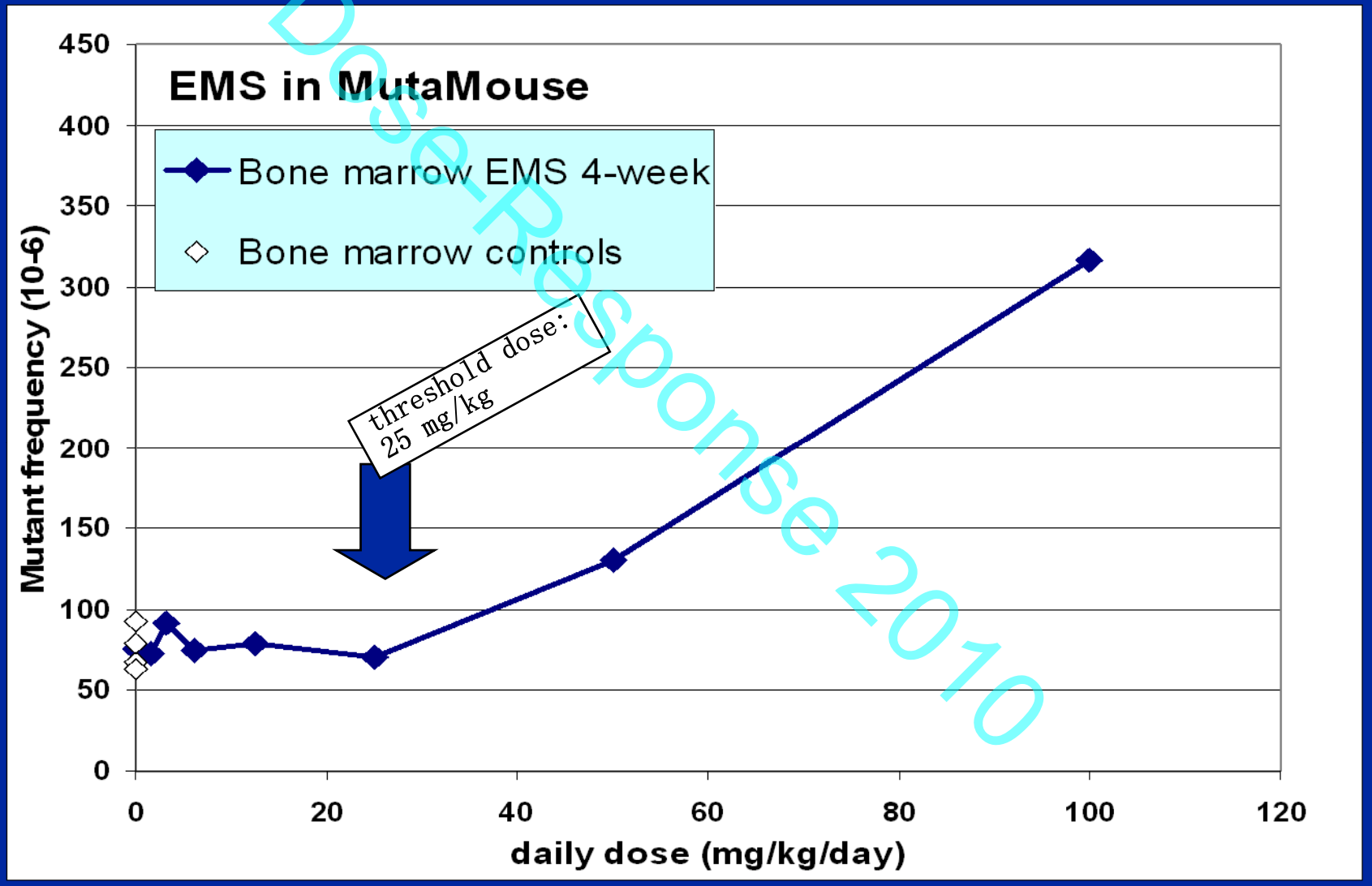
MutaMouse results: comparison EMS vs ENU (4-weeks treatment)



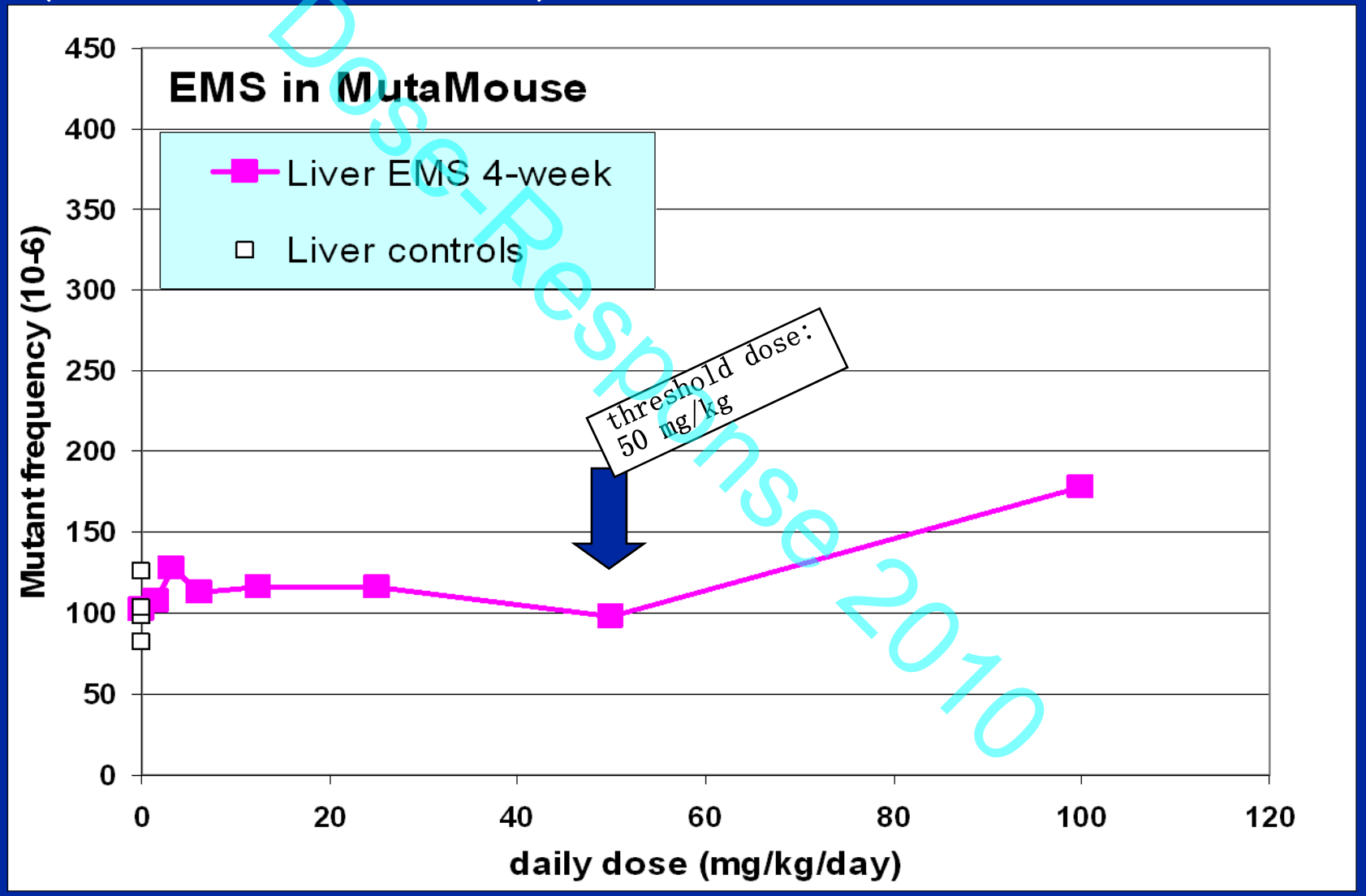




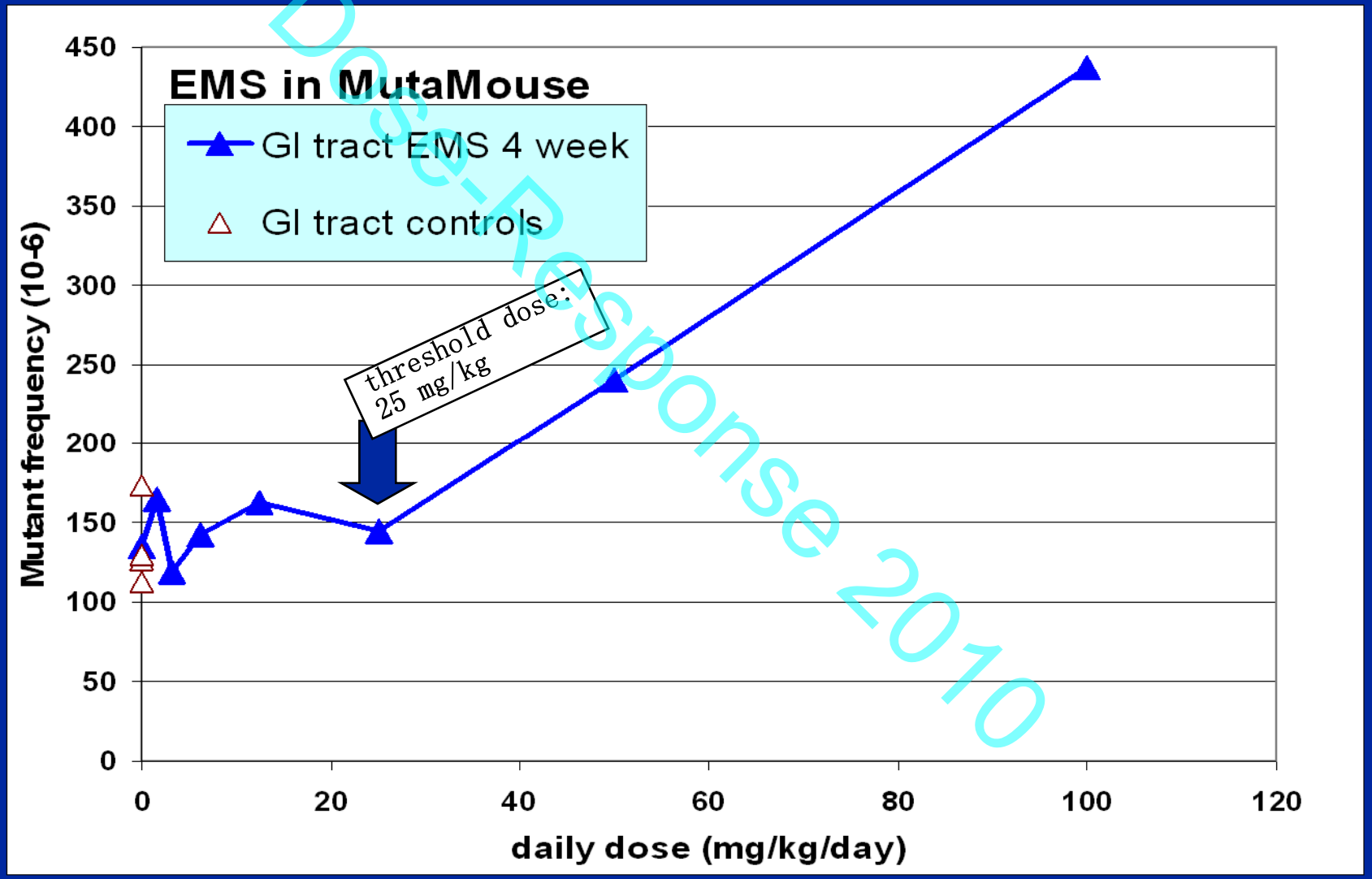
Gene mutations in bone marrow as function of dose (4-weeks treatment)



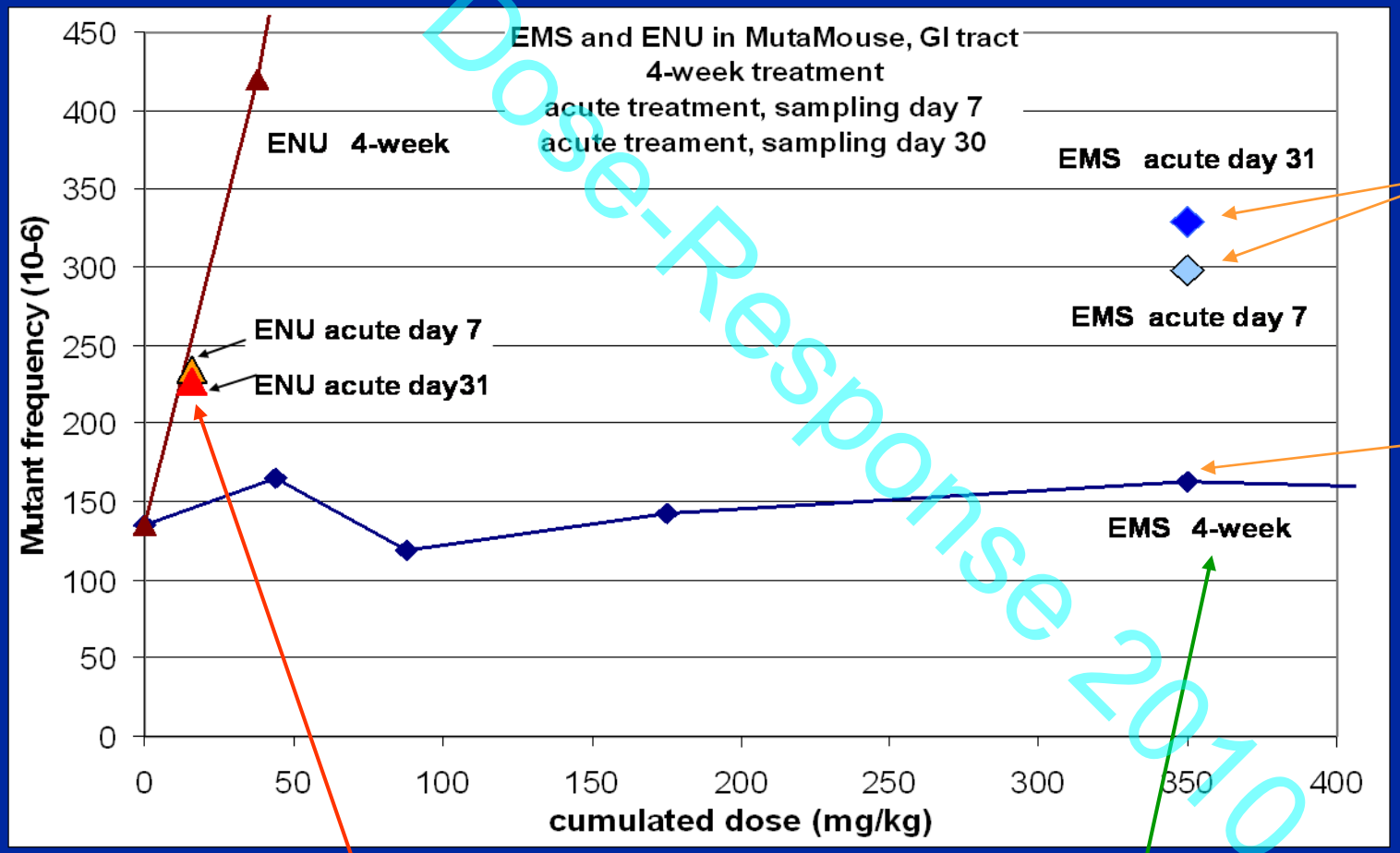
Gene mutations in liver as function of dose (4-weeks treatment)



Gene mutations in GI tract as function of dose (4-weeks treatment)



MutaMouse results: Cumulative versus acute treament: GI tract



350 mg/kg/day x 1 day
= 350 mg/kg

12.5 mg/kg/day x 28 days
= 350 mg/kg

ENU: effect independent of dose fractionation

EMS: dose fractionation abolishes the effect

Complete statistical approach (a)

1. Comparison of control groups (to allow cumulation)
2. Rejection of linear dose response relationship (entire dose range)
3. Acceptance of linear dose response relationship below the NOEL
4. Application of threshold software developed by Lutz and Lutz (2009)
   - to calculate the threshold values incl. confidence limits

Threshold analysis by hockeystick approach (Lutz and Lutz, 2009)

study	organ	NOEL (mg/kg)	td (mg/kg)	95% Confidence interval of td (mg/kg)
MNT	Bone marrow	80	89.812	56.665 to 118.245
Muta™mouse	Bone marrow	25	35.446	21.464 to 45.728
Muta™mouse	liver	50	51.314	25.670 to 99.997
Muta™mouse	GI-tract	25	24.51	12.966 to 38.513

confidence interval does not include ‚0'

Complete statistical approach (b)

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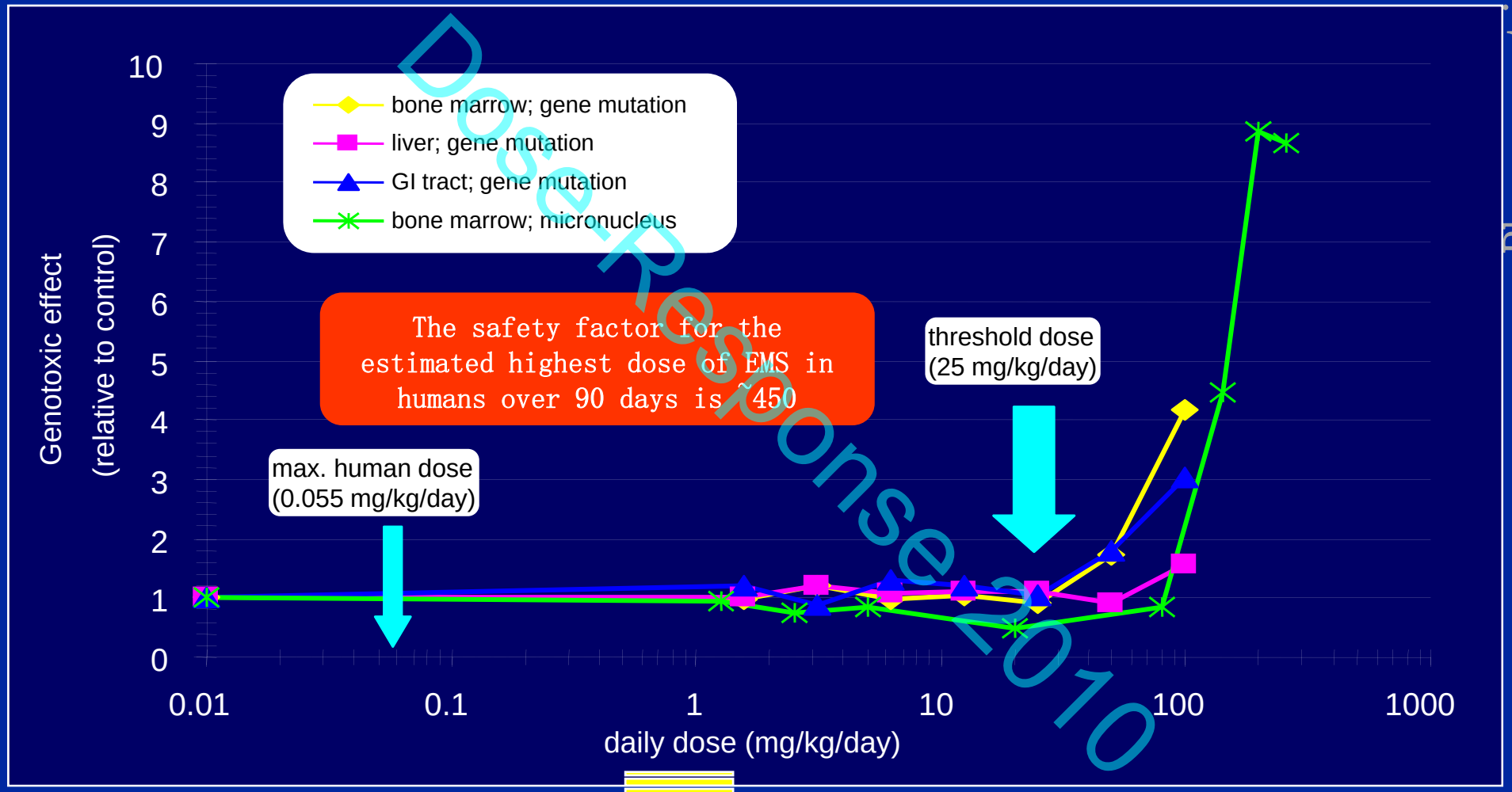
5. Analysis of slope in low dose region

study	organ	Slope at low dose region	95% Confidence interval of slope
MNT	Bone marrow	-0.10	-0.201 to -0.001
Muta™mouse	Bone marrow	-0.19	-1.19 to 0.81
Muta™mouse	liver	-0.10	-0.69 to 0.48
Muta™mouse	GI-tract	0.48	-0.96 to 1.92

slope is given as No. of MN/4000PCE/mg/kg, and mutation frequency ($x10^6$)/mg/kg, respectively

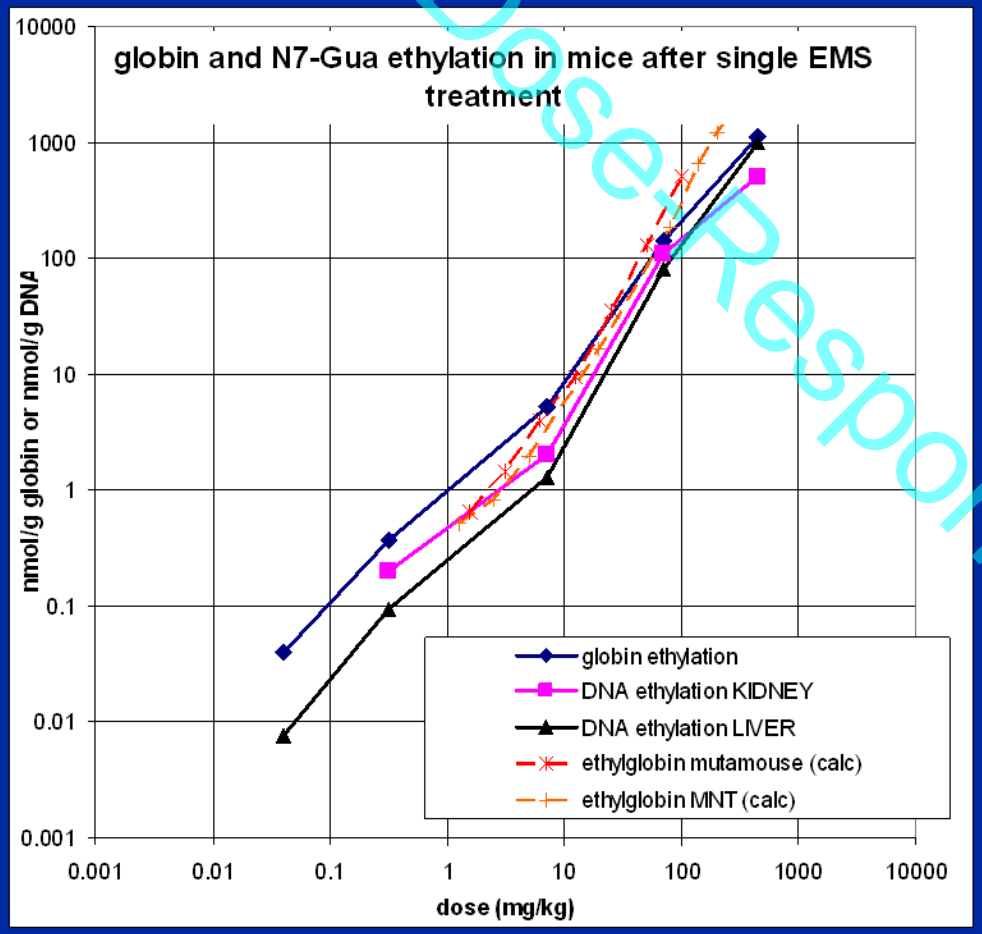
Summary of genotoxicity studies: Safety margin (dose based comparison)



Authorities were convinced → request for patient registry was withdrawn

DNA ethylations at threshold dose

based on Murthy et al (1984), Beranek (1990) and our data on ethylvaline



Dose response for N7-Gua ethylation and

total globin ethylation

EMS induced

380 000 *ethylations everyday in the DNA of each each liver cell at the threshold dose*

The cell can repair large amounts of DNA damages FULLY ERROR FREE

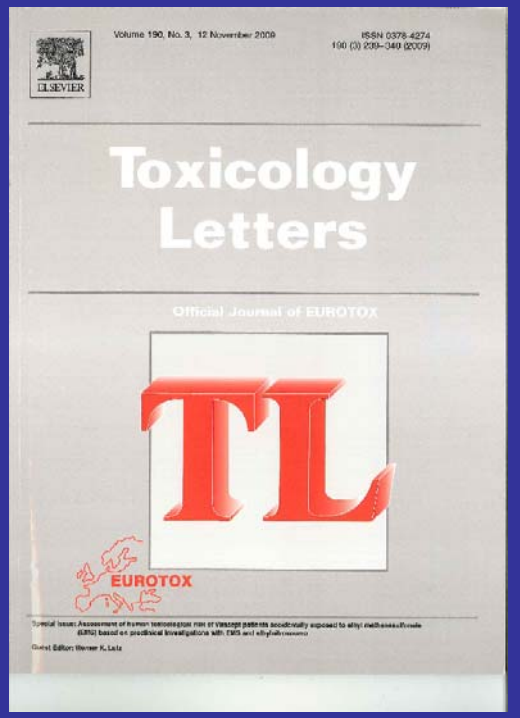
THERE IS A THRESHOLD FOR MUTAGENESIS BY DNA DAMAGING MUTAGENS !!!!

(at least for some)

The cell can repair large amounts of exogeneously induced DNA damages FULLY ERROR FREE

A paradigm shift in risk assessment of genotoxic carcinogens

The complete data (incl. ADME and general Tox studies) and detailed risk assessment are published as special issue in Tox. Letters



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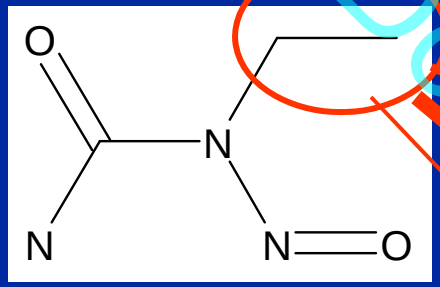
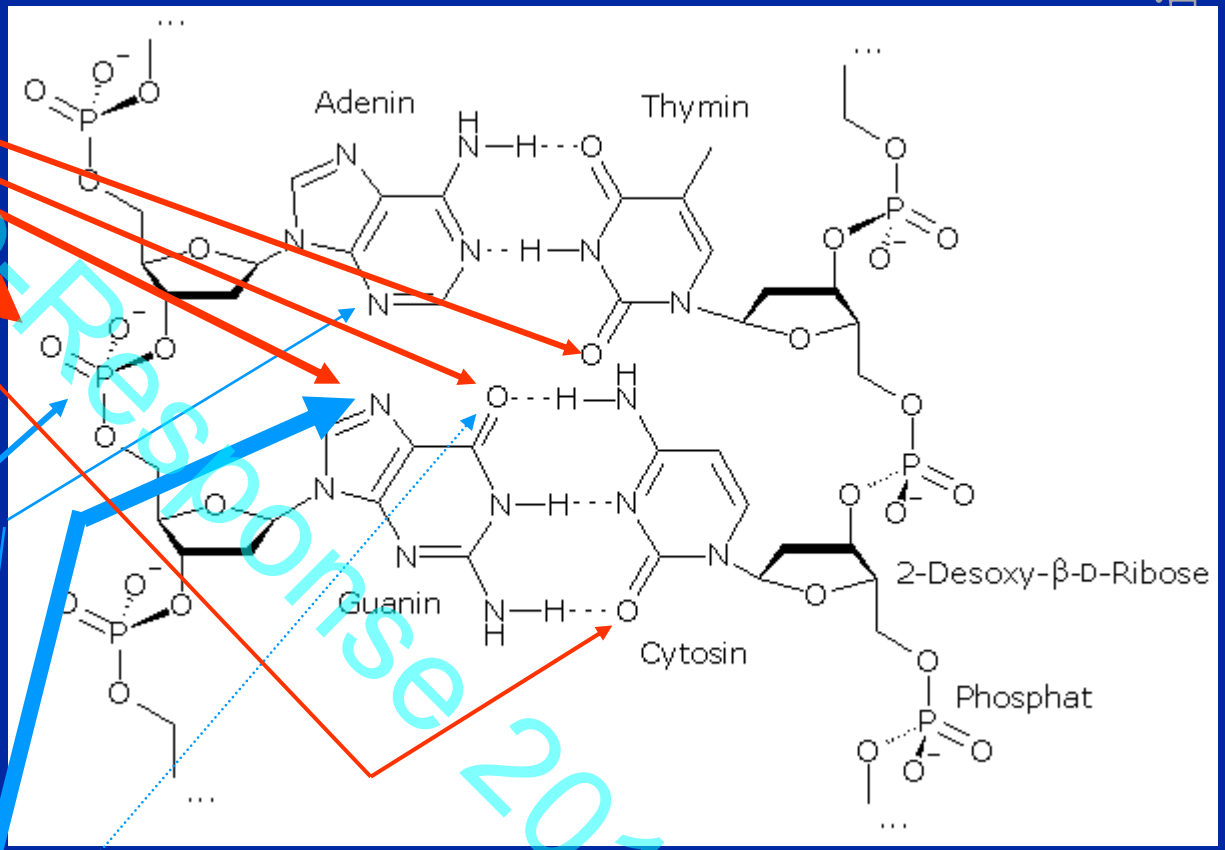
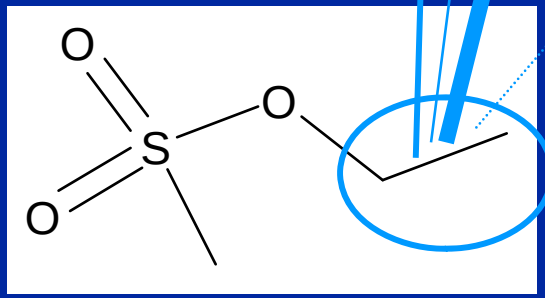
...and what about a threshold for ENU?



Ethylnitrosourea

ENU ethylates stronger at O^6-G; O^2-T, O^2-C





Ethylmethanesulfonate

EMS ethylates DNA predominantly at N^7-Guanine

Dose-Response 2010

Comparison of adduct formation:
based on ethylvaline adducts (our study) and localisation of adducts in DNA (Beranek et al 1990)

ENU induces about 60 fold more adducts at oxygen (O^6G,O^2T,O^4T,O^2C) than EMS at the same dose

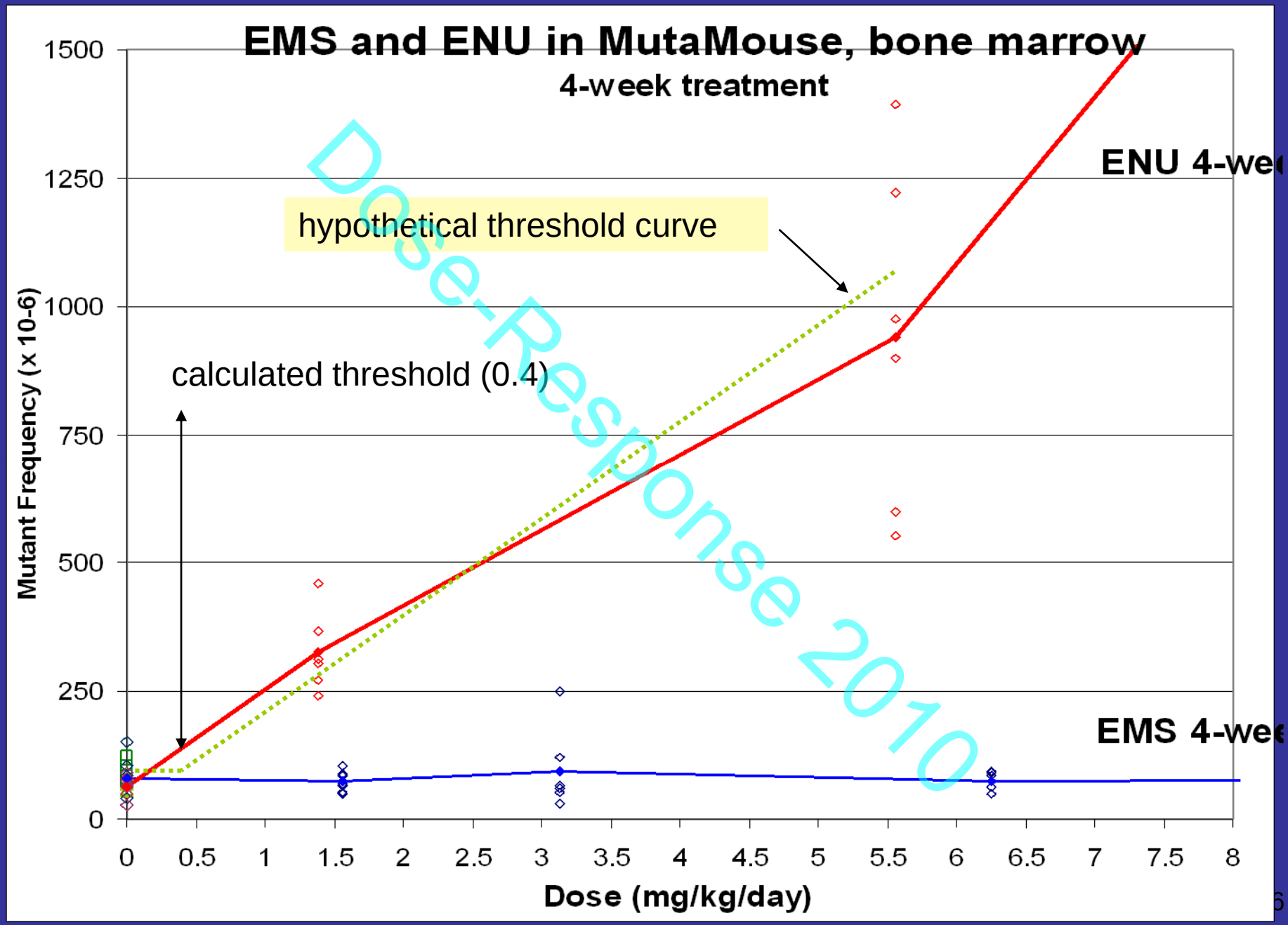
Table 1. DNA adduct profiles for MMS, MNU, EMS and ENU

Adduct	MMS	EMS	MNU	ENU
s value	>0.83	0.67	0.42	0.26
N^7-G	81–83	58–65	65–70	11–11.5
N^3-G	0.6	0.3–0.9	0.6–1.9	0.6–1.6
N^7-A	1.8	1.1–1.9	0.8–2	0.3–0.6
N^3-A	10.4–11.3	4.2–4.9	8–9	2.8–5.6
N^3-T	0.1	Nd	0.1–0.3	0.8
N^3-C	<1	0.4–0.6	0.06–0.6	0.2–0.6
O^6-G	0.3	2	5.9–8.2	7.8–9.5
O^2-T	Nd	Nd	0.1–0.3	7.4–7.8
O^4-T	Nd	Nd	0.1–0.7	1–2.5
O^2-C	Nd	0.3	0.1	2.7–2.8
Phosphotriesters	0.8	12–13	12–17	55–57

NOTE: Adapted from Beranek (13). Data are in percentages; all possible adducts were not included, so columns do not add up to 100%.
Abbreviation: Nd, not detected.

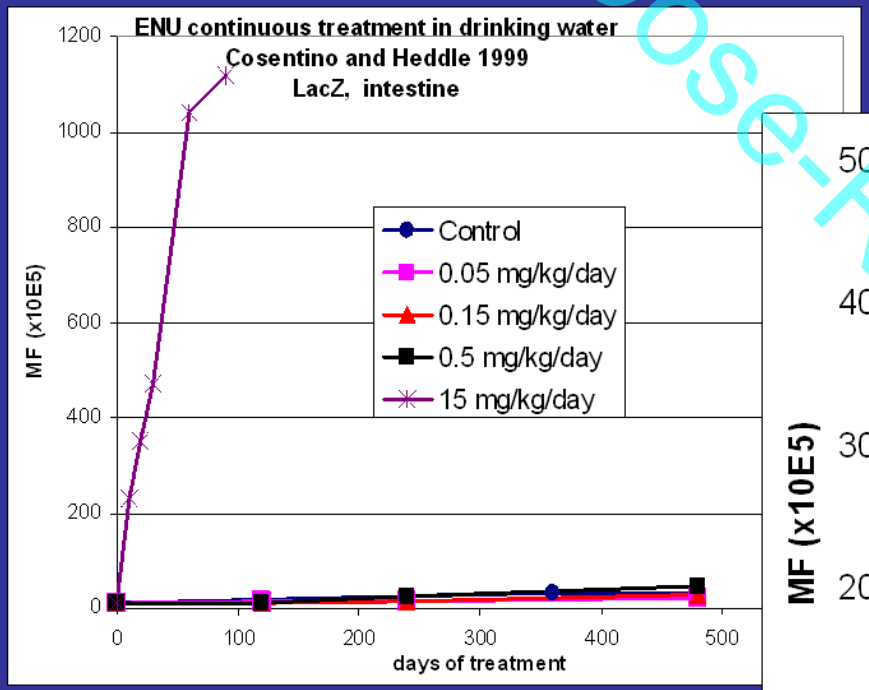
If thresholds arise due to oxygen adducts the threshold for ENU should be

25 mg/kg/day/60 = 0.4 mg/kg/day for gene mutations in bone marrow, GI tract
50 mg/kg/day/60 = 0.8 mg/kg/day for gene mutations in liver

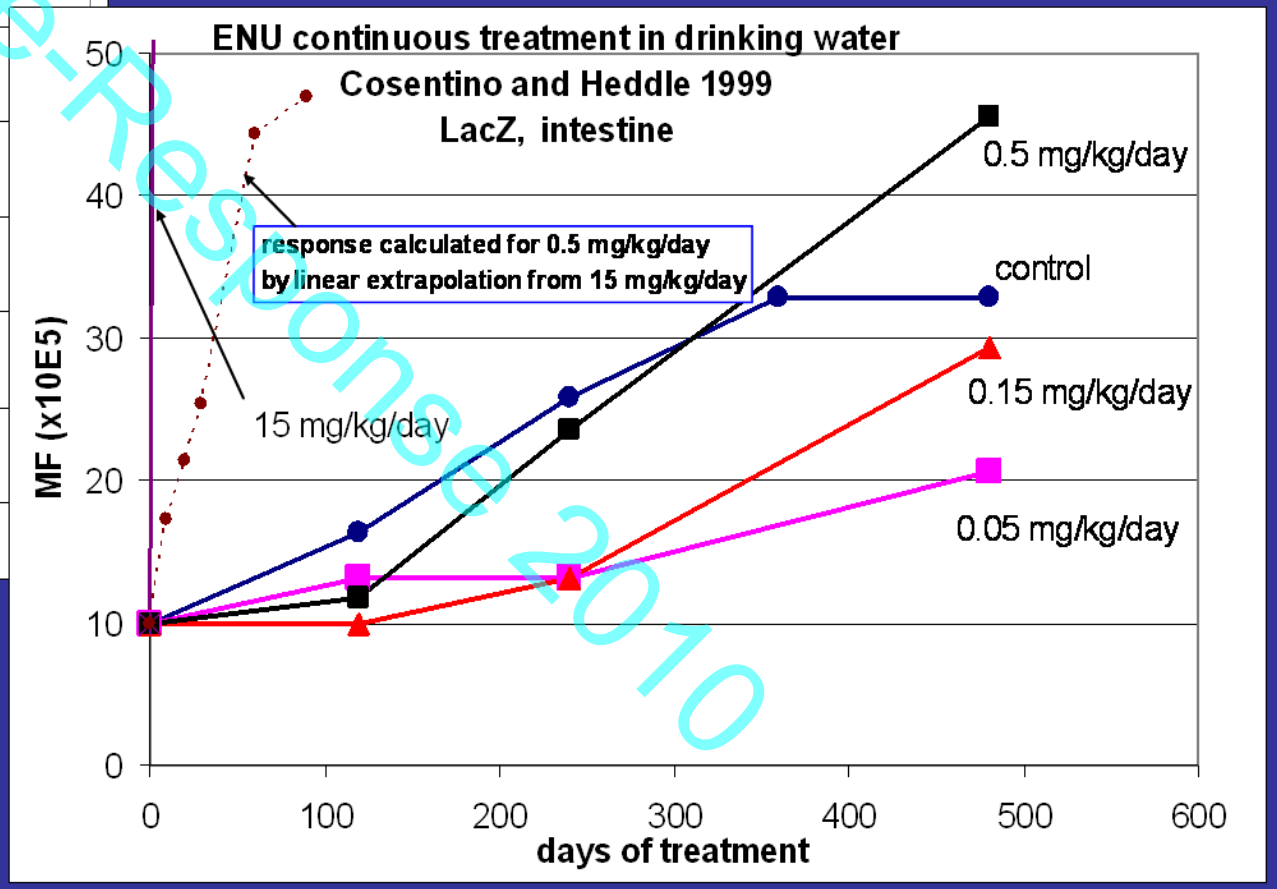

EMS and ENU in MutaMouse, bone marrow
4-week treatment
ENU 4-we
EMS 4-we
hypothetical threshold curve
calculated threshold (0.4)
Dose-Response 2010
Mutant Frequency (x 10-6)
1500
1250
1000
750
500
250
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
Dose (mg/kg/day)

in vivo data: Continuous ENU treatment in drinking water
Sampling after 120,240, 360, 480 days with 0.05 to 0.5 mg/kg/day
10, 20, 30, 90 days for 15 mg/kg/day
(redrawn from Cosentino and Heddle 1999)

LacZ Mutations

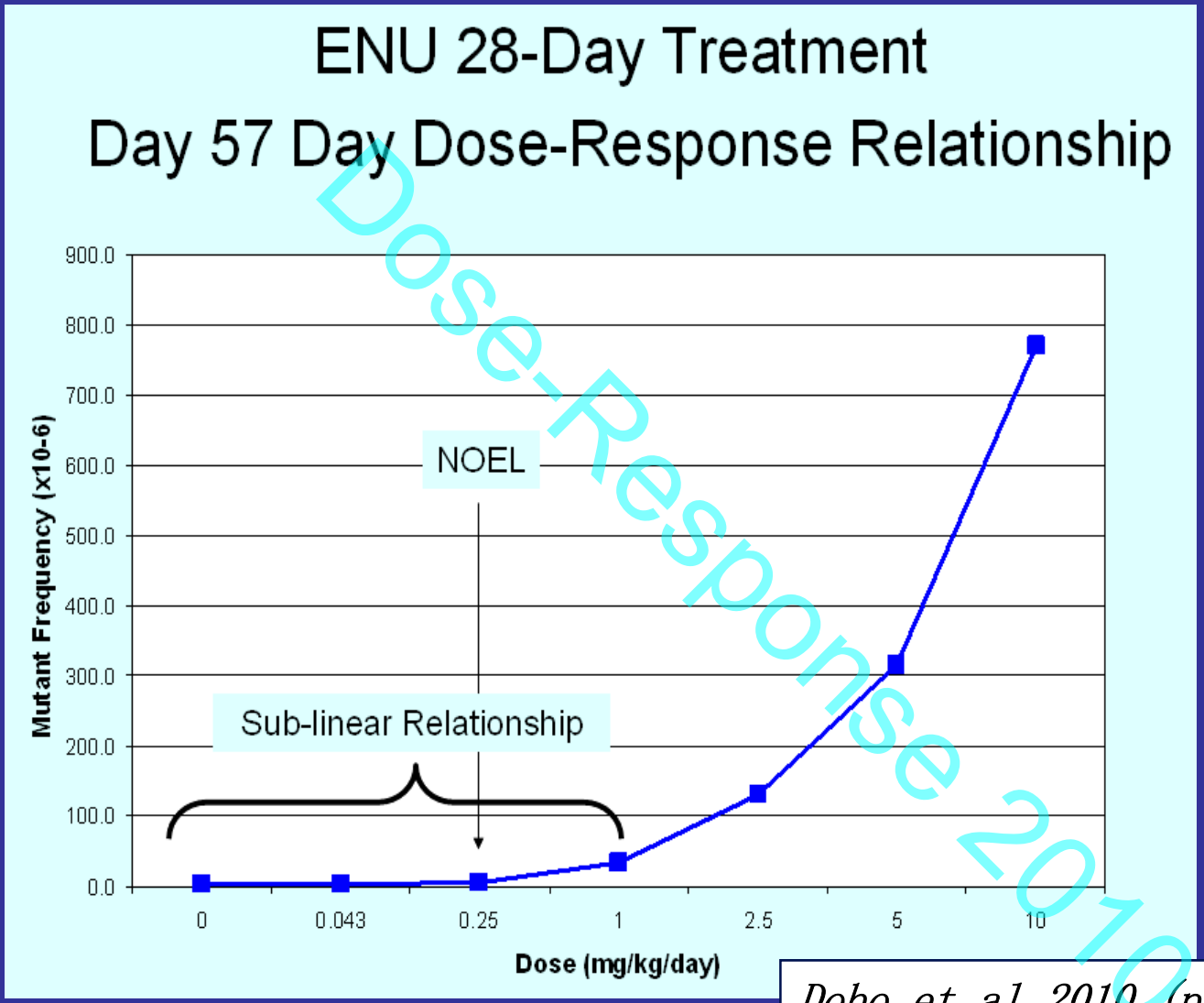


expansion of y-axis



→ at most marginal effect at 0.5 mg/kg/day, much lower than predicted by linear extrapolation

pigA gene mutation test in rats



Dobo et al 2010 (pers comm)

Also for ENU there is a threshold for in vivo induction of mu