

Risk assessment of genotoxic impurities: the case of EMS

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What happened:

- **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 (EMA, 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

Consequences:

- Maximal content of EMS in API: ca 2300 ppm
- Maximal content of EMS in tablets: ca 1000 ppm (TTC level: 0.6 ppm)
- Maximal dose of EMS in patients: 0.055 mg/kg
- Maximal duration of exposure: 3 months
- Number of patients: $\leq 40\ 000$

**What risk for adverse effects?
(cancer, birth abnormalities, heritable defects)**

What do we know from literature about EMS:

EMS is mutagenic

- belongs to the most often employed mutagens
- often used as reference mutagen when establishing a new test system
- the mechanism of action is well understood
- but dose response analysis to very low, and multiple doses is scarce

EMS is carcinogenic

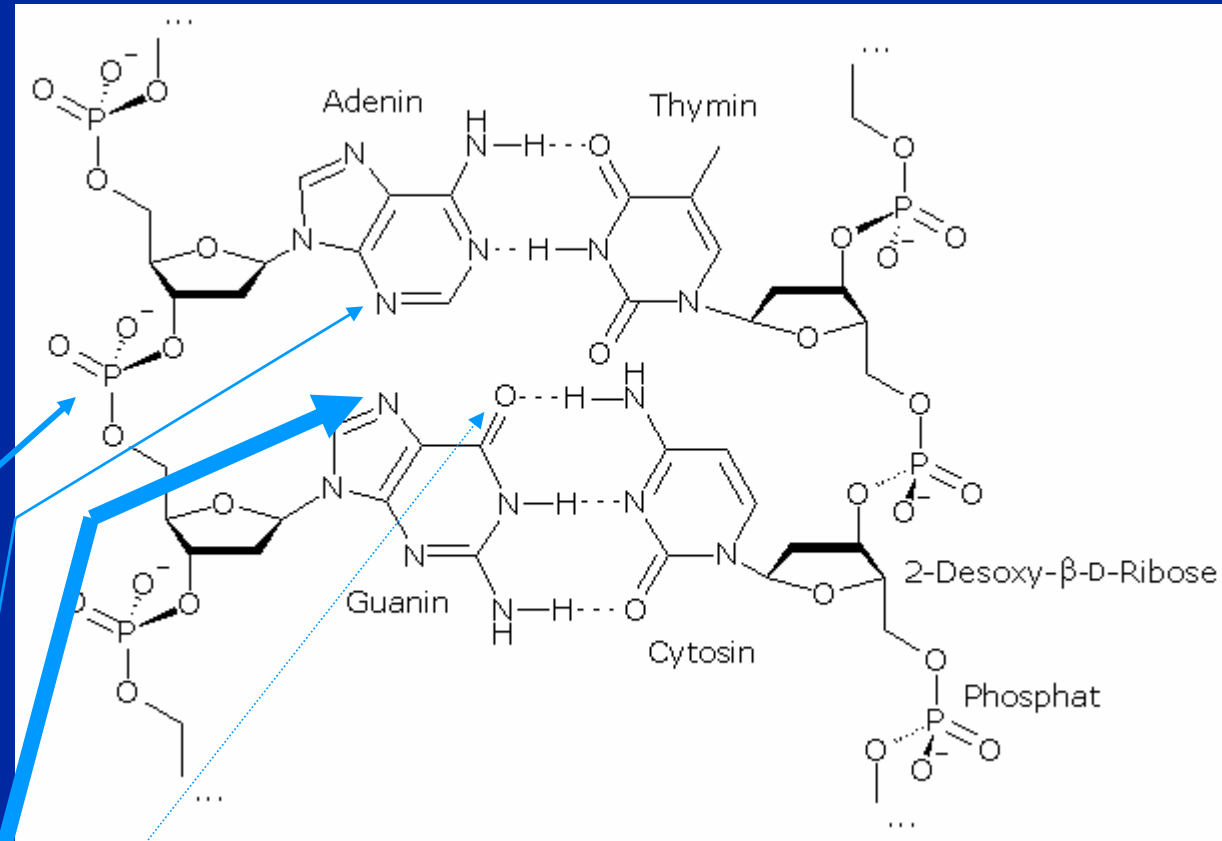
- carcinogenicity data are `patchy` (no NTP longterm study)

EMS is teratogenic

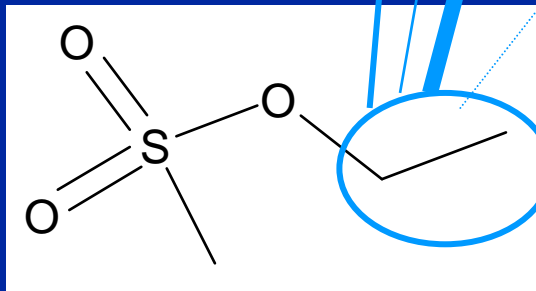
- extensively investigated by group of Platzek

EMS is a `laboratory chemical`

- there is no known environmental exposure -
--> no human data



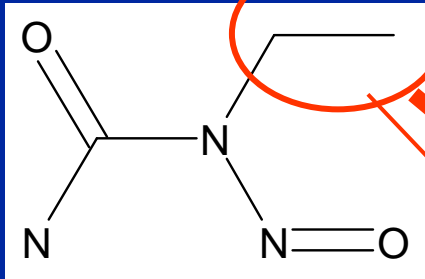
DNA damage by EMS



Ethylmethanesulfonate

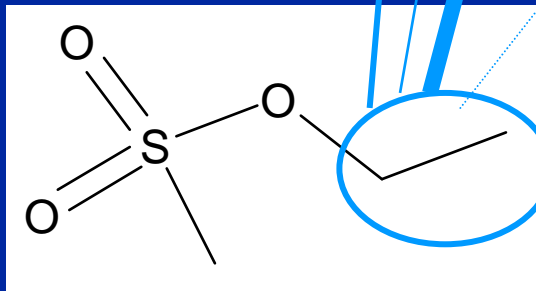
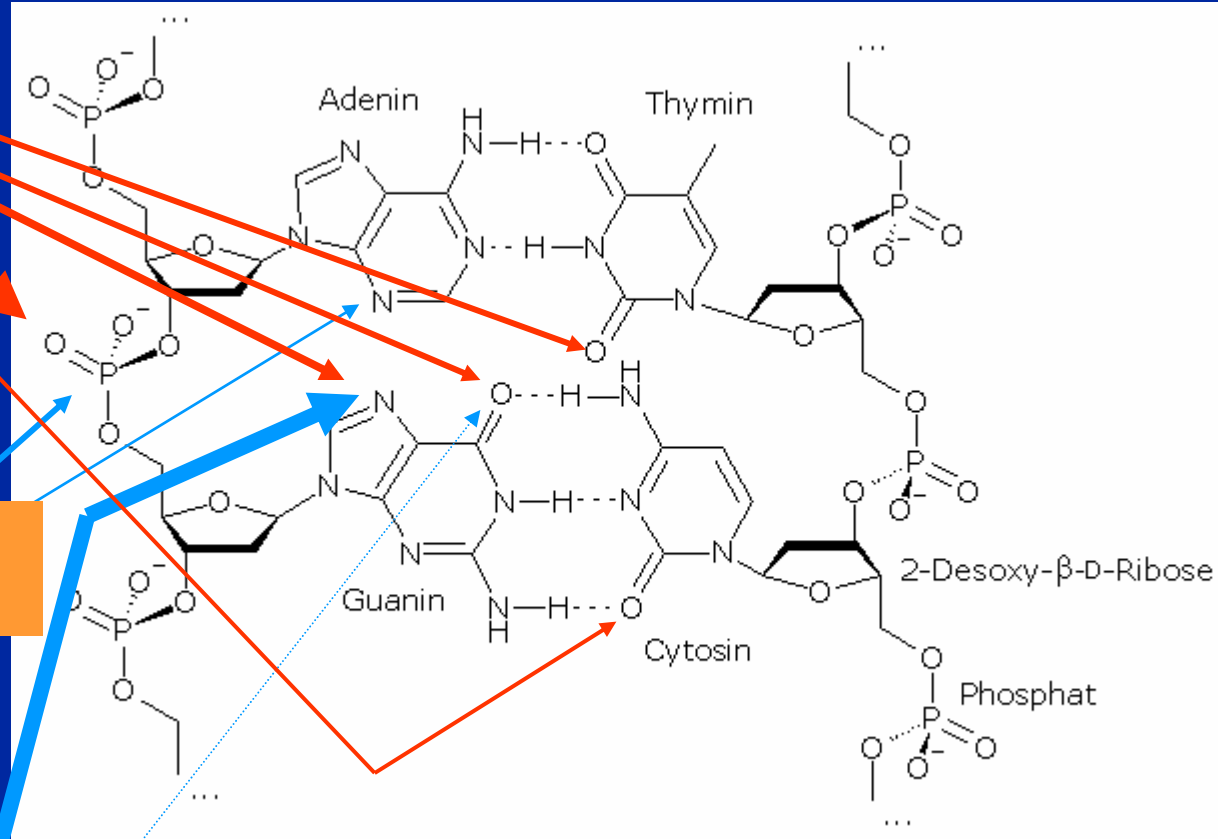
EMS ethylates DNA predominantly at N⁷-Guanine

DNA damage by ENU



Ethylnitrosourea

ENU ethylates stronger at O⁶-G; O²-T, O²-C

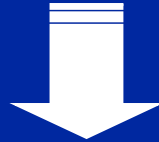


Ethylmethanesulfonate

EMS ethylates DNA predominantly at N⁷-Guanine

Akylators are thought to damage DNA in a largely random fashion

- the dose response for mutation is generally considered linear
- by default the dose response for cancer, teratogenicity is also thought to be linear



**every exposure carries a definite risk
(there is no safe dose)**

Based on available data (NTP cancer study with MMS) we estimated a maximal increase of the cancer incidence by about 1 in 10^4 patients

Chemical mutagenesis of mammalian cells can be quantified

Nature Vol. 269 27 October 1977

J. P. O'NEILL
A. W. HSIE

Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

dose response appears linear when plotted against concentration or exposure (conc. x time)

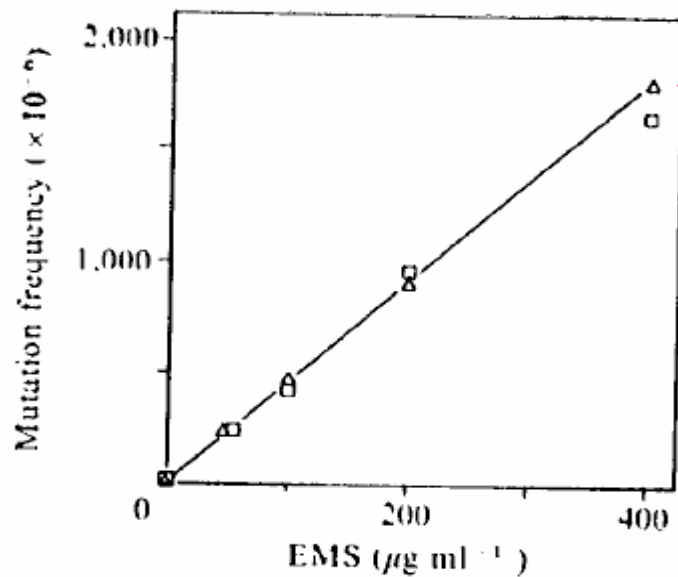


Fig. 2 Dose response of EMS-induced mutagenesis. Data from two separate experiments are presented; the average of the mutation frequencies at days 7, 9, 11, and 13 from Fig. 1 (○) (average spontaneous frequency $3.1 \cdot 10^{-6}$); and the frequencies for 24-h incubation time from Fig. 3 (△).

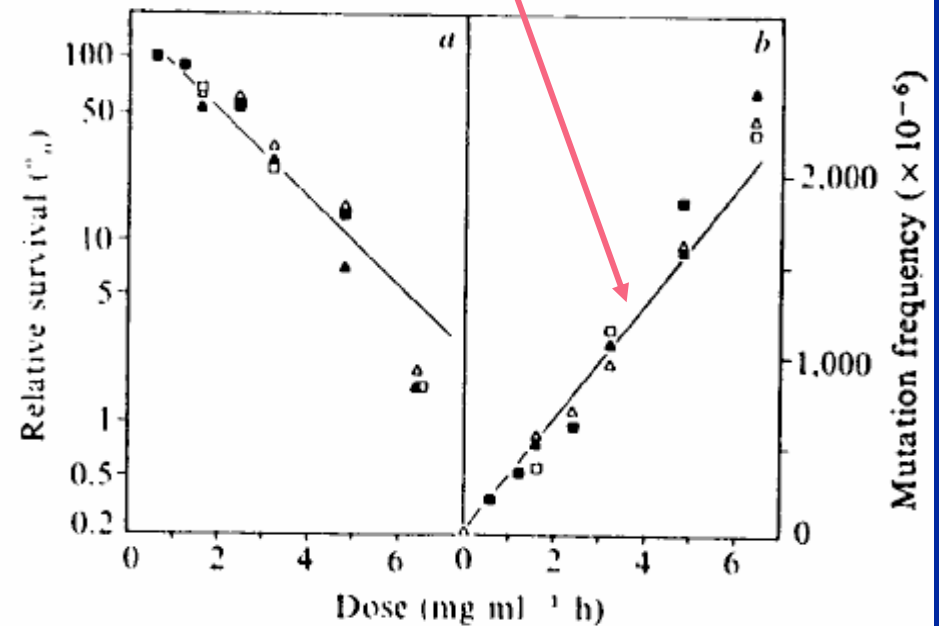


Fig. 4 Dosimetry for EMS cellular lethality (a) and induced mutation frequency (b). Cells were treated with various concentrations (mg ml^{-1}) of EMS for 2 (○), 4 (▲), 8 (□), or 12 h (■).

But the lowest concentration tested was 50 $\mu\text{g/ml}$

However,

Cellular defense mechanisms (scavenging, inactivation, repair) could reduce or abolish the effects at low dose levels;

→ sublinear dose response

→ **thresholded dose response**



exposure below threshold carry no risk
(there is a safe dose)

Mechanistic Influences for Mutation Induction Curves after Exposure to DNA-Reactive Carcinogens

Shareen H. Doak, Gareth J.S. Jenkins, George E. Johnson, Emma Quick, Elizabeth M. Parry, and James M. Parry

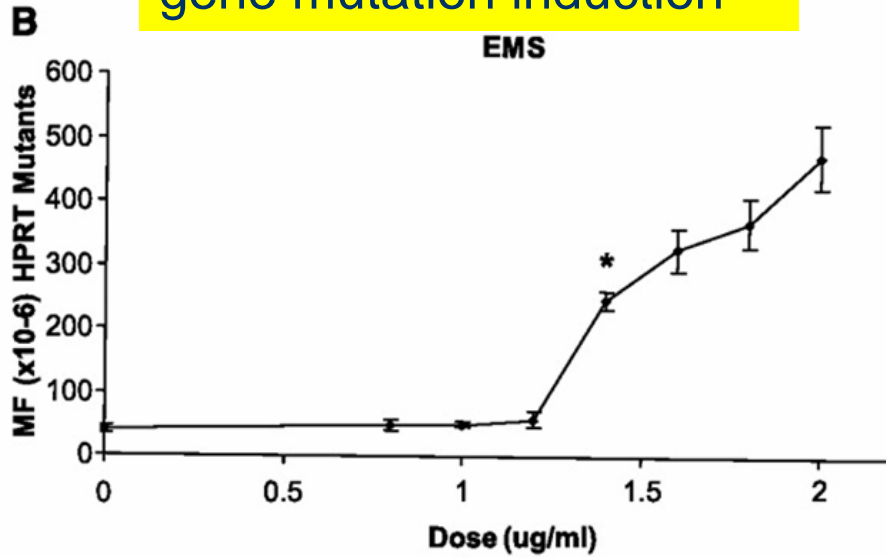
School of Medicine, University of Wales Swansea, Swansea, Wales, United Kingdom

Cancer Res 2007; 67: (8). April 15, 2007

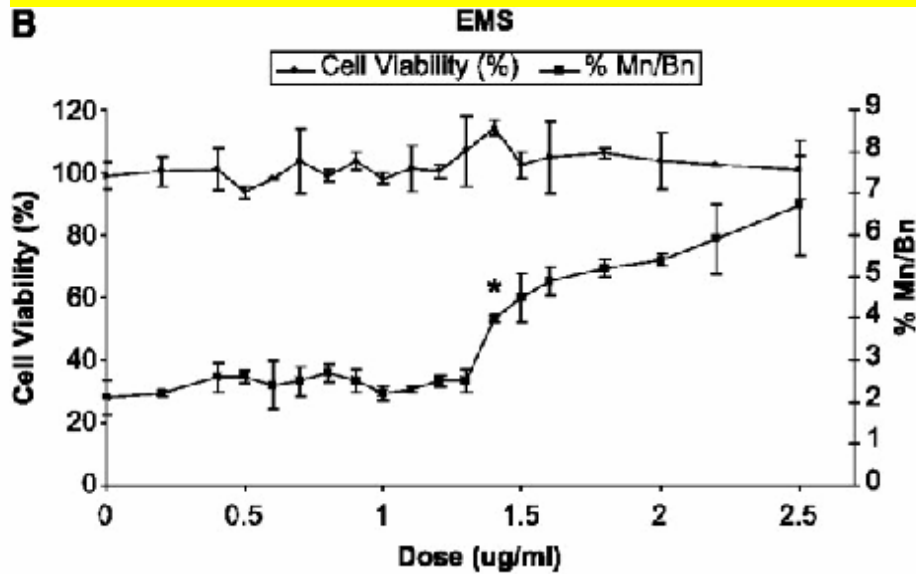
In vitro genotoxicity EMS



gene mutation induction

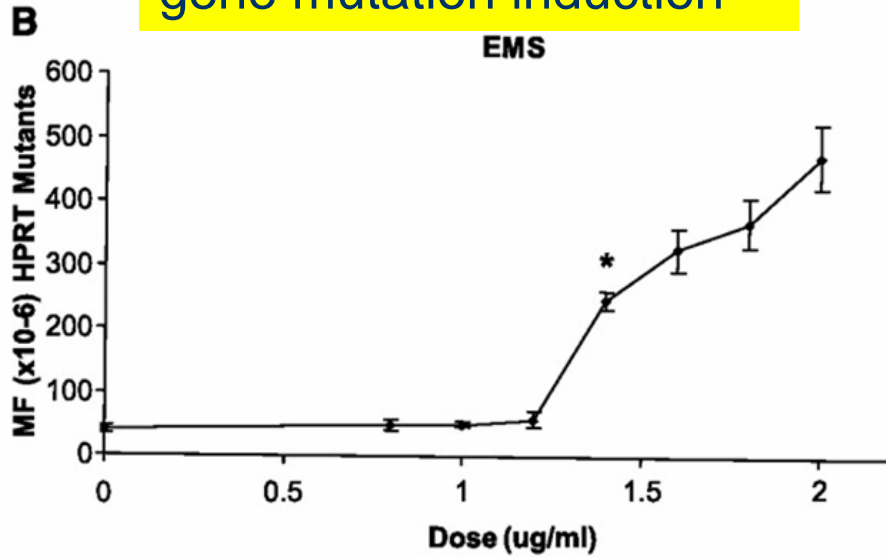


chromosomal damage induction



In vitro genotoxicity EMS

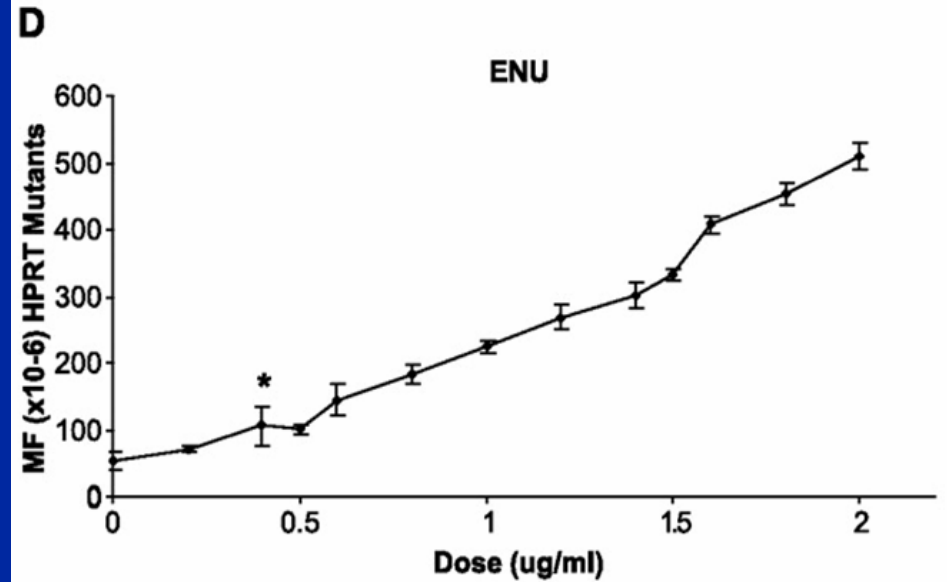
gene mutation induction



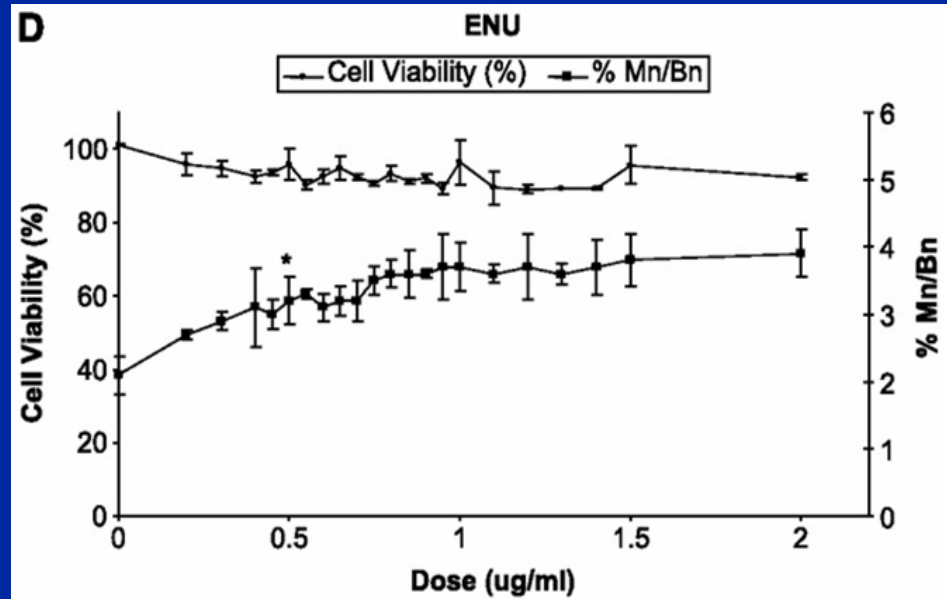
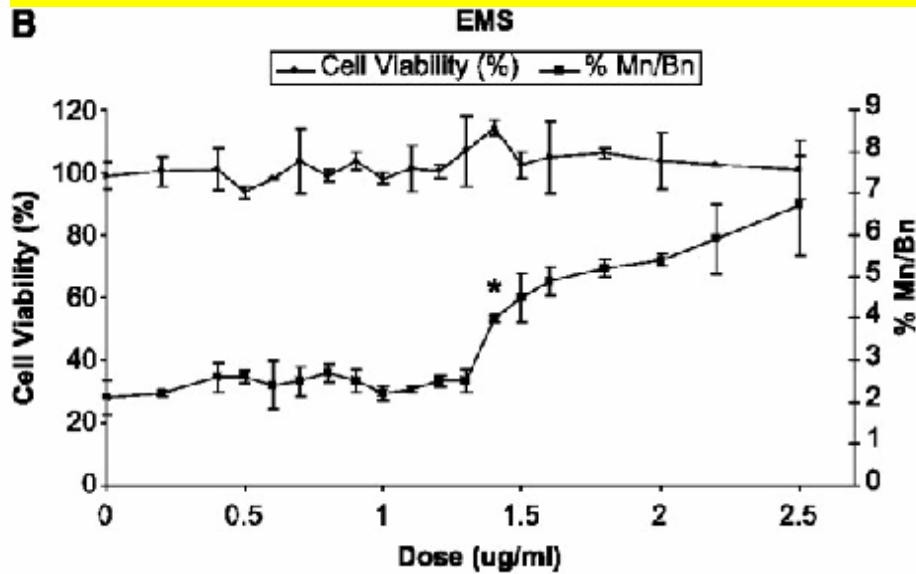
versus ENU



als



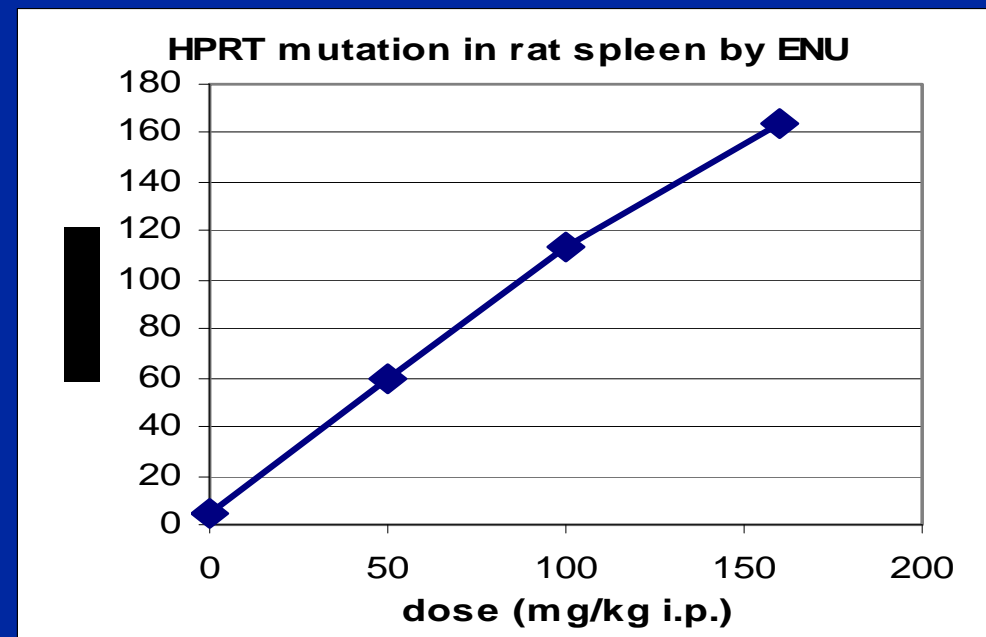
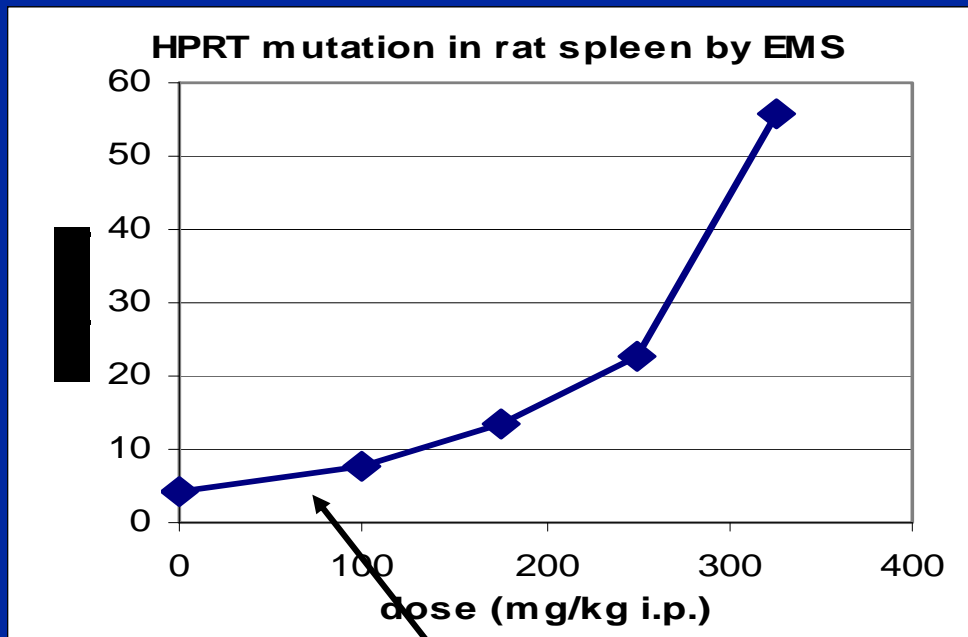
chromosomal damage induction



For risk assessment we need in vivo studies

- In a few studies EMS was tested in parallel to ENU and dose response curves were taken

In vivo gene mutation induction: EMS vs ENU



(data from Jansen et al 1997)

clearly sublinear!

But is it thresholded?

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



Non-clinical program to assess the risk of EMS

Study 1: General toxicity General 4-week toxicity study in the rat with EMS	Aim: To establish basic knowledge of EMS organ toxicity and clinical chemistry/ haematology assessments in conjunction with exposure data
Study 2: Genotoxicity (MNT) Induction of chromosomal damage in bone marrow of mice after repeat dosing (7 days) with EMS and ethyl nitrosourea (ENU)	Aim: To provide evidence of a threshold dose response for EMS in low doses for chromosome damage compared to a linear dose-effect for ENU
Study 3: Genotoxicity (MutaMouse) Induction of LacZ gene mutations in MutaMouse (transgenic model) after 28-day oral dosing with EMS and ENU	Aim: To provide evidence of a threshold dose response for EMS in low doses for gene mutations compared to a linear dose-response for ENU
Studies 4: Prediction to man Cross-species in vitro and in vivo evaluation of exposure to EMS (mouse, rat, monkey, human)	Aim: To retroactively facilitate exposure judgment in patients having taken elevated levels of EMS via Viracept

EMS Study 1: 4-wk General Toxicity Study in the Rat

– Purpose & Design

- Purpose: Systemic/organ toxicity of EMS (no published data)
 - Support assessment of absence of any primary organ toxicities in exposed Viracept patients
- Doses: 20 – 60 – 180 / 120 mg/kg/day p.o.
- Duration: 4 weeks (high dose 19 days)
- Exposure monitoring: Hemoglobin adducts

EMS: 4-wk general toxicity study in the rat - results

DNA- and RNA damage



- Hematopoiesis (white and red cell system)
- Spermatogenesis (testes, epididymides)
- Thymolymphatic system (thymus, lymph nodes)

Starvation



- Thymolymphatic system (thymus, lymph nodes)
- Other sexual organs
- Thyroid gland
- Pancreas
- Spleen

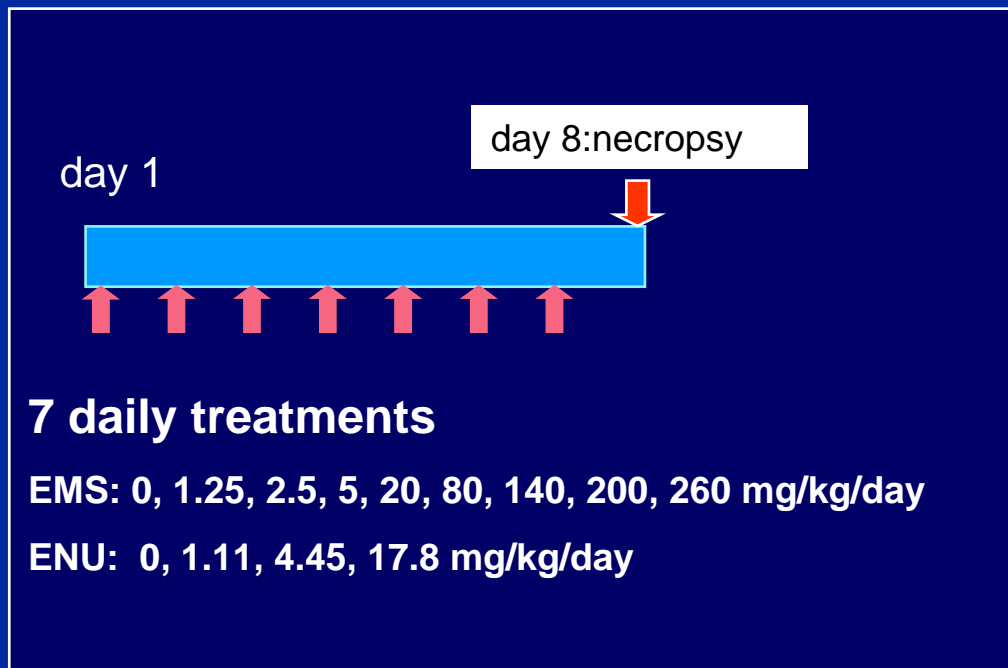
- No Observed Adverse Effect Level (NOAEL) = 20 mg/kg/day
- → All observed effects can be explained as consequence of genotoxicity of EMS or as secondary to starvation at high dose

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Study 2: Induction of chromosomal damage in bone marrow of mice

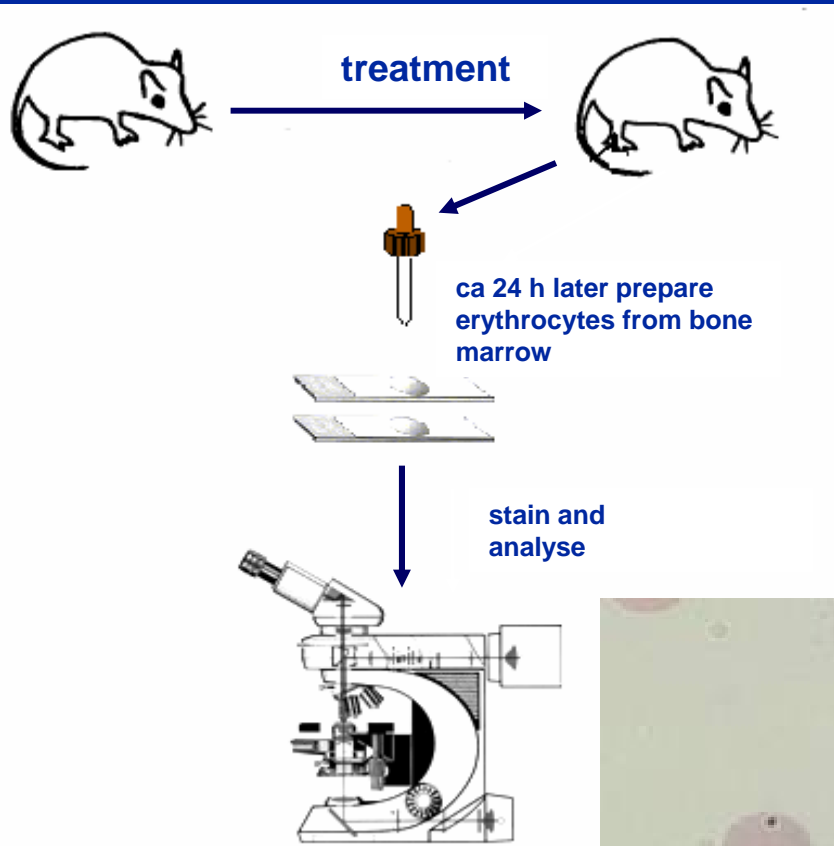


- Erythrocytes prepared from bone marrow for assessment of micronuclei
- Assessment of exposure via ethyl- adducts in terminal valine of hemoglobin

Micronucleus Test *in vivo* (mouse bone marrow)



- Detection of chromosome damage or damage to the spindle apparatus *in vivo*



Erythroblast

NUCLEAR DIVISION

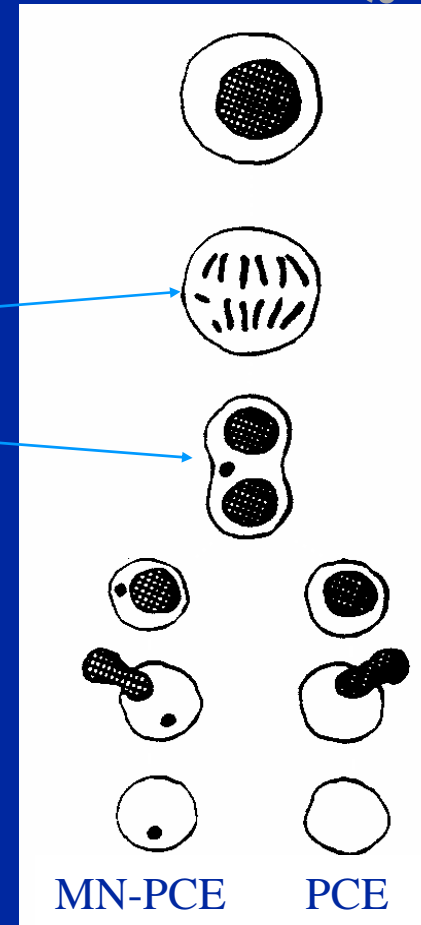
Chromosome fragment or unattached chromosome

Micronucleus

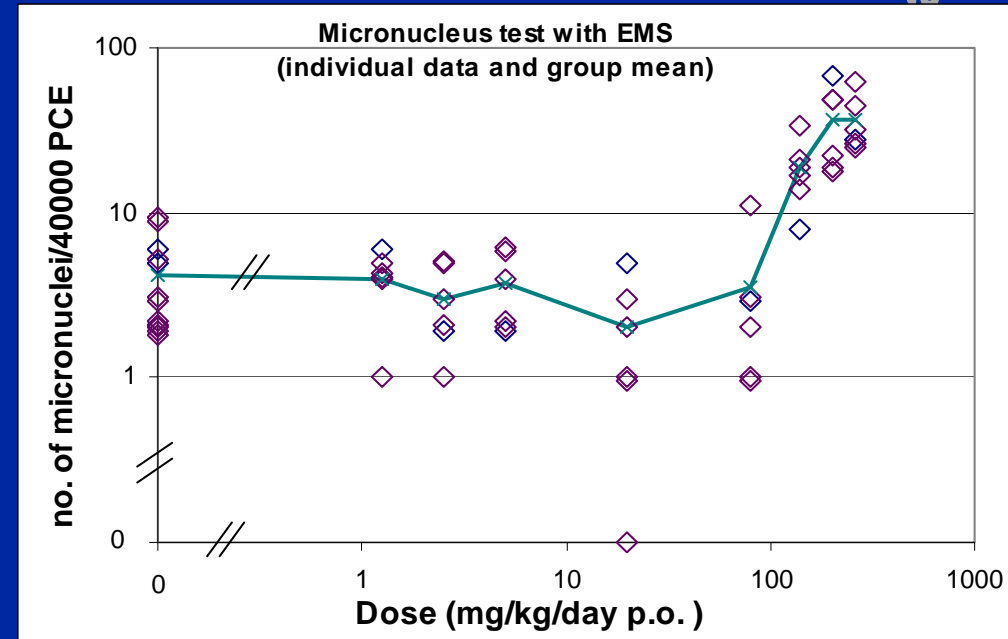
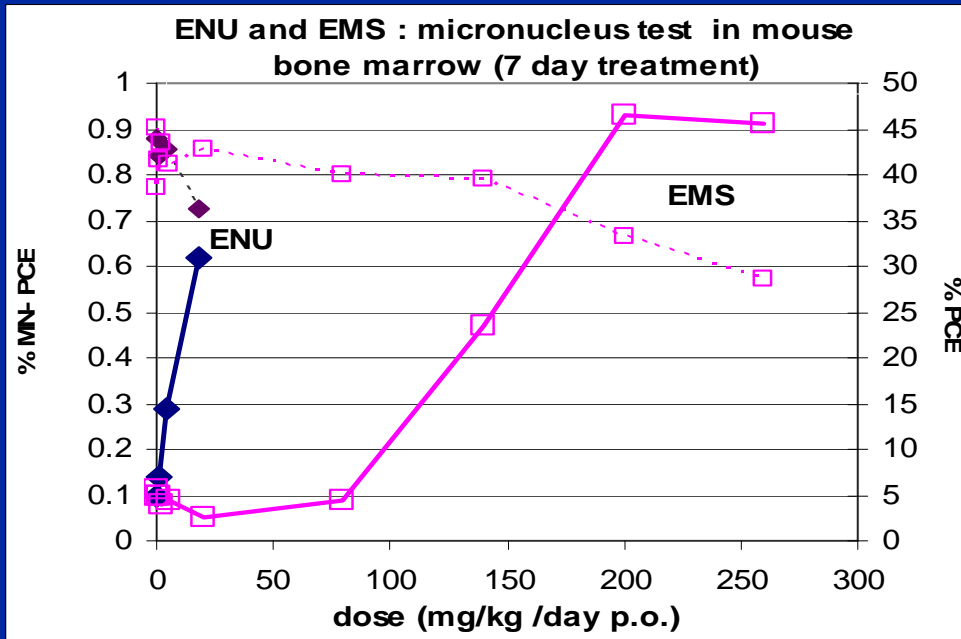
CELL DIVISION

EXPULSION OF NUCLEUS

Erythrocytes

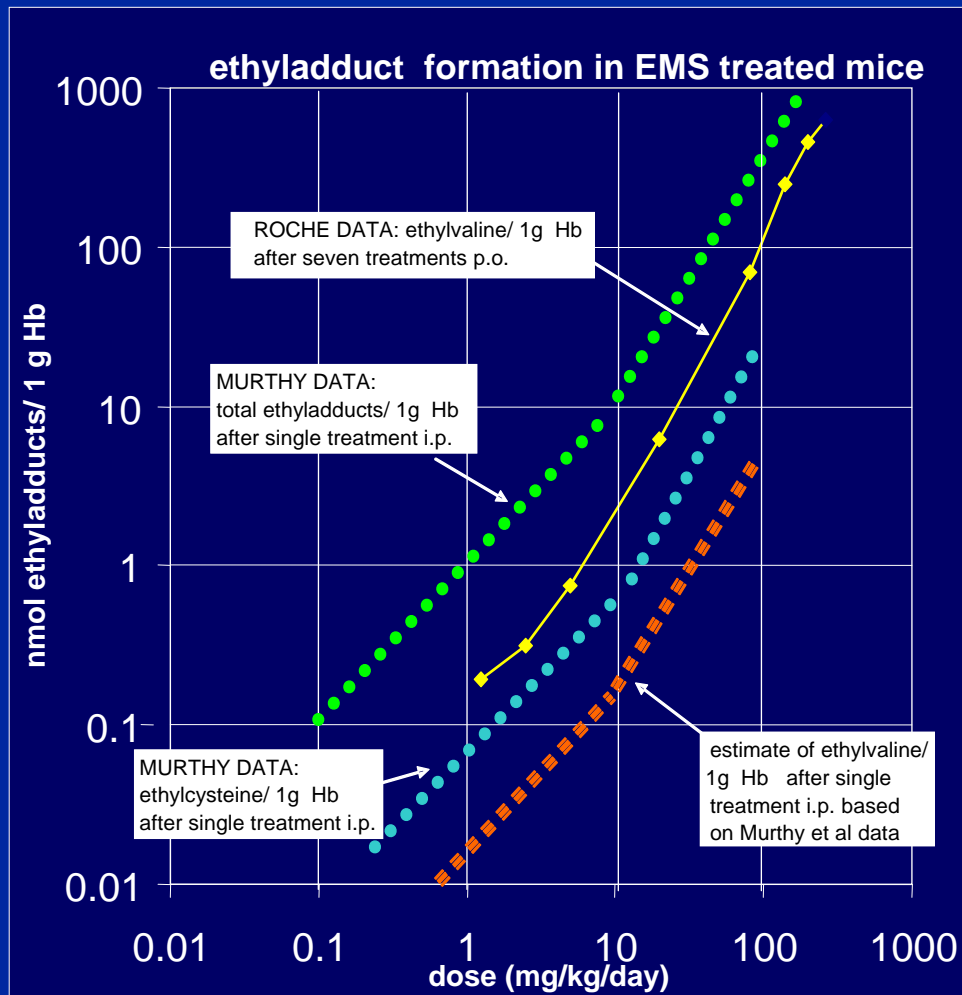


Micronuclei as a function of dose



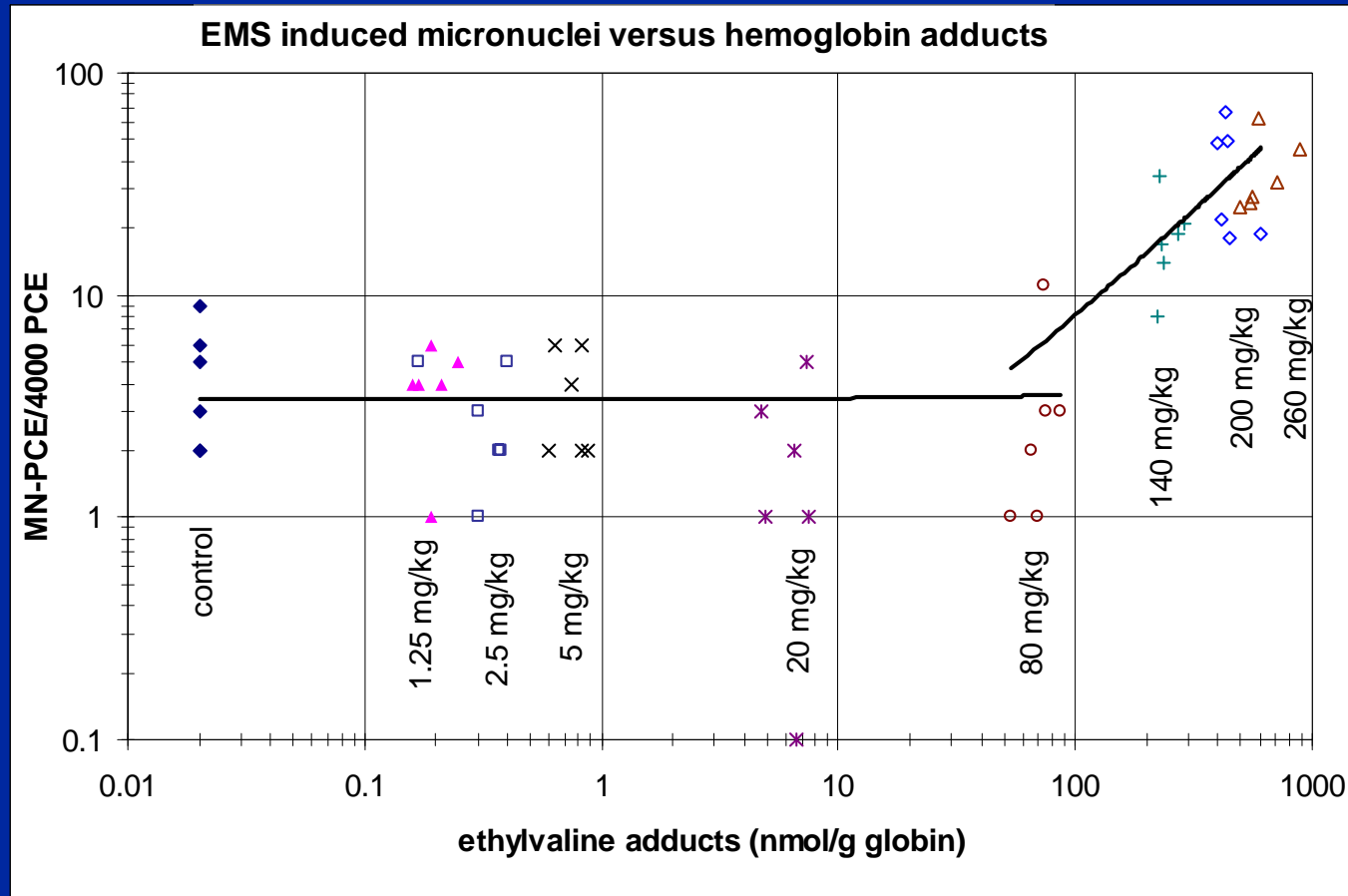
- EMS induces a thresholded dose response (80 mg/kg/day)
- ENU induces a linear response

Ethylvaline formation in globin as a biomarker of exposure



- Roughly linear increase at low doses, supralinear 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)



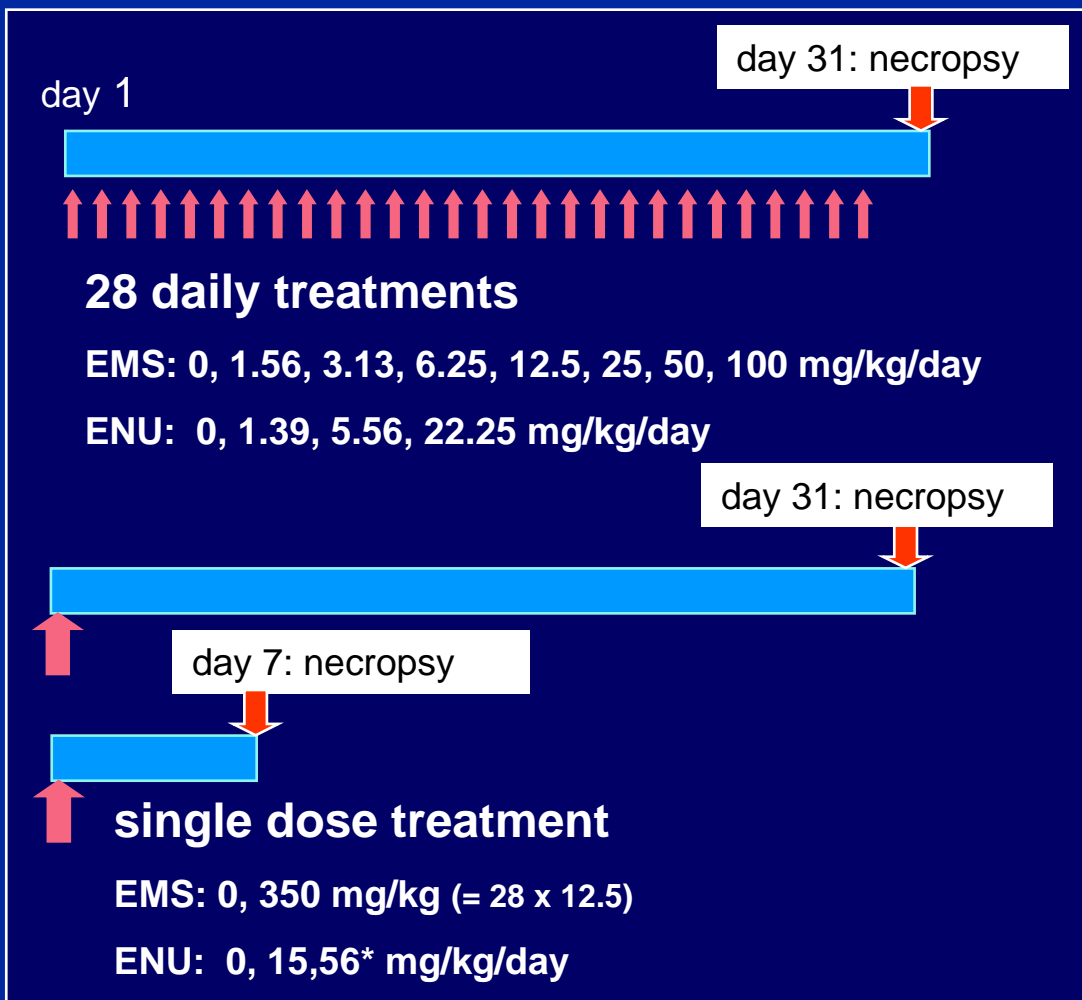
- No increase of micronuclei below ~100 nmol/g globin
(more than 1000 fold higher than background level of < 0.1 nmol/g globin)

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Study 3: Induction of LacZ gene mutations in MutaMouse (transgenic) model

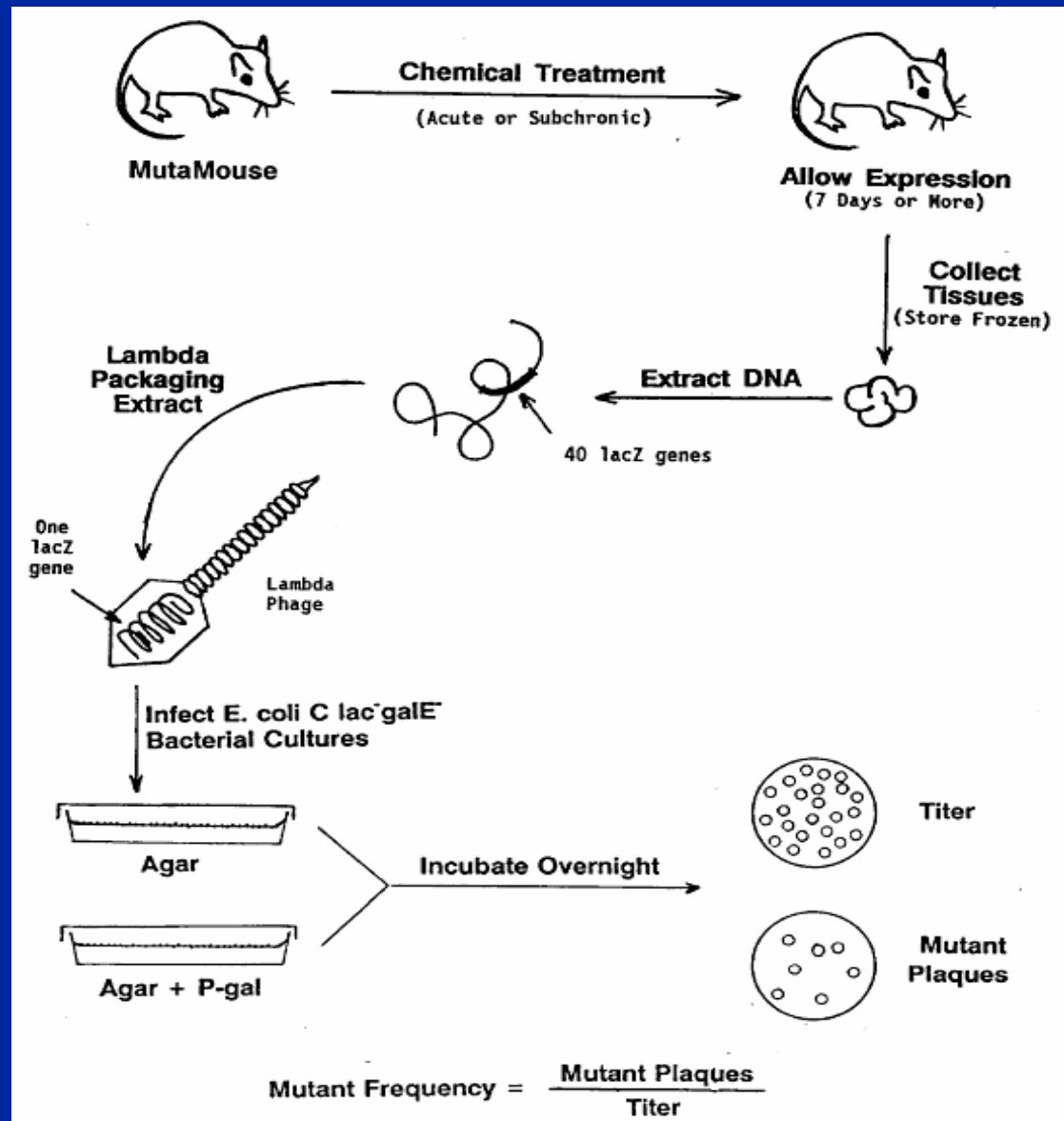


*planned was a tenfold higher dose (28 x 5.56 = 156 mg/kg)

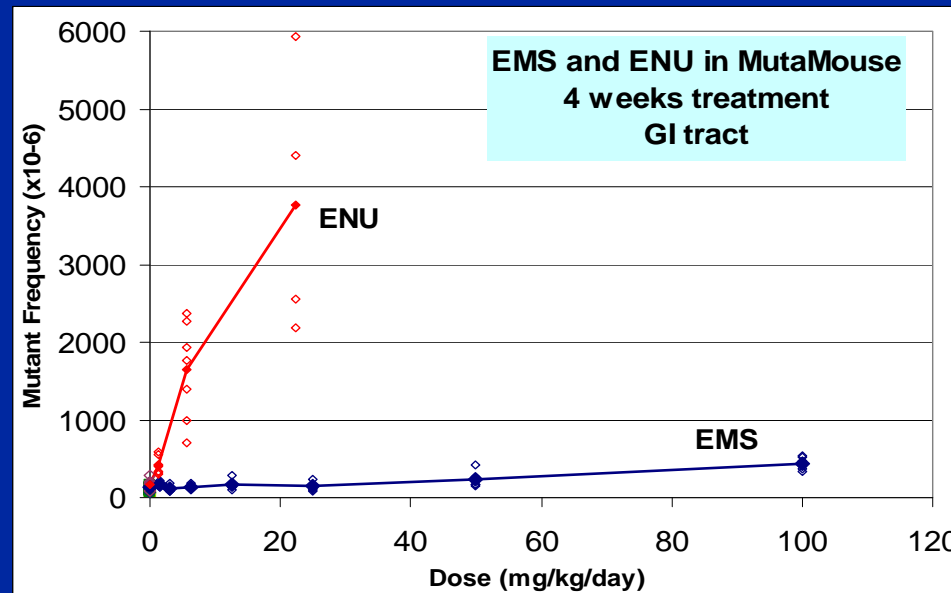
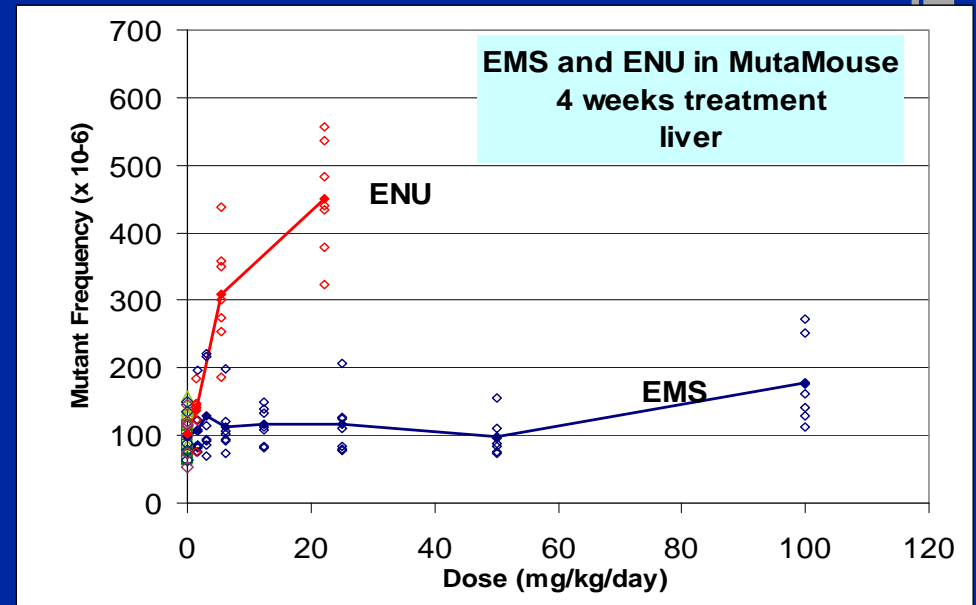
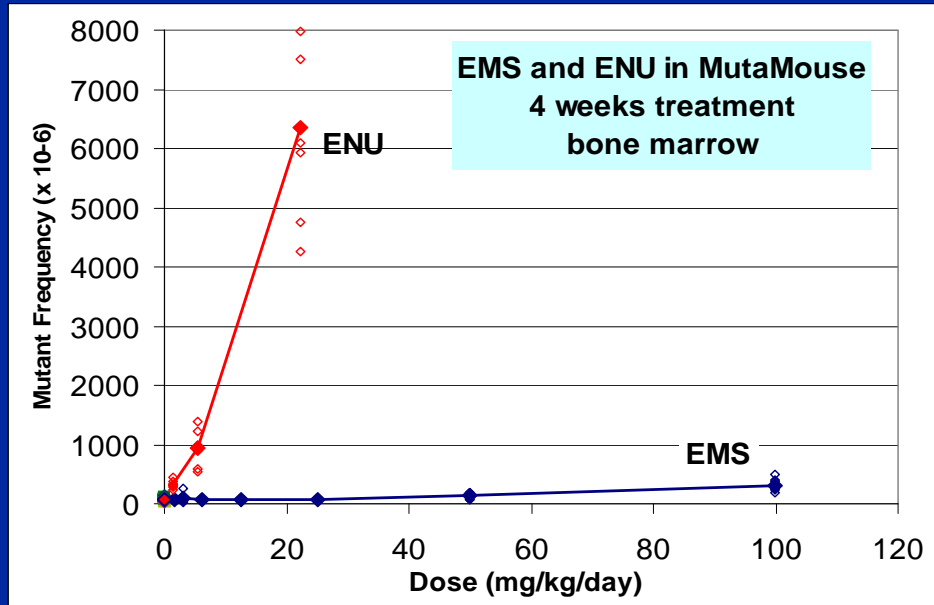


- DNA extracted from
 - bone marrow
 - liver
 - GI tract
- Assessment of exposure via ethyl- adducts in terminal valine of hemoglobin

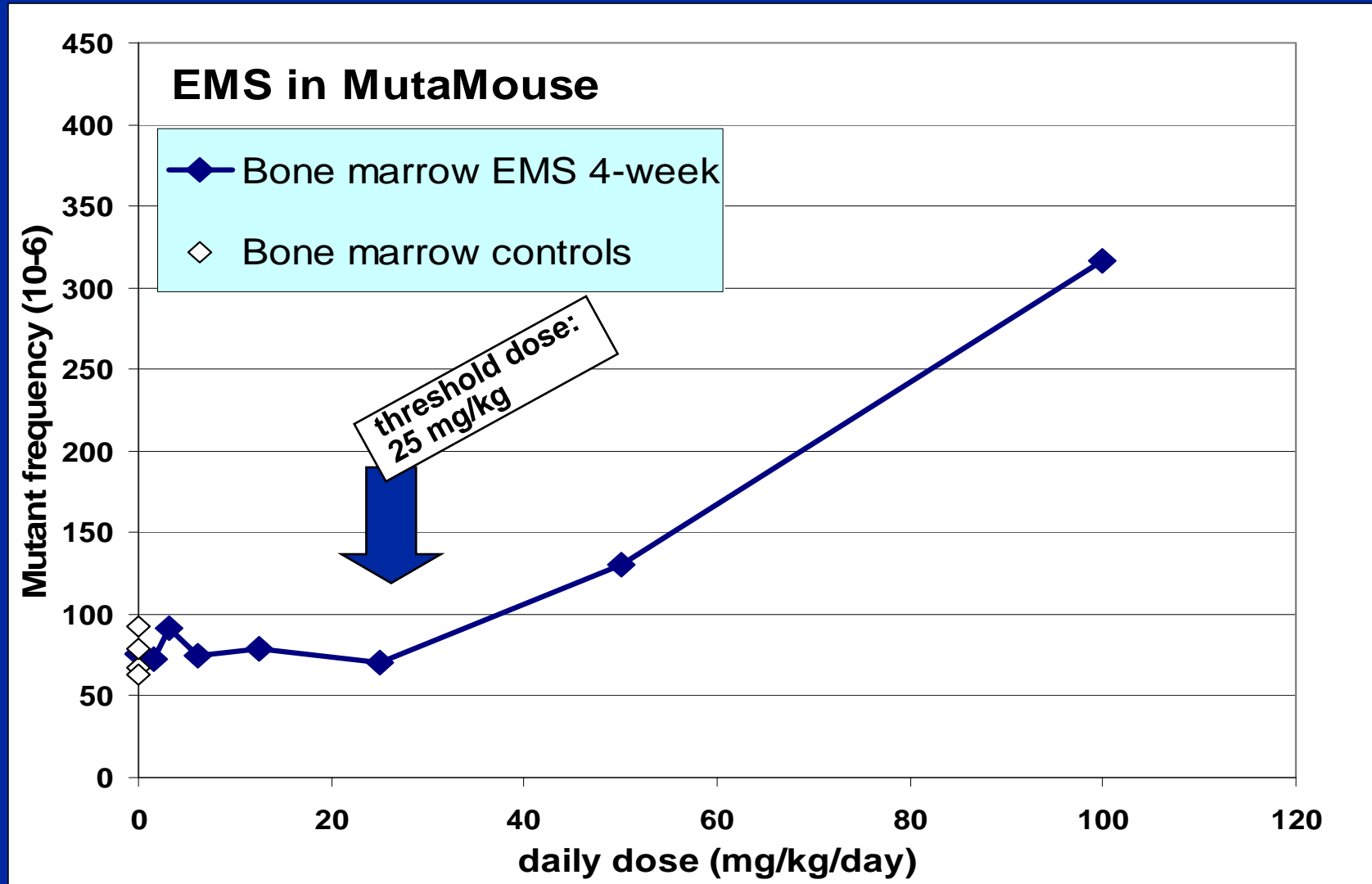
MutaMouse Test : *in vivo* test for gene mutations at transgenic lacZ locus



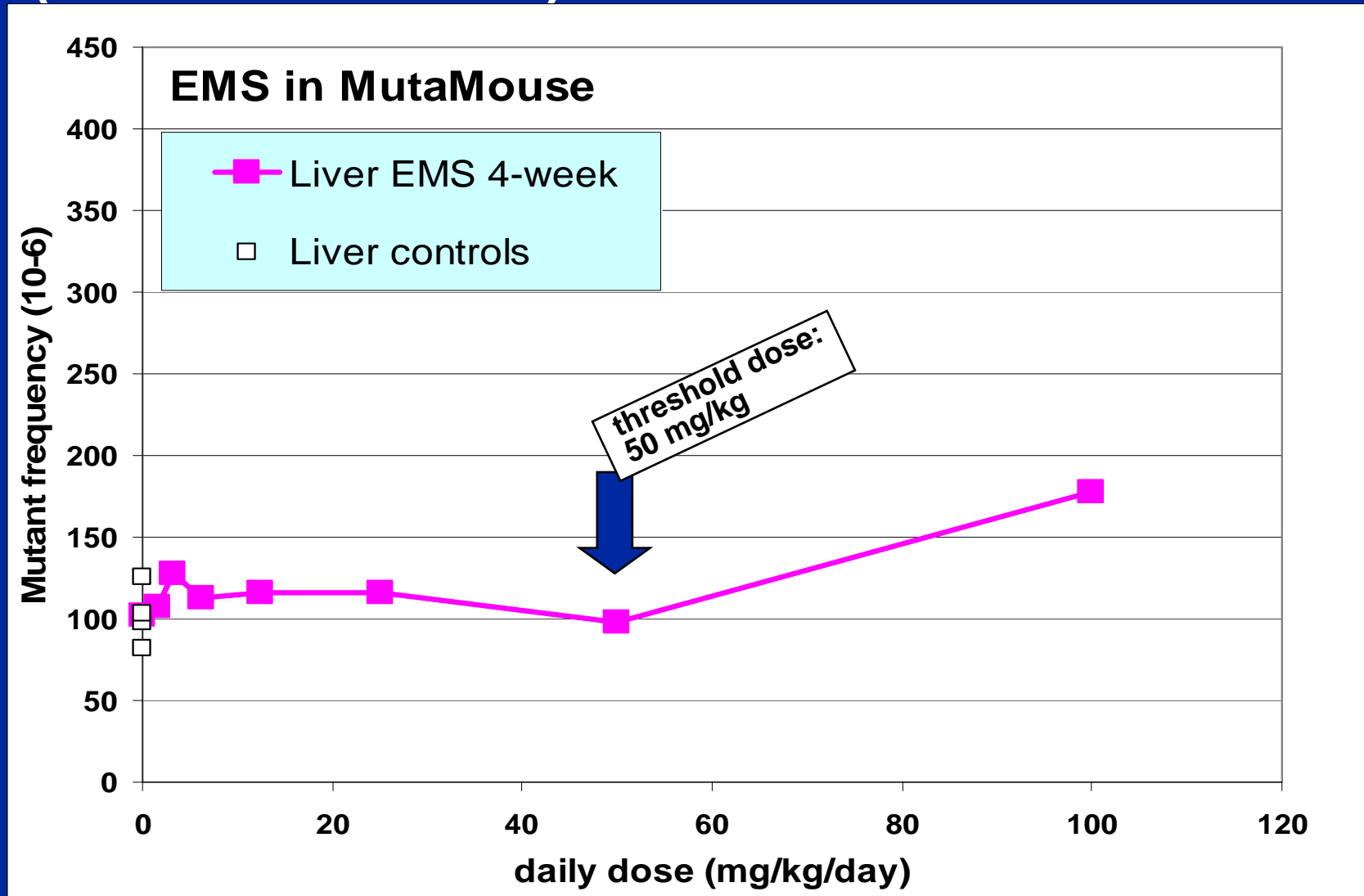
MutaMouse results: comparison EMS vs ENU (4-weeks treatment)



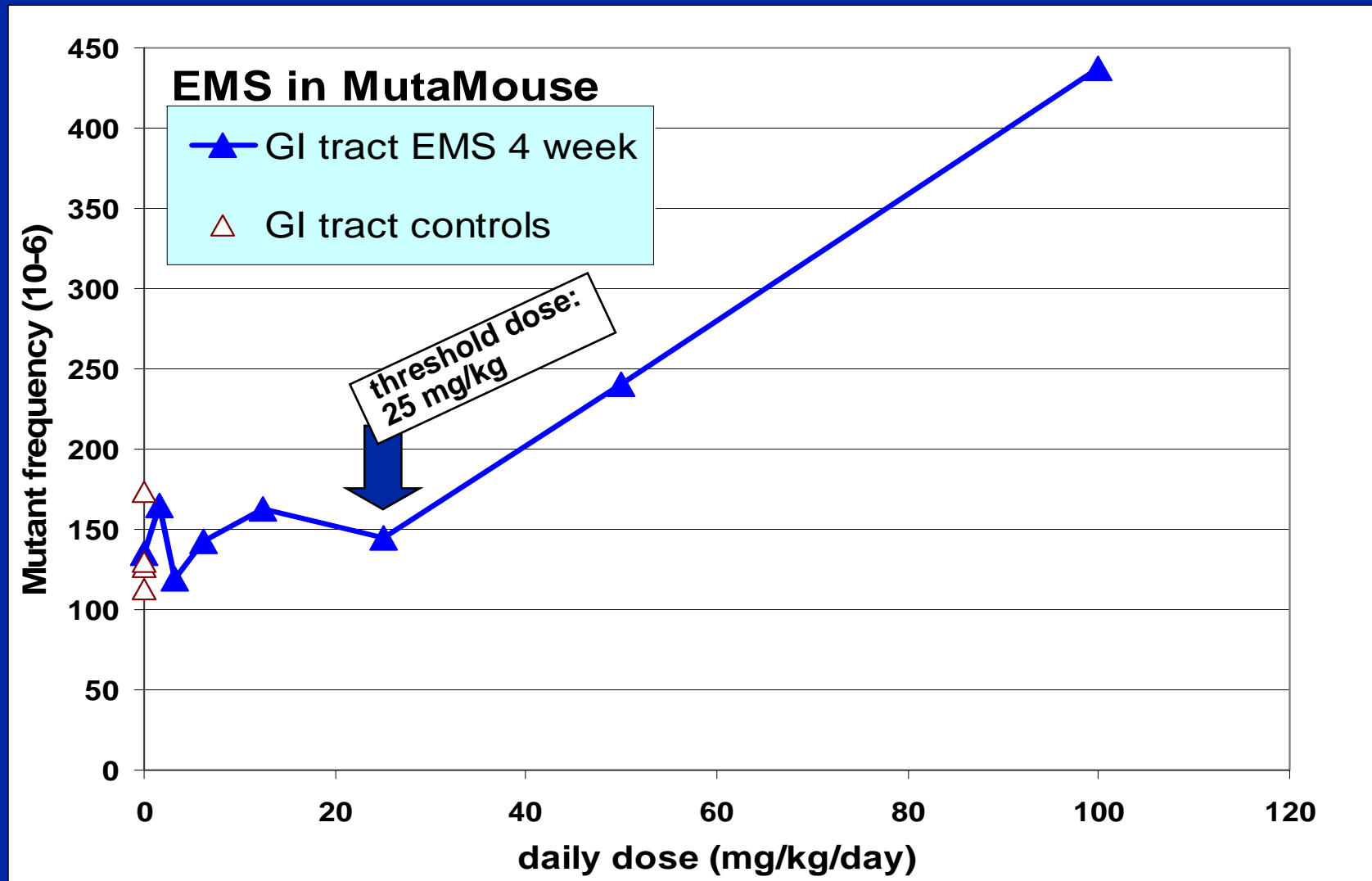
Gene mutations in bone marrow as function of dose (4-weeks treatment)



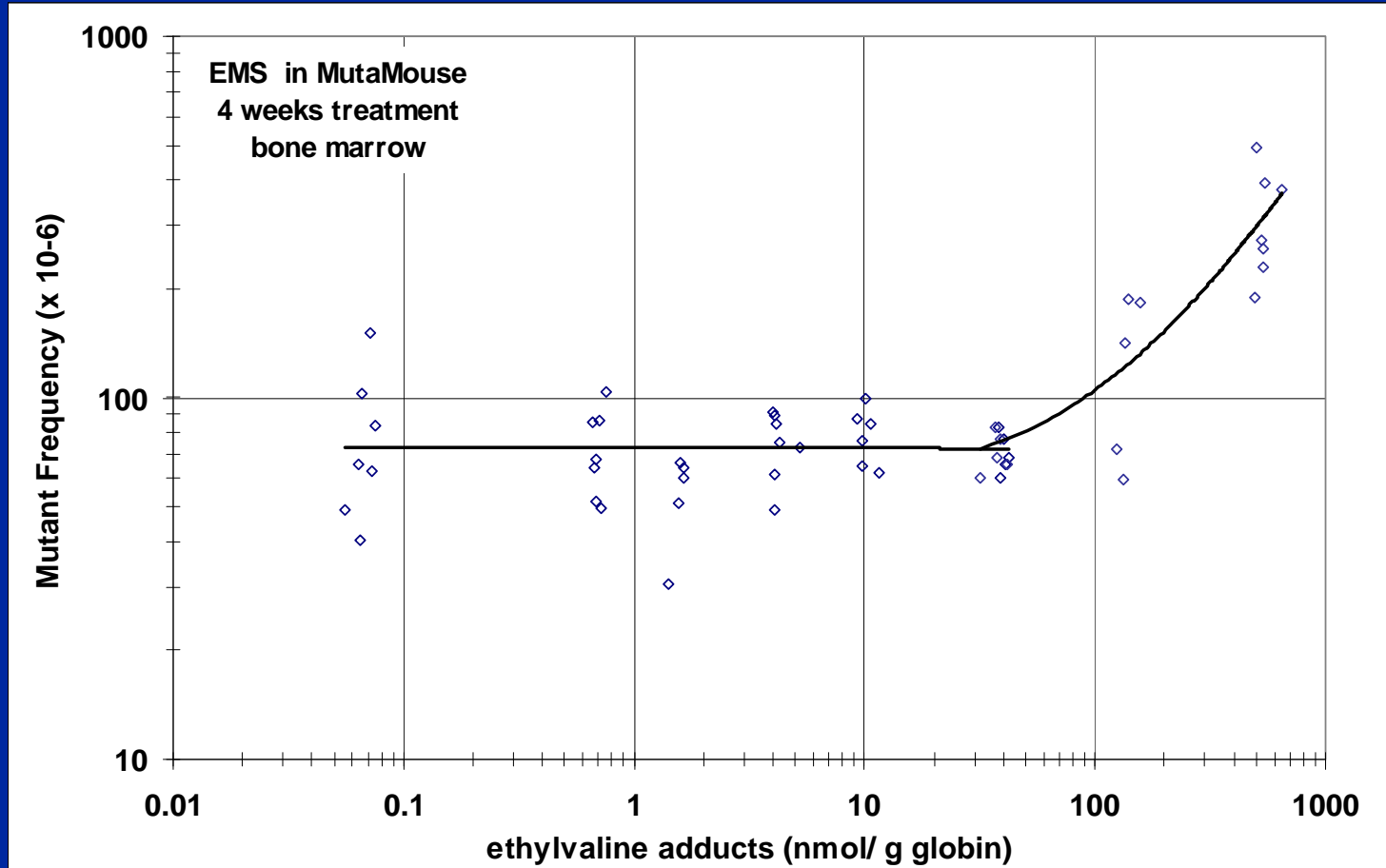
Gene mutations in liver as function of dose (4-weeks treatment)



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



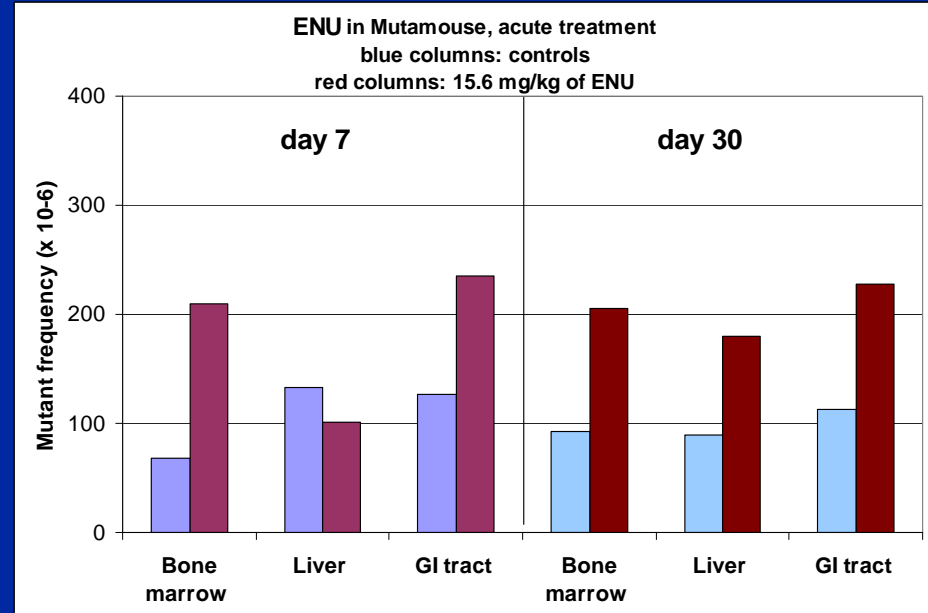
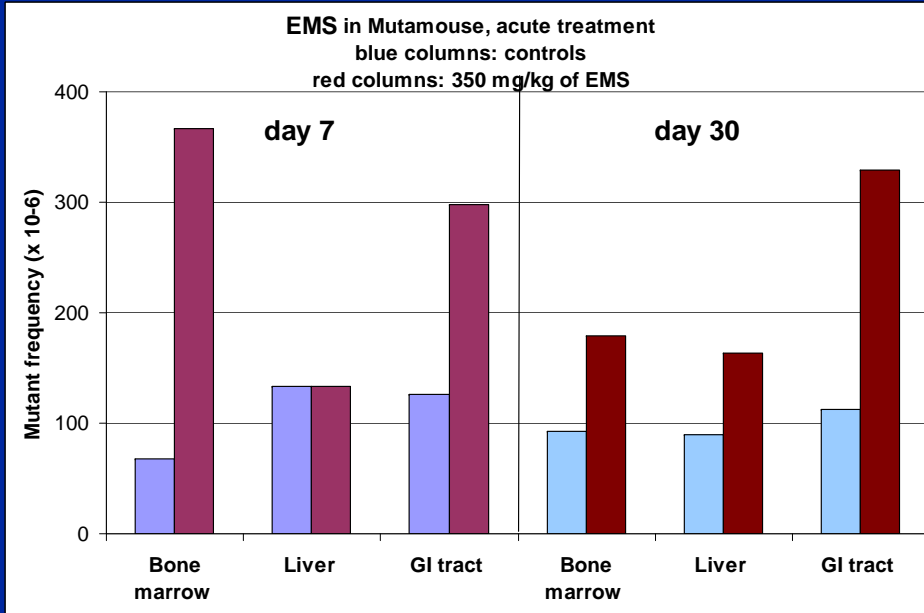
Gene mutations as function of exposure (ethyl-adduct levels) Bone marrow



Data from 7 animals deleted due to grossly deviating adduct levels

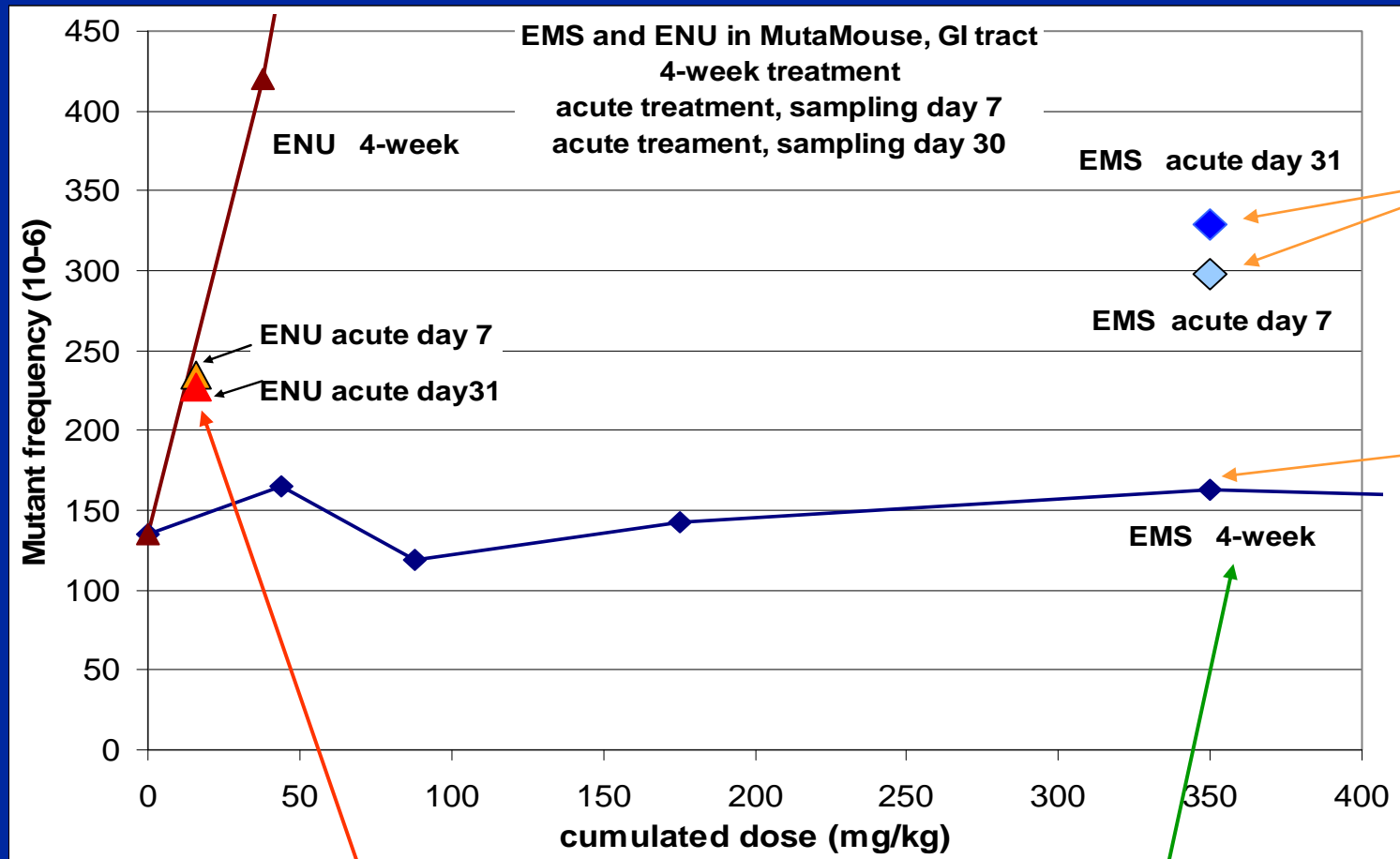
- No increase of micronuclei below ~40 nmol/g globin
(more than 400 fold higher than background level of < 0.1 nmol/g globin)

MutaMouse results: Acute treatments (sampling at 7 and 31 days) Comparison of EMS and ENU



- roughly similar effects at 350 mg/kg of EMS compared to 15.6 mg/kg of ENU
- ENU about 20 fold more potent than EMS (at max. applicable dose of EMS)
- no effect in liver at 7 day sampling, comparatively weak effect at 31 day sampling

MutaMouse results: Cumulative versus acute treatment: GI tract



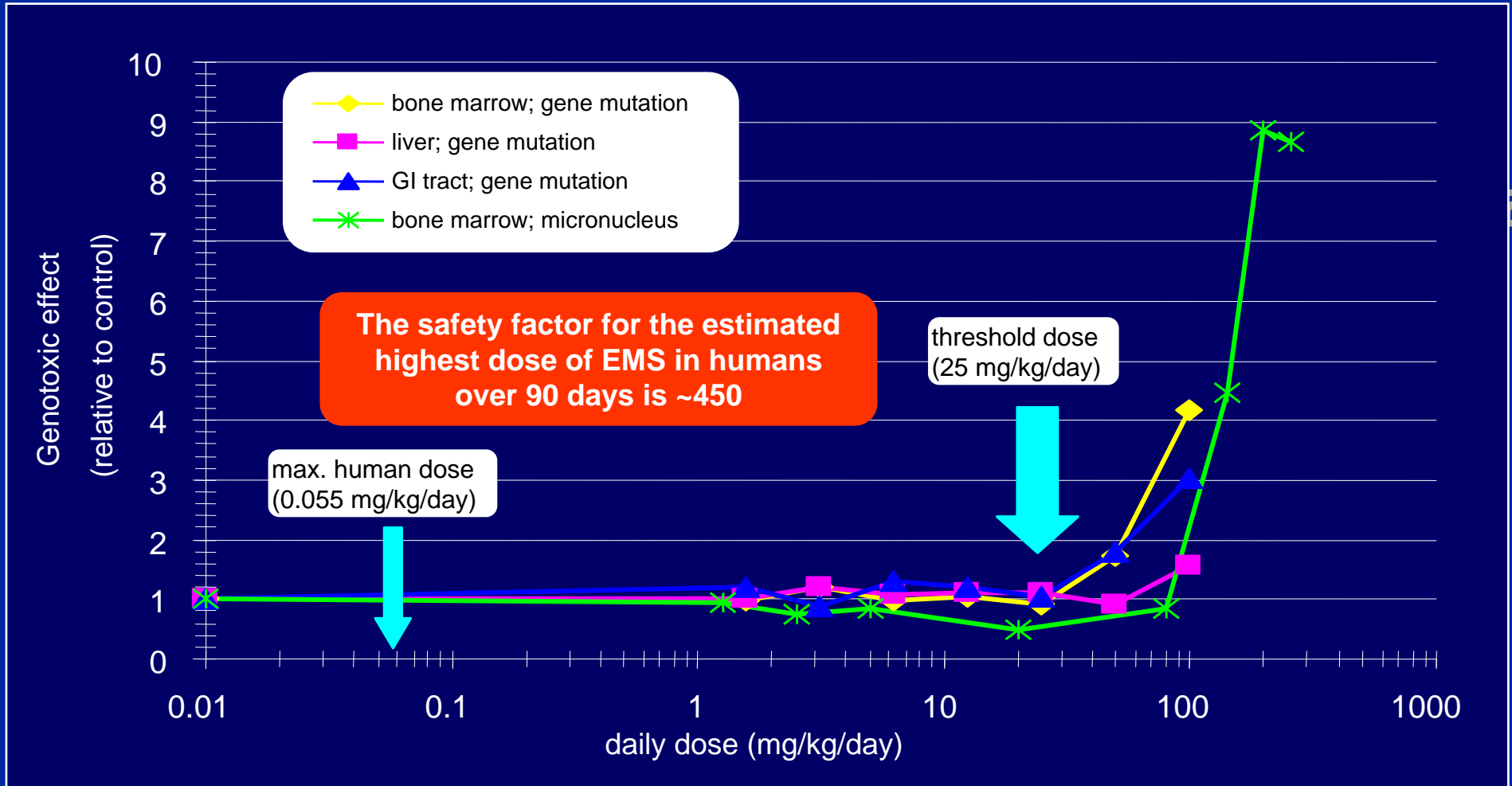
350 mg/kg/day x 1 day
= 350 mg/kg

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

ENU: effect independent of dose fractionation

EMS: dose fractionation abolishes the effect

Summary of genotoxicity studies: Evidence for threshold (dose based comparison)



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How the EMS results in animals can be translated to Viracept patients: modelling of patient exposure

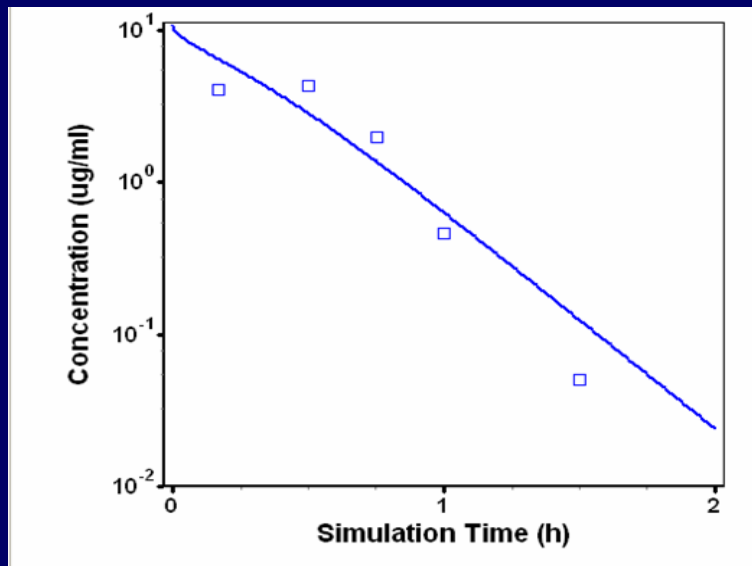
- Step 1:** PK studies (measure amount of drug in blood stream) with ^{14}C - EMS were conducted in animals (mice, rats, monkeys) and in vitro
- Step 2:** EMS PKs in animals was linked to a measure of tissue exposure (ethyl-globin)
- Step 3:** PK-Model was developed and validated using real data from mice
- Step 4:** Using data from Steps 1 through 4 we used this PK model to estimate patient exposure to EMS from taking Viracept



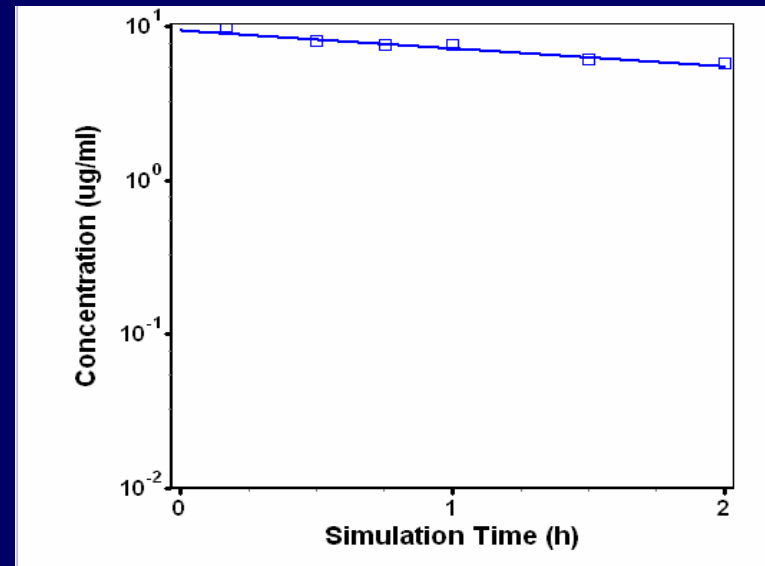
Safety factor calculated for exposure (AUC) and C_{max}

STEP 1: Pharmacokinetics of EMS in animals derived: distribution, clearance and half-life

Mouse, iv, 5 mg/kg

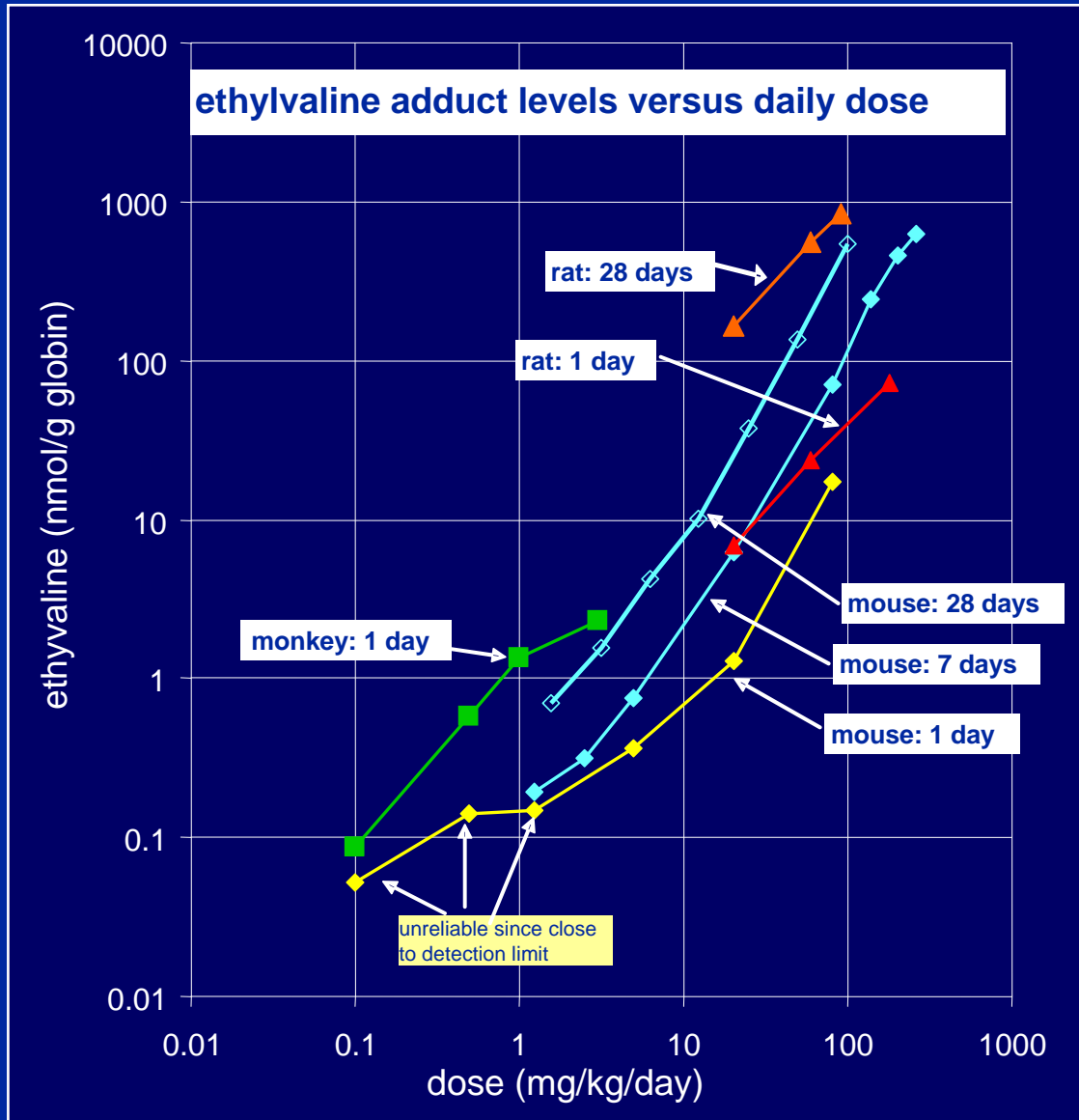


Rat, iv, 5 mg/kg



	Mice (1 and 5 mg/kg)	Rat (1 and 5 mg/kg)
Oral bioavailability	high	
Distribution (L/kg)	limited (0.47)	0.5
Clearance (ml/min/kg)	non linear (max: 51.4)	2.4
Half-life (hours)	0.2 (at low dose)	2.5

STEP 2: Dose-related increase in ethyl-Hb observed in mouse, rat and monkey

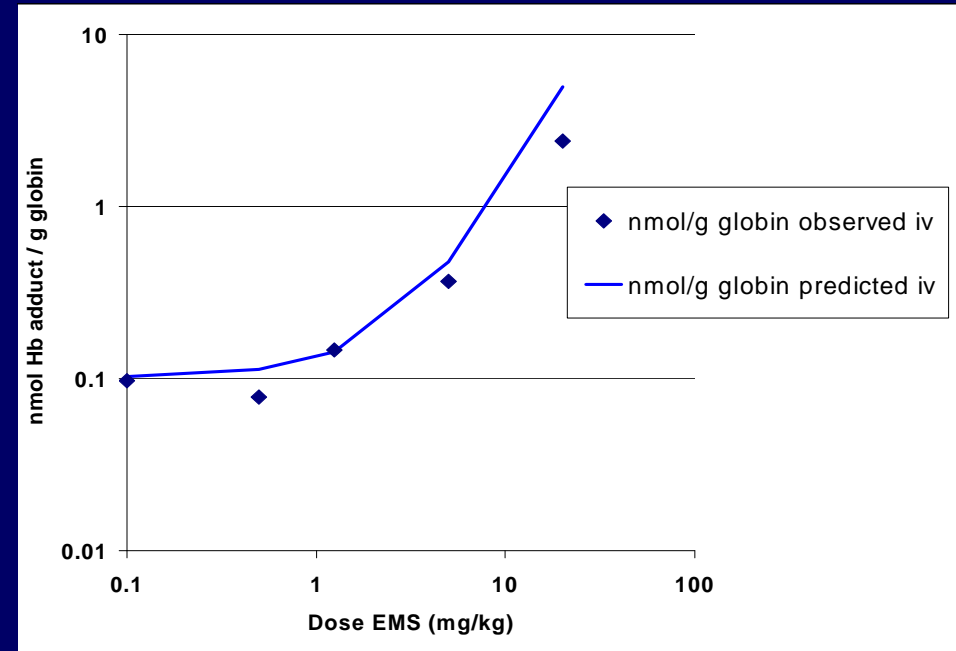
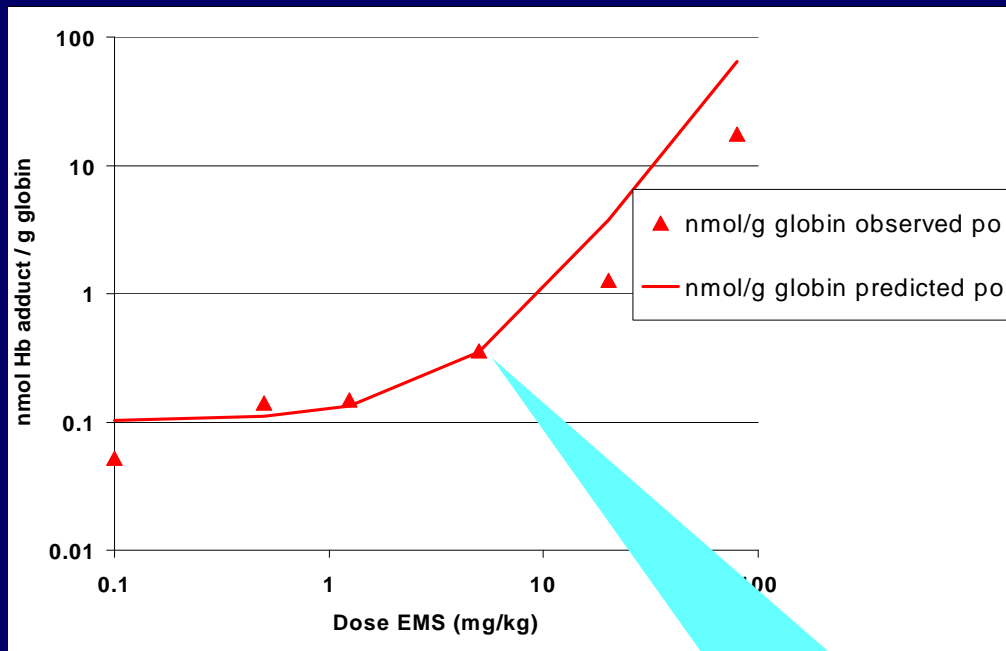


- Accumulation of Hb adducts consistent with Hb life span
- Higher Hb adduct levels in rat and monkey vs mice due to lower clearance (longer half-life) of EMS
- Higher Hb adduct levels expected in man

STEP 3: PK model was validated using real data from mice

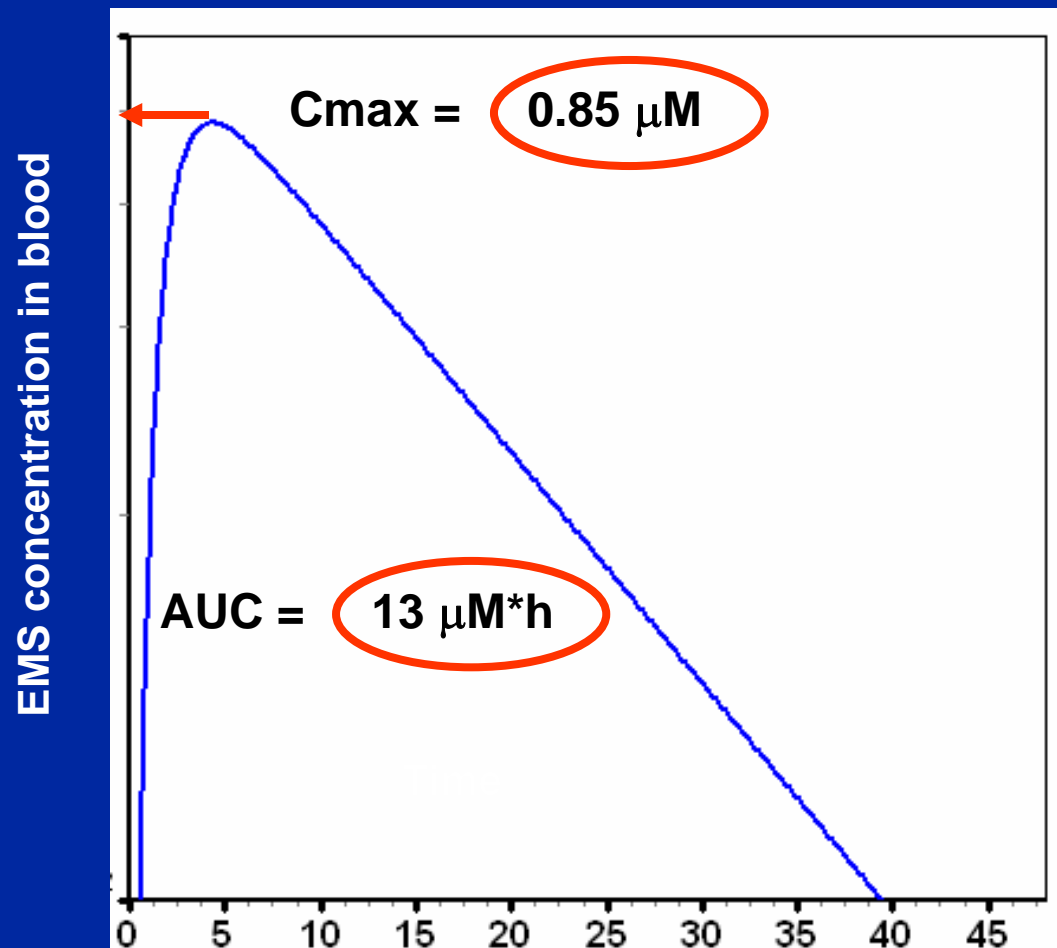


Mouse



Good simulation only with non linear clearance + baseline Hb adduct levels

STEP 4: Predicted pharmacokinetics of EMS in man (most conservative scenario with highest estimated EMS dose in Viracept **0.055 mg/kg**)

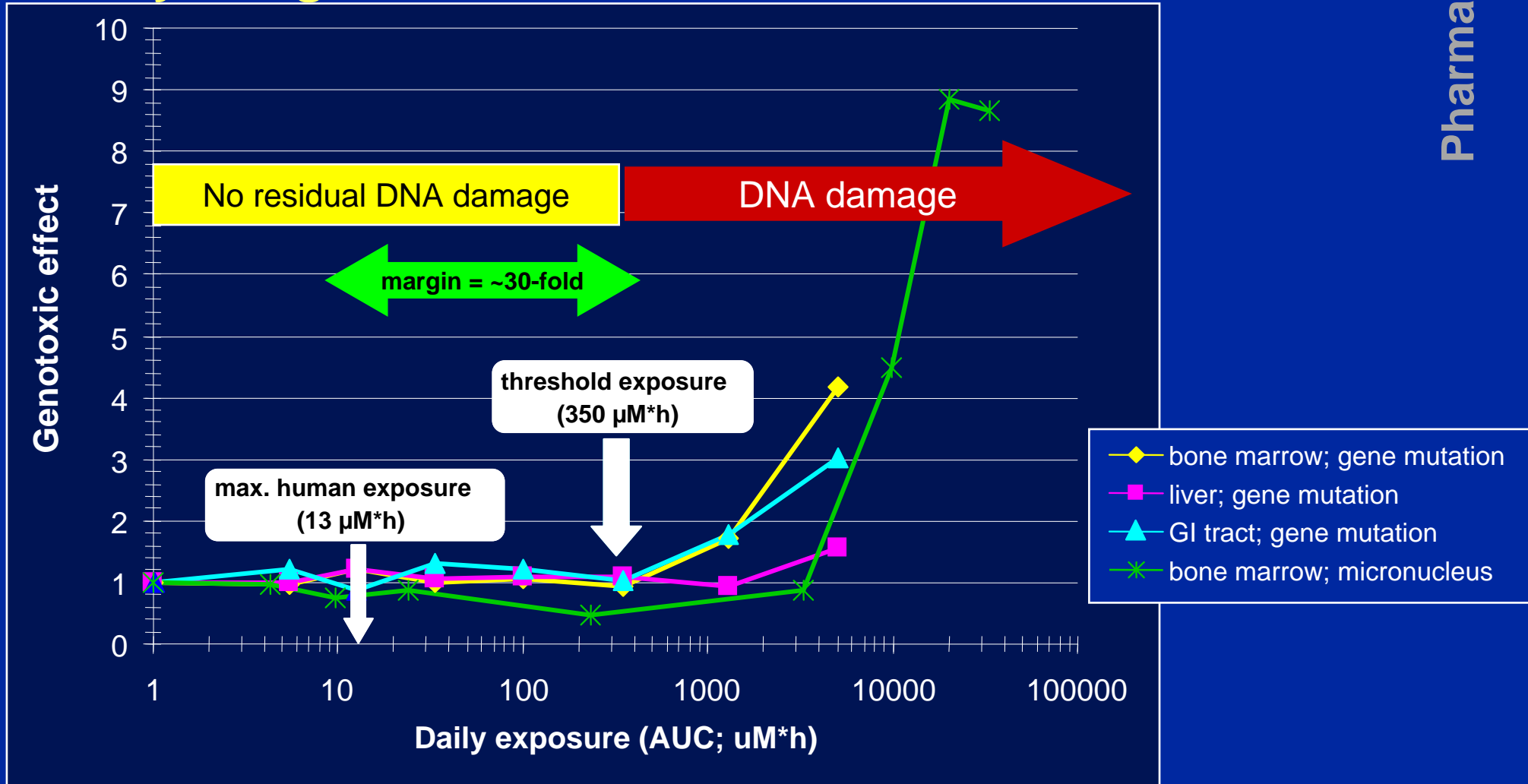


The prediction represents a *worst case* scenario:

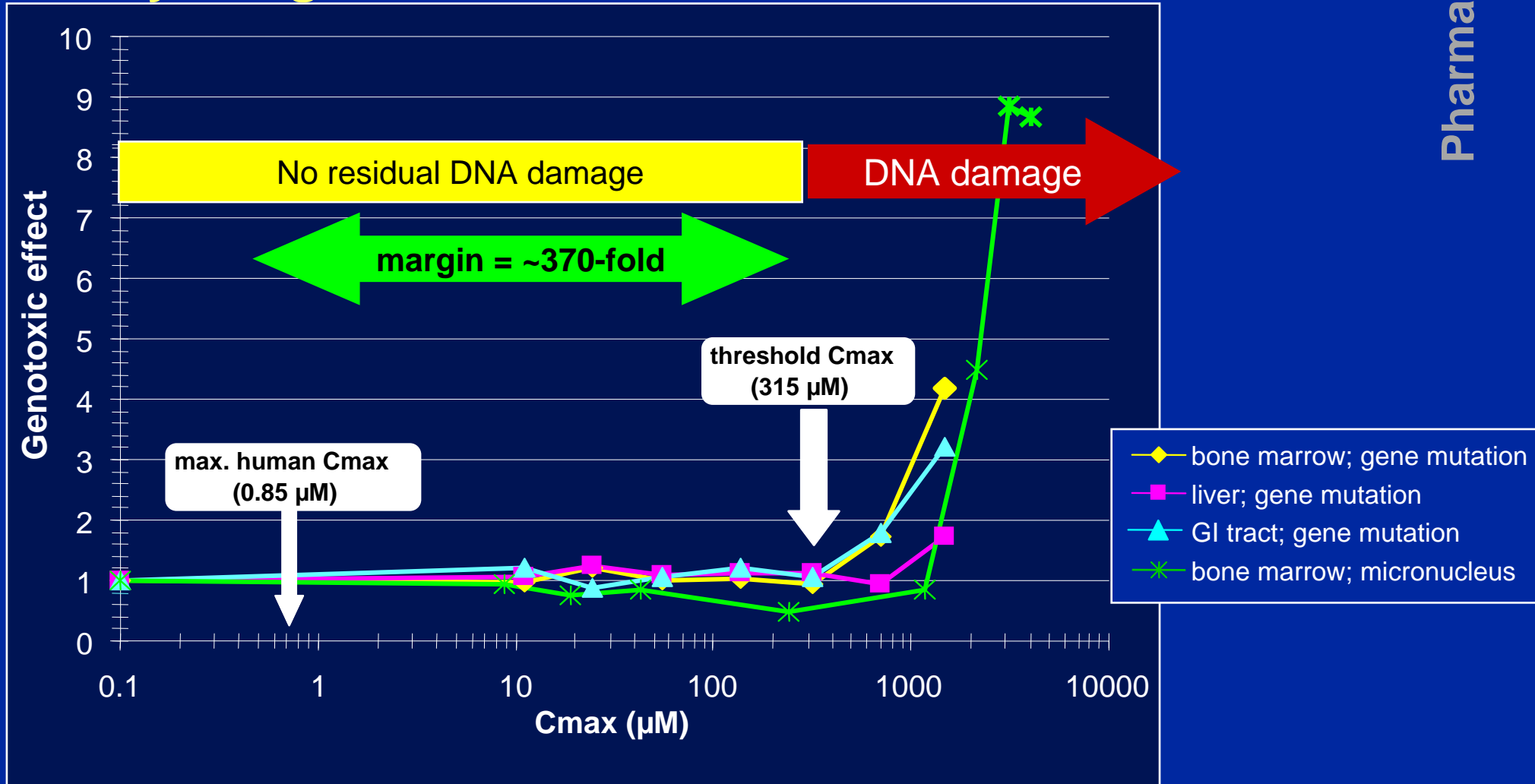
- a low clearance is assumed which is close to what is predicted just on the basis buffer stability, ignoring additional elimination processes
- the half-life is in the range of the half-life observed in buffer (i.e. 11 hours, it cannot be longer than that)

AUC in man predicted to be about 12 fold higher than in mice (in line with allometric scaling)

Risk assessment for Viracept patients calculated using **AUC** (measure of exposure) **Safety margin ~30-fold**



Risk assessment for Viracept patients calculated using **C_{max}** (measure of exposure) **safety margin ~370-fold**



What is the pivotal parameter?

- AUC- if the amount of DNA damage (total repair capacity) is decisive
- Cmax – if the time course of DNA damage (speed of repair) is decisive

Whatever the measure:

The exposure of the patients to contaminated Viracept carries no risk for elevated mutation

And, therefore, no risk for cancer, birth abnormalities, heritable defects

This conclusion was accepted by the authorities

The request for patient registries was withdrawn

What is the toxicological significance of these studies?

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

(at least for some)

Paracelsus is correct also for some DNA damaging mutagens

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

We have calculated that EMS induced everyday at the threshold dose

380 000 ethylations in the DNA of each each liver cell

A paradigm shift in genetic toxicology



Thanks to all Non-Clinical Contributors

Internal

- Axel Pähler
- Ruby Wiegand
- Heini Buergin
- Berthold Lausecker
- Eberhard Wall
- Herbert Birnboeck
- Michael Pantze
- Muriel Cordon-Federspiel
- Nicole Clemann
- Ursula Torriani
- Jürgen Gottowik

External:

- Marc Ballantyne (Covance)
- James Whitwell (Covance)
- Gabriele Leng (Bayer)
- Wolfgang Gries (Bayer)
- A. Eichinger-Chapelon (RCC)
- Dirk Flade (RCC)
- Michael Wall (statistics consultant)