



# *Acute-to-chronic extrapolation in vitro. Implications for the development of KERs.*

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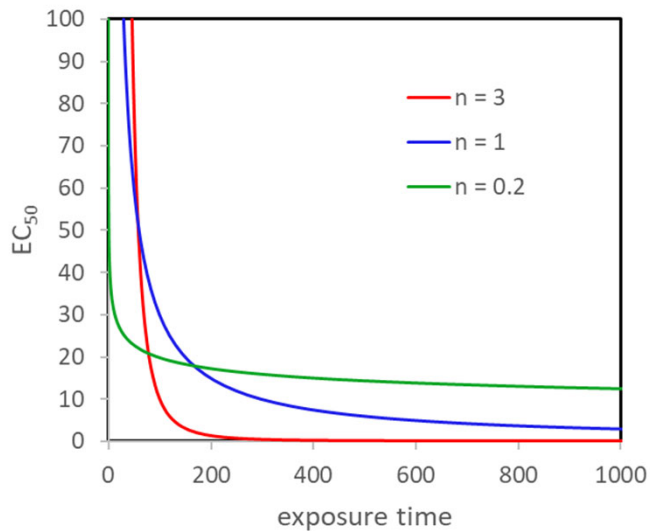
*European Commission - Joint Research Centre (JRC), Ispra, Italy*

*European Union Reference Laboratory for alternatives to animal  
testing (EURL ECVAM)*

# Outline

- Concentration-time responses - modified Haber's rule
- Our case study on liver cell line HepaRG
- Chronicity index and acute-to-chronic extrapolation
- Links with AOPs

## Isoeffect lines – relationship between the concentration (or dose) and the exposure time needed to achieve the same toxic effect - hyperbolic shape



$$C * t^n = constant$$

$$C = k * t^{-n}$$

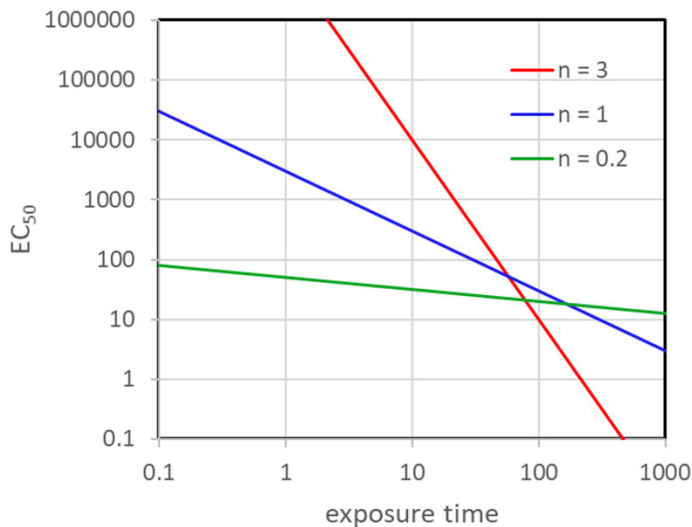
$$\ln C = \ln k - n * \ln t$$

*n* = 1 – Haber's rule

Fritz Haber – used this relationship to describe the effects of deadly gases used in World War I

The rule was used to set thresholds for toxic gases in industry, in phototoxicity, nuclear industry and medicine.

*n* ≠ 1 – modified Haber's rule  
(organism-chemical-effect specific)



Other variations used as well:

$$C^a * t = constant$$

$$C^a * t^b = constant$$

$$(C - C_0) * t^n = constant \text{ (if a threshold no effect } C_0 \text{ exists)}$$

$$(C - C_0)^a * t^b = constant \text{ (if a threshold no effect } C_0 \text{ exists)}$$

# Our case study on liver HepaRG line

5 chemicals + control

Rotenone

Tamoxifen

Aflatoxin B1

Cadmium chloride

Methylene dithiokyanate



Live cell imaging on HCI platform Cellomics over 86 hours in a live cell chamber (37 °C, 95% humidity, 5% CO<sub>2</sub>)

Image-iT® DEAD™ Green viability stain impermeant dye to healthy cells that becomes permeant when the plasma membrane of cells is compromised – marker of cell death.

After 86 hours, cells were stained with DAPI, fixed, imaged and counted. Their total counts were used for data normalisation.

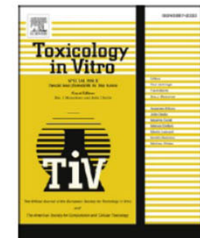
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Toxicology in Vitro

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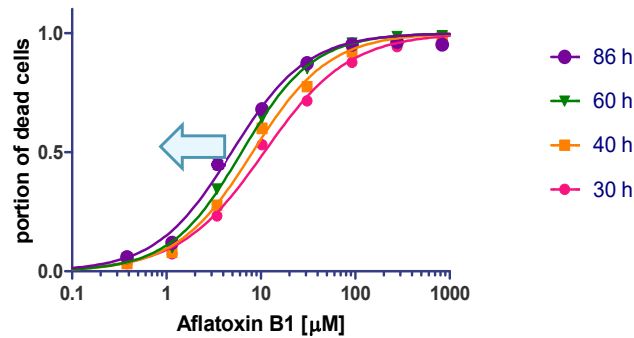
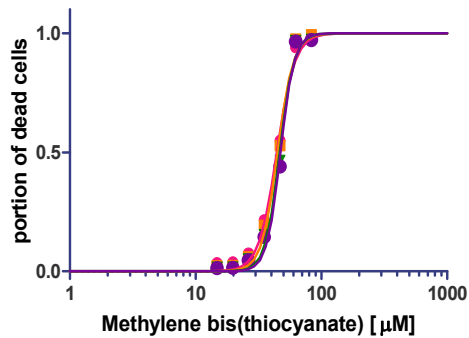
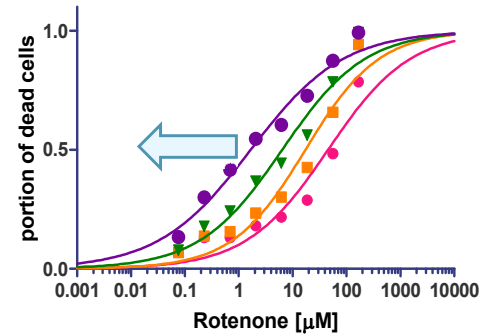
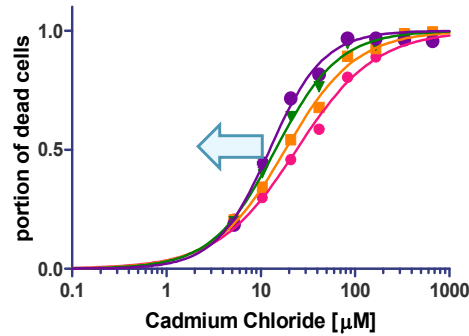
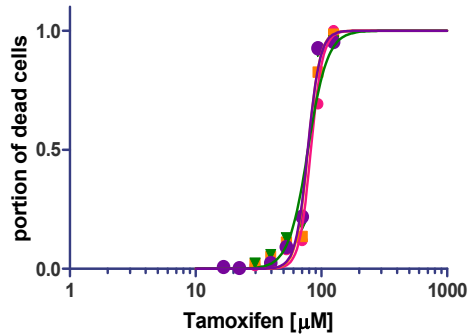
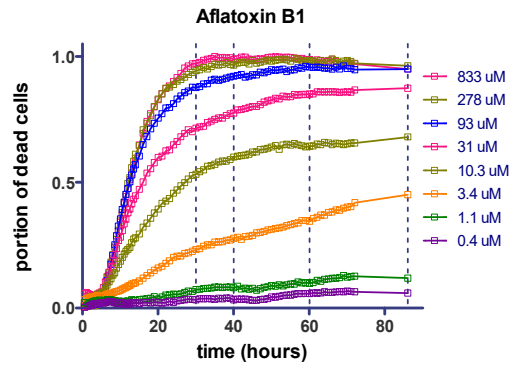
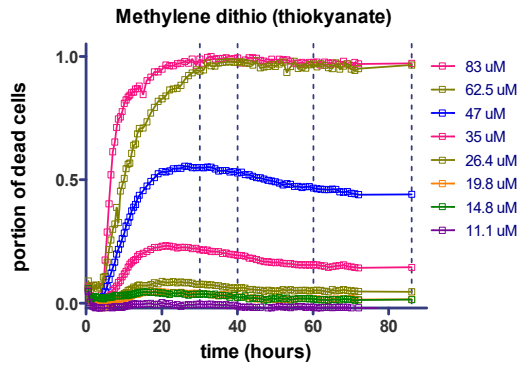
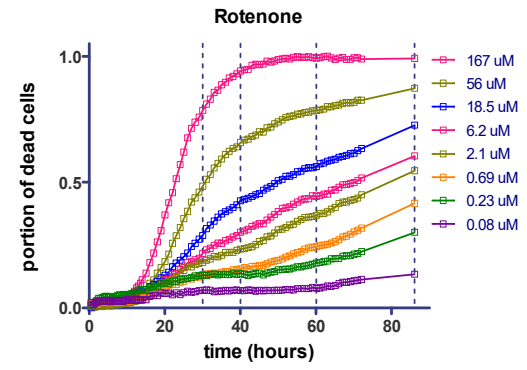
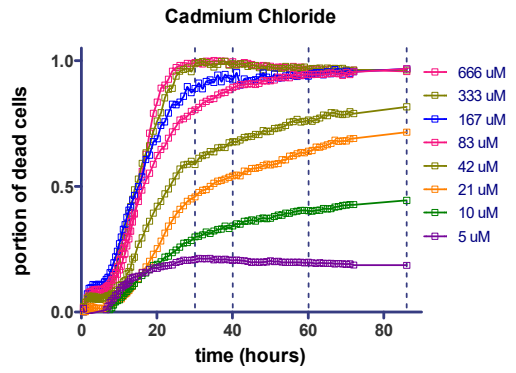
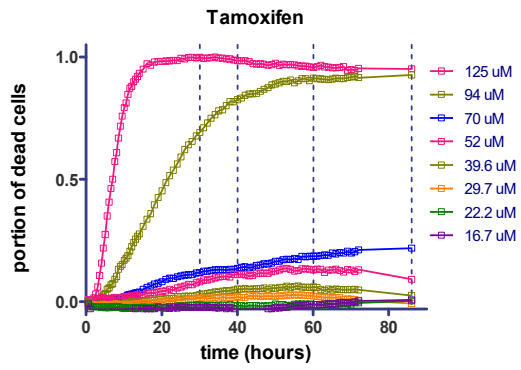


Extrapolating from acute to chronic toxicity *in vitro*

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Relationships between  $IC_{50}$  for which 50% mortality of HepaRG cells was observed and the exposure time (open circles). Solid lines show the modified Haber's rule fit extrapolated to longer exposure times. The intersections with the red vertical lines, corresponding to the typical lifespan of hepatocytes in humans (200–300 days), is used to estimate the in vitro chronic  $IC_{50}$ .

$$IC_{50}(t) = k t^{-n}$$

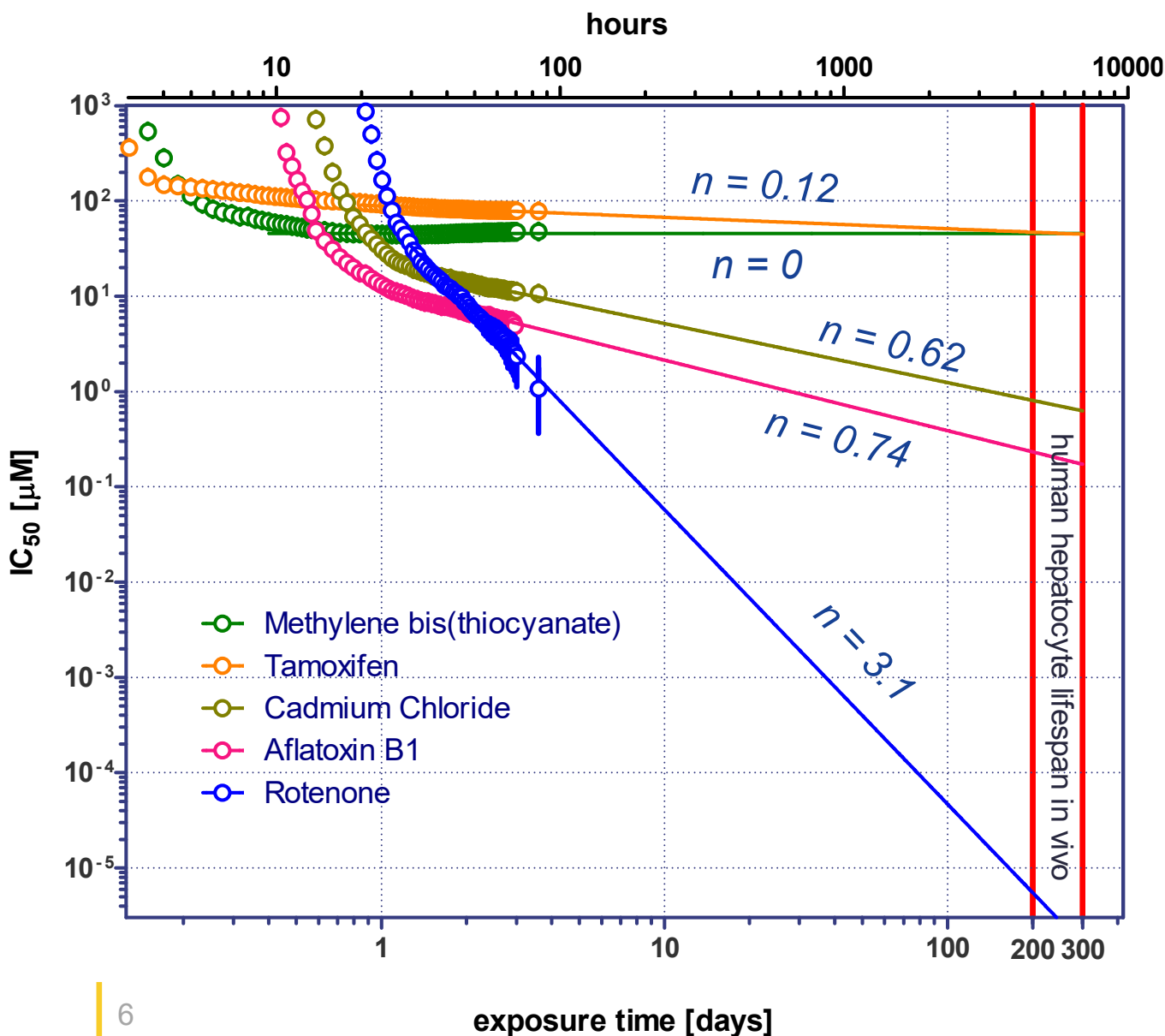
	n	k [ $\mu\text{M} \times \text{day}^n$ ]	$IC_{50}$ at 300 days [ $\mu\text{M}$ ]
Rotenone	3.1	70.3	$1.5 \times 10^{-6}$
Aflatoxin B1	0.74	11.7	0.17
Cadmium Chloride	0.62	21.5	0.63
Tamoxifen	0.12	88.5	45
Methylene bis(thiocyanate)	0	46	46

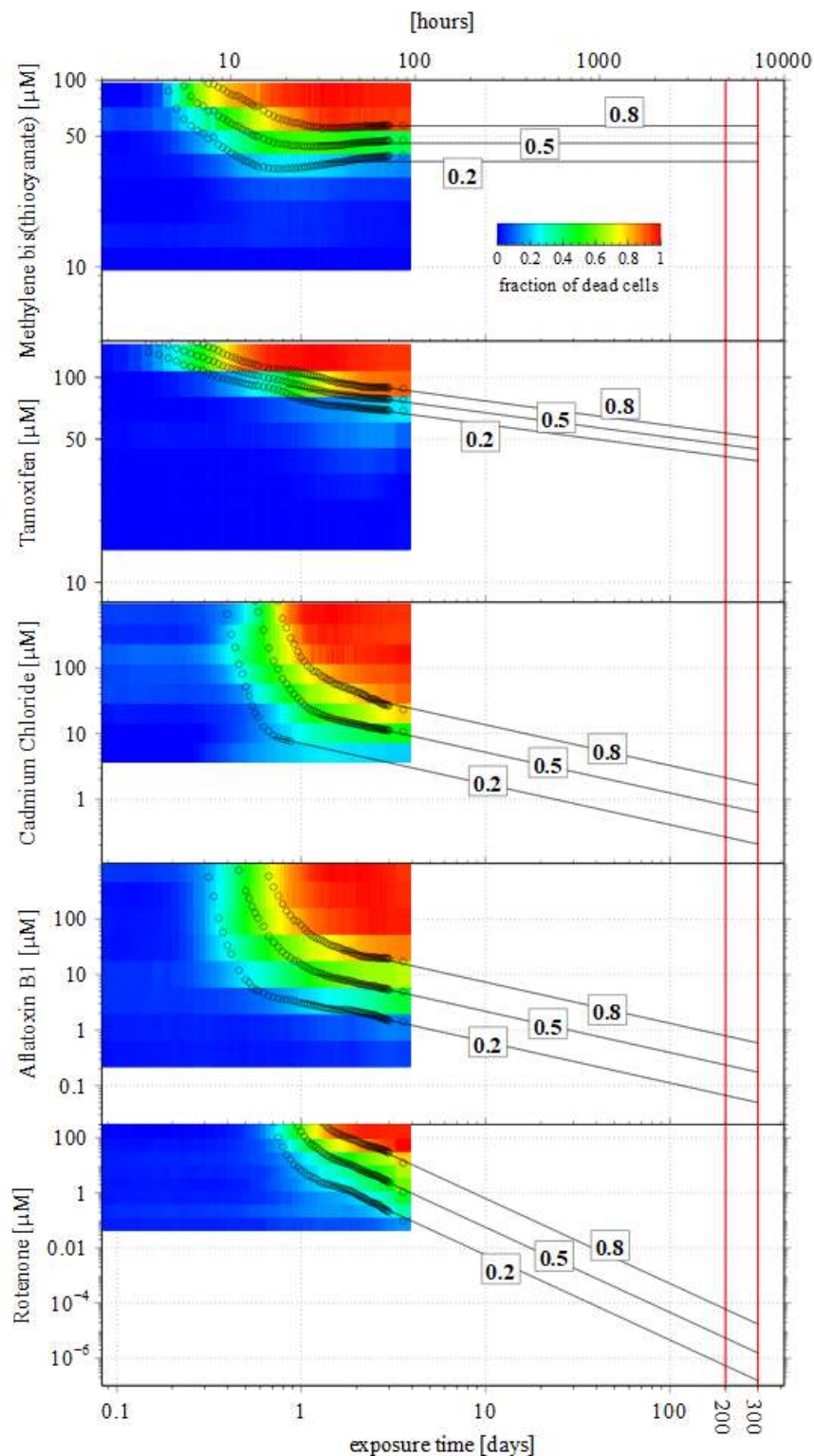
### *n* – chronicity index

*n* = 0 – longer exposure time does not worsen the toxic effect - acute only toxicity

*n* = 1 – Haber's rule – both the concentration and the exposure time contributes equally

*n* > 1 – effect significantly reinforced with exposure time





Colour-coded visualisation of the HepaRG cells mortality in concentration versus exposure-time domain with black circles showing the points of the isoeffect level (20%, 50%, and 80% cell death). Black lines show their extrapolation up to the typical lifespan of hepatocytes in humans. The extrapolation is used to estimate the in vitro chronic Point of Departure (cPoD) for each chemical.

By applying a safety margin, the No Effect Concentration (NEC) can be set.

Methylene dithiocyanate – pesticide, fungicide, disinfectant, only one case of acute liver failure documented (worker exposed to kgs of powder), metabolised in liver to cyanide and formaldehyde – no accumulation, therefore  $n = 0$

Tamoxifen – cytotoxic at high concentration, impair lipid metabolism at low concentration and can lead to steatosis (what is different toxic effect therefore another readout should be used to build corresponding "equal toxic effect line", for which we can expect different (bigger)  $n$

Cadmium Chloride – forms covalent bonds with many biomolecules, long life-time in organism, accumulative effect

Aflatoxin B1 – binds to RNA, DNA, and proteins what inhibits cellular processes but can be also metabolised, therefore partial detoxification is possible

Rotenone – irreversible inhibitor of complex I in mitochondria (cumulative effects with time), causes high ROS production (cumulative toxic effects with time), cell damage significantly reinforced over time

# Can the time-concentration responses be used to characterise the dynamics of AOPs and the Key Event Relationships (KERs) to develop 'quantitative' AOPs?

Motivation from:

Druckrey, H., Küpfmüller, K., 1949. Dosis und Wirkung – Beiträge zur theoretischen Pharmakologie. Cantor GmbH. Freiburg im Breisgau, Germany.

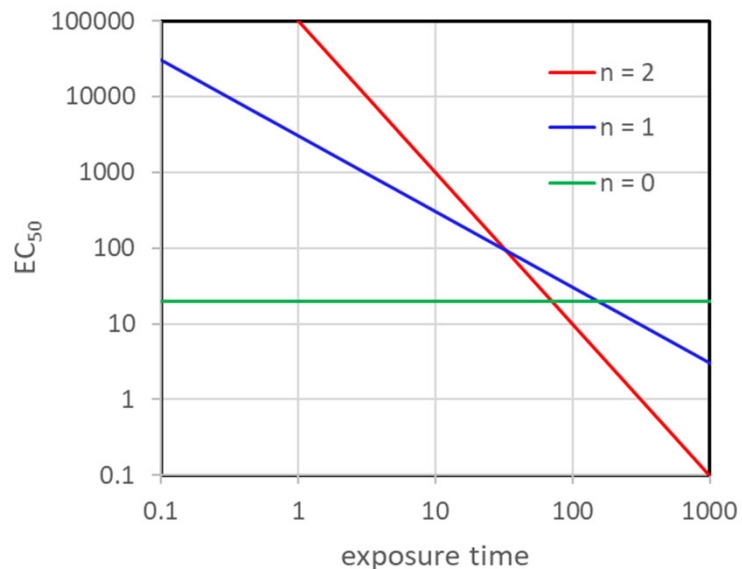
and

Tennekes et al, Toxicology 309 (2013) 39– 51

Dose–response characteristics according to Druckrey and Küpfmüller.

Reversibility of receptor binding	Bound receptors in relation to toxicant concentration	Reversibility of the effect	Effect in relation to bound receptor concentration	Effect in relation to toxicant concentration	Dose–response characteristics
$T_R \rightarrow 0$	$C_R \sim C$	$T_r \rightarrow 0$ $T_r \rightarrow \infty$	$E \sim C_R$ $E \sim \int C_R dt$	$E \sim C$ $E \sim \int C dt$	Dose-dependent $C \cdot t = \text{constant}^a$
$T_R \rightarrow \infty$	$C_R \sim \int C dt$	$T_r \rightarrow 0$ $T_r \rightarrow \infty$	$E \sim C_R$ $E \sim \int C_R dt$	$E \sim \int C dt$ $E \sim \int \int C dt$	$C \cdot t = \text{constant}^a$ Reinforced by time [ $C \cdot t^n = \text{constant}$ , with $n > 1$ ]

<sup>a</sup> Assuming that an exposure concentration  $c$  is kept constant and that, as a result, the toxicant concentration at the site of action  $C$  remains constant as well.



(i) If effect  $E \sim C$

Cmax scenario - only  $C$  defines the toxic effects)

$$\text{then } IC_{50}(t) = kt^0 = k \quad (n=0)$$

(ii) If effect  $E \sim Ct$

AUC scenario

$$\text{then } IC_{50}(t) = kt^1 = k \quad (n=1) \text{ Haber's rule}$$

(iii) If effect  $E \sim Ct^2$

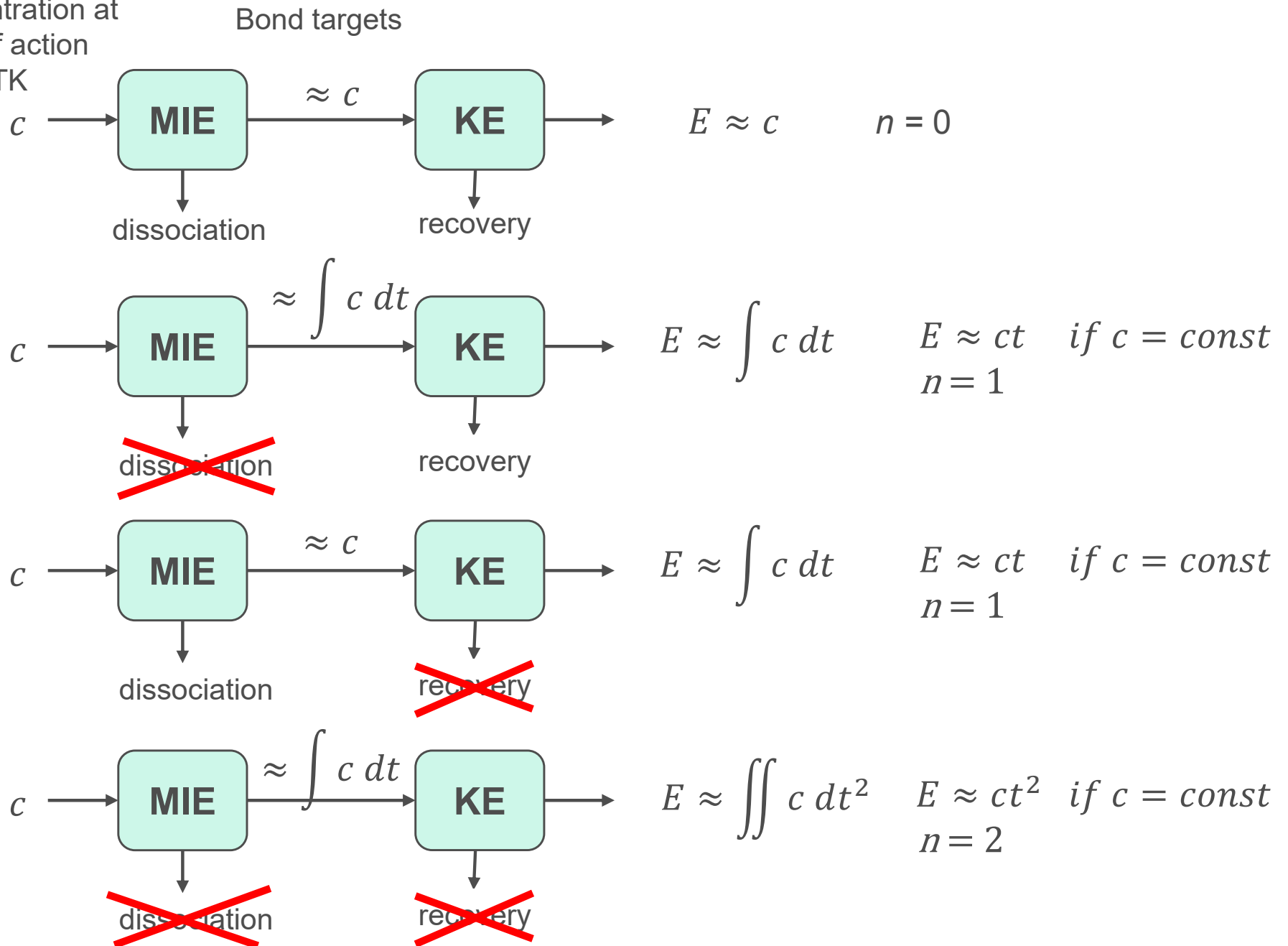
Area Under the AUC scenario

$$\text{then } IC_{50}(t) = kt^2 = k \quad (n=2)$$

the toxic effects are reinforced by time of exposure

# A general AOP - Molecular Initiating Event (MIE) + Key Event (KE)

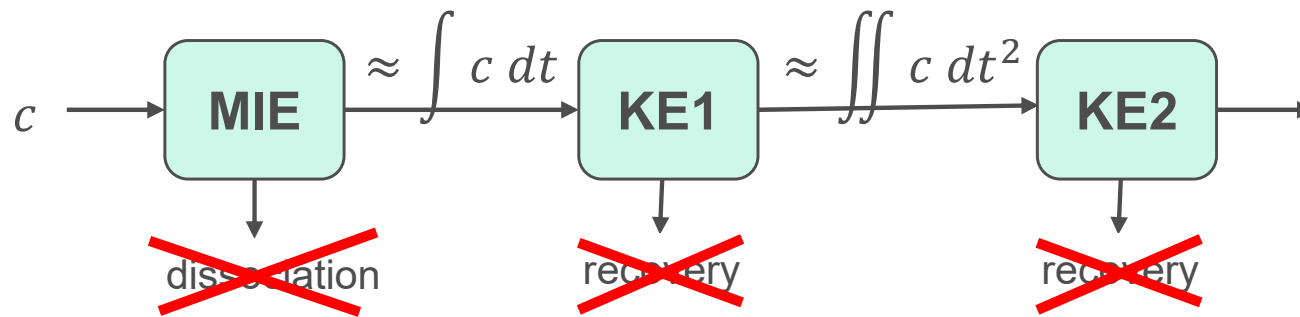
free concentration at the place of action  
- output of TK



## A general AOP - Molecular Initiating Event (MIE) + 2 Key Events (KE1 + KE2)

free concentration at  
the place of action  
- output of TK

Bond targets



$$E \approx \iiint c dt^3$$

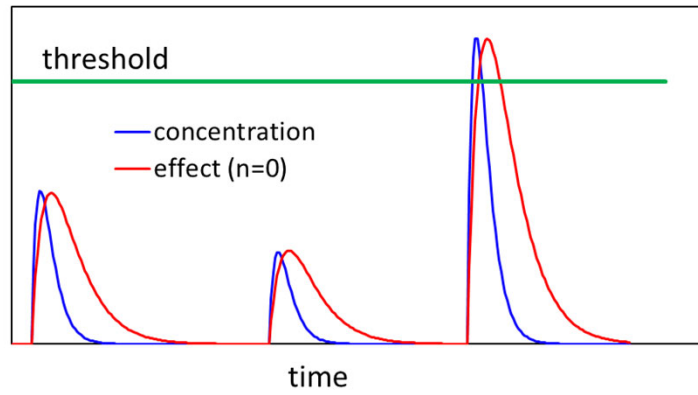
$$E \approx ct^3 \quad \text{if } c = \text{const}$$

$$n = 3$$

In the above, it was supposed that all the events (MIE, KEs) are linearly proportional to the concentration  $c$ , or to the output of the previous event - **first order** processes

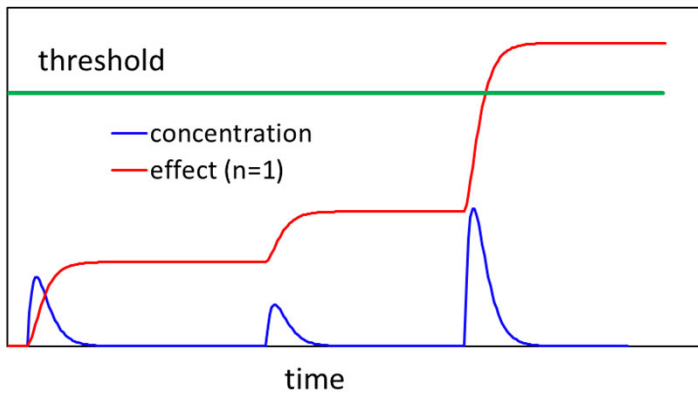
However, in general the relationship can be more complex, often expressed as a power function ( $\approx c^\alpha$ ) – **fractional order** processes (e.g. enzymatic Michaelis–Menten process, complex reaction mechanisms)

# Toxicodynamics in pulsed exposure scenario – if effect $\approx C t^n$ (linearly proportional to concentration)



for  $n = 0$   $E(t) \approx c(t)$

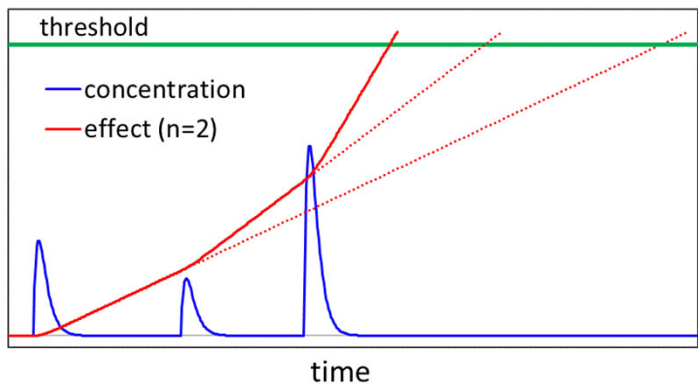
Reversible effect  
Equivalent of the  $C_{\max}$  scenario  
in pharmacokinetics terminology  
effect is proportional to  $C_{\max}$



for  $n = 1$   $E(t) \approx \int c(t) dt$

Permanent effect  
Equivalent of the 'Area Under the Curve' (AUC) in  
pharmacokinetics terminology  
effect is proportional to AUC

$E \approx ct$  in constant exposure scenario



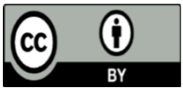
for  $n = 2$   $E(t) \approx \iint c(t) dt^2$

Effect continues raising after cessation of the cause:  $E(t) \approx t$

$E(t) \approx ct^2$  in constant exposure scenario

2<sup>nd</sup> and 3<sup>rd</sup> case ( $n \geq 1$ ) holds true only for zero initial conditions (no previous exposure)

# Thank you



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