Time variables and exposure in in vitro testing strategies

Peter Macko

Systems Toxicology Unit

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

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

Outline

- Concentration-time responses: case study on liver cell line HepaRG
- Time characteristic of events (MIE and KE) for AOPs
- Analytical model: a set of ordinary differential equations (ODEs)
- Implication for risk assessments

Abstract

In vitro methodologies serve as valuable alternatives to animal testing, forming integral components of novel approach methodologies for toxicological hazard and risk assessments. However, in vitro experiments often have limitations in terms of their duration, measurements of responses, and rarely consider more time points, which may result in the disregard of potential cumulative chronic effects over time. To address this issue, we propose an experimental design that not only characterizes the toxicodynamics of a response in relation to concentration but also incorporates the dimension of time. The concentration-time responses are modelled using a set of ordinary differential equations (ODEs). This approach enables the characterization of the dynamics of key events and their relationships, thus facilitating the development of quantitative adverse outcome pathways.

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_2)

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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Extrapolating from acute to chronic toxicity *in vitro*

Peter Macko *, Taina Palosaari, Maurice Whelan

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

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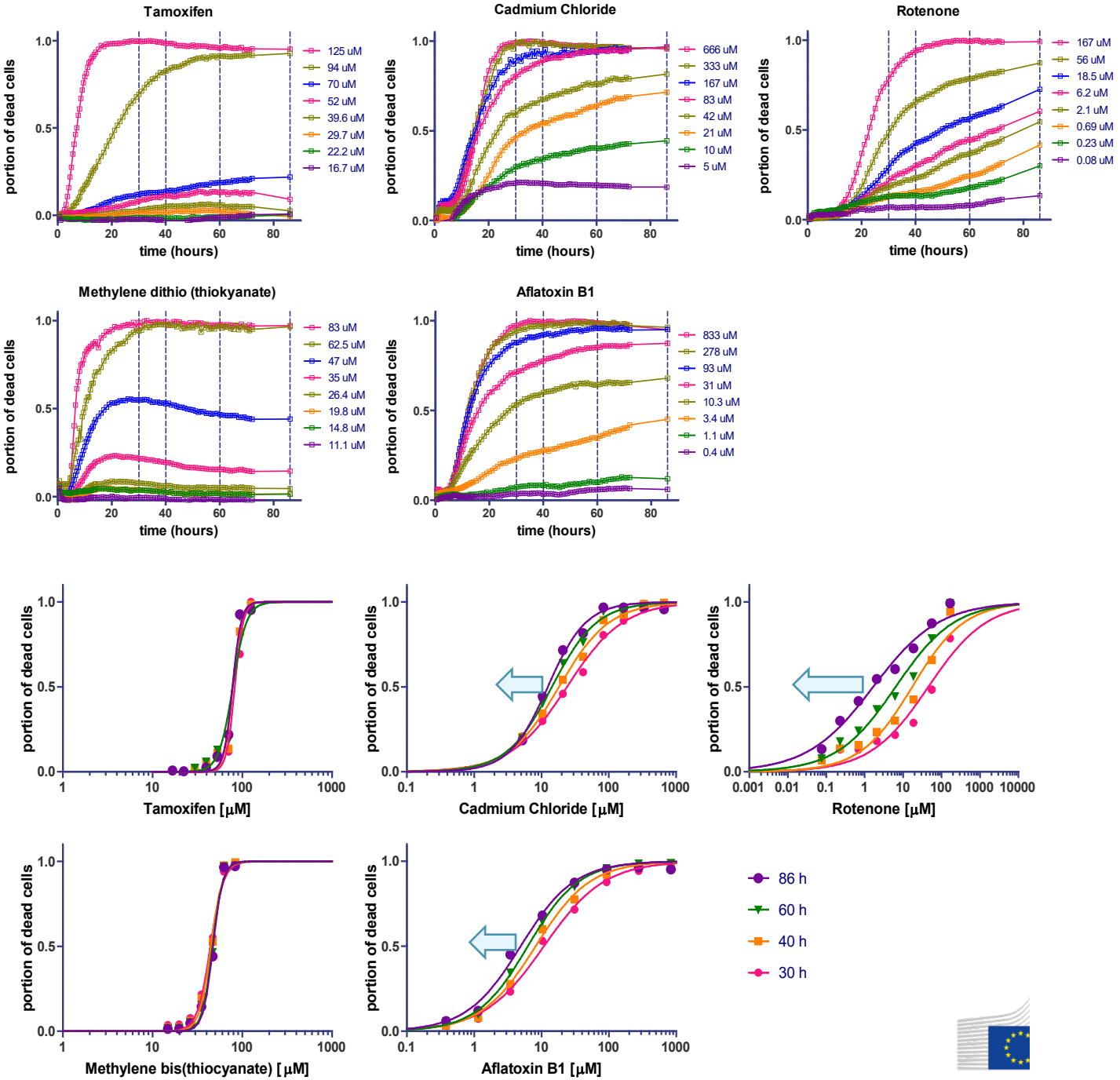
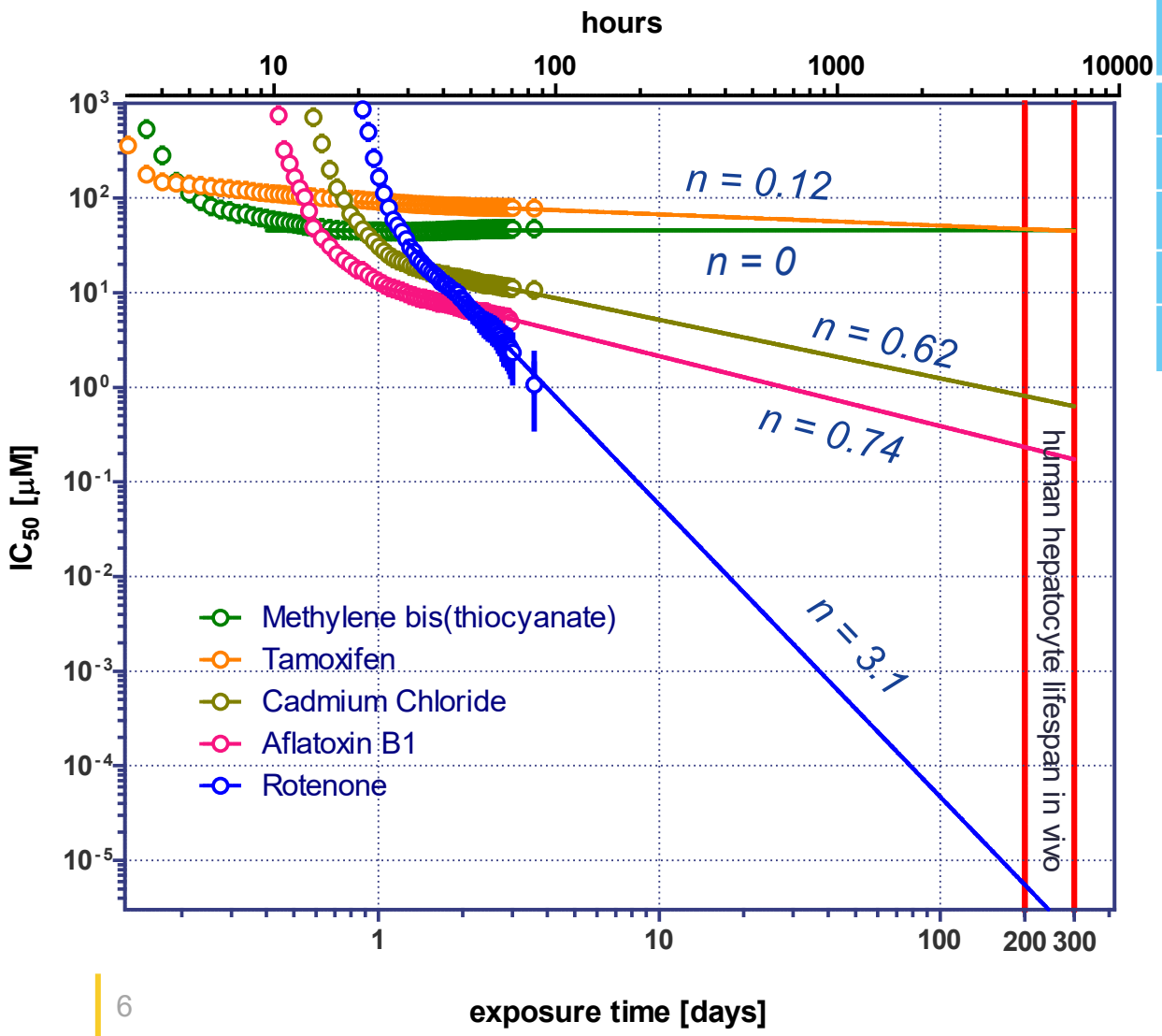

Tamoxifen
portion of dead cells
time (hours)
125 uM
94 uM
70 uM
52 uM
39.6 uM
29.7 uM
22.2 uM
16.7 uM
Cadmium Chloride
666 uM
333 uM
167 uM
83 uM
42 uM
21 uM
10 uM
5 uM
Rotenone
167 uM
56 uM
18.5 uM
6.2 uM
2.1 uM
0.69 uM
0.23 uM
0.08 uM
Methylene dithio (thiokyanate)
83 uM
62.5 uM
47 uM
35 uM
26.4 uM
19.8 uM
14.8 uM
11.1 uM
Aflatoxin B1
833 uM
278 uM
93 uM
31 uM
10.3 uM
3.4 uM
1.1 uM
0.4 uM
Tamoxifen [µM]
Cadmium Chloride [µM]
Rotenone [µM]
Methylene bis(thiocyanate) [µM]
Aflatoxin B1 [µM]
86 h
60 h
40 h
30 h

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}.

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



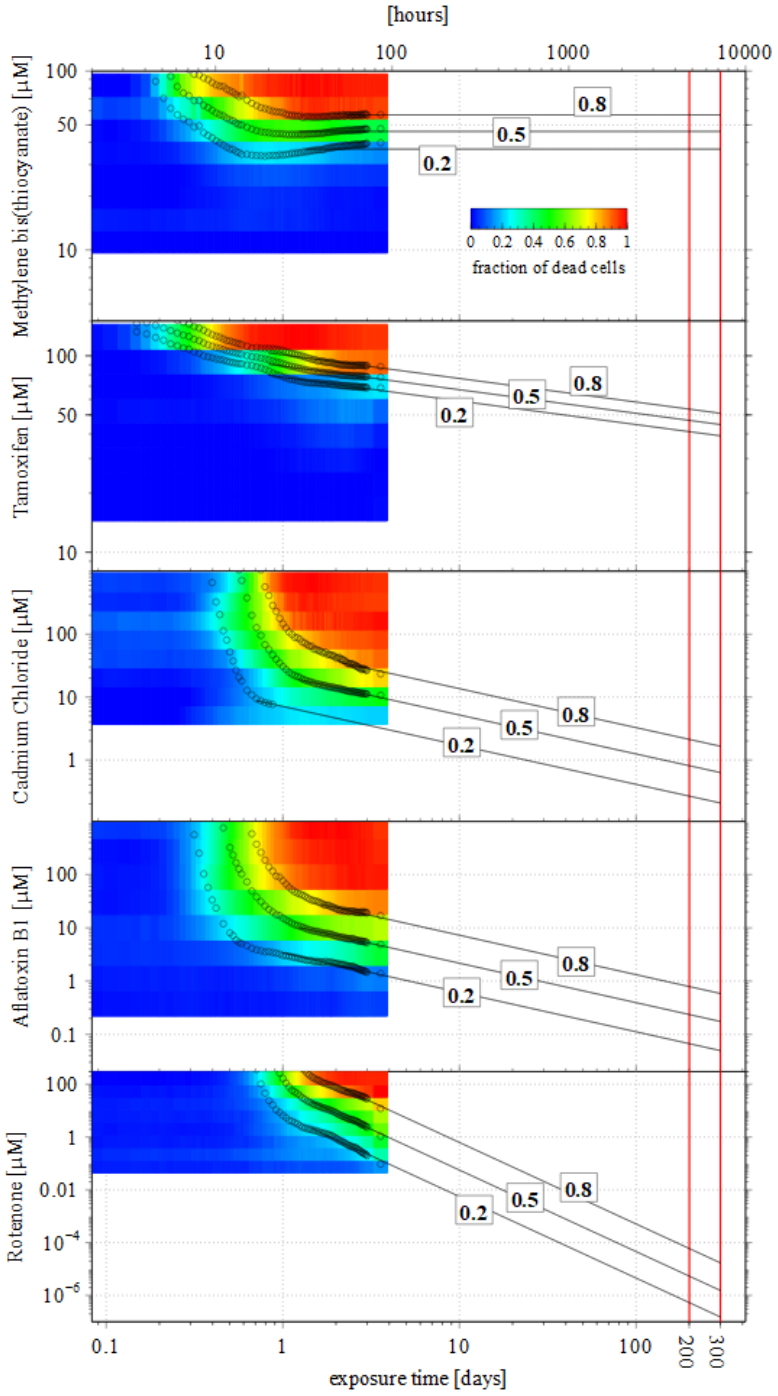
	n	k [µM×day^n]	IC_{50} at 300 days [µM]
Rotenone	3.1	70.3	1.5×10^{-6}
Aflatoxin B1	0.74	11.7	0.17
Cadmium Chloride	0.62	21.5	0.63
Tamoxifen	0.12	88.5	45
Methylene bis(thiocyanate)	0	46	46

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.

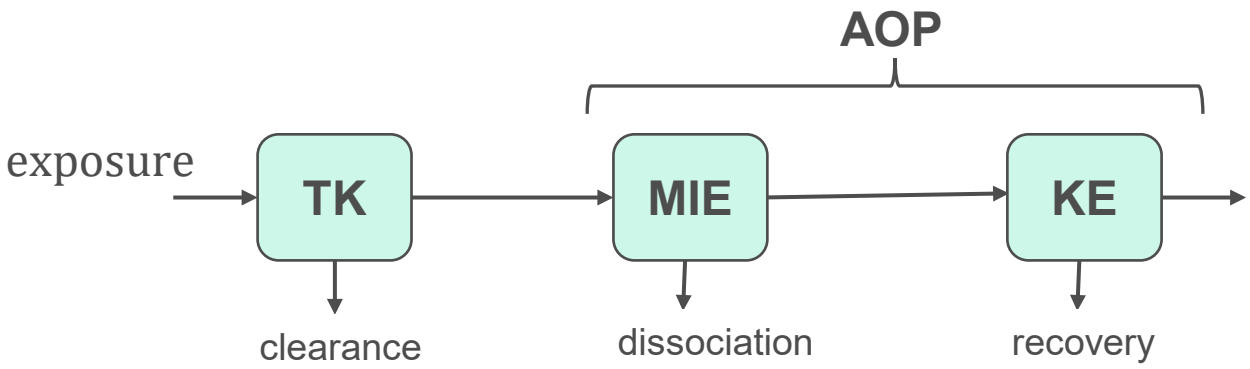
Methylene dithiocynate – pesticide, fungicide, disinfectant, only one case of acute liver failure documented (worker exposed to kgs of powder), metabolised in liver to cyanide and formaldehyde – no accumulation, therefore n = 0

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

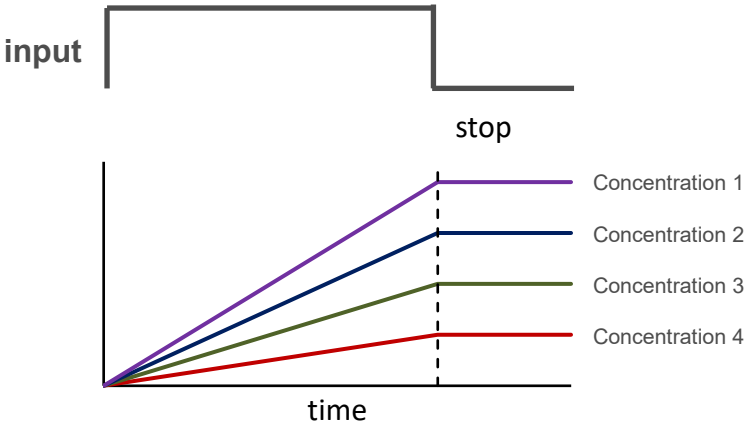


No clearance
Cumulation

No dissociation
∞ residence time

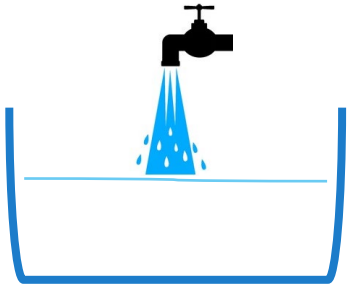
No recovery
permanent damage

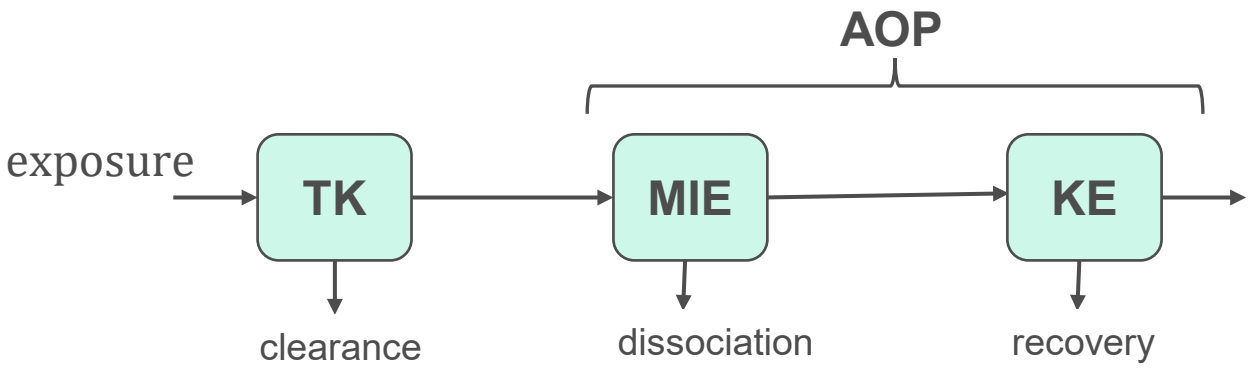
Events time-characteristics



$$E \approx \int c \, dt$$

equivalent of a tight container



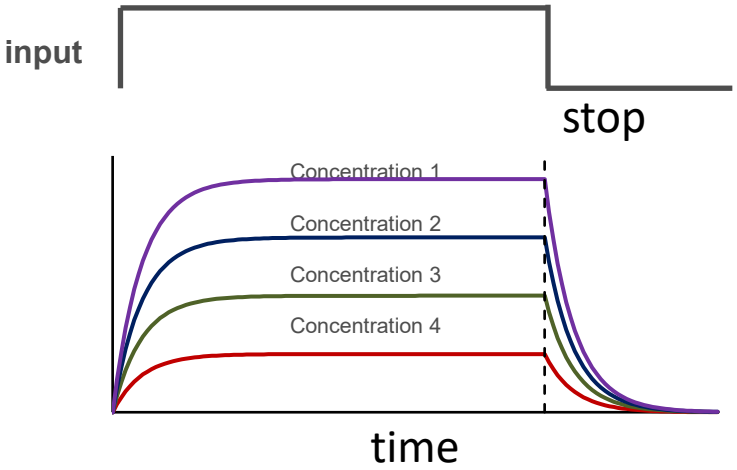


fast clearance
No cumulation

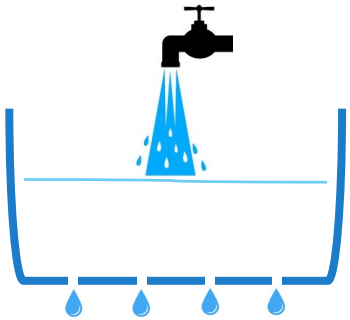
fast dissociation
short residence time

fast recovery
transient damage

Events time-characteristics

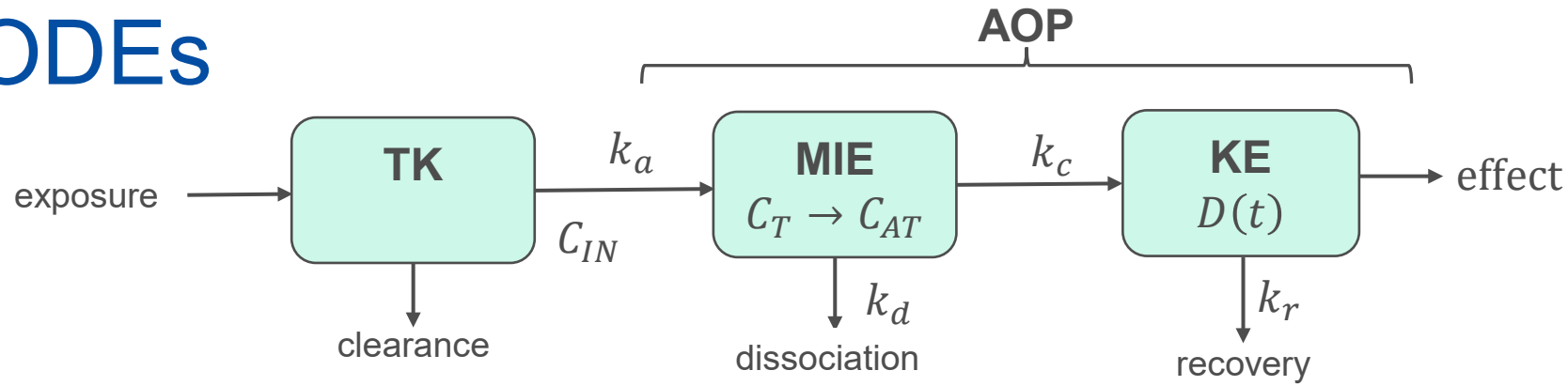


equivalent of a leaky container



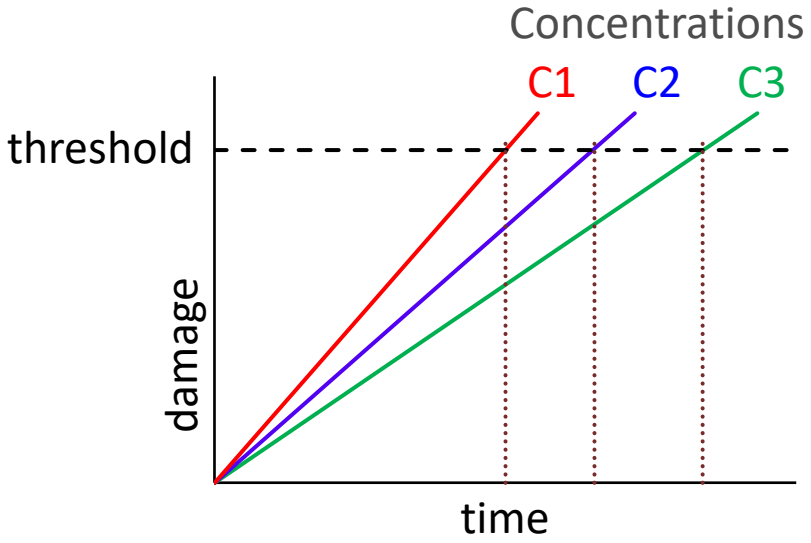
$$E \approx c$$

ODEs



$$\frac{dC_{AT}(t)}{dt} = k_a C_T C_{IN}(t) - k_d C_{AT}(t)$$

$$\frac{dD(t)}{dt} = k_c C_{AT}(t) - k_r D(t)$$



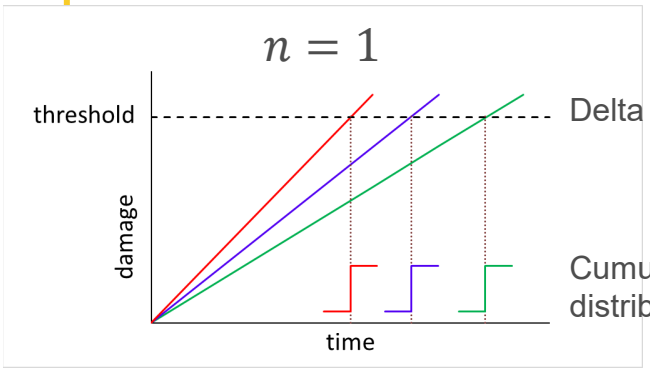
k_a - rate constant for binding to target
k_d - rate constant for dissociation [1/h]
k_c - rate constant for damage accrual [1/h]
k_r - rate constant for recovery [1/h]
C_T - concentration of targets
C_{IN} - concentration of toxicant
C_{AT} - concentration of targets affected by toxicant
$D(t)$ - damage

Equation 1 in case of saturation of targets:

$$\frac{dC_{AT}(t)}{dt} = k_a\big(C_T - C_{AT}(t)\big)C_{IN} - k_d C_{AT}(t) = k_a C_{IN} C_T - (k_a C_{IN} + k_d) C_{AT}(t)$$

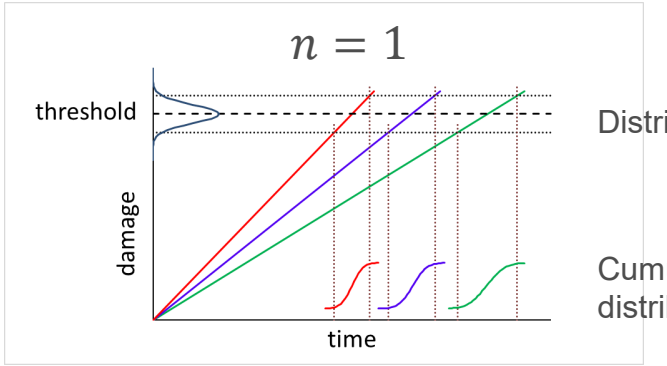
Saturation of targets	C_{IN}	Binding reversibility	Damage recovery	$D(t)$	$D(t)$ $t \to 0$	$D(t)$ $t \to \infty$	n $\lim_{t\to 0}$	n $\lim_{t\to \infty}$
N	constant C_{IN}	0	0	$k_a k_c C_T C_{IN} \frac{t^2}{2}$	$k_a k_c C_T C_{IN} \frac{t^2}{2}$	$k_a k_c C_T C_{IN} t^2$	2	2
N	constant C_{IN}	0	k_r	$k_a k_c C_T C_{IN} \frac{1}{k_r}\left(t - \frac{1}{k_r} + \frac{e^{-k_r t}}{k_r}\right)$	$k_a k_c C_T C_{IN} \frac{t^2}{2}$	$k_a k_c C_T C_{IN} \frac{t}{k_r}$	2	1
N	constant C_{IN}	k_d	0	$k_a k_c C_T C_{IN} \frac{1}{k_d}\left(t - \frac{1}{k_d} + \frac{e^{-k_d t}}{k_d}\right)$	$k_a k_c C_T C_{IN} \frac{t^2}{2}$	$k_a k_c C_T C_{IN} \frac{t}{k_d}$	2	1
N	constant C_{IN}	k_d	k_r	$k_a k_c C_T C_{IN} \frac{1}{k_r k_d}\left(1 + \frac{k_d e^{-k_r t}}{k_r - k_d} + \frac{k_r e^{-k_d t}}{k_d - k_r}\right)$	$k_a k_c C_T C_{IN} \frac{t^2}{2}$	$\frac{k_a k_c C_T C_{IN}}{k_d k_r}$	2	0
N	$C_{max} e^{-k_2 t}$	0	k_r	$k_a k_c C_T C_{max} \frac{1}{k_r k_2}\left(1 + \frac{k_2 e^{-k_r t}}{k_r - k_2} + \frac{k_r e^{-k_2 t}}{k_2 - k_r}\right)$	$k_a k_c C_T C_{max} \frac{t^2}{2}$	$\frac{k_a k_c C_T C_{max}}{k_2 k_r}$	2	0
N	$C_{max} e^{-k_2 t}$	0	0	$k_a k_c C_T C_{max} \frac{1}{k_2}\left(t - \frac{1}{k_2} + \frac{e^{-k_2 t}}{k_2}\right)$	$k_a k_c C_T C_{max} \frac{t^2}{2}$	$k_a k_c C_T C_{max} \frac{t}{k_2}$	2	1
N	$C_{max}(1 - e^{-k_1 t})$	0	0	$k_a k_c C_T C_{max}\left(\frac{1}{{k_1}^2} - \frac{t}{k_1} + \frac{t^2}{2} - \frac{e^{-k_1 t}}{{k_1}^2}\right)$	$k_a k_c C_T C_{max} \frac{k_1}{6} t^3$	$k_a k_c C_T C_{max} \frac{t^2}{2}$	3	2
N	$C_{max}(1 - e^{-k_1 t}) e^{-k_2 t}$	0	0	$k_a k_c C_T C_{max}\left(\frac{1}{(k_1 + k_2)^2} - \frac{1}{{k_2}^2} + \frac{t}{k_2} - \frac{t}{k_1 + k_2} + \frac{e^{-k_2 t}}{{k_2}^2} - \frac{e^{-(k_1 + k_2)t}}{(k_1 + k_2)^2}\right)$	$k_a k_c C_T C_{max} \frac{k_1}{6} t^3$	$\frac{k_a k_c C_T C_{max} k_1}{k_2(k_1 + k_2)} t$	3	1
N	$C_{max} e^{-k_2 t}$	k_d	k_r	$k_a k_c C_T C_{max}\left(\frac{e^{-k_r t}}{(k_2 - k_r)(k_d - k_r)} + \frac{e^{-k_2 t}}{(k_r - k_2)(k_d - k_2)} + \frac{e^{-k_d t}}{(k_r - k_d)(k_2 - k_d)}\right)$	tbc	tbc	2	0
N	$C_{max}(1 - e^{-k_2 t})$	k_d	k_r	$k_a k_c C_T C_{max} \frac{1}{k_d k_r}\left(1 + \frac{k_d k_2 e^{-k_r t}}{(k_r - k_2)(k_d - k_r)} + \frac{k_d k_r e^{-k_2 t}}{(k_2 - k_r)(k_d - k_2)} + \frac{k_r k_2 e^{-k_d t}}{(k_d - k_r)(k_2 - k_d)}\right)$	tbc	tbc	3	0
Y	constant C_{IN}	k_d	k_r	$\frac{k_c k_a C_{IN} C_T}{k_r(k_a C_{IN} + k_d)}\left(1 + \frac{(k_a C_{IN} + k_d) e^{-k_r t}}{k_r - (k_a C_{IN} + k_d)} + \frac{k_r e^{-(k_a C_{IN} + k_d)t}}{(k_a C_{IN} + k_d) - k_r}\right)$	tbc	tbc	∞	0
Y	constant C_{IN}	0	k_r	$k_c C_T \frac{1}{k_r}\left(1 + \frac{k_a C_{IN} e^{-k_r t}}{k_r - k_a C_{IN}} + \frac{k_r e^{-k_a C_{IN} t}}{k_a C_{IN} - k_r}\right)$	tbc	tbc	∞	1
Y	constant C_{IN}	0	0	$k_c C_T\left(t - \frac{1}{k_a C_{IN}} + \frac{e^{-k_a C_{IN} t}}{k_a C_{IN}}\right)$	tbc	tbc	∞	2

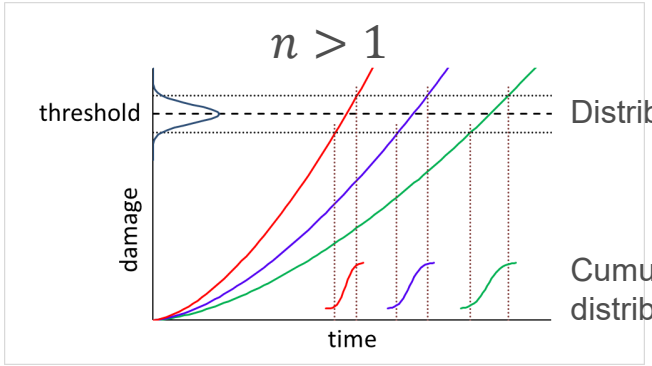
Principle of concentration and time response $\mathrm{Res(c,t)}$ formation

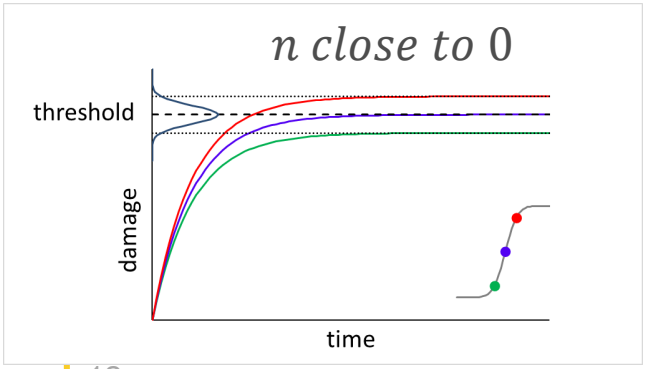


$$\mathrm{Res(c,t)} = \int_0^t DF\ (D(c,\tau) - D_{th})d\tau$$
$$\mathrm{Res(c,t)}\ = CDF(D(c,t) - D_{th})$$

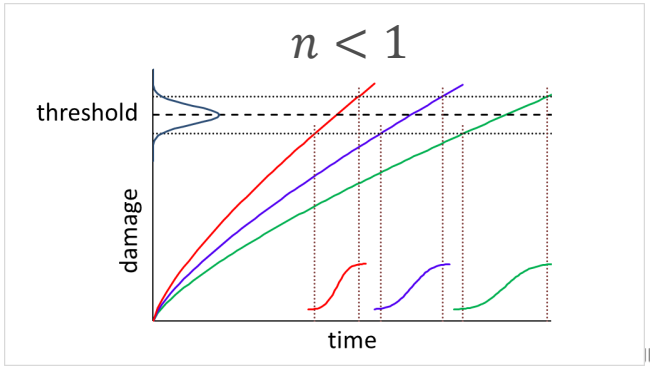
Log-normal *DF* is used

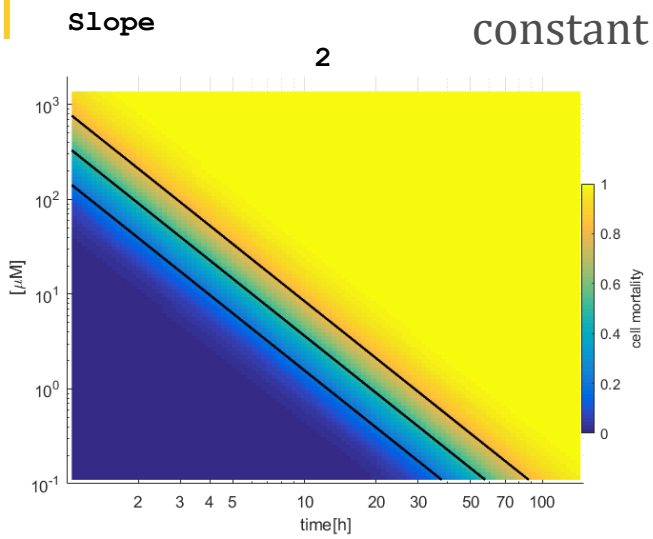




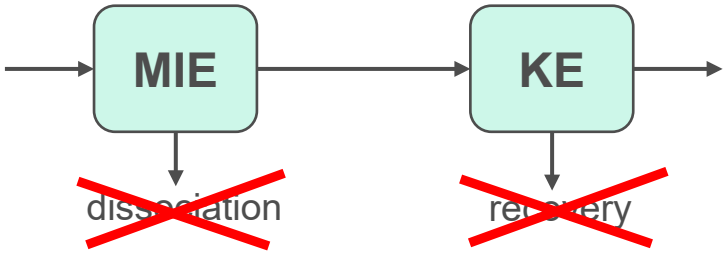


$$D \approx ct^n$$



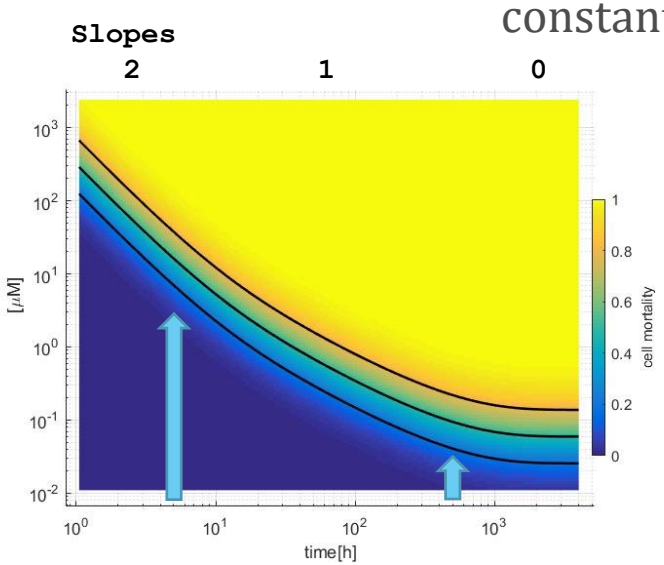


constant CIN

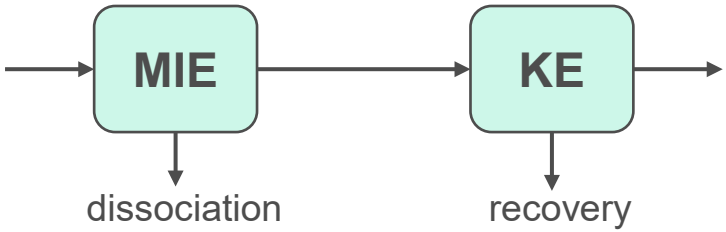


$$D(t) = k_a k_c C_T C_{IN} \frac{t^2}{2}$$

```
kd = 0; % dissociation rate (1/h)
kr = 0; % recovery rate (1/h)
```



constant CIN

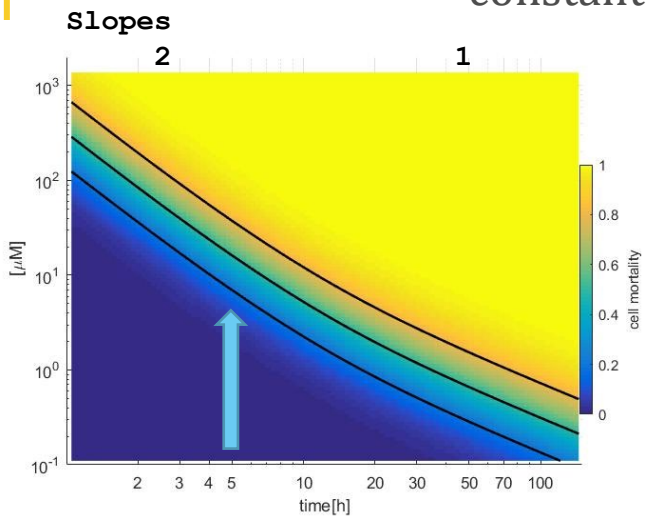


$$D(t) = k_a k_c C_T C_{IN} \frac{1}{k_r k_d}\left(1 + \frac{k_d e^{-k_r t}}{k_r - k_d} + \frac{k_r e^{-k_d t}}{k_\mathrm{d} - k_\mathrm{r}}\right)$$

```
kd = 1/5; % dissociation rate (1/h)
kr = 1/500; % recovery rate (1/h)
```

1/kd and 1/kr defines the time points where the isotox line bends and its slope changes

Symmetry in $D(t)$: kd = 1/500 & kr = 1/5 gives the same results



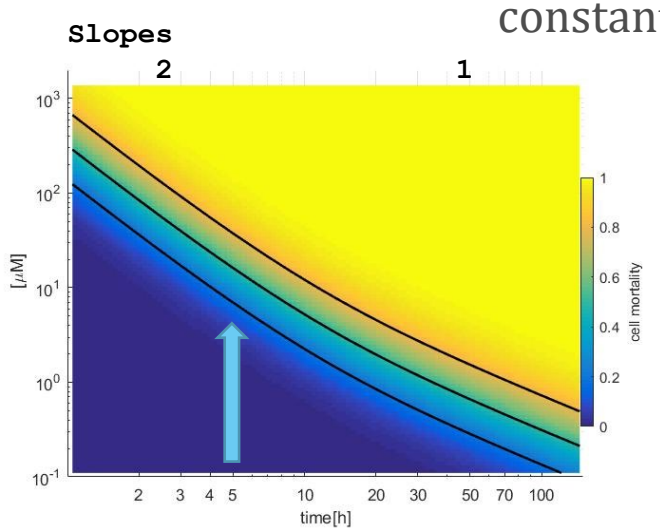
constant CIN → MIE → KE

~~dissociation~~

recovery

$$D(t) = k_a k_c C_T C_{IN} \frac{1}{k_r}\left(t - \frac{1}{k_r} + \frac{e^{-k_r t}}{k_r}\right)$$

```
kd = 0; % dissociation rate (1/h)
kr = 1/5; % recovery rate (1/h)
```



constant CIN → MIE → KE

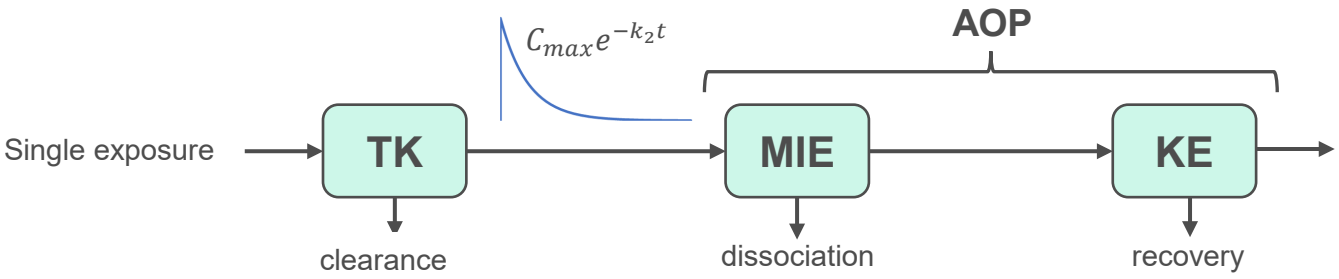
dissociation

~~recovery~~

$$D(t) = k_a k_c C_T C_{IN} \frac{1}{k_d}\left(t - \frac{1}{k_d} + \frac{e^{-k_d t}}{k_d}\right)$$

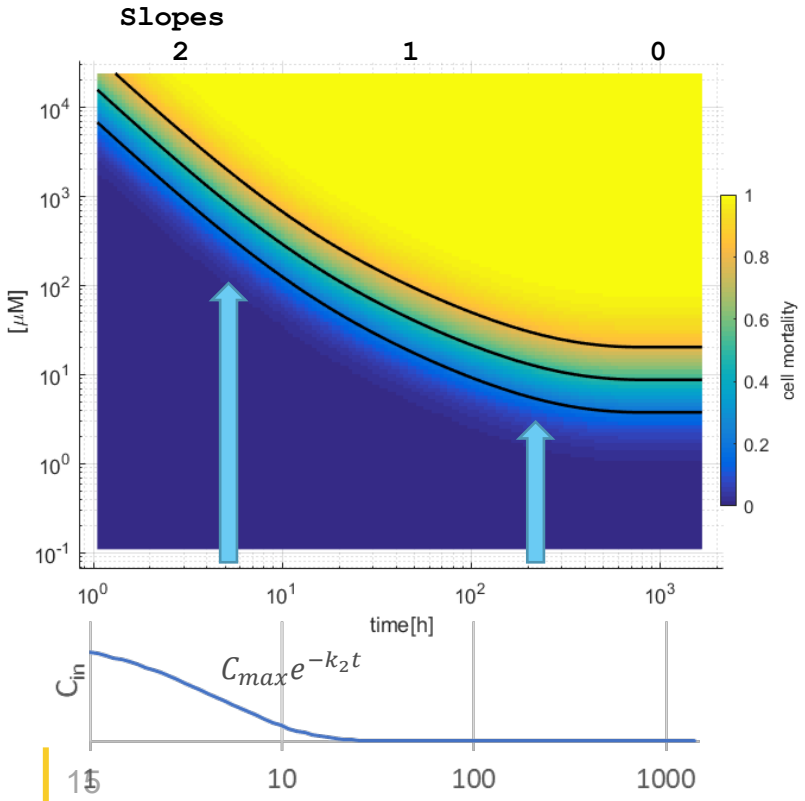
```
kd = 1/5; % dissociation rate (1/h)
kr = 0; % recovery rate (1/h)
```

1/kr or 1/kd defines the time points where the isotox line bends and its slope changes



the concentration decays to zero in time

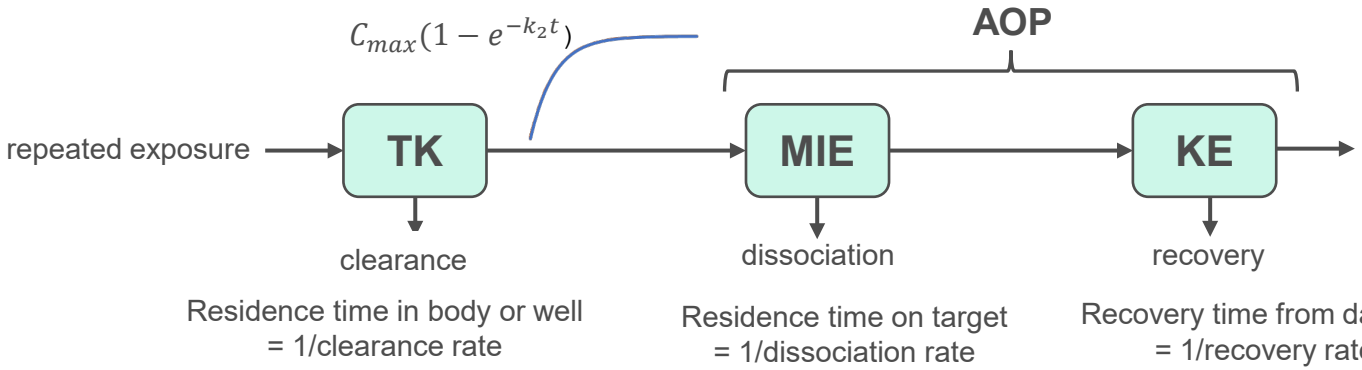
$$D(t) = k_a k_c C_T C_{max}\left(\frac{e^{-k_r t}}{(k_2 - k_r)(k_\mathrm{d} - k_r)} + \frac{e^{-k_2 t}}{(k_\mathrm{r} - k_2)(k_\mathrm{d} - k_2)} + \frac{e^{-k_\mathrm{d} t}}{(k_r - k_\mathrm{d})(k_2 - k_\mathrm{d})}\right)$$




```
kd = 1/200; % dissociation rate (1/h)
kr = 1/10000; % recovery rate (1/h)
k2 = 1/5; % TK clearance rate (1/h)
```

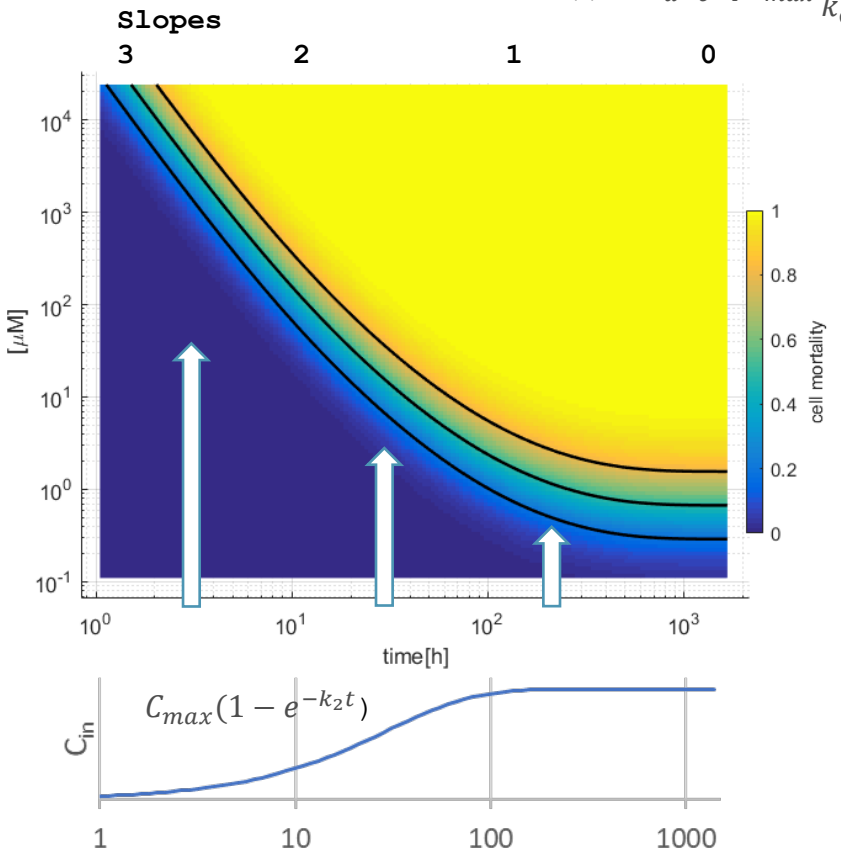
Symmetrical relation - kd, kr, k_2 interchangeable, Two larger of them, (here kd and k_2) define the bending points, where the slope changes

European Commission



the envelope of C_{in} follows ~ $C_{max}(1-e^{-k_2 t})$
the concentration reaches a steady state (C_{max}) in (~ $3/k_2$) hours

$$D(t) = k_a k_c C_T C_{max} \frac{1}{k_d k_r}\left(1 + \frac{k_d k_2 e^{-k_r t}}{(k_r - k_2)(k_d - k_r)} + \frac{k_d k_r e^{-k_2 t}}{(k_2 - k_r)(k_d - k_2)} + \frac{k_r k_2 e^{-k_d t}}{(k_d - k_r)(k_2 - k_d)}\right)$$




```
kd = 1/3; % dissociation rate (1/h)
kr = 1/200; % recovery rate (1/h)
k2 = 1/30; % TK clearance rate (1/h)
```

Symmetrical relation - kd, kr, k_2 interchangeable, They define the bending points, where the slope changes

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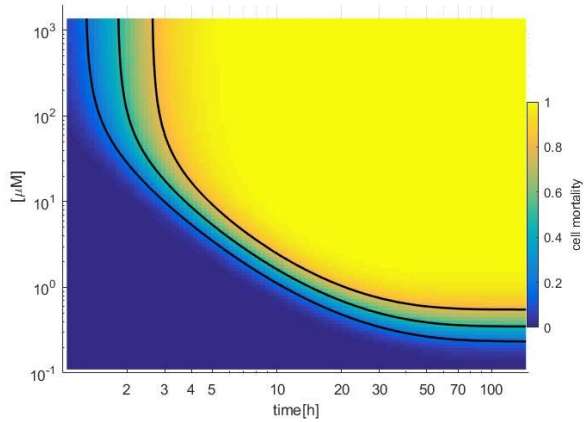
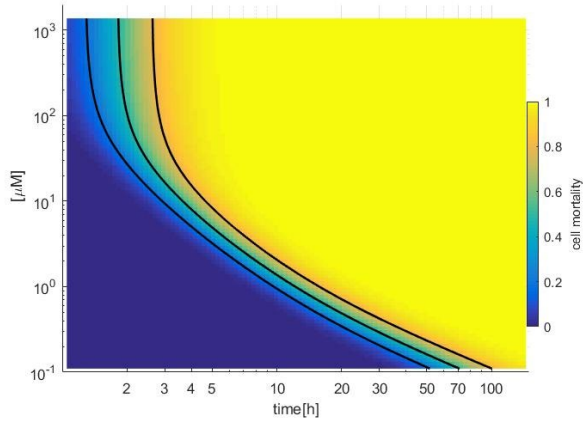
Saturation of targets – calculated for constant C_{IN}



- It has an impact on the left side of the graph, where its slope becomes ∞
- No impact on the right side

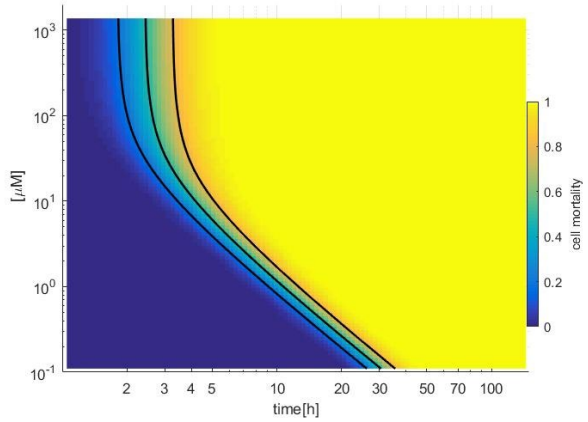
$$D(t) = \frac{k_c k_a C_{IN} C_T}{k_r (k_a C_{IN} + k_d)} \left(1 + \frac{(k_a C_{IN} + k_d) e^{-k_r t}}{k_r - (k_a C_{IN} + k_d)} + \frac{k_r e^{-(k_a C_{IN} + k_d) t}}{(k_a C_{IN} + k_d) - k_r} \right)$$

```
kd = 1/25; % dissociation rate (1/h)
kr = 1/5; % recovery rate (1/h)
ka = 1/20; % MIE binding rate
```



$$D(t) = k_c C_T \frac{1}{k_r} \left(1 + \frac{k_a C_{IN} e^{-k_r t}}{k_r - k_a C_{IN}} + \frac{k_r e^{-k_a C_{IN} t}}{k_a C_{IN} - k_r} \right)$$

```
kd = 0; %dissociation rate(1/h)
kr = 1/5; %recovery rate (1/h)
ka = 1/20; % MIE binding rate
```



$$D(t) = k_c C_T \left(t - \frac{1}{k_a C_{IN}} + \frac{e^{-k_a C_{IN} t}}{k_a C_{IN}} \right)$$

```
kd = 0; %dissociation rate (1/h)
kr = 0; %recovery rate (1/h)
ka = 1/20; % MIE binding rate
```

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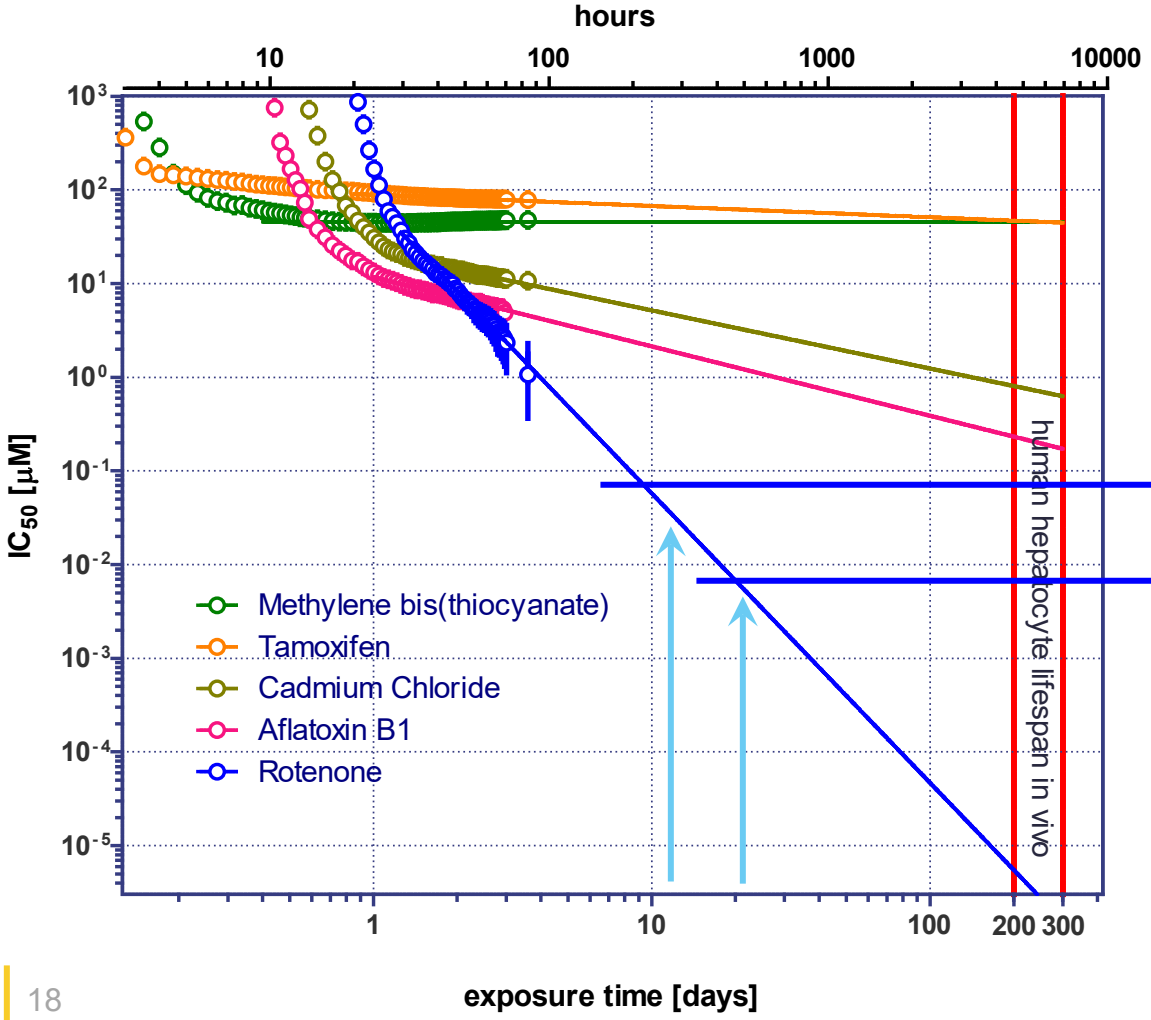
Implication for risk assessments

Recovery at the organ level: after transplantation, donor liver is back to normal in 6-8 weeks, characteristic recovery time is 1/3 x (6 to 8) = 2 to 3 weeks



Rotenone IRIS NOEL – chronic = 0.380 mg/kg-day oral Human (comptox.epa.gov)

0.078 uM

0.0078 uM

httk IVIVE supposing that Rotenone is not easily taken up through the gastrointestinal tract
– if 10% c = 0.078 uM, if 1% c = 0.0078 uM

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Conclusion

The analysis showed an insight into the description of the processes using their characteristic times (residence times in body or well (TK), residence time on the targets (MIE), and recovery time (KE)), which are the metrics used to quantify how quickly systems can return to their original states.

Limitation is that we supposed that all the events (TK, MIE, KEs) are the **first order** processes (linearly proportional to the concentration ***c***, or to the output of the previous event). In general, events are complex networks of reactions, sometimes can be described be e.g. as a power function ($\approx c^{\alpha}$) – **fractional order** processes (e.g. enzymatic Michaelis–Menten process, complex chain reaction mechanisms).

Only three consecutive events were supposed, need to address also a more complex networks of events.

Thank you

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