Baseline toxicity and volatility cut-off in reporter gene assays used for high-throughput screening

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Key words

Cell-based bioassays, reporter gene assays, high-throughput screening, narcosis, quantitative structure activity relationship, QSAR, volatility cut-off

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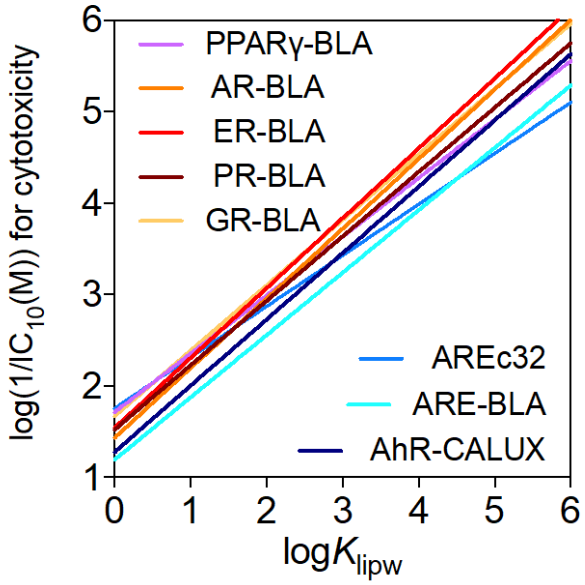


ABSTRACT

Most studies using high-throughput *in vitro* cell-based bioassays tested chemicals up to a certain fixed concentration. It would be more appropriate to test up to concentrations predicted to elicit baseline toxicity because this is the minimal toxicity of every chemical. Baseline toxicity is also called narcosis and refers to nonspecific intercalation of chemicals in biological membrane leading to loss of structure of membranes and impaired functioning of membrane-related processes such as mitochondrial respiration. In cells baseline toxicity manifests as cytotoxicity, which was quantified by a robust live-cell imaging method. Inhibitory concentrations for baseline toxicity varied by orders of magnitude between chemicals and were described by a simple quantitative structure activity relationship (QSAR) with the liposome-water partition constant as sole descriptor. The QSAR equations were remarkably similar for eight reporter gene cell lines of different cellular origin, six of which were used in Tox21. Mass-balance models indicated constant critical membrane concentrations for all cells and all chemicals with a mean of 69 mmol·kg_{lip}^{-1} (95%CI: 49 to 89), which is in the same

range as for bacteria and aquatic organisms and consistent with the theory of critical membrane burden of narcosis. The challenge of developing baseline QSARs for cell lines is that many confirmed baseline toxicants are rather volatile. We deduced from cytotoxicity experiments with (semi)volatile chemicals that only chemicals with medium-air partition constants >10000 L/L can be tested in standard robotic setups without appreciable loss of effect. Chemicals just below that cut-off showed cross-over effects in neighboring wells, whereas the effects of chemicals with lower medium-air partition constants were plainly lost. Applying the "volatility cut-off" to >8000 chemicals tested in Tox21 indicated that approximately 20% of Tox21 chemicals could have partially been lost during the experiments. We recommend applying the baseline QSARs together with volatility cut-offs for experimental planning of reporter gene assays, i.e., to dose only chemicals with medium-air partition constants > 10000 at concentrations up to the baseline toxicity level.

INTRODUCTION

The advent of high-throughput screening (HTS) with reporter gene assays has been instrumental for the shift towards *in vitro* methods in toxicity testing and risk assessment.[1, 2] For quantitative *in vitro* to *in vivo* extrapolation,[3] a comprehensive exposure assessment with measured freely dissolved concentrations in cell-based bioassays would be ideal. In 24-, 48- and 96-well plates, solid-phase microextraction methods have been implemented to quantify the freely dissolved concentrations of selected chemicals in cell-based bioassays,[4, 5] but it is not feasible yet to measure concentrations in 384- and 1536-well plates on a routine basis for the ten thousands of chemicals screened in programs like Tox21.[6] For effect assessment of environmental samples, such as surface water, wastewater, sediment, biota and human biomonitoring, we are faced with thousands or more diverse chemicals in one sample and there is no way to quantify them all analytically in all types of environmental samples, let alone in the bioassays. Provided we can transfer environmental mixtures in a defined way into the cellular test system,[7] we can estimate freely dissolved and cellular concentrations over the exposure time of an experiment by application of established mass balance models[8, 9] and kinetic information of cellular uptake[10] and binding to the multi-well plate.[11]

There remain two major issues that impede the implementation of HTS reporter gene assays in risk assessment and these refer to the loss of chemicals to the air in

common HTS setups and the need to define minimal toxicity (baseline toxicity). The latter is needed to define appropriate dosing concentrations and to interpret the cytotoxicity burst, which refers to the observation that, at concentrations close to cytotoxicity, cells activate numerous defense mechanisms, potentially leading to non-specific activation of reporter genes.[12, 13] Many different methods for cytotoxicity assessment[14] exist but not all of them are suitable for routine HTS.[15] Cytotoxicity assays are typically based on staining of cells or by quantifying metabolic function but artifacts are abundant,[15] especially when it comes to testing mixtures of environmental samples. We apply here a much simpler method, based on live-cell imaging, which is non-invasive and well compatible with testing of environmental samples.[16]

There are diverse set ups to dose volatile chemicals via the air phase in cell-based bioassays,[17] some of which were designed specifically for dosing via the air-liquid interface and most commonly applied to expose lung cells to aerosols and fine particles.[18] Mass balance models have also been applied to estimate the exposure in air-liquid interface cell system.[19, 20] None of these exposure systems are amenable to HTS using multi-well plates that are just covered with a plastic lid or a breathable sealant plus a plastic lid.

Semi-volatile organic chemicals are hard to dose via classical air-exposure systems because they are not volatile enough but they may still get lost or cause cross-contaminations in HTS bioassays. This grey zone remains to be clearly defined. Classic cellular bioassays dosed via the aqueous phase can also be set up without head-space, which is fairly easy for bacterial assays[21] but more challenging for the HTS bioassays in multi-well plates, where airtight systems are tedious and only work manually with syringe injections of the chemical to be dosed and minimal headspace, thereby typically compromising the cell viability and performance of the assay.[22] We have previously proposed an empirical “volatility cut-off” around a Henry constant of 10^{-6} atm m^3 mol^{-1}, corresponding to an air-water partition constant $K_{a/w}$ of $4 \cdot 10^{-5}$ L/L at 37°C (310K).[23] This cut-off was derived from a mass balance model expanded from Liu et al.[20] also accounting for binding of chemicals to medium proteins and lipids. A better determinant for the loss of effects of (semi)volatile chemicals is expected to be the medium-air partition constant ($K_{medium/air}$), and the terms “volatility cut-off” and “Henry constant cut-off” seem thus not optimal. An empirical $K_{medium/air}$ cut-off for effect losses due to evaporative losses of semi-volatile chemicals in standard test systems will thus be developed in this study. This will be accomplished by observing loss of

effects and cross-over of effects to other wells in combination with mass balance modelling.

In this study we opted against quantification of the exposure concentration in the cell assays. To measure loss processes in HTS systems by chemical analysis, we would have to modify the setup, which would not be a realistic HTS scenario. Hence we decided to quantify loss processes as loss of effect. While solid-phase microextration (SPME) methods have been developed[24] for this purpose and a practical workflow has been demonstrated using 96-well plates and reporter gene bioassays,[5] any measurement would interfere with the practical bioassay workflow of a typical cell-based bioassay. In an accompanying study Birch et al.[25] have measured the losses and cross-over of 24 volatile and semi-volatile chemicals from 3 different cell culture media in 96-well plates without cells. Both approaches taken together provided a strong line of evidence what is practically feasible. While exploring the domain of applicability, we also compared different dosing strategies, comparing conventional dosing using pipettes with dosing using a digital dispenser.[26]

Quantitative structure-activity relationships (QSAR) for prediction of baseline toxicity based on biomembrane-water partitioning constants (or proxies thereof, such as the octanol-water or liposome-water partition constant) have been developed for many aquatic organisms. Vaes et al.[27] developed a QSAR for non-polar and polar narcotics towards guppy fish with measured liposome-water partition constants $K_{lip/w}$[28] for 19 confirmed baseline toxicants (8 non-polar and 11 polar chemicals). They demonstrated that there is no difference in baseline toxicity between non-polar and polar chemicals and henceforth many groups have developed general baseline toxicity QSARs based on $K_{lip/w}$[29, 30] and the concept was also expanded to ionizable compounds by applying the ionization-corrected $D_{lip/w}$(pH).[31-33]

This group of 19 confirmed baseline toxicants was used to develop baseline toxicity QSARs for diverse reporter gene cell lines after those chemicals were excluded that would not pass the $K_{medium/air}$ cut-off. Another goal was to derive the critical membrane concentration for baseline toxicity in reporter gene cell lines. Each experimental nominal concentration can be converted to critical membrane concentration by mass balance modelling to check if baseline toxicity is uniform across cells. Provided we can confirm constant critical membrane concentrations, the mean of the critical membrane concentrations can be used to predict nominal cytotoxic concentrations and construct baseline toxicity QSAR for any given cell line and assay medium. Although baseline

toxicity constitutes the minimal toxicity any chemical has, it is important to know it, in order to define how specific effects are and to improve the planning of the dosing in HTS.

MATERIALS AND METHODS

Chemicals. The 19 chemicals from the original set of the Vaes et al.[27] were considered in this study (Table 1). This set of chemicals had been used to set up a baseline toxicity (narcosis) QSAR based on measured K_{lipw}[28] as chemical descriptor.

All chemicals in Table 1 were evaluated in the mass balance model, those with $K_{a/w}$ < 0.1 L/L were tested experimentally (Table S1), and those that passed the $K_{medium/air}$ cut-off of 10^4, which was derived experimentally as further detailed below, were included in the QSAR development.

Physicochemical Properties. The liposome-water partition constants $K_{lip/w}$ were experimentally determined by Vaes et al.[28] at 288 K using L-α-dimyristoylphosphatidylcholine as a model for membrane lipids (Table 1). Given the small temperature difference of only 2 K to the temperature, at which cell assays were performed (290 K), and because of the generally low temperature dependence of partition constants between condensed phases,[34] we did not apply a temperature correction. Bovine serum albumin served as surrogate for cell and medium proteins and the partition constants between proteins and water log $K_{protein/w}$ were estimated by a linear-solvation energy relationship (LSER) using eqation 1 from Endo et al.[35] and the chemical descriptors from the UFZ LSER database[36] (Table 1).

The air-water partition constants $K_{a/w}$ at 290K (Table 1) were determined from van't Hoff plots of log $K_{a/w}$ against 1/T, where the temperature dependence of Henry's law constant was estimated with the LSER given by Goss et al.[37]

The partitioning between assay medium and water $K_{medium/w}$ (Table 1) was calculated by a mass balance model for three types of media that were used for the bioassays. AhR-CALUX and AREc32 cells were tested in 90% DMEM and 10% FBS (volume fraction Vf_w = 99.09 %, Vf_{lip} = 0.0139 %, $Vf_{protein}$ = 0.89 %)[5], ARE-BLA was tested in 90% DMEM and 10% dialyzed FBS (dFBS; assuming the same Vf_{lip} and $Vf_{protein}$ as for AhR-CALUX and AREc32) and the assay medium for all other GeneBLAzer cell lines was 98% Opti-MEM supplemented with 2% charcoal-stripped FBS (csFBS; Vf_w = 99.51 %, Vf_{lip} = 0.0023 %, $Vf_{protein}$ = 0.49 %).[5]

The $K_{medium/w}$ were calculated with eq. 1 from the volume fractions Vf of lipids (Vf_{lip}), proteins ($Vf_{protein}$) and water (Vf_w) and the partition constants between lipids and water ($K_{lip/w}$) and proteins and water ($K_{protein/w}$).

$$K_{medium/w}=Vf_{lip,medium}K_{lip/w} + Vf_{protein,medium}K_{protein/w} + Vf_{w,medium} \quad (1)$$

The partition constants between medium and air $K_{medium/air}$ were calculated with Hess' law (eq. 2).

$$K_{medium/air}=\frac{K_{medium/w}}{K_{a/w}} \quad (2)$$

Table 1. Chemicals tested and their partition constants between liposomes and water $K_{lip/w}$, protein and water $K_{protein/w}$,air and water $K_{a/w}$ as well as between medium and water $K_{medium/w}$ and medium and air $K_{medium/air}$. The column "HTS setup?" indicates if the chemical can be safely run under HTS conditions (marked with yes), or not (marked with no). In the column "Included in QSAR?" the chemicals that were the training set of the QSAR are indicated and the "additional" refers to chemicals that were used to validate the applicability domain for the QSAR. In the column "polarity" we indicate the previous classification for polar and non-polar chemicals from the initial set of Vaes' baseline toxicants.[27] The chemicals are sorted from high to low $K_{medium/air}$ (calculated by eq. 1 and 2 without further temperature correction). Further information on the tested chemicals is given in the SI, Table S1.

Chemical	HTS setup?	Included in QSAR?	Polarity	log $K_{lip/w}$ [L/L][28]	log $K_{protein/w}$ [L/L] LSER	log $K_{a/w}$ [L/L] LSER	log $K_{medium/w}$ [L/L][a]	log $K_{medium/w}$ [L/L][b]	log $K_{medium/air}$ [L/L] [a]	log $K_{medium/air}$ [L/L] [b]
Temperature				*288 K*	*290 K*	*290 K*				
2-Phenylphenol	yes	training set	polar	3.46	2.99	-5.87	1.01	0.76	6.88	6.64
3-Nitroaniline	yes	training set	polar	2.17	2.13	-6.00	0.35	0.22	6.35	6.22
4-Chloro-3-methylphenol	yes	training set	polar	3.34	2.80	-4.52	0.84	0.61	5.36	5.14
4-Pentylphenol	yes	training set	polar	4.31	3.55	-3.77	1.55	1.27	5.31	5.03
2-Allylphenol	yes	training set	polar	3.06	2.46	-4.05	0.57	0.38	4.62	4.43
2,4,5-Trichloroaniline	yes	training set	polar	4.16	3.40	-3.05	1.41	1.13	4.46	4.19
2-Butoxyethanol	yes	training set	non-polar	0.60	0.71	-4.24	0.02	0.01	4.25	4.24
Aniline	no	excluded	polar	1.63	1.39	-3.82	0.08	0.05	3.91	3.87
Quinoline	no	excluded	polar	1.67	1.77	-3.68	0.18	0.11	3.86	3.79
Butan-1-ol	no	additional	non-polar	0.45	0.91	-3.03	0.03	0.01	3.06	3.05
Pentan-3-ol	no	additional	non-polar	0.995	1.00	-2.89	0.03	0.02	2.92	2.91
Nitrobenzene	no	additional	polar	2.01	1.99	-2.71	0.28	0.17	2.99	2.88
Hexan-1-ol	no	additional	non-polar	1.91	1.71	-2.75	0.16	0.10	2.92	2.85
2-Nitrotoluene	no	additional	polar	2.41	2.34	-2.48	0.48	0.32	2.96	2.80
N,N-Dimethylaniline	no	additional	polar	2.33	2.09	-2.33	0.33	0.20	2.66	2.53
2,4,5-Trichlorotoluene	no	excluded	non-polar	4.77	3.96	-0.61	1.96	1.67	2.57	2.28
1,3,5-Trichlorobenzene	no	excluded	non-polar	3.95	3.55	-0.36	1.53	1.27	1.89	1.62
Chlorobenzene	no	excluded	non-polar	2.81	2.53	-0.51	0.61	0.42	1.12	0.93
p-Xylene	no	Excluded	non-polar	2.98	2.64	-0.32	0.70	0.50	1.03	0.82

[a]90% DMEM with Glutamax and 10% FBS; [b]98% OptiMEM and 2% cs-FBS.

Cell Lines. The reporter gene assays and the cell line they were derived from are listed in Table 2. The GeneBLAzer cell lines[38,39] were obtained from Thermo Fisher (Schwerte, Germany), AREc32 cells[40] by courtesy of C. Roland Wolf, Cancer research UK, and AhR-CALUX cells[41] by courtesy of Michael Denison, UC Davis, USA. 30 µL of cell suspension containing the number of cells given in Table 2 were plated in each well of a black 384-well polystyrene microtiter plate with clear bottom (AREc32 #3764, all other cell lines BioCoat # 356663, Corning, Maine, USA) using a Multiflow Dispenser (Biotek, Vermont, USA) followed by 24h incubation at 37°C and 5% CO_2.

Previous experiments have demonstrated that these cells need 24h to adhere and to adapt to the new environment. Thus, the cell number stays virtually constant during that time[10] and we used the number of cells plated as the starting cell number. We measured the confluency of the cell layer in the plate directly before dosing corresponding to 24 h after seeding and again after 24±2 h after dosing. The average of the confluency was used to estimate the final cell number. The average of the difference between the plated cell number and the estimated final cell number was used for modeling (mean cell number in assay). The total volume of the cells in Table 2 and the volume fraction of water $Vf_{w,cell}$, proteins $Vf_{protein,cell}$ and lipid $Vf_{lipid,cell}$ of the GeneBLAzer cell lines were taken from Fischer et al.[9] and of AREc32 and AhR-CALUX from Henneberger et al.[24] The partition constants between cells and water (eq. 3) were calculated in analogy to the medium-water partitioning (eq. 1).

$$K_{cell/w}=Vf_{lip,cell}K_{lip/w} + Vf_{protein,cell}K_{protein/w} + Vf_{w,cell} \quad (3)$$

Table 2. Reporter gene cell lines evaluated and numbers of cells plated and averaged during the experiment in 384-well plates as well as the total volume of the cells and the apportionment into water, lipid and protein phases.

Reporter gene cell line	Derived from	Number of plated cells/ well	Estimated mean cell number in assay[a]	Total volume of cells V_{cell} (nL)	$Vf_{water,cell}$	$Vf_{protein,cell}$	$Vf_{lipid,cell}$
AREc32	MCF7	2500	4300±290	16.8	94.4%[c]	5.1%[c]	0.5%[c]
ARE-BLA	HepG2	5500	5820±310	18.2	87.4%[b]	9.5%[b]	3.2%[b]
AhR-CALUX (H4L7.5c2)	H4IIe	3000-3250	5360±750	21.8	93.9%[c]	5.5%[c]	0.6%[c]
PPARγ-BLA	HEK293H	4500-5500	5940±760	15.7	88.7%[b]	8.0%[b]	3.4%[b]
AR-BLA	HEK293T	4500-5000	5650±580	331.8	90.6%[b]	8.4%	1.0%[b]

ERα-BLA	HEK293T	3500-4250	5110±460	35.3	90.6%[b]	8.4%[b]	1.0%[b]
PR-BLA	HEK293T	4500-4750	5870±450	41.4	90.6%[b]	8.4%[b]	1.0%[b]
GR-BLA	HEK293T	4500-5000	6410±450	45.0	90.6%[b]	8.4%[b]	1.0%[b]

[a]*average between plated cells and final cell number after 24 h of* exposure; [b]Fischer et al.;[9] [c]Henneberger et al.[24]

Assay Medium. All cell lines were grown as described in previous work.[42-44] For the cytotoxicity assay we switched from growth medium to assay medium that was composed of 90% DMEM Glutamax with 10% FBS for AREc32 and AhR-CALUX, 90% DMEM with 10% dFBS and 0.1 mM NEAA und 25 mM HEPES for ARE-BLA and 98% Opti-MEM with 2% cs-FBS for all other GeneBLAzer cell lines. 100 U/mL Penicillin and 100 µg/mL Streptomycin were supplemented to the media. All media and FBS were purchased from Thermo Fisher (Schwerte, Germany).

Dosing Procedures. Liquid chemicals were dosed into medium as neat compounds. Of the baseline toxicants, only 3-Nitroaniline, 2,4,5-Trichloroaniline and 2-Phenylphenol were solids as well as the seven additional test chemicals and 20 mM to 0.5 M stock solutions were prepared in DMSO.

The dosing plates were prepared by dispensing different volumes of the liquids or DMSO stock solutions into 120 µL medium in 96-well plates using a Tecan D300e Digital Dispenser (Tecan, Crailsheim, Germany). Technical details and diverse bioassay applications of this dispenser that is based on inkjet technology are provided in the literature.[26, 45, 46] The dosing plates were sealed and shaken for 5-10 seconds prior to the dosing step.

The diluted test chemicals were dosed in duplicates by transferring two times 10 µL from two 96-well dosing plates into a 384-well plate that contained 30 µL medium and the number of cells given Table 2, using a 96-pipette head (Hamilton Microlab Star, Bonaduz, Schwitzerland). In routine HTS set up of the assays, a lid is placed on the plates during incubation. If no further information was given, this was the experimental set up of this study. We also evaluated if the loss of chemicals was reduced if the plate was sealed by a breathable foil (Biozym, Hessisch Oldendorf, Germany) during incubation for 24h.

For comparison, the dosing plates were also prepared with a Hamilton Robot (Star, Hamilton, Bonaduz, Switzerland) as previously described.[44] Briefly, for each chemical, 45µL of the chemical dissolved in assay medium were transferred from the

dosing vials into 45 µL of assay medium in one well of a clear 384-well plate (Corning, Maine, USA) followed by a 11-step serial dilution with a 1:2 dilution between each step. 10µL of the diluted samples were dosed into 384-well plate containing 30µL of medium with the cell numbers given in Table 2. A detailed visualization of the bioassay workflow is given in the SI, Section S2, Figure S1.

Quantification of Cytotoxicity. The confluency as a surrogate for the number of cells in each well of the 384-well plates was measured immediately before dosing and again after another 24h incubation at 37°C and 5% CO_2 using an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, USA). Image analysis of the confluency of the cell layer was performed using the IncuCyte S3 software, that provides tools for image processing and quantitative analysis. A method for analysis for each cell line was defined using a training set of images with different confluency (see Section S3 in the SI and Figure S2 for more details). Confluency served as surrogate for cell viability and proliferation and was expressed as "% inhibition of cell viability" as compared to unexposed cells.

$$\%\ \text{Inhibition of cell viability} = 100\% - \frac{\%\ \text{confluency (exposed cells)}}{\%\ \text{confluency (unexposed cells)}} \quad (4)$$

The SI, Section S4 and Figure S3, provides a comparison of cell viability testing using the Presto Blue® assay and the cell imaging. The live-cell imaging method has been used for cytotoxicity assessment of water samples in previous studies.[16, 47] We further compared the dosing by the digital dispenser of DMSO stocks and dosing of methanol stocks with automated pipetting (SI, Section S5, Figures S4 and S5) and found no differences, hence all concentration-response curves of a given chemical were evaluated together.

The inhibitory concentration for 10% reduction of cell viability/growth, i.e. cytotoxicity, IC_{10}, was determined from the linear portion of the concentration-response curve, which is below 30-40% inhibition.[48] The IC_{10} was calculated from the slope of the regression of % inhibition of cell viability against the dosed (nominal) concentration with eq. 5 and the standard error of IC_{10} was calculated with eq. 6.

$$IC_{10} = \frac{10\%}{\text{slope}} \quad (5)$$

$$SE\ (IC_{10}) \approx \frac{10\%}{\text{slope}^2} \cdot SE(\text{slope}) \quad (6)$$

The IC_{10} of DMSO are given for reference in the SI, Table S2.

Baseline toxicity QSAR. Baseline toxicity QSAR of the form given in eq. 7 were set up for all cell lines from a regression of experimental $\log(1/IC_{10})$ against $\log K_{lip/w}$.

$$\log(1/IC_{10}(M)) = \text{slope} \cdot \log K_{lip/w} + \text{intercept} \quad (7)$$

Mass balance model for 384-well plates with head space. We expanded the mass balance model developed previously[9] by an additional air compartment analogously to Liu et al.[20] Additional loss processes in HTS bioassays include binding to the plastic of the well plates and degradation. Binding to the plastic of the well plates was determined to be negligible under the test conditions.[11] The baseline toxicants used here were stable in other toxicity experiments[27] over longer duration, so we did not check stability in the present study.

The resulting mass balance equations for the fraction in air, f_a, in medium, f_{medium} and in the cells, f_{cell}, is given by eqs. (8-10).

$$f_a = \frac{1}{1 + \frac{K_{cell/w} V_{cell}}{K_{a/w} V_a} + \frac{K_{medium/w} V_{medium}}{K_{a/w} V_a}} \quad (8)$$

$$f_{medium} = \frac{1}{1 + \frac{K_{cell/w} V_{cell}}{K_{medium/w} V_{medium}} + \frac{K_{a/w} V_a}{K_{medium/w} V_{medium}}} \quad (9)$$

$$f_{cell} = \frac{1}{1 + \frac{K_{medium/w} V_{medium}}{K_{cell/w} V_{cell}} + \frac{K_{a/w} V_a}{K_{cell/w} V_{cell}}} \quad (10)$$

The cellular inhibitory concentration $IC_{10,cell}$ can then be predicted from the nominal inhibitory concentration IC_{10} by multiplying with f_{cell} and correcting for the volume ratios (eq. 11).

$$IC_{10,cell} = IC_{10} \cdot f_{cell} \cdot \frac{V_{medium} + V_{cell}}{V_{cell}} \quad (11)$$

Critical Membrane Concentrations. Within the cell compartment, the fraction in the membrane of the cell $f_{lip,cell}$ can be calculated with eq. 12. For simplicity, we use the liposome water partition constant $K_{lip/w}$ as the partition constant representative for all cellular lipids.

$$f_{lip,cell} = \frac{1}{1 + \frac{1}{K_{lip/w} V_{lip,cell}} V_{w,cell} + \frac{K_{protein/w} V_{protein,cell}}{K_{lipw} V_{lip,cell}}} \quad (12)$$

The critical membrane concentration $IC_{10,membrane}$ can then be derived from $f_{lip,cell}$ and $IC_{10,cell}$ by accounting also for the volumes of lipids in cells $Vf_{lipid,cell}$ (Table 2).

$$IC_{10,membrane} = IC_{10,cell} \cdot \frac{f_{lip,cell}}{Vf_{lipid,cell}} \quad (13)$$

Prediction of $K_{medium/air}$ for Tox21 chemicals. The names and physicochemical properties of 8947 chemicals tested in Tox21[49] were retrieved from the Chemistry Dashboard of the UP EPA[50] The $K_{a/w}$ was calculated from the K_{ow} and K_{oa} that had been predicted with OPERA.[51] We calculated the $K_{medium/air}$ from $K_{medium/w}$ and $K_{a/w}$ assuming a medium that contains 10% FBS. For screening purposes, the $K_{medium/w}$ can be estimated by very simple QSARs and a mass balance model (eq. 14) that only require the K_{ow} as sole input parameter to estimate protein binding $K_{protein/w}$ and lipid partitioning $K_{lip/w}$, together with some information on the medium composition.[52]

$$K_{medium/w} = 0.0046 \cdot K_{protein/w} + 0.00015 \cdot K_{lip/w} + 0.99525$$

$$= 0.0046 \cdot 10^{0.71 \cdot \log K_{ow} + 0.42} + 0.00015 \cdot 10^{1.01 \cdot \log K_{ow} + 0.12} + 0.99525 \quad (14)$$

This equation holds only for neutral organic chemicals but was nevertheless applied to all chemicals in Tox21 irrespective of their speciation.

RESULTS AND DISCUSSION

Loss processes to the air and cross-contamination of neighboring wells. For the standard HTS set up, we previously proposed a "volatility cut-off" corresponding to a Henry constant of 10^{-6} atm·m^3·mol^{-1}, corresponding to a $K_{a/w}$ of approximately $4 \cdot 10^{-5}$ ($\log K_{a/w}$ -4.4).[23] Here we explored this cut-off in more detail and if chemicals can cross-contaminate neighboring wells. To this end we dosed only the middle six rows of a 384-well plates with a dilution series of one chemical per plate and added medium only to the remaining rows. Detailed results are described in Section S6 of the SI. Briefly, Butoxyethanol ($\log K_{a/w}$ -4.24) showed uniform cytotoxicity in all wells dosed with the same concentration and no effects in neighboring unexposed wells (Figure S6A). Pentan-3-ol ($\log K_{a/w}$ -2.89) clearly showed a loss of cytotoxicity (Figure S6B) and N,N-Dimethylaniline ($\log K_{a/w}$ -2.33) cross-contaminated the unexposed wells or wells dosed with lower levels (Figure S6C).

Sealing the plate with a breathable foil instead of just placing a lid did not change the picture for Butoxyethanol but reduced the loss of effect for the two more volatile chemicals, however, could not avoid the cross-contamination of neighboring wells

(Figure S6). In the accompanying paper,[25] sealing did not reduce loss of chemicals but reduced cross-over.

The test chemicals were ranked in Table 1 according to their $K_{medium/air}$. Butoxyethanol had the lowest log$K_{medium/air}$ of 4.24/4.25 of all chemicals that appeared still retained in the plate during the experiment and therefore we tentatively defined a $K_{medium/air}$ cut-off of 10^4, which is evaluated more systematically in the next section.

Loss processes to the air: defining the physicochemical applicability domain of HTS reporter gene assays. The loss processes to air were not only determined by the volatility or vapor pressure of the compounds or the Henry constant but also by how much the medium components and cells retain the chemical and reduce the freely dissolved concentration, i.e., by the partition constant between medium and air $K_{medium/air}$. As Figure 1A indicates, only very hydrophilic chemicals showed a direct correlation between the $K_{medium/air}$ and the air water partition constants $K_{a/w}$ (dashed line). More hydrophobic chemicals such as 2-Phenylphenol, 4-Pentylphenol or 2,4,5-Trichloroaniline (Table 1, hydrophobicity expressed as log $K_{lip/w}$) deviated up to a factor of 10 from the one-to-one line indicated by a dotted horizontal line in Figure 1A and the deviation was slightly larger for the AhR-CALUX, AREc32 and ARE-BLA medium that contained a higher fraction of FBS, and therefore had a higher retaining capacity.

The vertical dotted line in Figure 1B indicates the $K_{medium/air}$ cut-off of 10^4, below which we had seen loss of chemicals and cross contamination of wells in our experiments as discussed above. This cut-off corresponds to 400 mL of air in equilibrium with the 40 µL of medium to reach a one-to-one distribution between medium and air.

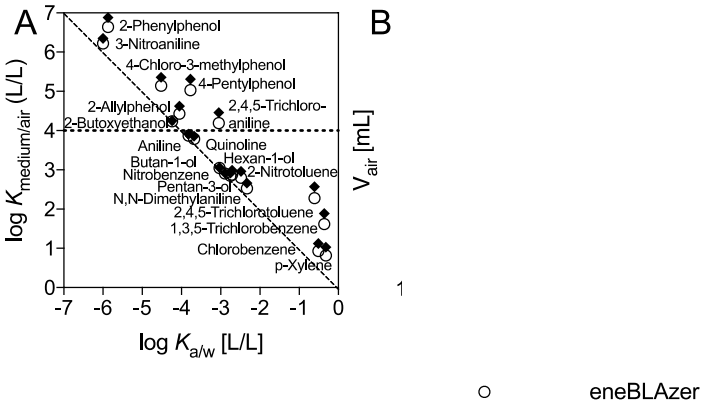


Figure 1A. Medium-air partition constants $K_{medium/air}$ *plotted against air-water partition constants* $K_{a/w}$ *for media with 10% and 2% FBS (AhR-CALUX, AREc32 and ARE-BLA with medium consisting of 90% DMEM Glutamax with 10%*

FBS (black diamonds) and the other GeneBLAzer assays with medium consisting of 98% Opti-MEM with 2% csFBS (open circles)). B. Volume of air to be equilibrated with 40 µL of media (same symbols as in A) to reach an one-to-one distribution between the two phases (phase ratio V_{medium}:V_{air} = 1 with V_{medium} = 40 µL). The horizontal line in A and the vertical line in B mark the $K_{medium/air}$ cut-off of 10^4.

The cells have only a very minor contribution to the overall partitioning of chemicals within the well and will not change the picture substantially unless they are metabolized.[9] There is a difference between the different media used, the medium for AREc32 and AhR-CALUX is supplemented with 10% FBS, which has a higher sorptive capacity than the GeneBLAzer medium supplemented with 2% FBS and hence we can expect that more chemicals can be retained in the AREc32 and AhR-CALUX assays (Figure 1A).

It is interesting to compare Aniline, Quinoline and 2,4,5-Trichloroaniline: From $K_{a/w}$ alone one would expect that 2,4,5-Trichloroaniline would be lost and cross-contaminate neighboring wells, while the others pose less of a problem. This is not the case, it is just the other way around. As is shown below in section "Baseline toxicity QSARs", 2,4,5-Trichloroaniline was a valid contributor to the baseline toxicity QSARs of all eight cell lines. In contrast, Aniline and Quinoline contaminated neighboring wells so badly that all plates that contained those two chemicals could not be evaluated (data not shown). These two chemicals were excluded from further experiments altogether. The $K_{medium/air}$ cut-off substantiates these empirical findings- 2,4,5-Trichloroaniline is above and Aniline and Quinoline are below the cut-off (Figure 1A).

The other semi-volatile chemicals with a log $K_{medium/air}$ between 2 and 3 (Butan-1-ol, Pentan-1-ol, Nitrobenzene, Hexan-1-ol, 2-Nitrotoluene and N,N-Dimethylaniline) were mainly lost without a strong cross contamination and were therefore included in further experiments in order to use the baseline QSAR analysis to estimate the degree of loss. We did not attempt to measure the cytotoxicity of the remaining four chemicals with log $K_{medium/air}$ < 2, they were only included in the thermodynamic analysis as reference. Note that Birch et al.[25] included a substantially higher number of such more volatile chemicals in their study of test substance losses and cross-over in 96-well plates.

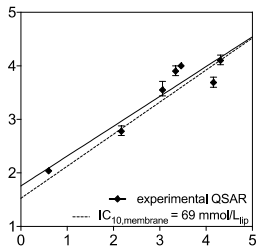
Loss processes to other system components. The losses due to binding to the multi-well plates are expected to be negligible because we recently demonstrated that the binding to polystyrene will only become significant within the 24h exposure for medium that is not supplemented with FBS due to the substantially lower

$K_{polystyrene/medium}$ than $K_{polystyrene/w}$ and very slow diffusion coefficients of chemicals in polystyrene.[11]

Likewise the "loss" due to cellular uptake is negligible in absence of biotransformation in the overall mass balance with fractions of chemicals in cells (eq. 7) of 0.06 to 3.1%. As we will show below, this does not mean that the effective cellular concentrations are low but just that the volumes of proteins and lipids of the medium are much higher than that of the cells (Table 2). It is vital to differentiate between mass balances, i.e., amounts and fractions in the different compartments, and concentrations in the different compartments (cell, medium, air).

Baseline toxicity QSARs. All concentration-cytotoxicity curves are plotted in the SI, Section S7, Figures S7 to S14. We obtained valid IC_{10} (Table S3) for only 7 out of the 19 chemicals in the dataset of Vaes et al.[27] after defining the $K_{medium/air}$ cut-off. This data size is relatively small for regression analysis with two fit parameters per cell line. However the entire set of 51 valid IC_{10} can be used to evaluate if critical membrane concentrations are constant across chemicals and cell lines.

All baseline QSARs are depicted in Figure 2 and the QSAR equations are given in Table 3. As anticipated, the QSARs of all tested cell lines were similar. The additional chemicals tested around the volatility cut-off are discussed in Section S8, some are still within the QSAR but they are clearly starting to get lost and were therefore not included in the QSAR training set.



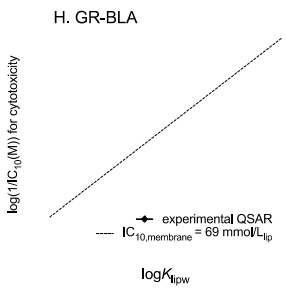


Figure 2. QSARs for baseline toxicity for all cell lines. The solid lines correspond to the best fit (equations are given in Table 3) and the dotted lines are the predicted IC_{10} and associated QSAR for the mass balance model, calculated with an internal critical membrane concentration of 69 mmol/L_{lip}.

Table 3. QSARs for baseline toxicity for all cell lines of the form log(1/IC_{10}(M)) = slope · logK_{lipw}+ intercept. If n = 6, 3-Nitroaniline had to be excluded due to poor quality of the concentration-inhibition curves..

Reporter gene cell line	Slope	intercept	R^2	n
AREc32	0.56±0.09	1.76±0.28	0.8906	7
ARE-BLA	0.68±0.08	1.19±0.26	0.9350	7
AhR-CALUX	0.73±0.10	1.28±0.31	0.9181	7
PPARγ-BLA	0.64±0.20	1.71±0.69	0.9523	6
AR-BLA	0.76±0.14	1.44±0.49	0.8755	6
ERα-BLA	0.76±0.10	1.54±0.42	0.8762	7
PR-BLA	0.70±0.16	1.52±0.11	0.8283	6
GR-BLA	0.72±0.13	1.67±0.42	0.8943	6

Critical membrane concentrations. The critical membrane concentrations $IC_{10,membrane}$ were calculated from nominal IC_{10} by eqn. 7, 8 9 and 10. As Figure 3A shows, there was no significant difference in $IC_{10,membrane}$ between cell lines (ANOVA, F=0.7853, P=0.6168) with a mean $IC_{10,membrane}$ of 69 mmol·L_{lip}^{-1} (95% CI; 49 to 89) and a median of 40 mmol·L_{lip}^{-1}.

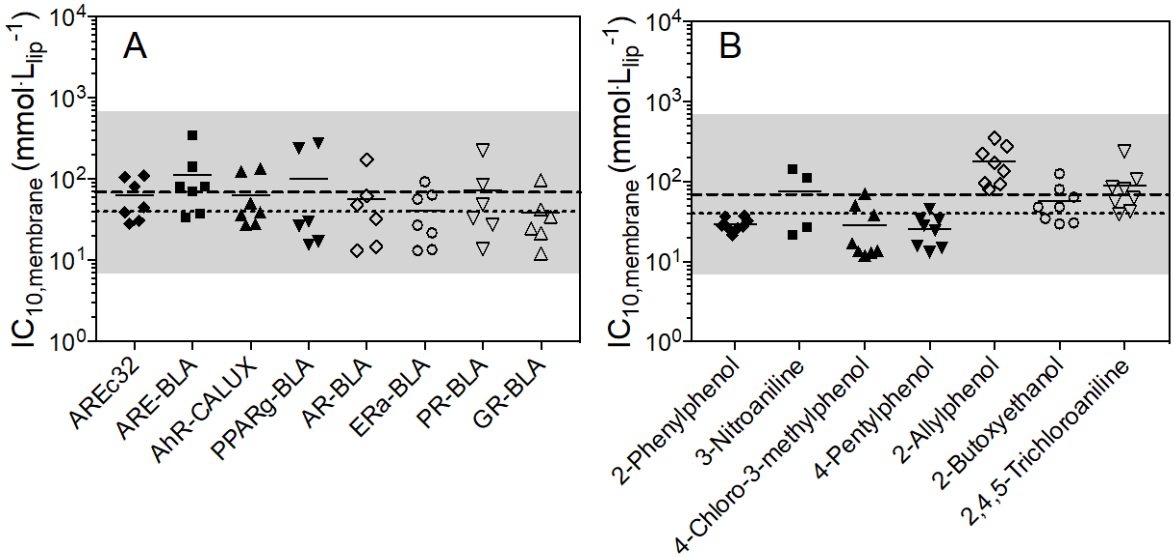


Figure 3. The critical membrane concentration $IC_{10,membrane}$ calculated from the measured IC_{10} using the mass balance model in the wells (eqs. 7-8) and the mass balance in the cells (eqs. 9-10). The short lines are the means

of the individual cell lines, the broken line is the mean of all data and the dotted line corresponds to the median. The grey bands correspond to a factor of 10 in each direction. A. $IC_{10,membrane}$ binned according to cell line, B. $IC_{10,membrane}$ binned according to chemical.

For comparison the critical membrane burdens for 50% mortality ILC_{50} were 118 $mmol \cdot kg_{lip}^{-1}$ (95% CI 64 to 173) for daphnia and 108 $mmol \cdot kg_{lip}^{-1}$ (95% CI 73 to 143) for fish.[31] Note the different units but in the literature the density of lipids is often assumed to be 1 $kg_{lip} \cdot L_{lip}^{-1}$ and hence the units can be used interchangeably. Although concentration response curves are not expected to be linear up to 50% effect but rather turn into a log-sigmoidal form above 30-40% of effect,[48] we can roughly estimate the $IC_{50,membrane}$ in the investigated cells as 345 $mmol \cdot kg_{lip}^{-1}$. The 95% CI of the ILC_{50} for aquatic animals are overlapping with the 95% CI of the cytotoxicity $IC_{10,membrane}$ and are only slightly lower than the estimated $IC_{50,membrane}$. It is interesting to note but does not come as a surprise that cytotoxicity in cell lines and lethality to aquatic organisms occurred at similar exposure levels. In the luminescent bacteria *Aliivibrio fischeri* 50% cytotoxicity occurred at a modelled membrane burden of approximately 200 $mmol \cdot kg_{lip}^{-1}$. This difference of a factor of two can be rationalized by the difference in effect level and by the much shorter exposure with *A. fischeri,* which was 30 min, while cytotoxicity toward human and rat cells was assessed after 24h in the present study.

A subset of Vaes' baseline toxicants[27] and additional chemicals were also tested for their effect to accelerate the decay of the membrane potential in isolated energy-transducing membranes, which is an indicator of the disturbance of membrane structure.[53] In that study, an effective membrane concentration of 300 $mmol \cdot kg_{lip}^{-1}$ lead to the critical effect, independent if polar or nonpolar or even charged organic compounds were tested, confirming the hypothesis of common mechanism of action of nonpolar and polar chemicals.

Due to the volatility cut-off, we could include only one of the nonpolar baseline toxicants of the initial set of Vaes' baseline toxicants.[27] This was Butoxyethanol, which was statistically not different from the other chemicals (t-test, P=0.4278, Welch-corrected t=0.8066, df=23.93). Butoxyethanol had a narrower confidence band and lay overall closer to the mean and median than the other tested chemicals (Figure 3B).

We can use the QSARs to predict nominal IC_{10} but an alternative approach is to backtrack eqs. 8-13 to derive expected cytotoxicity concentrations IC_{10} from the constant critical membrane burdens of approximately 70 $mmol \cdot kg_{lip}^{-1}$. Calculating the

IC_{10} either way prior to an experiment can also help define appropriate dosing concentrations, interpret toxicity data and support drug discovery.

Implications of the volatility cut-off for HTS. On first sight, the volatility cut-off appears at a quite high $K_{medium/w}$. For each 10000 molecules in medium one is in air (at equal compartment sizes) and still those chemicals escape the system. We analyzed the list of 8947 chemicals tested in Tox21[49] to identify how many of the chemicals that were included in Tox21 are likely to be lost while performing an bioassay experiment. This is only a thought experiment, the conditions under which the Tox21 assays were run, might have differed substantially from the setup that was applied to define the volatility cut-off but is useful to estimate the dimension of the problem associated to potential loss of chemicals in HTS systems.

As Figure 4A demonstrates, medium containing 10% FBS can retain some chemicals and the $K_{medium/air}$ can be orders of magnitude higher than the $K_{w/air}$. 81% of the Tox21 chemicals were above the threshold of log $K_{medium/air}$ = 4 (Figure 4B), and hence can be tested without any expected significant loss but the 19% that are below this threshold might have been partially lost. The $K_{medium/air}$ is not directly related to the K_{ow} (Figure 4B), and this is why the effect of the medium on $K_{medium/air}$ can be stronger or weaker depending on the K_{ow}.

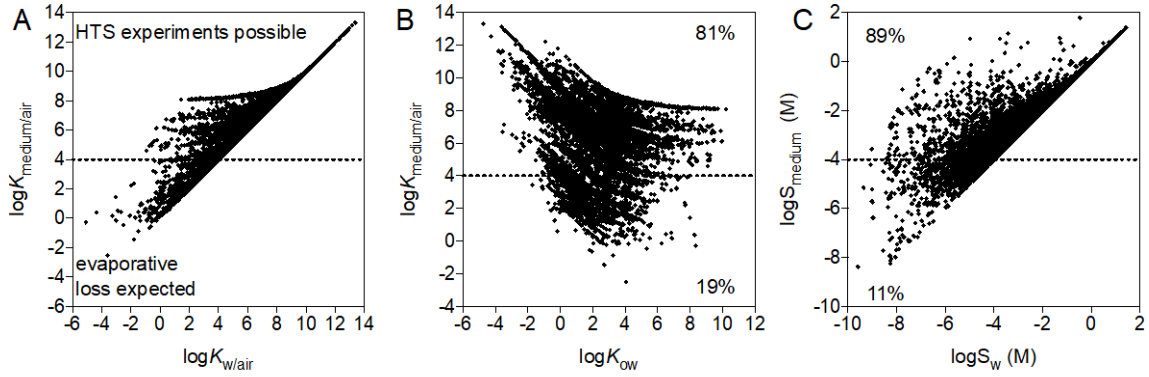


Figure 4. A. Relationship between $K_{medium/air}$ *and* $K_{w/air}$ *to demonstrate the retaining effect of medium. B. No relationship between the* $K_{medium/air}$ *cut-off and hydrophobicity, expressed as log*K_{ow}*. C. 89% of all chemicals had a solubility in medium above 100 µM and the solubility enhancement by medium components is dependent on the medium compositions (calculations in the figure for 10 % FBS).*

Implications of the baseline toxicity QSAR for dosing in HTS. In Tox21, chemicals were dosed from DMSO stocks to a maximum concentration of 100 µM in the final volume of 6 µL in the bioassays. We calculated, at which log$K_{lip/w}$ the IC_{10}(QSAR)

would be 100 µM using the QSAR equations in Table 3, which comes to log$K_{lip/w}$ 3.1 to 4.1 depending on the cell line. That means that chemicals with a log$K_{lip/w}$ below 3.1 to 4.1 were not tested up to their minimum toxicity if they were tested up to 100 µM. Cytotoxicity or effects occurring at rather high concentrations but still below baseline toxicity would not be detected (false negative).

On the other end of the spectrum, hydrophobic chemicals with log$K_{lip/w}$ > 4 could easily be accidentally overdosed if dosed up to 100 µM and might have precipitated in the bioassay. Not all chemicals are expected to be soluble at their baseline-toxic concentration. Especially hydrophobic chemicals with high melting point can often not be dosed up to concentrations where baseline toxicity would occur.[54] It is possible to estimate the solubility in bioassay medium from the aqueous solubility S_w by multiplying with the $K_{medium/w}$.[52] As Figure 4C shows, the medium solubility S_{medium} can be much higher than the S_w (calculations performed for medium with 10% FBS). Dosing at S_w risks again a false negative result because the medium enhances apparent solubility but also binds most of the dosed chemicals, hence one should rather aim at dosing up to S_{medium}. 89% of all Tox21 chemicals had a S_{medium} >100 µM and could have dosed higher in some of the assays.

One must keep in mind that the models presented here will have highest predictability for exactly the same experimental setups but can provide a guidance for similar bioassays and HTS set ups.

CONCLUSION

The analysis presented here will help to further improve HTS using reporter gene assays. HTS bioassays that use automated liquid handling or the D300 dispenser require multi-well plates to be open during handling. Also during incubation, the plates are typically not fully sealed but just covered with a plastic lid or a breathable sealant plus a plastic lid. We have demonstrated earlier that the same assays can also be run in a headspace-free set up[22] or with a defined headspace.[21] However, manual injection with syringes of the volatile chemicals is labor-intensive, oxygen deficiency can impact cell viability and it can also be challenging to keep CO_2 concentrations and pH constant in closed tests.[22] Systems with defined headspace have been successfully applied to biodegradation testing[55, 56] and have potential to be adapted to *in vitro* bioassays.

Paying attention to the volatility cut-off in routine HTS set ups can help to avoid artifacts and false negative results.

We refined a previously proposed Henry coefficient cut-off by accounting for the binding of chemicals to medium components, which retains chemicals in the assay system and defined a new robust $K_{medium/air}$ cut-off of 10000 L/L. Evidently, loss to the air is not the only loss process possible in HTS cell assays. Additional consideration should be given to the stability of chemicals in the test medium and binding to plastic multi-well plates.

Instead of dosing all chemicals at the same maximum concentration, it is more preferable to adjust dosing to the physicochemical properties and expected baseline toxic effects of the specific chemical and to dose up to or slightly exceeding what is predicted from the baseline toxicity QSARs. This might be logistically challenging and not possible for practical reasons in large HTS setups but it would be at least useful to bin chemicals into groups with physicochemical properties and test the bins in different ranges. By comparing experimental cytotoxicity with the QSAR, we can find out if the cytotoxicity is caused by baseline toxicity or occurs at much lower concentrations, which would then point to a specific mode of action.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at doi:xxxxxx. Additional sections on chemicals, the dosing procedures and the bioassay workflow, comparison of cell viability testing using the Presto Blue® assay and live-cell imaging, comparison of dosing with the digital dispenser with automated pipetting, loss processes to the air and contamination of neighboring wells, all concentration-response curves of all test chemicals in all assays and supplementary analyses.

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Notes

The authors declare no competing financial interest.

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