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Quantitative *In Vitro*-to-*In Vivo* Extrapolation: Nominal Versus Freely Dissolved Concentration

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KEYWORDS

QIVIVE, protein binding, pharmaceuticals, cell-based bioassays

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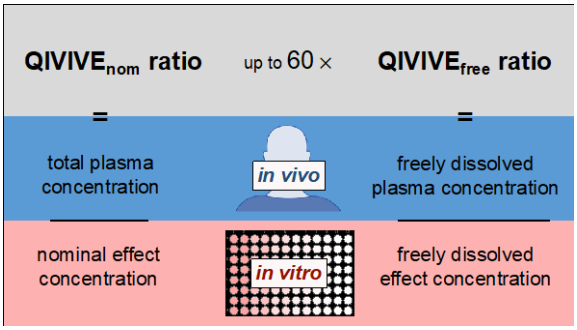

ABSTRACT

Discussions are ongoing, which dose metric should be used for quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE) of *in vitro* bioassay data. The nominal concentration of the test chemicals is most commonly used and easily accessible, while the concentration freely dissolved in the assay medium is considered to better reflect the bioavailable concentration but is tedious to measure. The aim of this study was to elucidate how much QIVIVE results will differ when using either nominal or freely dissolved concentrations. $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios, that is, the ratios of plasma concentrations divided by *in vitro* effect concentrations, were calculated for ten pharmaceuticals using previously published nominal and freely dissolved effect concentrations for the activation of the peroxisome proliferator-activated receptor gamma (PPARγ) and the activation of oxidative stress response. The $QIVIVE_{nom}$ ratios were higher than $QIVIVE_{free}$ ratios by up to a factor of 60. For four chemicals the risk of *in vivo* effects was classified as being high or low using the $QIVIVE_{nom}$ and for three chemicals using $QIVIVE_{free}$ ratios. Unambiguous classification was possible for nine chemicals by combining the $QIVIVE_{nom}$ or $QIVIVE_{free}$ ratios with the respective specificity ratios (SR_{nom} or SR_{free}) of the *in vitro* effect data, which helps to identify whether the specific effect was influenced by cytotoxicity. $QIVIVE_{free}$ models should be preferred as they account for differences in bioavailability between *in vitro* and *in vivo*, but $QIVIVE_{nom}$ may still be useful for screening the effects of large numbers of chemicals because it turned out to be generally more conservative. The use of SR of the *in vitro* effect data as second classification factor is recommended for $QIVIVE_{nom}$ and $QIVIVE_{free}$ models because a clearer picture can be obtained with respect to the likeliness that a biological effect will occur and that it is not caused by non-specific cytotoxicity.

INTRODUCTION

Effect data from cell-based *in vitro* bioassays are considered promising alternatives to animal testing, but are of little value for risk assessment without quantitative *in vitro*-to-*in vivo* extrapolation (QIVIVE).[1] In general, two different approaches can be used for QIVIVE of *in vitro* bioassay results from high-throughput testing.[2] The first approach compares the effect concentration from *in vitro* bioassays to a measured or modelled plasma concentration to assess the likelihood of *in vivo* effects.[3] The second approach, also called "reverse dosimetry", combines the data from *in vitro* bioassays with pharmacokinetic information on the chemicals to estimate the external dose (e.g., an oral equivalent dose) that would be required to cause an effect *in vivo*.[4] Previous studies have used QIVIVE models for the identification of potential endocrine disruptors,[5] risk assessment of food contaminants and additives[6] and of cosmetic ingredients.[7]

The majority of the published QIVIVE models rely on nominal *in vitro* effect concentrations (EC_{nom}). Nominal concentrations are easily accessible as they can be simply derived by dividing the amount of chemical dosed to the *in vitro* bioassay by the total volume of assay medium and cells. However, EC_{nom} does not account for the various partitioning and loss processes that influence the effective concentration of the test chemical. The advantages and disadvantages of several other dose metrics have recently been discussed and the choice of the dose metric may actually depend on the purpose of the QIVIVE.[8, 9]

The freely dissolved concentration in the assay medium (C_{free}) might be considered a better metric for QIVIVE because equal C_{free} in the assay medium *in vitro* and in plasma *in vivo* are expected to result in the same intracellular exposure.[10] Up to now only few studies have tried to improve QIVIVE by taking protein binding *in vitro* and *in vivo* into account.[11-13]

Mass balance models that consider protein, lipid and well plate plastic binding[14-17] can be used to derive C_{free} of a given chemical in an *in vitro* assay system. However, these models fail if C_{free} is not constant, but a function of the concentration of the test chemical due to saturable binding to medium proteins, e.g., for organic acids,[18] or a function of time due to volatilization or chemical degradation by abiotic processes or cellular metabolism, e.g., as recently shown for benzo[a]pyrene.[19] Non-linear protein binding, abiotic degradation, volatilization and cellular metabolism limit the possibility of retrospective correction of published nominal *in vitro* effect data. QIVIVE based on C_{free} therefore often requires experimentally determined freely dissolved effect concentrations (EC_{free}) and ideally also measured free fractions in plasma, but experimental EC_{free} are rarely published.

The present study used previously reported experimental EC_{nom} and EC_{free} of ten pharmaceuticals, two neutral and eight ionizable structures, and compared them to total ($C_{total,plasma}$) and freely dissolved therapeutic plasma concentrations ($C_{free,plasma}$) in humans. For the calculation of $C_{free,plasma}$ experimental plasma-water distribution ratios ($D_{plasma/w}$) were used. The aim of this study was to elucidate the differences in the results from QIVIVE models based either on nominal or freely dissolved concentrations. By using only experimental input parameters for QIVIVE uncertainties related to the use of prediction models were reduced to a minimum. Two *in vitro* reporter gene assays were used to illustrate the approach, the TOX21_PPARg_BLA_Agonist_ratio assay (in short PPARγ assay) and the AREc32 assay, which is very similar to the TOX21_ARE_BLA_Agonist_ratio assay.[20] These two assays are not necessarily the most relevant endpoints for the chemicals investigated but there were measured freely dissolved concentrations in these bioassays available.[18]

MATERIALS AND METHODS

QIVIVE models and theoretical considerations

For the comparison of *in vivo* and *in vitro* data the ratios between the total plasma concentration ($C_{total,plasma}$) and the nominal effect concentration ($EC_{10,nom}$ for the PPARγ assay or $EC_{IR1.5,nom}$ for the AREc32 assay) were used to derive the $QIVIVE_{nom}$ ratios (eq. 1). The $QIVIVE_{free}$ ratio is the ratio between the freely dissolved plasma concentration ($C_{free,plasma}$) and the freely dissolved effect concentration ($EC_{10,free}$ or $EC_{IR1.5,free}$, eq. 2). The terms "$QIVIVE_{nom}$ ratio" and "$QIVIVE_{free}$ ratio" were chosen in the present study as they allow more flexibility regarding the choice of plasma and *in vitro* effect concentrations compared to the previously used term "C_{max}-to-AC_{50} ratio".[3]

$$QIVIVE_{nom}\ ratio = \frac{C_{total,plasma}}{EC_{10,nom}\ (or\ EC_{IR1.5,nom})} \qquad \text{eq. 1}$$

$$QIVIVE_{free}\ ratio = \frac{C_{free,plasma}}{EC_{10,free}\ (or\ EC_{IR1.5,free})} \qquad \text{eq. 2}$$

In analogy to suggestions by Sipes et al.[3] three different thresholds were used in the present study to classify $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios and to assess whether the effects measured *in vitro* could be relevant for human health *in vivo* (Fig. 1). According to Sipes et al.[3] a ratio of ≥1 indicates that an *in vivo* effect of the chemical is likely. If the ratio is between 0.1 and 1 an effect is possible and below a ratio of 0.1 this possibility is remote. In the present study, chemicals with $QIVIVE_{nom}$ or $QIVIVE_{free}$ ratios ≥1 were classified as having a high risk of causing *in vivo* effects triggered by the effect of the *in vitro* assay, while chemicals with ratios <0.01 have a low risk. The term "risk" is used in this context as likelihood of an effect described by the given *in vitro* bioassay occurring *in vivo*. Ratios between 0.01 and 1 are in the area of uncertainty of the QIVIVE models, where a factor of 10 extrapolates from 10% effect to no effect and another factor of 10 accounts for differences between *in vitro* and *in vivo* cellular responses.

Ratio	Classification	Interpretation
≥1	high risk	the concentration in plasma may cause 10 % or 1.5-fold activation (or more) of the cellular process under investigation
0.1-1	uncertain	an extrapolation factor of 10 to extrapolate from 10 % effect to no effect/no activation was implicitly included
0.01-0.1		an additional factor of 10 accounts for uncertainty of extrapolation from *in vitro* to *in vivo* cellular effects
<0.01	low risk	no effects are expected

Figure 1. Thresholds for $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios adapted from Sipes et al.[3]

In theory, chemicals with high free fractions *in vitro* and *in vivo* will have almost identical $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios, while chemicals that show significant binding to medium and plasma components will have higher $QIVIVE_{nom}$ than $QIVIVE_{free}$ ratios, because human plasma has more proteins and lipids [21] than *in vitro* bioassay media.[16] This means that QIVIVE approaches based on nominal concentrations may overestimate the ratio between *in vivo* and *in vitro* concentrations and can therefore be considered more conservative or protective than QIVIVE models that use freely dissolved concentrations.[22] For example, a chemical, for which a nominal effect concentration of 10^{-6} M was measured in an *in vitro* assay, will have a $QIVIVE_{nom}$ ratio of 1 if the total plasma concentration reaches 10^{-6} M. Assuming that this chemical has a free fraction of 1 % in plasma and 10 % in the assay medium, the $QIVIVE_{free}$ ratio of this chemical would only be 0.1.

However, this only applies to chemicals that are stable in the *in vitro* assay system, which means that the total medium concentration (C_{total}) is similar to C_{nom}. The $QIVIVE_{free}$ ratio might actually be higher than the $QIVIVE_{nom}$ ratio if the $QIVIVE_{nom}$ ratio is calculated from a nominal effect concentration for a chemical that is prone to irreversible loss processes in *in vitro* bioassays (e.g., metabolism or volatilization) and the $QIVIVE_{free}$ ratio is calculated based on measured freely dissolved concentrations of this chemical. Using the same example as above, but assuming a loss

of chemical that leads to a significant reduction of C_{total} from 10^{-6} M to 10^{-8} M, the $QIVIVE_{free}$ ratio of the chemical would be 10. The detailed calculation for both examples can be found in the Supporting Information (Table S1).

One could argue that $QIVIVE_{free}$ should be preferred because $QIVIVE_{nom}$ ignores all partitioning and loss processes that influence the bioavailability in *in vitro* bioassays and, as demonstrated with the examples above, may therefore not always be more conservative than $QIVIVE_{free}$. Partitioning processes like protein and lipid binding are reversible processes, apart from a few exceptions. In contrast, loss processes like abiotic degradation, metabolism or volatilization are irreversible. Chemicals that are degraded or metabolized *in vitro* need a completely different QIVIVE approach, because metabolites and transformation products usually have a different biological activity compared to the parent compound, which does not allow a simple extrapolation. In the following, only chemicals were included for which stability during the *in vitro* bioassay had previously been confirmed experimentally.

Specificity ratios

While $QIVIVE_{nom}$ or $QIVIVE_{free}$ ratios <0.01 and >1 will lead to a straightforward conclusion, whether an effect measured with an *in vitro* bioassay is relevant *in vivo* as well, it remains unclear how to deal with chemicals with $QIVIVE_{nom}$ or $QIVIVE_{free}$ ratios from 0.01 to 1. A clearer picture might be obtained by taking a second factor into account such as the specificity ratio (SR) of the *in vitro* effect data.[20] SR is derived by dividing the effect concentration for cytotoxicity (i.e., the IC_{10}) by the effect concentration of the specific effect (i.e., the EC_{10} or $EC_{IR1.5}$). Chemicals showing effects with $1 \leq SR < 10$ are classified as acting moderately specific, while $SR > 10$ indicates a specific and $SR > 100$ a highly specific effect.[20] The SRs of the chemicals of the present study can be found in Table 1. For a graphical representation see Fig. S1.

All chemicals with $QIVIVE_{nom}$ or $QIVIVE_{free}$ ratios <0.01 were classified as “low risk”, irrespective of their SR. Similarly, all chemicals with SR <10 were classified as “low risk”, irrespective of their QIVIVE ratio. Chemicals with a high QIVIVE ratio (>1) that showed specific effects (SR>10) or with moderately high QIVIVE ratios (>0.01) that showed very specific effects (SR>100) were classified as having a high likelihood of causing *in vivo* effects.

Table 1. Nominal ($EC_{10,nom}$ and $EC_{IR1.5,nom}$) and freely dissolved effect concentrations ($EC_{10,free}$ and $EC_{IR1.5,free}$) and corresponding specificity ratios (SR_{nom} and SR_{free}) of the effects, logarithmic plasma-water distribution ratios ($\log D_{plasma/w}$), therapeutic total ($C_{total,\ plasma}$) and freely dissolved plasma concentrations ($C_{free,\ plasma}$) and calculated $QIVIVE_{nom}$ (eq. 1) and $QIVIVE_{free}$ ratios (eq. 2) for the test chemicals of this study.

Chemical	*In vitro* effect data[a]						*In vivo* exposure data					$QIVIVE_{nom}$ ratio	$QIVIVE_{free}$ ratio
	$IC_{10,nom}$ [M]	$EC_{10,nom}$ [M]	SR_{nom}	$IC_{10,free}$ [M]	$EC_{10,free}$ [M]	SR_{free}	$\log D_{plasma/w}$ (pH 7.4) [L/kg]	Ref	$C_{total,\ plasma}$ [M]	$C_{free,\ plasma}$ [M]	Ref		
Activation of PPARγ													
Caffeine	2.44×10^{-3}	3.48×10^{-4}	7.01	1.84×10^{-3}	2.89×10^{-4}	6.37	1.42	b	3.60×10^{-5}	1.39×10^{-5}	d	0.1035	0.0480
Lamotrigine	4.39×10^{-4}	1.02×10^{-4}	4.30	3.82×10^{-4}	9.81×10^{-5}	3.89	1.74	b	5.47×10^{-5}	1.24×10^{-5}	e	0.5354	0.1266
Diclofenac	-	2.12×10^{-6}	188*	-	2.75×10^{-7}	188**	3.87	b	6.98×10^{-7}	1.50×10^{-9}	f	0.3289	0.0054
Naproxen	-	1.77×10^{-5}	45.1*	-	4.62×10^{-6}	45.1**	3.41±0.11	c	2.17×10^{-4}	1.34×10^{-6}	g	12.3024	0.2897
Ibuprofen	3.16×10^{-4}	5.49×10^{-6}	57.6	1.50×10^{-4}	8.78×10^{-7}	171	3.71±0.08	c	2.42×10^{-4}	7.50×10^{-7}	h	44.1889	0.8541
Torasemide	1.84×10^{-4}	5.19×10^{-5}	3.55	7.19×10^{-6}	2.12×10^{-6}	3.39	3.55	b	3.27×10^{-5}	1.46×10^{-7}	i	0.6303	0.0689
Warfarin	2.42×10^{-4}	4.01×10^{-6}	60.5	1.04×10^{-4}	3.67×10^{-7}	285	3.35	b	3.90×10^{-6} ***	2.76×10^{-8} ***	j	0.9739	0.0752
Telmisartan	2.37×10^{-5}	1.67×10^{-7}	142.1	6.32×10^{-6}	2.58×10^{-8}	244.8	3.11±0.25	c	2.03×10^{-6}	2.48×10^{-8}	k	12.1759	0.9611
Chemical	*In vitro* effect data[a]						*In vivo* exposure data					$QIVIVE_{nom}$ ratio	$QIVIVE_{free}$ ratio
	$IC_{10,nom}$ [M]	$EC_{IR1.5,nom}$ [M]	SR_{nom}	$IC_{10,free}$ [M]	$EC_{IR1.5,free}$ [M]	SR_{free}	$\log D_{plasma,w}$ (pH 7.4) [L/kg]	Ref	$C_{total,\ plasma}$ [M]	$C_{free,\ plasma}$ [M]	Ref		
Activation of oxidative stress response													
Propranolol	1.47×10^{-4}	5.84×10^{-5}	2.52	1.47×10^{-4}	5.84×10^{-5}	2.52	2.35	b	1.32×10^{-7}	8.90×10^{-9}	l	0.0023	0.0002
Labetalol	8.90×10^{-5}	3.36×10^{-5}	2.65	8.90×10^{-5}	3.36×10^{-5}	2.65	2.27±0.14	c	5.02×10^{-7}	4.03×10^{-8}	m	0.0150	0.0012

[a]Ref 18. [b]Ref 21. [c]experimental data of the present study (±SD). [d]Ref 23. [e]Ref 24. [f]Ref 25. [g]Ref 26. [h]Ref 27. [i]Ref 28. [j]Ref 29. [k]Ref 30. [l]Ref 31. [m]Ref 32. *calculated using predicted $IC_{10,nom}$ for baseline toxicity ($SR_{baseline}$).[20] **SR_{free} was assumed to be equal to SR_{nom}. ***$C_{free,plasma}$ reported, $C_{total,plasma}$ calculated by eq. 5.

The SRs calculated from the nominal (SR_{nom}) and freely dissolved effect concentrations (SR_{free}) are identical if the freely dissolved effect concentrations are derived by simply multiplying the nominal effect concentrations with the free fraction of the chemicals in the assay medium. More precise was the approach taken here: the *in vitro* effect data from Huchthausen et al.[18] presented in Table 1 were derived by plotting the measured effects either against the nominal concentration dosed or against the measured freely dissolved concentrations in the individual wells. For these data, SR_{nom} and SR_{free} are only similar if the measured free fraction was constant, i.e., independent of the concentration of the chemical. This was the case for caffeine, lamotrigine, torasemide, propranolol and labetalol, but not for ibuprofen, warfarin and telmisartan, for which the free fraction was higher at cytotoxic concentrations than at effective concentration resulting in $SR_{free} > SR_{nom}$ (see Fig. 2 for exemplary concentration-response curves and Huchthausen et al.[18] for more detailed discussion). Increasing free fractions of a test chemical can be expected if the test chemical binds to a limited number of binding sites on the medium proteins, which become saturated at elevated concentrations of the chemical.

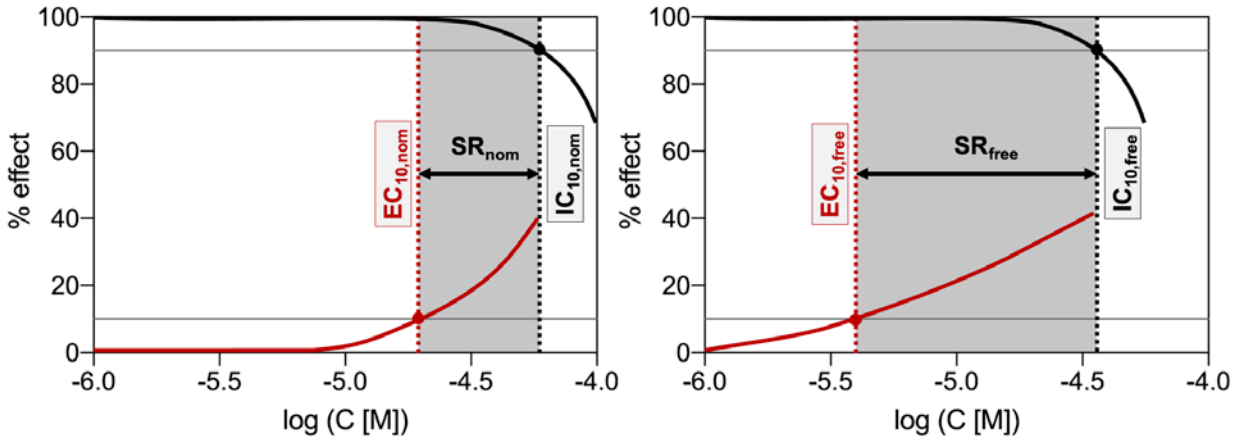

Figure 2. Exemplary comparison of specificity ratios calculated from nominal (SR_{nom}, left graph) and measured freely dissolved effect concentrations (SR_{free}, right graph) for a chemical that shows concentration dependent binding to proteins in *in vitro* bioassay medium.

For diclofenac and naproxen no $IC_{10,nom}$ and $IC_{10,free}$ for cytotoxicity was available and no SRs could be calculated. Instead, a theoretical $IC_{10,nom}$ for baseline toxicity was predicted for the PPARγ assay using the QSAR model from Escher et al.[33] (eq. 3) to derive $SR_{baseline}$ as a proxy for SR_{nom}.[20]

$$\log(1/IC_{10}[M]) = 0.64 \times \log D_{lip/w} + 1.71 \quad \text{eq. 3}$$

Experimentally determined logarithmic liposome-water distribution ratios ($\log D_{lip/w}$) at pH 7.4 of diclofenac (2.64[34]) and naproxen (2.17[35]) were used for the calculation. Because no data for the free fraction of diclofenac and naproxen at cytotoxic concentrations was available, SR_{free} of these chemicals was assumed to be equal to SR_{nom}.

Data collection

In vitro effect data for ten pharmaceuticals from Huchthausen et al.[18] were used (Table 1) including experimentally derived nominal and freely dissolved effect concentrations for the activation of the peroxisome proliferator-activated receptor gamma (PPARγ, $EC_{10,nom}$ and $EC_{10,free}$) for eight chemicals (caffeine, lamotrigine, diclofenac, naproxen, ibuprofen, torasemide, warfarin, telmisartan) and the activation of oxidative stress response ($EC_{IR1.5,nom}$ and $EC_{IR1.5,free}$) for two chemicals (propranolol, labetalol). All ten chemicals were tested in both assays, but only the active chemicals were used for QIVIVE. Measured therapeutic plasma concentrations from clinical studies were collected for all pharmaceuticals from various sources. Table 2 presents the originally published values and the type of plasma concentration used for the QIVIVE model. For the majority of the test chemicals only $C_{total,plasma}$ was reported and $C_{free,plasma}$ was calculated by eq. 4 using the plasma-water distribution ratio ($D_{plasma/w}$) of the test chemical and previously measured total protein and lipid mass ($m_{prot+lip,plasma}$ = 62.81 g) and water content ($V_{w,plasma}$ = 950.3 mL) of 1 L human plasma.[21]

$$C_{free,plasma} = \frac{C_{total,plasma} \times V_{total,plasma}}{V_{w,plasma} + D_{plasma/w} \times m_{prot+lip,plasma}} \quad \text{eq. 4}$$

If the therapeutic plasma concentration was reported as $C_{free,plasma}$, which was only the case for warfarin in the present study, $C_{total,plasma}$ was calculated analogously by eq. 5.

$$C_{total,plasma} = \frac{C_{free,plasma} \times (V_{w,plasma} + D_{plasma/w} \times m_{prot+lip,plasma})}{V_{total,plasma}} \quad \text{eq. 5}$$

Table 2. Therapeutic plasma concentrations (C_{plasma}) collected from the literature. Data were either published as peak/maximal plasma concentration (C_{max}), upper (TR_{high}) or lower limit of the therapeutic range (TR_{low}), steady-state unbound plasma concentration ($C_{ss,free}$), average whole plasma concentration at maximum therapeutic response ($C_{max,resp}$) or mean steady-state plasma concentration ($C_{ss,mean}$).

Chemical	M [g/mol]	C_{plasma}	Metric	C_{plasma} [M]	Reference
Caffeine	194.194	7 µg/mL (total)	C_{max}	3.60×10^{-5}	23
Lamotrigine	256.09	14 mg/L (total)	TR_{high}	5.47×10^{-5}	24
Diclofenac	318.129	222 ng/mL (total)	C_{max}	6.98×10^{-7}	25
Naproxen	230.263	50 mg/L (total)	TR_{low}	2.17×10^{-4}	26
Ibuprofen	206.285	50 mg/L (total)	TR_{high}	2.42×10^{-4}	27
Torasemide	348.421	11.4 µg/mL (total)	C_{max}	3.27×10^{-5}	28
Warfarin	308.333	0.0085 mg/L (free)	$C_{ss,free}$	2.76×10^{-8}	29
Telmisartan	514.629	1046 ng/mL (total)	C_{max}	2.03×10^{-6}	30
Propranolol	295.807	39 ng/mL (total)	$C_{max,resp}$	1.32×10^{-7}	32
Labetalol	328.412	165 ng/mL (total)	$C_{ss,mean}$	5.02×10^{-7}	32

Experimental determination of plasma-water distribution ratios

For the majority of the test chemicals, experimentally determined plasma-water distribution ratios ($D_{plasma/w}$) were available from a previous study.[21] For consistency, $D_{plasma/w}$ of telmisartan and labetalol was measured for the present study using the same experimental approach. For

naproxen and ibuprofen, the reported therapeutic plasma concentrations were rather high (50 mg/L in undiluted plasma), while the previous measurements of $D_{plasma/w}$ were performed with samples containing only 10 % plasma at much lower concentrations of the chemicals (1.5 mg/L). Because plasma protein binding of acidic pharmaceuticals like naproxen was found to be concentration-dependent,[26] $D_{plasma/w}$ of naproxen and ibuprofen was re-measured for the present study at therapeutic concentrations in undiluted plasma. Protein saturation and concentration dependence of $D_{plasma/w}$ can be expected if more than 10 % of the protein binding sites are occupied by the test chemicals (i.e., molar ratio chemical to protein (ν) >0.1). We estimated ν for all test chemicals using the equation from Henneberger et al.[36] and only naproxen and ibuprofen exceeded the threshold of 0.1 at therapeutic plasma concentrations.

Plasma-water distribution of telmisartan (Cayman Chemicals, 11615, purity ≥98%), labetalol (Sigma Aldrich, L1011, purity 98%), naproxen (Cayman Chemicals, 70290, purity ≥99%) and ibuprofen (Euro OTC, 700633, purity 99.6%) was determined by solid-phase microextraction as described previously.[21] Stock solutions were prepared in methanol (Chemsolute, 1485, UHPLC-MS grade) at 1 g/L for telmisartan and labetalol and at 10 g/L for naproxen and ibuprofen. For telmisartan and labetalol 4 µL of the stock solution were spiked into 1 mL of human plasma (Sigma Aldrich, P9523) leading to a total concentration of 4 mg/L in plasma. For naproxen and ibuprofen 4 µL of the stock solution were spiked into 796 µL of human plasma leading to a total concentration of 50 mg/L in plasma.

The plasma samples were pre-equilibrated for 24 h using an incubated orbital shaker (Thermo Fisher Scientific, MaxQ 6000) at 37°C and 250 rpm. Four replicates per chemical of pre-equilibrated, spiked plasma were filled into HPLC vials with inserts and a SPME fiber with C18/PAN coating (Sigma-Aldrich, 57281-U, conditioned 2 h in methanol and 20 min in water,

coating volume 173 nL for telmisartan, 69 nL for labetalol and 520 nL for ibuprofen and naproxen) was added to each sample. The samples were incubated with the SPME fibers for 24 h at 37°C and 1200 rpm (DMS-2500 from VWR or BioShake iQ from Quantifoil Instruments). SPME fibers were removed from the samples and the chemicals were desorbed at 1200 rpm for 2 h (DMS-2500 from VWR or BioShake iQ from Quantifoil Instruments) using 180 µL of a mixture of acetonitrile (Merck, 1.0017.2500, SupraSolv for GC ECD and FID,) and water (90/10) for telmisartan and labetalol and of methanol and water (50/50) for ibuprofen and naproxen. Control samples containing only phosphate buffered saline (PBS) and no plasma were run in parallel. Two replicates of control samples were extracted with SPME fibers and two replicates were not extracted. Concentrations of the chemicals in the control samples were similar to the expected $C_{free,plasma}$ (telmisartan, labetalol and ibuprofen 1 mg/L, naproxen 0.1 mg/L). The concentration of the chemicals in all fiber extracts and the PBS phase of the control samples was quantified using a liquid chromatography system (Agilent 1260 Infinity II) coupled to a triple quadrupole mass spectrometer (Agilent 6420) operating in MRM mode. More details on the instrumental analysis can be found in Tables S2 and S3 (analytical columns used, composition of eluents, MS parameters, limits of quantification (LOQ)). Calibration solutions were prepared for all chemicals in PBS and in the desorption solution.

The concentrations of the chemicals in the desorption solution and the volumes of desorption solution (V_{des}) and fiber coating (V_f) were used to derive the concentrations in the SPME fibers (C_f, eq. 6).

$$C_f = (C_{des} \times V_{des}) / V_f \quad \text{eq. 6}$$

The concentration measured in the PBS phase (C_w) of the control samples without plasma and the corresponding C_f were used to derive the fiber-water distribution ratio of the chemicals ($D_{f/w}$, eq. 7).

$$D_{f/w} = C_f / C_w \quad \text{eq. 7}$$

The $D_{plasma/w}$ of the chemicals was calculated from the C_f of the plasma samples, the total amount of chemical (n_{total}) in the sample and in the fiber (n_f), the total mass of proteins and lipids ($m_{prot+lip}$) and the volume of water (V_w) in the sample (eq. 8).

$$D_{plasma/w}\ (\text{pH7.4})\left[\frac{L_w}{kg_{prot+lip}}\right] = \frac{\frac{n_{total}}{n_f} \times D_{f/w} \times V_f - V_w - V_f \times D_{f/w}}{m_{prot+lip}} \quad \text{eq. 8}$$

RESULTS

$QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios and classification

The $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios are shown in Fig. 3. For the $QIVIVE_{nom}$ ratios, a clear classification, whether the effects measured *in vitro* are relevant for *in vivo* scenarios, was only possible for four of the ten test chemicals. The $QIVIVE_{free}$ ratio allowed a classification of three chemicals but most fell into the range of uncertainty. For naproxen, ibuprofen and telmisartan $QIVIVE_{nom}$ ratios were well above 1 and these chemicals are therefore expected to show activation of PPARγ *in vivo* at therapeutic plasma concentrations. In contrast, no chemical would be classified as "high risk" based on freely dissolved effect concentrations as no $QIVIVE_{free}$ ratio exceeded the threshold of 1. However, the $QIVIVE_{free}$ ratios of telmisartan (0.96) and ibuprofen (0.85) were very close to 1.

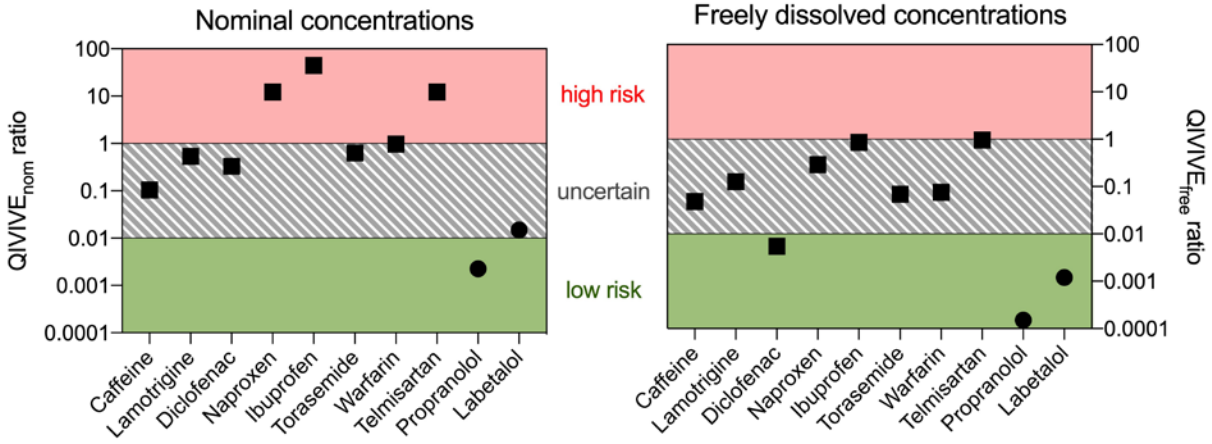

Figure 3. Calculated $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios for the test chemical of this study and classification according to scheme shown in Fig. 1.

For propranolol and labetalol, the two chemicals that showed induction of oxidative stress response *in vitro,* $QIVIVE_{free}$ ratios were well below 0.01 and no effects are expected *in vivo* at therapeutic plasma concentrations. The $QIVIVE_{nom}$ ratio of propranolol was also <0.01, while the ratio was slightly >0.01 for labetalol. $QIVIVE_{nom}$ ratios of six chemicals (caffeine, lamotrigine, diclofenac, torasemide, warfarin and labetalol) and $QIVIVE_{free}$ ratios of seven chemicals (caffeine, lamotrigine, naproxen, ibuprofen, torasemide, warfarin and telmisartan) were between 0.1 and 1, and no clear classification of the risk was possible.

Comparison of $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios

For the neutral and hydrophilic chemicals, caffeine and lamotrigine, $EC_{10,free}$ was very close to $EC_{10,nom}$[18] and only weak binding to human plasma was reported[21] and consequently $QIVIVE_{nom}$ and $QIVIVE_{free}$ gave similar ratios (within a factor of 4, Fig. 4).

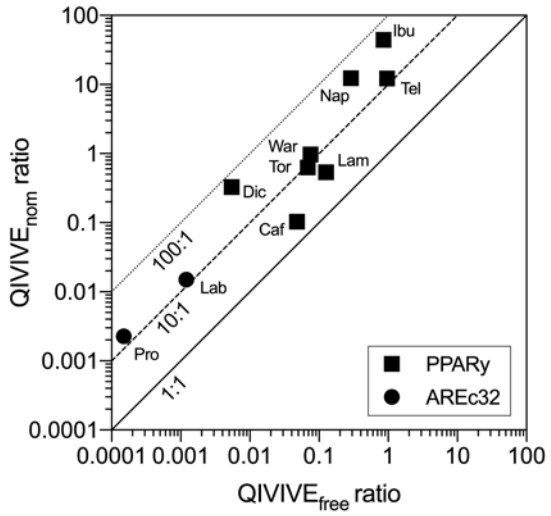

Figure 4. Comparison of $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios for the test chemical of this study: caffeine (Caf), lamotrigine (Lam), diclofenac (Dic), naproxen (Nap), ibuprofen (Ibu), torasemide (Tor), warfarin (War), telmisartan (Tel), propranolol (Pro), labetalol (Lab).

For diclofenac, naproxen, ibuprofen, torasemide, warfarin and telmisartan $QIVIVE_{nom}$ ratios were significantly higher than $QIVIVE_{free}$ ratios, between a factor of 9 (torasemide) and 60 (diclofenac). For propranolol and labetalol $EC_{IR1.5,free}$ was assumed to be identical to $EC_{IR1.5,nom}$, because the measured free fraction was high (>65 %) in the bioassay medium.[18] However, significant binding to human plasma was measured[21] and therefore $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios differ by a factor of 15 (propranolol) and 12 (labetalol).

Specificity ratios and two-dimensional classification

Six chemicals were classified as "uncertain" based on their $QIVIVE_{nom}$ ratio ($0.01<QIVIVE_{nom}$ ratio<1) and seven chemicals based on their $QIVIVE_{free}$ ratio ($0.01<QIVIVE_{free}$ ratio<1). An improved evaluation of the likelihood of *in vivo* effects was possible if the specificity ratio (SR) of the *in vitro* effect data was taken into account, considering that a baseline-toxic effect in an *in vitro* assay (SR<10) would not lead to risk if the chemical was in the "uncertain" range (Fig. 5). Only chemicals with 0.01<QIVIVE ratio<1 and 10<SR<100 could not be classified, but chemicals with a SR>100 would be considered "high risk" even if 0.01<QIVIVE ratio<1.

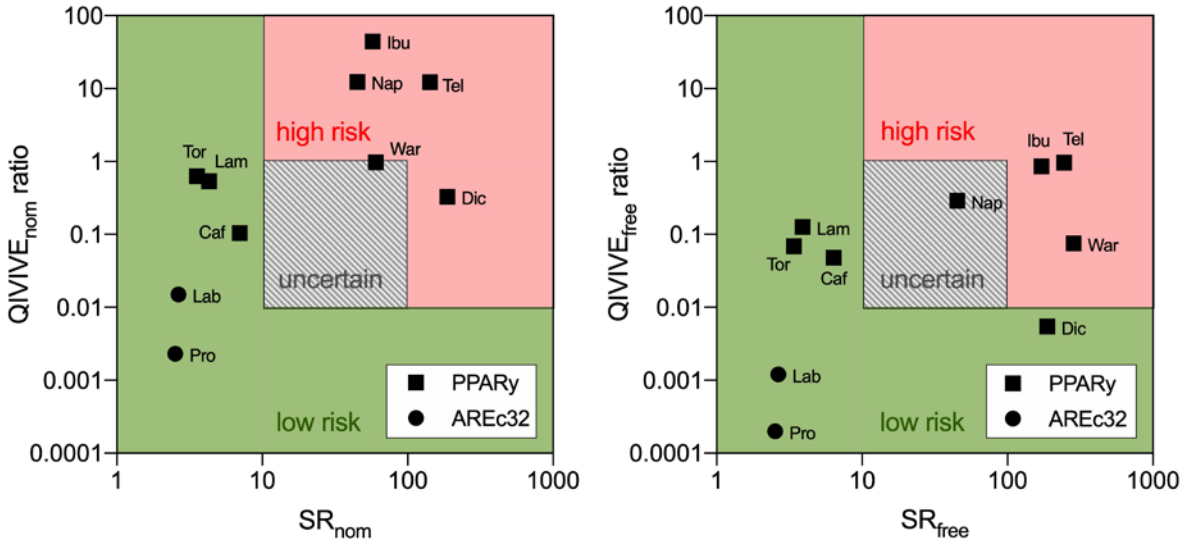

Figure 5. QIVIVE$_{nom}$ and QIVIVE$_{free}$ ratios versus specificity ratios of the nominal (SR$_{nom}$) and freely dissolved effect concentrations (SR$_{free}$) and resulting classification of the test chemicals.

In the present study, the dual-factor classification allowed a clear classification of the risk of nine of the ten test chemicals (Fig. 5). The chemicals that remained in the area of uncertainty were warfarin for QIVIVE$_{nom}$-SR$_{nom}$ and naproxen for QIVIVE$_{free}$-SR$_{free}$. Based on their QIVIVE$_{nom}$ ratios and SR$_{nom}$, four chemicals (diclofenac, naproxen, ibuprofen, telmisartan) and based on their QIVIVE$_{free}$ ratios and SR$_{free}$, three chemicals (ibuprofen, telmisartan, warfarin) were classified as having a high risk of causing *in vivo* effects. Five (caffeine, lamotrigine, torasemide, propranolol, labetalol) were identified as low-risk chemicals by QIVIVE$_{nom}$-SR$_{nom}$ and six chemicals (caffeine, lamotrigine, diclofenac, torasemide, propranolol, labetalol) by QIVIVE$_{free}$-SR$_{free}$.

QIVIVE$_{nom}$-SR$_{nom}$ and QIVIVE$_{free}$-SR$_{free}$ resulted in the same classification for seven chemicals (ibuprofen, telmisartan, caffeine, lamotrigine, torasemide, propranolol, labetalol). Telmisartan, a chemical that is known into activate PPARγ *in vivo*[37] was correctly classified as “high risk” by the QIVIVE$_{nom}$-SR$_{nom}$ and the QIVIVE$_{free}$-SR$_{free}$ approach. Warfarin was classified as “high risk” by QIVIVE$_{free}$-SR$_{free}$ but was classified “uncertain” by QIVIVE$_{nom}$-SR$_{nom}$, because the QIVIVE$_{nom}$

ratio was slightly below 1 (0.97). Naproxen was classified as "high risk" by $QIVIVE_{nom}$-SR_{nom} but was classified "uncertain" by $QIVIVE_{free}$-SR_{free}. The biggest discrepancy in classification was found for diclofenac, which was classified as "high risk" by $QIVIVE_{nom}$-SR_{nom} but was a low-risk chemical according to $QIVIVE_{free}$-SR_{free}. Interestingly, diclofenac was also the chemical with the highest difference between the $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratio (see previous section). Unfortunately, there are no studies available regarding the PPARγ activation in humans by acidic drugs like diclofenac, ibuprofen or warfarin, which would allow a better validation of the QIVIVE models.

DISCUSSION

In the present study, the calculated $QIVIVE_{nom}$ ratios were up to a factor of 60 higher than the corresponding $QIVIVE_{free}$ ratios. This is in line with the theoretical considerations stated in the "Materials and Methods" section. The classification of the test chemicals also differed between $QIVIVE_{nom}$ and $QIVIVE_{free}$ for some chemicals. The largest difference in classification was found for diclofenac, which was also the chemical with the largest difference between the $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratio.

$QIVIVE_{free}$ models based on experimentally derived freely dissolved effect and plasma concentrations should always be preferred as they account for differences in bioavailability of the chemicals between *in vitro* and *in vivo* systems. For practical reasons, $QIVIVE_{nom}$ may still be used for screening purposes because they are precautionary and not underestimating the risk. Chemicals that are classified as "high risk" by $QIVIVE_{nom}$ may then be further scrutinized by a more sophisticated $QIVIVE_{free}$ approach. The false negative classification of unstable chemicals as "low risk" chemicals by $QIVIVE_{nom}$ as discussed in the "Materials and Methods" section may be avoided by accompanying *in vitro* test batteries with *in silico* or *in vitro* tools that test for abiotic

stability (hydrolysis, photolysis, protein reactivity etc.) and cellular metabolism. Another factor that should be considered is the variation of the human plasma concentration as the outcome of the QIVIVE will differ depending on the type of plasma concentration used (e.g., C_{max}) and also depending on how much the concentration varies between different individuals and treatments.

Confidence in the QIVIVE results can also be increased by using the SR of the *in vitro* effect data as a second classification factor. Only chemicals with high $QIVIVE_{nom}$ or $QIVIVE_{free}$ ratios that showed specific or highly specific activation of a receptor will be classified as chemicals of high risk, while all chemicals with moderate specificity will be classified as low-risk chemicals. Using the two-dimensional classification, a better interpretation of the results of this study was possible as nine out of ten chemicals could be clearly classified as either low or high-risk chemicals. The dual-factor approach is also applicable if cytotoxicity data and consequently SR_{nom} and SR_{free} of the test chemicals are missing (e.g., for diclofenac and naproxen in the present study), because $SR_{baseline}$ can be calculated using previously published prediction models.[33]

As an alternative to measured freely dissolved concentrations *in vitro* and *in vivo*, measured total concentrations may be used, e.g., for neutral hydrophilic chemicals, for which QIVIVE based on nominal and freely dissolved effect concentrations gave very similar results. This was also recently highlighted in a case study for coumarin[7] where no correction for protein and lipid binding was applied, because the differences between the free fractions *in vitro* and *in vivo* were within a factor of 3. For chemicals that show strong binding to medium and plasma proteins, measuring the total concentration to exclude loss of test chemical may not be sufficient, as the difference between $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios may not be constant, but a function of the concentration of the chemical. This is the case if the test chemical shows non-linear binding to plasma proteins *in vivo*, e.g., naproxen in the present study, and/or to medium proteins *in vitro*, e.g., diclofenac and

naproxen in the present study (for a more detailed discussion see Huchthausen et al.[18]). This means that $QIVIVE_{free}$ ratios cannot simply be derived from $QIVIVE_{nom}$ ratios by a single conversion factor that accounts for the free fraction, as the free fraction may be a function of the concentration of the chemical.

ASSOCIATED CONTENT

The following files are available free of charge.

Examples calculation for $QIVIVE_{nom}$ and $QIVIVE_{free}$ ratios, liquid chromatography (LC) and mass spectrometry (MS) parameters of the test chemicals, additional figure showing the specificity ratios of the nominal (SR_{nom}) and freely dissolved effect concentrations (SR_{free}) (PDF).

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