# Does *In Vitro* Potency Predict Clinically Efficacious Concentrations?

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**The *in vitro* affinity of a compound for its target is an important feature in drug discovery, but what remains is how predictive *in vitro* properties are of *in vivo* therapeutic drug exposure. We assessed the relationship between *in vitro* potency and clinically efficacious concentrations for marketed small molecule drugs ($n$ = 164) and how they may differ depending on therapeutic indication, mode of action, receptor type, target localization, and function. Approximately 70% of compounds had a therapeutic unbound plasma exposure lower than *in vitro* potency; the median *ratio* of exposure in relation to *in vitro* potency was 0.32, and 80% had *ratios* within the range of 0.007 to 8.7. We identified differences in the *in vivo*–to–*in vitro* potency *ratio* between indications, mode of action, target type, and matrix localization, and whether or not the drugs had active metabolites. The *in vitro*–assay variability contributions appeared to be the smallest; within the same drug target and mode of action the within-variability was slightly broader; but both were substantially less compared with the overall distribution of *ratios*. These data suggest that *in vitro* potency conditions, estimated *in vivo* potency, required level of receptor occupancy, and target turnover are key components for further understanding the link between clinical drug exposure and *in vitro* potency.**

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ *In vitro* potency is commonly used alone, or in combination with preclinical *in vivo* data, to predict clinically therapeutic exposure levels. But there is limited knowledge regarding how predictive *in vitro* potency is in general to estimate the therapeutic drug exposure.

### WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This study addressed the general relationship between clinical unbound therapeutic concentrations and *in vitro* potency, how they may differ depending on therapeutic indication, mode of action, receptor type, target localization and function, and potential drug discovery and development implications from this analysis.

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The results emphasize that generic predictions of *in vivo* efficacious concentration based on *in vitro* potency may be highly variable and may lack biological significance. The data additionally identify sources to variability and their relative impact upon predictions of *in vitro*–to–*in vivo* clinically efficacious exposure levels.

### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ This might provide an enlightened awareness of factors to consider when relating therapeutic exposure to *in vitro* potency. It also provides the scientist with a quantitative level of confidence on how predictive *in vitro* potency alone is to estimate therapeutically efficacious drug exposure levels.

The *in vitro* affinity of a compound for its target is an important feature in drug discovery, but even more central is the question of how predictive *in vitro* properties are of *in vivo* therapeutic drug exposure. The underlying assumption is that a compound with high *in vitro* affinity and favorable *in vivo* pharmacokinetic properties may have a greater chance to deliver its intended clinical action in comparison with compounds with poor pharmacokinetic properties and *in vitro* potency.[1,2]

It is possible to at least partly hypothesize on desired onset, intensity, and duration of a pharmacological response based on a compound's mechanism of action. Insights into target occupancy relative to actual drug concentrations are also key parameters towards a therapeutic response.[3] Thus, for receptor antagonists and irreversible enzyme inhibitors, it is often assumed that high and/or extended target occupancy is essential, in contrast to agonists where these features are not necessary to elicit a clinical response. According to the law of mass action, drug concentrations ~ 3–5 times the *in vitro* potency (affinity constant $K_d$) would reach a target occupancy of about 80% and thereby possess a good chance to result in a pharmacological response.[4] Target turnover and transduction-coupling efficiency aspects are often neglected despite being equally important for *in vivo* ("open" system) effects. In some instances, pharmacokinetic–pharmacodynamic reasoning has been successful to

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remedy these shortcomings.[5–11] Clearly, capricious use of multiples of efficacious *in vitro* concentrations in the absence of a clear scientific rationale may jeopardize the selection process as well as onwards clinical drug development. In turn, this may lead to underpredictions or overpredictions and ultimately erroneous conclusions with respect to drug efficacy as well as therapeutic benefit/adverse event ratio estimates. Gleeson and coworkers assessed the generic relationship between therapeutic doses, *in vitro* potency, and physicochemical properties.[1] They concluded that high *in vitro* drug potency does not necessarily predict *in vivo* benefit, partly because of poorer pharmacokinetic properties.

The present study was undertaken to address, "What is the relationship between clinical unbound concentrations and *in vitro* potency, and how can drug discovery and development benefit from a better understanding of this linkage?" A survey of 164 drugs was done to identify important points to consider when assessing new molecular entities. The compilation includes pharmacokinetic properties (clearance, bioavailability, plasma protein binding, volume of distribution, half-life, and active metabolites), clinically unbound concentrations ($C_{u,ss}$), intracellular or extracellular target localization, *in vitro* binding affinities, type of *in vitro* property parameter (target binding, function; dissociation constant ($K_d$), inhibitory constant ($K_i$), concentration of drug producing 50% inhibition of maximal inhibition ($IC_{50}$), concentration of drug producing 50% of its maximum effect ($EC_{50}$)), mechanism of action, target type, and therapeutic area.

## MATERIALS AND METHODS

### Data compilation

Literature data comprising dosing regimen, pharmacokinetic properties, plasma protein binding, and *in vitro* potency were collected for a total of 164 clinically low molecular weight (<700 g/mol) approved drugs. The information gathered also included mechanism of action, therapeutic drug class, target type and location, and type of reported *in vitro* property ($K_d$, $K_i$, $IC_{50}$, $EC_{50}$). All data are available in the **Supplement** (**Tables S5, S6**).

### *In vitro* potency

Drug *in vitro* potency parameters ($K_d$, $K_i$, $IC_{50}$, and $EC_{50}$) were extracted from database and literature sources (for basic definitions and a more in-depth description of these pivotal parameters, please see the International Union of Basic and Clinical Pharmacology (IUPHAR) update on nomenclature in receptor pharmacology by Neubig *et al.* (2003)[12]). The publicly accessible ChEMBL (https://www.ebi.ac.uk/chembl/) and IUPHAR / British Pharmacological Society (BPS) Guide to Pharmacology (http://www.guidetopharmacology.org/) databases were the main resources for the *in vitro* potency data. If they could not be found from these sources, literature searches via PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) were done and data extracted from original publications. For all compounds, we stated if the *in vitro* potency was defined as either $IC_{50}$, $EC_{50}$, $K_i$, or $K_d$. For the final analyses, $K_i$ and $K_d$ were converted to $IC_{50}$ by a factor 2 assuming competitive inhibition and that substrate concentration is equivalent to its Michaelis-Menten constant.[13] The *in vitro* activity for the drugs were directly related to the primary target indication and whenever possible, only *in vitro* activity values were used based on the human receptor. This resulted in a total of 3963 *in vitro* potencies for the 164 compounds (**Table S5, Supplement**). For agents with more than one reported pharmacologically relevant *in vitro* potency target value and assay, the geometric mean value was used and precision was calculated.

### Clinical plasma concentrations and therapeutic dose

Pharmacokinetic properties including oral bioavailability, plasma clearance, volume of distribution, half-life, and plasma protein binding were obtained from Obach *et al.*[14], the US Food and Drug Administration (www.FDA.gov), the www.drugs.com database, and from peer-reviewed publications. Dose and dose frequencies were mainly obtained from the Swedish National Medical Product Register (NMPR; https://nsl.mpa.se/index_english.htm). Dosing regimen data on drugs not available in the NMPR database were obtained from the drugs.com website (https://www.drugs.com). Dose and dosing regimen were based on the particular therapeutic indication according to the Anatomical Therapeutic Chemical (ATC) classification system. In cases when there were different doses and frequencies available, the typical recommended dose was used. If it was stated that the dose needed to be highly individualized, the mean dose used or the dose level most commonly used was utilized. Details on each drug is available in **Supplemental Table S6**.

The majority of drugs (157 of 164 compounds in total) were intended for oral administration and chronic/subchronic ($n$ = 161 of 164) treatment.

Clinically unbound concentrations at steady state $C_{u,ss}$ were derived using

$$C_{u,ss} = \frac{F \cdot \text{Dose} \cdot f_u}{\text{Cl} \cdot \tau} \quad (1)$$

where $F$ corresponds to bioavailability, *Dose* to dose per occasion, *Cl* clearance, and $\tau$ the dosing interval.[15] The free fraction $f_u$ is the ratio of unbound to total plasma concentration, and is governed by density of binding sites on the plasma protein and drug-protein binding affinity. In the few cases where the drug was prescribed as a single dose administration only, we calculated the total area under curve (AUC), divided by 24 hours to convert this to an average drug exposure over 24 hours, to yield a clinical concentration measure. Peak unbound steady state plasma concentration ($C_{u,ss,max}$) at repeated dosing and trough unbound steady state concentration ($C_{u,ss,min}$) prior to next dose were also evaluated and are described in the **Supplement**. The ratio of unbound plasma concentrations to *in vitro* potency was calculated as $C_{u,ss}$/*in vitro* potency, and is denoted *Ratio*. Subset analyses were also performed based on different potency values (see **Supplement**).

### Additional drug descriptors

To provide further insight into the relationship between clinical unbound plasma concentration and *in vitro* potency, information was also gathered about

- Ligand target class—i.e., G protein-coupled receptor (GPCR), enzyme, ion channel, transporter/reuptake protein, or nuclear receptor
- Ligand property/mechanism of action—i.e., agonist, antagonist, inhibitor, or modulator
- Presence of pharmacologically active metabolites—i.e., (i) no active metabolites known to contribute to the pharmacological effect, (ii) known active metabolites, or (iii) active metabolites present, but unclear how they contribute to the pharmacological effect
- If the drug is given as a racemate or as the pure isomer
- Extracellular or intracellular localization of the drug target
- Drug therapeutic indication area according to the ATC classification system
- Type of reported *in vitro* potency measure—i.e., $K_d$, $K_i$, $EC_{50}$, or $IC_{50}$

The collated data set included a broad range of chemical and pharmacological classes with varying pharmacokinetic properties, modes

of action, ligand target type, and therapeutic indication (**Table S6, Supplement**).

### Exploratory and statistical analyses

Data of this analysis were presented graphically as histograms, cumulative frequency plots, and scatter plots. Comparisons between groups were done based on means of log-transformed data, and corresponding 85% confidence intervals of the mean value (i.e., precision). The 85% confidence intervals, rather than 90% confidence intervals, were used to assess differences between groups at the ~ 5% level with the assumption that Standard Errors between groups were approximately equal.[16] Additionally, the nonparametric Kolmogorov-Smirnov test (K-S test) was used to assess whether the different groups differed between each other based on their cumulative *Ratio* distribution profiles.[17]

Range and variability data were also analyzed. To this end, we assessed within-drug *in vitro* potency variability for each drug with three or more reported *in vitro* potency values. After establishing median values, we arithmetically generated corresponding variability distribution curves expressed as percentiles with the median set as the 50$^{th}$ percentile. Assuming that the drug data used in this exercise are indeed representative of the class in question, the derived percentile curves of *in vitro* potency provide an estimate of the typical distribution of within-drug class variability around the 50$^{th}$ percentile.

To evaluate variability within each drug target and mode of action, the ratios and the percentiles were normalized to the 50$^{th}$ percentile, and thereafter median percentiles were generated across the different classes of drugs.

To assess potential influences of covariates on the relationship between $C_{u,ss}$ and *in vitro potency*, a nonlinear mixed effects modeling approach was used. This was done according to the equation

$$C_{u,ss} = \text{slope} \times \textit{In vitro}\ \text{potency} + \text{Covariate} \times \left(\text{Covariate}_{\text{individual value}} - \text{Covariate}_{\text{median value}}\right) + \text{intercept} \quad (2)$$

where the parameters *slope, covariate*, and *intercept* were fitted to the data. $\text{Covariate}_{\text{individual } value}$ is the individual value for each drug and the $\text{Covariate}_{\text{median value}}$ is the median covariate value for the data set. An exponential (exp) error model was used (Observation = Prediction × $\exp^{(\text{Residual Error})}$). The inclusion of covariates was assessed by using the log likelihood ratio, in which the difference in the objective function value between the full and reduced models was asymptotically $\chi^2$ distributed. A decrease in the objective function value of 3.84 between two nested models (1 degree of freedom) was considered to indicate a statistically improved model ($P < 0.05$). Additionally, the 90% confidence interval of the covariate parameter was also assessed, if different from zero. Data were modeled using Phoenix WinNonlin 8.1 (Pharsight Certara, Princeton, NJ), using the naive-pooled method. The following covariates were evaluated: drug lipophilicity (logP), molecular weight, clearance, bioavailability, half-life, volume of distribution, unbound fraction in plasma, dosing frequency, and daily dose (**Supplement**, **Table S4**). Graphical generations, statistical analyses, and covariate analyses were either done using Microsoft Excel for Office 365 (Microsoft, Redmond, WA) or Phoenix WinNonlin 8.1 (Pharsight Certara, Princeton, NJ).

## RESULTS

### Overall relationship of Ratios

The primary results in terms of unbound clinical concentrations–to–*in vitro* potencies, denoted *Ratio(s)*, are shown in **Figure 1**, where log *Ratios* are plotted against frequency and cumulative frequency. Notably, ~ 70% of all *Ratios* are less than unity; the median ratio was 0.32, and fewer than 10% of compounds displayed *Ratios* exceeding 10.

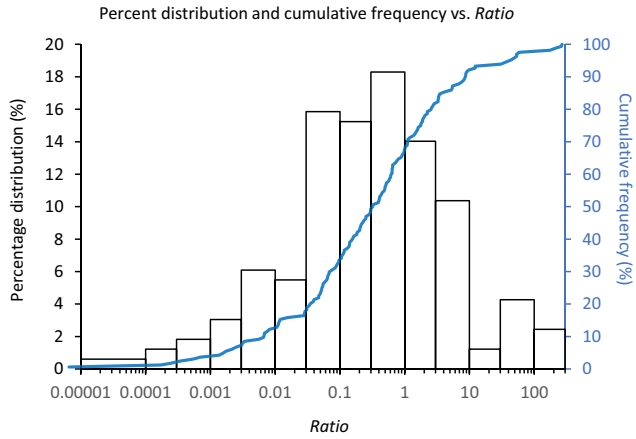


**Figure 1** Distribution of the unbound plasma concentrations–to–*in vitro* potency ratio of 164 compounds. ***Left axis***, divided into logarithmically increasing bins. Bins are divided into threefold categories. ***Right axis***, cumulative frequency vs. the ratio. [Colour figure can be viewed at wileyonlinelibrary.com]

### Ratios by therapeutic indication: ATC categories and overall comparison of drug target variabilities

Drugs categorized as antiinfectives (ATC code J) and antiparasitic/insecticides/repellents (ATC code P), represent a distinct group. These agents are enzymatic inhibitors with *Ratios* generally higher as compared with drugs for all other therapeutic indications (**Figure 2 and Table S2**). As the drugs in this group act on *non-human* cell targets, they were excluded from subsequent analyses.

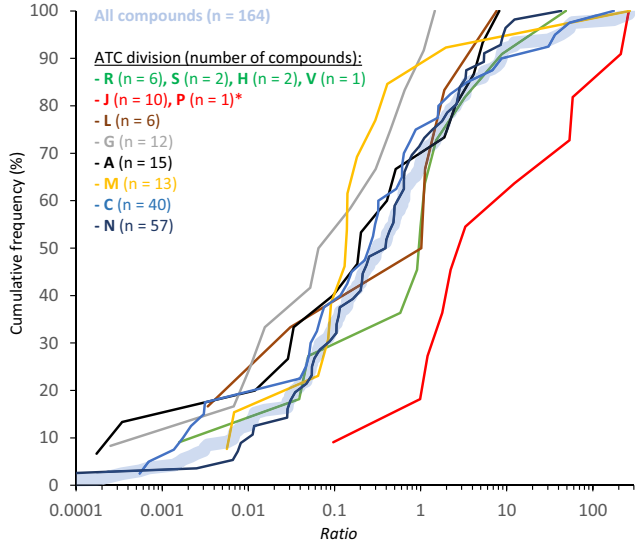


**Figure 2** Cumulative frequency vs. *Ratio* divided according to the Anatomical Therapeutic Chemical (ATC) Classification System. The pale wide blue line is for all compounds. A, alimentary tract and metabolism; C, cardiovascular system; G, genito-urinary system and sex hormones; H, systemic hormones excluding sex hormones, insulins; J, antiinfectives for systemic use; L, antineoplastic and immunomodulating agents; M, musculo-skeletal system; N, nervous system; P, antiparasitic products, insecticides and repellents; R, respiratory system; S, sensory organs; V, antidotes. *J, P group (red line) deviated significantly ($P < 0.05$) from the remaining data set based on the nonparametric Kolmogorov-Smirnov test. [Colour figure can be viewed at wileyonlinelibrary.com]

A relatively low *Ratio* variability was observed within some therapeutic classes e.g., genito-urinary system and sex hormones (ATC code G) and musculoskeletal system (ATC code M). When divided into second-level therapeutic subgroups (**Figure S4**) and removing drugs with potentially confounding contributions of reported active metabolites, 7 of 15 ATC groups had a prediction coefficient ($R^2$) of 0.63–0.92, when using a linear regression of the log-transformed data. The remaining nine groups showed an $R^2$ value of 0.41 or less. Notably, agents acting on the renin–angiotensin system (ATC code C09) displayed a very poor correlation ($R^2 = 0.03$, $n = 7$).

The overall *in vitro* assay variability derived from within-drug medians and 10$^{th}$ and 90$^{th}$ percentiles (centered around the corresponding 50$^{th}$ percentile) was 0.32 and 3.0, respectively, thus representing a 10-fold range (**Figure 4**). Ten targets in our database had four or more drugs per target and the same mode of action (a total of 55 compounds, e.g., 5-hydroxytryptamine (HT) reuptake inhibitors, 5-HT$_{1D}$ receptor agonists, etc.). In this target excerpt the absolute median 10$^{th}$ and 90$^{th}$ percentile (centered around the corresponding 50$^{th}$ percentile) potency *Ratio* varied between 0.25 and 13, respectively, among the groups (**Supplement**, **Figure S7**), thus representing an ~ 50-fold range.

For comparison, the global $C_{u,ss}$/*in vitro* potency *Ratio* variability across all classes of drugs (10$^{th}$ and 90$^{th}$ percentile centered around the corresponding 50$^{th}$ percentile) amounted to a 1,275-fold range (**Figure 4**).

### Ratios by mechanism of action or receptor type

Among the different modalities and mechanisms (excluding antimicrobial & insecticide agents, see above) the highest *Ratios* across all classes were found for reuptake/transporter inhibitors (**Figure 3**). Compounds in this class were primarily serotonin (5-HT) or mixed noradrenaline/5-HT transporter inhibitors. However, there was an overlap in the 85% confidence interval of *Ratios* compared with other classes (**Supplement**, **Table S1**), and hence, no statistically significant differences. The lowest and highest *Ratio* variability among analyzed classes was seen with GPCR antagonists and enzyme inhibitors, respectively.

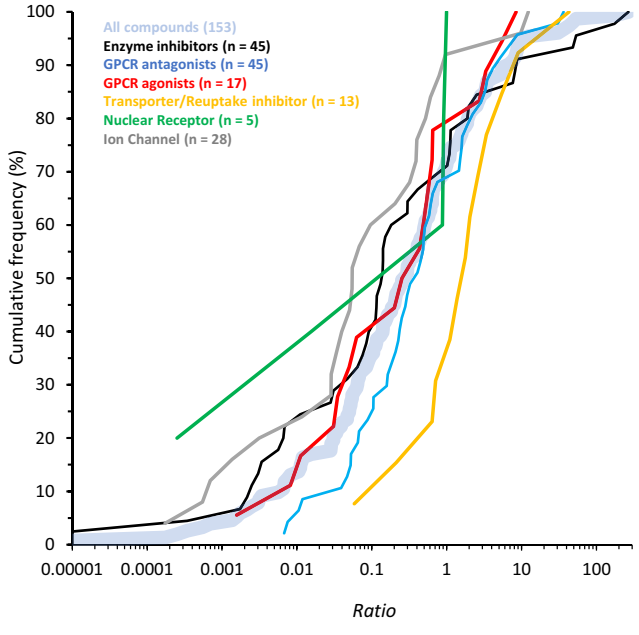


**Figure 3** Cumulative frequency of *Ratios* for compounds classified by mechanism of action and target type. The pale wide blue line is for all compounds. Antiinfectives for systemic use (ATC code J); antiparasitic products, insecticides, and repellents (ATC code P) were excluded. GPCR, G protein-coupled receptor. [Colour figure can be viewed at wileyonlinelibrary.com]

### Ratios by localization of target, the presence of active metabolites, and covariate analysis

Drugs aimed at intracellular targets tended to have lower *Ratios* compared with extracellular targets (**Figure 5**, left). However, 80% of agents for intracellular targets were enzyme inhibitors, and the mode of action therefore substantially different compared with the other groups of ligands.

Compounds with pharmacologically active metabolites had in general a lower *Ratio* compared with those with no reported active metabolites (**Figure 5**, right). No major differences were seen for pure isomer drugs vs. drugs given as racemic mixtures.

A number of covariates appeared to be influencing the ratios. The covariates' bioavailability and unbound fraction in plasma correlated positively, whereas logP and molecular weight correlated negatively to the *Ratios*. However, the residual error was only marginally improved irrespective of covariate examined (**Supplement**, **Table S4**).

The *Ratios* were also explored according to type of reported of *in vitro* potency ($EC_{50}$, $IC_{50}$, or $K_i$, $K_d$; see **Supplement Figure S6**). Based on the 10$^{th}$ to 90$^{th}$ percentile, *in vitro* $IC_{50}$ or $K_i$ values displayed numerically less variability as compared with $EC_{50}$ or $K_d$. When conducting this assessment for matched compounds, for *in vitro* $IC_{50}$ vs. $K_i$ (available for 90 compounds) and based on the 10$^{th}$ and 90$^{th}$ percentiles, *in vitro* $IC_{50}$ had a threefold lower range compared with $K_i$. For *in vitro* $EC_{50}$ vs. $K_i$ values (19 compounds) we observed a similar variability. For *in vitro* $IC_{50}$ vs. *in vitro* $EC_{50}$ (24 compounds) we observed an approximately twofold lower range for *in vitro* $EC_{50}$ vs. *in vitro* $IC_{50}$. All data are available in the **Supplement** (**Table S6**).

## DISCUSSION

### Overall assessment of Ratios

We sought to provide a more detailed insight into how drug *in vitro* target potency relates to clinically effective concentrations. Data on 164 registered drugs were collated and reviewed with respect to their clinically effective unbound plasma exposures to *in vitro* potency relationship *Ratio*. The *Ratios* were also assessed vs. therapeutic indication area, mode of action, receptor type, racemate or isomer, pharmacologically active metabolites, and intracellular or extracellular target location.

The relationship was stronger between *in vitro* potency and therapeutic unbound plasma exposure compared with daily dose (**Supplement Figure S2**), which is consistent with the generally accepted notion that the unbound plasma concentrations "drive" the pharmacological effect of small molecule drugs.[1,9,18]

A high variability was seen in the *Ratios* independently of drug classification. The accumulated observations demonstrate that

prediction of therapeutic exposure based on multiples of *in vitro* potency should be treated with caution since this metric is highly multifactorial. However, the findings may be used for statistical assessment to interpret drug exposure levels in *in vivo* pharmacological studies. Additionally, this probability assessment can further be divided into therapeutic indications, receptor type, and target localization.

### Therapeutic indication areas (ATC) and overall comparison of drug target variabilities

Antimicrobials (antiinfectives for systemic use, antiparasitic products, and insecticides and repellents; ATC classes J & P) showed a higher *Ratio* in contrast to ligands for human cell targets. Presumably this relates to the need to ensure adequate penetration of drug to the target site and to provide efficient eradication of the infectious (or parasitic) matter, thereby optimizing recovery.[19,20] It is worth recalling in this context, also, that agents like these treat the cause of disease rather than symptoms thereof. In turn, to achieve the desired therapeutic action, a saturation or supersaturation of the culprit microbial/parasitic target—accompanied by high $C_{u,ss}$ exposure—is therefore more probable by compounds in this class.

In certain classes of biological pathways and within certain indications, the *in vitro* potency can be fairly predictive of therapeutic exposure. Thus, dividing compounds into subgroups based on the therapeutic classification system ATC revealed several classes of drugs (e.g., drugs for acid-related disorders, cardiac arrhythmia, hypertension, beta-adrenoceptor blocking agents, analgesics, psycholeptics, and for obstructive airway diseases) with a high correlation between $C_{u,ss}$ and *in vitro* potency, although it should be noted that most of these correlations are based on a limited number of agents (**Supplement Figure S5, Table S2**).

Generally, the data suggest that the principal source of variability is *between targets* rather than within *in vitro* assays or within same drug target and mode of action. Thus, the median variability observed that could be attributed to *in vitro* assay contributions was only 10-fold, whereas the global variability range was 1,275-fold (**Figure 4**). Potential reasons for this difference are discussed further below.

### Reasons for variations in the ratio

The median *Ratio* across all drugs was below 1.0 but highly variable and independent of mechanism of action. Potential reasons include promiscuous drug action, suboptimal *in vitro* assay conditions (temperature, target expression, or *in vitro* test compound exposure), interference from pharmacologically active metabolites, and target turnover differences.

It is unlikely that the main reason for the variability is drug promiscuity. Although drug candidates can be promiscuous, the level seems to vary according to the data set used.[21–23] Our work did not address pharmacological effects *per se*, but rather clinically effective plasma concentrations and their relative value to *in vitro* potency of the claimed primary target. Many of the drugs examined are also considered selective for their primary target, particularly taking the unbound exposures into consideration. To minimize the risk of confounding promiscuity we assessed potency for the primary target indication only and strove to select the pharmacologically most relevant *in vitro* measures. Given this, we judge nonselectivity in drug target action to be a but minor confounder within this work, although we cannot entirely exclude that a smaller subset of drugs bind to other than the primary receptors and may add to the variability observed.

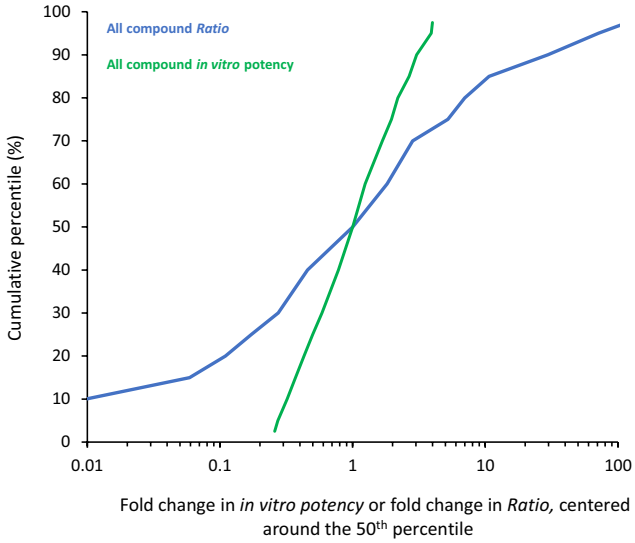


**Figure 4** Cumulative percentile distribution when centered around their 50th percentiles. Blue line is the percentile distribution for all compounds ($n$ = 164) of the *Ratio* when centered around the 50th percentile ($n$ = 164). Green line is the reported *in vitro* potency for the same drug centered around their 50th percentile for all compounds (128 compounds which had 3 or more reported *in vitro* potencies corresponding to a total of 3963 *in vitro* potencies) and the median distribution is depicted. [Colour figure can be viewed at wileyonlinelibrary.com]

Only a small portion of the spread in the observed *Ratios* within this work may be accounted for by *in vitro* variability. For several drugs in this work there were multiple reported *in vitro* potency values for one and the same target, and indeed the potency for 128 of 164 compounds was based on three or more data sources. For these compounds the median coefficient of variation of the geometric mean value was 185%, and the majority of the *in vitro* potency values had variabilities within a factor of ten. As mentioned elsewhere within this manuscript, sources of *in vitro* variability were likely multifactorial. Within this work we did not assess the influence of irreversible/covalent target *in vitro* binding;[24] neither did we mechanistically corroborate potential nonspecific binding in the *in vitro* system.

### Pharmacokinetic and target matrix considerations

Compounds with reported pharmacologically active metabolites displayed an approximately threefold leftward shift in the *Ratios* compared with compounds without (**Figure 5**, right graph). This emphasizes the need to account for the contribution of active metabolites for Ratio assessments and clinically effective concentrations.[25]

Our data suggest that intracellular vs. extracellular distribution may also be a factor relevant to consider in the drug discovery phase. Indeed, there was a trend towards smaller *Ratios* for intracellular

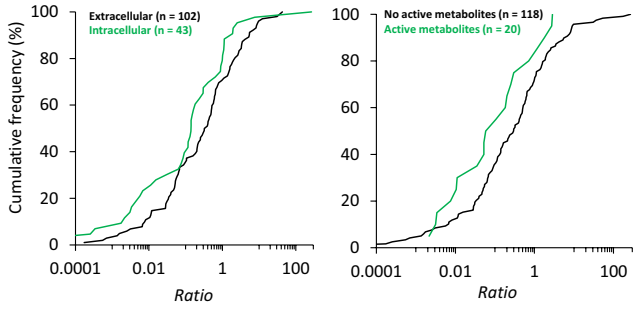


**Figure 5** Cumulative frequency vs. *Ratio*. **Left**, graph marked according to target location (intracellular, extracellular). Four compounds of unclear or mixed designation regarding extracellular vs. intracellular target localization were excluded. **Right**, graph marked according to known contribution of active metabolites or not. Five compounds were excluded that had reported metabolites, but it was unclear whether or not these contributed to the pharmacological effect. Antiinfectives for systemic use (ATC code J); antiparasitic products, insecticides, and repellents (ATC code P) were excluded. [Colour figure can be viewed at wileyonlinelibrary.com]

compared with extracellular targets. This said, in our database all drugs with intracellular targets were either enzyme- or nuclear receptor-acting agents, whereas agents for extracellular targets were either GPCR (58%), ion channel (23%), transporter/reuptake (12%), or enzyme-acting (8%) ligands, thus limiting interpretations on the impact of target localization as such. For comparison, a high *Ratio* has also been described to be required for an inhibitor of an extracellular (membrane-bound) enzyme to elicit a clinically pharmacological effect.[26] There have been efforts to improve the understanding of intracellular drug exposure and methods to assess this for preclinical drug candidates.[27–29] A deeper understanding of the relationship between free drug concentrations and distribution in plasma and within tissues is clearly needed.[30]

The steady-state unbound exposures ($C_{u,ss}$) were used for the *Ratio* calculations. Exploration of $C_{u,ss,max}$ and $C_{u,ss,min}$ vs. *in vitro* potency showed a larger variation when $C_{u,ss,min}$ instead of $C_{u,ss,max}$ or $C_{u,ss}$ was used (**Figure S3**). This is in line with the requirement for a high and sustained drug occupancy for therapeutic responses via many of the targets included in this study (e.g., enzymes and reuptake transporters; see, ref.3, but otherwise this analysis did not cast any further light on the general *Ratio* assessment.

### Covariate analyses

In the analysis of the impact of covariates upon the *Ratios,* drug bioavailability and unbound fraction in plasma were positively correlated, while logP and molecular weight were negatively correlated to *Ratios* (**Supplement**, **Table S4**). These changes were, however, mostly minor, and any interpretations of these analyses require caution. In general, evaluation of covariate influences would require further investigations and also be separately evaluated with a training and a test set to draw any conclusions with regard to their possible importance.

### *In vivo* potency vs. target turnover

It is evident that several factors contribute to the *in vivo* potency readout, not only linked to drug-related features. Indeed, an extended expression of *in vivo* potency $EC_{50}$ was recently derived, which incorporates both binding affinity ($K_d = k_{off}/k_{on}$, where $k_{on}$ and $k_{off}$ denote the second-order on and first order rate of ligand-receptor binding), ligand-target binding rates, and target turnover properties.[31] The *in vitro* drug potency measure primarily assesses binding affinities (e.g., the $K_d$ parameter) but typically neglects ligand-target kinetics and target turnover. Highly variable *Ratios* across compounds and targets are therefore not surprising.[31,32] A clear *in vitro*–to–*in vivo* correlation is not to be expected unless target-to-complex kinetics are constant or close to unity, and ligand-target removal is relatively slow (ligand-target complex elimination rate << ligand-target off-rate).[31,32] Failure to take target turnover and target-ligand complex characteristics into account is therefore likely to result in less predictable *in vivo* properties from *in vitro* potency readouts.

Based on the aforementioned, we surmise that the biological variation between therapeutic unbound exposure levels relative to *in vitro* potency predominantly originates from:

1. Substantial variability in the observed *in vivo* potency vs. *in vitro* drug affinity owing to the impact of *in vivo* target kinetics. For example, recent work by Gabrielsson *et al.*[31] quotes data excerpted from six literature studies. Recalculating *in vivo* $EC_{50}$–to–*in vitro* $K_d$ ($k_{off}/k_{on}$) *Ratios* from these studies results in ranges from 0.22 to 66, thus a factor of 300-fold difference in turnover rates among the targets in question. While these examples were large molecule (antibody) targets, similar findings have been reported also for small molecules. In particular, agents with targets in the circulation compartment and/or that have slow target dissociation rates $k_{off}$ are expected to show *in vivo* potencies highly dependent upon the corresponding target turnover rate ($k_{deg}/k_{on}$, where $k_{deg}$ is target degradation rate, see Gabrielsson *et al.*[31]
2. Differences in the level of *in vivo target* ***occupancy required*** *for clinical efficacy* across targets, dependent both upon the level of endogenous tone at the target, drug mechanism of action (e.g., GPCR agonist vs. antagonist), and target amplification properties (receptor reserve; see further studies, e.g., Grimwood & Hartig, 2009[3]). For example, a high efficacy agonist drug at the dopamine D2 receptor may elicit a near-maximal receptor response already at receptor occupancies <10–20%, whereas 70–80% receptor occupancy by antagonists at the same target is typically required for therapeutic efficacy in schizophrenia. It follows that marked differences in plasma concentrations are to be expected, depending on what level of receptor occupancy may be required for beneficial effects *in vivo*, even for the very same target site. The difference between 20 to 80% receptor occupancy usually corresponds to at least a 10-fold difference in drug exposure. A possible corollary is that the curve for GPCR agonists would be shifted ~ threefold to the right had *in vitro* $EC_{10}$ or $EC_{20}$ rather than $EC_{50}$ been used for $C_{u,ss}$ / *in vitro* potency calculations in this class. Conversely, the curve for GPCR antagonist agents would be shifted ~ threefold leftward had the *in* vitro $EC_{70}$ or $EC_{80}$ rather than $EC_{50}$ been used in the same manner.
3. Factors related to *the experimental model used* to determine *in vitro* potency (e.g., assay conditions, target binding, or

functional type of assay). For example, the 10th and 90th percentile factors were 0.31 and 3.3 of the geometric mean value when there were multiple *in vitro* potency values available for the same drug in our database; that is, roughly a 10-fold range.

These three reasons are the likely main sources of variability between *in vitro* potency and *in vivo* clinical therapeutic exposure relationships, and illustrate that a large variability between them is to be expected. Indeed, combining items *1–3* (i.e., 300-fold, 10-fold, and 10-fold range) would result in an even wider theoretical spread compared with the ~ 1300-fold range (10th to 90th percentile) of *Ratios* observed within the present work. As described above, in terms of the main sources of variability, we found that within–*in vitro* assay conditions vary the least (~ 10-fold), within-target varies moderately (~ 50-fold) and could vary less if having identical *in vitro* assay conditions, whereas cross targets vary the most (~ 1300-fold). Cross-target ratios are a conglomerate of variability in *in vitro* assays, desired level of occupancy, and target turnover, whereas within the same target and mode of action variability may be less dependent on differences in occupancy level.

The high variability in *Ratios* across compounds should also be considered in the context of safety assessments of drug candidates. Essentially, this analysis proposes that quantitatively predicting *in vivo* drug exposure in relation to any *in vitro* potency safety assessment should be carried out with great caution, and emphasizes the need to assess safety in the *in vivo* setting. This is in alignment with the success of early implementation of safety studies *in vivo* for preclinical drug candidates.[33]

### Overall conclusions

The aim of this work was to assess what factors may influence the precision of predictions from (low-molecular) drug *in vitro* potency to clinically efficacious (steady-state) unbound concentrations. Our analysis has

1. Described the overall distribution of *in vivo*–to–*in vitro Ratios* for a broad set of clinically launched drugs ($n = 164$) and their corresponding targets; 70% of these *Ratios* were found to be at or below unity
2. Indicated a modest trend to higher *Ratios* for drugs inhibiting transporter/reuptake sites, and to lower *Ratios* for drugs with known active metabolites or those acting at intracellular vs. extracellular targets; except for these trends, no major differences were distinguished among drug *Ratios* based on either substratification by means of the ATC classification scheme, type of target (enzyme, GPCR, ion channel, etc.), mechanism of action (agonist, antagonist, inhibitor, or modulator), or type of assay and measure used in the *in vitro* drug characterization ($EC_{50}$, $IC_{50}$, $K_d$, or $K_i$)
3. Identified some key factors that contribute to the global, nearly 1,300-fold, range of *Ratios* across all drugs examined; based on closer scrutiny of the components involved, the largest variability source appears to be *in vivo* biology related (e.g., wide range of *target* kinetics), whereas the *in vitro* assay variability appears to contribute the least, with drug-target occupancy-response variability adding to the total *Ratios* observed

These results underscore that predictions of *in vivo* efficacious concentration based on generic *in vivo–to–in-vitro Ratios* may be highly variable, and may even lack biological significance. To advance compound selection during drug discovery, we would like to stress the need to further develop more *in vivo*–like *in vitro* systems and setups, such as sophisticated cell/organ arrangements, microphysiological systems, and as well the usage of mechanistic-based pharmacokinetic–pharmacodynamic modeling.[34–39] The current analysis also strongly supports further *in vivo* pharmacological evaluation of test compounds, even if they display a low *Ratio*. Our data suggest that *in vitro* potency assay conditions, *in vivo* potency estimations from pivotal disease models, defining the required level of receptor occupancy, and probing *in vivo* target turnover properties are key components to the understanding of the links between clinical drug exposure and *in vitro* binding affinity.

### SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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### CONFLICT OF INTEREST

All authors declared no competing interests for this work.

### AUTHOR CONTRIBUTIONS

R.J.-L., S.H., and J.G. wrote the manuscript. R.J.-L., S.H., and J.G. designed the research. R.J.L performed the research. R.J.-L. performed the analysis.



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