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Short Communication

Fraction Unbound for Liver Microsome and Hepatocyte Incubations for All Major Species Can Be Approximated Using a Single Species Surrogate

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Running Title: Single Species Surrogate for Incubation Fraction Unbound

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Results/Discussion: 1488 words

Abbreviations: DDI, drug-drug interaction; DMEM, Dulbecco's modified eagle medium; $f_{u,\ hep}$, fraction unbound in hepatocytes; $f_{u,\ inc}$, fraction unbound in an in vitro incubation; $f_{u,\ liver}$, fraction unbound in liver homogenate; $f_{u,\ mic}$, fraction unbound in liver microsomes; IS, internal standard; IVIVC, in vitro to in vivo correlation; LogD, partition coefficient of a molecule between octanol and buffer at pH 7.4; LogP, partition coefficient of a molecule between octanol and water.

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Abstract

It is well-recognized that nonspecific binding of a drug within an in vitro assay (f_u) can have a large impact on in vitro to in vivo correlations of intrinsic clearance. Typically, this value is determined experimentally across multiple species in the drug discovery stage. Herein we examine the feasibility of using a single species (rat) as a surrogate for other species using a panel of small molecules representing highly diverse structures and physiochemical classes. The study demonstrated that 86% and 92% of the tested compounds measured in mouse, dog, monkey, and human were within two-fold of rat values for f_u in microsomes and hepatocytes, respectively. One compound, amiodarone, exhibited unique species-dependent binding where the f_u was approximately 10-fold higher in human microsomes and 20-fold higher in human hepatocytes compared to the average of the other species tested. Overall, these data indicate that using a single species (rat) f_u as a surrogate for other major species, including human, is a means to increase the throughput of measuring nonspecific binding in vitro.

Introduction

In small molecule drug discovery, microsomal and hepatocyte stability assays are commonly employed to predict in vivo metabolic clearance by utilizing various in vitro to in vivo correlation (IVIVC) methods (Obach, 1999; Austin et al., 2002). It is well-recognized that nonspecific binding of the test compound within an in vitro assay incubation, or fraction unbound in an incubation ($f_{u,\ inc}$), can have a large impact on these predictions (Obach, 1999; Heuberger et al., 2013). Further, fraction unbound in microsomes ($f_{u,\ mic}$) and hepatocytes ($f_{u,\ hep}$) is a critical parameter for improving intrinsic clearance estimations and, as such, is commonly measured. In addition, in vitro drug-drug interaction (DDI) regulatory guidance documents (EMA, 2012; FDA, 2012; FDA, 2017) emphasize application of the free fraction in estimating DDI potential for

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investigational new drug candidates. To address this, f_u will typically be measured across every species in which an in vitro clearance has been tested. Thousands of compounds may need to progress through the testing funnel in the drug discovery stage, and thus the investigation of $f_{u,\ inc}$ across multiple species can rapidly become a resource and labor-intensive endeavor, particularly with respect to reagents, in addition to the instrument and analyst time required.

In an effort to increase the efficiency of high throughput testing of this critical in vitro parameter, we asked the question: are there meaningful interspecies differences of microsomal and hepatocytic f_u such that testing all species, mouse, rat, dog, monkey and human is warranted? To date, there are limited published reports in which microsomal and hepatocyte binding has been rigorously investigated across species. Obach (Obach, 1997) demonstrated that $f_{u,\ mic}$ was equivalent across four species, rat, dog, monkey and human, using three probe compounds imipramine, propranolol and warfarin. Zhang et al (Zhang et al., 2010) also evaluated microsomal binding in these same species using several (thirty-two) clinical drugs and observed no species-specific differences with respect to $f_{u,\ mic}$. In a more recent publication, the fraction unbound in rat liver homogenate ($f_{u,\ liver}$) for a variety (twenty two) of compounds was consistent with $f_{u,\ liver}$ and cellular fraction unbound ($f_{u,\ cell}$) across other species (Riccardi et al., 2018). Despite these findings, it remains common practice to evaluate nonspecific binding across multiple species. In this work, we systematically evaluated $f_{u,\ mic}$ and $f_{u,\ hep}$ in the prototypical preclinical species (mouse, rat, dog, monkey and human) for a highly diverse panel of small molecules, ranging in charge state, such as acid, base, neutral, or zwitterion, and lipophilicity. Our findings demonstrate that rat liver microsomes and hepatocytes are a suitable surrogate for determining $f_{u,\ inc}$ in other species including human.

Materials and Methods

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Materials. A library containing the 36 compounds tested (listed in Table 1) was purchased as 10 mM stock solution in DMSO from Selleck Chemicals (Houston, TX). Mouse (male CD-1) and rat (male Sprague Dawley) liver microsomes with a pool size of 380 and 210 subjects, respectively, were purchased from Corning Life Sciences (Corning, NY). Dog (male beagle), monkey (male cynomolgus), and human (mixed gender, mixed race) liver microsomes with a pool size of 10, 3, and 50, respectively, were purchased from Gibco Biosciences (Dublin, Ireland). Male CD-1 (single donor), male Sprague Dawley rat (single donor), male beagle dog (single donor), male cynomolgus monkey (single donor), and mixed sex human (50-donor pool) cryopreserved hepatocytes were purchased from Bioreclamation IVT (Baltimore, MD). Dulbecco's modified eagle medium (DMEM) was purchased from Gibco (Dublin, Ireland).

Liver Microsome Nonspecific Binding Using Ultracentrifugation. Liver microsomes stocks (20 mg/mL protein content) were diluted in 100 mM Potassium Phosphate buffer, pH 7.4, to a final concentration of 0.25 mg/mL. To a 1 mL solution of microsomes, 1 µL of a DMSO stock solution of test compound (0.5 mM) was added to provide a 0.5 µM final concentration. The mixture of compound and microsomes was incubated for 45 min at 37°C. In triplicate, 200 µL aliquots were then centrifuged at 37°C for 3 hours at 627,000 *x g*. An aliquot of supernatant (50 µL) was removed and was transferred to 50 µL of 0.25 mg/mL blank microsomal mixture. For control (uncentrifuged) samples, 50 µL of microsome/compound mixture was added to 50 uL of blank microsomal filtrate. All samples were quenched with 0.3 mL of acetonitrile containing 10 µM tolbutamide as internal standard. Samples were vortexed and centrifuged at 3220 *x g* for 20 minutes. Supernatants were analyzed by LC-MS as described for hepatocyte experiments below.

Hepatocyte Nonspecific Binding using Ultracentrifugation. Cell suspensions ($0.5x10^6$ cells/mL) were prepared in 1x DMEM buffer plus 1 mM L-glutamine. Suspensions were freeze-

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thawed one time and cell viability (≤ 10%) was confirmed by trypan blue exclusion. Compound stocks were prepared in DMSO and added to an 800 µL hepatocyte suspension for a final concentration of 0.5 µM (0.5% DMSO). Following an equilibration for 15 min at 37°C, each suspension (200 µL) was transferred to polycarbonate tubes (7 x 20 mm) in duplicate and centrifuged at 100,000 rpm for 3 hrs at 37°C using an Optima TLX Ultracentrifuge (Beckman Coulter). To facilitate the calculation of fraction unbound, 50 µL of initial spiked hepatocyte suspensions were added in duplicate to 50 µL 1x DMEM buffer and quenched with 300 µL ACN containing 1 µM tolbutamide as an internal standard (IS). After centrifugation, 50 µL of supernatant was removed, added to 1x DMEM, and proteins were precipitated with ACN containing IS. Samples were then centrifuged for 10 min at 3220 *x g*.

LC-MS/MS and Data Analysis. Samples (1 µL) were injected onto a Kinetex C18 column (2.6 µm, 50 x 2.1 mm; Phenomenex) using a Shimadzu ultrafast-liquid chromatography system coupled to an AB Sciex Qtrap 5500 mass spectrometer. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) using a flow rate at 1 mL/min and a gradient as follows: 5% B for 0.8 min, 99% B for 0.5 min, and returned to 5% B to 1.5 min. Analytes were quantified using the analytical parameters described in supplementary section (Supplemental Table 1). Compound peak areas were integrated using Analyst 1.6.2 software and normalized to the IS.

The unbound fraction was calculated using the following equation:

$$Unbound\ fraction, F_u = \frac{Ultracentrifuge\ supernatant\ at\ 3\ h}{Spiked\ microsome\ or\ hepatocyte\ suspension\ at\ 0\ h}$$

Results and Discussion

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Correction for the $f_{u,\ inc}$ in liver microsomes and hepatocytes is expected to improve IVIVC in preclinical species and thus more accurately predict human clearance. As a consequence of this practice, free drug fraction is typically measured across multiple species. An alternative to a multispecies screening approach would be to select a representative species to measure f_u and use this value to scale cross species, which could add significant value to small molecule discovery research as the proposed method described herein has the capability of decreasing experimental resource burden by up to 5-fold. Riccardi et al. have recently recognized the utility of this approach. In their study, f_u data measured in liver homogenate suggested a single species surrogate (rat) may be appropriate to replace $f_{u,\ inc}$ determination in other species (Riccardi et al., 2018). However, to date, this observation has yet to be systematically tested mainly using a diverse library of small molecules in microsomes as well as hepatocytes isolated from all four major preclinical species in addition to human.

In line with the Riccardi publication, we selected rat as the comparator species to test our hypothesis. Rat is advantageous for two reasons. First, rat is often the initial preclinical species to use for in vivo PK studies, so binding experiments are very routinely performed to inform IVIVC. Secondly, the cost associated with rat microsomes and hepatocytes are markedly less expensive compared to other species, particularly human. If $f_{u,\ inc}$ is indeed identical across all prototypic species, then the choice of comparator species will not impact experimental results.

To assess the binding properties across a range of chemical space, we strategically selected a panel of 36 small molecules for investigation. Overall, each compound class (acid, base, neutral, and zwitterionic) was represented with at least six compounds and encompassed a range of lipophilicities (LogD ranging from -4 to 6). Calculated logD values were determined using ChEMBL algorithm developed by the European Bioinformatics Institute which can be found

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here: https://www.ebi.ac.uk/chembl/. Table 1 summarizes the values measured for $f_{u, mic}$ across five species: mouse, rat, dog, monkey and human. As anticipated, $f_{u, mic}$ values across compounds were quite diverse, ranging from tightly bound to highly free ($f_{u, mic}$ = 0.0039 to 1, 0.0046 to 1, 0.0016 to 1, 0.0027 to 1, and 0.027 to 1 for mouse, rat, dog, monkey and human, respectively). For an interspecies comparison, we selected rat $f_{u, mic}$ as the comparator on the x-axes, and plotted the fraction unbound of each preclinical species either mouse, dog, monkey, human on the y-axes (Figure 1). Using a two-fold ± margin cutoff (dashed lines), these graphs demonstrate that majority of compounds tested (85% in total) fell within two-fold of rat measurements for mouse, dog, monkey, and human (94, 83, 78, and 83%, respectively). Further analysis by compound class also revealed that the average $f_{u, mic}$ fold difference for all species relative to rat was consistently within two-fold for all classes (Supplemental Figure 1). These results indicate that using rat microsomal binding as a surrogate for all other species would provide a reasonable estimate to inform decisions in early drug discovery.

We then applied the same approach to test if hepatocyte binding exhibited a similar trend. Table 2 shows the $f_{u, hep}$ values for the same compound library tested across all five species. In general, the free fraction of molecules was somewhat greater in hepatocytes compared to microsomes, however, similar trends overall were observed. Moreover, measured $f_{u, hep}$ values were just as diverse and mirrored that observed in microsomes ($f_{u, hep}$ = 0.0023 to 1, 0.0081 to 1, 0.0060 to 1, 0.0062 to 1, and 0.076 to 1 in mouse, rat, dog, monkey and human, respectively). Figure 2 shows the results of using rat as the comparator species. Using a two-fold margin cutoff above and below (dashed lines), the graphs indicate a large majority of compounds (96% in total) fell within two-fold of rat measurements for mouse, dog, monkey, and human (89, 97, 100, and 97%, respectively). Further analysis by compound class also revealed that the average $f_{u, hep}$ fold difference for all

species relative to rat was consistently within two-fold for all classes (Supplemental Figure 1). These results indicate the same conclusion: using the hepatocytic binding in single species (rat) can be used as a reasonably accurate estimate for other species.

It should be noted however, that one compound, amiodarone, exhibited a distinctly different free fraction in human liver microsomes and hepatocytes compared to the other species tested. The fraction unbound was approximately 10-fold higher in human microsomes and 20-fold higher in human hepatocytes versus the average of mouse, rat, dog and monkey. This observation of distinctly higher binding in human relative to other species was also reported previously (Zhang et al., 2010). The authors argued that the observed interspecies difference of amiodarone microsomal binding cannot be explained on the basis of physicochemical properties, since a structurally similar tamoxifen, an amphipathic amine with similar lipophilicity (clogD 6.6) demonstrated less than threefold binding difference. Similarly, in our study, nicardipine with logD 4.6 demonstrated comparable binding (< 2-fold difference) across species which was in agreement with Zhang et al. argument that physicochemical properties alone cannot explain the binding difference of amiodarone. However, these data may reflect targeted binding of amiodarone to a specific protein that is either absent or expressed at a lower abundance in humans. Alternatively, since amiodarone is highly lipophilic and known to interact strongly with lipid bilayers (Rusinova et al., 2015), we hypothesis the difference in lipid composition between human and preclinical species may lead to the observed discrepancy in nonspecific binding. In line with this, previous measurements have shown human liver microsomes contain twice the amount of total lipid content relative to rat in addition to differential fatty acid composition (Benga et al., 1983). To our knowledge, the lipid and fatty acid compositions of other species has not been critically investigated. Follow up studies to understand the binding difference of amiodarone and similar

compounds is in progress and will be reported in due time. Amiodarone as an outlier demonstrates that although there is generally a lack of interspecies differences with respect to nonspecific microsomal and hepatocyte binding for most small molecules, there still may be a minority of compounds that exhibit pronounced species dependence, and thus caution should be exercised when interpreting discovery data, particularly for basic compounds with high lipophilicity. Hence, we recommend periodic spot-checking of compounds in a new chemical series to confirm no appreciable interspecies difference.

Besides the single species surrogate approach described herein, other resource-conserving approaches relying on computational methods have been evaluated. Several empirical relationships for the prediction of unbound fraction in microsomal incubations have been proposed (Austin et al., 2002; Hallifax and Houston, 2006; Turner et al., 2006). The empirical relationships were developed using same set of compounds and had demonstrated good predictability. More recently a fragment based empirical approach to predict microsomal binding was reported (Nair et al., 2016). The authors were able to reliably predict nonspecific binding of 114 of 120 compounds but the method was not successful to predict binding of steroids (neutral) or morphinan nucleus incorporating a 4-5 epoxy ring (base), indicating needs for further refinement on the predictive models. Additionally, a mechanistic tool to predict nonspecific binding of drugs in liver microsomes using a similar set of drugs was discussed (Poulin and Haddad, 2011) and the accuracy of prediction was found to be comparable to the empirical methods. The empirical relationships solely rely on lipophilicity parameters (logP/D) of the of drugs and experimental determination of the logP/D is recommended (Poulin and Haddad, 2011). The universal utility of these in silico approaches has not been well evaluated and up to 10-fold error on predictability was documented

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(Poulin and Haddad, 2011). Consequently so, in silico models are generally utilized as a complement to experimental measurements, not as a replacement for them (Gao et al., 2010).

In conclusion, microsomal and hepatocyte nonspecific binding was measured across mouse, rat, dog, monkey, and human using a chemically diverse library of 36 small molecules. Overall, 86% and 92% of the compounds measured in mouse, dog, monkey, and human were within two-fold of rat values for $f_{u,\,mic}$ and $f_{u,\,hep}$, respectively. One compound, amiodarone, exhibited unique species-dependent binding: the fraction unbound was approximately 10-fold higher in human microsomes and 20-fold higher in human hepatocytes compared to the average of other species. The aggregate of these data indicate that using a single species $f_{u,\,mic}$ and $f_{u,\,hep}$ as a surrogate for other species is sensible for most compounds. As such, we recommend measuring rat $f_{u,\,mic}$ and $f_{u,\,hep}$ in the drug discovery setting and using this value as a proxy for preclinical species and human. To exercise caution, we recommend periodically spot-checking compounds in a new chemical series to confirm no appreciable interspecies difference. Overall, this workflow will mitigate the resource burden in drug discovery, while maintaining integrity and confidence of IVIVC.

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Authorship Contributions

Participated in research design: Barr, Lade, Tran, Dahal
Conducted in vitro experiments: Barr, Lade, Tran, Dahal
Contributed new reagents or analytic tools: N/A
Performed data analysis: Barr, Lade, Tran, Dahal
Wrote or contributed to the writing of the manuscript: Barr, Lade, Tran, Dahal

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Legends for Figures

Figure 1. Comparison of fraction unbound ($f_{u, mic}$) in mouse, rat, dog, or monkey, and human liver microsomes for 12 acidic (closed circle), 12 basic (closed square), 6 neutral (open diamond) and 6 zwitterionic drugs (closed triangle). Solid and dashed lines represent lines of unity and twofold upper and lower bound limits, respectively.

Figure 2. Comparison of fraction unbound ($f_{u, hep}$) in mouse, rat, dog, or monkey, and human liver hepatocytes for 12 acidic (closed circle), 12 basic (closed square), 6 neutral (open diamond), and 6 zwitterionic drugs (closed triangle). Solid and dashed lines represent lines of unity and twofold upper and lower bound limits, respectively.

Tables

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Table 1. $F_{u, mic}$ for 36 compounds across 5 species. A, B, N, and Z correspond to acid, base, neutral, and zwitterionic compound classes, respectively. Values represent a mean of triplicate determinations; %CV was ≤ 20% for all compounds.

Compound	Class	LogD	Mouse	Rat	Dog	Monkey	Human
Bumetanide	A	-0.080	0.63	0.98	1.0	0.94	1.0
Cefazolin	A	-4.4	0.45	0.59	0.43	0.43	0.43
Cefoperazone	A	-1.1	0.79	0.53	0.55	0.78	1.0
Diclofenac	A	1.4	0.88	0.96	1.0	0.82	1.0
Fluvastatin	A	-2.2	1.0	1.0	1.0	0.78	1.0
Gemfibrozil	A	1.7	0.73	0.88	0.56	0.49	0.81
Glyburide	A	1.1	0.38	0.52	0.46	0.38	0.43
Ketoprofen	A	-0.16	0.59	0.6	0.63	0.67	0.74
Naproxen	A	0.35	0.61	0.77	0.49	0.31	0.39
Oxaprozin	A	0.090	0.16	0.24	0.19	0.11	0.28
Phenytoin	A	-0.71	0.5	0.495	1.0	0.87	1.0
Tenoxicam	A	-2.9	1.0	0.91	0.69	0.86	0.77
Amiodarone	B	5.9	0.0039	0.0046	0.0016	0.0027	0.035
Amitriptyline	B	2.7	0.24	0.21	0.26	0.21	0.18
Bupivacaine	B	2.9	1.0	0.99	0.45	0.66	0.85
Chlorpromazine	B	3.2	0.29	0.11	0.33	0.24	0.17
Clozapine	B	3.5	0.26	0.3	0.34	0.79	0.71
Disopyramide	B	-0.070	0.59	0.48	0.41	0.41	0.84
Haloperidol	B	2.9	0.47	0.63	0.57	0.50	0.35
Imatinib	B	2.5	ND	0.43	0.43	0.18	0.17
Imipramine	B	2.4	0.53	0.6	0.65	0.82	0.77
Metoprolol	B	-0.47	0.61	0.54	0.59	0.58	0.66
Nicardipine	B	4.6	0.067	0.12	0.18	0.098	0.13
Propranolol	B	0.79	0.2	0.26	0.21	0.23	0.3
Albendazole	N	3.0	1.0	1.0	0.51	0.93	1.0
Antipyrine	N	0.44	0.45	1.0	0.43	0.41	0.41
Dexamethasone	N	-4.6	1.0	1.0	0.94	0.91	0.67
Isradipine	N	3.7	0.66	0.71	0.86	0.64	0.27
Indapamide	N	2.0	0.79	0.53	0.55	0.78	1.0
Zidovudine	N	0.050	0.72	0.71	1.0	1.0	0.64
Doxorubicin	Z	-1.5	0.021	0.039	0.016	0.016	0.027
Levofloxacin	Z	-0.39	0.26	0.35	0.30	0.31	0.29
Methotrexate	Z	-5.1	0.018	0.025	0.028	0.010	0.040
Naltrexone	Z	1.6	0.84	0.85	0.73	0.81	0.59
Telmisartan	Z	3.49	0.56	0.65	0.54	0.58	0.56
Topotecan	Z	-0.32	0.88	0.68	0.61	0.59	0.59

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Table 2. $F_{u, hep}$ for 36 compounds across 5 species. A, B, N, and Z correspond to acid, base, neutral, and zwitterionic compound classes, respectively. Values represent a mean of duplicate determinations.

Compound	Class	LogD	Mouse	Rat	Dog	Monkey	Human
Bumetanide	A	-0.080	1.0	0.94	0.93	0.97	1.0
Cefazolin	A	-4.4	0.97	1.0	1.0	0.93	1.0
Cefoperazone	A	-1.1	0.14	0.15	0.18	ND	0.15
Diclofenac	A	1.4	0.84	0.95	0.65	0.72	0.97
Fluvastatin	A	-2.2	0.42	0.58	0.52	0.51	0.53
Gemfibrozil	A	1.7	1.0	1.0	1.0	1.0	1.0
Glyburide	A	1.1	1.0	1.0	0.69	0.80	0.84
Ketoprofen	A	-0.16	0.98	1.0	1.0	1.0	1.0
Naproxen	A	0.35	0.89	0.99	1.0	1.0	1.0
Oxaprozin	A	0.090	1.0	1.0	1.0	1.0	1.0
Phenytoin	A	-0.71	1.0	0.96	0.76	0.48	0.99
Tenoxicam	A	-2.9	1.0	1.0	1.0	1.0	0.93
Amiodarone	B	5.9	0.0023	0.0081	0.0060	0.0062	0.12
Amitriptyline	B	2.7	0.19	0.24	0.34	0.31	0.32
Bupivacaine	B	2.9	0.85	0.96	0.92	0.92	0.94
Chlorpromazine	B	3.2	0.055	0.14	0.12	0.14	0.17
Clozapine	B	3.5	0.33	0.50	0.45	0.45	0.50
Disopyramide	B	-0.070	1.0	0.98	0.97	1.0	0.99
Haloperidol	B	2.9	0.51	0.76	0.70	0.81	0.81
Imatinib	B	2.5	0.36	0.79	0.72	0.67	0.79
Imipramine	B	2.4	0.34	0.37	0.42	0.33	0.37
Metoprolol	B	-0.47	0.92	0.95	1.0	1.0	1.0
Nicardipine	B	4.6	0.038	0.062	0.051	0.034	0.076
Propranolol	B	0.79	0.71	0.69	0.86	0.94	0.82
Albendazole	N	3.0	0.55	0.70	0.68	0.69	0.74
Antipyrine	N	0.44	0.98	1.0	0.89	1.0	1.0
Dexamethasone	N	-4.6	0.82	0.95	0.96	0.95	0.95
Indapamide	N	3.7	0.14	0.15	0.18	ND	0.15
Isradipine	N	2.0	0.22	0.34	0.34	0.32	0.43
Zidovudine	N	0.050	1.0	1.0	0.98	0.94	0.97
Doxorubicin	Z	-1.5	0.023	0.086	0.031	0.11	0.11
Levofloxacin	Z	-0.39	0.94	0.98	0.97	0.99	0.98
Methotrexate	Z	-5.1	1.0	1.0	1.0	1.0	1.0
Naltrexone	Z	1.6	0.90	0.98	0.98	1.0	0.99
Telmisartan	Z	3.49	0.40	0.54	0.47	0.44	0.44
Topotecan	Z	-0.32	0.87	0.73	0.81	0.90	0.71

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Figures

Figure 1

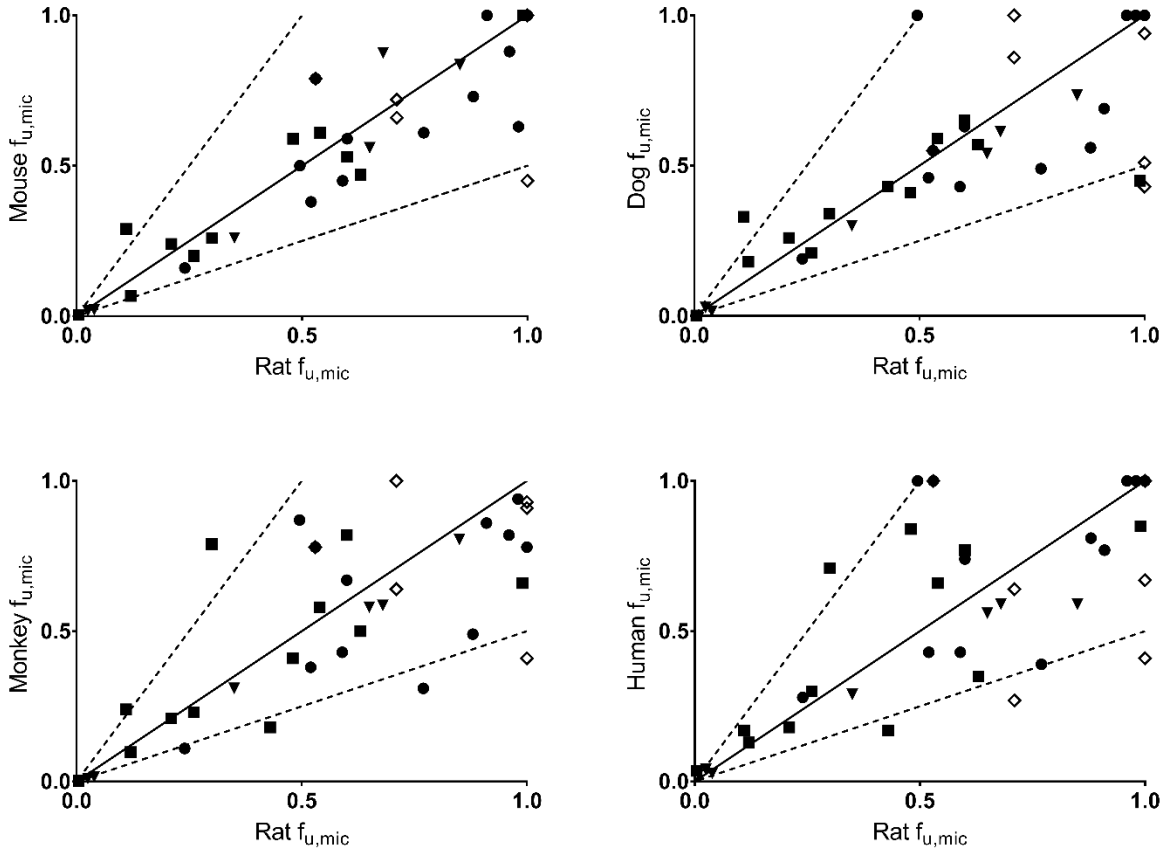

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Figure 2