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# Relating Molecular Properties and in Vitro Assay Results to in Vivo Drug Disposition and Toxicity Outcomes

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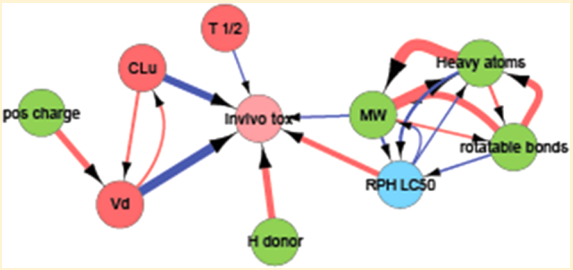
Supporting Information

**ABSTRACT:** A primary goal of lead optimization is to identify compounds with improved absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. A number of reports have linked computed molecular properties to desirable in vivo ADMET outcomes, but a significant limitation of these analyses is the failure to control statistically for possible covariates. We examine the relationship between molecular properties and in vitro surrogate assays vs in vivo properties within 173 chemical series from a database of 3773 compounds with rodent pharmacokinetic and toxicology data. This approach identifies the following pairs of surrogates as most predictive among those examined: rat primary hepatocyte (RPH) cytolethality/volume of distribution ($V_d$) for in vivo toxicology outcomes, scaled microsome metabolism/calculated logP for in vivo unbound clearance, and calculated logD/kinetic aqueous solubility for thermodynamic solubility. The impact of common functional group substitutions is examined and provides insights for compound design.




## INTRODUCTION

Lead generation and lead optimization efforts in drug discovery are time-consuming and costly, typically requiring 3.5 years and $12.5 million for successfully developing a clinical candidate.[1] Improving the in vivo ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of one or more chemical series represents a large proportion of the effort. Medicinal chemists routinely utilize molecular properties and in vitro ADMET assays to select the most promising molecules from a chemical series for further evaluation in vivo. The inherent assumption in applying molecular properties and/or ADMET surrogates is that the inexpensive method is useful for quantitative prediction or rank-ordering of molecules according to their in vivo properties.[2]

A number of studies have examined the relationship between computed molecular properties (e.g., Lipinski parameters[3]) and in vivo ADMET and other results[4−9] or attrition from clinical development.[10,11] Large collections of results from pharmaceutical company databases, analyzed with statistical techniques such as ANOVA, reveal statistically significant trends that are intuitive and frequently invoked as important criterion in assessing the ADMET properties of compounds. For example, the work of Gleeson[8] notes the relationship between increased molecular weight and decreased solubility across a large collection of molecules tested at GlaxoSmithKline. However, the analysis does not control for ionization state at neutral pH, or hydrophobicity, or any other attribute known to influence solubility. Statisticians involved in clinical trial design and analysis refer to such attributes as covariates, and patients are randomized to trial arms in a manner that removes their influence from the analysis (i.e., a designed experiment). Corporate databases of ADMET results are not populated from designed experiments, and retrospective analyses that make no attempt at controlling for covariates may suffer from the identification of spurious relationships. Noteworthy exceptions in the medicinal chemistry literature are the work of Kenny and Leach, where pairs of molecules differing by single functional groups are compared.[12,13] The required analysis framework is used extensively in the social sciences, where covariates are unknown, designed experiments are uncommon, and vigorous debates over cause and effect have spurred the use of approaches that establish relationships in a narrow context. In a notable example, Seymour Lipset's "modernization hypothesis" postulates a link between education and democracy, which seems intuitive and is borne out in an analysis of average education vs Freedom House democracy ratings across countries. Yet when examining changes in education over time within single countries, no correlation vs change in democracy ratings is found. Carefully devised studies reinforce the absence of any such relationships.[14]

As for the social sciences, pharmaceutical company databases were not constructed with a goal to identify all covariates in advance or control for them in a traditional manner. To minimize the role of covariates, we examined trends within each of 173 chemical series to identify factors connected with in vivo ADMET outcomes. Thus, individual factors required high correlation with in vivo properties across a large number of

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chemical series in order to emerge as important general predictors of those in vivo properties. In practical terms, our analytical approach provides guidance on the utility of various surrogates and how large a change is required with regard to the surrogate end point in order to achieve a given improvement of in vivo properties.

## RESULTS

The relationship between in vivo ADMET outcomes and molecular properties or surrogate assays (henceforth surrogates) was examined within 173 unique chemical series representing 3773 compounds (Supporting Information Figure 1). The in vivo studies comprise the short-term rodent studies used by many companies during lead optimization to identify molecules likely to represent viable clinical candidates for entry into Good Laboratory Practice (GLP) preclinical safety studies. These included repeat-dose toxicology studies of 4−14 days duration as well as single-dose pharmacokinetic rodent studies. Nonrodent data were not included in the analysis. The data set includes in vivo and in vitro ADMET results collected prior to declaring a clinical candidate (Table 1). Most, if not all, of the in vitro assays are commonly used across the pharmaceutical industry. A number of molecular properties (e.g., Lipinski parameters) were calculated from the molecular structure using in-house or commercial cheminformatics applications.

**Property Trends and Covariates.** We first repeated analyses described by Hughes et al.[4] that associate increased ClogP or decreased PSA to increased compound toxicity. For this analysis, "toxic" molecules produce adverse histological changes in one or more tissues (given in Supporting Information) or death at $C_{max}$-derived compound exposure of 10 $\mu$M or less. Similar to Hughes et al., our analysis reveals increased odds of negative outcomes for low PSA/high ClogP compounds vs high PSA/low ClogP compounds but with a markedly smaller effect (3.2-fold vs 6.1-fold in the Hughes analysis; Supporting Information Table 2). However, increased odds of negative toxicology outcomes for low vs high PSA compounds having ClogP < 3 are not observed in our data set (odds of 0.08 vs 0.11). The relationship between molecular weight and bioavailability noted by Gleeson[8] is not monotonic in our analysis (albeit with few observations in the extremes), whereas the molecular weight vs solubility trend is similar (Supporting Information Figure 2). When analyzed across our entire data set, Caco-2 permeability appears predictive of in vivo toxicity (Supporting Information Figure 3). These analyses do not control for covariates.

To reduce the effect of covariates in such data sets, we examined surrogate vs in vivo relationships within chemical series (i.e., not comparing compounds from different series). Ideally, surrogates quantitatively predict results from in vivo studies, defined by a high Pearson correlation coefficient ($r$). Less desirable but nonetheless useful, a surrogate may lack quantitative accuracy but correctly rank-orders compounds according to in vivo properties; we used Spearman's $\rho$ to measure rank-order correlation within a chemical series. Finally, a surrogate might at best qualitatively predict changes of in vivo properties, i.e., a large improvement in the surrogate property is more likely to be associated with a large improvement in the in vivo outcome than a small change in the surrogate.

To identify qualitative relationships between surrogates and in vivo outcomes, we identify all unique pairs of compounds from the same scaffold and order the two compounds such that the second has the higher (not necessarily better) value for a given surrogate (Table 1). Hence, all surrogate changes in our analysis are positive, and we examine the impact of increasing a surrogate end point on the in vivo outcome. For example, a scaffold with three compounds generates three unique pairs (A vs B, A vs C, and B vs C; Table 2). Different surrogate quantities have different natural scales; it is not evident whether increasing ClogP by 2 units is a larger change than increasing molecular weight (MW) by 100 Da. To compare surrogate increases on an equal footing, we calculate the 25th, 50th, 75th, and 90th percentile for each quantity across all pairs of compounds from the same scaffold. For MW, these values correspond to 16, 35, 64, and 99 Da; the median MW increase when comparing compounds from the same scaffold is 35 Da, whereas the largest 10% exceed 99 Da. We examined the relationship between a large number of in vivo and surrogate properties via percentile differences (Table 3)

**Surrogates vs in Vivo Toxicology Outcomes.** Toxicology results for a compound are summarized as the lowest observed adverse effect level (LOAEL): we identified the lowest dose that causes adverse histological changes or death in rat and assigned the corresponding compound concentration in plasma (i.e., $C_{max}$ value) associated with this dose. Small values denote compounds with unfavorable toxicology outcomes. Some compounds produce no adverse findings at the highest dose tested, and are reported with qualifiers ">" (e.g., LOAEL > 10.5 $\mu$M for a compound with no adverse findings that has $C_{max}$ = 10.5 $\mu$M at the highest dose tested).

We examined the ability of each surrogate to quantitatively (via Pearson $r$ or Spearman $\rho$) or qualitatively (via the pairs analysis) predict changes in LOAEL within a given chemical series. Because low-dose PK study results are available when selecting compounds for toxicology studies, we treated the PK parameters as possible surrogates with regards to toxicology predictions. While the quantitative agreement between surrogates and LOAEL is modest, several surrogates provided useful qualitative information (Figure 1): large increases in $V_d$ or $CL_u$ from low-dose rodent PK studies tend to result in significantly lower LOAEL values; the converse is observed for large increases in in vitro RPH cytolethality or AUC from low oral dose rodent PK studies. Among computed molecular properties, a large increase in molecular weight or heavy atom count tends to decrease LOAEL, whereas increasing hydrogen bond donors tends to increase LOAEL. In contrast to the analysis across all series (Supporting Information Figure 3), there is no apparent relationship between Caco-2 permeability and LOAEL within a given chemical series.

We investigated whether the best surrogates have overlapping roles in explaining LOAEL changes. Changes in $V_d$, $CL_u$, and oral AUC from rodent PK studies are highly correlated or anticorrelated within a chemical series, but those between $V_d$ and RPH $LC_{50}$, molecular weight, or H-donors are not (Supporting Information Tables 3 and 4). To determine their degree of independence in explaining LOAEL changes, we identified compound pairs exhibiting a small change for one surrogate (<50th percentile) and a large increase for a second surrogate (>75th percentile). The contributions of $V_d$ and RPH $LC_{50}$ toward explaining LOAEL changes overlap partially (~40%), while those from molecular weight or H-donor increases are mostly redundant with $V_d$ and/or RPH $LC_{50}$ (Supporting Information Figure 4). The average impact on LOAEL when simultaneously considering changes in $V_d$ and RPH $LC_{50}$ is given in Table 4. Increasing $V_d$ and decreasing RPH $LC_{50}$ >5-fold (i.e., both changes in the "wrong" direction)

**Table 1. Molecular Properties, In Vitro Surrogates, And in Vivo PK End Points**

| property[a] | description |
|---|---|
| | In Vivo Pharmacokinetics (PK) |
| log10 CL (mL/min/kg) | plasma clearance in rat following 1 or 3 mg/kg intravenous dose |
| log10 $CL_u$ (mL/min/kg) | plasma clearance in rat normalized by predicted[15] fraction unbound in plasma ($CL/f_{u,plasma}$) |
| log10 AUC (h·kg/mL) | area-under-the-curve from plasma concentration vs time plot following 3, 5, or 10 mg/kg oral dose in rat expressed in mg·h/mL, normalized by dose in mg/kg |
| log10 bioavailability (%) | oral bioavailability in rat: $(AUC/dose)_{oral}/(AUC/dose)_{iv} \times 100$ |
| log10 *T*1/2 (h) | half-life in rat following 3, 5, or 10 mg/kg oral dose |
| log10 Vd (mL/kg) | apparent volume of distribution ($V_{darea}$) in rat following 1 or 3 mg/kg intravenous dose (dose/initial plasma concentration) |
| | Physical Properties |
| log10 thermodynamic solubility pH 6−8 (M) | thermodynamic (or equilibrium) solubility at room temperature in pH 6−8 range measured by the classical shake flask or modified HPLC method,[2] expressed in mg/mL and converted to mol/L; quantifiable levels are expressed with qualifiers as <0.001 mg/mL and >2 mg/mL, respectively, or <3.5 $\mu$m and >2.8 mM after conversion |
| log10 kinetic solubility pH 7.4 ($\mu$M) | kinetic solubility at room temperature measured by adding 10 mM DMSO stock solution in pH 7.4 buffer and detection of precipitation via nephelometry; the highest soluble concentration is reported, up to a maximum of 100 $\mu$M (i.e., solubility >100 $\mu$M) |
| | In Vitro ADMET Surrogate Assays |
| Caco-2 permeability (% A−B transport) | A to B transport[16] in Caco-2 monolayer permeability assay, using LC/MS/MS compound detection following 3 h incubation of 10 $\mu$M compound concentration at 37 °C |
| human microsome metabolism (%) | human liver microsome metabolism by LC/MS/MS compound detection following a 30 min incubation of 2 $\mu$M test substance, 0.5 mM NADPH at 37 °C, 50 mM sodium phosphase pH 7.4 buffer, and 0.5 mg/mL protein; metabolism = 100-percent remaining |
| mouse microsome metabolism (%) | mouse liver microsome metabolism using above protocol |
| rat microsome metabolism (%) | Sprague−Dawley rat liver microsome metabolism using above protocol |
| log10 $CL_{int}$ from rat mic (mL/min/kg) | intrinsic clearance obtained by scaling rat microsome (mic) metabolism:[17,18] ln(100/percent remaining)/30 min) × (liver wt/body wt) × (protein/liver wt)/protein conc in incubation; for rat, (liver wt/body wt) = 40 g/kg; (protein/liver wt) = 45 mg/g; microsome metabolism is measured at 30 min only and log−linear loss is assumed |
| log10 $CL_{int,u}$ from rat mic (mL/min/kg) | above quantity divided by predicted fraction unbound in microsomes, to correct for the concentration of test substance susceptible to microsomal metabolism: $CL_{int}/f_{u,microsome}$ |
| log10 rat primary hepatocyte $LC_{50}$ ($\mu$M) | rat primary hepatocyte (RPH) cytothelality measured via lactate dehydrogenase (LDH) leakage from cells;[19−21] compounds are incubated at 100, 80, 60, 40, 20, 10, and 1 $\mu$M final concentration; following curve-fitting, the $LC_{50}$ indicates the concentration that causes 50% cytolethality compared to vehicle (DMSO) control; values with no significant toxicity at the highest concentration tested are expressed as $LC_{50}$ >100 $\mu$M |
| | In Vitro Pharmacology Assays |
| log10 $IC_{50}$ against target ($\mu$M) | Potency in primary pharmacological assay used in the corresponding discovery project, with nonquantified $IC_{50}$s expressed as $IC_{50}$ > 20 $\mu$M |
| | Calculated Molecular Properties |
| calculated basic p$K_a$ | ChemAxon[b] calculated p$K_a$ for bases |
| ClogD@pH = 7.4(Chemaxon) | ChemAxon[b] calculated log *D* at pH 7.4 |
| ClogP(Chemaxon) | ChemAxon[b] calculated log *P* |
| ClogP(Daylight) | Daylight/Biobyte calculated log *P*, Biobyte version 4.3 |
| H acceptor | no. of hydrogen bond acceptors (Lipinski rule) |
| H donor | no. of hydrogen bond donors (Lipinski rule) |
| Heavy atoms | no. of non-hydrogen atoms |
| MW | molecular weight |
| pos charge | no. of basic centers |
| PSA | topological polar surface area[22] |
| rotatable bonds | no. of rotatable bonds |

[a]Properties preceded by log10 are log10 transformed to increase normality of the data distribution. [b]ChemAxon toolkit version 5.1.7.



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**Table 2. Pairs Analysis Example: Molecular Weight vs Plasma Clearance Changes for a Chemical Series of Three Compounds[a]**

| | MW increase | | |
|---|---|---|---|
| compd pair | daltons | percentile range | log10 CL change |
| A vs B | 15 | 0–25 | 0.23 |
| A vs C | 75 | 75–90 | 0.11 |
| B vs C | 60 | 50–75 | −0.12 |

[a]Theoretical example for compounds A, B, and C sharing the same scaffold, having molecular weights of 350, 365, and 425 Da, respectively, and plasma clearance in rat equal to 35, 60, and 45 mL/min/kg, respectively. CL values are log10 transformed to normalize their distribution.

results in an average log10 LOAEL change of −1.37 (i.e., decreasing the LOAEL by ca. 24-fold). Consistent with this, the impact on LOAEL of increasing $V_d$ is blunted by an increase in RPH $LC_{50}$. The reverse logic also applies: decreasing $V_d$ and increasing RPH $LC_{50}$ > 5-fold gives an average improvement in LOAEL of 24-fold. The effect of $V_d$ and RPH $LC_{50}$ on toxicity is intuitive: $V_d$ measures the extent of distribution in tissue, while RPH $LC_{50}$ measures the intrinsic toxicity of the compound. Increasing $V_d$ and decreasing RPH $LC_{50}$ causes greater tissue exposure to an intrinsically more toxic compound.

**Surrogates vs in Vivo Unbound Clearance.** In addition to improving the toxicology profile of compounds, decreasing unbound clearance is a major challenge in lead optimization. Unbound clearance is obtained by dividing total clearance (CL) from low-dose iv studies with fraction unbound in plasma and disentangles the effects of reducing the rate of elimination via plasma protein binding (PPB) vs reducing the susceptibility to hepatic metabolism (the latter is viewed as solving a CL problem; the former is not[24]). A related quantity is unbound intrinsic clearance ($CL_{int,u}$) in a theoretical no flow, no PPB situation, obtained from total CL and fraction unbound in plasma using models of hepatic clearance.[17] These models assume that clearance is fully hepatic and give negative $CL_{int,u}$ values for compounds with CL in excess of hepatic flow. For this reason, we focus on the simpler $CL_u$ quantity.

In contrast to LOAEL, the utility of surrogates for explaining $CL_u$ is similar whether one considers quantitative (Pearson $r$, Spearman $\rho$) or qualitative correlations (large changes between compounds; Figure 2). Various measures of microsome metabolism are all predictive for $CL_u$ from low-dose rat PK studies; the scaled estimate of $CL_{int,u}$ which corrects for fraction unbound in microsomes is more predictive than the unscaled or uncorrected quantities. In addition, increasing ClogP or ClogD also predicts for increasing $CL_u$ within a chemical series. ClogP and $CL_{int,u}$ from microsome metabolism make additive contributions in explaining in vivo $CL_u$ (Table 5). The role of both quantities is consistent with expectation: microsome assays measure susceptibility to hepatic metabolism, and binding to plasma proteins and drug-metabolizing CYPs is enhanced when compound hydrophobicity is increased.[2] The lack of full concordance between $CL_{int,u}$ from microsome assays and in vivo $CL_u$ is expected when significant compound elimination occurs via non-CYP pathways. Other computed molecular properties have modest utility in predicting $CL_u$ changes.

**Surrogates vs in Vivo Bioavailability.** The bioavailability of a compound reflects its ability to enter systemic circulation via oral dosing relative to its plasma exposure from iv dosing. Adequate bioavailability is a requirement for oral drug delivery, and is first evaluated in rat. In vitro assays measured permeability in model systems, and properties such as polar surface area (PSA) and molecular weight have been linked to rat

**Table 3. Molecular Properties, In Vitro Surrogates and in Vivo End Points Evaluated between Pairs of Compounds from the Same Chemical Series**

| | difference for compound pairs from same series (percentiles)[b] | | | | |
|---|---|---|---|---|---|
| property[a] | 25th | 50th | 75th | 90th | compd pairs |
| In Vivo Toxicology | | | | | |
| log10 LOAEL (μM) | 0.29 | 0.64 | 1.08 | 1.52 | 1430 |
| In Vivo Pharmacokinetics (PK) | | | | | |
| log10 CL (mL/min/kg) | 0.16 | 0.35 | 0.65 | 1.03 | 47767 |
| log10 $CL_u$ (mL/min/kg) | 0.21 | 0.47 | 0.84 | 1.27 | 47767 |
| log10 AUC (mg·h/mL over dose in mg/kg) | 0.32 | 0.69 | 1.23 | 1.81 | 104991 |
| log10 bioavailability (%) | 0.2 | 0.43 | 0.8 | 1.23 | 26963 |
| log10 $T1/2$ (h) | 0.11 | 0.24 | 0.42 | 0.63 | 66156 |
| log10 $V_d$ (mL/kg) | 0.17 | 0.37 | 0.66 | 0.98 | 45877 |
| Physical Properties | | | | | |
| log10 thermodynamic solubility pH 6–8 (M) | 0.58 | 1.09 | 1.86 | 2.65 | 33398 |
| log10 kinetic solubility pH 7 (μM) | 0.14 | 0.32 | 0.68 | 0.74 | 14130 |
| In Vitro ADMET Surrogate Assays | | | | | |
| Caco-2 permeability (% A–B transport) | 2.63 | 7.4 | 15.09 | 23 | 55376 |
| human microsome metabolism (%) | 7.6 | 16.7 | 31.1 | 50.2 | 74311 |
| mouse microsome metabolism (%) | 7.4 | 18.2 | 37.3 | 58.7 | 74386 |
| rat microsome metabolism (%) | 8.4 | 19.6 | 37.8 | 58.2 | 74319 |
| log10 $CL_{int}$ from rat mic (mL/min/kg) | 0.17 | 0.37 | 0.66 | 1.02 | 74319 |
| log10 $CL_{int,u}$ from rat mic (mL/min/kg) | 0.21 | 0.46 | 0.82 | 1.28 | 74319 |
| log10 rat primary hepatocyte $LC_{50}$ (μM) | 0.18 | 0.44 | 0.68 | 0.92 | 3302 |
| In Vitro Pharmacology Assays | | | | | |
| log10 $IC_{50}$ against target (μM) | 0.19 | 0.43 | 0.77 | 1.2 | 112502 |
| Calculated Molecular Properties | | | | | |
| calculated basic pka | 0.31 | 1.02 | 1.74 | 2.46 | 38032 |
| ClogD@ pH=7.4(Chemaxon) | 0.42 | 0.92 | 1.66 | 2.59 | 123787 |
| ClogP(Chemaxon) | 0.39 | 0.84 | 1.48 | 2.25 | 123787 |
| ClogP(Daylight) | 0.4 | 0.87 | 1.56 | 2.44 | 123787 |
| H acceptor | 0 | 1 | 2 | 2 | 123787 |
| H donor | 0 | 0 | 1 | 1 | 123787 |
| Heavy atoms | 1 | 2 | 4 | 7 | 123787 |
| MW | 16 | 35 | 64 | 99 | 123787 |
| pos charge | 0 | 0 | 1 | 1 | 123787 |
| PSA | 5 | 12.4 | 22 | 33.9 | 123787 |
| rotatable bonds | 0 | 1 | 2 | 3 | 123787 |

[a]See Table 1 for abbreviations. Several result types are log10 transformed to normalize their distribution prior to statistical analysis; increases of 0.3, 0.48, 0.7, and 1 on a log10 scale correspond to fold increases of 2, 3, 5, and 10 in the nontransformed values. [b]Pairs of compounds from the same scaffold are compared with respect to a given end point, and the increase that corresponds to the given percentile is shown in the table.


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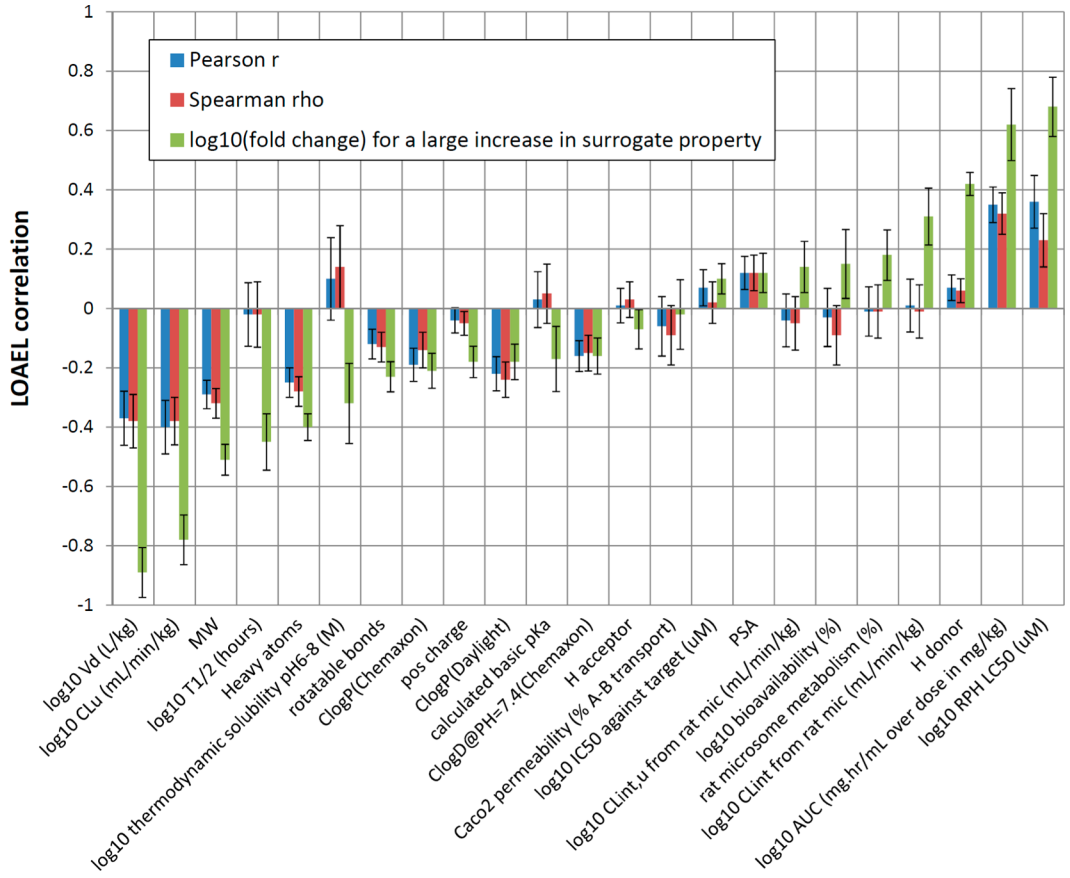


**Figure 1.** Relationship between ADMET surrogates ($X$-axis) and lowest-observed adverse effect level (LOAEL; $Y$-axis) from rat toxicology studies; small LOAEL values are unfavorable. The Pearson $r$ and Spearman $\rho$ correlation coefficients were calculated for each chemical series and surrogate end point against log10 LOAEL and averaged across all chemical series having five or more compounds with both values defined;[23] log10(fold change) denotes the average difference in log10(LOAEL) for compound pairs from the same chemical series that exhibit a large difference in a given surrogate end point (i.e., a difference that exceeds the 75th percentile). Values of 1 and −1 denote a 10-fold increase and 10-fold decrease in LOAEL for the compound with large surrogate value relative to that with the smaller value. The error bars correspond to the standard error of the correlation coefficient across all chemical series or the standard error across all compound pairs.

**Table 4. Average (Standard Error) log10 LOAEL Change for a Given Change in $V_d$ and RPH $LC_{50}$**

| | RPH $LC_{50}$ fold change | | | |
|---|---|---|---|---|
| $V_d$ fold change | decrease >5 | decrease 2.5−5 | decrease 1−2.5 | increase |
| increase 1−2.5 | −0.90 (0.23) | 0.02 (0.21) | −0.27 (0.13) | −0.13 (0.10) |
| increase 2.5−5 | −0.73 (0.38) | −0.41 (0.26) | −0.47 (0.21) | −0.50 (0.18) |
| increase >5 | −1.37 (0.15) | −0.85 (0.20) | −0.57 (0.35) | −0.57 (0.23) |

bioavailability in other work.[5,8] We examined the quantitative and qualitative correlation between surrogates and rat bioavailability in the manner described above (Figure 3). In contrast to LOAEL and $CL_u$, the best surrogates have modest utility for predicting changes in bioavailability. On average, decreasing PSA by 22 Å or increasing thermodynamic solubility 72-fold (i.e., changes beyond the 75th percentile) lead to a modest 2-fold improvement in bioavailability. Because bioavailability is a function of solubility, intestinal permeability, and plasma CL, the limited predictive utility of Caco-2 permeability used alone is expected.

**Surrogates vs Thermodynamic Solubility.** Screen hits frequently have low solubility, which hinders their reliable characterization using biological assays and formulation for in vivo studies.[2] The standard shake-flask approach for thermodynamic solubility measurement is laborious and consumes milligrams of material. In response, kinetic solubility from DMSO stock solution and various molecular properties are frequently used prior to thermodynamic solubility determination.[2]

When examined via quantitative and qualitative correlation approaches, the surrogates that predict changes in thermodynamic solubility within a chemical series provide few surprises: increasing basic $pK_a$, the number of positive charges, or kinetic solubility tends to increase thermodynamic solubility, while increasing ClogD decreases solubility (Figure 4). Of note, decreasing RPH $LC_{50}$ is strongly associated with increasing solubility, underscoring the risks of increasing $pK_a$/introducing charged groups with regards to toxicity. While all these quantities may be thought redundant for explaining thermodynamic


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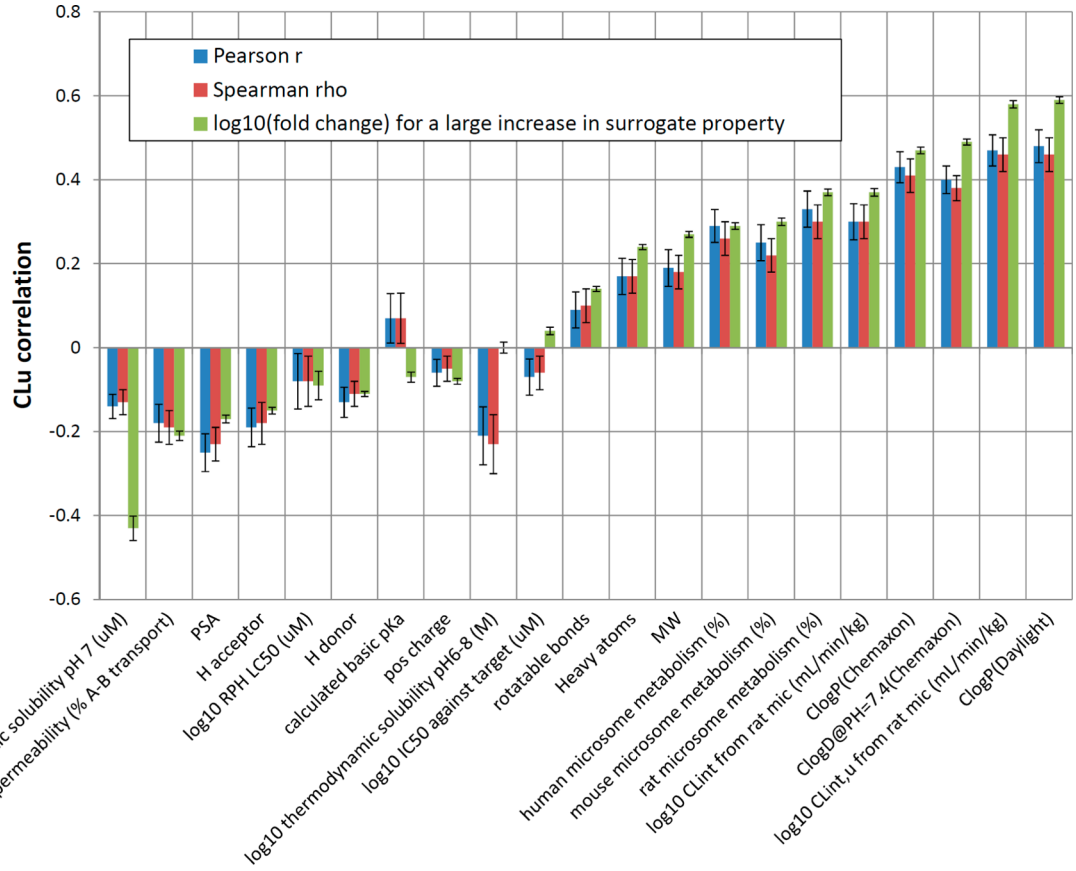


**Figure 2.** Relationship between ADMET surrogates (*X*-axis) and unbound clearance from low-dose rat iv PK studies ($CL_u$; *Y*-axis); Pearson *r* and Spearman $\rho$ denote the average Pearson *r* and Spearman $\rho$ correlation coefficients between each surrogate and log10($CL_u$) calculated across all chemical series having five or more compounds; log10(fold change) denotes the average difference in log10($CL_u$) for compound pairs from the same chemical series that exhibit a large difference in a given surrogate end point; see Figure 1 and the results text for details.

**Table 5. Average (Standard Error) log10 $CL_u$ Change from Low-Dose Rat iv PK Study for a Given Change in Scaled Microsome Stability Corrected for Fraction Unbound in Microsomes and ClogP**

| | ClogP (Daylight) | | | | | |
|---|---|---|---|---|---|---|
| $CL_{int,u}$ fold change | decrease >1.6 | decrease 0.9−1.6 | decrease 0−0.9 | increase 0−0.9 | increase 0.9−1.6 | increase >1.6 |
| increase 1−3 | −0.29 (0.03) | −0.14 (0.02) | 0.00 (0.01) | 0.15 (0.01) | 0.30 (0.01) | 0.48 (0.02) |
| increase 3−6 | −0.22 (0.04) | −0.04 (0.03) | 0.12 (0.02) | 0.28 (0.01) | 0.43 (0.02) | 0.66 (0.02) |
| increase >6 | −0.12 (0.06) | 0.03 (0.05) | 0.12 (0.02) | 0.41 (0.02) | 0.55 (0.02) | 0.95 (0.01) |

solubility, the identification of compound pairs where ClogD is relatively unchanged but other surrogates are substantially increased or decreased shows that their contributions are not fully redundant (Supporting Information Figure 5). Increasing kinetic solubility and decreasing ClogD leads to larger increases in thermodynamic solubility than increasing either property alone (Table 6). Increasing H donors, PSA, or MW have no appreciable effects on solubility.

**Relating Measured End Points to Molecular Properties and Functional Group Substitutions.** Several predictive properties are either in vitro assays or in vivo properties themselves (e.g., Vd vs LOAEL). We repeated the pairs analysis by treating each property in Table 1 as "target" and identified other properties positively or negatively correlated with the target. When examined via a network representation, several intuitive clusters of end points emerge (Figure 5). This can be used to select molecular properties correlated with a given in vitro or in vivo end point.

To examine the impact of common functional group substitutions on in vivo properties, we identified 16868 pairs of compounds from the same chemical series differing by one group using MIMIC.[25] This includes both terminal substitutions (e.g., −F to −Cl) and linker substitutions (e.g., replacing a bridging phenyl with pyridine) and yields 11218 unique substitutions. The majority of these substitutions are uncommon and not amenable to statistical analysis: 77.6% are exemplified only once, and a further 19.8% are exemplified 2−4 times. We focused on 20 substitutions exemplified 9 or more times among compounds with measured in vivo $CL_u$ and $V_d$ (Table 7). Addition of halides and alkyl groups to aromatic carbons tend to modestly increase $CL_u$, $V_d$, and bioavailability (0.1 corresponds to a ∼25% increase). Substitutions to aromatic methoxy tend to decrease $CL_u$ and $V_d$; substitutions from aromatic Cl to $CF_3$ and $CF_3$ to $C(Me)_3$ tend to increase $CL_u$ and decrease $V_d$ and bioavailability. Aliphatic substitutions from methyl to ethyl to isopropyl increases $CL_u$ and $V_d$. Aliphatic substitutions from


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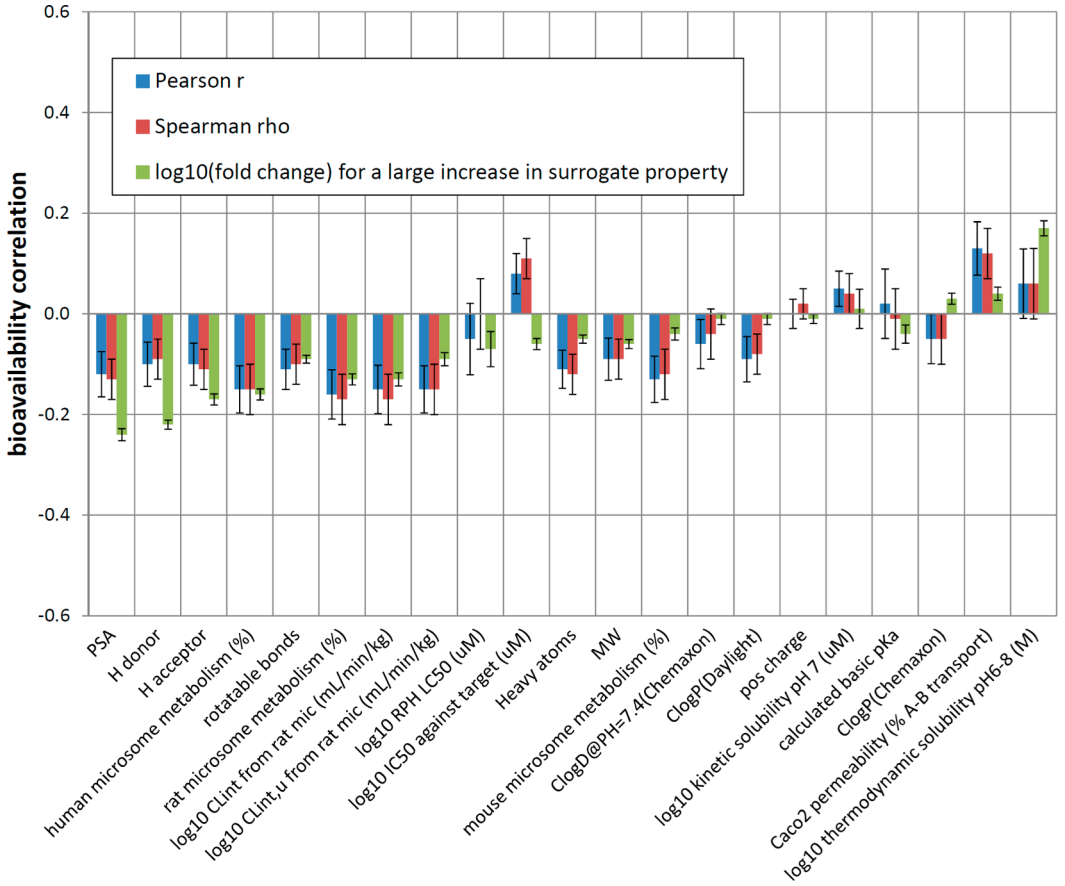


**Figure 3.** Relationship between ADMET surrogates (*X*-axis) and bioavailability from low-dose rat PK studies (*Y*-axis); Pearson *r* and Spearman $\rho$ denote the average Pearson *r* and Spearman $\rho$ correlation coefficients between each surrogate and log10(bioavailability) calculated across all chemical series having five or more compounds; log10(fold change) denotes the average difference in log10(bioavailability) for compound pairs from the same chemical series that exhibit a large difference in a given surrogate end point; see Figure 1 and the results text for details.

hydroxyl to methoxy or secondary to tertiary amines increase $CL_u$. Most common substitutions have very modest effects on these properties, comparable in magnitude to the average change in pharmacological activity from the substitution: the proportion of top 20 substitutions inducing a >10-fold change for most in vivo or in vitro end points ranges from 0.5% (RPH $LC_{50}$) to 8% ($CL_{int,u}$), not substantially different from the proportion inducing a >10-fold change in pharmacological activity (4%). The exceptions are thermodynamic solubility (14%) and LOAEL (21%), although the latter is based on only 24 substitutions. More interesting chemical substitutions emerge when examining those associated with large changes (Scheme 1), but their lower frequency prevents more rigorous statistical analysis.

## DISCUSSION AND CONCLUSIONS

We have examined the utility of various molecular properties or in vitro surrogate assays for predicting in vivo ADMET properties of compounds using a large data set generated over several years of lead optimization at Lilly. Studies of this nature have been previously published, and certain global property trends are not observed within chemical series (e.g., decreasing bioavailability or decreasing solubility vs increasing molecular weight,[8] increasing ClogP, and decreasing PSA vs toxicology outcomes[4]). We believe this to be caused by the "clumpy" nature of such data sets, characterized by many close analogues within distinct SAR series. Comparing within-series vs global property trends for our results reveals greater discrepancy for the toxicology study data than the pharmacokinetics (PK) data (Supporting Information Figure 6). The former is significantly more "clumpy" due to the focused nature of the SAR when routine in vivo toxicology work begins, whereas characterization of the PK properties of different chemotypes and/or significant SAR exploration at the early stages of a program is common. The nature of these data sets will be further biased by varying interest in target classes over time because different target classes tend to bind molecules with distinct molecular properties.[26] For example, a data set dominated by biogenic amine G-protein coupled receptors and nuclear receptors will be populated by small cationic molecules and large lipophilic molecules. Resolving the role of molecular weight or lipophilicity via standard approaches (*t* tests, ANOVA, etc.) assumes that the other property (and other covariates) have similar means in each group.

Our approach does not resolve causation among multiple end points that are difficult to modulate independently in lead optimization (e.g., changes in $V_d$ and $CL_u$, both of which are predictive for LOAEL). It may be argued that distinguishing cause vs correlation is unnecessary. This is only true if both properties cannot be varied independently. A role in predicting solubility ascribed to molecular weight might in fact be related to lipophilicity. Such trends may appear robust: an analysis of molecular weight vs solubility across our data set suggests that


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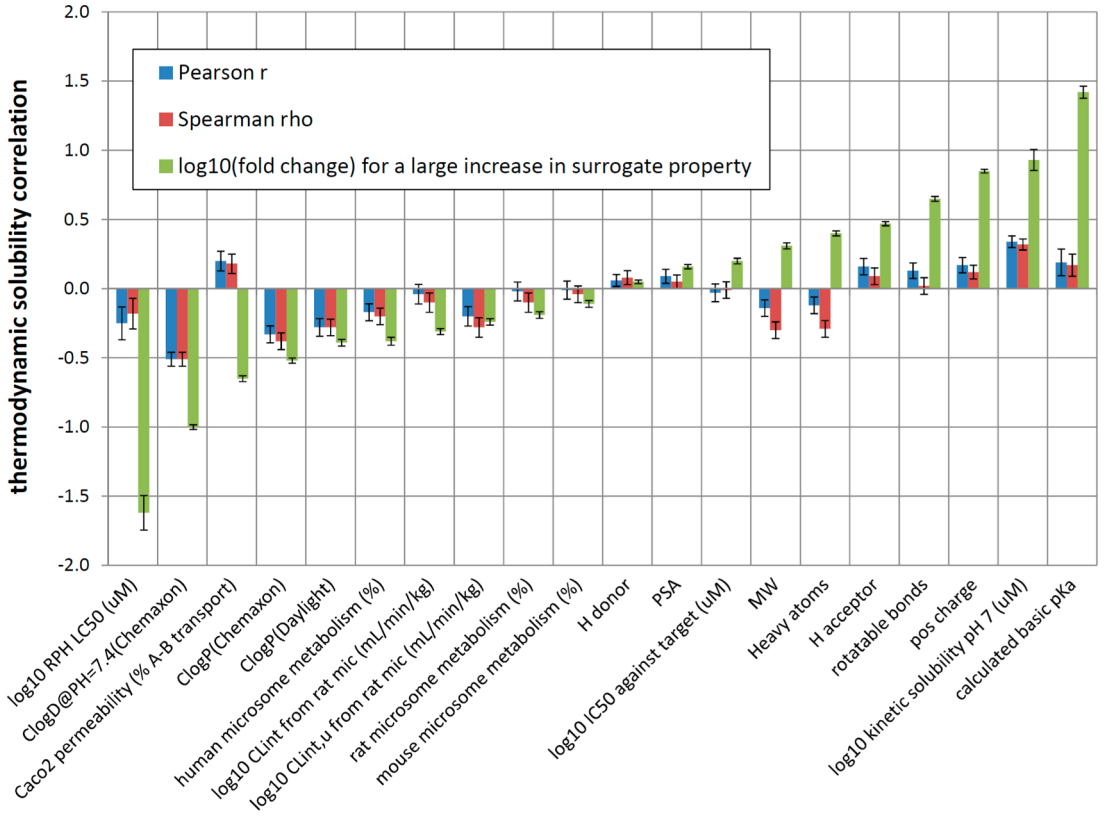


**Figure 4.** Relationship between ADMET surrogates (*X*-axis) and thermodynamic solubility (TS; *Y*-axis); Pearson $r$ and Spearman $\rho$ denote the average Pearson $r$ and Spearman $\rho$ correlation coefficients between each surrogate and log10(TS) calculated across all chemical series having five or more compounds; log10(fold change) denotes the average difference in log10(TS) for compound pairs from the same chemical series that exhibit a large difference in a given surrogate end point; see Figure 1 and the results text for details.

**Table 6. Average (Standard Error) log10 Thermodynamic Solubility Change for a Given Change in ClogD and Kinetic Solubility**

| | kinetic solubility pH 7-fold change | | | | |
|---|---|---|---|---|---|
| ClogD change | decrease >5 | decrease 2−5 | decrease 1−2 | increase 1−2 | increase >2 |
| 0−0.9 | −0.82 (0.18) | −1.01 (0.09) | −0.28 (0.06) | −0.08 (0.06) | 0.50 (0.11) |
| 0.9−1.7 | −1.19 (0.15) | −1.39 (0.10) | −0.72 (0.07) | −0.19 (0.08) | 0.53 (0.26) |
| >1.7 | −0.99 (0.11) | −1.81 (0.07) | −0.98 (0.10) | −0.48 (0.14) | 0.10 (0.40) |

molecular weight <300 Da confers higher solubility than 300−500 Da (Supporting Information Figure 2). A closer examination reveals that the average ClogD in each group is 0.1 and 2.4, respectively, a very large difference. As we showed by analyzing trends within chemical series, decreasing molecular weight while keeping ClogD constant actually decreases solubility slightly. In a similar vein, the commonly used analysis of ranges (i.e., "binning") applied to our entire data set of toxicology outcomes supports using Caco-2 permeability as a toxicology surrogate, yet the analysis of permeability changes within chemical series shows that it has no utility in optimizing toxicology properties in a medicinal chemistry program. In practical terms, we suggest validating the utility of global property trends within chemical series before applying them broadly in lead optimization.

The utility of surrogate systems such as LDH leakage[19,20] or microsome stability[17,18] for rank-ordering marketed drugs according to their in vivo toxicity and PK properties is well established. This study corroborates their robustness for selecting the most promising compounds within chemical series and provides a reference point when considering predictive accuracy vs the intuitive nature of molecular properties in compound design. Functional group substitutions that lead to large changes in these properties can be used to suggest structural modifications, but this approach is hampered by the need to have many examples of a substitution in different series to have confidence in the effect. Another approach replaces in vitro assay results with in silico predictions when large in vitro surrogate data sets are available for quantitative structure−activity relationship (QSAR) model development.

The results in Tables 4−6 provide guidelines for lead optimization programs, where in vivo ADMET results and surrogates are available for one or more compounds. In lead generation, one must usually interpret surrogates in the absence of in vivo study results when selecting scaffolds for further study. This analysis does not yield the probability of observing a certain in vivo outcome given the absolute values of the surrogates (e.g., what is the probability of high $CL_u$ given the measured rat microsome stability and ClogP of a molecule?). One possible approach is to select the


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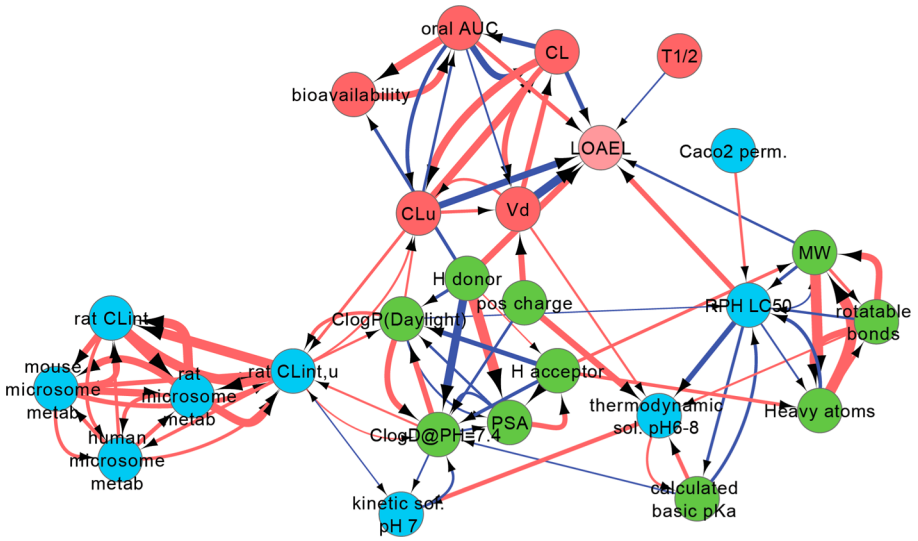


**Figure 5.** Network representation of relationships between in vivo (red nodes), in vitro (blue nodes), and molecular properties (green nodes), with positive/negative correlations between surrogate vs target pairs denoted with red/blue edges. Edges denote property pairs for which large changes in the surrogate property explain 40% or more of large changes in the target property (width proportional to strength of relationship). The pairs analysis used in Figures 1−4 was repeated using each property as the target (*Y*-axis), and all other properties as potential surrogates (*X*-axis). The *Y*-axis scale is normalized in the 0−100 range using the target property as the surrogate for explaining itself (i.e., division by values in diagonal for Supporting Information Table 4).

**Table 7. Average and Standard Error of log10(fold change) for the 20 Most Common Substitutions**

| substitution | $CL_u$ | $V_d$ | bioavailability | $IC_{50}$ against target | $CL_u$ and $V_d$ pairs[b] | bioavailability pairs[b] |
|---|---|---|---|---|---|---|
| H → F | 0.11 (0.04) | −0.03 (0.04) | 0.1 (0.07) | 0.06 (0.04) | 96 | 65 |
| H → Cl | 0.00 (0.12) | 0.09 (0.07) | 0.12 (0.14) | 0.07 (0.07) | 28 | 14 |
| F → Cl | 0.13 (0.05) | 0.04 (0.05) | 0.20 (0.10) | 0.08 (0.05) | 42 | 24 |
| H → Me | 0.12 (0.09) | 0.09 (0.07) | 0.07 (0.10) | −0.08 (0.07) | 38 | 18 |
| Me → Et | 0.21 (0.10) | 0.04 (0.09) | −0.22 (0.12) | 0.02 (0.08) | 15 | 9 |
| iPr → cycloPr | −0.37 (0.08) | 0.31 (0.10) | 0.19 (0.10) | −0.07 (0.07) | 9 | 6 |
| Me → F | −0.24 (0.09) | −0.06 (0.08) | 0.11 (0.15) | 0.03 (0.08) | 23 | 15 |
| Me → Cl | −0.24 (0.10) | 0.24 (0.08) | 0.42 (0.15) | −0.12 (0.07) | 22 | 8 |
| H → OMe | 0.12 (0.12) | 0.17 (0.15) | −0.15 (0.09) | 0.06 (0.10) | 13 | 11 |
| Me → OMe | −0.24 (0.11) | −0.09 (0.12) | 0.1 (0.15) | 0.10 (0.12) | 10 | 8 |
| F → OMe | −0.16 (0.12) | −0.44 (0.11) | 0.05 (0.11) | −0.01 (0.13) | 13 | 11 |
| Cl → OMe | −0.15 (0.1) | −0.26 (0.10) | −0.05 (0.18) | −0.06 (0.12) | 15 | 7 |
| Cl → $CF_3$ | 0.13 (0.13) | −0.06 (0.14) | −0.04 (0.10) | 0.15 (0.09) | 13 | 7 |
| $CF_3$ → $C(Me)_3$ | 0.28 (0.16) | −0.07 (0.16) | −0.48 (0.23) | 0.01 (0.06) | 12 | 5 |
| OH → OMe[a] | 0.45 (0.14) | −0.36 (0.12) | −0.36 (0.26) | −0.32 (0.08) | 10 | 6 |
| Me → Et[a] | 0.07 (0.09) | 0.1 (0.08) | −0.19 (0.07) | −0.06 (0.09) | 13 | 12 |
| Me → iPr[a] | 0.39 (0.18) | 0.02 (0.19) | −0.13 (0.18) | 0.06 (0.10) | 9 | 9 |
| Et → iPr[a] | 0.57 (0.12) | 0.27 (0.13) | −0.11 (0.07) | −0.03 (0.09) | 13 | 8 |
| $NH(Me)$ → $N(Me)_2$[a] | 0.25 (0.13) | 0.11 (0.07) | 0.33 (0.26) | 0.21 (0.06) | 9 | 6 |
| $N(Me)_2$ → morpholino[a] | −0.13 (0.13) | 0.55 (0.12) | 0.1 (0.25) | −0.17 (0.08) | 11 | 9 |

[a]Substitutions on aliphatic carbons; other substitutions on aromatic carbons. [b]The number of pairs where both compounds have the property defined and differ only by the given substitution; LOAEL statistics not presented due to inadequate representation (<9 times) for all substitutions except H → F.

surrogates predictive *within* chemical series with the expectation that they are predictive *across* series and analyze absolute value ranges in the same manner reported by Hughes et al. and Gleeson[4,8] (Supporting Information Tables 5,6). This work supports the predictive utility of simple properties like ClogP (for $CL_u$, thermodynamic solubility) and molecular weight and hydrogen bond donors (for LOAEL) but argues against a rigid implementation of the rule of 5[3] and its more stringent derivates[10,27,28] that ignores other surrogates with higher predictive utility for ADMET outcomes.

## EXPERIMENTAL SECTION

**Defining Chemical Scaffolds.** We identified 3816 compounds evaluated in standard low-dose rodent pharmacokinetic and/or acute toxicology studies, targeting 99 proteins from eight gene families. Then 3773 compounds were assigned to 173 chemical series (having two or more compounds) by structure clustering with an in-house cheminformatics application followed by manual review and adjustments, grouping together scaffolds where their relationship is easily recognized (e.g., permutations of heteroatoms within rings, ring enlargement, etc.). While distinguishing scaffolds can be subjective in general, our data set minimizes ambiguities because


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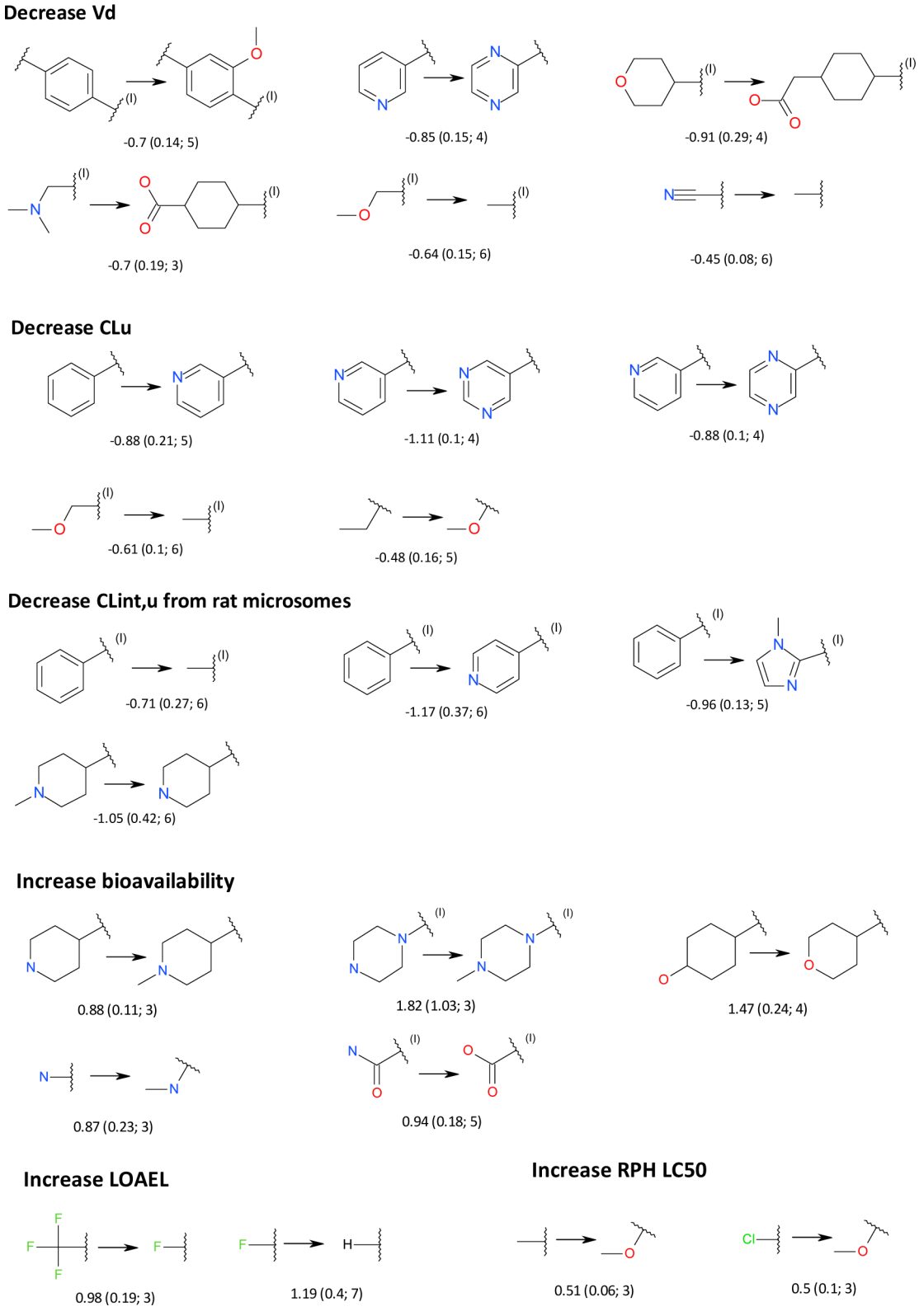
**Scheme 1. Chemical substitutions Associated with Large Changes in Vitro and in Vivo Properties**[a]




[a]The following notation denotes the effect on log10(fold change) for the given substitution: average (standard error; *N*).

chemical optimization of pharmacological activity before the use of in vivo ADME studies results in chemical series with a well-defined core.

**Computed Molecular Property Calculations.** The ChemAxon toolkit version 5.1.7 was used to calculate basic p$K_a$, ClogP and ClogD. Likewise, Biobyte 4.3 was used to calculate Daylight/Biobyte ClogP. The topological polar surface area was calculated with an in-house implementation of the method described by Ertl et al.[22] Counts of hydrogen bond donors, acceptors, and rotatable bonds were calculated as described by Veber et al.[5] Positive charge was calculated by summing the number of occurrences for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each of the following functional groups: aliphatic amine, 4-aminopyridine, amidine, guanidine, imine, imide; only one basic center is assigned for aliphatic amines separated by one or two methylene groups.

**In Vivo Studies.** All studies adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and were performed with approval from the Lilly Animal Care and Use Committee. Standard low-dose pharmacokinetic (PK) studies employed single oral doses of 3, 5, or 10 mg/kg and single iv doses of 1 or 3 mg/kg administered to male rats; Sprague–Dawley CD IGS rats were used for 95% of compounds and Fischer 344 or Lewis rats for the remainder. While compound formulation varied, 40% of iv doses were administered in captisol 20% w/v buffered to pH 2 with 25 mM NaPO4 buffer; 70% of oral doses were administered as suspensions in 1% carboxymethylcellulose or hydroxyethylcellulose w/v with 0.25% polysorbate 80 v/v and 0.05% antifoam v/v in purified water. Blood samples were obtained via tail bleeds or cannula at 0, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h postdose and compound levels analyzed by LC/MS/MS. Pharmacokinetic parameters were derived by fitting the concentration vs time profile, and geometric means were calculated for three animals in the dose group. Commonly used study protocols at Lilly have been described elsewhere.[29] Although in vivo study protocols are variable across discovery projects, they are conserved within a project to allow the reliable comparison of compounds. Compounds belonging to the same chemical series mostly originate from a single discovery effort, minimizing the influence of protocol variation in our analysis.

Toxicology study data in our analysis originates from 4–14 day multidose studies using oral administration to Sprague–Dawley CD IGS rats, usually with three female rats per dose.[30] A corresponding toxicokinetic study (in different animals) was conducted using the same study protocols with the purpose of determining associated compound plasma levels. To simplify the analysis, we identified the lowest dose (if any) that results in animal death or prescheduled sacrifice due to poor condition or adverse histological changes in one or more animals when examining tissue from adrenal glands, heart, ileum, jejunum, kidneys, liver, lung, pancreas, spleen, or stomach. For this work, we define an adverse change as one that would present a significant concern when observed in the absence of other histological findings in the tissue. For example, we classify liver necrosis as adverse and liver vacuolation (a possible indicator of phospholipidosis) as nonadverse. A complete list of histological changes considered adverse is provided in the Supporting Information Table 1. For a dose to be classified as nonadverse, all animals in the group must survive until scheduled necropsy and present no adverse histology findings in the 10 tissues noted above. The lowest observed adverse effect level (LOAEL) of each compound is obtained from the TK study by reporting the $C_{max}$ (total) of plasma concentration of the dosed compound corresponding to the lowest toxic dose.

**Statistical Analysis.** Computer code in perl is provided in Supporting Information to calculate Pearson and Spearman correlations within series and to identify pairs in the last quartile for each surrogate. A number of end points can produce qualified results (e.g., RPH $LC_{50}$ > 100 $\mu$M; see Table 1), which limited our ability to compare compounds. For the within-series analysis, pairs where both compounds have qualified results were excluded; pairs where one value is qualified and the other is defined and 3-fold larger or smaller than the qualified result were included in the analysis, and the qualifier was ignored (e.g., $LC_{50}$ > 100 $\mu$M and $LC_{50}$ = 30 $\mu$M values are separated by >3-fold, and the value 3.3 is used in the analysis). For in vivo and in vitro results evaluated in multiple experiments, the average value was used (after log10 transformation where applicable). Averages and standard errors were calculated in TIBCO Spotfire version 3.1. The complete data set used for analysis is provided in Supporting Information.

The network analysis for property–property relationships was created with Cytoscape v. 2.8.3 (www.cytoscape.org) using weighted spring embedding and small manual adjustments.

## ■ ASSOCIATED CONTENT

### Supporting Information

Adverse histology findings, tables, figures, computer code and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

A special waiver on disclosing the structures of compounds was requested and granted by the editors.

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

RPH, rat primary hepatocyte; $V_d$, apparent volume of distribution; ANOVA, analysis of variance; GLP, Good Laboratory Practice; CL, clearance; $CL_u$, unbound clearance; LDH, lactate dehydrogenase; $C_{max}$, maximal plasma concentration; LOAEL, lowest observed adverse effect level; TS, thermodynamic solubility; TK, toxicokinetic

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