

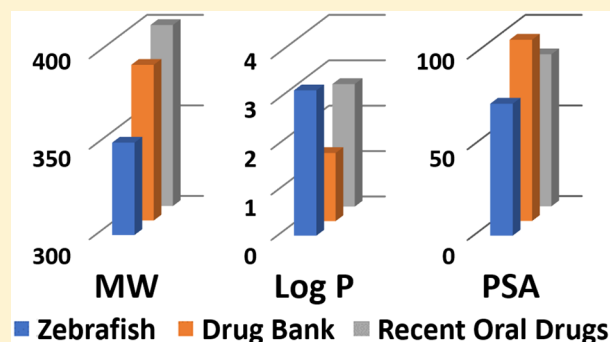
Do Zebrafish Obey Lipinski Rules?

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Supporting Information

ABSTRACT: The use of zebrafish in whole organism phenotypic assays has become a valuable strategy throughout the drug discovery process. Zebrafish assays can be used not only to screen libraries of compounds at the earliest stages but also to evaluate advanced leads for their effects on specific biological pathways or for toxicity. However, when confronted with inactivity of a compound in a zebrafish assay, there are little data that can be used to judge if the compound is truly biologically inert or inactive due to a lack of permeability into the model organism. While medicinal chemistry principles suggest parameters that are predictive of human oral bioavailability, cellular permeability, and even bacterial permeability, there have been no such parameters developed for zebrafish absorption. To address this question, we compiled a set of 700 compounds reported in the literature to be active in zebrafish assays, evaluated their properties, and compared them to properties derived from a set of historical drugs and a set of recently approved oral drugs. While some properties overlap, the averages and 10th and 90th percentiles of molecular weight, octanol–water partition coefficient (logP), H-bond counts, and polar surface area for zebrafish-active compounds are statistically different from those of known drugs. This analysis should be useful to scientists interpreting structure–activity relationships based on data from zebrafish assays and help to inform the translation from fish to mammals.

KEYWORDS: Zebrafish, physicochemical properties, drug-like properties, permeability, Lipinski Rule-of-Five, phenotypic assays



Whole organism phenotypic screening has become an important complement to more traditional target-based and cell-based assays throughout the drug discovery process.¹ Benefits include the ability to evaluate multiple parameters, such as biological effects, permeability, and toxicity, in a single assay. Zebrafish (*Danio rerio*), in particular, have been widely used in this regard. Their small size, transparent embryos, ease and low cost of breeding, rapid development, evolutionary similarity to humans, and well understood genetics make zebrafish a powerful model organism for screening of small molecules, and for evaluating advanced leads for effects in a biological pathway^{2–5} or for toxicity.⁶ Indeed, zebrafish have been used as models for a variety of disease states including kidney disease,⁷ cancer,⁸ and central nervous system disorders,⁹ among others.

In most cases, small molecules are dispensed in the fish water, offering continuous exposure of the zebrafish to drug substance.¹⁰ Small molecules can also be directly injected into the fish to ensure exposure, but this approach is not common, particularly in a screening paradigm.⁴ When dispensed in tank water, compound absorption occurs either through permeation of the skin layer, transport through gills, or, when using embryos, via absorption through the yolk sac. After treatment, effects are monitored and/or quantified. When no effects are

observed, conclusions are either (1) the compound was absorbed and had no effect on the biological system or the organism (inactive) or (2) the compound was not absorbed by the fish, and therefore, conclusions about its effects on the biological target or the organism cannot be made. The ability to make this distinction can have important consequences; for example, when zebrafish assays are used to assess safety, and lack of permeability could be incorrectly interpreted as lack of toxicity.

There is an abundance of literature on which physical properties of small molecules predict human oral bioavailability. Lipinski and co-workers correlated preferred values for molecular weight ($MW \leq 500$), lipophilicity as measured by octanol–water partition coefficients ($\log P \leq 5$), and the numbers of hydrogen bond donors ($HBD \leq 5$) and acceptors ($HBA \leq 10$) to oral bioavailability (Lipinski Rules, Rule of Five¹¹). Veber et al. suggested that total polar surface area ($tPSA \leq 140 \text{ \AA}^2$) of the molecule and the number of rotatable bonds (≤ 10) could predict oral bioavailability.¹² Others have described characteristics of molecules that are important for

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cell permeability¹³ and even bacterial permeability.¹⁴ However, there is little that correlates physical properties such as molecular weight, lipophilicity, and solubility to zebrafish absorption. In this absence, the assumption is usually made that the properties that are predictive of human/mammalian absorption will translate to fish; moreover, since the ultimate goal is to treat mammals, not fish, designing for human oral bioavailability is appropriate. However, significant physiological differences between fish and mammals raise questions about the validity of this assumption, and the absence of data may lead to incorrect conclusions about whether a compound is truly biologically inert or did not permeate the fish, thereby complicating any structure–activity relationship (SAR) analysis or potentially leading to false conclusions about the toxicity of small molecules.

To address this knowledge gap, we assembled structures of compounds reported in the literature to exhibit biological activity in zebrafish assays and made the assumption that biological activity necessitated permeability/absorption into this model organism (whether it be passive or active). We then calculated and predicted a series of properties and property descriptors and asked (1) what is the range of drug-like properties [e.g., molecular weight (MW), logP, polar surface area (PSA), number of rotatable bonds, H-bond donors (HBD), H-bond acceptors (HBA), solubility, and permeability] exhibited by compounds that are absorbed by zebrafish?; (2) do the general characteristics of zebrafish-active compounds differ from the standard physicochemical property guidelines for oral bioavailability used by medicinal chemists or from those of known drugs?; and (3) can we develop a set of guidelines that can be used to interpret zebrafish assay data? This report details our analysis.

To compile a set of zebrafish-permeable compounds, we used keywords such as “zebrafish assay” and “zebrafish screen” to search the Scifinder and Pubmed databases for publications reporting data on small molecules’ activity in zebrafish assays. We also directly searched medicinal chemistry and chemical biology journals such as *ACS Chemical Biology*, *ACS Medicinal Chemistry Letters*, *Bioorganic & Medicinal Chemistry*, *Bioorganic & Medicinal Chemistry Letters*, *European Journal of Medicinal Chemistry*, *Journal of Medicinal Chemistry*, and *MedChemComm*. In total, 158 unique publications were identified, and the structures of active compounds were extracted from them. To supplement the literature data set, we reviewed Pubchem for data reported in zebrafish assays. We eliminated assays designated as “screening” as they typically report high false positive rates. Thirty-one compounds labeled as “active” in four different confirmatory or secondary assays (AIDs 691, 1137, 504373, 652284) were added to the data set. While it is likely that some publications were not captured, we believe the number and diversity of compounds identified (*vide infra*) provide a representative data set. Figure 1 outlines the data compilation and curation process.

Structures of compounds, administered in tank water, that were reported to be active in a zebrafish embryo assay, regardless of potency, degree of efficacy, or time of exposure were collected. We eliminated structures that contained metals or were large peptides or proteins, and those that bypassed the absorption process by administration via injection or other direct method. Structures were prepared as SMILES strings (simplified molecular-input line-entry system); salt counterions and any duplicates were removed. Applying these criteria, 700 unique small molecules (“zf 700”) were identified (see SI).

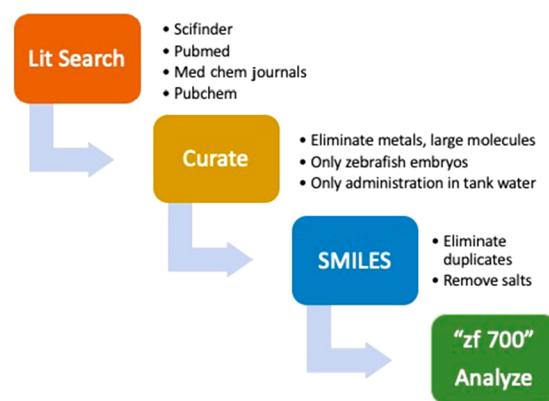


Figure 1. Process for data accumulation and curation.

We calculated MW, logP (as AlogP), HBD, HBA, number of rotatable bonds, and molecular PSA.¹⁵ Table 1 shows the range and averages for these properties for the zf 700. Not unexpectedly, the averages all fall within the typical Lipinski/ Veber guidelines: MW = 351; AlogP = 3.2; HBD = 1.4; HBA = 4.1; rotatable bonds = 4.4; and tPSA = 72.8 Å. Notable, however, is the wide range of values that appear to be compatible with zebrafish absorption, particularly molecular weight (110 to 1291 Da), AlogP (−14.0 to +7.9), and tPSA (0 to 551 Å). Other properties evaluated, including HBD, HBA, and rotatable bonds, also reveal a very wide range associated with zebrafish active compounds. While some of the extreme values are represented only sparsely, this data suggests that compounds with properties far outside what is typically considered drug-like can, in some cases, be permeable to zebrafish.

To ensure that disproportionate representation of multiple close structural analogs in the zf 700 set were not skewing the averages, we used a computational algorithm (“Affinity Propagation Clustering”¹⁶) that clustered the 700 based on structural similarity. This algorithm identified 87 exemplars (see SI), representative of the structural diversity of the entire set, for which we calculated the same values for comparison with the full zf 700 set. An independent evaluation confirmed the diversity of this Exemplar 87 set (SI). As seen in Table 1, even though the ranges are narrower, the averages calculated for the 87 exemplars is, in most cases, very similar to those generated from the full set of 700. This analysis provides evidence that the presence of large numbers of closely related compounds in the zf 700 set did not bias the calculated averages.

We calculated the 10th and 90th percentiles of the values in Table 1 for the zf 700 set to interrogate the boundaries that can be applied to predict zebrafish absorption. For comparison, we also completed a similar analysis of a set of historical small molecule drugs (approved and experimental, regardless of route of administration) derived from DrugBank ($n = 1395$)¹⁷ and a set of >400 oral drugs approved by the FDA in a recent 20-year (1998–2017) span.¹⁸ This data set (Table 1) highlights the differences between the characteristics of the majority of zebrafish-permeable compounds and characteristics of approved drugs, and by extension the properties medicinal chemists typically strive for when developing drug-like and/or orally available molecules. Inclusion of the 1998–2017 set allows for specific comparison with orally administered drugs, and also takes into account some of the considerable debate in the literature on which properties reflect “drug-likeness.”¹⁸

Table 1. Calculated Properties of Zebrafish Active, Small Molecule Drugs, and Recently Approved Oral Drugs^a

	zf 700 Average	zf 700 Range	Exemplar Average Range (n=87)	zf 700 10 th percentile	zf 700 90 th percentile	Small Molecule Drugs ¹⁷ Average 10 th – 90 th percentiles (n=1395)	FDA Approved Oral NCEs 1998–2017 ¹⁸ Average 10 th –90 th percentiles (n=409)
Molecular Weight	351	110 – 1291	365 129 – 958	206	489	386* 182* – 599*	400* 213* – 571*
AlogP	3.2	-14.0 – +7.9	3.3 -2.2 – +7.8	1.3	5.3	1.5* -2.3* – +4.9	2.7* -0.1* – +4.9*#
H-Bond Donor	1.4	0 – 13	1.5 0 – 8	0	3	2.2* 0* – 4*	2.0* 0* – 4*
H-Bond Acceptor	4.1	0 – 23	4.5 0 – 14	1	7	5.1* 1 – 10*	6.4* 2 – 10.2*
Rotatable bonds #	4.4	0 – 35	5.0 0 – 23	0	9	6.1* 1* – 12*	6.5* 2* – 11*
Molecular Polar Surface Area (Å)	72.8	0 – 551	74.8 0 – 208	20.2	123.5	100.6* 32.0* – 186.3*	88.1* 33.9* – 144.1*

^aSmall molecule drugs derived from DrugBank;¹⁷ 1998–2017 values adapted from Table 5 in ref 18; *p* values for means based on one-way ANOVA using GraphPad Prism7 software and posthoc analysis using Tukey's multiple comparison test; *p* values for 10th and 90th percentile values are based on nonparametric Wilcoxon signed-rank test and are relative to zf 700; *p* < 0.05 considered significant, indicated with*; #AlogP value for 1998–2017 drug set based on Stardrop clogP.

The majority of zebrafish-permeable compounds fall within the molecular weight range of 200–500 Da, with an average MW of 351. These numbers are shifted to lower values compared to both drug groups, and by ~50 Da compared to the recent oral drug set. This shift is most apparent at the higher end of the molecular weight range with the 90th percentile of the zf 700 set (489 Da) being ~100 Da lower than the two drug sets (599 and 571 Da), but the 10th percentiles being very close. The molecular weight differences reached statistical significance for all values.

Also divergent are the AlogP values. The zf 700 average (3.2) is significantly higher than the two drug groups (1.5 and 2.7 for the small molecule and recent oral drug sets, respectively), tending toward greater lipophilicity. The 90th percentile for the zf 700 is also greater than the same values for the historical and recent oral drugs (5.3 vs 4.9 for both drug sets); however, for the 90th percentiles, only the difference between the zebrafish and recent oral drug set was significant. This preference for higher lipophilicity could be attributed to zebrafish-active molecules being predominantly absorbed through the yolk sac, which contains high concentrations of lipids.

In accordance with the higher values for AlogP, the HBD and HBA counts for the zf 700 are also shifted toward more lipophilic properties. The 90th percentiles for HBD and HBA are 3 and 7, respectively, with averages of 1.4 (HBD) and 4.1 (HBA) vs the more typical limits (90th percentile) for HBD of 4 and HBA of 10 for both the small molecule and recent oral drugs. The averages for the two drug sets (HBD, 2 for both; HBA, 5.1 and 6.4 for DrugBank and recent oral drugs, respectively) were significantly higher than the zf 700 averages. Consistent with the trend toward more lipophilic characteristics, the average PSA of the zebrafish active set was 72.8 Å,

and its 90th percentile equaled 123 Å; both significantly lower than the two drug categories. The rotatable bond count average (4.4) is also lower in the zf 700 set compared to the drug sets (6.1 and 6.5), as are the 10th and 90th percentile values.

Overall, zebrafish-absorbed molecules tend to be more lipophilic than known drugs, and in a most cases, their physicochemical properties fall within a narrower range of values. Based on this analysis, in particular the 90th percentiles (which were loosely applied to derive the Lipinski rules), we suggest that compounds most likely to be absorbed by zebrafish will have the following characteristics:

- MW ≤ 500
- clogP ≤ 5.3
- HBD ≤ 3
- HBA ≤ 7
- tPSA ≤ 124 Å
- rotatable bonds ≤ 9

To develop a further understanding of the characteristics of zebrafish-permeable compounds, we predicted¹⁹ additional properties such as solubility, various cellular permeability rates, and number of predicted metabolites. Table 2 shows those results and a comparison to the DrugBank drug set.¹⁷ Of note, the same analysis with the exemplar set was, again, consistent with the full set of 700 compounds (data not shown). Solubility trended toward lower values (log *S* average = -4.6 M) compared to typical goals of log *S* > -4.2 M²⁰ and the drug set (log *S* = -3.7 M), perhaps confirming the shift toward greater lipophilicity. This result was somewhat unexpected given that the zebrafish actives were administered in tank water, which requires aqueous solubility at relatively high concentrations (typically ≥10 μM) with low concentrations

Table 2. Predicted Properties of zf 700 and Small Molecule Drugs

Property	zf700	Small Molecule Drugs ¹⁷
<i>Recommended Value</i>	Average (10th – 90th percentile)	Average (10th – 90th percentile)
Solubility^a (M) -6.5 – +0.5	-4.6 (-7.1 – -2.3)	-3.7* (-6.6* – -0.6*)
Caco-2 Permeability^a (nm/sec) <25=poor;>500=high	1885 (99 – 4886)	772* (3* – 2164*)
MDCK Permeability^a (nm/sec) <25=poor;>500=high	1485 (56 – 5229)	819* (2* – 1970*)
Number Metabolites^a [1-8]	3.4 (1 – 7)	4.2 (1 – 8)
MetStabOn²²	Low: 0 Medium: 691 High: 9	

^aValues predicted for 684/700 compounds; *p* values for means based on one-way ANOVA using GraphPad Prism7 software and posthoc analysis using Tukey's multiple comparison test; 10th and 90th percentiles calculated using R. *p* values for 10th and 90th percentile values are based on nonparametric Wilcoxon signed-rank test and are relative to zf 700; *p* < 0.05 considered significant, indicated with *.

(typically <0.5% or less) of cosolvents (e.g., DMSO, EtOH, MeOH) present.¹⁰

Averages for cellular permeability predictions (Caco-2 and MDCK cell lines) were far above the >500 nm/s value that is generally characterized as “excellent”, as were the averages calculated for the small molecule drug set. Of interest, however, the 10th percentiles for both cell lines for the zf 700 set were also well above the “poor” boundary of <25 nm/s, with values equal to 99 and 56 nm/s, respectively. This metric is at variance from the historic drug set, where the 10th percentiles for both Caco-2 (3 nm/s) and MDCK (2 nm/s) permeability fell into the “poor” descriptor. This suggestion that adequate compound permeability is more important for fish embryo absorption than mammalian cell absorption is not unexpected and could be due to a number of factors including differences between mammalian and fish membrane composition or in the activity of ABC efflux transporters.²¹ However, this analysis suggests that predicted permeability in either Caco-2 or MDCK cell lines may be useful in evaluating the potential of compounds to be absorbed into zebrafish, and that activity is unlikely to be observed with compounds predicted to be poorly permeable (i.e., <25 nm/s).

Finally, we considered whether zebrafish-actives would be distinguished from other sets by differences in predicted metabolism. Since the zebrafish yolk is highly metabolically active²² and assays are often run over the course of days, one could hypothesize that activity in zebrafish assays requires metabolic stability. The prediction of numbers of metabolites indicated that the zf 700 set (average = 3.4; 10th percentile =

1; 90th percentile = 7) fell within the recommended value of 1–8 and was only marginally different from values calculated for small molecule drugs (Table 2).

To evaluate this hypothesis further, we used the open source program MetStabOn²³ to predict human metabolic stability. Of the 700 zebrafish-active compounds, 0 were predicted to exhibit high metabolic stability, and 691 were predicted to have medium metabolic stability; low metabolic stability was predicted for only nine compounds. Based on this analysis, one can conclude that zf-active compounds, as a whole, are not exceptionally stable compared to typical drugs.

We believe this analysis should be useful when interpreting data from small molecule testing in zebrafish assays; however, there are some limitations to the data set. First, practical limitations in zebrafish testing/assay capacity and/or incorporation of zebrafish assays late in the drug discovery process may have resulted in compounds in the zf 700 data set being prechosen for good/excellent drug-like properties, rather than for a more diverse sampling. For example, when used for screening, libraries that very often contain known drugs¹⁰ or molecules that are more drug-like than lead-like are frequently used. In other instances, our literature search identified advanced compounds, already optimized for good drug-like properties, that were evaluated in zebrafish assays for specific effects, such as toxicity. In this way, compounds in the zf 700 data set may not represent the broadly diverse scaffolds that are common in more traditional HTS libraries. However, if a preselection of compounds had influenced the data, we would have expected no or minimal difference between the zf 700 set and the known drug sets, rather than the significant differences observed.

Second, our criteria for inclusion was binary (active/inactive) and considered neither potency nor degree of efficacy. While most compounds in the zf 700 set exhibited activity at concentrations of 10–20 μM, some were active at concentrations as high as 100 μM. Therefore, future studies should focus on how potencies correlate with properties. Third, due to a lack of data, we do not understand what precludes absorption into zebrafish and can only infer the properties that zebrafish active molecules possess.

Despite these caveats, the reported analyses should be of value to medicinal chemists who rely on SAR data from zebrafish assays to guide drug discovery efforts and translation into mammals. Importantly, our results provide guidelines to aid in determining which compounds are likely to be absorbed by zebrafish and, as a result, can subsequently be used to rationalize when an “inactive” report in a zebrafish assay should be attributed to a lack of biological activity versus a lack of permeability. The observation that zebrafish-actives display a different range of the properties typically considered drug-like should help focus medicinal chemistry design that relies on zebrafish assay data and also ensure that optimization of activity in fish can be translated to humans.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsmchemlett.9b00063](https://doi.org/10.1021/acsmchemlett.9b00063).

Methods (PDF)

Zf 700: identity, SMILES, references, calculated properties, QikProp predictive properties, statistical analysis (XLSX)

Eighty-seven exemplars: identity, SMILES, calculated properties, statistical analysis (XLSX)

DrugBank small molecules: identity, SMILES, calculated properties QikProp predictive properties (XLSX)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AlogP, atom based logP prediction; HBA, hydrogen bond donor; HBA, hydrogen bond acceptor; MDCK, Madin–Darby Canine Kidney cells; QPlogS, predicted aqueous solubility; QPPCaco, predicted apparent Caco-2 permeability; QPPMDCK, predicted apparent MDCK permeability; SMILES, simplified molecular-input line-entry system; PSA, polar surface area; zf, zebrafish

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