Hydrophobicity of HIV Protease Inhibitors by Immobilized Artificial Membrane Chromatography: Application and Significance to Drug Transport

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Purpose. The feasibility of using hydrophobicity measurements as screens for intracellular availability in T-cells or intestinal permeability in Caco-2 cells was examined.

Methods. *T-cell experiments*: Cells were counted, collected, then incubated with drug solution at 37°C. At selected time intervals, uptake was quenched by transferring a sample into oil, followed by rinsing, lysis of cells, protein precipitation and analysis by HPLC. *Caco-2 cell experiments*: Cells were grown on plastic dishes for 7–10 d, then rinsed and incubated with drug solution at 37°C. Uptake was quenched, cells were lysed, protein precipitated and drug was analyzed by HPLC. *IAM chromatography*: Stock solutions were injected onto an IAM column for HPLC. Mobile phase consisted of varying amounts of acetonitrile in buffer (pH 7.4). The capacity factor, k'_{IAM}, was calculated using citric acid to measure the void volume and was obtained by extrapolation to pure buffer.

Results. Nine HIV protease inhibitors were studied for uptake by CEM T-cell suspensions or Caco-2 cell monolayers. Capacity factors (log) between IAM and C-18 columns were positively correlated for this series. Caco-2 uptake rates correlated well with T-cell uptake rates when normalized by protein mass. Single-variable regression using IAM or C-18 columns was acceptable for analysis of T-cell data. Correlation coefficients between T-cell uptake and log k'_{IAM} or log $k'_{C\text{-}18}$ were not improved with multivariable regression. Correlation between Caco-2 uptake and log k'_{IAM} was enhanced when molecular weight and hydrogen-bonding potential were included in multivariable regression analysis (from r^2 of 0.39 to 0.91). Correlations obtained using log k'_{IAM}, log $k'_{C\text{-}18}$ or log distribution coefficient (log D) were comparable when regressed against Caco-2 uptake using this approach. Calculated log partition coefficient (ClogP) provided the poorest correlation in the multivariable analysis (r^2 = 0.57 for T-cell uptake and r^2 = 0.71 for Caco-2 cell uptake).

Conclusions. Uptake of HIV protease inhibitors by T-cell suspensions or Caco-2 cell monolayers was positively correlated. Uptake by T-cell suspensions was adequately described by hydrophobicity alone. Description of uptake by Caco-2 cell monolayers required multivariable regression analysis in which molecular weight and hydrogen bonding were included. Experimental measures of hydrophobicity (log k'_{IAM}, log k'_{C18} and log D) were superior to ClogP in the correlation analysis.

KEY WORDS: hydrophobicity; Caco-2 cell uptake; T-cell uptake; IAM; immobilized artificial membrane; HIV protease inhibitors.

INTRODUCTION

Hydrophobicity is inextricably linked to the transport of molecules across biological membranes. The optimal descriptors of hydrophobicity and membrane interaction have been the object of intense study on the basis of providing predictive values for passive, biological transport (1–6). Measurements of hydrophobicity have included partition and distribution coefficients using various organic phases, partitioning into liposomes, as well as chromatographic retention on reversed-phase HPLC columns. This work describes application of an Immobilized Artificial Membrane (IAM) column composed of phosphatidylcholine (PC) headgroup packing material to the determination of hydrophobicity and description of biological transport. This particular approach using IAM was developed by Pidgeon et al. (7) and includes both hydrophobic and electrostatic contributions to membrane interaction.

New elements and new needs are driving the investigation of hydrophobicity descriptors in pharmaceutical industry. These elements can be traced, not surprisingly, to the routine application of mass screening and combinatorial chemistry. These techniques in drug discovery have resulted in orders-of-magnitude increases in the numbers of compounds being presented for biopharmaceutical characterization. As a result, the ideal hydrophobicity measurement is not only accurate, descriptive and reproducible, but is also fast, facile, and amenable to automation.

To achieve therapeutic efficacy, drug discovery candidates in the HIV protease inhibitor area needed to overcome successive delivery barriers: absorption through the intestinal mucosa after oral administration and penetration into T lymphocytes to reach the site of action at the budding virion. Since transport was critical to the design and success of these agents, effort was directed at devising methods by which transport could be readily assessed. Several methods of hydrophobicity determination were investigated, including capacity factors on IAM and reversed-phase columns, distribution coefficients and calculated partition coefficients. These methods were examined for correlation with uptake by CEM T-cell suspensions and by Caco-2 cell monolayers.

METHODS

Materials

HIV protease inhibitors PD1 to PD10R were obtained from the Chemical & Biological Information Library at Parke-Davis Pharmaceutical Research (Ann Arbor MI). Structures of PD1 to PD10R are shown in Fig. 1. PD10R is QC.Asn.Phe ψ[CH(OH)CH_2N]-PIC.NHtBu where QC is quinoline-2-carbonyl and PIC is piperidine-2(S)-carbonyl (8).

Cell Culture

CEM-T4 lymphocytes were obtained from AIDS Research and Reference Reagent Catalog (#117) and were grown in

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ABBREVIATIONS: Capacity factor, k'; Immobilized Artificial Membrane, IAM; acetonitrile, CH_3CN.

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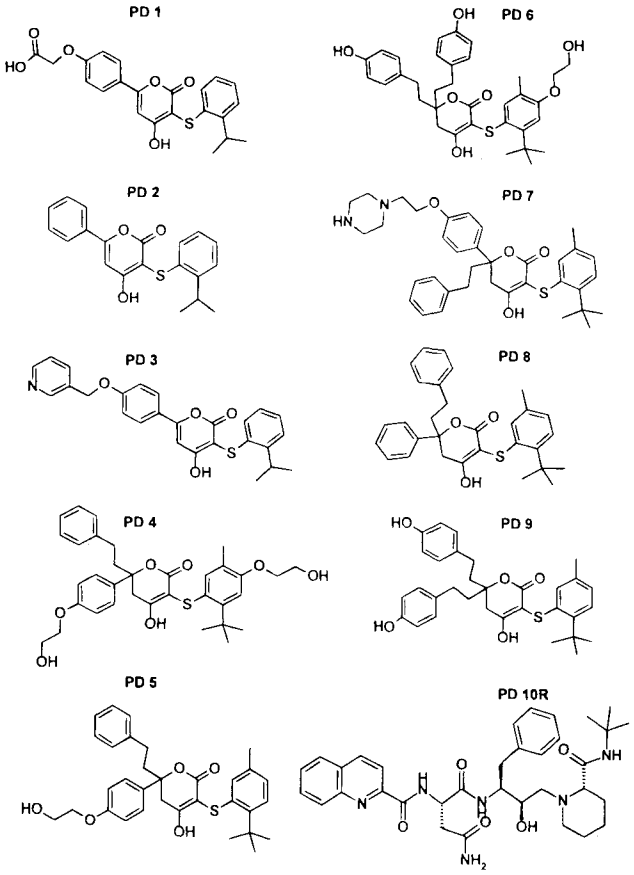

Fig. 1. Chemical structures of the pyrone-(PD 1-3), dihydropyrone-(PD4-9) and peptide-based (PD10R) HIV protease inhibitors.

suspension in media consisting of RPMI 1640 (Celox Corp. Hopkins MN), HEPES (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 ug/mL). Cell concentrations of 1 to 3 × 10^6 cell/mL were passed every 3 to 4 days at 1:20 dilution.

Caco-2 cells (passage numbers 36 to 46) were obtained from American Type Culture Collection (Rockville MD). Cells were seeded at 2.5 × 10^5 cells/well in 6-well plastic cluster trays and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis MO), L-glutamine (1%) and Minimum Essential Medium Non-Essential Amino Acids (1%, Gibco/BRL, Grand Island NY). Cells were confluent for use in uptake studies within seven to ten days.

Cellular Uptake Experiments

For T-cell uptake experiments, the T-cell concentration was measured and the cells were collected. Cell concentrations were adjusted to 4 to 10 × 10^7 cells/mL. After centrifugation at 1000 × g for 5 min, the growth medium was removed and cells were washed, then resuspended in MOPS buffer (3-[N-morpholino]propanesulfonic acid, pH 7.4). At time zero, the drug solution (1.35 mL) was mixed with the T-cell suspension (0.15 mL) and incubated at 37°C. Drug concentrations ranged from 0.5 to 43.7 μM, depending on the solubility of the drug. At selected time points out to 15 min, samples (0.20 mL) were removed from the incubation medium and spiked into a microcentrifuge tube containing 0.05 mL oil (dibutyl phthalate:dioctyl phthalate, 4:1) (9). Samples were spun immediately at 16,000 × g for 20 s. Drug solution and oil were carefully removed. The cell pellet was washed once with ice-cold MOPS, followed by centrifugation at 16,000 × g for 20 s. After removal of the supernatant, 0.05 mL water was added to the sample tube and it was sonicated for 2 to 30 min. Acetonitrile (0.05 mL) was added to precipitate proteins, then the sample was centrifuged for 20 min. The supernatant was transferred to an HPLC vial for analysis.

For Caco-2 cell uptake experiments, growth media was removed and the cell monolayer was rinsed with MOPS buffer, then incubated with drug solution in MOPS (pH 7.4) at 37°C. Multiple compounds were consistently incubated simultaneously in the Caco-2 uptake experiments. Total drug concentration was less than 50 μM. At selected time points out to 15 min, the incubation solution was removed, and the cells were washed with ice-cold MOPS buffer. After removal of the buffer, 0.5 mL water was added to each well and the cells were sonicated for 20 min. The resulting suspension was collected and an equal volume of CH_3CN was added, followed by centrifugation. The supernatant was collected for analysis by HPLC.

Uptake rates were calculated from the linear portion of the uptake curve. Uptake units were mole/min-10^6 cells or mole/min-mg protein, normalized to 0.100 mM incubation concentration of drug. Replicates of at least three were performed.

Reference Compounds

^{3}H-propranolol and an orally-active, peptide-based protease inhibitor, PD10R, were used as reference compounds. The radiolabelled propranolol was analyzed by liquid scintillation counting. PD10R was analyzed by the HPLC methods detailed below.

Capacity Factor (k′) Determination

Capacity factors were obtained on both an Immobilized Artificial Membrane. PhosphatidylCholine ($^{\delta G}$IAM.PC) column (10 cm × 4.6 mm, 5 μm particle size, Regis Chemical, Morton Grove, IL) and a Hypersil C18 column (10 cm × 2.1 mm, Hewlett Packard) using UV detection at 216 nm. Stock solutions were prepared in CH_3CN and injected onto the column. Mobile phase consisted of Dulbecco's phosphate-buffered saline (10 mM phosphate, pH 7.4) and CH_3CN. The high hydrophobicity of many of the compounds necessitated the use of CH_3CN as an organic modifier to elute the compounds in a timely manner. When this was the case, each compound was injected under isocratic conditions over a range of mobile phase compositions (eg, 15% CH_3CN:85% Dulbecco's to 25% CH_3CN:75% Dulbecco's) and capacity factors (k′) were determined:

$$k' = (t_R - t_0)/t_0$$

where t_R is the retention time of the test compound and t_0 was the retention of the void volume marker, citric acid. Capacity factors from four to five mobile phase compositions were used in plots of log capacity factor *versus* percent CH_3CN to calculate k′ by extrapolation in aqueous mobile phase, k'_{IAM} and k'_{C18}, on the respective columns. The r^2 values for all linear regression plots were > 0.98. Data were normalized for daily column performance using k′ for phenytoin.

Reversed-Phase HPLC Analysis

Two sets of chromatographic conditions were used. For analysis of samples from T-cell experiments, a C18 column was employed. The gradient mobile phase conditions consisted of 60 to 75% CH_3CN and 40 to 25% water containing 0.1% triethylamine (TEA). Samples from the Caco-2 studies used a phenyl column with gradient mobile phase conditions ranging from 50 to 60% CH_3CN and 50 to 40% water containing 0.1% TEA. Mobile phases were adjusted to pH 3.0 with H_3PO_4. The wavelength used for the analysis ranged from 255 to 265 nm.

Calculated and Other Parameters

Hydrogen bonding (H-bonding) capacity was calculated using the method put forth by Stein (10). Partition coefficients (ClogP) were calculated using the MedChem program (PC Models, v 4.42, Daylight Chemical Information Systems) (11). Distribution coefficients (log D) were determined in saline:octanol at pH 7.4 by Robertson Microlit Laboratories, Inc. (Madison NJ) using a pH-titration method (12).

Statistical Analyses

Excel (Microsoft, Seattle WA) and JMP (SAS Institute, Cary NC) were used for statistical and multivariable regression analyses.

RESULTS AND DISCUSSION

Hydrophobicity Parameters

Hydrophobicity was assessed by calculation (ClogP), octanol:water distribution (log D), or by chromatographic means using reversed-phase (log k'_{C18}) or IAM (log k'_{IAM}) columns. Hydrophobicity values are depicted in Figure 2. ClogP values represent distribution of the nonionized species and are generally several log units higher than the experimental values obtained at pH 7.4. In this particular chemical series, experimental values were influenced by the pyrone or dihydropyrone hydroxyl group pK_a in the range of 5.5 to 6.5. As illustrated in Figure 2, hydrophobicity values for each protease inhibitor ranked as follows:

$$\text{ClogP} > \log k'_{C18} > \log k'_{IAM} \geq \log D$$

The curves closely paralleled each other in most cases. The greatest divergence was noted for the log D value for PD1. Log D for PD1 dropped precipitously relative to the other parameters, resulting in the only negative hydrophobicity parameter. PD1 was the only compound in this set that had a double negative charge at physiological pH.

Cell Uptake Studies

Two reference compounds, propranolol and PD10R, were used to benchmark the uptake rates of the PD protease inhibitors based on reported *in vivo* performance. Approximately 100% of propranolol was available after absorption (13). PD10R is a peptide-based HIV protease inhibitor that has demonstrated clinical efficacy after oral administration (8). PD10R has a molecular weight of 617 and 11 potential hydrogen bonding sites, suggesting that its fraction absorbed in humans will be low. Comparison of the PD protease inhibitors to the reference compounds provided a basis for projecting whether transport into intestinal cells or into target cells would limit delivery of inhibitors to the virus.

In T-cells, the uptake of PD10R was 0.51 nmol/min-mg protein-0.1 mM, less than $^1/_2$ that of propranolol (1.29 nmol/min-mg protein-0.1 mM) (Table I). Uptake of the clinical candidate, PD10R, into T-cells thus reflected a minimum acceptable uptake rate for the PD protease inhibitors to be evaluated against. As shown in Table I, only one protease inhibitor (PD1) had an uptake rate substantially lower than PD10R, while four had uptake rates within two-fold of PD10R (PD2, PD4, PD6,

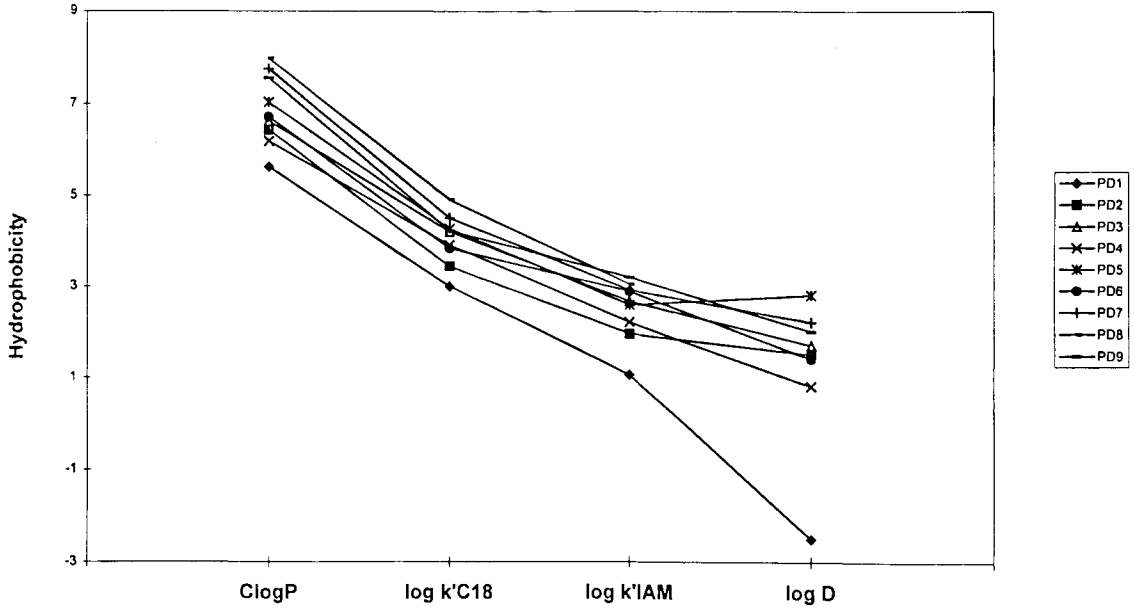

Fig. 2. Comparison of ClogP, log k'_{C18}, log k'_{IAM} and log D for the set of nine HIV protease inhibitors.

Table I. Summary of T-Cell and Caco-2 Cell Uptake Studies

Key	T-cell uptake[a]	Caco-2 cell uptake[a]
PD1	0.013 ± 0.002	0.13 ± 0.04
PD2	0.55 ± 0.10	5.99 ± 1.40
PD3	3.25 ± 1.10	12.95 ± 4.13
PD4	0.24 ± 0.02	0.47 ± 0.10
PD5	16.3 ± 0.89	4.76 ± 0.08
PD6	0.38 ± 0.06	0.54 ± 0.19
PD7	0.75 ± 0.06	1.18 ± 0.12
PD8	7.45 ± 2.81	21.71 ± 4.3
PD9	5.14 ± 0.81	3.40 ± 1.91
Propranolol	1.29 ± 0.51	3.44 ± 0.39
PD10R	0.51 ± 0.06	0.28 ± 0.18

[a] Uptake at pH 7.4 in nmol/min-mg protein-0.100 mM.

PD7). The remaining four protease inhibitors (PD3, PD5, PD8, PD9) showed considerably higher uptake. Mitigating factors such as protein binding, stability, and activity against the virus were not accounted for in these studies; however, it can be concluded that transport into target cells should not be rate-limiting for eight of nine PD protease inhibitors.

The uptake rate of PD10R into Caco-2 cells was 0.28 nmol/min-mg protein-0.1 mM), while that of propranolol was more than ten-fold higher (Table I). Uptake rates at least equal to those of PD10R should be sufficient to achieve antiviral activity in the absence of dissolution rate or stability limitations. All of the PD protease inhibitors had Caco-2 cell uptake rates greater than PD10R with the exception of PD1, previously noted as being doubly anionic at physiological pH. Five of the protease inhibitors (PD2, PD3, PD5, PD8, PD9) had uptake rates equal to or greater than that of propranolol. Propranolol is completely absorbed from the intestine, although extensively metabolized during first-pass. Therefore, these compounds were all candidates for oral administration, with the exception of PD1.

The two cell systems were dissimilar to each other physically in that T-cell incubations were conducted with cells grown in suspension, while Caco-2 cells were grown in monolayers. The predominantly spherical T-cells were bathed in incubation media. The highly differentiated Caco-2 cells were complete with tight junctions between the cells and presented only the apical, microvillar surface to the incubation media. None the less, it was anticipated that driving forces should be similar for uptake into the two cell types. Surface area was considered constant in each system, which was normalized for the amount of protein. There was a reasonable correlation between uptake into T-cells and Caco-2 cells (Table I), with a slope close to unity (1.1) and r^2 of 0.71.

Modeling and Correlations

The complete result set for single-variable analysis of the data is presented in Table II. This matrix shows that all measures of hydrophobicity (log k'_{IAM}, log k'_{C18}, log D), as well as ClogP, correlated well with each other. For uptake into T-cell suspensions, there was a positive correlation with hydrophobicity (Table II). Values ranged from 0.74 to 0.90, with ClogP being the lowest and log D the highest. With the exception of log D ($r = 0.74$), hydrophobicity was poorly correlated with uptake into Caco-2 cell monolayers (0.56 to 0.74).

Given that transport across biological membranes is not solely a function of hydrophobicity (14,15), the compound set was examined for ranges in molecular weight and hydrogen-bonding. The molecular weights of the protease inhibitors were 338 to 601, and hydrogen-bonding capability was 2.5 to 8.5. Multivariable analysis was conducted in which log T-cell or Caco-2 cell uptake was regressed against hydrophobicity (log k'_{IAM}, log k'_{C18}, log D or ClogP), molecular weight and hydrogen-bonding.

The results of multivariable analysis are shown in Table III. For T-cell uptake, there was little or no improvement in correlation beyond single-variable analysis. Neither hydrogen bonding (A_2) or molecular weight (A_3) were statistically significant variables for T-cell uptake when log k'_{IAM}, log k'_{C18}, or log D were used for the variable representing hydrophobicity (A_1). There was no satisfactory correlate when Clog P was used for hydrophobicity. Log D was clearly superior to log k'_{IAM} and log k'_{C18} in this analysis.

Conversely, multivariable analysis greatly improved the correlation of hydrophobicity with uptake by Caco-2 cells (Table III). When log k'_{IAM} represented hydrophobicity (A_1), both hydrogen-bonding (A_2) and molecular weight (A_3) were needed to describe the uptake process, and r^2 then rose to 0.91. Using log k'_{C18} or log D for hydrophobicity, molecular weight was again significant, but hydrogen-bonding was rejected. Only when ClogP was used for hydrophobicity was the correlation with Caco-2 uptake not considered significant. Furthermore, using the molecular weight term to adjust Caco-2 permeability increased the correlation between Caco-2 and T-cell uptake from $r^2 = 0.71$ (Table II) to 0.90 (Table III).

From this analysis, the three experimentally determined hydrophobicity measures, log k'_{IAM}, log k'_{C18} and log D, provided useful and comparable correlates of HIV protease inhibitor uptake by T-cell suspensions or Caco-2 cell monolayers. The calculated partition coefficient, ClogP, was less useful in both cases. Of the three experimental measures, we found log k'_{IAM} most accessible to our own use and most facile in obtaining results. Log D was a more time-consuming approach usually reserved for fewer compounds later in development. In the discovery mode, log D determinations were contracted to an external resource. Log D measurements by pH titration can also be problematic for poorly water-soluble compounds. Log k'_{C18} provided reliable correlations that were virtually indistinguishable from log k'_{IAM} in this series; however, the C18 column represents only hydrophobic contributions, while the IAM.PC column contains both hydrophobic and electrostatic contributions to membrane interaction.

CONCLUSIONS

Uptake of HIV protease inhibitors by T-cell suspensions or Caco-2 cell monolayers was positively correlated, although some differences were noted. Uptake by T-cell suspensions could be adequately described by hydrophobicity alone. Uptake by Caco-2 cell monolayers required multivariable regression analysis in which molecular weight and hydrogen bonding were included. Experimental measures of hydrophobicity (log D, followed by log k'_{IAM} and log k'_{C18}) were useful in the correlation analysis, whereas ClogP was not. With a large set of compounds to lend robustness to the multivariable model, log k'_{IAM} could be used to screen and select compounds for T-cell

Table II. Correlation Matrix (r values) for HIV Protease Inhibitors

Variable	log k'_{IAM}	log k'_{C18}	*log D	ClogP	log T-cell	log Caco-2	H-bond	MW
log k'_{IAM}	1.000							
log k'_{C18}	0.862	1.000						
*log D	0.848	0.847	1.000					
ClogP	0.865	0.900	0.793	1.000				
log T-cell	0.784	0.808	0.901	0.742	1.000			
log Caco-2	0.562	0.658	0.740	0.605	0.844	1.000		
H-bond	0.013	−0.308	−0.202	−0.269	−0.334	−0.686	1.000	
MW	0.542	0.448	0.367	0.354	0.160	−0.299	0.578	1.000

Note: n = 9 compounds except for *log D (n = 8 compounds).

Table III. Multivariable Regression Summary of Dependent Variables for Log T-Cell and Caco-2 Cell Uptake

Log T-Cell[a]	A_0	A_1	A_2	A_3	n	R^2	s	F(p<)
Log k'_{IAM}	−2.77	1.11			9	0.61	0.63	11.13 (0.012)
Log k'_{C18}	−5.42	1.34			9	0.65	0.60	13.13 (0.008)
Log D	−0.76	0.52			8	0.81	0.44	25.9 (0.002)
ClogP	−4.84	0.67			9	0.52	0.70	7.5 (0.03)
Log Caco-2	−0.36	1.09			9	0.71	0.54	17.3 (0.004)
	−2.77	1.26		−0.0047	9	0.90	0.35	26.4 (0.001)
Log Caco-2[b]								
Log k'_{IAM}	−1.20	0.61			9	0.32	0.65	3.22 (0.12)
	0.97	1.12		−0.0069	9	0.83	0.35	14.7 (0.005)
	0.78	0.94	−0.13	−0.0044	9	0.91	0.28	16.7 (0.005)
Log k'C18	−3.09	0.85			9	0.43	0.59	5.34 (0.05)
	−1.83	1.28		−0.0060	9	0.87	0.30	20.68 (0.002)
Log D	−0.17	0.31		−0.0044	8	0.55	0.49	7.3 (0.036)
	1.95	0.40			8	0.90	0.26	21.63 (0.004)

[a] Log T-cell = A_0 + A_1*Hydrophobicity + A_2*H-bonding + A_3*MW · H-bonding and MW were not significant at $p > 0.1$.
[b] Log Caco-2 = A_0 + A_1*Hydrophobicity + A_2*H-bonding + A_3*MW · ClogP gave no significant correlations at $p > 0.1$.

and Caco-2 transport, thus reducing the number of actual cell experiments needed.

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