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Chemical Substituent Effect on Pyridine Permeability and Mechanistic Insights from Computational Molecular Descriptors

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Abstract

The objective was (1) to evaluate the chemical substituent effect on Caco-2 permeability, using a congeneric series of pyridines, and (2) compare molecular descriptors from a computational chemistry approach against molecular descriptors from the Hansch approach for their abilities to explain the chemical substituent effect on pyridine permeability. The passive permeability of parent pyridine and 14 mono-substituted pyridines were measured across Caco-2 monolayers. Computational chemistry analysis was used to obtain the following molecular descriptions: solvation free energies, solvent accessible surface area, polar surface area, and cavitation energy. Results indicate that the parent pyridine was highly permeable, and that chemical substitution was able to reduce pyridine permeability almost 20-fold. Substituent effect on permeability provided the following rank-order: $3\text{-COO}^- < 4\text{-NH}_2 < 3\text{-CONH}_2 < 3\text{-Cl} < 3\text{-CHO} < 3\text{-OH} < 3\text{-CH}_2\text{OH} < 3\text{-C}_6\text{H}_5 < 3\text{-NH}_2 < 3\text{-CH}_2\text{C}_6\text{H}_5 < 3\text{-C}_2\text{H}_5 < \mathbf{3\text{-H}} < 3\text{-CH}_3 < 3\text{-F} < 4\text{-C}_6\text{H}_5$. This substituent effect was better explained via molecule descriptors from the computational chemistry approach, than explained by classic descriptors from Hansch. Computational descriptors indicate that aqueous desolvation, but not membrane partitioning per se, dictated substituent effect on permeability.

Keywords

chemical substituent; permeability; computational chemistry; Caco; 2; QSAR; aqueous desolvation

Introduction

Modern drug discovery methods require the rational design of favorable oral absorption and bioavailability during compound development. In silico approaches to screen for oral absorption and/or intestinal permeability offer great potential to achieve this goal^{1–4}. Such computer-based methods have increasing utilization due to their abilities to predict absorption/permeability of diverse compounds from compound structure^{5–16}. Interestingly, and in contrast to traditional quantitative-structure activity relationship (QSAR) methods in drug design, there is limited data that measures the influence of chemical substituents on drug intestinal permeability. Anderson and colleagues observed functional groups had the following rank-order effect on the intrinsic permeability of substituted p-toluic acids and p-methylhippuric acids across artificial lipid bilayers: $\text{-CONH}_2 < \text{-COOH} < \text{-OH} < \text{-CH}_2\text{OH} < \text{-Cl} < \text{-H}$ ¹⁷. Given the general lack of information concerning chemical group effects on permeability, one objective of the present study was to evaluate chemical substituent effect on Caco-2 permeability, using a congeneric series of pyridines. Caco-2 monolayers were selected

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as a permeability model because (a) biological bilayers may be expected to exhibit a sensitivity to chemical substituents that differs from the sensitivity of an artificial bilayer¹⁸ and (b) Caco-2 monolayers are widely used to assess lead compound permeability and predict oral absorption¹⁹. A congeneric series of pyridine was selected since pyridine is a common scaffold in real drug structure²⁰.

A second objective was to compare the relative abilities of molecular descriptors from a computational chemistry approach versus those from the Hansch approach, to explain the chemical substituent effect on pyridine permeability. Classical Hansch parameters π , σ , and E_s have been widely employed to describe substituent effect on drug activity²¹ and would appear to serve as a reference to evaluate a computational chemistry approach to explain functional group effects. The computational approach taken here included solute-solvent interactions (e.g. solute-water interactions), since aqueous desolvation of solute is a potentially rate-limiting step in membrane permeation²². To date, the majority of computational methods that describe permeability in terms of molecular descriptors only consider the solute, and not explicit solute-solvent interactions. To compare the computational chemistry approach and the Hansch approach, we have measured the permeabilities of a series of substituted pyridines through Caco-2 cells as well as obtained computational and Hansch-based molecular descriptors for the respective compounds. Regression analysis between the experimental data and both types of descriptors was then performed to evaluate the two approaches. A model for the molecular events dictating the permeability of substituted pyridines was obtained and highlights the computational chemistry approach to better explain pyridine permeability.

Experimental Section

Materials

Fifteen pyridines were purchased from Aldrich Chemical Co. (Milwaukee, MI). ¹⁴C-Mannitol was purchased from New England Nuclear (Boston, MA). Dulbecco's Modified Eagles Media (DMEM) and Hank's Balanced Salt Solution (HBSS) were obtained from Sigma Chemical Co. (St. Louis, MO). Nonessential amino acids (NEAA), fetal bovine serum (FBS), trypsin, penicillin-streptomycin, and HEPES buffer were purchased from Biofluids Inc. (Rockville, MD). Caco-2 cell line (passage number 17) was obtained from American Type Culture Collection (Rockville, MD).

Cell culture and Caco-2 permeability measurement

Caco-2 cells were grown in T-150 flasks at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity, as previously described²³. Growth medium consisted of DMEM, 10% FBS, 1% penicillin-streptomycin, and DEAA and was adjusted to pH 7.2 with 0.1 N NaOH. Cells (between passage number 35 and 48) were trypsinized using 0.25% trypsin and 0.2% EDTA solution. The cells were seeded on Costar Transwell inserts (0.4mm, 4.71 cm²) at a seeding density of 1×10^5 cells/cm² and were cultured for 21–25 days prior to utilization in conducting transport studies.

Monolayers with TEER values of at least 850 Ω cm² in the culture media at room temperature were used for permeability studies. Transport studies were conducted in HBSS at pH 6.8. For apical-to-basolateral (A-B) studies, a 1 mM substituted pyridine solution (1.5 mL) was placed in the apical chamber and 2.5 mL of HBSS was added to the basolateral chamber. At pre-determined time intervals, the filter was carefully moved to a new basolateral chamber, in order to sample flux over time. Aliquots were withdrawn from the basolateral chamber and analyzed to determine the pyridine mass that permeated across the Caco-2 cell monolayer. Basolateral-to-apical (B-A) studies were performed in a similar manner; samples were withdrawn from apical chamber at selected time intervals and replaced with an equal amount of HBSS. At the

end of each experiment, the monolayer integrity was assessed via a ^{14}C -mannitol permeability study. Mannitol permeability was less than 1×10^{-6} cm/sec, indicating acceptable monolayer integrity. As monolayers were used from four passages, the permeability of pyridine was assessed on each of the four occasions; pyridine permeability did not vary with occasion (ANOVA $p = 0.9$). Permeability was calculated from:

$$P_{\text{eff}} = \frac{dC_R}{dt} \frac{V_R}{AC_0} \quad (1)$$

where V_R is the volume of the receiver compartment, A is the membrane surface area, and C_0 is the donor concentration. Mass balance analysis was conducted to determine the percent of initial mass that could be accounted for at the conclusion of the study. For each pyridine, mass balance ranged within 80% to 105%, which was deemed acceptable. Permeability is reported as mean ($\pm$ SEM).

Computational chemistry methods

A computational chemistry approach was applied to derive several molecular descriptors. Calculations were performed using the semiempirical quantum chemical program AMSOL 5.4²⁴. Along with parent pyridine, each molecule consisted of a pyridine ring with a substituent at the 3' position or 4' position (Fig. 1). Since Caco-2 permeability studies were performed at pH 6.8, the ionization state of each compound was assigned based on its experimental pK_a value. For the few pyridines with no experimentally reported pK_a , their computationally-estimated pK_a values were used²⁵.

Geometries of the 15 compounds were fully optimized using the semiempirical Austin Model 1 (AM1)²⁶ Hamiltonian in the gas phase at the appropriate ionic state. For congeners whose substituents have rotational freedom, potential energy surfaces of the associated dihedral angle were determined and the conformer of the lowest energy was selected. Free energies of solvation in water and hexane were calculated with the AM1-SM2²⁷ and AM1-SM4²⁸ models, respectively. For calculations in the solvents, the previously optimized geometry from the gas phase was adopted. Reoptimization of the structure in the presence of the solvent had only a minor impact on the geometry and energetics, compared to the considerable computational cost (results not shown).

Four types of physicochemical properties were calculated. First, the solvation free energies in water (ΔG_w) and hexane (ΔG_h) were obtained as the difference between the total free energies in water or hexane, respectively, and the gas phase heat of formation. Second, solvent accessible surface area (SASA) of each compound was calculated in the presence of water or hexane using Lee and Richards method, where a sphere representing the solvent molecule was used to probe along the van der Waals surface^{29,30}. Third, polar surface area (PSA) was defined as the sum of solvent accessible surface area of all chlorine, fluorine, nitrogen, and oxygen atoms and hydrogens covalently attached to these heteroatoms³¹, as determined in the AM1-SM2 and AM1-SM4 calculations. SASAs and PSAs were calculated using the conformation at the global energy minimum for each tested compound. Since the substituents in this pyridine series were relatively rigid and small, variation in SASA or PSA due to substituent flexibility was assumed to be negligible. Fourth, the free energy for creating a cavity in the solvent to accommodate a solute, and for the changes in dispersion interactions and solvent structure that accompany the solvation process, were evaluated for each compound²⁸. This parameter was denoted cavitation energy or CDS, and was computed for both water and hexane as solvent. Additionally, the number of hydrogen bonds (HB) was counted as the number of lone electron pairs (i.e. number of hydrogen bond acceptors) and hydrogens attached to an N or O (i.e. hydrogen bond donors). HB was counted via an in-house script.

Simple linear regression analysis of computational chemistry parameters

As an intermediate step in deriving a final model that employed multiple linear regression to relate permeability to computational chemistry parameters, simple (i.e. single) linear regression was performed between permeability and each individual computational chemistry molecular descriptor [i.e. ΔG_w , $SASA_w$, PSA_w , $fPSA_w$, CDS_w , HB , ΔG_h , $SASA_h$, PSA_h , $fPSA_h$, CDS_h , and molecular weight (MW)]. This analysis was conducted in order to elucidate the general influence of each parameter on permeability. Regression was performed using SYSTAT ver 7.0 (SPSS Inc., Chicago, IL).

Hansch parameters

For each substituent, classical Hansch parameter values for π , σ , and E_s were obtained from a comprehensive reference²¹. For any one substituent, this reference cites several reported numerical values for π , σ , and E_s . The median value was used (Table 1).

Briefly, π , σ , and E_s characterize substituent hydrophobicity, electronic withdrawing ability, and molecular size, respectively. π , σ , and E_s values are relative to hydrogen, whose π , σ , and E_s values are zero. π is generally the difference of log P values between the substituted compound and its parent. Positive π values represent lipophilic substituents, relative to hydrogen. σ measures the substituent's ability to attract electrons. Positive σ values representing electron withdrawing character, rather than electron donating character. E_s is a measure of the substituent's steric bulk. Since substituents are generally larger than hydrogen and hinder reactivity, E_s values are generally negative. As discussed in Results and Discussion, the parameter π_- was devised. π_- only allows for Hansch π values that are negatively-valued; positively-valued π were set to zero for π_- . Similarly, the parameter π_+ was devised, with π_+ only allowing for Hansch π values that are positively-valued; negatively-valued π were set to zero for π_+ .

Comparison of computational chemistry versus Hansch molecular descriptors to explain substituent effect

Caco-2 permeability values were regressed via multiple linear regression against the molecular descriptor values from both the computational chemistry approach and from Hansch. Regression was performed using the MLR module of SYSTAT. In the computational chemistry approach, the following molecular descriptors were considered: ΔG_w , ΔG_h , $SASA_w$, $SASA_h$, PSA_w , PSA_h , $fPSA_w$, $fPSA_h$, CDS_w , CDS_h , and MW. The square of each of these parameters was also considered. Forward step-wise regression was used to arrive at a model^{32,33}. Regression was also pursued via the parameter reduction method^{32,33}. Briefly, in the parameter reduction method, a subset of descriptors that were not collinear were identified and subjected to further analysis; the other descriptors were eliminated since each was collinear with one of the retained descriptors. The degree of collinearity between each pair of molecular descriptors was assessed via Pearson's coefficient. As described in the Appendix, all molecular descriptors except ΔG_w were collinear (i.e. Pearson's $r > 0.75$) with at least one other non-squared descriptor, such that only four descriptors were selected for further consideration (ΔG_w , PSA_w , ΔG_h , CDS_h , along with PSA_w^2). Each squared descriptor (i.e. descriptor raised to the power 2) was collinear with its non-squared descriptor. As described in the Results and Discussion, the final model included ΔG_w and PSA_w^2 .

In the Hansch approach, the following molecular descriptors were considered: π , π_+ , π_- , σ , and E_s , as well as their squares. Both forward step-wise regression and the parameter reduction method were used to arrive at a model that related permeability to Hansch parameters.

Predictability of the final model was evaluated by cross-validation via the "leave-one-out" method. The model was rederived using the remaining 14 compounds, which was subsequently

employed to predict the permeability of the excluded compound. In this regard, 14 compounds served as a training set and the to-be-predicted compound served as a test compound. This procedure was repeated until the permeability of each compound had been predicted once (i.e. until each compound served as the test compound). Model predictability was assessed through

$$Q^2 = 1 - \frac{\sum (y_{pred} - y_{obs})^2}{\sum (y_{obs} - y_{mean})^2} \quad (2)$$

where Q^2 is the cross-validated r^2 value. Models that perfectly predict observed values result in $Q^2 = 1$, while models that predict as poorly as chance alone result in $Q^2 = 0$. For permeability models with two or more descriptors, partial regression analysis was used to assess the relative importance of each descriptor to modulate permeability^{32,33}.

Results and Discussion

Substituent effect on pyridine permeability across Caco-2 monolayers

The permeabilities of parent pyridine and its 14 mono-substituted congeners were determined in the A-B and B-A directions. For each pyridine, A-B permeability was within 20% of the B-A permeability, indicating each compound was effectively not transported by any active influx or efflux mechanism. Hence, only A-B permeability values were further considered and reflect passive Caco-2 permeability values.

In Table 2, substituted pyridines exhibited a wide range of permeabilities. Permeability values ranged from 6×10^{-6} cm/sec to 130×10^{-6} cm/sec. Parent pyridine provided a relatively high permeability of 107×10^{-6} cm/sec. The ionized pyridines (i.e. 3-carboxy pyridine and 4-amino pyridine) exhibited the lowest permeability, while nonpolar alkyl substituents tended to yield the highest values. The rank-order substituent effect on pyridine permeability was: $3\text{-COO}^- < 4\text{-NH}_2 < 3\text{-CONH}_2 < 3\text{-Cl} < 3\text{-CHO} < 3\text{-OH} < 3\text{-CH}_2\text{OH} < 3\text{-C}_6\text{H}_5 < 3\text{-NH}_2 < 3\text{-CH}_2\text{C}_6\text{H}_5 < 3\text{-C}_2\text{H}_5 < \mathbf{3\text{-H}} < 3\text{-CH}_3 < 3\text{-F} < 4\text{-C}_6\text{H}_5$. There was a major ring position effect on amino substitution. 4-NH₂ substitution substantially reduced permeability more than 3-NH₂ substitution. Also of note, 3-C₆H₅ substitution reduced permeability relative to parent pyridine, while 4-C₆H₅ substitution increased permeability. This observation is consistent with the membrane transport model where minimal cross-sectional area hinders transport³⁴.

Rank-order results are similar to results of Mayer et al., who measured the intrinsic permeability of substituted p-toluic acids and p-methylhippuric acids¹⁷. Six substituents were common to both the present study and the work by Mayer et al.¹⁷, who presented data in terms of intrinsic permeability by normalizing apparent permeability for the degree of ionization. Without this normalization, rank-order apparent permeabilities from Mayer et al. are: $\text{-COO}^- < \text{-CONH}_2 < \text{-OH} < \text{-CH}_2\text{OH} < \text{-Cl} < \text{-H}$ ^{17,35}. This rank-order is similar to the present study of pyridine permeability across Caco-2 monolayers, which showed: $3\text{-COO}^- < 3\text{-CONH}_2 < 3\text{-Cl} < 3\text{-OH} < 3\text{-CH}_2\text{OH} < 3\text{-H}$. The difference between the present study and Mayer et al.¹⁷ is 3-Cl in the present study reduced permeability to a greater extent than did 3-OH and 3-CH₂OH. Nevertheless, there is good rank-order agreement for the substituent effect between studies.

It is notable that the present pyridine studies employed Caco-2 monolayers, while Mayer et al.¹⁷ used artificial lipid systems, namely large unilamellar vesicles for p-methylhippuric acid studies and planar egg lectin/decane bilayers for p-toluic acid studies. From the six substituents common to the three data sets, substituents modulated Caco-2 permeability 17-fold. These same substituents modulated permeability over 1,000-fold across artificial lipid systems¹⁷. These observations point towards biological bilayers (e.g. Caco-2 monolayers) as being less sensitive to substituent effects, relative to artificial membrane systems. Using substituent p-

methylhippuric acids, Xiang and Anderson¹⁸ found that incorporation of a membrane protein into egg-based phosphatidylcholine bilayer compressed the range of substituent effect on permeability. Hence, it is possible here that membrane proteins within the Caco-2 apical membrane contribute to reduce the sensitivity of Caco-2 membranes to substituent effects, relative to artificial bilayers.

It should be noted that parent pyridine exhibited a relatively high Caco-2 permeability. Pyridine permeability across filters with no cells was $125(\pm 9) \times 10^{-6}$ cm/sec. Hence, only 14% of the resistance for pyridine transport across Caco-2 cells was due to the Caco-2 monolayer, with the remaining 86% due to the “aqueous boundary layer” [i.e. filter plus stagnant diffusion layer]³⁶. Figure 2 illustrates pyridine transport across a lipid bilayer, including the potential role of the aqueous boundary layer (i.e. step 1) to limit permeability. Because of aqueous boundary layer-controlled transport of parent pyridine, there was greater allowance for substituents to decrease pyridine permeability than to increase permeability. In Table 2, substituents were able to markedly reduce permeability, but only increase permeability very slightly. Hence, the chemical substituent effect on Caco-2 permeability in Table 2 reflects a substituent effect for a high permeability parent.

Molecular descriptor values from the computational chemistry approach

Table 2 lists molecular descriptor values from computational chemistry calculations. Free energies of solvation were negatively-valued, reflecting the thermodynamic favoring of all pyridines to be solvated by both water and hexane. Compared to ΔG_h , ΔG_w showed a much wider range of values, with the ionized pyridines possessing large, negative ΔG_w values.

As expected, substitution increased solvent accessible surface area. $SASA_h$ was typically 90 Å² larger than $SASA_w$, since hexane is larger than water. Substitution either increased polar surface or had no effect. As expected, PSA_w was larger than PSA_h since water is a smaller probe than hexane, such that water more thoroughly probed the van der Waals surfaces of the polar atoms. PSA_w varied 3-fold across substituents, while PSA_h varied 5-fold. Since PSA_w values were larger than corresponding PSA_h values, $fPSA_w$ was larger than $fPSA_h$.

As expected, cavitation energy was dependent on solvent and pyridine substitution. All CDS_h were negatively-valued (i.e. thermodynamically favorable), while CDS_w was either positively-valued or negatively-valued, depending upon substituent. Cavity formation in water without subsequent solute insertion was energetically unfavorable due to the loss of water-water hydrogen bonding, while cavitation in nonpolar solvents (e.g. hexane) was entropically favorable, since nonpolar solvent molecules became less ordered. When pyridine was placed in an aqueous cavity, the unfavorable thermodynamics from cavitation was compensated by favorable solute-solvent dispersion interactions, such that polar substituents (e.g. 3-COO⁻ and 3-CONH₂ in Table 2) provided thermodynamically favorable values (i.e. $CDS_w < 0$). Meanwhile, nonpolar substituents (e.g. 3-CH₃ and 3-C₆H₅) resulted in CDS_w values being approximately zero or greater than zero. Unlike in water, each pyridine in hexane exhibited favorable cavitation energy, in part since solvent-solvent hydrogen bond loss was not an issue and since favorable dispersion may be introduced with pyridine insertion into the cavity. Hydrogen bond number and molecular weight are also listed in Table 2.

Computational chemistry molecular descriptors to explain substituent effect

Simple (or single) linear regression was performed in order to elucidate the influence of each computational parameter on permeability. Table 3 lists regression results. ΔG_w was the parameter with the strongest linear relationship with permeability ($r^2 = 0.63$), while ΔG_h was not correlated with permeability. After ΔG_w , PSA_w and PSA_h showed the second and third strongest relations with permeability ($r^2 = 0.57$ and 0.52 , respectively). Consequently,

permeability correlated with each $fPSA_w$ and $fPSA_h$, although not as strongly as PSA_w and PSA_h . These favorable results for PSA are consistent with previous findings between permeability and PSA^{5–8,10}. Artursson and colleagues appear to suggest that the ability of PSA to predict permeability is underpinned by PSA's hydrogen bonding character¹. Total solvent accessible surface area (SASA) in water and hexane did not correlate with permeability, as previous studies found^{1,6}.

As a single parameter, the number of hydrogen bonds (HB) provided a modest correlation ($r^2 = 0.29$), given the apparent simplicity of this parameter, where each hydrogen bond is assumed to provide the same interaction strength. Molecular weight had no effect on permeability, perhaps since molecular weight only varied over a narrow range.

The forward step-wise regression approach and the parameter reduction method each yielded the following as the best model to relate permeability to the computational molecular descriptors:

$$P_{app} = A \Delta G_w + B PSA_w^2 + C \quad (3)$$

Table 4 summarizes the model. The majority of the substituent effect on permeability can be attributed to ΔG_w and the square of PSA_w ($r^2 = 0.80$). It should be noted that raw, and not mean, permeability values were used in regression analysis. Figure 3 illustrates observed and predicted pyridine permeabilities. Cross-validation analysis showed the model to be predictive ($Q^2 = 0.77$). ΔG_w and PSA_w^2 were not strongly collinear ($r^2 = 0.29$).

Equation 3 reflects the above discussion, where parent pyridine was recognized as highly permeable, and chemical substituents significantly reduced permeability, but did not increase permeability appreciably. In eqn 3, $C = 116 \times 10^6$ cm/sec is consistent with the observed parent pyridine permeability of $125(\pm 9) \times 10^{-6}$ cm/sec across filters without a cell monolayer. The constant C reflects aqueous boundary layer-controlled transport, under the hydrodynamic conditions applied (50 rpm). The positive-valued coefficient for ΔG_w and the negatively-valued coefficient for PSA_w^2 indicate that addition of a chemical substituent onto parent pyridine will generally reduce permeability, except for nonpolar alkyl substituents. Parent pyridine (i.e. -H substituent) possessed a slightly negative ΔG_w and a small PSA_w , such that parent pyridine was highly permeable (107×10^{-6} cm/sec in Table 2). Substitution that yielded pyridines with very favorable free energies of aqueous solvation (i.e. $\Delta G_w \ll 0$) and/or substitution that result in pyridines with large PSA_w reduced permeability significantly.

Partial regression analysis indicated that ΔG_w and PSA_w^2 were about equally influential on permeability, but varied with substituent. Permeability was reduced by approximately 1×10^{-6} cm/sec for each 1 kcal/mol in ΔG_w , as well as for each $25 \text{ \AA}^2$ of PSA_w . For example, 3-COO⁻ and 4-NH₂ resulted in the lowest permeabilities due to significant ΔG_w effects, as well as through PSA_w . 3-CONH₂ and 3-Cl resulted in low permeabilities, largely through the substituents' effects on PSA_w^2 .

Of note is the position effect for the amino substituent. Because of resonance effects, 4-NH₂ resulted in the pyridine ring nitrogen to be ionized at pH 6.8, while 3-NH₂ did not have this effect. While 4-NH₂ and 3-NH₂ have essentially the same PSA_w , 4-NH₂ has a much more thermodynamically favorable ΔG_w than 3-NH₂, resulting in the 4-NH₂ pyridine to have a much lower permeability than 3-NH₂ pyridine. On the other hand, the computational chemistry model was not successful in predicting the positional effect for C₆H₅, since the computational model failed to capture the important molecular shape differences between 4-C₆H₅ pyridine and 3-C₆H₅ pyridine. The para substitution of 4-C₆H₅ provides a more "narrow" solute, as compared to the meta substituted 3-C₆H₅ pyridine. The present experimental results are consistent with narrow solutes being more permeable than wider solutes³⁴.

Model based upon Hansch parameters

The forward step-wise regression approach and the parameter reduction method each yielded the following as the best model to relate permeability to Hansch parameters:

$$P_{app} = A\pi + C \quad (4)$$

Table 4 summarizes the model. Substituent effect on permeability was largely attributed to π_- ($r^2 = 0.58$). Cross-validation analysis showed the model to be moderately predictive ($Q^2 = 0.32$). Molecular descriptors from the computational chemistry model (eqn 3) provided a better model than one based on Hansch parameters (eqn 4). Equation 4 reflects discussions above that substituents can largely only lower parent pyridine permeability. π_- is either negatively-valued or zero, and is never positively-valued. π_- assumes the values of π that are negative; substituents with positively-valued π are assigned a π_- value of zero. Permeability was reduced by approximately 1×10^{-6} cm/sec for each 0.05 π_- .

Comparison of computational chemistry versus Hansch approach to explain substituent effect

One objective of this work was to compare the relative abilities of molecular descriptors from a computational chemistry approach and molecular descriptors from the Hansch approach to explain chemical substituent effect on pyridine permeability. The computational approach performed better than the Hansch method in three ways. First, the computational approach provided a better model fit ($r^2 = 0.80$ for eqn 3 versus $r^2 = 0.58$ for eqn 4) and better predictability ($Q^2 = 0.77$ versus $Q^2 = 0.34$). The basis for this better performance by computational descriptors is discussed below, along with insight provided by the computational descriptors.

Second, the computational approach performed better since the Hansch approach was practically more complex. π_- was considered only after the construction of the computational chemistry-based model and after considerable examination of the Hansch parameters π , σ , and E_s . Use of only parameters π , σ , and E_s lead to a poorer model ($r^2 = 0.51$) than considering π_- alone. Meanwhile, eqn 3 was achieved through common regression methods and was more straightforward to obtain, even though the computational approach considered more variables (i.e. ΔG_w , $SASA_w$, PSA_w , $fPSA_w$, CDS_w , HB , ΔG_h , $SASA_h$, PSA_h , $fPSA_h$, CDS_h , and MW).

This difference between the computational and Hansch approaches leads to a third relative benefit of the computational chemistry-based approach: molecular descriptors from the computational approach provide more insight than Hansch parameters. The Hansch approach implicated reduced partitioning as the basis for chemical substituent effect on pyridine permeability. However, the computational approach more specifically identified aqueous desolvation, and not overall partitioning. This advantage of greater specificity of the computational approach is discussed immediately below.

Insight from computational molecular descriptors

The solubility-diffusion model outlines the physicochemical basis for solute permeability across a lipid membrane^{37–39}. This model highlights (a) solute partitioning from solution into the membrane and (b) solute diffusion across the membrane as kinetic steps in membrane permeation. The solubility-diffusion model is generally expressed as

$$P_m = \frac{K_m D_m}{d_m} \quad (5)$$

where P_m is the solute permeability across the membrane, K_m is the partition coefficient of the solute between the solvent and membrane, D_m is the diffusion coefficient of the solute in the

membrane, and d_m is the membrane thickness. As written, eqn 5 implicates membrane homogeneity of K_m , D_m , and d_m to reflect average membrane properties. Lipid bilayers, such as cell plasma membranes, are heterogeneous^{40,41}. Anderson and colleagues^{42,43} have elucidated that the ordered hydrocarbon chain region of a bilayer is the barrier domain for polar solutes, such that

$$P_m = \frac{K_{bd/w} D_{bd}}{d_{bd}} \quad (6)$$

where $K_{bd/w}$ is the barrier domain/water partition coefficient, D_{bd} is the diffusion coefficient of the solute in the barrier domain, and d_{bd} is the barrier domain thickness.

The partition coefficient can be estimated from the thermodynamics of solute solvation into each phase. Figure 4 illustrates the thermodynamic cycle for the partitioning of a pyridine between an aqueous phase and a lipid phase. The partition coefficient is

$$\log K = \frac{-1}{2.303RT} (\Delta G_{lipid} - \Delta G_{aq}) \quad (7)$$

where K is the partition coefficient between the lipid and aqueous phases, ΔG_{aq} is the free energies of solvation from the gas phase into water, ΔG_{lipid} is the free energy of solvation from the gas phase into lipid. Using eqn 7, $\log K$ was calculated from ΔG_w and ΔG_h . Table 5 shows the strength of linear associate between several variables. $\log K$ is, of course, perfectly correlated with $\Delta G_h - \Delta G_w$. However, the substituent effect on $\log K$ is completely explained by ΔG_w ($r^2 = 0.99$), with ΔG_h having no impact on $\log K$ ($r^2 < 0.01$).

These relationships indicate that pyridine permeability was not controlled by partitioning per se, but controlled by aqueous desolvation. In Table 5, π was able to explain only 51% of the substituent effect on permeability. Substituents with positively-valued π did not appreciably increase permeability, due to parent pyridine's high permeability. In Table 5, π_- provided $r^2 = 0.58$ with permeability. π_- produced $r^2 = 0.62$ with permeability when 4-NH₂ was dropped; the Hansch parameter π cannot account for 4-NH₂'s modulation of the ring nitrogen protonation state. The correlation between ΔG_w and permeability ($r^2 = 0.63$ without 4-NH₂) essentially matched the correction between π_- and permeability, since ΔG_w explains π_- . Figure 5 illustrates the relation between π and ΔG_w . For the majority of substituents, π can be explained by ΔG_w , particularly for $\pi \leq 0$ ($r^2 = 0.95$). These results mimic the essentially complete dependence of $\log K$ on ΔG_w . Given this relationship and the observation that π_- constituted the best Hansch model to explain pyridine permeability (eqn 4), it appears that aqueous desolvation, and not partitioning per se, is the kinetic barrier for permeation by pyridines. Partitioning can be considered to be composed of solute aqueous desolvation, with subsequent insertion of the solute into the membrane (Fig. 2 and 4). Only aqueous desolvation was able to limit pyridine permeability. Pyridine insertion into the membrane did not limit pyridine permeability, as evidence by the lack of correlation between pyridine permeability with either ΔG_h or CDS_h (Table 3).

Permeability analysis that considers the aqueous boundary layer³⁶ suggests that the nine least permeable pyridines (i.e. 3-COO⁻, 4-NH₂, 3-CONH₂, 3-Cl, 3-CHO, 3-OH, 3-CH₂OH, 3-C₆H₅, and 3-NH₂) exhibited transport that was globally controlled by the Caco-2 monolayer. The remaining pyridines were aqueous boundary layer-controlled. Control of pyridine permeability by these nine substituents is based upon the ability of each of these substituents to hinder pyridine desolvation from water, and generally occurred when $\Delta G_w < -8$ kcal/mol. In Table 5, correlation analysis between ΔG_w and π re-iterates this finding. The linear r^2 for ΔG_w versus π was 0.95 for hydrophilic substituents (i.e. $\pi < 0$), excluding 4-NH₂.

The above analysis indicated that molecular descriptors from the computational chemistry approach were more insightful than Hansch descriptors. The computational approach included solvent-solute interactions (i.e. solvent-pyridine interactions). For example, free energies of solvation and cavitation energies were computed. The vast majority of computational methods that describe permeability in terms of molecular descriptors only consider the solute, and not explicit solvent-solute interactions, even though aqueous desolvation has been implicated in determining solute permeability²². Computational descriptors highlight ΔG_w and PSA_w ² (eqn 3) as surrogates for transport resistance across Caco-2 monolayers. Since ΔG_w directly measures aqueous solvation thermodynamics, ΔG_w is interpreted to reflect the aqueous desolvation barrier. Artursson and colleagues appear to suggest that the ability of PSA to predict permeability is underpinned by PSA's hydrogen bonding character¹. Hence, PSA_w is also interpreted here to reflect the aqueous desolvation barrier. These results suggest that pyridine permeability is controlled by desolvation of the pyridine from water, rather than global pyridine partitioning. Specifically, substituent effect on permeability was mediated through the substituents' modulating effect on pyridine aqueous desolvation.

It should be noted that these substituent results would not be expected to apply to parent compounds that, unlike pyridine, are lowly permeable. For example, a parent structure with low permeability due to an already high aqueous solvation energy and high polar surface area would not be expected to be sensitive to the substituent pattern observed here for pyridine. Rather, lead structures that are lipophilic and high membrane permeability, which occurs frequently in drug design, would be more likely to follow the substituent pattern observed here.

In summary, the objective was (1) to evaluate the chemical substituent effect on Caco-2 permeability, using a congeneric series of pyridines, and (2) compare the relative abilities of molecular descriptors from a computational chemistry approach and molecular descriptors from the Hansch method, to explain the chemical substituent effect on pyridine permeability. The passive permeability of 15 members of a congeneric pyridine series were measured across Caco-2 monolayers. Results indicate a 20-fold effect of chemical substitution on Caco-2 permeability, where substituents more frequently reduced permeability than increased permeability, in part reflecting high parent pyridine permeability. The pyridines were also subjected to computational chemistry analysis to characterize each pyridine in terms of several molecular descriptions, including solvation free energies in water and polar surface area in water. Permeability was regressed against the molecular descriptors from computational chemistry, as well as classic Hansch parameters. The observed substituent effect was better explained via the molecule descriptors from the computational chemistry, than classic descriptors from Hansch. Computational descriptors suggest that aqueous desolvation, but not membrane partitioning per se, was the mechanism by which chemical substituents modulated pyridine permeability.

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Appendix. Intermediate results from multiple linear regression analysis

This appendix provides intermediate results in conducting multiple linear regression of permeability against computational molecular descriptors via the parameter reduction method. The parameter reduction method was carried out in addition to forward stepwise regression, since forward step-wise regression does not guarantee arrival at the best model.

In the parameter reduction method, a subset of descriptors that are not collinear are identified and subjected to further analysis; the other descriptors are eliminated since each is collinear with one of the retained descriptors. The degree of collinearity between each pair of molecular

descriptors was assessed via Pearson's coefficient from SYSTAT. All molecular descriptors except ΔG_w were collinear (i.e. Pearson's $r > 0.75$) with at least one other non-squared descriptor (data not shown). Each squared descriptor (i.e. descriptor raised to the power 2) was collinear with its non-squared descriptor. ΔG_w , PSA_w , ΔG_h , and CDS_h were selected for further consideration, by virtue of their simplicity (compared to squared terms) and their collective collinearity with all other parameters. PSA_w and PSA_h were essentially perfectly collinear, with Pearson $r = 0.99$. Although either could have been selected, PSA_w was selected over PSA_h , since PSA_w is frequently used to explain permeability.

Table 6 shows the single regression analysis results of permeability versus each ΔG_w , PSA_w , ΔG_h , CDS_h , and PSA_w^2 . As described in the Results and Discussion, the final model included ΔG_w and PSA_w^2 .

R = H, CH₃, C₂H₅, CH₂OH, C₆H₅, CH₂C₆H₅, CHO, COO⁻, CONH₂, F, Cl, OH, NH₂

R' = C₆H₅, NH₂

Fig. 1.
Pyridine analogs.

Fig. 2.

Solute transport across a lipid bilayer. For solutes with high bilayer permeability, diffusion across the “aqueous boundary layer” is rate-limiting. At the bilayer level, the solubility-diffusion model for membrane transport highlights (a) solute partitioning from solution into the membrane and (b) solute diffusion across the membrane barrier domain as kinetic steps in membrane permeation (37–39). Partitioning is composed of aqueous solute desolvation and subsequent solute insertion into the membrane barrier domain. Legend: 1 – diffusion across the aqueous boundary layer; 2 – aqueous solute desolvation; 3 – solute insertion into barrier domain; 4 – solute diffusion across barrier domain; 5 – solute partition from membrane.

Fig. 3. Observed and predicted permeability values. Predicted values were based upon eqn 3 (i.e. computational approach). Error bars represent SEM and illustrated when sufficiently large to drawn clearly.

Fig. 4.

Thermodynamic cycle for the partitioning of a pyridine between an aqueous phase and a lipid phase. ΔG_{aq} and ΔG_{lipid} are the free energies of solvation from the gas phase into water and lipid, respectively. ΔG_K is the free energy of partitioning from the aqueous phase into lipid, and is equal to $\Delta G_{lipid} - \Delta G_{aq}$. In the context of Fig. 2, solute transport will require solute partitioning via (a) aqueous solute desolvation [step 2 in Fig. 2] and (b) subsequent solute insertion into barrier domain [step 3 in Fig. 2]. Figure 4 indicates the two component processes of partitioning are determined by ΔG_{aq} and ΔG_{lipid} , respectively.

Fig. 5. Relation between π and ΔG_w . For a majority of substituents, π can be explained by ΔG_w , particularly for hydrophilic substituents (i.e. $\pi < 0$). The large π of 3-CH₂C₆H₅, 3-C₆H₅, and 4-C₆H₅ (open circles) is not reflected by ΔG_w since the hydrophobicity of the substituents is benefited from their favorable ΔG_h . In contrast to 3-NH₂, 4-NH₂ (open triangle) did not follow the general pattern between π and ΔG_w , due to the inability of π to anticipate resonance effects on the pyridine ring nitrogen.

Table 1

Hansch properties values.

Substituent	π	π_+	π_-	σ	E_s
3-COO ⁻	-4.36	0	-4.36	-0.10	-0.55
4-NH ₂	-1.19	0	-1.19	-0.66	-0.61
3-CONH ₂	-1.49	0	-1.49	0.28	-0.55
3-Cl	0.53	0.53	0	0.37	-0.97
3-CHO	-0.65	0	-0.65	0.35	-0.55
3-OH	-0.67	0	-0.67	0.12	-0.55
3-CH ₂ OH	-0.62	0	-0.62	0.00	-1.21
3-C ₆ H ₅	1.96	1.96	0	0.06	-3.43
3-NH ₂	-1.19	0	-1.19	-0.16	-0.61
3-CH ₂ C ₆ H ₅	2.01	2.01	0	-0.08	-1.51
3-C ₂ H ₅	1.02	1.02	0	-0.07	-1.31
3-H	0.00	0.00	0	0.00	0.00
3-CH ₃	0.50	0.50	0	-0.07	-1.24
3-F	-0.17	0	-0.17	0.34	-0.55
4-C ₆ H ₅	1.96	1.96	0	-0.01	-3.43

Table 2

Caco-2 permeability values and computational property values. Computational properties include pK_a of the pyridine nitrogen, free energy of solvation (ΔG, kcal/mol), solvent accessible surface area (SASA, Å²), polar surface area (PSA, Å²), fraction of PSA (fPSA), cavitation energy (CDS, kcal/mol) and number of hydrogen bonds (HB). The environment is indicated by the subscripts w (water) or h (hexane). Polar surface area is the sum of surface area from nitrogen, oxygen, fluorine, chlorine and their attached hydrogens. fPSA = PSA/SASA.

Substituent	Permeability ×10 ⁶ (cm/s) ^c	pK _a	ΔG _w (kcal/ mol)	SASA _w (Å ²)	PSA _w (Å ²)	fPSA _w	CDS _w (kcal/ mol)	HB _d	ΔG _h (kcal/ mol)	SASA _h (Å ²)	PSA _h (Å ²)	CDS _h (kcal/ mol)	MW
<i>a</i> ₃ -COO ⁻	6.40 (0.33)	4.82 ^b	-71.97	283.1	172.5	0.61	-7.2	3	-5.64	358.6	164.0	-2.95	122.1
<i>a</i> ₄ -NH ₂	29.1 (1.1)	9.11 ^b	-71.11	248.3	130.2	0.52	-4.22	4	-6.26	329.8	125.3	-3.28	95.1
3-CONH ₂	52.9 (1.1)	4.48	-14.09	288.6	179.0	0.62	-6.23	5	-7.18	367.1	174.6	-3.54	122.1
3-Cl	60.2 (5.5)	2.84 ^b	-4.59	257.3	141.2	0.55	-1.06	1	-5.17	333.4	127.3	-3.85	113.5
3-CHO	65.9 (5.0)	0.97	-8.14	263.4	120.3	0.46	0.26	2	-5.72	343.9	100.0	-3.28	107.1
3-OH	78.0 (0.4)	6.13	-9.36	242.6	121.9	0.50	-5.24	3	-5.62	322.4	102.8	-2.79	95.6
3-CH ₂ OH	81.2 (1.3)	4.78	-8.91	265.3	119.5	0.45	-4.94	3	-5.43	356.8	104.4	-3.43	109.1
3-C ₆ H ₅	85.9 (0.7)	3.34	-4.89	322.7	51.6	0.16	0.17	1	-8.63	438.6	35.5	-6.65	155.2
3-NH ₂	95.4 (1.1)	7.52	-9.53	247.3	129.7	0.52	-5.15	4	-6.65	329.9	124.8	-3.29	95.1
3-CH ₂ C ₆ H ₅	101 (1)	6.36	-4.94	346.2	51.7	0.15	0.35	1	-9.47	470.0	35.6	-7.32	169.1
3-C ₃ H ₅	106 (1)	5.35	-3.83	265.5	51.7	0.19	-0.12	1	-5.61	374.2	35.6	-4.11	107.2
3-H	107 (8)	5.25	-4.39	216.1	51.7	0.24	-0.54	1	-4.54	306.1	35.6	-2.92	79.1
3-CH ₃	109 (4)	5.70 ^b	-4.06	241.3	51.7	0.21	-0.3	1	-5.11	341.5	35.6	-3.54	93.1
3-F	129 (9)	1.94	-3.24	230.0	96.4	0.42	0.26	2	-3.55	317.0	99.6	-2.26	98.1
4-C ₆ H ₅	130 (7)	5.40	-4.96	322.8	51.8	0.16	0.15	1	-8.68	438.8	35.7	-6.66	155.1

^aThe 3-COOH substituent (pK_a = 2.00) and the pyridine nitrogen of 4-amino pyridine were treated as ionized in water and neutral in hexane.

^b Experimental pK_{as} from the Handbook of Chemistry and Physics, 84th Edition.

^c Mean (SEM)

^d Hydrogen bond number (HB) included lone electron pairs (i.e. hydrogen bond acceptors) and hydrogens attached to an N or O (i.e. hydrogen bond donors).

Table 3

Single linear regression results.

Computational molecular descriptor	Qualitative relationship with permeability ^a	r ²
ΔG_w	Positive ^b	0.63
SASA _w	Zero	< 0.01
PSA _w	Negative	0.57
fPSA _w	Negative	0.48
CDS _w	Positive	0.44
HB	Negative	0.29
ΔG_h	Zero	< 0.01
SASA _h	Zero	0.02
PSA _h	Negative	0.52
fPSA _h	Negative	0.47
CDS _h	Zero	0.06
MW	Zero	< 0.01

^a Qualitative relationship indicates the sign of the coefficient. Positive and negative indicate an increasing or decreasing, statistically significant (i.e. $p > 0.05$) linear relationship between the molecular descriptor and permeability, respectively. Zero indicates a lack of a significant (i.e. $p < 0.05$) linear relationship.

^b ΔG_w values (and ΔG_h values) were negative for each pyridine.

Table 4

Final models from the computational and Hansch approaches.

	Computational approach	Hansch approach
Model and model parameters	$P_{app} = A \Delta G_w + B \text{PSA}_w^2 + C$ where $A = 0.801(\pm 0.124) \times 10^6 \text{ cm/sec per kcal/mol}$, $B = -0.00174(\pm 0.00029) \times 10^6 \text{ cm/sec per } \text{\AA}^2$, and $C = 116(\pm 4) \times 10^6 \text{ cm/sec}$	$P_{app} = A \pi_w + C$ where $A = 23.3(\pm 3.1) \times 10^6 \text{ cm/sec}$ and $C = 97.0(\pm 4.0) \times 10^6 \text{ cm/sec}$
Fit	$r^2 = 0.80$ $p \ll 0.001$ for all parameters	$r^2 = 0.58$ $p \ll 0.001$ for all parameters
Validation	$Q^2 = 0.77$	$Q^2 = 0.34$

Table 5

Correlation coefficient for the linear relationship between selected parameters. Results indicate aqueous desolvation to underpin substituent effect on permeability, including the basis for permeability's apparent dependence on Hansch lipophilicity.

Relationship	Linear r^2
logK versus $\Delta G_w + \Delta G_h$	1.0
logK versus ΔG_w	0.99 ($r^2 = 0.75$ if 3-COO ⁻ and 4-NH ₂ excluded)
logK versus ΔG_h	0.01
ΔG_w versus ΔG_h	< 0.01
Permeability versus π	0.51
Permeability versus π_-	0.58
Permeability versus π_- (exclude 4-NH ₂)	0.62
Permeability versus logK	0.63
Permeability versus ΔG_w	0.63
ΔG_w versus π [logK versus π]	0.50 [0.53]
ΔG_w versus π (exclude all data with $\pi > 0$ data) as well as 4-NH ₂ [logK versus π (exclude all data with $\pi > 0$ as well as 4-NH ₂ data)]	0.95 [0.93]

Table 6

Results from simple regression analysis of permeability as a function of individual computational parameters.

Parameter	r^2	Coefficient $\times 10^{6a}$	p-value
ΔG_w	0.63	1.20 (± 0.14) (cm/sec per kcal/mol)	< 0.001
PSA_w	0.57	-0.573 (± 0.077) (cm/sec per Å)	< 0.001
PSA_w^2	0.60	-0.00276 (± 0.00034) (cm/sec per Å ²)	< 0.001
ΔG_h	< 0.01	0.27 (± 3.36) (cm/sec per kcal/mol)	< 0.01
CDS_h	0.06	-5.71 (± 3.46) (cm/sec per kcal/mol)	0.10

^a Mean ($\pm$ SEM)