Predicting Drug Absorption from Molecular Surface Properties Based on Molecular Dynamics Simulations

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Purpose. To develop an efficient method for generating representative conformations for calculation of the conformationally dependent molecular surface area, and to investigate the relation between this parameter and the permeability in Caco-2 cells.

Methods. High temperature molecular dynamics (MD) simulations were used to obtain 1000 conformations of six beta-blocking agents and their prodrugs. The Boltzmann averaged (B.a.) polar surface area of the 1000 conformations was correlated to the apparent permeability coefficients (P_{app}) for transport across filter-grown Caco-2 cells.

Results. Sampling of 1000 conformations during the MD simulations was sufficient for obtaining a representative set of conformations. The B.a. polar water accessible surface area (PWASA) yielded an excellent linear correlation with P_{app} for both series of compounds under study ($R^2 = 0.98$). Thus, the improved permeability of the prodrugs could be explained by a reduced PWASA. The improvement of permeability after derivatization correlated positively with the size of the non-polar water accessible surface area—suggesting a synergistic effect of the cyclopropyl and the non-polar parts of the molecule to shield the polar parts from contact with water.

Conclusions. An efficient method for generating the representative conformations for calculation of the B.a. polar surface area has been established. An excellent linear correlation explaining the improved permeability of the prodrugs was obtained.

KEY WORDS: molecular dynamics; conformational analysis; polar surface area; absorption; Caco-2 cells.

INTRODUCTION

The importance of considering the absorption properties of new chemical entities (NCE) early in the process of drug development has been recognized (1–5). It is attractive to be able to predict the permeability from molecular characteristics derived entirely from theoretical calculations. That may obviate experimental determinations of permeability and even the actual synthesis of compounds with poor predicted permeability. The two main factors determining the passive transport of a solute across a biological membrane are the hydrogen bonding capacity and the size of the solute (3). Within homologous series of compounds the hydrogen bonding potential may appear to be the only determining factor (3) as seen for peptides (6–8) and β-blocking agents (9). In molecular terms, the hydrogen bonding potential may be expressed as the polar part of the molecular surface area. In the first study relating this parameter to permeability, a reasonable inverse relationship was found between the polar surface area and the blood-brain barrier permeability using only one conformation of each of structurally diverse compounds (10). However, more recent studies indicate that the predictive quality is improved when taking into account the conformational variability of the polar surface area of flexible compounds (9,11). In an analogous fashion, it has been shown that calculated partition coefficients of flexible molecules depend on the conformation (12). Recently, the importance of conformation for absorption has been elaborated: small lipophilic peptides with a strong tendency to adopt folded conformations in solution were absorbed 100-fold faster in Caco-2 cells than peptides without this tendency (13). A sigmoidal relationship between the conformationally dependent polar surface area and absorption fraction in humans (11) has been reported. This may suggest that a certain polar surface area is limiting for transcellular transport so that the lower part of the sigmoidal curve corresponds to pure paracellular transport. Such a limiting polar surface area has been found for other classes of compounds as well (14,15). In these two latter studies the conformationally dependent polar surface area was unsuccessful in predicting the absorption of peptidomimetics, possibly because the absorption was primarily paracellular.

In order to take into account the conformational variability of the polar surface area, it is necessary to do a conformational analysis of each compound. We have chosen high temperature molecular dynamics, (MD) for generating representative conformations (16). By representative conformations it is inferred that, provided that the conformational space is covered sufficiently, the generated conformers account for the structural properties of the molecule. In an MD simulation, a term of kinetic energy is added to the overall steric energy of the molecule so as to move the atoms relative to each other. If enough energy is added, the potential energy barrier between different conformations may be overcome and, thus, different conformers are generated.

In this paper, we are reporting the use of an efficient molecular dynamics method for calculating the conformationally dependent polar surface area which exhibits an excellent inverse correlation with permeability in Caco-2 cells for two series of compunds.

MATERIALS AND METHODS

Model Compounds and Transport Studies

A total of eleven compounds from two different series were used as model compounds. The first series of compounds consisted of six beta-blocking agents of varying lipophilicity, the second series of the corresponding O-cyclopropane carbox-

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ABBREVIATIONS: TVDW, total van der Waal's surface area; PVDW, polar van der Waal's surface area; NPVDW, non-polar van der Waal's surface area; VDWVOL, van der Waal's volume; TWASA, total water accessible surface area; PWASA, polar water accessible surface area; NPWASA, non-polar water accessible surface area; P_{app}, apparent permeability coefficient in Caco-2 cells; MD, Molecular dynamics; E_{rel}, Steric energy relative to the global energy minimum conformation.

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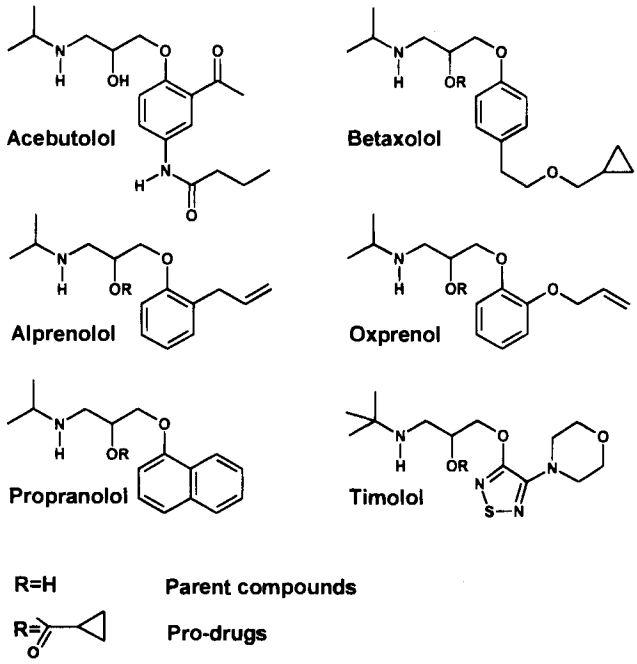

Fig. 1. Molecular structure of the model compounds: six β-blocking agents (series 1) and five corresponding O-cyclopropane carboxylic acid ester prodrugs (series 2).

ylic acid ester prodrugs (Fig. 1). The physico-chemical properties and apparent permeability coefficient (P_{app}) for transport across Caco-2 monolayers of all compounds taken from Hovgaard *et al.* (17) are listed in Table I. Detailed procedures for synthesizing the bioreversible prodrugs and transport across Caco-2 cells have previously been published (17).

Calculation of Molecular Size Parameters

All calculations were performed using the Tripos force field (Sybyl version 6.2) (18). All energy minimizations were employing a convergence criteria of 0.005 kcal/mol/Å. The dielectric constant, ε, was set to 4 to indirectly account for the effect of solvation on the intramolecular interactions. Each compound was built with the common side chain in an extended conformation and subjected to a preliminary energy minimization without considering electrostatics. The ester group of the prodrugs was built in an s-*trans* conformation. Calculation of electrostatical potential derived partial charges were performed in Spartan (19) by the semiempirical method AM1 (20) with the amines taken to be in their neutral form. Finally, the molecules were energy minimized in Sybyl with the electrostatic interactions included.

Table I. Physicochemical Properties and Apparent Permeability Coefficients (P_{app}) for Transport Across Caco-2 Monolayers for the Eleven Model Compounds (17)

Compound	ID	P_{app}($\times 10^6$cm/s) ± STDS	MW	log D[a]
Acebutolol	A	4.45 ± 0.45	372.93	−0.04
Alprenolol	B	75 ± 7.7	285.84	0.31
Betaxolol	C	48.5 ± 8.8	343.94	0.28
Oxprenolol	D	65.5 ± 2.2	301.84	0.45
Propranolol	E	82.4 ± 5.1	295.84	1.38
Timolol	F	44.3 ± 2.8	432.52	−0.04
Alprenolol ester	b	108 ± 4.7	433.44	2.78
Betaxolol ester	c	96.5 ± 9.2	491.54	0.63
Oxprenolol ester	d	97.2 ± 13.7	485.94	1.98
Propranolol ester	e	104 ± 6.1	411.94	3.02
Timolol ester	f	78.9 ± 4.5	500.54	1.74

[a] Partition coefficient between n-octanol and buffer pH 7.4.

Generation of Conformations

The energy minimized structures were used as starting conformations for MD simulations (16) with sampling every picosecond (ps). It consisted of a 10 ps heating period and 1000 ps productive sampling at a temperature of 1000 K—thus generating 1000 conformations for each compound. Due to the high simulation temperature, the generated structures were slightly distorted and, therefore, all conformations were energy minimized, yielding 1000 representative conformations.

Calculation of the Boltzmann Weighted Molecular Properties

Pearlmans analytical algorithm Savol3 (21) for calculating molecular surface and volume parameters was used to calculate the following properties of all conformations of the compounds: total van der Waals surface area (TVDW), polar van der Waal's surface area (PVDW), van der Waal's volume (VDWVOL), total water accessible surface area (TWASA) and polar water accessible surface area (PWASA). The van der Waal's surface area is defined by the overlapped vdW radii of the molecule, whereas the water accessible surface area is defined by the surface tracked out by the center of a hypothetically spherical solvent molecule (here: water) rolling over the entire molecule (22). Formally, this corresponds to adding the radius of the solvent molecule to the vdW radius of the atoms—for water this is taken to be 1.4 Å. The polar surface area was defined as the surface contribution from all oxygen and nitrogen atoms plus the hydrogen atoms attached to them (9), i.e. the atoms capable of forming hydrogen bonds.

The molecular size parameters for each compound were weighted according to the probability, P_j, of each conformation j as given by a normalized Boltzmann distribution at 310 K (37°C) (eq. 1):

$$P_j = \exp(-\Delta E_j/RT)/\sum_j^N \exp(-\Delta E_j/RT) \qquad (1)$$

where ΔE_j is the relative steric energy of the j'th conformation, R is the gas constant, T is the temperature (in Kelvin) and N is the total number of conformations. All calculations (MD, energy minimizations, Savol calculations) were performed by means of spl (Sybyl programming language) macros—thus allowing for a semi-automated procedure.

Data Analysis

The correlation between the Boltzmann weighted parameters and P_{app} was analyzed by means of multiple linear regression using Modde 3.0 (23). The significance level was 5%. Squared correlation coefficients (R^2) as well as the predictive

powers (Q^2) were calculated, where the former expresses the percentage of variation explained by the regression whereas the latter expresses the predictive power according to leave-one-out cross validation.

As already mentioned by Hovgaard *et al.* (1995) (17), pro-acebutolol was the only prodrug for which neither the purity nor the fraction being degraded during the transport studies were determined. Therefore, this compound was excluded from the analysis.

RESULTS AND DISCUSSION

Sampling of the Conformational Space

A prerequisite for using molecular dynamics for conformational analysis is that the entire conformational space is covered. For this, two requirements have to be fulfilled: 1) sufficient kinetic energy is added to overcome all possible energy barriers between the conformations and 2) the simulation is carried out for a sufficiently long time to allow visiting of all minima.

It has been shown that 1000 K is required to obtain ring inversion in 6-membered rings (16) where the energy barriers are typically in the range of 10 kcal/mol. In the β-blockers, the rotatable bonds are typically sp3-sp3 hybridized carbon-carbon or carbon-nitrogen bonds for which energy barriers in the range of 3 kcal/mol can be expected. The assumption that a temperature of 1000 K is sufficient is confirmed when examining Figure 2. As an example of the sampling efficiency of the simulation, this figure shows the distribution of two torsional angles in the side chain of 1000 conformations of propranolol before and after energy minimization. The conformational space is sampled as would be expected from the nature of the side chain—the energetically favored torsional angles being ±60° and 180°. None of the expected torsional angles are missing from the plot. Therefore, it may be concluded that the selected combination of kinetic energy input and simulation time is sufficient to sample the conformational space. A similar conclusion could be drawn from plots of the corresponding torsional angles as well as other torsional angles obtained after MD simulations of the rest of the compounds (results not shown).

A plot of preferred conformations employing just two torsional angles like the one in Figure 2 does not show if all combinations of torsional angles for the entire molecule have been covered. To verify that, it is necessary to analyze properties which vary with the conformation of the entire molecule such as the molecular size parameters. Figure 3 shows the trajectory of the PWASA during a 1000 ps simulation of propranolol (energy minimized conformations). When dividing the simulation into 10 equally large time intervals each containing 100 conformations, a one-way ANOVA reveals a significant difference in the mean PWASA ($p = 0.04$), whereas there is no statistical difference between the mean PWASA of five 200 ps intervals (one-way ANOVA, $p = 0.52$). On the other hand, if calculating the Boltzmann weighted PWASA instead of the mean PWASA there is a significant difference between the two first and the three last 200 ps intervals (one way ANOVA, $p = 0.045$). This means that 200 ps is sufficient to obtain a stable mean PWASA, i.e. a stable simulation, but not a stable Boltzmann weighed PWASA, i.e., sampling for 1000 ps is necessary. When including more and more conformations in the sampling interval, more low energy confomations are sampled. This stabilizes the B.w. PWASA due to the larger number as also discussed by Palm *et al.* (9).

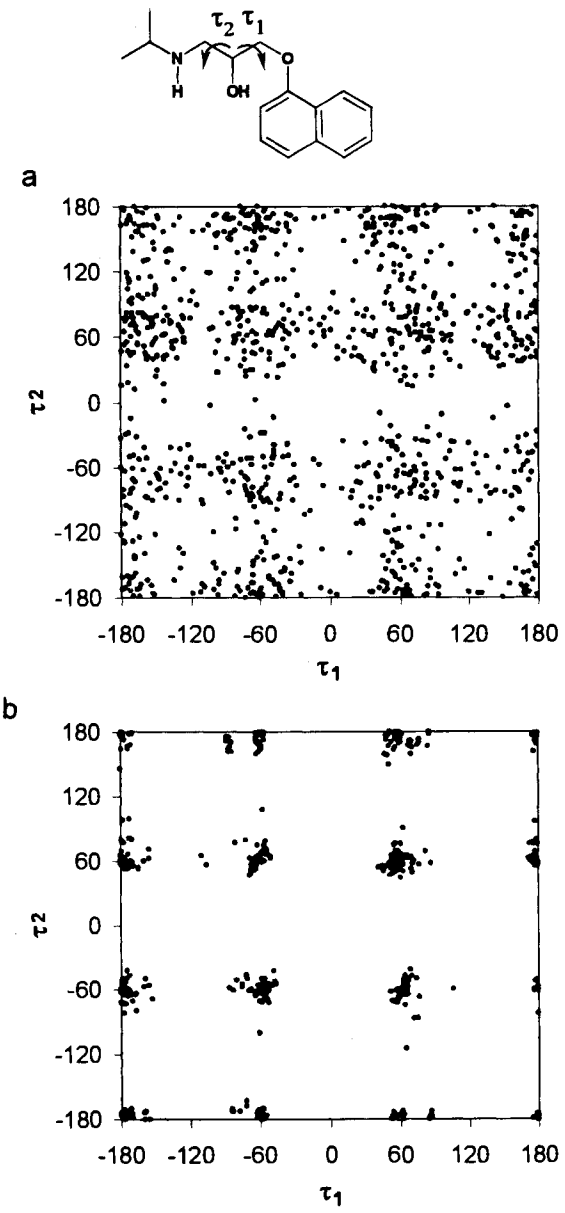

Fig. 2. Distribution of the torsional angles τ_1 and τ_2 of 1000 conformations of propranolol before (a) and after (b) energy minimization. As expected from the nature of the side chain, the preferred torsional angles are ±60° and 180°.

The 1000 ps MD simulation has been repeated for six of the compounds: acebutolol, betaxolol, propranolol, pro-alprenolol, pro-oxprenolol, and pro-timolol. A two-tailed paired t-test showed no significance of the Boltzmann weighted size parameters being different between the replicates. The standard deviation of PWASA between the simulations ranged from 0.05% to 4%. This finding supports the above finding that the combination of 1000 K and 1000 ps sampling is appropriate for sampling the conformational space and thus the variation of molecular size parameters with conformation. As long as the number of conformations is sufficient, it is not necessary to optimize the minimum number of conformations, but it is definitely interest-

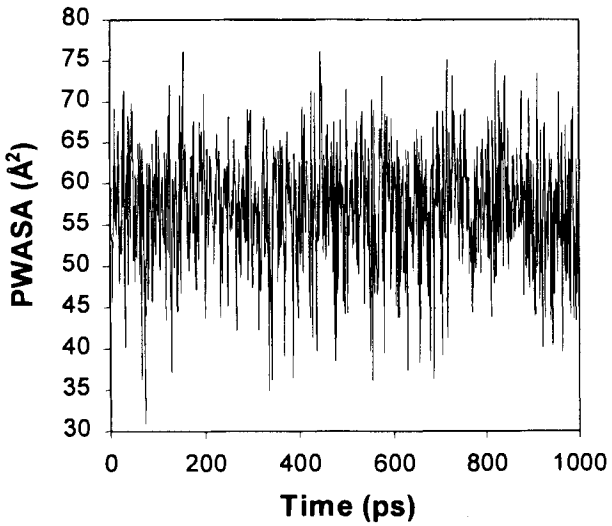

Fig. 3. Trajectory of the PWASA of propranolol during a 1000 ps MD simulation (energy minimized conformations).

ing to do so in the future in order to minimize computational time. For more flexible compounds probably more than 1000 conformations would be needed.

It could be argued that sorting according to e.g. 3-D diversity criteria could reduce the 1000 conformations. However, this is not feasible as numerous low-energy minima with similar but not identical conformations exist. By sorting, there would be a risk of losing structural information and, therefore, we have chosen to use all 1000 conformations.

Correlation of Boltzmann Weighted Parameters with Permeability

Table II shows the results of linear regressions of each of the Boltzmann weighted parameters with log P_{app}. The best linear correlation with the transport data was found with PWASA, whereas the correlation with PVDW was much poorer. This is in conflict with the findings of Palm *et al.* (9), who obtained almost identical R^2 values for the linear correlation of Boltzmann weighted PVDW and PWASA (0.99 and 0.96 respectively) to log P_{app} for six β-blocking agents—two of which are also part of the present investigation (oxprenolol and alprenolol). The most obvious explanation for this difference is that this group was studying only one series of compounds while the present investigation includes two different series.

In Figures 4a and b, log P_{app} is plotted versus PWASA and PVDW, respectively. From Figure 4b, an explanation for the poor correlation with PVDW can be inferred: the two series of drugs are defining two separate lines which can be attributed to the fact that the PVDW increases upon esterification. In contrast to that, the PWASA is explaining the permeability of the parent compounds as well as the prodrugs from one single regression line (cf. Fig. 4a). The increased permeability of the prodrugs can thus be explained by a reduction of the PWASA. The differential effects of esterification on PVDW and PWASA are illustrated by Figure 5 picturing the PVDW and PWASA of similar conformations of propranolol and pro-propranolol. The observation that PWASA is a better descriptor of the hydrogen bonding potential than PVDW is appealing in that the former takes into account the conformationally dependent steric hindrance for making hydrogen bonds with water which is not the case for PVDW.

The ratio PWASA/NPWASA explains the magnitude of log P_{app} to about the same extent as PWASA itself, but it's predictive power is somewhat poorer than that of PWASA. The same relationship was noticed by Palm *et al.* (9). This indicates that the balance between the non-polar and polar parts may be of as great importance is the PWASA itself. A similar balance is theoretically expressed by the traditionally used lipophilicity parameter, log D. This parameter does not predict the permeability of the two series under study ($R^2 = 0.33$, $Q^2 = -0.03$), and thus the calculated ratio seems to be superior to the experimental value in this regard.

The molecular size as expressed by the vdW volume is not contributing significantly to explain the permeability when included in a linear combination with PWASA. These results are only partly in accordance with the conclusions of van de Waterbeemd *et al.* (3) saying that membrane permeability is primarily determined by the hydrogen bonding potential and

Table II. Boltzmann Averaged Molecular Descriptors Used for the Regression Analysis on P_{app} in Caco-2 Cells

ID	PWASA (Å²) [range]	PWASA/NPWASA (%)	PVDW (Å²) [range]	PVDW/NPVDW (%)	VDWVOL (Å³)
A	128.9 [99.9–168.8]	29.2	85.5 [80.2–90.4]	27.1	272.9
B	52.4 [24.5–73.5]	12.1	43.3 [40.4–45.5]	15.9	213.5
C	65.3 [26.8–92.6]	13.5	54.0 [44.2–56.7]	16.1	265.5
D	56.5 [28.8–86.4]	12.9	53.2 [48.9–55.3]	19.4	220.5
E	55.7 [30.9–76.2]	13.1	43.9 [40.4–45.5]	16.7	213.1
F	69.9 [44.0–93.5]	16.0	57.4 [54.4–60.7]	19.8	240.5
b	35.9 [8.9–78.0]	7.0	47.2 [39.2–50.0]	13.8	265.0
c	45.1 [21.8–94.8]	8.8	57.2 [48.8–59.9]	16.7	272.1
d	43.2 [7.7–77.8]	8.8	47.7 [48.8–59.7]	14.4	264.6
e	44.0 [12.7–74.6]	7.8	57.8 [39.4–50.0]	14.3	313.4
f	55.2 [17.7–94.8]	10.3	62.2 [53.3–65.2]	17.3	292.1
R^2	0.98	0.95	0.72	0.87	0.00
Q^2	0.93	0.81	−0.01	0.54	−0.33

Note: In brackets is given the range for all 1000 conformations. VDWVOL varies less than 0.5% so the range has been omitted. Results from the regression analysis are reported as squared correlation coefficients (R^2) and predictive powers (Q^2) by the leave-one-out method.

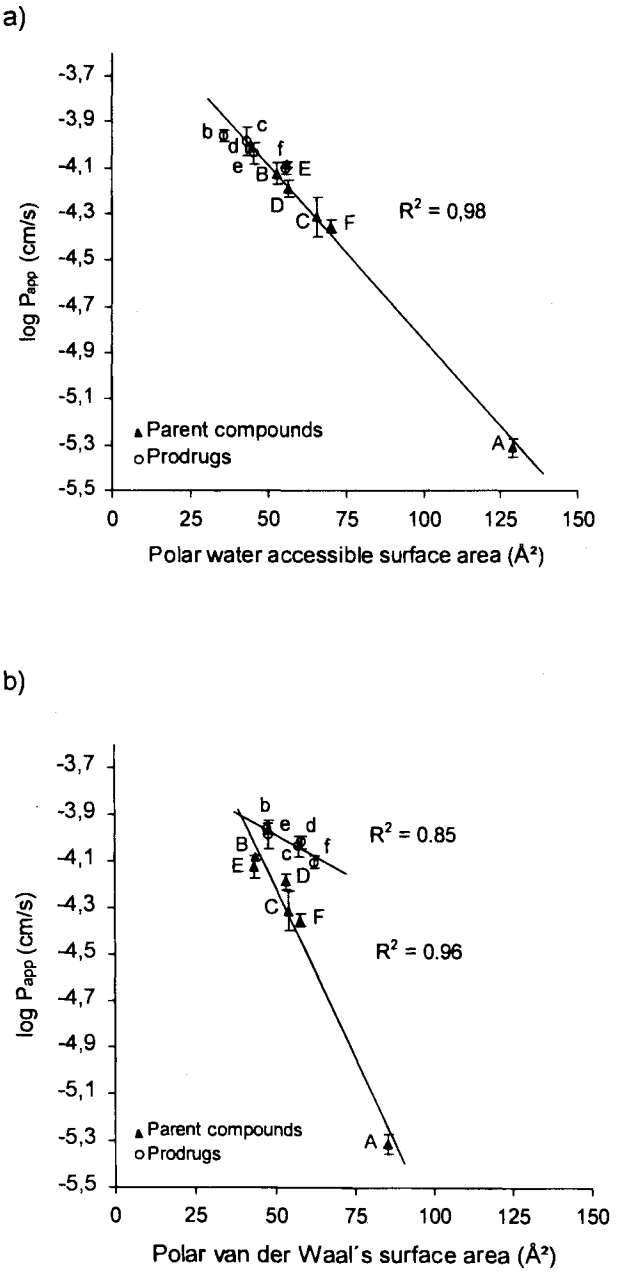

Fig. 4. Log P_{app} plotted versus the Boltzmann weighted polar water accessible surface area (a) and polar van der Waal's surface area (b). PWASA explains the permeability of both series of compounds from one single regression line. PVDW increases upon esterification and the two series define two different regression lines.

secondarily by the molecular size. The range of molecular size of the model compounds used here may be too narrow to see an effect. The range of vdW volume was 213-313 Å^3 versus 125-364 Å^3 in the study by van de Waterbeemd *et al.* (3).

Unfortunately, the P_{app} of acebutolol and the nearest compound, timolol, differ by a factor of ten so that acebutolol contributes relatively much to the regression analysis. Palm *et al.* (9) has shown that the correlation between the polar surface area and log P_{app} can be extended beyond oxprenolol to reach a polar surface area comparable to the one of acebutolol (atenolol). Therefore, it is reasonable to assume that no serious error is made when we are interpolating from betaxolol to acebutolol. If excluding acebutolol from the regression analysis, the range of P_{app} is narrowed and thus the explanatory power is reduced ($R^2 = 0.92$). Nevertheless, a similar model to explain the permeability is obtained, i.e. PWASA is the main explanatory descriptor and no additional information is gained when including molecular size descriptors in the regression analysis.

The two nitrogens of the 1,2,5-thiadiazole ring of timolol and pro-timolol were not included in the definition of the polar surface area as they are regarded as being very reluctant hydrogen bond acceptors. The pKa-value of the 1,2,5-thiadiazole is −4,90 (24). This extremely low basicity is ascribed to the inductive effect of the ring sulfur in the alpha-position which withdraws electrons from the nitrogen lone pair so that the pi-orbital in the ring is of high electron density and the lone pair electron density on the nitrogen atoms is correspondingly low (24). Furthermore, a search for the 1,2,5-thiadiazole moiety in the Cambridge Structural Database (version October 1996) (25) suggested that the two nitrogens are not obvious hydrogen-bond acceptors in crystal structures. Of 103 crystal structures, only one had the right orientation and distance between the donor (an amino group) and the acceptor atom. The suggested hydrogen bond would be rather weak as the distance was longer than 3 Å. This example indicates that the defintion of the polar surface area may be refined so as to take into account the strength of the hydrogen bonds.

Being amines, both series of compounds are highly ionized at physiological pH. However, in the calculations only desolvation properties of the uncharged species are considered. This is a formal error, which is not readily solvable as it is an inherent problem to handle charged species adequately in molecular mechanics calculations. However, it does not seem to influence the results here, which may again be attributed to the similarity of the compounds. On the other hand, it could also be due to the fact that only the uncharged species can cross the epithelium transcellularly. The rate determining step may be the desolvation of the uncharged species and not the deprotonation of the charged species. Such an explanation would also be in concordance with the results. As judged from the magnitude of the P_{app} values, the compounds under study predominantly use the transcellular route.

Effect of Esterification

Even though the chemical modification of the side chain is identical for the five β-blockers, the magnitude of the improvement of the P_{app} (ΔP_{app}) after esterification is varying. ΔP_{app} can be explained and predicted quite well by the size of the Boltzmann weighted non-polar water accessible surface area, NPWASA:

$$\Delta P_{app} = 23.7\ (\pm 3.6)\ \text{NPWASA} + 32.2\ (\pm 1.3) \qquad (2)$$

$$(n = 5\ R^2 = 0.94\ Q^2 = 0.84\ \text{df} = 3)$$

This result may arise from a combined and perhaps even synergistic effect of the cyclopropyl group and the non-polar parts of the adjacent substituents on the aromatic ring to shield the polar groups of the molecule.

The increased permeability of the prodrugs is not merely a result of an increase in the lipophilicity of the drug, but also a reduction of the hydrophilicity of specific functional groups.

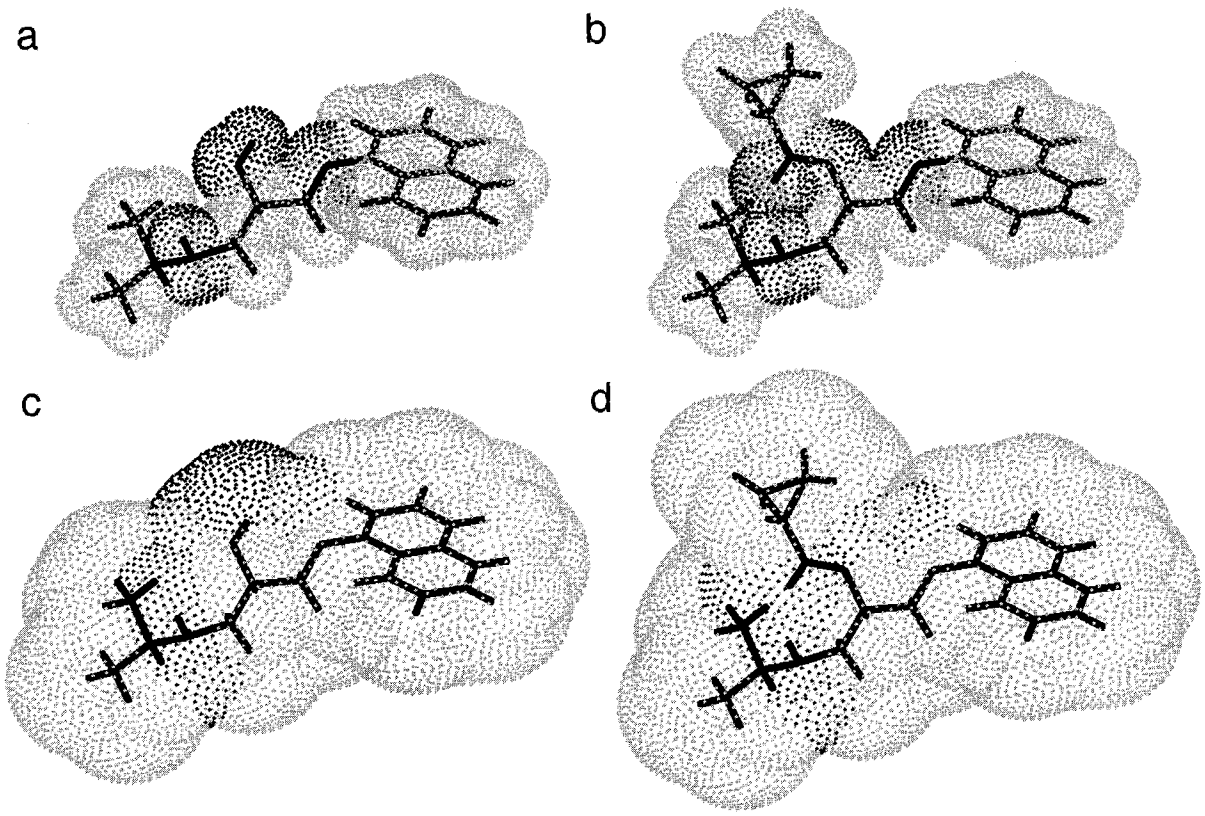

Fig. 5. Illustration of the differential effects of esterification on the size of PWASA and PVDW shown for similar conformations of a parent compound (propranolol) and a prodrug (pro-propranolol). Legend. **a** : vdW surface of the parent compound. **b**: vdW surface of the prodrug. **c**: water accessible surface area (WASA) of the parent compound. **d**: WASA of the prodrug. Dark shading: polar surface area, light gray shading: non-polar surface area. The cyclopropyl shields part of the PWASA of the ester oxygens and the amine reducing the PWASA from 55.2 $Å^2$ to 31.6 $Å^2$. The reduced PWASA thus explains the improved permeability of the prodrug. The PVDW is not reduced by the presence of the cyclopropyl (44.7 vs 44.7 $Å^2$).

In this case, the cyclopropane moiety is shielding the ester oxygens and partly the amine from contact with water and is thus facilitating the transport across the cell membrane.

It has been proposed that simply counting the number of potential hydrogen bond donors and -acceptors gives an estimate of the permeability (3,6). Esterification alters the number of potential hydrogen bonds to a similar extent for all the compounds under study and thus this parameter can not be used for a quantitative prediction of the beneficial effects of derivatization. Clearly, the improved permeability is dependent on a conformational effect on the PWASA as discussed above. The conformationally averaged hydrogen bonding parameter of PWASA is of potential use for predicting the effect on permeability of other kinds of derivatization and possibly identifying which one is the optimal one.

Relevance of Boltzmann Weighting

Boltzmann weighting of the molecular size parameters does not seem to yield any extra information compared to weighting each conformation equally by 1/1000 (for PWASA: $R^2 = 0.985$, $Q^2 = 0.950$). On the other hand, the mean PWASA is statistically different between high-energy and low-energy conformations (for propranolol: 373 conformations with $E_{rel} <$ 2.5 kcal/mol, mean PWASA 56.4 $Å^2$; 373 conformations with 2.5 kcal/mol $< E_{rel} <$ 3.56 kcal/mol, mean PWASA 57.4 $Å^2$; two-tailed t-test $p = 0.044$), which explains the observation that the mean PWASA is consistently higher than the Boltzmann weighted. Nevertheless, this has no influence on the predictive power of the PWASA; which may be attributed to the large number of conformations leveling out the differences between the high- and low-energy conformations. Thus 1000 conformations seem to be sufficient to level out the differences between Boltzmann weighting and averaging, while 200 are not according to the above findings.

Still, it must be regarded as being more correct to use the Boltzmann weighted parameters. At a temperature of 1000 K the high energy levels are more populated than at 310 K. Therefore, when doing a MD simulation at 1000 K, the high energy conformations are sampled more frequently and thus are slightly overrepresented. The error introduced by this procedure is smaller when calculating the Boltzmann weighting than when averaging the 1000 conformations.

Starting Conformation

When using the PWASA of the starting conformation, an R^2 of 0.978 and Q^2 of 0.912 is obtained. It may thus seem superfluous to do the conformational analysis. However, the starting conformation of the common side chain was the same

for all the compounds, which may not be that different after all. The error of using just one conformation for predicting absorption would be elaborated if using a series of more structurally diverse flexible compounds. The existence of several preferred conformations different from an extended one could seriously deterioate the predictive value. At least for this class of compounds an initial estimate of the transport properties can be obtained from the PWASA of a conformation with all the side chains in an extended conformation.

CONCLUSIONS

When it comes to predicting the effect of esterification on P_{app}, the starting conformation is no longer useful. If trying to use the same model as above (NPWASA) the predictive power is negative. In order to do a more detailed analysis of the relationship between molecular properties and permeability, it is of utmost importance to do a conformational analysis—especially when the effects as here are synergistic and not additive.

In conclusion, we have demonstrated how a semi-automated conformational analysis can be used to obtain detailed information about the absorption properties of two different series of drug molecules. An excellent predictive equation for absorption from one single molecular parameter was obtained, for which the importance of conformation was suggested but not unambigiously proven. However, the improvement of absorption after a chemical modification was shown to be related to conformationally dependent molecular properties, for which conformational analysis was crucial. It remains to be seen if these relationships hold for more structurally diverse compounds like peptides. Work is currently under way in our laboratory to elucidate this matter.

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