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High-throughput evaluation of relative cell permeability between peptoids and peptides

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ABSTRACT

Peptides are limited in their use as drugs due to low cell permeability and vulnerability to proteases. In contrast, peptoids are immune to enzymatic degradation and some peptoids have been shown to be relatively cell permeable. In order to facilitate future design of peptoid libraries for screening experiments, it would be useful to have a high-throughput method to estimate the cell permeability of peptoids containing different residues. In this paper, we report the strengths and limitations of a high-throughput cell-based permeability assay that registers the relative ability of steroid-conjugated peptides and peptoids to enter a cell. A comparative investigation of the physicochemical properties and side chain composition of peptoids and peptides is described to explain the observed higher cell permeability of peptoids over peptides. These data suggest that the conversion of the monomeric residues in peptides to an *N*-alkylglycine moiety in peptoids reduced the hydrogen-bonding potential of the molecules and is the main contributor to the observed permeability improvement.

1. Introduction

Peptides are excellent ligands for proteins and, in particular, are often capable of targeting regions of proteins not easily recognized by traditional small molecules, such as protein interaction surfaces. However, the practical utility of peptides as drugs or tools for chemical biology is limited by their sensitivity to proteases and their lack of cell permeability. Therefore, there is considerable interest in the development of compound classes with the protein-binding properties of peptides, but more favorable pharmacokinetic properties. Towards this goal, we and others have demonstrated that peptoid[1] (oligo-N-substituted glycines) libraries[2,3] are excellent sources of protein-binding ligands.[4–7] As expected, peptoids are not sensitive to proteases or peptidases.[8]

We have reported previously a cell-based assay in which entry of a peptoid- or peptide-steroid conjugate triggers the expression of a reporter gene in a dose-dependent fashion,[9] allowing one to compare the relative cell permeability of various peptide- or peptoid-steroid conjugates.[9] We have found that the movement of these compounds into cells requires passive diffusion and does not appear to involve any form of active transport.[10] Using this assay, we demonstrated that many peptoid tetramers from a combinatorial library are quite cell permeable, in that the peptoid-steroid conjugate induces reporter gene expression in the cell-based assay almost as well as the steroid alone. In other words, many tetrameric peptoids do not diminish the cell permeability of the attached steroid. Many octameric peptoids were also found to be cell permeable by this criterion, though less so than the tetramers, as expected.

We have also employed this assay to carry out careful comparisons of the relative cell permeability of a small number of isomeric peptides and peptoids. These experiments, which involved titration of the indicator cells with different concentrations of the

Abbreviations: AEEA, acetyl-ethyleneglycol-ethyl-amine; BBB, blood–brain barrier; DBD, DNA-binding domain; Dex, dexamethasone; DIC, diisopropylcarbodiimide; DIPEA, diisopropylethylamine; EC_{50}, half-maximal effective concentration; Fmoc, fluorenyl-methoxy-carbonyl; GR, glucocorticoid receptor; HATU, *O*-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HoAt, 1-hydroxy-7-azabenzotriazole; HoBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Hsp, heat shock protein; IC_{50}, half-maximal inhibitory concentration; LBD, ligand-binding domain; log*P*, octanol–water partition coefficient; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; OxDex, oxidized derivative of dexamethasone; PR, permeability ratio; PSA, polar surface area; SDex, isothiocyanate derivative of dexamethasone; TFA, trifluoroacetic acid; TPSA, topological polar surface area

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peptide- and peptoid-steroid conjugates, confirmed that peptoids are anywhere from 3- to 30-fold more permeable than the analogous peptide, depending on the size of the molecule.[11]

We were curious to determine if this conclusion was valid in general for peptoids and peptides with a wide diversity of side chains. In this report, we employ the cell-based permeability assay in a high-throughput mode to address this issue. The large number of compounds employed in this study precluded carrying out titrations and demanded a single-point analysis for each compound. In this paper, we address the technical issue inherent in carrying out such a screen and also consider various physical models to rationalize the results. In general, the data support the idea that peptoids are generally more cell permeable than peptides and that this difference can be attributed largely to the absence of the highly polar N–H main chain bond in peptoids.

2. Results and discussion

2.1. Library design and synthesis

Our previous studies with steroid-conjugated peptoid and peptide analogs demonstrated that as the length of the molecule increases from a dimer to an octamer, the cell permeability decreases.[9–11] Based on this observation, we decided to synthesize libraries of tetramers for our current high-throughput comparison study as a compromise between maintaining the diversity of the libraries and retaining decent permeability within the molecules. The peptoid libraries were synthesized using the 'sub-monomer' synthesis[3] in a conventional split-and-pool approach. The amines employed in this chemistry contained cationic, anionic, hydrophobic, and neutral moieties (Fig. 1A). The peptide libraries displayed comparable side chains (Fig. 1B) and were constructed by standard solid-phase Fmoc chemistry. High-capacity polystyrene macrobeads were used to generate a sufficient amount of compound per bead (~80 nmol) for the cell permeability assay upon cleavage from the beads, in a one bead per well manner.

The generic structure of the libraries was OxDex-AEEA-X_4-β-Ala, where β-alanine is the invariant C-terminal residue, and OxDex is the steroid capping molecule at the N-terminus conjugated to the four variant monomers (X) via an acetyl-ethyleneglycol-ethyl-amine (AEEA) linker (Fig. 1A and B). OxDex is product of oxidative cleavage of the dexamethasone side chain.[12] Figure 1C shows the amide form of OxDex used to determine its IC_{50} value, a measure of relative binding affinity to the glucocorticoid receptor (GR). Quality control analyses were performed on both libraries by subjecting randomly selected beads from each library to high-performance liquid chromatography (HPLC), followed by mass spectrometry (MS) analysis. Nine of the ten beads showed a clear major product peak in the HPLC traces with 90% purity and a single dominant peak was observed in the mass spectra in the expected mass range. Note that OxDex was linked to the peptide or peptoid during solid-phase synthesis, and uncoupled steroid was removed by thorough washing. Thus, the solutions are not contaminated by free steroid, which would, of course, skew the results of the permeability assays. Furthermore, we have shown previously that the steroid-peptoid or -peptide linkage is stable in cell culture medium.[10,11]

2.2. High-throughput cell permeability assay

The high-throughput cell permeability assay employed in this study has been described elsewhere.[9,10] A schematic of the system is depicted in Figure 2. Briefly, the OxDex conjugates were exposed to HeLa cells transfected with three plasmids. One encodes for a fusion protein containing the Gal4 DNA-binding domain, the GR ligand-binding domain and the VP16 transactivation domain (Gal4DBD-GRLBD-VP16). The apo form of this protein is sequestered in the cytoplasm in its inactive form through a tight interaction with heat shock protein 90 (Hsp90) in the absence of ligand. This interaction is disrupted by an influx of the steroid, which binds to the GR LBD, allowing the fusion protein to translocate into the nucleus. It then drives the expression of firefly luciferase expression by activating the Gal4-responsive firefly luciferase reporter gene carried by the second plasmid. We have shown that the affinity of different peptide- and peptoid-steroid complexes for the GR LBD differs only modestly, thanks to the presence of the β-alanine linker between the variable sequence and the steroid.[9,10] Therefore, expression of the firefly luciferase is dependent on the permeability of the steroid-conjugated molecule. The third plasmid carries a constitutively expressed *Renilla reniformis* luciferase gene that serves as a transfection control. The ratio of firefly luciferase activity (compound-dependent) to *Renilla* luciferase activity (compound-independent internal control) is a reflection of the concentration of steroid conjugates that have successfully permeated the cell membrane into the cell. This ratio compensates for the well-to-well variability that could potentially arise during

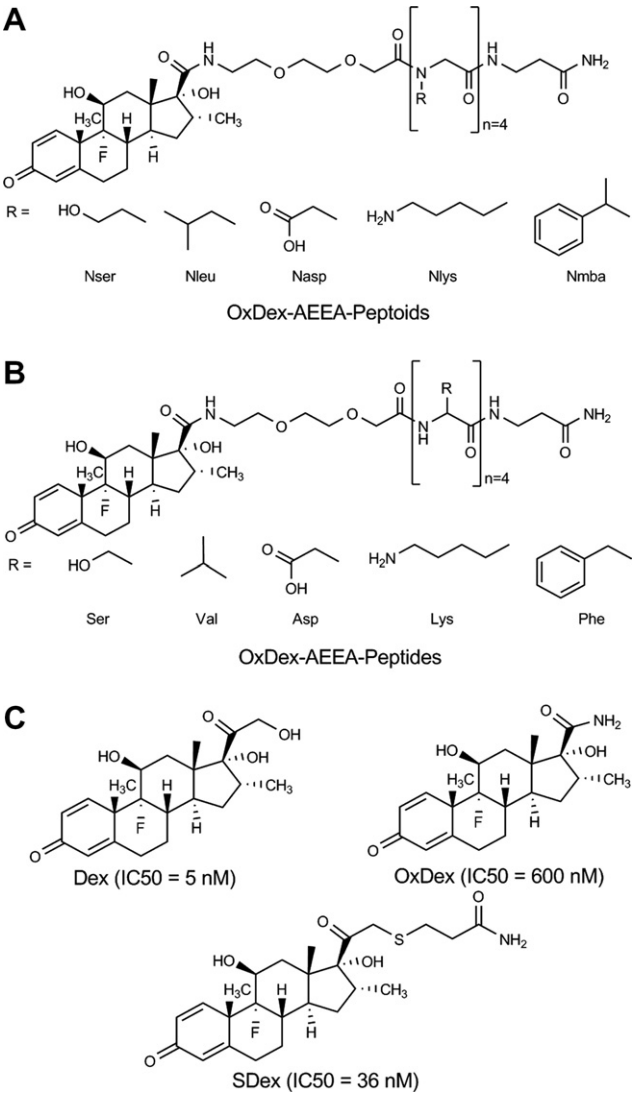

Figure 1. Chemical entities used in high-throughput study. Generic chemical structure of the OxDex-conjugated (A) peptoid and (B) peptide libraries and the side chain moieties incorporated. (C) Chemical structure of dexamethasone and its amine derivatives, OxDex and SDex.

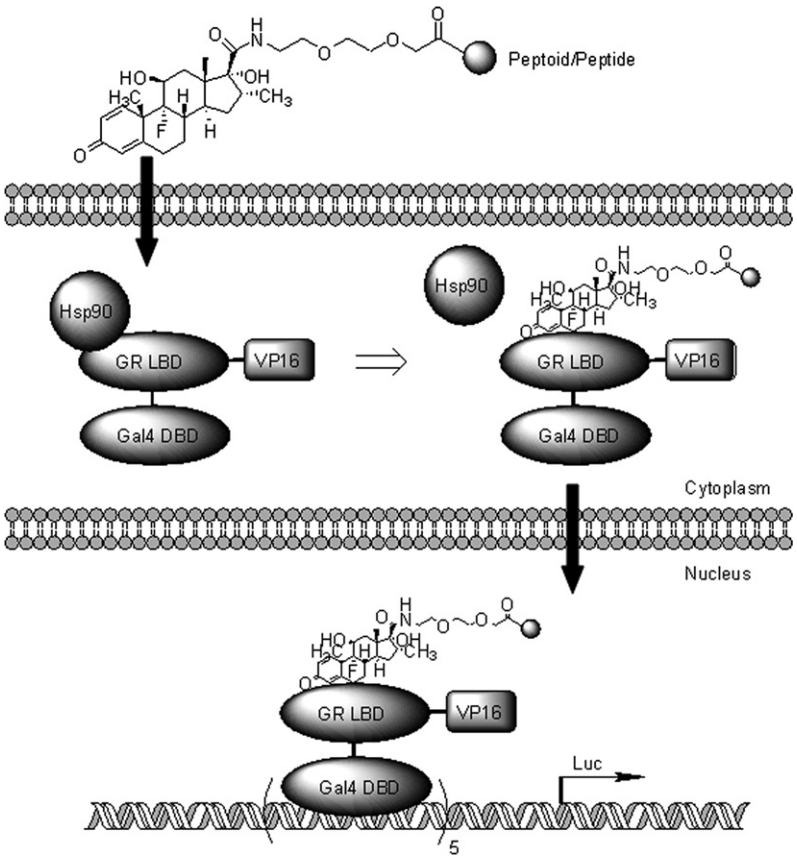

Figure 2. Schematic illustration of the cell permeability assay used in the high-throughput study of steroid conjugates. Refer text for details.

readouts. Four 96-well plates from each library were used in this comparison study. To address the possibility of plate-to-plate variability, the permeability ratio of each compound in each plate was normalized to the plate's ratio of the negative (no compound) and positive (dexamethasone) controls.

2.3. Technical issues

A total of eight 96-well plates (four from each library) were assayed simultaneously using the same batch of HeLa cells to minimize variability due to cell preparation. For practical reasons, a Tecan SpectraFluor Plus plate reader was chosen for the luminescence activity readout due to the high-throughput nature of the study. Titration of steroid-conjugated peptoid and peptide analogs for comparison between the Tecan and Sirius luminometer (Berthold Detection Systems) revealed that the reduced sensitivity of the Tecan resulted in lower luminescence readout values than the luminometer. Fortunately, the Tecan plate reader preserved the relative difference between the molecules and produced comparable EC_{50} values (luciferase induction) for the same molecules (Table 1 and Supplementary Material), albeit with a reading two orders of magnitude lower than that of the single-tube luminometer.

Table 1
Luminescence readout comparison between Tecan and luminometer

Compound	Luciferase induction[a], EC_{50} (μM)	
	Luminometer	Tecan
PO4HT	6.9 ± 2.5	7.2 ± 4.3
PI4HT	30.3 ± 8.2	35.5 ± 11.6

[a] Luciferase induction (EC_{50}) values obtained from the individual titration curve of tetramer peptoid (PO4HT) and peptide (PI4HT). Refer Supplementary Material for titration curves.

In the actual study, the final concentration of OxDex-conjugated peptoid or peptide exposed to HeLa cells in each well was about 100 μM since the construct OxDex-AEEA-$CONH_2$ was shown to have an EC_{50} value of <50 μM.[9] Therefore, at this concentration, we expect the OxDex conjugates to show at least half-maximum induction of luciferase expression, allowing us to clearly distinguish between permeable and impermeable compounds. Because of the large number of molecules analyzed in this study, it was not feasible to examine multiple compound concentrations to obtain a titration curve. Therefore, it was imperative to determine beforehand that the readouts will be in the linear, and not the saturated, part of such a titration curve. As shown in Figure S3 in the Supplementary Material, this appeared to be the case for two particular peptoids that had been identified previously as having relatively high cell permeability.[9] However, for two peptoids that had been previously identified as being poorly cell permeable, only a small amount of reporter gene induction was observed at an extracellular concentration of 100 μM and it is not clear if this value is in the linear range (Fig. S3). Based on this observation, we assume that all of the single-point readings for peptoids and peptides that score as relatively permeable reflect the linear part of the titration curve for each compound, but that this assumption may not be valid for some peptoids that are unusually cell impermeable. Of course, another complicating factor is that in the analysis of the library, we cannot be certain that the concentration of each peptoid- or peptide-steroid conjugate is the same due to possible differences in synthesis efficiency, though we believe that these differences are not large, based on the aforementioned analysis of several compounds chosen randomly from the library.

2.4. Comparison of peptoids to peptides

The current study compared the relative cell permeability of 350 steroid-conjugated peptoids and 350 steroid-conjugated peptides. The permeability ratio for each compound per well was calculated as (Firefly luciferase luminescence/*Renilla* luciferase luminescence)/(Blank negative control luminescence/ Dexamethasone positive control luminescence). The average permeability ratio from each class of molecules was subject to a 2-tail *t*-test using SAS. The statistical analysis showed that the two groups of compounds passed the equality of variance requirement and had a statistically significant difference with a *P*-value of <0.01 when the average permeability ratio of peptoids (0.0118) was compared to the average permeability ratio of peptides (0.0059) (Fig. 3 and Supplementary Material).

The relative permeability investigated here obviously reflects that of the steroid conjugates and may not reflect the true permeability of the parent compounds. However, any assay involving labeling of the molecule of interest suffers from this limitation, including confocal fluorescence microscopy. Thus, we restrict our comments in the discussion below to relative statements comparing one set of steroid-substituted molecules to another.

2.5. Comparison of physicochemical properties with permeability

The prediction of absorption, distribution, metabolism, and excretion (ADME) properties of organic molecules continue to play a critical role in the drug discovery process and it is now possible to make reasonable estimates of the permeability of organic compounds based on their molecular structures. In an attempt to delineate the factors that are responsible for the observed difference in permeability between peptoids and peptides, we investigated a number of physicochemical properties that have been shown to affect permeability.[13–15] We first considered highly cell permeable peptoids and peptides that have a permeability ratio at least two standard deviations greater than the average permeability ratio of its class. The composition of the peptoids and peptides that scored as such was identified by mass spectrometry.

The physicochemical parameters examined include lipophilicity, polar surface area (PSA), hydrogen-bonding capacity (hydrogen bond acceptors and donors), molecular size, molecular volume, and molecular rigidity, which were calculated using Molinspiration Cheminformatics as reported elsewhere.[13]

2.5.1. Lipophilicity

A traditional molecular transport descriptor that is used to model permeability is the *n*-octanol–water partition coefficient ($\log P_{oct}$ or simply $\log P$). Log*P* is still widely used as a measure of hydrophobicity or lipophilicity that affects membrane penetration and permeability,[14–17] with a higher log*P* value indicating greater lipophilicity. The computational log*P* (*C*log*P*) values in this study take into consideration the intramolecular hydrogen-bonding contribution and charge interactions. In our study, peptoids appear more cell permeable and tend to have a lower *C*log*P* value than peptides, suggesting peptoids are slightly less lipophilic than peptides. The average *C*log*P* value of peptoids in the high-throughput study is lower than that of peptides (−2.67 vs −1.90) (Table 2a).

This trend of lower *C*log*P* value with an observed higher permeability is in line with the study of steroid transport across Caco-2 monolayer cells by Faassen and co-workers[18] and other work.[19] This phenomenon could be attributed to the peptoid residues, which as imino acids are more hydrophilic than the corresponding amino acid counterparts based on the Liu–Deber hydropathy scale.[20] It is known that excessive lipophilicity is a common cause of poor solubility[21] and thus leads to poor cell permeability. In converting the residue to an *N*-alkylglycine moiety, the N-atom becomes tertiary and more basic, likely increasing the water solubility of the peptoids. Although a good predictor of permeation across biological membranes, lipophilicity is not the sole determinant of cell permeability and other factors have been shown to play a role in affecting the overall permeability of a molecule.

2.5.2. Polar surface area (PSA)

In recent years, the PSA of a molecule has emerged as a key predictor of permeability. PSA is defined as the sum of the van der Waals (or solvent-accessible) surface areas of oxygen and nitrogen

Table 2a
Mean value of selected molecular descriptors in high-throughput study

Compound	PR[a]	*C*log*P*	M_W[b]	Nrotb[c]	Molecular volume (Å^3)
Peptoids	0.0221	−2.67	1061.65	30	976.69
Peptides	0.0134	−1.90	1074.00	29	985.49

[a] PR is the mean permeability ratio obtained from the highly permeable OxDex-conjugated compounds ($\geqslant 2\sigma$ above average permeability ratio, $n = 12$ for peptoids and $n = 12$ for peptides).
[b] M_W represents the molecular weight.
[c] Nrotb is the number of rotatable bonds.

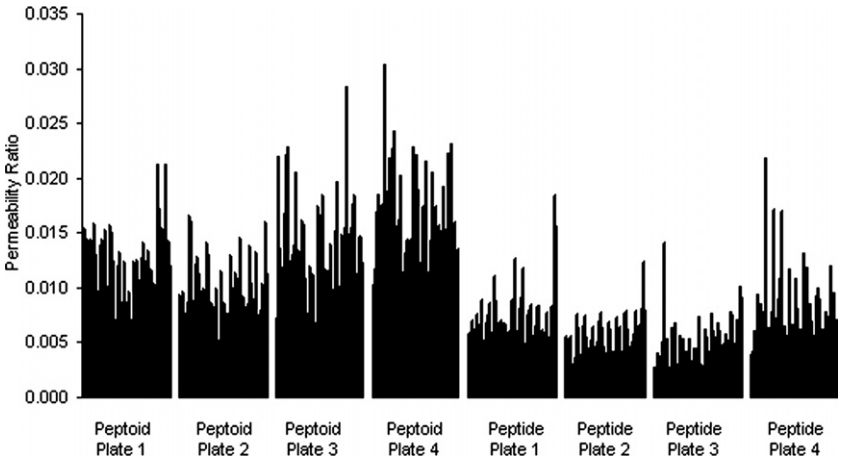

Figure 3. High-throughput comparison of relative cell permeability between peptoids and peptides. Permeability ratio = (Firefly luciferase luminescence/Renilla luciferase luminescence)/(Blank negative control luminescence/Dexamethasone positive control luminescence).

atoms, including attached hydrogens.[22] The topological polar surface area (TPSA) was calculated to investigate this parameter in our study. It is computationally up to three times faster and comparable to the classical 3D PSA. In our high-throughput study, the average TPSA value of the highly permeable peptoids of 335.30 Å^2 is also lower than the highly permeable peptides' average TPSA value of 358.80 Å^2 (Table 2b). This is a relatively small difference and may play only a modest role in the observed differences in the permeability of peptides and peptoids. To the extent that it does contribute, this means that overall, peptoids have less polar groups exposed to solvents than peptides, which in turn suggests that the resulting lower TPSA value might contribute to the higher cell permeability of peptoids over peptides. This trend is also present within the OxDex-conjugated peptoids with differing permeabilities. Peptoids with a lower TPSA tend to have a higher permeability ratio (Fig. 4).

Literature suggests that permeability is optimal when PSA is <120 Å^2 based on a study of commercially available drugs.[23] Although all the molecules in our study have a PSA > 120 Å^2, the trend of lower PSA conferring higher permeability is observed, though the effect is subtle. PSA has been used to predict passage though the blood–brain barrier (BBB), flux across Caco-2 monolayers, and human intestinal absorption,[22–24] and succeeded in providing good correlation with experimental transport data.[13] In our study, the lower TPSA in peptoids can be attributed to the conversion of the backbone amide from a secondary to a tertiary nitrogen which eliminates the very polar amide bond found in peptides. Consequently, the tendency for a hydration shell to form around the peptoid is reduced. It is postulated that polar groups resist desolvation when they move from an aqueous extracellular environment to the more lipophilic interior of membranes. The PSA thus may reflect at least part of the desolvation energy for breaking the solute:water interaction necessary in membrane transport. Specifically, the higher PSA in peptides may indicate the greater desolvation energy required to overcome the strong amide:water interactions in peptides.

Table 2b
Hydrogen-bonding capacity parameters in high-throughput study

Compound	PR[a]	TPSA (Å^2)	H-bond acceptors	H-bond donors	Total H-bonds[b]
Peptoids	0.0221	335.30	23	9	32
Peptides	0.0134	358.80	22	14	36

[a] PR is the mean permeability ratio obtained from the highly permeable OxDex-conjugated compounds ($\geqslant 2\sigma$ above mean permeability ratio, $n = 12$ for peptoids and $n = 12$ for peptides).
[b] Total H-bonds is the sum of H-bond acceptors and donors.

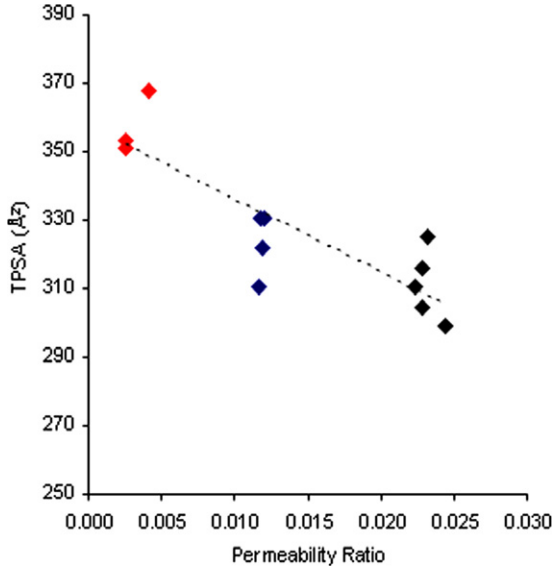

Figure 4. Trend in TPSA in OxDex-conjugated peptoids. Peptoids with permeability ratio at least 2σ below (red), above (black), or at (blue) mean permeability ratio value.

2.5.3. Hydrogen-bonding capacity

The polar functionalities of the PSA parameter of a compound can be related to its hydrogen-accepting and hydrogen-donating ability, with hydrogen-bonding being one of two main components of lipophilicity. The peptoids and peptides have an equal number of hydrogen acceptors but the peptoids have a lower number of hydrogen donors (average of 9 hydrogen donors in peptoids against 14 in peptides) (Table 2b), resulting in a reduced total hydrogen-bonding capacity (sum of hydrogen acceptors and donors). This correlated with increased permeability. A modest trend of this sort is observed within the OxDex-conjugated peptoids (Fig. 5). Since the structural difference between peptoids and peptides mainly affects the hydrogen-bonding potential of peptoids, we further investigated whether it could be the main physicochemical parameter responsible for the higher permeability seen in peptoids. Highly permeable molecules from both classes with comparable *C*log*P* values were compared for differences in hydrogen-bonding capacity. We found that the TPSA and hydrogen-bonding capacity were decreased in peptoids, specifically in a reduction of hydrogen bond donor potential (Table 2c). This reduction that accompanies higher permeability seen in peptoids supports the notion that lipophilicity can affect cell permeability only to a certain extent whereas the hydrogen-bonding potential might assume a more determinant role, as suggested previously.[25]

Hydrogen-bonding capacity bears such significance that it constitutes two of Lipinski's Rule of Five[26] of drug design. It has been found that the hydrogen-bonding capacity of a drug solute correlates reasonably well with passive diffusion.[27,28] An increased N–H bond count for both acceptors and donors tends to worsen permeability.[29,30] To further substantiate this argument, compounds with high hydrogen forming potential, such as peptides with their amide groups as small as di- and tripeptides, have

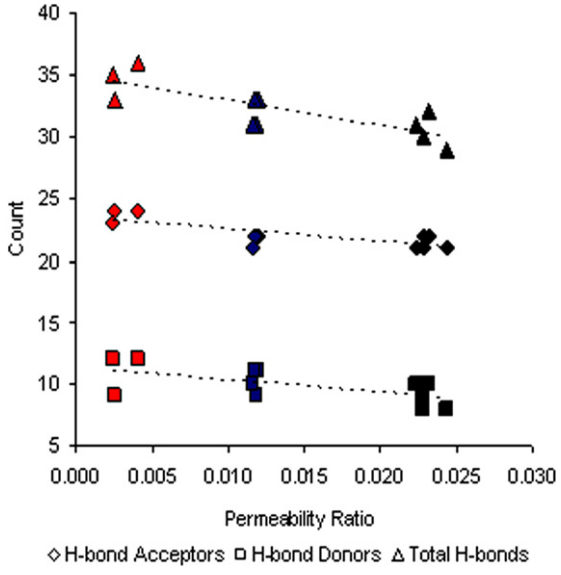

Figure 5. Trend in hydrogen-bonding capacity in OxDex-conjugated peptoids. Peptoids with permeability ratio at least 2σ below (red), above (black), or at (blue) mean permeability ratio value. Total H-bonds is the sum of H-bond acceptors and donors.

Table 2c
Comparison of Clog*P* and hydrogen-bonding capacity parameters in high-throughput study

Compound	PR[a]	Clog*P*	TPSA (Å^2)	H-bond acceptors	H-bond donors	Total H-bonds[b]
Peptoids	0.0229	−1.70	319.03	22	9	31
Peptides	0.0134	−1.38	353.71	22	13	35

[a] PR is the mean permeability ratio obtained from the highly permeable OxDex-conjugated compounds ($\geqslant 2\sigma$ above mean permeability ratio) with comparable Clog*P* values ($n = 7$ for peptoids and $n = 6$ for peptides).
[b] Total H-bonds is the sum of H-bond acceptors and donors.

minimal distribution through the BBB, while compounds possessing a tertiary nitrogen show a high degree of brain permeation.[31] Indeed, tertiary nitrogen is a feature of many central nervous system drugs.[31] Hydrogen-bonding potential might thus constitute the limiting step in cell permeation.

Molecules with very polar amide bonds like peptides have greater hydrogen-bonding interactions with the surrounding water. As a result, the desolvation energy required to break these interactions in order for the peptides to transfer from the hydrophilic, aqueous environment to the hydrophobic, non-hydrogen-bonding membrane interior is substantially increased. Moreover, once the solute:water interaction is overcome, cell permeability can be further hindered by the binding of the molecules to the lipid-rich layer of cell membranes through the donation of hydrogen-bonds as it approaches the polar surface of the membrane and desolvates as it moves into the lipid portion.[32] In fact, the hydrophilic part of lipids contains hydrogen-bonding acceptor groups which may hinder the transbilayer insertion of the high hydrogen-donating molecules and prevent their transport across a cellular membrane via tight binding. Hence, the greater hydrogen-donating potential of peptides over peptoids might be the cause of lower permeability seen in peptides. It is therefore not surprising that modifications that result in the reduction of hydrogen-bond-donating capacity, such as conformational facilitation of intramolecular hydrogen bonds[32–38] and the absence of hydrogen donors altogether from the tertiary amines in the case of peptoids, will facilitate membrane permeation.

2.5.4. Molecular size, volume and rigidity

Molecular size is the second basic component of solubility and permeability. Molecular weight is a surrogate for other properties, including molecular volume and rigidity. The simplest measure of molecule rigidity is by determining the number of rotatable bonds present. In our high-throughput study, molecular size and volume are comparable between the OxDex-conjugated peptoids and peptides. Similarly, there is no difference in the number of rotatable bonds between the two classes of molecules under investigation (Table 2a). Even though these molecular properties have been implicated in cell permeability,[39] they appear to play a minimal role in the permeability difference observed between peptoids and peptides in this study.

2.5.5. Side chain composition

To determine if specific side chain characteristics are preferred over others in the more permeable molecules, the prevalence of each side chain used in the library was probed. The molecular formulas of the highly cell permeable molecules from both classes were predicted from their molecular ion mass obtained via mass spectrometry, the generic molecular structure, and the mass of the five residues used in the library using an in-house program. The side chains were then confirmed via tandem mass spectrometry sequencing, and the frequency of occurrences tabulated. It appears that the highly cell permeable peptoids and peptides consist of 1.5 times more hydrophilic residues than hydrophobic ones (Fig. 6A and B), where hydrophilic residues are the positively charged lysine and the negatively charged aspartic acid. Phenylalanine and valine constitute the hydrophobic residues. Of the 12 highly permeable molecules from each class, a large majority of them are charged (11 for peptoids, 10 for peptides). Out of these charged molecules, seven peptoids and nine peptides have two or more charged residues (Supplementary Material).

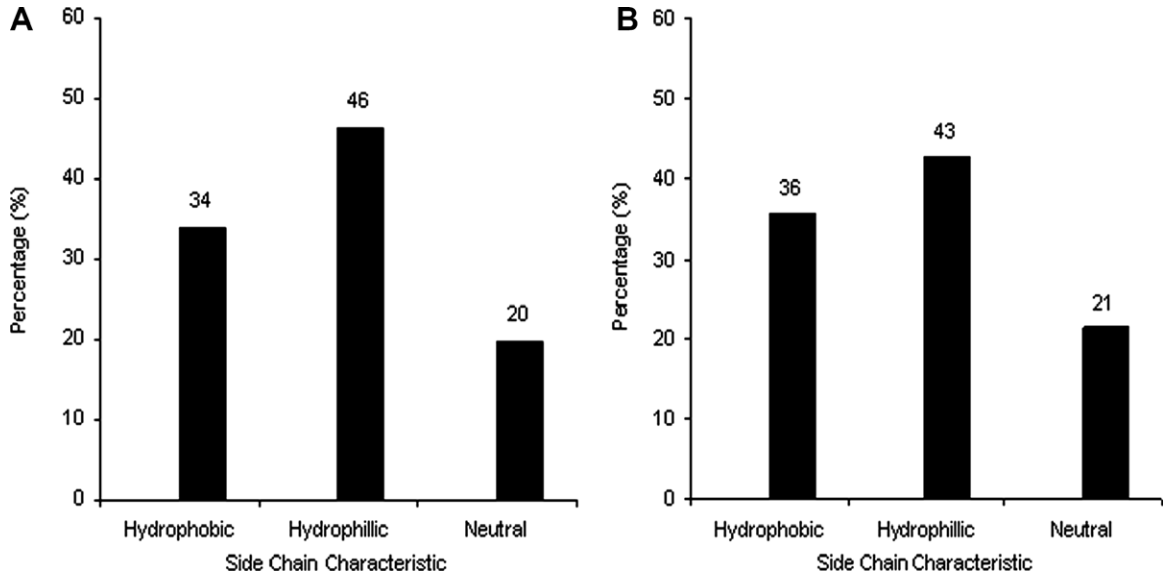

Figure 6. Side chain characteristic prevalence of highly permeable ($\geqslant 2\sigma$ above mean permeability ratio) (A) peptoids and (B) peptides. Hydrophobic residues are Nleu and Nmba, hydrophilic residues are Nasp and Nlys, and neutral residue is Nser for peptoids. Hydrophobic residues are Val and Phe, hydrophilic residues are Asp and Lys, and neutral residue is Ser for peptides.

The observation that the more cell permeable molecules tend to consist of more charged residues than hydrophobic ones corresponds well with an increasing body of evidence supporting ion partitioning over neutral molecules, as substantiated by studies suggesting ionic species may contribute significantly to transport across Caco-2 monolayers.[38,40] Since our cell permeability assay uses steroid conjugates, the charges on the highly permeable molecules may serve to increase solubility of the molecules in the presence of the hydrophobic steroid. Amphiphilicity (the combination of the hydrophilic and hydrophobic parts of a molecule) may in itself influence cell permeability and deserves further investigation.[21]

2.5.6. Physicochemical property evaluation of SDex conjugates

A similar evaluation of the above parameters was performed on the SDex conjugates used in the study by Kwon et al.[11] (Fig. 7). SDex is a higher affinity GR ligand than is OxDex (Fig. 1C) and thus shifts the titration curve to lower compound concentrations. The relative permeability comparison between peptoids and peptides in this set of conjugates involved far fewer compounds but was based on careful titrations of the exact isomeric analogs. Specifically in Kwon's study, a series of peptoid and peptide conjugates containing leucine and homoserine side chains and varying in length from dimers to octamers were synthesized and their relative cell permeability compared using the same cell-based reporter assay employed in our high-throughput study.[11] It was found that peptoids were more cell permeable than peptides, with shorter conjugates exhibiting higher permeability. The physicochemical properties discussed above for the high-throughput study of OxDex conjugates were calculated for this set of SDex-conjugated analogs and their values compared between peptoids and peptides. Not surprisingly, the trends observed in the single-point readout but more extensive set of molecules in the OxDex conjugates were mirrored in the SDex-conjugated molecules (Figs. 8–10, Tables 3a and 3b).

3. Conclusion

Undoubtedly, the overall permeability of a molecule is determined by the delicate balance of numerous parameters that are clearly interrelated such that changing one will affect the others. To date, there exists no prediction method for this crucial attribute in drug design. In this study, we evaluated whether a cell-based permeability assay, when conducted in high-throughput mode, could contribute to helping us understand the differences in cell permeability of peptides and peptoids. As mentioned in the introduction, careful previous studies from our laboratory of a few compounds have shown that peptoids are anywhere from 3 to 30 times more permeable than comparable peptides, depending on the compound. The single-point nature of the assays conducted here appear to flatten this difference somewhat as, on average, the peptoids were found to be about twice as permeable as the peptides. Nonetheless, the general trend held, allowing us to attempt to correlate various aspects of the molecular characteristics of some of the molecules with the observed relative cell permeability. Of special interest is the reduction in hydrogen-bond-donating potential of peptoids, which appears to be the dominant factor accounting for the increased cell permeability.

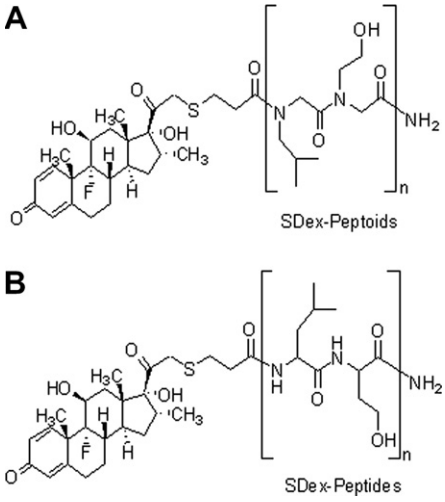

Figure 7. Chemical entities used in analog study. Generic chemical structure of the SDex-conjugated analogs of (A) peptoids and (B) peptides, where $n = 1, 2, 3$, or 4.

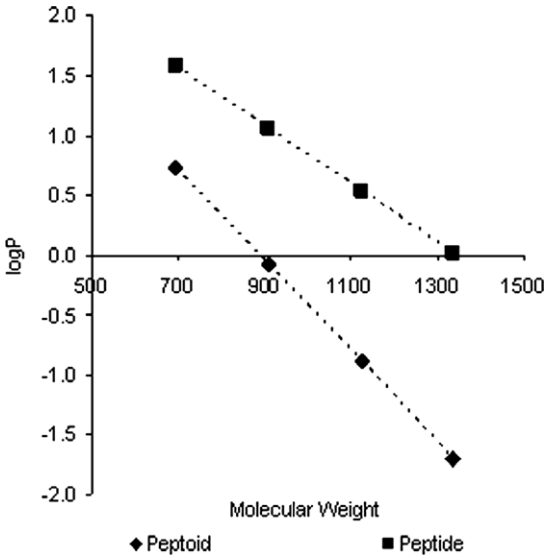

Figure 8. Comparison of *C*log*P* value of SDex-conjugated peptoid (PO*n*) and peptide (PI*n*) analogs. Dimers (PO2, PI2), tetramers (PO4, PI4), hexamers (PO6, PI6), and octamers (PO8, PI8) are represented by their molecular weight.

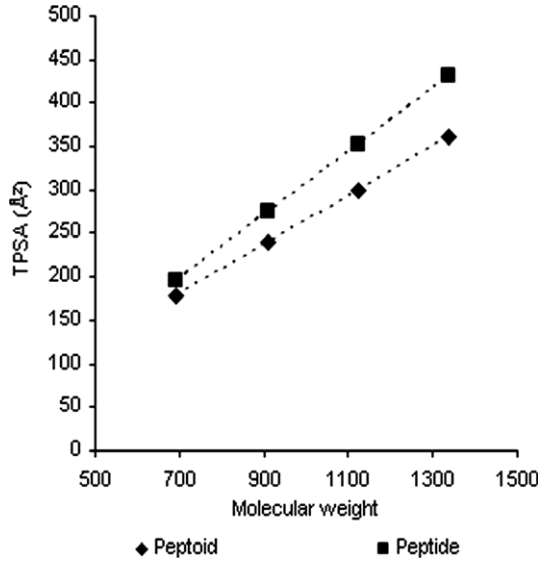

Figure 9. Comparison of TPSA value between SDex-conjugated peptoid (PO*n*) and peptide (PI*n*) analogs. Dimers (PO2, PI2), tetramers (PO4, PI4), hexamers (PO6, PI6), and octamers (PO8, PI8) are represented by their molecular weight.

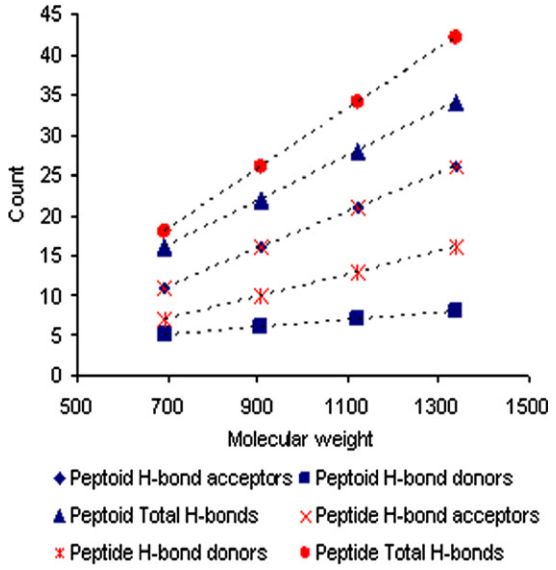

Figure 10. Comparison of hydrogen bond capacity between SDex-conjugated peptoid (PO*n*) and peptide (PI*n*) analogs. Dimers (PO2, PI2), tetramers (PO4, PI4), hexamers (PO6, PI6), and octamers (PO8, PI8) are represented by their molecular weight. Total H-bonds is the sum of H-bond acceptors and donors.

Table 3a
Mean value of selected molecular descriptors in analog study

Compound[a]	*C*log*P*	Molecular weight	Number of rotatable bonds	Molecular volume (Å^3)
PO2	0.73	693.88	14	637.57
PO4	−0.08	908.14	22	843.08
PO6	−0.89	1122.41	30	1048.59
PO8	−1.70	1336.67	38	1254.10
PI2	1.58	693.88	14	636.86
PI4	1.05	908.14	22	841.66
PI6	0.53	1122.41	30	1046.46
PI8	0.01	1336.67	38	1251.26

[a] All molecules represented here are SDex-conjugated analogs of peptoids (PO*n*) and peptides (PI*n*), where molecule length $n = 2, 4, 6$, or 8.

4. Experimental

4.1. Reagents and instrumentation

All chemicals and reagents in organic synthesis were purchased from Sigma–Aldrich. For library synthesis, Polystyrene A-RAM macrobeads (500–560 μm, 0.55 mmol/g) were from Rapp Polymere. Rink amide AM resins (200–400 mesh, capacity: 0.71 mmol/g) were from NOVAbiochem. For solid-phase synthesis, *N*-(9-Fluorenylmethoxy-carbonyl)-acetyl-ethyleneglycol-ethyl-amine (Fmoc-AEEA-OH), *O*-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and 1-hydroxy-7-azabenzotriazole (HoAt) were from Applied Biosystems. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HoBt) were from SynPep. Diisopropylethylamine (DIPEA) and 2,6-lutidine were from Sigma–Aldrich. All Fmoc amino acid monomers were from SynPep and Advanced NOVAbiochem. *O*-*tert*-Butyl ethanolamine was from CSPS Pharmaceuticals. Glycine *tert*-butyl ester acetate was from NOVAbiochem. Diaminobutane, isobutylamine, (*R*)-methylbenzylamine, bromoacetic acid, and diisopropylcarbodiimide (DIC) were from Sigma–Aldrich. Cell culture media and transfection reagents were purchased from Invitrogen. Preparative HPLC was performed on a Waters Binary HPLC system with a C18 reverse-phase column with the gradient elution of water/acetonitrile with 0.1% trifluoroacetic acid (TFA). Mass spectrometry (MALDI-TOF) was performed on a Voyager-DE PRO biospectrometry workstation (Applied Biosystems) with α-cyano-4-hydroxycinnamic acid as matrix.

Table 3b
Hydrogen-bonding capacity parameters in analog study

Compound[a]	M_W[b]	TPSA (Å^2)	H-bond acceptors	H-bond donors	Total H-bonds[c]
PO2	693.88	178.54	11	5	16
PO4	908.14	239.38	16	6	22
PO6	1122.41	300.23	21	7	28
PO8	1336.67	361.08	26	8	34
PI2	693.88	196.12	11	7	18
PI4	908.14	274.54	16	10	26
PI6	1122.41	352.96	21	13	34
PI8	1336.67	431.39	26	16	42

[a] All molecules represented here are SDex-conjugated analogs of peptoids (PO*n*) and peptides (PI*n*), where molecule length $n = 2, 4, 6$, or 8.
[b] M_W represents the molecular weight.
[c] Total H-bonds is the sum of H-bond acceptors and donors.

4.2. Syntheses of OxDex and SDex

OxDex-COOH (Dex-17β-carboxylic acid) and SDex-COOH (Dex-21-thiopropionic acid) were synthesized based on previously published procedures.[9,10]

4.3. Syntheses of OxDex-peptoid and OxDex-peptide libraries

Both peptoid and peptide libraries were constructed on Polystyrene A-RAM macrobeads (500–560 μm, 0.55 mmol/g) from Rapp Polymere. Peptoid library synthesis was performed as described previously with slight modifications.[2] The synthesis of peptoids under microwave conditions was performed in a 1000 W Whirlpool microwave oven (model MT1130SG) with 10% power. Peptide library synthesis was performed using standard solid-phase synthesis methods. The synthesis of peptides was performed in a New Brunswick Scientific Innova 4000 incubator shaker. Standard glass peptide synthesis vessels (Chemglass) were used for the synthesis in the incubator shaker and in the microwave oven. Upon completion of the library syntheses, Fmoc-AEEA-OH and OxDex-COOH were coupled to the beads using standard solid-phase synthesis methods. The libraries were sorted into 96-well plates in a one bead per well fashion and cleaved with trifluoroacetic acid:water (95:5 vol/vol) at room temperature with slight agitation for 2 h. The TFA was evaporated under hoods and the compounds were resuspended in 20 μl 50% acetonitrile in water. One-fourth (5 μl) of the compounds per bead was aliquoted into a separate 96-well plate for sequencing purposes. The remaining three-fourths (~60 nmol) of compounds were dried and resuspended in 11.5 μl 10% DMSO in water for cell culture experiments. About 10 nmol of each compound was used so that each well contained about 100 μM of each OxDex-capped molecule in 100 μl of cell culture media.

4.4. Syntheses of SDex-peptoid and SDex-peptide analogs

SDex-conjugated peptoid and peptide analogs were synthesized as described previously.[11] The analogs were capped with SDex-COOH steroid.

4.5. Plasmids, cell culture, transfection and luciferase assay

Procedures were performed as described previously.[9–11] HeLa cells were grown in 96-well plates for the high-throughput study.

4.6. In vitro competition GR-binding assays and high-throughput cell permeability assay

Procedures were performed as described previously.[9–11]

4.7. Permeability ratio determination

The permeability ratio (PR) was obtained by dividing the luminescence reading from the firefly luciferase activity to the luminescence reading from the *Renilla* luciferase activity. The PR was then normalized to the negative (no compound) and positive (dexamethasone) control ratios from each plate. PR = (Firefly luminescence/*Renilla* luminescence)/(No compound luminescence/Dexamethasone luminescence).

4.8. Statistical analyses

The mean luminescence reading for the internal control *Renilla* luciferase was obtained from all eight plates. Compounds with a *Renilla* luminescence reading that lies greater than ±2 standard deviations from the mean were omitted from the study. The mean normalized PR for each class of compounds was calculated from the remaining compounds. The 2-tail *t*-test on mean PR between peptoids and peptides was performed using the statistical software SAS.

4.9. Peptoid and peptide sequencing

Compounds that have a normalized PR that is at least two standard deviations greater than the mean ratio were analyzed using the Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics to obtain the molecular ion mass. The possible monomeric composition of the molecule was predicted from an in-house program written in Perl using the molecular ion mass, the known generic structure of the libraries, and the residue mass of the monomers. Tandem mass spectrometry sequencing on the same instrument was performed to confirm the sequences.

4.10. Physicochemical property computations

All physicochemical property calculations were obtained using Molinspiration Cheminformatics (http://www.molinspiration.com/cgi-bin/properties). Log*P* prediction is based on group contributions and takes into account the intramolecular hydrogen-bonding contribution to log*P* and charge interactions. TPSA calculation is based on the summation of tabulated surface contributions of polar fragments (atoms regarding also their environment) and provides results of practically the same quality as the classical 3D PSA, but is two to three orders of magnitude faster.[13]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.074.

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