Intestinal Absorption Screening of Mixtures from Combinatorial Libraries in the Caco-2 Model

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Purpose. Understanding how chemical structures influence transport across the intestinal mucosa will greatly enhance the discovery of orally available drugs. In an attempt to accelerate defining such relationships between structure and transport, six arbitrary mixtures of N-substituted glycine (NSG) peptoids containing 24 physicochemically diverse compounds were evaluated in the Caco-2 model of intestinal absorption.
Methods. Samples were analyzed by HPLC and the areas of the peaks representing the components of each mixture were summed to measure "aggregate" apparent permeability coefficients (P_{app}), a score of the influence of the common structural element within each mixture towards absorption. Mass spectrometry was used to identify the chemical structure of Caco-2 permeable compounds.
Results. Three linear trimeric mixtures were examined and, for each mixture, none of the components was detected in receiver chambers. It was concluded that the components of these mixtures each had a P_{app} value less than 0.8×10^{-6} cm/sec, a permeability less than mannitol. Three dimeric mixtures were examined and they exhibited aggregate P_{app} values of 9.2×10^{-6}, 14×10^{-6} and 6.9×10^{-6} cm/sec. These transport rates reflected the transport of most of the components of each mixture. Furthermore, the components of the dimeric mixtures which were transported at a rate greater than mannitol were apparently transported by passive mechanisms.
Conclusions. This study demonstrated that mixtures can be used to study structure-transport relationships in the Caco-2 model. The information obtained from this type of study will be integrated into the design of future chemical libraries. Other potential uses of chemical mixtures with the Caco-2 model are also discussed.

KEY WORDS: Caco-2; intestinal absorption; structure-transport relationships; structure-activity relationships (SAR); mixtures; combinatorial chemistry.

INTRODUCTION

The development of molecular diversity technologies has dramatically accelerated the process of discovering new pharmacophores (1). An example of technology are oligomeric N-substituted glycine (NSG-) peptoids (2). NSG-peptoids are prepared automatically by a robotic instrument employing a highly efficient solid-phase synthesis that generates equimolar mixtures of compounds (3). Any of thousands of primary amines can be used as building blocks allowing for tremendous diversity and complexity. Since several potent phaRmacophores have been discovered from libraries of NSG-peptoids (4), and NSG-peptoids are generally resistant to proteolytic enzymes (5), they are attractive candidates for development as orally bioavailable therapeutics.

Methods that expedite the development of lead drug candidates derived by combinatorial synthesis and combinatorial screening paradigms will enhance the drug discovery process. One such approach is the synthesis of combinatorial libraries that are biased towards intestinally permeable compounds. To generate such libraries, an understanding of the relationship between structure and intestinal absorption is required. To date, this issue has been approached by systematically studying the transport of individual molecules, generally using a related set of analogs, across Caco-2 monolayers (6–9). We questioned whether this process could be accelerated by simultaneously measuring multiple analytes in the Caco-2 model (see ref. 10–13 for review of Caco-2 model). Thus, mixtures containing 24 physicochemically diverse compounds produced by combinatorial synthesis (4) were examined. By testing mixtures of this nature in the Caco-2 model, the influence on absorption of the common structural element within each mixture was rapidly discerned because, in essence, the analysis of one mixture was equivalent to studying 24 diverse analogs at one time. The results are discussed in terms of the use of mixtures to study relationships between structure and intestinal absorption, and how screening mixtures in the Caco-2 model could be further implemented in the drug discovery process.

MATERIALS AND METHODS

Materials

NSG-peptoids were robotically synthesized at Chiron Corp. (Emeryville, CA) as previously described (4) and provided as solutions of 1 mM per compound in dimethylsulfoxide (DMSO). [^{14}C]mannitol, [^{14}C]testosterone and [^{3}H]prazosin with specific activities of 56.7 mCi/mmol, 57.3 mCi/mmol and 71.8 Ci/mmol, respectively, were purchased from NEN Research Products, Boston, MA. [^{14}C]phenylalanine with a specific activity of 59.0 mCi/mmol was purchased from Amersham Corp., Arlington Heights, IL. Valyl-valine dipeptide and phenylalanine were purchased from Sigma Chemical Co., St. Louis, MO. Caco-2 cells, at passage 22, were provided as a gift from the laboratory of Dr. Ronald Borchardt at the University of Kansas. All tissue culture media components were from Irvine Scientific, Santa Ana, CA. Tissue culture flasks, plates and BioCoat Collagen I cell culture inserts were from Collaborative Biomedical Products, Bedford, MA. HPLC grade heptane sulfonic acid and acetonitrile were purchased from Fisher Scientific, Pittsburgh, PA. All synthetic reagents were of reagent grade.

Initial Caco-2 Permeability Screening

Caco-2 cells were maintained, and transport studies were performed as previously described (11) with the following modifications. Caco-2 cells between passage 30 and 40 were seeded at a density of 2–3 $\times 10^6$ cells/cm^2 on 1.05-cm i.d. BioCoat

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; RP-, reversed phase; LC/MS, liquid chromatography/mass spectrometry; val-val, valyl-valine dipeptide, PBS, Dulbecco's phosphate buffered saline; DMSO, dimethylsulfoxide, HEPES; N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid].

Collagen I inserts (1 μm pore size) and an acetate transport buffer (ATB; 50 mM sodium acetate, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.9 mM calcium chloride, 1.9 mM magnesium chloride, 50 mM mannitol, 0.1 % glucose, pH 6.5) was used for transport studies. Experiments were initiated by addition of 0.8 ml of ATB (pH 6.5) containing 1/100 volume NSG-peptoid mixture (which yielded 10 μM/compound final concentration) and approximately 1×10^5 dpm/ml of [^{14}C]mannitol, or containing 1×10^5 dpm/ml of drug plus 1% DMSO, to the apical (AP) chamber of each insert that already had 1.0 ml of receiver buffer (ATB, pH 6.5, supplemented with 1% DMSO) in the basolateral (BL) chamber. Radiolabeled mannitol, a marker for paracellular transport, was co-incubated with all NSG-peptoid samples to monitor monolayer integrity. Cell inserts were incubated at 37°, and at 60 and 120 minutes inserts were transferred to a new well containing 1.0 ml of fresh receiver buffer. Each experiment was performed in triplicate.

Evaluation of Mechanism of Transport

For apical-to-basal (AP→BL) transport, experiments were performed as above except with the following changes. AP chambers were maintained at pH 6.0 with ATB, pH 6.0, and BL chambers at pH 7.4 with HEPES transport buffer (HTB), pH 7.4, which differed from ATB only in that HEPES was substituted for sodium acetate. For basolateral-to-apical (BL→AP) transport, compounds were added to HTB (pH 7.4) and experiments were initiated by adding 0.8 ml of this buffer to the BL chamber of each insert which already had 1 ml of ATB (pH 6.0) supplemented with 1% DMSO in the AP chamber. Phenylalanine and val-val donor samples were made to 1 mM and 5 mM, respectively, with unlabeled drug. Receiver samples were collected at a single time-point of 60 minutes. Each experiment was performed in triplicate.

Data Analysis

Transport of radioactive drugs was quantitated by liquid scintillation counting. Transport of NSG-peptoids was quantitated by RP-HPLC on a system optimized for narrow-bore chromatography. The system consisted of Dynamax HPLC software to control Rabbit HPLC pumps (Rainin Instrument Co., Inc., Emeryville, CA) equipped with a 200 μl volume static mixer (Michrom BioResources, Auburn, CA), an autosampler (Alcott, Norcross, GA), a UV detector (Applied Biosystems, Palo Alto, CA) and 0.005 inch i.d. tubing throughout the system. In brief, after samples were made 0.5% in heptane sulfonic acid and 0.2% in formic acid, 500μl was injected onto a C_{18} (2.1 × 150 mm) column protected with a guard cartridge (Vydac, Hesperia, CA) and the analytes were eluted with a linear gradient of acetonitrile in a mobile phase of 0.2% heptane sulfonic acid/0.1% formic acid at 200 μl/min. Elution of NSG-peptoids was detected by absorbance at 210 nm. Calibration standards of each NSG-peptoid mixture were generated by 2-fold serial dilutions of the donor sample with fresh receiver buffer then treated and injected onto the HPLC as described above. For each mixture analyzed, the same peak integration parameters were used to integrate all chromatograms of that mixture. The region integrated and summed was tailored for each mixture based on the peaks that changed height with the different calibration standards. The sum of the peak areas at 210 nm was plotted versus the concentration of peptoid mixture and standard curves were fit to the average of duplicate sets of standards using $1/y^2$ weighted linear regression analysis. Apparent permeability coefficients (P_{app}) were determined from the equation:

$$P_{app} = \frac{V_r}{AC_0} (dC/dt)$$

where V_r is the volume in the receiver chamber (cm^3), A is the area of the membrane (cm^2), C_0 is the initial concentration of the solute in the donor sample (mM), and dC/dt is the permeability rate (mM/sec).

Mass Spectral Analysis

The chemical structure of Caco-2 permeable compounds was identified by LC/MS analysis of both donor and receiver samples utilizing a Michrom BioResources microbore HPLC system coupled to a LCQ ion-trap mass spectrometer equipped with an electro-spray interface (Finnigan MAT, San Jose, CA). In brief, after samples were made 0.2% in formic acid, 100μl was injected onto a Monitor C_{18} (1 × 150 mm) column (Column Engineering, Ontario, CA) and the analytes were eluted with a linear gradient of acetonitrile in a mobile phase of 0.1% formic acid. Elution of NSG-peptoids was serially detected by absorbance at 210 nm followed by ion scanning from 150 to 600 atomic mass units.

Physico-Chemical Characteristics

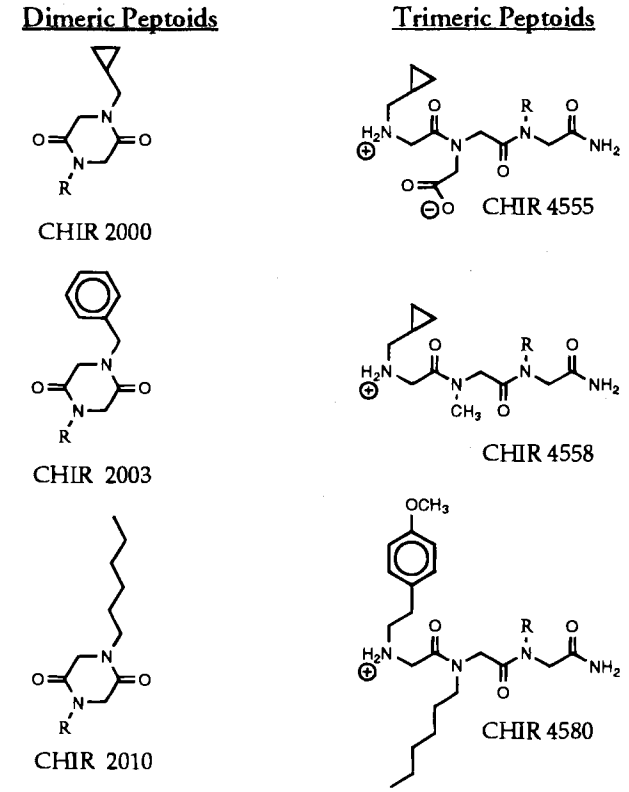
Calculations of apparent octanol/buffer distribution coefficient (Log $D_{o/b}$) and pKa were performed using PrologD v2.0 and pKalc v3.1 software, respectively, from CompuDrug Chemistry Ltd., Budapest, Hungary.

RESULTS AND DISCUSSION

Description and Quantitation of Mixtures of NSG-Peptoids

The chemical structures of the NSG-peptoids analyzed in the Caco-2 model are shown in Figure 1. Each mixture was derived from the same set of 24 side chains and contained that set in the variable position. This set of sidechains was chosen to maximize molecular diversity based on the following criteria: 1) *chemical functionality*, which refers to the functional groups that are represented in each structure; 2) *receptor binding descriptors*, which include radius (sterics), acidity, basicity, hydrogen-bond accepting, hydrogen-bond donating, and aromaticity; 3) *topological indices*, which refers to shape, flexibility, branching, and arrangement of cycles; and 4) *lipophilicity*, such as calculated octanol/buffer partition coefficient (14). As shown in Figure 2 and summarized in Table 1, each mixture contained compounds that varied with respect to lipophilicity (calculated octanol/buffer distribution coefficient), molecular weight, acidity, and basicity. Thus, based on these parameters, each mixture should have exhibited the effects of a fairly wide range of physicochemical parameters on intestinal transport of the common structural element within that mixture.

In order to simplify quantitative comparisons between mixtures, each mixture was treated as a single component such that the values used for quantitation represented the sum of the



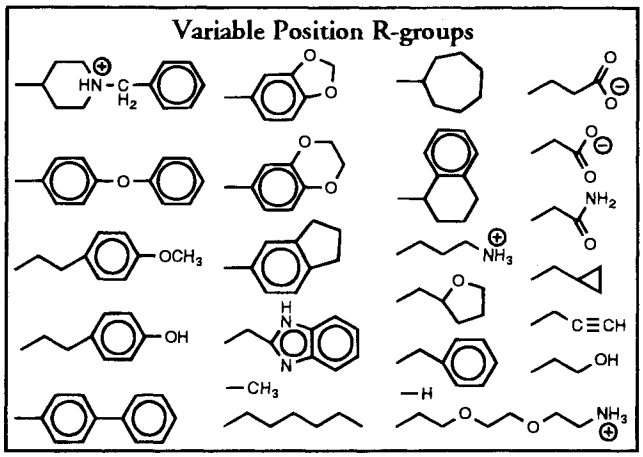


Fig. 1. Chemical structures of NSG-peptoid mixtures analyzed for permeability across Caco-2 monolayers. Chemical functional groups that are charged at neutral pH are denoted by a circled positive or negative sign. Variable position side chains are denoted by R.

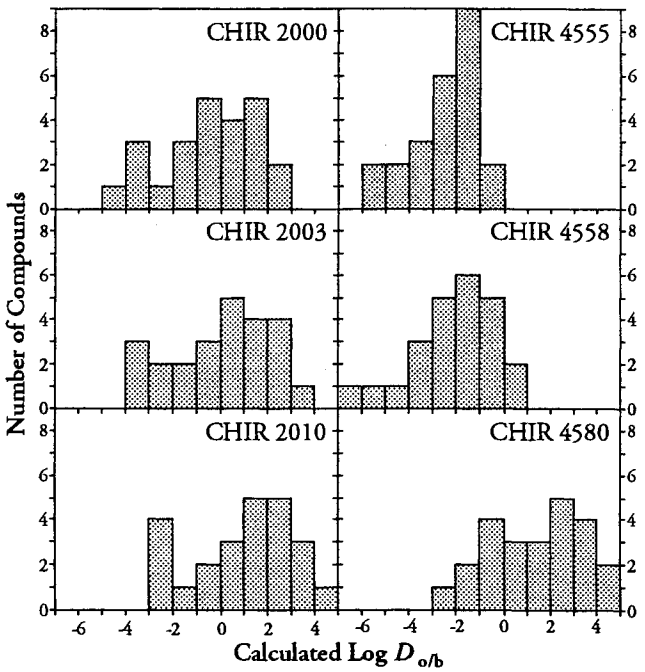


Fig. 2. Histogram analysis of calculated distribution coefficients within mixtures tested in the Caco-2 model. Distribution coefficients between octanol and buffer (Log $D_{o/b}$) were calculated with a buffer containing 150 mM NaCl at pH 7.4 and include ionized species.

individual peak areas. This type of quantitation permitted the calculation of aggregate apparent Caco-2 permeability coefficients (P_{app}) that reflected more the influence of the common structural element within each mixture on absorption. Figure 3 shows a typical chromatogram of a mixture with the region that was integrated and summed defined by verticle hatched lines. In most cases, the 24 compounds that made up each mixture were not completely resolved by the HPLC methodology employed. Thus, it was deduced that some of the peaks represented 2 or more compounds. Standard curves used for quantitation were linear to at least 30 nM per compound with an R^2 of 0.99, and each point had an average difference of approximately 10% between duplicate samples. This limit of quantitation corresponded to a minimum P_{app} of 0.8×10^{-6} cm/sec that could have been measured for each component in a mixture.

Caco-2 Permeability of Mixtures of NSG-Peptoids

Caco-2 permeability values of the NSG-peptoid mixtures analyzed are listed in Table I. For the three linear, trimeric mixtures analyzed, CHIR 4555, CHIR 4558 and CHIR 4580, no compounds were detected in receiver samples at 60 and 120 minutes while the permeability of the mannitol in these samples was unaffected indicating that the monolayers were intact. Based on the minimum measurable P_{app} described above, this observation showed that, for these mixtures, the permeability coefficient of each component was below 0.8×10^{-6} cm/sec, or below the permeability of mannitol (1.0×10^{-6} cm/sec). In addition, the lack of any detectable components in the receiver samples suggested that significant formation of permeable metabolites did not occur. Thus, it appears that many trimeric, linear NSG-peptoids with side chains combinations like methylcyclopropane/acetic acid, methylcyclopropane/methyl, or 4-hydroxyphenethyl/hexyl side chains will be transported across Caco-2 monolayers at a rate below that of mannitol.

Dimeric diketopiperazine (DKP) mixtures CHIR 2000, CHIR 2003 and CHIR 2010 exhibited intermediate aggregate P_{app} values of 9.2×10^{-6}, 15×10^{-6} and 6.9×10^{-6} cm/sec, respectively, as compared to an average P_{app} value of 1.0×10^{-6} cm/sec for the mannitol in these samples (indicating intact monolayers) and a P_{app} value 28×10^{-6} cm/sec for testosterone. Linear transport was observed for each of these NSG-peptoid mixtures over 60 and 120 minutes (data not shown) suggesting that measurements were taken under steady-state conditions. To

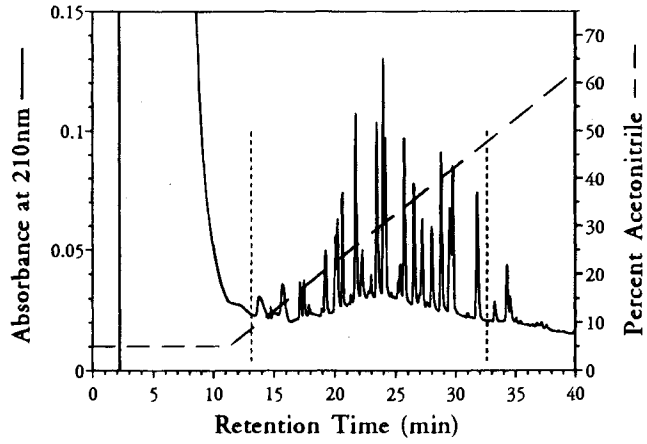


Fig. 3. Reversed phase HPLC analysis of NSG-peptoid mixture CHIR 2003. Percent acetonitrile was corrected for delay volume and represents the concentration of acetonitrile at the detector. Vertical dashed lines define the beginning and end of the region integrated and used for quantitation.

examine whether the aggregate P_{app} values reflected transport of a few or most of the components of a mixture, chromatograms of donor and receiver samples were compared. As shown in Figures 4A–C, the similarity of the RP-HPLC profiles of donor and receiver samples suggested that, while some selectivity was observed, most of the components of the mixtures were detected in receiver samples and contributed to each aggregate permeability measurement.

LC/MS analysis of donor and receiver samples was performed to identify the molecular entities in the HPLC peaks integrated for quantitation and to identify the structures of the compounds that were not transported across the Caco-2 monolayers. The only compounds not detected in the receiver sample of CHIR 2000 were the ones that contained either propionic acid or methyl groups at the variable position side chain. For 2010, compounds containing propionic acid, acetic acid, propylamine and ethoxyethoxyethylamine side chains at the variable position were not detected in the receiver sample. These data suggested that these components of CHIR 2000 and CHIR 2010 exhibited P_{app} values less than 0.8×10^{-6} cm/sec. All parent compounds were detected in the receiver sample of CHIR 2003. These results suggested that DKP NSG-peptoids containing at least one non-polar side chain will generally be permeable to Caco-2 monolayers. LC/MS analysis also revealed that several additional peaks were present in all samples that represented contaminants from the synthesis (see peaks not measured in Figures 4A–C). Furthermore, Figures 4A–C show the appearance of a few new, but small peaks that were unique to the receiver sample of each mixture which suggested the occurrence of some low level metabolism. However, these peaks of unidentified molecules represented less than 10% of the aggregate peak area measured for each sample and did not significantly contribute to aggregate P_{app} values.

Retention during reversed phase chromatography is a measure of polarity such that greater retention time corresponds to lesser polarity. Relative Caco-2 permeability of the parent compounds was plotted across the HPLC profile of CHIR 2000, CHIR 2003 and CHIR 2010 to evaluate the influence of polarity on the permeability of the common structural element within each mixture. As shown in Figures 4A–C, no relationship existed between this measure of polarity and Caco-2 permeability within these mixtures. It is interesting to note, however, that the largest fluctuation in permeability tended to occur relatively early in the RP-HPLC profile, which included the elution positions of the compounds within each mixture not detected in the receiver samples by LC/MS (see Figures 4A and C). These observations suggested that the relatively polar compounds in each mixture exhibited the greatest variable in terms of Caco-2 permeability, while the less polar compounds exhibited more consistent permeability.

Determination of Transport Mechanism for CHIR 2000, CHIR 2003, and CHIR 2010

To determine whether the components of CHIR 2000, CHIR 2003 and CHIR 2010 were passively or actively transported across Caco-2 monolayers, a simple, universal method was desired that would discriminate between these transport mechanisms. It has previously been shown that compounds actively transported via the amino acid, dipeptide, or bile acid transporters exhibited transport polarity with transport predominantly in the AP → BL direction (15–17). Furthermore, for the amino acid and dipeptide transporters, AP → BL transport was shown to be pH dependent, demonstrating that transport was

Table I. Characteristics of Mixtures Containing 24 NSG-Peptoids

Mixture	Template Structure	Molecular Weight Range[a]	Calculated Log $D_{o/w}$ Range[b]	Calculated Acid p Ka[c]	Calculated Base p Ka[c]	Aggregate Caco-2 P_{app}[d] (cm/sec x 10^{-6})
CHIR 2000	cyclic/dimeric	168–341	(−4.2)–2.7	3.4–9.9	4.6–9.8	9.2 ± 1.8
CHIR 2003	cyclic/dimeric	204–377	(−3.7)–3.1	3.4–9.9	4.6–9.8	15 ± 2.7
CHIR 2010	cyclic/dimeric	198–371	(−2.8)–4.1	3.4–9.9	4.6–9.8	6.9 ± 3.0
CHIR 4555	linear/trimeric	300–473	(−5.2)–(−0.61)	3.3	8.4–9.8	<0.8 (ND)[e]
CHIR 4558	linear/trimeric	256–429	(−6.04)–0.78	3.4–9.9	8.4–9.8	<0.8 (ND)
CHIR 4580	linear/trimeric	392–565	(−2.5)–4.5	3.4–10.1	7.6–9.8	<0.8 (ND)

[a] Values indicate the range of molecular weights in a pool.
[b] Values indicate the range of calculated distribution coefficients in octanol/buffer of ionized species in a pool using 150 mM NaCl at pH 7.4 buffer.
[c] Values indicate the range of calculated p Ka values of acids or bases in a pool.
[d] P_{app} values represent the average and 95% confidence interval of triplicate measurements.
[e] Not detected in the receiver chamber. Values shown are relative to the lowest quantifiable standard.

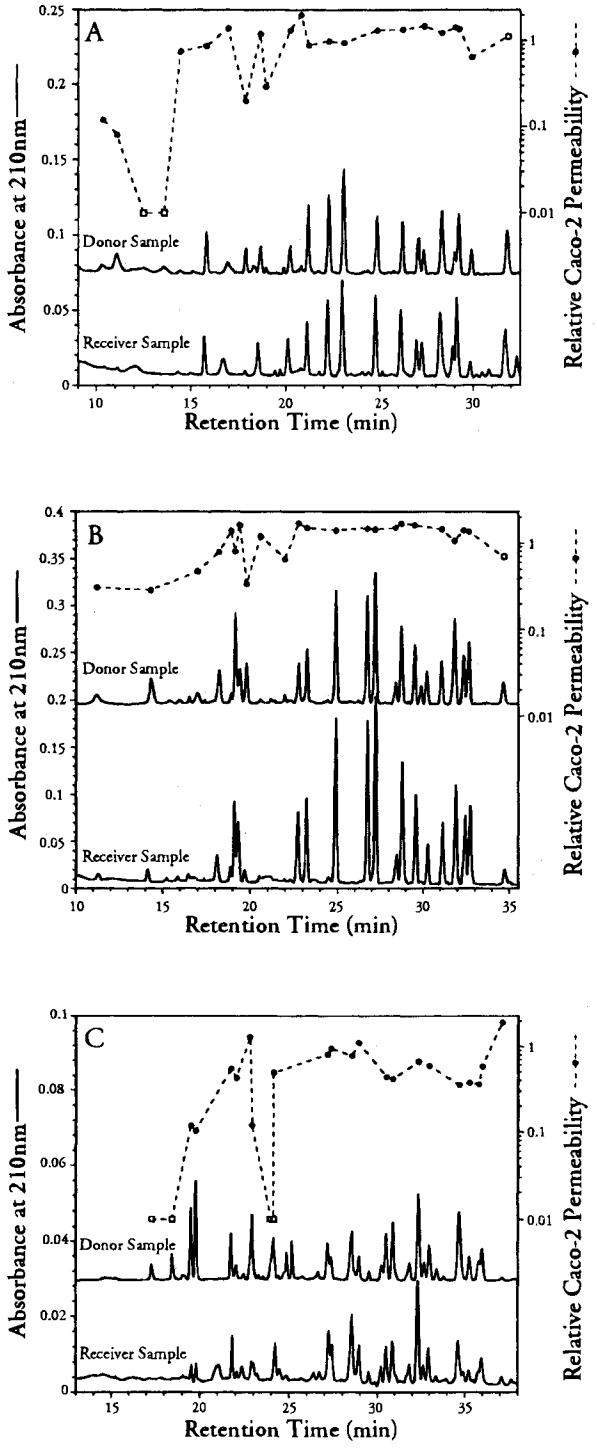


Fig. 4. Comparison of RP-HPLC profiles of donor and receiver samples of NSG-peptoid mixtures CHIR 2000 (A), CHIR 2003 (B) and CHIR 2010 (C). Relative permeability values for parent compounds verified by mass spectral analysis of these chromatograms were defined as the ratio of individual peak area in a receiver sample relative to a donor sample. Peaks representing two co-eluting compounds are denoted by open circles (○). Peaks representing compounds that were undetected in the receiver sample by mass spectral analysis are denoted by open boxes (□).

driven by an inwardly directed proton gradient. Based on these observations, the use of transport directionality under an AP → BL proton gradient was investigated as a screen for transport mechanism. As summarized in Table II, flux of phenylalanine was approximately 8-fold greater in the AP → BL direction compared to the BL → AP direction. Similarly, flux of val-val was approximately 10-fold greater in the AP → BL direction. The transport rates observed for these actively transported compounds were similar to previously reported values (15,17). Flux of a predominately paracellularly transported compound, mannitol, and two predominately transcellularly transported compounds, prazosin and testosterone, were not significantly influenced by transport direction, suggesting that transport by the paracellular or transcellular routes was not direction dependent. Therefore, these data demonstrated that transport polarity can be used to discriminate between passive and active transport across Caco-2 monolayers.

The effect of transport direction on the flux of CHIR 2000, CHIR 2003 and CHIR 2010 is summarized in Table II. For each of these mixtures, flux in the BL → AP direction was equivalent to flux in the AP → BL direction. In addition, RP-HPLC profiles of the components transported in the BL → AP direction were identical to profiles of the components transported in the AP → BL direction (data not shown). Thus, for CHIR 2000, CHIR 2003 and CHIR 2010, all of the components transported across Caco-2 monolayers at a rate greater than mannitol apparently were transported by passive diffusion. The transcellular route was likely the predominant diffusion pathway for these compounds since the rate of diffusion across Caco-2 monolayers by the paracellular pathway is inversely proportional to molecular weight (due to the molecular sieving effect of tight intercellular junctions) (18) and virtually all components of CHIR 2000, CHIR 2003 and CHIR 2010 were larger than mannitol. Moreover, based on the aggregate P_{app} values of CHIR 2000, CHIR 2003 and CHIR 2010 and the LC/

Table II. Effect of Transport Direction on Flux Across Caco-2 Monolayers

Compound or Mixture	Transport Rate (percent/hr/cm²)	
	AP → BL[a]	BL → AP[b]
Phenylalanine[c]	1.2 ± 0.27	0.16 ± 0.032*
Val-Val[c]	0.14 ± 0.27	<0.014 (ND)[d]*
Mannitol	0.21 ± 0.015	0.14 ± 0.012
Prazosin	3.0 ± 1.6	1.91 ± 0.35
Testosterone	9.4 ± 1.6	6.1 ± 2.4
CHIR 2000	4.3 ± 0.42	4.0 ± 0.74
CHIR 2003	6.8 ± 1.2	7.9 ± 2.5
CHIR 2010	3.4 ± 1.2	3.5 ± 0.50

[a] Compound was added to the apical chamber (pH 6.0) and appearance in the abasolateral chamber (pH 7.4) was measured. Values represent the average and 95% confidence interval of triplicate measurements.
[b] Compound was added to the basolateral chamber (pH 7.4) and appearance in the apical chamber (pH 6.0) was measured. Values represent the average and 95% confidence interval of triplicate measurements.
[c] Measured under steady-state conditions at a substrate concentration at or above the reported K_m of the transporter (6,11).
[d] Not detected in the receiver chamber. Value shown is relative to the lowest quantifiable standard.
* Significantly different ($p < 0.05$) from AP → BL transport rate by Student's t test.

MS results described above, it was concluded that dimeric NSG-peptoids with DKP backbones containing a non-polar side chain will generally be readily transported across Caco-2 monolayers by passive mechanisms, and with an order of potential transport of benzyl>methylcyclopropane≈hexyl. Furthermore, the physicochemical diversity among all of the dimeric compounds examined suggested that the DKP backbone facilitated Caco-2 permeability.

CONCLUSIONS

We have shown that mixtures synthesized by combinatorial methods can be used effectively to study structure-transport relationships very rapidly in the Caco-2 model. Since HPLC analysis of Caco-2 samples is an inexpensive and automated assay method, it is feasible to measure aggregate P_{app} values for large numbers of mixtures. Each aggregate P_{app} value is considered a score of the influence of the common structural element within each mixture on intestinal transport, and these values are being used to generate a database that summarizes the influence of various structural elements on transport. Structural elements that exhibit low aggregate P_{app} values are considered unbeneficial to intestinal transport. Furthermore, mass spectrometry facilitates the identification of specific compounds in a mixture, providing a means of elucidating the influence of specific side chain combinations on Caco-2 transport. The information gained from these types of analyses will be incorporated into the design of new synthetic libraries resulting in libraries that are biased towards intestinally permeable compounds.

As combinatorial chemistry continues to flourish, the complexity and diversity of libraries continues to increase leading to the development of new paradigms for more rapid drug discovery (4,19). In the paradigm described by Zuckermann *et al.* (4), high-affinity ligands for the α_1-adrenergic and μ-opiate receptors were discovered by screening large equimolar mixtures of NSG-peptoids in competitive radioligand-binding assays, then re-screening successively smaller sub-mixtures generated by iterative re-synthesis until individual active components were identified (a process referred to as "deconvolution"). When trying to identify orally available drug candidates, the use of an intestinal absorption screen at a mixture stage in the deconvolution process might further accelerate the drug discovery process. For example, we have tested pharmacologically active mixtures of up to 88 compounds in the Caco-2 model and analyzed receiver compartment samples both for transport rate and pharmacological activity (unpublished data). This process of coupling screens for therapeutic activity with GI permeability using mixtures has the potential to rapidly identify compounds that are both biologically active and orally available. Thus, only compounds that are potentially orally bioavailable are further pursued as drug candidates. Screening paradigms such as this are anticipated to bridge the gap between technological advancements in combinatorial chemistry and the introduction of combinatorially derived molecules into the clinic.

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