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International Journal of Pharmaceutics 210 (2000) 29–44

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

A rapid screening system to determine drug affinities for the intestinal dipeptide transporter 2: affinities of ACE inhibitors

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Received 3 December 1999; received in revised form 7 August 2000; accepted 24 August 2000

Abstract

Purpose: To assess the affinities of a series of ACE inhibitors for the di/tri/oligopeptide transport system (DTS) using a rapid in vitro system. *Methods:* Monolayers of Caco-2 cells were cultured in plastic wells for 7–9 days and the uptake of Gly-[^{3}H]L-Pro was used as an affinity probe. Gly-[^{3}H]L-Pro (50 nM), together with excess L-Pro (10 mM), to suppress uptake of any [^{3}H]L-Pro produced by degradation of the probe, was incubated with the test compound (usually 1 mM) at pH 6 for 3-mins. The uptake of radiolabel was determined by liquid scintillation counting. *Results:* A 2-dimensional six-domain model of the transporter based on the structure of a phosphinate ACE inhibitor (SQ-29852) was constructed to facilitate interpretation of the competitor affinities. The SQ-29852 molecule was divided into six binding domains (A–F) based on functional groups within these regions and the effects of structural variation in four of these domains (A, C–E) were explored. A series of dipeptide-like compounds varying within specific domains were selected from a large number of commercially available ACE inhibitors and SQ-29852 analogues. Domain A had a preference for an uncharged group, with bulky hydrophobic groups reducing affinity. Domain C exhibited a preference for a positive charge over a neutral function, with the space this functional group occupies contributing to affinity. Domain D favoured lipophilic residues and domain E retained activity when the carboxylic acid was esterified. *Conclusion:* The test system is able to reveal structure-activity relationships of peptidomimetic agents and may well serve as a design tool to optimise affinity for the DTS.

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0378-5173/00/$ - see front matter

PII: S0378-5173(00)00564-0

Keywords: ACE inhibitors; Caco-2 cells; Dipeptide transporter system (DTS); Dipeptides; Peptidomimetic agents; Structure–activity relationships

1. Introduction

In addition to its nutritional importance, the dipeptide transporter system (DTS) is responsible for the absorption of certain drugs across the intestinal barrier due their similarity to the natural dipeptide substrates. For example, the oral absorption of some angiotensin-converting enzyme (ACE) inhibitors (Hu et al., 1995; Swaan et al., 1995; Thwaites et al., 1995; Marino et al., 1996; Nicklin et al., 1996; Swaan and Tukker 1997; Hu and Amidon, 1988; Friedman and Amidon, 1989a,b) and several cephalosporins (Dantzig and Bergin, 1990; Dantzig et al., 1992; Inui et al., 1992; Oh et al., 1992; Gochoco et al., 1994; Dantzig, 1997; Terada et al., 1997; Bretschneider et al., 1999) is suggested as being in part facilitated by this system. The role of the DTS in the absorption mechanisms for these compounds was discovered after their selection as clinical candidates and information regarding the structural requirements needed for a molecule to bind to the DTS is limited. Previous competition studies have used a variety of probes (e.g. cephalosporins) and animal models (e.g. rat and rabbit brush-border membrane vesicles [BBMV] and perfusion (Okanu et al., 1986)), resulting in a series of investigations that are not directly comparable. Usually, a large concentration of competitor (10–20 mM) has been employed and this may result in saturation of the system, preventing the relative activity of competitors from being ranked. However, we have recently demonstrated that by using a lower competitor concentration (1 mM) a series of structurally similar compounds (dipeptides) could be ranked with regard for affinity for the transporter in a rapid throughput screening (Moore et al., 1995, 2000).

The DTS has been shown to be involved with drug uptake and/or transport in Caco-2 cells (Bailey et al., 1996; Nicklin et al., 1996; Gan and Thakker, 1997 Dantzig, 1997; Terada et al., 1997; Raessi et al., 1999; Bretschneider et al., 1999; Schoenmakers et al., 1999) however, structural information required for recognition by the transporter is sparse. It has been reported that a free α-amino group is not essential for recognition by the DTS (Bai et al., 1991; Swaan and Tukker, 1997; Moore et al., 2000). The presence of a net charge on dipeptides has been reported to have no effect on interaction with the DTS (Hidalgo et al., 1995). However, we have shown that dipeptides with a net negative charge have less affinity than those in a zwitterionic state (Moore et al., 2000). Also, the presence of a negatively charged carboxylic acid moiety has been suggested to be essential for transport (Swaan and Tukker, 1997). The C-terminal peptide bond has been shown not to be essential for recognition by the transporter but is essential for transport. This was demonstrated by the inhibition of amoxicillin transport across rat intestine by enalapril and its reduced peptide analogue enamipril whilst concentration dependent flux was seen (Schoenmakers et al., 1999). Intriguingly, Han et al. (1998) have recently shown that amino acid esters of nucleosides are substrates for the dipeptide transporter despite having no structural features in common with the natural substrates. To help resolve these issues, and to provide enhanced structure–activity relationships, an in-depth systematic study is required, whereby a large number of structurally different potential competitors from related series, are screened for interaction with the DTS. This would allow the structural demands for binding to the transporter to be defined, offering drug discovery and development programmes the prospect of designing new orally absorbed peptide-like drugs with enhanced bioavailability via improved recognition to the DTS. A system using Caco-2 cells and glycyl-[^{3}H]L-proline (Gly-[^{3}H]L-Pro) suitable for rapid throughput screening, has been designed to allow affinity of potential competitors for the DTS to be ranked (Moore et al., 2000). Our aim in the present study was to explore the structural requirements for recognition by the DTS using this screening model. Approximately forty compounds were selected from ACE-in-

hibitors, both commercially-available and from 5000 dipeptide-like SQ-29852 analogues available from the in-house library at Bristol-Myers Squibb, with the selection criteria being based on structural variations within specific molecular domains.

2. Materials and methods

2.1. Materials

Culture media and conditions were as described previously (Moore et al., 2000). The ACE inhibitors, benazepril and benazeprilat were a kind gift from Dr R. Webb, Ciba (Summit, NJ), and cilazapril was kindly provided by Roche (Welwyn Garden City, Hertfordshire, UK). All other compounds used in this series of experiments were from the in-house library of Bristol-Myers Squibb, Princeton, NJ.

2.2. Cell culture

Caco-2 cells (passage 17) were purchased from the American Type Tissue Culture Collection, Rockville, MD, and cultured as previously described (Moore et al., 2000). For uptake studies, the cells used were between passages 25 and 36 and grown on 24-well tissue culture plates (2 cm^2) at a density of 8×10^4 cells cm^{-2} (1.6×10^5 cells $well^{-1}$). The growth media (M1) (2 ml) was renewed every 48 h and the monolayers were used after 7–9 days of growth.

2.3. Gly-[^{3}H]L-Pro uptake into Caco-2 cells in the presence of competitors

The methods employed and validation of the system have been described previously (Moore et al., 2000). Briefly, the incubation media (M2) contained 50 nM of the probe Gly-[^{3}H]L-Pro, 10 mM L-Pro, 2% DMSO, and 1 mM of competitor, unless otherwise stated. The pH of M2 was checked after any additions and corrected to pH 6 with HCl (1M) or NaOH (1M) solutions if required. SQ-29852 was tested at 1 mM on each experimental day, in order to check the reproducibility and viability of the system. Cells were washed (0.5 ml for 5 min) with M2 at 37°C and then incubated with the incubation solution (250 μl) for 3 min, at 37°C with agitation by an orbital shaker (50 cycles min^{-1}). The plates were then transferred to the cold table (4°C) and the apical solutions removed. The monolayers were washed (2×500 μl $\times$ 5 min) with PBS stop-solution (4°C), and then harvested in 1 ml 1% (v/v) TX-100 in double-distilled water. The apical solutions, washings and solubilised cells were collected, added to 15 ml Ecolite$^+$ cocktail and assessed for [^{3}H]-content by liquid scintillation counting [(LSC), Hewlett Packard, Model 2500]. The competitors were initially screened at a 1 mM concentration for inhibition of Gly-[^{3}H]L-Pro uptake, and thus their affinity for the binding site of the DTS. For the competition studies where the % inhibition of Gly-[^{3}H]L-Pro uptake was less than 20% or greater than 80%, the experiment was repeated with the competitor at a higher or lower concentration respectively, solubility and material supply permitting.

2.4. Data analysis

The data sets are expressed as the mean K_i (mM) $\pm$ standard deviation (S.D.) from at least three monolayers. In order to quantify the effect of competitors on the active component of Gly-[^{3}H]L-Pro uptake, the inhibitions (%) were corrected for non-specific, passive, uptake ($\sim$15%) by subtracting the amount of uptake seen in the presence of 5 mM unlabelled Gly-L-Pro. The K_i value was calculated from Eq. (1) (Moore et al., 2000) assuming competitive binding.

$$K_i = \mathrm{IC}_{50} = \frac{[I]}{(r-1)}, \quad (1)$$

where K_i (mM) is the transport inhibition constant — equivalent to the concentration required to reduce interaction by 50% (IC_{50}), r is the ratio of the uptake rate in the absence (v_0) of an inhibitor to the value in the presence of (v_i) of an inhibitor ($r = v_0/v_i$) and [I] (mM) is the inhibitor concentration.

Where K_i values were greater than the test concentration used, results are italicised with the

compound ideally requiring further testing at a higher concentration in order to produce a more accurate value; very poor interactions are expressed as >20 mM,. Analysis of variance with the Dunnett (comparison with control SQ-29852) Tukey (comparison of all values with each other) post-tests, or *t*-tests, were used as appropriate.

3. Results and discussion

3.1. ACE inhibitors

There has been significant interest regarding the role of the DTS in the absorption mechanisms of the peptide-like ACE inhibitors across the gastrointestinal tract (Thwaites et al., 1995; Friedman and Amidon 1989a,b; Hu et al., 1995; Swaan et al., 1995; Morrison et al., 1996). Recently, the diacid ACE inhibitors (enalaprilat, benazeprilat, quinaprilat and fosinoprilat) have been shown to be able to bind to the DTS, but are not substrates for transport, whereas the inactive ester prodrugs are transported (Swaan and Tukker, 1997). This suggests that the second carboxylic acid group, which possesses a negative charge, possibly abolishes transport (Swaan and Tukker, 1997). Schoenmakers et al. (1999) showed that a dipeptide bond is essential for transport but is not a requirement for recognition. All of the commercially available ACE inhibitors tested produced some degree of inhibition of the probe (Table 1), producing K_i values which varied from 0.11 ± 0.01 to >20 mM and indicating that all have some degree of interaction with the DTS. This, however, does not imply that active transport is an essential feature for in vivo absorption. For example, fosinopril had the greatest affinity for the transporter (K_i 0.11 ± 0.01 mM) but reports state that absorption in rats (single-pass perfusion method) is by a passive mechanism which does not involve the DTS (Friedman and Amidon, 1989b). However, active transport may well occur but this may be swamped by the major passive component during in vivo studies at therapeutic concentrations.

SQ-29852, which has been shown to have carrier-mediated uptake in Caco-2 cells (Nicklin et al., 1992, 1996), produced inhibition of the probe (Moore et al., 2000). A molecular modelling study (Swaan and Tukker, 1997) has suggested that the second carboxylic acid function of lisinopril, which in the case of enalaprilat has been shown to abolish transport, is stabilised by internal bond formation with the amino group of the lysyl side-chain thus facilitating the observed active transport. Such an effect can also occur in the case of SQ-29852. Quinapril, fosinoprilat and benazepril had equivalent affinities for the transporter, with no significant difference between K_i values at the confidence limits tested (95%). Quinapril transport has been shown to involve the DTS in Caco-2 cells, with a contribution from a non- saturable pathway (Hu et al., 1995). The fact that quinapril transport could only be inhibited by 50–55% with various inhibitors, and cephalexin had a much greater affinity for the DTS in Caco-2 cells, suggests a major passive component (Hu et al., 1995). However, in the present system, quinapril demonstrated a much greater affinity for the DTS than cephalexin (K_i 0.94 ± 0.23 mM and 9.55 ± 0.51 mM respectively, Moore et al., 2000). This difference may be due to use of a different Caco-2 clone, or the influence of specific experimental models, i.e. uptake versus transport. Fosinoprilat showed a 10-fold lower K_i value than its prodrug (fosinopril) which produced a value of 1.03 ± 0.04 mM.

Similarly, the esterified prodrug, benazepril, showed greater affinity for the DTS compared to its free diacid non-prodrug benazeprilat (K_i 1.07 ± 0.09 mM and 5.02 ± 1.45 mM respectively). Benazepril uptake by rat everted intestinal rings has found to include a carrier-mediated component whereas uptake of benazeprilat was found to be passive (Kim et al., 1994). As previously suggested for enalapril and enalaprilat, benazaprilat may simply be blocking the active site on the DTS and therefore preventing uptake of the displaceable probe, without being a substrate for transport itself (Swaan and Tukker, 1997). Alternatively, it may have a lower affinity for the DTS and therefore, in vivo, the active component is not discernible in presence of the larger passive component. Cilazapril, which has a bicyclic (seven- and six-membered fused) ring system in

Table 1
The effect of a series of ACE inhibitors on the uptake of 50 nM Gly-[^{3}H]L-Pro[a]

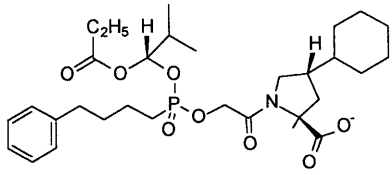
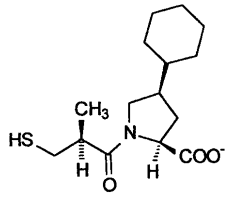
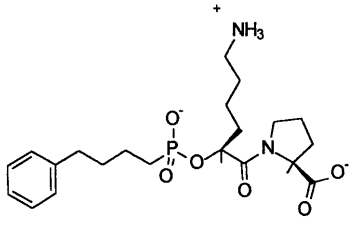
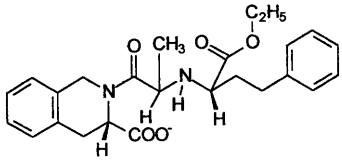
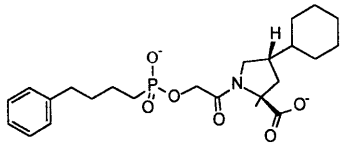
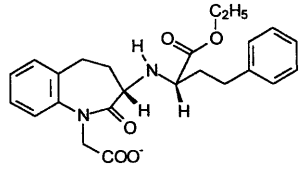
Compound	Conc (mM)	% Inhibition mean [S.D.]	K_i (mM) mean [S.D.]	
Fosinopril		0.1	47.28 [1.11]	0.11 [0.01]
4-Cyclohexylcaptopril		1	60.28 [1.53]	0.66 [0.04]
SQ-29852		1	60.51 [6.73]	0.68 [0.17]
Quinapril		1	52.03 [5.70]	0.94 [0.23]
Fosinoprilat		1	49.31 [0.94]	1.03 [0.04]
Benazepril		1	48.49 [2.15]	1.07 [0.09]

Table 1 (*Continued*)

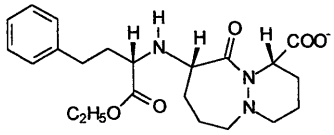
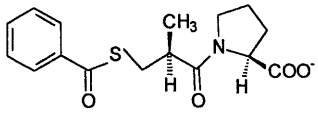
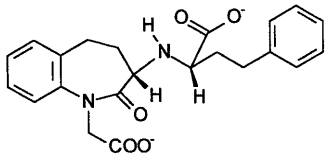
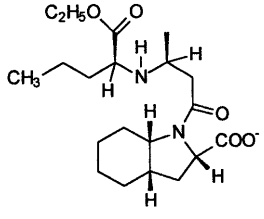
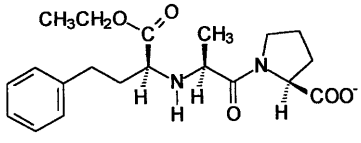
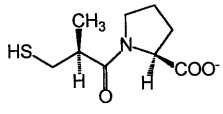
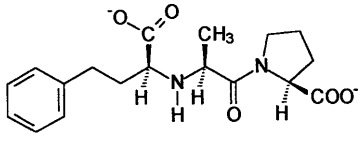
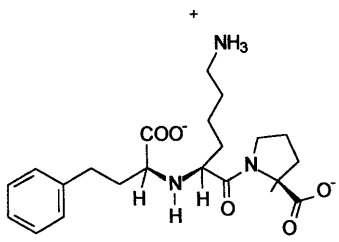
Compound	Conc (mM)	% Inhibition mean [S.D.]	K_i (mM) mean [S.D.]	
Cilazapril		1	43.27 [5.53]	*1.34 [0.29]*
S-Benzoylcaptopril		1	41.58 [5.20]	*1.43 [0.33]*
Benazaprilat		5	50.68 [7.96]	5.02 [1.45]
Perindopril		5	47.43 [6.07]	5.66 [1.34]
Enalapril		10	33.32 [2.21]	>20
Captopril		10	29.40 [2.59]	>20
Enalaprilat		10	25.43 [4.76]	>20

Table 1 (*Continued*)

Compound	Conc (mM)	% Inhibition mean [S.D.]	K_i (mM) mean [S.D.]	
Lisinopril		10	24.09 [3.72]	>20

[a] For those compounds which show affinity for the DTS (i.e. excluding enalapril, captopril, enalaprilat and lisinopril where $K_i > 20$ mM) only benazeprilat and perindopril were significantly less active ($P < 0.01$) than the control (SQ-29852) using analysis of variance and the Dunnett post-test; fosinopril was more active ($P < 0.05$) when benazeprilat and perindopril were discounted. Monolayers were washed (1×0.5 ml (Ap) $\times 5$ min) with M2. They were incubated with M2 containing 50 nM Gly-[3H]L-Pro, 10 mM L-Pro in 2% DMSO at pH 6 in the presence of (0.1–10 mM) ACE inhibitor. Data are corrected for 15% non-specific uptake and presented as mean% inhibition $\pm$ S.D. for three monolayers and mean $K_i \pm$ S.D. mM.

place of the proline ring, showed a high level of affinity for the transporter, demonstrating that the proline ring is not essential for recognition. The prodrug perindopril produced 47.43 ± 6.07% inhibition of the probe compound when tested at 5 mM; however, the active moiety, perindoprilat, was without an effect at 1 mM (data not shown).

Enalapril, an alanyl-L-proline analogue and prodrug, has a non-passive absorption mechanism via the DTS in rat intestinal tissue in vitro (Swaan et al., 1995). Morrison et al. (1996) showed that this drug had a low affinity for the transporter and failed to inhibit the uptake of Gly-[^{3}H]L-Pro by Caco-2 cells at a concentration of 1 mM although 10 mM produced inhibition. The non-competitive inhibition by enalapril of cephradine uptake (a cephalosporin and a substrate of the DTS) has also been reported (Yuasa et al., 1994). Both enalapril and enalaprilat showed similar degrees of affinity for the DTS, producing inhibition values of 33.32 ± 2.21 and 25.43 ± 4.96% respectively at a 10 mM concentration. Although enalaprilat is reported to be absorbed passively in rat intestines, affinity for the transporter has been previously reported (Swaan et al., 1995). It has been proposed that the second negative group in enalaprilat has a deleterious effect on transport but does not prevent binding to the receptor (Swaan and Tukker, 1997). Present results are in agreement with this, as indicated by the observed inhibition, and suggest that enalaprilat is blocking the transporter site of the DTS, rather than being a true substrate.

The absorption mechanism of captopril has been the subject of debate. It was originally shown, in human pharmacokinetic studies, to have concentration-independent absorption, implying a passive pathway (Duchin et al., 1982). However, coadministration with food reduced the absorption by 35% — perhaps suggesting a saturable carrier-mediated mechanism (competition for the transporter with the products of the intraluminal digestion of dietary proteins) or the result of the formation of poorly absorbed disulphides from captopril and food proteins (Singhvi et al., 1982; Williams and Sugerman, 1982). Concentration-dependent absorption and inhibition with dipeptides and cephradine has been observed in a rat study suggesting an active process via the DTS (Hu and Amidon, 1988). This was confirmed in studies that showed captopril absorption to be via a carrier-mediated process (Zhou and Li Wan Po,

1994). Captopril has also been shown to produce acidification of the intracellular environment in Caco-2 cells suggesting H^+-flow into the cells as a result of uptake via the DTS (Thwaites et al., 1995). Captopril transport probably has both passive and active components but here belongs to the group of compounds with the least affinity for the transporter. The 4-cyclohexyl and 4-benzoyl analogues of captopril both produced better inhibition of the probe when compared to the parent compound. This suggests that lipophilic functional groups on the proline ring may enhance interaction with the transporter.

There are also conflicting reports on the absorption mechanisms of the lysyl-L-proline-like ACE inhibitor, lisinopril. In Caco-2 cell monolayers, the transport has been shown to be independent of the H^+ co-transport system (DTS) and appears to involve a paracellular pathway (Thwaites et al., 1995). However, lisinopril was absorbed by a non-passive mechanism and to a very low extent in a rat perfusion model (Friedman and Amidon, 1989a). Swaan et al. (1995) have also demonstrated an active process for lisinopril transport in an in vitro system using rat intestine. Intermolecular hydrogen-bond formation causes an increase in affinity for the carrier when compared to the structurally-related diacid enalaprilat. The present study does not find a significant difference in the inhibition values for enalapril, enalaprilat and lisinopril and all exhibit low affinity for the transporter in the model used ($K_i > 20$ mM).

The esterified prodrugs, benazapril and fosinopril, showed greater affinity for the DTS than the non-prodrug forms. Enalapril, however, showed similar affinity to its prodrug enalaprilat. Prodrugs are developed in order to increase oral absorption of the parent drug by increasing lipophilicity and, thus, improving passive uptake. From the present work, increasing the lipophilicity by esterification also appears to improve recognition by the DTS, which may, thus, be an important feature for interaction.

3.2. SQ29852 analogues

A 2-dimensional domain model (Fig. 1) of the DTS binding site based on the structure of SQ-29852, was constructed in order to facilitate the selection of potential competitors. The theoretical binding site was divided into six binding domains (A–F) and compounds were selected based on their structural variations in localised regions. The results shown have been selected in order to illustrate the structural dependence of the interactions. No 3-dimensional structure assumptions were employed. All of the analogues tested had the same structural feature in domains B and F, thus preventing investigation into binding requirements of these regions; the number of compounds with variations in domain E was small, thus limiting the extent of that investigation.

3.2.1. Domain A

The structural requirements of binding region A (Fig. 1) were explored by replacing the phenylbutyl group in SQ-29852 with various other substituents (Table 2). Compound **1** has an amino-*n*-hexyl group (weak base) that gives domain A a positive charge and makes the molecule a double zwitterion. This shows a reduction in affinity for the DTS (large increase in the K_i value compared to SQ-29852). This suggests that a large hydrophobic group is preferred in this region, as previously suggested in a putative model designed for transport recognition sites with the DTS through molecular modelling studies (Swaan and Tukker, 1997). Compound **2**, which further has the the aminobutyl (lysyl residue) group in domain C replaced by a methyl group, shows no affinity for the transporter, even though it has the same overall net charge as SQ-29852. The second negative carboxylic acid group has been reported to have a unfavourable effect on binding (Swaan and Tukker, 1997). However, in SQ-29852 and **1**, the amino group of the aminobutyl chain may be able to form internal hydrogen bonds, which neutralise this effect and allow binding to occur. This is reflected in inhibition of the dipeptide probe in the present system. It may be that the aminohexyl side-chain in domain A is not able to hydrogen bond with the phosphinic acid residue, perhaps due to transannular interactions in a medium-size ring; this results in a single free carboxylic acid function analogous to lisinopril. Based on the size

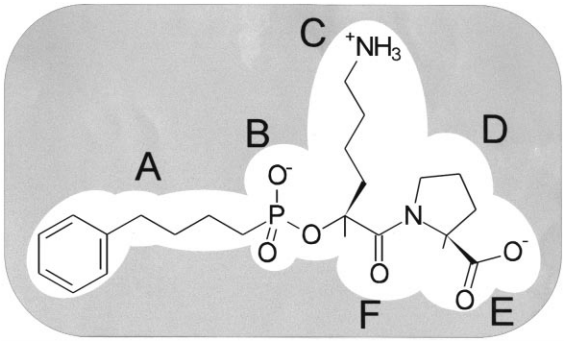


Fig. 1. Theoretical two-dimensional six-domain model of the DTS binding site based on the SQ-29852 structure.

of ring structures formed via hydrogen bonding, the hydrogen bonds with the phosphinic acid function or the proline carboxylic acid in SQ-29852 should be more favoured than the formation of hydrogen bonds between the aminohexyl side-chain of domain A in **2** and either the phosphinic acid function or the proline carboxylic acid function of this compound. This and the higher lipophilicity of domain A of SQ-29852 possibly contribute to the reduced affinity of **2** for the DTS relative to SQ-29852. Compound **3**, which has an additional phenylketo group in domain A shows a reduction in inhibition of the probe compared to SQ-29852-but not to the extent seen with **1** and **2**. An aromatic amide group in the A domain (**4**) also showed reduced recognition, compared to SQ-29852, but showed greater affinity than **1**. Whether these effects are due to steric interactions in the (now) bulky A domain or whether the substituent groups in question have electron redistribution effects which influence intra-molecular hydrogen bonding could not be clarified in the present work.

In summary, region A preferred an uncharged group for optimal binding but the of bulky hydrophobic groups in this region diminished recognition relative to SQ-29852. Interestingly, this type of structural feature has been shown to enhance interaction between ACE-inhibitors and ACE (Pascard et al., 1991).

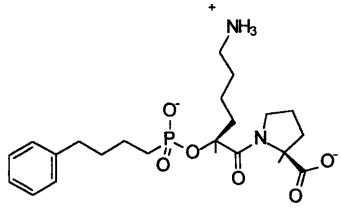
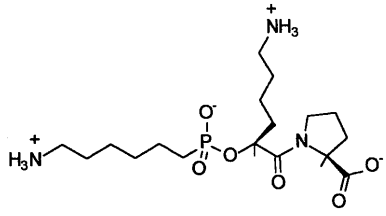
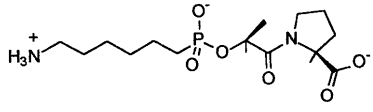
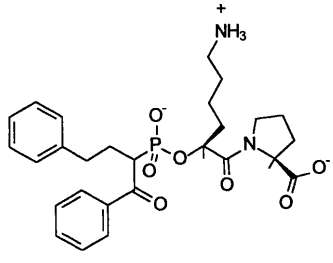
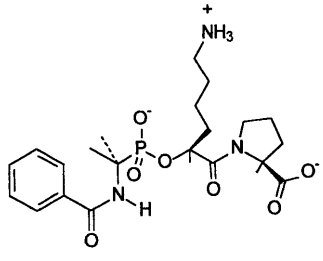
3.2.2. Domain C

Compound **5** (Table 3), has the lysyl side-chain amino group extended by a single methylene group, compared with SQ-29852, which results in the total loss of inhibition at the concentration tested (1 mM). This suggests that the binding site in this region may not be large enough to accommodate this extended structure. When the amine group is removed (**6**), the affinity is again reduced compared to the control (SQ-29852). This suggests that a positive charge in this region is preferred and supports the idea of intermolecular hydrogen-bond formation to override the deleterious effect of the second negatively charged function (Swaan and Tukker, 1997). A similar result has been demonstrated in vivo using lisinopril analogues. The ornithine analogue of lisinopril (positive charge in domain C) was well absorbed in rats, whereas, the *N*-acetyl analogue (neutral function in domain C) displayed poor oral absorption (Patchett, 1993). Further replacement of the amine with a guanadino group (**7**), which is a strong base ($pK_a \sim 12.5$), shows a reduction in binding when compared to SQ-29852. However, extending the chain from aminobutyl (**7**) to aminopentyl (**8**) increases affinity. Compound **5** has a 5-carbon chain in the C domain and recognition was virtually abolished but, when the amine is replaced with a guanadino group (**8**), affinity for the DTS returns. This suggests that the chain-length alone is not an important parameter, but that the space occupied and the surface area available for interaction with the transporter binding site is a key factor. A similar effect has been observed in rats in vivo, with the oral absorption of the guanadino analogue of lisinopril, being lower than that of the parent compound (Patchett, 1993), thus illustrating that the present model can reflect in vivo data.

3.2.3. Domain D

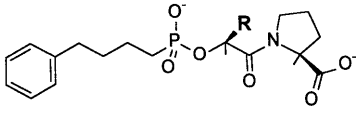
Structural requirements for domain D were explored by using a series of SQ-29852 analogues where substitutions were made at position 4 of the proline ring (Table 4) and where the proline ring was substituted by other functional groups (Table 5). Substitutions in the D domain produced an

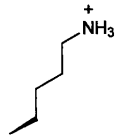
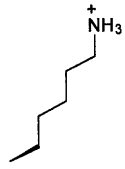
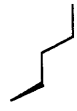
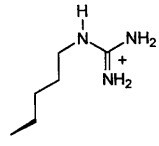
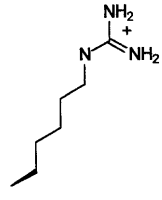
Table 2
Evaluation of binding domain A

Compound	Structure	Conc (mM)	% Inhibition mean [S.D.]	K_i mM mean [S.D.]
SQ-29852		1	60.51 [6.73]	0.68 [0.17]
1		10	18.84 [3.01]	>20
2		1	−1.15 [4.68]	*y*[a]
3		1	24.86 [2.95]	*3.06 [0.51]*
4		10	42.03 [3.62]	*13.91 [2.16]*

[a] *y* corresponds to a % inhibition value which is too small to be converted into a realistic K_i value.

Table 3
Evaluation of binding domain C



Compound	Substituent (R)	Conc(mM)	% Inhibition Mean [S.D.]	K_i mM Mean [S.D.]
SQ-29852		1	60.51 [6.73]	0.68 [0.17]
5		1	0.63 [5.37]	y^a
6		10	88.97 [2.14]	1.24 [0.27]
7		1	25.08 [5.79]	*3.14 [1.01]*
8		1	38.23 [2.39]	*1.62 [0.16]*

[a] y corresponds to a % inhibition value which is too small to be converted into a realistic K_i value.

increase in recognition with the DTS when compared to SQ-29852. All of the analogues tested, with functional groups at position 4 of the proline ring (Table 4), had better affinity for the transporter than the control compound. Compound **9** (OH replacing H) appeared to show a slight increase in affinity for the DTS; however, the K_i value was not statistically different from that of the control ($P = 0.05$). When a lipophilic, aliphatic group, (e.g. cyclohexyl, **10**) is added to the 4-position on the proline ring, there is at least a 10-fold decrease in K_i compared to the control

compound. Compound **11**, which is the stereoisomer, also had greater affinity than SQ-29852, but affinities for the two stereoisomers were not significantly different. Compound **12** (CH_3 at position 4) also showed enhanced affinity. Extension, with a benzyl substituent (**13**) yielded no extra enhancement. Substitution with a 4-thiomethyl group (**14**) produced $\sim$10-fold increase in affinity over the control. In contrast, its stereoisomer (**15**) suffered a reduction in recognition (five-fold increase in K_i compered to **14**), indicating some stereochemical preference at this position. The K_i of a 2,5-dithiocyclopentyl substituent (**16**) was similar to that of the control but the 4-dithiomethyl group (**17**) had the greatest affinity for the DTS from all the compounds tested, producing $67.71 \pm 1.69\%$ inhibition at 0.025 mM (K_i 0.01 mM).

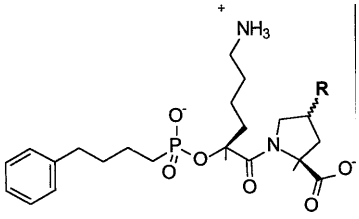
Variations in the proline ring (Table 5) also show a marked improvement in recognition by the DTS. With a 5-methyl substituent (**18**), affinity is marginally but not significantly ($P = 0.080$) improved. However, a positional specificity is apparent, as this compound (**18**) has a lower DTS affinity than the 4-methyl isomer (**12**, Table 4; $P = 0.002$). However, when methyl groups are at both positions 4 and 5 (**19**), an accumulative increase is apparent with a four-fold enhancement of activity (K_i) over the control. Both 4,5-cyclopentyl (**20**) and 4,5-cyclohexyl ring fusion (**21**) with the proline ring, show almost identical affinities-over 10-fold increases compared to SQ-29852-and illustrate the dependence upon lipophilicity in this region. Compound **22**, which has an *N*-cyclohexyl glycine group in place of the proline residue, and **23** which has an analogous *N*-benzocyclopentyl group, both showed an increase in recognition compared to control but both have lower affinities for the DTS than **20** and **21**. This may perhaps be accounted for by the greater rigidity within the more active compounds, which may favour more efficient binding to the transporter to be maintained. These results suggest that a hydrophobic constrained group is preferred in domain D. Additionally, sulphur substituents at position 4 have been shown to be compatible with the DTS, and improve recognition. However, the 2,5 dithiocyclopentyl group (**16**) did not follow this trend. This structure is more conformationally constrained than **17** but exhibits a weaker interaction. This may indicate a relatively precise steric tolerance in domain D.

3.2.4. Domain E

Exploration of the structural requirements for domain E (carboxylic acid) were limited due to the small number of analogues available. The benzoyl ester of the proline carboxylic acid group in SQ-29852 (data not shown) led to an increase in affinity (K_i of 0.05 ± 0.01 mM versus 0.68 ± 0.17 mM). This is in contrast to previous literature that states the presence of a negatively charged carboxylic acid moiety is essential for transport (Hidalgo et al., 1995; Swaan and Tukker, 1997). Further analogues with specific structural variations in domain E are required to clarify the structural demands of this region.

Previous structure-activity relationships (SAR) for the DTS are sparse and comparisons with previous studies are limited. We have recently shown that a free α-amino group is not essential for binding (Moore et al., 2000), which is in agreement with dipeptide transport investigations carried out using rat intestinal segments (Hidalgo et al., 1995) and molecular modelling studies (Swaan and Tukker, 1997). A hypothetical peptide structure for recognition and transportation by the DTS, has previously been proposed, through conclusions drawn from a series of studies investigating cephalexin uptake into rabbit small intestinal brush-border membrane vesicles (BBMV) and photoaffinity labelling of the oligopeptide transporter protein (Kramer et al., 1995). It was suggested that our domain D should be a small, electrically neutral substituent such as H, CH_3 or vinyl. However, we have shown that large lipophilic, aliphatic groups such as cyclohexyl (**11**) result in higher affinities than the parent compound SQ-29852. Kramer et al. (1995) suggest that the region we designate domain C must not include a negatively charged group. We propose, based on the present study, that a group positively charged at physiological pH, such as aminobutyl, has better affinity than a neutral

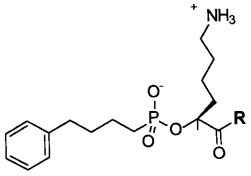
Table 4
Evaluation of binding domain D, using analogues of SQ-29852[a]

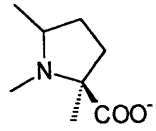
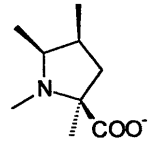
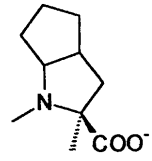
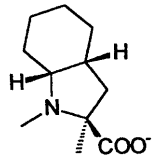
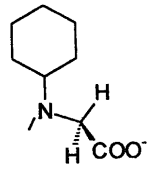
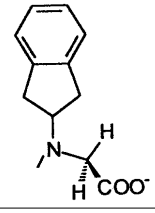


Compound	Substituent (R)	Conc (mM)	% Inhibition Mean [S.D.]	K_i mM Mean [S.D.]
9		1	67.69 [5.41]	0.48 [0.12]
10		0.1	66.29 [7.67]	0.05 [0.02]
11		1	91.68 [3.54]	0.09 [0.04]
12		1	81.05 [1.66]	0.23 [0.03]
13		1	88.24 [2.10]	0.13 [0.03]
14		0.1	66.44 [1.85]	0.05 [0.00]
15		1	78.52 [1.85]	0.27 [0.03]
16		1	58.05 [3.02]	0.73 [0.09]
17		0.025	67.71 [1.69]	0.01 [0.00]

[a] All compounds, with the exception of **16**, were significantly different from the control (SQ-29852) using analysis of variance and the Dunnett post-test; all but **9** ($P<0.05$) were significant at the $P<0.01$ level.

Table 5
Evaluation of binding domain D, using SQ-29852 analogues[a]



Compound	Substituent (R)	Conc (mM)	% Inhibition mean [S.D.]	K_i mM mean [S.D.]
18		1	67.36 [2.24]	0.49 [0.05]
19		1	86.53 [0.21]	0.16 [0.00]
20		0.1	62.37 [1.31]	0.06 [0.00]
21		0.1	61.62 [3.78]	0.06 [0.01]
22		1	75.43 [2.31]	0.33 [0.04]
23		0.5	67.51 [1.42]	0.24 [0.02]

[a] Attachment to the core structure is through the nitrogen atom of all functional groups. All compounds were significantly different from the control (SQ-29852) using analysis of variance and the Dunnett post-test; all but **18** ($P<0.05$) were significant at the $P<0.01$ level.

function. Further work to study this is in progress. The chain length of this group also seems important in the extent of recognition with the DTS. For the A domain in our model, we found that a neutral group, such as phenyl is preferred over positively charged moieties (amino group).

4. Conclusion

A model designed for rapidly screening a large number of potential substrates for interaction with the DTS binding site has been applied to a number of ACE inhibitors and their structural analogues. A two-dimensional six-domain (A–F) model was used to select compounds and variation in four of these domains (A, C–E) was evaluated. Domain A showed a preference for an uncharged group for optimum interaction with the DTS. Domain C showed a preference for a positive charge over a neutral function in this region with some additional size-dependence. All substitutions made to the proline ring (position 4; domain D) resulted in better affinity for the DTS compared to SQ-29852. Some stereochemical preference for the configuration of the substituent functional group was illustrated. For example, the presence of a small aliphatic substituent such as thiomethyl at position 4 on the proline ring *cis* to the proline carboxylate dramatically increased affinity relative to the *trans* analogue. This difference is lost when larger groups are substituted (e.g. cyclohexyl). Interestingly, in a recent molecular modelling publication regarding determinants of recognition for the DTS, this structural aspect was not addressed (Swaan and Tukker, 1997). The validity of the model is confirmed through reference to in vivo data on the bioavailability of ACE-inhibitor analogues (Patchett, 1993) and the information provided here may prove a basis for further work leading to structural optimisation and the improvement of potential clinical candidates for oral delivery, through targeting to the DTS. On the basis of data collected to date, compound 17 may be someway towards this goal.

Acknowledgements

The authors thank the Biotechnology and Biological Sciences Research Council for the award of a post-graduate research studentship (CASE) to V.A. Moore and Bristol-Myers Squibb for additional funding. Also kind thanks go to Novartis and Roche for the provision of the benazepril and benazeprilat, and cilazapril respectively.

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