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Cellular uptake of a radiolabelled analogue of neurotensin in the Caco-2 cell model

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Abstract

Neurotensin is a linear tridecapeptide that elicits a variety of physiological responses in the brain, including hypothermia and antinociception, and reduced levels have been linked to schizophrenia. Previously in our laboratory we developed a truncated neurotensin derivative, KK13. This hexapeptide exhibited key pharmacokinetic and behavioural characteristics of an antipsychotic and elicited central effects after oral administration. To examine the potential mechanism(s) of uptake, a radioactive analogue of KK13 (*KK13) was synthesized, characterized, and evaluated in the Caco-2 cell model of the human intestinal epithelium. Results suggested that uptake of *KK13 was a time-dependent passive process. A general linear trend in uptake was demonstrated over the concentration range (10 μM–1 mM) tested, and uptake was neither pH- nor sodium-dependent. Finally, after 60 min, intact *KK13 was identified associated with the cell components, providing further evidence for uptake and stability of the peptide.

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Introduction

A major obstacle to the development of peptides and peptidomimetics as therapeutic compounds has been their inability to cross biological membranes, in particular, the blood–brain barrier (BBB) and the intestinal epithelium. Strategies designed to improve the membrane permeability of peptides have focused primarily on modulating specific physicochemical properties to facilitate barrier crossing; these include lipophilicity, size, charge state, in-vivo stability, and three-dimensional conformation (Burton et al 1996; Knipp et al 1997; Okumu et al 1997). Transport studies in the Caco-2 cell model of the human intestinal epithelium have demonstrated the importance of solute–solvent hydrogen bonds; the free energy required to desolvate a peptide inversely correlates with the permeability of the peptide (Conradi et al 1991; Okumu et al 1997; Borchardt 1999). Technology developed in our laboratory focuses on the synthesis of non-natural analogues of the cationic amino acids arginine (Arg) and lysine (Kennedy et al 2000). This technology is designed to favourably modulate pharmacokinetic properties in bioactive peptides, including enhancement of barrier permeability. Recently, we identified analogues of neurotensin (NT), a centrally active peptide implicated in schizophrenia (Kinkead et al 1999; Binder et al 2001), that elicit central nervous system (CNS) effects after oral administration (Hadden et al, submitted). These peptides are derivatives of the active C-terminal portion of NT, Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13} [NT(8–13)], and are the first of this class to demonstrate significant antipsychotic properties in animal models after oral dosing, indicating that their unique effects are potentially mediated through improved intestinal absorption. Determining the mechanism(s) responsible for the improved oral bioavailability of these analogues would establish a solid foundation for further development of orally active peptides.

Caco-2 cells, derived from a human colorectal carcinoma, spontaneously differentiate into polarized cells that exhibit well-developed microvilli and brush-border enzymes, including peptidases (Hilgers et al 1990). Caco-2 cells also express the proteins responsible for active transport of amino acids, peptides, and sugars. A strong correlation between uptake in the Caco-2 cell model and oral bioavailability in-vivo has been identified (Artursson & Karlsson 1991). These features make the cells an

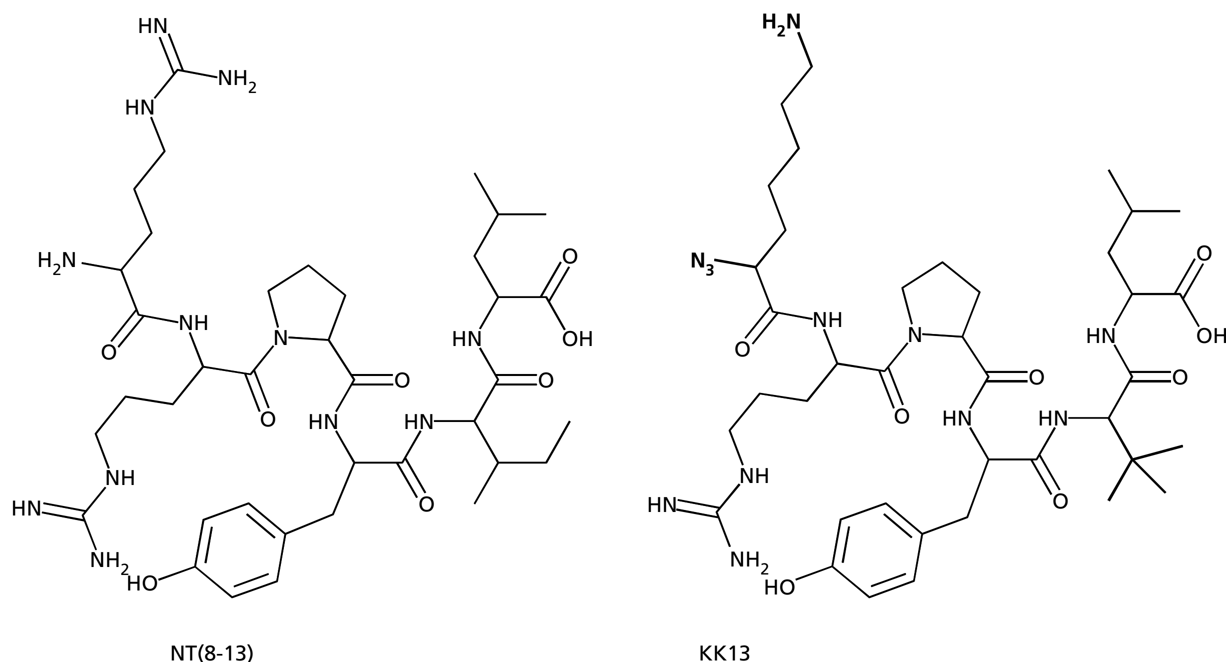

Figure 1 Structural comparison of NT(8–13) and KK13. Bold portions of KK13 represent structural differences from native NT(8–13).

excellent model to study the uptake and transport phenomena of the human small intestine, in which uptake and transfer mechanistic studies can be performed.

KK13 is a lead compound in the development of NT(8–13) analogues as novel antipsychotic drugs (Figure 1) (Kokko et al 2005). It maintains nanomolar binding affinity to neurotensin receptor 1 (NTR_1), the receptor associated with the antipsychotic effects of neurotensin. In addition, KK13 elicits hypothermia and attenuates D-amphetamine-induced hyperlocomotion, two hallmarks of potential antipsychotic compounds, after oral administration. Therefore, KK13 was chosen as a prototype for evaluating the in-vitro cellular uptake of the NT(8–13) analogues via the Caco-2 cell model. A radioactive analogue of KK13 (denoted *KK13) was synthesized for initial examination of the occurrence and potential mechanism of cellular uptake of the NT(8–13) derivatives. The time- and concentration-dependent uptake of *KK13 was examined. In addition, the uptake of *KK13 under reduced pH, sodium-free and ATP-depletion conditions was measured. Finally, RP-HPLC was employed to analyse the solubilized Caco-2 cell components for *KK13 and potential *KK13 degradation products.

Materials and Methods

Synthesis of *KK13

L-Proline (20.7 mg, 0.18 mmol) (Advanced Chemtech) was dissolved in 450 μL of a 10% sodium carbonate solution to which had been added 5 mL H_2O:EtOH (98:2) containing 250 μCi L-[U-^{14}C]proline (Moravek, Brea, CA). Fmoc-N-hydroxysuccinimide (Fmoc-Osu) (100 mg, 1.5 equiv.) in 3 mL dimethoxyethane (DME) was added dropwise to the stirring amino acid solution. The reaction was allowed to stir for 12 h at room temperature and the DME removed in-vacuo. The remaining aqueous solution was diluted with 10 mL H_2O and extracted with saturated n-butanol (4 × 10 mL). The butanol extracts were combined and concentrated to give a pale oil. Residual Fmoc-Osu was removed on silica gel eluting with MeOH:CH_2Cl_2 (50:50). Crude Fmoc-Proline-OH* was used without further purification in peptide synthesis. The synthesis, purification, and characterization of *KK13 was as described for the unlabelled peptide by Kokko et al (2003). The specific activity of *KK13 was 41.6 μCi (51 mg).

Cell culture

Caco-2 cells from American Type Culture Collection (Manassas, VA) were cultured in Eagle's minimum essential medium (MEM) (Cellgro; Mediatech, Herndon, VA) fortified with 1% nonessential amino acids (Mediatech), 10% fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA), 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (Mediatech). Cells were grown in a controlled, humidified atmosphere of 5% CO_2 at 37°C. Cells used for these experiments were from passage 70 to 90.

Cellular uptake methods

For all cellular uptake studies, confluent monolayers were grown in 6-well plastic plates (Falcon; Becton Dickinson,

Franklin Lakes, NJ). Culture medium was replaced three times a week and cells were used 7–10 days post-seeding. Fresh culture media was added 24 h before uptake experiments. One hour before initiating experiments, culture medium was aspirated and monolayers were pre-incubated twice for 30 min with uptake buffer. Time- and concentration-dependence experiments were performed in Hanks' balanced salt solution (HBSS) (Mediatech) with 25 mM HEPES, pH 7.4. Uptake experiments examining pH-dependence were performed in HBSS with 25 mM MES, pH 5.4. In uptake experiments designed to examine sodium-dependence, phosphate-buffered saline (PBS;140 mM Na^+) was replaced with sodium-free PBS brought to equal osmolarity with choline chloride (140 mM). In uptake experiments designed to examine ATP-dependence, cells were ATP-depleted by pretreatment with PBS containing 10 mM sodium azide and 10 mM 2-deoxy-D-glucose. Following the pre-incubation period, buffer was aspirated and replaced with 1 mL uptake buffer containing *KK13. After the specified incubation time, uptake buffer was removed rapidly and the monolayers rinsed three times with ice-cold buffer. Cells were dissolved overnight in 1 mL 0.1 M NaOH.

After complete dissolution of the cells, a sample was removed to determine the protein concentration using the Lowry method (Lowry et al 1951). Hionic-Fluor (10 mL, Packard Bioscience, Meriden, CT) was added to the dissolved cell solution and the samples counted using a Tricarb 2900TR Liquid Scintillation Analyzer (Packard). The statistical significance of differences between treatments was evaluated via an unpaired Student's *t*-test with $P < 0.05$ denoting a significant response. Data was the mean from one experiment performed in triplicate. Separate experiments, performed in triplicate, produced analogous results (data not shown).

RP-HPLC assay

The general procedure for the uptake assays was followed as described above. After complete dissolution of the cells, solvent was removed in-vacuo and the cell components reconstituted in mobile phase and analysed via RP-HPLC. In addition, pure *KK13 was analysed under the same conditions to serve as an elution standard. A linear gradient of 0%–100% B over 30 min at a constant flow rate of 3 mL min^{-1} was used. Fractions were taken every minute and upon completion of the gradient, solvent was removed in-vacuo. Each fraction was dissolved in 1 mL of a 50:50 H_2O:acetonitrile solution, 10 mL liquid scintillation fluid was added, and the fractions counted for radioactivity.

Results

Time-dependent uptake of *KK13

Uptake of *KK13 (200 μM) by the Caco-2 cells was a time-dependent process reaching equilibrium at 60 min (Figure 2A). The initial level of radioactivity measured at time zero was consistent with rapid binding to the cell surface (Kuwayama et al 2002; Vaidyanathan & Walle 2003); however, the continual increase in *KK13 measured over the 60-min time course suggested uptake into the cell. In subsequent experiments analysing the effects of varying treatments on uptake, data was obtained at 5 min as this time point was within the linear range where any specific mechanisms of uptake, if involved, would be active.

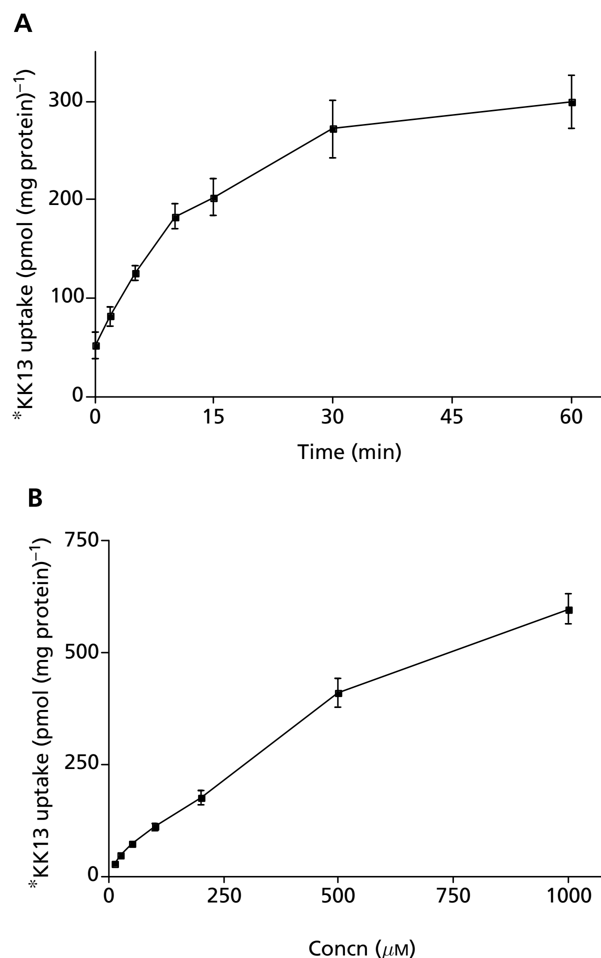

Figure 2 Time- (A) and concentration-dependent (B) cellular uptake of *KK13. A. Monolayers were incubated with *KK13 (200 μM) for the designated time period. B. Monolayers were incubated with the noted concentration of *KK13 for 5 min. Each point represents the mean ± s.d. of an experiment performed in triplicate. Experiments were repeated and analogous results obtained (data not shown).

Concentration-dependent uptake of *KK13

To determine the concentration-dependence of the uptake of *KK13, uptake was evaluated at 5 min across the concentration range 10 μM–1 mM (Figure 2B). Uptake of *KK13 over this range followed a general linear trend. However, without measuring uptake at concentrations greater than 1 mM, no definite conclusions could be made. Higher concentrations were unattainable experimentally.

Effect of varying treatments on *KK13 uptake

The effects of pH, sodium, and ATP on the cellular uptake of *KK13 were determined. Decreased pH had no effect on mean uptake values (Figure 3A). Experiments performed in sodium-free PBS did not produce a significant response (Figure 3B). Uptake under ATP-depletion conditions resulted in a significant increase in mean uptake (Figure 3C) ($P < 0.01$).

RP-HPLC assay

Analysis of *KK13 using the standard gradient described above demonstrated that intact peptide eluted from 21–28 min (Figure 4A). Mass spectral analysis using matrix-assisted lasser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) verified that these fractions were intact *KK13. After a 2-min uptake experiment, a radioactive peak corresponding precisely to intact *KK13 was identified in the solubilized cell components (Figure 4A). Analysis of the cell components after a 60-min uptake experiment revealed an additional fraction containing radioactivity (Figure 4B). Mass spectral analysis of the solubilized cell components with MALDI-TOFMS was not possible due to low concentrations of *KK13; however, the HPLC retention time was consistent with the compound being a fragment of the parent peptide.

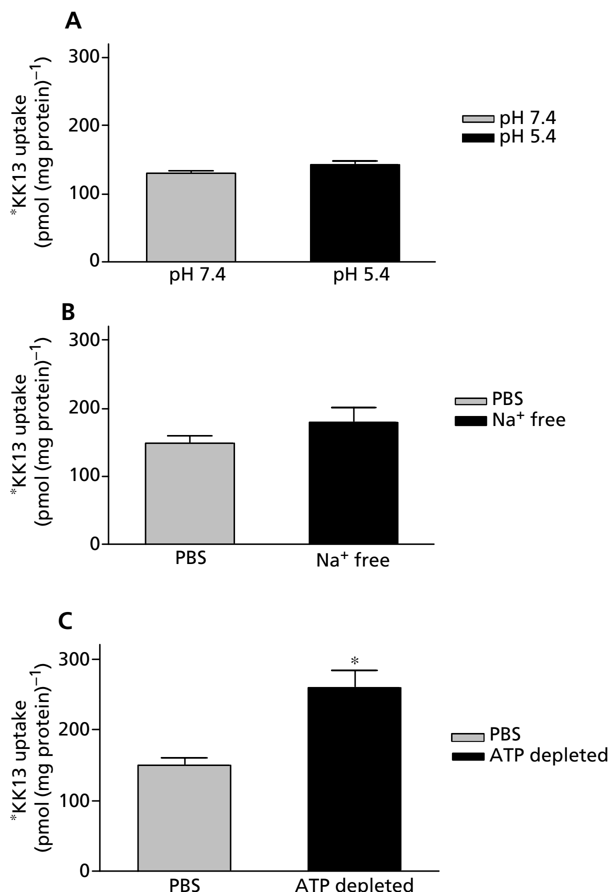

Figure 3 Effects of reduced pH (A), sodium-free medium (B), or ATP-depletion (C) on the cellular uptake of *KK13. Monolayers were incubated with *KK13 (200 μM) under varying conditions as noted in the methods section. Data represent mean ± s.d. of an experiment performed in triplicate (*$P < 0.01$). Experiments were repeated and analogous results obtained (data not shown).

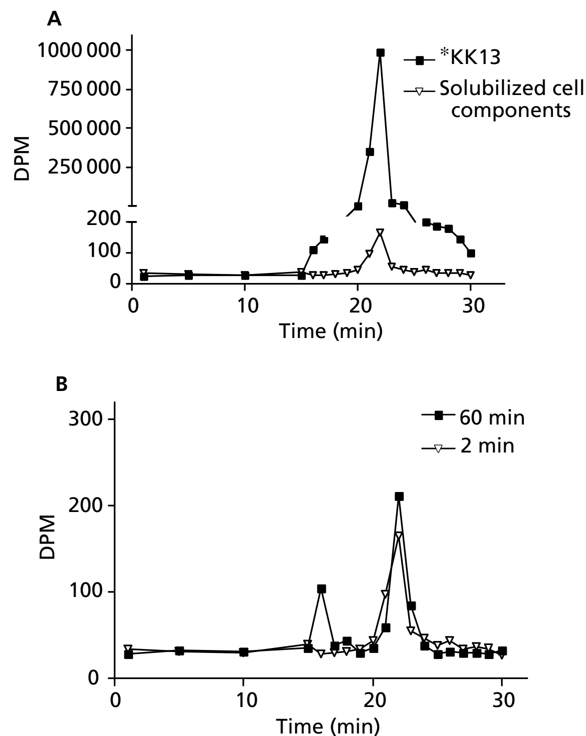

Figure 4 RP-HPLC analysis of the solubilized cell components after *KK13 uptake. A. Comparison of standard *KK13 elution with cell components after a 2-min incubation. B. Comparison of cell components after 2- and 60-min incubation. After uptake experiments, solubilized cell components were analysed via RP-HPLC using the standard gradient described in the methods section.

Discussion

To examine the occurrence and potential mechanism of cellular uptake of the NT(8–13) derivative KK13, Caco-2 cells, a well-established model of the intestinal epithelium, were utilized. When the cells are grown to confluence in regular six-well plates, only the apical membrane, which is well characterized with respect to both absorptive and efflux transporters, is accessible. As noted above, the NT(8–13) analogues developed in our laboratory were the first neurotensin derivatives to elicit CNS effects after oral administration. These preliminary studies were intended to provide information to aid in the development of future peptide analogues with enhanced oral activity.

The concentration of *KK13 used for these uptake studies, 200 μM, was chosen based on the amount available to the rat in-vivo after gavage administration. The

concentration of a 20 mg kg^{-1} dose of KK13, delivered in saline (1 mL kg^{-1}), was 24 mM. The stomach volume in the rat has been estimated to be 11.3 mL/250 mg (Davies & Morris 1993). As gavage dosing ensures direct administration into the stomach, a concentration of approximately 2 mM should be available to the small intestine in-vivo. Therefore, assuming variability in rat size, stomach volume, and peptide absorbed, 200 μM was a physiologically relevant concentration to study *KK13 uptake in-vitro.

From this study, no direct evidence verified the presence of *KK13 within the cell; however, certain findings suggested that the peptide was taken up by the Caco-2 cells in a time-dependent manner. Firstly, washing the Caco-2 cells three times with cold buffer following the designated incubation period removed any loosely bound peptide. Secondly, small molecules that adsorb to the surface of Caco-2 cells do so rapidly (less than 1 min) (Kuwayama et al 2002; Vaidyanathan & Walle 2003). The association kinetics of [^{3}H]neurotensin in rat brain membranes demonstrated that specific binding reached saturation by 10 min (Goedert et al 1984). In addition, maximal levels of nonspecific binding were reached rapidly (approximately 2 min) and remained constant over the 60-min time course (Goedert et al 1984), indicating that all modes of binding were complete at 10 min. Finally, the binding kinetics of other peptide neurotransmitters, including somatostatin, substance P, and neuropeptide Y, parallel those determined for neurotensin (Unden et al 1985). Since the time-dependent uptake of *KK13 did not reach a steady-state equilibrium until 60 min (Figure 2), the results of these studies suggested that the peptide was entering the Caco-2 cells in a time-dependent manner rather than strictly adsorbing to the cell surface.

The concentration-dependence observed for the uptake of *KK13 followed a general linear trend across the concentration range tested (Figure 3). These data supported non-saturable uptake of *KK13; however, they did not conclusively exclude saturable uptake mechanisms. Isoforms of monocarboxylate transporter demonstrated linear transport up to 1 mM and did not show saturation until substrate concentrations of 3 mM and higher were reached (Hadjiagapiou et al 2000). In the *KK13 experiments, limited quantities of peptide made the examination of uptake at concentrations greater than 1 mM unfeasible. Reduced pH had no effect on *KK13 uptake, excluding pH-dependent transporters such as peptide transporter 1 and monocarboxylate transporter 1 from further consideration. In addition, mean uptake values in sodium-free buffer were consistent with control uptake values, which excluded the sodium-dependent transporters SGLT1 and OCTN as well. Interestingly, ATP-depletion conditions produced a significant increase in *KK13 accumulation. The increased accumulation of *KK13 observed in the ATP-depleted cells was consistent with the peptide being a substrate for an active efflux pump. This result was not unexpected as some hydrophobic peptides are substrates for P-glycoprotein (Sharma et al 1992; Sharom et al 1995). In addition, cyclic peptidomimetics are actively effluxed from Caco-2 cells by P-glycoprotein and multidrug resistant protein 2 (Tang & Borchardt 2002a, b).

To assess the stability of *KK13 in cell culture, an RP-HPLC assay analysing the solubilized cell components for intact peptide was developed. After a 2-min uptake experiment, intact *KK13 was identified in the cell components. The HPLC fraction from the solubilized cells that contained peak radioactivity values corresponded precisely with the elution fraction of *KK13. Analysis of the cell components after a 60-min uptake experiment revealed an additional radioactive fraction (16 min). The major radioactive peak after 60 min corresponded to intact *KK13; however, a comparison of the amount of radioactivity detected in the two fractions suggested that approximately one third of the peptide had degraded.

There are four peptidases known to be responsible for neurotensin and NT(8–13) inactivation in the gastrointestinal tract: angiotensin converting enzyme (ACE), and three endopeptidases (EP24.11, EP24.15, and EP24.16) (Checler 1991). Two of these peptidases, ACE (Checler 1991) and EP24.11 (Shrimpton et al 2002), are located in plasma. ACE and EP24.11 have also been identified in the brush-border membranes of Caco-2 cells (Howell et al 1992). KK13 was stable in plasma for greater than 24 h (Kokko et al 2003). These two enzymes do not cleave KK13 in plasma, therefore it was unlikely that they were responsible for the additional radioactive peak identified in the 60-min uptake experiment, suggesting that *KK13 degradation was occurring within the Caco-2 cells. EP24.15 and EP24.16 are primarily located in the cytosol of brain, small intestine, and kidney (Wu et al 1997). Neurotensin cleavage fragments consistent with these two enzymes were found in HT29 cells, another cell model of the intestine derived from a colon adenocarcinoma (Checler et al 1988). This suggested a role for EP24.15 and EP24.16 in the degradation of neurotensin in HT29 cells and their possible role in the degradation of NT(8–13) in Caco-2 cells. Since these two enzymes are primarily located within the cytosol, this supported the contention that the Caco-2 cells had taken up *KK13.

Inspection of the structure of *KK13 provided further support that the peptide was being taken up by the Caco-2 cells. Restricting the conformational flexibility of linear hexapeptides through cyclization has been used as a strategy to increase permeability in Caco-2 cells. The cyclized analogues demonstrated increased lipophilicity and decreased molecular radius compared with the corresponding linear peptide, and in subsequent tests showed increased transport across Caco-2 cells (Okumu et al 1997). Further evaluation of the cyclized peptides provided evidence for the presence of a well-defined secondary structure, specifically a β-turn (Gangmar et al 1996; Okumu et al 1997). In addition, the major conformer in solution of a cyclic prodrug containing an acyloxyalkoxy-linker exhibited an intramolecular hydrogen bond, effectively reducing the hydrogen-bonding potential of the compound (Gangmar et al 1996). Subsequent studies supported the findings that peptides exhibiting a β-turn structure in solution were more lipophilic and would permeate more readily Caco-2 cell monolayers via the transcellular

route (Knipp et al 1997). A recently proposed secondary structure for NT(8–13), a β-turn around Pro^{10} that promotes an intramolecular hydrogen bond between the phenolic oxygen of Tyr^{11} and a guanidinium proton of Arg^{9} (Pang et al 1996), suggested that NT(8–13) analogues that maintain these features might exhibit improved absorption via the transcellular route.

Conclusions

This study did not conclusively establish a specific mechanism responsible for the uptake of *KK13, however a non-saturable mechanism was supported. The uptake of *KK13 followed a general linear trend over the concentration range tested and was not pH- or sodium-dependent. Traditionally, the pharmacokinetic characteristics of a peptide implied that an active mechanism was necessary for permeating the plasma membrane. More recently, it has been established that a rigid conformation and reduced hydrogen bonding potential improves transcellular diffusion in the Caco-2 cell model. *KK13 retained the structural elements of NT(8–13) that were predictive of a Type 1 β-turn and formed a constrained intramolecular hydrogen bond. The uptake of *KK13 supported the contention that these structural characteristics were necessary for developing future NT(8–13) analogues with improved oral bioavailability. In addition, this platform could potentially be applied to other peptides and peptidomimetics to design orally-active compounds.

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