N-Acylated α-Amino Acids as Novel Oral Delivery Agents for Proteins

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A series of *N*-acylated α-amino acids were synthesized and shown to improve the oral delivery of two protein drugs, salmon calcitonin (sCT) and interferon-α. Forty-five compounds in this series were tested in vivo in rats and primates. A significant positive correlation was found between the log *P* of the acylated amino acids and the decrease in serum calcium following oral dosage of sCT in rats. Such a correlation was not found for interferon-α. These derivatized amino acids only weakly inhibited the activity of trypsin or leucine aminopeptidase. Histological examinations of rat intestinal tissue after oral dosing of acylated amino acid/protein combinations revealed no detectable pathology.

Introduction

The gastrointestinal tract has evolved to be a tremendously efficient tissue for absorbing processed nutrients that are essential for the organism while excluding many harmful materials from entering the body. To accomplish this goal, the system presents many barriers that can inhibit the oral delivery of proteins and large peptides. Acid-induced hydrolysis in the stomach, enzymatic degradation throughout the gastrointestinal tract, and bacterial fermentation in the colon are among these barriers. Other barriers to delivery include poor solubility in the intestinal environment and lack of permeation through the epithelial cells. The latter can exclude the passage of compounds across the tissue based on size, charge, and/or lipophilicity. Given these barriers, it is not surprising that oral absorption of protein and peptide drugs has been considered impossible and, at best, difficult. As a consequence, these drugs are administered as parenteral formulations.

In spite of the numerous obstacles described above, there have been numerous reports of attempts to increase the oral absorption of proteins.[1,2] In general, two strategies have been pursued: chemical alteration of the molecule itself to result in a prodrug[3] or the development of formulations that act on membranes to exploit physiological or nonphysiological transport mechanisms. The former approach has had limited success and has been restricted to the preparations of derivatives that improve resistance to enzymatic degradation or alter physicochemical properties, such as hydrogen bonding and/or lipophilicity.[4–7] One problem with this approach is that it requires synthesis of new chemical entities, which can have altered efficacy. The second approach has focused on improving permeability either by the use of general penetration enhancers that probably alter membrane permeability in a nonspecific manner or by the use of nonspecific protease inhibitors. However, these systems are highly inefficient and have been shown to cause transient to long-lasting membrane damage. Such membrane effects have the added disadvantage of allowing nonselective transport of toxic materials. Efforts have also been made to exploit known transport mechanisms such as the peptide transporter, the B12 receptor, M cell uptake, and lipid absorption pathways.[8]

We have discovered an alternative approach to drug delivery based on low molecular weight acylated amino acids which facilitate gastrointestinal absorption of proteins in both rats and primates. Herein we exemplify this system with a low molecular weight model peptide and a high molecular weight model protein, namely salmon calcitonin (sCT, ~3500 Da) and interferon-α (~20 000 Da).

The work reported here is novel and based primarily upon unpublished research[9,10] and, as such, requires a brief description of the rationale that led to the present studies, serendipity notwithstanding. In the late 1980s, Steiner and Rosen in our laboratories discovered that microspheres formed from thermally condensed α-amino acid mixtures could be used to promote oral drug delivery.[11] In general, four amino acids were pyrolyzed in a high boiling solvent. The final products isolated from these processes as dry powders were very complex mixtures consisting mainly of di-, tri-, and tetrapeptides and an uncharacterized polymer component.[12,13] Efforts to separate the thermal condensate into its many individual components using a variety of chromatographic techniques proved difficult and time-consuming. However, we did determine that the small, peptide components of the thermal condensate contained mainly hydrophobic amino acids and the polymeric portion was primarily hydrophilic. We were also able to identify the former, lipophilic mixture of compounds as the component responsible for microsphere formation and subsequent oral drug delivery.

During the course of this work, we were interested in identifying an inexpensive, commercially-available supply of α-amino acids. Thus, we found that hydrolyzed soy protein contained 16 of the 20 natural α-amino acids. However, thermal condensation of this mixture did not generate microsphere-forming material, but rather a hydrophilic mixture that was too complex for analysis.

We then attempted to focus on a method for the generation of low molecular weight, hydrophobic α-amino acid compounds with the ability to form microspheres. Hydrolyzed soy protein again provided a ready source of inexpensive starting materials for the preparation of compounds of this type. In order to increase the lipophilicity of the amino acids in the soy hydrolysate, we derivatized these compounds with phenylsul-

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fonyl chloride. The resulting mixture of phenylsulfonamides proved to have good microsphere-forming properties.[14] Although this material was heterogeneous, it was significantly less complex than its thermal condensate precursor and could be used to encapsulate and orally deliver sCT in rats and primates.[14]

During the course of these experiments, we discovered that sCT absorption could be enhanced significantly by the addition of empty microspheres to the sCT–microsphere preparation.[15] In defining the limits of this dose–response relationship for the delivery agent (empty microspheres), we encountered procedural difficulties in dosing highly concentrated *microsphere suspensions*. In order to overcome these problems, a *solution* of phenylsulfonylated soy hydrolysate and sCT was prepared. Dosing of this solution orally also facilitated sCT absorption in rats and primates.[15] Thus, microspheres were not necessary to promote the oral delivery of sCT.

Chemical analysis of the microspheres prepared from the phenylsulfonylated hydrolyzed soy protein showed five major components: the *N*-phenylsufonamides of valine, leucine, phenylalanine, lysine, and arginine. Indeed, reaction of a mixture of these five amino acids with phenylsulfonyl chloride produced a product remarkably similar, both chemically and in *in vivo* activity, to that obtained using soy protein hydrolysate as the reaction starting material. With the discovery that (phenylsulfonyl)-α-amino acids had the ability to facilitate the oral delivery of protein drugs,[35] we initiated the work described herein.

Salmon calcitonin is a single-chain polypeptide hormone of 32 amino acid residues[16,17] which regulates a number of physiological processes including calcium ion levels in the blood.[18] The calcitonin amino acid sequence varies greatly among species; however, two features of the molecule remain constant: a carboxy-terminal prolinamide and a disulfide bridge between cysteines at positions 1 and 7. Therapeutically, sCT appears to be more potent than human calcitonin because it has a higher circulating half-life and greater affinity for the receptor.[19] Clinically, calcitonin is indicated in the treatment of symptomatic Paget's disease,[20] hypercalcemia[21,22] and postmenopausal osteoporosis.[23]

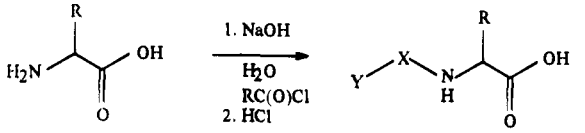
Interferon-α is a lymphokine consisting of 165 amino acids with nonspecific, antiviral activity.[24–26] It is thought to bind to specific receptors on cell surfaces.[27] Once bound, a cascade of intracellular events occurs which results in the inhibition of viral replication in virus-infected cells. Cellular proliferation is also effected. Overall, the immune response of the cells is changed.[28] Interferon-α can be produced commercially by bacterial fermentation of a genetically engineered human leukocyte plasmid. Clinically, interferon-α is indicated in the treatment of hepatitis,[29] hairy-cell leukemia,[30] and AIDS-related Kaposi's sarcoma.[31]

Although significantly dissimilar, sCT and interferon-α may be delivered orally in rodents and primates when dosed in solutions containing one of the acylated α-amino acids described herein.

Results and Discussion

Synthesis. The acylated α-amino acids listed in Tables 1–3 were prepared by standard techniques[32–34] in either aqueous or organic solvents. In general, aqueous reaction conditions include the dissolution of an α-amino acid in aqueous sodium hydroxide followed by the addition of a selected acid chloride (Table 1). After the mixture was stirred at room temperature for about 2 h, the product was isolated by precipitation from the acidified reaction mixture and purified by recrystallization. A representative process in organic solvents is the dissolution of equimolar amounts of an α-amino acid and triethylamine in tetrahydrofuran, followed by the addition of a selected acid chloride. After being stirred at room temperature for about 4 h, the reaction mixture

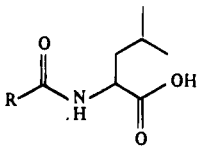
Table 1. *N*-(Phenylsulfonyl)- and *N*-Benzoyl-α-amino Acids



compd[a]	R (amino acid)	X	Y	mp[b] (°C)
1	H (glycine)	C(O)	phenyl	
2	CH_3 (alanine)	SO_2	phenyl	
3	*i*-Pr (valine)	SO_2	phenyl	
4	*i*-Pr (valine)	C(O)	phenyl	
5	*i*-Bu (leucine)	SO_2	phenyl	
6	*i*-Bu (leucine)	C(O)	phenyl	
7	CH_2Ph (phenylalanine)	SO_2	phenyl	
8	CH_2Ph (phenylalanine)	C(O)	phenyl	
9	CH_2Ph (phenylalanine)	C(O)	cyclohexyl	170–171
10	$CH_2(4\text{-}C_6H_4OH)$ (tyrosine)	C(O)	phenyl	
11	CH_2CO_2H (aspartic acid)	C(O)	phenyl	
12	$(CH_2)_2CO_2H$ (glutamic acid)	C(O)	phenyl	
13	$(CH_2)_4CO_2H$ (lysine)	SO_2	phenyl	
14	$(CH_2)_4CO_2H$ (lysine)	C(O)	phenyl	
15	$(CH_2)_3CO_2H$ (ornithine)	C(O)	phenyl	
16	$(CH_2)_3NHC(NH)NH_2$ (arginine)	SO_2	phenyl	
17	$(CH_2)_3NHC(NH)NH_2$ (arginine)	C(O)	phenyl	
18	$(CH_2)_3NHC(NH)NH_2$ (arginine)	C(O)	cyclohexyl	CHN
19	CH_2OH (serine)	SO_2	phenyl	223–225

[a] Compounds **1–8** and **10–17** were purchased from Bachem Biosources, Inc., Philadelphia, PA. [b] Mp for lit. compounds. (CHN) indicates acceptable combustion analysis for new compounds.

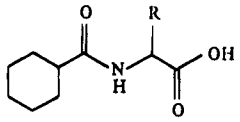
Table 2. Derivatized Leucines for Oral Delivery of Calcitonin



compd	R	peak reduction in serum calcium	HPLC t_R (min)	mp[b] (°C)
20	cyclohexyl	−21.82 ± 3.23	21.95	153–55
21	2-methylcyclohexyl	−18.27 ± 4.89	23.81	CHN
22	3-methylcyclohexyl	−13.69 ± 3.52	24.39	CHN
23	4-methylcyclohexyl	−15.52 ± 5.89	24.57	109–11
24	cycloheptyl	−19.76 ± 4.20	23.50	160–1
25	cyclopentyl	−6.28 ± 7.8	20.59	147–9
26	cyclopropyl	−3.87 ± 5.68	16.20	CHN
27	2-carboxycyclohexyl	−10.47 ± 8.67	18.66	CHN
6[c]	benzoyl	−12.9 ± 2.6		*c*
28[d]	3-methoxyphenyl	−7.9 ± 4.6		*d*
29[d]	2-nitrophenyl	−6.61 ± 2.2		*d*
30[d]	3-nitrophenyl	−14.5 ± 3.6		*d*
31[d]	4-nitrophenyl	−11.8 ± 7.0		*d*
32 (D-isomer)	cyclohexyl	−18.32 ± 6.99	21.97	154–55
33	$(CH_2)_2$cyclohexyl	−14.97 ± 5.0	25.93	CHN
18/20/36	1:2:1 ratio	−33.9 ± 1.7		*e*

[a] The dose of compound was 400 mg/kg, and the dose of calcitonin was 10 μg/kg. The data for compounds **25–28** were normalized to this dose. Control experiments showed that the compounds themselves had no effect on serum calcium levels, and calcitonin dosed alone did not cause a hypocalcemic response. [b] Mp for lit. compounds. CHN indicates acceptable combustion analysis for new compounds. [c] Purchased from Bachem Biosciences, Philadelphia, PA. [d] Cited in ref 35. [e] Total compound dose was 800 mg/kg.

Table 3. *N*-Cyclohexanoylamino Acids for the Oral Delivery of Interferon-α



compd	R	interferon-α[a] (pg/mL)	mp[b] (°C)
9	CH_2Ph	611 ± 126	170–171
18	$(CH_2)_3NHC(NH)NH_2$	2107 ± 2016	CHN
20	*i*-Bu	1124 ± 763	153–155
34	*s*-Bu	340 ± 350	(CHN)
35	$(CH_2)_4NH_2$	3340 ± 2939	oil
36	$CH_2(4\text{-}C_6H_4OH)$	3422 ± 5277	177–9, CHN
37	$(CH_2)_3NHC(O)NH_2$	1922 ± 1371	CHN
38	CH_2(imidazole)	3374 ± 1773	79–81
39	Ph	3808 ± 3203	180–182
		2598 ± 1423[c]	
		434 ± 288[d]	

[a] Compounds **9**, **18**, **20**, **35**, **36**, **38**, and **39** were dosed at 800 mg/kg, and interferon-α was dosed at 1 mg/kg. Compounds **34** and **37** were dosed at 400 mg/kg, and interferon-α was dosed at 0.5 mg/kg. [b] Mp for lit. compounds. CHN indicates acceptable combustion analysis for new compounds. [c] Compound **39** was dosed at 400 mg/kg, and interferon-α was dosed at 500 μg/kg. [d] Compound **39** was dosed at 400 mg/kg, and interferon-α was dosed at 250 μg/kg.

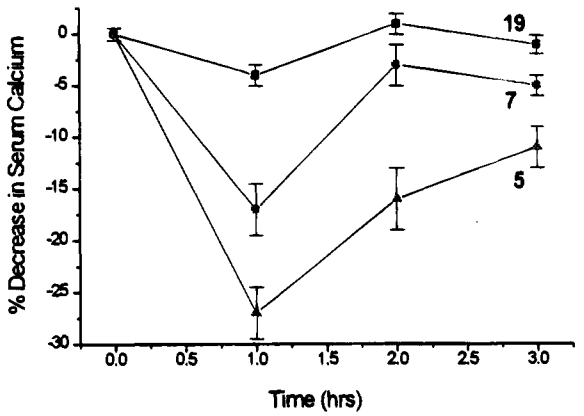


Figure 1. Oral delivery (PO) of sCT in rats using *N*-(phenylsulfonyl)serine (**19**), *N*-(phenylsulfonyl)leucine (**5**), and *N*-(phenylsulfonyl)phenylalanine (**7**) as measured by a decrease in serum calcium at a drug dose of 30 μg/kg and a compound dose of 300 mg/kg.

is quenched and the product purified by recrystallization. All of the compounds reported herein were prepared by either of these two methods with the exception of compound **16**. This material was prepared by acylation of *O*-benzyltyrosine benzyl ester, followed by hydrogenolysis.

Lead Identification and Structure–Activity Relationships. The compounds listed in Table 1 were prepared[35] and tested[14,15] in rats for their abilities to effect the oral delivery of sCT and interferon-α. In this series, *N*-(phenylsulfonyl)leucine (**5**) provided the most efficient oral delivery of sCT and *N*-cyclohexanoylarginine (**18**) produced the most consistent oral delivery of interferon-α. Figure 1 shows the results using compounds (**5**, **7**, **19**) selected from Table 1 for oral sCT delivery in the rat and shows a reduction in serum calcium, the expected physiological response to calcitonin,[36] over time. Although very similar structurally, *N*-(phenylsulfonyl)serine (**19**) does not effect the oral delivery of calcitonin; this performance is generally representative of all of the hydrophilic α-amino acid derivatives tested. *N*-(Phenylsulfonyl)phenylalanine (**7**)

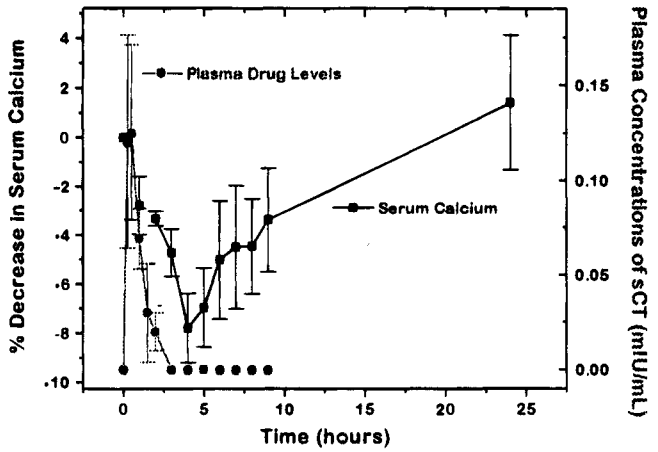


Figure 2. Oral delivery (PO) of sCT in primates using a 2:1:1 ratio by weight of *N*-cyclohexanoylleucine (**20**), *N*-cyclohexanoyltyrosine (**36**), and *N*-cyclohexanoylarginine (**18**) as measured by a decrease in serum calcium and serum sCT levels at a drug dose of 30 μg/kg and a total compound dose of 800 mg/kg.

allows the oral delivery of sCT, but is not as effective as *N*-(phenylsulfonyl)leucine (**5**). The performance of compound **7** is similar to that of all of the derivatized aromatic α-amino acids tested. Attempts to use these *N*-acylated amino acid derivatives to deliver interferon-α in rats met with more limited success. The leads which emerged from the initial experiments were compounds **9** and **18**, both of which produced detectable serum drug concentrations in the test animals (Table 3) and will be discussed later.

With the discovery that **5** facilitates the oral delivery of sCT and that two *N*-cyclohexanoyl-α-amino acids could promote the oral delivery of interferon-α, albeit to a lesser extent, a program to explore these leads was initiated. Fifteen *N*-terminal leucine amides were prepared by reaction of leucine with a variety of aromatic and cyclic acid chlorides (Table 2). Each compound was tested for its ability to facilitate the oral delivery of sCT in rats. In this model, the most efficient single compound in this series was *N*-cyclohexanoylleucine (**20**). The dose of this delivery agent was varied over the range of 400, 300, and 200 mg/kg at a constant sCT dose of 10 μg/kg. The serum calcium levels decreased by 21.82 ± 3.23, 18.61 ± 3.8, and 16.23 ± 8.9%, respectively.

During the course of our experiments, a mixture of **18**, **20**, and **36** in a ratio of 1:2:1 by weight also produced a significant reduction in serum calcium in the rat. On the basis of these data, this mixture was used to demonstrate the oral delivery of calcitonin in primates (Figure 2). Determination of serum sCT concentration and the subsequent decrease in serum calcium provided both pharmacokinetic and pharmacodynamic measure of drug delivery in this experiment. The data clearly show that serum calcium decreases as a response to circulating sCT.

These data suggest that aromatic amide derivatives are significantly less efficient vehicles than the alicyclic amides for sCT delivery. The aromatic amide compounds (**6**, **28–31**) caused an average decrease in serum calcium of about 9%, while the cyclic amides (**20-24**, **32–33**) caused an average decrease in serum calcium of about 18%. Of the alicyclic amides tested, the maximum response was obtained from *N*-cyclohexanoylleucine (**20**); this molecule consists of a six-carbon saturated

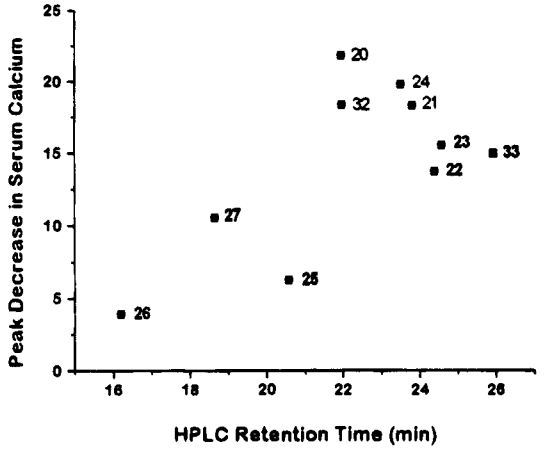


Figure 3. Correlation of oral sCT delivery (PO) in rats, as measured by a reduction in serum calcium, with compound log *P*, as measured by HPLC retention time.

ring in the amide moiety. Those compounds having more than six carbons in the amide group (**21**–**24**, **27**, **33**) were slightly less active; those compounds having fewer than six carbons in the amide group (**25**, **26**) were much less active. This optimal activity for the six-carbon amides suggests that carrier lipophilicity is at least one of the factors effecting the oral delivery of sCT and that a six-carbon amide group results in the most efficacious balance of hydrophobic/hydrophilic properties.

In order to explore this hypothesis, we attempted to correlate our in vivo data of peak hypocalcemia values to define biological response with HPLC retention times as a measure of apparent log *P* (Table 2, Figure 3). Although the data do not define a linear correlation (r = 0.66), the overall trend suggests that, within this series of compounds, those compounds with higher log *P* values are more effective in producing the oral delivery of calcitonin. The weak correlation supports the contention that log *P* is important but not sufficient for drug delivery. There are other factors which play critical roles in promoting absorption. Further insight into this was obtained during our lead optimization studies with interferon-α.

A series of *N*-cyclohexanoylamino acid amides was prepared to study further the oral delivery of interferon-α (Table 3). These compounds were based upon the promising activity of *N*-cyclohexanoylamino acids (**9** and **18**) discussed earlier. In this series (Table 3), maximal drug delivery was obtained using *N*-cyclohexanoylphenylglycine (**39**) which consistantly produced (n = 118 rats) the least variability and the highest serum drug concentrations of the compounds tested. Other compounds in this series showed activity (**35**, **36**, **38**), albeit less that that of **39**.

In a dose–response study, the amounts of delivery agent **39** and interferon-α required to produce measurable serum drug concentrations in the rat were found to be about 400 mg/kg and 250 μg/kg, respectively (Table 3). Compound **39** was also used to demonstrate the feasibility of oral interferon-α delivery in primates. Serum drug levels of about 3000 pg/mL were obtained (Figure 4) after a single oral dose of a solution of delivery agent **39** and drug. For comparison, clinically significant interferon-α levels in humans are reported[37] to be 90–580 pg/mL.

On the basis of the performance of compound **39** with interferon-α, a series of derivatized phenylglycines was

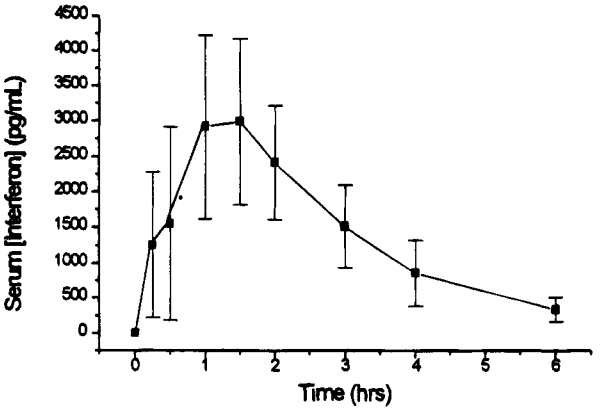
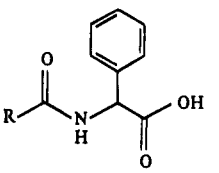


Figure 4. Oral delivery (PO) of interferon-α in cynomolgous monkeys following a single dose of an aqueous solution of compound **39** (800 mg/kg) and interferon-α (1.0 mg/kg).

Table 4. Derivatized Phenylglycines for Oral Interferon-α Delivery



compd	R	interferon-α[a] (pg/mL)	HPLC t_R (min)	mp[b] (°C)
39	cyclohexyl	3808 ± 3203	21.68	180–182
40	cyclopentyl	4707 ± 4039	19.81	127–9 CHN
41	cycloheptyl	4066 ± 6008	23.40	185–7 CHN
42	methylcyclohexyl	6401 ± 5763	23.91	99–102 CHN
43	$(CH_2)_2$cyclohexyl	3026 ± 1639	30.45	137–9 CHN
44	phenyl	8427 ± 2838	20.03	198–200
45	2-hydroxyphenyl	757 ± 581	21.29	117–9 CHN
none	none	688 ± 173		

[a] Compounds **39**–**44** were dosed at 800 mg/kg, and interferon-α was dosed at 1 mg/kg. Compound **45** was dosed at 400 mg/kg, and interferon-α was dosed at 0.5 mg/kg. Reported drug levels are peak values. [b] Mp for lit. compounds. CHN indicates acceptable combustion analysis for new compounds.

next prepared (Table 4). Of these eight compounds, *N*-methylcyclohexanoylphenylglycine (**42**) and *N*-(benzoylphenyl)glycine (**44**) are the most efficient for the oral delivery of interferon-α, producing the highest peak values. These compounds have seven and six carbons in their amide groups, respectively. Another amide having a seven-carbon amide group (**41**) does not perform as well as **42**. Amides having less than (**40**) six carbons or more than seven carbons (**43**) also do not perform as well. For example, **40** and **41** produce high peak drug concentrations; however, the variability is substantial. These data suggest that the hydrophobic/hydrophilic balance of these compounds may be only partially responsible for drug delivery.

The relationship between log *P* and drug delivery measured as peak serum concentration was examined for the interferon-α data. In this case, no correlation was found to exist between these two parameters (Table 4, Figure 5). Thus, it appears that although for the small polypeptide sCT, which lacks tertiary structure, log *P* correlates reasonably well with the ability of *N*-acylated α-amino acids to effect oral protein delivery; in the case of the large, structurally complex protein

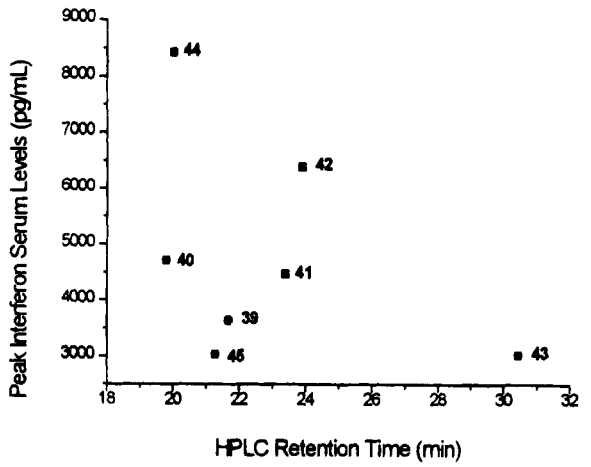


Figure 5. Correlation of oral interferon-α delivery (PO) in rats, as measured by serum drug levels, with compound log *P*, as measured by HPLC retention time.

interferon-α, the relationship between log *P* and drug delivery is less clearly defined. This observation implies, not surprisingly for complex molecules, that other influential factors play an important role in the ability of *N*-acylated α-amino acids to deliver protein drugs orally.

Enzyme Inhibition Studies. Having thus established that these compounds promote oral delivery of both sCT and interferon-α, a preliminary study of the means by which the compounds deliver these protein drugs was undertaken. Initial experiments were designed to investigate if these derivatized amino acids function as potent enzyme inhibitors, which might protect the drug from enzymatic digestion and effect safe transit from the stomach to the intestine, where absorption occurs. While this is a possibility because acylated amino acid derivatives have been reported to be inhibitors of numerous peptidases and carboxylases,[38-40] it is not the case here.

The potential enzyme inhibitory activities of compounds **20** and **39** were measured because they were the most efficient compounds for promoting the oral delivery of calcitonin and interferon-α, respectively. In an effort to mimic in vivo conditions, crude enzyme preparations of USP pancreatin and porcine intestinal peptidase were used to evaluate the effect of the delivery agent compounds on proteolytic enzymes. These two enzyme preparations were chosen as general models for the many proteolytic enzymes found in the gastrointestinal tract. Benzoyl-L-arginine *p*-nitroanilide was selected for study with pancreatin because it is a specific substrate[41] for the trypsin component of the pancreatin. Alanine *p*-nitroanilide was chosen because it is a specific substrate for the leucine amino peptidase (LAP)[42] component of peptidase.

The derivatized amino acids are only weak enzyme inhibitors as compared to Bowman Burke inhibitor (BBI), a known inhibitor of trypsin,[43] and actinonin, a known LAP inhibitor[44] (Table 5). Compound **20** gives 39% inhibition of trypsin and 61% inhibition of LAP at the very high concentration of 10 mg/mL, while BBI inhibits trypsin 100% at 100 μg/mL and actinonin inhibits LAP 100% at 20 μg/mL. Likewise, a concentration of 10 mg/mL of compound **39** is required to achieve 44% inhibition of trypsin and 8% inhibition of LAP. This minimal enzyme inhibitory activity provides strong evidence that the compounds presented here effect oral absorption by a mechanism or mechanisms other than classical inhibition of digestive enzymes. Indeed, other laboratories have shown that enzyme inhibition alone is not sufficient for oral absorption of proteins.[45,46]

Table 5. Enzyme Inhibition Studies

compd	concn	% trypsin inhibition[a]	% LAP inhibition[b]
20	10 mg/mL	39	61
39	10 mg/mL	44	8
BBI	100 mg/mL	100	
actinonin	20 mg/mL		100

[a] Assayed with 1 mg/mL pancreatin and 300 μM benzoyl-L-arginine *p*-nitroanilide as substrate at 37 °C for 12 min. [b] Assayed with 0.1 mg/mL peptidase and 300 μM alanine *p*-nitroanilide as a substrate at 37 °C for 12 min.

Another possible, but undesirable, mechanism for protein transport across intestinal membranes is general permeation enhancement, as discussed in the introduction. Most penetration enhancers have been shown to alter, and frequently damage, the membrane.[47] Furthermore, a general permeation enhancer would, by definition, enable indiscriminate transport of substances across the membrane.

In order to address the issue of potential membrane damage by the *N*-acylated amino acids, histopathological examinantions of rats dosed orally with compounds **20** and **39** were conducted. Autopsy focusing on the gastrointestinal tracts of the animals followed by histological examination indicated no pathology (see Experimental Section). These studies suggest that drug transit across the intestinal membranes is not the result of mucosal damage.

To test further the transport specificity of this system and the lack of general permeation enhancement, the oral delivery of USP heparin, a polysaccharide structurally dissimilar from peptides, was conducted using each of the two compounds that were the most effective for protein delivery (Figure 6). USP heparin has a molecular weight range of 5−20K and poor oral bioavailability.[48] Evidence of heparin absorption after oral dosing with solutions of heparin and **20** or **39** was monitored by clotting times (APTT). Neither *N*-cyclohexanoylleucine (**20**) nor *N*-cyclohexanoylphenylglycine (**39**) produced significant increases in APTT (>2× base line), even at high heparin doses of 100 mg/kg (as compared to an effective subcutaneous dose of 2.5 mg/kg). Thus, compounds that enable the oral delivery of sCT and interferon-α do not deliver a polysaccharide drug of similar size. If drug transport were mediated by membrane penetration enhancement, the oral delivery of drugs such as heparin would most likely be facilitated.

Conclusion

We report here a novel method of facilitating the in vivo absorption of proteins and peptides through the gastrointestinal membrane in two animal species, rodents and primates. Taken together, the structural and biological data support the view that the observed protein transport cannot be explained through classical protease inhibition or general penetration enhancement. These data suggest the possibility that specific compound/protein interactions cause transport across the gastrointestinal membrane. Our future studies will exam-

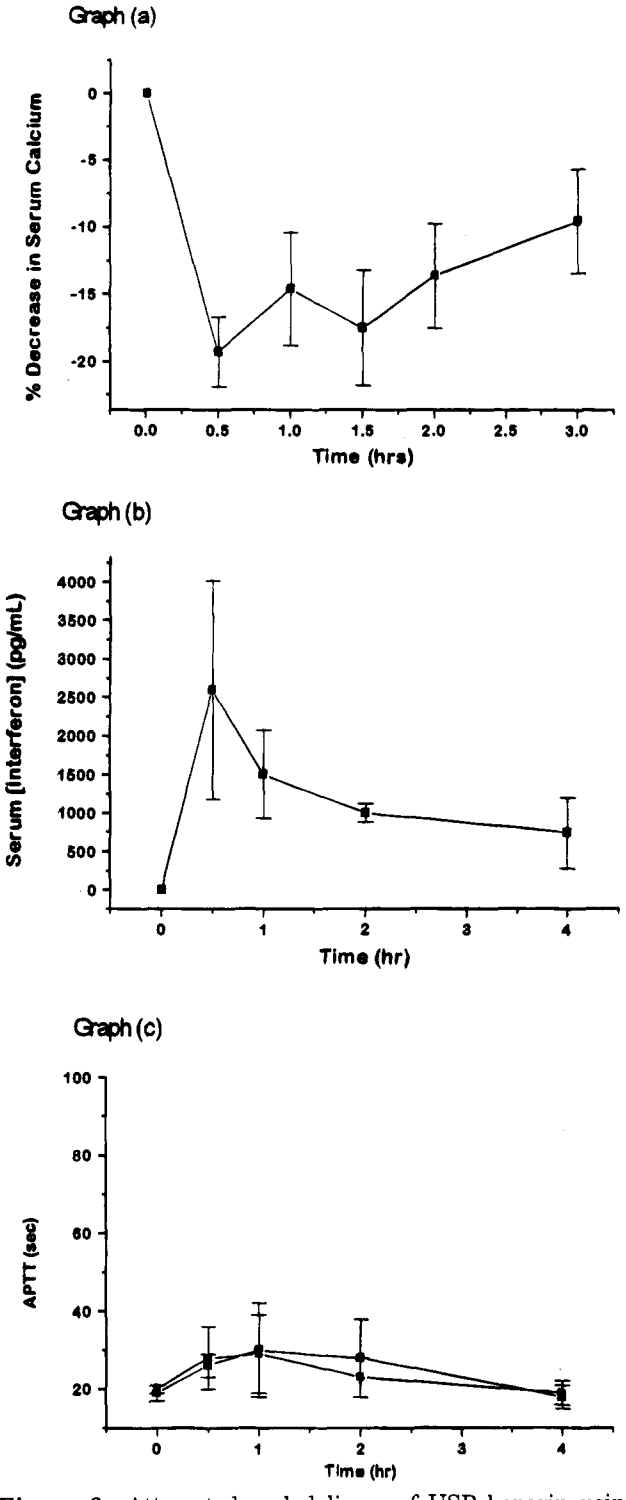


Figure 6. Attempted oral delivery of USP heparin using *N*-cyclohexanoylleucine (**20**) and *N*-cyclohexanoylphenylglycine (**39**), compounds effective in oral protein delivery: (a) oral delivery (PO) of sCT using **20**; (b) oral delivery (PO) of interferon-α using **39**; (c) attempted oral delivery (PO) of USP heparin using **20** and **39**.

ine in more detail the mechanism of protein transport effected by these compounds.

Experimental Section

Chemistry. Compounds 1, **2**, **4**, **6**, **8**, and 11–**17** were purchased from Bachem Biosciences, Inc., Philadelphia, PA. NMR spectra were recorded at 300 MHz in either D_2O or DMSO-d_6. Combustion analyses were performed by Microlit Laboratories, Madison, NJ and are within acceptable limits (C, H, N ±0.4%) Thin layer chromotography was performed using E. Merck Kieselgel 60 F-254 plates. Reactions were monitored by high-pressure liquid chromotography on a Vydac 25 × 4.6 mm protein and peptide column using a gradient of 0–50% acetonitrile in water with 0.1% trifluoroacetic acid. Melting points were performed using a Mel-Temp II from Laboratory Devices, are uncorrected, and are in agreement with the literature values.

General Procedure for the Preparation of Derivatized Amino Acids. The following procedure was used to prepare the acylated amino acids described herein. The preparation of *N*-cyclohexanoylphenylglycine (**39**) is given as a representative example. (*S*)-Phenylglycine (50.0 g, 331 mmol) was dissolved with stirring in aqueous sodium hydroxide (414 mL, 2 N) in an open flask. The resulting solution was cooled to about 10–15 °C in an ice/water bath, and cyclohexanecarbonyl chloride (44.2 mL, 331 mmol) was added dropwise, maintaining the reaction temperature at about 10–15 °C. After the addition was complete, the reaction solution was stirred for 2.5 h at room temperature. The pH of the reaction mixture was adjusted to 9.5 with aqueous hydrochloric acid (37%), and the unreacted phenylglycine was separated as a white solid and removed by filtration. The pH of the filtrate was then further lowered to 4.5 and crude **39** precipitated from solution. This solid was removed by filtration and recrystallized from methanol to give *N*-cyclohexanoyl-(*S*)-phenylglycine (**39**, 12 g, 34%) as a white crystalline solid: mp 180–182 °C; ^{1}H NMR (300 MHz, d_6-DMSO) δ 12.0 (s, 1H, OH), 7.8 (s, 1H, NH), 7.4 (m, 2H, Ph), 7.2 (br s, 3H, Ph), 4.8 (s, 1H, C*H* α to NH), 2.3 (m, 1H, ring CH α to C=O), 1.8 (br m, 5H, cyclohexane), 1.2 (br m, 5H, cyclohexane). Anal. ($C_{15}H_{19}NO_3$) C, H, N.

Enzyme Inhibition Studies. Trypsin, porcine intestinal mucosal peptidase, and pancreatin were purchased from Sigma Chemical Co. UV–vis spectra were recorded on a Model U-2000 instrument equipped with a water-jacketed six-cell chamber for temperature control from Hitachi Instruments, Danbury, CT.

Inhibition of Trypsin. The following method was used to measure trypsin inhibition. Trypsin activity was assayed by monitoring the conversion of *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) to *p*-nitroaniline at 400 nm at 37 °C. The assay solution, having a final volume of 1.0 mL, consisted of 50 mM potassium phosphate, pH 7.6, 300 μM BAPNA, and test compounds at 10 mg/mL and 1 mg/mL USP pancreatin. The stock solution of BAPNA (2 mM) was prepared in DMSO. Test compounds were added to the assay solution prior to enzyme addition. Assays were initiated by the addition of substrate following a 10 min preincubation of enzyme and test compound. A change in absorbance was recorded over 12 min. Assays lacking test compounds served as controls. The reaction rates were compared, and the percent inhibition due to the presence of test compounds was calculated at 12 min.

Inhibition of Leucine Aminopeptidase (LAP). LAP activity was measured as described above by substituting 300 μM alanine *p*-nitroanilide for BAPNA.

Animal Experiments. All animal experimental procedures and protocols were approved in advance by the Emisphere Institutional Animal Care and Use Commitee. Male Sprague–Dawley rats weighing 125–150 g were used to demonstrate the oral delivery of sCT. Male Sprague–Dawley rats weighing 250–325 g were used to demonstrate the oral delivery of interferon-α. Each experiment was performed on a group of six to seven rats. The rats were housed under standard conditions with free access to water. All of the rats in these studies were anesthetized with 44 mg/kg ketamine and 0.5 mg/kg thorazine immediately prior to dosing. The rats were administered the dosing solutions by oral gavage. Serum calcium was determined by quantitation with a Calcium Kit available from Sigma Chemical Co. The data are reported as mean ± standard error. Salmon calcitonin was obtained from Sandoz, Ltd., Basel, Switzerland. Interferon-α concentrations were measured by an ELISA assay for human interferon-α from Biosource International, Camarillo, CA. The data is reported as mean ± standard deviation. Histology studies were performed by Pharmaco LSR, East Millstone, NJ.

General Protocol for Rat Experiments. The following protocol is a general description of the rat experiments performed as part of these studies. The oral dosing of salmon calcitonin with (**20**) is a representative example. An sCT

dosing solution was prepared by dissolving compound **20** (400 mg) in water (2.9 mL). The pH of the solution was adjusted to 7.2 with aqueous sodium hydroxide (1.0 N), and sCT (10 μg) was added. Water was then added to bring the final volume to 4 mL. Six rats were given 2 mL/kg of this sCT dosing solution. The total dose of sCT was 10 μg/kg, and the total dose of acylated amino acid compound was 400 mg/kg. Control groups, each containing six rats, were administered a solution of sCT or a solution of compound **20**. All of the groups were dosed at the same time. Blood samples were collected serially from the tail artery.

General Protocol for Primate Experiments. The following protocol is a general description of the primate experiments performed as part of these studies. Three Rhesus monkeys weighing 4–5 kg were used. The monkeys were fasted overnight and placed in primate restraint chairs for dosing and blood sampling. Conscious monkeys received a solution of sCT and a combination of compounds **18**, **20**, and **36** (1:2:1 by weight) by nasogastric gavage, and blood samples were collected from saphenous vein catheters. Blood samples were collected at 1 and 0.5 h before dosing and 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h after dosing for serum calcium determination.

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