Brief Articles

Novel Lipoamino Acid- and Liposaccharide-Based System for Peptide Delivery: Application for Oral Administration of Tumor-Selective Somatostatin Analogues

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Received February 5, 1999

Lipoamino acid and liposaccharide conjugates of somatostatin analogue TT-232 were synthesized to modify the physicochemical properties of the parent peptide. The relative position, the number, and the nature of the lipid and/or saccharide moieties were varied. Experiments in vitro clearly showed that many compounds modified at the N- and/or C-terminus with lipid or sugar moieties retained the biological activity of the parent compound. An interesting construct was synthesized containing lipid and sugar units at opposite ends of the somatostatin analogue, so that the entire molecule could be considered as an amphipathic surfactant.

Introduction

In recent years, many potent somatostatin analogues have been developed as antisecretory and antiproliferative hormones (Sandostatin or Octreotide of Sandoz, Somatuline of Biomeasure, and RC-160 of Debiopharm), acting longer than the native hormone. They have been used in the treatment of hormone-dependent tumors; however, their administration as antitumor agents is limited because of their side effects.[1−3] A unique somatostatin structural derivative with a five-residue ring TT-232 (**1a**) has been developed in our laboratory. This analogue did not inhibit the release of growth hormone (GH) or gastric acid but exhibited very strong antiproliferative activity. Around 90% inhibition was achieved on breast carcinoma, prostate tumor, pancreatic, leukemia, melanoma, and gastric tumor cell lines, suggesting that TT-232 is a promising and selective antitumor agent.

In order for a peptide to be of significant value as a drug, there are several potential problems that must be overcome: peptides are poorly transported across cell membranes, oral delivery is difficult due to absorption barriers in the gut, and proteolytic enzymes degrade the peptide.[5] We have developed a novel drug-delivery system for the oral administration of drugs and peptides, which are normally poorly absorbed or biologically unstable. The method involves combining the peptide or drug with a lipoamino acid (LAA) or liposaccharide (LS), which can act as a carrier. LAAs combine the properties of amino acids with those of lipids; thus combining them with a peptide or drug provides a means of transporting the compound into the body in a stable and biologically active form.[6−8] These findings confirm the principle that conjugation with one or more LAAs or LSs has the capacity to increase the uptake of molecules across epithelium in the gut and skin and to increase their resistance to breakdown by enzymes. We have demonstrated that various peptides can be administered orally as LS conjugates.

In the present work we report our synthetic efforts to generate LAA- and LS-conjugated analogues of the tumor-selective somatostatin analogue TT-232 in order to improve its physicochemical and pharmacokinetic properties as well as its bioavailability.

Results and Discussion

Structure−activity studies on TT-232 showed that some modifications on either or both ends of the molecule did not affect the biological activity of the parent compound.[9] Furthermore, radiolabeled octreotide analogues using a chelator at the N-terminus for complexation of the radionuclide showed favorable effects on tumor growth and survival.[10] It was expected that terminal extensions of the heptapeptide with lipid-like compounds would not result in the loss of biological activity, so a series of lipophilic conjugates of TT-232 have been synthesized with one or more LAAs at either the N- or C-terminus of the parent peptide. The combination of LAAs with TT-232 resulted in bifunctional

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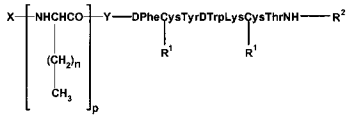
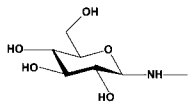
10.1021/jm9910167 CCC: $18.00
Published on Web 09/03/1999

Chart 1

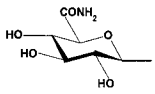


	X	p	n	Y	R^1	R^2
1a	H	0	0	-	disulphide bridge	H
1b	H	1	7	-	disulphide bridge	H
1c	H	1	9	-	disulphide bridge	H
1d	H	1	9	-Gly-Gly-	disulphide bridge	H
1e	**Sug**-CO-$(CH_2)_2$-CO-	1	11	-	disulphide bridge	H
1f	H	0	0	-	Acm	H
1g	H	1	15	-	Acm	H
1h	H	1	17	-	Acm	H
1i	H	2	7	-	Acm	H
1j	H	1	9	-GABA-Gly-Gly-	Acm	H
1k	**Sug**-CO-$(CH_2)_2$-CO-	1	11	-	Acm	H
1l	H	0	0	-	Acm	**LA**-Gly-OH
1m	H	0	0	-	Acm	**SA**
1n	H	1	9	-	Acm	**SA**
1o	H	1	11	-	Acm	**SA**

Sug:



SA:



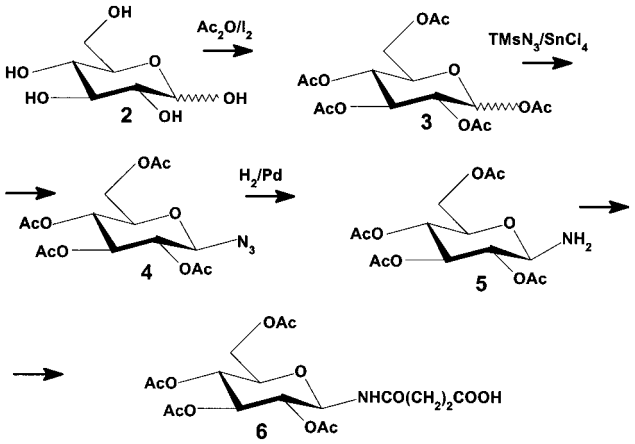
LA: -CH[$(CH_2)_7$-CH_3]CO-; Acm: $CH_2NHCOCH_3$

conjugates, in which the long alkyl side chains conferred lipophilicity, whereas the peptide part was hydrophilic. The LAAs and the somatostatin analogue were either coupled directly, resulting in conjugates **1b**, **1c**, and **1g**−**1i**, or coupled through a linker moiety, resulting in compounds **1d**, **1e**, and **1j**−**1l** (Chart 1).

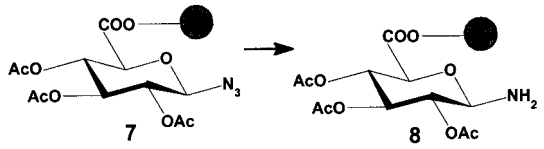
Although conjugation of LAAs improves the lipophilicity necessary for crossing biological membranes, it can have an adverse effect on the water solubility. The conjugate has to show a certain degree of water solubility, to allow absorption from the gastrointestinal tract. As a consequence, further analogues were synthesized which incorporated sugars, alone or in combination with LAAs, to improve water solubility (**1e**, **1k**, **1m**−**1o**). The relative position, nature, and number of saccharide and lipidic portions can be altered to modify and optimize the absorption profiles of the conjugates. Sugar conjugation can serve not only to improve physicochemical properties of parent compounds, such as solubility, but also to allow the conjugate to utilize active transport uptake systems (e.g. the Na^+-dependent D-glucose transporter)[11] and help to target the compound (e.g. sialyl Lewis-X sugars, elevated β-glucuronidase expression in some tumors).[12]

The conjugation of a sugar to the N-terminus of the peptide required the design and synthesis of a stable carbohydrate building block with a free carboxyl-containing side chain at the anomeric position. An α,β-D-glucopyranose (**2**) based building block was synthesized by first fully O-acetylating the starting sugar (**3**) and then forming the anomeric azide (**4**). The azide was

Scheme 1. Synthesis of Glucopyranose-Based Building Block



Scheme 2. Synthesis of Glucuronic Acid-Based Building Block



then catalytically reduced to the corresponding amine (**5**), which was reacted with succinic anhydride to produce the desired free carboxyl-containing sugar building block (**6**) (Scheme 1).

The sugar unit was coupled to the N-terminus of the peptide chain using standard peptide-coupling methods. Removal of the protecting groups and cleavage of the peptides from the resin then produced the target conjugates. Conjugation of a sugar at the C-terminus required a sugar with both a free carboxyl function (for resin attachment) and a free amino function (for first amino acid coupling). 1-Azido-2,3,4-tri-*O*-acetyl-1-deoxy-β-D-glucuronic acid[8] was coupled to the resin resulting in construct **7**, and the azide group was reduced to provide compound **8** with an amino function (Scheme 2). Peptide chain extension was then carried out to give compound **1m**. The glycopeptides described above were also conjugated with LAAs, producing peptides with amphipathic character (**1e**, **1k**, **1n**, **1o**).

Antiproliferative Activity of the Somatostatin Analogues. The LAA or carbohydrate conjugation, carried out on either the cyclic (**1a**) or the linear (**1f**) heptapeptide, in most cases, did not result in the loss of the antiproliferative activity of the parent peptide (Table 1). In the cyclic series, depending on the tumor cell line, the new analogues expressed full (**1b**, SW620 cell line; **1e**, A2058 cell line) or somewhat decreased (35−70%) antiproliferative activity. Only in one case (**1c**, PC3 cell line) was the biological activity very low. There was no clear correlation between the biological activity and the length of the side chain of the LAA, or the presence of a spacer between the N-terminus of the molecule and the ligand, or the presence of an additional carbohydrate moiety (**1e**). The linear peptide **1f** was less active than the cyclic form **1a**, but in most cases the conjugation of the lipid at either the N- or C-terminus resulted in the restoration of the strong antiproliferative activity. The lipophilicity of compound **1f** was varied by

Table 1. In Vitro Antiproliferative Effect of TT-232 Conjugates on Various Tumor Cell Lines[a]

	% inhibition[b]						
compd	SW620	PC3	HT29	A2058	A431	Faza 967 clone 2	Faza 967 clone 2/T1
1a	90 ± 8	90 ± 12	76 ± 11	96 ± 6	100	100	100
1b	95 ± 21	42 ± 11					
1c	65 ± 17	5 ± 8					
1d			50 ± 10	35 ± 13	48 ± 8	33 ± 5	43 ± 4
1e		55 ± 7	30 ± 10	100			
1f			80 ± 24	30 ± 12	34 ± 10	10 ± 5	0
1g			20 ± 18	60 ± 18	83 ± 7	93 ± 3	88 ± 10
1h			50 ± 6	34 ± 9		71 ± 15	87 ± 3
1i			60 ± 10	68 ± 10		96 ± 4	95 ± 5
1j			100	75 ± 3	100	92 ± 2	70 ± 10
1k			39 ± 1	62 ± 7	32 ± 7	61 ± 3	50 ± 7
1l			93 ± 4	97 ± 7	76 ± 10	92 ± 6	90 ± 5
1m			85 ± 15	88 ± 10	90 ± 12	93 ± 4	90 ± 4
1n			36 ± 8	80 ± 9	50 ± 8	19 ± 4	29 ± 3
1o			18 ± 15	2 ± 13	10 ± 6	23 ± 6	17 ± 10

[a] Applied dose of the conjugates: 50 μg/mL. Treatment lasted for 24 h. Values were determined at least in three parallels and are the mean ± SD. [b] Percent inhibition compared to the values of untreated cells (0%).

modifying its N-terminus. This was achieved by changing the nature and the number of LAAs and by the incorporation of a spacer (**1j**) or carbohydrate moiety (**1k**). The best of these analogues was compound **1j** in which the side chain of the LAA was the shortest and a long spacer was inserted between the lipidic unit and the peptide. Additional conjugation of a carbohydrate moiety resulted in increased water solubility and a decrease in antiproliferative activity. Modification of the C-terminus of the linear peptide **1f** by a LAA (resulting in **1l**) or by a carbohydrate moiety (resulting in **1m**) gave conjugates with strong antitumor activity. Modifications which were favorable on either end of the molecule (**1j** for N-terminal LAA moiety, **1m** for C-terminal carbohydrate moiety) resulted in a considerable loss of antiproliferative activity when applied to both ends of the molecule at the same time (**1n**, **1o**). The most promising compounds (**1k**−**1m**) will be employed in the development of an orally active analogue of the potent antitumor peptide TT-232 (**1a**).

Caco-2 Cell Experiments. On the basis of the systematic conjugational strategies to improve bioavailability and in vitro antitumor activity of the conjugates, several compounds were selected for studies of intestinal epithelial permeability.

First the potential toxicity of the compounds was investigated, using [^{14}C]mannitol as a marker molecule. Increased permeability of Caco-2 cell monolayers to the hydrophilic marker molecule mannitol usually reflects a decrease in the monolayer integrity.[13] Peptides **1f** and **1l** (0.2 mM) did not affect the permeability of Caco-2 cell monolayers for [^{14}C]mannitol, and the P_{app} values for [^{14}C]mannitol did not differ significantly from those of controls after 1- or 3-h exposure (Table 2). On the contrary, monolayers exposed to 0.2 mM **1g** and **1k** for the same time interval had significantly higher (ca. 2−4 times higher) P_{app} values for [^{14}C]mannitol than controls (data not shown). After the concentration of **1g** and **1k** was decreased from 0.2 to 0.1 mM, no increase in P_{app} was noticed after 1-h exposure and only a minor increase after 3-h exposure to Caco-2 cell monolayers (Table 2). Therefore, a 1-h exposure time and a working concentration of 0.1 mM was chosen in the transport studies with ^{3}H-labeled **1g** and **1k**. The transport of ^{3}H-labeled **1f** and **1l** across Caco-2 cell monolayers was studied for the same time interval at a concentration of 0.2 mM, to ensure the highest possible radioactivity was available for the transport.

Table 2. Effect of **1f**, **1l**, **1g**, and **1k** on P_{app} of [^{14}C]Mannitol in Caco-2 Cell Monolayers[a]

peptide	concn (mM)	exposure time (h)	$P_{app} \times 10^7$ (cm/s) ± SD	p
control	0	1	1.84 ± 0.50	
		3	1.01 ± 0.10	
1l	0.2	1	1.83 ± 0.89	ns
		3	1.10 ± 0.23	ns
1g	0.1	1	1.94 ± 0.69	ns
		3	1.67 ± 0.41	<0.05
1k	0.1	1	1.59 ± 0.43	ns
		3	1.62 ± 0.32	<0.05

[a] Mean values ± SD (n = 4).

The transepithelial electrical resistance (TEER) measurements were used as an additional integrity marker.[13] When the integrity of epithelial cell barrier decreases, the flux of ions across the epithelium increases and hence TEER decreases. No significant decrease in TEER was observed in monolayers exposed to peptides at the indicated concentrations, as compared to control monolayers (data not shown). On the basis of these results, the transport experiment with radioactively labeled **1l**, **1f** and **1g**, **1k** was performed at concentrations of 0.2 and 0.1 mM, respectively.

Transport of ^{3}H-Labeled Peptides Across Caco-2 Cell Monolayers. Previous studies have shown that in order to be well-absorbed, a drug should have a permeability coefficient in Caco-2 monolayers in the order of 1×10^{-6} cm/s.[14] Compound **1k** fulfilled this criterion ($P_{app} = 0.86 \times 10^{-6}$), while the P_{app} values of compounds **1l** and **1h** were more than 10-fold lower (7×10^{-8}, 9×10^{-8}) and the transport of compound **1g** was below the detection limit. According to our previous predictions, the permeabilities of compounds **1l** and **1f** were likely to be on the order of zero to a few percent. We conclude that compounds with a structural motif similar to that of **1k** are most likely to be well-absorbed in vivo.

Conclusion

Modifying the N- and C-termini of TT-232 with LAA or LS resulted in amphipathic surfactant-like molecules

with improved stability and bioavailability. These studies support the proposal that an initial lead compound with poor pharmacokinetics can be developed with improved absorption profiles, improved biological half-life, and improved resistance to degradation and could still effectively and selectively kill cancer cells.

Experimental Section

Synthesis, purification, and characterization of the peptides were carried out as previously described.[8] Mass spectra were run on a Fisons VG-TOF spectrometer using matrix-assisted laser desorption ionization (MALDI) or a VG Analytical ZAB-SE instrument, using fast atom bombardment (FAB) ionization, or a Finnigan MassLab Navigator quadrupole mass spectrometer, using electrospray ionization. N_2 flow, 300 L/h; temperature, 180 °C; cone voltage; 49 V. The spectral data and the details for the synthesis of 1,2,3,4,6-penta-*O*-acetyl-α,*β*-D-glucopyranose (**3**), 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl azide (**4**), 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosylamine (**5**) and *N*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)succinamide (**6**) are provided as Supporting Information.

In Vitro Antiproliferative Effect of Conjugates on Human Tumor Cell Lines. The conjugates (**1b**−**1o**) and control TT-232 (**1a**) were incubated with various tumor cell lines for 24 and 48 h, at varying concentrations (20, 50, 100 *μ*g/mL). Visualization of the antitumor activity was achieved by employing a spectrophotometric assay using a tetrazolium salt (MTT),[15] which was converted to a colored derivative (Formazan) only by surviving cells and not those killed by the analogues. The OD of the Formazan produced was measured at 570 nm. The tumor cell lines used were SW620 (colonic), PC3 (prostatic), HT29 (colonic), A2058 (melanoma), and A431 (epidermoid carcinoma) cells.

Intestinal Epithelial Absorption of Peptides 1f, 1g, 1k, and 1l. Absorption studies were performed using differentiated human intestinal epithelial cell monolayers cultivated in permeable cell culture inserts. The experimental details on the cell culture, the determination of the integrity of Caco-2 cell monolayers after exposure to peptides and transepithelial electrical resistance, and the examination of the transport of ^{3}H-labeled peptides **1f**, **1g**, **1k**, and **1l** across Caco-2 cell monolayers where carried out as previously described.[16−18] The experimental details are given in the Supporting Information and are available in the World Wide Web edition of this Journal.

Statistics. Results are expressed as mean values ± SD. Statistical differences between two mean values were evaluated by unpaired two-tailed *t*-test.

Acknowledgment. This work was supported in part by a grant from the British Council (No. GB-43/95), the Hungarian National Research Foundation (OTKA T026385 and OTKA T026388), and Inco-Coppernicus European Grant (PL 966087).

Supporting Information Available: Synthesis, purification, and characterization of the peptides and compounds **3**−**6**, in vitro antiproliferative effect of conjugates on human tumor cell lines, intestinal epithelial absorption of peptides, HPLC retention time of compounds **1a**−**1o**, MS data of compounds **1a**−**1o**, and microanalysis of compounds **3**−**6**. This information is available free of charge via the Internet at http://pubs.acs.org.

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JM9910167