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Lipophilization of somatostatin analog RC-160 with long chain fatty acid improves its antiproliferative and antiangiogenic activity *in vitro*

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1 The therapeutic potential of the somatostatin analogue RC-160 having antiproliferative activity, is limited by its short serum half life. To overcome this limitation, fatty acids namely butanoic acid and myristic acid were conjugated to the N-terminal residue of RC-160. The lipophilized derivatives of RC-160 were synthesized, purified by reverse phase HPLC and characterized by ES-mass spectroscopy. The antiproliferative activity of lipophilized derivatives of RC-160 on the growth of MIA-PaCa2 (human pancreatic carcinoma), DU145 (human prostate carcinoma), ECV304 (human umbilical chord endothelioma), as well as their antiangiogenic activity was evaluated *in vitro*. The relative stability of myristoyl-RC-160 towards degradation by proteases and serum was also determined.

2 Myristoyl-RC-160 exhibited significantly higher antiproliferative efficacy than RC-160, on the above cell lines ($P<0.01$). Receptor binding assays, demonstrated that the affinity of RC-160 towards somatostatin receptors remains unaltered by myristoylation. Unlike RC-160, the myristoylated derivative was found to have significantly greater resistance to protease and serum degradation ($P<0.01$). Myristoyl-RC-160 exhibited significantly greater antiproliferative activity on ECV304, than RC-160 ($P<0.01$). Myristoyl RC-160 could also inhibit capillary tube formation more efficiently than RC-160 in a dose dependent manner, suggesting that it possessed enhanced antiangiogenic activity *in vitro* ($P<0.001$).

3 Lipophilization of RC-160 with long chain fatty acids like myristic acid endows it with improved antiproliferative and antiangiogenic activity, stability and therapeutic index.
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Keywords: Somatostatin; RC-160; lipophilization; antiproliferative activity; antiangiogenic activity; protease-resistance

Abbreviations: CAM, Chicken chorioallantoic membrane; DMSO, dimethyl sulphoxide; EDTA, ethylene diamine tetraacetic acid; EGF, epidermal growth factor; EGTA, ethylene glycol bis (β-aminomethylether)-N,N,N,N tetraacetic acid; ES-mass, electron spray mass spectrometry; Fmoc, 9-flourenylmethoxycarbonyl; GH, growth hormone; GTPγS, guanosine-5-O-(3-thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piparazineethane sulphonic acid; IGF, insulin like growth factor; $InsP_3$ inositol triphosphate; PMSF, phenylmethylsulfonylflouride; RP-HPLC, reverse phase HPLC; SCLC, small cell lung cancer; SSTR, somatostatin receptor; TFA, triflouroacetic acid; TLS, tube like structures; VIP, vasoactive intestinal peptide

Introduction

The delivery of therapeutic peptides to the site of action remains a major challenge even today. The hydrophilic nature of most peptide drugs severely limits their permeability across lipophilic biomembranes. Peptides are rapidly degraded by proteolytic enzymes present in the cell membrane and blood (Dutta, 1993). The half life of most of the bioactive peptides in serum as well as in the cellular microenvironment is extremely small. This has led to the research focused on the design of peptide analogues with increased bioactivity, permeability and plasma half life. An alternate strategy has been to devise long-acting sustained release preparations by entrapping peptides within poly-(DL-lactide-co-glycollide) microspheres, or by using miniosmotic pumps to ensure sustained release of the therapeutic peptide (Dutta, 1993).

RC-160, an analogue of the cyclic neuropeptide somatostatin, is one of the most extensively studied peptides, for its antiproliferative and antisecretory activity (Pollak *et al*., 1998). The antiproliferative activity of RC-160

(D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2)

has been demonstrated in a number of experimental models of pancreatic, prostate, renal and mammary cancers (Qin *et al*., 1995; Jungwirth *et al*., 1998), suggesting its high antineoplastic therapeutic potential.

The biological effects of somatostatin are mediated through high affinity, G-protein coupled receptors on target cells (Reisine *et al*., 1995), which belong to five distinct subtypes (SSTRx, x = 1–5). The inhibitory effect of RC-160 on tumour growth may be mediated directly by SSTR's on cancer cells, or induced indirectly by the inhibition of growth factors such as EGF, IGF-1, etc. induced proliferation of cancer cells (Liebow *et al*., 1989; Catteneo *et al*., 1996). The antineoplastic effect of RC-160 may also be attributed to its ability to inhibit angiogenesis (Barrie *et al*., 1993; Woltering *et al*., 1997).

Although RC-160 is longer acting than somatostatin, its biological half life of less than 2 h requires either frequent administration, or sustained release formulation, to attain enhanced antiproliferative activity and a long term suppression of hormones like GH and IGF-1 (Radulovic *et al*., 1993; Szepeshazi *et al*., 1992). The administration of these antitumour agents has also been limited by their pleotropic nature and side effects like inhibition of gall bladder emptying which leads to increased incidence of cholesterol gallstones (Lamberts *et al*., 1996). The acute administration of somatostatin has been found to produce receptor desensitiza-

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tion which results in diminished therapeutic response and induction of tolerance (Lamberts *et al*., 1996). Hence, there is a need for further modification of these somatostatin analogues to increase their stability and improve their therapeutic index.

Lipophilization of bioactive peptides is a novel strategy to increase their stability and biological availability. Unlike sustained release formulations, lipophilization can also impart enhanced receptor selectivity to peptides. The attachment of fatty acids to peptides like VIP leads to the generation of highly potent and selective peptide analogues (Gozes *et al*., 1995; 1996), which have provided novel tools in drug design for Alzheimer's disease and in various forms of cancer (Moody *et al*., 1997). Similarly, the attachment of fatty acids to the N-terminal residue of insulin, tetragastrin, TRH improves their bioavailability and absorbability across cell membranes (Muranishi *et al*., 1992).

The objective of the present study was to evaluate the effect of lipophilization on the antineoplastic activity of RC-160, and to determine whether such derivatization of RC-160 could confer better resistance to protease degradation, leading to increased half life and enhanced antiproliferative activity. The effect of varying carbon chain length on the activity of lipophilized RC-160 was also investigated. Another important objective of the study is to obtain an insight into the ability of lipophilized RC-160 to inhibit tumour angiogenesis, relative to RC-160 *in vitro*.

Methods

Chemicals

Rink amide resin and Fmoc amino acids were purchased from Bachem California, U.S.A. All growth media, antibiotics were purchased from Gibco BRL, U.S.A. Fetal calf serum (FCS) was obtained from Biological Industries, Israel. Matrigel was obtained from Collaborative Biomedical Products, U.S.A. All other chemicals were obtained from Sigma Chemical Co. U.S.A.

Synthesis, purification and characterization of peptides

Peptide synthesis was carried out, according to the manual solid phase strategy, employing optimum side chain protection (Fields & Noble, 1990). The cysteine residues were protected by the trityl group. Butanoic acid and myristic acid were coupled to the N-terminal residue of RC-160 while it was bound to the resin, using a combination of DIPCDI and HOBT, normally employed for extension of the peptide chain. The reaction was allowed to proceed overnight. The intramolecular disulphide bond was formed by dissolving 4 mg of the crude peptide in 3 ml DMSO, in a 12 : 1 v v^{-1} of acetic acid water buffer (pH = 6, adjusted by ammonium bicarbonate). The reaction mixture was stirred overnight. The oxidation reaction was monitored on an HPLC system, equipped with a semipreparative C_{18} reverse phase column (Nihon Waters, Japan), using gradients established between 0.1% triflouroacetic acid (TFA) and acetonitrile containing 0.1% TFA (v v^{-1}). The yield of the disulphide oxidation reaction was about 9–20%. The parent peptide was characterized by N-terminal sequencing. The molecular weights of the lipophilized peptides and the parent peptide were ascertained by electron spray mass spectrometry.

Cell culture

MIA-PaCa2 (human pancreatic carcinoma cell line) was obtained from National Centre of Cell Sciences, Pune. DU145 (human prostate carcinoma cell line) and ECV304 (human umbilical chord endothelioma cell line) were obtained from the ATCC, Rockville, Maryland, U.S.A. MIA-PaCa2 and DU145 were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 25 mM HEPES, 100 units ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 40 μg ml^{-1} gentamycin and 10% FCS. ECV304 was maintained in M-199 supplemented with 2 mM glutamine, 20% FCS and antibiotics, as detailed above. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Receptor binding assay

^{125}I-[Tyr^1]-somatostatin-14 (Du Pont Nen, U.S.A.), of specific activity 2000 Ci $mmol^{-1}$ was used to measure somatostatin-14 binding sites on whole cell preparations (Qin *et al*., 1995). Cells were seeded to 24-well culture plates (5×10^4 cells $well^{-1}$) for 36 h. The cells were washed once in binding buffer consisting of RPMI-1640, 0.1% BSA, 10 mM $MgCl_2$, 1 mM EGTA, 0.25 mM PMSF and 10 μg ml^{-1} aprotinin. Cells were incubated with 0.5 nM of ^{125}I-[Tyr^1]-somatostatin-14 in the presence or absence of various concentrations of RC-160 and unlabelled lipopeptides, for 2 h. The cells were subsequently washed four times with ice cold binding buffer and lysed using 0.3 N NaOH. The radioactivity of the cellular lysate was measured using a gamma counter (LKB Wallac, Finland). Nonspecific binding was determined in the presence of 1 μM of somatostatin-14. The IC_{50} values were calculated from displacement assays and the inhibitory constant was calculated by the method of Cheng & Prusoff (1973).

The coupling of somatostatin receptor subtypes with G-proteins was investigated by incubating the cells with 100 μM of GTPγS. Experiments involving pertussis toxin were performed by treating the cells for 18 h with 50 ng ml^{-1} pertussis toxin prior to the binding assay.

Proliferation assay

Human cancer cells (MIA-PaCa2 and DU145) cultured to 70% confluence were harvested using 0.05% trypsin-EDTA solution and replated in medium supplemented with 10% FCS, in 96-well microtiter tissue culture plates at a density of 5000 cells $well^{-1}$. The plates were incubated for 36 h to allow complete reattachment of the cells. Subsequently, cells were incubated with serum free medium for 36–48 h to unmask the effect of endogenous growth factors. Thereafter, cells were stimulated with 16 nM EGF in the presence or absence of peptide analogues for 36 h. All lipopeptides were dissolved in serum free media containing 1% DMSO. The analogues were added twice a day during the 36 h incubation (Cattaneo *et al*., 1996). Cells stimulated with EGF in serum-free medium containing 0.1% DMSO (Genzyme Corporation, U.S.A) served as the controls for the assay. [^{3}H]-thymidine (1 μCi $well^{-1}$; specific activity 2 Ci $mmol^{-1}$, Du Pont Nen, U.S.A.) was added during the last 6 h of incubation. The cells were harvested and collected on glass fibre filters using an automatic cell harvester (Skatron Instruments, Norway). The radioactivity incorporated was measured using a β-counter (Wallac Oy, Finland).

Endothelial cell proliferation assay

Human endothelial cells, ECV304 were plated in M-199, supplemented with 20% FCS in 96-well microtiter plates at a density of 5000 cells $well^{-1}$. After complete reattachment of the cells, the medium was changed to M-199 supplemented with 2.5% FCS. The analogues were

added every 12 h, for a period of 72 h. The cells were pulsed with [^{3}H]-thymidine (1 μCi well^{-1}; specific activity 2 Ci mmol^{-1}) for the last 6 h of incubation. The cells were harvested and collected on glass fibre filters using an automatic cell harvester and the radioactivity was measured using a β-counter.

In vitro *angiogenesis*

Matrigel (Collaborative Biomedical Products, U.S.A.) was used to promote the differentiation of endothelial cells into capillary tube-like structures (Hughes, 1996). 100 μl of thawed neat Matrigel was added to 96-well tissue culture plates and incubated for 60 min at 37°C to allow polymerization. 1×10^4 ECV304 cells were seeded on the gels, in medium containing various concentrations of lipopeptides and RC-160 (Hughes, 1996). The cells were maintained in standard culture conditions for 48 h. The peptides were added daily during the 48 h incubation. The inhibition in capillary tube formation was quantitated using a Nikon Diaphot-300 phase contrast microscope (Nikon, Japan) coupled to an image analyser (Leading Edge, Australia).

Protease sensitivity

The peptide was dissolved in 0.1 M ammonium bicarbonate, 1 M EGTA buffer (pH = 8.0), containing 1% DMSO. The crude bovine pancreatic protease was added to the peptide solution (ratio 1 : 50 w w^{-1}). The reaction mixture was incubated at 37°C for 36 h (Marshak, 1996). The progress of the digestion was monitored, using a C_{18} reverse phase column, on an HPLC system.

In vitro *serum sensitivity*

The peptide was dissolved in 0.1 M ammonium bicarbonate, 1 M EGTA buffer (pH = 8.0), containing 1% DMSO, at a concentration of 1 mg ml^{-1}. 100 μl of peptide solution was added to 1 ml of normal mouse serum. The reaction mixture was incubated at 37°C for 36 h. The reaction was monitored by RP-HPLC. At various time intervals, aliquots of the reaction mixture were taken and equal volumes of acetonitrile was added to precipitate the proteins present therein. The suspension was spun at 10,000 r.p.m. and the supernatant was injected into an HPLC system, equipped with a C_{18} reverse phase Deltapak column (Nihon Waters, Japan).

Statistical analysis

All data are expressed as the mean ± s.e.mean. Mean values between the treatment group and the control group were analysed by a two-way ANOVA (Micro Cal Origin Ver. 2.0). Data was considered significant when a two-tailed value of P was less than 0.05.

Results

Synthesis, purification and characterization of lipophilized RC-160

RC-160 and its lipophilized derivatives were synthesized by manual solid phase peptide synthesis, using the Fmoc strategy (Fields & Noble, 1990). After the peptide was cleaved from the resin, the intramolecular disulphide bond was formed by DMSO oxidation, purified on an HPLC system using a reverse phase C_{18} column (Tam *et al.*, 1992). The yield of the disulphide oxidation reaction was between 9–20%. The peptides were characterized by electron spray mass spectrometry. There was a good agreement between the predicted molecular weights (butanoyl RC-160 = 1201.51, myristoyl RC-160 = 1341.76) and the molecular weight actually obtained (butanoyl RC-160 = 1200.60, myristoyl RC-160 = 1340.4).

Binding of RC-160, and lipophilized RC-160 on somatostatin receptors on MIA-PaCa2 and DU145

The binding of ^{125}I-[Tyr^1]-somatostatin-14 was studied in intact cells. The specific binding of the radiolabelled peptide was studied both at 4°C as well as at 25°C. The amount of ^{125}I-[Tyr^1]-somatostatin-14 bound to the cells at 4°C, was higher than that of cells at 25°C (Figure 1A). Conse-

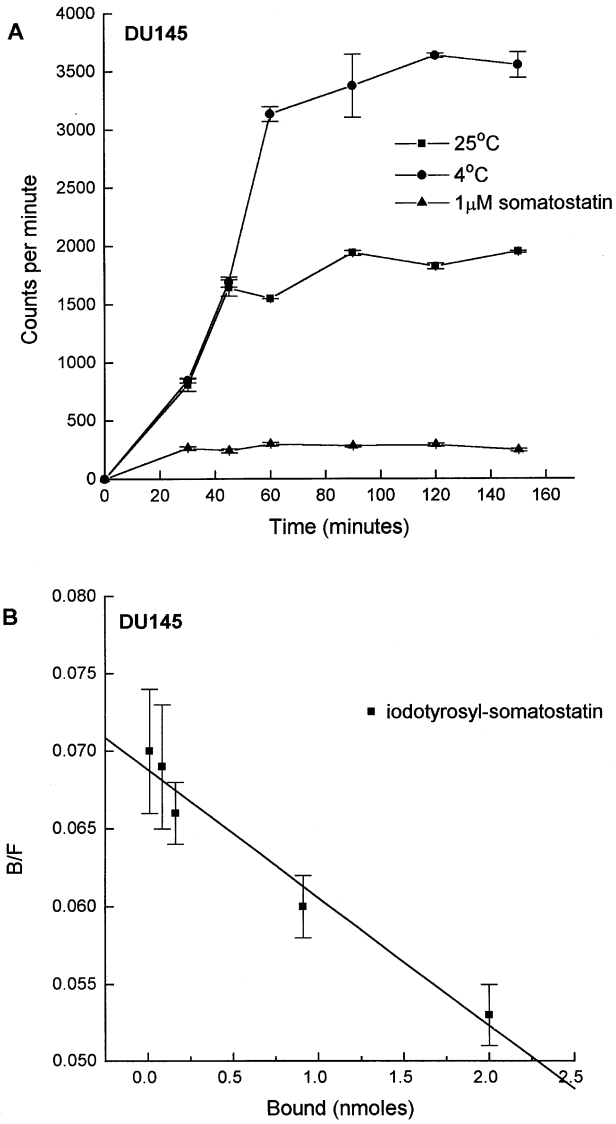

Figure 1 Time course of the specific binding of ^{125}I-[Tyr]^1-somatostatin to the human prostate cancer cell line DU145. The cells were incubated for various time periods at 4°C or 25°C with 0.5 nM of ^{125}I-[Tyr]^1-somatostatin. The non-specific binding was determined by incubating the cells with 0.5 nM ^{125}I-[Tyr]^1-somatostatin, in the presence of 1 μM unlabelled somatostatin (A). Scatchard plot of the specific binding of ^{125}I-[Tyr]^1-somatostatin to DU145 cells, indicating the presence of a single class of specific binding sites (B).

Table 1A Specific binding of ^{125}I-[Tyr^1]-somatostatin to the cancer cell lines *in vitro*. Scatchard analysis was used to determine the nature of binding sites as well as the B_{max} values.

SNo	*Cell line*	K_d (nM)	$\pm$*s.e.mean*	$B_{max}/5\times10^4$ *cells* (fmol)	$\pm$*s.e.mean*
(1)	MIA-PaCa2	2.35	$\pm$0.089	40.07	$\pm$2.13
(2)	DU145	1.26	$\pm$0.051	8.625	$\pm$0.83
		3.61[1]	$\pm$0.241	52.69[4]	$\pm$2.21
(3)	ECV304	0.17[2]	$\pm$0.014	3.47[3]	$\pm$0.32

[1]Low affinity; [2]High affinity; [3]Low capacity; [4]High capacity.

Table 1B Displacement of ^{125}I-[Tyr^1]-somatostatin binding by lipopeptides and RC-160. The cells were incubated with 0.5 nM ^{125}I-[Tyr]-somatostatin, in the presence of the peptides, for 2 h at 4°C.

		DU145			*MIA-PaCa2*			*ECV304*		
SNo	*Lipopeptides*	IC_{50} (μg/ml)	$\pm$*s.e.mean*	K_i (μM)	IC_{50} (μg/ml)	$\pm$*s.e.mean*	K_i (μM)	IC_{50} (μg/ml)	$\pm$*s.e.mean*	K_i (μM)
(1)	RC-160	0.045	+0.003	0.052	0.037	+0.001	0.039	0.052	+0.002	0.276[1] 0.048[2]
(2)	Butanoyl-RC-160	0.23	+0.018	0.268	0.29	+0.026	0.29	0.15	+0.0	0.750[1] 0.144[2]
(3)	Myristoyl-RC-160	0.043	+0.002	0.045	0.037	+0.002	0.026	0.048	+0.002	0.222[1] 0.042[2]

[1]For low affinity somatostatin receptors, [2]For high affinity somatostatin receptors.

quently, all binding assays were performed at 4°C, for 2 h. Scatchard analysis indicated the presence of only one class of specific binding sites on MIA-PaCa2 and DU145 (Figure 1B). The K_d and B_{max} values for each class of binding sites, in each of the cell lines are shown in Table 1A. The presence of specific somatostatin receptors on the above cancer cell lines was confirmed by displacement assays using excess of cold peptide. RC-160, butanoyl-RC-160 as well as its myristoylated counterpart could effectively displace ^{125}I-[Tyr^1]-somatostatin-14, bound to the tumour cells, in a dose dependent manner (Figure 2). The concentration of peptide which causes a 50% inhibition of binding of ^{125}I-[Tyr^1]-somatostatin-14 to the above cell lines (IC_{50}) and the inhibitory constant K_i are shown in Table 1B.

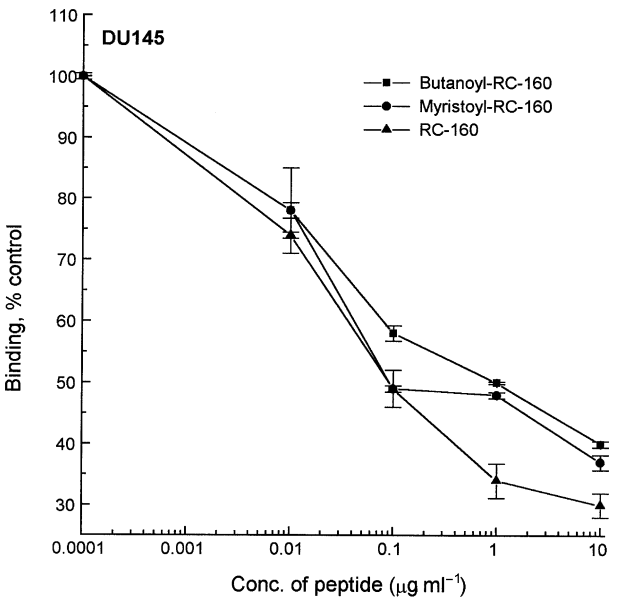

Figure 2 Displacement of bound ^{125}I-[Tyr]^1-somatostatin from cells by RC-160 and its lipophilized derivatives, in the human prostate carcinoma cell line DU145. Cells were incubated with 0.5 nM of ^{125}I-[Tyr]^1-somatostatin and the cold peptides at the above mentioned concentrations for 2 h. The excess radioactivity was washed off. Subsequently the cells were lysed and cell associated radioactivity was read in a gamma counter.

Antiproliferative activity of RC-160 and its lipophilized derivatives

RC-160 could inhibit the proliferation of all the above cell lines *in vitro*. In the presence 1 μg/ml of RC-160, a significant suppression of cell growth was obtained ($P<0.001$), in DU145 (Table 2); the cell growth was decreased by 49$\pm$2.2%. In the case of the pancreatic adenocarcinoma cell line MIA-PaCa2, at 37$\pm$2.5% inhibition of cell growth was observed at 10 ng ml^{-1} of RC-160 (Table 2).

Figures 3 and 4A show the antiproliferative activity of myristoyl-RC-160, as compared to butanoyl-RC-160 and RC-160 in DU145 and ECV304 cell lines. Table 2 shows the relative efficacy of lipophilized RC-160 in DU145, MIA-PaCa2 and ECV304 cell lines. The antiproliferative efficacy of RC-160 seems to increase with myristoylation, for MIA-PaCa2 ($P<0.05$) and DU145 ($P<0.001$). Myristoyl-RC-160 is about 10 fold more effective in the pancreatic carcinoma cell line MIA-PaCa2 (Table 2), as compared to the prostate carcinoma cell line DU145 ($P<0.01$). However, myristoyl-RC-160 does not display enhanced antiproliferative activity, in terms of an increased reduction in cell growth, as compared to RC-160. The suppression in cell proliferation induced by myristoyl-RC-160 (the inhibition of cell growth being 45$\pm$4%) is similar to RC-160 in magnitude, however the effect is displayed at about a 1000 fold lower concentration, than RC-160, in DU145. In the pancreatic carcinoma cell line MIA-PaCa2, myristoyl-RC-160 manifests antiproliferative activity (the suppression of cell growth being 33$\pm$2.01%) at about a 100 fold lower concentration, as compared to RC-160 (Table 2). Similarly, in the human umbilical chord endothelioma cell line ECV304, myristoyl-RC-160 displays antiproliferative activity at about a 1000 fold lower concentration compared to RC-160 ($P<0.01$). However butanoyl-RC-160 is equipotent to RC-160 in all the cell lines studied.

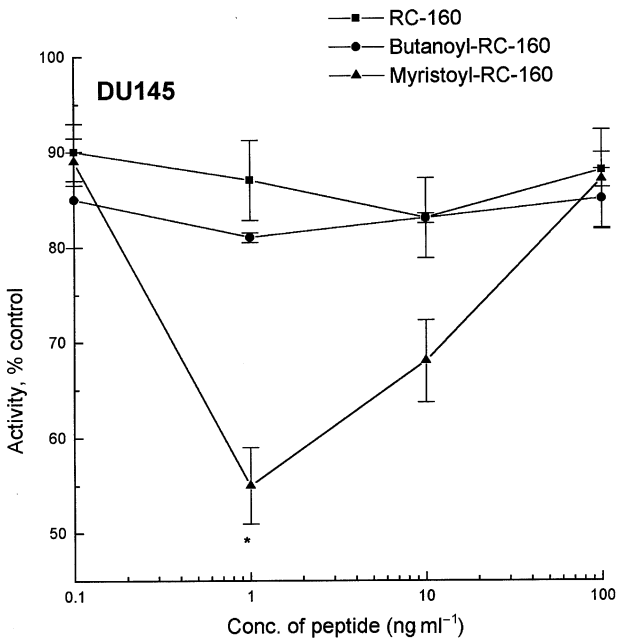

Figure 3 Antiproliferative activity of RC-160 and its lipophilized derivatives on the human prostate carcinoma cell line DU145 *in vitro*. Cells were rendered quiescent by serum starvation. The cells were incubated with EGF alone or with 16 nM EGF and the peptides. Cells stimulated with EGF alone served as the controls for the assay. The peptides were added every 12 h for a period of 36 h. The cells were pulsed with tritiated thymidine and subsequently lysed and counted. *Concentration of myristoyl-RC-160 displaying the maximum antiproliferative activity. ***$P<0.001$ (between RC-160 and control). ***$P<0.001$ (between myristoyl-RC-160 and control). **$P<0.01$ (myristoyl-RC-160 versus RC-160).

Table 2 The relative efficacy of lipophilized RC-160 in pancreatic and prostate cell lines *in vitro*, as compared to unmodified RC-160. The antiproliferative activity of the lipopeptides in the human umbilical cord endothelioma cell line ECV304, as compared to RC-160 is also shown

			Conc. of peptide showing maximum antiproliferative activity (ng ml^{-1})		
SNo	*Lipopeptides*	*Chain length*	*DU145*	*MIA-PaCa2*	*ECV304*
(1)	RC-160	0	1000	10	1000
(2)	Butanoyl-RC-160	4	1000	10	1000
(3)	Myristoyl-RC-160	14	1.0	0.1	1.0

Antiangiogenic activity of lipophilized RC-160 in vitro

RC-160 inhibited the proliferation of the human endothelial cell line, ECV304; the cell counts were found to decrease by $40\pm2.05\%$ at the optimum concentration of 1 μg ml^{-1} ($P<0.001$) (Table 2). Myristoyl RC-160 showed the maximum antiproliferative efficacy on ECV304 cells ($P<0.01$), the inhibition of cell growth being $45\pm0.5\%$ at a concentration of 1 ng ml^{-1} (Figure 4A). As already mentioned earlier, the inhibition in endothelial cell growth induced by the myristoyl-RC-160 was not significantly different from butanoyl RC-160 and RC-160. However, myristoyl-RC-160 exhibited antiproliferative activity at about a 1000 fold lower concentration than butanoyl RC-60 or RC-160 itself ($P<0.01$). The antiangiogenic activity of the peptides *in vitro* was assessed by using the 'Matrigel model' system. Unlike HUVEC cells, ECV304 cells exhibit a highly consistent and reproducible network formation, irrespective of passage (Hughes, 1996), when seeded on Matrigel. Both myristoyl-RC-160 and RC-160 inhibited the formation of tube like structures (TLS) in a dose dependent manner (Figure 4B). The inhibition of angiogenesis was quantitatively measured using an image analyser (Figure 4C and Table 3) which clearly showed that myristoyl-RC-160 was a better inhibitor of angiogenesis than RC-160 ($P<0.001$). Myristoyl RC-160 inhibited the formation of tube like structures (TLS) to the same magnitude (approx. $46\pm4.6\%$) as compared to butanoyl-RC-160 and RC-160 ($49\pm0.96\%$ inhibition and $42\pm0.74\%$ inhibition in TLS formation respectively), however, the antiangiogenic activity was manifested at about a 10 fold lower concentration, as compared to RC-160.

The antiangiogenic activity of RC-160 *in vitro*, seems to be mediated by the presence of specific somatostatin receptors on endothelial cells (Table 1A). Cold RC-160 and lipopeptides are able to displace the receptor bound radiolabelled somatostatin, in a dose dependent manner (Table 1B), on ECV304 cells. GTPγS and pertussis toxin were able to significantly block the binding of ^{125}I-[Tyr^1]-somatostatin to the receptor ($P<0.001$), indicating the coupling of somatostatin receptors with a pertussis toxin sensitive GTP binding regulatory protein (Figure 5).

Protease and serum sensitivity of lipopeptides versus RC-160

Lipophilization of peptides is designed to protect the peptide against proteolytic degradation in serum. The stability of myristoyl-RC-160, as compared to RC-160, was assessed by evaluating their susceptibility towards crude bovine pancreatic protease and serum (Figure 6A,B). The proteolytic degradation was monitored on a HPLC system, using a C_{18} reverse phase column. Myristoyl-RC-160 exhibited greater stability to protease digestion, even after 24 h, as compared to RC-160. In the presence of normal mouse serum, RC-160 was degraded within 2 h, whereas myristoyl-RC-160 was clearly detectable in serum, even after 24 h.

Discussion

The antiproliferative activity of somatostatin is mediated by membrane associated G-protein coupled receptors (SSTRx, x = 1 – 5) (Reisine & Bell, 1993; Reisine *et al*., 1995). The receptor subtype SSTR2 mediates the antiproliferative effect of RC-160 *in vitro*, by activation of a phosphotyrosine phosphatase (Buscail *et al*., 1994; 1995). The role of SSTR2 in the negative control of cell proliferation is further strengthened by the presence of this subtype in human breast cancer, SCLC pancreatic cancer, which respond *in vitro* and *in vivo* to the effect of somatostatin analogues (Reubi *et al*., 1994). Hence, understanding the molecular interactions between RC-160 and SSTR2 is crucial for designing analogues with better activity and stability profile than RC-160.

Molecular modelling has been done to elucidate the critical amino acid residues mediating the interaction of peptide analogues with SSTR2 (Liapakis *et al*., 1996; Kaupman *et al*., 1995). The ligand binding site of SSTR2 was demonstrated to be located in a pocket formed by hydrophobic regions. The putative binding pocket of SSTR2 is lined with hydrophobic residues which are involved in lipophilic interactions with the

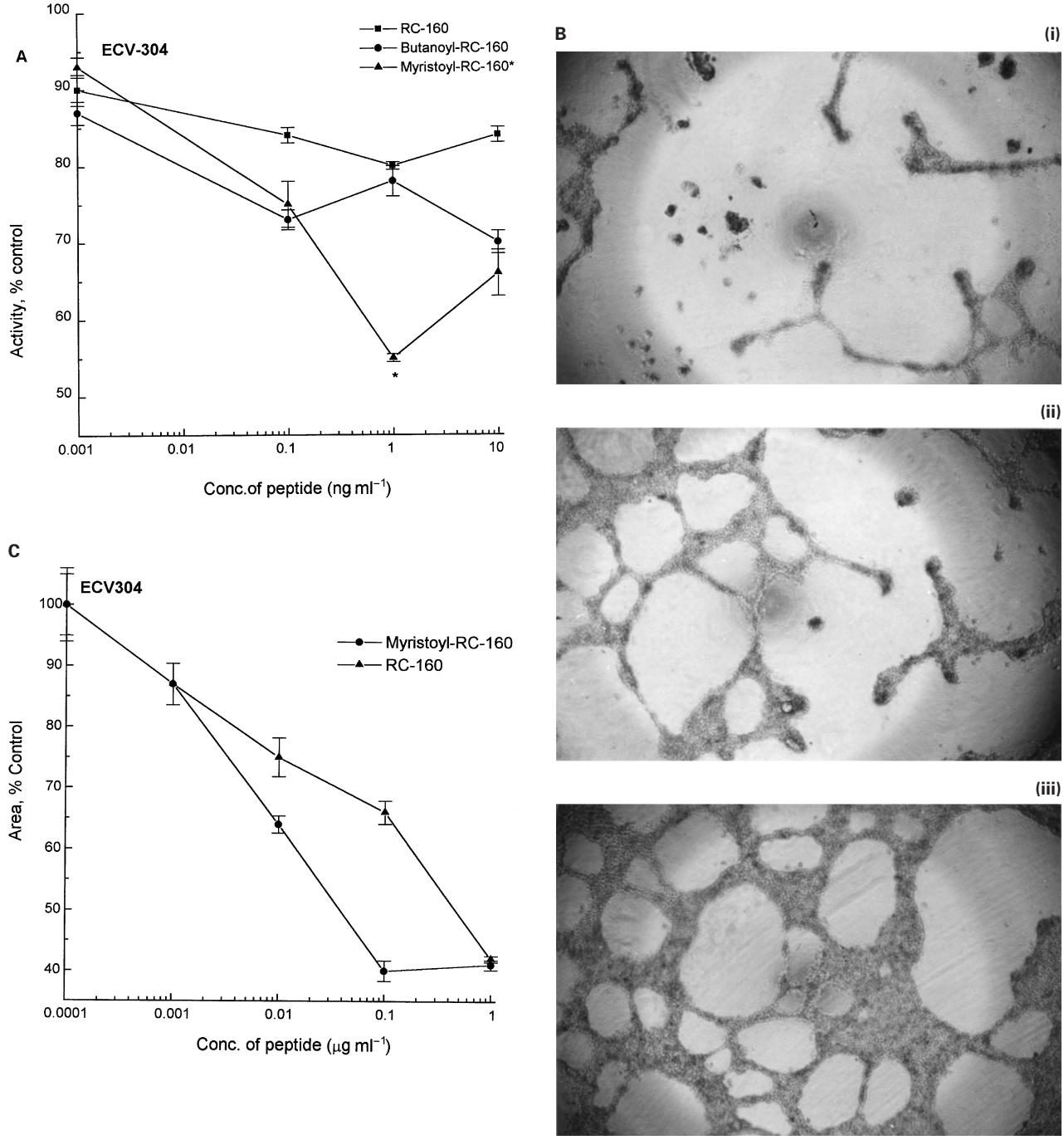

Figure 4 (**A**) Antiproliferative activity of RC-160 and its lipophilized derivatives, on the human endothelial cell line ECV304 *in vitro*. Cells were incubated with the peptides for 72 h in the presence of 2.5% FCS. The peptides were added twice daily. The inhibition in cell growth was estimated by pulsing the cells with tritiated thymidine. *Concentration of myristoyl-RC-160 displaying the maximum antiproliferative activity. ***$P<0.001$ (between RC-160 and control). ***$P<0.01$ (between myristoyl-RC-160 and control). **$P<0.01$ (myristoyl-RC-160 versus RC-160). (**B**) Inhibition of the formation of capillary tube like structures *in vitro*, by 0.1 μg ml^{-1} myristoyl-RC-160 (i) and 1 μg ml^{-1} RC-160 (ii) relative to the control panel (iii). Network formation was achieved by culturing human endothelial cells (ECV304) on Matrigel. The inhibition in tube formation was assessed by light microscopy. (**C**) Dose dependent inhibition of myristoyl-RC-160 and RC-160, in the formation of tube like structures on Matrigel. Human endothelial cells (ECV304) were seeded on polymerized Matrigel to promote the differentiation of endothelial cells to capillary tube-like structures. The inhibition in TLS formation was quantitated using an image analyser. ***$P<0.001$.

aromatic residues of octreotide. The interaction of SSTR2 with somatostatin agonists appear to be primarily hydrophobic in character. Hence, increasing the hydrophobicity of RC-160 should increase the receptor affinity of RC-160, which should improve its biological activity.

The receptor affinity of myristoyl RC-160 (as indicated by the IC_{50} values) is not significantly different from RC-160, in MIA-PaCa2 & DU145. Contrary to our belief, the receptor affinity does not seem to be significantly influenced by the increased hydrophobicity of RC-160. This can be explained by the fact that there seems to be sufficient structural homology amongst G-protein-linked peptide receptors to accommodate peptides of varying sizes at the binding site; receptor selective properties can be conferred by optimizing side chain structure and stereochemistry of the amino acids (Bass *et al*., 1996). This is illustrated by the apparent utility

Table 3 Effect of lipopeptides and RC-160 on the formation of capillary tube like structures *in vitro*. The inhibition of capillary tube formation, induced by the peptides, was quantitated with an image analyser.

SNo	*Lipopeptides*	*Dosage* (μg/ml)	*% inhibition of capillary tube structures* in vitro	$\pm$*s.e.mean*
(1)	RC-160	1.0	42.4	$\pm$0.74
(2)	Butanoyl-RC-160	1.0	49.04	$\pm$0.96
(3)	Myristoyl-RC-160	0.1	46.5	$\pm$4.6

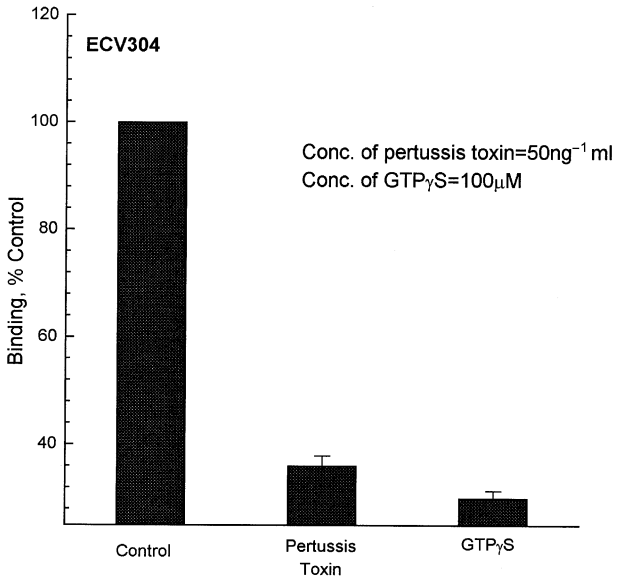

Figure 5 Abrogation of the binding of ^{125}I-[Tyr]^1-somatostatin in the presence of 100 μM of GTPγS and 50 ng ml^{-1} of pertussis toxin in the human umbilical chord endothelioma cell line ECV304. The cells were incubated with ^{125}I-[Tyr]^1-somatostatin in the presence of of 100 μM GTPγS. Experiments involving pertussis toxin were performed by treating the cells for 18 h with 50 ng ml^{-1} pertussis toxin prior to the assay. The receptor binding assay was done as specified earlier. The excess radioactivity was washed off. The cells were subsequently lysed and counted. ***$P<0.001$.

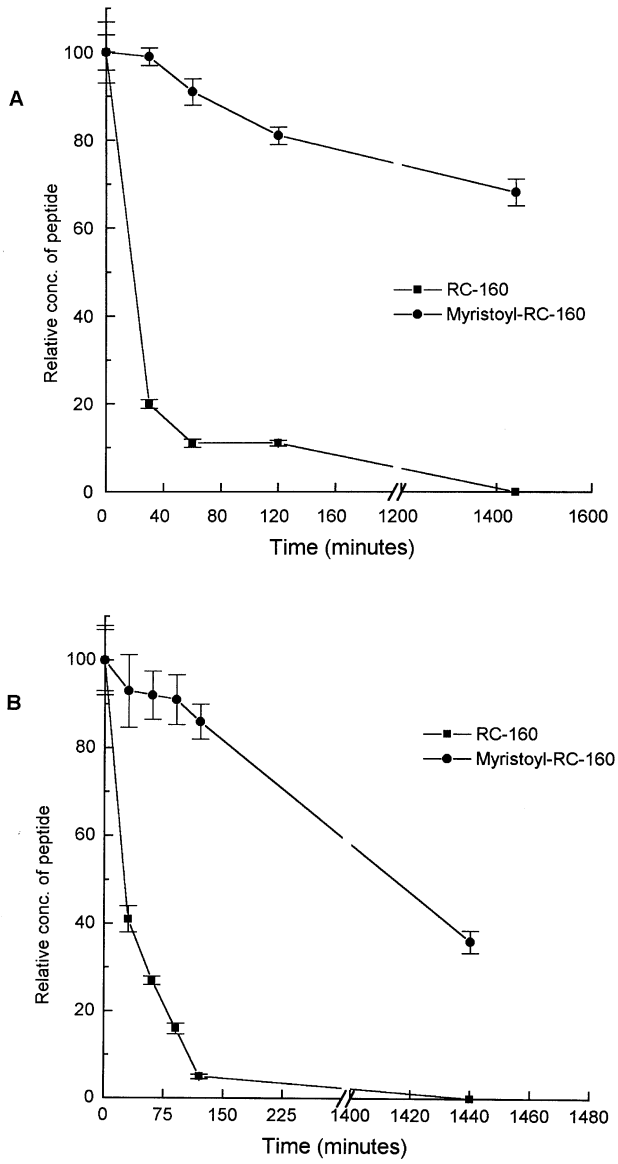

Figure 6 (**A**) Susceptibility of myristoyl-RC-160 and RC-160 towards crude bovine pancreatic protease. The peptides were incubated with these proteases, in digestion buffer of pH = 8, at 37°C. The digestion reaction was monitored at different time points, using C_{18} reverse phase column, on an HPLC system. ***$P<0.01$. (**B**) Serum half life of myristoyl-RC-160 versus RC-160. The peptides were incubated in normal mouse serum at 37°C. The reaction mixture was diluted 1 : 1 with acetonitrile to precipitate the proteins. It was spun at 10,000 r.p.m. and the clear supernatant was injected into a RP-HPLC system, equipped with a C_{18} column. **$P<0.01$

of the somatostatin scaffold to develop highly selective and potent agonists and antagonists to a variety of other G-protein coupled receptors. Antagonists to NK-1 receptor, oxytocin and neuromedin B have been synthesized by modification of somatostatin agonists (Hruby 1987; Hirschman *et al.*, 1996; Orbuch *et al.*, 1993). However, the receptor affinity of butanoyl-RC-160 for the somatostatin receptor is lower than RC-160. The attachment of butanoic acid to RC-160 possibly causes a perturbation in the conformation of RC-160, which reduces its receptor affinity. The size of the fatty acyl group, being conjugated to RC-160, appears to be a crucial factor governing the efficacy of the lipopeptides. Fatty acids of chain lengths ranging from 4–18 were conjugated to RC-160; the antiproliferative and antiangiogenic activities of these lipopeptides were determined (data unpublished). It was found that conjugation of RC-160 to long chain fatty acids such as myristic acid, resulted in the maximum improvement of its antiproliferative and anti-angiogenic activity *in vitro*.

The antiproliferative activity of RC-160 is dependent on the relative expression of somatostatin receptor subtypes on various cancer cell lines. The antiproliferative activity of lipophilized RC-160 seems to increase with increasing hydrophobicity of the peptide. All the three peptides inhibited tumour cell proliferation to the same magnitude, the cell growth being decreased by approx. 30–45%. However, myristoyl RC-160 inhibits tumour cell growth at about a 1000 fold lower concentration than butanoyl-RC-160 and RC-160 on the prostate carcinoma cell line DU145. In the pancreatic carcinoma cell line MIA-PaCa2, myristoyl RC-160 suppresses cell growth at about a 100 fold lower

concentration, as compared to RC-160, and butanoyl-RC-160. Controversial results were obtained in various laboratories on the antiproliferative activity of RC-160 on MIA-PaCa2 *in vitro*. Gillespie *et al*. (1992) could detect neither somatostatin receptors, nor an effect of RC-160 on the growth of MIA-PaCa2 *in vitro*. By contrast, Radulovic *et al*. (1993) found somatostatin receptors on MIA-PaCa2 and the growth of the same cell line to be inhibited by RC-160 *in vitro* and *in vivo*. However, we detected somatostatin receptors on MIA-PaCa2. We also observed that RC-160 inhibited EGF stimulated proliferation of MIA-PaCa2. Such differences in the somatostatin receptor status, might be due to handling, source of media, passage number etc. Moreover, the assay protocol used (Cattaneo *et al*., 1996) by us, involved the treatment of cells with RC-160 every 12 h, unlike the above published reports. It is known that the dose response of RC-160, to inhibit cell growth is biphasic in nature. A similar phenomena is observed in case of myristoyl-RC-160, where the antiproliferative activity is only manifested in a narrow range of hormone concentration. According to Delesque *et al*. (1995) high doses of somatostatin can stimulate $InsP_3$ formation, which can abrogate its antiproliferative activity. Since, butanoyl RC-160 possessed lower bioactivity, as compared to myristoyl RC-160, the latter was chosen for all further studies.

Myristoyl RC-160 exhibits antiproliferative activity on the endothelial cell line ECV304 at about a 1000 fold lower concentration, as compared to RC-160. Hence, we conjectured that it may be a better inhibitor of angiogenesis than RC-160. Somatostatin agonists have been shown to inhibit angiogenesis in the CAM model (Barrie *et al*., 1993). In the present study, we have demonstrated the inhibitory effect of RC-160 and the lipopeptides, using the 'Matrigel system' for angiogenesis, using the endothelial cell line ECV304. Human umbilical vein endothelial cells (HUVECs) are mostly used *in vitro* angiogenesis assays. The heterogeneity of various HUVEC isolates and their short life span, can make critical interpretation of data difficult. Unlike HUVEC isolates, ECV304 cells exhibit a highly consistent and reproducible network formation, when seeded on Matrigel, which has been shown to be a model for *in vitro* angiogenesis (Hughes, 1996). The antiangiogenic activity profile exhibited by myristoyl-RC-160 and RC-160, seems to be similar to their antiproliferative activity (Table 3). The quantitative inhibition in the formation of TLS, exhibited by myristoyl-RC-160 is similar to RC-160, however myristoyl RC-160 inhibits the formation of TLS at about a 10 fold lower concentration, as compared to and butanoyl-RC-160 and RC-160.

The antiproliferative action of lipopeptides, as well as RC-160, seems to be mediated through specific high affinity somatostatin receptors on target cells. Both butanoyl-RC-160 and myristoyl-RC-160 are able to displace ^{125}I-[Tyr^1]-somatostatin from the binding sites, on all the three cell lines. Scatchard analysis indicated the presence of one class of binding sites on MIA-PaCa2 and DU145, whereas ECV304 expresses two classes of binding sites. One showed high affinity ($K_d = 104.6$ pM) and low capacity ($B_{max} = 3.22$ fmol 5×10^4 cells^{-1}), and the other had low affinity ($K_d = 3.33$ nM) and high capacity ($B_{max} = 54.14$ fmol 5×10^4 cells^{-1}). The anti-angiogenic activity of these lipopeptides is mediated by high affinity somatostatin receptors on endothelial cells. The addition of a non-hydrolyzable GTP analogue GTPγS, as well as pertussis toxin blocked the binding of ^{125}I-[Tyr^1]-somatostatin to its receptor. RC-160 and its myristoylated derivative seem to interact with somatostatin receptors on endothelial cells *via* a pertussis toxin sensitive GTP binding protein.

The relative stability of these lipopeptides versus RC-160 was assessed by treating them with crude bovine pancreatic protease. It was observed that RC-160 was degraded at a much faster rate than myristoyl-RC-160. A similar trend is observed regarding the serum half life of both these peptides. RC-160 is degraded in normal mouse serum, within 2 h, whereas myristoyl RC-160 is detectable in serum even after 24 h. This seems to suggest that the myristoyl moiety of the lipopeptide shields the peptide against enzymatic degradation in serum. This may explain the improved activity of myristoyl-RC-160, as compared to RC-160, discussed above. Myristoyl-RC-160 does not get degraded by proteases in the serum, so is present in the cellular microenvironment for a greater length of time as compared to RC-160. The enhanced biological stability of myristoyl-RC-160 makes it possible for it to display equivalent antiproliferative and antiangiogenic activity at a significantly lower concentration than RC-160.

The biological activity of lipophilized-RC-160 has been described above and compared to the activity of RC-160. It seems that lipophilization of RC-160 with long chain fatty acids like myristoyl RC-160 is a good strategy to increase its stability, as well as enhance its antiproliferative and antiangiogenic activity. However, the crucial issue remains whether lipophilized RC-160 will have greater antiproliferative activity, as compared to RC-160 *in vivo*. The antineoplastic activity of lipophilized RC-160 on tumour growth *in vivo* (in nude mouse model) is being investigated currently in our laboratory. However, the above data suggests that myristoylation of RC-160 lowers the optimum dose required for the inhibition of cancer cell growth without compromising its efficacy, thereby increasing the scope of improving its therapeutic index.

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