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Somatostatin Receptor Expression in Lung Cancer

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Experimental evidence suggests that somatostatin analogues may have a role to play in the management of lung tumours. We evaluated membrane preparations of nine small cell lung cancer (SCLC) cell lines and of tumour samples from 3 patients with non-small cell lung cancer (NSCLC), 1 patient with an atypical carcinoid and another with a bronchial carcinoid for the presence of specific binding sites for RC–160, a potent growth inhibitory octapeptide analogue of somatostatin. Specific binding was noted on six of nine SCLC lines. Radio-receptor assay on the cell line NCI H 69 showed evidence of two specific binding sites for RC–160, one with high affinity and the other with low affinity. Binding sites were also found on all five tumour samples. Scatchard analysis indicated the presence of a single class of receptors with high affinity in each case. Histological assessment of the resected specimens before binding assay showed them to be comprised of tumour cells and necrotic tissue, stroma and/or inflammatory cells. Therefore, the specific binding of RC–160 may be to tissues other than the tumour cells. In 3 patients, from whom the tumour samples were obtained, radiolabelled somatostatin analogue scintigraphy using [^{111}In] pentetreotide was performed prior to surgery. In all cases, the radiolabel localised the disease. This study demonstrates the presence of specific binding sites for RC–160 in SCLC. Furthermore, the detection of specific binding *in vitro* and *in vivo* in NSCLC and intrapulmonary carcinoids demonstrates that these tumours contain cells which express specific binding sites for somatostatin. These results suggest that RC–160 may have a role to play as a therapeutic agent in lung cancer.

Key words: somatostatin, receptor, RC–160, [^{111}In] pentetreotide, lung cancer
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INTRODUCTION

IN VITRO studies have demonstrated that 50–75% of small cell lung cancer (SCLC) tumours express specific high affinity binding sites for somatostatin. Somatostatin analogues inhibit vasoactive intestinal polypeptide-induced cyclic adenosine monophosphate accumulation in SCLC cells, the clonal growth of SCLC cell lines in culture and the growth of SCLC xenografts in athymic nude mice. These results suggest that somatostatin receptors on SCLC are functional, and that the effect of somatostatin on SCLC proliferation is exerted through interference with growth pathways within the cell [1–6]. As a result of the receptor and growth inhibitory studies, somatostatin analogues are currently being evaluated as radiodiagnostic and therapeutic agents in SCLC. Employing radiolabelled somatostatin analogues as the scintigraphic agent, SCLC tumours have been successfully imaged in 62.5–100% of patients evaluated to date [1, 7, 8]. One small study, on the value of octreotide in the treatment of SCLC prior to chemotherapy, yielded equivocal results and demonstrated that this agent did not interfere with the subseqent chemoresponsiveness of the disease [4].

Whilst *in vitro* studies have failed to demonstrate the presence of somatostatin receptors in non-small cell lung cancer (NSCLC), somatostatin analogue treatment has inhibited NSCLC xenograft growth *in vivo* [1–6].

RC–160 is a potent octapeptide analogue of somatostatin. Like octreotide, it is a highly potent inhibitor of growth hormone

release [9]. RC–160 activates tyrosine phosphatase, thereby slowing tumour growth stimulated by tyrosine kinase [10]. RC–160 has been demonstrated to inhibit the growth of pancreatic, colorectal, prostatic and breast tumours *in vivo*, and is currently being evaluated in clinical trials [10–14]. The purpose of this study was to evaluate lung tumours for the presence of specific binding sites for RC–160, and thereby lay the groundwork for the assessment of this agent as a possible therapeutic modality in lung cancer.

MATERIALS AND METHODS

RC–160 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH_2) was supplied by Debiopharm S.A. (Lausanne, Switzerland). Radioisotope Na[^{125}I] was purchased from Amersham (Arlington, Illinois, U.S.A.. ^{111}Indium-labelled diethylenetriaminopentaacetic acid-linked octreotide, [^{111}In] pentetreotide (OctreoScan 111) was supplied by Mallinckrodt Medical (The Netherlands). Other peptides and chemicals were purchased from Sigma (St Louis, Missouri, U.S.A.), Bachem (Torrance, California, U.S.A.), Pierse (Rockford, Illinois, U.S.A.) and Baxter (Muskigon, Michigan, U.S.A).

Buffers

The homogenisation buffer consisted of 0.3 M sucrose, 25 mM Tris base, 0.25 mM phenylmethylsulphonylfluoride (PMSF), 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N,n - tetraacetic acid (EGTA), 10 mM monothioglycerol and Trasylol (aprotonin) 10 000 kallikrein inactivator units (KIU)/l, pH 7.5

The incubation buffer for the binding of radioiodinated RC–160 was composed of 50 mM Tris base, 5 mM $MgCl_2$, 0.25 mM PMSF, Trasylol 100 000 KIU/l, bacitracin 20 mg/l and 0.2% bovine serum albumin (BSA), pH 7.5

The wash buffer was the same as the incubation buffer, but without BSA.

Radioiodination of RC–160

Radioiodinated RC–160 was prepared by the chloramine-T method as previously described, with some modifications [15, 16]. Radioiodination of 5 μg of RC–160 in 20 μl of 0.5 M phosphate buffer (pH 7.5) was carried out with approximately 1 mCi of Na[^{125}I] for 40 s at room temperature. The reaction was initiated with 10 μg of chloramine-T in 10 μl and terminated by adding 50 μg L-cysteine (in 10 μl 0.01 N HCl). The labelled RC–160 was purified by reversed phase HPLC on a VYDAC C–18 column (Hesperia, California, U.S.A.), using 0.1% aqueous trifluoracetic acid (TFA) as solvent A and 0.1% TFA in 70% aqueous acetonitrile as solvent B. Elution was carried out by a linear gradient of 45–65% B in 30 min. The effluent was monitored by a UV detector at 280 nm and a flow-through radioactivity detector constructed from a ratemeter (SML–2, Technical Associates, Canoga Park, California, U.S.A.) in our laboratory. One–millilitre fractions were collected in borosilicate glass tubes, containing 30 μg/ml bacitracin and 10 mg/ml bovine serum albumin (BSA) in 1 ml of 0.25 M phosphate buffer (pH 7.5). The fraction, corresponding to the mono-iodinated compound and identified by elution position, radioactivity and UV peak, was stored at −70°C until use. The ionidation procedure yielded between 500 and 700 μCi of mono-iodinated RC–160 with a specific activity of 1700–2000 Ci/mmol.

Tissues

Specimens of NSCLC and intrapulmonary carcinoid tissue were obtained from fresh, surgically-resected tumours. The samples were placed on ice, snap-frozen in liquid nitrogen and stored at −70°C until evaluation. SCLC samples were obtained from cell cultures of the NCI cell lines H 69, H 82, H 249, H 345, H 510, H 524 and H 526 and of the cell lines HX 149 and HC 12 kindly provided by the Institute of Cancer Research (London and Surrey, U.K.). The cell lines were cultured in RPMI–1640 with L-glutamine (Gibco) and 5% fetal calf serum in humidified air with 5% CO_2 at 37°C until sufficient cells were present for harvesting. The cultures were centrifuged at 1000 *g* for 5 min. The supernatant was decanted, and the cells were washed once in PBS. Following the second wash and removal of the supernatant, the cells and residual PBS were transferred to a 2–ml Eppendorf tube. This was centrifuged at 1000 *g* for 5 min and the supernatant then removed. The resultant pellet was snap frozen in the tube and stored at −70°C until evaluation.

Prior to preparing membranes from the NSCLC and carcinoid tumour specimens, 10–μm fresh-frozen sections were cut, mounted on precleaned slides and stained with haematoxylin and eosin. The crossing points of a microscope ocular net that coincided with connective tissue or necrosis in each of the tumour sections were counted. The ratio of these points to the number of all points represented the ratio of the volume of connective tissue or necrosis to the volume of the tumour tissue. The inflammatory cells were not counted. The type of inflammatory cell which formed the main component of the infiltrate and the pattern of infiltration (diffuse, focal) was determined. The degree of the infiltration was estimated as slight, moderate or marked.

Membrane preparation

The tissue and cell line samples were thawed on ice. The tissue samples were cut into slices prior to homogenisation. All samples were homogenised in five times their volume of homogenisation buffer on ice using an Ultra-Turrax homogeniser (Tissumizer, Tekmar, Cincinnati, Ohio, U.S.A.) at maximum speed for 3–5 s. The homogenate was centrifuged at 500 *g* for 15 min at 4°C to remove nuclear debris. The supernatant containing the crude membrane fraction was ultracentrifuged at 40 000 *g* for 60 min at 4°C (Beckman L8–80M ultracentrifuge, Beckman instruments inc., Palo Alto, California, U.S.A.). The final pellet was resuspended in wash buffer and used for the receptor binding studies. Protein concentration was determined by the Bio-Rad protein assay kit (Richmond, California, U.S.A.) according to Bradford [17].

Receptor binding of peptides

The binding assay of RC–160 was conducted as described previously with some modifications [18]. Binding reactions were performed in 12 × 75–mm borosilicate glass tubes (Curtis Matheson Scientific, Inc.) for 2 h at room temperature using a competive-inhibition method. To assess specific binding, 50 μl of membrane fraction containing 30–70 μg of protein were incubated with 50 μl of [^{125}I]RC–160 (corresponding to between

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Table 1. *RC–160 binding characteristics*

Patient no. or cell line	Tumour	Specific binding	*Kd* (nM)	*B*max (pmol/mg protein)
H69	SCLC	Positive	*Kd*1 = 0.4 *Kd*2 = 77	*B*max 1 = 0.35 *B*max 2 = 11.4
No. 1	SCC	Positive	8.1	0.9
No. 2	SCC	Positive	6.6	0.5
No. 3	BC	Positive	13	3.8
No. 4	SCC	Positive	6	1.9
No. 5	AC	Postive	9.5	1.1

SCLC, small cell lung cancer; SCC, squamous cell carcinoma; BC, bronchial carcinoid; AC, atypical carcinoid. Radio receptor assay revealed a single class of high affinity binding sites in the SCC and carcinoid samples and both a high affinity and low affinity binding site in the SCLC cell line NCI H69. The primary tumours in patient nos. 1–3 were imaged with OctreoScan 111.

45 and 70 000 cpm) as radioligand with 50 µl of incubation buffer in the presence or absence of unlabelled RC–160. Therefore, the final incubation volume in each tube was 150 µl. The tests were performed in duplicate or triplicate on at least two occasions. The reaction was terminated by rapid filtration through glass fibre filters (Whatman GF/B) prewetted in assay buffer, and presoaked in 0.5% polyethylenimine solution to minimise filter adsorption. A semi-automatic harvesting system was employed (Cambridge Technology, Massachusetts, U.S.A.) with three 5 s washes. The radioactivity of the filters was counted in a gamma-counter (Micromedic Systems Inc., Huntsville, Alabama, U.S.A.) Evidence of specific binding was defined as a difference between total and non-specific binding equivalent to > 15% of total binding.

Displacement curves were obtained on the cell line H 69 and on the non-small cell and two carcinoid tumours by incubating 50 µl of membrane fraction containing 30–70 µg of protein with 50 µl of [^{125}I]RC–160 as above and 50 µl of increasing amounts of unlabelled peptide (10^{-6} to 10^{-10} M). Each assay point was performed in duplicate or triplicate if sufficient tissue was available. The specificity of the binding was demonstrated using somatostatin–14 and unrelated peptides, such as bombesin/gastrin releasing peptide (GRP), D–Trp^6–LHRH (luteinising hormone releasing hormone) and epidermal growth factor (EGF) at 10^{-6} M as competitors.

Mathematical analysis of binding data

Final binding parameter estimates were calculated by the LIGAND computer programme of Munson and Rodbard [19], as modified by McPherson [20]. To determine the types of receptor binding, dissociation constants (*Kd*) and the maximal binding capacity of receptors (*B*max), the binding data were also analysed by the Scatchard method [21].

[^{111}In]Pentetreotide scintigraphy

The imaging studies were performed with Ethical Committee approval and with informed, written, patient consent.

The [^{111}In]pentetreotide was administered as an intravenous bolus injection. Prior to injection, the pentetreotide was labelled with ^{111}In in the nuclear medicine department as a single step procedure and percentage binding was calculated. Percentage binding was > 97% in all cases. One vial of the radiolabel, equivalent to ^{111}MBq, was administered to each patient.

Scintigraphic images were obtained 4 and 24 h postadministration using gamma cameras with medium-energy parallel-hole collimators. Two energy peaks were used, 171 and 245 keV, each with a 20% window. Images of the thorax and abdomen were obtained at 4 h. Images of the head and neck, thorax, abdomen and pelvis were obtained at 24 h. The planar images were followed by single photon emission computed tomography (SPECT) acquisitions of the thorax and liver using an IGE 400 ACT gamma camera employing an elliptical 360° orbit with 64 projections at 20 s per projection.

RESULTS

Of the nine SCLC cell lines evaluated for the presence of binding for RC–160, a significant difference between total and non-specific binding was seen in six—NCI H69, NCI H249, NCI H345, NCI H510, NCI H524 and HC 12. Therefore, evidence for the presence of specific receptors for RC–160 was seen in 66% of the samples studied. Scatchard analysis of the displacement curve of the cell line, NCI H69, revealed two specific binding sites, one of high affinity (*Kd* = 0.4 nM) and low capacity (*B*max = 0.35 pmol/mg protein) and the other of low affinity (*Kd* = 77 nM) and high capacity (*B*max = 11.4 pmol/mg protein) (Figure 1, Table 1).

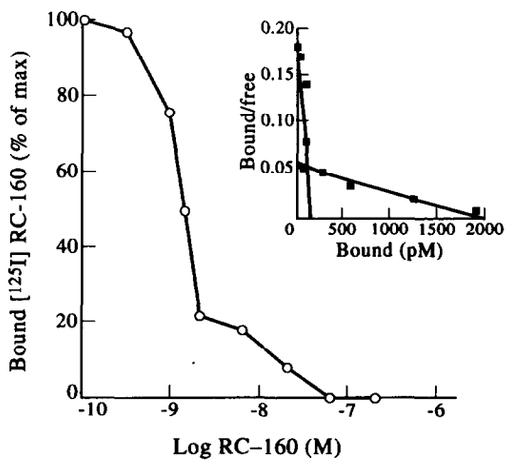

Figure 1. Displacement of [^{125}I] RC–160 binding by increasing concentrations of unlabelled RC–160 using membranes of the SCLC cell line NCI H 69. The Scatchard plot of the same binding data is also shown (inset). Each data point represents the mean of duplicate determinations. Two specific binding sites are seen, one of high affinity and one of low affinity.

Table 2. Histological characteristics of the NSCLC and bronchial carcinoid tumours analysed for the presence of RC–160 receptors

Patient	Tumour	% tumour tissue	% stroma	% necrosis	Inflammatory infiltrate
No. 1	SCC	60	40	55–60	Moderate, focal lymphocytic around tumour
No. 2	SCC	80	20	2–3	Slight
No. 3	BC	60	40	0	None
No. 4	SCC	50	50	0	Diffuse, granulocytic
No. 5	AC	90	10	0	None

SCC, squamous cell carcinoma; BC, bronchial carcinoid; AC, atypical carcinoid.

The NSCLC and bronchial carcinoid specimens were comprised of tumour cells and necrotic tissue, stroma and/or inflammatory cells. Details of the composition of the tumours are outlined in Table 2. Scatchard analysis revealed the presence of a single class of high affinity binding sites on each of the five specimens studied. Figure 2 shows a representative example of [^{125}I]RC–160 binding to the NSCLC membrane preparations studied.

The specificity of these receptors was demonstrated by the observation that while somatostatin–14 completely displaced the radioligand, none of the functionally unrelated peptides tested could inhibit the binding at 10^{-6} M concentration.

[^{111}In] Pentetreotide scintigraphy was performed in 2 patients with NSCLC and 1 with a bronchial carcinoid prior to surgery. In all 3 cases positive images were obtained.

In case 1, a patient with a squamous cell lung carcinoma (Table 1), [^{111}In] pentetreotide imaging suggested a large stage II tumour (Figure 3). Formal standard staging, including a full blood count, renal, liver and bone biochemistry, CxR and CT scan of the thorax and upper abdomen, suggested that the tumour also involved hilar lymph nodes and was abutting on to the pleura. At surgery, the tumour mass was stuck to the pleural surface and to the aorta, necessitating an extrapleural chest wall

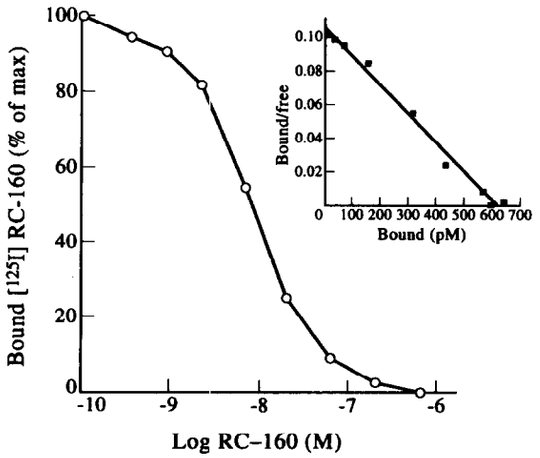

Figure 2. Displacement of [^{125}I] RC–160 binding by increasing concentrations of unlabelled RC–160 using membranes of the NSCLC sample from patient 4. The Scatchard plot of the same binding data is also shown (inset). Each data point represents the mean of triplicate determinations. A single class of high affinity binding site is seen.

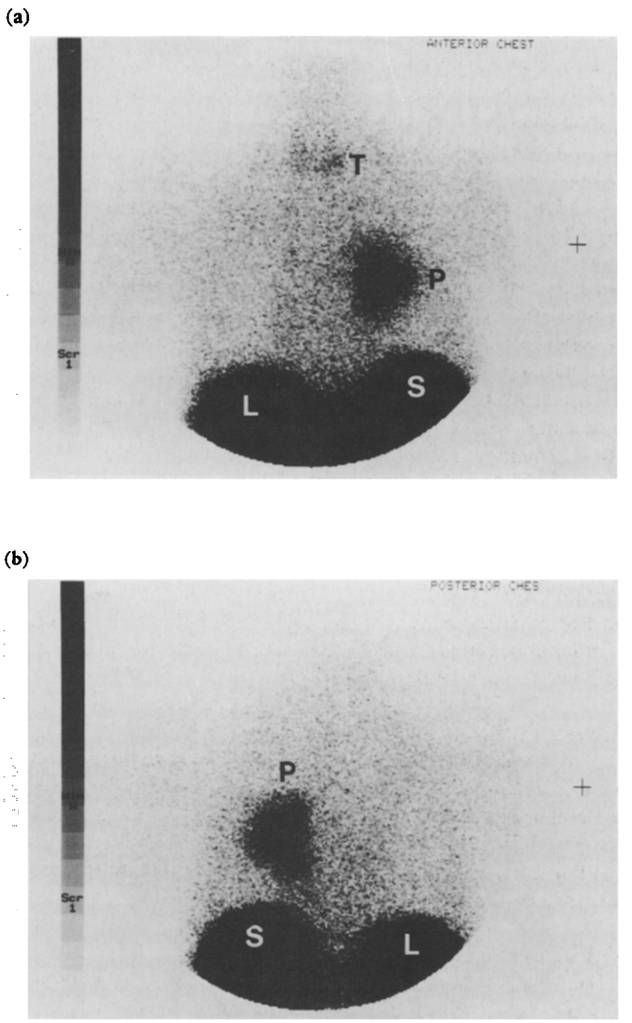

Figure 3. Anterior (a) and posterior (b) planar images of the thorax of patient no.1, 24 h after administration of [^{111}In]pentetreotide. A large stage II tumour (P) is demonstrated in the left lung extending from the hilum into the lung parenchyma. At surgery, a 10–cm mass was found which was stuck to the pleura and aorta. Histological evaluation of the resected specimen showed the resection margins, pleural surface and hilar lymph nodes to be free of malignant disease. Physiological uptake is noted in the thyroid (T), liver (L) and spleen (S).

and subadventitial aortic wall dissection. Review of the resected specimen, however, revealed that the pleural surface and bronchial resection margins were actually free of tumour and that none of the resected lymph nodes were involved. These findings confirmed the staging as indicated by the scintigraphy.

In case 2, also a patient with squamous cell carcinoma (Table 1), scintigraphy suggested a stage IIIb tumour. Following surgery, tumour was found to involve the pleura consistent with stage III disease. However, only one local lymph node was invaded with tumour. Interestingly, all of the resected lymph nodes had evidence of granulomatous disease deemed as likely to be secondary to infective tuberculosis. However, no organisms were detected in the specimen.

In case 3, a patient with a bronchial carcinoid tumour (Table 1), SPECT imaging revealed a small 1×1.5 cm lesion in the right lung in the region of the hilum. This was confirmed on examination of the resected specimen where a locally invasive carcinoid was found in the bronchus intermedius. Regional lymph nodes were free of disease.

DISCUSSION

Despite improvements in our understanding of the biology of lung cancer, SCLC and NSCLC remain diseases with a poor prognosis. Although SCLC is sensitive to both chemotherapy and radiotherapy, median survival for patients with limited stage disease is in the region of 16 months with an overall 5-year survival of 7–20%. With extensive stage SCLC, the median survival is 9 months with few cures. The outlook for patients with NSCLC is likewise poor. The majority of NSCLC patients are either unsuitable for surgery or have inoperable disease at presentation. Furthermore, NSCLC is relatively resistant to chemotherapy, with an overall 20–30% response rate and only a marginal survival benefit. Therefore, novel approaches to treatment are required [22–25].

SCLC is characterised by the expression of neuroendocrine markers. Recent work has also shown that approximately 20% of NSCLC tumours have neuroendocrine characteristics, and that this subgroup may represent the NSCLC which is sensitive to chemotherapy [1, 22]. Experimental evidence suggests that neuroendocrine tumours may express receptors or high-affinity binding sites for somatostatin [26]. In keeping with this has been the finding *in vitro* that between 50 and 75% of SCLC tumours have specific high-affinity binding sites for somatostatin. Furthermore, SCLC disease sites have been localised through scintigraphic imaging with the radiolabelled somatostatin analogues [^{123}I]Try^3 octreotide and [^{111}In]pentetreotide which bind to tissues expressing somatostatin receptors [1, 7, 8]. To date, no receptors for somatostatin have been demonstrated on NSCLC cell lines and tumour samples *in vitro* through both membrane binding assays and autoradiography [2–6].

Specific binding, suggestive of receptor sites for radiolabelled RC–160, was noted on 66% of the SCLC cell lines evaluated in this study. Scatchard analysis of the displacement curve of the cell line NCI H 69 revealed evidence of two specific binding sites, one of high affinity and low capacity and the other of low affinity and high capacity. The high affinity binding site is in keeping with previous studies. Taylor and colleagues described a single binding site for [^{125}I-Try^{11}]somatostatin–14 to membranes prepared from NCI H 69 with a $Kd = 0.59$ nM and Bmax = 0.173 pmol/mg protein [2]. Furthermore, whilst we did not evaluate resected SCLC samples in this study, Reubi and colleagues demonstrated a single binding site for the somatostatin analogue [^{125}I]204–090 on membranes prepared from an SCLC fresh frozen tumour sample with $Kd = 0.53$ nM and Bmax = 0.189 pmol/mg [3]. The significance of the low affinity binding site requires further evaluation.

This is the first study to evaluate somatostatin analogue binding to bronchial carcinoid tissue. The finding of a single class of specific high affinity binding sites in both specimens in this study is not unexpected given that their counterparts in the gastrointestinal tract are characterised by the expression of somatostatin receptors [26].

The interesting result was the finding of a single class of specific high affinity binding sites in each of the NSCLC tumour samples in this series. The tissues examined were composed of a number of cell types including tumour cells, stroma, inflammatory cells and necrotic tissue (Table 2). Therefore, it is possible that the NSCLC cells themselves are not expressing specific somatostatin binding sites, as there may be uptake of the radiolabel by other cells within the tumour mass. For example, lymphocytes are known to express somatostatin receptors [27].

Prior to surgery, [^{111}In]pentetreotide scintigraphy was performed on 3 of the patients, 2 with NSCLC and 1 with a bronchial carcinoid, from whom resected tissue was analyzed for the presence of specific binding for RC–160. Scintigraphy localised and correctly staged the disease in 2 patients. In the third case, an NSCLC patient, uptake of the radiolabel in the mediastinum was found. Following surgery, histological examiniation of resected mediastinal lymph nodes revealed granulomatous changes without evidence of tumour invasion. Uptake in the granulomas is not unexpected as *in vivo* receptor imaging of granulomatous disease such as sarcoidosis, tuberculosis and Wegener's granulomatosis has been positive in the majority of cases studied [28].

As a result of these findings, whilst we cannot say with certainty that NSCLC tumours cells themselves express somatostatin receptors, the expression of somatostatin receptors in tumour tissue makes possible the assessment of radiolabelled somatostatin scintigraphy as a staging modality in the pretreatment evaluation of patients with NSCLC. Five subtypes of somatostatin receptor have recently been cloned and functionally characterised. They are members of a distinct sub-family of GTP-binding protein-coupled seven-helix transmembrane spanning receptors, being similar in their amino acid sequences, but differing in extracellular amino terminal and intracellular carboxy-terminal domains. While all five subtypes bind somatostatin–14 and somatostatin–28, they show major differences in their affinity for somatostatin analogues. The patterns of expression of mRNA for these receptor subtypes and their distributions are also different, but are often overlapping in various tisses [29–33]. Analysis of RC–160 binding to three subtypes of the somatostatin receptor have been performed revealing high affinity binding to receptor subtype 2 (SSTR2), but low affinity to the others [33, 34]. The results of this study suggest that SCLC tumours may express somatostatin receptor subtypes with high and low affinity for RC–160. Furthermore, our findings raise the possibility that NSCLC and pulmonary carcinoid tumours may also express high affinity somatostatin receptors. The high affinity receptor detected on NCI H69 and on the NSCLC and carcinoid tumours in this study may be similar to SSTR2. Evaluation of SCLC and NSCLC/carcinoid samples for somatostatin receptor subset mRNA may answer these questions.

Somatostatin analogues are currently being assessed as possible therapeutic agents in SCLC and NSCLC with encouraging results. As discussed earlier, whilst the growth inhibitory effects

may be direct, they may also be indirect through the inhibition of growth factors released from other tissues, such as IGF–1 and epidermal growth factor [4, 35]. The demonstration of specific binding of RC–160 to the SCLC and NSCLC and pulmonary carcinoids in this study lays the groundwork for the evaluation of this potent, growth inhibitory peptide in the treatment of lung cancer.

In conclusion, this study demonstrates that SCLC tumour cells express specific binding sites for the potent growth inhibitory somatostatin analogue RC–160. The results in the NSCLC and pulmonary carcinoid specimen studies suggest that these tumours contain cells which have specific receptors for somatostatin. As the tumours samples were of mixed histology, the specific binding of RC–160 may be to tissues other than the tumour cells. Successful detection of the three NSCLC and carcinoid tumours assessed with [^{111}In]pentetreotide scintigraphy indicates that this radiological technique may have a role to play in the clinical evaluation of these patients prior to treatment. These results also suggest the merit of formal assessment of RC–160 in the treatment of lung cancer.

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