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A targeted cytotoxic somatostatin (SST) analogue, AN-238, inhibits the growth of H-69 small-cell lung carcinoma (SCLC) and H-157 non-SCLC in nude mice

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Abstract

Recently, we developed a cytotoxic analogue of somatostatin (SST), AN-238, in which the SST carrier peptide RC-121 was linked to 2-pyrrolinodoxorubicin (2-pyrrolino-DOX) (AN-201), a potent derivative of doxorubicin. AN-238 can be targeted to SST receptors (SSTRs) on tumours. In the present study, we evaluated the effects of AN-238 on the growth of H-69 small-cell lung carcinoma (SCLC) and H-157 non-SCLC xenografted into nude mice. High affinity binding sites for SST are present in H-69 SCLC and were now detected in H-157 non-SCLC xenografts, but not in H-157 cells. A strong expression of the human SSTR subtype 2 (*hSSTR-2*) and a weaker expression of subtype 5 (*hSSTR-5*) was found in H-69 SCLC cells, but not in H-157 non-SCLC cells. However, a strong expression of mRNA for mouse (*m*)*SSTR-2* could be detected in H-157 xenografts. AN-238 effectively inhibited the growth of H-69 SCLC tumours in nude mice. Twenty-six days after a single injection of AN-238 at 200 nmol/kg, the volume of H-69 tumours was decreased by approximately 55% ($P < 0.05$) compared with the controls, while AN-201 at the same dose was highly toxic and produced only a minor tumour inhibition. To evaluate the potency of multiple doses of AN-238, nude mice bearing H-69 SCLC received three injections of AN-238 at 150 nmol/kg on days 1, 12 and 28. In the period of 42 days after the first injection, the growth rate of H-69 tumours was approximately 50% lower than that of controls. In nude mice bearing H-157 non-SCLC tumours, a single i.v. administration of AN-238 at 200 nmol/kg inhibited tumour volume by 91% after 28 days ($P < 0.01$ compared with controls). AN-201 was toxic and ineffective at the same dose. Two injections of AN-238 at 150 nmol/kg given on days 1 and 18 produced 83% inhibition of H-157 tumour growth ($P < 0.01$ versus controls). AN-238 given as a single dose of 200 nmol/kg induced necrosis, while two injections of 150 nmol/kg induced apoptosis in the tumour tissue. Our results indicate that targeted cytotoxic SST analogue AN-238 could be considered for therapy of both SCLC and non-SCLC.

Keywords: Lung cancer; Targeted analogue; Chemotherapy; Receptor; Tumour inhibition; Doxorubicin

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the Western world [1,2]. Although some progress has been made for the management of patients with lung cancer, the need for the development of novel treatment modalities persists. Lung cancer consists of four histological entities: small-cell lung cancer (SCLC), which accounts for approximately 20% of lung cancer cases, and three other subtypes (squamous-cell carcinoma, adenocarcinoma and large-cell carcinoma) that are grouped together because of similar clinical management, and known as non-SCLC [1]. Surgery is the therapy of choice for non-SCLC, while chemotherapy and/or radiotherapy are preferred for SCLC, because SCLC patients have either locally advanced disease or distant metastases and surgery is only rarely possible [1].

Targeting of cytotoxic agents to tumour cells is a modern approach in the treatment of various cancers, which should improve the efficacy of therapy and decrease the toxicity [3]. Typically, targeted hybrids consist of a toxic moiety conjugated to a carrier possessing a high affinity for cancer cells. Enzymes, antigens or receptors that are expressed predominantly on the sur-

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face of cancer cells may be used as targets to increase the selectivity of therapy [3,4]. Recently, we developed a cytotoxic somatostatin (SST) analogue AN-238 [5], which consists of the SST carrier octapeptide RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2) covalently linked to 2-pyrrolinodoxorubicin (2-pyrrolino-DOX) (AN-201), a derivative of doxorubicin, 500–1000 times more potent than the parent compound [6]. AN-238 fully retains the cytotoxic activity of the radical and the receptor binding affinity of the peptide carrier [5], and inhibits significantly the *in vivo* growth of various tumours that express receptors for SST, such as prostate [7,8], breast [9], brain [10] and renal cancers [11].

The expression of receptors for SST in the majority of SCLCs and in a subset of non-SCLC, has been reported by several investigators [12–15]. Previously, we have detected the presence of high affinity binding sites for SST in NCl-H-69 SCLC cells [15,16]. In addition to the expression of SST receptors by the cancer cells, the successful imaging of many SST receptor-negative non-SCLCs by radiolabelled SST analogues revealed the expression of binding sites for SST by activated leucocytes, proliferative neuroendocrine cells around the tumours and peritumoral veins [17–19]. These findings indicate that therapy based on the targeting of cytotoxic agents to SST receptors on lung tumours could be superior to conventional chemotherapy. In the present study, we evaluated the effects of cytotoxic SST analogue, AN-238, on the growth of NCl-H-69 SCLC and NCl-H-157 non-SCLC cells xenografted into nude mice.

2. Materials and methods

2.1. Peptides and cytotoxic agents

The SST octapeptide analogue RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2) and 2-pyrrolino-DOX (AN-201) were synthesised in our laboratory as described in Refs. [5,6]. The cytotoxic conjugate AN-238 was made by coupling one molecule of AN-201-14-*O*-hemiglutarate to the amino terminus of [Lys (N-(9-fluorenyl)-methoxycarbonyl)^5]RC-121, followed by deprotection and purification [5]. Before the intravenous (i.v.) injection, the compounds were dissolved in 20 μl of 0.1 N aqueous acetic acid and diluted with 5% (w/v) aqueous D-mannitol solution (Sigma, St Louis, MO, USA) to the final volume.

2.2. Cell culture

The human SCLC cell line NCI-H-69 was obtained from American Type Culture Collection (Manassas, VA, USA) and the non-SCLC cell line NCI-H-157, from H. Oie (NCI-Navy Medical Oncology Branch, Bethesda, MD, USA). These cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 4 mM L-glutamine, 50 units ml^{-1} penicillin G sodium, 50 μg ml^{-1} streptomycin sulphate, 0.125 μg ml^{-1} amphotericin B and 10% fetal bovine serum (H-69) or newborn calf serum (H-157) at 37°C in a humidified 95% air/5% carbon dioxide atmosphere. Cells were passaged weekly and routinely monitored for mycoplasma contamination using a detection kit (Boehringer Mannheim, Mannheim, Germany). All culture media components were purchased from Gibco (Grand Island, NY, USA).

2.3. Animals

Five- to 6-week-old male athymic (NCr nu/nu) nude mice were obtained from the National Cancer Institute (NCI) (Bethesda, MD, USA). The mice were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12-h light/12-h dark schedule and were fed autoclaved chow and water *ad libitum*. All experiments were performed in accordance with institutional ethical guidelines for the care and use of experimental animals.

2.4. Experimental protocol

In the first experiment, xenografts were initiated by subcutaneous (s.c.) injection of 10^7 NCl-H-69 SCLC cells into the right flanks of three male mice. Tumours resulting after 2 weeks in donor animals were aseptically dissected and mechanically minced. Three mm^3 pieces of tumour tissue were transplanted s.c. by trocar needle into 40 mice under methoxyflurane (Metofane, Pittman-Moore, Mundelein, IL, USA) anaesthesia. The take rate was approximately 85%. Three weeks after transplantation, when tumours had grown to a volume of approximately 350 mm^3, mice were divided into five groups of 6–7 mice per group, and received the following treatment as i.v. injections in the tail vein: group 1, control, vehicle solution (5% mannitol); group 2, cytotoxic radical AN-201 at a dose of 200 nmol/kg given on day 1; group 3, cytotoxic analogue AN-238 at a dose of 200 nmol/kg given on day 1; group 4, cytotoxic radical AN-201 at a dose of 150 nmol/kg given on days 1, 12 and 28; group 5, cytotoxic analogue AN-238 at a dose of 150 nmol/kg given on days 1, 12 and 28.

In the second experiment, xenografts were initiated by s.c. injection of 6×10^6 NCl-H-157 non-SCLC cells into the right flanks of three male mice. Tumours resulting after 10 days in donor animals were aseptically dissected and mechanically minced. Three mm^3 pieces of tumour tissue were transplanted s.c. by trocar needle into 44 mice under methoxyflurane (Metofane, Pittman-Moore, Mundelein, IL, USA) anaesthesia. The take rate was 100%. Two weeks after transplantation, when tumours had grown to a volume of approximately 100 mm^3, mice

were divided into six groups, of 7–8 mice per group, and received the following treatment as a single i.v. injection in the tail vein: group 1, control, vehicle solution (5% mannitol); group 2, cytotoxic radical AN-201 at a dose of 200 nmol/kg; group 3, unconjugated mixture of the cytotoxic radical AN-201 and the carrier RC-121 at a dose of 200 nmol/kg; group 4, cytotoxic analogue AN-238 at a dose of 200 nmol/kg; group 5, carrier peptide RC-121 at a dose of 200 nmol/kg; group 6, cytotoxic analogue AN-238 at a dose of 150 nmol/kg. Eighteen days after the initiation of the treatment, group 6 received a second injection of AN-238 at 150 nmol/kg.

In the first experiment, mice from groups 1–3 were sacrificed 26 days after the initiation of the treatment, while mice from groups 4 and 5 were sacrificed after 42 days. The second experiment was terminated 28 days after the initiation of the therapy. Mice were anaesthetised with methoxyflurane and killed by decapitation. In both experiments, tumour volume was measured 1–2 times a week using microcallipers [20], and changes in body weights and deaths related to toxicity were recorded. At the end of the experiments, autopsy was performed and the tumour weights were measured. Samples of each tumour were fixed in formalin for histological examination.

2.5. Histological methods

Tumour samples were processed as previously described [20]. The extent of necrosis was evaluated with the point-counting method on tumour slides stained with haematoxylin-eosin. For the measurement of the number of mitotic and apoptotic cells, 10 high power fields were considered and the numbers of mitotic and apoptotic cells per 1000 cells (mitotic and apoptotic indices) were calculated in haematoxylin-eosin stained slides. For demonstration of the nuclear organiser region (NOR) in tumour cell nuclei, the argyrophilic NOR (AgNOR) method of Chiu and colleagues [21] was used with a small modification [22]. The silver stained black grains in 50 cells of each tumour were counted and the AgNOR number per cell was calculated.

2.6. Receptor binding assay

The presence of receptors for SST in the membranes of H-157 tumours and membrane fraction of cultured H-157 cancer cells was determined by ligand competition assays using [^{125}I]-labelled RC-160 and [^{125}I]-labelled [Tyr^{11}]SST-14, as previously reported [7,9,11,15,19]. The LIGAND PC computerised curve-fitting program of Munson and Rodbard [23] was used to determine the types of receptor binding, the maximal binding capacity (B_{max}) of the receptors and the dissociation constant (K_d) values.

2.7. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from H-69 SCLC and H-157 non-SCLC xenografts, and cells cultured *in vitro* and subjected to RT-PCR analysis for SST receptor subtypes 2 and 5 as previously described [8]. For amplification from first strand cDNAs, gene-specific primers for human *(h)SSTR2* (sense, 5′-ATGGACATG-GCG-GATGAGCCACT-3′; antisense, 5′-TACTGGTTTGG-AGGTCTCCATTGA-3′), *hSSTR5* (sense, 5′-CGTCT-TCATCATCTACACGG-3′; antisense, 5′-GGCCAGG-TTGACGATGTTGA-3′). [8] and human *B-actin* (internal control) (sense, 5′-TCATGAAGTGTGACG-TGGAC-3′; antisense, 5′-ACCGACTGCTGTCACCT-TCA-3′) [24] were used.

Total RNA for 5 H-157 control tumours were subjected to digestion with DNase to eliminate the possibility of DNA contamination before performing the RT-PCR reaction for the amplification of mRNA for *hSSTR-3*. The sequences of gene-specific primers for *hSSTR-3* were (sense, 5′-TCAGTCACCAACGTCTA-CATCC-3′; antisense, 5′-ACGCTCATGACAGTCA-GGC-3′) [25].

The presence of mouse SST receptor subtypes 2 and 5 (*mSSTR-2* and *mSSTR-5*) was also evaluated in the human H-157 non-SCLC xenografts and cultured cells. For the amplification from first strand cDNAs, gene-specific primers for *mSSTR-2* (sense, 5′-TCCTCTGG-AATCCGAGTGGG-3′; antisense, 5′-CCGGTTGGC-ACAGCTGTTG-3′) [26], *mSSTR-5* (sense, 5′-ATGG-AGCCCCTCTCTTTGG-3′; antisense, 5′-CAGTAGG-AGACAGCATTC-3′) [27] and mouse *β-actin* (internal control) (sense, 5′-GTCACCCACACTGTGCCCATCT-3′; antisense, 5′-ACAGAGTACTTGCGCTCAGGAG-3′) [28] were used.

2.8. Statistical analysis

The data are expressed as the mean±standard error of the mean (SEM). Statistical evaluation of the data was performed by the Student's *t*-test (two-tailed). Differences were considered statistically significant when $P < 0.05$. *P* values shown are against the control group unless otherwise stated. The SigmaPlot computer software (Jandel, San Rafael, CA, USA) was used for the preparation of the figures.

3. Results

3.1. Tumour inhibition and toxicity

The effects of cytotoxic SST analogue AN-238 on tumour volume, tumour weight and animal mortality in athymic nude mice bearing H-69 SCLC tumours or

Table 1
Effect of cytotoxic SST analogue AN-238 and cytotoxic radical AN-201, on tumour volume, tumour weight and animal mortality in athymic nude mice bearing H-69 SCLC tumours[a]

	Tumour volume (mm^3) on day 26	Final tumour volume (mm^3) on day 42	Tumour weight (g)	Dead animals/ total animals
Control	3841 ±615		4±0.7	0/7
AN-201 (200 nmol/kg)	2379±994		2.4±0.7	4/6
AN-238 (200 nmol/kg)	1730.1±474*		1.8±0.5*	2/6
AN-201 (150 nmol/kg) days 1, 12 and 28	2575.5±659	5102±992	N.D.	4/7
AN-238 (150 nmol/kg) days 1, 12 and 28	1040.5±307.1**	2166±587.3***	N.D.	1/7

N.D., not determined.
[a] Initial tumour volumes were 326.4±90.7 to 385±88. For mice injected with multiple doses of cytotoxic agents, two values of tumour volume are shown, one on day 26 and the other on day 42.
*$P < 0.05$, **$P < 0.001$ against control, ***, $P < 0.05$ compared with group treated with AN-201.

H-157 non-SCLC are shown in Tables 1 and 2, respectively. In the first experiment, AN-238 was tested in mice bearing H-69 SCLC tumours. Twenty-six days after the injection of AN-238 at 200 nmol/kg, tumour volume and weight both decreased by approximately 55% ($P < 0.05$) compared with the controls (Table 1, Fig. 1a). AN-201 administered at equimolar dose produced only a marginal tumour inhibition and had a higher toxicity than AN-238. Only two of six mice died in the AN-238 treated group, while the mortality related to toxic effects was 66% (four of six animals) in the group injected with AN-201. A potent inhibition of tumour growth was also produced by three injections of AN-238 at 150 nmol/kg. As shown in Table 1 and Fig. 1a, 42 days after the initiation of the therapy, the rate of tumour growth in animals injected three times with AN-238 at 150 nmol/kg, was decreased to approximately 50% that of the controls. Thus, on day 42, the final tumour volume in this group was 2166±587 mm^3, which corresponds to the tumour volume of the controls at around day 21. In experiment 1, administration of 3 doses of AN-201 was toxic and significantly ($P < 0.05$) less effective than AN-238 in inhibiting tumour growth. The tolerance of nude mice to the cytotoxic analogue AN-238 administered three times was higher than that to an equimolar dose of the cytotoxic radical AN-201. Only one of seven mice died in the group that received three doses of AN-238, but four of the seven animals were dead in the group injected three times with AN-201, 42 days after the initiation of treatment.

In the second experiment, a single i.v. injection of AN-238 at 200 nmol/kg inhibited significantly ($P < 0.01$) the growth of H-157 non-SCLC tumours xenografted into nude mice. Twenty-eight days after the injection, tumour volume and tumour weight of H-157 xenografts in mice treated with AN-238 were inhibited by 91% ($P < 0.01$) and 88% ($P < 0.01$), respectively, compared with the controls (Table 2, Fig. 2a). Tumour inhibition produced by the treatment with equimolar dose of AN-201 given alone or as a mixture with carrier RC-121 was not significant and accompanied by high toxicity: Three of seven animals injected with AN-201 and four of eight mice treated with a mixture of AN-201 and RC-121 died from toxicity, while only one of seven mice was dead in the AN-238 treated group (Table 2). The carrier peptide RC-121 was not toxic, but had no tumour inhibitory effect (Table 2, Fig. 2a). Significant tumour growth inhibition was also produced by AN-238 administered twice at 150 nmol/kg at an interval of 17 days: 28 days after the initiation of the therapy, tumour volume and tumour weight in mice treated twice with AN-238 at 150 nmol/kg were significantly ($P < 0.01$) inhibited by 83 and 77%, respectively, compared with controls. None of the animals died in the group injected twice with AN-238 at 150 nmol/kg.

Table 2
Effect of cytotoxic SST analogue AN-238, cytotoxic radical AN-201, carrier peptide RC-121 and mixture of RC-121 and AN-201 on tumour volume, tumour weight and animal mortality in athymic nude mice bearing H-157 non-SCLC tumours

	Initial tumour volume (mm^3)	Final tumour volume (mm^3)	Tumour weight (g)	Dead animals/ all animals
Control	94±25.7	4583±1017	4.3±0.9	0/7
AN-201 (200 nmol/kg)	101±50.3	3659±1939	3.3±1.5	3/7
AN-201 + RC-121 (200 nmol/kg)	104±43.3	2891±1035	3±0.9	4/8
AN-238 (200 nmol/kg)	130±25.8	411±150.6*	0.5±0.2*	1/7
RC-121 (200 nmol/kg)	112±38	4131±1088	3.8±1	0/8
AN-238 (150 nmol/kg) days 1 and 18	111±49.4	801±253*	1±0.4*	0/7

*$P < 0.01$ against control.

Losses in body weights occurred in all groups treated with cytotoxic agents, but the body weights of the surviving animals returned to normal by the end of the experiment (Figs. 1b and 2b).

3.2. Histological findings

3.2.1. Experiment 1

H-69 SCLC were undifferentiated tumours consisting of relatively small epithelial cells. Extensive necrosis was present in most tumours. A significant ($P < 0.05$) reduction in the AgNOR numbers, by approximately 11% versus controls, was observed in the tumours from mice treated with AN-238 at 200 nmol/kg. No significant differences in the % area of necrosis, the apoptotic index and the ratio of apoptotic to mitotic indices were detected between groups.

3.2.2. Experiment 2

H-157 non-SCLCs were undifferentiated tumours consisting of relatively large uniform epithelial cells with large round nuclei containing prominent nucleoli. Necrotic areas were significantly more ($P < 0.05$) extended in the tumours from mice treated once with 200 nmol/kg of AN-238, and apoptosis was significantly

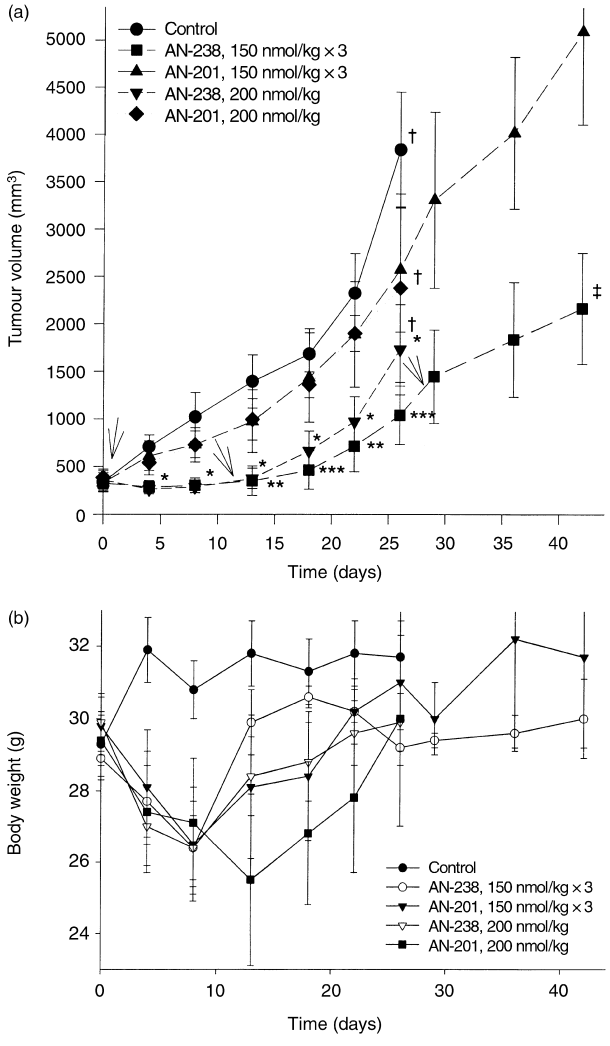

Fig. 1. (a) Changes in tumour volume in athymic nude mice bearing H-69 SCLC tumours after treatment with a single dose at 200 nmol/kg or three doses at 150 nmol/kg of cytotoxic SST analogue AN-238 or cytotoxic radical AN-201 given on days 1, 12 and 28. Arrows indicate the time of injection of cytotoxic agents; (b) changes in body weights of athymic nude mice bearing H-69 SCLC tumours after treatment with a single dose at 200 nmol/kg or three doses at 150 nmol/kg of cytotoxic SST analogue AN-238 or cytotoxic radical AN-201 given on days 1, 12 and 28. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control. †Mice from the control group and mice from the groups injected once with AN-238 and AN-201 were sacrificed on day 26. ‡$P < 0.05$ versus AN-201-treated group.

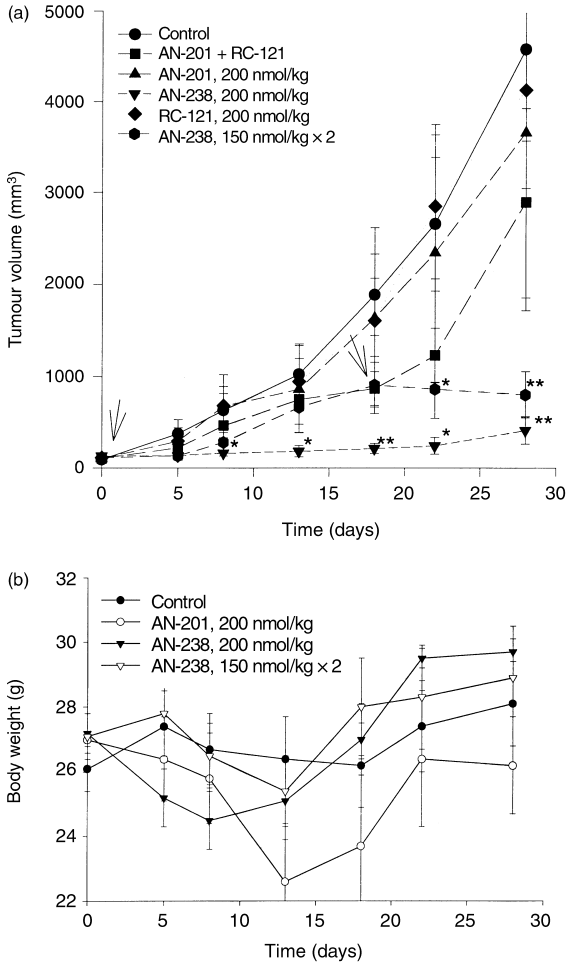

Fig. 2. (a) Changes in tumour volume in athymic nude mice bearing H-157 non-SCLC tumours after treatment with a single dose at 200 nmol/kg of the cytotoxic SST analogue AN-238, the cytotoxic radical AN-201 the carrier RC-121, an unconjugated mixture of AN-201 and the carrier RC-121 or two doses of AN-238 at 150 nmol/kg given on days 1 and 18. Arrows indicate the time of injection with cytotoxic agents; (b) changes in body weights in athymic nude mice bearing H-157 non-SCLC tumours after treatment with a single dose at 200 nmol/kg of cytotoxic SST analogue AN-238 and cytotoxic radical AN-201 or two doses of AN-238 at 150 nmol/kg given on days 1 and 18. $*P < 0.05$, $**P < 0.01$ versus control.

Table 3
Effect of cytotoxic SST analogue AN-238 on various histopathological parameters of H-157 non-SCLC tumour cells in athymic nude mice

	% Area of necrosis	Apoptotic index	Ratio of apoptotic to mitotic indices	AgNOR numbers
Control	40±16	6.0±2	0.34±0.17	6.0±0.1
AN-238 (200 nmol/kg)	79±6*	4.0±1	0.27±0.04	5.25±0.05*
AN-238 (150 nmol/kg) days 1 and 18	53±10	16±2*	1.83±0.43*	4.75±0.05**

AgNOR, argyrophilic NOR.
*$P < 0.05$, **$P < 0.01$.

($P < 0.05$) enhanced in the tumours from mice injected twice with AN-238 at 150 nmol/kg (Table 3) as compared to the controls. The ratio of apoptotic to mitotic indices was also increased significantly ($P < 0.05$) in the group treated twice with AN-238 compared with controls. A significant reduction in the AgNOR numbers was also found in the tumours of mice treated with AN-238 at 200 nmol/kg ($P < 0.05$) and in the group given AN-238 twice at 150 nmol/kg ($P < 0.01$) compared with controls (Table 3).

3.3. SST receptor assays

In membranes of four out of five H-157 tumours tested from the control group of Experiment 2, radioreceptor analysis revealed a single class of high affinity binding sites for RC-160 with a mean dissociation constant (K_d) of 8.66±1.55 nM and a mean maximal binding capacity (B_{max}) of 574±49.8 fmol/mg membrane protein. In contrast, using complete displacement analyses with either [^{125}I]-labelled RC-160 or [^{125}I-Tyr^{11}]SST-14 as radioligands, we could not detect the presence of binding sites for SST in the cell membrane fraction of cultured H-157 non-SCLC cells.

3.4. Expression of mRNA for SSTR-2 and SSTR-5 in H-69 SCLC and H-157 non-SCLC cell lines

Using gene-specific primers, we investigated the expression of mRNA for *hSSTR-2* and *hSSTR-5* in H-69 SCLC and H-157 non-SCLC cell lines. RT-PCR analyses revealed a strong expression of mRNA for *hSSTR-2* and a weaker expression for *hSSTR-5* in H-69 SCLC cells cultured *in vitro* (Fig. 3). In H-69 SCLC xenografts, the expression of mRNA for *hSSTR-2* was detected in five of six samples and that for *hSSTR-5* in one of six tumour specimens. The expression of mRNA for *hSSTR-2* or *hSSTR-5* was not detected in H-157 non-SCLC cells either cultured *in vitro* or xenografted into nude mice (Fig. 3).

To identify a RT-PCR product that can explain the binding of RC-160 to H-157 non-SCLC tumours, the expression of mRNA for *hSSTR-3* and *mSSTR-2* and *mSSTR-5* was also investigated. Neither the cells nor the xenografts contained mRNA for hSSTR-3 (data not shown). As expected, no expression of mRNA for mouse *β-actin*, *mSSTR-2* and *mSSTR-5* was found in cultured human H-157 cells (data not shown; Fig. 4). In H-157 non-SCLC xenografts, a strong expression of mRNA for *mSSTR-2* was detected in six of six tumour specimens (Fig. 4). The expression of mRNA for *mSSTR-5* was only weakly detectable in the same samples (data not shown). Nude mouse brain cortex was used as a positive control for the RT-PCR analyses (Fig. 4.).

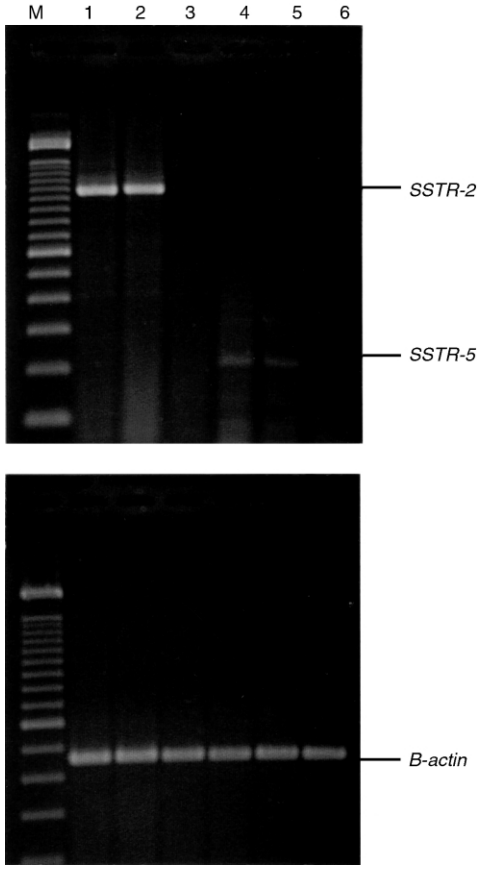

Fig. 3. Representative electrophoresis of reverse transcriptase-polymerase chain reaction (RT-PCR) products showing the expression of mRNA for *SSTR-2* (upper panel, lanes 1–3), *SSTR-5* (upper panel, lanes 4–6) and *B-actin* (lower panel) in H-69 SCLC and H-157 non-SCLC cell lines. Lanes 1 and 4, positive controls; lanes 2 and 5, H-69 SCLC; lanes 3 and 6, H-157 non-SCLC. M, DNA size marker.

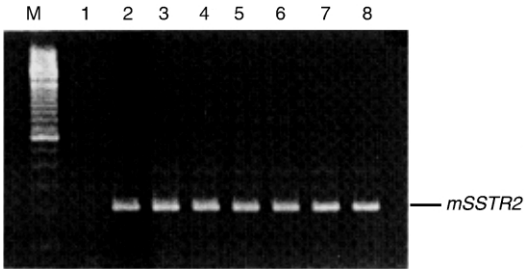

Fig. 4. Electrophoresis of reverse transcriptase-polymerase chain reaction (RT-PCR) products showing the expression of mRNA for the mouse (*m*)*SSTR-2* 217bp product in H-157 xenografts (lanes 3–8), and H-157 cells cultured *in vitro* (lane 1). Lane 2, mouse brain cortex (positive control); M, DNA size marker.

4. Discussion

Targeted chemotherapy represents a modern approach in the treatment of various cancers, which should enhance the efficacy of the cytotoxic antineoplastic agents, decrease the toxicity and likely allow a dose escalation. Recently, we developed a cytotoxic analogue of SST, AN-238 containing 2-pyrrolino-DOX (AN-201), a potent derivative of doxorubicin. When AN-238 was tested in experimental models of brain, prostate, renal and breast cancers, it induced a greater inhibition of tumour growth and showed a lower toxicity than AN-201 [3,7–11]. The receptor-specific action of AN-238 was also demonstrated by the findings that pre-treatment with high doses of SST octapeptides can block its effects [10,29]. In the present study, we showed that the cytotoxic SST analogue AN-238 inhibits significantly the growth of H-69 SCLC and H-157 non-SCLC xenografted into nude mice, while the cytotoxic radical AN-201 was more toxic and produced only a non-significant tumour inhibition. In both experiments, multiple injections of AN-238 at 150 nmol/kg, were well tolerated by the animals and produced a strong tumour growth inhibition. In H-157 non-SCLC tumours in particular, tumour regression was observed after the second injection of AN-238 at 150 nmol/kg, on day 18. Histological analysis showed that the AgNOR numbers significantly decreased in tumours of mice treated with AN-238. NORs are sites of genes that encode for ribosomal RNA and are associated with argyrophylic non-histone acidic proteins that can be detected by the AgNOR method. AgNOR numbers correlate with cell proliferation rate. The decrease in the AgNOR numbers in the tumours of mice treated with AN-238 is consistent with the inhibition of tumour growth. Histological examination of H-157 tumours revealed that AN-238 injected once at 200 nmol/kg resulted in about a 2-fold increase in the necrotic areas, while two injections of AN-238 at 150 nmol/kg induced apoptosis. This difference is probably due to the variation in the dosages used. It is well known that cytotoxic agents, when administered at low concentrations induce apoptosis, while at higher concentrations they can cause necrotic changes [30].

A significantly better growth inhibition of H-69 SCLC tumours by targeted cytotoxic SST analogue AN-238, compared with cytotoxic radical AN-201, can be explained by the presence of receptors for SST. It has previously been shown by us and other groups that H-69 xenografts, in accord with the majority of primary SCLC, express binding sites for SST [12–16]. It has been also demonstrated that H-69 tumours xenografted into nude mice accumulate radiolabelled SST analogues, used for imaging SST receptor-positive cancers [31]. Our work indicates that AN-238 can be targeted to H-69 cells that express SST receptors, producing significant inhibition of tumour growth. The prevalent subtype of SSTR, identified by us in H-69 SCLC, was *SSTR-2*. A weaker expression of *SSTR-5* was also detected in H-69 SCLC cells cultured *in vitro*, but was present in only one of six xenografts.

In contrast to H-69 SCLC, in our earlier investigation we could not detect binding sites for SST in membrane fractions of H-157 non-SCLC xenografted into nude mice [16]. However, when in the present study, we tested the binding of SST analogue RC-160 to the membrane fraction of H-157 xenografts, the receptor analysis revealed that four of five tumours examined showed high affinity SST binding sites. Interestingly, neither RC-160 nor [Tyr^{11}]SST-14 displayed specific binding to membranes of cultured H-157 cells. The expression of SST receptors by non-SCLC remains controversial. While non-SCLC are described by many investigators as SST receptor- negative cancers [12,13,32,33], Eden and Taylor [34] detected mRNA for SST receptor subtype 2 in two non-SCLC cell lines. Adding to the complexity of the issue, Siegfried and colleagues [35] recently showed that under certain conditions, non-SCLC may acquire characteristics, such as the expression of gastrin releasing peptide (GRP), a hormone associated with the growth of various tumours including SCLC. Nevertheless, the absence of SST receptors in one of five H-157 tumours tested in our study suggests a heterogeneity regarding the expression of SST receptors on H-157 non-SCLC. In the present study, we demonstrated that mRNA for *hSSTR-2*, *hSSTR-3* and *hSSRT-5* were absent in the H-157 cells and tumour xenografts.

In addition to the possible direct action of AN-238 on the H-157 cells, a different mechanism may also account for the very strong antitumour activity of AN-238 in this model. It has been demonstrated that in spite of the lack of SST-analogue binding to non-SCLC cells, imaging of tumours in patients with non-SCLC can be performed successfully in the vast majority of the cases [17,18]. This was explained in part by the expression of SST receptors in peritumoral veins [19] causing accu-

mulation of the radioligand around the tumours. Recently, Albini and colleagues [36] reported that SST strongly inhibited the growth of a Kaposi's sarcoma xenograft in nude mice, although expression of SST receptors by the tumour cells *in vitro* was not detected. These investigators showed that SST inhibited endothelial cell growth *in vitro* and suppressed angiogenesis *in vivo*. They also showed that both endothelial cells and monocytes express receptors for SST [36]. Based on these observations, the strong antitumour effect of cytotoxic SST analogue AN-238 on H-157 non-SCLC reported here, could also be due to targeting of AN-238 to tumoral and/or peritumoral veins, which could inhibit neovascularisation and induce apoptosis in tumours. Indeed, about a 3-fold induction of the apoptotic index was found in the tumours of mice injected twice with AN-238 at 150 nmol/kg. A recent demonstration of very efficacious treatment of large MDA-MB-435 breast cancer xenografts by peptide-doxorubicin conjugates directed to the tumour vasculature also supports this view [37]. To investigate whether host cells such as those of tumour vasculature could be the target for AN-238 in H-157 xenografts, we tested tumour samples for the expression of mRNA for *mSSTR-2* and *mSSTR-5* and found a strong band for the former, and a weaker band for the latter. As expected, these two signals and mRNA for the mouse *β-actin* were absent in cultured H-157 cells. This indicates that the cells were not contaminated with mouse tumour cells and that the mouse mRNAs detected by us in the xenografts originated from the host, possibly from the vasculature. In conclusion, although non-SCLC tumours may not express SSTR, under a clinical setting AN-238 could be targeted to SSTR in tumoral and peritumoral veins, as indicated above.

Although the rate of mortality was much higher in animals receiving AN-201, than in mice treated with AN-238, the initial loss in body weights in both groups (Figs. 1b and 2b) indicates a similar toxicity pattern for the two drugs. This is in line with our previous results obtained in nude mice [3]. However, Copenhagen rats bearing Dunning AT-1 rat prostatic carcinoma can tolerate a dose of AN-238 about 3 times higher than that of AN-201 [7]. This can be explained by interspecies differences in serum carboxyesterase (EC.3.1.1.1) activity. These enzymes are responsible for partial hydrolysis of the conjugate in blood, and the release of AN-201 before the targeting is completed. In a most recent study [29], we also demonstrated that by suppressing the carboxylesterase activity in nude mice, about twice the maximum tolerated dose of AN-238, containing AN-201 could be administered without compromising the animals. The stability of the ester bond, which couples the cytotoxic radical to the peptide carrier, in the serum of nude mice with suppressed esterase activity was still significantly lower than in human serum (half life = 70 min versus 120 min, respectively) [29]. This suggests that an even better targeting of AN-238 could be achieved in patients. Our previous results in experimental models demonstrated that a longer half-life of the conjugate allows the administration of higher doses, which is most important, considering that the antitumour effect of AN-238 is dose-dependent [3,7,10].

Collectively, this study shows that the cytotoxic SST analogue AN-238, consisting of 2-pyrrolino-doxorubicin, a superactive derivative of doxorubicin linked to the SST octapeptide analogue RC-121, can be targeted to SCLC and non-SCLC tumours producing potent inhibition of tumour growth. Our results suggest that targeted chemotherapy with cytotoxic SST analogues such as AN-238 could be a promising new modality for the treatment of lung cancer.

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