

Regression of U-87 MG Human Glioblastomas in Nude Mice after Treatment with a Cytotoxic Somatostatin Analog AN-238¹

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ABSTRACT

Receptors for somatostatin (SST) found on brain tumors could be used for targeting of chemotherapeutic agents. This study was conducted to investigate the effects of targeted cytotoxic SST analogue AN-238, consisting of 2-pyrrolinodoxorubicin (AN-201), a potent derivative of doxorubicin (DOX) linked to somatostatin analogue RC-121, on the growth of SST receptor-positive U-87 MG human glioblastomas. Nude mice bearing U-87 MG xenografts received i.v. saline or equimolar doses of AN-238 or AN-201 (150 nmol/kg). Experiments also included groups that were administered RC-121 prior to the injection of AN-238, and groups injected with AN-162, a cytotoxic SST analogue similar to AN-238 but containing DOX instead of AN-201. Tumor volume, weight, and burden were determined. The effect of AN-238 and AN-201 on the survival time of nude mice bearing orthotopically implanted U-87 MG tumors was also evaluated. The binding of AN-238 to U-87 MG tumors was determined by radioreceptor assay and SST receptor (SSTR) subtype by reverse transcription-PCR. Nineteen days after a single administration of AN-238 the growth of U-87 MG tumors in nude mice was significantly inhibited ($P = 0.00168$), whereas two injections of AN-238 produced the regression of tumors ($P = 0.0046$). AN-201 was toxic and ineffective at the same dose. The antitumor effect of AN-238 could be blocked by pretreatment of the tumor-bearing mice with RC-121. The mean survival time of nude mice inoculated orthotopically with U-87 MG cells into the brain was significantly prolonged by treatment with AN-238 ($P = 0.0099$). AN-162 failed to inhibit significantly the growth of U-87 MG xenografts. High affinity binding sites for SST and

mRNA for SST-2 receptor subtype were detected in U-87 MG tumors. Cytotoxic SST analogue AN-238 can be targeted to SST receptors on U-87 MG human glioblastomas to produce powerful inhibition of growth.

INTRODUCTION

Malignant glioblastomas represent the most common type of primary brain tumor in adults and are considered incurable (1, 2). Therapeutic modalities for malignant gliomas, including surgery, radiation, and chemotherapy, are of limited effectiveness, and novel treatment modalities must be explored. A direct targeting of cytotoxic agents to cancer cells represents a modern approach to the treatment of malignant gliomas and other cancers, because it should improve tumor inhibition and decrease toxicity (3). Analogues of peptide hormones for which high affinity binding sites are present on the surface of tumor cells can be used as carriers for cytotoxic radicals. These radicals are linked covalently to free functional groups on the peptide with the preservation of the binding affinity. It is well established that brain tumors express high levels of somatostatin (SST)³ receptors, especially the SST-receptor (SSTR) subtype 2 (SSTR-2) (4–8). Expression of SSTRs on blood vessels in and around the tumors has also been demonstrated (6).

Recently we developed a cytotoxic SST analogue AN-238 (9), consisting of the SST carrier octapeptide RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH₂) covalently linked to 2-pyrrolinodoxorubicin (AN-201), a derivative of doxorubicin (DOX), which is 500–1000 times more potent *in vitro* than the parent compound (10). This analogue fully retains the cytotoxic activity of the radical and the receptor binding affinity of the peptide carrier (9) and has been previously shown to inhibit significantly the growth of various tumors that express SST receptors (11–13).

In this study, we evaluated the antiproliferative effects of targeted cytotoxic SST analogue AN-238 *in vitro* and *in vivo* in mice bearing s.c. xenografts of human malignant glioblastoma cell line U-87 MG, which expresses high affinity binding sites for SST (14). In addition, we studied the effect of AN-238 on the survival time of mice with U-87 MG cells inoculated orthotopically into the brain. A comparison of the antiproliferative activity of AN-238 and AN-162, a targeted SST analogue containing DOX, was also performed in nude mice bearing s.c. xenografts of U-87 MG human glioblastomas.

MATERIALS AND METHODS

Peptides and Cytotoxic Agents. The SST analogue RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH₂) and AN-201

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³The abbreviations used are: SST, somatostatin; SSTR, SST-receptor; SSTR-2, subtype 2; DOX, doxorubicin; AN-201, 2-pyrrolinodoxorubicin.

were synthesized in our laboratory as described (10, 15). The cytotoxic conjugates AN-162 and AN-238 were made by coupling *N*-(9-fluorenyl)methoxycarbonyldoxorubicin-14-*O*-hemiglutarate or AN-201-14-*O*-hemiglutarate, respectively, to the NH₂ terminus of [Lys-(*N*-(9-fluorenyl)methoxycarbonyl)⁵]RC-121, followed by deprotection and purification (9). Before the 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) to the final volume.

Cell Culture and Cytotoxicity *in Vitro*. The human malignant glioblastoma cell line U-87 MG was obtained from American Type Culture Collection (Manassas, VA) and cultured as described previously (14). 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). The cytotoxic activity of the analogues was determined *in vitro* using a colorimetric assay based on quantitation of biomass by staining cells with crystal violet (16). IC₅₀ values, defined as the drug concentration that inhibited cell growth by 50% compared with untreated control cultures, were calculated from three independent experiments, consisting of seven replicate tests each, after exposure of U-87 MG cells to cytotoxic agents for 116 h.

Animals. Male athymic (NCR nu/nu) nude mice, 5–6 weeks old, were obtained from the National Cancer Institute (Bethesda, MD). 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 accord with institutional ethical guidelines.

Experimental Protocol. U-87 MG malignant glioblastomas growing exponentially were implanted into five male nude mice by s.c. injection of 5×10^6 cells in the right flank. Tumors resulting after 2 weeks in donor animals were aseptically dissected and mechanically minced. Pieces of tumor tissue (3 mm³) were transplanted (s.c.) by trocar needle into 100 mice under methoxyflurane (Metofane, Pittman-Moore, Mundelein, IL) anesthesia. The take rate was 100%. In the first part of the experiment we used tumors that had grown to approximately 400–500 mm³. Mice were divided into five groups, of five to eight 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 150 nmol/kg; group 3, unconjugated mixture of the cytotoxic radical AN-201 and the carrier RC-121 at a dose of 150 nmol/kg; group 4, cytotoxic analogue AN-238 at a dose of 150 nmol/kg; group 5, carrier peptide RC-121 at a dose of 150 nmol/kg. For the second part of the experiment, we selected 19 mice bearing large tumors of approximately 900 mm³. The mice were divided into two experimental groups of 9 and 10 animals each and received within 10 days two sequential i.v. injections of AN-201 or AN-238, respectively, at 150 nmol/kg. In the third part of the experiment, mice bearing U-87 MG xenografts, measuring approximately 450 mm³, were divided into two experimental groups and pretreated with RC-121 before the injection of AN-238 as follows: group 1 ($n = 10$ mice), RC-121 s.c. at 50 μg/day per animal for 7 days prior to the i.v. injection of AN-238 at 150 nmol/kg; group 2 ($n = 5$ mice), RC-121 as a

single s.c. injection of 500 μg per animal, 4 h before the administration of AN-238 at 150 nmol/kg. Three mice were sacrificed after treatment with RC-121 for 7 days for receptor studies. Another experiment was performed in two groups of four mice each, bearing U-87 MG xenografts measuring 500–600 mm³. The first group received an i.v. injection of AN-238 at 150 nmol/kg, and the second group received AN-162 at 13.75 μmol/kg. Eight days after the injection, the mice treated with AN-238 were sacrificed, while the mice treated with AN-162 were anesthetized and their tumors were partially removed. Twelve days later, when their tumors reached a volume of approximately 500 mm³, mice were divided into two groups of two mice each, and each group received AN-238 at 150 nmol/kg or AN-162 at 13.75 μmol/kg.

During all experiments, tumor volume was measured twice a week using microcallipers (14), and changes in body weights and deaths related to toxicity were recorded. At the end of the experiments, autopsy was performed and the tumor weights were measured. Samples of each tumor were either fixed in formalin for histological examination or snap-frozen at –70°C for preparation of membranes for receptor assay and for extraction of RNA for reverse transcription-PCR.

Survival Test. U-87 MG cells were inoculated into the brains of 33 six-week old athymic male NCR nu/nu nude mice as described previously (14). Briefly, mice were anesthetized with methoxyflurane (Metofane, Pittman-Moore, Mundelein, IL). A midline incision was made over the anterior aspect of the cranium and the scalp retracted to the right. A guarded 26-gauge needle was used to drill a hole 3–4 mm deep in the skull, 3 mm to the right of the midline, just anterior to the coronal suture. Using a syringe (Hamilton, Reno, NV) 15 μl of 0.9% sterile sodium chloride containing 5×10^4 U-87 MG glioblastoma cells was injected and the scalp was closed with surgical skin staples. Two days after the inoculation of the cells, mice were randomly divided into three experimental groups of 11 mice each and received the following treatment as a single i.v. injection: group 1, control, vehicle solution (5% mannitol); group 2, cytotoxic radical AN-201 at a dose of 150 nmol/kg; group 3, AN-238 at a dose of 150 nmol/kg.

Histological Methods. Tumor samples were processed as described previously (17). The extent of necrosis was evaluated with the point-counting method on tumor slides stained with H&E. 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 H&E-stained slides.

Receptor Assay. Binding characteristics of SSTRs on membrane fractions of U-87 MG tumors were determined by ligand competition assays using ¹²⁵I-labeled RC-160 as reported previously (9). The LIGAND PC computerized curve-fitting program (18) was used to determine the type of receptor binding, the dissociation constant (K_d), and the maximal binding capacity (B_{max}) of receptors (18). Receptor binding affinity of cytotoxic SST analogue AN-238 as well as its carrier peptide RC-121 to tumor membranes was measured in displacement experiments based on competitive inhibition of ¹²⁵I-RC-160 binding using various concentrations of AN-238 and RC-121 (10^{-6} – 10^{-12} M). IC₅₀, defined as the dose causing 50% inhibi-

Table 1 Effect of cytotoxic SST analogue AN-238, cytotoxic radical AN-201, carrier peptide RC-121, and mixture of RC-121 and AN-201 on tumor volume, tumor weight, and animal mortality in athymic nude mice bearing U-87MG human glioblastomas

Treatment	Tumor volume (mm ³)		Tumor burden ^a	Mortality ^b
	Initial	Final		
Control	516 ± 172	4782 ± 1012	117.5 ± 20.3	0/5
AN-201	447 ± 214	3133 ± 1256	94.2 ± 40.5	1/6
RC-121 + AN-201	466.8 ± 113	5200 ± 713	148.1 ± 20.9	1/5
AN-238	535.2 ± 190	867.1 ± 313.3	23.5 ± 8.1	0/6
RC-121	593.2 ± 255	3769 ± 626 ^c		
RC-121 (chronically) ^d + AN-238	455 ± 177.3	596 ± 206	20.9 ± 6	0/7
RC-121 (acute) ^e + AN-238	413.8 ± 155	3054.9 ± 1097	92.6 ± 30.3	0/5
AN-238 (days 0, 11)	890.7 ± 378.1	637.5 ± 248.3	26.2 ± 10.9	1/9
AN-201 (days 0, 11)	941.1 ± 304.9	3274.2 ± 794.9	112.9 ± 25.7	3/10

^a Tumor weight (mg)/body weight (g).

^b Number of dead animals/number of total animals.

^c On day 15, the tumor volume of 3 of 4 animals exceeded 3500 mm³, and the animals were sacrificed. Thus, the tumor volume on day 15 is shown and no data are available on tumor burden.

^d Mice were treated daily for 7 days with RC-121 (50 µg/day per animal s.c.) prior to the administration of AN-238 at 150 nmol/kg.

^e Mice received RC-121 at 500 µg s.c. 4 h before the administration of AN-238 at 150 nmol/kg.

tion of ¹²⁵I-labeled RC-160 binding, was calculated by a computerized curve-fitting program (19).

RNA Extraction and Reverse Transcription-PCR. Total RNA was isolated using the RNazol B reagent (TEL-TEST, Inc., Friendswood, TX) following the manufacturer's instructions. The quantity and the quality of the RNA was assessed by spectrophotometry at 260 and 280 nm. For the reverse transcription 1 µg of total RNA was added in a test tube containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM of each deoxyribonucleoside triphosphate, 2.5 µM random hexamers, 1 unit of RNase inhibitor and double distilled H₂O in a final volume of 19 µl. Following heating for 10 min at 65°C and quenching on ice, 2.5 units of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Norwalk, CT) was added and the reaction mixture was incubated for 10 min at room temperature following an incubation at 42°C for 1 h. The reaction was terminated by heating at 95°C for 5 min and quenching on ice. Subsequently, cDNA was amplified by the PCR as follows: 1 µl of the cDNA was amplified in a 50-µl solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each deoxynucleotide triphosphate, 2.5 units of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) and 0.4 mM of each primer. The primers used were 5'-TCCTCT-GACTTCAACAGCGACACC-3' and 5'-TCTCTCTTCTCT-TGTGCTCTTGG-3' for human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), 5-ATGGACATGGCGGATGAGC-CACT-3' and 5'-TACTGGTTTGGAGGTCTCCATTGA-3 for SSTR-2, and 5'-CGTCTTCATCATCTACACGG-3' and 5'-GGCCAGGTTGACGATGTTGA-3 for SST receptor subtype-5. Samples were denatured at 94°C for 5 min and then subjected to 40 cycles of 94°C for 1 min (denaturation), 62°C for 1 min (annealing), and 72°C for 1 min (extension) for SSTR-2; 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for SSTR-5; or 25 cycles of 94°C for 35 s, 60°C for 40 s, and 72°C for 40 s for hGAPDH. This was followed by a final extension at 72°C for 5 min, using a DNA thermal cycler (Perkin-Elmer model 2400). The number of cycles was determined in preliminary experiments to be within the exponential range of PCR amplification. Five µl of each PCR product was

electrophoresed in a 8% polyacrylamide gel and stained with silver.

Statistical Analysis. The data are expressed as the mean ± SE. Statistical evaluation of data were performed by Student's *t* test (two-tailed). Differences were considered statistically significant when *P* ≤ 0.05. SigmaPlot computer software (Jandel, San Rafael, CA) was used for preparation of figures.

RESULTS

Tumor Inhibition and Toxicity. The first part of the experiment was designed to compare the antitumor effects and toxicity of single i.v. injections of cytotoxic radical AN-201, targeted cytotoxic SST analogue AN-238, carrier peptide RC-121, and the unconjugated mixture of RC-121 and AN-201 in nude mice bearing xenografts of U-87 MG glioblastomas. The results are summarized in Table 1. Administration of a single i.v. injection of AN-238 at 150 nmol/kg produced a significant tumor growth inhibition. Nineteen days after the injection, the tumor volume in animals treated with AN-238 was significantly (*P* = 0.00168) reduced to 867 ± 313 mm³ as compared with the control group, which measured 4782 ± 1012 mm³; this corresponded to a growth inhibition of 82% (Fig. 1A). In contrast, tumors in mice treated with an equimolar dose of AN-201 given alone and as an unconjugated mixture with RC-121 grew steadily and measured 3133 ± 1256 mm³ and 5200 ± 713 mm³, respectively, 19 days after the injection. Treatment with AN-201 alone or together with RC-121 also resulted in the death of 1 of 6 and 1 of 5 animals, respectively (Table 1). The carrier peptide RC-121 was not toxic, but had no tumor inhibitory effects after a single i.v. injection of 150 nmol/kg. 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 (Fig. 2).

In the same experiment, selected mice bearing very large U-87 MG tumors measuring approximately 900 mm³ received two sequential injections of AN-238 or AN-201 at a dose of 150 nmol/kg each, administered at an interval of 10 days. As shown in Table 1 and Fig. 1B, the final tumor volume in the mice

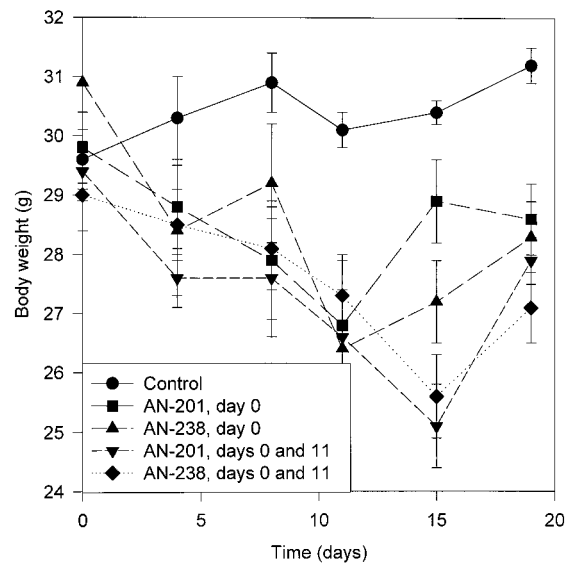
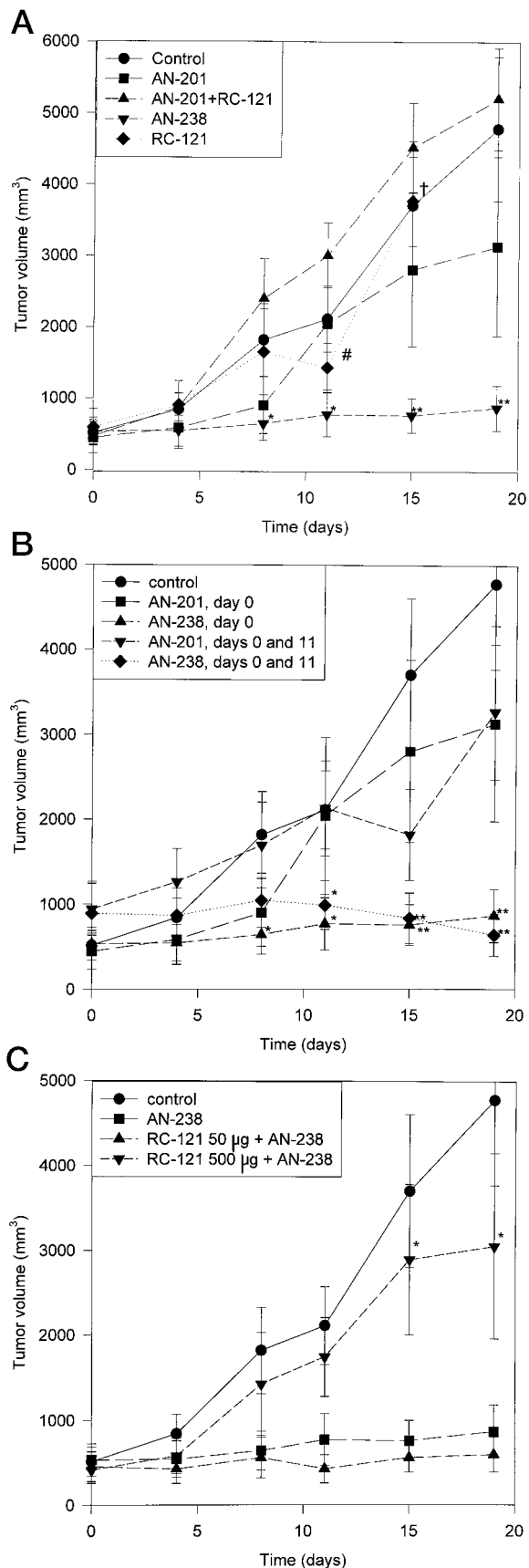


Fig. 2 Changes in body weights of nude mice bearing U-87 MG human glioblastoma and treated with 1 or 2 i.v. injections of AN-238 or AN-201 at 150 nmol/kg.

treated twice with AN-238 regressed to $637.5 \pm 248.3 \text{ mm}^3$ ($P = 0.00046$) from an initial tumor volume of $890.7 \pm 378 \text{ mm}^3$, corresponding to a decrease of 30%. The U-87 MG tumors in animals treated with cytotoxic radical AN-201 grew steadily even after the administration of the second injection, reaching $3274 \pm 795 \text{ mm}^3$ 19 days after the first injection of the cytotoxic radical. The tumor volume in this group was 510% higher than that in mice treated with an equimolar dose of AN-238. AN-201 was also more toxic than AN-238. Three of 10 animals died in the group that received AN-201, but only 1 of 9 animals was dead after treatment with AN-238. Significant losses of body weights were recorded in all animals, but the surviving animals started to recuperate before the end of the experiment (Fig. 2).

Effect of Pretreatment with RC-121 on the Antitumor Activity of AN-238. In the third part of this experiment, mice bearing approximately 450-mm^3 xenografts of U-87 MG gli-

Fig. 1 Effect of treatment with cytotoxic SST analogue AN-238 on the growth of U-87 MG human glioblastomas xenografted into nude mice. A, changes in the tumor volume in nude mice after treatment with a single i.v. injection of cytotoxic SST analogue AN-238, cytotoxic radical AN-201, unconjugated mixture of AN-201 and the carrier peptide RC-121, or the carrier peptide RC-121 at 150 nmol/kg doses. #, the sharp decrease in the average tumor volume on day 11 in the RC-121-treated group was due to an animal bearing a tumor that became necrotic and subsequently disintegrated. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control †. On day 15, the tumor volume of 3 of 4 animals exceeded $3,500 \text{ mm}^3$ and the animals were sacrificed. Thus, the tumor volume on day 15 is shown. B, changes in the tumor volume in nude mice treated with 1 or 2 i.v. injections of AN-238 or AN-201 at 150 nmol/kg. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control. C, effect of pretreatment with RC-121 administered s.c. at $50 \mu\text{g/day}$ per animal for 7 days or at $500 \mu\text{g/animal}$ 4 h before the administration of AN-238 at 150 nmol/kg. Vertical bars, SEM. *, $P = 0.051$ versus the AN-238-treated group and not significant versus control.

Table 2 Effect of treatment with cytotoxic SST analogue AN-238^a and cytotoxic radical AN-201^a on the percent area of necrosis of U-87MG glioblastomas transplanted into nude mice

Treatment	% area of necrosis
Control	38 ± 13
AN-238 (day 0)	44 ± 7
AN-201 (day 0)	31 ± 10
AN-238 (days 0 and 11)	63 ± 8
AN-201 (days 0 and 11)	34 ± 8
RC-121 (500 µg) s.c. 4 h before AN-238	43 ± 4
RC-121 (50 µg/day) s.c. for 7 days before AN-238	77 ± 11 ^b

^a Cytotoxic SST analogue AN-238 and cytotoxic radical AN-201 were injected i.v. at 150 nmol/kg once (day 0) or twice (days 0 and 11).

^b $P = 0.041$.

blastomas were pretreated s.c. with the carrier peptide SST analogue RC-121, before a single i.v. administration of AN-238 at 150 nmol/kg. RC-121 was given at a dose of 50 µg/day per animal for 7 days or at 500 µg per animal 4 h before the injection with AN-238. The experiment in which RC-121 was administered chronically at a low dose was performed to ascertain whether pretreatment with RC-121 could produce a higher tumor inhibition than AN-238 alone, by a possible up-regulation of the SST receptors on the U-87 MG tumors. Acute administration of RC-121 at a high dose was intended to produce a blockade of the SST receptors on the tumor cells that could decrease or abolish the antitumor effect of AN-238.

As shown in Table 1 and Fig. 1C, pretreatment with RC-121 for 7 days followed by a single i.v. injection of AN-238 at 150 nmol/kg did not produce a higher tumor inhibition than administration of AN-238 without pretreatment with RC-121. However, the necrotic area of tumors from pretreated animals was increased by 102% as compared with the tumors from the untreated control mice ($P = 0.041$), whereas the area of necrosis in the tumors from mice that received AN-238 alone was only 16% higher (not significant) than that of the controls (Table 2). SST analogue carrier peptide RC-121, administered s.c. at a dose of 500 µg 4 h before the injection with AN-238 to nude mice bearing xenografts of U-87 MG glioblastomas, blocked the tumor inhibitory effects of the cytotoxic SST analogue AN-238 the resulting inhibition being not significant *versus* control (Fig. 1C). The inhibition of tumor growth produced by AN-238 alone was 82%, while administration of a high dose of RC-121 before the injection with AN-238 reduced the tumor inhibition to 37% ($P = 0.051$ *versus* AN-238-treated group). This tumor inhibition was similar to that obtained with the cytotoxic radical alone, being 32% *versus* the controls (Table 1).

Comparison of the Antitumor Activities of AN-238 and AN-162. This experiment was designed to compare the antiproliferative activity of AN-238 with that of AN-162, a cytotoxic SST analogue containing DOX. Nude mice bearing s.c. xenografts of U-87 MG glioblastomas were given a single i.v. injection of AN-162 at 13.75 µmol/kg or AN-238 at 150 nmol/kg. According to our previous experience with AN-162, a single dose of 13.75 µmol/kg represents the maximum tolerated dose, which is equivalent to 8 mg/kg of DOX. Fig. 3 shows that 8 days after the treatment with AN-162 tumors grew from 566 ± 72

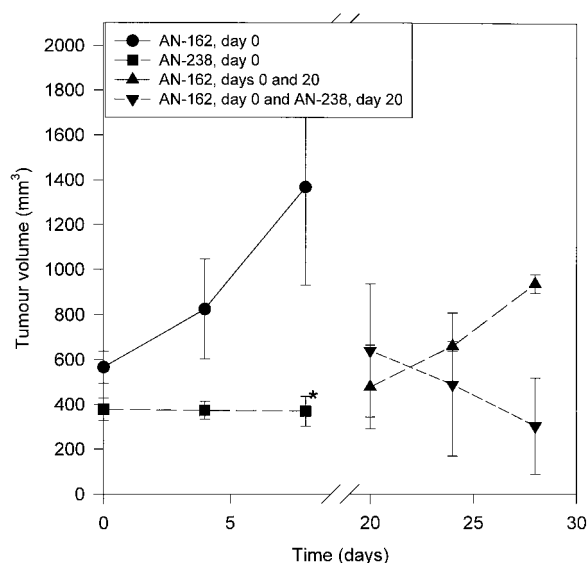


Fig. 3 Changes in tumor volume in athymic nude mice bearing s.c. xenografts of U-87 MG malignant glioblastomas after treatment with 150 nmol/kg of AN-238 or 13.75 µmol/kg of AN-162. Vertical bars, SEM. *, $P = 0.0281$. On day 8, tumors from the AN-162-treated mice were partially removed, and when new tumors on day 20 reached a volume of approximately 500 mm³, mice were divided into two experimental groups and treated with AN-162 or AN-238.

mm³ to 1368 ± 439 mm³, while AN-238 produced a significantly stronger antitumor effect ($P = 0.0281$), virtually arresting tumor growth. The loss of body weights caused by AN-162 was slightly higher than that caused by AN-238, being 13% (3.6 g) *versus* 8.5% (2.5 g), respectively. This implies that administration of a higher dose of AN-162 could have been lethal.

To investigate whether AN-238 could inhibit the growth of tumors in mice that were already treated unsuccessfully with AN-162, U-87 MG tumors from mice treated with AN-162 were partially removed surgically, but small pieces representing about 10% of the tumors were left and allowed to grow. Twelve days later, when tumors reached a volume of approximately 500 mm³, mice were divided into two groups, which received AN-238 at 150 nmol/kg or AN-162 at 13.75 µmol/kg. As shown in Fig. 3, the tumors from mice treated with AN-162 continued to grow, while the tumors from mice treated with AN-238 regressed by 50% within 8 days.

Effect of AN-238 on the Survival Time of Mice Bearing Orthotopically Implanted U-87 MG Cells. The life span of mice inoculated orthotopically with 5×10^4 U-87 MG cells was prolonged significantly by a single administration of cytotoxic SST analogue AN-238, as compared to untreated mice. The mean survival times of mice in the control group, and groups treated with AN-238 or AN-201 at 150 nmol/kg were 23.8 ± 2 , 33.7 ± 3.1 , and 29.4 ± 2.5 days, respectively (Fig. 4). Thus, treatment with AN-238 increased the mean survival time of mice inoculated orthotopically with U-87 MG glioblastomas by 10 days ($P = 0.00987$), which represents an extension in their life span by 42%. Treatment with cytotoxic radical AN-201 at 150 nmol/kg also increased the survival time by 5.5 days *versus* the controls, but this was not significant statistically ($P = 0.0853$).

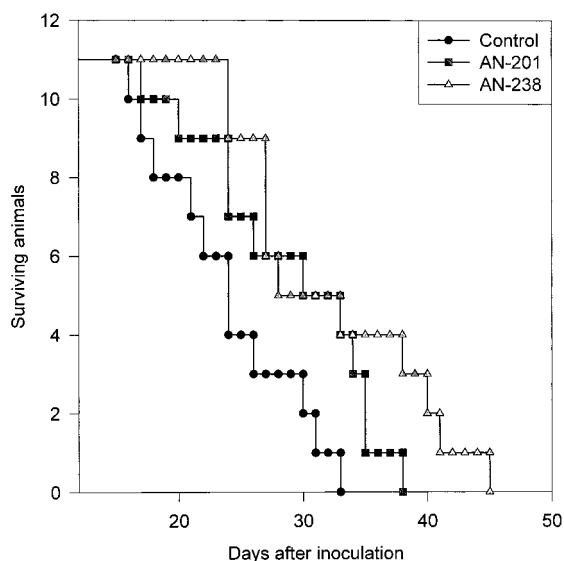


Fig. 4 Effect of systemic treatment with cytotoxic radical AN-201 or cytotoxic SST analogue AN-238 on the survival time of nude mice inoculated orthotopically with U-87 MG glioblastoma cells.

Cytotoxicity in Vitro. Antiproliferative activities of cytotoxic SST analogues AN-238 and AN-162 and the corresponding cytotoxic radicals AN-201 and DOX were evaluated in U-87 MG cells cultured in vitro. The drug concentrations for AN-238 and AN-162 that inhibited cell growth by 50% as compared to control (IC_{50}) were 1.6×10^{-10} M and 2.5×10^{-8} M, respectively. Unconjugated cytotoxic radicals AN-201 and DOX had IC_{50} values similar to their corresponding SST analogues, being 1.7×10^{-10} M and 1.9×10^{-8} M, respectively.

Histological Findings. In some areas, U-87 MG tumors consisted of relatively small elongated cells arranged in bundles. In other areas, the cells were larger and more rounded containing pale nuclei with big nucleoli. As shown in Table 2, all groups that received AN-238 had a larger area of necrosis than the controls. The most extensive necrosis was found in tumors from the mice treated with AN-238, after 1 week of pretreatment with RC-121. The area of necrosis in tumors from mice treated with AN-201 was essentially equal to that of the controls. Mitotic and apoptotic indices, as well as their ratio were not altered by the treatment with cytotoxic SST analogue AN-238 or cytotoxic radical AN-201 (data not shown).

Receptor Binding Studies and Reverse Transcription-PCR in U-87 MG Tumors. Ligand competition assay showed a single class of high affinity binding sites for SST in membranes of U-87 MG glioblastoma xenografts with a mean dissociation constant (K_d) of 9.77 ± 2.17 nM and a mean maximal binding capacity (B_{max}) of 835 ± 130 fmol/mg membrane protein (Fig. 5A). After a single or repeated treatment with AN-238 there were no significant changes in the binding affinities ($K_d = 6.95 \pm 0.95$ and 7.79 ± 2.33 nM, respectively) and the capacities ($B_{max} = 784 \pm 153$ and 715 ± 154 fmol/mg protein, respectively) of the SST receptors. Similarly, K_d and B_{max} values were not affected when AN-238 treatment was performed after pretreating the tumor bearing mice with a high

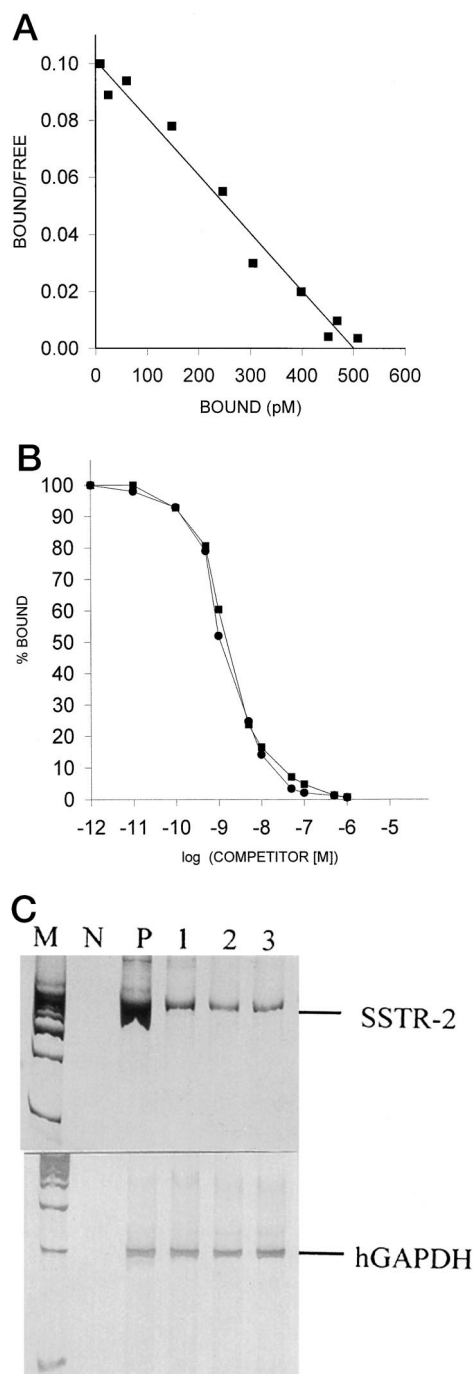


Fig. 5 A, representative example of Scatchard plots of ^{125}I -RC-160 binding to the membrane fraction isolated from untreated U-87 MG tumors. Specific binding was determined as described. Each point represents the mean of triplicate determinations. B, representative displacement of ^{125}I -RC-160 binding to membrane fractions of U-87 MG tumors by increasing concentrations of RC-121 (●) and cytotoxic somatostatin analogue AN-238 (■). 100% specific binding is defined as the difference between binding in the absence and in the presence of 10^{-5} M RC-160. Each point represents the mean of triplicate determinations. C, polyacrylamide gel electrophoresis of reverse-transcribed and subsequently PCR-amplified mRNAs for SSTR-2 and hGAPDH in three representative xenografts of U-87 MG human glioblastomas from untreated nude mice (lanes 1–3); M, molecular weight DNA marker; N, negative control; P, positive control (NCI-H-69 human small cell lung carcinoma).

dose of the carrier peptide RC-121 ($K_d = 10.5 \pm 3.25$ nM; $B_{max} = 930 \pm 146$ fmol/mg protein). In three animals treated with RC-121 alone at a dose of 50 μ g/day for 7 days and then sacrificed, we found no statistically significant changes in the binding affinities ($K_d = 9.85 \pm 2.58$ nM), although the concentration of receptors for SST was slightly, but not significantly higher (1041 ± 97 fmol/mg membrane protein) than in the control group.

To assess the potency of cytotoxic SST analogue AN-238 and its carrier peptide RC-121 to displace radiolabeled RC-160 from receptors for SST on U-87 MG tumors, heterologous displacement experiments were performed. Our results show that the concentration of RC-121 required to inhibit 50% of the specific binding of 125 I-RC-160 (IC_{50}) was 2.83 ± 0.17 nM (Fig. 5B). Cytotoxic SST analogue AN-238 retained the high binding affinity of the peptide hormone portion of the conjugate to membrane receptors for SST in U-87 MG tumors, displaying a similar IC_{50} value of 3.67 ± 0.46 (Fig. 5B).

The expression of SSTR subtype in U-87 MG glioblastomas was then investigated. Using reverse transcription-PCR analysis, mRNA for SSTR subtype 2, but not for SSTR subtype 5, was found in xenografts of U-87 MG glioblastomas from untreated animals (Fig. 5C).

DISCUSSION

Targeted chemotherapy represents a modern approach to the treatment of various human tumors because it should enhance the efficacy, decrease the toxicity, and possibly allow a dose escalation. In view of the findings that high affinity binding sites for SST are expressed in the membranes of various human tumors, we developed a cytotoxic analogue of SST, AN-238 containing the potent derivative of DOX, 2-pyrrolinodoxorubicin, covalently linked to the SST analogue carrier peptide RC-121. In the present study, the antitumor activity of AN-238 was evaluated in nude mice bearing xenografts of human U-87 MG glioblastomas, a rapidly growing brain tumor that expresses receptors for SST (14).

Our results demonstrate that AN-238 can powerfully inhibit the growth of large (approximately 500 mm³) U-87 MG tumors by some 80% without severe toxicity, while the unconjugated cytotoxic radical AN-201 has no significant effect on tumor growth and is more toxic. We also found that a single administration of AN-238 at a nontoxic dose of 150 nmol/kg arrested growth of very large (approximately 900 mm³) U-87 MG xenografts, while a second injection of AN-238 at the same dose resulted in a 30% regression. Nineteen days after the initiation of the therapy, the experiment had to be terminated because the tumor volume in the controls was very high (4782 ± 1012 mm³). Thus, although a third administration of AN-238 would have been worthy of testing, it could not be applied.

It has been observed in U-87 MG tumors that chronic treatment with SST analogue RC-160 somewhat increased the binding capacity of SST receptors (14). To test whether such an increase could enhance the antiproliferative activity of AN-238, SST carrier analogue RC-121 was administered for 7 days at 50 μ g/day per animal prior to the administration of AN-238. The resulting slight, but not significant up-regulation of SST receptors by pretreatment with RC-121 did not further enhance significantly the antitumor activity of AN-238. However, histolog-

ical examination revealed that tumors from the RC-121 pretreated group had significantly ($P = 0.041$) higher content of necrotic cells than did the tumors from the untreated mice or tumors from mice treated only with AN-238.

When RC-121 was injected s.c. at the high dose of 500 μ g per animal, 4 h before the administration of AN-238 at 150 nmol/kg, the antitumor effect of AN-238 was abolished and the resulting tumor inhibition was similar to that of cytotoxic radical AN-201. It is likely that the excess of RC-121 occupied the SST receptors on the tumors, while the unbound cytotoxic SST analogue AN-238 was hydrolyzed by carboxylesterase enzymes (EC 3.1.1.1) in blood releasing unconjugated AN-201. This finding supports strongly the concept that the antitumor effect of AN-238 is receptor-mediated.

Although AN-238 was less toxic than AN-201 in these experiments on the basis of animal mortality, the trends in the loss of body weights in animals treated with AN-238 or AN-201 (Fig. 2) indicate a similar toxicity pattern for the two compounds. This is in agreement with our previous finding that the maximum tolerated doses of both compounds are similar in male nude mice, about 175–200 nmol/kg for single i.v. injections, and are determined by hematopoietic toxicity (12). However, Copenhagen rats bearing R-3327-AT-1 rat Dunning prostatic carcinoma tolerate a single i.v. injection of 300 nmol/kg without apparent toxicity, whereas 115–125 nmol/kg of AN-201 is lethal (11). Because in AN-238 an ester bond is used to link AN-201 to the carrier peptide, different tolerance in these species could be explained by variations in the carboxylesterase enzyme activity. We found that one of our cytotoxic luteinizing hormone-releasing hormone analogues, AN-152, in which DOX is linked to the carrier peptide through the same ester bond, had a half-life of 10 min in nude mouse serum, 30 min in Copenhagen rat serum, and 120 min in human serum at 37°C *in vitro* (12, 20). The longer half-life of the conjugate in the blood may allow a better targeting, and apparently the DNA-intercalating antiproliferative agent AN-201 does not cause side effects that are life-threatening after being absorbed by noncancerous SST receptor-positive tissues. A large difference in the carboxylesterase activities in rodents and human beings is well known (21). Mice can tolerate a much higher dose of organophosphate acetylcholinesterase (EC 3.1.1.7.) inhibitors than primates, because these toxic agents also react with carboxylesterases present at much higher levels in mice, but the inhibition of these enzymes causes no toxicity (21). In accordance with our hypothesis that the extremely high esterase activity is responsible for the toxicity pattern of AN-238 in mice, we found recently that healthy nude mice without tumors can tolerate two consecutive i.v. injections of 400 nmol/kg AN-238 without severe toxicity after treatment with organophosphate esterase inhibitors.⁴ Because of the very low esterase activity in human beings, we can speculate that it might be possible to give even much

⁴ A. Nagy, A. Plonowski, and A. V. Schally. Stability of cytotoxic luteinizing hormone-releasing hormone conjugate (AN-152) containing doxorubicin 14-*O*-hemiglutarate in mouse and human serum *in vitro*: implications for the design of preclinical trials. Proc. Natl. Acad. Sci. USA, 97: 829–834, 2000.

higher doses of AN-238 to humans. This is important because, as we demonstrated previously (11) and also in this study, the effect of AN-238 is dose-dependent.

The antitumor effects of AN-238 were also compared to those of AN-162, its counterpart cytotoxic SST analogue containing DOX instead of the highly potent 2-pyrrolinodoxorubicin. Under our experimental conditions, AN-162 could not inhibit the growth of large U-87 MG tumors, while treatment with AN-238 resulted in the stabilization of the tumor growth. In this experiment both AN-238 and AN-162 were used at equitoxic doses. In fact, the loss of body weights in the AN-162 treated animals was higher than that in the mice treated with AN-238, indicating that administration of a higher dose of AN-162 could be lethal. The striking difference in the antitumor activity of these two cytotoxic SST analogues could be due to differences in the mechanism of action of DOX and AN-201 and/or the limited number of receptors on tumors available for targeting. When these tumors, which have been unsuccessfully treated with AN-162, were subjected to therapy with AN-238, they regressed by 50% within 8 days, demonstrating that the 2-pyrrolinodoxorubicin-containing analogue still had full antiproliferative activity on U-87 MG tumors even after pretreatment of these tumors with the DOX-containing analogue, that could have resulted in the selection of cells resistant to chemotherapy. This is in agreement with our previous findings that AN-201 and AN-238 showed very strong antitumor effect on MX-1 human mammary carcinoma, which is resistant to DOX (13).

The difference in the potency between these cytotoxic agents was also demonstrated by *in vitro* studies in which AN-201 and AN-238 exhibited approximately 150 times lower IC_{50} values than DOX and AN-162, respectively, in this cell line. No difference was found in the antiproliferative activity between the cytotoxic radicals and their corresponding cytotoxic SST analogues *in vitro*. This was probably due to a fast hydrolysis of AN-238 *in vitro*.

An intratumoral injection of anticancer agents has been previously suggested for the treatment of brain tumors (22, 23), whereas recently, the locoregional administration of the SST analogue ^{90}Y -labeled DOTA⁰-D-Phe¹-Tyr³-octreotide was tested successfully in glioma patients (24). We felt that it was still worthwhile to evaluate whether systemic administration of AN-238 could extend the life span of nude mice bearing U-87 MG tumors inoculated orthotopically into the brain. Mice treated with a single nontoxic dose of 150 nmol/kg of AN-238 had their life span extended by 42% ($P = 0.00987$), as compared to that of the untreated mice, suggesting that AN-238 may penetrate to a certain extent the tumor blood-brain barrier when systemically administered. This is in agreement with the results of a previous study showing that somatostatin octapeptide analogue RC-160 (vapeotide, octastatin) could penetrate the weakened blood-brain tumor barrier in KHT sarcomas (25). AN-201 did not significantly affect the survival of mice with orthotopic implants of U-87 MG tumors.

The radiolabeled ligand competition assay confirmed the high affinity binding of cytotoxic SST analogue AN-238 to the membrane fraction of U-87 MG xenografts, which is in agreement with the detection of mRNA for SST receptor subtype 2 in these tumors. Thus U-87 MG glioblastomas can be considered

representative of primary brain tumors that express high levels of SSTRs and especially SSTR-2. The high affinity binding sites for SST analogues were preserved after two injections of AN-238, indicating that repeated treatment by targeted cytotoxic SST analogue could be applied.

Histological analysis showed that the antitumor activity of AN-238 was associated with an induction of necrosis, while the apoptotic index remained unaffected by the treatment. This is likely due to the high cytotoxic activity of this agent, which is at least 90 times more potent *in vivo* and approximately 150 times more potent *in vitro* than AN-162, the corresponding DOX-containing analogue, and may induce necrosis rather than apoptosis (26) in this cell line.

In conclusion, we have shown that cytotoxic SST analogue AN-238, consisting of 2-pyrrolinodoxorubicin, a superactive derivative of DOX, linked to the SST octapeptide analogue RC-121, could be targeted to malignant glioblastomas that express receptors for SST, producing regression of even very large tumors, while 2-pyrrolinodoxorubicin itself is ineffective and more toxic. Additional experiments, involving additional models of glioblastomas and various dosage regimens, are required to establish the full spectrum of antitumor effects of AN-238 and to predict more accurately the subset of primary glioblastomas that might respond to such therapy. Our results suggest that targeted chemotherapy with cytotoxic SST analogues such as AN-238 could be a promising new modality for the management of malignant brain tumors.

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Regression of U-87 MG Human Glioblastomas in Nude Mice after Treatment with a Cytotoxic Somatostatin Analog AN-238

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