Inhibition of PC-3 Human Androgen-independent Prostate Cancer and Its Metastases by Cytotoxic Somatostatin Analogue AN-238

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ABSTRACT

We evaluated whether AN-238, the cytotoxic analogue of somatostatin (SST) consisting of the radical 2-pyrrolinodoxorubicin (AN-201) linked covalently to the SST octapeptide carrier RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH₂), could be used for targeting human primary and metastatic prostate carcinomas that express SST receptors (SSTRs). The antitumor activity and toxicity of AN-238 and its components were first characterized in nude mice bearing s.c. xenografts of PC-3 human androgen-independent prostate cancer. In experiment 1, AN-238 was injected i.v. at 200 nmol/kg when the mean volume of s.c. tumors was about 30 mm³. Administration of AN-238 inhibited tumor growth, as shown by a 74% decrease in tumor volume and by a 71% reduction in tumor weight after 7 weeks as compared with the control group. AN-201 at an equimolar dose did not show any antitumor activity. The mortality was 14.3% (one of seven mice) in the AN-238-treated group and 47% (three of seven mice) in mice that received AN-201. In experiment 2, two i.v. injections of AN-238 at 150 nmol/kg were given 10 days apart when the tumors measured 65–70 mm³. A significant inhibition of tumor volume (62.3%; P < 0.001) and tumor weight (61.1%; P < 0.01) was observed after 4 weeks of treatment. AN-201, given alone at the same dose or coadministered with RC-121, had no significant effect on PC-3 tumors. The suppression of tumor growth induced by AN-238 was accompanied by a significant enhancement of apoptosis (P < 0.01). There were similar side effects in all treated groups, which included a transient loss of body weight and leukopenia. The effectiveness of AN-238 in a metastatic model was then investigated in animals implanted orthotopically with 2 × 10⁶ PC-3 cells. Two i.v. injections of AN-238 or AN-201 at 150 nmol/kg were administered 10 days apart at 10 weeks after intraprostatic inoculation of PC-3 cells. After 4 weeks of treatment, the mean weight of primary tumors in animals receiving AN-238 was 77% lower (P < 0.01) than that in controls. This reduction was also significantly greater (P < 0.05) than that in animals given AN-201, which showed only a 34% inhibition (nonsignificant versus controls). All control animals and four of six (67%) mice treated with AN-201 developed metastases in the lymph nodes; however, no lymphatic spread of cancer was found in the AN-238-treated group. Using reverse transcription-PCR analysis, we demonstrated the expression of SSTR2 and SSTR5 in intraprostatic tumors and their metastases in lymph nodes as well as in s.c. tumors. The present study demonstrates the high efficacy of SSTR-targeted chemotherapy in a model of advanced human androgen-independent prostatic carcinoma, as shown by the inhibition of primary tumors and their metastases by the cytotoxic SST analogue AN-238.

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignant neoplasm among American men and remains the second leading cause of cancer-related deaths (1, 2). In spite of major refinements in diagnosis, a significant number of prostate cancers are only detected at an advanced stage of the disease, with no possibility of cure by radical prostatectomy (2, 3). The principal approach to management of disseminated carcinoma of the prostate has continued to be essentially unchanged since 1941, when the effectiveness of androgen ablation was first reported by Huggins and Hodges (4). Although the rate of response to androgen deprivation is high (80%), the mean duration of the remission is only 18–36 months, and patients eventually die of hormone-refractory disease (5, 6).

Conventional chemotherapy for prostate cancer that is no longer responsive to androgen deprivation was initially found to produce only marginal responses (7) but is now being reevaluated with interest as new agents and better supportive care become available (reviewed in Ref. 8). Nevertheless, palliation still remains the primary goal of current chemotherapy, and overall response rates are low and are associated with general toxicity. To overcome the problem of toxicity, attempts have been made to achieve site-specific drug delivery and improve the therapeutic index. Targeted chemotherapy using potent cytotoxic radionuclide conjugated to carrier molecules, such as antibodies (9, 10) or analogues of peptide hormones (reviewed in 11), that can be specifically recognized by tumor cells may improve therapy of androgen-independent tumors.

Various tumors, including carcinoma of the prostate, express receptors for peptides such as LH-RH and SST (6, 11–14). Preclinical studies have shown that SST and its octapeptide analogues can inhibit the growth of prostate cancer and other malignancies (6, 11, 15–18). SSTRs were found on surgical specimens of primary and metastatic lesions of prostate cancer and visualized in vivo by scintigraphy with ¹¹¹In-labeled octreotide (19, 20). Some clinical improvement was observed in patients with relapsed prostate cancer who were treated with SST analogues (21–24). The successful use of radiolabeled SST analogues for in vivo imaging of SSTR-positive tumors (20, 25, 26), as well as our own previous experience with cytotoxic analogues of LH-RH (6, 11) and a prototype peptide conjugate consisting of methotrexate linked to SST analogue RC-121 (27), encouraged us to design and synthesize a series of modern targeted cytotoxic analogues of SST (28). These conjugates consist of doxorubicin or its potent derivative 2-pyrrolinodoxorubicin (AN-201) (29) linked covalently to carrier SST octapeptide analogues such as RC-121 (30). The hybrids thus formed retain both the receptor affinity and the cytotoxic activity of their respective components (28, 31). Recently, Koppán et al. (31) demonstrated that one of these cytotoxic analogues, AN-238 containing AN-201 conjugated to RC-121, inhibited the growth of SSTR2-positive Dunning R-3327-AT-1 anaplastic rat prostate cancer at non-toxic doses.

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4 The abbreviations used are: LH-RH, luteinizing hormone-releasing hormone; SST, somatostatin; SSTR, SST receptor; hSSTR, human SSTR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; NOR, nuclear organizer region; BW, body weight.
The aim of the present study was to investigate whether SSTRs found on PC-3 human hormone-independent prostatic cancer (14, 18, 32) could be used for targeted SST chemotherapy. Two pilot experiments in nude mice bearing s.c. PC-3 xenografts were first carried out to determine the tolerance, antitumor activity, and histological changes induced by cytotoxic analogue AN-238 and its components. Subsequently, because the PC-3 cell line shows a high metastatic potential after orthotopic implantation into nude mice (33, 34), we used this clinically relevant model to evaluate the effect of AN-238 on intraprostatic tumors and on the development of metastases in the lymph nodes. The expression of SSTR2 and SSTR5 in primary tumors and metastatic lesions was also investigated.

MATERIALS AND METHODS

Peptide and Cytotoxic Agent

The SST analogue RC-121 (t-Phe-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH2) was synthesized in our laboratory as described previously (30). The cytotoxic conjugate AN-238 was made by coupling one molecule of 2-pyrrolidinodoxorubicin-14-O-hemiglutarate to the NH2 terminus of [Lys(Fmoc)]5RC-121, followed by deprotection and purification (28). Before i.v. injection, all compounds were dissolved in 20 μL of 0.1 N acetic acid and diluted with 5% (w/v) aqueous D-mannitol (Sigma Chemical Co., St. Louis, MO) solution.

Animals

Male athymic (Ncr nu/nu) nude mice, approximately 6 weeks old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD), housed in laminar airflow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule, and fed autoclaved standard chow and water ad libitum. All experiments were performed in accordance with institutional guidelines on animal care.

Experimental Protocols

The PC-3 human androgen-independent prostatic carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as described previously (18).

s.c. Implantation. PC-3 tumors were transplanted s.c. as described previously (18). Experiment 1 was started when tumors had grown to a volume of approximately 30 mm3 and were still in the latent phase of growth. Three groups of seven mice each received single i.v. injections of AN-201 at 200 nmol/kg, AN-238 at 200 nmol/kg, or vehicle (controls). Tumor volumes (length × width × height) and BWs were measured weekly. Blood samples were collected once a week from the tail vein using the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ) to determine total WBC counts. After 7 weeks, mice were euthanized under anesthesia, and tumor burden (mg tumor weight/g body weight) was measured.

Additionally, samples of each tumor were collected once a week from the tail vein using the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ) to determine the number of WBCs. After 7 weeks, mice were euthanized under anesthesia, and tumor burden (mg tumor weight/g body weight) was measured.

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Histological Procedure

Six-μm-thick sections of paraffin-embedded tumor specimens were stained with H&E. The numbers of mitotic and apoptotic cells/1000 cells were counted on PC-3 tumors and metastatic lesions was also investigated.

RNA Isolation and RT-PCR

Total RNA was isolated using the Micro RNA Isolation Kit (Stratagene, La Jolla, CA) and quantified spectrophotometrically at 260 and 280 nm. First-strand cDNA was reverse-transcribed from total RNA with Moloney murine leukemia virus reverse transcriptase. Distilled water was used as a negative control. Initially, 3 μg of RNA were incubated with 300 ng of random primer in diethylpyrocarbonate-treated water in a total volume of 38 μL for 5 min at 65°C and then cooled slowly to room temperature. The RNA-primer complex was then incubated in 50 μL of reaction mixtures containing 50 units of Moloney murine leukemia virus reverse transcriptase, 40 units of RNase inhibitor, 1 mM each deoxyribonucleotide triphosphate, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl2, for 60 min at 37°C, followed by a 5-min incubation at 94°C. The same reaction mixture without reverse transcriptase was used as the negative control to exclude the contamination of genomic DNA. After reverse transcription, 5 μL of the reaction product were subjected to PCR amplification. PCR was performed in a final volume of 100 μL containing 0.5 μM (NH4)2SO4, 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween 20, 800 μM deoxyribonucleotide triphosphates (200 μM each of dATP, dGTP, dCTP, and dTTP), 2.0 mM MgCl2, 2.5 units of Taq DNA polymerase, and 150 ng of each of the sense and antisense primers. All of the reagents used for RT-PCR were purchased from Stratagene, with the exception of Taq DNA polymerase (ISCA:BioExpress, Kaysville, UT). For amplification from first-strand cDNAs, gene-specific primers for hSSTR2 (sense, 5′-ATGGACATGGGCGGATGGCCACCT-3′; antisense, 5′-TACTGTTTGTGACCTCATGCAA-3′), hSSTR5 (sense, 5′-CGTCTTCATCATCTACACGG-3′; antisense, 5′-GGCCAGGTTGAGCAGTGA-3′), and human GAPDH (internal control) were used (36–38). Plasmids containing cDNA for hSSTR2 or hSSTR5 were used as a positive control. Additionally, specimens of H-69 human small cell lung carcinoma and HT-29 human colon cancer served as positive controls for the in vivo expression of SSTR2 and SSTR5, respectively. PCR was performed in a DNA thermal cycler (Model 2400; Perkin-Elmer, Norwalk, CT). After denaturation at 94°C for 5 min, samples were subjected to 45 cycles comprising 94°C for 1 min, 62°C for 1 min, and 72°C for 1.5 min for SSTR2; 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for SSTR5; or 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for GAPDH and followed by a final extension at 72°C for 5 min. PCR products were separated electrophoretically on an 8% polyacrylamide gel and stained with silver. The exponential character of PCR for SSTR5 was checked after 30, 35, and 40 cycles, and the 35 cycle time point was chosen for semiquantitation of the SSTR5 bands using a scanning densitometer (Model GS-700; Bio-Rad) coupled with the Bio-Rad PC analysis software.

Statistical Analysis

The data are expressed as the mean ± SE. The statistical analyses were performed using Student’s two-tailed t test. The χ2 test was used to evaluate the differences in the incidence of lymph node metastases. Differences were considered significant at P < 0.05.
In the first experiment, we evaluated the antitumor activity of the cytotoxic radical AN-201 given as a single injection at 200 nmol/kg (maximum tolerated dose for male nude mice) and an equimolar dose of the cytotoxic SST analogue AN-238. Seven weeks after the administration of AN-238, the volume of PC-3 tumors (53.4 ± 8.0 mm³) was significantly smaller than that in controls that measured 203.2 ± 38.2 mm³, corresponding to a 74% inhibition (P < 0.01; Fig. 1A). No inhibition of tumor growth was observed in animals that received AN-238. Distant hematogenous metastasis was found in one of seven animals treated with AN-238. In contrast, no metastases were found in any of the six mice given AN-201.

In experiment 2, treatment was initiated when s.c. tumors had grown to a volume approximately twice as large as that in experiment 1. To reduce the toxicity and increase the cumulative amount of the drug, the mice were given two i.v. injections of 150 nmol/kg BW of cytotoxic radical AN-201 alone, an unconjugated mixture of AN-201 and carrier SST analogue RC-121, or the cytotoxic hybrid AN-238, all administered on days 1 and 10. As shown in Fig. 1B, after 4 weeks of therapy, AN-238 significantly inhibited the growth of PC-3 tumors, as evidenced by a tumor volume of 164.9 ± 32.6 mm³ compared with controls that measured 437.2 ± 35.3 mm³ (a 62.3% reduction; P < 0.001). Treatment with AN-238 also extended the tumor doubling time to 28.6 ± 7.1 days versus 11.1 ± 0.8 days in controls (P < 0.05). Tumor weight was similarly reduced by 61.1% (P < 0.01), and tumor burden was reduced by 60.1% (P < 0.01) as compared to controls (Table 1). AN-201, alone or coadministered with RC-121, had no significant effect on the final volume of PC-3 tumors, as compared with controls. Similarly, at the end of the experiment, no differences in tumor weight, tumor burden, and tumor volume doubling time were observed between groups treated with injection solvent, AN-201, or its unconjugated mixture with RC-121 (Table 1). No toxicity-related deaths occurred during the experiment in any of the groups. On day 14, the reduction in BW in all treated groups was less than 10% of the initial values, but the WBC count was significantly decreased in groups given AN-201, the unconjugated mixture, and AN-238 by 80%, 67%, and 83%, respectively. However, 2 weeks later, neither the BW nor the WBC count of the treated animals differed significantly from the control values.

**Effect of AN-238 and AN-201 on the Growth of Orthotopic PC-3 Tumors and the Development of Metastases.** Ten weeks after intraprostatic inoculation, two i.v. injections of AN-201 or AN-238 were administered at doses of 150 nmol/kg, 10 days apart. Four weeks after the first injection, the tumor weight in animals receiving AN-238 was 188 ± 20 mg, corresponding to a 77% reduction (P < 0.01) as compared with control tumors that weighed 830 ± 144 mg (Table 2). The weight of tumors in the group treated with AN-238 was 91 ± 14 mg (a 90% reduction; P < 0.001), significantly lower than that in animals given AN-201 (547 ± 140 mg). The effect of AN-201 on the weight of primary tumors (a 34% reduction) was not significant as compared with the control group. All control animals developed metastases into regional (retroperitoneal) and distant lymph nodes, whereas in AN-201-treated mice, the incidence of lymphatic spread was 67% (four of six mice). In contrast, no metastases were found in any of the six mice given AN-238. Distant hematogenous metastasis was found in one of seven control animals (in the lungs), but not in the AN-201- or AN-238-treated groups. The side effects of AN-238 and AN-201 were similar in both groups, the leukocyte count returned to normal values within 2 weeks. Neither compound significantly affected the BWs of surviving animals.

**RESULTS**

**Inhibition of the Growth of s.c. Xenografts of PC-3 Tumors by AN-238.** In the first experiment, we evaluated the antitumor activity of the cytotoxic analogue of SST AN-238 or its components on the growth of s.c. xenografts of PC-3 human hormone-independent prostate cancer in nude mice. In experiment 1 (A), single i.v. injections of 200 nmol/kg AN-238 or AN-201 were given when tumor volume was 30 mm³. The mortality caused by AN-238 and AN-201 was 14.3% and 43%, respectively. In experiment 2 (B), two injections of 150 nmol/kg AN-201, AN-238, or the mixture of AN-201 and the carrier were given 10 days apart when the initial tumor volume had reached 65–70 mm³. No deaths related to toxicity occurred. Arrows, day of injection; bars, SE, * P < 0.05 versus control; **, P < 0.01 versus control; ***, P < 0.001 versus control and P < 0.05 versus AN-201.

**Fig. 1.** The effect of cytotoxic analogue of SST AN-238 or its components on the growth of s.c. xenografts of PC-3 human hormone-independent prostate cancer in nude mice. In experiment 1 (A), single i.v. injections of 200 nmol/kg AN-238 or AN-201 were given when tumor volume was 30 mm³. The mortality caused by AN-238 and AN-201 was 14.3% and 43%, respectively. In experiment 2 (B), two injections of 150 nmol/kg AN-201, AN-238, or the mixture of AN-201 and the carrier were given 10 days apart when the initial tumor volume had reached 65–70 mm³. No deaths related to toxicity occurred. Arrows, day of injection; bars, SE, * P < 0.05 versus control; **, P < 0.01 versus control; ***, P < 0.001 versus control and P < 0.05 versus AN-201.

(75.5 mg ± 37.5; P < 0.05). The mortality related to toxic effects was 43% (three of seven mice) in mice given AN-201 and 14.3% (one of seven animals) in animals treated with AN-238. On day 7, the average WBC counts in the AN-238-treated group and in the surviving animals in the AN-201 group were reduced by 71% (P < 0.01) and 53% (P < 0.05), respectively. In both groups, the leukocyte count returned to normal values within 2 weeks. Neither compound significantly affected the BWs of surviving animals.

In experiment 2, treatment was initiated when s.c. tumors had grown to a volume approximately twice as large as that in experiment 1. To reduce the toxicity and increase the cumulative amount of the drug, the mice were given two i.v. injections of 150 nmol/kg BW of cytotoxic radical AN-201 alone, an unconjugated mixture of AN-201 and carrier SST analogue RC-121, or the cytotoxic hybrid AN-238, all administered on days 1 and 10. As shown in Fig. 1B, after 4 weeks of therapy, AN-238 significantly inhibited the growth of PC-3 tumors, as evidenced by a tumor volume of 164.9 ± 32.6 mm³ compared with controls that measured 437.2 ± 35.3 mm³ (62.3% reduction; P < 0.001). Treatment with AN-238 also extended the tumor doubling time to 28.6 ± 7.1 days versus 11.1 ± 0.8 days in controls (P < 0.05). Tumor weight was similarly reduced by 61.1% (P < 0.01), and tumor burden was reduced by 60.1% (P < 0.01) as compared to controls (Table 1). AN-201, alone or coadministered with RC-121, had no significant effect on the final volume of PC-3 tumors, as compared with controls. Similarly, at the end of the experiment, no differences in tumor weight, tumor burden, and tumor volume doubling time were observed between groups treated with injection solvent, AN-201, or its unconjugated mixture with RC-121 (Table 1). No toxicity-related deaths occurred during the experiment in any of the groups. On day 14, the reduction in BW in all treated groups was less than 10% of the initial values, but the WBC count was significantly decreased in groups given AN-201, the unconjugated mixture, and AN-238 by 80%, 67%, and 83%, respectively. However, 2 weeks later, neither the BW nor the WBC count of the treated animals differed significantly from the control values.

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**In Vivo Expression of SSTR.** Using gene-specific primers, we investigated the expression of hSSTR2 and hSSTR5 in PC-3 prostate cancers growing orthotopically, in their metastases in lymph nodes, and in tumors transplanted s.c. (Fig. 2). The presence of mRNAs for SSTR2 and SSTR5 was detected in all samples. Whereas the expression of SSTR2 was fairly low, the intensity of the signal for SSTR5 varied. A strong expression of SSTR5 was detected in the specimens of orthotopic and s.c. tumors, whereas the level in metastatic lesions...
The expression of SSTR was found on a high percentage of specimens of human prostate cancers (13, 19). SSTR1 and SSTR5 were identified as the major subtypes present on primary tumors (13, 19). In metastatic lesions, the predominant subtypes of SSTR appear to be SSTR2 and SSTR5 (20). Functional SSTRs detected on the PC-3 human hormone-refractory prostate cancer cell line (42) were shown to bind the SST octapeptide analogue RC-160 and its radiolabeled derivatives with high affinity (18, 25, 43). Intraperitoneally implanted PC-3 tumors produce spontaneous metastases into the lymph nodes and, as a late event, also produce hematogenous spread into the lungs and other organs (33, 34). In the present study, we showed that orthotopic PC-3 tumors and their metastases, as well as tumors grown s.c., express SSTR2 and SSTR5. This is in line with the previous in vitro detection of mRNA for SSTR2 in PC-3 cells (14, 16). However, the detection of mRNA for SSTR5 and the distinct pattern of expression in orthotrophic and metastatic PC-3 lesions have not been reported previously. Because these two subtypes of SSTR bind SST octapeptides such as RC-121 and RC-160 that are used as carriers for cytotoxic radical AN-201 (28, 30, 31, 44), our findings indicate that the orthotopically implanted PC-3 tumor is an appropriate model for investigating the efficacy of SSTR-targeted chemotherapy because the results obtained would be likely to be applicable to a clinical setting. Two preliminary experiments with s.c. xenografts were performed to evaluate the antiproliferative effect of AN-238 and its cytotoxic radical AN-201 after administration at different stages of tumor growth and to optimize the dosage regimen. Our results indicate that a single i.v. injection of AN-238 at a dose of 200 nmol/kg BW during the early phases of growth, when the tumors were less than 30 mm³ in size, strongly suppressed tumor progression for at least 7 weeks. Tumor weight in the AN-238-treated group was decreased by 71%, and tumor volume was decreased by 74% as compared to the controls. In another experiment, two applications of AN-238 at 150 nmol/kg were given 10 days apart when the tumors were approximately twice as large. Four weeks after the initiation of treatment, tumor weight and tumor burden were about 60% lower than in the control group. In both experiments, cytotoxic radical AN-201 did not affect tumor growth when given as a single drug at equimolar doses or coadministered with SST analogue RC-121 in an unconjugated mixture. Comparable results were observed in the previous study with Dunning R-3327-AT-1 rat prostate cancer that expressed SSTR2 (31). Because only a weak expression of SSTR2 was found in PC-3 tumors, it is likely that a significant difference between the effects of AN-201 and AN-238 can be attributed to a specific delivery of the cytotoxic radical by SST carrier primarily to SSTR5 on tumor cells. Because the expression of SSTR5 was also demonstrated in cultured epithelial cells from specimens of human prostatic adenocarcinoma (13), it is reasonable to

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Table 1. The effect of cytotoxic radical AN-201, an unconjugated mixture of AN-201 and carrier RC-121, and cytotoxic SST analogue AN-238 on the growth of PC-3 human hormone-independent prostate cancer xenografted s.c. into nude mice (experiment 2)

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>Tumor volume (mm³)</th>
<th>Tumor doubling time (days)</th>
<th>Tumor weight (mg/g body mass)</th>
<th>Tumor burden (mg/g body mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final (% inhibition)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>71.3 ± 8.6</td>
<td>437.2 ± 35.3</td>
<td>11.1 ± 0.8</td>
<td>321.8 ± 37.3</td>
</tr>
<tr>
<td>AN-201</td>
<td>65.0 ± 9.2</td>
<td>389.3 ± 79.5 (10.1%)</td>
<td>12.8 ± 2.1</td>
<td>301.5 ± 70.5 (6.3%)</td>
</tr>
<tr>
<td>Mixture of AN-201 and RC-121</td>
<td>67.0 ± 10.7</td>
<td>401.4 ± 100 (8.2%)</td>
<td>12.1 ± 2.0</td>
<td>310.0 ± 87.2 (3.7%)</td>
</tr>
<tr>
<td>AN-238</td>
<td>70.2 ± 9.2</td>
<td>164.9 ± 32.6 (62.3%)</td>
<td>28.6 ± 7.1⁶</td>
<td>125.1 ± 37.1² (61.1%)</td>
</tr>
</tbody>
</table>

* Two i.v. injections of 150 nmol/kg BW of each compound were given on day 1 and day 10. The experiment lasted for 4 weeks. Values are the mean ± SE.

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Table 2. The effect of cytotoxic radical AN-201 and cytotoxic SST analogue AN-238 on the growth of orthotopically implanted PC-3 human prostate cancer in nude mice

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>Tumor weight (mg/g body mass)</th>
<th>Incidence of lymph node metastases</th>
<th>Incidence of distant metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>830 ± 140 (100%)</td>
<td>777 (100%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>AN-201</td>
<td>547 ± 130 (34%)</td>
<td>472 (29%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>AN-238</td>
<td>189 ± 20³ (77%)</td>
<td>8.69 ± 1.13³ (76%)</td>
<td>0⁹,c,e (0%)</td>
</tr>
</tbody>
</table>

* Treatment was started 10 weeks after inoculation and consisted of two i.v. injections of 150 nmol/kg BW AN-201 or AN-238 given 10 days apart. The experiment was terminated 4 weeks after the first injection. Values are the mean ± SE. χ² test was used to evaluate differences in the incidence of metastasis into the lymph nodes.

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**DISCUSSION**

The idea of chemotherapy targeted to SSTR on tumors using SST analogues as carriers for cytotoxic radicals is an extension of our work on cytotoxic LH-RH analogues (6, 11) and is based on Paul Ehrlich’s concept of magic bullets (41). According to this hypothesis, SST-mediated targeting of cytotoxic radicals would allow the delivery of an increased dose of cytotoxic agents to SSTR-positive tumor tissue without compromising normal cells. Following this approach, we recently developed modern hybrid analogue AN-238 consisting of 2-pyrrolinozoxorubicin (AN-201) and carrier RC-121 (28), and Kopšán et al. (31) have shown it to be highly effective in SSTR2-positive anaplastic Dunning R-3327 AT-1 rat prostate cancer at a nontoxic dose. In contrast, the cytotoxic radical and the carrier, administered separately and as an unconjugated mixture, were ineffective and toxic (31).
assume that the targeted cytotoxic SST analogue AN-238 could be of value for the therapy of patients with prostate cancer.

In experiment 3, AN-238 and AN-201 were administered twice at 150 nmol/kg doses to mice inoculated orthotopically with PC-3 cells. The treatment was started 10 weeks after implantation, when some of the primary tumors were palpable through the abdominal wall and were likely to develop metastases (34). Four weeks after the initiation of treatment, the weight of primary tumors treated with AN-238 was inhibited by 77% as compared with the controls. A stronger inhibitory effect of AN-238 on the progression of orthotopic tumors than on those implanted s.c. may be explained in part by a better vascularization of the host organ and, consequently, a better penetration of the drug to the neoplastic tissue. Because blood vessels surrounding the primary and metastatic tumors were shown to possess a high density of SSTRs (45), the host peritumoral vascular system may be also a possible target for AN-238. A better blood supply to orthotopic tumors could also explain a small but not significant response to cytotoxic radical AN-201 observed in our experiment. Another encouraging observation of this experiment was that treatment with the cytotoxic SST analogue AN-238 completely eliminated the lymphatic and hematogenous spread of PC-3 cancers. A possible interpretation of this finding could be that by arresting the growth of primary tumors, treatment with AN-238 prevented the dissemination of the cancer. However, a more likely mechanism may involve direct targeting of the radical to already established micrometastases that express SSTR2 and SSTR5, leading to their complete regression. The expression of SSTRs on PC-3 tumors after repeated treatment with AN-238 was unchanged, in accord with previous observations that treatment with the cytotoxic SST analogue does not affect the expression of SSTR in tumor tissue (31). This finding would allow the use of multiple dosage regimens. As shown in experiments 2 and 3, such a repetitive treatment schedule with smaller doses appears to be less toxic and permits the use of a higher cumulative dose.

Although the mortality rate in experiment 1 was much higher in mice that received 200 nmol/kg AN-201 (47%) than in the group treated with AN-238 (14.3%), the decreases in the WBC count and the changes in BW in animals treated with both types of cytotoxic compounds were very similar in all three experiments. Because AN-201 is linked to the carrier peptide through an ester bond, similar toxicity patterns of AN-238 and AN-201 can be explained by partial deconjugation of the radical from the conjugate by nonspecific esterases. However, in our previous studies in the rat Dunning model, AN-238 was nontoxic at 300 nmol/kg, whereas AN-201 caused 90% mortality at 115 nmol/kg (31). As we pointed out earlier (28), this can be explained by the differences between the nonspecific esterase activities in the rat and the mouse. Thus, in an in vitro experiment described previously (28), we compared the deconjugation of doxorubicin from a cytotoxic LH-RH analogue (AN-152) consisting of

Fig. 2. The expression of mRNA for hSSTR2, hSSTR5, and human GAPDH in PC-3 human androgen-independent prostate cancer as revealed by RT-PCR. Representative samples from untreated animals are shown. Lanes 1–2, orthotopic PC-3 tumors; Lanes 3–4, metastases into lymph nodes; Lanes 5–6, s.c. PC-3 tumors; Lane M, molecular marker (100-bp DNA ladder); Lane N, negative control; Lane P1, positive control cDNA plasmids. Lane P2, positive control for the detection of in vivo expression of SSTR2 (H-69 human small cell lung carcinoma) and SSTR5 (HT-29 human colon cancer).

Fig. 3. Sections of s.c. xenografts of PC-3 human androgen-independent prostate cancer treated with injection solvent (A), cytotoxic radical AN-201 (B), or cytotoxic SST analogue AN-238 (C). Two i.v. injections of each compound at 150 nmol/kg BW were given on days 1 and 10. The incidence of apoptotic (arrows) and mitotic cells was evaluated 4 weeks after the initiation of therapy. Treatment with AN-238 resulted in a significant enhancement of apoptosis in tumors. Sections were stained with H&E. Magnification, ×460.
doxorubicin-14-O-hemiglutarate linked to [d-Lys<sup>6</sup>]<sup>2</sup>LH-RH in mouse, rat, and human sera. Using HPLC analysis, we found that 50% deconjugation occurred in 10 min in mouse serum, 30 min in rat serum, and about 2 h in human serum at 37°C. However, it should be emphasized that in spite of a high esterase activity in the mouse, AN-238 still produced significantly better inhibition of growth of PC-3 tumors than AN-201. On the basis of our observations on species-specific differences in esterase activity and the results from the rat Dunning model, it could be speculated that AN-238 would be even more efficacious in human beings.

Our study demonstrates that the cytotoxic SST analogue AN-238 has an increased antitumor activity and a smaller toxicity than its radical AN-201, indicating that a localized delivery of the cytotoxic radical to the tumor tissue and metastasis can be achieved when it is linked to RC-121. This more selective delivery could be attributed to a targeting action of the SST carrier. This view is in accordance with biodistribution studies with <sup>111</sup>In-labeled octreotide injected i.v. into mice bearing PC-3 tumors that showed a rapid clearance from the blood with a tumor:nontumor uptake ratio of 2:1 at 4 h after injection (43). In addition, several radiolabeled derivatives of octreotide were shown to accumulate in AT-20 pituitary tumors in nude mice at 6, 24, and 48 h after i.v. injections (46). Other biodistribution studies demonstrated a fast and significant accumulation of a <sup>90</sup>Y-labeled SST analogue designed for targeted radiotherapy in pancreatic tumors expressing SSTR (47). It has been shown that the in vivo uptake of radioactivity in SSTR-positive tissues at various time points after the administration of a radioligand also depends on its rate of metabolism in these tissues as well as in the liver and kidneys, which are the major sites for clearance of the octapeptide SST derivatives (46, 48). Preliminary clinical studies have shown no or only low-grade toxicity to the pituitary, kidney, and bone marrow after the administration of radiolabeled SST analogues to cancer patients, whereas a significant reduction in tumor progression was achieved (47, 49). Radiotherapy with <sup>111</sup>In-labeled pentetreotide is well-tolerated and provides an effective therapy in some subjects with SSTR-expressing tumors (50). In view of a wide distribution of SSTRs in the gastrointestinal tract and other tissues (6, 11, 12, 17), it cannot be excluded that therapy with AN-238 would produce some side effects. However, clinical findings suggest that SSTR-targeted radiotherapy may not affect the ability of the progenitor cells to replace the damaged cells (49, 50). Our observations that treatment with a cytotoxic LH-RH analogue containing AN-201 caused only a transient deterioration of the anterior pituitary function in rats also imply that in nonproliferating endocrine cells, the cytotoxic peptide analogues are more likely to impair the receptor-coupled signal transduction mechanism rather than induce permanent cell damage (11). Nevertheless, an appropriate replacement therapy could further alleviate the symptoms of gastrointestinal and endocrine dysfunctions. The mechanism of action of cytotoxic SST analogues is the subject of intense investigations in several laboratories. It is assumed that an SST analogue containing a cytotoxic radical is internalized after binding to the membrane receptors (46). Because very low concentrations of cytotoxic radical AN-201 and analogue AN-238 as compared to doxorubicin are used in vivo, the development of new, sensitive methods is necessary to perform comparative biodistribution studies.

The histological study revealed that growth suppression of PC-3 prostate cancers achieved by treatment with cytotoxic SST analogue AN-238 is mainly due to an increase in the number of cells undergoing apoptosis. This is of particular interest because androgen-refractory prostate cancer is characterized by the acquisition of apoptosis-resistant genotypes, and its progression may be a result of dysfunction in programmed cell death and not of excessive proliferation (51). According to recent reports, the concentration of doxorubicin that can induce effective apoptosis in histocultured specimens of human prostate cancer is about 2 orders of magnitude higher than its clinically achievable plasma levels (52). Thus, it could be surmised that apoptosis induced by AN-238 may be a result of SSTR-mediated accumulation of doxorubicin derivative AN-201 in PC-3 tumors. However, the mechanisms underlying this phenomenon require further investigation.

In conclusion, the present study demonstrates a high efficacy of SSTR-targeted chemotherapy in a model of disseminated human androgen-independent prostatic carcinoma. It is likely that SSTR-mediated delivery of cytotoxic analogue to tumor tissue allows the activation of apoptosis, which is known to be impaired in hormone-refractory prostate cancer. The use of cytotoxic SST analogue AN-238 could provide a novel and effective therapeutic approach to management of patients with advanced prostatic carcinoma who relapsed androgen ablation.

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Inhibition of PC-3 Human Androgen-independent Prostate Cancer and Its Metastases by Cytotoxic Somatostatin Analogue AN-238

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