Effective treatment of experimental DU-145 prostate cancers with targeted cytotoxic somatostatin analog AN-238

ARTUR PLONOWSKI, ANDREW V. SCHALLY, ATTILA NAGY, BAODONG SUN and GABOR HALMOS

Endocrine Polypeptide and Cancer Institute, Veterans Administration Medical Center, New Orleans,
LA 70112-1262; Section of Experimental Medicine, Department of Medicine,
Tulane University School of Medicine, New Orleans, LA 70112-1262, USA

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Abstract. We evaluated the effectiveness of targeted cytotoxic analog of somatostatin (SST) AN-238, consisting of 2-pyrrolidinodoxorubicin (AN-201) linked covalently to SST octapeptide carrier RC-121 in DU-145 human androgen-independent prostate cancers xenografted into nude mice. We also investigated the expression of mRNAs for SST receptor subtypes 2A and 5 (sst2 and sst5) in DU-145 tumors. After 8 weeks of treatment, AN-238 practically arrested the proliferation of DU-145 cancers. The tumor volume in nude mice that received 4 injections of AN-238 at the dose of 110 nmol/kg was 63 ±6.7 mm³, nearly 4 times smaller than that in controls which measured 249 ±36.3 mm³ (p<0.001). Treatment with AN-238 lowered tumor weight by 68% (p<0.01) compared with the control group and extended the tumor volume doubling time to 184 ±69.4 days, versus 37 ±6.6 days in controls (p<0.05). No toxicity-related deaths occurred during treatment with AN-238. Cytoxic radical AN-201 administered alone or in an unconjugated mixture with carrier RC-121 inhibited the growth of DU-145 tumors only after the third and fourth injection and was toxic. The expression of mRNA for sst2 and sst5 was detected in all specimens of control DU-145 tumors and in tumors treated with AN-238. The present study demonstrates the high efficacy of SST-receptor-targeted chemotherapy in a model of human androgen-independent prostate carcinoma.

Introduction

In spite of a significant refinement in early detection of prostate cancer, approximately 10-20% of patients present with metastatic lesions at the time of diagnosis (1,2). In addition, locally advanced cancer is found in nearly one-half of surgically treated patients with clinically organ-confined tumors (2,3). In recurrent or advanced disease, all therapies based on androgen deprivation can produce a clinical remission, but will not prevent the ultimate progression to an androgen-refractory stage (4). No effective treatment is currently available for androgen-independent prostate cancer (5,6), because of its intrinsic chemoresistance and antitumor mechanisms, although some clinical response can be achieved with combined regimens of docetaxel, estramustine and carboplatin (7). To improve the effectiveness of chemotherapy we introduced a new therapeutic approach based on targeted delivery of cytotoxic agents using peptide carriers for which receptors are present on neoplastic cells (8,9).

The successful use of radiolabeled somatostatin (SST) analogs for imaging and treatment of various SST receptor-expressing tumors and demonstration of SST receptors on surgical specimens of prostate cancer (10-14) provide a rationale for testing the effectiveness of modern cytotoxic SST analogs in preclinical models of androgen-independent prostate cancers. Because these cytotoxic SST conjugates retain the receptor binding affinity of their peptide carriers (19,20), it is assumed that the uptake of these compounds from the bloodstream, mediated by SST receptors, should lead to their selective accumulation within the tumors. As a result, these agents may overcome multidrug resistance characteristic of these cancers (9). Thus, in previous work with rat Du-165 R-3327 AT-1 and human PC-3 hormone-independent prostate carcinomas that express SST receptors (20,21), we showed that cytotoxic analog of somatostatin AN-238 (19), consisting of 2-pyrrolidinodoxorubicin (AN-201) linked covalently to SST octapeptide carrier RC-121 (22,23), strongly inhibits tumor growth in vivo and increases the incidence of apoptosis. In both models, cytotoxic radical AN-201 was ineffective and toxic (20,21). Our recent work indicates that more than 66% of locally advanced prostate cancers express SST receptors (18). To further corroborate the usefulness of AN-238 for the targeted therapy of prostate cancers, in the present study we evaluated its effectiveness in DU-145 human androgen-independent prostate cancer, which has a higher proliferative potential than the previously used PC-3 cell line (24). We also investigated the expression of mRNAs for SST receptor subtypes in DU-145 cancers, and the binding affinity of targeted analog AN-238 to SST receptors on the tumor membranes.

Correspondence to: Professor Andrew V. Schally, Veterans Administration Medical Center, 1601 Perdido Street, New Orleans, LA 70112-1262, USA

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Materials and methods

Peptides and cytotoxic agent. The SST analog RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH₂) was synthesized in our laboratory (23). The cytotoxic conjugate AN-238 was made by coupling one molecule of 2-pyridylcarboxylic acid-14-0-hemiglutarate to the amino terminus of [Lys(Fmo)c]RC-121 followed by deprotection and purification (19). Cytotoxic radical AN-201 was prepared as described (22). Before i.v. injection, the compounds were dissolved in 20 µl of 0.1 N acetic acid and diluted with 6% (weight/volume) aqueous D-mannitol (Sigma Chemical Co., St. Louis, MO) solution.

Cell line and animals. DU-145 human androgen-independent prostate carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as described previously (25). Male athymic (Ncr nu/nu) nude mice, approximately six weeks old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) and 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 for animal care.

Experimental protocol. Subcutaneous xenografts of DU-145 prostate cancer were initiated by injection of 5x10⁶ cells into the right flank of four nude mice. Resulting tumors were transplanted subcutaneously into nude mice as described previously (25). When the xenografts reached 45-50 mm³ in size, mice were randomly divided into five groups for treatment with cytotoxic SST analog AN-238 (n=8), cytotoxic radical AN-201 (n=7), carrier SST analog RC-121 (n=5), unconjugated mixture of AN-201 and RC-121 (n=5), or vehicle solution (control, n=7). On day 1, all compounds were administered through the jugular vein at the dose of 150 nmol/kg. The treatment was repeated on days 11, 21, and 42. Tumor volumes (length x width x height x 0.5236) and body weights were recorded weekly. Total leukocyte count (WBC) was determined manually on day 14 using a Unopette microcollection kit (Beckton Dickinson, Franklin Lakes, NJ). Eight weeks after the initiation of the treatment, mice were euthanized and tumors were excised and weighed. Inhibition of tumor growth was calculated using the following formula: 100% x (TVol_control - TVol_treatment)/TVol_control, where TVol = Final tumor volume. Initial tumor volume. Tumor specimens were snap-frozen and stored at -70°C until preparing membranes for receptor assay and extracting RNA for RT-PCR.

Receptor binding assay. Binding of octapeptide RC-160 to SST receptors on tumor membrane preparations was determined with ligand competition assays using [³²P]labeled RC-160, as reported (18,23). Receptor binding affinity of cytotoxic SST analog AN-238 to tumor membranes was measured in displacement experiments based on competitive inhibition of [³²P]RC-160 binding using various concentrations of AN-238 (10⁻⁸ to 10⁻⁴ M). IC₅₀ value was calculated using a computerized curve-fitting program and is defined as a dose of AN-238 causing 50% inhibition of [³²P]RC-160 binding (56).

Statistical analysis. The data are expressed as the mean ± SE. The statistical analyses were performed using the two-tailed Student's t-test, p<0.05 being considered significant.

Results

In vivo studies. Treatment with cytotoxic SST analog AN-238 or its components, radical AN-201 and carrier RC-121, was started when subcutaneous DU-145 tumors had grown to a volume of 40-50 mm³. The compounds were administered on days 1, 11, 21, and 42 at the dose of 150 nmol/kg. The inhibitory effect of AN-238 on tumor growth became significant as early as one week after the first injection as shown in Fig. 1. During the remaining seven weeks of treatment, AN-238 practically arrested the proliferation of DU-145 cancers, as revealed by a 93.9% tumor growth inhibition. At the end of the experiment, the mean tumor volume in mice receiving AN-238 was 6146 ± 7 mm³, being about 4 times smaller, than that in controls, which measured 2491 ± 363 mm³ (p<0.001). Treatment with AN-238 extended the tumor volume-doubling time to 184.1 ± 89.4 days.
Table I. The effect of cytotoxic SST analog AN-238 and its components AN-201 and RC-121 on the growth of DU-145 human prostate cancer in nude mice.

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>Tumor volume (mm³)</th>
<th>Tumor growth inhibition (%)</th>
<th>Tumor volume doubling time (days)</th>
<th>Tumor weight (mg) (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final (± inhibition)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47.3±5.2</td>
<td>249.1±63.3</td>
<td></td>
<td>134.7±20.1</td>
</tr>
<tr>
<td>AN-201, 4x150 nmol/kg</td>
<td>40.4±7.3</td>
<td>126.3±30.9</td>
<td>57.4</td>
<td>57.8±15.8</td>
</tr>
<tr>
<td>RC-121, 4x150 nmol/kg</td>
<td>48.6±10.9</td>
<td>211.4±45.4</td>
<td>19.4</td>
<td>7.1±3.6</td>
</tr>
<tr>
<td>Mixture AN-201 and RC-121, 4x150 nmol/kg each</td>
<td>49±6.8</td>
<td>110.5±20.9</td>
<td>69.6</td>
<td>30.6±4.2</td>
</tr>
<tr>
<td>AN-238, 4x150 nmol/kg</td>
<td>50.1±5.2</td>
<td>63.4±6.7</td>
<td>93.9</td>
<td>14.4±6.9</td>
</tr>
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</table>

*The compounds were administered i.v. on days 1, 11, 21, and 42. *The inhibition of tumor growth was calculated by the following formula: 
100% x (ΔTVol_final - ΔTVol_initial)/ΔTVol_initial where ΔTVol = Final tumor volume - Initial tumor volume. *p<0.05 vs. control. *p<0.01 vs. control.

Table II. The tolerance to cytotoxic SST analog AN-238 and its components in nude mice bearing s.c. xenografts of DU-145 human prostate cancer.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mortality (%)</th>
<th>Final body weight (g) and (% loss)</th>
<th>WBC on day 14 (counts/mm³) and (% reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/6</td>
<td>32.7±1.3</td>
<td>8668±877</td>
</tr>
<tr>
<td>AN-201</td>
<td>4/7</td>
<td>27.3±1.8</td>
<td>2502±302</td>
</tr>
<tr>
<td>RC-121</td>
<td>0/5</td>
<td>31.4±1.0</td>
<td>8250±1224</td>
</tr>
<tr>
<td>Mixture AN-201 and RC-121</td>
<td>1/5</td>
<td>27.3±1.6</td>
<td>2530±414</td>
</tr>
<tr>
<td>AN-238</td>
<td>0/8</td>
<td>27.5±0.5</td>
<td>1961±351</td>
</tr>
</tbody>
</table>

*Number of dead mice/total number treated. *p<0.05 vs. control. *p<0.01 vs. control. 

versus 32.7±1.3 days in controls (p<0.05). Tumor weight was similarly lowered by 68% (p<0.01) compared with the control group (Table I). No toxicity-related deaths occurred during the treatment with AN-238, and the final body weights were decreased by approximately 15% compared with the controls.

Cytotoxic radical AN-201 administered alone or in an unconjugated mixture with carrier RC-121 significantly reduced the growth of DU-145 tumors only after the third and fourth injection (Fig. 1). At the end of the experiment, the final tumor volume in mice receiving AN-201 was 49% smaller (p<0.01) compared with the controls, and a 56% (p<0.01) reduction in tumor volume was also found in animals treated with an unconjugated mixture of AN-201 and RC-121 (Table I). However, these antitumor effects were accompanied by high mortality. Four of 7 mice in the AN-201 group and 1 of 5 animals that received the mixture died of toxicity (Table II). The mean body weights of surviving mice in both groups were about 16% lower than those in the control group. After the second injection, a marked decrease in WBC count was observed in mice receiving AN-238, AN-201 alone, or the mixture of AN-238 and RC-121. Neither antitumor effects nor toxicity were observed in mice treated with the carrier peptide RC-121.

Expression and binding affinity of somatostatin receptors

The expression of mRNA for sst₂₅ and sst₅ was detected in all specimens of control DU-145 tumors and in tumors treated with AN-238. Two weeks after the fourth injection of AN-238, the levels of mRNA for sst₂₅ and sst₅ did not differ significantly from the control group (Fig. 2). Binding studies with radiolabeled RC-160 showed a single class of specific, high-affinity (K_d = 8.18±0.63 nM), moderate-capacity (B_max = 11.42±10.75 fmol/mg protein) binding sites on membranes of DU-145 tumors. Neither the affinity (K_d =
Figure 2. The expression of mRNA for SSTα and SST, subtypes of SST receptors in DU-145 human androgen-independent prostate cancer as revealed by RT-PCR analysis. Representative samples from the control group or from animals treated with cytotoxic analog of SST AN-238 are shown. mRNA for human GAPDH (internal control) was detected in all tumors. M, molecular marker; N, negative control.

7.3±0.96 nM, nor the binding capacity (B_max = 1126±89.5 fmol/mg protein), was affected by treatment with AN-238 compared with SST receptor characteristics in control tumors. The concentration of cytotoxic SST analog AN-238 required to inhibit the binding of [125I]RC-160 by 50% (IC_{50}) was 15.8±0.1 nM, indicating a high binding affinity of AN-238 to SST receptors.

Discussion

Anthracycline antibiotics such as doxorubicin and daunorubicin are among the most powerful anticancer agents endowed with activity against a broad spectrum of epithelial cancers. After an i.v. administration, these low molecular weight compounds are readily distributed to nearly all body compartments including tumor tissue (27). Because the antitumor efficacy of anthracyclines depends on their concentration in neoplastic tissue, various approaches were tried to modify their pharmacokinetics in order to achieve a more intense and selective delivery to the tumor. Most of these approaches are based on the passive intratumoral retention of large-molecule formulations of doxorubicin, such as liposomes or polymer carrier conjugates (28,29). Alternatively, doxorubicin or its derivatives can be linked to peptide carriers for targeted delivery to cells that express their respective receptors (8,9). Receptor-mediated uptake and internalization of cytotoxic peptide analogs should allow a higher accumulation of cytotoxic compounds in receptor-positive cancer cells and thus may overcome the mechanisms of chemoresistance (9,30).

The presence of SST receptors was detected in a high percentage of human primary prostate cancer specimens (18). About 90% of tumors express SST1 as revealed by RT-PCR, while 70% express SST2 and only 30% are positive for SST3. In metastatic lesions, the predominant subtypes of SST receptors appear to be SST2 and SST3 (14,15). The presence of SST1 and SST2 subtypes in DU-145 model, demonstrated in the present study, corresponds to the pattern of SST receptor subtypes detected previously in PC-3 prostate cancer (21). Thus, in regard to the expression and properties of SST receptors, DU-145 and PC-3 models can be considered representative of metastatic lesions of prostate cancer, from which these two lines were originally derived. High affinity binding sites for SST octapeptide analog RC-160 and its radiolabeled derivatives were also detected in PC-3 and DU-145 androgen-independent prostate cancer cell lines (13,16,25). We showed that cytotoxic analog AN-238 binds with high-affinity to the membranes of DU-145 tumors which is in agreement with the results for RC-160, obtained by radioligand binding assay. This implies that in a clinical setting, the targeted SST analog AN-238 should also preferentially bind to disseminated cancerous lesions.

In a previous study we reported that cytotoxic SST analog AN-238 strongly inhibited the growth of PC-3 human hormone-independent prostate cancers, while its unconjugated radical was ineffective and toxic (21). A good tolerance to two applications of AN-238 at 150 nmol/kg in nude mice bearing PC-3 tumors and the preservation of tumoral SST receptors after the treatment encouraged us to escalate the dosage regime. Thus, in the present study with DU-145 human hormone-independent prostate cancer, we doubled the cumulative dose of AN-238, applying four injections at 150 nmol/kg. This schedule was even more effective than that in PC-3 model, virtually arresting the growth of DU-145 tumors. After eight weeks of treatment with AN-238, tumor growth was inhibited by 95% and tumor-volume doubling time was extended 6-fold. Partial responses were also observed in mice receiving equimolar doses of AN-201, either as a single drug or in an unconjugated mixture with the carrier peptide RC-121. However, the third and the fourth injection of AN-201 caused a severe loss in body weight and led to animal deaths. A narrow 'therapeutic window' for 2-pyridylamidoxorubicin (AN-201) is characteristic of intensely potent daunorubicin-modified doxorubicin derivatives (31), and was also observed in other studies with various models of human prostatic cancer (21,32). Thus, an increased antitumor activity of AN-238 and tolerance to the treatment can be attributed to targeted delivery of the drug to tumor cells expressing SST receptors.

A good tolerability to the cumulative dose of 600 nmol/kg of conjugate AN-238, as opposed to high mortality caused by radical AN-201, indicates that targeted chemotherapy with cytotoxic analogs of SST is less toxic and permits a dose escalation. However, some specific side effects, such as myelosuppression, can be still observed in nude mice treated with cytotoxic SST analog AN-238 (9,21,33). We showed previously that, as a result of high activity of serum carboxyl-esterases in nude mice, the ester bond linking the cytotoxic moiety to the carrier peptide can be partially hydrolyzed in the blood stream before the targeting is completed (9,34). Because the stability of our cytotoxic analogs in human serum is approximately six times higher than that in mouse serum (34), it is expected that in patients the tolerance to targeted analogs will be better than in nude mice. In previous experiments, we did not observe a specific toxicity of cytotoxic SST analogs to organs that express SST receptors, such as...
as the primate gland or kidneys (33). DNA-intercalating antitumor agents, such as 2-pyridylmethyl-2H)-benzoxaborole (AN-238), affect mainly cells with a high mitotic activity, and the damage to well-differentiated cells with slow turnover ratio is probably smaller than that inflicted on neoplastic tissue. In addition, the resting cells in the gastrointestinal tract can eventually replace the damaged cells, restoring the normal organ function. Accordingly, clinical studies showed only low-grade toxicity to the primate, kidneys, and the bone marrow after administration of high doses of radiolabeled SST analogs to cancer patients (17). Moreover, an appropriate hormonal replacement therapy can further alleviate a possible dysfunction of the endocrine and alimentary systems.

In conclusion, the present study demonstrates a high efficacy of cytotoxic SST analog AN-238 in a model of human androgen-independent prostate cancer. Our results suggest that chemotherapy with AN-238 targeted to SST receptors on tumors might improve the management of prostate cancer patients with metastatic disease, who no longer respond to androgen ablation.

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References


