Inhibition of Metastatic Renal Cell Carcinomas Expressing Somatostatin Receptors by a Targeted Cytotoxic Analogue of Somatostatin AN-238

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ABSTRACT

The effectiveness of chemotherapy targeted to somatostatin (SST) receptors based on cytotoxic SST analogue AN-238, consisting of 2-pyrrolinodoxorubicin (AN-201) linked to SST carrier octapeptide, was investigated in human renal cell carcinomas (RCCs). SST receptors, which showed high-affinity binding for AN-238, were found in the SW-839 RCC line with sst subtype and in the 786-0 RCC line, which expressed the sst subtype. Caki-1 RCC, which does not express sst or sst subtype, was used as a negative control for testing the specificity of SST receptor targeting. Using microsatellite analysis, AN-238 was shown to selectively inhibit the proliferation of 786-0 line, but not the Caki-1 RCC line in vitro. The effects of three i.v. injections of 150 nmol/kg of AN-238 or AN-201, given on days 1, 8, and 21, were evaluated in groups of nude mice bearing s.c. xenografts of SW-839 and 786-0 RCC. After 5 weeks, the volumes of SW-839 and 786-0 RCC tumors were decreased by 67.2 (P < 0.05) and 78.3% (P < 0.01), respectively, whereas AN-201 had no significant effect on tumor growth. The inhibition of SST receptor-negative Caki-1 tumors by AN-238 was only marginal. To investigate the efficacy of SST receptor-targeted chemotherapy in metastatic RCC, three i.v. injections of AN-238 or AN-201 at 150 nmol/kg were given at biweekly intervals to nude mice implanted with 786-0 tumors under the renal capsule. After 6 weeks, the weight of orthotopic tumors treated with AN-238 (55.3 ± 44.3 mg) was significantly lower (87% reduction; P < 0.001) than that in the control group (414.2 ± 41.0 mg) or in animals given AN-201 (270.2 ± 60.3 mg; P < 0.05). Five of six animals (83%), both in the control and the AN-201 group, developed metastases to lymph nodes, but only one of seven mice (14%) given AN-238 showed lymphatic spread. Lung metastases were found in 83% of controls and 50% of AN-201 treated animals, but none occurred in mice treated with AN-238. This study demonstrates that targeted cytotoxic SST analogue AN-238 provides an effective therapy for chemoresistant neoplasms such as RCC. Because most clinical RCCs express SST receptors, this treatment modality might be beneficial to patients with metastatic disease.

INTRODUCTION

In the past two decades, the incidence rate of RCC has increased by >45% (1). In the United States alone, ~30,000 new cases of RCC and 12,000 deaths from this malignancy were estimated for 1999 (2). Approximately one-third of the RCC cases have documented metastasis at the time of the initial presentation, and ~50% of patients with presumably organ-confined disease who are treated with radical nephrectomy are expected to develop metastases within 10 years (3). The prognosis for metastatic RCC remains dismal because of its resistance to both chemotherapy and radiotherapy (4). Immunotherapy of advanced RCC has only a marginal effectiveness, whereas its side effects substantially decrease the quality of life (5–7).

To overcome the problem of chemoresistance, efforts have been made to use cytotoxic drugs in combination with agents that can reverse the MDR mechanism (8). This approach, however, has led only to an increased toxicity to normal organs that are protected by MDR, such as bone marrow or brain (9). Alternatively, the MDR in cancer cells can be abolished by enhanced local accumulation of a cytotoxic agent. This can be achieved by targeted delivery of cytotoxic radicals coupled to carriers such as monoclonal antibodies or analogues of peptide hormones that preferentially bind to malignant cells (10, 11). Thus, cytotoxic hybrids of peptide hormones such as LHRH, bombesin, or SST that can be targeted to their respective receptors on various tumors were shown to be more potent and less toxic than their unconjugated cytotoxic radicals (11). One of these cytotoxic analogues, AN-238, consisting of 2-pyrrolinodoxorubicin (AN-201) linked to carrier SST analogue RC-121, was recently demonstrated to strongly inhibit experimental SST receptor-positive tumors that are resistant to conventional chemotherapeutics (12, 13).

Several previous reports demonstrated that most kidney neoplasms express receptors for SST (14–16). Thus, 72% of specimens of human RCC were shown to bind the SST octapeptide analogue 125I-[Tyr]-octreotide with high affinity (14). PCR analysis of another series of 13 samples of human RCC revealed that subtype 2 of the SST receptor (sst2) was expressed in all of the specimens, whereas sst1 and sst4 were expressed in 85 and 46% of the specimens, respectively (15). In a clinical setting, the scintigraphy with radiolabeled octreotide was useful for the visualization of RCC in patients, especially in cases with multiple lesions (16). These findings suggest that targeted chemotherapy with cytotoxic SST analogues may have potential application in SST receptor-positive RCC.

In the present study, we investigated the effectiveness of SST receptor-targeted chemotherapy in experimental models of human RCC. We first showed that cytotoxic SST analogue AN-238 binds selectively to SST receptor-positive RCC lines and inhibits their proliferation in vitro. We then demonstrated that analogue AN-238 inhibits the growth and metastatic dissemination of RCC in vivo. These findings, which suggest that targeted chemotherapy based on AN-238 could be considered for patients with advanced, SST receptor-positive RCC, are described herein.

MATERIALS AND METHODS

Chemicals

The SST analogue RC-121 (d-Phe-Cys-Tyr-Trp-Lys-Val-Cys-Thr-NH2) was synthesized in our laboratory as described previously (17). The cytotoxic conjugate AN-238 was made by coupling one molecule of 2-pyrrolinodoxorubicin-14-0-hemiglutarate to the NH2 terminus of (Lys-Fmoc)9-RC-121 followed by deprotection and purification (18). The cytotoxic radical AN-201 was prepared as described previously (19). GHRH [hGHRH(1-29)NH2] was synthesized in our laboratory. RC-160 (Vapreotide) was obtained from Debio (Lausanne, Switzerland).

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4 The abbreviations used are: RCC, renal cell carcinoma; MDR, multidrug resistance; LHRH, luteinizing hormone-releasing hormone; SST, somatostatin; GHRH, growth hormone-releasing hormone; RT-PCR, reverse transcription-PCR; BW, body weight; GH, growth hormone; CE, carboxylesterase.

MATERIALS AND METHODS

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Cell Lines and Animals

SW-839, 786-0, and Caki-1 human RCC cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as described previously (20). Male athymic (Ncr nude) nude mice, 6 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.

RNA Isolation and RT-PCR

The expression of SST2A, SST3, and glyceraldehyde 3-phosphate dehydrogenase (internal control) in RCCs was examined by RT-PCR as described previously (12), using gene-specific primers for hss2A (sense, 5'-AGTGACATGGCGATGGACCCACT-3'; antisense, 5'-TACTGGTGATGGAGTTCTCAATTGA-3'), hss3 (sense, 5'-CGTCTTCATCRTCACAACGGG-3'; antisense, 5'-GGCGAGGTGAGCGATGGTA-3'). PCR products were separated electrophoretically on 8% polyacrylamide gels and stained with silver.

Receptor Binding Assay

Binding of octapeptide RC-160 to SST receptors on tumor membrane preparations was determined by ligand competition assays using 125I-labeled RC-160, as reported previously (13). Binding of the cystototic SST analogue AN-238 to tumor membranes was measured in displacement experiments based on competitive inhibition of 125I-RC-160 binding using various concentrations of AN-238 (10⁻⁶–10⁻¹² M). The IC₅₀ was calculated by a computerized curve-fitting program, and is defined as the concentration causing a 50% inhibition of 125I-RC-160 binding (13).

In Vitro Targeting and Microsatellite Analysis

In vitro targeting to SST receptor-positive RCC was demonstrated using microsatellite analysis as described in detail previously (21). The method is based on the assumption that the intensity of cell line-specific alleles containing the D3S2418 microsatellite marker is proportional to the number of viable cells. Briefly, cocultured 786-0 cells (10⁵) expressing SST3 and SST receptor-negative Caki-1 cells (4×10⁵) were exposed to 10 nm AN-201 or 10 nm AN-238 for 30 min and 1 h. Untreated cells were used as controls. After the treatment, the cells were washed twice with PBS and cultured for an additional 7 days (cells exposed to AN-201) or 4 days (cells treated with AN-238) in fresh medium that did not contain cytotoxic agents. Genomic DNA was extracted by the standard phenol-chloroform method. Microsatellite marker D3S2418 was obtained from Research Genetics (Huntsville, AL). Fifty ng of DNA were amplified as described previously (21). Five μl of each PCR product were electrophoresed in a 10% polyacrylamide gel, stained with silver, and semi-quantified using a scanning densitometer (Model GS-700; Bio-Rad, CA). The ratio of Caki-1/786-0-specific bands for microsatellite marker D3S2418 was calculated to identify the cell line that was affected preferentially by the treatment.

Experimental Protocols

s.c. Implantation. All tumors were transplanted s.c. as described previously (20). Experiments 1 (SW-839 RCC line) and 2 (786-0 RCC line) were started when tumors had grown to a volume of ~60–70 mm³. In each experiment, three groups of seven to eight mice received three i.v. injections of AN-201 at 150 nmol/kg, AN-238 at 150 nmol/kg, or vehicle only (controls), administered on days 1, 8, and 21. Cytotoxic compounds were dissolved in 20 nmol/kg, AN-238 at 150 nmol/kg, or vehicle only (controls), administered on days 1, 8, and 21. Cytotoxic compounds were dissolved in 20 nmol/kg, AN-238 at 150 nmol/kg, or vehicle only (controls, n = 8) on day 1, followed by a second administration on day 8. BW and tumor volume were recorded weekly, and the experiment was terminated after 4 weeks. Tumor specimens were snap-frozen and stored at ~70°C for the receptor binding assay and extraction of RNA for RT-PCR.

Orthotopic Implantation (Experiment 4). For the evaluation of SST receptor-targeted chemotherapy in a model of disseminated RCC, mice were implanted orthotopically with 786-0 tumors. Tumor tissue, harvested aseptically from donor animals after 8 weeks of s.c. growth, was cleaned of connective tissue and necrotic parts, weighed, mechanically minced in RPMI 1640, and pushed through a medium-mesh tissue sieve. The tissue suspension was centrifuged for 10 min at 1000 rpm; the supernatants were then removed, and the pellets were resuspended in RPMI 1640 at a final concentration of 500 mg tumor tissue/ml. Mice received injections of 0.02 ml of the suspension (corresponding to 10 mg of tumor tissue) under the capsule of the left kidney. Two weeks later, animals were randomly divided into three groups for treatment with AN-238 (n = 7), AN-201 (n = 6), or vehicle solution (n = 6). The compounds were administered three times at a dose of 150 nmol/kg BW at intervals of 2 weeks. Mice were killed 14 days after the last injection (8 weeks after implantation) or when they became moribund. At the autopsy, tumor weight was calculated by subtracting the weight of the right (normal) kidney from the weight of the left kidney (implanted with tumor). Mice were carefully checked under a dissecting microscope for the presence of gross metastases in lymph nodes, lungs, and other organs.

Evaluation of Toxicity

The toxicity was monitored by weekly measurement of BW. At the end of experiment 2, myelotoxicity was estimated by total leukocyte count (WBC), and pituitary function was assessed by determining the GHRH(1-29)NH₂-stimulated release of GH. Trunk blood was collected 15 min after i.v. injection of GHRH at 2 μg in 100 μl of 5% mannitol, and serum GH levels were measured by RIA as described previously (20).

Statistical Analysis

The data are expressed as the mean ± SE. The differences between groups were assessed with the Student’s two-tailed t test. Fisher’s exact test was used for comparison of the incidence of metastases. Differences were considered significant at P < 0.05.

RESULTS

Gene Expression and Binding Properties of SST Receptors in Human RCC. Using gene-specific primers, the expression of SST2A and SST3 was investigated in human SW-839, 786-0, and Caki-1 RCCs grown in nude mice (Table 1). We found that SW-839 expressed mRNA for SST2A but not for SST3. In contrast, mRNA for SST3 but not for SST2A could be detected in 786-0 tumors. High-affinity binding sites for 125I-labeled RC-160 were found in tumor membrane preparations of both lines (Table 1). Cytotoxic SST analogue AN-238 also exhibited high-affinity binding to membranes of both lines, as expressed by IC₅₀ values of 7.38 ± 0.68 nm for SW-839 and 2.04 ± 0.25 nm for 786-0 (Table 1). Caki-1 renal cancers did not express mRNA for SST2A and SST3. No binding of radiolabeled RC-160 was detected on cell membranes of Caki-1 tumors.

In Vitro Targeting. Selective in vitro cytotoxicity of AN-238 against cells expressing SST receptors was demonstrated by analysis

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Binding of 125I-RC-160</th>
<th>Binding of AN-238, IC₅₀ (nm)</th>
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<tbody>
<tr>
<td>SW-839</td>
<td>+</td>
<td>8.64 ± 0.56</td>
</tr>
<tr>
<td>786-0</td>
<td>+</td>
<td>6.33 ± 0.33</td>
</tr>
<tr>
<td>Caki-1</td>
<td>-</td>
<td>4.82 ± 0.30</td>
</tr>
</tbody>
</table>

*Values are means ± SE.

mRNA: present; –, not detected; NP, not performed.

*IC₅₀ is defined as the concentration of AN-238 causing a 50% inhibition of 125I-RC-160 binding to tumor membranes.
Effects of AN-238 and AN-201 on Growth of s.c. Xenografts of SW-839, 786-0, and CAKI-1 RCCs. Fig. 2 presents the effect of cytotoxic SST analogue AN-238 and its radical AN-201 on the growth of three human RCC xenografts in murine hosts. In sst<sup>2A</sup>-positive SW-839 tumors and in the 786-0 line expressing sst<sub>5</sub>, the treatment was started when tumors had reached 60–70 mm<sup>3</sup> and consisted of three i.v. injections of 150 nmol/kg of AN-201 or AN-238 on days 1, 8, and 21 (Fig. 2, A and B). In both models, the inhibition of tumor progression was already visible after two injections of AN-238. This inhibitory effect was further reinforced after the third injection. At the end of the experiments, the volume of SW-839 tumors was decreased by 67.2% (P < 0.05 versus control), whereas the average size of the 786-0 RCC was reduced by 78.3% (P < 0.01 versus control). Treatment with AN-238 significantly prolonged the tumor doubling time from 13 ± 2.2 to 22.2 ± 3.2 days (P < 0.05) in the SW-839 model, and from 10 ± 1.0 to 31.1 ± 7.1 days (P < 0.05) in 786-0 tumors, as compared with controls. In both experiments, the unconjugated cytotoxic radical AN-201 had no significant effect on tumor growth, the reduction in tumor volume being only 27% in SW-839 line and 40% in 786-0 RCC line (Fig. 2, A and B).

In the SST receptor-negative CAKI-1 model, the treatment was started when tumors had grown to ~30 mm<sup>3</sup> in size. Two injections of 150 nmol/kg of AN-238 exerted only a minor inhibition of tumor growth, which was comparable to the effect of AN-201 (Fig. 2C). Thus, there was a 32% reduction in final tumor volume in AN-238-treated animals and a 23% decrease in mice given AN-201, compared with the controls.

Inhibition of Orthotopic Growth and Development of Metastases of 786-0 RCC. The treatment, consisting of three i.v. injections of AN-238 or AN-201 at 150 nmol/kg, was given to nude mice at biweekly intervals starting 2 weeks after orthotopic implantation of 786-0 RCC. Six weeks after the initiation of treatment, three of seven mice receiving AN-238 were found to be tumor free, whereas in three other animals, the tumor mass was <30 mg (Table 2). One tumor in this group was disproportionately large (320 mg) and did not express SST receptors, as demonstrated later by a radioligand binding assay. The mean weight of AN-238-treated tumors was 55.3 ± 44.3 mg, corresponding to an 87% reduction (P < 0.001) compared with control tumors, which measured 414.2 ± 41.0 mg (Fig. 3, A, B, and C). The weight of tumors in the group treated with AN-238 was also

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**Fig. 1.** In vitro targeting of AN-238 to the sst<sub>5</sub>-positive 786-0 human RCC cell line as demonstrated by the D3S2418 microsatellite alleles assay. A, PCR products of DNA isolated from 786-0 cells (Lane 1), SST receptor-negative CAKI-1 cells (Lane 2), and cocultured 786-0 and CAKI-1 cells, untreated (Lane 3), treated with 10 nM AN-238 for 30 min (Lane 4) or 1 h (Lane 5), and treated with 10 nM AN-201 for 30 min (Lane 6) or 1 h (Lane 7). a and b indicate the alleles specific for CAKI-1 and the 786-0 line, respectively, and the ratio of their intensity reflects the proportion of each cell line in the coculture (B). The marked increase of the relative intensity of the a band versus the b band in Lanes 4 and 5 indicates selective cytotoxicity of AN-238 toward the 786-0 cell subpopulation.

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**Fig. 2.** Effect of SST receptor-targeted chemotherapy on the growth of s.c. xenografts of SW-839 (A), 786-0 (B), and CAKI-1 (C) human RCCs in nude mice. Animals bearing SW-839 and 786-0 SST receptor-positive tumors (A and B, respectively) received three i.v. injections of 150 nmol/kg of cytotoxic SST analogue AN-238 ( ), cytotoxic radical AN-201 (▲), or vehicle solution (control; □). The treatment was started when tumor volume had reached 60–70 mm<sup>3</sup> and was administered on days 1, 8, and 21. In the experiment with the SST receptor-negative CAKI-1 line (C), two injections of 150 nmol/kg of AN-201, AN-238, or placebo were given 7 days apart when the initial tumor volume was 30 mm<sup>3</sup>. Arrows indicate the days of injection; bars, SE; *, P < 0.05 versus control; **, P < 0.01 versus control.
effects of AN-201 and AN-238 on the pituitary reserve of GH, using i.v. stimulation with GHRH. The levels of GH evoked by GHRH in mice treated with AN-238 or AN-201 were lowered nonsignificantly by 25 and 12%, respectively, compared with the control (Table 3).

**DISCUSSION**

Chemotherapy targeted to SST receptors was developed to achieve a more efficacious and selective drug delivery to malignant lesions (11, 18) and to reduce peripheral toxicity. It is assumed that the binding of cytotoxic SST analogues such as AN-238 to SST receptor-positive cancer cells should lead to a greater accumulation of the cytotoxic radical in the tumor compared with that produced by a passive diffusion of traditional chemotherapeutics from the blood stream. High intratumoral concentrations of a cytotoxic drug, which could be achieved by targeted delivery, are thought to abolish the MDR and trigger the death of cancerous cells. Thus, in previous studies with androgen-independent prostate cancers expressing SST receptors, we observed a strong inhibition of tumor growth and an increased incidence of apoptotic cells after treatment with AN-238 (12, 13). These results demonstrated the efficacy of targeted chemotherapy in SST receptor-positive tumors that are resistant to traditional chemotherapy. Because a high percentage of human RCCs can express SST receptors (14, 15), we evaluated in the present study whether the cytotoxic SST analogue AN-238 could have therapeutic effects on this highly chemoresistant malignancy.

Two RCC lines used in the present study, SW-839 and 786-0, showed high-affinity binding sites for radiolabeled SST analogue RC-160, which is structurally similar to the carrier octapeptide RC-121 used in cytotoxic SST analogue AN-238. The receptor subtypes were identified as sst2A in SW-839 and sst5 in 786-0 cancers. In accordance with a previous report on the lack of binding of SST octapeptides to CAKI-1 RCC (20), we were not able to detect the

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**Table 2 The effect of cytotoxic SST analogue AN-238 and radical AN-201 on the growth of orthotopically implanted 786-0 human RCC in nude mice**

<table>
<thead>
<tr>
<th>Group and treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Weight of primary tumors (mg)</th>
<th>Incidence of metastases to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE (% reduction)</td>
<td>Lymph nodes</td>
</tr>
<tr>
<td>Control</td>
<td>245.7 ± 390.4 (41.4 ± 41.0)</td>
<td>5/6</td>
</tr>
<tr>
<td>AN-201</td>
<td>100.3 ± 194.7 (270.2 ± 60.3)</td>
<td>5/6</td>
</tr>
<tr>
<td>AN-238&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0 (55.3 ± 44.3&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>0/7&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Treatment started 2 weeks after implantation and consisted of three i.v. injections at 150 nmol/kg given biweekly. The experiment was terminated 6 weeks after the first injection.

<sup>b</sup> In three mice, only the residual scar tissue was found at implantation site.

<sup>c</sup> No SST binding was detected on this tumor. The large orthotopic tumor was accompanied by metastases to lymph nodes.

<sup>d</sup> P < 0.001 versus control, and P < 0.05 versus AN-201 (two-tailed t-test).

<sup>e</sup> P < 0.05 versus control, and P < 0.05 versus AN-201 (Fisher’s exact test).

<sup>f</sup> P < 0.01 versus control (Fisher’s exact test).

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**Table 3 General and SST receptor-specific side effects of treatment with cytotoxic radical AN-201 or cytotoxic SST analogue AN-238 in nude mice bearing xenografts of human 786-0 RCC**

<table>
<thead>
<tr>
<th>Group and treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>General side effects</th>
<th>SST receptor-specific side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW, g (% of control)</td>
<td>WBC, counts/mm&lt;sup&gt;3&lt;/sup&gt; (% of control)</td>
</tr>
<tr>
<td>Control</td>
<td>29.1 ± 0.97</td>
<td>9432 ± 1210 (94%)</td>
</tr>
<tr>
<td>AN-201</td>
<td>27.3 ± 0.66</td>
<td>4840 ± 920 (51%)</td>
</tr>
<tr>
<td>AN-238&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.1 ± 1.0</td>
<td>4180 ± 485 (44%)</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Three i.v. injections of 150 nmol/kg of AN-201 or AN-238 were given on days 1, 8, and 21. The side effects were evaluated 2 weeks after the last injection. Values are means ± SE.

<sup>b</sup> The pituitary reserve of GH was estimated on the basis of serum GH level after stimulation with GHRH (2 µg i.v.).

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<sup>c</sup> P < 0.05 versus control (two-tailed t-test).
expression of \textit{sst}_A or \textit{sst}_5 in this cell line. This allowed us to use as a negative control for testing the specificity of SST receptor targeting \textit{in vitro} and \textit{in vivo}. Thus, the proportion of SST receptor-positive RCC lines chosen for this study (two of three) corresponds roughly to the incidence of \textit{\textsuperscript{125}I-}{\textsuperscript{Tyr}}-octreotide-prefering binding sites (72\%) demonstrated on the specimens of human RCC (14). Among SST receptor subtypes detected on surgical specimens of RCC, \textit{sst}_2 appears to be the most common, whereas \textit{sst}_1 and \textit{sst}_5 occur less frequently (15). However, no data are available on the incidence of \textit{sst}_5.

The mRNA encoding for \textit{sst}_5, which displays a moderate binding of SST octa- peptides (22), was not detected in RCC specimens (15). In accordance with the results for RC-160, obtained by radioligand binding assays and the pattern of expression of SST receptor subtypes, we showed high-affinity binding of AN-238 to membranes of SW-839 and 786-0 tumors.

Using the analysis of alleles for the D3S2418 microsatellite marker in cocultured 786-0 and CAKI-1 lines, we demonstrated the selective cytotoxicity of AN-238 against cells expressing SST receptors. Cytotoxic SST analogue AN-238 preferentially decreased the number of \textit{sst}_5-positive 786-0 cells. In contrast, an equimolar amount of the radical AN-201 did not affect preferentially any of the two lines. This finding supports the hypothesis that accumulation of a cytotoxic drug mediated by SST receptors can overcome the chemoresistance in neoplastic cells.

A comprehensive series of \textit{in vivo} experiments was conducted to evaluate the efficacy and specificity of SST receptor-targeted chemotherapy in human RCC. A significant inhibition of tumor growth after treatment with cytotoxic SST analogue AN-238 was observed in the \textit{sst}_2A-positive SW-839 line and in 786-0 tumors that express \textit{sst}_5. In both models, equimolar doses of unconjugated cytotoxic radical AN-201 exerted only a nonsignificant effect. In accord with the lack of binding of octapeptide SST analogues to CAKI-1 RCC \textit{in vitro}, the effect of AN-238 on the growth of CAKI-1 tumors was marginal and comparable to the inhibition caused by the radical AN-201. These results indicate that the tumor inhibition achieved by AN-238 depends on the specific binding of the cytotoxic conjugate by SST receptor-positive tumors and that both \textit{sst}_1 and \textit{sst}_5 are essentially equally effective in mediating the antitumoral effect of AN-238. This also implies that chemotherapy targeted to SST receptors may be effective in cancers that express predominantly \textit{sst}_5, such as colon or primary prostate carcinomas (23, 24).

In experiment 4, we evaluated the efficacy of SST analogue AN-238 in a model of disseminated RCC. Six weeks after initiation of the treatment, the mean weight of 786-0 primary tumors treated with AN-238 was reduced by 87\% compared with controls. In three of seven mice, the tumors appeared to be eradicated, and only residual connective tissue was found at the implantation site. In one mouse, however, no response was observed, which was later explained by the lack of SST binding sites on this particular tumor, as determined by a radioligand assay. Loss of SST receptors after several passages of experimental tumors was observed previously (25) and is a result of the clonal growth and heterogeneity of neoplastic tissue. A slightly stronger effect of AN-238 on the growth of orthotopic tumors compared with its action on s.c. xenografts was noted in an earlier study (12), and is most likely caused by better vascularization and hence a higher drug supply to the neoplastic tissue. The targeting of AN-238 to tumor vasculature expressing \textit{sst}_2 may also account for a better response of orthotopic tumors (26). The cytotoxic radical AN-201 affected the progression of orthotopic 786-0 tumors only nonsignificantly. Another promising observation is that the treatment with AN-238 almost completely eliminated the lymphatic and hematogenous spread of 786-0 cancers. A potent inhibition of tumor dissemination by cytotoxic SST analogue AN-238 was similarly observed in ortothopic PC-3 human prostate cancers expressing \textit{sst}_2A and \textit{sst}_5 (12). The mechanisms of inhibition of metastatic lesions may include the growth arrest of primary tumors, thus preventing the dissemination of the cancer and/or targeting to already established micrometastases, leading to their regression. Because metastases of human RCC express SST receptors as demonstrated by scintigraphy with radiola- beled octreotide (16), it could be expected that targeted chemotherapy with AN-238 may significantly improve the management of disseminated RCCs.

In the present study, we observed similar side effects of AN-238 and AN-201 administered at the cumulative dose of 450 nmol/kg. Because AN-201 is linked to the carrier peptide through an ester bond, comparable toxicity patterns of both cytotoxic compounds in mice suggest a rapid deconjugation of the radical from the conjugate by serum CE (EC 3.1.1.1). CE activity in mouse serum is \textasciitilde10-fold higher than in human serum (27). Thus, in a recent study we investigated the tolerance to AN-238 and AN-201 in tumor-free nude mice with CE activity suppressed by diisopropyl fluorophosphate (28). We found that the tolerance to AN-238 could be doubled, whereas the tolerance to AN-201 was not affected (28). Under these conditions, the half-life in mouse serum of cytotoxic LHRH analogue AN-152, consisting of doxorubicin-14-O-hemiglutamate linked to \textsuperscript{\textit{\alpha}-Lys\textsuperscript{\textit{\beta}}}LHRH, was extended from 20 to \textasciitilde70 min, which is still much shorter than the 120 min observed in human serum. Because the tolerance to AN-238 depends on its stability in serum, it could be expected that AN-238 will be even less toxic in human beings than in nude mice.

The study of the tolerance of AN-238 in tumor-free nude mice after pharmacological inhibition of CE activity also suggests that the lower general toxicity of AN-238 is the result of drug disposition to normal tissues expressing SST receptors (28). This is in accordance with the \textit{in vivo} uptake of radioactivity by SST receptor-positive organs after administration of radiolabeled SST analogues (29–31). To evaluate the possible side effects of targeting SST receptors in normal tissues, we investigated in the present study the function of the pituitary gland in animals treated with a cumulative dose of 450 nmol/kg of AN-238 or AN-201. Both compounds diminished nonsignificantly the GH response to GHRH compared with the controls, but the pituitary reserve of GH in mice given AN-238 was lower than in animals given AN-201. Likewise, a selective impairment of the pituitary function was observed in rats after administration of the cytotoxic LHRH analogue AN-207, which contains AN-201 (32). However, the pituitary insufficiency caused by AN-207 is only transient, and recovery occurs in 3 weeks. Similarly, a good tolerance to AN-238 at cumulative doses of 800–900 nmol/kg by nude mice with suppressed CE activity suggests that the side effects of SST receptor-targeted chemo- therapy are much less harmful than the general adverse reactions to unconjugated cytotoxic radicals, such as myelosuppression (28). This view is also supported by preliminary clinical studies that showed no or only low-grade toxicity to the pituitary, the kidneys, and the bone marrow after administration of radiolabeled SST analogues to cancer patients, whereas a significant reduction in tumor progression was achieved (31, 33, 34).

In conclusion, we demonstrate here that the cytotoxic SST analogue AN-238 strongly inhibits the growth of human RCCs expressing SST receptors. Because chemotherapy with AN-238 can be also targeted to SST receptor-positive RCC metastases, this therapeutic modality might be of benefit to patients with residual cancer tissue after radical nephrectomy or with metastatic disease.

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Inhibition of Metastatic Renal Cell Carcinomas Expressing Somatostatin Receptors by a Targeted Cytotoxic Analogue of Somatostatin AN-238

Artur Plonowski, Andrew V. Schally, Attila Nagy, et al.

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