A tumor-selective somatostatin analog (TT-232) with strong in vitro and in vivo antitumor activity

(apoptosis/cell proliferation/tyrosine kinase/xenograft)


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ABSTRACT We report a series of new in vitro and in vivo data proving the selective antitumor activity of our somatostatin structural derivative, TT-232. In vitro, it inhibited the proliferation of 20 different human tumor cell lines in the range of 50–95% and induced a very strong apoptosis. In vivo TT-232 was effective on transplanted animal tumors (Colon 26, B16 melanoma, and S180 sarcoma) and on human tumor xenografts. Treatment of MDA-MB-231 human breast cancer xenografted in mice with low submaximal doses of TT-232 [0.25 and 0.5 mg/kg of body weight (b.w.)] caused an average 80% decrease in the tumor volume resulting in 30% tumor-free animals surviving for longer than 200 days. Treatment of prostate tumor (PC-3) xenografted animals with 20 mg/kg of b.w. of TT-232 for 3 weeks resulted in 60% decrease in tumor volume and 100% survival even after 60 days, while 80% of nontreated animals perished. We have demonstrated that TT-232 did not bind to the membrane preparation of rat pituitary and cortex and had no antiserotory activity. TT-232 was not toxic at a dose of 120 mg/kg of b.w. in mice. Long-term incubation (24 h) of tumor cells with TT-232 caused significant inhibition of tyrosine kinases in good correlation with the apoptosis-inducing effect. The level of p53 or KU86 did not change following TT-232 treatment, suggesting a p53-independent apoptotic effect. Preincubation of human breast cancer cells (MDA-MB-453) with TT-232 for 2 h decreased the growth factor receptor autophosphorylation. All of these data suggest that TT-232 is a promising and selective antitumor agent.

Somatostatin, a natural tetradecapeptide, inhibits both the growth hormone release (1) and various other endocrine secretions (i.e., glucagon, insulin, and gastrin) (2, 3). It inhibits or regulates several cell functions as well, and it has also been discovered to be an important endogen antiproliferative agent (4–8). In recent years, many potent somatostatin analogs have been developed as antisecretory and antiproliferative hormones (Sandostatin or Octreotide of Sandoz, Somatuline of Biomedasure, and RC-160 of Debiopharm), acting longer than the native hormone, and they have been used in the treatment of hormone dependent tumors, but their administration as antitumor agents is limited because of their side effects (9–13). Lots of efforts have been made to obtain a tumor selective somatostatin analog but without any significant success.

Previously we reported a unique, potent tumor-selective somatostatin analog of a five-residue ring structure: D-Phe-Cys-Tyr-d-Trp-Lys-Cys-Thr-NH₂ (TT-232). This analog had practically no growth hormone (GH) release inhibitory activity either in superfused rat pituitary cells or in rats in vivo. TT-232 showed a strong antiproliferative effect both in vitro and in vivo (14–16) and induced apoptosis in a pancreatic tumor cell line (17) as demonstrated for other somatostatin analogs (18, 19). This compound has a special structure, its backbone behaving very differently from that observed in the GH active analogs (20). TT-232 inhibited tyrosine kinase activity of some human colon tumor cell lines, and this inhibition correlated well with the inhibition of cell proliferation (14). When incubating colon tumor cells with this peptide for short periods of time (10–100 min), no significant inhibition of tyrosine kinases was observed, but a significant stimulation of tyrosine phosphatases could be detected with a biphasic dose dependency (21). TT-232 has been tested on 60 various human tumor cell lines by the National Cancer Institute of the National Institutes of Health. It was found that at a higher dose (100 μM) TT-232 had a very strong antiproliferative effect on 58 cell lines, while TT-248, one of our "traditional type" somatostatin analogs, which in other respect showed similar biological effects to that of RC-160 or Sandostatin had practically no effect on the same cell lines (unpublished results). Somatostatins activate protein kinase C and stimulate the inositol trisphosphate level in colon tumor cells following a long term incubation (22, 23). So it may be concluded that TT-232 interacts with various signaling pathways in tumor cells, and the interrelationship of these pathways may determine the antiproliferative effect via the induction of apoptosis and the inhibition of mitosis. In vivo, TT-232 has been very effective in the Lewis lung tumor, spleen-liver metastasis model in mice, causing a 70% inhibition in the number of metastasis (17).

To test the possibility whether TT-232 could be a tumor-selective drug, several new in vitro and in vivo studies, as well as mechanism of action studies have been carried out, and the results of these studies are presented here. The objective of these studies were to investigate what kind of tumor cells could be the primary target of TT-232, to investigate the selectivity of TT-232 considering the traditional somatostatin-like effects, and to investigate the interrelationship of tyrosine kinase inhibitory, antiproliferative, and apoptosis-inducing effect of TT-232. The other objectives of these studies were to investigate the in vivo antitumor effect of TT-232.

MATERIALS AND METHODS

Peptides. d-Phe-Cys-Tyr-d-Trp-Lys-Cys-Thr-NH₂ (TT-232) and β-Asp(Ind)-Cys-Tyr-d-Trp-Lys-Val-Cys-Thr-NH₂ (TT-256) were synthesized by Dr. P. Dargatz, University of Rochester, Rochester, N.Y. TT-232 was purified by reverse-phase-HPLC on an Ultra-Performance Liquid Chromatography system (Waters, Milford, Mass.) using a Phenomenon C18 analytical column (250 × 4.6 mm) and an analytical ultradispenser. Peptides were monitored at 214 nm.

Abbreviations: GH, growth hormone; b.w., body weight.

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Tumor Cell Culture. The cells were obtained from American Type Culture Collection and cultured with RPMI medium 1640 or Leibovitz’s L-15 medium supplemented with 10% fetal calf serum (all from SEBAK, Aidenbach, Germany). The cells were kept at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

In Vitro Antitumor Assays. Approximately 350,000 cells were cultured in 2 ml of culture medium in a well (6-well plates were used, Greiner, Nuringen, Germany) and incubated for 24 h with different doses of TT-232 in the absence of fetal calf serum. After trypsinization and dispersion, the cells were dyed with trypan blue and counted in a hemocytometer.

Study of Apoptosis. Tumor cells (10⁵/ml) were treated with various doses of TT-232 for 8–24 h. Cell count was determined regularly at 24, 48, and 72 h. Triplicate determinations per dose and time points were performed both in cases of treated and untreated cell cultures. Three coverslips per dose and time point were fixed in ethanol/acetic acid (3:1) and stained with hematoxylin and eosin. The specific morphology of apoptotic cells (nuclear pyknosis or karyorrhexis, cytoplasmic shrinkage, apoptotic bodies in the cytoplasm of nonapoptotic tumor cells) was identified by the hematoxylin and eosin staining and by routine transmission electronmicroscopic investigation. The ratio of apoptotic and mitotic figures was determined by counting 500 cells. Immunohistochemical reaction using Apop-Tag kit (26) (Oncor) was applied to detect in situ the new 3'-OH DNA ends characteristic to apoptosis.

Tyrosine Kinase Activity Assay. The assay was carried out as described earlier (27) using a synthetic peptide substrate (E₁₁G₁). The results were expressed as incorporated pmol of ³²P per mg of protein per min.

Animals and Experimental Procedures for in Vivo Tests. Colon 26 tumor fragments (from Karolinska Institute, Stockholm) were s.c. transplanted into BALB/C inbred mice; B16 melanoma cell suspension (also from Karolinska Institute) was transplanted into BDF₁ hybrid mice; S180 sarcoma tumor fragments (Chester Beatty Cancer Research Institute, London) were transplanted into Swiss outbred mice. Ten animals were in each group (21–23 g). The i.p. treatment with different doses of TT-232 (0.5, 1, 2, 5, 10, 25 mg/kg body weight (b.w.) twice a day) was initiated 1 day after the tumor transplantation and continued till the first animal perished. Lifetime changes were compared with untreated tumor-bearing animals, and the size of solid tumors was measured with a caliper two or three times weekly. Tumor volume was calculated using the formula %b × 0.6 × π/6 where a means the shorter diameter, which is perpendicular to b the longest diameter of a tumor (28).

Human MDA-MB-231 breast and PC-3 prostate tumor cell lines obtained from American Type Culture Collection were xenografted in immunodeficient mice. Female 6–7-week-old inbred CBA/Ca mice from our specified pathogen-free breeding were used. Pieces of adequate tumor tissue (1–1.5 mm³) were transplanted s.c. Development of immunosuppression in normal CBA/Ca mice was carried out by thymectomy, whole body irradiation (19.5 Gy), and bone marrow transplantation. Two or three weeks after tumor transplantation, tumors had grown to a volume of approximately 0.1 cm³, the mice were randomized and divided into experimental groups (10 mice per group). TT-232 was administered in a saline vehicle containing 0.1% acetic acid s.c. on the side opposite of the tumor once or twice daily at doses of 0.25, 0.5, 5, 10, and 20 mg/kg of b.w. Treatments of breast and prostate tumor-bearing animals with TT-232 were started on days 21 and 18, respectively, after tumor transplantation. Drug administration was applied con-

RESULTS

In Vitro Antiproliferative Effect of TT-232 on Human Tumor Cell Lines. Table 1 summarizes the results. TT-232 showed at least 50% antiproliferative effect on the examined cell lines; however, around 90% inhibition was achieved on breast carcinoma, prostate tumor, pancreatic, leukemia, melanoma, and gastric tumor cell lines. The effect of TT-232 and RC-160 was compared on the proliferation of PC-3 and HT-29 tumor cell lines. In a representative experiment on PC-3 cell line, TT-232 at 30 μg/ml dose showed a 90 ± 2% decrease in the number of tumor cells, but RC-160 resulted in only a 29 ± 2% decrease. On HT-29 cell line, the inhibition caused by TT-232 was about 72 ± 5%, and by RC-160 it was only 25 ± 3% when both
Table 1. *In vitro* antiproliferative effect of TT-232 on different tumor cell lines

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Inhibition of cell proliferation (%)</th>
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<tbody>
<tr>
<td>1. MCF7 breast (16)*</td>
<td>87 ± 12</td>
</tr>
<tr>
<td>2. BSMZ breast</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>3. SK-BR-3 breast</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>4. MDA-MB breast</td>
<td>44 ± 6</td>
</tr>
<tr>
<td>5. PC-3 prostate</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>6. DU 145 prostate (14)*</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>7. SW620 colon (14)*</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>8. HT-29 colon (14)*</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>9. COLO 205 colon</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>10. A-549 lung</td>
<td>59 ± 6</td>
</tr>
<tr>
<td>11. Lewis Lung Carcinoma</td>
<td>97 ± 5</td>
</tr>
<tr>
<td>12. P818 pancreatic</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>13. P818-4 pancreatic (17)*</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>14. HT-58 lymphoma</td>
<td>84 ± 8</td>
</tr>
<tr>
<td>15. K-562 leukemia</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>16. WM 938 melanoma</td>
<td>81 ± 7</td>
</tr>
<tr>
<td>17. M1 melanoma</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>18. SK-OV-3 ovary</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>19. 4-1ST gastric</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>20. M27 rat carcinoma</td>
<td>67 ± 11</td>
</tr>
</tbody>
</table>

The applied dose of TT-232 was 20–30 μg/ml, and the treatment lasted for 24 h.

*These data have already been published. The inhibitory potency of TT-232 is shown around the IC50 values of TT-232 on each cell line. IC50 values were between 20 and 40 μM and were determined in several but at least in two independent experiments. Values are the mean ± SD.

peptides were applied at the same 30 μg/ml dose and cells were treated for 24 h.

**In Vivo Antitumor Effect of TT-232.** TT-232 strongly inhibited the tumor growth of transplanted Colon 26 tumor reaching a 70% inhibition at the low submaximal dose of 750 μg/kg of b.w. of TT-232. Even at the dose of 15 μg/kg of b.w. more than 60% inhibition was achieved by the 24th day relative to control animals (Fig. 1).

Transplanted B16 melanoma was also inhibited with about 50 ± 8% by 750 μg/kg of b.w. of TT-232 after 19 days. A smaller dose (15 μg/kg of b.w.) resulted in 25 ± 7% inhibition of tumor growth.

S180 sarcoma tumor growth was inhibited with about 50 ± 10% by 750 μg/kg of b.w. of TT-232.

Treatment for 30 days with 0.25 and 0.5 mg of TT-232 per kg of b.w. once a day significantly decreased the xenografted MDA-MB-231 tumor volume, and the complete regression of tumors was observed at some animals in both dose groups. Fig. 2 illustrates the tumor growth curves of five mice in the 0.5 mg/kg of b.w. dose group, where about 80% tumor growth inhibition was observed compared with the control animals.

TT-232 treatment for 30 days resulted in two tumor-free surviving animals for longer than 200 days out of eight mice at the dose of 0.25 mg/kg of b.w. and three out of eight mice in the 0.5 mg/kg of b.w. dose group.

In the literature to perform the *in vivo* assays, a very broad dose range of the various somatostatin analogs has been used (ranging from 25–150 μg/mice = 1.25–60 mg/kg of b.w.). In the very unresponsive PC-3 xenograft we used higher doses of TT-232 (5, 10, and 20 mg/kg of b.w.), and at 20 mg/kg of b.w. dose, a 60% inhibition of tumor growth was achieved after 3 weeks of treatment (Fig. 3). Lower doses of TT-232 inhibited the tumor growth less. Treatment with 20 mg/kg of b.w. of TT-232 for 3 weeks resulted in 100% survival 60 days post tumor transplantation, while in the control group the survival was only 20% (one mouse out of five). No difference was found in the percentage of survival among the different, but lower dose groups; all groups showed 60% survival.

**Toxicology Studies with TT-232.** Further *in vivo* studies where higher doses of TT-232 were to be applied (about 10–20 mg/kg of b.w.) necessitated toxicology studies with TT-232. These experiments showed that a lethal dose for TT-232 could not be determined because beyond a small and temporary body weight loss (10% compared with the control), no death was observed even when this peptide was applied at a dose of 120 mg/kg of b.w. A significant growth in the weight of uterus (about 70%) and a decrease in the weight of lungs (about 30%) could be observed. The weight of other organs was not affected by the treatment with different doses up to 120 mg/kg of TT-232. The hematological parameters and the qualitative blood picture were not affected either. TT-232 even at 120 mg/kg of b.w. dose had no effect on the number and the qualitative picture of the bone marrow cells. Histological studies of different organs from TT-232 treated mice did not show significant modification in the tissues.

**Studies Proving the Selectivity of TT-232.** Previously we have reported that *in vitro* 10−8 M of TT-232 did not inhibit the GH releasing hormone-induced GH release, and in *in vivo* assays in rats no decrease of initial serum GH levels was
observed by TT-232 at the applied doses of 1 and 4 μg/100 g (16).

Binding studies with TT-232 have also been carried out and compared with the binding of [Tyr³]octreotide. The binding of [¹²⁵I-Tyr³]octreotide to the membrane preparation of rat pituitary (maximum binding capacity was 2614 fmol/mg of membrane protein) could be displaced in a dose-dependent manner with unlabeled [Tyr³]octreotide but not with TT-232. Similar results were obtained with the rat cortex membrane preparation (data not shown). The fact that TT-232 did not bind either to the pituitary or to the brain receptors supports the previous finding that it has no effect on GH release.

It has been reported that somatostatin inhibits the secretion of gastric acid (2). We found that TT-232 did not inhibit the secretion of gastric acid; meanwhile results shown in Fig. 5 indicate that RC-160 and TT-248 two “traditional type” somatostatin analogs induced significant inhibition (90%) of gastric acid secretion when administered concurrently with different doses (0.25 μg/kg; 1 μg/kg; 4 μg/kg) of pentagastrin.

Mechanism of Action Studies with TT-232. In the present work, we have demonstrated that TT-232 induces apoptosis (programmed cell death) in human colon (HT-29 and SW620), pancreatic (P818), leukemia (K-562), melanoma (WM 938/B, M-1, EP), and lymphoma (HT-58) tumor cell lines. The occurrence of apoptosis was followed by light- and electron-microscopic as well as by immunohistochemical studies. In a representative experiment, the apoptotic index increased about 70 times in M-1 melanoma, 60 times in K-562 leukemia, 13 times in P818 pancreatic cells, seven times in HT-29 human colon tumor cells, and three times in PC-3 prostate tumor cells after 48 h incubation with 10 μg/ml of TT-232. In case of WM melanoma and EP melanoma cells, five and four times increase of apoptotic index has been observed after 24 h of incubation with the same dose of TT-232 (Fig. 5). In some of the above described cell lines, further increasing the dose of TT-232 and the time of the incubation cell lysis occurred, which is probably due to a so far unknown mechanism resulting in destruction of the structure of cell membranes as a consequence of apoptosis. We have investigated the effect of 10 and 30 μg/ml TT-232 on the level of wild-type p53 protein in P818 pancreatic and in HT-29 colon tumor cells and found no alterations in p53 level. We also investigated the effect of TT-232 on the translocation of the KU86 protein from the cytosol to the nucleus (KU86 is a nuclear somatostatin receptor associated with p53) and found no significant effect, suggesting that TT-232 induces apoptosis on a p53-independent way.

TT-232 strongly inhibited tyrosine kinases (75%) after long term incubation (24 h) in SW620 colon tumor cell lines (Fig. 6), this effect correlates well with the antiproliferative and apoptosis inducing effect of TT-232.

In MDA-MB-453 human breast cancer cells, different doses (0.1, 1, or 30 μg/ml) of TT-232 and Sandostatin resulted in a decrease in the phosphotyrosine content of the proteins. At 30 μg/ml dose, no bands of phosphotyrosine could be visualized at the immunoblot analysis in the case of TT-232, while Sandostatin also caused a decrease in the phosphotyrosine content but to a much smaller extent.

Stability Studies with TT-232. The stability of TT-232, both in solid (lyophilized) form and in aqueous solution, was investigated during storage at different temperatures. Samples stored for various time periods were analyzed for TT-232 content as well as for degradation products using HPLC methods (31). TT-232 was stable at least for 36 days in solid (lyophilized) form kept at 22°C. In sterile aqueous solution it was stable at least for 40 days when kept in refrigerator, for 35 days at ambient temperature, and for 2 days at 37°C.

DISCUSSION

A somatostatin structural derivative (TT-232) has been developed in our laboratory with strong antiproliferative effect but no GH release inhibitory activity (14–16). In the present paper, we report a series of new in vitro and in vivo studies demonstrating that TT-232 is the first tumor-selective and
releasing inhibitory and antiserotonin activity, we believe that the antiproliferative and apoptosis inducing effect of TT-232 is mediated through a still unknown receptor. Known somatostatin analogs like RC-160 or Sandostatin exert their antiproliferative effect through the STR2 receptor, which is also present in the pituitary (38). We are currently working on the isolation of the TT-232 receptor from tumor cells with affinity chromatography.

In *in vivo* experiments, relatively low doses of TT-232 have been applied, although our *in vitro* data show that for the dramatic antiproliferative and apoptosis-inducing effect, a critical dose—which also depends on the cell type—has to be reached. TT-232 had strong *in vivo* antitumor activity even at relatively low doses, which give us very good perspectives for the application of higher doses, reaching the critical threshold dose for the induction of apoptosis. Toxicity studies clearly demonstrated that TT-232 does not show toxic side effects even at a dose of 120 mg/kg of b.w.

In conclusion, TT-232 has good perspectives as a potent and selective antitumor drug.

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**Fig. 6.** Dose dependence of TT-232 stimulated tyrosine kinase activity on SW620 colon tumor cells. SW620 cells were incubated with various concentrations of TT-232 for 24 h at 37°C. Each value is representative of at least three separate experiments. The control (100%) value denotes a tyrosine kinase activity of 0.137 32P/min/mg of protein; *P < 0.05; **P < 0.005; ***P < 0.001.