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A Specifically Radiolabeled Somatostatin Analog with Strong Antitumor Activity Induces Apoptosis and Accumulates in the Cytosol and the Nucleus of HT29 Human Colon Carcinoma Cells

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The new heptapeptide somatostatin analog TT-232 decreases proliferation of HT-29 human colon carcinoma cells in vitro by reducing mitotic and increasing apoptotic activity. We have synthesized and characterized a specifically tritium labeled ^{3}H-Tyr3-TT-232 (30 Ci/mmol) to investigate the effect and the fate of this antitumor peptide on human colon tumor cells. ^{3}H-labeled TT-232 could be detected on the cell surface, on cytoplasmic membranes and also in the nucleus of HT-29 cells, 1–6 h after the administration of 0.5 and 50 μg/mL [^{3}H]TT-232. Binding and internalization of TT-232 to human colon tumor cells at a relatively high dose provide further evidence for the existence of low-affinity somatostatin receptors in such cells, which might mediate the apoptosis-inducing effect. Our data suggest the possible use of TT-232 in the treatment of human colon tumors.

Key Words: Somatostatin analog; antitumor activity; apoptosis; colon tumor; internalization.

Introduction

The inhibitory effect of somatostatin and luteinizing hormone-releasing hormone (LHRH) analogs on the multiplication of human tumor cell populations has been attributed to the enhancement of apoptosis *(1–7)*. Specific binding sites for somatostatin analogs *(8,9)* and hypothalamic peptide analogs *(10,11)* were found in various tumor cells and in fibroblast *(12)*. Binding of a radiolabeled tumor-selective gonadotropin-releasing hormone (GnRH) analog to PC-3 human prostate cancer cells and the induction of apoptosis by this analog has been reported by our group *(13)*.

Somatostatin was reported to induce apoptosis of MCF-7 human breast adenocarcinoma cells as measured by flow cytometry *(14)*. The somatostatin analog octreotide induced apoptosis associated with a rapid, time-dependent induction of wild-type p53 and an increase in Bax, but not associated with the induction of p21 or c-Myc. The DNA fragmentation in this cell line is due to selective activation of a cation-insensitive acidic endonuclease *(15)*. As other authors demonstrated, no G_1 cell-cycle arrest has been found in the case of octreotide-induced apoptosis, which is signaled uniquely through human SSTR-3 *(16)*. The role of SSTR-1 in growth inhibition of CHO-K1 cells also has arisen, because the stimulation of the receptor increases a phosphotyrosine phosphatase activity in the cells *(17)*. In GH3 rat pituitary tumor cells, the proliferation inhibiting effect of 1 μg/mL octreotide was demonstrated to be the result of a block in progression from the G_0 to G_1 phase to the S phase, but no apoptotic cells have been found *(18)*.

Recently we have demonstrated that our new tumor-selective somatostatin analog; TT-232 ([$Cys^2 \rightarrow Cys^6$] H-D-Phe-Cys-[^{3}H]-Tyr-D-Trp-Lys-Cys-Thr-NH_2), induces very strong apoptosis in various kinds of tumor cells. The evidence of apoptosis has been demonstrated by electron microscopy, DNA gel electrophoresis (ladder formation), and TUNEL (*in situ* DNA end-labeling) reaction *(19)*. The antiproliferative and apoptosis-inducing effect of TT-232 was the best among several analogs tested *(19,20)*. In the present work, HT-29 human colon tumor cell cultures were treated with the new somatostatin analog TT-232, and its

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effect on the mitotic and apoptotic activity was studied. We have synthesized a specifically tritium-labeled TT-232, and this radiolabeled hormone was administered to HT-29 cells in order to study the binding of this compound to various cellular compartments. Specific binding sites for somatostatin analogs *(10,11)* and hypothalamic peptide hormones on various kinds of tumor cells have been reported *(13,21,22)*. Also it was suggested in the literature that peptide hormones might have a physiological effect after internalization *(23–25)*. The aim of our work was to investigate the effect and the fate of TT-232 in HT-29 human colon tumor cells.

Results

Antiproliferative and Apoptosis Inducing Effect of TT-232

The antiproliferative and apoptosis-inducing effects of TT-232 on HT29 human colon tumor cells were shown by classical morphological characteristics, revealed on HE-stained preparations (Fig. 1) and previously confirmed by electron microscopy, DNA-ladder, and TUNEL reaction as well as by positive fluorescence after Hoechst 33258 staining. Cell number, apoptotic index, and mitotic index after TT-232 treatment of HT-29 cells are shown on Fig. 2A–C. Apoptotic index started to increase at h 12 after TT-232 treatment and reached 48% at h 18 and 72.5% at h 24. The number of mitotic cells decreased from 2% to 1% at h 12 and no mitotic figures could be detected at h 24. Although the viable cell number doubled in the first 24 h, significant decrease in cell number was observed in case of 50 μg/mL TT-232 treatment at h 18 (74% vs control) and at h 24 (88.4% vs control).

The propidium iodide staining showed a marked increase of stained cells in treated sample (Fig. 3). There were very few dead cells in untreated control and the majority of cells were positive 12 h after 50 μg/mL TT-232 treatment. This method unspecifically detects the loss of viability without an extra morphological data.

The *in situ* end labeling of fragmented DNA showed some apoptotic nucleus in the control, but an extended cell death in treated culture. The positivity of the nuclei (brown) correlated well with the morphology, which showed a condensation and shrinkage (Fig. 4)

FACS Analysis

FACS analysis of the dose and time-dependence of TT-232 induced DNA fragmentation (sub-G_1 fraction) with (Fig. 5A) or without (Fig. 5B) serum is shown. In the treated cultures, a transient and moderate increase was in the G_0-G_1 and decrease in the S-phase fraction at 12–14 h. During the 20 h of incubation, the control values were between 8% and 18%. The ratio of apoptotic and fragmented nuclei reached about 75% in the case of 50 μg/mL TT-232 treatment in the serum-free medium after 2 h incubation and moved between 65% and 82% during the first 24 h. In the presence of serum 50 μg/mL TT-232 induced DNA fragmentation reached maximum after 10 h incubation, and remained about 55% up until 24 h. Lower doses of TT-232 caused a local maximum (about 35%) in DNA fragmentation at 8–10 h and went back to the control values.

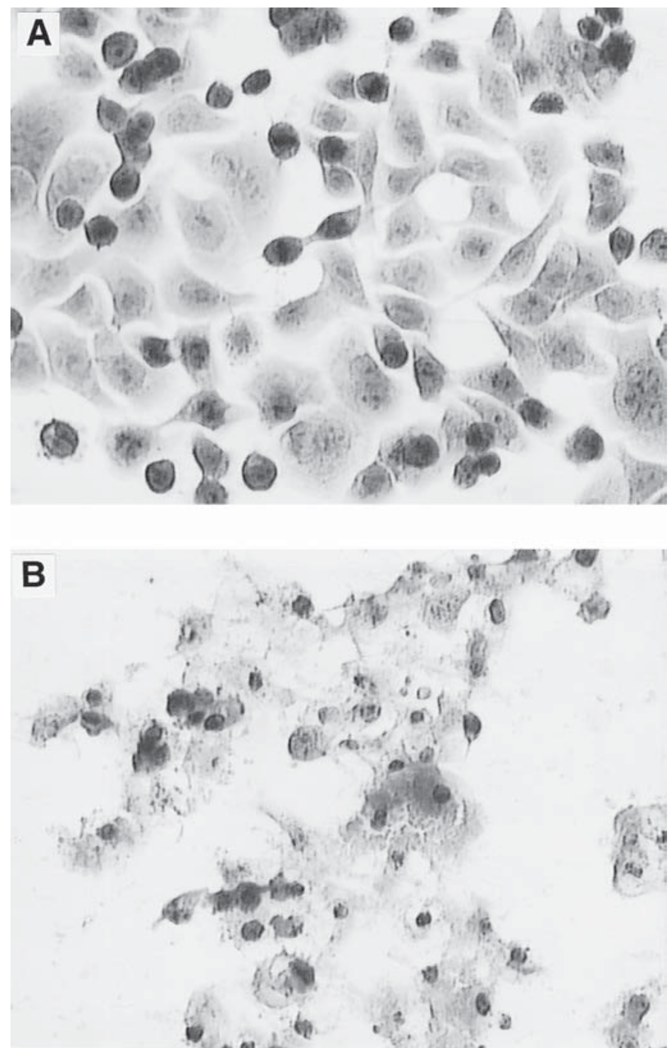

Fig. 1. Hematoxilin-eosin staining of HT-29 colon carcinoma cells. **(A)** Untreated cells. Lots of mitotic cells (dark) could be seen. **(B)** Cells were treated with 50 μg/mL TT-232 for 12 h in the presence of serum. The cell structure is changed, the nuclei show condensation and decrease in volume.

Labeling of HT-29 Cells with [^{3}H] TT-232

After 1 h of treatment with 0.5 μg/mL [^{3}H]TT-232, marked labeling over the cell surface, the cytoplasm, and especially over the nucleus was observed (Fig. 6B). At 6 h these values decreased, but were still considerably high (Fig. 6D) and nearly equaled those of cells that were treated for 1 h with 50 μg/mL [^{3}H] TT-232. Interestingly, the 1-h values after the high dose (Fig. 6C) were significantly lower than the 1-h values after the low dose.

After 6 h of treatment with the high dose 50 μg/mL [^{3}H] TT-232, the labeling over the cell surface, cytoplasm, and nucleus (Fig. 6E) increased in comparison to the 1-h values and exceeded the grain counts taken at the same time after 0.5 μg/mL [^{3}H]TT-232 treatment. These relations were

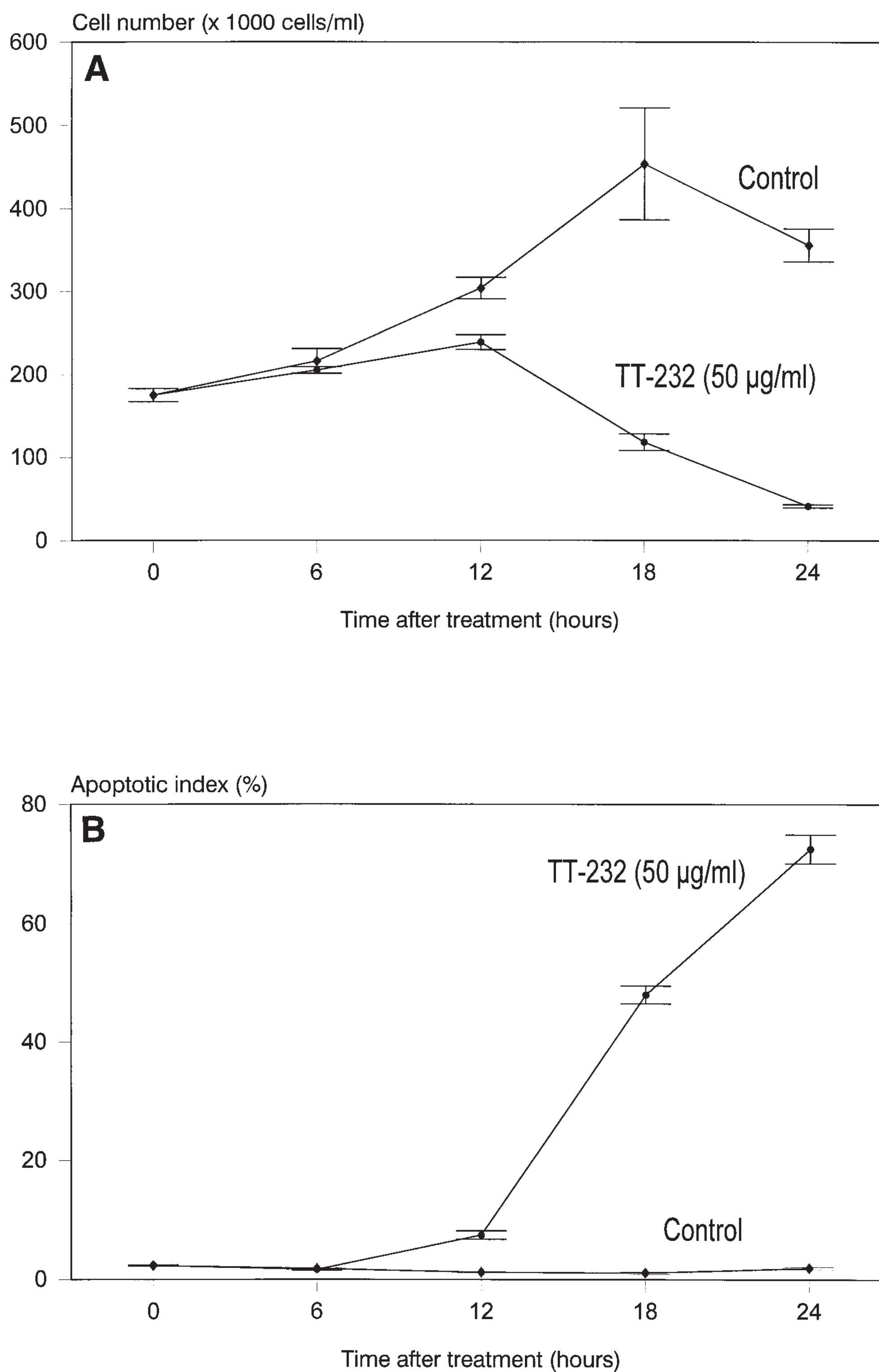

Fig. 2. HT-29 human colon carcinoma cell culture after TT-232 (50 µg/mL) treatment. **(A)** Viable cell number. **(B)** Apoptotic index.

equally true when the grain count was calculated per cell or according to length of cell surface or to cytoplasmic and nuclear area (Table 1). The cells of the sample with the combined treatment ([^{3}H] TT-232 and "cold" TT-232) did not show labeling (Fig. 6A).

Discussion

Our studies demonstrated that treatment with TT-232 results in an increase of apoptosis, and a decrease in mitotic activity and viable cell number in HT-29 human colon carcinoma cell culture.

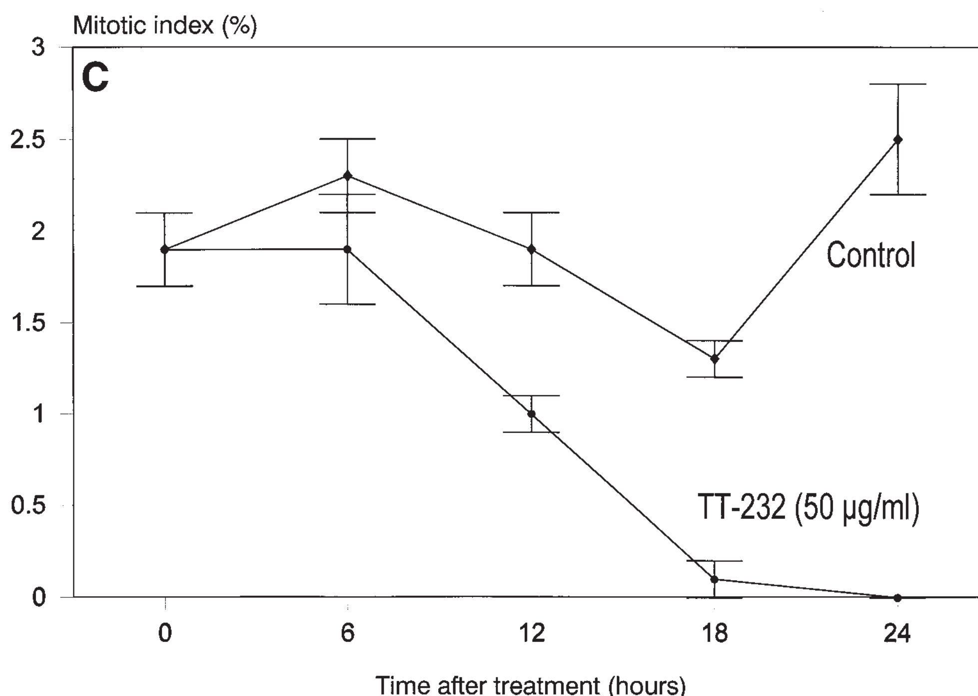

Fig. 2. *(continued)* **(C)** Mitotic index.

The apoptosis-inducing effect of the somatostatin analog TT-232 suggests the presence of somatostatin receptors in HT-29 cells. GnRH and somatostatin receptors were detected earlier in various types of tumor cells by biochemical and morphological methods, and a possible intracellular receptor for these hormones was suggested *(26)*. Until now five somatostatin receptor subtypes have been cloned (SSTR1–5) *(15)*, and recently, the KU86 autoantigen, which forms a regulatory complex with p53 and DNA dependent kinase, was claimed to be an intracellular somatostatin receptor *(25)*. Since TT-232 was found to have strong antiproliferative effect on more than 80 human tumor cell lines, including the NCI panel of cells *(19)*, we cannot expect a high-affinity binding site on all these cell lines, but it was logical to suppose that a low-affinity binding site or an intracellular receptor might mediate the strong apoptosis-inducing effect of TT-232. In our earlier work, we have demonstrated that somatostatin analogs affect KU86 distribution in HT29 human colon tumor cells *(27)*. In the present study, the binding of a specifically tritium-labeled somatostatin analog to the cell surface, cytoplasm, and nucleus of the tumor cells suggests evidence for somatostatin receptors in HT-29 cells. Previous studies using iodine- or indium-labeled somatostatin analogs demonstrated the binding of these analogs to HT 29 colon tumor cells. However, these derivatives from a chemical point of view cannot be considered identical to the original peptide. Although no intracellular binding was found in these experiments, several data suggested that peptide hormones might get into the cell.

According to our previous studies, GnRH receptors are also present on the cell membranes and in the nucleus *(23,28)* of tumor cells. The dose dependency and time of labeling with [^{3}H]TT-232 showed a more intensive binding of this compound after 60 min with a relatively low dose (0.5 µg/mL) compared to the application of 50 µg/mL. Therefore it may be assumed that the higher dose caused down-regulation or saturation of the receptors mediating internalization. At h 6, the labeling increased in parallel with the dose of [^{3}H]TT-232.

The morphologic studies showed a dose-dependent increase in the apoptotic index with a maximum at the 8th h. The DNA fragmentation measured by flow cytometer reached the maximum values at the same time, but the levels in case of higher dose remained elevated. The difference between the morphological (H&E) counts and FACS analysis in the levels of apoptosis came from the differences of techniques. The flow cytometer counts the early dying cells with fragmented DNA before they show remarkable changes in morphology. The dying cells detached from the plate surface float and escape from the phagocytosis by neighbor cells, and show an accumulation of dead cells in the cell culture. When the cells were treated with a high dose of TT-232 without a serum, the very early increase in sub-G$_1$ fraction suggests a different apoptosis-inducing mechanism than in the other cases.

As postulated, peptide hormone treatment of tumor cells bearing receptors for these hormones may activate a region of DNA representing one or more of the genes responsible for cell suicide. The determination of peptide hormone

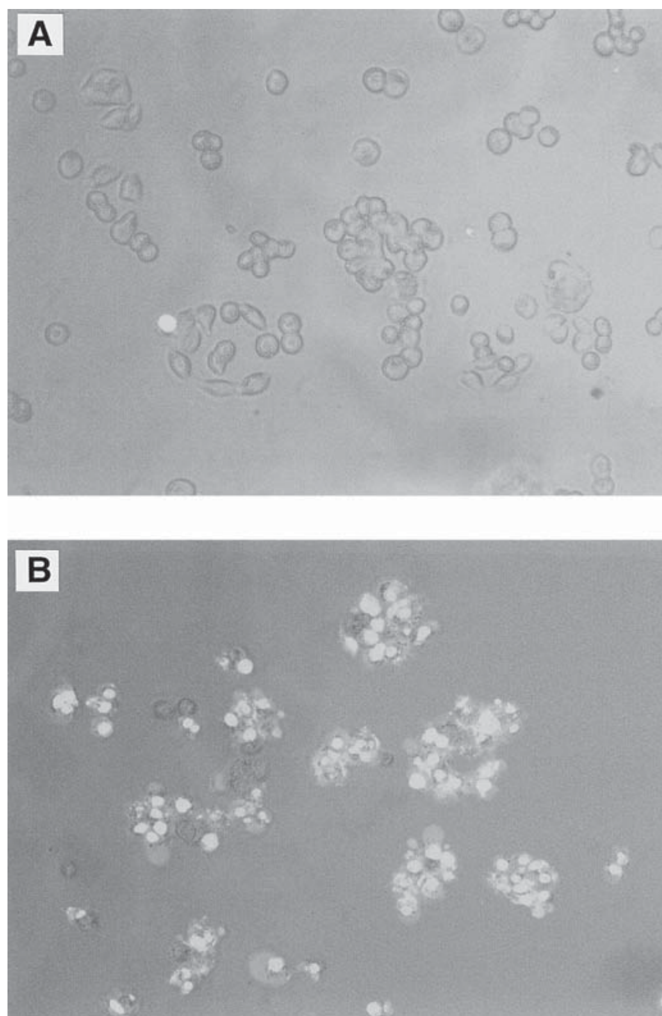

Fig. 3. Propidium iodide staining of HT-29 cells. The image was made using a confocal laser-scanning microscope to detect a fluorescent signal. The origin of gray background was a phase contrast layer. **(A)** Untreated cells. **(B)** Cells were treated with 50 μg/mL TT-232 for 12 h in the presence of serum. Majority of the cells show a positively marked nucleus.

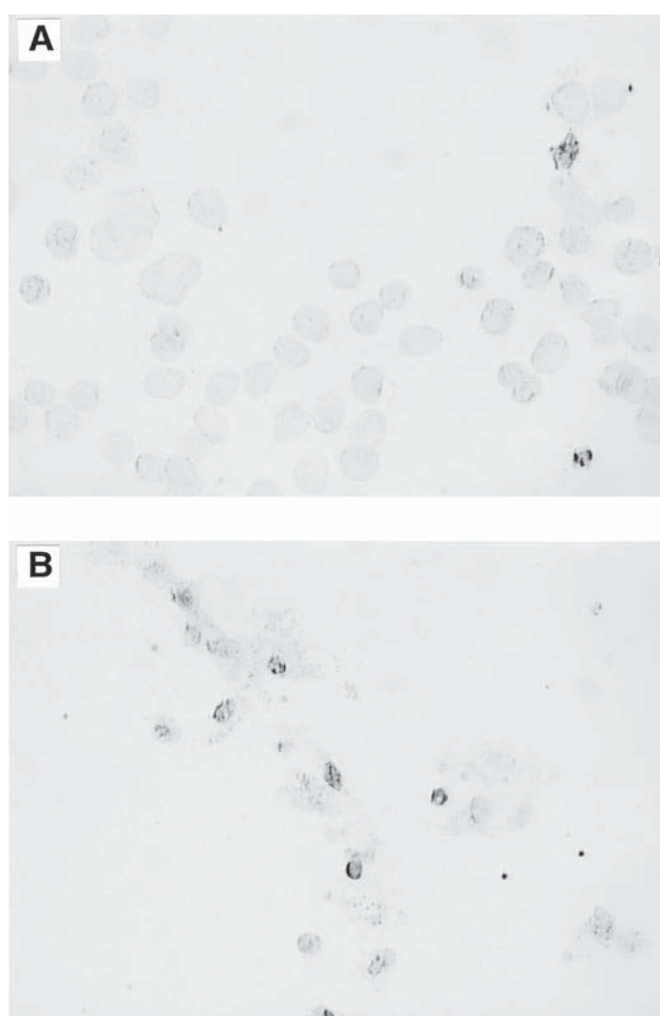

Fig. 4. *In situ* end labeling of the fragmented DNA by ApopDetek kit. **(A)** Untreated cells. **(B)** Cells were treated with 50 μg/mL TT-232 for 12 h in the presence of serum. The dark nuclei and the typical morphology provide evidence of apoptosis in a high percentage of cells.

receptors, including intracellular receptors mediating apoptosis, may be useful also in human tumor therapy in order to rationalize the choice of drug. Binding and intracellular accumulation as well as the induction of apoptosis by TT-232 in colon tumor cells point to the possible effective use of this compound in the treatment of human colon tumors.

Materials and Methods

Synthesis of Specifically Tritium-Labeled TT-232 ([Cys^2→Cys^6] H-D-Phe-Cys-[^3H]-Tyr-D-Trp-Lys-Cys-Thr-NH_2)

TT-232 was synthesized by solid phase peptide synthesis as has been described previously *(20)*. The [3,5 diiodo Tyr]^3 TT-232 analog was synthesized on benzhydrylamine resin (1.25 g, 0.6 mmol/g, Bachem, Bubendorf, Switzerland) with Solid Phase Peptide Synthesis (Boc/Bzl strategy). The side chain protecting groups were as follows: Bzl for Thr, 4-Me-Bzl for Cys, Z for Lys; the side chain of Boc-(3,5-diiodo)-tyrosine was not protected. The cleavage of the peptide from the resin and deprotection were performed simultaneously by using liquid HF (0°C, 60 min). The disulfide bridge formation was made by oxidation with 0.03 *M* iodine/methanol solution in 95% acetic acid. The crude product was purified in 50% acetic acid on a Sephadex G-25 column and by medium pressure liquid chromatography (gradient elution in 10% acetic acid up to 30% 2-propanol.). The purified peptide was analyzed by TLC, HPLC (more than 98% purity), amino acid analysis, and FAB-MS (the expected M+H value).

The specific tritium-labeling of TT-232 was carried out by heterogenic catalytic dehalotritiation of [3,5 diiodo Tyr]^3-TT-232 analog precursor peptide, using tritium gas (Technobexport, Russia); 2.95 mg (~ 2 μmol) of the

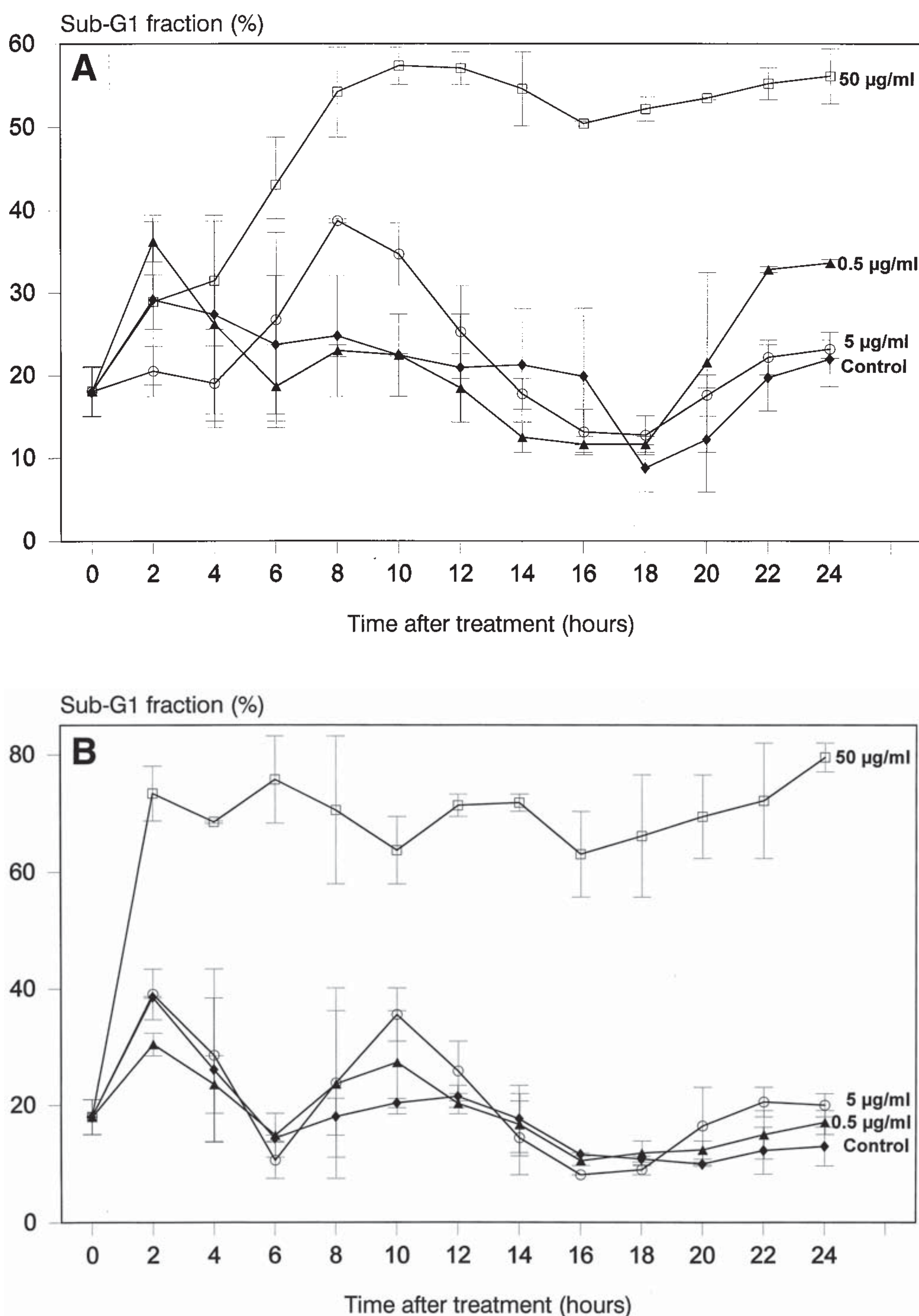

Fig. 5. The effect of TT-232 treatment on HT-29 human colon carcinoma cell line, on the sub-G_1 fraction of cells by flow cytometer. Data represent the number of events with the same size and decreased DNA content compared to G_1-phase cells. Values are the means of three samples of a representative experiment. **(A)** Treatment with various concentrations of TT-232 in presence of serum. **(B)** Treatment with various concentrations of TT-232 without serum.

diiodo-tyrosine- (Dit) containing precursor peptide ([$Cys^2 \rightarrow Cys^6$] H-D-Phe-Cys-Dit-D-Trp-Lys-Cys-Thr-NH_2) was dissolved in 400 μL of distilled water and 600 μL of dimethylformamide (DMF), and 15.4 mg of Pd/$CaCO_3$ (10% Pd, Merck 80716) catalyst was added. The reaction vessel was connected to the tritiation equipment, it was cooled by liquid nitrogen, and was evacuated. Tritium gas was expanded into the reaction vessel. The mixture was stirred at room temperature for 120 min. The reaction was terminated by freezing the solution and adsorbing the unreacted tritium gas on phyrophoric uranium. The catalyst was removed by filtration, washed by 0.5 % AcOH (acetic acid), the labile tritium atoms were eliminated by repeatedly evaporating ethanol:water (1:1) mixture from the

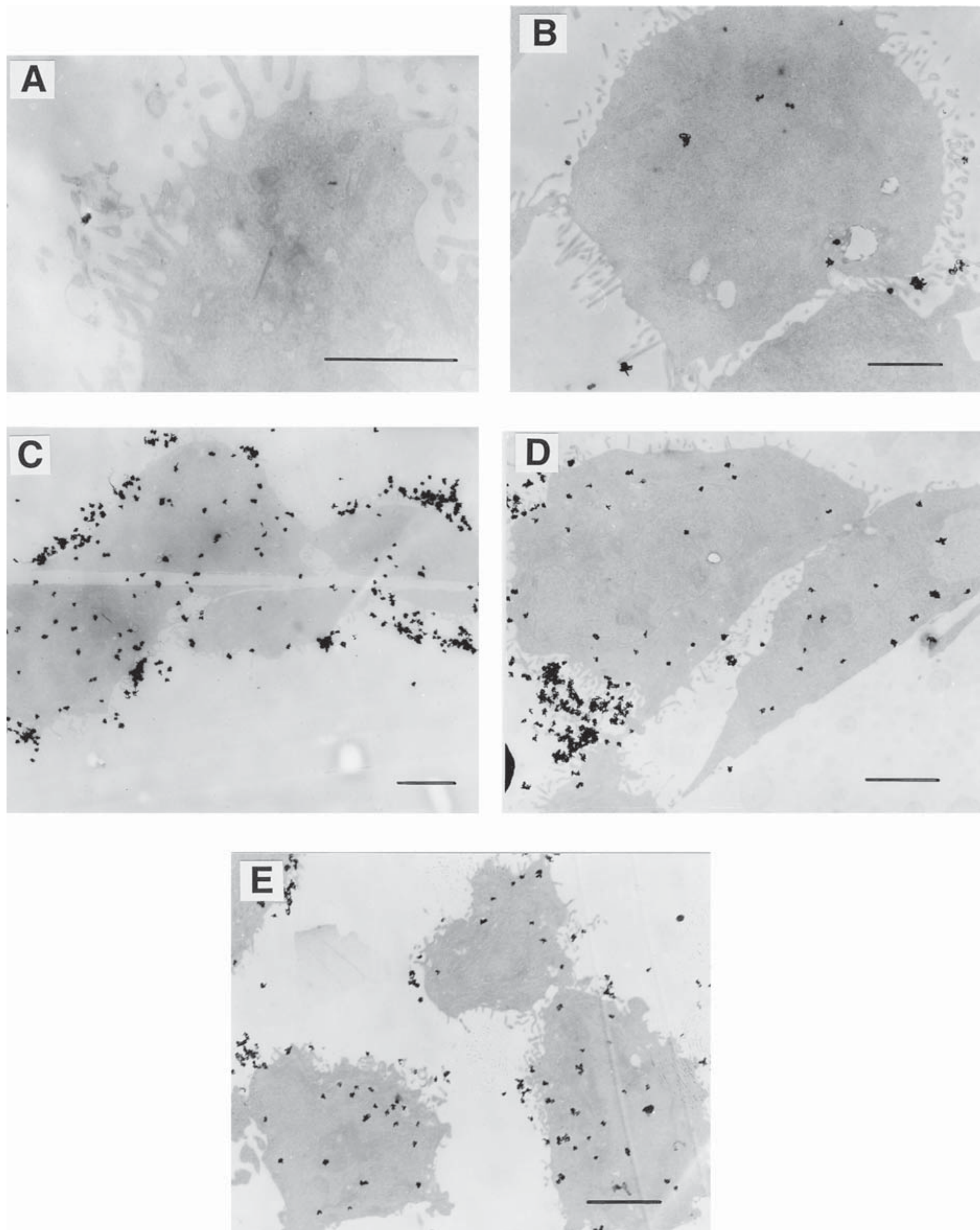

Fig. 6. **(A)** Untreated HT-29 human colon carcinoma cells. Very few grains could be seen and are attached to the villous cell. Scale bar = 3 μm. **(B)** HT-29 human colon carcinoma cells treated in vitro with 0.5 μg/mL tritiated TT-232 for 1 h. Numerous grains can be seen over the villous, rather active appearing cell surface as well in the cytoplasm and nuclei of most tumor cells. Scale bar = 3 μm. **(C)** HT-29 human colon carcinoma cells treated in vitro with 50 μg/mL tritiated TT-232 for 1 h. A few grains are attached to the villous cell processes and appear also in the cytoplasm and in the nucleus of the tumor cell(s). Scale bar = 2 μm. **(D)** HT-29 human colon carcinoma cells treated in vitro with 0.5 μg/mL tritiated TT-232 for 6 h. Fewer silver grains are seen over the surface of "active" tumor cells with (villous or bleb-like cytoplasmic protrusions). Compare with the effect of shorter treatment (1 h) with the same dose of the somatostatin analog (Fig. 6B). The cytoplasms as well as the nuclei of the tumor cells are "labeled" medium to high. **(E)** HT-29 human colon carcinoma cells treated in vitro with 50 μg/mL tritiated TT-232 for 6 h. An accumulation of grains can be seen over the numerous, thin, villous cytoplasmic projections of one tumor cell (left). The other tumor cell (right) has a smoother, slightly labeled cell surface. In both tumor cells, a moderate to high number of grains is located over the cross-section of the cytoplasm as well as over the nuclei. Scale bar = 3 μm.

Table 1
Number of Silver Grains Counted on EM Micrographs Over Different Cellular Compartments of HT-29 Human Colon Carcinoma Cells Treated with 50 μg/mL (high) and 0.5 μg/mL (Low) Doses of Tritiated TT-232 for 1 and 6 H, Respectively

	Cell surface		Cytoplasm		Nucleus	
Treatment	Grains, 100 μm^2	Grains, cell	Grains, 100 μm^2	Grains, cell	Grains, 100 μm^2	Grains, cell
Control	0.7 ± 0.4	0.2 ± 0.05	2.4 ± 0.9	0.5 ± 0.1	0.6 ± 0.3	0.2 ± 0.1
1 H low	37.4 ± 5.4	12.9 ± 1.7	58.1 ± 7.5	26.8 ± 3.0	77.1 ± 9.3	17.0 ± 2.4
1 H high	15.2 ± 1.4	4.5 ± 0.4	26.1 ± 2.1	9.0 ± 1.2	35.5 ± 5.4	6.2 ± 1.1
6 H low	12.4 ± 1.7	3.7 ± 0.5	28.4 ± 3.1	8.4 ± 1.2	52.2 ± 6.9	7.6 ± 1.0
6 H high	20.7 ± 2.1	6.1 ± 0.6	53.6 ± 3.4	13.9 ± 0.9	67.4 ± 9.0	7.7 ± 0.9

[a]Values are means ± S.E.M. $N = 30$ (number of cells evaluated in each experimental group).

radiolabeled product. The crude material was dissolved in 0.5 % AcOH, and the total radioactivity was measured by (Searle Delta 300) liquid scintillation counter (LSC), using Triton-X-100 2:1 cocktail (28.14 mCi, 1.04 GBq).

The reaction product was analyzed by reversed phase high performance liquid chromatography (RP-HPLC) (Jasco) on Vydac 218TP54 C_{18} column, at $\lambda = 275$ nm, using gradient elution (0–5 min. 10% of A, 5–25 min. 10–25% of A, 25–35 min. 25-40% of A, 35–40 min. 40–10% of A; A: 0.1% TFA (trifluoroacetic acid)/acetonitrile, B: 0.1% TFA/water).

The product of the tritiation reaction contained four peptides: about 15% of [$Cys^2 \rightarrow Cys^6$] H-d-Phe-Cys-[^{3}H]Tyr-D-Trp-Lys-Cys-Thr-NH_2, 53% of H-D-Phe-Ala-Tyr-D-Trp-Lys-Ala-Thr-NH_2), 27% of [$Cys^2 \rightarrow Cys^6$] H-D-Phe-Cys-Mit-D-Trp-Lys-Cys-Thr-NH_2 (Mit: monoiodotyrosine), and about 5% of [$Cys^2 \rightarrow Cys^6$] H-D-Phe-Cys-Dit-D-Trp-Lys-Cys-Thr-NH_2. These resulting peptides were identified by liquid chromatography-mass spectrometry (LC-MS) (Finnigan TSQ 700) using the products of the inactive experiment.

The [$Cys^2 \rightarrow Cys^6$] H-D-Phe-Cys-[^{3}H]Tyr-D-Trp-Lys-Cys-Thr-NH_2 peptide was purified by RP-HPLC, and its specific activity was determined (21.52 Ci/mmol, 796 MBq/mmol). The purified, labeled peptide was stored in 2-mL aliquots (1 mCi/mL, 37 MBq/mL) under liquid nitrogen.

Treatment of Cell Culture with TT-232 and Determination of Apoptosis

HT-29 (ATCC No. HTB38) human colon adenocarcinoma cells *(29)* were cultured in six-well Greiner (Kremsmünster, Austria) plates using RPMI and 10% fetal calf serum. The cell number/mL was 10^5 at plating (2 mL/well). TT-232 (50 μg/mL) treatment was performed 24 h after plating. This dose was chosen on the basis of our previous experiments, in which TT-232 was selected from other somatostatin analogs, which showed less or no effect on cell proliferation *(19)*. At that time, the culture medium (containing serum) was removed. TT-232 was dissolved in serum-free medium, and thereafter no serum was used. The medium was not changed over either the treated or the untreated (control) cultures. It should be mentioned that proliferation of HT-29 cells continues even in serum-free medium at least for 48 h. Cell counts were determined at various points in time after treatment up to 36 h. At the same points in time, samples growing on cover slips in six-well plates were fixed in methanol and stained with H&E. From each treated and control group, triplicate samples were taken at each time-point for cell count and determination of apoptotic index. The ratio of mitotic to apoptotic figures was determined according to morphologic criteria *(30)*. The mitotic and apoptotic index was calculated by counting 2000 cells/slide.

For detection of cell death, propidium iodide staining on unfixed cells has been used. One well, containing a glass cover slip, was treated with 50 μg/mL TT-232 for 12 h in serum containing medium and one remained untreated. After washing the cells with phosphate-buffered saline (PBS), they were incubated in 10 μg/mL propidium iodide for 5 min at room temperature and were examined by a confocal laser-scanning microscope (MRC-1024, Bio-Rad, London, UK) at low resolution. The phase-contrast layer has been used as a gray background and the propidium iodide signal as red.

TUNEL reaction (ApopDetek Cell Death Assay System, Enzo, NY) has been used to show the DNA fragmentation *in situ*. The method is based on the activity of terminal dezoxitimidine transferase enzyme activity, which marks the free ends of the fragmented DNA with biotinilated dUTP tails.

Flow Cytometry

Cells were plated in 24-hole Grainer plates (Kremsmünster, Austria) using RPMI medium supplemented with 10% bovine serum (Sigma). The cell number at plating was 1.5 × 10^5. Treatment with TT-232 (0.5–5–50 ≤g/mL) was performed 24 h after plating, when the culture medium was removed and cells were treated in serum-free or serum-containing medium. Triplicate samples of control and treated cultures were examined in every 2 h, during the 24-h treatment.

The medium of the corresponding hole was sucked off and put into FACS tube. The monolayer of cultured cells was washed with 0.9% sodium chloride and treated for 20 min with EDTA (0.5). The suspended cells were also put in the same FACS tube. The tubes were centrifuged (1500 rpm for 3 min), and the supernatant was discarded. The cells were resuspended in 1 mL of –20°C ethanol and stored at –20°C.

The cells suspended in ethanol were centrifuged (1500 rpm for 3 min) and resuspended in 1 mL pH 7.8 phosphate-citrate buffer, to remove the smaller DNA fragments. The buffer contained 100 µg/mL RNAse (Sigma). After 20 min of incubation at 25°C the samples were treated with 10 µg/mL ethidium bromide (Sigma). A FACStar flow cytometer (Beckton-Dickinson) was used to determine proportion of cells in various phases of the cell cycle as well as apoptotic cells with fragmented DNA (sub-G_1 fraction). Six thousand cells per sample were examined, with 300–400 cell/min flow rate *(28,29)*. Using this method by gating the events on the forward scatter and fluorescence diagram (dot-plot) we could select only those cells which size was similar to the normal cells. That is why the debris and possible necrotic cells do not included in the results.

Ultrastructural and Autoradiographic Procedures

HT-29 cells were cultured in Greiner flasks containing 3000 µL RPMI (Sigma) supplemented with 10% FCS (Flow, Irwine, Scotland). Cell number at dilution was 10^6/2-mL flask. Twenty four hours after dilution the medium was discarded and replaced by serum-free RPMI containing 50 µg/mL and 0.5 µg/mL [^{3}H]TT-232, respectively. Two flasks per dose and a control culture (0.5 mg/mL [^{3}H]TT-232 + 50 mg/mL "cold" TT-232) were treated. Treatment was stopped at h 1 (one flask per dose) and at h 6 (one flask per dose).

Cells were collected and fixed in glutaraldehyde (1%) and post-fixed in O_SO_4 (2%), embedded in Polybed (Polysciences, USA). Ultrathin sections (40 nm) were cut, using an LKB microtome (Stockholm, Sweden). Ultrathin sections were collected on Formvar coated slides covered with evaporated carbon. Kodak fine grain emulsion (for EM) was diluted 1:3 with bidistilled water and the slides were covered with a thin layer of the melted emulsion at 40°C using the "dipping" method. Following exposure for 12–14 wk, the silver grains were developed by D-19 developer at 18°C for 5 min and fixed in 20% Na-trisulfate (3 min at 4°C). The ultrathin sections were mounted on 50-mesh copper grids and examined under a JEOL 100B electron microscope (Tokyo, Japan).

The average number of grains over different cellular compartments (100-µm cell surface, 100 nm^2 cytoplasm, 100 nm^2 nucleus) as well as the average number of grains/cell over the cell surface, cytoplasm and nucleus were counted on EM micrographs. In each experimental group 30 cells were evaluated and the counts were given as means ± SEM. For statistical evaluation MS Excel software was used.

Acknowledgments

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