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Growth Inhibitory Effect of the Somatostatin Structural Derivative (TT-232) on Leukemia Models

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Abstract. *TT-232 is a structural derivative of the natural signal inhibitory peptide somatostatin, with selective antiproliferative and anti-inflammatory properties. TT-232 activates SSTR receptors (primarily the SSTR-1), which leads to irreversible cell cycle arrest, followed by secondary induction of apoptosis. TT-232 has passed phase I clinical trials without toxicity and significant side-effects. We examined the antiproliferative effect in vitro and the antitumor effect in vivo of TT-232 on leukemia cell lines. During in vivo experiments, we evaluated the therapeutic efficacy of TT-232 in various long-term administration routes; traditional injection versus infusion treatment via an inserted Alzet minipump on P-388 mice and HL-60 human leukemia models. Treatment with TT-232 started after development of the disease. In vitro, TT-232 inhibited the proliferation of P-388 mice lymphoid cells and HL-60 human promyelocytic leukemia cells in the range of 46%-97% with 24-hour treatment and 82%-100% with 48-hour treatment. Cells were treated with 30 μg/ml and 60 μg/ml dose of TT-232. With the same in vivo models, the best results were achieved when TT-232 was applied by infusion treatments. The infusion treatment with TT-232 produced 50%-80% inhibition of growth and resulted in 20%-40% long-term and leukemia-free survivors. TT-232 showed dose-, time- and administration mode-dependent antileukemia activity in vitro and in vivo, both on rodent and human models. Our results suggest that TT-232 is a promising new antileukemia agent.*

The present study was designed to examine the efficacy of the novel somatostatin analog (TT-232) in various leukemia models. Somatostatin, a natural tetradecapeptide, inhibits both the growth hormone release and various endocrine secretions (*i.e.* glucagon, insulin, gastrin). It inhibits or regulates several cell functions also being an important endogen antitumor agent (1-4). TT-232 is a structural heptapeptide analog of somatostatin, (D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH2) but, in contrast to the parent hormone and its "traditional" analogs, this compound has strong and selective growth-inhibitory antitumor potential without the wide-ranging endocrine side-effects. It also has a strong anti-inflammatory and neurogenic inflammation inhibitory activity. The molecule has been shown to have unique conformational characteristics, selective binding properties to the 1st and 4th subtypes of somatostatin receptors (SSTR1 and 4) and to the intracellular receptor: pyruvate kinase M2. Its mechanism of action is in line with the new era of molecular medicine called signal-transduction therapy, where "internal communication" of cells is corrected without interfering with basic cell functions and machinery. TT-232 has been shown to inhibit proliferation and induce apoptosis both *in vitro* and *in vivo* in various types of tumor cells, but also in activated lymphocytes. The molecular mechanism of these biological activities has been linked to both short-term activation of intracellular tyrosine phosphatases and long-term inhibition of tyrosine kinases (5). Short-term (30 min) exposure of cells to TT-232 activates SSTR receptors (primarily the SSTR-1), which leads to irreversible cell cycle arrest in G1/S followed by secondary induction of apoptosis (6). In contrast, continuous incubation with TT-232 leads to direct induction of active cell death independently from SSTR-mediated signaling (7).The mechanism of action and the signaling cascade of TT-232 in A431 epidermoid carcinoma cells has been fully elucidated (8-11). In our previous *in vitro* and *in vivo* experiments, the antitumor efficacy of the novel somatostatin analog was studied on different tumor models (12-17). The antitumor activity of TT-232 has been found to be associated with induction of programmed cell death (apoptosis) in tumor cells, resulting in highly selective elimination of tumor tissue. TT-232 induced apoptosis in a time- and dose- dependent manner and inhibited mitosis of the cell population, that paralleled apoptosis by both biochemical and morphological parameters (18-20).The TT-232-induced inhibition of the

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growth-promoting tyrosine kinase signal could be coupled to the induction of the regular cell cycle with an apoptotic end. The role of tyrosine kinase inhibition in the induction of apoptosis has been well demonstrated, while our recent studies proved that an EGFR selective tyrosine kinase inhibitor induced a non-apoptotic programmed cell death. Tyrosine kinase inhibitors and signal transduction therapy opens a new era in the treatment of leukemia, representing the first targeted molecular therapy which is able to target abnormal cells without damaging normal cells, as compared with traditional antineoplastic drugs (21-23).

The objectives of these studies were to investigate the therapeutic efficacy of TT-232 on rodent and human leukemia models *in vitro* and *in vivo*. *In vitro,* we investigated the effect of TT-232 on P-388 mice lymphocytic and HL-60 human promyelocytic leukemia cell lines. Cells were treated with a 30μg/ml and 60μg/ml dose of TT-232 for 24 and 48 hours. *In vivo,* we studied the therapeutic efficacy of the novel somatostatin analog on P-388 mice and HL-60 human tumor-bearing mice applying the injection and infusion treatments of TT-232. The antineoplastic activity of TT-232 was evaluated on the basis of survival time and tumor growth inhibition.

Table I. *In vitro antiproliferative effect of TT-232 on leukemia cell lines.*

Cell line	Inhibition of cell proliferation (%)			
	24 hours		48 hours	
	30 μg/ml	60 μg/ml	30 μg/ml	60 μg/ml
P-388 rodent lymphocytic leukemia	46±2	82±3	82±1	92±1
HL-60 human promyelocytic leukemia	59±3	97±2	99±0	100±–

The applied doses of TT-232 were 30 and 60 μg/ml. The treatment periods were 24 and 48 hours. Values are the mean±SD

Materials and Methods

Compound. TT-232 was dissolved in buffer solution (pH=4.1) containing 0.1M acetic acid, 0.1M sodium acetate, 3% mannit and diluted with distilled water. The stock solution proved to be stable at 37˚C over 3 weeks.

In vitro studies

Cell cultures: The cells (P-388 mice lymphocytic and HL-60 human promyelocytic leukemia) were obtained from the American Type Culture Collection and cultured in RPMI medium 1640 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Gibco BRL). Cells were kept at 37˚C and subcultured twice a week.

Antitumor assay: Approximately 800,000 cells were cultured in 3 ml RPMI medium 1640 in a well (6-well plates were used, GRAINER, Kremsmünster, Germany) and incubated for 24 and 48 hours with different doses of TT-232. The cells were dyed with trypan blue and counted in a hemocytometer.

In vivo studies

Animals: Female inbred BDF1 and artificially immunosuppressed CBA/Ca mice, from a specified pathogen-free (SPF) breeding of the Department of Experimental Pharmacology, National Institute of Oncology (Budapest, Hungary), were used for these experiments. Development of immunosuppression was achieved with thymectomy, whole body irradiation with 9.5 Gy and bone marrow transplantation. The animals were fed with a sterilized standard diet (Biofarm, Budapest, Hungary) and had free access to tap water *ad libitum*. They were kept in macrolon cages at 23-25˚C (40-50% humidity), with a lighting regimen of 12/12 hours light/dark. The animals used in these experiments were cared for according to the "Guiding Principles for the Care and Use of Animals" based upon the Helsinki declaration and they were approved by the local ethical committee. In our experiments we utilized 5-10 mice/group.

Tumors cells: P-388sc lymphocytic leukemia cells (obtained from Wodinsky I., Arthur Little and Co, Cambridge, MA, USA) and HL-60 promyelocytic leukemia (obtained from the National Institute of Hematology and Immunology, Budapest, Hungary) were used.

Administration route and treatment schedule: The *i.p.* and *s.c.* injections were applied twice a day for 14 days (14xq12hx2d).The *s.c.* and *i.v.* infusion treatments using Alzet osmotic minipumps (Model 2002) were carried out for 14 or 28 days. On the basis of our previous experiments, we found that the optimum dose of TT-232 was 15μg/kg twice a day in the case of injection treatment. The injected dose (15 μg/kg twice a day) equals a 0.6 μg/day infusion treatment with osmotic minipumps. In the case of the HL-60 leukemia human model, the injected doses were 15, 3 and 0.3 mg/kg of b.w. of TT-232. The doses of 3.0 and 12.0 μg/day of TT-232 were applied *via* infusion using the Alzet osmotic minipump. In the case of TT-232 infusion for 28 days, we utilized successively two minipumps (24-25).

Transplantation of the tumors: $5x10^6$ P-388 leukemia cells per mouse and an optimal fragment ($(2-5)x10^2$mg) of HL-60 promyelocytic leukemia tumor/mouse were transplanted *s.c.* into the intrascapular region of the mice. Treatment with TT-232 started after development of the tumor. In all cases, vehicle solution was used as control. The ratio of the volume/body weight was 0.1ml/10g. Administration of TT-232 with the Alzet type osmotic minipumps was carried out as instructed by the manufacturer. The animals were anesthetized by Na-pentobarbital at a dose of 50mg/kg, *i.p.*

Evaluation, statistical analysis. The animals were weighed and the tumor volumes were measured with a microcaliper on every 2nd or 3rd day. The tumor volume was calculated with the following formula: $V= \pi/6 \times L \times D^2$ (V=tumor volume, L=longest diameter, D= diameter perpendicular to L). Survival times related to that of the controls were recorded. Experimental data were subjected to computerised statistical analysis of variance with the Student-Newman-Keuls test. Statistical significance was accepted at $p<0.05$ levels.

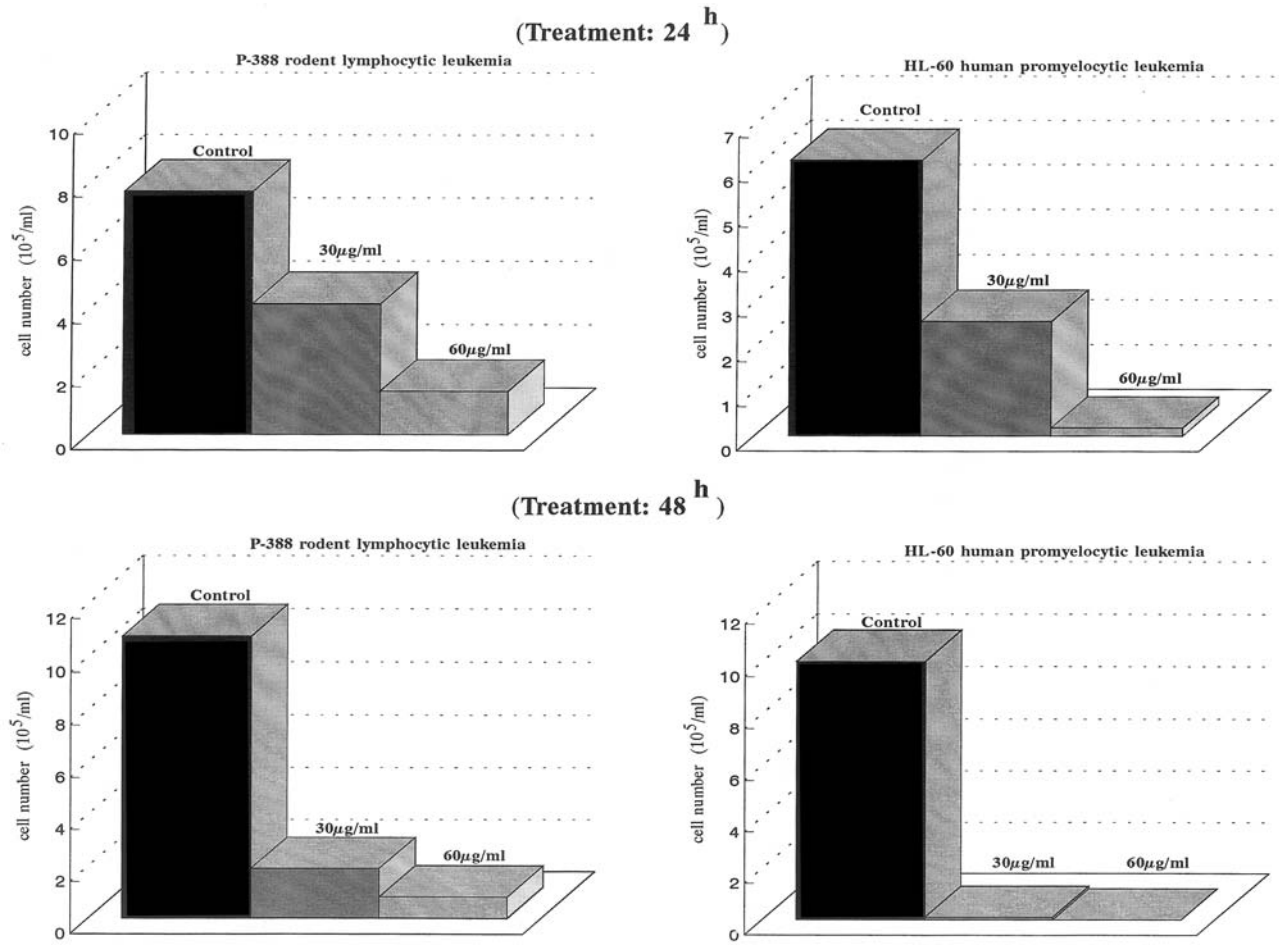
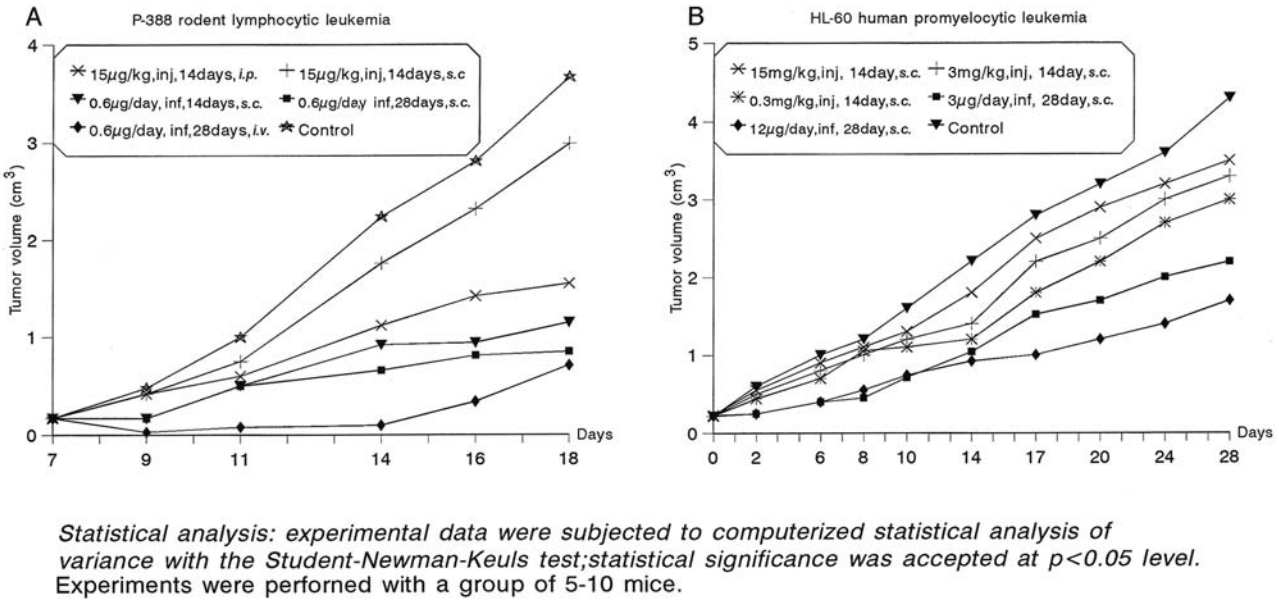


Figure 1. *In vitro activity of TT-232 on different leukemia cell lines.*



Statistical analysis: experimental data were subjected to computerized statistical analysis of variance with the Student-Newman-Keuls test;statistical significance was accepted at p<0.05 level.
Experiments were performed with a group of 5-10 mice.

Figure 2. *Antitumor efficacy of the somatostatin analog (TT-232) on rodent and human leukemia tumor models.*

Table II. *Influence of the different administration routes and treatment schedules on the therapeutic effect of the somatostatin structural derivative (TT-232) in rodent and human leukemia models.*

Tumor type	Treatment	Dose	Treatment Schedule	Treatment route	Survival time M. S.D.	T/C %	Survivors[1]/ Total	Survival T/C x 100
		15 μg/kg	12hx2dx14	*i.p.*	20.8±1.3/10 mice	112	0/10	–
P-388sc	injection	15 μg/kg	12hx2dx14	*s.c.*	19.8±1.6/10 mice	106	0/10	–
rodent		Control	–	–	18.6±1.3/10 mice	100	0/10	–
lymphocytic								
leukemia	infusion	0.6 μg/day	14 days	*s.c.*	23.0±2.6/10 mice	115	0/10	–
		0.6 μg/day	28 days	*s.c.*	33.0±2.1/8 mice	165[2]	2/10	20
		0.6 μg/day	28 days	*i.v.*	34.8±3.3/8 mice	174[2]	2/10	20
		Control	–	–	20.0±1.8/10 mice	100	0/10	–
		0.3 mg/kg	qd x 14	*s.c.*	61.6±1.7/5 mice	144	0/5	–
	injection	3 mg/kg	qd x 14	*s.c.*	56.4±1.4/5 mice	132	0/5	–
HL-60 human		15 mg/kg	qd x 14	*s.c.*	53.9±1.6/5 mice	126	0/5	–
promyelocytic		Control	–	–	42.8±1.2/5 mice	100	0/5	–
leukemia								
	infusion	3 μg/day	28 days	*s.c.*	47.0±3.2/3 mice	105	2/5	40
		12 μg/day	28 days	*s.c.*	48.0±1.2/4 mice	107	1/5	20
		Control	–	–	44.7±3.9/5 mice	100	0/5	–

[1]Tumor-free

[2]Statistical significance was accepted at $p<0.05$ level. Experiments were performed with a group of 5-10 mice.

Results

In vitro studies. Figure 1 and Table I show the significant antiproliferative effect of the novel somatostatin structural derivative (TT-232) on P-388 mice lymphocytic and HL-60 human promyelocytic leukemia cell lines. When the P-388 cell line was treated with TT-232 for 24 hours at doses of 30 μg/ml and 60 μg/ml, the number of tumor cells was decreased by 46±2% and 82±3%. In the case of treatment for 48 hours, the inhibition caused by TT-232 was about 59±3% and 97±2%. On the HL-60 cell line, 30 and 60 μg/ml doses of TT-232 for 24 hours produced a very significant antiproliferative effect (82±1 and 92±1%). When this line was treated with 30μg/ml and 60μg/ml doses of TT-232 for 48 hours, a dramatic inhibition of the growth of HL-60 leukemia cells was achieved (99±0% and 100±0%).

In vivo studies. The antitumor effect of TT-232 on P-388 rodent lymphocytic leukemia tumor: Figure 2A demonstrates the effect of TT-232 on P-388 mice lymphoid leukemia using injection and infusion treatment. The long-term TT-232 treatment given as *i.p.* or *s.c.* injection influenced the growth of the P-388 tumor to different extents (61% and 25%). Figure 2A shows that the two *s.c.* infusion (14 and 28 days) treatments resulted in 71% and 80% tumor inhibition. The *i.v.* infusion for 28 days resulted in 82% growth inhibition. The effect of TT-232 on survival time is presented in Table II. *I.v.* and *s.c.* injections failed to influence the survival of the P-388 leukemia tumor-bearing mice. The subcutaneous infusion for 14 days had virtually no influence on the survival of tumor-bearing mice. The TT-232 given in *s.c.* and *i.v.* infusion for 28 days produced tumor-free, long-term survivors (over 80 days) in 20% of all mice. The mean survival time of the remaining animals was much higher (165% and 174%) than that of the untreated control group.

The antitumor effect of TT-232 on HL-60 human promyelocytic leukemia tumor: We investigated the inhibitory effect of TT-232 *via* injection and infusion treatment on a HL-60 promyelocytic leukemia tumor model. When we applied TT-232 at a dose of 15 mg/kg with *s.c.* injection for 14 days, a moderate (26%) tumor growth-inhibitory effect was observed. The 3 mg/kg and 0.3 mg/kg, *s.c.* injection treatment for 14 days resulted in 32% and 44% tumor inhibitory effects. On the basis of tumor growth curves, a significant inhibitory activity of this novel somatostatin analog (TT-232) was observed following long-term infusion using Alzet 2002 tip. osmotic minipumps implanted *s.c.* The tumor inhibitory activity of TT-232 following infusion treatment for 28 days was 50% (3 μg/day) and 60% (12 μg/day) with Alzet tip. implanted minipumps (Figure 2B). The effect of TT-232 on survival times is presented in Table II. The *s.c.* infusion of TT-232 using *s.c.* implanted Alzet tip. osmotic minipumps for 28 days resulted is tumor-free survival in 40% (3 μg/day) and in 20% (12 μg/day) of the treated animals, respectively.

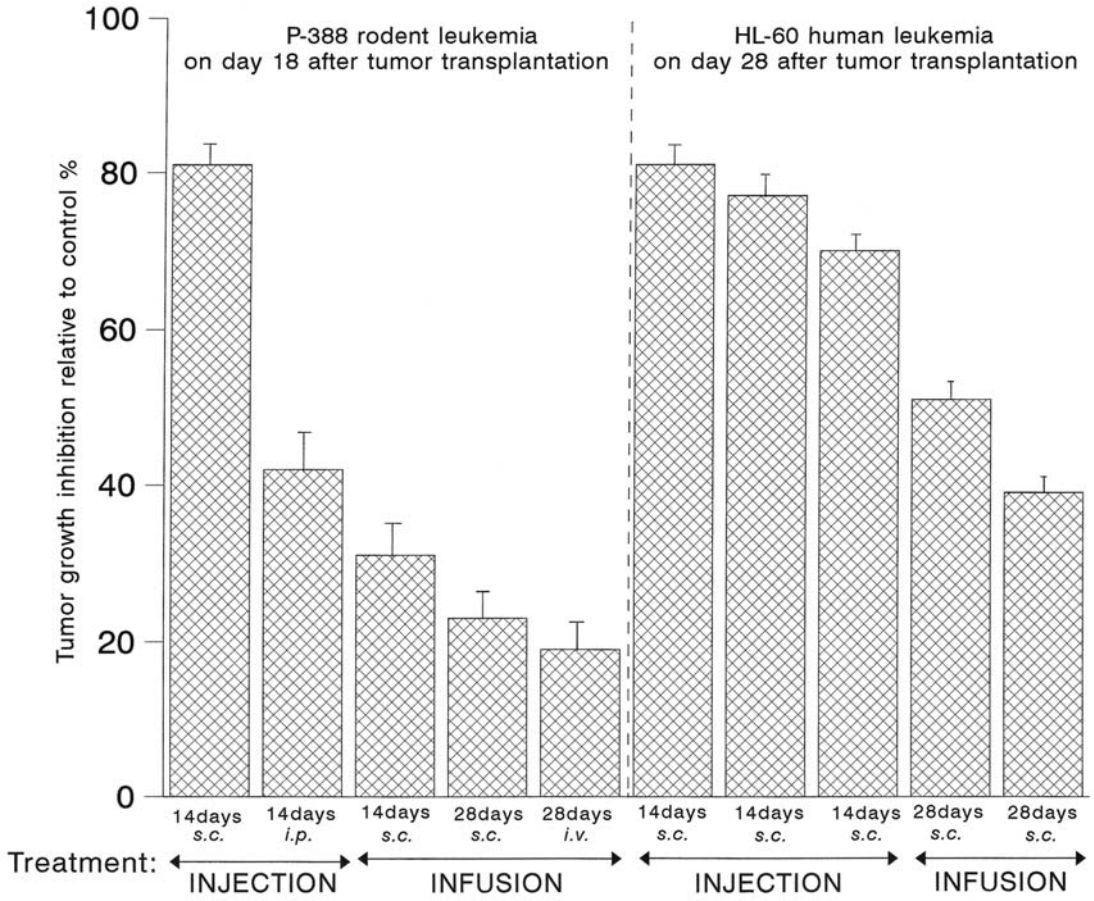


Figure 3. *Tumor growth inhibitory effect of TT-232 applied by various administration routes in leukemia tumor models.*

Discussion

TT-232 has been developed as a signal-transduction inhibitor drug candidate targeting oncological applications, while increasing evidence on the molecular pharmacology of its action, along with extensive preclinical efficacy studies, have demonstrated a strong anti-inflammatory effect and potential therapeutic indication also(26-27).The overall results of the numerous safety studies showed that TT-232 is a molecule of low toxicity: no accumulation, allergic or mutagenic effects were seen. The most significant feature is that TT-232 does not effect the vital function or morphology of tissues, as most cytotoxic agents do.

In the present paper, we examined the activity of TT-232 *in vitro* and *in vivo* on P-388 mice lymphoid and HL-60 human promyelocytic tumor leukemia models. *In vitro,* the antiproliferative effect of the novel somatostatin structural derivative was very significant. It inhibited the proliferation of P-388 mice lymphoid and HL-60 human promyelocytic leukemia cells in the range of 46%-97% with treatment for 24 hours and 82%-100% with 48-hour treatment.The results of our experiments *in vitro* demonstrated that HL-60 tumor promyelocytic leukemia cells have similar sensitivity to treatment with TT-232 as P-388 mice lymphoid tumor cells. *In vivo,* we demonstrated the efficacy of TT-232 on the same leukemia tumor models transplanted in mice (P-388 mice lymphoid leukemia and HL-60 human promyelocytic leukemia). The tumor growth inhibitory effect of TT-232 on these leukemia tumor models proved to be significant. *In vivo*, with the P-388 mice tumor, the infusion of TT-232 by Alzet osmotic minipump resulted in 70%-80% tumor growth inhibition and 20% tumor-free survival. In the HL-60 human leukemia tumor model, long-term infusion treatment with TT-232 caused a 50% and 60% decrease in tumor volume and resulted in 20% and 40% tumor-free animals. Our experiments demonstrated that, in different leukemia tumor models, the application of high doses of TT-232 by infusion treatment results in a therapeutically significant tumor growth inhibition (Figure 3). We applied a long-term infusion of TT-232 using the Alzet osmotic minipumps (Model 2002) in order to maintain a low dose of the hormone in the circulation for a longer time period. The frequent and long-lasting repetition

of the novel somatostatin analog injection enhanced the therapeutic efficacy of the somatostatin analog, however, these serial injections represent significant stress to the animals and require precautions in terms of drug administration. To reduce and eliminate the above-mentioned problem, we used an Alzet osmotic minipump inserted *s.c.*. Infusion from inserted Alzet minipumps maintains a constant drug level, resulting in a well-defined, consistent pattern of drug exposure throughout the period of drug administration. These studies suggest that TT-232 is a potent inhibitor of leukemia tumor *in vitro* and *in vivo* and suggest infusion treatment as a beneficial application in clinical practice.

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