INTERNATIONAL JOURNAL OF ONCOLOGY 27: 169-174, 2005

Inhibition of experimental U-118MG glioblastoma by targeted cytotoxic analogs of bombesin and somatostatin is associated with a suppression of angiogenic and antiapoptotic mechanisms

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Received November 17, 2004; Accepted January 28, 2005

Abstract. Human gliomas express receptors for bombesin and somatostatin that can be used for targeted chemotherapy. In the present study, the efficacy and the mechanism of action of cytotoxic bombesin analog AN-215, and cytotoxic somatostatin analog AN-238 were investigated in U-118MG human glioblastomas xenografted into nude mice. The expression of vascular endothelial growth factor (VEGF) and the apoptotic markers Bcl-2 and Bax was analyzed by Western blotting. The toxicity was evaluated by measuring leukocyte levels and body weights. Treatment with AN-215 or AN-238 reduced tumor growth by ~50%, and diminished the levels of VEGF by 45 and 75%, respectively. The relative ratio of Bcl-2 to Bax proteins was decreased by ~90%, indicating a net apoptotic gain and efficacy of treatment. Specific receptors for bombesin and somatostatin were found in U-118MG tumors. Our results suggest that targeted cytotoxic analogs, AN-215 and AN-238, could be useful for the treatment of human glioblastomas.

Introduction

Malignant glioblastomas represent the most common type of primary brain tumors in adults (1,2). Surgery, radiation, and chemotherapy are of limited effectiveness in the treatment of this disease. Therefore, new therapeutic strategies must be explored (1,2).

New approaches to improve the efficacy of chemotherapy and radiotherapy are based on targeting cytotoxic agents or radionuclides to specific receptors expressed on tumor sites (3,4). Low grade glioblastomas (astrocytomas), express high affinity receptors for the neurohormones bombesin/gastrin releasing peptide (GRP) and somatostatin (5-8). Consequently, analogs of these peptides could be used for targeted tumor therapy. The feasibility and efficacy of such a targeted therapy of brain tumors were demonstrated by Merlo *et al* (9), who successfully treated patients with astrocytomas by a loco-regional infusion of a ^{90}Y-labeled derivative of the somatostatin octapeptide, octreotide. Recently, we developed a cytotoxic analog of bombesin AN-215 and a cytotoxic hybrid of somatostatin AN-238 for the targeted therapy of various tumors that express receptors for these peptide hormones. We also demonstrated the increased efficacy of these analogs over the cytotoxic radical 2-pyrrolinodoxorubicin (AN-201), which they contain (4,10-12). AN-201 is a superactive derivative of doxorubicin (DOX) and is 500-1000 times more potent *in vitro* than its parent compound, and non-cross-resistant with DOX (13-15). In view of the resistance of brain tumors to chemotherapeutic agents including DOX, the targeting of AN-201 to brain tumors may be especially advantageous (8). This has been demonstrated recently in U-87 MG human glioblastomas, which did not respond to therapy with a cytotoxic somatostatin analog containing DOX, but AN-238, containing AN-201, caused the shrinkage of even large U-87 MG tumors, 600-900 mm^3 in size (8). In another study, we showed that treatment with cytotoxic bombesin analog AN-215 also significantly inhibited the growth of U-87 MG tumors (16). In the present investigation, we tested another model of human glioblastoma, U-118MG, that can be grown in nude mice, in order to further evaluate the efficacy of targeted chemotherapy of brain tumors with AN-238 and AN-215.

The neovascularization plays a critical role in the growth of tumors (17), and the angiogenic factor vascular endothelial growth factor (VEGF) was documented to be involved in this process (18). The overexpression of VEGF has been demonstrated in various cancers including brain tumors (18), and shown to be a negative prognostic factor in patients with lung, breast, and gastric cancers (18).

Apoptosis plays an important function in the cell homeostasis, and its inhibition could contribute to tumorigenesis. The members of the Bcl-2 family exert a major role in the regulation of apoptosis, acting either as inhibitors (e.g. Bcl-2,

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Abbreviations: DOX, doxorubicin; GRP, gastrin releasing peptide; VEGF, vascular endothelial growth factor; TGR, tumor growth reduction; WBC, white blood cells

Key words: targeted therapy, cytotoxic bombesin analog, cytotoxic somatostatin analog, brain tumors, VEGF, Bcl-2, Bax

Bcl-xl, Mcl-1, or promoters (e.g. Bcl-xs, Bax, Bak, Bad) (19). An increase in the ratio of antiapoptotic and proapoptotic Bcl-2 family proteins through an overexpression of Bcl-2 and/or down-regulation of Bax, has been correlated with a poor prognosis for patients with brain tumors (20,21).

We evaluated the antitumor effects and the toxicity of targeted cytotoxic bombesin and somatostatin analogs and the non-targeted cytotoxic agent AN-201 in nude mice bearing xenografted U-118MG human glioblastomas. Because the mechanism of action of cytotoxic peptide analogs is not fully understood, we investigated the effect of these compounds on VEGF and apoptotic markers such as Bcl-2 and Bax.

Materials and methods

Peptides and cytotoxic agents. The cytotoxic somatostatin analog AN-238 was synthesized by coupling 1 molecule of 2-pyrrolino-DOX-14-*O*-hemiglutarate to the NH_2 terminus of [Lys(fluorenylmethoxycarbonyl)^5]RC-121, followed by deprotection and purification (11). Cytotoxic bombesin analog AN-215 was prepared by a similar procedure (10). The cytotoxic radical, AN-201 was synthesized as described (15). Bombesin antagonist RC-3095 and somatostatin analog RC-121 were synthesized in our laboratory using standard peptide chemistry. For the injection, the cytotoxic compounds were dissolved in 20 µl of 0.01 N acetic acid and diluted with 6% (weight/volume) aqueous D-mannitol (Sigma, St. Louis, MO).

Cell line and animals. Human glioblastoma cell line U-118MG, obtained from American Type Culture Collection (Manassas, VA), was cultured in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Male athymic nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD), and maintained under pathogen-limited conditions. To obtain donor animals with tumors, 2 nude mice received s.c. injections of cell suspension containing $2x10^7$ U-118MG cells. The developed tumors were dissected and minced, and 3 mm^3 pieces of tumor tissue were transplanted s.c. to both flank areas of the experimental animals. When the tumors became measurable, control and experimental groups were formed with animals bearing tumors about the same size. Tumor volumes and body weights were measured every 4 days. Antitumor activity of the compounds was evaluated by calculation of tumor growth reduction (TGR) using the following formula: TGR% = 100-100 x (T-t)/(C-c), where t is the volume of treated tumors at the beginning of therapy, T is the volume of the same tumors at the end of the experiment, c is the volume of controls at the start of treatment, and C is the volume of controls at the end of the experiment. Thirty-six days after treatment, mice were sacrificed, and various organs were removed and weighed. The number of white blood cells (WBC) was determined by using the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ) Tumors were excised, weighed, snap-frozen and stored at -70˚C until further analyses. All animal experiments were reviewed by the institutional animal care and use committee and were performed in accordance with institutional guidelines for animal care.

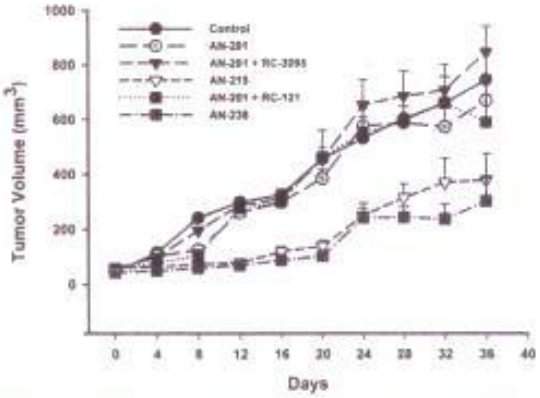

Figure 1. Changes in tumor volumes in nude mice bearing subcutaneous xenografts of U-118MG glioblastomas during therapy with cytotoxic peptide analogues AN-215 and AN-238, and cytotoxic radical AN-201 as well as mixtures of AN-201 with RC-3095 or RC-121. Data represent means ± SE. $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ vs. control.

Experimental protocol. The experiment was started when tumors had grown to approximately 50 mm^3 in volume. Mice were divided into groups containing 8 animals each, and received single injections of 200 nmol/kg of the compounds into the jugular vein as follows: Group 1, control, vehicle solution, group 2, AN-201; group 3, unconjugated mixture of AN-201 and bombesin/GRP antagonist RC-3095; group 4, AN-215; group 5, unconjugated mixture of AN-201 and somatostatin carrier RC-121; and group 6, AN-238.

Receptor binding assays. The binding characteristics of receptors for somatostatin and bombesin/GRP were determined in tumor membrane fractions of control and treated animals. For *in vitro* binding studies, radioiodination of [Tyr^4]bombesin and somatostatin analog RC-160, and separation of the monoiodinated radioligands by high-performance liquid chromatography were performed (22,23). To characterize membrane receptors for bombesin/GRP and somatostatin, ligand competion assays were performed, based on binding of the radiolabeled ligands to tumor membrane homogenates (22,23). The ligand PC^2 computerized curve-fitting program was used to determine the type of receptor-binding, dissociation constant (K_d) and maximal binding capacity (B_{max}) of the receptors (22,23).

Western blotting assays. Protein-matched samples (40 µg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE). Proteins were immunodetected on nitrocellulose membranes using a chemiluminescence detection system (Amersham, Arlington Heights, IL). The membranes were incubated overnight at 4˚C in 5% non-fat dry milk in TBS-Tween. The blots were probed with polyclonal antibodies (1:1000) to VEGF (1-147), Bax (N-20), and monoclonal antibody (1:1000) to Bcl-2, before the incubation with horseradish peroxidase-conjugated secondary antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA) and the exposure to the chemiluminescence substrate. The protein

Table I. Effects of therapy with cytotoxic peptide analogues AN-215 and AN-238, and cytotoxic radical AN-201 as well as mixtures of RC-3095 or RC-121 with AN-201 on growth of U-118MG human glioblastomas xenografted in nude mice.

Groups	Tumor weight (g)	Final tumor volume (mm^3)	TGR (%)	WBC[c]
Control	1.56±0.13	744±95	0.00±8.0	15,400±2,926
AN-215	1.10±0.14[a]	379±95[a]	54.0±8.6[b]	11,300±1,920
AN-238	0.90±0.07[a]	302±82[a]	63.0±11.6[b]	11,600±1,800
AN-201	1.36±0.11	668±96	12.0±1.9	13,500±2,025
AN-201 + RC-3095	1.51±0.05	846±95	0.00±9.5	14,900±2,300
AN-201 + RC-121	1.44±0.15	589±102	24.0±2.9	12,900±1,950

[a]$p<0.05$ and [b]$p<0.01$ vs. control. TGR, tumor growth reduction. [c]WBC was determined on day 36 at the end of the study.

Table II. Binding characteristics of receptors for bombesin/GRP and somatostatin on membranes of U-118MG human glioblastomas.

Bombesin/GRP receptors			Somatostatin receptors		
	K_d (nM)	B_{max} (fmol/mg protein)		K_d (nM)	B_{max} (fmol/mg protein)
Control	1.32±0.07	493.5±83.1	Control	6.79±0.35	294.2±40.9
AN-215	1.78±0.12	447.9±49.2	AN-238	6.12±0.23	277.2±48.4
AN-201	1.28±0.09	488.3±72.5	AN-201	5.98±1.07	304.2±53.6
AN-201 + RC-3095	1.48±0.11	507.2±64.3	AN-201 + RC-121	6.94±0.58	281.5±23.3

Binding assays were performed with radiolabeled [Tyr^4]bombesin or RC-160, as described in Materials and methods. K_d, dissociation constant; B_{max}, maximal binding capacity.

bands were quantified by normalizing the signals of different proteins to β-actin signal (1:2000, Santa Cruz) using the Kodak EDAS 290 imaging system with Kodak 1D Image Analysis Software (Kodak, Rochester NY).

Statistical analyses. The SigmaStat Software was used for the statistical analysis of data. Results were evaluated by one way ANOVA and the Bonferroni and Fisher tests. Results were considered statistically significant when $p<0.05$.

Results

Effects of therapy on U-118MG glioblastoma in vivo. Treatment of nude mice bearing U-118MG tumors with cytotoxic bombesin analog AN-215 nearly stopped tumor growth for about 16 days ($p<0.001$). After day 20, the inhibitory effect on tumor volumes lessened, but a significant suppression ($p<0.05$) in the tumor weight and final tumor volume compared to controls was still observed on day 36, at the end of the study (Fig. 1 and Table I). The tumor growth reduction after AN-215 was 54% ($p<0.01$), compared to controls (Table I). Therapy with cytotoxic somatostatin analog AN-238 also initially produced a very strong inhibition ($p<0.001$), virtually nullifying tumor growth for about 20 days. The suppressive effect of AN-238 continued at a somewhat lower rate and the final tumor volume and weight were significantly ($p<0.05$) reduced, compared to controls. The tumor growth reduction rate was 63% ($p<0.001$) after treatment with AN-238. No significant inhibitory effects were observed in response to cytotoxic radical AN-201, and mixtures of AN-201 with bombesin antagonist RC-3095, or somatostatin carrier RC-121 at the same doses (Fig. 1 and Table I). No significant differences in the WBC count were observed in any of the treatment groups compared to controls (Table I). Significant ($p<0.05$) losses of body weights of about 10% were observed in all treated groups on day 4 after treatment, but one week later, the body weights in all the animals almost completely recovered.

Receptor-binding studies. Radiolabeled [Tyr^4]bombesin was bound to a single class of specific, high affinity receptors for bombesin/GRP in U-118MG human glioblastoma tumors (Table II). Using radioiodinated somatostatin analog RC-160 as a radioligand, high affinity receptors for somatostatin were also detected in all tumors investigated (Table II). Treatment with AN-215 and AN-238 did not change significantly the binding characteristics of receptors compared to controls.

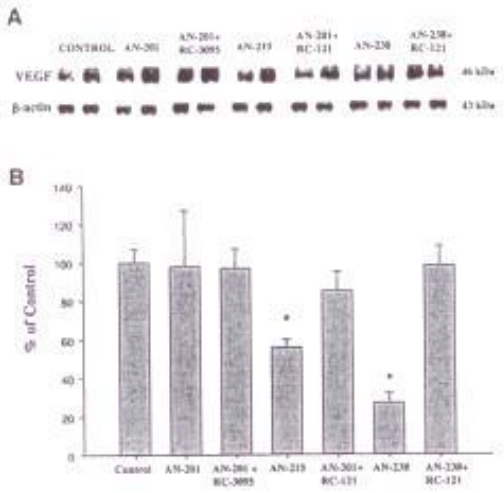

Figure 2. (A) Western blotting for VEGF in U-118MG glioblastoma. (B) Levels of VEGF protein expressed as a percentage of control. The experiments were done in triplicate. Data are expressed as means ± SE. *p<0.05 vs. control

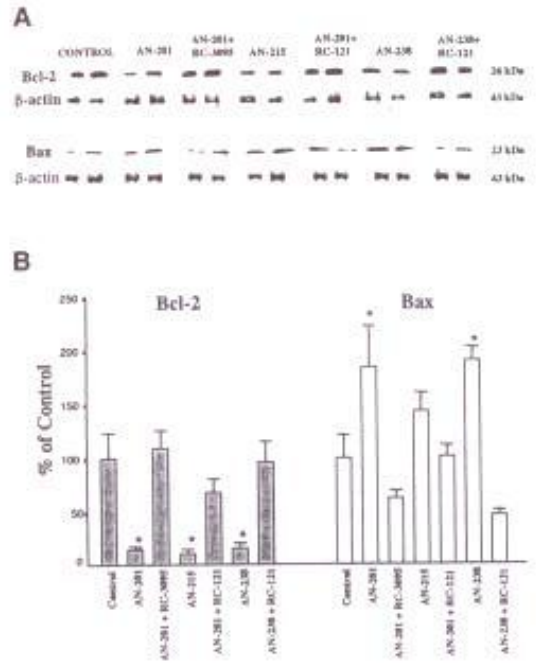

Figure 3. (A) Western blotting for Bcl-2 and Bax in U-118MG tumors. (B) Bcl-2 and Bax were normalized to β-actin and expressed as percentage of control. Experiments were done in triplicate. Data are means ± SE. *p<0.05 vs. control.

Effects of cytotoxic compounds on the expression of VEGF in U-118MG human glioblastoma. VEGF expression in U-118MG tumors was investigated by Western blot analysis. The levels of VEGF protein were reduced significantly ($p<0.05$) by 45% in the group receiving AN-215 and by 73% in the AN-238-treated group, compared to controls (Fig. 2). On the contrary, the cytotoxic radical AN-201, and mixtures of AN-201 and bombesin antagonist RC-3095, or somatostatin carrier RC-121, had no effect on the level of VEGF in U-118MG tumors (Fig. 2).

Modulation of the expression of Bcl-2 and Bax proteins in tumors treated with cytotoxic compounds. The levels of Bax and Bcl-2 proteins in U-118MG tumors were determined by Western blot analysis. As shown in Fig. 3, the levels of Bcl-2 were significantly ($p<0.05$) decreased by treatment with all three cytotoxic compounds, AN-215, AN-238 and AN-201, while the levels of Bax protein were significantly ($p<0.05$) increased by AN-201 and AN-238. Treatment with the cytotoxic bombesin analog AN-215 also elevated the Bax protein levels by 43%, but this effect was not significant statistically. In all three groups, the ratio of the Bcl-2 to Bax proteins, which is a parameter of a net apoptotic gain, was about 90% lower than the control values, indicating the effectiveness of treatment. No significant effects on the levels of Bcl-2 and Bax were observed after administration of the mixtures of AN-201 with RC-3095 or RC-121 (Fig. 3).

Discussion

The presently available treatment modalities for malignant glioblastomas, including surgery combined with radiotherapy or chemotherapy, are of limited effectiveness (2,9). The resistance of glioblastoma cells to radio- or chemotherapy is among the reasons for the low response rates. To improve the efficacy and lower the toxicity of systemic chemotherapy we developed targeted cytotoxic analogs of somatostatin and bombesin (10,11,13). Previously, we demonstrated the efficacy of these analogs on xenografts of U-87 MG human glioblastoma in nude mice, which express receptors for both hormones (8,16). The non-targeted cytotoxic radical, 2-pyrrolino-DOX (AN-201), contained in these conjugates, was ineffective, despite the fact that it can kill cells that are resistant to DOX (8,13,15,16). In these studies, we demonstrated that the increased effectiveness of AN-215 and AN-238 compared to AN-201 was due to targeting to receptors for somatostatin and bombesin expressed on U-87 MG tumors. That targeting results in a local accumulation of radioactivity in the tumors (24), was demonstrated with radiolabeled bombesin and somatostatin analogs in experimental animal models, and in patients with various bombesin/GRP and somatostatin receptor-positive cancers. Similarly, the targeting of cytotoxic conjugates can lead to an increased concentration of the cytotoxic agent in tumor tissue.

In the present study, we tested the antitumor effects of AN-215 and AN-238 on xenografts of U-118MG human glioblastoma, in which we found high affinity receptors for bombesin/GRP and somatostatin by ligand competition assays. Both AN-238 and AN-215 showed a very strong inhibitory effect on tumor growth, in the first 2-3 weeks of treatment. Each analog was still producing significant tumor suppression two weeks later, at the termination of the study. In contrast, the non-targeted cytotoxic agent, AN-201 and mixtures of AN-201

with bombesin antagonist RC-3095, or somatostatin octapeptide RC-160 were ineffective. These results are similar to those obtained previously with the U-87 MG tumors (8,16). The 'straight' bombesin and somatostatin analogs RC-3095 and RC-121, respectively, used in the study in the unconjugated mixtures with AN-201 were administered at very low doses and only once at the beginning of the study, and therefore hormonal effects on the tumors were not anticipated. It has been shown previously that hormonal therapy with somatostatin and bombesin analogs can influence brain tumors, but only after long-term administration of high doses of these peptides (11,25). No signs of toxicity were observed in any of the treatment groups as determined by measurements of body weights and WBC counts, demonstrating that the antitumor effects were obtained without causing a severe toxicity-related stress to the animals.

In this experiment, and in the previous work with the U-87 MG model, we have shown that the high efficacy of both AN-215 and AN-238 is due to a targeted delivery of the analogs to their respective receptors (8,16). To further investigate the mechanism of action of the cytotoxic peptide analogs, we evaluated the effects of treatment on the levels of certain proteins involved in the growth of glioblastomas. Neovascularization plays a crucial role in the development of brain tumors, and VEGF was proposed as one of the major angiogenic factors involved (26). Glioblastomas express high levels of VEGF, which is often up-regulated during the progression of the disease (27,28). It was demonstrated that the inhibition of the expression of VEGF causes a suppression of tumorigenicity of glioblastoma cells in immunodeficient mice (29). Thus, in this study we investigated the effects of AN-215 and AN-238 on the expression of the VEGF protein. Our results reveal that therapy with AN-215 and AN-238 causes a significant reduction in the expression of VEGF protein in U-118MG tumors, indicating that the mechanism of action of these analogs involves the suppression of angiogenesis.

An overexpression of the anti-apoptotic Bcl-2 protein has been shown to enhance cellular survival by inhibiting programmed cell death (30), and the ratio of the Bcl-2, and pro-apoptotic Bax protein is positively correlated with a poor prognosis for patients with brain tumors (20,21). Because most chemotherapeutic agents induce cell death through apoptotic mechanisms (30), we investigated the expression of the Bcl-2 and Bax proteins after therapy with cytotoxic analogs in U-118MG tumors. Our results show that administration of targeted cytotoxic analogs AN-215 and AN-238, as well as cytotoxic radical AN-201 down-regulates the expression of the Bcl-2 protein by about 85%. In addition, the levels of the Bax protein were significantly increased in the groups treated with AN-238 and AN-201. AN-215 also raised the Bax protein level by 43%, but this was not statistically significant. However, the ratio of the Bcl-2 and Bax levels was reduced in all these groups by about 90% compared to controls. A low Bcl-2:Bax ratio is an indicator of a net apoptotic gain and a good prognosis for the patients (20,21). When AN-201 was administered in a physical mixture with bombesin antagonist RC-3095 or somatostatin octapeptide analog RC-160, different effects were observed than when AN-201 was injected alone. As this was noted in the case of both mixtures and in two different studies with the Bcl-2 and the Bax proteins, we assume that AN-201 might have been inactivated by RC-3095 and RC-121 in the mixture. It is conceivable that the latent aldehyde function in the pyrrolino moiety of AN-201 (15) formed an aldehyde-ammonia adduct with the free amino groups present in the two peptides, masking and inactivating the reactive functionality in AN-201.

The fact that AN-201 had no significant effect on the tumor volumes and weights indicates that suppression of the Bcl-2: Bax ratio alone may not be enough to inhibit the growth of U-118MG tumors. While cytotoxic radical AN-201 had no effect on the levels of the angiogenic growth factor, VEGF, both AN-215 and AN-238 significantly suppressed its expression. The antiangiogenic effects of AN-215 and AN-238 may be explained by a direct effect of the analogs on endothelial cells of new blood vessels that express receptors for somatostatin and binding sites for bombesin (24,31,32). These results indicate that targeted cytotoxic somatostatin analog AN-238 and bombesin conjugate AN-215, produce an antiangiogenic effect by a down-regulation of VEGF expression as well as a strong decrease in the relative ratio of the apoptotic proteins Bcl-2:Bax leading to an inhibition of growth of the U-118MG glioblastomas.

In conclusion, this study in U-118 MG model of glioblastomas xenografted into nude mice extends the findings on effectiveness of targeted cytotoxic bombesin analog AN-215 and somatostatin analog AN-238 for therapy of brain tumors. Our work also shows that the targeted cytotoxic analogs act by decreasing the levels of the angiogenic VEGF protein, and reducing the ratio of the apoptotic Bcl-2 and Bax proteins. The results of this study, and our previous investigations provide a rationale for the use of cytotoxic analogs AN-215 and/or AN-238 for the treatment of patients with brain tumors expressing receptors for bombesin/GRP and somatostatin. Both targeted cytotoxic agents are scheduled for clinical trials in the near future.

Acknowledgements

The study was supported by the Medical Research Service of the Veterans Affairs Department and by a grant from Zentaris AG, Frankfurt on Main, Germany, to Tulane University School of Medicine (all to A.V.S.). We thank Patricia Armatis for the excellent technical assistance.

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