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Effective treatment of H838 human non-small cell lung carcinoma with a targeted cytotoxic somatostatin analog, AN-238

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Abstract. The accumulation of radioactive somatostatin analog [^{111}In]pentetreotide in non-small cell lung cancer (non-SCLC) during scintigraphy of patients provides a rationale for investigating the efficacy of somatostatin receptor-based chemotherapy in non-SCLC. Consequently, in this study, we evaluated the antitumor effects of cytotoxic somatostatin analog AN-238 on H838 human non-SCLC xenografted into nude mice in comparison with its cytotoxic radical, 2-pyrrolinodoxorubicin (AN-201). The expression of messenger RNA (mRNA) for human somatostatin receptor subtypes 2 ($hsst_2$) and 5 ($hsst_5$) in H838 cells, and tumors was also investigated using reverse-transcription polymerase chain reaction (RT-PCR). Somatostatin receptors on H838 tumors were characterized by ligand competition assay using radiolabeled somatostatin analog, RC-160. Three i.v. injections of AN-238 at 150 nmol/kg, given on days 1, 7 and 21, resulted in a significant ($p<0.05$) tumor growth inhibition, the final tumor volume being 60% smaller than in the controls. The tumor doubling time was also extended significantly ($p<0.05$) from 9.65±0.56 days in the controls to 17.52±3.3 days. Only one of 8 mice died due to toxicity. In contrast, cytotoxic radical AN-201 was ineffective and more toxic, killing 2 of 7 animals. mRNA for $hsst_2$ was found in H838 xenografts, but not in H838 cells from which the xenografts originated. Interestingly, H838 cells grown in a special, serum-free medium did express mRNA for $hsst_2$. mRNA for $hsst_5$ was not found in any samples tested. Binding studies demonstrated the presence of high affinity (K_d = 7.3±1.2 nM) binding sites for RC-160 with a mean maximal binding capacity (B_{max}) of 953.3±45.3 fmol/mg protein. AN-238 at 3.14±0.93 nM concentration displaced 50% of radiolabeled RC-160 binding to somatostatin receptors in H838 tumors. Our results indicate that patients with inoperable non-SCLC may benefit from chemotherapy targeted to somatostatin receptors based on AN-238.

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Key words: lung cancer, tumor targeting, chemotherapy, somatostatin receptor, doxorubicin

Introduction

Lung cancer is the leading cause of cancer-related deaths in the Western world and in the US alone about 150,000 people die each year from this malignancy (1,2). The estimated number of deaths due to lung cancer is more than 900,000 annually world-wide (3). About 20% of lung cancers are small cell lung carcinomas (SCLC), while other types including squamous cell carcinomas, adenocarcinoma, and large cell carcinomas, are classified as non-SCLC. The main treatment modality for non-SCLC is surgery, but a high percentage of patients present with inoperable tumors. In addition, non-SCLC is relatively resistant to chemotherapy (4). Consequently, it is mandatory to explore new, more efficient therapeutic modalities for the management of non-SCLC.

Targeted chemotherapy is a modern approach for the treatment of cancers, offering a higher efficacy and a lower toxicity compared to standard systemic chemotherapy (5). Cytotoxic agents can be linked to carrier molecules with high affinity to receptors specifically expressed or overexpressed on tumors (5). Based on these considerations, recently we developed a series of targeted cytotoxic peptide hormone conjugates containing the cytotoxic agents doxorubicin (DOX), or its derivative, 2-pyrrolino-DOX (AN-201), which is about 1,000 times more potent (5,6). One of these conjugates, AN-238 consists of 2-pyrrolino-DOX-14-*O*-hemiglutarate linked to a well characterized somatostatin octapeptide carrier, RC-121. AN-238 was shown to effectively inhibit the growth of a wide variety of somatostatin receptor-positive tumors including prostatic, ovarian, breast, renal, pancreatic and colon cancers, brain tumors, SCLC, and non-SCLC (7-10).

Although the presence of somatostatin receptors on SCLC is well established, expression of these receptors on non-SCLC cells is somewhat controversial. Thus, clinical studies with non-SCLC patients revealed that scintigraphic imaging with radiolabeled somatostatin analogs can visualize a very high percentage of primary lesions, but autoradiographic studies on cryostat sections of surgically removed specimens show no specific binding sites for the radiotracer on tumor cells (11,12). Other studies indicate that several non-SCLC cell lines express receptors for somatostatin (13-17). In a

previous study with H-157 human non-SCLC, we found no messenger RNA (mRNA) expression for human somatostatin receptor subtype 2 ($hsst_2$) and subtype 5 ($hsst_5$), but tumors grown from these cells in nude mice showed a high affinity binding of radiolabeled RC-160 (18). Because the tumors expressed the mRNA for mouse sst_2, it was assumed that the receptors are expressed by tumor vasculature (18). Treatment of these tumors with AN-238 induced a >90% growth inhibition. Denzel and Reubi also found high affinity binding sites for somatostatin in peritumoral blood vessels in various tumors including non-SCLC (19), and growing vascular endothelial cells were shown to express sst_2 (20). Collectively, these findings suggest that somatostatin receptors associated with non-SCLC could be used for targeted therapy by cytotoxic somatostatin analogs such as AN-238.

In this study, we demonstrate the efficacy of targeted chemotherapy with AN-238 in yet another human non-SCLC, H838, xenografted into nude mice. We also reveal interesting expression patterns of $hsst_2$ in these cells grown *in vitro* and *in vivo* in mice.

Materials and methods

Peptide and cytotoxic agents. The somatostatin octapeptide analog RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2) was synthesized in our laboratory as described (21). The cytotoxic conjugate AN-238 was made by coupling one molecule of 2-pyrrolino-DOX-14-*O*-hemiglutarate to the NH_2 terminus of [Lys(Fmoc)^5]RC-121 followed by deprotection and purification (7). The cytotoxic radical AN-201 was prepared as described (6). For the injection, the compounds were dissolved in 20 µl of 0.01 N acetic acid and diluted with 5% (w/v) aqueous D-mannitol (Sigma, St. Louis, MO).

Cell line, animals and tumors. Human non-SCLC cell line NCI-H838, was obtained from American Type Culture Collection (Manassas, VA) and maintained in culture using Dulbecco's modified Eagle's medium (DMEM) with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Some H838 cells were grown to 85% confluence and washed from the media described above followed by the addition of a serum-free N_2E medium in which the cells were grown for 48 h. These cells were used only for the determination of mRNA expression for $hsst_2$. The N_2E medium used consists of DMEM with 2 mM L-glutamine, 1 µg/ml transferrin, 30 nM selenium, 20 nM progesterone and 100 µM putrescine (22) and a mixture of protease inhibitor cocktail P 1860 (Sigma, St. Louis, MO). All media components were from Gibco, (Gaithersburg, MD) or Sigma.

Male athymic (Ncr nu/nu) nude mice, approximately 6 weeks old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD), and housed in laminar air-flow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule, and fed autoclaved standard chow and water *ad libitum*. Xenografts were initiated by s.c. injection of 15×10^6 H838 cells into the right flanks of 5 male nude mice. Tumors resulting after 5 weeks were aseptically dissected, mechanically minced, and 3-mm^3 pieces of tumor tissue were transplanted s.c. with a trocar needle. All experiments were carried out in accordance with institutional guidelines for animal care.

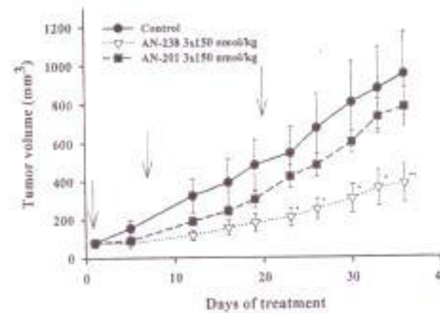

Figure 1. The effects of cytotoxic somatostatin analog AN-238 and cytotoxic radical AN-201 on the growth of s.c. xenografts of H838 non-SCLC tumors in nude mice. Treatment consisting of 3 i.v. injections of the respective compounds at 150 nmol/kg of BW, was started when the tumor volume reached an average of 77 mm^3 (arrows indicate the days of injections, vertical bars show SE; *p=0.05 or **p<0.05 vs. control).

Experimental protocols. The study was started when tumors had grown to an average of 77 mm^3 in volume. The animals were randomly divided into three groups: group 1, control, composed of 7 mice received vehicle solution; group 2 (7 mice), was given cytotoxic radical AN-201; and group 3, consisting of 8 mice, was injected with cytotoxic somatostatin conjugate AN-238. The injections were administered through the jugular vein on days 1, 7, 21. The cytotoxic compounds were given at a dose of 150 nmol/kg of body weight (BW). Tumor volume (length x width x height x 0.5236) and BW were measured twice a week. Five days after the injections of the cytotoxic compounds or vehicle, blood samples were collected from the tail vein using the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ) to determine the total leukocyte (WBC) counts. The experiment was terminated on day 36. The mice were euthanized under anesthesia, tumors were excised and weighed. Tumor specimens were snap-frozen and stored at -70°C until the extraction of total RNA for reverse transcription-polymerase chain reaction (RT-PCR), and receptor binding studies. Tumor volume doubling time was calculated between days 1 and 36 using the formula as described by Smolev *et al* (23):

$$\frac{\text{Days of treatment}}{[\log(\text{final vol}) - \log(\text{initial volume})]/\log 2}$$

Evaluation of toxicity. General toxicity was evaluated on the basis of WBC, mortality rate and changes in BW.

Histologic methods. Samples of tumor tissues were fixed in 10% buffered-formalin. The specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO). Six µm thick sections were cut and stained with hematoxylin-eosin. Mitotic

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AN-238	77.12±17.47	383.46±100.15[b] (60)	17.52± 3.3[b]	460±108[c]

[a]Three i.v. injections of 150 nmol/kg of BW of each compound were given on days 1, 7, 21. The experiment was terminated on day 36. The values are means ± SE. [b]$p<0.05$ vs. control. [c]$p=0.05$ vs. control.

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RNA extraction and RT-PCR. Total RNA was isolated using the Stratagene MicroRNA Isolation Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Following precipitation, RNA samples were quantified by spectrophotometry at 260 and 280 nm. First-strand cDNA was reverse-transcribed from total RNA and resulting cDNA transcripts were PCR-amplified using the GeneAmp RNA PCR Core Kit (Perkin-Elmer, Norwalk, CT) with gene-specific primers for ß-actin or GAPDH (internal control), $hsst_2$ and $hsst_5$ as described (18). Aliquots of 15 µl of each amplification reaction were electrophoretically separated on a 1.8% agarose gel, stained with ethidium bromide, and visualized under UV light on a transilluminator. Negative controls containing RNA were run in parallel to confirm that samples were not contaminated with genomic DNA.

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Statistical analysis. The histologic data were evaluated by one way analysis of variance (ANOVA) and the treated groups compared to the control by Dunnett's test. Tumor volumes and weights are expressed as mean ± SE. Differences between mean values were evaluated by two-tailed Student's t-test, $p \le 0.05$ being considered significant.

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Histology. Histologically, H838 cancer is an undifferentiated tumor consisting of relatively large cells having round and pale nuclei with a few nucleoli. Some scattered large multinuclear cells can be also seen. Most tumors showed extensive focal or confluent necrosis. The quantitative histologic data are shown in Table III.

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