Effective Inhibition of Experimental Human Ovarian Cancers with a Targeted Cytotoxic Bombesin Analogue AN-215

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ABSTRACT

Purpose: To determine whether the cytotoxic analogue of bombesin/gastrin-releasing peptide (GRP) AN-215 can inhibit the in vivo growth of four human ovarian cancer cell lines. AN-215 consists of 2-pyrrolinodoxorubicin (AN-201), a superactive derivative of doxorubicin linked to a bombesin antagonist carrier des-D-Tpi-RC-3095. This conjugate binds strongly to receptors for bombesin/GRP and can be targeted to tumors that express these receptors. Bombesin/GRP receptors are found in 77% of human ovarian cancer specimens.

Experimental Design: Nude mice bearing xenografts of ES-2, SKOV-3, OV-1063, and UCI-107 human ovarian carcinomas were treated with AN-215. The antitumor effects and the toxicity were determined. The expression of bombesin receptor subtypes was measured by reverse-transcriptase PCR analysis, and the presence of bombesin/GRP receptors was determined by radioligand binding assays.

Results: AN-215 significantly (P < 0.05) inhibited growth of ES-2, OV-1063, and UCI-107 tumors, prevented the metastatic spread of ES-2 cancers, and prolonged the survival of nude mice bearing i.p. ES-2 xenografts. Cytotoxic radical AN-201, the unconjugated mixture of bombesin antagonist RC-3095 and AN-201 or RC-3095 alone had no significant effects. Blockade of bombesin/GRP receptors abolished the effect of AN-215. The expression of bombesin/GRP receptors was not changed after repeated treatment with AN-215.

Conclusions: Our findings indicate that targeted chemotherapy with cytotoxic bombesin/GRP analogue AN-215 can inhibit ovarian tumors, which express bombesin/GRP receptors. AN-215 might provide a new treatment modality for women with advanced ovarian carcinoma.

INTRODUCTION

Ovarian cancer is the most common cause of death from gynecologic cancers in the United States (1). In more than two thirds of the cases, patients have advanced disease when diagnosed. Advanced epithelial ovarian cancer is currently treated by cytoreductive surgery and chemotherapy. The introduction of cisplatin in the 1970s was associated with a substantial survival benefit (2). A further progress in the management of advanced-stage disease was made by the incorporation of paclitaxel into the chemotherapeutic regimen, which produced a significant improvement in both progression-free and overall survival rates (3, 4). Chemotherapy with paclitaxel/carboplatin is associated with a lower toxicity than the paclitaxel/cisplatin regimen, whereas being similar with respect to efficacy and survival (5). Thus, the preferred treatment for patients with advanced-stage disease is the paclitaxel/carboplatin combination. However, after an initial response (6), patients may eventually experience a recurrence of the disease (7), which is reflected by 5-year survival rates of 37% for the International Federation of Gynecology and Obstetrics stage III and 25% for International Federation of Gynecology and Obstetrics stage IV disease (8). In addition, the systemic administration of cytotoxic agents usually produces toxic side effects (2, 4). Consequently, the therapy of late-stage ovarian cancer remains a challenge and new treatment approaches are needed.

The discovery of specific receptors for growth factors and peptide hormones on tumor cells has led to the development of monoclonal antibodies and peptides conjugated to radionuclides or cytotoxic radicals for the targeted therapy of some cancers (9–12). The results of various preclinical studies indicate that chemotherapy based on doxorubicin or 2-pyrrolino-doxorubicin conjugated to peptide analogues, which can be targeted to receptors on tumor cells, is more efficacious and less toxic than the respective unconjugated cytotoxic radicals (11). Significant inhibition of experimental ovarian carcinomas was achieved using cytotoxic analogues of luteinizing hormone–releasing hormone (LHRH; refs. 13–17) and somatostatin (18). However, for patients with ovarian carcinoma expressing no or only a low level of LHRH and somatostatin receptors, cytotoxic peptide conjugates targeted to receptors for other peptides might be required.

Bombesin-like peptides, such as the gastrin-releasing peptide (GRP), were initially reported as autocrine growth factors in some human small cell lung carcinomas (19–22). However, recent studies suggest that bombesin/GRP is also involved in the pathogenesis of pancreatic, prostatic, and breast cancers (10, 23–26). Specific receptors for bombesin/GRP have been shown in various human cancers and cancer cell lines (10, 12, 17, 25–28).
Molecular cloning revealed the presence of three distinct bombesin receptors in mammals. Bombesin receptor subtype-1 (GRPR/BRS-1) is preferentially activated by GRP, whereas neuromedin B binds to NMRR/BRS-2 with high affinity (29). Bombesin receptor subtype-3 (BRS-3) is considered an orphan receptor with an unknown natural ligand (29).

Recently, we determined the expression of bombesin receptors in 22 human ovarian cancer specimens and found that 77% expressed mRNA for the GRP-R. Eight of 11 samples tested bound radiolabeled [Tyr4]bombesin, which is a well-known, high-affinity ligand for GRP receptor (GRPR; ref. 30). These binding sites could be used for targeting cytotoxic bombesin analogues to ovarian cancers. Several years ago, a cytotoxic analogue of bombesin, AN-215, was developed in our laboratory. AN-215 consists of 2-pyrrolino-doxorubicin (AN-201), a highly active derivative of doxorubicin (31), covalently linked to the bombesin-like carrier octapeptide RC-3094, which corresponds to des-D-Tpi-RC-3095 bombesin antagonist (32). AN-215 has a high affinity to GRPR/BRS-1 receptors on Swiss 3T3 cells and retains the antiproliferative activity of its cytotoxic moiety, which is 500 to 1,000 times more potent than doxorubicin (32).

The aim of this study was to evaluate the antitumor effects of the cytotoxic bombesin conjugate AN-215, in four human ovarian cancer cell lines xenografted into nude mice, using different regimens and treatment schedules.

MATERIALS AND METHODS

Peptides and Cytotoxic Radical. Cytotoxic radical 2-pyrrolino-doxorubicin (AN-201) and the cytotoxic bombesin analogue AN-215 consisting of 2-pyrrolino-doxorubicin-14-O-hemiglutarate linked to the amino terminal of Gln-Trp-Ala-Val-Gly-His-Leu-$\omega$(CH2-NH)-Leu-NH2 (RC-3094) were synthesized in our laboratory as described (32). Bombesin antagonist RC-3095 [D-Tpi6, Leu13]$\omega$(CH2NH)14Leu]bombesin was also synthesized in our laboratory (33). The compounds were dissolved in 5% (w/v) aqueous d-mannitol solution (Sigma, St Louis, MO) before i.v. injection.

Cell Lines. Three human ovarian cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). ES-2 cell line is well differentiated clear cell ovarian carcinoma derived from a tumor of a 47-year-old African American woman (34). SKOV-3 cell line is a moderately differentiated ovarian adenocarcinoma derived from the ascitic fluid of a 64-year-old Caucasian woman (35). OV-1063 cell line, which originated from a metastatic papillary cystadenocarcinoma of the ovary of a 57-year-old woman (36), is positive for bombesin binding sites and expresses mRNA for all three bombesin receptor subtypes (30).

The UCI-107 human epithelial ovarian cancer cell line originated from a patient with papillary adenocarcinoma and was provided by Dr. A. Manetta (University of California, Irvine Medical Center, Orange, CA). UCI-107 cells possess binding sites for bombesin and express mRNA for GRPR and the NMRR subtypes (30).

All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, penicillin (100 units/mL), streptomycin (100 $\mu$g/mL), and amphotericin B (250 g/L).

The cells were grown at 37°C in a humidified 95% air 5% carbon dioxide atmosphere, passed weekly and routinely monitored for Mycoplasma contamination using a detection kit (Boehringer Mannheim, Mannheim, Germany). All culture media were purchased from Life Technologies (Grand Island, NY).

Animals. Five- to 6-week-old female athymic nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (Bethesda, MD). The animals were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12-hour light/12-hour dark schedule and were fed autoclaved chow and water ad libitum.

Experimental Protocol. Ten million cells from each cell line growing exponentially were inoculated s.c. into both flanks of five female nude mice. Tumors resulting after 4 weeks in donor animals were aseptically dissected and mechanically minced. In all experiments, except experiment 3, pieces of tumor tissue, about 3 mm³, were transplanted s.c. into the experimental animals by a trocar needle. When tumors had reached an appropriate size, the treatment was initiated. Experimental animals received equimolar doses of AN-215 and its cytotoxic radical AN-201 as a single (experiments 1-4) or multiple (experiments 5 and 6) injections into the jugular vein. Control animals were given vehicle solution (5% d-mannitol). Tumor volume (length × width × height × 0.5236) and body weight were measured weekly. The total leukocyte count (WBC) was determined with the Unopette microcollection kit (Becton Dickinson, Franklin Lakes, NJ).

In experiments 1 and 2, mice bearing ES-2 tumors received a single injection of AN-215 and its radical at a dose of 250 nmol/kg. In experiment 1, two additional experimental groups were included, receiving either unconjugated mixture of the cytotoxic radical AN-201 and bombesin antagonist RC-3095 or RC-3095 at an equimolar dose. In experiment 2, an additional group of mice received 200 $\mu$g of the bombesin antagonist RC-3095 i.v. 15 minutes before the i.v. injection of cytotoxic analogue AN-215 at 250 nmol/kg to block the bombesin receptors. In experiments 3 and 4, mice received a single dose of AN-201 and AN-215 at 200 nmol/kg. In experiment 3, female nude mice were injected i.p. with 2 $\times$ 10⁷ ES-2 human ovarian cancer cells. After 14 days, symptoms of ascites were present in all animals and the treatment was initiated. The experiment was designed as a survival study and continued until the death of all animals. Experiment 4 was carried out with large tumors originating from the very aggressive UCI-107 cell line. Each experimental group was sacrificed for ethical reasons when the mean tumor size reached ~2,500 mm³. In experiments 5 and 6, animals bearing OV-1063 and SKOV-3 tumors were given AN-215 and AN-201 at multiple doses starting with a loading dose of 200 nmol/kg on day 1 and 150 nmol/kg on day 15 in experiment 5 and on days 15 and 22 in experiment 6.

At the end of each experiment (except experiment 3), mice were killed under anesthesia, tumors were excised and weighed, and a necropsy was done. Tumor specimens were snap frozen and stored at −70°C. All experiments were in accordance with the institutional guidelines for the welfare of animals in experiments.
Local Toxicity. Healthy nude mice were injected with control vehicle solution, AN-215 or AN-201 at 250 nmol/kg. On day 5, mice were sacrificed and the gastrointestinal organs were excised and fixed in 10% buffered formalin for histologic studies.

Fixed specimens were embedded in paraplast (Oxford Labware, St. Louis, MO), and 6-μm-thick sections of the stomach, the duodenum, and the jejunum were cut and stained with H&E. Specimens were examined by light microscopy for any kind of cellular damage.

Receptor Binding Assay. Preparation of the membrane fractions from ES-2 and SKOV-3 human ovarian cancers grown in nude mice was carried out as reported (37). Binding characteristics of bombesin/GRP receptors on tumor membrane preparation from control animals and animals after treatment with AN-215 and AN-201 were determined by ligand competition assay using 125I-labeled [Tyr4]bombesin, as described (37).

RNA Isolation and Reverse Transcriptase-PCR Analysis. Total RNA was extracted from tumors by using the TRI-Reagent Kit (Sigma-Aldrich Co., St. Louis, MO), following the manufacturer’s instructions. RNA was subjected to reverse-transcription using the RNA PCR core kit (Applied Biosystems, Norwalk, CT) according to the manufacturer’s instructions. Two micrograms of RNA were transcribed into cDNA in a final volume of 50 μL. All PCR reactions were done in an Applied Biosystems PCR system 2700 (Applied Biosystems). For amplification of cDNA transcripts, gene-specific primers for GRPR, NM33D, BRS-3, and β-actin were used as described in detail (28). The nested PCR for GRPR was done using 1 μL from the first PCR reaction mixture and carried out under the same thermocycler conditions.

Ten-microliter aliquots of the PCR products were separated on 1.8% agarose gel and visualized by ethidium bromide using a gel documentation system (Kodak, Rochester, NY). The intensity of each band was normalized against β-actin after background correction. A total RNA control was used in each PCR to rule out genomic DNA contamination.

Statistical Analysis. Data are expressed as means ± SE. Differences between mean values were evaluated by two-tailed Student’s t test. P < 0.05 was considered significant. To compare the survival time in experiment 3, one-way ANOVA was done additionally using Bonferroni’s test for post hoc comparisons between the groups.

RESULTS

Antitumor Effects and Toxicity of Cytotoxic Bombesin Analogue AN-215 on ES-2 Ovarian Cancers. In experiment 1, a single dose of AN-215 at 250 nmol/kg significantly (P < 0.05) inhibited the growth and also significantly (P < 0.05) prolonged the tumor-doubling time of ES-2 human ovarian cancers. In the animals treated with AN-215, tumor volume was reduced by 60.3% (P < 0.05) and tumor weight by 39.5% (P < 0.05), 21 days after the injection whereas equimolar doses of the cytotoxic radical AN-201, a mixture of AN-201 and bombesin antagonist RC-3095 and RC-3095 alone had no significant effects on any variable (Fig. 1; Table 1).

In experiment 2, a single administration of AN-215 at 250 nmol/kg significantly (P < 0.05) suppressed the growth of ES-2 human ovarian cancers. The final tumor volume was significantly decreased (P < 0.05) from day 15 until the end of the experiment on day 29 (Fig. 2). Tumor weights in the group treated with AN-215 were 57.1% lower than in controls (P < 0.05), in accord with the results of experiment 1. The effect of AN-215 could be blocked by injecting 200 μg of the bombesin antagonist RC-3095, 15 minutes before the administration of AN-215. The unconjugated cytotoxic radical AN-201 again had no significant effects on tumor growth (Fig. 2; Table 1). In this study, a total of nine animals developed paraaortic lymph node metastases. Three of these were in the control group, four in the AN-201 group and two in the group blocked with RC-3095. None of the animals treated with AN-215 developed metastatic disease.

In experiment 3, the survival time after i.p. implantation of ES-2 human ovarian cancer cells was significantly (P < 0.05) prolonged by 53.1% compared with controls, following a single dose of 200 nmol/kg of AN-215. An equimolar dose of AN-201 prolonged survival time only by 11%, which was not statistically significant (Fig. 3). Animals treated with AN-215 lived significantly (P < 0.05) longer than those injected with AN-201. Mean survival times were 5.3 ± 1.2 days in the controls, 5.9 ± 1.2 days in the AN-201 group and 8.1 ± 2.6 days in the AN-215-treated group.

Effect of AN-215 on UCI-107 Ovarian Cancers. Mice with relatively large initial tumors measuring 389 to 435 mm3 were used in this study (experiment 4). A single dose of AN-215 significantly (P < 0.05) inhibited the growth of UCI-107 ovarian cancers on day 8 of the experiment, causing 59.2% inhibition of tumor volume, whereas AN-201 produced only a 22.8% inhibition, which was not significant (Fig. 4). Tumor doubling time, calculated on day 8, was also significantly prolonged by AN-215, but not by AN-201. On that day, control animals had to be sacrificed as their mean tumor volume had reached 2,390.7 ± 589.3 mm3. On day 11, the AN-201-treated group was sacrificed, as the mean tumor volume was 2,652.2 ± 595.5
mm³. The size of the tumors in the AN-201 group was significantly \( (P < 0.05) \) greater than in animals given AN-215. The group treated with AN-215 was sacrificed on day 17 as mean tumor size reached 2,634.7 ± 307.5 mm³ (Fig. 4; Table 1).

### Antitumor Effects of AN-215 on OV-1063 Ovarian Cancers

In experiment 5, administration of AN-215 at a dose of 200 nmol/kg on day 1 and 150 nmol/kg on day 15 significantly \( (P < 0.05) \) inhibited the growth of OV-1063 human ovarian cancer xenografts from day 8 until the end of the experiment, the final tumor volume being 62% smaller than in controls. Tumor doubling time was also significantly \( (P < 0.05) \) prolonged by AN-215. The final tumor weight was inhibited by 59% (*P* < 0.05). No significant effect on tumor growth was observed in animals treated with equimolar doses of AN-201 (Fig. 5; Table 1).

In experiment 6, administration of AN-215 at doses of 200 nmol/µg on day 1 and 150 nmol/µg on days 15 and 22 inhibited the growth of SKOV-3 human ovarian cancers by 65.9%, but this effect was statistically not significant (Table 1). AN-201 induced a 35% inhibition, also not significant.

### Side Effects and Toxicity

In all experiments, the loss of body weight after therapy with AN-215 or AN-201 ranged from 2.6% to 7.1% on day 8. In experiment 1, body weights of animals treated with AN-215 were significantly decreased \( (P < 0.05) \) on days 8 and 15 (a loss of 4.1%). AN-201 also significantly \( (P < 0.05) \) lowered these weights on day 8. Both AN-215 and AN-201 significantly \( (P < 0.05) \) suppressed the WBC on days 4 (Table 1) and 10. In animals given AN-215, WBC were still significantly \( (P < 0.05) \) suppressed on day 20. In all other experiments, cytotoxic radical AN-201 induced a greater suppression of WBC than the analogue AN-215. One animal died in the group treated with AN-215.

In experiment 2, significant \( (P < 0.05) \) weight loss was observed on day 8, both in the AN-201 group and the group blocked with RC-3095. AN-215, AN-201, and blockade of AN-215 with RC-3095 significantly \( (P < 0.05) \) suppressed the WBC on day 8. One animal each died in groups treated with AN-215 and AN-201.

In experiment 4, on day 8 body weights were significantly \( (P < 0.05) \) decreased in animals treated with AN-215, WBC on day 8. One animal each died in groups treated with AN-215 with RC-3095 significantly \( (P < 0.05) \) inhibited the growth of OV-1063 human ovarian cancer xenografted into nude mice.

### Table 1  Effects of therapy with cytotoxic analogue of bombesin (AN-215) on the growth of human ovarian carcinoma xenografted into nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor weight [mg] (% inhibition)</th>
<th>Tumor doubling time [d]</th>
<th>WBC on day 8 [cells/mm³]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>646.3 ± 86.6</td>
<td>5.6 ± 0.3</td>
<td>13,477 ± 2,647*</td>
</tr>
<tr>
<td>AN-215</td>
<td>391.2 ± 63.3 (39.5)*</td>
<td>8.9 ± 0.8*</td>
<td>4,565 ± 658*</td>
</tr>
<tr>
<td>AN-201</td>
<td>577.3 ± 73.3 (10.7)</td>
<td>6.9 ± 0.6</td>
<td>3,326 ± 176*</td>
</tr>
<tr>
<td>Mixture</td>
<td>723.7 ± 124.0 (+12.0)</td>
<td>6.5 ± 0.6</td>
<td>NM</td>
</tr>
<tr>
<td>RC-3095</td>
<td>648.9 ± 120.1 (+0.4)</td>
<td>6.5 ± 0.5</td>
<td>NM</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>2,528.7 ± 580.1</td>
<td>6.56 ± 0.3</td>
<td>11,200 ± 1,738</td>
</tr>
<tr>
<td>AN-215</td>
<td>1,084.1 ± 93.6 (57.1)*</td>
<td>8.33 ± 0.4</td>
<td>4,648 ± 1,392*</td>
</tr>
<tr>
<td>AN-201</td>
<td>1,675.1 ± 244.8 (33.8)</td>
<td>7.1 ± 1.6</td>
<td>4,208 ± 385*</td>
</tr>
<tr>
<td>Blockade</td>
<td>1,774.6 ± 188.4 (29.8)</td>
<td>7.7 ± 2.3</td>
<td>4,620 ± 225*</td>
</tr>
<tr>
<td><strong>Experiment 6</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>1,684.7 ± 934.4</td>
<td>11.5 ± 4.8</td>
<td>10,313 ± 685</td>
</tr>
<tr>
<td>AN-215</td>
<td>574.3 ± 130.2 (65.9)</td>
<td>19.0 ± 4.9</td>
<td>8,965 ± 452</td>
</tr>
<tr>
<td>AN-201</td>
<td>1,080.6 ± 262.7 (35.9)</td>
<td>10.6 ± 1.2</td>
<td>6,545 ± 645</td>
</tr>
<tr>
<td><strong>Experiment 5</strong></td>
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<tr>
<td>Control</td>
<td>2,914.7 ± 561.9</td>
<td>6.4 ± 0.6</td>
<td>10,285 ± 664</td>
</tr>
<tr>
<td>AN-215</td>
<td>1,209.6 ± 370.6 (58.5)*</td>
<td>8.5 ± 0.7*</td>
<td>8,965 ± 452</td>
</tr>
<tr>
<td>AN-201</td>
<td>2,516.7 ± 411.6 (13.7)</td>
<td>6.2 ± 0.4</td>
<td>6,518 ± 621*</td>
</tr>
<tr>
<td><strong>Experiment 4</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>—</td>
<td>3.5 ± 0.1</td>
<td>10,258 ± 665</td>
</tr>
<tr>
<td>AN-215</td>
<td>—</td>
<td>5.8 ± 0.5*</td>
<td>8,690 ± 2,294</td>
</tr>
<tr>
<td>AN-201</td>
<td>—</td>
<td>3.8 ± 0.2</td>
<td>4,373 ± 912*</td>
</tr>
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</table>

Abbreviation: NM, not measured.

*Measured on day 4.

\(|P < 0.05| \) (two-sided Student’s *t* test).
In experiment 5, animals treated with AN-201 showed a significantly (P < 0.05) suppressed WBC on days 8, 15, and 22. One animal died in both the AN-215 and AN-201 group.

In experiment 6, body weights were significantly lower on day 29 (P < 0.05) after treatment with AN-215, compared with controls (a reduction of 9.4%). AN-201 significantly (P < 0.05) suppressed the WBC on days 8 and 29.

Microscopic examinations of the gastrointestinal tract showed small changes 5 days after the treatment with AN-215 or AN-201 at 250 nmol/kg compared with controls. Few apoptotic cells, some mononuclear cell infiltration and a slight increase of mitotic cells were detectable in the mucosa of stomach, duodenum, and jejunum with no obvious differences between the two treatment regimens.

In vivo Expression and Characteristics of Bombesin Receptors. Using gene-specific primers, the expression of mRNA for three known subtypes of the bombesin receptor was investigated by reverse transcriptase-PCR in ES-2 and SKOV-3 ovarian cancer xenografts. mRNAs were detected for all three receptor subtypes in both cell lines, but the levels of mRNAs for BRS-3 and GRPR were low in SKOV-3 cells. The expression of mRNA for the GRPR in SKOV-3 cells was shown by nested PCR (Table 2).

High-affinity and low-capacity binding sites for 125I-labeled [Tyr4]bombesin were detected on membrane fractions of ES-2 and SKOV-3 ovarian cancers. AN-215 given in single or multiple doses did not change the binding characteristics of bombesin/GRP receptors in either cell line (Table 2).

DISCUSSION

The involvement of bombesin/GRP in the growth of small cell lung cancer (SCLC) and breast, ovarian, pancreatic, and other cancers, prompted the development of antagonistic analogues of this peptide family for the hormonal treatment of these neoplasms (10). Peptide analogues that target bombesin receptors on malignant tissues can be also used as carriers for radionuclides (12) or cytotoxic moieties (10, 11). Thus, various radionuclide derivatives of bombesin have been proposed for imaging or targeted radiotherapy of bombesin receptor–positive tumors (12, 38–40). For the targeted chemotherapy of bombesin receptor–positive cancers, we developed a cytotoxic hybrid of a bombesin-like peptide linked to 2-pyrrolino-doxorubicin. This conjugate coded AN-215 retains high affinity to bombesin receptors and a potent antiproliferative activity (32). AN-215 has been shown more effective than its nontargeted cytotoxic radical, AN-201 in preclinical models of various malignancies, such as SCLC, gastric carcinoma, prostate cancer, and glioblastoma (41–44). Recently, the synthesis of paclitaxel derivatives of bombesin analogues has been reported (45).

mRNA for the GRPR has been detected in 77% of human ovarian cancer specimens and in ovarian cancer cell lines OV-1063, UCI-107, and ES-2 (33, 42, 46). Sun et al. (28) also showed the presence of the two other receptor subtypes, NMBR and BRS-3, in human ovarian cancer specimens. However, the binding of a radiolabeled bombesin-like peptide was only detected in specimens that expressed mRNA for the GRPR. Targeted cytotoxic bombesin analogue AN-215 was designed to bind with high affinity to this receptor subtype. Accordingly, in a recent study AN-215 inhibited the growth of only the human gastric cancer cell line AGS that expresses the GRPR, but was ineffective in human gastric cancer cell lines Hs 746T and NCI-N87, which are GRPR negative (44).

Thus, in the current study, we investigated the antitumor activity of cytotoxic bombesin analogue AN-215 in four human ovarian cancer cell lines focusing on GRPR. AN-215 significantly inhibited the growth of ES-2, OV-1063, and UCI-107 human ovarian cancer xenografts, whereas cytotoxic radical AN-201 did not exert a significant inhibition. In xenografted SKOV-3 human ovarian cancers, a 60% suppression of tumor growth was obtained with AN-215, but it was not statistically significant. This may be due to the fact that SKOV-3 had the lowest concentration of bombesin receptors among the four cell lines tested and showed only a weak expression of GRPR mRNA. Nevertheless, AN-215 was still twice as potent inhibitor as cytotoxic radical AN-201 in this model.
Unconjugated bombesin antagonist RC-3095 alone or a mixture of AN-201 and RC-3095 given at 250 nmol/kg did not significantly suppress the growth of ES-2 cancer xenografts, indicating that the effect of AN-215 is not related to the antagonistic hormonal activity of the conjugate on bombesin receptors, but rather to its ability to deliver AN-201 to cancerous cells.

Nevertheless, it should be mentioned that in a previous study, daily administration of bombesin antagonist RC-3095 at a dose of 20 μg (~700 nmol/kg; ref. 46), significantly reduced the volume of ES-2 ovarian cancer, implying that bombesin/GRP could be a growth factor for ovarian cancers. As the receptors for bombesin/GRP are present in most ovarian cancers, the existence of an autocrine/paracrine loop cannot be excluded. That AN-215 works as a targeted cytotoxic agent, rather than a hormone is supported by the fact that the antitumor effect of AN-215 could be blocked by injecting a high dose of bombesin antagonist RC-3095 before the administration of AN-215.

In experiment 2, AN-215 prevented the lymphatic spread of ES-2 cells, probably by targeting GRPR on metastatic cells and destroying the micrometastases. In agreement with this result, in a previous study we found that targeting AN-207 to LHRH receptors inhibits the metastatic spread of LHRH receptor-positive MDA-MB-435 breast cancers (47). Thus, targeted chemotherapy seems to be effective in destroying micrometastases expressing the target receptor.

Because late-stage ovarian cancer is associated with a dismal prognosis, we investigated the antitumor effect of AN-215 in animal models with a high tumor load and peritoneal carcinomatosis. In experiment 4, AN-215 was given when the mean tumor size of UCI-107 human ovarian cancer xenografts had reached about 400 mm³. In spite of the large initial tumor size, AN-215 significantly inhibited the growth of this very aggressive cancers. Control animals had to be killed on day 8 because their tumors became too large. Animals treated with AN-201 had to be sacrificed on day 11 for the same reason, whereas mice receiving AN-215 could be kept alive until day 17, which represented a prolongation of survival by >50%.

Treatment of mice bearing i.p. grown ES-2 cancers with AN-215 prolonged the survival time significantly (P < 0.05). Using one-way ANOVA and Bonferroni’s test for post hoc comparisons, the experimental groups were directly compared and the survival time of animals treated with AN-215 proved to be significantly (P < 0.05) prolonged by 37.7% compared with mice injected with AN-201.

The toxicity of cytotoxic bombesin analogue AN-215 and its radical AN-201 was compared with respect to the incidence of deaths, body weight loss and WBC. Only six deaths occurred among a total of 117 experimental animals, three deaths after treatment with AN-215 and two deaths after AN-201, but they could not be related to toxicity. Myelotoxicity is usually the most serious side effect and the dose-limiting factor of chemotherapy. In most experiments, cytotoxic radical AN-201 caused a stronger suppression of the WBC than the analogue AN-215. The decrease in WBC after treatment with 250 nmol/kg of AN-215 occurred probably because of a high esterase activity in serum of mice, which can cause the cleavage of the ester bond in AN-215 and release AN-201. Thus, AN-201 in the circulation could reach a level, which causes a significant drop in WBC, as seen after treatment with 150 or 200 nmol/kg doses of AN-201. It was previously shown in our laboratory, that the inhibition of the esterase activity in mice significantly decreases the toxicity of AN-215 (42, 48). Because the esterase activity is much lower in humans than in mice (48), the toxicity of AN-215 (due to AN-201) is expected to be reduced in patients. Nevertheless, as GRPR are known to be expressed in the gastrointestinal tract, some side effects in the alimentary system could be anticipated. Therefore, we

![Fig. 5 Effect of cytotoxic bombesin analogue AN-215 and cytotoxic radical AN-201 on the growth of OV-1063 human ovarian cancers in nude mice. Initial tumor volumes were 54.9 to 56.1 mm³. Animals were given AN-215 and AN-201 at multiple doses starting with a loading dose of 200 nmol/kg on day 1 and 150 nmol/kg on day 15.](Image)

Table 2 mRNA expression for GRPR, NMBR, and BRS-3 and binding characteristics of bombesin/GRP receptors in ES-2 and SKOV-3 human ovarian cancers xenografted in nude mice (experiments 1 and 6)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>$K_d$ (nmol/L)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
<th>GRPR (mRNA)</th>
<th>NMBR (mRNA)</th>
<th>BRS-3 (mRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES-2</td>
<td>Control</td>
<td>1.33 ± 0.07</td>
<td>586.1 ± 10.9</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AN-215</td>
<td>1.54 ± 0.49</td>
<td>548.7 ± 36.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AN-201</td>
<td>1.17 ± 0.19</td>
<td>601.2 ± 40.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Control</td>
<td>0.54 ± 0.03</td>
<td>153.4 ± 33.3</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AN-215</td>
<td>0.65 ± 0.14</td>
<td>138.7 ± 29.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AN-201</td>
<td>0.51 ± 0.12</td>
<td>150.8 ± 38.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Binding characteristics were obtained from 12-point displacement experiments. $[^{125}]$[Tyr$^4$]BN was used as radioligand. All values represent mean ± SE of three independent experiments, each done in duplicate or triplicate. +, weak expression; ++, strong expression.
histologically examined samples of stomach, duodenum, and jejunum after treatment with AN-215 or AN-201 at the highest dose used in this study. However, only small cellular changes were observed in both groups on day 5, when toxic side effects were expected to be maximal. Thus, it is reasonable to expect relatively minor side effects to the gastrointestinal system.

We investigated different dose regimens of AN-215 to establish the most favorable treatment protocol. Initially (experiments 1 and 2), mice were injected with AN-215 at 250 nmol/kg, as this dose was well tolerated by female mice in the case of our cytotoxic somatostatin and LHRH analogues AN-238 and AN-207 both containing the cytotoxic radical AN-201 (14, 49). However, a prolonged myelosuppression at this dosage prompted us to decrease the initial dose to 200 nmol/kg, which significantly inhibited the very aggressive UCI-107 tumors without causing a significant decrease of the WBC (experiment 4). Finally, it could be shown that administering AN-215 at multiple doses on days 1, 15 (experiment 5) and days 1, 15, and 22 (experiment 6) is feasible and effective. In terms of efficacy, no pronounced difference was observed between a single dose of 250 nmol/kg, a single dose of 200 nmol/kg and multiple doses of 200 and 150 nmol/kg of AN-215. However, the last two treatment schedules were associated with a lower toxicity.

We also evaluated the changes in GRPR expression after treatment with AN-215, because a reduction in receptor levels would lead to a decreased antitumor effect in subsequent treatment cycles but found no down-regulation of GRPR after treatment with AN-215.

In conclusion, our results show that three of four human ovarian cancer cell lines tested expressed receptors for bombesin/GRP in adequately high concentrations for an efficacious tumor inhibition in response to therapy with targeted cytotoxic bombesin analogue AN-215. As AN-215 induced a significant prolongation of survival time in the ES-2 ascites model, prevented the lymphatic spread of ES-2 ovarian cancers, and was effective on large initial ovarian tumors of ~400 mm³ in the very aggressive UCI-107 cell line, these findings suggest that AN-215 may be useful for the treatment of advanced and metastatic disease. Further development is in progress and targeted cytotoxic bombesin/GRP analogue AN-215 should be available for clinical trials in the near future.

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Jörg B. Engel, Gunhild Keller, Andrew V. Schally, et al.


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