# Therapy of experimental hepatic cancers with cytotoxic peptide analogs targeted to receptors for luteinizing hormone-releasing hormone, somatostatin or bombesin

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**As there is no effective systemic therapy for advanced hepatocellular carcinoma (HCC), we investigated the presence of receptors for somatostatin, bombesin and luteinizing hormone-releasing hormone (LHRH) in SK-Hep-1 human hepatic carcinoma and the effects of cytotoxic analogs of somatostatin (AN-238), bombesin (AN-215) and LHRH (AN-207) on the growth of this tumor. Nude mice bearing SK-Hep-1 HCCs were treated with AN-238, AN-215, AN-207 and their combination, or cytotoxic radical 2-pyrrolinodoxorubicin (AN-201). Tumor growth reduction was determined and cell proliferation characteristics and apoptosis were studied by histologic analysis. The expression of receptors for somatostatin, bombesin and LHRH was investigated by radioreceptor assays and immunohistochemistry. High-affinity binding sites for somatostatin, bombesin and LHRH were detected in SK-Hep-1 cancers. All three cytotoxic peptide analogs inhibited growth of SK-Hep-1 tumors and decreased the cell proliferation rate. Combination therapy with two or three cytotoxic analogs resulted in the strongest tumor inhibition. Receptors for somatostatin, bombesin and LHRH are expressed in SK-Hep-1 human HCC. Cytotoxic peptide analogs targeted to these receptors inhibit growth of this tumor. Targeting to multiple receptors enhances the efficacy of therapy. The results of our study encourage additional experimental investigations to permit the introduction of these cytotoxic analogs into clinical trials.** *Anti-Cancer Drugs* **19:349–358 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Hepatocellular carcinoma (HCC) is one of the commonest neoplasms in the world with the highest rates in southeast Asia and Sub-Saharan Africa [1–4]. In the western world the incidence of HCC is relatively low, but it has been rising [5–7]. In developed countries, surgical resection and liver transplantation are the preferred treatments for early stage HCC [3,7–10]. High dose radiotherapy can be also used in patients with localized tumors [11,12], but most hepatic tumors are diagnosed at an advanced stage [7,9]. Systemic chemotherapy is not effective [8]. The general association of HCC with an underlying serious liver disease further complicates therapy [10]. Only a recent investigation demonstrated a statistically significant improvement in the survival of patients treated with sorafenib, a multikinase inhibitor, which now becomes the first-line treatment option for advanced HCC [13].

In the past decade, great advancements have been made in the identification of cellular events responsible for cancer development and progression [7,14,15]. Targeting these pathways may provide new therapeutic modalities for HCC [14,15]. One approach consists of targeted chemotherapy and is based on the fact that receptors for various peptide hormones such as somatostatin, bombesin or luteinizing hormone-releasing hormone (LHRH) are expressed in relatively high concentrations on human tumors. Such hormones can be used as carriers to deliver cytotoxic agents to cancer cells that express the specific receptors. Thus, we developed a series of novel targeted cytotoxic analogs of somatostatin, bombesin and LHRH that consist of doxorubicin (DOX) or its superactive derivative, 2-pyrrolino-DOX (AN-201) coupled to a peptide hormone carrier [16–18]. Targeted therapy of nude mice, bearing various human cancers, with cytotoxic somatostatin analog AN-238, bombesin analog AN-215 and LHRH analogs AN-207 or AN-152 powerfully

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inhibited growth of tumors that expressed receptors for the carrier hormone, and had lesser side effects than treatment with the cytotoxic radical [18–20].

As HCC has a very poor prognosis and the development of new treatment modalities is essential, we investigated whether SK-Hep-1, a human HCC, expresses receptors for somatostatin, bombesin and LHRH, and whether treatment with cytotoxic peptide hormone analogs can inhibit growth of this tumor in nude mice.

## Materials and methods

### Materials

AN-238 was synthesized by coupling 2-pyrrolino-DOX-14-*O*-hemiglutarate (AN-201) to the N-terminus of somatostatin analog [Lys(fluorenylmethoxycarbonyl)$^5$]RC-121 [21]. Cytotoxic bombesin analog AN-215 was made by conjugating the same cytotoxic moiety to the N-terminal of bombesin antagonist RC-3094 [22]. Cytotoxic LHRH analog AN-207 was prepared in the similar way by using the same cytotoxic compound (AN-201) and [D-Lys$^6$]LHRH as carrier [23,24]. For the injection, the cytotoxic compounds were dissolved in 20 µl of 0.01 N acetic acid and diluted with 6% (w/v) aqueous D-mannitol.

### Animals and tumors

Athymic nude mice (Ncr *nu*/*nu*) were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, Maryland, USA) and maintained under pathogen-limited conditions. Human HCC cell line SK-Hep-1 was purchased from the American Type Culture Collection (Manassas, Virginia, USA) and cultured as recommended by it.

Pieces of SK-Hep-1 tumor tissue were transplanted subcutaneously to both flanks of the experimental animals. All animal experiments were done in accordance with institutional ethical guidelines for animal care.

### Experimental protocol

Five experiments were carried out. The groups, treatments schedules and doses are summarized in Table 1.

Combination therapy groups were treated with AN-238 + AN-215 in Experiment 3 and with AN-238 + AN-215 + AN-207 in Experiment 5. The mode of administration is explained in the footnotes of Table 1. The dissolved compounds were injected into the jugular vein in the volume of 0.2 ml/20 g of body weight. The controls received vehicle only. Doses and treatment schedules were based on previous studies in our laboratory [17,18]. Body weights and tumor sizes (length × width × height × 0.5236) were measured weekly. Antitumor activity of the compounds was evaluated by calculating tumor growth reduction. At the termination of the studies, the mice were killed by decapitation under Nembutal (60 mg/kg) anesthesia.

**Table 1** Design of the experiments with SK-Hep-1 human hepatic cancers in nude mice[a]

| No. of experiment | Treatment (groups) | Dose (nmol/kg) | Treatment on days | No. of mice per group | Period between transplantation of tumors and first treatment (days) | Mean tumor volume at time of first treatment ($mm^3$) | Duration of experiment (days from first treatment to end) |
|---|---|---|---|---|---|---|---|
| 1 | Control | 150 | 1, 15, 29, 43 | 7 | 37 | 29 | 91 |
| | AN-238 | | | | | | |
| | AN-201 | | | | | | |
| 2 | Control | | | | | | |
| | AN-215 | 150 | 1, 15, 36, 51 | 7 | 60 | 27 | 76 |
| 3 | Control | | | | | | |
| | AN-215 | | | | | | |
| | AN-238 | | | | | | |
| | Combination 1/2 doses each[b] | 150 | 1, 15, 29, 43 | 9 | 42 | 48 | 50 |
| | Combination alternately[c] | | | | | | |
| 4 | Control | | 1, 11, 22, 31, | | | | |
| | AN-207 | 100 | 40, 51, 82, 93, | 10 | 61 | 49 | Survival experiment[f] |
| | AN-201 | | 113, 267 | | | | |
| 5 | Control | | | | | | |
| | AN-238 | 150 | 1, 15, 30 | 8 | 43 | 53 | 47 |
| | AN-215 | | | | | | |
| | AN-207 | | | | | | |
| | Combination 1/3[d] | | | | | | |
| | Combination alternately[e] | | | | | | |

[a]Male nude mice were used in Experiments 1–4 and females in Experiment 5.
[b]AN-215 + AN-238, 75 nmol/kg each, mixed.
[c]150 nmol/kg AN-238 on days 1 and 29; 150 nmol/kg AN-215 on days 15 and 43.
[d]AN-238 + AN-215 + AN-207, 50 nmol/kg each, mixed.
[e]150 nmol/kg AN-238 on day 1; 150 nmol/kg AN-215 on day 15; 150 nmol/kg AN-207 on day 30.
[f]The mice either died or were killed when they became weak or moribund. One mouse receiving AN-207 survived and the growth of the two tumors in this animal was followed up for about 1 year. The cancer started regrowing after 200 days, and once more treatment on day 267 with a higher dose of AN-207 (200 nmol/kg) temporarily stopped the growth of tumors.

### Histologic and immunohistologic analysis

The methods of histologic studies, the calculation of mitotic and apoptotic indices and the demonstration and calculation of the argyrophilic nucleolar organizer regions (AgNORs) have been described previously [25]. The number of AgNOR granules is an indicator of cell proliferation. For immunohistochemical analysis of receptors in paraffin-embedded tumor tissues, rabbit polyclonal primary antibodies Novus Biologicals (Littleton, Colorado, USA) at 10 μg/ml concentrations were used. The slides were incubated in antibodies to somatostatin receptor type 2 (s6 + 2) and bombesin/gastrin-releasing peptide (GRP) receptor for 60 min at room temperature and to somotostatin receptor type 5 (s6 + 5) and gonadotropin-releasing hormone (LHRH) receptor overnight at 4°C. Before the incubation in antisera to somotostatin receptor type 5, GRP and gonadotropin-releasing hormone receptor, the slides in sodium citrate buffer (10 mmol/l, pH 6.0) containing 0.05% Tween 20 were heated in a pressure cooker for 1 min. For the detection, Vectastain Elit ABC kit and NovaRed substrate (both from Vector Labs, Burlingame, California, USA) were used.

### Receptor assays

The binding characteristics of receptors for somatostatin, bombesin/GRP and LHRH were determined in tumor membrane fractions of control mice as described [23,25,26]. For in-vitro ligand competition studies, radioiodination of somatostatin analog RC-160, [$Tyr^4$]bombesin and [D-$Trp^6$]LHRH, and separation of the monoiodinated radioligands by high-pressure liquid chromatography were carried out [23,25,26].

### In-vitro studies

For the analysis of in-vitro effects of the compounds on cell proliferation, SK-Hep-1 cells were incubated with AN-238, AN-215, AN-207 or AN-201, each at 35 nmol/l concentration, for 60 min. After 48 h, cell viability was determined by incubation with 96 $AQ_{ueous}$ Cell Proliferation Assay reagent (Promega, Madison, Wisconsin, USA) for 2 h. The absorbance was measured at 550 nm using a Dynex plate reader (Dynex Technologies, Chantilly, Virginia, USA). For receptor blocking, the cells were preincubated with 1 μmol/l somatostatin analog RC-160, bombesin antagonist RC-3095 or LHRH antagonist cetrorelix for 30 min before adding the cytotoxic analogs to the media.

The induction of apoptosis was measured by detection of apoptosis-related peptide poly(ADP ribose) polymerase (PARP) using standard Western blotting assay. Briefly, proteins from SK-Hep-1 cells treated with 1.5 μmol/l or 35 nmol/l AN-238, AN-215, AN-207 or AN-201 were separated by 10% SDS–PAGE and incubated with anti-PARP p85 fragment polyclonal antibody (1 : 400; Promega Corporation, Madison, Wisconsin, USA) or β-actin monoclonal antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, California, USA) at 4°C overnight. The bands were visualized with enhanced chemiluminescence Western blot detection system (Amersham, Arlingon Heights, Illinois, USA) and quantified with ChemiImager 4000 (Alpha Innotech Corp., San Leandro, California, USA). Relative protein levels were normalized versus the corresponding levels of β-actin.

### Statistical analysis

SigmaStat software (Jandel Scientific, San Raphael, California, USA) was used for the statistical evaluation. The data were analyzed by analysis of variance and the groups compared using Dunnett's method.

## Results

### Effects of treatment with cytotoxic compounds *in vivo*

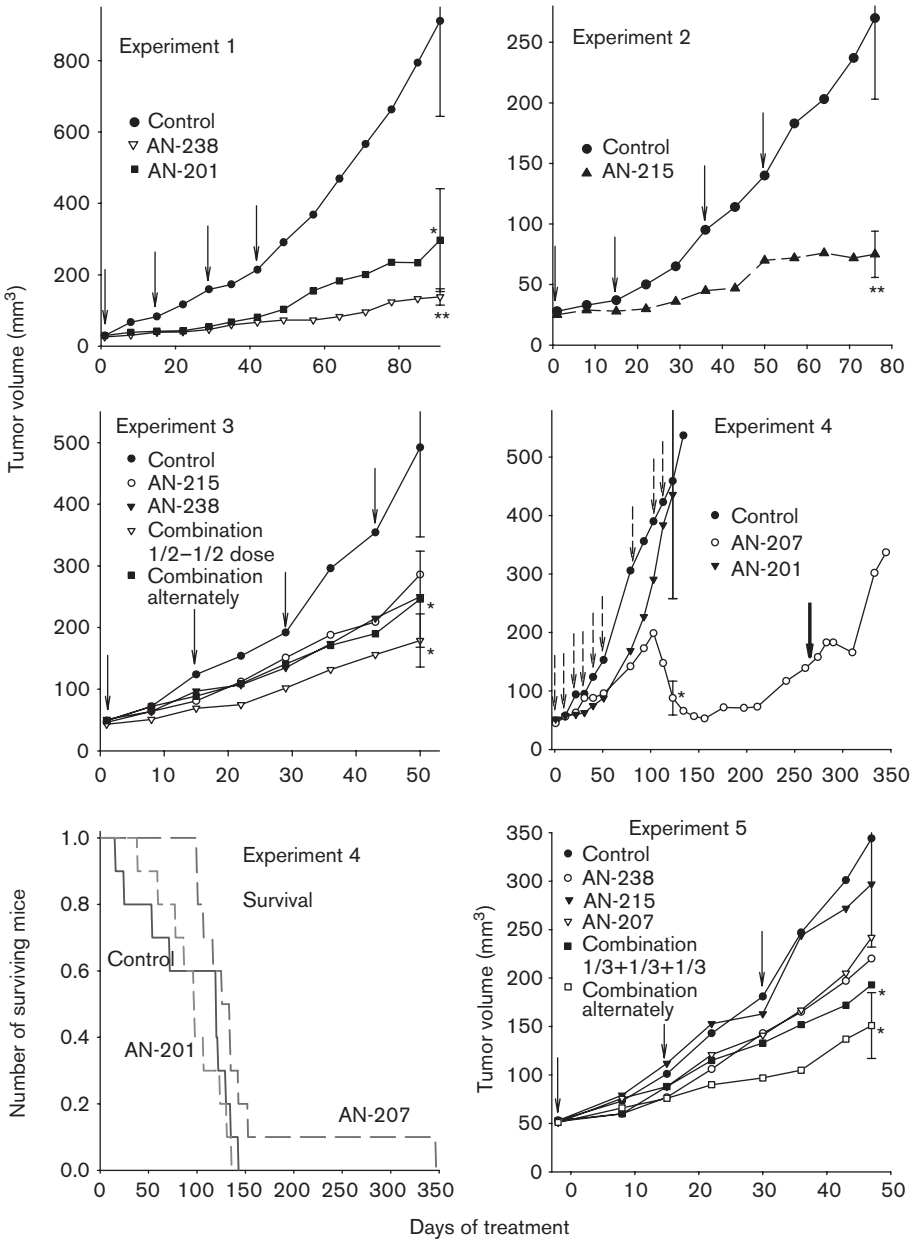
In Experiment 1, treatment with AN-238 significantly decreased tumor volume from day 49 and treatment with AN-201 from day 79 as compared with controls (Fig. 1 and Table 2). Treatment with AN-238 significantly reduced the number of mitoses and AgNOR scores in tumors and increased the apoptotic indices (Table 3). Body weights of mice receiving AN-201 were significantly smaller than those in the other two groups and spleen weights were reduced in both the treated groups.

In Experiment 2, treatment with AN-215 resulted in a strong inhibition of tumor growth (Fig. 1 and Table 2). Mitotic indices and AgNOR numbers were significantly decreased by AN-215, but apoptosis was not affected (Table 3). Body weights were found slightly ($< 10\%$) decreased 1 week after each injection of AN-215, but the weights were recovered by the end of the experiment (data not shown). Organ weights were not changed by the therapy.

In Experiment 3, tumor volume was significantly smaller in the groups treated with a combination of the two compounds (Fig. 1 and Table 2). Histologically, treatment with AN-215 decreased mitotic indices and combination therapy reduced AgNOR numbers (Table 3). Body weights were not significantly changed by the treatments at the end of the experiment, but liver, spleen and heart weights were smaller in the group treated with AN-238 (data not shown).

In Experiment 4, mean tumor volume in the group treated with AN-207 was significantly smaller ($P = 0.025$) than control volume on day 123. Treatment with AN-201 had no effect on tumor growth (Fig. 1 and Table 2). The median survival of control mice was 120 days. Median survival of the mice was extended by AN-207 to 134 days and shortened by AN-201 to 97.5 days. The log-rank test showed that the survival of animals treated with AN-207 was significantly ($P = 0.0195$) longer than that of the

**Fig. 1**




Effect of treatment with cytotoxic somatostatin (AN-238), bombesin (AN-215) and luteinizing hormone-releasing hormone (AN-207) analogs or their combination, and cytotoxic radical AN-201 on growth of SK-Hep-1 human hepatocellular cancers xenografted into nude mice. The arrows indicate days and doses of treatment (—— 150 nmol/kg, - - - - - 100 nmol/kg, ▬▬ 200 nmol/kg). Vertical bars: standard error. *$P<0.05$, **$P<0.01$ versus control. In Experiment 4 also survival of mice is shown.

animals receiving AN-201. Therapy with AN-207 also significantly ($P = 0.0291$) extended the survival of mice compared with control tumorous animals in the first 3 months of the experiment. No histologic examination was done in this experiment.

In Experiment 5, treatment with AN-238 and AN-207 resulted in a moderate inhibition of tumor growth and AN-215 was practically ineffective. The combination of the three compounds provided the best results (Fig. 1 and Table 2). Histologic analysis showed decreases in AgNOR numbers and mitotic indices in the groups with the smallest tumor sizes (Table 3). Body and organ weights were not different among the groups, only liver weights were found to have decreased in the mice treated with single agents (data not shown).

**Table 2 Effect of treatment with cytotoxic analogs of somatostatin (AN-238), bombesin (AN-215), luteinizing hormone-releasing hormone (AN-207) and their combination, and cytotoxic radical AN-201 on growth characteristics of SK-Hep-1 human hepatocellular cancers xenografted into nude mice**

| Experiment/group | Tumor growth reduction[a] (%) | Number of mice that died during the experiment | Final tumor volume ($mm^3$) on day | Tumor doubling time (days) | Tumor weights (mg) |
|---|---|---|---|---|---|
| Experiment 1 | | | 91 | | |
| Control | | 0 | 911 ± 267 | 25.6 ± 4.0 | 710 ± 193 |
| AN-238 | 87 | 0 | 138 ± 23* | 58.0 ± 12.9* | 136 ± 24* |
| AN-201 | 70 | 3 | 297 ± 144* | 41.3 ± 5.6 | 253 ± 123* |
| Experiment 2 | | | 76 | | |
| Control | | 4 | 275 ± 67 | 31.8 ± 9.5 | 215 ± 78 |
| AN-215 | 80 | 2 | 75 ± 19** | 82.8 ± 24.2* | 84 ± 29 |
| Experiment 3 | | | 50 | | |
| Control | | 1 | 492 ± 145 | 15.5 ± 0.9 | 301 ± 23 |
| AN-215 | 46 | 2 | 286 ± 86 | 21.5 ± 1.7 | 234 ± 59 |
| AN-238 | 55 | 1 | 250 ± 48 | 27.5 ± 4.6 | 322 ± 69 |
| Combination 1/2 + 1/2 | 69 | 2 | 179 ± 43* | 57.2 ± 23.6* | 182 ± 45 |
| Combination Alternately | 56 | 3 | 256 ± 78* | 25.4 ± 3.0 | 127 ± 46 |
| Experiment 4 | | | 123 | Until day 123 | |
| Control | | | 431 ± 201 | 38.9 ± 3.9 | Not measured |
| AN-207 | 89 | | 88 ± 29* | 207.3 ± 110.9* | |
| AN-201 | 4 | | 419 ± 185 | 53.6 ± 13.6 | |
| Experiment 5 | | | 47 | | |
| Control | | 6 | 343 ± 102 | 30.0 ± 6.4 | 368 ± 155 |
| AN-238 | 42 | 5 | 219 ± 37 | 27.9 ± 2.0 | 249 ± 44 |
| AN-215 | 16 | 2 | 297 ± 96 | 29.0 ± 5.8 | 334 ± 138 |
| AN-207 | 34 | 1 | 242 ± 110 | 37.4 ± 9.6 | 237 ± 126 |
| Combination 3 × 1/3 | 52 | 4 | 193 ± 42* | 59.8 ± 16.6* | 214 ± 73 |
| Combination Alternately | 66 | 4 | 152 ± 34* | 52.1 ± 9.3* | 214 ± 66 |

Values are mean ± standard error; *$P<0.05$; **$P<0.01$ versus control.
[a]Tumor growth reduction (TGR) was calculated using the formula $TGR\% = 100 - 100 \times (T - t)/(C - c)$, 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 the treatment and $C$ is the volume of controls at the end of the experiment.

**Table 3 Effect of therapy with cytotoxic analogs of somatostatin (AN-238), bombesin (AN-215), luteinizing hormone-releasing hormone (AN-207) and their combination and cytotoxic radical (AN-201) on histological growth characteristics of SK-Hep-1 human hepatocellular cancers in nude mice**

| Experiment/group | Interval between last treatment and killing (days) | Mitotic index | Apoptotic index | AgNOR count |
|---|---|---|---|---|
| Experiment 1 | | | | |
| Control | 50 | 9.4 ± 1.4 | 8.4 ± 0.8 | 6.42 ± 0.30 |
| AN-238 | | 5.4 ± 1.0* | 12.0 ± 0.7* | 4.84 ± 0.29* |
| AN-201 | | 5.7 ± 1.0 | 11.8 ± 1.9 | 5.43 ± 0.13 |
| Experiment 2 | | | | |
| Control | 30 | 4.3 ± 0.9 | 7.0 ± 0.9 | 6.78 ± 0.13 |
| AN-215 | | 1.5 ± 0.3* | 6.8 ± 1.1 | 5.58 ± 0.08* |
| Experiment 3 | | | | |
| Control | 6 | 3.6 ± 0.6 | 12.7 ± 3.4 | 5.31 ± 0.13 |
| AN-215 | | 1.7 ± 0.2* | 6.0 ± 0.5 | 4.85 ± 0.17* |
| AN-238 | | 2.4 ± 0.7 | 10.0 ± 1.1 | 4.84 ± 0.07* |
| Combination 1/2 + 1/2 | | 2.2 ± 0.4 | 9.8 ± 1.0 | 4.43 ± 0.14* |
| Combination alternately | | 2.5 ± 0.5 | 8.3 ± 0.8 | 4.93 ± 0.10* |
| Experiment 5 | | | | |
| Control | 20 | 5.5 ± 0.8 | 9.2 ± 1.6 | 6.61 ± 0.10 |
| AN-238 | | 3.9 ± 0.7 | 9.9 ± 0.5 | 5.11 ± 0.10* |
| AN-215 | | 5.1 ± 0.9 | 9.4 ± 1.0 | 6.32 ± 0.13 |
| AN-207 | | 5.0 ± 0.5 | 12.3 ± 0.7 | 5.70 ± 0.09* |
| Combination 1/3 + 1/3 + 1/3 | | 3.1 ± 0.5* | 11.5 ± 0.3 | 4.92 ± 0.11* |
| Combination alternately | | 3.3 ± 0.4* | 10.0 ± 0.7 | 4.85 ± 0.07* |

Values are mean ± standard error; *$P<0.05$ versus control.
AgNOR, argyrophilic nucleolar organizer region.

### Receptor analysis

Using radiolabeled RC-160 as a radioligand, specific high-affinity and low-capacity receptors for somatostatin were detected in control SK-Hep-1 tumor samples. Radiolabeled [$Tyr^4$]bombesin was bound to a single class of specific high-affinity and moderately high-capacity bombesin/GRP receptors in untreated SK-Hep-1 cancer samples. Similarly, high-affinity and low-capacity receptors for LHRH were demonstrated in tumor membranes of control HCC samples by using radiolabeled [D-$Trp^6$]LHRH as a radioligand. Receptor binding data are shown in Table 4.

Immunohistochemical investigation similarly showed that subtypes 2 and 5 somatostatin receptors and receptors for GRP and LHRH were present in tumor cells. Immunohistochemistry provides valuable information about the localization of receptors and the distribution of antibody-expressing cells. Figure 2 demonstrates some characteristic immunohistochemical patterns of receptor expression in SK-Hep-1 cancers.

### In-vitro studies

The effects of AN-238, AN-215, AN-207 and AN-201 on apoptosis and cell proliferation were analyzed. Strong immunoreactive bands of about 85 kDa, specific for apoptosis-related cleaved PARP, were seen in western blots from all treated cells, but not in the control cells

(Fig. 3). No significant differences were found in the apoptosis-inducing effects of the four cytotoxic compounds *in vitro*.

The four agents also uniformly inhibited proliferation of SK-Hep-1 cells. A preincubation with the respective peptides decreased the antiproliferative effect of the cytotoxic hormone analogs (Fig. 4), but none of the blocking peptides altered the effect of AN-201 (data not shown).

## Discussion

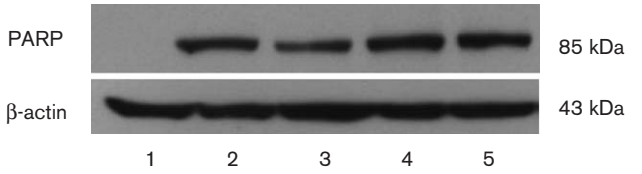
The expression of somatostatin receptors in the livers of patients with various hepatic diseases, including HCC, was verified several years ago [27,28]. Later, specific subtypes of somatostatin receptors were identified in HCC and liver cirrhosis [29,30]. Recently, Reubi *et al.* [28] detected somatostatin binding in 41% of 59 human HCCs by receptor autoradiography, but found no binding in normal liver tissue. Somatostatin receptors were demonstrated also in HCC cell lines, such as Hep-G2, Hep-3B and SMMC-7721 [26,30–33]. Based on the ineffectiveness of systemic cytotoxic therapy on HCC and the presence of receptors for somatostatin in hepatic tumors, several groups tested somatostatin analogs for treatment of patients with HCC [34]. The results were variable. Dimitroulopoulos *et al.* [35] and Samonakis *et al.* [36] reported an improvement in survival and quality of

**Table 4 Binding characteristics of receptors for somatostatin, bombesin and LHRH on membranes of SK-Hep-1 human hepatocellular cancers transplanted into nude mice**

| Receptors | $K_d$ (nmol/l) | $B_{max}$ (fmol/mg protein) |
|---|---|---|
| Somatostatin | 6.17 ± 0.41 | 293.2 ± 68.7 |
| Bombesin | 7.45 ± 0.61 | 999.2 ± 93.9 |
| LHRH | 7.22 ± 0.94 | 530.9 ± 46.9 |

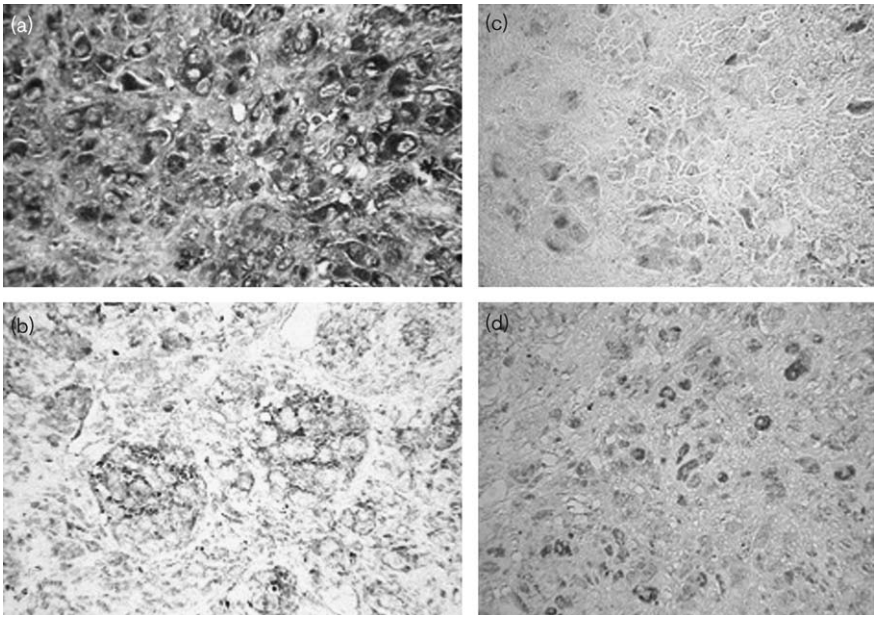
Binding assays were carried out with radiolabeled RC-160, [Tyr$^4$]bombesin and [D-Trp$^6$]LHRH as described in Materials and methods.
Binding characteristics were obtained from 12-point displacement experiments. Values are mean ± standard error of three independent experiments, each done in duplicate or triplicate.
LHRH, luteinizing hormone-releasing hormone.

**Fig. 3**



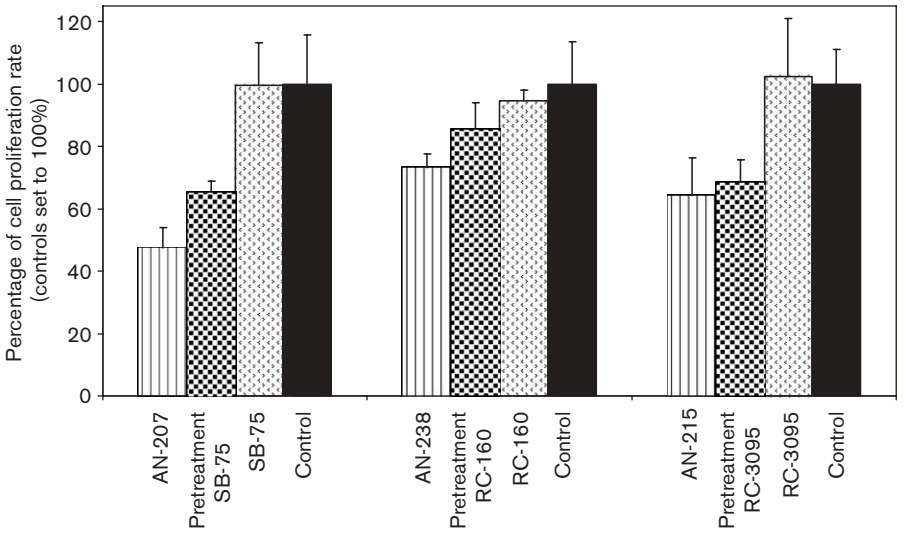

Expression of cleaved poly(ADP ribose) polymerase (PARP) in SK-Hep-1 cells detected by western blotting as described in Materials and methods. Lanes: 1, control; 2, AN-201; 3, AN-238; 4, AN-215; 5, AN-207. Apoptosis induction was clearly demonstrated in all treated samples, but not in control cells. The experiments were repeated with identical results.

**Fig. 2**




Immunohistochemical demonstration of various peptide hormone receptors in human SK-Hep-1 cancers in nude mice. The staining procedure is described in Materials and methods. Cells expressing various receptors were quite evenly scattered in the tumors, but cells with sst5 formed smaller or larger groups in some areas. sst2 seemed to be the most frequent receptor, appearing in about 80% of cancer cells, whereas LHRH and GRP receptors were present in about 30–50% of tumor cells. Mostly a diffuse or spotted cytoplasmic staining was observed but sst5 often showed a marked reaction on the surface of cells. (a) Subtype 2 somatostatin receptors, most cells show dark cytoplasmic staining (methylgreen counterstain, ×180). (b) Subtype 5 somatostatin receptors, a pronounced cell membrane reaction is apparent (×250). (c) GRP receptors, scattered cells exhibit strong diffuse or focal cytoplasmic staining (×150); and (d) LHRH receptors, the receptor containing cells stained with variable intensity (×120). LHRH, luteinizing hormone-releasing hormone; GRP, gastrin-releasing peptide.

**Fig. 4**




Demonstration of the viability of SK-Hep-1 cells treated with various cytotoxic hormone analogs by the CellTiter 96 $AQ_{ueous}$ One Solution Cell Proliferation Assay as described in Materials and methods. Significant decreases in cell proliferation were induced by AN-207 ($P=0.003$), AN-238 ($P=0.007$) and AN-215 ($P=0.001$) compared with controls. The antiproliferative effect of AN-207 was significantly reduced by pretreatment with cetrorelix ($P=0.003$) and that of AN-238 by RC-160 ($P=0.047$), but pretreatment with RC-3095 caused only a slight and not significant ($P=0.483$) reduction in the effect of AN-215.

life of patients with HCC after treatment with somatostatin analogs, octreotide or lanreotide, and Rahmi *et al.* [37] described complete remission in one patient. In contrast, others found no antitumor activity [31,38].

The expression of somatostatin receptors on various tumors allows a possible targeted therapy with cytotoxic somatostatin analogs such as AN-238. This compound, similarly to its carrier somatostatin analog molecule, binds preferentially to sst2 and sst5, and shows moderate affinity to sst3 [19]. AN-238 inhibited the growth of a wide variety of experimental human tumors expressing somatostatin receptors [16–20].

Our study demonstrates the expression of high-affinity receptors for somatostatin in SK-Hep-1 tumors and the presence of both sst2 and sst5, which are the most important subtypes for binding of somatostatin analogs. AN-238 had a greater inhibitory effect on growth of hepatic cancer than cytotoxic radical AN-201 and was less toxic.

Bombesin/GRP receptors are expressed on a wide variety of tumors including prostatic, breast and ovarian cancers [17], but their occurrence in HCC has not been investigated. Some experimental studies indicated that bombesin is present in rat hepatocellular tumors [39,40]. Bombesin also increased phosphorylation of intracellular proteins in human liver cancer samples [41]. The occurrence of bombesin receptors on HCC [28,42], however, has not been reported.

We detected high-affinity receptors for bombesin/GRP in SK-Hep-1 human HCC grown in nude mice. Cytotoxic bombesin analog AN-215 powerfully inhibited tumor growth in Experiment 2, but was somewhat less effective in Experiment 3, and had little effect in Experiment 5.

Receptors for LHRH were described earlier on Hep-G2 cells [43–45], but this is the first report on their expression in SK-Hep-1 tumors. Antiandrogens and antiestrogens have been tried for the treatment of patients with HCC, but the results were essentially negative [3,46–50].

Cytotoxic LHRH analog AN-207 strongly inhibited growth of SK-Hep-1 cancers in nude mice and significantly extended the survival of nude mice. Cytotoxic radical AN-201 was ineffective.

Histologic characteristics of proliferation were changed by various treatments. AgNOR scores, which are good indicators of cell proliferation rate, showed good correlations with growth inhibition indicated by tumor volume, and mitotic indices were also lower in some treated groups.

The disparity in the efficacy of the therapy in the five experiments may be owing to the differences in tumor passages, as the experiments were done consecutively in the order in which they are numbered. In addition, the experimental settings were also different in the five

studies (length of experiments, sizes of tumors at the start of treatment, the intervals between last treatment and sacrifice of mice, etc.) as shown in Tables 1 and 3.

As many normal tissues such as the gastroenteropancreatic system, the kidneys and pituitary also express high-affinity receptors for somatostatin, bombesin and LHRH, some side effects of treatments with cytotoxic hormone analogs were expected. No receptor-specific toxicity was observed in our earlier studies with AN-238 or AN-215, probably because they were used in relatively low doses [19]. We have never observed intestinal toxicity caused by these cytotoxic compounds, despite the expression of receptors and relatively high cell turnover rate of the intestinal mucosa [18]. Earlier we showed that treatment with cytotoxic LHRH analog AN-207 caused only temporary damage to the gonadotroph cells in the pituitary [19].

The possible hepatic consequences of cytotoxic therapy targeted to somatostatin receptors were discussed previously [26]. We have never encountered hepatic toxicity in our in-vivo experiments with cytotoxic analogs of somatostatin, bombesin and LHRH at therapeutic doses. The effect of cytotoxic peptides on the livers with cirrhosis, however, is not known and has to be clarified in further investigations.

The binding of the cytotoxic peptide to the receptor is followed by internalization [16–20]. The active agent is the cytotoxic radical, which interacts with cytoplasmic and nuclear components, and affects primarily rapidly proliferating cells. The internalization process was demonstrated on several experimental models [18–20]. Thus, a specific internalization of cytotoxic LHRH analogs in rat pituitary and human ovarian, endometrial and breast cancer cells was shown by real-time optical tracking methods [51–54]. The mechanism of targeting, however, is effective only *in vivo*. In cell culture, cytotoxic compound AN-201, added to the medium, is readily available for the cells and rapidly enters the cells without receptor involvement. Therefore, specific methods are needed to demonstrate *in vitro* the receptor-specific action of our cytotoxic hormone analogs. In earlier in-vitro studies with cytotoxic bombesin and LHRH analogs, we could demonstrate specific, receptor-mediated action by microsatelite analysis [55,56]. Although this targeting mechanism has not yet been demonstrated with cytotoxic somatostatin analogs, the localization of primary and metastatic tumors by scintigraphic techniques using radiolabeled somatostatin analogs strongly supports this theory [17].

Another important factor of targeting is the stability in serum of the linker between the carrier and the cytotoxic agent. Our investigations show that the half-life of cytotoxic analog AN-152 in human serum is about 2 h, which seems to be adequate for targeting [57].

In-vitro studies reported in this investigation demonstrate the apoptosis-inducing action of the cytotoxic compounds on SK-Hep-1 cells by western blotting and show the 85-kDa fragment of PARP that results from caspase cleavage. The four compounds, including AN-201, had similar antiproliferative and apoptosis-inducing activity *in vitro*. This finding is understandable in view of the information cited above that the targeting mechanism is only effective *in vivo*. The pretreatment with the corresponding noncytotoxic peptides, however, significantly decreased the antiproliferative effect of the cytotoxic hormone analogs, confirming the receptor-mediated action of these compounds even *in vitro*.

As most tumors express receptors for various peptide hormones [58,59], treatment with combinations of two or three cytotoxic hormone analogs seems to be reasonable. Such targeted combination treatment may further increase inhibitory action on tumors. In this study, therapy with a combination of two or three cytotoxic analogs was also more powerful than therapy with single cytotoxic agents. In a clinical setting, combination therapy and the doses will also depend on the types and concentrations of the receptors on tumors. For tumors that strongly express single receptors for a respective peptide, combination therapy may not have advantages over treatment with single agents [60]. Targeted therapy with a combination of various cytotoxic analogs, however, may be preferable for treating tumors with a lower concentration of multiple hormonal receptors. Our immunohistochemical study showed that the overlapping of various peptide receptors in tumor cells may explain why sometimes the efficacy of combination therapy only moderately exceeds the effects of single agents.

In conclusion, our findings demonstrate that cytotoxic analogs of somatostatin, bombesin and LHRH and their combinations strongly inhibit growth of SK-Hep-1 human HCC in nude mice. Some of our cytotoxic hormone analogs are already in phase II clinical trials. Our results encourage further investigations on the in-vivo action of these compounds on other types of liver cancers and on nontumorous livers to permit the introduction of the compounds into clinical trials on HCC.

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