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Report

Effective treatment of advanced estrogen-independent MXT mouse mammary cancers with targeted cytotoxic LH-RH analogs

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Key words: breast cancer, cell death, cell proliferation, doxorubicin, LH-RH analogs, targeted chemotherapy

Summary

Cytotoxic agents linked to hormonal carriers provide new approaches to tumor therapy, and LH-RH receptors expressed by breast cancers can be used for targeting chemotherapeutic compounds. In the present study, large, advanced estrogen-independent MXT mouse mammary cancers were treated with cytotoxic LH-RH analog AN-152 containing doxorubicin (DOX) or AN-207 incorporating superactive derivative 2-pyrrolino-DOX (AN-201). These cytotoxic hybrid molecules were administered once i.v., close to their maximum tolerated doses, at various time intervals after transplantation of tumors. The cytotoxic LH-RH analogs and the radicals alone, given at earlier stages of tumor development, inhibited growth of MXT cancers. Cytotoxic LH-RH conjugate AN-207 had significantly stronger effect than its respective cytotoxic radical, particularly when larger tumors were treated, causing 95%, 89%, 100% and 96% tumor growth reduction when administered on days 1, 7, 10 or 14, respectively. AN-152, AN-201, and DOX, given on day 14, were virtually ineffective. Histological characteristics of tumor cell proliferation and cell death were analyzed in large MXT cancers 1–4 days after treatment with AN-207 and AN-201. AgNOR scores were decreased and apoptotic indices increased after treatment of tumors with AN-207 or AN-201, but enhanced apoptosis and decreased AgNOR numbers persisted longer in the case of AN-207. In contrast to AN-201, AN-207 also increased the extent of necrosis in tumors. In conclusion, on the basis of its powerful inhibitory effect on the aggressive MXT mouse mammary tumor, the cytotoxic LH-RH analog AN-207 could be considered for treatment of advanced human mammary carcinomas that express LH-RH receptors.

Abbreviations: LH-RH: luteinizing hormone-releasing hormone; NOR: nucleolar organizing region; AgNOR: argyrophilic NOR; HPLC: high-performance liquid chromatography; DOX: doxorubicin; i.p.: intraperitoneally; i.v.: intravenously; TGR: tumor growth reduction

Introduction

Breast cancer is the most common malignancy among women in the Western world. Statistical projections indicate that in 1998 about 180,000 new breast cancer cases will be detected and over 43,000 women will die of breast carcinoma [1]. A recent slight fall in breast cancer mortality may be the result of an increased use of mammography and improvements in systemic adjuvant therapy of advanced cases [2]. However, most study groups agree that in the past decade the survival of patients with advanced breast cancer remained relatively constant [3–5]. Anthracyclines are the most active drugs for treatment of advanced breast carcinoma but their effects are limited by toxicity and tumor resistance, and the 5-year survival rate of patients with metastatic disease is still below 20% [5–7]. In recent trials, the use of doxorubicin (DOX) and paclitaxel in combination therapy produced only a slight improvement in the treatment of stage IV breast carcinoma [6, 8]. These disappointing results in chemotherapy of breast cancer indicate the need to develop new treatment modalities and explore new active drugs and combinations [3–6].

Targeted therapy is a modern approach to increase clinical efficacy and decrease toxicity. Targeted therapy requires an adequate delivery system making possible the recognition of tumors and an effector molecule for killing tumor cells [9]. New discoveries in the field of molecular oncology revealed new perspectives for targeting specific molecules to cancer cells [10]. Various types of antibodies or hormones have been tested as delivery systems, and cytotoxic drugs, toxins, or radionuclides have been used as effectors [9, 11]. Antigens, enzymes, or receptors that are present predominantly in tumor cells can be used for targeting [12]. Various studies, reviewed in [11, 13], indicate that more than 50% of human breast cancers express LH-RH receptors.

On the basis of the presence of receptors for LH-RH on breast, endometrial, ovarian, and prostatic cancers [11, 13], we developed a new class of targeted antitumor agents by linking cytotoxic radicals to LH-RH analogs [11]. Recently, we coupled DOX-14-*O*-hemiglutarate to [D-Lys^6]LH-RH to form cytotoxic analog AN-152. An even more potent hybrid molecule (AN-207) contains 2-pyrrolino-DOX (AN-201), a daunosamine-modified derivative of DOX, which is 500–1000 times more active *in vitro* than its parent compound, conjugated to the same carrier [14, 15]. These new cytotoxic LH-RH analogs powerfully inhibited the growth of various experimental tumors including estrogen-independent MXT mouse mammary cancers [16].

Animal models are critical for the evaluation of new therapies, and each model has specific advantages and disadvantages [17]. The MXT mouse mammary cancer is a very reproducible model. The tumors grow fast and invasively, contain little necrosis, and kill the mice regularly between days 18 and 24 after transplantation. In previous studies, we started therapy one day after transplantation because of the highly malignant characteristics of estrogen-independent MXT cancers and a short life span of mice bearing these tumors. In the present study, we evaluated the effects of the cytotoxic LH-RH analogs AN-207 and AN-152 on the growth of large, well-developed estrogen-independent MXT cancers, considering that the treatment of advanced cancers is the most difficult task and the greatest challenge for oncologists. In earlier studies, cytotoxic compounds were administered i.p. Because of possible differences in absorption of DOX, AN-201, AN-152, and AN-207 from the sites of i.p. injections, in the present investigation the compounds were administered i.v. to allow more exact comparison of the antitumor activities of the analogs and the cytotoxic radicals.

Materials and methods

Materials

LH-RH agonist [D-Lys^6]LH-RH (pyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2) was synthesized in our laboratory by solid-phase methods and purified by high-performance liquid chromatography (HPLC). DOX × HCl salt was purchased from Aldrich (Milwaukee, WI). AN-152 (DOX-14-*O*-hemiglutarate coupled to [D-Lys^6]LH-RH), AN-201 (2-pyrrolino-DOX), and AN-207 (conjugate of [D-Lys^6]LH-RH and 2-pyrrolino-DOX-14-*O*-hemiglutarate) were prepared as described [14, 15]; the chemical structures are shown in Figure 1. The compounds were dissolved in 6% (w/v) mannitol in water shortly before injection.

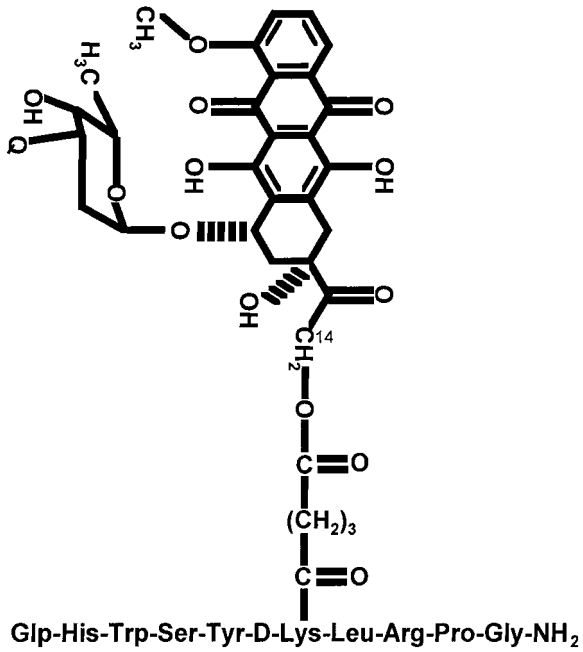

Figure 1. The structures of LH-RH analogs AN-152 and AN-207. [D-Lys^6]LH-RH is linked through a glutaric acid spacer to the 14-OH group of doxorubicin (Q = NH_2) to form AN-152, or to 2-pyrrolinodoxorubicin (Q = N) to produce AN-207. (Modified from Proc Natl Acad Sci USA 93: 7269–7273, 1996 [15]. Copyright (1996), National Academy of Sciences (USA). Reprinted with permission of Proceedings of the National Academy of Sciences, USA.)

Animals and tumors

Female B_6D_2F1 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The maintenance of animals, the source and transplantation method of MXT(3.2)Ovex mammary carcinoma, and the calculation of tumor volume were reported [18]. All experiments were performed according to institutional ethical guidelines.

Experimental protocol

One day after transplantation of tumors, the mice were randomly divided into groups. In Experiment I, all groups contained nine animals each. Control mice were given injection solvent (6% mannitol in water). The experimental mice received equimolar amounts (20.75 μmol/kg) of AN-152 (46.38 mg/kg) or DOX (12.0 mg/kg) which were injected once into the jugular vein but on different days, the groups being treated with the compounds 1, 7, 10 or 14 days after transplantation of tumors. In Experiment II, the groups consisting of 10 mice each were given equimolar amounts (250 nmol/kg) of AN-207 (563 μg/kg) or AN-201 (175 μg/kg) once i.v. 1, 7, 10 or 14 days after transplantation of tumors and the control group received the solvent. Body weights of the mice were measured twice weekly. In Experiment III, the mice were divided into three groups. Eleven days after transplantation, when the tumors were well-developed, 12 mice received 250 nmol/kg of AN-207 i.v., another group of 12 mice was treated with 250 nmol/kg of AN-201 i.v., and six control animals were injected with the vehicle. Three mice in each treated group were sacrificed 1, 2, 3 or 4 days after injection, and the controls 1 and 4 days after treatment.

Tumor growth reduction (TGR) was calculated according to the formula: $\text{TGR}\% = 100 - 100 \times (T - t)/(C - c)$, where t = mean volume of treated tumors at time of therapy, T = mean volume of treated tumors at the day of measurement after treatment, c = mean volume of control tumors at the time of therapy, C = mean volume of control tumors at the day of measurement after treatment (for tumors treated on day 1 and day 7, $t = c = 0$). In Experiments I and II, the mice started dying after day 18. The decreased number of surviving mice, particularly in the control groups, did not allow a comparative evaluation of tumor sizes after day 21. Tumor volume measured on day 21 contained a few censored data, which were automatically handled by SigmaStat computer program by using a general linear model. Mean survival time of mice in treated groups (T) was compared to that of controls (C) and the result expressed as $T/C\%$. In Experiments I and II in which survival was also studied, the animals that developed large tumors usually died within 1–2 days. In Experiment III, the mice were sacrificed by decapitation under Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL) anesthesia. The tumors were removed, cleaned, weighed, and processed for histological examination.

Histological methods

The tumor samples were processed as described [18]. For the measurement of the number of mitotic and apoptotic cells in slides stained with hematoxylin and eosine, six high-power fields were evaluated, and the numbers of mitotic and apoptotic cells per 1000 cells were calculated and expressed as mitotic and apoptotic indices, respectively. For the determination of the extent of necrosis in tumors, the crossing points of a microscope ocular net that coincided with necrosis in the slide made at the largest cross-section of each tumor were counted. The ratio of these points to the number of all points above the tumor represented the percentage area of necrosis. The AgNOR method was used as described [19]. The number of AgNOR granules is an indicator of cell proliferation. The silver-stained black dots in 50 cells of each tumor were counted and the mean AgNOR number per cell was calculated.

Statistical methods

The SigmaStat and SigmaPlot software were used for the statistical evaluation of data and the preparation of figures. Tumor volume changes were surveyed by two-way repeated measures analysis of variance (ANOVA), and the groups were examined by Tukey's multiple comparison test. Survival data of the groups were studied by Kruskal–Wallis one way ANOVA on ranks. Histological data were evaluated by Duncan's multiple range test.

Results

The effects of AN-152 and DOX administered i.v. in doses of 20.7 μmol/kg on tumor volume of MXT estrogen-independent mammary cancers are shown in Figure 2. Both compounds significantly inhibited growth of MXT cancers. The two compounds had similar effects on tumor growth. Tumor volume data are

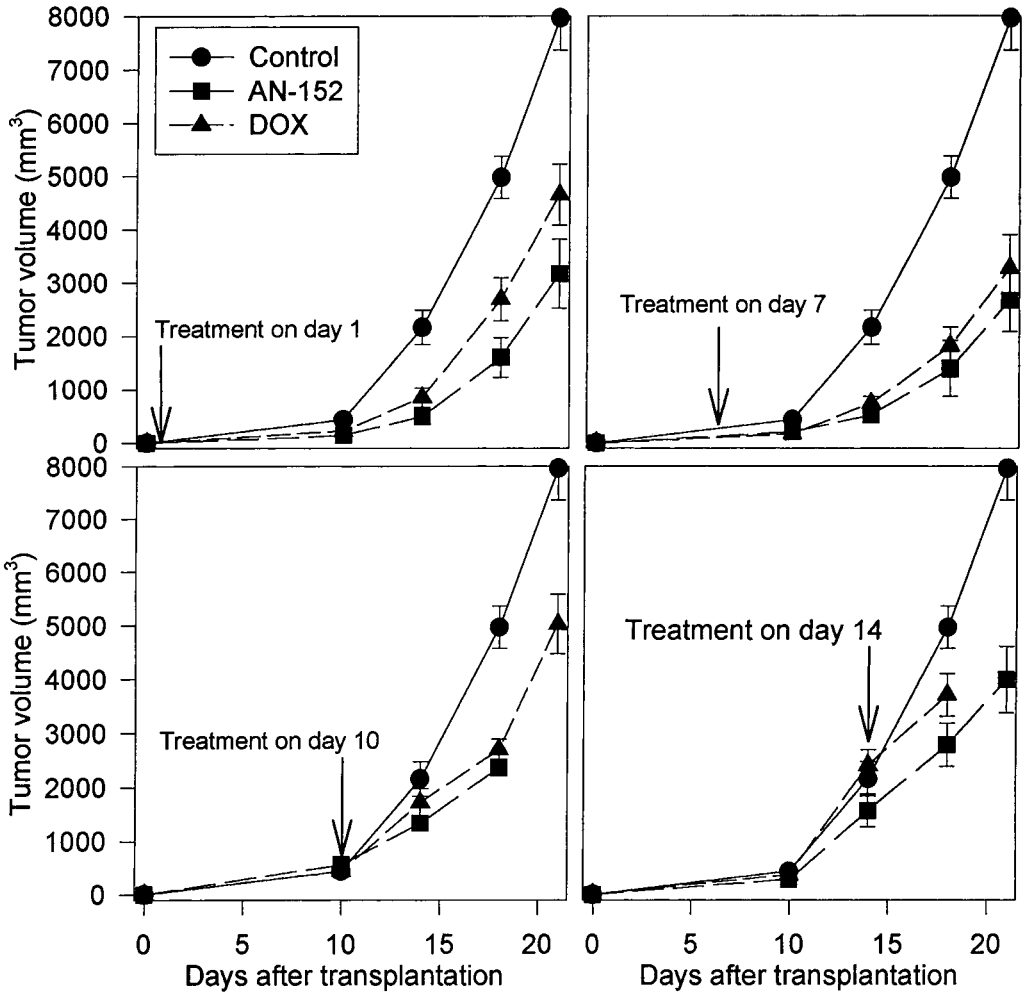

Figure 2. Effects of a single injection of 20.75 μmol/kg of AN-152 or doxorubicin given i.v. on different days after transplantation on tumor volume of estrogen-independent MXT cancers. Deaths of mice treated with AN-152 on day 10 and of animals receiving DOX on day 14 did not permit calculations of volume on day 21.

presented in Table 1. The survival of the mice was significantly extended in groups 2, 3 and 7 which were treated with AN-152 on day 1 or 7 and with DOX on day 7. The survival data are shown in Table 1 and Figure 3.

In the second experiment, mice bearing estrogen-independent MXT mouse mammary cancers were treated i.v. with 250 nmol/kg of AN-207 or AN-201 at different time intervals after tumor transplantation. The changes in tumor volume are shown in Figure 4. The difference in tumor inhibitory effect of the two compounds was relatively small when they were given on the first day after transplantation. However, AN-207 had a significantly stronger inhibitory action than the cytotoxic radical AN-201 when it was administered on days 7, 10 or 14. Tumor volume and survival data are shown in Table 2. The survival of mice with MXT cancers was significantly extended by treatment with AN-207 on day 10 and 14 or with AN-201 on day 1 (Figure 5, Table 2). In each group, the decrease in body weight after treatment was less than 15%, and the mice regained the original weights within 4–7 days.

In Experiment III, the mice were treated with AN-207 and AN-20111 days after transplantation of tumors, and they were sacrificed 1, 2, 3, or 4 days later to monitor changes in cancers after therapy. Mitotic indices and AgNOR scores are shown in Figure 6. Mitotic index was mainly unchanged, and decreased significantly only 4 days after administration of AN-207. Treatment with AN-207 as well as AN-201

Table 1. Effects of a single i.v. injection of 20.7 μmol/kg of cytotoxic LH-RH analog AN-152 or cytotoxic radical DOX made on different days on growth of estrogen-independent MXT mouse mammary cancers and on survival of mice

Group	Treatment on day	Tumor volume (mm^3) and tumor growth reduction (%)								Mean survival T/C (%)
		Day 10		Day 14		Day 18[d]		Day 21[d]		
		mm^3	%	mm^3	%	mm^3	%	mm^3	%	
1. Control		442 ± 82		2162 ± 317		4977 ± 395		7966 ± 606		100
2. AN-152	1	142 ± 59	67	500 ± 133	77[a,c]	1601 ± 370	68[b]	3171 ± 655	60[b]	123[e]
3. AN-152	7	206 ± 41	53	527 ± 131	76[a,c]	1725 ± 521	65[b,c]	2266 ± 580	72[b,c]	125[e]
4. AN-152	10	493 ± 22		1343 ± 100	51[c]	2371 ± 97	59[b]	1860 ± 826	82[b,c]	99
5. AN-152	14	315 ± 58		1571 ± 307		2365 ± 402	72[b]	3998 ± 614	58[b]	106
6. DOX	1	227 ± 55	49	853 ± 179	61[c]	2687 ± 404	46[b]	4648 ± 566	42[b]	111
7. DOX	7	178 ± 28	60	745 ± 127	66[c]	1818 ± 343	63[b]	3275 ± 633	59[b]	125[e]
8. DOX	10	438 ± 103		1012 ± 254	67[c]	2705 ± 188	50[b,c]	3491 ± 553	59[b]	109
9. DOX	14	372 ± 50		2216 ± 284		3443 ± 394	56[a]	4021 ± 570	52[b,c]	99

Tumor volume data are mean ± SE.
[a]$p < 0.05$, [b]$p < 0.001$ compared with control; data were evaluated by two-way repeated measures ANOVA, and the groups were compared by a multiple comparison procedure (Tukey test).
[c]Difference from previous measurement is not significant (Tukey test), which means growth inhibition.
[d]Missing data as a result of death of some mice, particularly in Groups 4 and 9, were automatically handled by SigmaStat by using a general linear model.
[e]$p < 0.05$ compared with control (Kruskal–Wallis one-way ANOVA on ranks).

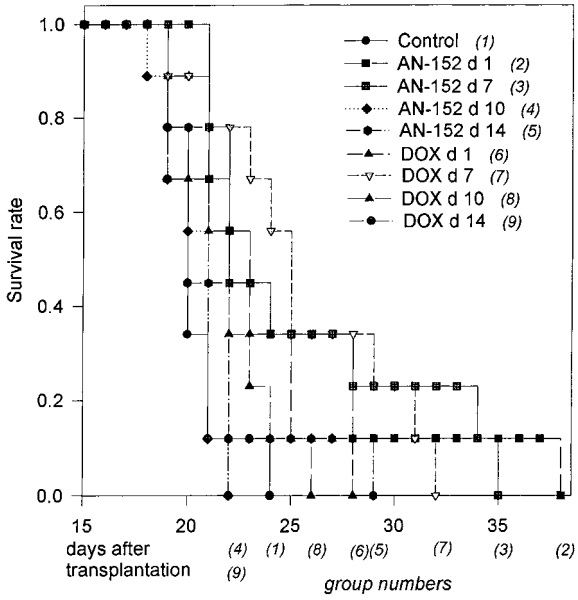

Figure 3. Survival of mice in Experiment 1. For easier identification, the ends of the lines are marked with group numbers in italics and parentheses.

significantly reduced AgNOR counts in tumors. The decrease in AgNORs persisted for 4 days after treatment with AN-207, but returned to control levels 3 days after therapy with AN-201. The extent of necrosis was significantly increased in the tumors treated with AN-207, but was not changed by AN-201 (Figure 7). A higher number of apoptotic cells was found in MXT cancers two days after therapy with either compound. The enhanced apoptosis persisted for at least 4 days in the group receiving AN-207, but gradually returned to control level by day 4 after treatment with AN-201 (Figure 7). The ratio of apoptotic to mitotic indices shows a significant difference between the effect of AN-207 and AN-201, 3 and 4 days after treatment (Figure 7).

Discussion

Since a relatively high proportion of human breast, ovarian, endometrial, and prostate cancers contains binding sites for LH-RH [11, 16, 18, 20, 21], these receptors can be used for targeting of anticancer drugs. Thus, chemotherapeutic agents linked to LH-RH analogs can be delivered more specifically to cells that have LH-RH receptors. Some cytotoxic compounds including DOX may not even need to enter targeted cells since they can act on cell surfaces and might also kill neighboring cells that do not have the targeted receptors [22].

Cytotoxic LH-RH analogs AN-152 and AN-207 preserve both the high binding affinity of the carrier to LH-RH receptors and also the antiproliferative activity of the respective cytotoxic radicals DOX and AN-201 [15]. AN-201, a daunosamine-modified

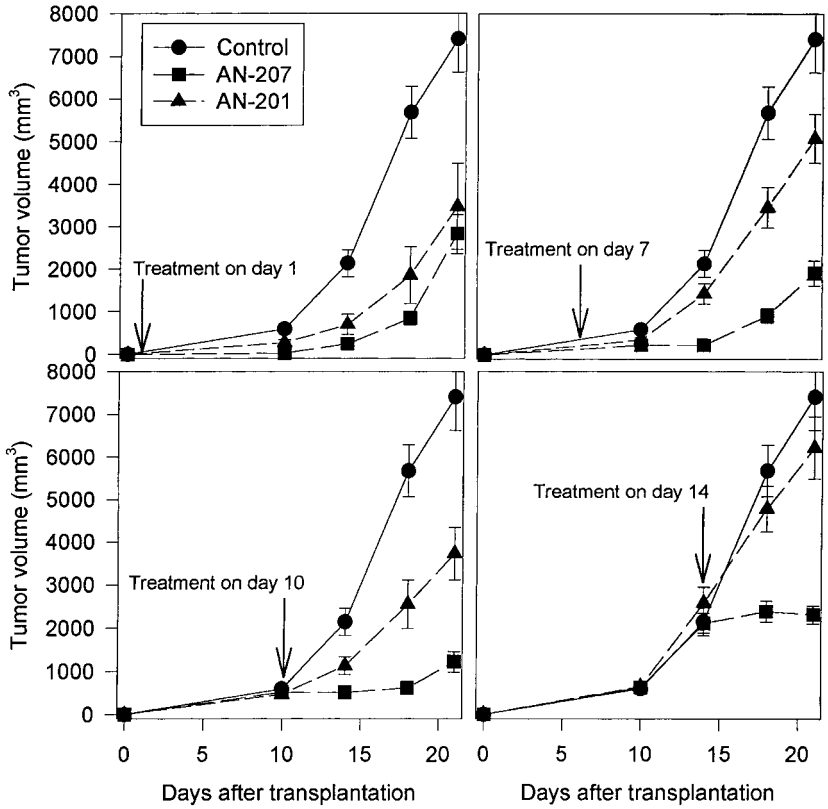

Figure 4. Effects of a single injection of 250 nmol/kg of AN-207 or AN-201 given i.v. on different days after transplantation on tumor volume of estrogen-independent MXT cancers.

Table 2. Effects of a single i.v. injection of 250 nmol/kg of cytotoxic LH-RH analog AN-207 or cytotoxic radical AN-201 made on different days on growth of estrogen-independent MXT mouse mammary cancers and on survival of mice

Group	Treatment on day	Tumor volume (mm^3) and tumor growth reduction (%)								Mean survival T/C (%)
		Day 10		Day 14		Day 18		Day 21		
		mm^3	%	mm^3	%	mm^3	%	mm^3	%	
1. Control		598 ± 77		2144 ± 318		5682 ± 609		7411 ± 781		100
2. AN-207	1	29 ± 11	95	250 ± 47	88[c]	849 ± 156	85[b,c]	2831 ± 457	62[b]	106
3. AN-207	7	230 ± 45	62	227 ± 43	89[c]	918 ± 170	84[b,c,d]	1918 ± 297	74[b,c,d]	107
4. AN-207	10	515 ± 104		513 ± 94	100[c]	620 ± 124	98[b,c,d]	1219 ± 231	90[b,c,d]	129[e,f]
5. AN-207	14	627 ± 125		2111 ± 225		2386 ± 242	92[b,c,d]	2312 ± 212	96[b,c,d]	120[e,f]
6. AN-201	1	279 ± 70	53	703 ± 134	67[c]	1861 ± 673	67[b]	3482 ± 1016	53[b]	129[e]
7. AN-201	7	353 ± 63	41	1450 ± 238	32	3463 ± 480	39	5078 ± 569	31	112
8. AN-201	10	474 ± 76		1128 ± 201	58[c]	2553 ± 564	59[b]	3735 ± 617	52[b,c]	113
9. AN-201	14	649 ± 112		2583 ± 372		4788 ± 540	38	6222 ± 726	31	108

Tumor volumes are mean ± SE.
[a] $p < 0.05$, [b] $p < 0.001$ compared with control; data were evaluated by two-way repeated measures ANOVA, and the groups were compared by a multiple comparison procedure (Tukey test).
[c] Difference from previous measurement is not significant (Tukey test), which means growth inhibition.
[d] $p < 0.05$ comparing the groups receiving AN-207 and AN-201 given on the same day.
[e] $p < 0.05$ compared with control (Kruskal–Wallis one-way ANOVA on ranks).
[f] $p < 0.05$ comparing groups receiving AN-207 and AN-201 given on the same day (Mann–Whitney rank sum test).

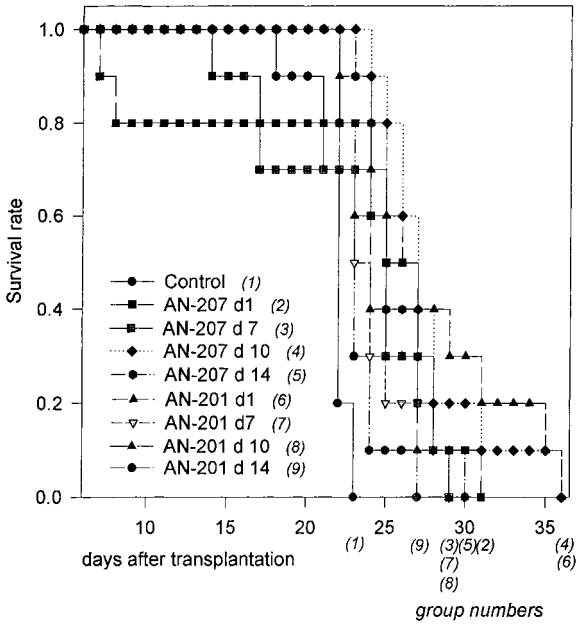

Figure 5. Survival of mice in Experiment II. Group numbers in italics and parentheses show the end of each line when all animals died in the group.

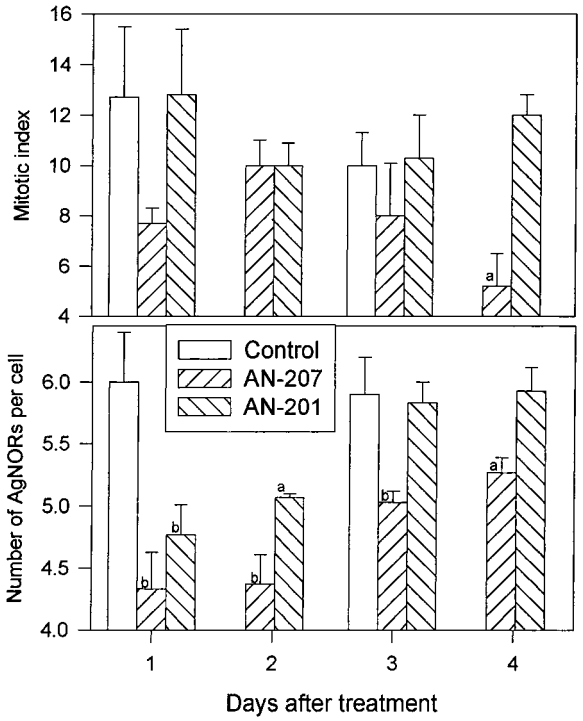

Figure 6. Effects of a single injection of 250 nmol/kg of AN-207 or AN-201 on mitotic index and AgNOR counts in estrogen-independent MXT mouse mammary cancers. $^{a}p < 0.05$, $^{b}p < 0.01$ compared with control (Duncan's test).

derivative of DOX, is about 500–1000 times more active *in vitro* than its parent compound, and its high activity is preserved after conjugation to the LH-RH carrier. In the present study *in vivo*, DOX, AN-201, and AN-152 had essentially the same inhibitory effect on MXT mouse mammary cancers. Cytotoxic LH-RH analog AN-152 and DOX, administered at their maximum tolerated doses, both significantly inhibited tumor growth. AN-207 and AN-201 were also administered at about their maximum tolerated dose level which is approximately 80 times smaller on a molar basis than that of DOX. When AN-201 was administered on day 1, it had a strong inhibitory effect on tumor growth that was similar to that of AN-207. Since two mice receiving AN-207 on day 1 died relatively early on days 7 and 8, the mean survival of mice in the group treated with AN-201 on day 1 was even longer than of animals treated with cytotoxic conjugate AN-207. The differences between the cytotoxic hormone analog and the cytotoxic radical alone were more conspicuous when the effects of AN-207 and AN-201 on larger tumors were compared. AN-207 had a significantly stronger inhibitory effect on tumors than AN-201, particularly when given 10 or 14 days after transplantation. It is especially noteworthy that treatment of tumors that measured 500–600 mm^3 or over 2000 mm^3 with AN-207 resulted in 100% and 96% inhibition, respectively, while AN-201 was practically ineffective on such large tumors. It is apparent that a single injection of AN-207 can stop growth of advanced cancers for about 10–14 days, but the tumors start regrowing after this period. The findings that AN-207 was more powerful at 250 nmol/kg than AN-152 at 20.7 μmol/kg while AN-201 and DOX had similar effects at the same respective 1:80 dose ratio could be explained as follows: Cytotoxic agents reach tumor cells randomly after injection. At the doses applied, about 80 times fewer molecules of AN-201 could be expected to accumulate in MXT cells than DOX molecules, but since the activity of AN-201 is higher, their effects at this 1:80 hypothetical ratio are similar. When the targeting approach is used, cytotoxic LH-RH analogs accumulate preferentially on LH-RH receptors on MXT tumors. A greater tumor inhibition produced by AN-207 resulting from targeting implies that the molar proportion of AN-201 reaching MXT cells when linked to the LH-RH carrier might have been higher than 1:80, the original hypothetical ratio. A higher efficacy of AN-207 as compared to AN-

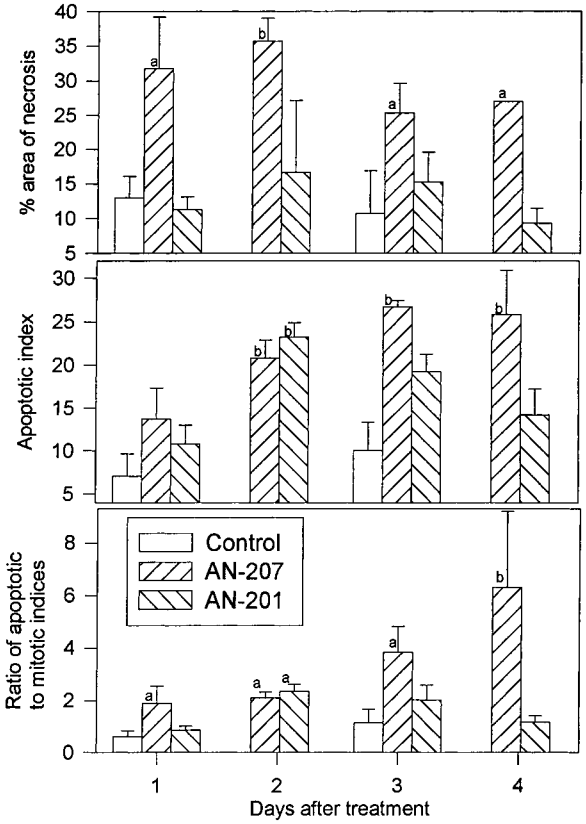

Figure 7. Effects of a single injection of 250 nmol/kg of AN-207 or AN-201 on the extent of necrosis, on the number of apoptotic cells, and on the ratio of apoptotic to mitotic indices in hormone-independent MXT mouse mammary cancers. [a] $p < 0.05$, [b] $p < 0.01$ compared with control (Duncan's test)

152 also suggests that the number of available LH-RH receptors on MXT tumors may be restricted.

The survival of mice was also significantly extended in most treated groups. However, the treatment with AN-207 on day 14 resulted in severe necrosis and ulceration of very large tumors, and several mice died in the group. This might have been caused by toxic substances liberated from decomposing tissue products. The size of tumors in this group were much smaller than those in controls at the time of deaths.

Adverse toxicity may affect rapidly proliferating cells, first of all in the hemopoietic system, and in those tissues that have a high concentration of LH-RH receptors, such as the pituitary. Earlier we demonstrated that treatment with AN-207 caused a temporary selective damage of the pituitary gonadotroph cell function, which, however, was fully recovered after two weeks [23]. Toxic effects of the treatments were monitored by regular body weight measurements. Previous studies showed that body weights of mice were decreased parallel with white blood cell and platelet counts after treatment with AN-207 and AN-201 [24]. Body weights were decreased only slightly in the present experiments showing that the treatment was not toxic.

Due to a very fast growth of hormone-independent MXT cancers and a short survival of mice with these tumors, we deemed it necessary in earlier studies to treat small cancers, usually starting on the first day after transplantation. Successful treatment of tumors at an early stage of development is an important goal. The aim of adjuvant chemotherapy is to eradicate cancers of microscopic sizes or scattered individual tumor cells that may be present at various sites at the time of surgical removal of primary cancers. However, the availability of an effective treatment modality for larger tumors may be even more important since therapy of advanced cancers is one of the greatest challenges for today's oncology. Targeting cytotoxic drugs to cancers may provide such a modality by enhancing the efficacy of therapy and reducing the toxicity. The presence of LH-RH receptors in estrogen-independent MXT tumors was demonstrated previously [16]. We also showed that cytotoxic LH-RH analogs accumulate in MXT breast cancers with a maximum of 3 h after injection [25]. Targeting theory is likewise supported by the findings on other experimental tumors. Thus, AN-152 inhibited growth of OV-1063 ovarian tumors that express receptors for LH-RH, but was not effective on UCI-107 ovarian cancers that do not have LH-RH receptors [26].

Histological examination of MXT cancers at various intervals after treatment revealed several differences in the effects of targeted cytotoxic LH-RH analog AN-207 and its cytotoxic radical AN-201. None of the compounds significantly affected the number of mitotic cells in tumors, but AN-207 caused lower mitotic indices 4 days after treatment. Although both compounds decreased AgNORs significantly within 2 days after therapy, AN-207 had a stronger and more protracted effect on AgNORs than AN-201. AgNOR numbers are good indicators of cell proliferation rate in tumors [27], reflecting cellular events occurring in different phases of cell cycle than mitosis. Thus AgNORs are independent parameters that do not necessarily correlate with mitotic indices. Targeted cytotoxic LH-RH analog AN-207 had a definite and sustained enhancing effect on apoptosis in MXT cancers, in contrast to a shorter temporary increase caused by AN-201. Treatment with AN-207 also resulted in

a more extensive necrosis in MXT cancers, while the size of necrosis in tumors treated with AN-201 remained similar to that in the control group.

The change in a tumor mass is the result of the difference in cell proliferation and cell loss. Cell destruction can occur by programmed cell death (apoptosis) or necrosis. Several cytotoxic drugs induce apoptosis in tumor cells. Apoptosis is inhibited by overexpression of Bcl-2, or p53-gene mutation, which are common phenomena in malignant cells. These genetic changes may explain why some tumors are or eventually become resistant to a chemotherapy which acts through induction of apoptosis [28].

The exact mechanism of action of AN-201 and AN-207 is presently being investigated. DOX and its derivatives are known to produce various effects on cells including DNA-intercalation, or interactions with topoisomerase I and II leading to covalent binding of these enzymes to DNA and subsequent DNA breaks, membrane alterations through lipid peroxidation, and generation of toxic free radicals [29]. DOX and derivatives also induce apoptosis in most cells. However, it is not clear whether apoptosis is an independent cell death mechanism or a reflection of membrane and DNA lesions [29]. In our study, AN-207 had a longer-lasting stimulatory effect on apoptosis in MXT cancers than AN-201. In addition, an even more marked difference was found in their ability to induce necrosis in tumors: AN-201 had no significant effect, but AN-207 caused extensive necrosis in cancers. Genetic changes mentioned above can make tumor cells resistant to treatments causing apoptosis, but no resistance can develop to agents causing necrosis. The development of necrosis presumably needs stronger cytotoxic impacts, and considering the complex pathways of programed cell death, apoptosis can be triggered by weaker effects. Targeting of cytotoxic compounds may concentrate chemotherapeutic radicals in tumors to levels that may result in necrosis [30].

This is the first report on the strong inhibitory effect of a cytotoxic LH-RH analog on advanced MXT mouse mammary cancers, while at this stage of tumor progression treatment with very high doses of DOX or 2-pyrrolino-DOX was ineffective. On the basis of these promising results, targeted cytotoxic LH-RH analog AN-207 could be considered for further development aimed at treatment of patients with advanced breast cancers that express LH-RH receptors.

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References

1. Landis SH, Murray T, Bolden S, Wingo PA: Cancer Statistics, 1998. CA Cancer J Clin 48: 6–29, 1998
2. Chu KC, Tarone RE, Kessler LG, Ries LAG, Hankey BF, Miller BA, Edwards BK: Recent trends in U.S. Breast cancer incidence, survival and mortality rates. J Natl Cancer Inst 88: 1571–1579, 1996
3. Smith G, Henderson IC: New treatments for breast cancer. Semin Oncol 23: 506–528, 1996
4. Goldhirsch A, Gelber D: Endocrine therapies of breast cancer. Semin Oncol 23: 494–505, 1996
5. Hudis CA, Norton L: Adjuvant drug therapy for operable breast cancer. Semin Oncol 23: 475–493, 1996
6. Gehl J, Boesgaard M, Paaske T, Jensen BV, Dombernowsky P: Paclitaxel and doxorubicin in metastatic breast cancer. Semin Oncol 23 (Suppl 15): 35–38, 1996
7. Hortobagyi GN: Anthracyclines in the treatment of cancer. Drugs 54 (Suppl 4): 1–7, 1997
8. Awada A, Van Vreckem A, Paridaens R, Bruning PF, Klijn J, Tomiak E, Vinholes J, Roy JA, Kusenda Z, Hoctin Boes G, Van Glabbeke M, Piccart MJ: EORTC-IDBBC (Investigational Drug Branch for Breast Cancer): 5 years of European collaboration in new drug development for breast cancer. Eur J Cancer 33: 1173–1176, 1997
9. Begent RHJ: UICC/CRC conference on targeted cancer therapy. Int J Cancer 55: 355–358, 1993
10. Magrath IT: Targeted approaches to cancer therapy. Int J Cancer 56: 163–166, 1994
11. Schally AV, Nagy A, Szepeshazi K, Pinski J, Halmos G, Armatis P, Miyazaki M, Comaru-Schally AM, Yano T, Emons G: LH-RH analogs with cytotoxic radicals. In: Filicori M, Flamigni C (eds) Treatment with Gn-RH analogs: controversies and perspectives. Parthenon, Carnforth, UK, 1996, pp 33–44
12. Siegall CB: Targeted toxins as anticancer agents. Cancer 74: 1006–1012, 1994
13. Schally AV: Hypothalamic hormones: from neuroendocrinology to cancer therapy. Anticancer Drugs 5: 115–130, 1994
14. Nagy A, Armatis P, Schally AV: High yield conversion of doxorubicin to 2-pyrrolinodoxorubicin, an analog 500–1000 times more potent: Structure-activity relationship of daunosamine-modified derivatives of doxorubicin. Proc Natl Acad Sci USA 93: 2464–2469, 1996
15. Nagy A, Schally AV, Armatis P, Szepeshazi K, Halmos G, Kovacs M, Zarandi M, Groot K, Miyazaki M, Jungwirth A, Horvath J: Cytotoxic analogs of luteinizing hormone-releasing hormone containing doxorubicin or 2-pyrrolinodoxorubicin, a derivative 500–1000 times more potent. Proc Natl Acad Sci USA 93: 7269–7273, 1996

16. Szepeshazi K, Schally A, Nagy A, Halmos G, Groot K: Targeted cytotoxic luteinizing hormone releasing hormone (LH-RH) analogs inhibit growth of estrogen-independent MXT mouse mammary cancers *in vivo* by decreasing cell proliferation and inducing apoptosis. Anticancer Drugs 8: 974–987, 1997
17. Clarke R: Animal models of breast cancer: Their diversity and role in biomedical research. Breast Cancer Res Treat 39: 1–6, 1996
18. Szepeshazi K, Milovanovic S, Lapis K, Groot K, Schally AV: Growth inhibition of estrogen-independent MXT mouse mammary carcinomas in mice treated with an agonist or antagonist of LH-RH, an analog of somatostatin, or a combination. Breast Cancer Res Treat 21: 181–192, 1992
19. Szepeshazi K, Korkut E, Schally AV: Decrease in AgNOR number in Dunning R-3327 prostate cancer after treatment with LH-RH agonist and antagonist. Am J Pathol 138: 1273–1277, 1991
20. Schally AV, Comaru-Schally AM: Hypothalamic and other peptide hormones. In: Holland JF, Frei E, III, Bast RC, Jr, Kufe DE, Morton DL, Weichselbaum RR, (eds) Cancer Medicine, 4th edn. Williams and Wilkins, Baltimore, MD, 1997, pp 1067–1086
21. Eidne KA, Flanagan CA, Harris NS, Millar RP: Gonadotropin-releasing hormone (GnRH)-binding sites in human breast cancer cell lines and inhibitory effects of GnRH antagonists. J Clin Endocrinol Metab 64: 425–432, 1987
22. Varga JM: Hormone-drug conjugates. Methods Enzymol 112: 259–269, 1985
23. Kovacs M, Schally AV, Nagy A, Koppan M, Groot K: Recovery of pituitary function after treatment with a targeted cytotoxic analog of luteinizing hormone-releasing hormone. Proc Natl Acad Sci USA 94: 1420–1425, 1997
24. Koppan M, Nagy A, Schally AV, Plonowski A, Halmos G, Arencibia JM, Groot K: Targeted cytotoxic analog of luteinizing hormone-releasing hormone AN-207 inhibits the growth of PC-82 human prostate cancer in nude mice. Prostate 38: 151–158, 1999
25. Szepeshazi K, Schally AV, Halmos G, Szoke B, Groot K, Nagy A: Effect of a cytotoxic analog of LH-RH (T-98) on the growth of estrogen dependent MXT mouse mammary cancers: correlations between growth characteristics and EGF receptor content of tumors. Breast Cancer Res Treat 40: 129–139, 1996
26. Miyazaki M, Nagy A, Schally AV, Lamharzi N, Halmos G, Szepeshazi K, Groot K, Armatis P: Growth inhibition of human ovarian cancers by cytotoxic analogues of luteinizing hormone-releasing hormone. J Natl Cancer Inst 89: 1803–1809, 1997
27. Derenzini M, Trere D: Importance of interphase nucleolar organizer regions in tumor pathology. Virchows Arch B Cell Pathol 61: 1–8, 1991
28. Granville DJ, Carthy CM, Hunt DWC, McManus BM: Apoptosis: Molecular aspects of cell death and disease. Lab Invest 78: 893–913, 1998
29. Mansat V, Laurent G, Levade T, Bettaieb A, Jaffrezou J-P: The protein kinase C activators phorbol esters and phosphatidylserine inhibit neutral sphingomyelinase activation, ceramide generation, and apoptosis triggered by daunorubicin. Cancer Res 57: 5300–5304, 1997
30. Kiaris H, Schally AV: Apoptosis vs. necrosis: Which should be the aim of cancer therapy? Proc Soc Exper Biol Med 222: 87–88, 1999

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