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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

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Tumor inhibitory action and the optimal dosage regimens of highly potent targeted cytotoxic luteinizing hormone releasing hormone (LH-RH) analogs containing doxorubicin (DOX) or 2-pyrrolino-DOX (AN-201) were tested in female BDF mice bearing estrogen independent MXT mouse mammary cancers. The effects were compared to those obtained with the cytotoxic radicals DOX or AN-201 alone. Analog AN-207, formed by linking 2-pyrrolino-DOX to [D-Lys^6]LH-RH, and analog AN-152, produced by conjugation of DOX to the same carrier, given i.p. as a single injection or repeatedly 2 days apart at their maximum tolerated doses (MTDs) resulted in a 89–93% inhibition of tumor growth. Equimolar amounts of the cytotoxic radicals were toxic. AN-207 and AN-152 likewise had stronger tumor inhibitory effects than their respective cytotoxic radicals AN-201 or DOX alone, when compared at the lower doses corresponding to MTDs of the radicals. Histological evaluation indicated that decreased cell proliferation (shown by mitotic index and AgNOR counts) as well as increased apoptosis (demonstrated by histological and biochemical methods) both contributed to tumor suppression caused by the cytotoxic hormone analogs. Specific, high-affinity LH-RH receptors were present on MXT tumor samples of control untreated mice, but no binding sites for LH-RH could be found on tumor membranes after treatment with the cytotoxic LH-RH analogs. The results suggest that these powerful targeted cytotoxic LH-RH analogs could be considered for treatment of human mammary cancers having receptors for LH-RH.

Key words: Apoptosis, cytotoxic LH-RH analogs, experimental breast cancer, LH-RH receptors, targeted cancer therapy.

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Introduction

For the past several decades, chemotherapy has been one of the main modalities for the systemic treatment of cancer. Antineoplastic drugs are used as single agents or as combinations of various cytotoxic compounds with different mechanisms of action.[1–3] Chemotherapy can also be used as an adjuvant to surgery, radiotherapy or hormonal treatment.[1–3] Cancer chemotherapy is primarily limited by two factors, i.e. drug resistance of cancer cells and toxicity to normal cells.[4] The discovery of drugs that selectively affect tumor cells has long been a goal of chemotherapy. In recent years, a better understanding of molecular characteristics of cancer cells has led to a new approach based on targeting specific agents to tumor cells. Certain molecules, such as antigens, enzymes and receptors, present on tumor cells may be used as targets to increase the selectivity of chemotherapy. Various types of receptors, including those for hormones and growth factors, have been detected on cancer cells. After binding specific ligands, these receptors activate intracellular signaling mechanisms that influence cell proliferation. Among the receptors for hormones on tumor cells, those that bind luteinizing hormone releasing hormone (LH-RH) have been widely investigated.[5,6] Thus, receptors for LH-RH have been found in several human mammary carcinoma cell lines[7,8] and in a high percentage of human breast cancer samples.[9,10] LH-RH receptors have been also detected in specimens of human prostate cancers,[11] and in LNCaP and DU-145 human prostate cancer lines.[11–13] Most human ovarian and endometrial cancer cells likewise contain receptors for LH-RH.[14,15] In addition, experimental animal tumors such as estrogen-independent MXT mouse mammary

carcinomas[16,17] have LH-RH receptors. These receptors may mediate direct effects of LH-RH analogs on tumors. The existence of such direct effects is now supported by various observations such as occasional responses to LH-RH agonists seen in postmenopausal women, and the inhibitory effect of LH-RH analogs on human breast cancer cell lines *in vitro* and estrogen independent mouse mammary cancers *in vivo*. [5,6]

On the basis of the presence of specific receptors for LH-RH on tumor cells, we developed a new class of targeted antitumor agents by linking various cytotoxic radicals to LH-RH analogs.[5] According to the theoretical assumptions, tumor cells with LH-RH receptors that bind these conjugates would be preferentially killed, while normal cells that do not have receptors would be less affected.[5,6] Early cytotoxic compounds contained nitrogen mustard and metal complexes.[5,6] Later, to increase antitumor activity of the cytotoxic analogs, an alkylating agent melphalane, anthraquinone derivative hydroxymethyl anthraquinone, anticancer antibiotic adriamycin and antimetabolite methotrexate were linked to suitably modified analogs of LH-RH.[5,6] Most of these conjugates showed high binding affinities to LH-RH receptors and preserved the hormonal activity of the carrier molecule.

Some of these earlier cytotoxic analogs suppressed proliferation of several tumor cell lines *in vitro*, and inhibited growth of Dunning R-3327 H prostate cancers[5,6] and MXT estrogen independent or dependent mouse mammary cancers.[16-18] These cytotoxic LH-RH analogs had stronger tumor inhibitory effects than the carrier molecules or the cytotoxic compounds alone.

A high binding affinity to LH-RH receptors and the ability to be linked to cytotoxic compounds at the lysine side chain make [D-Lys^6]LH-RH an excellent carrier. Thus, to increase the antitumor effects of cytotoxic LH-RH analogs, we then searched for more potent cytotoxic compounds suitable for linking to the hormone and for the best coupling method that preserved the characteristics of both molecules.[19,20] Doxorubicin (DOX), a cytotoxic anthracycline antibiotic, is one of the most widely used anticancer drugs.[21] DOX binds to DNA by specific intercalation. Since 14-*O*-esters of DOX are relatively stable and retain the cytotoxic activity of DOX,[22] we used a dicarboxylic acid ester derivative, DOX-14-*O*-hemiglutarate for producing LH-RH–DOX conjugates (AN-152). Furthermore, conversion of these conjugates to more potent hybrid molecules (AN-207) with 2-pyrrolino-DOX, a daunosamine-modified derivative of DOX, which is 500–1000 times more active than its parent compound, was accomplished by a reaction with 4-iodobutyraldehyde.[20] Cytotoxic analogs AN-152 and AN-207 showed very strong cytotoxic activity *in vitro* and high binding affinity to LH-RH receptors on various cell lines.[20]

In this study, we report the evaluation of these new LH-RH analogs containing DOX or 2-pyrrolino-DOX in mice bearing transplanted estrogen independent MXT mouse mammary cancers. We have chosen this cancer model for the first *in vivo* evaluation of our cytotoxic compounds because the tumor take is always 100%, and the tumor is fast growing and invasive, killing the mice regularly between days 18 and 24 after transplantation. All these characteristics render this tumor model more advantageous for initial tests than most human cancers transplanted into nude mice. Since the same model was used for evaluation of our early cytotoxic LH-RH analogs, this makes a comparison possible.[16-18] Several pilot studies and more elaborate experiments were performed to determine optimal dosage regimens and toxic doses in mice, and to compare the effects of the conjugates with those of the cytotoxic radicals alone.

Materials and methods

Materials

LH-RH agonist [D-Lys^6]LH-RH (Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2) was synthesized in our laboratory by solid-phase methods and purified by HPLC. DOX-HCl salt was purchased from Aldrich (Milwaukee, WI). The following substances were used for treatment of the animals: DOX, AN-152 (DOX-14-*O*-hemiglutarate coupled to [D-Lys^6]LH-RH), AN-201 (2- pyrrolino-DOX), AN-207 (conjugate of [D-Lys^6]LH-RH and 2-pyrrolino-DOX-14-*O*-hemiglutarate) and T-107 (*N*-glutaryl-DOX coupled to [D-Lys^6]LH-RH). The preparation of AN-152, AN-201 and AN-207 was reported.[19,20] T-107, another analog of LH-RH containing DOX, was synthesized by coupling *N*-glutaryl-DOX to the hormone carrier.[23]

Animals and tumors

Female B_6D_2F1 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD) and were maintained as described.[16] All experiments were performed according to institutional ethical guidelines. MXT(3.2)ovex mammary carcinoma was originally obtained from Dr AE Bogden (Biomeasure, Hopkinton, MA) and was regularly transplanted in our laboratory. The methods of tumor transplantation and calculation of tumor volume were reported.[16]

Experimental protocol

One day after transplantation of tumors, the mice were randomly divided into groups and the therapy was initiated. The compounds used for treatment were dissolved in 50 µl 0.1% trifluoroacetic acid (TFA) (pH 2.0) and diluted with distilled water. The mice were injected with 0.2 ml solution/20 g body weight i.p. The control animals received vehicle only. The experimental groups, treatment doses and schedules of injections are shown in the respective tables. In Experiment I, for treatment of three groups, Alzet osmotic pumps (Alza, Palo Alto, CA) Model 2002 releasing 11.5 µl/day were used and implanted i.p. The filling and implantation of pumps were performed according to manufacturer's recommendations.

The groups consisted of five mice in the orientation experiments (Experiment II, III and IV), but in the complete experiments the groups contained 10 animals (Experiment I, V and VI). Body weights and tumor volume were determined regularly. Tumor volume reduction (TVR) (percentage of tumor growth inhibition) was calculated on the last day of animal survival, according to the equation:

$$\text{TVR}(\%) = (100 - \text{median tumor volume of treated group/median tumor volume of control group}) \times 100$$

In the orientation experiments, median survival time (MST) of mice in treated groups (T) was compared to that of the control group (C) and the results expressed as $T/C\%$, where

$$T/C\% = \text{MST of treated group/MST of control group} \times 100.$$

A dose resulting in less than 10% deaths was considered to be the MTD. The highest dose, having strong tumor inhibitory effect, but causing no mortalities, was regarded as optimal dose for BDF mice with MXT cancers.

In Experiments I, V and VI, the mice were sacrificed on day 18 by exsanguination under Metofane (methoxyflurane; Pitman-Moore, Mundelein, IL) anesthesia. The blood of animals was collected for determination of serum hormone levels. The tumors were removed, cleaned, weighed and processed for histology and receptor studies.

Histological methods

The processing of tumor samples was performed as described.[16] For the measurement of the number of mitotic and apoptotic cells, four high power fields were considered, and the numbers of mitotic and apoptotic cells per 1000 cells (mitotic and apoptotic indices) were calculated. Hematoxylin & eosin stained slides were used for the quantitative evaluation of apoptotic cells. For an accurate recognition, the apoptotic changes seen on these slides were compared to those detected by two special histochemical techniques. The TACS™ 1 Klenow *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD) was applied according to the manufacturer's instruction. As a second method, 5-bromo-2-deoxyuridine (BrdU) was used for *in situ* end labeling of DNA fragments and the bound BrdU was detected by immunohistochemical procedure as described by Aschoff *et al.*[24] BrdU, terminal deoxynucleotidyl transferase (TdT), monoclonal anti-BrdU, anti-mouse IgG-biotin conjugate, ExtrAvidin peroxidase conjugate and diaminobenzidine tablets were purchased from Sigma (St Louis, MO).

Analysis of DNA fragmentation

DNA fragmentation was studied using three tumor samples from each group treated with AN-201, AN-207, AN-152 and DOX, as well as from the controls in Experiments V and VI. Extraction of DNA and determination of the ratio of small DNA fragments to total (intact + fragmented) DNA was performed as described by Kyprianou and Isaacs.[25] The fractions were examined by agarose gel electrophoresis. The chemicals for DNA extraction were purchased from Sigma. DNA concentrations were measured using Kodak DNA Quik Strips (Eastman, New Haven, CT).

Receptor studies

LH-RH binding sites were analyzed in Experiments V and VI. Assays were performed on individual tumors using altogether 86 samples in the two experiments. Pieces of MXT cancers were frozen and stored at −70°C until analyzed for LH-RH receptors. Radioiodination of [D-Trp^6]LH-RH, preparation of membranes from tumor tissue, receptor binding assays and sources of the chemicals were described.[26]

Serum LH and estradiol levels

In Experiments V and VI, serum LH was measured by radioimmunoassay using materials provided by the National Hormone and Pituitary Program (NHPP, Rockville, MD) (rLH-RP-3/AFP-7187B; rLH-1-

9/AFP-10250C; anti-rat LH-RIA-S-10/AFP 571487). Serum estradiol levels were determined in the same experiments as described.[8]

Statistical analyses

Statistical evaluation of data was performed by Duncan's Multiple Range Test. SigmaPlot computer software (Jandel, San Rafael, CA) was used for preparation of the figures.

Results

Effects of AN-207 (2-pyrrolino-DOX linked to [D-Lys^6]LH-RH) and AN-201 (2-pyrrolino-DOX) on tumor growth and survival

As shown in Table 1, 55 nmol/kg of AN-207 given on two consecutive days each week for 3 weeks with 5 days pause inhibited tumors more effectively than 22 nmol/kg for 5 days followed by a 2 day pause, although the total amounts of AN-207 given during the 3 weeks period were the same in the two groups. The mice tolerated 154 nmol/kg of AN-207 injected once a week for 2 weeks and this dose had a very strong tumor inhibitory effect (Figure 1), resulting in tumor-free survival of two mice (followed for 6 months). AN-207 at 220 nmol/kg dose given on days 1 and 8, however, was toxic. AN-201 (2-pyrrolino-DOX) administered according to the same schedule and at equimolar amounts, showed much weaker antitumor effect and higher toxicity than AN-207.

The results of a pilot experiment performed in the search for the optimal dose of AN-207 are summarized in Table 2. AN-207 administered three times at 75 nmol/kg had only a weak inhibitory effect on the tumors. AN-207, given three times at 125 nmol/kg or twice at 150 nmol/kg (days 1 and 4), resulted in a strong tumor inhibition, but it was toxic, causing no extension of survival. AN-207 administered twice at 150 or 175 nmol/kg at 2 or 3 week intervals inhibited powerfully tumor growth and extended the survival of

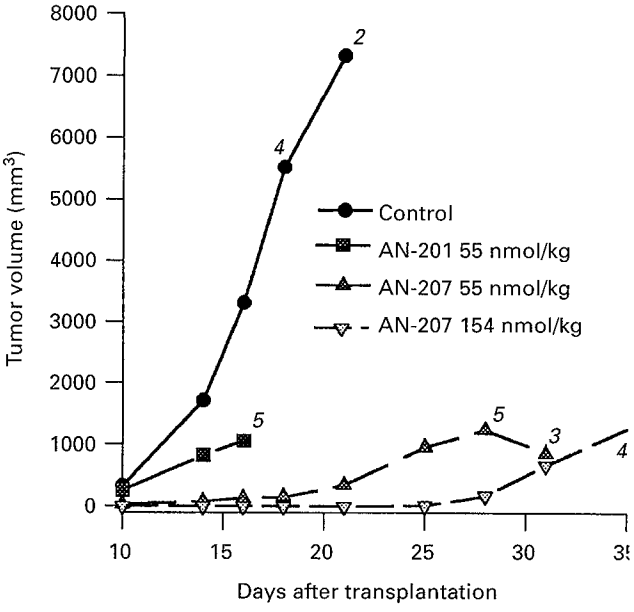

Figure 1. Tumor volume changes in selected groups of Experiment II. Treatment schedule is shown in Table 1. The numbers in italics indicate surviving animals (initially five mice per group).

Table 1. Experiment II: effect of treatment with cytotoxic LH-RH analog AN-207, 2-pyrrolino-DOX AN-201 and DOX on growth of estrogen independent MXT cancers and on survival of mice (n=5); see tumor volume changes of selected groups in Figure 1

Treatment	Daily dose		Injections i.p. on days	Total amount (nmol/kg)	Survival (*T/C*%)	Tumor volume reduction (%, day 16)	No. of mice without tumor/surviving mice on day			
	nmol/kg	mg/kg					10	14	18	31
AN-207	22	0.051	1–5	330	119	78	1/5	1/5	1/5	0/1
AN-201	22	0.016	8–12 15–19	330	95	35	0/5	0/5	0/4	0
AN-207	55	0.128	1,2	330	148	96	3/5	2/5	2/5	0/3
AN-201	55	0.04	8,9 15,16	330	81	68	1/5	1/5	0/2	0
AN-207	154	0.357	1,8	308	205	100	4/5	5/5	4/5	2/4
AN-207	220	0.51	1,8	440	67		5/5	0	0	0
DOX	22880	14.96	1,8,15	68640	95	97	1/4	1/4	1/4	0
Control	–	–	–	–	100	0	0/5	0/5	0/4	0

animals (Figure 2). One mouse in the 175 nmol/kg group survived for 1 year without a tumor.

Effects of higher doses of AN-207 and AN-201, administered as single bolus injections, were evaluated in Experiment IV, shown in Table 3. AN-207 at doses of 300 and 250 nmol/kg had a strong inhibitory effect on tumors, but was toxic, causing deaths of most mice within 2 weeks after treatment. AN-201 at doses of 100 and 110 nmol/kg was also toxic. The dose of 110 nmol/kg of AN-201 showed a toxicity similar to 250 nmol/kg AN-207, but had weaker tumor inhibitory effect (Figure 3).

In Experiment VI (Table 4 and Figure 4), both AN-207 and AN-201 were injected twice at two different doses determined according to the results of the previous experiments. The higher dose (110 nmol/kg) appeared to be optimal for the cytotoxic hormone analog AN-207 and the lower dose (55 nmol/kg) was close to the MTD for the cytotoxic moiety AN-201. To allow a comparison, AN-207, AN-201 and the carrier were given in equimolar and also equitoxic amounts. Treatment with 110 nmol/kg AN-207, administered twice, resulted in a significant inhibition of tumor growth without significant toxicity. The death of a single mouse on day 3 was probably accidental, because no other signs of toxicity were apparent in this group. At the end of the experiment, two mice in this group showed only tumors of microscopic sizes. AN-207 administered twice at 55 nmol/kg dose also significantly reduced tumor growth, but to a lesser extent than the higher dose. In the group that received twice 110 nmol/kg of cytotoxic radical AN-201, body weights were significantly decreased between days 7 and 10, the median survival time was reduced to 9 days and only a moderate inhibition of tumor growth was obtained. AN-201 given twice at 55 nmol/kg was not toxic, but had no significant effect on the tumors. An unconjugated mixture of AN-201 and [D-Lys^6]LH-RH in equimolar amounts had no effect on tumor growth and was toxic. At the end of the experiment, body, heart and liver weights were significantly lower in the groups treated with the higher dose of AN-201. Ovarian and uterine weights were similar in the treated and control groups.

Some proliferation characteristics of treated and untreated tumors were compared histologically. The data are shown in Table 5. The number of mitotic cells was significantly smaller only in the group receiving 55 nmol/kg of AN-207. An enhancement of apoptosis was

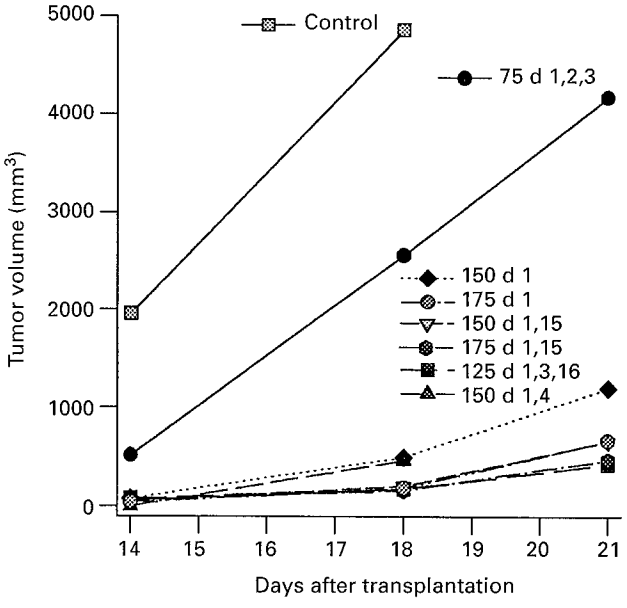

Figure 2. Tumor volume changes in Experiment III. All treated groups received AN-207. The numbers show doses in nmol/kg and the days of administration. Survival data are shown in Table 2.

Table 2. Experiment III: effect of treatment with cytotoxic LH-RH analog AN-207 on growth of estrogen independent MXT cancers and on survival of mice (n=5); tumor volume changes are shown in Figure 2

Treatment	Daily dose		Injections i.p. on days	Total amount (nmol/kg)	Survival (*T/C*%)	Tumor volume reduction (%, day 18)	No. of mice without tumor/surviving mice on day			
	nmol/kg	mg/kg					14	18	21	24
AN-207	75	0.175	1,2,3	225	110	56	0/5	0/5	0/4	0/2
AN-207	125	0.291	1,3,16	375	110	96	4/5	2/3	2/3	1/1
AN-207	150	0.350	1,4	300	90	90	0/3	1/3	0/1	0/1
AN-207	150	0.350	1,15	300	152	96	1/5	1/5	1/5	0/5
AN-207	150	0.350	1,22	300	138	91	2/4	1/4	1/4	0/4
AN-207	175	0.410	1,15	350	133	97	3/5	2/5	0/4	0/4
AN-207	175	0.410	1,22	350	162	96	4/5	1/5	1/5	1/5
Control	–	–	–	–	100	0	0/5	0/5	0/3	0

Table 3. Experiment IV: effect of treatment with cytotoxic LH-RH analog AN-207 and radical AN-201 injected i.p. on day 1 on growth of estrogen independent MXT cancers and on survival of mice (n=5); changes in tumor volume are shown in Figure 3

Treatment	Daily dose		Total amount (nmol/kg)	Survival (*T/C*%)	Tumor volume reduction (%, day 10)	No. of mice without tumor/surviving mice on day			
	nmol/kg	mg/kg				10	13	17	20
AN-207	250	0.58	250	62	99	3/4	0/1	0	0
AN-207	300	0.70	300	33	–	1/2	0/1	0/1	0/1
AN-201	100	0.07	100	95	51	1/5	0/5	0/3	0/1
AN-201	110	0.08	110	62	48	1/4	0/1	0	0
Control	–	–	–	100	0	0/5	0/5	0/5	0/5

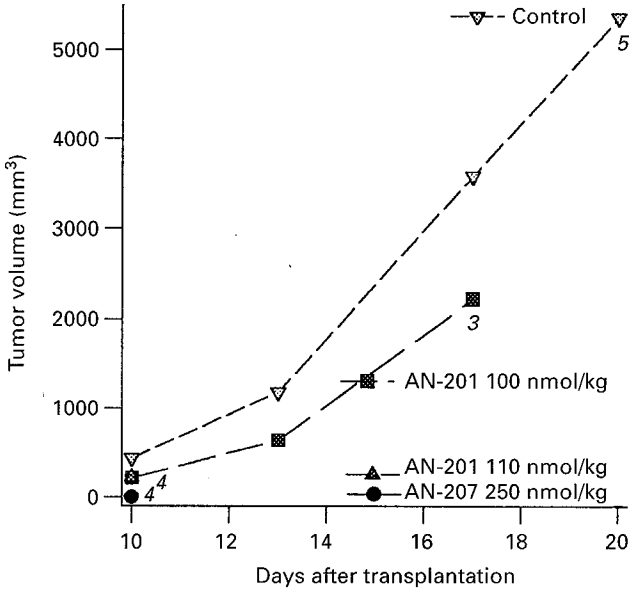

Figure 3. Changes in tumor volume after a single treatment with AN-207 or AN-201. The numbers in italics represent surviving mice (initially n=5). See also Experiment IV, Table 3.

also observed in this group and in the group treated with 55 nmol/kg of AN-201. In the group that received 110 nmol/kg AN-201, only two tumors remained for histological examination, which did not permit an evaluation. Cell proliferation rates, demonstrated by AgNOR counts, were decreased in cancers treated with AN-207 or AN-201. Serum LH and estradiol levels were similar in treated and control animals.

Effects of AN-152 (DOX-14-*O*-hemiglutarate coupled to [D-Lys^6]LH-RH) and DOX on tumor growth and survival

Our earlier cytotoxic analogs of LH-RH have been administered according to regimens for peptide hormones not for chemotherapeutic agents, i.e. given repeatedly or continuously. In Experiment I, we tried these modes of administration, using relatively low doses of AN-152, DOX and the carrier [D-Lys^6]LH-RH. The results are shown in Table 6. There were no signs of toxicity, and only DOX caused a slight decrease in heart, liver, spleen and kidney weights. However, all treatments resulted only in a weak inhibition of tumor growth. In Experiment II (Table 1), an overdose of DOX had a strong inhibitory effect on tumor growth, but could not eradicate these cancers. All the mice receiving DOX died between days 20 and 31.

In the preliminary experiment IV, shown in Table 7, various doses of AN-152 and DOX were tested. AN-152 at doses of 34–38 μmol/kg i.p. produced a strong tumor inhibitory effect without apparent toxicity (Figure 5). DOX used at similar levels inhibited tumor growth, but due to toxicity, the results could not be evaluated after day 10. Analog T-107 at similar or higher molar doses was not toxic, but had no effect on tumors.

In Experiment V, an effective dose of AN-152 (32.5 μmol/kg) was used i.p. and compared with an equimolar amount of DOX. Other groups of animals were treated with the MTD of DOX (20 μmol/kg) or with an equimolar dose of AN-152. The results are shown in Table 8 and Figure 6. Treatment with AN-152 resulted in a powerful, dose-dependent inhibition of tumor growth. There were no signs of toxicity and the body weights of mice remained stable. The early deaths of single mice in each of these groups on day 3 were probably accidental, because the mortality due to toxic effects usually occurred later, from about day 5. DOX at a dose of 32.5 μmol/kg also effectively inhibited tumor growth, but was toxic. DOX at 20 μmol/kg caused a partial inhibition of tumor growth, but resulted in the deaths of four mice. A single injection of an equimolar amount (32.5 μmol/kg) of the carrier [D-Lys^6]LH-RH had no effect on tumors and was not toxic. Treatment with a mixture of equimolar amounts of DOX and [D-Lys^6]LH-RH resulted in early

Table 4. Experiment VI: effect of treatment with cytotoxic LH-RH analog AN-207 and radical AN-201 injected i.p. on days 1 and 3 on growth of estrogen independent MXT cancers and on survival of mice (n=10); tumor volume changes are shown in Figure 4, histological data in Table 5 and receptor results in Table 9

Treatment	Daily dose		Total amount (nmol/kg)	Survival[a] (median)	Tumor volume reduction (%, day 17)	No. of mice without tumor/surviving mice on day			Tumor weights (g)
	nmol/kg	mg/kg				10	14	18	
AN-207	110	0.25	220	–	89[b]	3/9	3/9	1/9	0.83[b]
AN-201	110	0.078	220	9.0	48[b]	0/3	0/2	0/2	2.06[b]
AN-207	55	0.13	110	–	62[b]	0/10	0/10	0/10	3.02[b]
AN-201	55	0.039	110	–	22	0/10	0/10	0/10	4.79
AN-201+ [D-Lys[6]]LH-RH	110+ 110	0.078+ 0.176	220+ 220	14.5	10	0/8	0/4	0/2	6.33
Control	–	–	–	–	0	0/10	0/10	0/10	5.52

[a]Surviving mice were sacrificed on day 18.
[b]$p<0.01$ versus control.

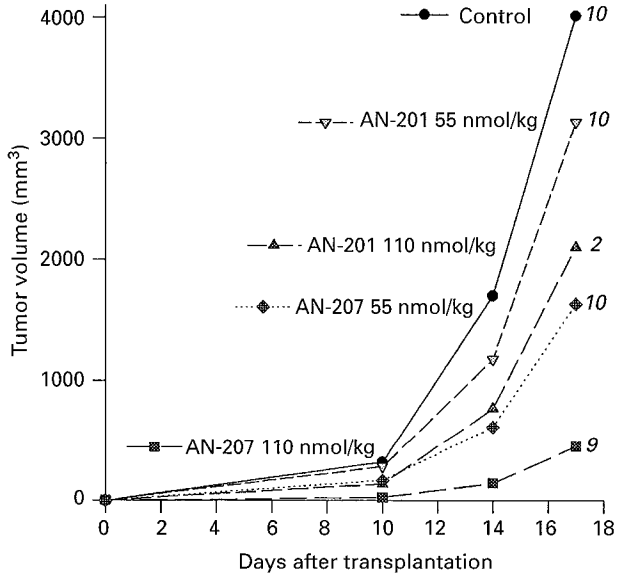

Figure 4. Tumor volume changes in Experiment VI. The numbers in italics on the right of the graph show surviving mice (initially n=10). Tables 4, 5 and 9 contain other data from this experiment.

deaths of all animals and the effects on cancers could not be evaluated. Liver and spleen weights were decreased in mice receiving AN-152 or DOX. Heart weights were lower after treatment with the 32.5 μmol/kg of DOX, but were higher in the groups treated with AN-152 than in controls (data not shown). Ovarian and uterine weights were unchanged.

The quantitative histological data are shown in Table 5. Treatment with AN-152 increased the frequency of apoptosis and decreased AgNOR numbers, but decreased the number of mitotic cells only in the case of the higher dose. DOX caused only small, not significant changes in these parameters. Serum LH and estradiol levels of the mice were determined at the end of the experiment. LH and estradiol levels in treated groups did not differ significantly from control values.

DNA fragmentation

The amount of small DNA fragments was expressed as a percentage of the total DNA. Using three tumor samples from each group in Experiments V and VI, the quantity of fragmented DNA was 16.7% in the control group, which was similar to 18.5% in the group treated with AN-201 and 17.0% in the animals that were given DOX. However, the relative amount of fragmented DNA was increased to 24.8% after treatment with AN-207 and to 25.0% in the group receiving AN-152.

LH-RH receptor levels

The results of analyses of receptors for LH-RH are shown in Table 9. LH-RH receptors could be found in about half of the untreated tumors at the end of the experiments. The maximum binding capacity of the receptors was 687 and 597 fmol/mg membrane protein in control tumors from Experiments V and VI, respectively. However, no receptors for LH-RH were detectable in tumor membranes after treatment with the cytotoxic LH-RH analogs AN-207 or AN-152. Receptors for LH-RH were found in some of the tumors treated with the cytotoxic radicals AN-201 or DOX. The numbers of mice surviving by the end of the

Table 5. Experiments V and VI: effect of i.p. treatment with cytotoxic LH-RH analogs AN-207 and AN-152, as well as DOX, AN-201 and [D-Lys^6]LH-RH on histological characteristics of estrogen independent MXT cancers; other data of these experiments are shown in Tables 4, 8 and 9 and Figures 4 and 6

Groups	Doses[a]	Mitotic index	Apoptotic index	Ratio of apoptotic to mitotic indices	Number of AgNORs per nucleus
Experiment VI					
AN-207	110 nmol/kg	14.6±1.9	15.1±1.5	1.18±0.20	5.13±0.33[c]
AN-207	55 nmol/kg	11.3±1.9[b]	16.6±1.8[b]	2.14±0.63[b]	5.06±0.11[c]
AN-201	55 nmol/kg	13.4±1.5	16.3±0.8[b]	1.39±0.18	5.23±0.13[c]
AN-201+ [D-Lys^6]LH-RH	110 nmol/kg+ 110 nmol/kg	16.3±1.2	14.4±1.2	0.91±0.08	5.99±0.17
control	–	16.8±1.1	11.4±1.0	0.72±0.09	6.43±0.15
Experiment V					
AN-152	32.5 μmol/kg	5.3±1.5[b]	16.8±2.1[b]	6.16±2.67[c]	5.04±0.15[c]
DOX	32.5 μmol/kg	9.5±1.2	13.7±2.2	1.47±0.26	5.80±0.23
AN-152	20 μmol/kg	10.3±1.6	17.3±1.7[b]	2.21±0.45	5.14±0.13[c]
DOX	20 μmol/kg	11.4±2.2	11.0±1.1	1.29±0.37	5.75±0.11
[D-Lys^6]LH-RH	32.5 μmol/kg	12.3±1.2	13.2±0.8	1.25±0.26	5.88±0.19
control	–	9.6±0.6	11.9±1.2	1.28±0.18	6.24±0.23

[a]Injections i.p. on days 1 and 3 (Experiment VI), and on day 1 (Experiment V).
[b]$p<0.05$; [c]$p<0.01$. Values are means±SE.

Table 6. Experiment I: effect of treatment with DOX, AN-152 and [D-Lys^6]LH-RH on growth of estrogen independent MXT cancers and on survival of mice (n=10)

Treatment	Daily dose		Injections i.p. on days	Total amount (μmol/kg)	Tumor volume reduction (%, day 18)	No. of mice without tumor/surviving mice on day			Tumor weights
	μmol/kg	mg/kg				10	14	18	(g)
DOX	4.31	2.5	1,2,8,9	17.25	43	1/10	0/10	0/10	2.82
DOX	1.15	0.665	op	17.25	45	1/10	1/10	0/9	3.25
AN-152	1.15	2.57	op	17.25	39	0/10	0/10	0/10	3.98
[D-Lys^6]LH-RH	1.15	1.84	op	17.25	60	0/10	0/10	0/9	3.01
Control	–	–	–	–	0	2/12	0/11	0/10	5.63

op=osmotic pump.

Table 7. Experiment IV: effect of i.p. treatment on day 1 with cytotoxic LH-RH analogs AN-152 and T-107 as well as DOX injected i.p. on day 1 on growth of estrogen independent MXT cancers and on survival of mice (n=5); tumor volume changes of selected groups are shown in Figure 5

Treatment	Daily dose		Survival (*T/C*%)	Tumor volume reduction (%, day 10)	No. of mice without tumor/surviving mice on day				
	μmol/kg	mg/kg			10	13	17	20	25
AN-152	34	76	148	100	3/4	2/4	2/4	1/4	1/4
AN-152	35.5	79.35	138	98	2/4	1/4	1/4	1/4	1/4
AN-152	38	84.9	–[a]	98	2/5	1/5	1/5	–[a]	–[a]
DOX	32.5	18.85	62	80	0/3	0/2	0/1	0/1	0
DOX	35	20.3	81	94	1/3	1/3	0/2	0	0
DOX	37.5	21.75	33	93	0/1	0	0	0	0
T-107	37.5	83.8	105	7	0/5	0/5	0/5	0/4	0
T-107	42.5	95	100	−7	0/5	0/5	0/5	0/4	0
Control	–	–	100	0	0/5	0/5	0/5	0/5	0

[a]Three mice in the group were sacrificed on day 17.

experiments were low in the groups receiving higher doses of the cytotoxic radicals, but at least some of the MXT cancers retained binding abilities for LH-RH after treatment with lower doses of AN-201 or DOX.

Discussion

Targeted tumor therapy in a broad sense includes all selective manipulations on cancers that may not affect non-tumorous sites of the body. Several concepts of targeting have been utilized such as the use of anti-tumor antibodies, the inactivation of genes or gene products involved in oncogenesis, interference with tumor-specific metabolic pathways or inhibition of angiogenesis, which is essential for tumor growth.[4,27] A special approach to targeted tumor therapy is based on selective delivery systems that would concentrate various cytotoxic drug molecules in cancer cells by recognizing specific structures in these tumors. The toxic molecules can be plant or bacterial toxins,[27,28] chemotherapeutic drugs[5,6,29] or radionuclides.[27] Spe-

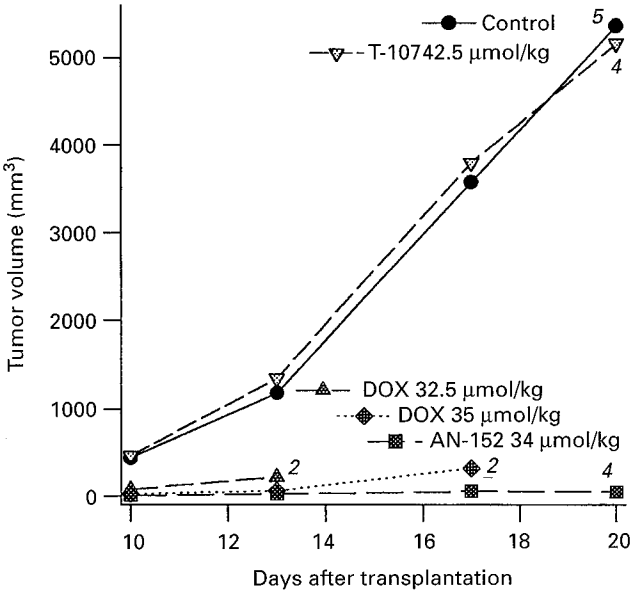

Figure 5. Tumor volume changes in selected groups of Experiment IV. Numbers in italics show surviving animals (initially n=5). Details of data are shown in Table 7.

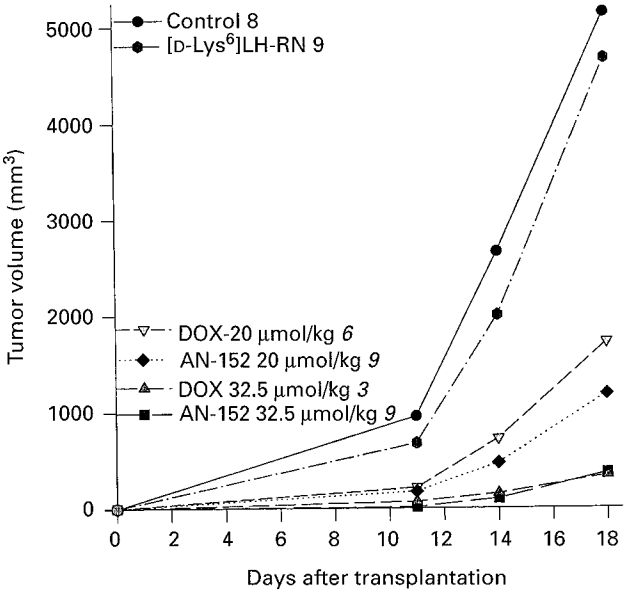

Figure 6. Tumor volume changes in experiment V. Other data are shown in Tables 5, 8 and 9. The numbers in italics represent surviving mice on day 18 (initially n=10).

Table 8. Experiment V: effect of treatment with cytotoxic LH-RH analog AN-152, DOX and [D-Lys^6]LH-RH injected i.p. on day 1 on growth of estrogen independent MXT cancers and on survival of mice (N=10); tumor volume changes are shown in Figure 6, histological data in Table 5 and receptor data in Table 9

Treatment	Daily dose		Total amount	Survival (median)	Tumor volume reduction	Tumor weights	No. of mice without tumor/surviving mice on day		
	μmol/kg	mg/kg	μmol/kg		(% day 18)	(g)	11	14	18
AN-152	32.5	72.65	32.5	NA	93[b]	0.59[b]	3/9	2/9	0/9
DOX	32.5	18.85	32.5	NA	93[b]	0.52[b]	1/4	0/3	0/3
AN-152	20.0	44.7	20.0	NA	81[b]	2.10[b]	0/9	0/9	0/9
DOX	20.0	11.6	20.0	NA	66[a]	2.54[b]	0/7	0/7	0/6
[D-Lys^6]LH-RH	32.5	52.0	32.5	NA	7	6.42	0/9	0/9	0/9
DOX+ [D-Lys^6]LH-RH	32.5+ 32.5	18.85+ 52.0	32.5+ 32.5	6.0	–	–	1/1	0	0
Control	–	–	–	NA	0	7.30	0/8	0/8	0/8

NA, the animals were sacrificed at day 18.
[a]$p<0.05$; [b]$p<0.01$ versus control.

Table 9. Experiments V and VI: effect of i.p. treatments with cytotoxic LH-RH analogs AN-207 and AN-152, as well as AN-201 and DOX on binding characteristics of LH-RH in membranes of estrogen independent MXT cancers; other data of the experiments are in Tables 4, 5 and 8 and Figures 4 and 6

Treatment	No. of tumors	Receptor status		B_{max} (fmol/mg protein)	K_d (nM)
		Positive	Negative		
Experiment VI					
AN-207 110 nmol/kg	9	0	9	ND	ND
AN-201 110 nmol/kg	2	0	2	ND	ND
AN-207 55 nmol/kg	10	0	10	ND	ND
AN-201 55 nmol/kg	10	3 (2)	5	565.3±76.0	7.70±2.11
AN-201+D[Lys^6]LH-RH	2	1	1	581.8	11.6
control	10	4	6	597.2±29.2	6.24±0.47
Experiment V					
AN-152 32.5 μmol/kg	9	0	9	ND	ND
DOX 32.5 μmol/kg	3	0	3	ND	ND
AN-152 20 μmol/kg	9	0 (2)	7	ND	ND
DOX 20 μmol/kg	6	1	5	670.6	4.00
[D-Lys^6]LH-RH	9	2 (2)	5	684.4±56.4	8.87±2.66
control	7	3	4	686.7±40.6	7.54±2.11

ND, not detectable. Numbers in brackets show the numbers of tumors with moderate receptor binding.

cific antibodies, hormones and growth factors may be used as delivery molecules.[5,28,27] Ideally, the toxic molecules specifically delivered to cancer cells would kill these tumor cells while normal cells could be spared.[5]

Since relatively high percentages of human breast, ovarian, endometrial and prostate cancers contain receptors for LH-RH,[5,6,13,14] LH-RH analogs that bind specifically to these receptors can be used as carrier molecules for cytotoxic radicals. Thus, sensitive normal tissues that lack LH-RH binding sites can be spared from toxic effects of chemotherapy.[5] Recent studies demonstrated that both the antiproliferative activity of the cytotoxic radicals and the high binding affinity of the carrier to LH-RH receptors are fully preserved in the cytotoxic LH-RH analogs AN-152 and AN-207.[20] *In vitro*, in MCF-7 human breast cancer line and MXT estrogen independent mouse mammary carcinoma line, the cytotoxic activities of AN-152 and AN-207 corresponded to those of DOX and AN-201, respectively. However, DOX and AN-152 were cytotoxic at 10^{-7} M concentrations while AN-207 and AN-201 were effective at much lower concentrations of 3×10^{-10} M.[20] In this study we examined the effects of both classes of compounds *in vivo* in mice bearing estrogen independent MXT breast tumors. Pilot experiments and survival studies showed that AN-207 at a dose between 100 and 175 nmol, given twice, had the strongest antitumor effect without significant toxicity. A dose of 110 nmol of AN-201 seemed to have a toxicity similar to 250 nmol/kg of AN-207. The optimal dose of AN-152, resulting in a 93% inhibition of tumors without apparent toxicity, was about 34 μmol/kg. A comparable tumor inhibition was caused only by highly toxic doses of DOX of the order of 32.5 μmol/kg. This corresponds to 18.9 mg/kg, which is about 50% higher than the usual MTD of DOX.[30–32] In the *in vivo* experiments reported herein, the optimal doses of AN-152 and AN-207 were 32.5 and 2×110 nmol/kg, respectively. Treatment with these doses resulted in essentially similar inhibition of tumors. The advantage of AN-207 is that a dose about 150 times smaller than that of AN-152 can achieve the same effect. On the basis of the conclusions of present experiments, AN-207 was also successfully used for treatment of experimental Dunning prostate cancers in rats.[33]

Our earlier cytotoxic LH-RH analogs inhibited tumor growth by a combined hormonal and cytotoxic action.[16–18] Using DOX or AN-201 as antineoplastic radicals strongly increases the cytotoxicity of the hybrid molecules and the hormonal activity becomes subordinate. These new compounds require a regimen of administration different from that used for the earlier cytotoxic LH-RH analogs. At the doses and schedules of administration used in the present study, AN-207 and AN-152 had no significant long lasting hormonal activity. Serum LH, estradiol levels and sex organ weights were not changed at the end of the experiments.

The activity of the cytotoxic LH-RH analogs is determined not only by the antineoplastic radicals, but also by the chemistry of the coupling of the two molecules. DOX can be linked to peptides by using

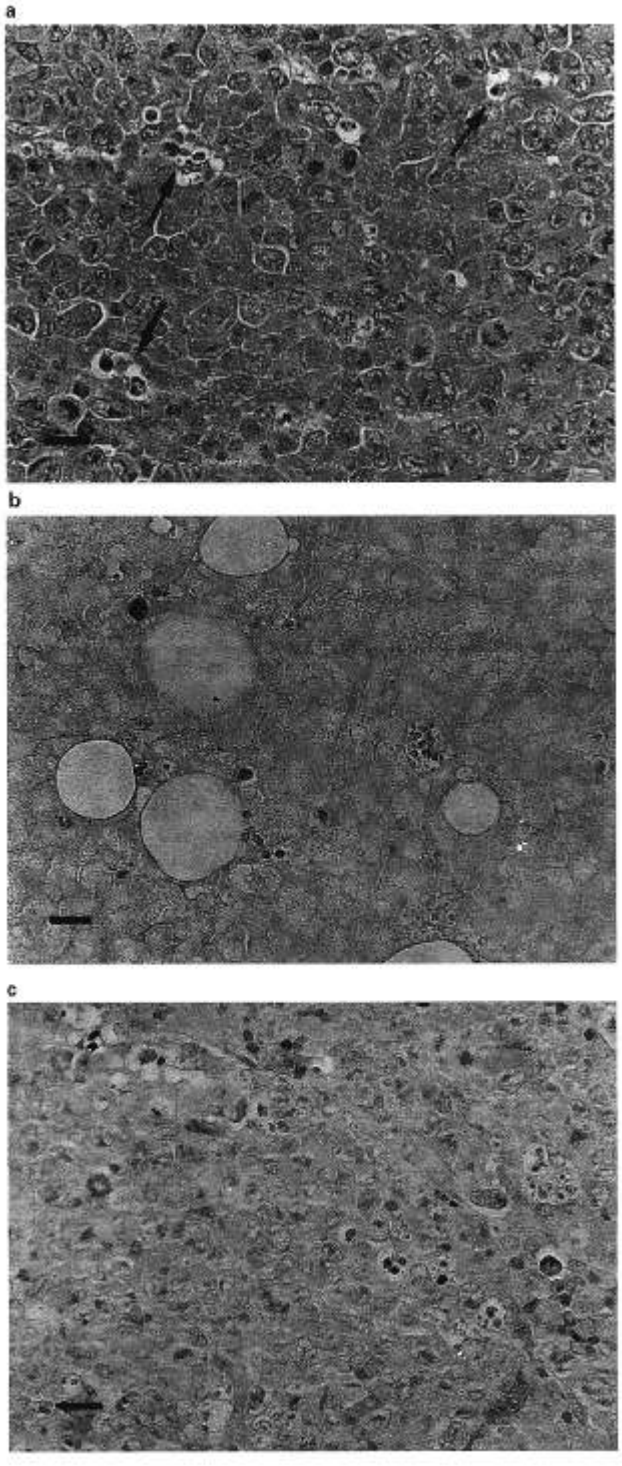

Figure 7. Estrogen-independent MXT mouse mammary cancers, scale bars=15 μm. (a). Several apoptotic cells (arrows) and mitotic figures can be seen. Untreated tumor, hematoxylin & eosin. (b) A tumor treated with AN-207. Pyknotic nuclei containing fragmented DNA shown as dark spots with the BrdU binding technique. (c) Nuclear fragments, some phagocytosed by neighboring cells in an MXT cancer after treatment with AN-152. DNA fragments that incorporated BrdU appear dark.

various chemical reactions. Because 14-*O*-esters of DOX are relatively stable and allow the retaining of the cytotoxic activity of DOX, we used DOX-14-*O*-hemiglutarate for the formation of peptide–DOX conjugates.[20] Earlier analogs, such as T-107, synthesized by coupling *N*-glutaryl-DOX to [D-Lys^6]LH-RH, show poor activity or no activity. The neutralization of the daunosamine nitrogen of DOX in this analog results in a loss of cytotoxic activity as shown by the results of our Experiment IV *in vivo* or earlier results *in vitro*.[23]

Both AN-207 and AN-152 had more potent tumor inhibitory effects than the respective cytotoxic moieties AN-201 or DOX, and were less toxic. The carrier [D-Lys^6]LH-RH administered in equimolar doses had no effect on tumors. AN-201 and DOX given together with [D-Lys^6]LH-RH acted similarly to the cytotoxic moieties alone, the mixtures being very toxic. It should be also emphasized that AN-207 and AN-152 inhibited tumor growth much more powerfully than our earlier cytotoxic LH-RH analogs. Our work shows that in the estrogen independent MXT cancer, a very aggressive tumor model, over 90% tumor reduction could be achieved and the cancers showed a complete remission in some animals.

Estrogen independent MXT mouse mammary cancer is a fast growing tumor and a useful model for initial *in vivo* testing of antitumor agents. Monitoring tumor volume changes gives a good indication of the dynamics of antitumor activity. Analysis of histological characteristics of cell proliferation at the end of the experiments provides information about the duration of the antitumor effect. Determinations of the mitotic index and AgNOR count are simple and proper methods for characterization of cellular proliferation. The cell loss by apoptosis was measured by calculating apoptotic indices in tumors using histological methods. Apoptotic alterations in hematoxylin & eosin stained slides can be easily recognized. In parallel studies, electron microscopy or histochemical techniques can be used for comparison on a limited number of samples. *In situ* end-labeling techniques are widely used for the detection of apoptotic cells.[34–36] Recently, BrdUTP[37] or BrdU[24] were used for *in situ* terminal transferase tailing of fragmented DNA. The BrdU tails are detectable with immunohistochemistry using monoclonal antibodies to BrdU. The method described by Aschoff *et al.*[24] was used for Epon-embedded semi-thin sections. In the present study, we successfully employed this method for paraffin sections and found it to be as efficacious as the procedures based on TUNEL or ISNT methods.

Previously we introduced the concept of the ratio of apoptotic to mitotic indices, which provides simulta-

neous measurement of cell gain and cell loss in tumors.[18] Moreover, the determination of both of these characteristics in the same microscopic fields eliminates possible errors caused by variations in thickness of slides and cellularity of various areas of tumors. These parameters showed that both decreased cell proliferation (mitoses and AgNORs) and enhanced apoptosis contributed to tumor growth suppression produced by AN-207 and AN-152.

Several chemotherapeutic agents act through induction of apoptosis.[38–40] Etoposid and bleomycin cause apoptosis of *in vitro* cultured fibroblasts[41] or in lymphoblastic leukemia cells[42] and cisplatin produces apoptosis in testicular germ cell tumors.[43] The effects of anthracyclines are mediated, at least in part, by the induction of apoptosis.[44] Suppression of apoptosis, e.g. by expression of specific genes, leads to cell survival and provides an explanation for resistance to chemotherapy.[45] However, derivatives of cytotoxic compounds may have different mechanisms of action. DOX, a topoisomerase II inhibitor, causes mostly cleavage of double-stranded DNA. In contrast, an intensively potent derivative of DOX, methoxymorpholinyl DOX, inhibits both topoisomerases I and II, resulting in predominantly single-stranded DNA cleavage.[46] In our study, analyzing apoptosis about 2 weeks after administration of the drugs, we found that DOX did not change the frequency of apoptotic cells. However, treatment with AN-201 (2-pyrrolino-DOX) resulted in an enhancement of apoptosis, and therapy with cytotoxic peptides AN-207 and AN-152 had similar effects.

Cell proliferation rates, as shown by AgNORs, were lower in the groups receiving AN-207 and AN-152, and mitotic activity was also reduced in some groups receiving the cytotoxic LH-RH analogs. These results indicate that the inhibition of cell proliferation and enhancement of apoptosis caused by AN-207 and AN-152 still occur 17 days after the treatments with these analogs.

Two classes of LH-RH receptors were detected in 52% of human breast cancers, one class being of high affinity.[9] Estrogen-independent MXT cancers, like human tumors, show heterogeneity with respect to their receptor content. Although one tumor was always used for transplantation in each experiment, only about 50% of well developed MXT cancers contained receptors for LH-RH (present study) or for EGF.[18] Since 86 tumor samples were analyzed for the receptors, these data appear to be reliable. Unfortunately, individual receptor analysis could not be performed before treatment. The absence of LH-RH receptors on tumor membranes after treatment with the cytotoxic LH-RH analogs is an interesting phenomenon which requires further studies. It could be caused by receptor down-regulation, the damage to specific binding sites or simply by the killing of cells containing receptors for LH-RH. The clarification of the dynamics of the disappearance and eventual recovery of receptors is essential for establishing the regimens for treatments with cytotoxic LH-RH analogs.

The experiments reported herein provide initial characteristics of *in vivo* antitumor effects of our new cytotoxic LH-RH analogs. The results demonstrate that analogs AN-152 and AN-207 containing DOX or a new very potent DOX derivative, AN-201, respectively, have a much stronger inhibitory effect on tumor growth and are less toxic than the cytotoxic radicals alone. Additional studies are required to further clarify their *in vivo* effects and to elucidate the detailed mechanism of action as well as the importance of LH-RH receptors in the tumor inhibitory effect.

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