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Review Article

Cytotoxic analogs of luteinizing hormone-releasing hormone (LHRH): A new approach to targeted chemotherapy

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Introduction

The hypothalamic decapeptide, luteinizing hormone-releasing hormone (LHRH), was first identified and characterized in physiological and clinical studies by Schally *et al*. some 30 years ago (1, 2). During the following decade, hundreds of agonistic analogs were synthesized. Some of them, including [D-Trp^6]LHRH (triptorelin), which was developed in our institute, contain a hydrophobic D amino acid substitution in position 6 of the LHRH sequence and are 50-100 times more potent than LHRH itself and also have prolonged half-lives (3). Physiological and clinical studies revealed that chronic treatment with superactive agonistic LHRH analogs or large doses of synthetic LHRH results in desensitization of pituitary gonadotrophs and suppression of sex steroid production (4). Based on these findings, LHRH agonists were introduced for the treatment of certain steroid hormone-dependent conditions like leiomyomas and endometriosis and malignancies such as breast and prostate cancers. Antagonists of LHRH such as cetrorelix (developed in our institute) were also demonstrated to be effective tools for the suppression of the pituitary-gonadal axis and can be used for the treatment of sex steroid-dependent conditions and malignancies (4, 5).

In addition to suppressing the pituitary-gonadal axis, LHRH agonists and antagonists can inhibit the growth of certain cancer cell lines *in vitro*. Consequently, a direct LHRH receptor-mediated antiproliferative effect of these analogs on tumor cells was proposed (5-7). Extensive research with experimental human cancer lines and tumor specimens from patients revealed that receptors for LHRH are present on a high percentage of breast, prostate, ovarian and endometrial cancers (5). These receptors were found to be identical with those expressed by the gonadotroph cells of the anterior pituitary (8). The presence of receptors for LHRH on certain cancers and their absence in most normal tissues provide a rationale for the design of cytotoxic LHRH conjugates consisting of agonistic or antagonistic analogs of LHRH as carrier molecules linked to a cytotoxic agent (9, 10). Nontargeted chemotherapeutic drugs have been used for about 7 decades in cancer therapy singly or in combination with various agents having different mechanisms of antiproliferative action. Chemotherapy is also utilized as an adjuvant to surgical resection. However, the use of chemotherapeutic agents is limited by their toxicity to normal cells and acquired or intrinsic resistance of cancerous cells to the drugs. Targeting cytotoxic agents to LHRH receptors on tumor cells should be associated with a lower toxicity and a higher local accumulation of the drug in tumor tissues which may improve efficacy and possibly help overcome intrinsic resistance (9).

The first series of cytotoxic LHRH hybrid molecules was synthesized in our institute in the late 1980s (9, 11, 12). By the middle of the 1990s, we developed a novel class of targeted cytotoxic LHRH analogs containing doxorubicin (DOX) or its intensely potent derivative, 2-pyrrolino-DOX (9, 13, 14). Very recently, others also described targeted antineoplastic agents based on LHRH

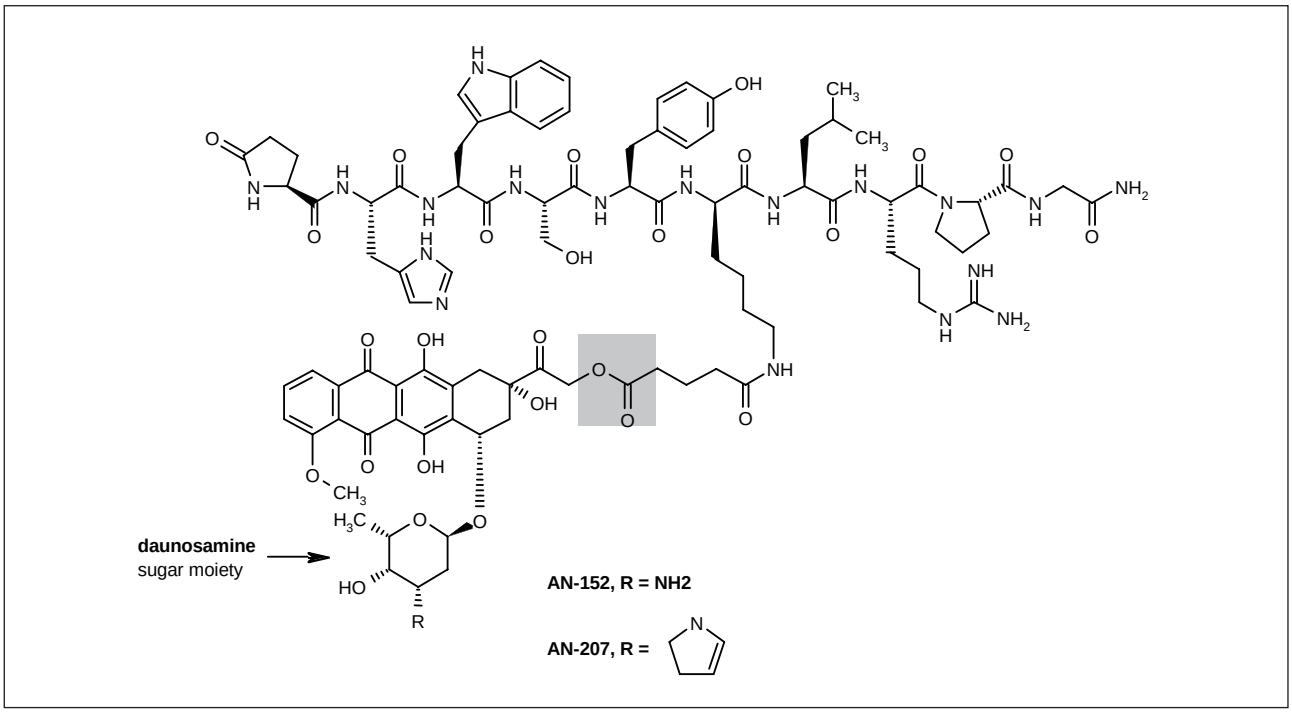

Fig. 1. Molecular structures of cytotoxic LHRH analogs AN-152 containing cytotoxic radical DOX (R=NH_2) and AN-207 incorporating AN-201 (R=2-pyrrolino). The 14-*O*-glutaryl ester of the cytotoxic radicals is linked to the epsilon amino group of [D-Lys^6]LHRH, the carrier peptide. The ester bond is indicated by a shaded area. (Modified from Proc Natl Acad Sci 1996, 93: 7269-73. Reprinted with permission. Copyright: National Academy of Science USA 1996.)

(15, 16). This review will focus on the design, synthesis and mechanism of action of our analogs and their antitumor effects in experimental models. Two of these conjugates, AN-152, consisting of [D-Lys^6]LHRH linked to DOX-14-*O*-hemiglutarate and AN-207, its superactive counterpart containing 2-pyrrolino-DOX (AN-201), have been tested extensively in preclinical studies (9) (Fig. 1). Clinical phase I trials with AN-152 have been planned to start this year.

Design and synthesis of cytotoxic LHRH analogs

Early prototypes

In designing a cytotoxic LHRH conjugate, it is essential to preserve the receptor binding affinity of the new hybrid entity and retain the cytotoxicity of the antineoplastic agent. It is well known that substitution of the Gly moiety in position 6 of the native LHRH sequence with D amino acids results in superactive analogs with high binding affinity to receptors for LHRH and with a longer plasma half-life. Bulky, hydrophobic side chains at this position were also shown to be preferable (3, 9). Thus, in our early attempts to form cytotoxic LHRH analogs, D-melphalan (D-Mel), a DNA-alkylating nitrogen mustard derivative of phenylalanine, was substituted into position 6 of LHRH or its antagonistic analogs. Some of these peptides such as agonist [D-Mel^6]LHRH and antagonist [Ac-D-Nal(2)^1,D-Phe(4-Cl)^2,D-Pal(3)^3,Arg^5,D-Mel^6,D-Ala^{10}]-LHRH showed high-affinity binding to receptors for LHRH on membrane preparations of Dunning R-3327-H rat prostate cancer and human breast and prostate cancer specimens (12). Mel was also linked to the epsilon amino side chain of D-Lys^6 in agonistic or antagonistic analogs of LHRH with a preservation of binding affinity. Some of these peptides showed moderate cytotoxicity *in vitro* in LHRH receptor-positive human breast and prostate cancer cell lines (17).

The bulk tolerance of LHRH analogs at the side chain of D-Lys^6 was utilized to form early cytotoxic LHRH analogs containing a variety of cytotoxic agents. These include the alkylating agent cisplatin, heavy metals such as Ni and Cu, the antimetabolite methotrexate and anthracycline analogs DOX and 2-hydroxymethylanthraquinone (9). To form conjugates with cisplatin, the D-Lys^6 side chain was acylated with diaminopropionic acid which in turn was reacted with K_2PtCl_4 in aqueous media (11). Heavy metal complexes were made similarly, by reacting the diaminopropionyl side chain with complexes of salicylaldehyde and Cu(II) or Ni(II) (11). One molecule of methotrexate could be coupled to the side chain of D-Lys^6, but carriers with a diaminopropionyl or diaminobutyryl spacer on the D-Lys^6 moiety allowed the attachment of 2 molecules of methotrexate to an LHRH analog. The conjugation was originally performed by nonspecific activation of the α and γ carboxyl groups of the glutamic acid moiety in methotrexate by diisopropyl car-

bodiimide (17). The method was later shown to produce 4 products containing D and L diastereomers of the α- and the γ-glutamyl derivatives (18). A procedure for the most preferable γ-L-glutamyl conjugates was also developed utilizing the α *tert*-butyl ester derivative of methotrexate for the selective coupling (18). One or two molecules of the glutaryl ester of 2-hydroxymehylanthraquinone could be linked similarly to the appropriate amino side chains of LHRH carriers (17).

Based on *in vitro* cytotoxicity assays, two of these early prototypes consisting of the agonist [D-Lys^6]LHRH and cytotoxic radicals methotrexate or 2-hydroxymehylanthraquinone were selected for preliminary *in vivo* oncological studies on the treatment of estrogen-dependent and -independent MXT mouse mammary carcinomas in BDF mice. The results showed that although the conjugates were somewhat more effective than the carrier peptide alone, further improvements in the chemical linkage were required to enhance the efficacy of the therapy (9, 19, 20). It also seemed to be necessary to select more potent cytotoxic agents. Thus, we began to focus our attention on one of the most potent and most widely used antitumor agents, DOX. An early analog, in which DOX was linked to the LHRH carrier through a glutaric acid spacer that formed a carboxamide bond with the daunosamine nitrogen in DOX, was about 1000 times less active than DOX *in vitro* and inactive *in vivo* at a high dose (9, 13, 21). These results and data by others indicated that the positive charge of the daunosamine nitrogen may be essential to preserve the cytotoxic activity of DOX (9, 14). Thus, a different chemical linkage had to be considered to produce conjugates containing DOX with preserved cytotoxicity. One such attempt involved a glutamic acid spacer instead of glutaric acid. In these conjugates, the α carboxyl group of the glutamic acid spacer was used to form an amide bond with the daunosamine nitrogen and the γ carboxyl group to acylate the D-Lys^6 side chain of the carrier peptide. The α amino group of the spacer was free to provide a positive charge near the now neutralized daunosamine nitrogen. Although these analogs were about 10 times more potent *in vitro* than those with a glutaric acid spacer (unpublished data), they were still 100 times less active than DOX and were considered impractical due to the large amounts of the LHRH conjugate necessary to produce an antitumor effect.

Highly potent new cytotoxic LHRH analogs

Because 14-*O*-esters of DOX were shown to preserve the full cytotoxic potential of DOX, we synthesized a series of LHRH analogues containing DOX-14-*O*-hemiglutarate (13). This chemical linkage also left the daunosamine moiety intact, allowing for modifications of the cytotoxic agent at this functionality (Fig. 1). Thus, in search of more active DOX derivatives, we synthesized a variety of daunosamine-modified analogues (14) that could be linked through their 14-*O*-glutaryl esters to the amino side chains of LHRH agonist or antagonist carriers (13). In these derivatives of DOX, the daunosamine nitrogen was incorporated into a 5- or 6-membered ring. Our most active analogue was 2-pyrrolino-DOX (AN-201), which is up to 2000 times more potent then DOX *in vitro* in cell lines that are not resistant to DOX (13, 14, 22, 23). The high-yield conversion of DOX to its superactive derivative, AN-201, was effected by a novel chemical reaction described in detail previously (14, 24). Briefly, the trifluoroacetic acid salt of DOX was dissolved in dimethyl formamide and reacted with a 5- to 10-fold excess of 4-iodobutyraldehyde. The conversion is almost 100%, completed within minutes after the addition of a tertiary base. DOX-14-*O*-hemiglutarate can be similarly reacted with 4-iodobutyraldehyde to form 2-pyrrolino-DOX-14-*O*-hemiglutarate (23, 24). This compound can be coupled to the D-Lys^6 side chains of LHRH carriers to form superactive cytotoxic LHRH conjugates such as AN-207. Interestingly, cytotoxic LHRH conjugates with DOX could also be converted to AN-201-containing analogues after reacting with 4-iodobutyraldehyde, without undesired side reactions. Thus, AN-152 was reacted with an excess of 4-iodobutyraldehyde followed by the addition of a tertiary base to form AN-207 in 60-70% yield (13).

The binding affinities of these cytotoxic LHRH analogues to receptors for LHRH on membrane preparations of rat anterior pituitary were determined in competitive binding assays. Radioiodinated [D-Trp^6]LHRH was used as radioligand and the binding affinities were expressed as IC_{50} values, the concentration of unlabeled analogue required to displace 50% of the binding of the radioligand. Thus, the IC_{50} values for AN-152, AN-207 and the carrier, [D-Lys^6]LHRH were found to be similar being 2.29, 5.59 and 2.26 nM, respectively (13).

Mechanism of action of cytotoxic LHRH conjugates

Targeting to LHRH receptors

It is assumed that after systemic injection, the binding of cytotoxic LHRH analogues to receptors for LHRH on cancerous cells would result in a local accumulation of the agents in tumor tissues. This would be followed by internalization and the release of the cytotoxic agent in the lysosomes. Because the internalization of LHRH agonists into pituitary cells was shown to be much faster than that of the antagonists (25), we concentrated our efforts on studying the targeted action of cytotoxic LHRH agonists. Thus, the tissue distribution of an early cytotoxic LHRH conjugate (T-98), consisting of 2-hydroxymethylanthraquinone linked to the agonist carrier [D-Lys^6]LHRH, was investigated in estrogen-dependent MXT mouse mammary carcinoma (20). In this study we demonstrated that 3 h after a s.c. injection of radiolabeled T-98, a significantly higher accumulation of radioactivity was found in the tumors than in LHRH receptor-negative tissues such as skeletal muscles. High-affinity binding and internalization of radiolabeled T-98, which could be inhibited by an excess of unlabeled T-98, was also shown in rat pituitary cells *in vitro* (26).

The mechanism of action of our modern cytotoxic LHRH agonist AN-152 containing DOX was investigated in LHRH receptor-positive and -negative human ovarian and endometrial cancer cell lines. The autofluorescence of DOX was used to follow the internalization and transport of AN-152 in the different cellular compartments by confocal laser scanning microscopy (27). It was revealed that AN-152 accumulated only in the nuclei of LHRH receptor-positive cells, while unconjugated DOX entered the nuclei of all cell types independently of their receptor status. The entry of AN-152, but not DOX, could be blocked by an excess of the LHRH agonist, [D-Trp^6]-LHRH. In addition, a significantly higher fluorescence signal could be detected in the nuclei of LHRH receptor-positive cells after treatment with AN-152 than with an equimolar amount of DOX. Accordingly, AN-152 was significantly more cytotoxic than DOX in these cells (27).

Similar results were obtained in human estrogen-dependent MCF-7 breast cancer cells *in vitro*. Newly developed two-photon emission fluorophores were linked to the free amino group of the daunosamine moiety of AN-152 or to the epsilon amino group of the carrier [D-Lys^6]LHRH. The low energy required for the excitation of these fluorophore tags allows "real-time" optical tracking of the conjugate and the carrier in the different compartments of living MCF-7 cells (28-31). Receptors for LHRH on MCF-7 cells could be upregulated by preincubation with epidermal growth factor (EGF) or bombesin and downregulated by the somatostatin octapeptide RC-160 (29, 31). The entry and cytotoxic activity of AN-152 was found to be dependent on the levels of LHRH receptors and could be blocked by an excess of [D-Trp^6]LHRH. In cells pretreated with EGF, binding and internalization of the labeled AN-152 or the carrier peptide took place within a few minutes (29, 30). While the labeled carrier peptide was localized mainly in the cytosols of MCF-7 cells, with only a small fraction appearing in the nuclei, the fluorescent label linked to AN-152 was found only in the nuclei (30). Because the label was coupled to the daunosamine nitrogen of DOX through a stable carboxamide linkage, it is most likely that the DOX moiety was also present in the nucleus where the two-photon fluorophore signal was detectable. No entry of labeled AN-152 could be observed in LHRH receptor-negative UCI-107 human ovarian cancer cells (29).

The effects of the targeted cytotoxic LHRH conjugate AN-207 and its nontargeted radical AN-201 were tested *in vitro* in mammalian COS-7 cells stably transfected with recombinant human LHRH receptors and compared to those in parental COS-7 cells that do not express endogenous LHRH receptors (32). AN-207 was found to selectively induce apoptosis in receptor-positive cells as measured by DNA fragmentation and the expression of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins. Assays on cell proliferation showed similar results. This selective effect of AN-207 could be partially blocked by the LHRH antagonists cetrorelix or antide. In contrast to AN-207, AN-201 showed no selectivity and was less effective in inhibiting the proliferation of LHRH receptor-positive COS cells (32). *In vitro* targeting of AN-207 to LHRH receptor-positive ES-2 human ovarian cancer cells was also demonstrated by coculturing these ES-2 cells with LHRH receptor-negative UCI-107 human ovarian cancer cells. Semiquantitative PCR analyses of microsatellite markers specific for ES-2 or UCI-107 cells revealed that AN-207 had a selective antiproliferative effect on ES-2 cells, while AN-201 killed both cell types indiscriminately (33).

Stability of 14-O-glutaryl esters of DOX and AN-201

The 14-*O*-glutaryl esters of DOX or AN-201 used in AN-152 and AN-207, respectively, can be hydrolyzed by nonspecific carboxylesterase enzymes (EC 3.1.1.1), which are ubiquitous. The activity of these enzymes in the serum is responsible for partial hydrolysis of the conjugates in the circulation, releasing a certain percentage of the cytotoxic agents before the targeting is completed (24, 34). To determine the stability of the ester bond used in our conjugates, we incubated AN-152 *in vitro* in mouse, rat and human serum at 37 °C in humidified air containing 5% CO_2. Our results indicate that the half-lives of AN-152 in mouse, rat and human serum are about 20, 60 and 120 min, respectively (9, 24, 34). We also demonstrated that a prolongation of the half-life of this ester bond in serum results in a more efficient delivery of the cytotoxic radicals to LHRH receptor-positive target cells and consequently, a lower toxicity. Thus, the tolerance of tumor-free healthy nude mice to AN-207 could be increased by about 50% to 300 nmol/kg of body weight by pretreatment of mice with an efficient esterase inhibitor, diisopropyl fluorophosphate. It was demonstrated that the addition of diisopropyl fluorophosphate to mouse serum *in vitro* at a concentration corresponding to its maximum tolerated dose (MTD) i.v., prolonged the half-life of the ester bond from 20 min to about 70 min, which is much closer to the 120 min half-life found in human serum (34). Another example of a longer half-life of a cytotoxic conjugate accompanied by a lower toxicity was demonstrated in the same model with the cytotoxic somatostatin analogue AN-238, consisting of AN-201-14-*O*-hemiglutarate linked to the somatostatin octapeptide carrier RC-121. Thus, in the nude mouse model with suppressed esterase activity, the tolerance of mice to AN-238 could be increased by 100%. This improvement in tolerance to AN-238 was reversed by blocking the receptors for somatostatin with a high dose of the somatostatin octapeptide analog RC-160 (34).

While AN-238 can be given to nude mice with suppressed esterase activity twice at a dose of 400 nmol/kg, all animals die after a single injection of the LHRH analogue AN-207 at 400 nmol/kg. These observations are in accord with the findings that receptors for somatostatin are more prevalent in normal tissues than receptors for LHRH. Based on these results we can assume that the cytotoxic LHRH analogue AN-207 may be even less toxic and more efficient in patients than in experimental nude

mouse models. The lower toxicity accompanying a better targeting also suggests that the possible damage caused to LHRH receptor-positive normal cells by AN-207 is much less detrimental to the host than the damage caused to the rapidly proliferating bone marrow cells by the nontargeted cytotoxic radical AN-201. In agreement with this view, we found that 2 weeks after treatment of rats with the MTD of AN-207, the pituitary function of the animals fully recovered (35). Both AN-207 and the carrier [D-Lys^6]LHRH decreased the levels of mRNA for LHRH receptors in the rat pituitary and induced a 10- to 100-fold elevation of serum LH levels in male and ovariectomized rats, respectively (36). The changes in serum LH caused by AN-207 and the carrier were similar and reversible, indicating that the carrier portion of AN-207 is responsible for these effects. As expected, the stimulated release of growth hormone and thyrotropin were not affected significantly by AN-207 (35, 36).

Carboxylesterase enzymes also play an important role in releasing the cytotoxic agents after binding, before internalization or inside the target cells. In case the drugs are released after binding, but before entering the cells, LHRH receptor-negative cells in the tumors adjacent to receptor-positive cells may also be affected by the higher drug concentration produced by targeting. This "bystander" effect may be advantageous in the treatment of cancers in which the receptors are not expressed evenly in each and every tumor cell.

DOX and AN-201

DOX is still one of the most widely used chemotherapeutic agents, with the broadest spectrum of antitumor activity (37). The exact mechanism of action of DOX is not fully understood, but it is well established that DOX can intercalate into the DNA and inhibit topoisomerase II, leading to DNA strand breaks in dividing cells (38). This explains why DOX shows some selectivity towards rapidly dividing cancerous cells and also accounts for certain side effects such as myelosuppression. The main dose-limiting toxicity of DOX, however, is not myelosuppression but cardiomyopathy, which is believed to be due to generation of reactive oxygen species mediated by the quinone moiety of the anthracycline molecule in the presence of iron ions (39). DOX was also shown to kill cancerous cells without entering the nucleus, through binding to membrane phospholipids (40). In addition to toxic effects, the clinical efficacy of DOX is further impeded by intrinsic or acquired multidrug resistance of cancerous cells (41). In contrast to DOX, AN-201 may need to enter the cell nucleus to exert its high antiproliferative activity, which can be attributed to the ability of its latent aldehyde function to form a covalent bond with an amino group of a guanine base in close vicinity to the DNA intercalation site (14, 24). Preliminary results indicate that similarly to cyanomorpholino-DOX or some other daunosamine-modified derivatives of DOX, AN-201 is also not cross-resistant with DOX (24). Possibly due to their low maximum tolerated doses, these superactive DOX derivatives were found to be noncardiotoxic (14, 24, 42, 43). Not surprisingly, the dose-limiting toxicity of these powerful DNA intercalating and cross-linking agents is myelosuppression (9, 43).

Treatment of experimental cancers expressing LHRH receptors

Breast cancer

Breast cancer is the second leading cause of cancer-related deaths among women in the Western world. Although the introduction of screening mammography and adjuvant treatments significantly improved the cure rate, estrogen-independent metastatic breast cancer is still incurable. In view of the fundamental role of adjuvant chemotherapy in the treatment of breast cancer, the development of more efficient cytotoxic agents with fewer side effects is most important (44). Receptors for LHRH on human breast cancer cell lines were reported more than a decade ago (6). Binding assays performed in our institute in the late 1980s demonstrated the presence of high-affinity binding sites for radiolabeled [D-Trp^6]LHRH in 260 of 500 human breast cancer specimens (45). These data provide a firm basis for the use of cytotoxic LHRH analogues to treat breast cancers. The binding affinities of AN-152 and AN-207 to receptors for LHRH on human breast cancer specimens were recently characterized by IC_{50} values of 7.45 ± 0.61 and 6.15 ± 0.56 nM, respectively (46).

The antitumor effects of AN-152 and AN-207 were first studied in estrogen-independent MXT mouse mammary carcinoma in BDF mice in comparison with DOX and AN-201, their respective cytotoxic radicals (21). Although only about 40% of the MXT control tumors expressed receptors for LHRH, both AN-152 and AN-207 were significantly more effective and less toxic than equimolar doses of DOX or AN-201, respectively (Tables I and II). Both cytotoxic LHRH analogues caused about 90% inhibition of tumor growth. Interestingly, no binding sites for LHRH could be detected on membrane fractions of tumors treated with the LHRH conjugates. When these agents were administered to mice bearing MXT tumors at a more advanced stage, initial tumor size being over 2000 mm^3, only AN-207 showed a significant effect (47).

A very impressive demonstration of targeting to LHRH receptors on breast cancers was achieved after a single i.v. injection of AN-207 at 250 nmol/kg of body weight, equivalent to MTD, to nude mice bearing xenografts of estrogen-independent, MX-1 human mammary carcinoma resistant to DOX (48). A complete regression of the tumors in 10 of 10 animals was observed after treatment, without major toxic effects and the animals remained tumor free even after 60 days. In contrast, after an initial regression, large tumors developed by day 60 in 8 of 10 mice treated with AN-201 at the same dose. In this group there was only 1 tumor-free survivor and 1 mouse died

due to toxicity (Table II). Receptors for LHRH on MX-1 control tumors were characterized by a dissociation constant (K_D) of 6.5 ± 1.3 nM and a concentration (B_{max}) of 185.4 ± 44.9 fmol/mg membrane protein.

Treatment of nude mice bearing LHRH receptor-positive, estrogen-independent MDA-MB-231 human breast cancers with a single i.v. injection of AN-207 at 250 nmol/kg caused an initial regression of tumors in 8 of 13 animals. A significant (p <0.01 *vs*. control) inhibition of tumor volume was maintained during the first 3 weeks of the study (Table II). After 3 weeks, receptors for LHRH could not be detected in tumors treated with AN-207, but these receptors were again detectable 60 days after the injection as characterized by K_D and B_{max} values of 5.68 ± 1.57 nM and 472.1 ± 109.4 fmol/mg membrane protein. The receptor characteristics of control tumors at that time were similar (K_D = 7.02 ± 0.76 nM; B_{max} = 649.0 ± 36.0 fmol/mg membrane protein). In the groups treated with AN-201, or a mixture of AN-201 and the carrier at the same dose, tumors grew steadily (44).

Similar results were obtained in MDA-MB-435 human estrogen-independent mammary carcinomas implanted orthotopically in the mammary fat pad of nude mice (49). While, a single dose of 250 nmol/kg or 2 injections of AN-207 at 150 nmol/kg both resulted in about a 70% inhibition of tumor growth compared to controls, the tumors grew steadily after treatment with AN-201 at the same doses (Table II). In addition, 6 of 8 animals in the controls, 3 of 8 mice in the group given a single dose of AN-201, but none of the AN-207-treated animals developed lymphatic metastases. This high efficacy of AN-207 could be partially blocked by pretreatment of nude mice with a large dose of the agonist [D-Trp^6]LHRH 1 h before administration of the conjugate. Under these conditions, 2 of 8 mice developed metastases in the lymph nodes and the toxicity of AN-207 was increased to that of AN-201 (49). The level of mRNA for LHRH receptors showed a 26% (p <0.05 *vs*. control) decrease 33 days after treatment with AN-207, but by day 48 it returned to the level of the controls. The binding characteristics for LHRH receptors in control tumors were K_D = 7.21 ± 1.6 nM and B_{max} = 577.7 ± 125.8 fmol/mg membrane protein.

These results demonstrate that cytotoxic analogues of LHRH should be effective in patients with estrogen-independent breast cancers.

Ovarian cancer

Carcinoma of the ovary is usually diagnosed at an advanced stage, consequently, the overall cure rate of ovarian cancer is very low. The main modality for the treatment of this malignancy is cytoreductive surgery combined with chemotherapy based on antineoplastic agents such as cisplatin, cyclophosphamide, paclitaxel or DOX, mostly in combination regimens (50). Because receptors for LHRH were found on a high percentage of human ovarian cancer specimens, it is reasonable to suggest that cytotoxic LHRH analogues may be more effective and less toxic for the management of this malignancy than the cytotoxic agents alone (9, 51, 52). Thus, AN-152 was tested in LHRH receptor-positive OV-1063 and receptor-negative UCI-107 human ovarian cancer cell lines xenografted into nude mice (50). The binding of radioiodinated [D-Trp^6]LHRH to OV-1063 control tumors was characterized by a K_D value of 9.56 ± 1.45 nM and a

Table I: Effects of treatment with the cytotoxic LHRH analog AN-152 and its cytotoxic radical DOX on LHRH receptor-positive experimental tumors.

	AN-152		DOX	
Tumor models	Dose*	Effects	Dose*	Effects
Breast				
MXT (mouse) (21)	35.5 μmol/kg ip	98% growth inhibition, 1 of 5 animals died of toxicity	35.5 μmol/kg ip	98% growth inihibition, 5 of 5 animals died of toxicity
Ovarian				
OV-1063 (human) (50)	20.6 μmol/kg ip	84% growth inihibition, no toxicity	20.6 μmol/kg ip	No significant growth inhibition, 6 of 9 animals died of toxicity
NIH:OVCAR-3 (human) (10)	15 μmol/kg iv	Tumors regressed by ~30%, no toxicity	15 μmol/kg iv	Tumors increased by ~25%, no deaths occurred
	35 μmol/kg iv	Tumors regressed by ~30%, no toxicity	35 μmol/kg iv	Tumor growth was arrested, 3 of 5 animals died of toxicity
ES-2 (human) (55)	17.25 μmol/kg iv	Significant inhibition of growth and factors associated with growth, no toxicity	17.25 μmol/kg iv	No significant effects, 1 mouse died
Endometrial				
HEC-1B (human) (10)	15 μmol/kg iv	Tumors regressed by ~25%, no toxicity	15 μmol/kg iv	Tumors increased by ~40%, no deaths occurred
	35 μmol/kg iv	Tumors regressed by ~20%, no toxicity	35 μmol/kg iv	1 tumor increased by ~ 40%, 4 of 5 animals died of toxicity

*Only one dose was given. The numbers in brackets indicate the references from which the data was taken.

Table II: Effects of treatment with the cytotoxic LHRH analog AN-207 and its cytotoxic radical AN-201 on LHRH receptor-positive experimental tumors.

	AN-207		AN-201	
Tumor models	Dose	Effects	Dose	Effects
Breast				
MXT (mouse) (21)	110 nmol/kg ip x 2	89% growth inhibition, 1 of 10 animals died of toxicity	110 nmol/kg ip x 2	98% growth inhibition, 8 of 10 animals died of toxicity
MXT (mouse) (47)	250 nmol/kg iv	Arrested growth of tumors >2000 mm^3, no toxicity	250 nmol/kg iv	No effect on tumors >2000 mm^3, no toxicity
MX-1 (human) (48)	250 nmol/kg iv	10 of 10 animals cured, no toxicity	250 nmol/kg iv	1 of 10 animals cured, 1 of 10 animals died of toxicity
MDA-MB-231 (human) (44)	250 nmol/kg iv	Initial regression in 8 of 10 mice, no toxicity	250 nmol/kg iv	The tumors grew steadily, no toxicity
MDA-MB-435 (human orthopical) (49)	150 nmol/kg iv x 2	68% growth innhibition, mice, no toxicity	150 nmol/kg iv x 2	The tumors grew steadily, no toxicity
Ovarian				
OV-1063 (human) (53)	150 nmol/kg ip x 2	65% growth inhibition, no toxicity	150 nmol/kg ip x 2	Effects could not be evaluated, 9 of 10 animals died of toxicity
Prostate				
Dunning R-3327-H (rat) (60)	50 nmol/kg ip x 3	8 cm^3 tumors regressed by 50%, no toxic deaths	50 nmol/kg ip x 3	Effects could not be evaluated, all animals died
PC-82 (human) (63)	200 nmol/kg iv	~70% growth inhibition, significant reduction of PSA levels, 1 of 8 mice died of toxicity	200 nmol/kg iv	~35% growth inhibition, no reduction of PSA levels, 3 of 8 mice died of toxicity
MDA-PCa-2b (human) (64)	200+150 nmol/kg iv	63% growth inhibition, significant reduction of PSA levels, no toxicity	200 nmol/kg iv x 1	No significant growth inhibition, 3 of 7 mice dided of toxicity

The numbers in brackets indicate the references from which the data was taken.

B_{max} value of 488.5 ± 23.5 fmol/mg membrane protein. AN-152 was highly efficient in inhibiting the growth of OV-1063 tumors after single i.p. injections of doses equivalent to 12 and 20.3 mg/kg DOX causing > 84% decreases in tumor volumes and weights after 4 weeks (Table I). The tumor burden was lowered and the tumor doubling time extended significantly in both groups compared with controls. No toxicity was observed in the group given the low dose of AN-152, but 2 of 9 animals died in the group receiving the high dose. AN-152 was also very effective at a single dose equivalent to 16 mg/kg DOX or 2 injections of 12 mg/kg without toxicity. Interestingly, receptors for EGF were downregulated as a result of treatment and the receptors for LHRH could not be detected in tumors treated with AN-152 at the termination of the study (50). In contrast, DOX at the dose of 12 mg/kg of body weight, killed 6 of 9 animals and had no significant antitumor effect. The importance of receptors for LHRH on tumor cells was demonstrated by the lack of activity of AN-152 at a dose equivalent to 12 mg/kg DOX on UCI-107 tumors.

Animals with OV-1063 and UCI-107 tumors were also treated with AN-207 and AN-201 (53). A single i.p. administration of AN-207 at 250 nmol/kg or 2 injections at 150 nmol/kg, both representing MTDs, had significant tumor growth inhibitory effects as evidenced by reduction in tumor volume and tumor burden as well as extension of tumor doubling time (Table II). mRNAs for LHRH receptors and EGF receptors were significantly reduced in tumors treated with AN-207. Binding sites for LHRH were not detectable by radioreceptor assay and the capacity of receptors for EGF was significantly downregulated in tumors treated with AN-207 (53). One of 9 animals died in the group given a dose of 250 nmol/kg of body weight, but no deaths occurred in the group receiving 2 doses of 150 nmol/kg. Again, AN-207 had no antitumor effect in UCI-107 tumors, supporting the concept that receptors for LHRH are crucial for the targeted action of AN-207. All animals treated with an i.p. injection of AN-201 or the mixture of AN-201 and the carrier [D-Lys^6]LHRH at 250 nmol/kg of body weight, died within 7 days and only 1 of 10 animals survived the 2 i.p. injections of AN-201 at 150 nmol/kg of body weight.

Although the toxic side effects of AN-207 are less serious than those of AN-201 and independent of the route of administration, a much higher tolerance of mice to AN-201 is observed when it is administered i.v. rather than i.p. (9). This could be explained by a possible specific toxicity of AN-201 to the liver. It is well known that virtually all of a compound that is absorbed by the gastrointestinal tract would go to the liver via the hepatic portal venous system (54) and most of an i.p. injection is taken

up by the capillaries in the gastrointestinal tract. In contrast, if a compound is absorbed by any other route, only about 30% of it would reach the liver before being distributed by the general circulation (54). Because there are no receptors for LHRH on liver cells (10), AN-207 cannot enter these cells and accordingly, mice have a similar tolerance to AN-207 given by the i.v. or the i.p. route. This demonstrates another possible advantage of the targeted conjugate, especially in view of the fact that debulking surgery in ovarian cancers is usually followed by administration of chemotherapeutic agents into the peritoneal cavity to prevent metastatic spread of the disease.

The antitumor effects of AN-152 were also assessed in nude mice bearing LHRH receptor-positive NIH:OVCAR-3 and LHRH receptor-negative SK-OV-3 human ovarian cancers (10). One week after i.v. injections of AN-152 at doses equivalent to 8.7 or 20.3 mg/kg DOX, the volume of NIH:OVCAR-3 tumors regressed significantly (Table I). There were no changes in body weights or toxicity-related deaths. In contrast, DOX was less effective and caused the death of 3 of 5 animals in the group receiving the high dose. Although 8 days after treatment with AN-152 the density of LHRH binding sites was reduced to 63 ± 26% of the control values, this decrease was not significant, indicating that the targeted therapy to LHRH receptors can be repeated (10). Using the same dosage, AN-152 was ineffective in the SK-OV-3 model.

The superior effects of AN-152 compared to DOX were also demonstrated in LHRH receptor-positive ES-2 human epithelial ovarian cancers (55). Four weeks after a single i.v. injection of AN-152 at a dose equivalent to 10 mg/kg DOX, the tumor volume was significantly reduced compared with controls. In addition, the levels of mRNAs for various factors and oncogenes associated with unfavorable prognosis and progressive tumor development were also reduced significantly by AN-152. Thus, mRNAs for EGF receptors, vascular endothelial growth factor and oncogenes c-*fos* and c-*jun* were reduced by 49, 48, 55 and 58%, respectively (Table I). The mRNA for Her-2/neu (c-erbB-2), an orphan receptor of the EGF receptor-family, was not detectable after treatment with AN-152. In contrast, DOX had no effect on tumor growth and showed only nonsignificant decreases in these genes (55). Our findings suggest that cytotoxic analogues of LHRH should be tried in patients with epithelial ovarian cancers.

Endometrial cancer

Endometrial cancer is the most frequently diagnosed gynecological malignancy in the Western world. Although most cases are detected at an early stage and the cure rate is high, steroid receptor-negative tumors in elderly patients and at advanced stages require improved treatment modalities (27). Targeted cytotoxic LHRH analogues may provide such improvement because receptors for LHRH were found in about 80% of human endometrial cancer specimens (56). To evaluate this potential, single i.v. injections of AN-152 were given at doses equivalent to 8.7 or 20.3 mg/kg DOX to nude mice bearing xenografts of HEC-1B human endometrial cancers (10). One week after treatment, a significant shrinkage of the tumor volume was observed at both doses (Table I). The treatments had no toxic side effects. Eight days after therapy with AN-152 the density of LHRH binding sites was reduced to 75 ± 18.6% of the control values, but this decrease was not significant (10). At the same concentrations, DOX caused no shrinkage of tumors and only 1 of 5 animals survived the therapy with the high dose (10).

In a preliminary study, we had similar results with AN-207 in HEC-1A human endometrial carcinoma in nude mice (unpublished data).

Prostate cancer

Carcinoma of the prostate is the most common noncutaneous malignancy in men. The prognosis of patients with relapsed androgen-independent prostate cancer is very poor and there is no effective treatment available at present (4, 5). Because more than 80% of human prostate cancers express high-affinity binding sites for LHRH, targeting cytotoxic agents to these receptors may improve the efficacy of the treatment (57, 58). The results of a most recent study showing an increased incidence of the expression of mRNA for LHRH receptors in hormone-refractory human prostate cancer specimens indicate that treatment with the targeted cytotoxic LHRH analogues AN-152 or AN-207 may benefit patients who no longer respond to palliative androgen deprivation therapy (59).

The antitumor efficacy of AN-207 was first evaluated in the androgen-dependent LHRH receptor-positive Dunning R-3327-H prostate carcinoma model in Copenhagen rats (60). Binding sites for radioiodinated [D-Trp^6]LHRH in control tumors were characterized by a K_D of 11.9 ± 0.65 nM and a B_{max} of 1110 ± 120 fmol/mg membrane protein. Three consecutive i.p. injections of AN-207 at 50 nmol/kg of body weight doses caused about a 50% regression of large initial tumors measuring approximately 8 cm^3 in size (Table II). No animals treated with AN-207 died in this experiment, while all rats receiving the second i.p. injection of radical AN-201 at the same dose died of toxicity. In a second experiment, AN-207 at the same dosing schedule again caused a 50% regression of large initial tumors measuring about 4 cm^3 in size. Two of 10 animals died in this study, indicating that this dose was the MTD for AN-207 given to rats i.p. Three consecutive i.p. injections of AN-207 at 25 nmol/kg of body weight arrested tumor growth without any side effects. Tumor burden was also significantly reduced by AN-207. Receptors for LHRH could not be detected in tumors from the groups treated with AN-207. Binding capacities for EGF receptors in tumors were also significantly downregulated by AN-207 (60). Six of 10 animals receiving 3 injections of AN-201 at a dose of 25 nmol/kg died. Despite the effect of AN-201 on tumor weight, due to the very low body weights of the surviving rats, the

tumor burden in this group was not significantly changed (60). In these experiments, AN-207 and AN-201 were given i.p.

In another study, AN-207 and AN-201 were injected i.v. at doses of 150 nmol/kg to rats bearing LHRH receptor-positive androgen-independent Dunning R-3327-AT-1 prostate cancers. After 3 weeks, AN-207 caused a significant inhibition of tumor growth, which was accompanied by a slight decrease in body weight. In contrast, all animals in the group receiving AN-201 died due to toxicity (61). The MTD for AN-201 by the i.v. route was later determined to be 110 nmol/kg in rats (24, 36, 62). Another study in which AN-207 was given i.v. to rats as a single injection indicates that its MTD is about 175 nmol/kg (36). This sharp difference in the tolerance to i.v. injections of AN-207 and AN-201 was not observed in mice where the MTDs for both compounds are about 200 nmol/kg in male and 250 nmol/kg in female nude mice (9, 24). The explanation for this distinction may be found in the fact that the esterase activity in mice is higher than in rats, obscuring the differences between the targeted conjugate and the cytotoxic radical. In accord with this view is our finding that the tolerance to a single i.v. injection of AN-207 can be increased by 50% to 300 nmol/kg in male nude mice with esterase activity suppressed by diisopropyl fluorophosphate, while the esterase inhibitor does not affect the tolerance to AN-201 (34). It has been shown that a longer half-life of the conjugate in serum results in a more completed targeting and a lower toxicity (24, 34). In the nude mouse model with suppressed esterase activity, the half-life of the ester bond linking the cytotoxic radical to the peptide carrier is similar to that found in rat serum, 70 and 60 min, respectively (24, 34).

In another study, a single i.v. injection of AN-207 at 200 nmol/kg of body weight was given to nude mice bearing LHRH receptor-positive androgen-dependent PC-82 human prostatic cancers (63). Radioreceptor assays revealed high-affinity binding sites for radioiodinated [D-Trp^6]LHRH with a K_D of 7.63 ± 0.82 nM and B_{max} of 495.9 ± 5.26 fmol/mg membrane protein in control tumors. After treatment with AN-207, an initial regression of tumor volumes was observed, which lasted for more than 2 weeks. Eight weeks after the injection, tumor volume, weight and burden were still significantly (p <0.01) inhibited compared to controls (Table II). Serum PSA values were also reduced significantly (p <0.01). At the end of the study, mRNA for LHRH receptor was found in all tumors, even in those treated with AN-207. Only 1 of 8 animals died due to toxicity. In contrast, 3 of 8 mice died after treatment with AN-201 and after 8 weeks, tumor volume, weight and burden as well as serum PSA values were not significantly different in the surviving mice, compared with controls. Single injections of the carrier peptide or the mixture of the carrier and AN-201 at the same doses were also not effective and the latter regimen was toxic, killing 3 of 9 mice (63).

In a most recent study, we evaluated the efficacy of AN-207 in MDA-PCa-2b experimental prostate cancer, which was derived from a bone metastasis of a patient who relapsed after androgen ablation (64). MDA-PCa-2b is a clinically relevant new experimental model, which is representative of human prostatic carcinoma disseminated to the bone and progressing despite androgen withdrawal. In contrast to the PC-82 line, these cells express mutated androgen receptors, which render them responsive to progesterone and corticosteroid and less sensitive to androgen. mRNA for LHRH receptors was found in MDA-PCa-2b control tumor samples. Because an i.v. injection of AN-207 at a dose of 200 nmol/kg was well tolerated by mice bearing relatively large tumors (> 300 mm^3 in size), a second injection of AN-207 was given at a dose of 150 nmol/kg 2 weeks later. Four weeks after the initiation of the therapy, tumor volume, weight and tumor doubling time as well as serum PSA levels were significantly reduced compared to controls (Table II). None of the animals died after the administration of a total dose of 350 nmol/kg of AN-207 (64). In sharp contrast, a single i.v. injection of cytotoxic radical AN-201 at a dose of 200 nmol/kg killed 3 of 7 animals. Consequently, a second injection was not given to the surviving mice. Four weeks after the administration of AN-201, there were no significant effects on tumor growth in this group compared with controls. Our results demonstrate a high efficacy of cytotoxic analogues of LHRH in prostate cancer models and suggest the merit of therapeutic trials in patients with relapsed prostate cancers.

Other cancers

Receptors for LHRH have been found in some experimental tumors that do not depend on gonadal steroids or the gonadotropins. These cancers include the nitrosamine-induced pancreatic cancers in hamsters (65), human pancreatic cancer line MIA-PaCa-2 (66), Colo 205 human colon cancers (16), hamster cheek pouch carcinoma model of oral cancer (67), KB human oral cancer and HEp-2 human laryngeal cancers (68). In addition, binding sites for radiolabeled LHRH agonists were also found in human specimens of pancreatic cancer (69) and nervous system tumors (70).

Some evidence suggests that the expression of LHRH receptors in tumors is mediated by an imbalance of tyrosine kinase activity, which is characteristic of many malignancies (71). In the chemically induced hamster cheek pouch carcinoma model it was observed that the appearance of LHRH receptors was associated with an increase in tyrosine kinase activity at an early stage of carcinogenesis (71). A stimulation of LHRH receptor expression by EGF was demonstrated in various cancer lines (29, 31, 66). Treatment of certain cancer cells with EGF also induced, through the tyrosine kinase activity of its receptor, the phosphorylation of a 60 kD protein, which corresponds in size to the LHRH receptor (66). Based on these observations, it can be assumed that a wide variety of cancers that overexpress EGF receptors may also express receptors for LHRH at certain stages of tumoral development (66, 67, 71). A possible role of EGF recep-

tors in the upregulation of LHRH receptors seems to be further supported by our findings that, in some cases, after treatment of experimental cancers with cytotoxic LHRH analogues AN-207 or AN-152, the downregulation of EGF receptors was accompanied by the disappearance of LHRH receptors (50, 53, 60). It should be mentioned, however, that the receptors for LHRH can be detected again at a later time point, suggesting that the targeted therapy to LHRH receptors can be given repeatedly (44).

Receptors for LHRH in cancers that are not classically dependent on gonadal steroids could also be utilized as targets for cytotoxic LHRH analogues. Thus, in KB and HEp-2 cells, a much stronger antiproliferative effect of AN-152 was demonstrated *in vitro* compared with DOX (68). A presently ongoing study using AN-152 and AN-207 in LHRH receptor-positive HT-29 human colon cancers in nude mice indicate that both agents have stronger antitumor effects than DOX or AN-201, their respective cytotoxic radicals (Szepesházi K., Schally, A.V., Nagy, A. *et al*., in preparation).

Based on these results, determination of the incidence of LHRH receptors in a variety of tumors may be warranted.

Conclusions

The preclinical studies reviewed in this paper strongly support the concept of using targeted cytotoxic LHRH analogues such as AN-152 and AN-207 for the treatment of tumors expressing receptors for LHRH. AN-152 is an attractive choice for clinical development because of the well-characterized nature of its cytotoxic radical, DOX (72). Nevertheless, AN-207 is also a good candidate for clinical development based on various important considerations (73). The effective, nontoxic dose of AN-207 is about 1% of that of DOX, which may allow the treatment of relapsed patients who have already received a maximum cumulative dose of DOX (450 mg/m^2) which is determined by its cardiotoxicity. The much higher efficacy of AN-207 may also be advantageous in tumors expressing low levels of LHRH receptors. In addition, AN-207 may be more effective than AN-152 in the treatment of tumors that are resistant to DOX (48).

Collectively, the use of the targeted cytotoxic LHRH analogues AN-152 and AN-207 could significantly enhance the efficacy of chemotherapy and reduce its toxicity and thus improve the quality of life of patients with cancers that express receptors for LHRH.

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