Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7269–7273, July 1996
Medical Sciences

Cytotoxic analogs of luteinizing hormone-releasing hormone containing doxorubicin or 2-pyrrolinodoxorubicin, a derivative 500–1000 times more potent

(targeted chemotherapeutic agents/hybrid molecules/receptor binding/antitumor activity)

ATTILA NAGY*†, ANDREW V. SCHALLY*†‡, PATRICIA ARMATIS*, KAROLY SZEPESHAZI*†, GABOR HALMOS*†, MAGDOLNA KOVACS*†, MARTA ZARANDI*†, KATE GROOT*, MASAHIRO MIYAZAKI*†, ANDREAS JUNGWIRTH*†, AND JUDIT HORVATH*†

*Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, and †Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146

Contributed by Andrew V. Schally, March 19, 1996

ABSTRACT **Doxorubicin (DOX) and its daunosamine-modified derivative, 2-pyrrolino-DOX, which is 500–1000 times more active than DOX, were incorporated into agonistic and antagonistic analogs of luteinizing hormone-releasing hormone (LH-RH). The conjugation of DOX with LH-RH analogs was performed by using *N*-(9-fluorenylmethoxycarbonyl)-DOX-14-*O*-hemiglutarate, a dicarboxylic acid ester derivative of DOX. Coupling this derivative covalently to the ε-amino group of the D-Lys side chain of agonist [D-Lys^6]LH-RH or antagonistic analog Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2 [where Nal(2) = 3-(2-naphthyl)alanine, Pal(3) = 3-(3-pyridyl)alanine, and Phe(4Cl) = 4-chlorophenylalanine] was followed by the removal of the 9-fluorenylmethoxycarbonyl protective group to yield cytotoxic derivatives of LH-RH analogs containing DOX. From these DOX containing LH-RH hybrids, intensely potent analogs with daunosamine-modified derivatives of DOX can be readily formed. Thus, cytotoxic LH-RH agonist containing DOX (AN-152) can be converted in a 66% yield by a reaction with a 30-fold excess of 4-iodobutyraldehyde in *N,N*-dimethylformamide into a derivative having 2-pyrrolino-DOX (AN-207). Hybrid molecules AN-152 and AN-207 fully preserve the cytotoxic activity of their radicals, DOX or 2-pyrrolino-DOX, respectively, *in vitro*, and also retain the high binding affinity of the peptide hormone portion of the conjugates to rat pituitary receptors for LH-RH. These highly potent cytotoxic analogs of LH-RH were designed as targeted anti-cancer agents for the treatment of various tumors that possess receptors for the carrier peptide. Initial *in vivo* studies show that the hybrid molecules are much less toxic than the respective cytotoxic radicals incorporated and significantly more active in inhibiting tumor growth.**

The use of antineoplastic agents has been, for many decades, the basis for an effective treatment of various disseminated cancers. However, one of the limitations of chemotherapy is that the systemic administration of an effective dose of a wide variety of cytotoxic drugs is restricted by their nonselective toxic effect on cells other than cancerous ones. A more specific local delivery by targeting of highly active cytotoxic agents to tumor cells, based on Paul Ehrlich's idea of "magic bullets" (1), offers an approach that might overcome these drawbacks. Macromolecules such as monoclonal antibodies, proteins, or peptide hormones for which specific receptors are located on cancerous cells, have been widely used as carriers for conjugation to suitable cytotoxic radicals (2–9). The presence of receptors for luteinizing hormone-releasing hormone (LH-RH) on membranes of various tumor cells makes analogs of LH-RH, which can bind to these receptors, excellent choices as carrier molecules (3). Several cytotoxic LH-RH conjugates have been developed and tested by our group during the past decade (3, 5–8). These hybrid molecules consisted of agonistic, antagonistic, and shortened analogs of LH-RH linked to a wide variety of cytotoxic agents like cisplatin, melphalan, methotrexate, or doxorubicin (DOX) (3, 5–8).

DOX is still the most widely used anticancer drug, with the broadest spectrum of antitumor effects (10). DOX can also be linked to other macromolecular carriers, such as monoclonal antibodies or proteins, to form targeted analogs with reduced toxicity (2, 4, 9, 11–14). Several synthetic procedures have been developed for the formation of such conjugates without loss of activity of the cytotoxic radical. Some noteworthy approaches include the sodium periodate oxidation followed by reductive alkylation at the daunosamine sugar moiety (11), the formation of ester bonds and C-N linkages between 14-bromodaunorubicin and proteins or poly-L-amino acids (12, 13), and the use of acid-sensitive (lysosomotrop) (2, 14) or enzyme-sensitive spacer arms (9). Analogs of LH-RH with DOX, previously developed in our laboratories, were synthesized by coupling *N*-glutaryl-DOX to the hormone carriers (7, 8). Unfortunately, the neutralization of the daunosamine nitrogen of DOX in these analogs resulted in severe loss of cytotoxic activity (7, 8, 15).

In this paper we describe the use of *N*-(9-fluorenylmethoxycarbonyl) (*N*-Fmoc)-DOX-14-*O*-hemiglutarate for formation of cytotoxic LH-RH analogs containing DOX. Conversion of these LH-RH–DOX hybrids to conjugates incorporating 2-pyrrolino-DOX, a daunosamine-modified derivative of DOX, which is 500–1000 times more active than its parent compound (16), is also reported. The cytotoxic LH-RH analogs thus obtained were tested *in vitro* to determine their cytotoxic and hormonal activities and binding affinities to LH-RH receptors. Some very promising results of preliminary *in vivo* tests are also discussed.

MATERIALS AND METHODS

Materials. DOX HCl salt, glutaric anhydride, piperidine, and 1-hydroxybenzotriazole were purchased from Aldrich.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DOX, doxorubicin; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; *N*-Fmoc, *N*-(9-fluorenylmethoxycarbonyl); Fmoc-OSu, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide; Nal(2), 3-(2-naphthyl)alanine; Pal(3), 3-(3-pyridyl)alanine; Phe(4Cl), 4-chlorophenylalanine; TFA, trifluoroacetic acid.

‡To whom reprint requests should be addressed at: Veterans Affairs Medical Center, 1601 Perdido Street, New Orleans, LA 70146.

N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) was bought from Peninsula Laboratories. Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent was obtained from Peptides International. 4-Iodobutyraldehyde was prepared as described (16). LH-RH agonist carrier, [D-Lys^6]LH-RH, was synthesized in our laboratories as described (7). LH-RH antagonist carrier, Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2 [where Nal(2) = 3-(2-naphthyl)alanine, Pal(3) = 3-(3-pyridyl)alanine, and Phe(4Cl) = 4-chlorophenylalanine] (5, 6) was a gift from Asta Medica, Frankfurt, Germany.

Synthesis. *Preparation of* N-*Fmoc-DOX-14-*O-*hemiglutarate.* DOX HCl salt (50 mg, 86 μmol) was dissolved in 1 ml of *N,N*-dimethylformamide (DMF), and Fmoc-OSu (30 mg, 90 μmol) was added, followed by 30 μl (172 μmol) of *N,N*-diisopropylethylamine (DIPEA). After 3 hr, the solvent was evaporated *in vacuo*, and the residue was crystallized by trituration from 0.1% aqueous trifluoroacetic acid (TFA) (vol/vol). The crystals were collected by filtration and washed once with cold diethyl ether to remove traces of excess Fmoc-OSu. After drying in a desiccator, 62 mg of 98% pure *N*-Fmoc-DOX was obtained (yield, 94%). This intermediate was reacted overnight with glutaric anhydride (11.4 mg, 100 μmol) in 1 ml of anhydrous DMF in the presence of DIPEA (26.1 μl, 150 μmol). The solvent was evaporated *in vacuo*, and the residual oil was solidified by trituration from 0.1% aqueous TFA (vol/vol).The material thus obtained contained ≈75% *N*-Fmoc-DOX-14-*O*-hemiglutarate, 18% unreacted *N*-Fmoc-DOX, and 7% of other impurities as assessed by analytical HPLC. This crude product was used without a further purification for the formation of peptide-DOX conjugates. For purification by HPLC, 50 ml of 60% aqueous acetonitrile containing 0.1% TFA was used to dissolve the crude material. Purification resulted in 45.7 mg of 98% pure end product (yield, 64%).

General procedure for the preparation of LH-RH analogs containing DOX. [D-Lys^6]LH-RH TFA salt (60 mg, 37.5 μmol) and *N*-Fmoc-DOX-14-*O*-hemiglutarate (75% pure; 45 mg, 37.5 μmol) were dissolved in 1 ml of DMF, and BOP reagent (22 mg, 50 μmol), 1-hydroxybenzotriazole (13.5 mg, 100 μmol), and DIPEA (52 μl, 300 μmol) were added. After stirring for 1 hr, the solvents were evaporated *in vacuo*, and the residual oil was crystallized by trituration from 3 ml of ethyl acetate and then washed twice by 3 ml of ethyl acetate. The resulting crude solid (90 mg) was dissolved in 3 ml of DMF and 300 μl of piperidine was added. After 5 min, the reaction mixture was placed in an ice bath and acidified by the addition of a mixture containing 300 μl of TFA, 700 μl of pyridine, and 2 ml of DMF. The solvents were evaporated *in vacuo*, and the residual oil was solidified by trituration from ethyl acetate. The crude solid thus obtained was dissolved in 1 ml of 70% aqueous acetonitrile containing 0.1% TFA (vol/vol), diluted with 3 ml of 0.1% aqueous TFA, and applied on semipreparative HPLC. Isolation of the main product resulted in 40 mg of 98% pure [D-Lys^6(DOX-14-O-glutaryl)]LH-RH (AN-152) (yield, 48%). Cytotoxic LH-RH antagonist analog containing DOX (AN-241) was similarly prepared.

General procedure for the preparation of LH-RH analogs containing 2-pyrrolino-DOX. AN-152 (11.2 mg, 5 μmol) was dissolved in 200 μl of DMF, and 30 mg (150 μmol, 30-fold excess) of 4-iodobutyraldehyde (16) was added, followed by 3 μl (17 μmol) of DIPEA. After 30 min, 10 μl of glacial acetic acid was used to acidify the reaction mixture, which was then added dropwise into 3 ml of 0.1% TFA in water. This aqueous solution was extracted with 1 ml of hexane and applied on HPLC to obtain 7.6 mg of 99% pure [D-Lys^6(2-pyrrolino-DOX-14-O-glutaryl)]LH-RH (AN-207) (yield, 66%). Cytotoxic LH-RH antagonist analog containing 2-pyrrolino-DOX (AN-243) was similarly prepared.

Purification. The final purification of all the crude products was carried out on a Beckman model 342 semipreparative HPLC system, using an Aquapore Octyl (250 × 10 mm; pore size, 300 Å; and particle size, 15 μm) column. The solvent system consisted of two components—0.1% TFA in water and 0.1% TFA in 70% aqueous acetonitrile—and was used in linear gradient mode.

Analytical HPLC. A Beckman analytical HPLC system equipped with model 168 diode array detector and SYSTEM GOLD chromatography software (Beckman) was used to check the purity and to monitor the chemical reactions. The column was Dynamax C_{18} (250 × 4.6 mm; pore size, 300 Å; and particle size, 12 μm).

Analysis. Bruker ARX300 NMR spectrometer (300MHz ^{1}H frequency and 75MHz ^{13}C frequency) and electrospray mass spectrometer Finnigan-MAT TSQ 7000 were used for the structural identification of the peptide conjugates.

Cytotoxicity Assay. The MCF-7 human breast cancer cell line, used for the determination of the antiproliferative activity of the cytotoxic LH-RH derivatives, was obtained from the American Type Culture Collection. MXT hormone-independent mouse mammary carcinoma cell line was a gift from Günther Bernhardt (University of Regensburg, Regensburg, Germany). For the evaluation of the activity of the analogs in both cell lines, a colorimetric cytotoxicity assay in microtitration plates was used based on quantification of biomass by staining cells with crystal violet. The results of this assay correlate very well with the determination of cell number (17).

LH-Releasing and LH-RH-Inhibiting Activities. These activities were evaluated *in vitro* by using a superfused rat pituitary cell system (18, 19).

Receptor Binding. Binding affinities of the analogs to receptors for LH-RH on rat pituitary were determined in competitive binding experiments using ^{125}I-labeled [D-Trp^6]LH-RH as radioligand as described (20).

RESULTS

Chemistry. *N*-Fmoc-DOX-14-*O*-hemiglutarate was prepared and used for formation of cytotoxic LH-RH analogs containing DOX. *N*-Fmoc-DOX was obtained in a high yield (94%) by reacting DOX with Fmoc-OSu in DMF. For the preparation of *N*-Fmoc-DOX-14-*O*-hemiglutarate, we developed a procedure using glutaric anhydride in the presence of DIPEA in anhydrous DMF. Under these conditions, *N*-Fmoc-DOX could be converted to its 14-O-hemiglutarate in a good yield (≈75% as assessed by HPLC). Purification by HPLC led to the isolation of 98% pure end product in 64% yield. Because of a very bad solubility of *N*-Fmoc-DOX-14-*O*-hemiglutarate in organic solvents containing water, the unpurified crude material was used for the synthesis of larger quantities of peptide–DOX conjugates. The selection of the carrier analogs, with the highest binding affinities to LH-RH receptors, was based on our previous studies (5–7). Thus, [D-Lys^6]LH-RH (Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2) was selected as the agonistic LH-RH carrier and Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2 as the antagonistic LH-RH carrier. *N*-Fmoc-DOX-14-*O*-hemiglutarate was coupled covalently to the ε-amino group of the D-Lys residue of the respective peptide sequences. The Fmoc protecting group was removed by 10% piperidine in DMF (vol/vol), and the reaction mixture was quenched after 5 min with a mixture of TFA and pyridine in anhydrous DMF. Cytotoxic analogs of LH-RH-containing DOX were obtained in a satisfactory yield of ≈50% after purification by HPLC. These conjugates were converted to analogs containing 2-pyrrolino-DOX by an adaptation of a convenient reaction developed by us for the derivatization of DOX (16). Thus, cytotoxic LH-RH agonist analog containing DOX (AN-152) was reacted with a 30-fold excess of 4-iodobutyraldehyde in DMF in the

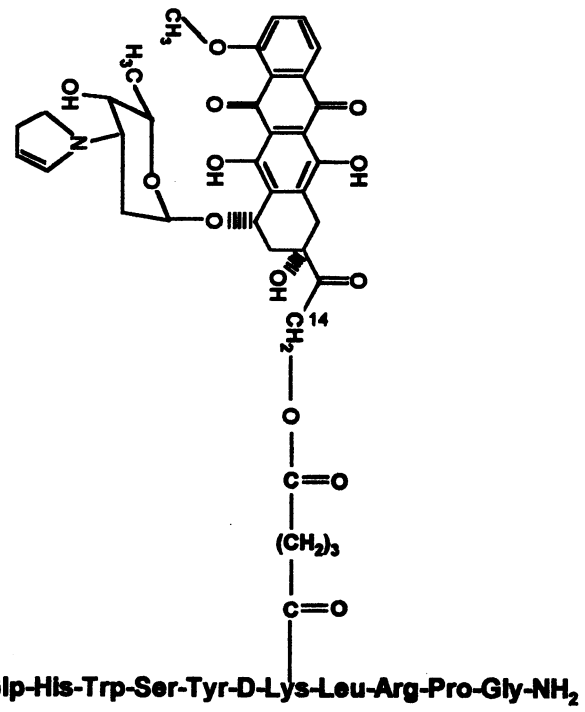

FIG. 1. Molecular structure of AN-207. [D-Lys^6]LH-RH is linked through the ε-amino group of its D-Lys moiety and a glutaric acid spacer to the 14-OH group of 2-pyrrolino-DOX (AN-201).

presence of a tertiary base to form an analog with 2-pyrrolino-DOX (AN-207) (Fig. 1). The reaction is completed in 15 min, and the yield is 66% after purification by HPLC. Cytotoxic LH-RH antagonist analog with DOX (AN-241) was converted similarly to a derivative containing 2-pyrrolino-DOX (AN-243).

Cytotoxicity. Tables 1 and 2 show that, in MCF-7 human breast cancer line and MXT mouse mammary carcinoma line, the antiproliferative activity *in vitro* of the cytotoxic radicals DOX and 2-pyrrolino-DOX (AN-201) is fully preserved after incorporation into LH-RH agonistic analog [D-Lys^6]LH-RH. Thus, the cytotoxic activities *in vitro* of AN-152 and AN-207 correspond respectively to those of DOX and AN-201. LH-RH antagonistic conjugate AN-241 containing DOX was only slightly less active than DOX itself, but hybrid AN-243 with AN-201 was ≈5 times less active than 2-pyrrolino-DOX in MCF-7 lines.

Receptor Binding Affinity: Agonistic and Antagonistic Properties. The results in Table 3 indicate that cytotoxic agonistic analogs of LH-RH, AN-152, and AN-207 fully preserve the high binding affinity of the carrier molecule [D-Lys^6]LH-RH to receptors for LH-RH on rat pituitary. In superfused rat pituitary cell system, both cytotoxic conjugates, AN-152 and AN-207, caused a greater stimulation of LH release than that induced by the carrier. The data presented in Table 4 demonstrate that cytotoxic antagonistic analog AN-241 with DOX maintained the high binding affinity of the carrier antagonist to receptors for LH-RH on rat pituitary membranes, but its 2-pyrrolino-DOX counterpart, AN-243, showed ≈10 times lower affinity for these receptors than the carrier. When the LH-RH-inhibiting activities of the carrier antagonist and its cytotoxic derivatives were compared in the superfused rat pituitary cell system, both AN-241 and AN-243 were found to have definite LH-RH inhibitory activities. AN-243 was a weaker inhibitor when the pituitary cells were exposed to it for only 9 min before the administration of LH-RH, but when the time of exposure was extended to 30 min, the LH-RH inhibitory activity of AN-243 was found to be similar to that of the carrier (Table 4).

DISCUSSION

Drug targeting is a modern approach that is being tried to overcome the problem of nonselective toxic effects of systemic chemotherapy (2–9). Drug targeting is based upon the selectivity of carrier molecules for specific binding sites in tumor tissues (2–9). Chemotherapeutic compounds and toxins can be covalently attached to various carriers including hormones for which receptors are present on cancer cells or to antibodies that preferentially recognize tumor cells (3). Specific binding sites for LH-RH are present in ≈50% of breast cancer specimens as well as ≈80% of ovarian cancers, 77% of endometrial carcinomas, and a very high percentage of prostate cancers (3, 21). LH-RH receptors were found even on pancreatic cancers (3). Thus, the specific binding of LH-RH to

Table 1. Inhibitory effects of DOX, its daunosamine modified derivative, 2-pyrrolino-DOX (AN-201), and their conjugates with agonistic LH-RH carrier* (AN-152 and AN-207, respectively) and antagonistic LH-RH carrier† (AN-241 and AN-243, respectively) on the growth of MCF-7 human mammary carcinoma cell line *in vitro*

Compound	Incubation time, hr	T/C value on MCF-7 cell line					
		3×10^{-10} M	10^{-9} M	3×10^{-9} M	10^{-8} M	3×10^{-8} M	10^{-7} M
Doxorubicin	70				98	82	54
	120				95	66	33
AN-152	70				111	89	63
	120				78	55	28
AN-241	70				104	95	72
	120				93	81	44
AN-201	70	50	−3	−18			
	120	26	2	−9			
AN-207	70	49	6	−32			
	120	30	−3	−11			
AN-243	70	103	109	96			
	120	92	27	−7			

Cells were incubated in Eagle's improved minimal essential medium (IMEM) containing 5% heat-inactivated dextran-coated charcoal-treated fetal bovine serum in 96-well plates. Relative cell number in treated and control plates was determined by crystal violet staining and was expressed as T/C values where T/C = T-C_0/C-C_0 × 100 [T = absorbance of treated cultures, C = absorbance of control cultures, and C_0 = absorbance of cultures at the start of incubation (t = 0). The measured absorbance is proportionate to the cell number.] Negative T/C values indicate a cell number smaller than the number originally seeded at t = 0—i.e., a cytocidal effect. At concentrations ranging from 3×10^{-10} M to 3×10^{-9} M, AN-152, AN-241, and DOX have no effect.

*Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2.

†Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2.

Table 2. Inhibitory effects of DOX, its daunosamine modified derivative, 2-pyrrolino-DOX (AN-201), and their conjugates with agonistic analog [D-Lys^6]LH-RH (AN-152 and AN-207, respectively) on the growth of MXT estrogen-independent mouse mammary carcinoma cell line *in vitro*

Compound	Incubation time, hr	T/C value							
		3×10^{-11} M	10^{-10} M	3×10^{-10} M	10^{-9} M	3×10^{-9} M	10^{-8} M	3×10^{-8} M	10^{-7} M
Doxorubicin	26						85	90	59
	50						74	60	43
AN-152	26						87	91	73
	50						71	59	50
AN-201	28	90	78	56					
	69	52	6	−13					
AN-207	28	91	78	64					
	69	59	15	−11					

Cells were incubated in RPMI 1640 medium containing 6 mM L-glutamine and 10% fetal bovine serum in 96-well plates. Relative cell number in treated and control plates was determined by crystal violet staining and was expressed as T/C values as defined in Table 1. At concentrations ranging from 3×10^{-11} M to 3×10^{-9} M, AN-152, and DOX are not effective.

certain tumor cells has been used in our laboratories for the design of targeted cytotoxic LH-RH analogs that contained various cytotoxic radicals such as melphalan, cisplatin, methotrexate, and DOX (5–8). Unfortunately, the early LH-RH analogs containing DOX did not retain the full activity of this cytotoxic agent as a result of neutralization of the daunosamine nitrogen by the glutaric acid spacer used for the conjugation. It is very important in the design and synthesis of targeted anticancer agents to preserve the biological activity of both the carrier and the cytotoxic radical and to protect the conjugate from enzymatic degradation until it reaches the target cells. DOX can be linked, by using various chemical reactions (2–4, 9, 11–14), without loss of its cytotoxic activity, to carrier molecules that would deliver it to cancer cells. Because 14-*O*-esters of DOX are stable and retain the cytotoxic activity of DOX (12–13, 22, 23), we used a dicarboxylic acid ester derivative, *N*-Fmoc-DOX-14-*O*-hemiglutarate, for the formation of peptide–DOX conjugates. Although a classical procedure for preparation of DOX-14-*O*-esters involves 14-bromo- or 14-iododaunorubicin, which can react with the sodium salt of a carboxylic acid (22, 23), we prepared *N*-Fmoc-DOX-14-*O*-hemiglutarate by reacting *N*-Fmoc-DOX with glutaric anhydride. Undesired esterification of the 4′-hydroxyl group of the daunosamine moiety by the anhydride is reduced to <4% as a result of steric hindrance caused by the bulky Fmoc group, which protects the adjacent 3′-amino function. The crude material thus obtained contained ≈75% *N*-Fmoc-DOX-14-*O*-hemiglutarate, 18% unreacted N-Fmoc-DOX, and 7% of other impurities as assessed by analytical HPLC. This crude product was used for coupling the cytotoxic radical to the D-Lys side chain of the peptide carriers. Because the main impurity, *N*-Fmoc-DOX, does not interfere with the coupling reaction, no undesired side reactions were encountered when using the unpurified material. Removal of the Fmoc protecting group led to cytotoxic analogs containing DOX. The daunosamine moiety of DOX in these conjugates remains intact, making possible the formation of conjugates with daunosamine-modified derivatives of DOX, such as 2-pyrrolino-DOX (Fig. 1.), which is 500-1000 times more active than its parent compound (16). To create such superactive targeted cytotoxic LH-RH analogs, the high yield reaction developed by us for the conversion of DOX to 2-pyrrolino-DOX (16) was adapted for the conversion of peptide–DOX conjugates. Thus, cytotoxic LH-RH agonist analog, AN-152 was reacted with a 30-fold excess of 4-iodobutyraldehyde to form AN-207, an analog containing 2-pyrrolino-DOX, in a 66% yield. In spite of being present in an excess, the haloaldehyde reagent reacts mostly with the vicinal amino alcohol function of the daunosamine moiety of the conjugate. The unprotected side chains of Ser, His, Arg, or Trp are left practically intact.

Table 3. Hormonal activities on LH release and receptor binding of cytotoxic agonistic analogs of LH-RH, AN-152 ([D-Lys^6]LH-RH carrying DOX), and AN-207 ([D-Lys^6]LH-RH carrying 2-pyrrolino-DOX) and the carrier peptide, [D-Lys^6]LH-RH

Compounds	Hormonal activity*	IC_{50}† values for rat pituitary receptors, nM
AN-152	15	2.29
AN-207	10	5.59
[D-Lys^6]LH-RH	8	2.26

*LH responses relative to LH-RH. LH responses to the analogs were determined in dispersed rat pituitary cell superfusion system as described (18).

†Binding affinities of the analogs to receptors for LH-RH on rat pituitary were determined in competitive binding experiments using ^{125}I-labeled [D-Trp^6]LH-RH as radioligand as described (20). The binding affinities were expressed by IC_{50} values, the concentration of unlabeled analog required to inhibit 50% of the specific binding of the radio ligand.

Our results (Tables 1–3) demonstrate that both the antiproliferative activity of the cytotoxic radicals and the high binding affinity of the carrier to pituitary LH-RH receptors are fully preserved in cytotoxic agonistic analogs of LH-RH, AN-152, and AN-207. The binding of cytotoxic analogs AN-152 and AN-207 to human cancer specimens will be reported elsewhere. In accordance with our previous findings (7), these analogs induce a greater LH release than the carrier because of the bulky apolar cytotoxic moiety linked to the side chain of D-Lys at position 6 of [D-Lys^6]LH-RH. Cytotoxic activity of antagonistic LH-RH analog AN-241 *in vitro* is only slightly smaller than that of DOX, whereas AN-243 is ≈5 times less active than 2-pyrrolino-DOX (Table 2). Similarly, the binding affinity of AN-243 to receptors for LH-RH on rat pituitary membrane fractions is lower than that of AN-241. AN-243 is also less active than AN-241, and their peptide carrier in inhibiting the LH-release induced by LH-RH on superfused rat pituitary cell system, when the cells are preincubated with the antagonists for 9 min before the addition of LH-RH. Interestingly, after a 30-min preincubation, AN-243 shows an LH-RH inhibitory activity comparable with that of the carrier, in spite of its lower binding affinity to receptors for LH-RH (Table 4).

Because AN-152 and AN-207 completely preserve the respective cytotoxic activities of DOX and 2-pyrrolino-DOX *in vitro* and also fully retain the high binding affinity of the carrier molecule to receptors for LH-RH on rat pituitary, these two analogs were selected for a further evaluation *in vivo* on various animal tumor models. Initial studies *in vivo* show that AN-152 and AN-207 are much less toxic and significantly more potent in inhibiting the growth of tumors, including prostatic, mammary, and ovarian cancers, which possess receptors for LH-RH, than the respective cytotoxic drugs, DOX and 2-pyrrolino-DOX. Inhibition of the growth of tumors that do not

Table 4. LH-RH-inhibiting activity and receptor binding affinity of Ac-D-Nal(2)-D-Phe(4Cl)-D-Pal(3)-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2 (ANT) carrier peptide and its conjugates with DOX (AN-241) and 2-pyrrolino-DOX (AN-243)

Peptide	% inhibition of LH response					IC_{50}*, nM
	0 min	30 min	60 min	90 min	120 min	
	After 9-min exposure to the antagonists					
ANT	100	96	53	47	47	1.43
AN-241	58	75	65	58	53	5.59
AN-243	14	46	36	31	33	12.66
	After 30-min exposure to the antagonists					
ANT	100	75	50	40	32	
AN-243	74	54	46	40	31	

Rat pituitary cells were exposed to the antagonists (10 nM) for 9 or 30 min, followed by an additional 3 min of administration of the antagonist together with LH-RH (5 nM) (0-min response). LH-RH (5 nM) was also administered for 3 min after 30, 60, 90, and 120 min. The amount of LH released into the system at different time points after the administration of the antagonists was compared with the LH release induced by administration of LH-RH (5nM) before incubation with the antagonists.
*IC_{50} values regarding receptors for LH-RH on rat pituitary were determined as defined in Table 3.

express receptors for LH-RH was also studied. In strong support of the theory that these cytotoxic LH-RH analogs act as targeted chemotherapeutic agents, we found that the cytotoxic peptide conjugates were not superior to the free cytotoxic radicals in tumors devoid of LH-RH receptors. These studies will be the subject of subsequent publications.

The presence of receptors for LH-RH on various human tumors, including mammary, ovarian, endometrial, and prostatic cancers (3), makes highly active targeted cytotoxic analogs of LH-RH, such as AN-152 and AN-207, potential candidates for practical clinical use for the treatment of these malignancies.

We thank Prof. J. Engel, Dr. M. Bernd, Dr. E. Busker, and Dr. A. Müller (Degussa and Asta Medica, Frankfurt am Main, Germany) for mass spectra and NMR analyses and for their help in preparation of this manuscript. We thank Katalin Halmos, Elena Glotser, Dora Rigo, Amy Giroir, and Harold Valerio for their experimental assistance. Some work described in this paper was supported by the Medical Research Service of the Veterans Affairs.

1. Ehrlich, P. (1956) in *The Collected Papers of Paul Ehrlich*, eds. Himmelweite, F., Marquardt, M. & Dale, H. (Pergamon, Elmsford, NY), Vol. 1, pp. 596–618.
2. Casazza, A. M., Trail, P. A. & Hellstrom, K. E. (1994) *Ann. Oncol.* **5,** 703–708.
3. Schally, A. V., Nagy, A., Szepeshazi, K., Pinski, J., Halmos, G., Armatis, P. Miyazaki, M., Comaru-Schally, A-M., Yano, T. & Emons, G. (1996) in *Treatment with GnRH Analogs: Controversies and Perspectives*, eds. Filicori, M. & Flamigni, C. (Parthenon, Carnforth, U.K.), pp. 33–44.
4. Varga, J. M., Asato, N., Lande, S. & Lerner, A. B. (1977) *Nature (London)* **267,** 56–58.
5. Bajusz, S., Janaky, T., Csernus, V. J., Bokser, L., Fekete, M., Srkalovic, G., Redding, T. W. & Schally, A. V. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6313–6317.
6. Bajusz, S., Janaky, T., Csernus, V. J., Bokser, L., Fekete, M., Srkalovic, G., Redding, T. W. & Schally, A. V. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6318–6322.
7. Janaky, T., Juhasz, A., Bajusz, S., Csernus, V., Srkalovic, G., Bokser, L., Milovanovic, S. R., Redding, T. W., Rekasi, Z., Nagy, A. & Schally, A. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 972–976.
8. Janaky, T., Juhasz, A., Rekasi, Z., Serfözö, P., Pinski, J., Bokser, L., Srkalovic, G., Milovanovic, S. R., Redding, T. W., Halmos, G., Nagy, A. & Schally, A. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10203–10207.
9. Trouet, A., Masquelier, M., Baurain, R. & Deprez-De Campeneere, D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 626–629.
10. Weiss, R. B., (1992) *Semin. Oncol.* **19,** 670–686.
11. Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R. & Sela, M (1975) *Cancer Res.* **35,** 1175–1181.
12. Zunino, F., Gambetta, R., Vigevani, A., Penco, S., Geroni, C. & Di Marco, A. (1981) *Tumori* **67,** 521–524.
13. Zunino, F., Savi, G., Giuliani, F., Gambetta, R., Supino, R., Tinelly, S. & Pezzoni, G. (1984) *Eur. J. Cancer Clin. Oncol.* **20,** 421–425.
14. Shen, W. C. & Ryser, H. J. P. (1981) *Biochem. Biophys. Res. Commun.* **102,** 1048–1054.
15. Zunino, F., Gambetta, R., Di Marco, A. & Zacarro, A. (1972) *Biochim. Biophys. Acta* **227,** 489–498.
16. Nagy, A., Armatis, P. & Schally, A. V. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2464–2469.
17. Reile, H., Birnböck, H., Bernhardt, G., Spruss, Th. & Schönenberger, H. (1990) *Anal. Biochem.* **187,** 262–267.
18. Vigh, S. & Schally, A. V. (1984) *Peptides* **5,** 241–247.
19. Csernus, V. J. & Schally, A. V. (1991) in *Neuroendocrine Research Methods*, ed. Greenstein, B. (Harwood, London), pp. 66–103.
20. Halmos, G., Schally, A. V., Pinski, J., Vadillo-Buenfil, M. & Groot, K. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2398–2402.
21. Emons, G. & Schally, A. V. (1994) *Hum. Reprod.* **9,** 1364–1379.
22. Horton, D. & Priebe, W. (1983) *J. Antibiot.* **36,** 1211–1215.
23. Israel, M., Modest, E. J. & Frei, E. (1975) *Cancer Res.* **35,** 1365–1368.