REVIEW

Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumors

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Abstract

In view of non-specific toxicity of most chemotherapeutic agents against normal cells, the development of targeted chemotherapy is warranted. Efficient targeting of chemotherapeutic drugs to the cancerous area could be of great benefit for patients with advanced or metastatic tumors. Targeted cytotoxic peptide conjugates are hybrid molecules composed of a peptide carrier which binds to receptors on tumors and a cytotoxic moiety. New cytotoxic analogs of LHRH, AN-152 in which doxorubicin (DOX) is linked to [D-Lys^6]LHRH, and AN-207 which consists of 2-pyrrolino-DOX (AN-201) coupled to the same carrier, show high-affinity binding and are much less toxic and more effective *in vivo* than their respective radicals in inhibiting tumor growth in LHRH receptor-positive models of human ovarian, mammary, or prostatic cancer. These results suggest that targeted cytotoxic LHRH analogs such as AN-207 could be considered for treatment of these cancers. The presence of receptors for bombesin-like peptides on a wide variety of tumors prompted us to use some of our bombesin/gastrin-releasing peptide antagonists as carrier molecules. Cytotoxic bombesin analogs, such as AN-215 containing AN-201, might find application in the treatment of small cell lung carcinoma (SCLC), and colorectal, gastric, pancreatic, mammary, and prostatic cancers. Since somatostatin receptors are found in various human neoplasms and the receptor subtypes to which octapeptide analogs bind with high affinity have been identified, we synthesized several cytotoxic somatostatin analogs including AN-162 and AN-238 containing DOX and 2-pyrrolino-DOX respectively, linked to octapeptide RC-121. Cytotoxic somatostatin analog AN-238 efficaciously inhibits growth of human breast or prostate cancers expressing somatostatin receptors-2 and -5 and can be used for receptor-targeted chemotherapy. Cytotoxic somatostatin analogs might also find applications for the therapy of human pancreatic, colorectal, and gastric cancer as well as brain tumors and non-SCLC. Cytotoxic compounds linked to analogs of hormonal peptides like LHRH, bombesin, and somatostatin that can be targeted to certain tumors possessing receptors for those peptides could be an important addition to oncological armamentarium.

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Introduction

For more than five decades, chemotherapy has been the main modality for systemic treatment of advanced or metastatic cancers (1, 2). Cytotoxic drugs are used as single agents or as combinations of several antitumor agents with different mechanisms of action (1, 2). Chemotherapy can also be applied as adjuvant to surgical resection and radiotherapy (1, 2). Cancer chemotherapy is limited by intrinsic or acquired multi-drug resistance of tumor cells and toxicity to normal cells (1–3). Although the effectiveness of chemotherapy can be increased by escalating the doses, the option for dose elevation is always restricted by the toxicity of chemotherapeutic drugs (1–3). A more selective delivery of the cytotoxic agents to the primary tumors and their metastases would allow a dose escalation and reduce the peripheral toxicity (3).

One hundred years ago, after detecting the specificity of antigen–antibody interactions, Paul Ehrlich created the concept of a 'magic bullet' for cancer therapy consisting of a molecule selective for a targeted cell that was linked to a highly toxic group (Fig. 1) (4). Thus, based on the assumption that tumors possess specific antigens, Ehrlich conceived the idea that antibodies could deliver toxic compounds to tumor cells and eradicate them (4). Ten years later, in 1908, Paul Ehrlich received the Nobel Prize in Medicine in recognition for his work on immunity and not for the concept of 'magic bullets'. The idea of 'magic bullets'

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> **"Bodies which possessed a particular affinity for a certain organ...as a carrier by which to bring therapeutically active groups to the organ in question."**
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> **P. Ehrlich (1898)**

Figure 1 From the writings of Paul Ehrlich (4) and *Collected Studies on Immunity*, vol. 2, pp 442–447. New York: Wiley Press, 1906.

made a major impact on medicine and chemistry, but remained essentially unexplored for many decades. With the advent of monoclonal antibodies (5) which recognize antigens associated with many types of human cancer, important advances in targeted therapy were made (6, 7). The 'magic bullet' approach was used for the construction of monoclonal antibody–toxin conjugates called immunotoxins and other chimeric toxins (7–9). These complexes consisted of cell-binding proteins, particularly monoclonal antibodies or growth factors like epidermal growth factor (EGF), conjugated to various toxins of bacterial or plant origin. These toxins included *Pseudomonas* exotoxin, diphtheria toxin, and ricin which act by irreversibly arresting the synthesis of protein in eukaryotic cells (8, 9). The highly specific antibodies deliver toxins into the cell, where they exert their cytotoxic effects. EGF, interleukins, and other growth factors or hormones coupled to toxins can also kill cells having respective receptors and induce remission of tumors (8–10). Initially, immunotoxins and chimeric toxins were made by chemically coupling antibodies or receptor-binding proteins to toxins but, more recently, recombinant DNA technology has been used (8, 9). Similarly, doxorubicin (DOX) and other chemotherapeutic agents can also be conjugated to monoclonal antibodies which recognize antigens expressed on human cancers or to growth factors (11). Thus, it is now well established that 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 (6–12). Such conjugates are designed to deliver cytotoxic agents more selectively to cancer cells. Ideally, tumor cells that bind these conjugates would be killed while normal cells that do not have the receptors would be spared (12).

Hormonal chemotherapeutic agents

The first hormonally targeted chemotherapeutic agents developed for the treatment of prostate cancer and breast cancer used estrogenic steroid molecules as carriers for various alkylating agents (13, 14). Nitrogen mustard compounds were chemically coupled to carrier estrogens for enhancing selectivity and cytotoxicity on estrogen receptor-positive cells (13, 14). The cytotoxic estrogens, such as estracyte (estramustine), accumulate in the rat prostate due to the presence in the ventral lobe of the prostate of a protein that binds estramustine with high affinity and high capacity (13, 14). However, estramustine has low affinity for the conventional estrogen receptors. Estracyte has been used clinically in patients with hormone refractory prostate cancer and in women with advanced carcinoma of the breast, but objective response rates are low. However, the use of other hormonal carriers that could increase the efficacy of chemotherapeutic agents appeared to be worthy of extensive exploration.

In other early studies, alkylating agents of the nitrogen mustard type, such as chlorambucil (Chl) and melphalan were incorporated into several peptide hormones, including bradykinin (15), luteinizing hormone (LH)-releasing hormone (LHRH) (16), and angiotensin II (17). Such analogs were expected to bind irreversibly to the receptors, producing a sustained blockade. However, incorporation of chlorambucil into fragments of bradykinin and related peptides did not produce an irreversible inhibitor of bradykinin receptors (15). Similarly, [Chl-D-Phe^1,D-Phe^2,D-Trp^{3,6}]LHRH showed much less antagonistic activity than the non-alkylating Ac-D-Phe^1 analog (16), and [D-Lys(Chl)^6]LHRH had agonistic and not antagonistic activity. One of the early conjugates containing DNA intercalator anthracyclins was reported by Varga (18). In this conjugate, daunomycin was linked to the N-terminal amino group of aspartic acid and ε-amino groups of Lys residues in β-melanocyte-stimulating hormone. The conjugate was able to kill melanoma cells *in vitro* (18).

Our interest in developing cytotoxic peptides arose more than 10 years ago (12). The 'magic bullet' approach was utilized by us for the synthesis of a large number of cytotoxic analogs of hypothalamic peptides (12, 19–22). These consisted of diverse hormonal analogs, mostly of LHRH, conjugated to a variety of chemotherapeutic agents. The high specificity of peptide receptors was utilized in an attempt to deliver these agents to the tumor cells where they might exert their cytotoxic effects (19–22). It was hoped that such hybrids could selectively destroy classes of cells containing specific receptors for several peptide hormones found on various cancer cells and serve as specific therapeutic agents for cancer treatment (12). The development of targeted hybrids directed against specific cell membrane receptors has been an extremely active area of our research. Earlier phases of this work were reviewed previously (12). Recent advances in the synthesis and evaluation of cytotoxic peptides in our laboratory may make it possible to put the concept of 'magic bullets' on firm foundations. These studies are described herein.

Cytotoxic LHRH analogs

Receptors for LHRH

Agonistic analogs of LHRH have been widely used in oncology and gynecology for nearly two decades (reviewed in references 23–26). Modern LHRH antagonists like Cetrorelix became clinically available more than 5 years ago, have been evaluated in various oncological and gynecological trials, and are awaiting regulatory approval (23, 25, 26). The mechanism of action of both agonistic and antagonistic analogs is mainly based on the inhibition of pituitary and gonadal functions (23, 24), and medical castration produced by chronic administration of LHRH analogs accounts for most benefits derived from the treatment (24). However, there are also many findings indicating that LHRH agonists and antagonists exert direct effects on tumor cells (23). The evidence for direct action of LHRH analogs on tumors is based on clinical results, the detection of high-affinity binding sites for LHRH in various cancers, and the inhibitory effects of analogs on tumor cell lines in cultures (for reviews see 23, 24, 27). Specific membrane receptors for LHRH have been found in various animal and human cancers (23, 24, 28). Thus, high-affinity binding sites for LHRH are present in most human prostate cancer samples (29–31). Receptors for LHRH were also detected in PC-82, LNCaP, and DU-145 human prostate cancer lines and Dunning rat prostate cancers (29–33). The expression of mRNA for LHRH receptors was also found in PC-82, DU-145 and LNCaP tumors and in most human prostate cancer specimens (31, 33, 34). Several investigators reported the presence of LHRH receptors in various human mammary carcinoma cell lines including MCF-7 and MDA-MB-231 (35–37). We detected high-affinity LHRH binding sites in more than 50% of human breast cancer samples (38). LHRH receptors were similarly found in about 80% of human ovarian epithelial cancer specimens and in EFO-21 and EFO-27, and OV-1063 human ovarian cancer lines (24, 27, 39–41). In nearly 80% of human endometrial carcinomas (42) and in HEC-1A and Ishikawa endometrial cancer lines, the presence of high-affinity membrane receptors for LHRH was also established (24, 27, 43). LHRH receptors on human cancers appear to be similar to pituitary LHRH receptors (44). The expression of LHRH receptor gene in human breast, endometrial, and ovarian tumors and respective cancer cell lines was also demonstrated by RT-PCR using the specific human LHRH receptor primers (27, 40, 44, 45). These findings provided support for the development of chemotherapy targeted to LHRH receptors on tumors and a rationale for the use of approaches based on cytotoxic LHRH analogs in malignancies in which specific receptors for LHRH are found. Thus, on the basis of the presence of specific receptors for LHRH on tumor cells, we started development of a new class of targeted antitumor agents by linking various cytotoxic radicals to LHRH analogs.

Design and synthesis of targeted cytotoxic analogs of LHRH

It was revealed many years ago that substitution of the Gly amino acid residue at position 6 of LHRH (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) by various D-amino acids results in very potent analogs of LHRH with high binding affinity (46). In our early attempts to create cytotoxic analogs of LHRH, D-melphalan (D-3-{p-[bis(2-chloroethyl)amino]phenyl}-alanine), an alkylating nitrogen mustard derivative of D-phenylalanine, was incorporated at position 6 of LHRH (19). This cytotoxic LHRH analog showed binding affinity to rat pituitary, human breast and prostate cancer and rat Dunning prostate tumor cell membranes similar to that of the superactive LHRH agonist [D-Trp[6]]LHRH (19). Antagonistic analogs containing Ac-D-Nal(2)[1], D-Phe(pCl)[2], D-Pal(3)[3] or D-Trp[3], Arg[5], D-Mel[6] and D-Ala[10] substitutions were also produced (19). Interestingly, the analog containing D-Trp[3] substitution displayed very low binding affinity to receptors for LHRH while its counterpart with D-Pal(3)[3] showed very high binding affinity (19). Among the numerous synthetic agonistic and antagonistic analogs of LHRH, those having a D-Lys moiety at position 6 offer an amino side-chain for convenient attachment of various cytotoxic compounds. It turned out that even bulky molecules could be linked to the ε-amino group of the D-Lys[6] moiety without significant loss of the high binding affinity of the peptide portion to receptors for LHRH. This bulk tolerance was exploited in our attempts to create cytotoxic LHRH hybrids in which diverse cytotoxic radicals were attached covalently to the D-Lys side-chain of the LHRH carrier agonists or antagonists (21, 47). The cytotoxic compounds included alkylating agent melphalan, DNA strand-breaker cross-linking agent cisplatin, antimetabolite methotrexate and anthracycline derivative 2-(hydroxymethyl)anthraquinone. Complexes of heavy metals such as Cu and Zn were also incorporated by sophisticated chemistry (20). In some of these early conjugates, DNA intercalating antibiotic doxorubicin (DOX), the most widely used anticancer agent, was linked to LHRH analogs using a glutaric acid spacer which formed carboxamide bonds between the daunosamine nitrogen of DOX and the ε-amino group of the D-Lys[6] moiety of the carrier (21). Unfortunately, the antiproliferative activity of DOX within these hybrids was greatly reduced due to the modification by the linkage. However, DOX can be linked by various chemical reactions to macromolecular carriers without a severe loss of its antitumor activity. Some noteworthy approaches include the sodium periodate oxidation followed by reductive alkylation at the daunosamine

sugar moiety (48), the use of spacer arms that are acid-sensitive (lysosomotrop) (49, 50) or enzyme-sensitive (51). The formation of ester bonds and C-N linkages between 14-bromodaunorubicin and proteins or poly-L-amino acids has also been reported to result in conjugates with preserved cytotoxic activity (52, 53). Since 14-*O*-esters of DOX are stable and known to have similar antitumor effects to DOX, we prepared *N*-Fmoc-DOX-14-*O*-hemiglutarate which was in turn coupled to the D-Lys side-chain of the LHRH analog carriers (54). The cytotoxic hybrids obtained after deprotection fully preserved the *in vitro* cytotoxicity of DOX and the binding affinity of the LHRH carriers.

Because the daunosamine moiety of DOX is intact in conjugates containing DOX-14-*O*-hemiglutarate, we developed several daunosamine-modified derivatives of DOX that could be linked to the carriers by the same chemistry. Our efforts led to the development of a new derivative of DOX, 2-pyrrolino-DOX (AN-201), which is 500–1000 times more active *in vitro* than its parent compound (55). To form this analog, DOX was reacted with a 30-fold excess of 4-iodobutyraldehyde in dimethylformamide. The conversion of DOX to 2-pyrrolino-DOX is virtually 100% and takes place within minutes. The same reaction could be utilized for the conversion of LHRH analogs containing DOX to the corresponding analogs with AN-201 (54). Thus, AN-152 in which DOX is linked to [D-Lys^6]LHRH was reacted with a 30-fold excess of 4-iodobutyraldehyde in dimethyl-formamide and converted in a high yield to superactive

Glp-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH₂

Figure 2 Molecular structure of cytotoxic LHRH analog AN-207. [D-Lys^6]LHRH 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) (54) Copyright (1996) National Academy of Sciences, USA. This figure is reprinted with permission from *Proceedings of the National Academy of Sciences of the USA.*

analog AN-207 containing 2-pyrrolino-DOX (Fig. 2). Both the antiproliferative activity of the cytotoxic radicals and the high binding affinity of the carrier to LHRH receptors are fully preserved in the cytotoxic LHRH analogs AN-152 and AN-207 (54). *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 (54). However, DOX and AN-152 were cytotoxic at 10^{-7} mol/l concentrations while AN-207 and AN-201 were effective at much lower concentrations of 3×10^{-10} mol/l (54). These new cytotoxic analogs of LHRH, AN-152 and AN-207 showed high-affinity binding to membranes of human breast cancer specimens and MCF-7 and MDA-MB-231 human breast cancer cell lines displaying IC$_{50}$ values in nanomolar concentration range (IC$_{50}$ = 2–13 nmol/l) (45).

Oncological tests *in vivo* on cytotoxic LHRH analogs

In an early *in vivo* study, rats bearing Dunning R-3327-H prostate adenocarcinomas were treated with prototypes of hybrid cytotoxic LHRH analogs containing anthraquinone or methotrexate (56). Large doses of these early cytotoxic analogs had moderate antitumor effects and caused a slightly greater tumor growth inhibition than the carrier peptide alone. Free anthraquinone or methotrexate given in equimolar doses were ineffective (56). Various cytotoxic LHRH analogs were also shown to inhibit the growth of estrogen-dependent and -independent MXT mouse mammary carcinoma *in vivo* (57). Because relatively large doses were used, it is likely that earlier cytotoxic LHRH analogs inhibited tumor growth by a combined hormonal and cytotoxic action (56, 57).

Ovarian cancer

In an initial *in vivo* study with the new cytotoxic analog AN-152, we demonstrated that the conjugate given intraperitoneally was more effective and less toxic than equimolar doses of DOX in the treatment of LHRH receptor-positive OV-1063 human ovarian cancers in nude mice (41). The growth of OV-1063 ovarian tumors in nude mice was inhibited significantly 4 weeks after treatment with AN-152 as based on reduction in tumor volume, even at the lowest dose tested (20.6 μmol/kg); the toxic effects of an equivalent dose of DOX caused substantial mortality (41) (Table 1). High-affinity receptors for LHRH and EGF were found on cell membranes of OV-1063 cancers; however, after *in vivo* treatment with AN-152, LHRH receptor-binding sites were not detectable and EGF receptors were reduced in number (41). AN-152 did not inhibit the growth of LHRH receptor-negative UCI-107 human ovarian carcinoma in nude mice. This indicates that
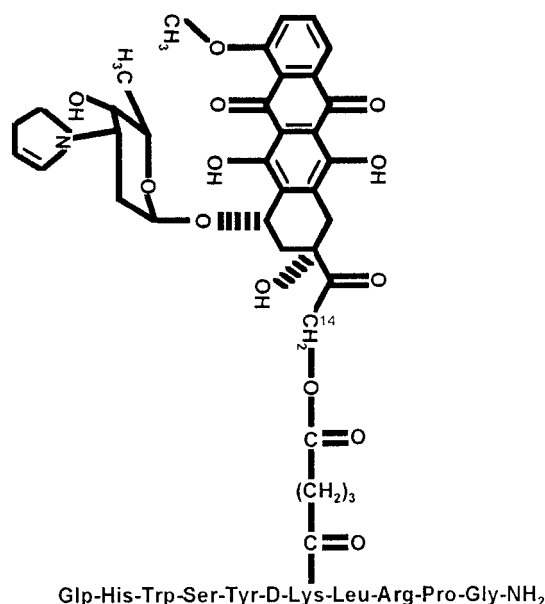
Table 1 Effects of cytotoxic LHRH conjugates AN-152 and AN-207 as well as their respective cytotoxic radicals DOX and 2-pyrrolino-DOX (AN-201) on various human and rodent LHRH receptor-positive tumors.

	Ovarian cancer OV-1063 (human)[1]	Breast cancers		Prostate cancers	
		MXT (mouse)	MX-1 (human)[1]	Dunning R-3327-H (rat)	PC-82 (human)[1]
AN-152					
Dose	20.6 μmol/kg once, i.p.	35.5 μmol/kg once, i.p.	Not tested	Not tested	Not tested
Effect	Powerful 84% growth inhibition	Powerful 98% growth inhibition			
Toxicity	No side-effects	1 of 5 animals died			
DOX					
Dose	20.6 μmol/kg once (12 mg/kg) i.p.	35.5 μmol/kg once, i.p.	Not tested	Not tested	Not tested
Effect	Not significant	Powerful 94% growth Inhibition			
Toxicity	6 of 9 animals died	5 of 5 animals died			
AN-207					
Dose	150 nmol/kg twice, i.p.	110 nmol/kg twice, i.p.	250 nmol/kg once, i.v.	50 nmol/kg three times, i.p.	200 nmol/kg once, i.v.
Effect	Significant growth inhibition	89% growth inhibition	10 of 10 animals cured	All tumours regressed	Significant growth inhibition
Toxicity	No side-effects	1 of 10 animals died	Transient fall in WBC[2]	Significant loss of body weight	1 of 8 animals died
AN-201					
Dose	150 nmol/kg twice, i.p.	110 nmol/kg twice, i.p.	250 nmol/kg once, i.v.	50 nmol/kg twice, i.p.	200 nmol/kg once, i.v.
Effect	Not significant	48% growth inhibition	1 of 10 animals cured	–	Not significant
Toxicity	9 of 10 animals died	8 of 10 animals died	1 of 10 animals died	All animals died	3 of 8 animals died

[1] Xenografted into nude mice; [2] white blood cell.
Based on data from References 41, 58, 59, and 61.

the presence of receptors is crucial for the improved antitumor effect of the conjugate.

In another study aimed at the development of better methods for treatment of human epithelial ovarian cancers, we investigated the effect of cytotoxic analog of LHRH (AN-207), cytotoxic radical 2-pyrrolino-DOX (AN-201), the carrier [D-Lys[6]]LHRH, and the unconjugated mixture of AN-201 and the carrier on the growth of the LHRH receptor-positive OV-1063 human epithelial ovarian cancers (58) (Table 1). We determined that the growth of OV-1063 tumor was inhibited significantly by intraperitoneal administration of 150–250 nmol/kg doses of AN-207, but not by carrier [D-Lys[6]]LHRH (58). Cytotoxic radical AN-201 and its mixture with carrier were toxic at these doses and killed all animals. Leukopenia, which is among the known side-effects of DOX and its derivatives, was also found to occur in animals treated with AN-207. Three days after administration of AN-207 at a dose of 5 nmol/20 g, white blood cell (WBC) counts of nude mice decreased by 36.3%, but after 7 days WBC values became normal. After treatment with AN-207, receptors for LHRH were not detectable, EGF receptor levels declined, and expression of mRNA for LHRH receptors and EGF receptors was decreased (58). We concluded that

targeted cytotoxic LHRH analog AN-207 is less toxic than equimolar doses of its radical 2-pyrrolino-DOX and that it can effectively inhibit ovarian tumor growth. These two studies (41, 58) indicated that targeted chemotherapy based on analogs such as AN-152 and AN-207 may improve the management of ovarian cancer. Preliminary results also indicate that cytotoxic analog AN-207 inhibits growth of HEC-1A human endometrial cancers xenografted into nude mice.

Breast cancer

In an initial study on breast cancer, we tested tumor inhibitory action of targeted cytotoxic LHRH analogs AN-152 and AN-207 at various dose regimens in female BDF mice bearing estrogen-independent MXT mouse mammary cancers (59) (Table 1). The effects were compared with those obtained with the cytotoxic radicals DOX or AN-201 alone. Analog AN-207 and analog AN-152 given intraperitoneally as a single injection or repeatedly 2 days apart at their maximum tolerated doses resulted in a 89–93% inhibition of tumor growth (59). Equimolar amounts of the cytotoxic radicals were toxic. AN-207 administered twice at a dose between 100 and 175 nmol/kg had the strongest

antitumor effect without significant toxicity. The optimal dose of AN-152, resulting in a 93% inhibition of tumors without apparent toxicity, was about 35 μmol/kg (59). The advantage of AN-207 is that a dose about 150–200 times smaller than that of AN-152 can achieve the same effect.

The regimen of treatment for these new compounds is based on only one or two injections in contrast to continuous or daily administration used for the earlier cytotoxic LHRH analogs (57). At the doses and schedules of administration used in the present study, AN-207 and AN-152 had no significant long-lasting hormonal activity and serum LH, estradiol levels, and sex organ weights were not changed at the end of the experiments. Specific, high-affinity LHRH receptors were present on MXT tumor samples of control untreated mice, but no binding sites for LHRH could be found on tumor membranes after treatment with the cytotoxic LHRH analogs.

In another investigation, nude mice bearing MX-1 hormone-independent DOX-resistant human breast cancers were injected intravenously with 250 nmol/kg doses of AN-201, AN-207 (Table 1) or the unconjugated mixture of AN-201 and [D-Lys^6]LHRH (60). Radioreceptor assays showed high-affinity binding sites for LHRH on MX-1 tumor cell membranes and the expression of mRNA for LHRH receptors was also found in tumors (60). Tumor growth and changes in hematological parameters were followed. AN-207 caused a complete regression of MX-1 tumors in all animals which remained tumor-free for at least 60 days after treatment (60). In contrast, therapy with AN-201 or the unconjugated mixture of AN-201 and [D-Lys^6]LHRH produced only transitory regression of MX-1 tumors. AN-201 caused animal deaths and significantly greater leukopenia than AN-207. These results suggest that targeted cytotoxic LHRH analogs such as AN-207 could be considered for treatment of advanced or metastatic breast cancers in women even for DOX-resistant tumors after the presence of receptors for LHRH is established (60).

Prostate cancer

Extensive investigations with new cytotoxic analogs of LHRH were also carried out in various models of prostate cancer. In the initial study, the effects of cytotoxic analog of LHRH, AN-207, were evaluated in rats bearing hormone-dependent Dunning R-3327-H prostate carcinomas (61) (Table 1). In the first experiment, we administered AN-207 intraperitoneally at a dose of 50 nmol/kg. AN-201 was also given at the same dose as a single drug and as an unconjugated mixture with [D-Lys^6]LHRH. Following the second administration of 50 nmol/kg AN-201 alone or mixed with the carrier, all rats died with signs of general toxicity, but all animals treated with the conjugate AN-207 survived. After 5 weeks of treatment with a total dose of

150 nmol/kg AN-207, the prostate tumors regressed to about one-half of their initial volume while tumors in the control group continued to grow (61). In another experiment, we compared the efficacy and toxicity of three intraperitoneal injections of 25 nmol/kg AN-201 or 25 nmol/kg and 50 nmol/kg AN-207. After 5 weeks of therapy, the tumors of rats treated with 50 nmol/kg AN-207 regressed. The reduction in tumor volume produced by 25 nmol/kg AN-201 and 25 nmol/kg AN-207 was similar, but AN-201 was still toxic in contrast to AN-207 (61). High-capacity LHRH receptors were found in the membranes of untreated Dunning tumors, but after treatment with AN-207 they could no longer be detected. This work showed that the cytotoxic analog AN-207 is much less toxic than its antineoplastic radical (AN-201) and significantly more active in inhibiting prostate tumor growth (61). This was the first demonstration that cytotoxic LHRH analog AN-207 can be an effective agent in treatment of prostate cancer (61).

In another study, we investigated the effect of cytotoxic analog AN-207 on the growth of LHRH receptor-positive PC-82 human prostate cancer xenografted into nude mice (31) (Table 1). Analog AN-207, radical AN-201, carrier [D-Lys^6]LHRH, or a mixture of [D-Lys^6]LHRH and AN-201 were injected intravenously once at doses of 200 nmol/kg. Tumor growth, body weight, total WBC counts and serum prostate-specific antigen (PSA) were determined (31). Eight weeks after administration of cytotoxic analog AN-207 there was a major reduction in tumor volume and tumor burden and a decrease in serum PSA levels as compared with controls. Only 12% of animals treated with AN-207 died. Cytotoxic radical AN-201 caused a minor and not significant reduction in tumor volume with no change in serum PSA and killed 40% of mice due to toxicity. Carrier [D-Lys^6]LHRH and unconjugated mixture of [D-Lys^6]LHRH and AN-201 caused no changes in tumor growth (31). Injection of AN-207 had no significant effect on the body weight but resulted in a fall in WBC and in platelet count after 1 week as compared with controls. This fall was smaller than that produced by AN-201 and the decrease in platelet count and WBC was no longer significant 2–3 weeks after treatment (31). A significantly stronger antiproliferative action and a much lower toxicity of AN-207, as compared with AN-201, in nude mice bearing xenografted PC-82 tumors could be attributed to a more selective delivery of analog AN-201 to PC-82 tumor cells (31). This view is supported by the presence of high-affinity binding sites for LHRH as well as the expression of mRNA for LHRH receptors in these tumors (31). Additional experimental studies and toxicological tests are required to further evaluate cytotoxic analogs of LHRH before they are used clinically, but targeted cytotoxic analogs of LHRH, such as AN-207, could eventually be used for the treatment of advanced prostate cancer after the relapse. Because of the presence of receptors for LHRH on a high percentage

of prostate cancers, targeted chemotherapy based on cytotoxic analogs of these peptides should be more efficacious and less toxic than the currently used systemic chemotherapeutic regimens and might permit an escalation in doses. In addition, cytotoxic analogs of LHRH might also be indicated for primary therapy of patients with advanced prostate cancer, thus extending the oncological uses of LHRH analogs from the current palliation toward an eventual cure (23).

Cytotoxic analogs of bombesin/ gastrin-releasing peptide (GRP)

The bombesin-like peptides comprise a large family of peptides found in amphibians and man (62). After the tetradecapeptide bombesin was isolated from frog skin, two mammalian bombesin-like peptides were characterized, GRP which is a 27-amino acid peptide, and neuromedin B which is related to amphibian ranatensin (62). The carboxyl-terminal decapeptide of GRP is similar to that of bombesin and possesses all the biological activity of bombesin. The link between bombesin/GRP and small cell lung carcinoma (SCLC) was discovered by Cuttitta *et al.* (63) who found that SCLC cells both secrete and respond to bombesin-like peptides. Bombesin-like peptides are produced also in other cancers, such as breast, prostatic, and pancreatic cancer (23). The findings that bombesin/GRP function as autocrine growth factors for SCLC (63), and likely for other tumors (23), stimulated several laboratories, including ours, to synthesize receptor antagonists for hormonal treatment of these malignancies.

Four receptor subtypes associated with the bombesin-like peptides have been described and cloned (62, 64, 65). These subtypes consist of the GRP-preferring subtype (62), the neuromedin B-preferring subtype (62), the bombesin receptor subtype 3 (BRS-3) present in lung cancer cell lines, the natural specific ligand of which is not yet known (64), and bombesin receptor subtype 4 (BRS-4 or BB4) that has a higher affinity for bombesin than for GRP (65). Specific receptors for bombesin/GRP have been demonstrated in human breast cancer and prostate cancer biopsies and in various human lung cancer, breast cancer, gastric and pancreatic cancer cell lines (23, 66, 67).

Bombesin/GRP antagonist such as RC-3095 (D-Tpi^6,-Leu$^{13}\Psi$(CH$_2$NH)-Leu^{14}-bombesin (6–14)) and related analogs block the binding of bombesin to the receptors on Swiss 3T3 cells and various human cancers (23, 68, 69). These nonapeptide antagonists inhibit the growth of MXT breast cancers in mice, nitrosamine-induced pancreatic cancers in hamsters, and various human cancer lines such as HT-29 colon cancer, PC-82, PC-3 and DU-145 prostate cancers, MKN-45 gastric carcinoma, CFPAC-1 and SW-1990 pancreatic cancer, H-69 SCLC, and MDA-MB-231 and MCF-7 MIII breast cancers xenografted into nude mice (23, 67, 68). More powerful antagonists, such as

Hca^6,Leu^{13},Ψ(CH$_2$N)Tac^{14}-BN(6–14) (RC-3940-II) show a higher binding affinity to the receptors on tumor cells and greater antitumor activity than RC-3095 (70). Ongoing clinical studies should determine the possible application of bombesin/GRP antagonists in treatment of various cancers (23, 68).

The presence of receptors for bombesin-like peptides on a wide variety of tumors prompted us to use some of our powerful bombesin/GRP antagonists as carrier molecules for targeting cytotoxic agents to tumor cells (71). We assumed that such cytotoxic bombesin analogs would be more potent than the straight bombesin/GRP antagonists and that they could produce a complete tumor regression and not merely a palliative stabilization.

The chemistry developed for the preparation of highly active cytotoxic LHRH hybrids containing DOX or AN-201 was used for the synthesis of cytotoxic bombesin-like analogs (71). To produce conjugates with high binding affinity to receptors for bombesin/GRP on various tumors, we selected as carriers our bombesin antagonists containing the amino acid sequence 6–14 of bombesin and a reduced peptide bond between residues 13 and 14 (69, 70). The cytotoxic radicals were linked to the amino terminal of these peptides (71). The resulting conjugates showed binding affinities to bombesin/GRP receptors on Swiss 3T3 cells comparable with that of their respective carriers (71). Conjugates consisting of the cytotoxic radical and bombesin 7–14 carriers such as AN-215 (Des-D-Tpi RC-3095 linked to 2-pyrrolino-DOX-14-O-hemiglutarate), that is octapeptide analogs 1 amino acid shorter than the RC class of antagonists, showed the highest binding affinity to receptors for bombesin/GRP ($K_D \approx 1$ nmol/l) (71). The cytotoxic bombesin analogs and their corresponding cytotoxic radicals exerted similar inhibitory effects on the *in vitro* growth of CFPAC-1 human pancreatic cancer, DMS-53 human lung cancer, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines that have receptors for bombesin/GRP. In DMS-53 cells, the activity of 2-pyrrolino-DOX and its conjugates was ≈ 2500 times higher than that of DOX and its hybrids (71).

Preliminary *in vivo* experiments on nitrosamine-induced pancreatic cancers in golden hamsters indicated that cytotoxic bombesin analog AN-215 had significant antitumor activity and lower toxicity than the unconjugated cytotoxic radical.

We then evaluated whether bombesin receptors could be used for targeting cytotoxic bombesin analogs to H-69 SCLC *in vivo* (72). Male nude mice bearing xenografted H-69 SCLC cell line received an intravenous injection of AN-215 or AN-201 (72). The growth of SCLC H-69 tumors was significantly inhibited by the treatment with 200 nmol/kg AN-215 as compared with the control groups, while equimolar doses of the cytotoxic radical AN-201 were toxic and produced only a minor tumor inhibition. mRNA for bombesin

receptors was detected in H-69 tumors and bombesin/ GRP binding sites were shown on H-69 tumor membranes (72). This supports the concept that cytotoxic bombesin analog AN-215 was preferentially targeted to H-69 SCLC tumors. Collectively, these results demonstrate that the cytotoxic bombesin analog AN-215 could be used for targeted therapy of tumors that express bombesin receptors such as SCLC (71). In addition to SCLC, cytotoxic bombesin analogs might also find application in the treatment of colorectal, gastric, pancreatic, mammary, prostatic, brain tumors, and other cancers (71).

Cytotoxic analogs of somatostatin

Tetradecapeptide somatostatin has many biological actions and appears to be an endogenous antiproliferative agent (23, 73, 74). The oncological potential of somatostatin has been appreciated for more than 20 years, but its half-life is very short, so that its therapeutic use is impractical (23). Several groups synthesized somatostatin analogs with more selective and prolonged activities (23, 74–77). Among these analogs, D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-OL (SMS-201–995, Sandostatin, octreotide) (75) and D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-NH$_2$ (RC-160, Vapreotide, Octastatin) (76) and BIM-23014 (Somatulin; D-Nal(2)-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH$_2$) are most active (77). RC-160 is much more potent than Sandostatin or BIM-23014 in oncological tests *in vitro* due to its higher affinity for somatostatin receptors (78). Most studies on potential clinical applications in oncology were carried out with these three analogs. Attempts are being made to use modern somatostatin analogs for the therapy of human breast cancer, prostate cancer, carcinoma of exocrine pancreas, colorectal cancer, gastric cancer as well as brain tumors and lung cancers (reviewed in references 23, 73, 74, 77).

Antitumoral action of somatostatin analogs could be direct or indirect (74, 77). The indirect mechanism would operate through a suppression of the growth hormone (GH) release from the pituitary and the resulting inhibition of the hepatic production of insulin-like growth factor-I (IGF-I) (74, 77). The fall in IGF-I could inhibit growth of various tumors since IGF-I and -II and other growth factors, including EGF, appear to be involved in the proliferation of neoplastic cells (74, 77). An antineoplastic action of a somatostatin analog is defined as 'direct' if it is a consequence of binding of the analog to somatostatin receptors present on tumor cells (77). High- or low-affinity somatostatin-14 receptors were identified in various normal tissues and human neoplasms, including brain tumors, pituitary tumors, gastrointestinal tumors, breast cancers, SCLC and non-SCLC cell lines, pancreatic cancers, colorectal cancer, human prostate cancers, and in human ovarian cancers (23, 77, 79–83). More recently, somatostatin binding sites have been classified with respect to receptor subtype. Five subtypes of somatostatin receptor, SSTR-1

to SSTR-5, have been cloned and functionally characterized (84–88). They all bind somatostatin-14 and somatostatin-28 with similar affinity but show major differences in their affinities for various somatostatin analogs (77). Sandostatin (Octreotide) and RC-160 have a low affinity for SSTR-1, but both possess a high binding affinity for receptor subtype SSTR-2 (89). RC-160 also exhibited moderate to high affinities for SSTR-3 and -5 and low affinity for SSTR-4 (90). Since mRNAs of receptor subtypes are variably expressed in different cancers, a precise determination of receptor subtypes in tumor tissue is necessary before therapy with analogs (89, 90). Thus, a recent report by Buscail *et al.* (91) indicates that most human pancreatic and colorectal cancers do not express SSTR-2, but SSTR-5 is still expressed.

Radioiodinated analogs of somatostatin such as [^{111}In-DTPA-D-Phe^1]-octreotide (Octreoscan) have been used clinically for the localization of tumors containing receptors for somatostatin (92). Various primary tumors, both neuroendocrine or non-neuroendocrine, containing high numbers of somatostatin receptors, and metastases can be visualized by scintigraphy (92). Work is also in progress on application of somatostatin analogs labeled with appropriate radionuclides such as ^{186}Rhenium or ^{90}Yttrium in cancer therapy (93). A better approach might consist of targeting chemotherapeutic agents linked to somatostatin analogs to receptors for somatostatin in certain cancers (23).

In an initial study, an early cytotoxic analog AN-51 consisting of methotrexate linked to the N-terminal of somatostatin octapeptide analog D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH$_2$ (RC-121) was tested in nude mice bearing transplanted MIA PaCa-2 human pancreatic cancers (22). The treatment with AN-51 inhibited tumor growth, whereas methotrexate or RC-121 administered singly had no significant effect (22). AN-51 also showed high specific binding affinity to receptors for somatostatin on rat cortex, Dunning R 3327 rat prostate cancers and MIA-PaCa-2 human pancreatic cancers (22). These findings indicated a bulk tolerance of the carrier for modification at the amino terminal. Thus, to create cytotoxic somatostatin hybrids containing DOX or AN-201, we used octapeptide analogs RC-160 and RC-121 developed in our institute (76). N-Fmoc-DOX-14-O-hemiglutarate was linked to the amino terminal of [Lys(Fmoc)^5]RC-121 to form, after removal of the Fmoc protection, the cytotoxic somatostatin analog AN-162 containing DOX (94). In contrast to cytotoxic analogs of LHRH or bombesin containing DOX, AN-162 could not be converted to 2-pyrrolino-DOX analog by a reaction with 4-iodobutyraldehyde because of side reactions (94). To prepare cytotoxic somatostatin analogs containing AN-201, DOX-14-O-hemiglutarate was synthesized and reacted with 4-iodobutyraldehyde to yield 2-pyrrolino-DOX-14-O-hemiglutarate. This moiety was in turn coupled to the amino terminal of [Lys(Fmoc)^5]RC-121. After removal of the Fmoc
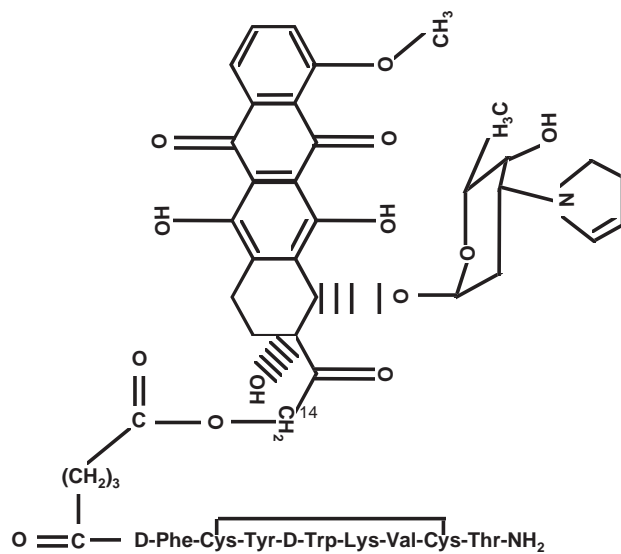
Figure 3 Molecular structure of cytotoxic somatostatin analog AN-238. Somatostatin analog RC-121 is linked through the α-amino group of its D-Phe moiety and a glutaric acid spacer to the 14-OH group of 2-pyrrolino-DOX (AN-201) (94). Copyright (1998) National Academy of Sciences, USA. This figure is reprinted with permission from *Proceedings of the National Academy of Sciences of the USA.*

protecting group, AN-238 (Fig. 3) was obtained in a moderate yield (94). AN-238 was shown to bind with high affinity to SSTRs on membrane preparations of rat pituitary and Dunning AT-1 rat prostatic tumors. *In vitro* tests on human cancer cell lines, including MKN-45 gastric cancer, MDA-MB-231 breast cancer, PC-3 prostate cancer, and MIA PaCa-2 pancreatic cancer, demonstrated that the antiproliferative activity of the cytotoxic radicals in these analogs was retained (94). In H-345 human SCLC cell line, conjugates of RC-121 similarly preserved the cytotoxic activity of their radicals. AN-238 also inhibited the GH-releasing hormone- or forskolin-induced GH release from superfused rat pituitary cells at similar nanomolar concentrations as the carrier RC-121 (94).

Oncological studies on AN-238 were carried out in animal models of breast and prostate cancers to determine its toxicity and potency in inhibiting tumor growth (Table 2). Thus, the effects of targeted cytotoxic somatostatin analog (AN-238) on tumor growth and hematologic parameters were studied in three human breast cancer models (95). The models included estrogen-independent MDA-MB-231 and MX-1 cancers and the estrogen-sensitive MCF-7-MIII tumors. High-affinity receptors for somatostatin and mRNA for both SSTR-2 and SSTR-5 were found in all three types of tumors (95) in accord with an earlier study by Evans *et al.* (96) of gene expression on SSTR subtypes in human breast cancers. Nude mice bearing xenografts of these cancers were injected intravenously with

250 nmol/kg doses of cytotoxic radical AN-201, cytotoxic somatostatin analog AN-238, or the unconjugated mixture of AN-201 and carrier RC-121. A significant inhibition of growth of MDA-MB-231, MX-1 and MCF-7-MIII tumors was observed 1 week after injection of a single dose of cytotoxic analog AN-238 (Table 2). The volumes and weights of MCF-7-MIII tumors and MDA-MB-231 tumors were still reduced 60 days after treatment with AN-238. AN-238 also caused a complete regression of MX-1 tumors in 50% of animals which remained tumor-free 60 days after treatment (95). In contrast, MDA-MB-231 and MCF-7-MIII tumors continued to grow after treatment with cytotoxic radical AN-201 and MX-1 tumors regressed only temporarily. The toxicity of AN-201 was much greater than that of AN-238 as measured by animal deaths, loss of body weight, and leukopenia. These results demonstrated that cytotoxic somatostatin analog AN-238 efficaciously inhibits growth of human breast cancers expressing SSTRs-2 and -5 and can be used for receptor-targeted chemotherapy (95).

We also evaluated the effects of AN-238 on the growth of androgen-independent Dunning R-3327-AT-1 prostate cancers in Copenhagen rats (97) (Table 2). Specific high-affinity receptors for somatostatin were found on Dunning R-3327-AT-1 tumor membranes by radio-ligand binding assay and were identified by RT-PCR as SSTR subtype 2 (97). Administration of cytotoxic radical AN-201 at single intravenous doses of 115–150 nmol/kg caused severe thrombocytopenia and loss of body weight and resulted in a 90–100% mortality. In contrast, a single intravenous injection of analog AN-238 at a dose of 300 nmol/kg was non-toxic and remarkably potent in inhibiting the growth of Dunning AT-1 tumors resulting in an 85.9% reduction in tumor volume 4 weeks after injection. Even a low dose of 115 nmol/kg AN-238 caused a 40% reduction in tumor volume (97). Treatment with AN-238 prolonged the survival time of tumor-bearing rats by 76.5%. No reduction in tumor growth was observed with carrier RC-121, but after the injection of unconjugated mixture of AN-201 and RC-121 at doses of 300 nmol/kg, all rats died in 4 days. Our study indicates that cytotoxic somatostatin analog AN-238 can be targeted to somatostatin receptors on tumors and produces a powerful inhibition of the growth of Dunning AT-1 prostate cancer at doses which are non-toxic, while its cytotoxic component, 2-pyrrolino-DOX, is toxic and ineffective (97). Our findings suggest that targeted cytotoxic somatostatin analogs could be more efficacious and less toxic than presently used systemic chemotherapeutic agents for the therapy of patients with advanced metastatic prostate cancer who no longer respond to androgen deprivation (97).

In a recent study, we evaluated whether AN-238 could be used for targeting human primary and metastatic prostate carcinomas that express SSTR-2 and SSTR-5 (98). The antitumor activity of AN-238 was

Table 2 Effects of cytotoxic somatostatin conjugate AN-238 and its cytotoxic radical 2-pyrrolino-DOX (AN-201) on various human and rat somatostatin receptor-positive tumors.

	Breast cancers			Prostate cancer	
	MDA-MB-238 (human)[1]	MCF-7-MIII (human)[1]	MX-1 (human)[1]	Dunning AT-1 (rat)[1]	PC-3 (human)[1]
AN-238					
Dose	250 nmol/kg once, i.v.	250 nmol/kg once, i.v.	250 nmol/kg once, i.v.	115–300 nmol/kg once, i.v.	150 nmol/kg twice, i.v.
Effect	Initial regression Significant growth inhibition	Initial regression Significant growth inhibition	5 of 10 animals cured	40% inhibition at 115, 80% at 300 nmol/kg	62–77% growth inhibition No metastases
Toxicity	Transient fall in WBC and body weight	Transient fall in WBC and body weight	Transient fall in WBC and body weight	Non-toxic	Transient fall in WBC and body weight
AN-201					
Dose	250 nmol/kg once, i.v.	250 nmol/kg once, i.v.	250 nmol/kg once, i.v.	110–125 nmol/kg once, i.v.	150 nmol/kg twice, i.v.
Effect	No effect	No effect	1 of 10 animals cured	Not significant at 110 nmol/kg	No effect, metastases in 4 of 6 mice
Toxicity	Severe fall in WBC and body weight	1 of 8 animals died	1 of 10 animals died	Lethal at 115–125 nmol/kg, non-toxic at 110 nmol/kg	Transient fall in WBC and body weight

[1]Xenografted into nude mice.
Based on data from References 95 and 97.

first investigated in nude mice bearing subcutaneous xenografts of PC-3 human androgen-independent prostatic cancer. AN-238 injected once i.v. at 200 nmol/kg induced a 74% decrease in tumor volume and a 71% reduction in tumor weight after 7 weeks. AN-201 at an equimolar dose did not show any antitumor activity (98). Two i.v. injections of AN-238 at 150 nmol/kg, 10 days apart, inhibited tumor volume by 62.3% and tumor weight by 61.1% after 4 weeks of treatment. The suppression of tumor growth induced by AN-238 was accompanied by a significant enhancement of apoptosis (98). The effectiveness of AN-238 in a metastatic model was then investigated in animals implanted orthotopically with PC-3 cells. Two i.v. injections of AN-238 at 150 nmol/kg, 10 days apart, reduced the weight of primary tumors by 77% ($P < 0.01$) after 4 weeks of treatment (98). All control animals developed metastases into lymph nodes; however, no lymphatic spread of cancer was found in the AN-238-treated group. RT-PCR analysis demonstrated an expression of SSTR-2 and SSTR-5 in intraprostatic tumors, their metastases into lymph nodes, as well as in subcutaneous tumors (98). This study demonstrated a high efficacy of SSTR-targeted chemotherapy in a model of disseminated human androgen-independent prostatic carcinoma (98). The use of cytotoxic somatostatin analog AN-238 could provide an effective therapy for patients with advanced prostatic carcinoma who relapsed androgen ablation.

Other studies in progress show that growth of various human pancreatic, colorectal and gastric cancers in nude mice as well as glioblastomas and non-SCLC can be suppressed by cytotoxic somatostatin analogs. Thus cytotoxic somatostatin analogs might find applications for the therapy of human breast cancer, prostate cancer, pancreatic, colorectal and gastric cancer as well as brain tumors and non-SCLC.

Discussion

It should be emphasized that cytotoxic peptide conjugates like AN-238 or AN-207 containing AN-201 are very active intravenously in 200 nmol/kg doses corresponding to 0.4–0.45 mg/kg or 8–9 μg/20 g mouse. The estimated human doses would be about 22.5 mg/50 kg.

The mechanism of action of these cytotoxic analogs is the subject of intense investigations in several laboratories. It is assumed that a peptide containing a cytotoxic radical may be bound to the membrane receptors and internalized. Thus, a cytotoxic LHRH analog T-98 containing an anthraquinone derivative coupled to [D-Lys⁶]LHRH is internalized by rat pituitary cells (99). A similar internalization could occur in cancer cells which contain receptors for LHRH. After endocytosis, such a compound could interfere with intracellular events in cancer cells. Analogs containing DOX may also kill cancer cells by membrane action on the cell surface without entering the cell (18, 100, 101).

The damage inflicted by cytotoxic analogs to pituitary cells secreting LH, follicle-stimulating hormone, and GH would not be deleterious to the cancer patient since hypophysectomy has been used for treatment of some cancers (23). These studies indicate that cytotoxic LHRH analogs such as AN-207 have a selective and transient effect on LH cells but not on GH and prolactin cells while cytotoxic radical AN-201 non-selectively damages the pituitary cells (102). A possible damage to other cells, e.g. corticotrophs, thyrotrophs, could also be alleviated by replacement therapy.

Conclusions

Cytotoxic compounds linked to analogs of hormonal peptides like LHRH, bombesin, and somatostatin can be targeted to certain tumors possessing receptors for those peptides and therefore are more selective for killing cancer cells. Cytotoxic peptide analogs are much less toxic than the respective chemotherapeutic agents and thus could be an important addition to oncological armamentarium. The results of experimental studies in ovarian, mammary, and prostate cancer models reviewed herein demonstrate the capability of cytotoxic analogs of LHRH and somatostatin to inhibit growth and even cause regression of various tumors. Our work supports the view that the use of targeted chemotherapy may permit an escalation of doses and improve the treatment outcome. It is also possible that repeated administration of cytotoxic analogs may totally eradicate some cancers. The approach based on cytotoxic analogs of LHRH, bombesin, and somatostatin requires additional experimental work and remains to be tested clinically. Nevertheless, our work indicates that the advances in cytotoxic peptide analogs are likely to result in major improvements in clinical management of various cancers.

Acknowledgements

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