HIGHLIGHT

Cytotoxic luteinizing hormone-releasing hormone conjugates and their use in gynecological cancer therapy

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Endometrial cancer is the most frequent malignant gynecological tumor in the western world. In most cases, it is diagnosed at an early stage when surgery alone or in combination with radiotherapy can achieve high cure rates (1). However, steroid receptor-negative tumors in elderly women or at advanced stages are rarely cured (2–4). Ovarian cancer is less frequent than endometrial cancer, but it is the most common cause of death from gynecological neoplasms (5). Effective regimens for surgery and first line cytotoxic chemotherapy are established, but in advanced or relapsed cases curative or palliative treatments with long term efficacy remain to be established (6–8). In both endometrial and ovarian cancer, new therapeutic strategies are required that are well tolerated and more efficacious.

Cytotoxic drugs are used as single agents or as combinations of several antitumor agents with different mechanisms of action. This chemotherapy is limited by its toxicity to normal cells. A more selective delivery of the cytotoxic agents to the tumor cells would allow the use of increased doses and would reduce the toxicity to normal cells.

In earlier studies it was shown that breast, ovarian, endometrial, pancreatic, and prostatic cancers express specific binding sites for luteinizing hormone-releasing hormone (LHRH) (9–16). Although these LHRH binding sites had a molecular mass comparable with that of pituitary LHRH receptor, their binding characteristics were of the low-affinity/high-capacity type (11, 15–17). Later, it became evident that in breast, ovarian, endometrial and prostate cancer cell lines, as well as in respective biopsy samples two types of LHRH binding sites exist, one of low affinity and high capacity, the other of high affinity and low capacity. The latter is comparable to the pituitary LHRH receptor (11, 15, 16). In 1992, the cloning, sequencing and expression of the human pituitary LHRH receptor was reported (18). In the same publication, the authors reported the expression of mRNA for the human LHRH receptor in the breast cancer cell line, MCF-7. These findings stimulated intensive research leading to the demonstration of LHRH receptor gene transcripts in ovarian and endometrial cancer cell lines and in about 80% of the respective primary tumors (16, 19–21). In ovarian and endometrial cancer specimens and cell lines expressing mRNA for the pituitary LHRH receptor, high-affinity/low-capacity binding sites were found closely related to the pituitary LHRH receptor (19–23). Kakar

et al. (24) demonstrated that the nucleotide sequence of LHRH receptors in human breast and ovarian tumors is identical to that found in pituitary. Data available today suggest that about 50% of breast cancers (25) and approximately 80% of ovarian and endometrial cancers express high-affinity binding sites for LHRH. For prostate cancer fewer findings have been published (15), but systematic investigations might lead to comparable results.

Since over 80% of human ovarian and endometrial cancers express receptors for LHRH, but most of human normal tissues do not express LHRH receptors (P Völker, C Gründker & G Emans, unpublished results), LHRH receptors on tumor cells might be used for targeted chemotherapy. This should improve antitumor effects and reduce side effects compared with systemic conventional cytotoxic chemotherapy (26). LHRH analogs covalently linked to cytotoxic radicals could bind specifically to LHRH receptors with their peptide moiety and act as chemotherapeutic agents after internalization of the ligand-receptor complex into cancer cells or by acting at the membrane level. In this fashion, these conjugates can selectively affect those cells that possess LHRH receptors and thus exert fewer side effects than unconjugated cytotoxic agents (27). A great variety of cytotoxic analogs containing different LHRH agonists and antagonists and different cytotoxic compounds including melphalan, cisplatin, methotrexate and cyclophosphamide derivatives have been synthesised in the past years (28–31). Most of these compounds preserved their LHRH analog action and were internalized into cells expressing receptors. Major problems, however, were caused by the instability of these compounds and the lack of preservation of their cytotoxic action (31–35). New cytotoxic analogs made of LHRH analogs having a D-Lys moiety in position 6, seem to solve these problems. This amino acid offers an amino side chain for convenient attachment of various cytotoxic compounds. It turns out that even bulky molecules could be linked to the ϵ -amino group of the D-Lys⁶ moiety, without significant loss of binding affinity of the peptide portion to the receptors for LHRH (35–36).

Doxorubicin is the most widely used cytotoxic agent with a broad spectrum of antitumor activity (37). The strong antiproliferative effect of doxorubicin is mainly due to its ability to induce apoptosis. In some early

conjugates, doxorubicin was linked to LHRH analogs using a glutaric acid spacer which formed carboxamide bonds between the daunosamine nitrogen of doxorubicin and the ϵ -amino group of the D-Lys⁶ moiety of the carrier (38). The antiproliferative activity of doxorubicin was greatly reduced due to modification by the linkage. Since 14-*O*-esters of doxorubicin are stable and known to have similar antitumor effects to doxorubicin, doxorubicin-14-*O*-hemiglutarate was prepared which was, in turn, coupled to the D-Lys⁶ side chain of the LHRH analog carriers (36). The cytotoxic hybrids obtained after deprotection fully preserved the cytotoxicity of doxorubicin and the binding affinity of the LHRH carriers. Recently, encouraging results have been made with the cytotoxic analogs AN-152 ([D-Lys⁶]-LHRH linked to doxorubicin) and AN-207 ([D-Lys⁶]-LHRH linked to 2-pyrrolinodoxorubicin, a derivative of doxorubicin which is 500–1000 times more potent than its parent compound) (36). Results from proliferation assays suggest that, in LHRH receptor-positive ovarian and endometrial cancer cell lines, the effects of AN-152 are mediated through LHRH receptors. In these experiments, the cytotoxic effect of AN-152 was blocked by an excess of LHRH antagonists in the fashion of competitive inhibition. In 2 LHRH receptor-negative cell lines tested, no indication of receptor-mediated action of AN-152 could be observed (39). Since most human normal tissues do not express LHRH receptors, a drug like AN-152 might provide the same cytotoxic activity as its cytotoxic moiety, doxorubicin, to the LHRH receptor-positive tumors while having significantly less effect on LHRH receptor-negative normal tissues. Observations using a confocal laser scanning microscope show a receptor-mediated action of AN-152 in LHRH receptor-positive cell lines, indicated by detection of AN-152 in the nucleus of these cell lines which could be nullified by an excess of [D-Trp⁶]-LHRH. In LHRH receptor-negative cell lines, AN-152 could not be detected in the nucleus.

In rat pituitary cells AN-207 selectively and reversibly damages gonadotropes (40). In nude mice with an ovarian carcinoma xenograft expressing LHRH receptors, treatment with AN-207 significantly inhibited tumor growth, while the cytotoxic residue alone (2-pyrrolino-doxorubicin) in equivalent doses was toxic to the animals and had no significant effect on tumor growth. Treatment with AN-207 downregulated receptors for LHRH and decreased epidermal growth factor receptor levels as well as expression of their mRNA (41). AN-152 had similar effects in LHRH receptor-positive OV-1063 ovarian cancer cell lines xenografted into nude mice. But no effect on tumor volume in LHRH receptor-negative UCI-107 cell lines was observed (42). AN-207 and AN-152 significantly inhibited tumor growth in LHRH receptor-positive MXT mouse mammary tumors while their respective cytotoxic residues alone were toxic to the animals without affecting tumor growth. In addition, a decrease

of mitotic action and increasing apoptosis has been shown after treatment with cytotoxic LHRH analogs (43, 44). On the basis of its powerful inhibitory effect on the aggressive MXT mouse mammary tumor, AN-207 could be considered as a treatment for advanced human breast cancers that express LHRH receptors.

A major problem of cytotoxic analogs is their stability *in vivo*. In long-time proliferation assays a growth inhibitory action of AN-152 could also be shown in receptor-negative cell lines. AN-152 might not be stable for the whole time that it is present in medium containing fetal calf serum (39). The antiproliferative effect of AN-152 in LHRH receptor-negative cell lines might be due to free doxorubicin, liberated from the peptide moiety by hydrolysis or enzymatic cleavage. In preliminary studies *in vitro* it was found that in serum of nude mice, rats, and humans the half-life ($t_{1/2}$) of AN-152 was 10, 30, and 120 min respectively (36, 45). Serum carboxylesterase enzymes partially hydrolyse these conjugates in the circulation, releasing the cytotoxic radical before the targeting is complete. The very high carboxylesterase activity in nude mice, which is about 10 times higher than in the human (46) can be inhibited by the carboxylesterase inhibitor diisopropyl fluorophosphate. The addition of diisopropyl fluorophosphate to mouse serum *in vitro* results in a prolonged $t_{1/2}$ of AN-152 to 70 min and a better targeting for the cytotoxic conjugates (47). Since the disintegration of AN-152 can be slowed down by the carboxylesterase inhibitor diisopropyl fluorophosphate and, in addition, the effects of free doxorubicin are well known and doxorubicin has already been used in tumor therapy for some time, AN-152 should be useable as a first line tumor cell specific chemotherapeutic drug. It is different in the case of AN-207. The daughter product, AN-201, is many times more toxic than doxorubicin. Newer examinations show that AN-207 disintegrates very fast without the action of carboxylesterase enzymes. AN-207, therefore, does not seem to be suitable for first line chemotherapy.

In conclusion these data suggest a selective receptor mediated action of the cytotoxic LHRH analogs that could lead to target cell specific cytotoxic chemotherapy with the possibility of dose intensification and reduction of toxicity. The results of experimental studies in breast, ovarian and endometrial cancer models demonstrate the capability of cytotoxic LHRH analogs to inhibit growth and even cause regression of these tumors.

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