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Effect of luteinizing hormone-releasing hormone analogs containing cytotoxic radicals on growth of estrogen-independent MXT mouse mammary carcinoma *in vivo*

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Cytotoxic luteinizing hormone-releasing hormone (LH-RH) analogs, AJ-004 (agonist [D-Lys^6]LH-RH linked to methotrexate (MTX)), T-98 ([D-Lys^6]LH-RH coupled to glutaryl-2-(hydroxymethyl)anthraquinone) (G-HMAQ) and T-121/B (antagonist containing two residues of G-HMAQ) were tested in female BDF$_1$ mice bearing MXT ((3.2)/Ovex) estrogen-independent mammary tumors. All three cytotoxic LH-RH analogs, administered from Alzet Osmotic Minipumps for 3 weeks, produced a significant inhibition of tumor growth. The effects of T-98 and T-121/B were superior to those obtained by treatment with equimolar doses of cytotoxic moiety anthraquinone or the LH-RH carrier alone. We assume that cytotoxic LH-RH analogs have a combined hormonal and cytotoxic activity with a reduced toxicity after administration *in vivo*. This is the first demonstration of *in vivo* tumor inhibition by targeted LH-RH analogs bearing cytotoxic radicals.

Key words: Anti-cancer drugs, cytotoxic compounds, hormonal carriers, LH-RH analogs, MXT mammary tumor.

Introduction

Luteinizing hormone-releasing hormone (LH-RH) agonists have been shown to inhibit the growth of estrogen-dependent mammary tumors in women with breast cancer[1–5] and in experimental models[6,7] by inducing a state of ovarian hormone ablation. LH-RH antagonists also inhibit the growth of estrogen-dependent mammary cancers in mice.[8] Much evidence suggests that in addition to the inhibitory effect on the pituitary–gonadal axis, some of these analogs may have direct action on tumor cells.[1,3,9–15] Clinical experience shows that some postmenopausal women may respond to therapy with LH-RH agonists.[1,3,9–15] The inhibitory effect of LH-RH agonists and antagonists was demonstrated *in vitro* on breast cancer cell lines.[9,10,12,13,15] Direct effects of these analogs could be mediated by specific receptors for LH-RH found on human and experimental breast cancer cells.[13–15,19] In human breast cancers, there is no correlation between estrogen receptors and LH-RH receptors and more than half of estrogen-receptor-negative specimens show binding sites for LH-RH.[14] In our previous studies,[20] we have demonstrated that agonist [D-Trp^6]LH-RH and LH-RH antagonist SB-75 are also able to inhibit the growth of estrogen independent MXT mouse mammary tumors.

On the basis of the presence of specific receptors for LH-RH on tumor cells and the premise of direct action of these analogs, we have developed new classes of antitumor drugs, linking cytotoxic radicals to LH-RH agonists or antagonists.[3,21] The chemistry of our early compounds and preliminary *in vitro* results were recently reported.[22,23] Extensive synthetic effort and many *in vitro* and *in vivo* investigations are required to develop a stable and practical cytotoxic analog based on an effective chemotherapeutic agent coupled to an active hormonal carrier. Such analogs could exert the effects of agonists or antagonists and, at the same time, act as chemotherapeutic agents. Their design was based on the assumption that a peptide containing a cytotoxic radical could be bound to tumor cell membrane receptors and after internalization would interfere with intracellular events in cancer cells.[3,21,23]

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This study describes the *in vivo* tests on three of our recently synthesized cytotoxic LH-RH analogs in mice bearing hormone-independent MXT mouse mammary tumors. Two compounds contained methotrexate (MTX) or 2-(hydroxymethyl)anthraquinone-hemiglutarate(G-HMAQ) linked to the LH-RH agonist, [D-Lys^6]LH-RH, and one analog had LH-RH antagonist T-147 as a carrier, which was coupled to G-HMAQ. All three analogs were previously found to bind to LH-RH receptors on breast cancer cells and to inhibit [^{3}H]thymidine incorporation into DNA in these cells.[24,25]

Materials and methods

Peptides and cytotoxic agents

The LH-RH agonist [D-Trp^6]LH-RH (pyro-Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2) was synthesized by solid phase methods and supplied by Debiopharm SA (Lausanne, Switzerland). Microcapsule formulation of this peptide in biodegradable poly(DL-lactide-co-glycolide) was prepared by Dr P Orsolini at Cytotech SA (Martigny, Switzerland) using a phase-separation process.[1,3,8] This sustained release formulation of lot G 476 in an aliquot of 21 mg maintained a continuous release of 21 μg/day of the analog for 4 weeks.

The agonistic analog [D-Lys^6]LH-RH (pyro-Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2) was synthesized in our laboratory by solid phase methods and purified by HPLC. The antagonistic LH-RH analog T-147, Ac-D-Nal(2)-D-Phe(4Cl)-D-Trp-Ser-Arg-D-Lys[A_2-pr(Ac)$_2$]-Leu-Arg-Pro-D-Ala-NH_2 was also designed and synthesized in our laboratory. G-HMAQ was prepared by reacting 2-(hydroxymethyl)anthraquinone with glutaric anhydride (both from Aldrich Chemical Co., Milwaukee, WI, USA). LH-RH agonist–cytotoxic conjugate AJ-004 was synthesized by coupling methotrexate (L(+)amethopterin, Sigma Chemical Co., St Louis, MO, USA) to [D-Lys^6]LH-RH. The coupling was made by linking the γ-carboxyl group of the glutamic acid residue in methotrexate to the free ε-amino group in [D-Lys^6]LH-RH. T-98 was prepared from [D-Lys^6]LH-RH and G-HMAQ by a single coupling step. Cytotoxic analog T-121/B was prepared by conjugation of two G-HMAQ molecules to the deacetylated form of T-147 by a single coupling step. The cytotoxic LH-RH conjugates were purified by HPLC and shown to be homogeneous. MTX LPF (Lederle Parenterals Inc., Carolina, Puerto Rico) 25 mg/ml injection solution was diluted for treatment.

Animals

A total of 140 female B6D2F$_1$ mice weighing 19.7 ± 1.3 g were maintained as previously described.[7,8,20] All experiments were performed according to institutional ethical guidelines.

Tumor

The source of the estrogen-independent MXT (3.2)/Ovex mammary carcinoma, the method of tumor inoculation and calculation of tumor volumes were previously described.[7,8,20] Tumor doubling time was calculated according to Smolev *et al.*[26]

Experimental protocol

Two days after implantation of tumors the mice were randomly divided into groups and the therapy was initiated. Three experiments were performed.

Experiment I
(1) Control tumorous animals, injection vehicle only.
(2) Methotrexate, 6 μg/day/animal.
(3) [D-Lys^6] LH-RH, 21 μg/day/animal.
(4) AJ-004, 25 μg/day/animal.
(5) [D-Trp^6]LH-RH, 21 μg/day/animal.
(6) Surgical bilateral ovariectomy.

Experiment II
(1) Control tumorous animals, injection vehicle only.
(2) G-HMAQ, 6 μg/day/animal.
(3) [D-Lys^6]LH-RH, 27 μg/day/animal.
(4) T-98, 31 μg/day/animal.
(5) Surgical bilateral ovariectomy.

Experiment III
(1) Control tumorous animals, injection vehicle only.
(2) G-HMAQ, 6 μg/day/animal.
(3) T-147, 25 μg/day/animal.
(4) T-121/B, 33 μg/day/animal.
(5) Surgical bilateral ovariectomy.

The doses used in groups (2), (3) and (4) in experiments I and II represent equimolar amounts of the compounds. In experiment III, the doses of the carrier T-147 and the cytotoxic analog T-121/B, selected on the basis of the required antagonistic activity, were equimolar, but the dose of free

G-HMAQ had to be reduced to 0.6 molar ratio because of the toxicity of this compound.

The groups consisted of 10 animals except for group (5) in experiments II and III, which had only five mice. For sustained delivery, microcapsule formulation of [D-Trp^6]LH-RH was used in group (5) in experiment I, and osmotic pumps Model 2002 releasing 0.48 μl/h (Alza Co., Palo Alto, CA, USA) implanted s.c. were employed in the other groups in all three experiments. Continuous release of drugs from this model is guaranteed for 14 days, but by calculating the filling volume, a sufficient supply for 16 days can be expected. The filling of the pumps and the implantation were carried out according to manufacturer's recommendations. The peptides were dissolved in 50% propylene glycol in water. The mice were anesthetized with Metofane (Pitman Moore, Washington Crossing, NJ, USA) and the pump implanted in the lateral-dorsal area using aseptic techniques. Control mice were implanted with pumps filled only with the solvent. [D-Trp^6]LH-RH microcapsules were suspended in 0.7 ml of injection vehicle solution containing 2% (w/v) carboxymethylcellulose and 1% (v/v) Tween 80 in water.[7,8] The suspension was mixed thoroughly using a Vortex mixer and injected subcutaneously through an 18-guage needle. Tumor volumes were measured 11, 14, 17 and 19 days after transplantation and the experiments were terminated on day 19. Histological examination and determination of serum LH and GH levels were carried out as previously described.[7,8,20] Mitotic and apoptotic cells in 10 high-power fields were counted and their number per square millimeter of tumor slide area was calculated. For demonstration of the nucleolar organizer region (NOR) in tumor cell nuclei, the AgNOR method of Chiu *et al.*[27] was used with small modifications. The silver stained black dots in 50 cells of each tumor were counted and the AgNOR number per cell was calculated.

Receptor Assays

Membrane preparation, radioiodination of peptides and receptor binding of [D-Trp^6]LH-RH were performed as previously described.[7,8,14]

Statistical methods

A computer program of Duncan's multiple range test and Student's *t*-test were used for statistical evaluation of data.

Results

Tumor growth and pathology

Tumor volume changes are shown in Figures 1–3. Three of 10 animals treated with G-HMAQ died in both experiments II and III. Tumor weights measured at the end of the experiments and doubling time of tumors are shown in Table 1. A significant inhibition of tumor growth was found in all treated groups in experiment I. MTX and cytotoxic analog AJ-004 caused similar tumor growth inhibition. The carrier [D-Lys^6]LH-RH also decreased tumor weight in experiment II, but the reduction was not statistically significant. While

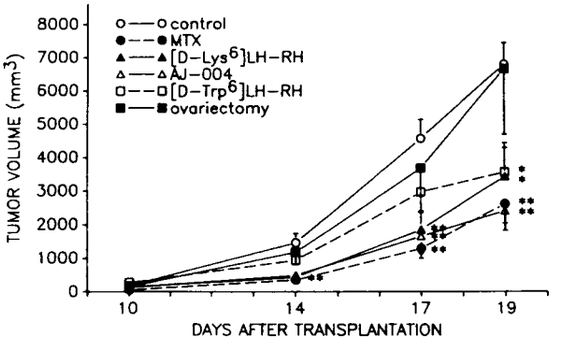

Figure 1. Volume changes of estrogen independent MXT mouse mammary tumors during treatment with [D-Trp^6]LH-RH, MTX, carrier [D-Lys^6]LH-RH or cytotoxic LH-RH analog AJ-004, and after ovariectomy. Data are means of groups in experiment I. $^*p < 0.05$, $^{**}p < 0.01$. The vertical lines show SE.

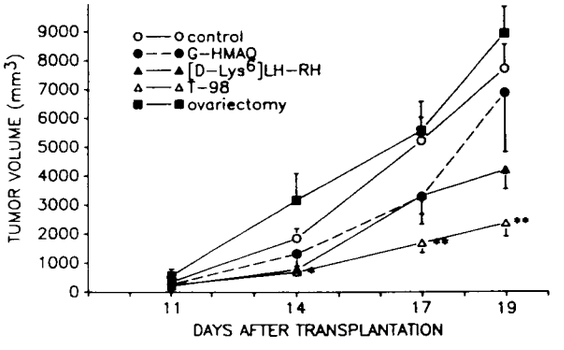

Figure 2. Volume changes of MXT (3.2)/Ovex estrogen independent mouse mammary tumors during treatment with G-HMAQ, [D-Lys^6]LH-RH or cytotoxic analog T-98, and after surgical ovariectomy. Data are means of groups in experiment II. The vertical lines show SE. $^*p < 0.05$, $^{**}p < 0.01$. On day 19, T-98 group differed significantly ($p < 0.05$) from G-HMAQ or [D-Lys^6]LH-RH groups.

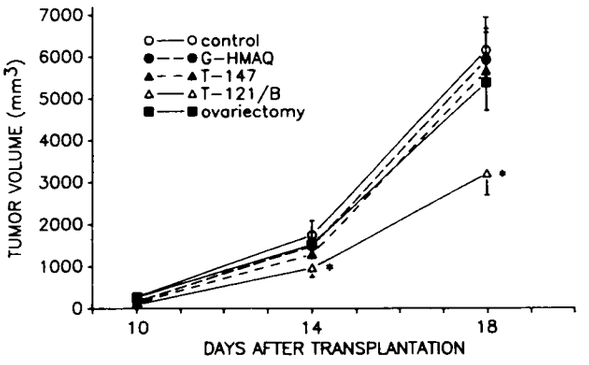

Figure 3. Volume changes of MXT (3.2)/Ovex estrogen independent mouse mammary tumors during treatment with G-HMAQ, carrier T-147 or cytotoxic analog T-121/B, and after ovariectomy. Data are means of groups in experiment III. The vertical lines show SE. *$p < 0.05$. On day 18, T-121/B group differed significantly ($p < 0.05$) from G-HMAQ or T-147 groups.

G-HMAQ alone had no effect on tumors, treatment with T-98 resulted in a highly significant inhibition. In experiment III, treatment with the cytotoxic LH-RH antagonist T-121/B significantly inhibited tumor growth, while the carrier T-147 alone caused only small suppression and G-HMAQ had no effect. Surgical ovariectomy caused no changes in volume or weights of tumors in all three experiments.

The histological characteristics of tumors were described previously.[20] The MXT (3.2)/Ovex tumor is a very fast-growing, highly anaplastic tumor, the basic histologic structure of which was similar in all groups. The number of mitotic cells was not significantly changed by the treatments. Apoptosis was found frequently in the tumors. Scattered cells showing shrinkage of cytoplasm with dark, shrunken, often fragmented nuclei, occurred even among cells of the control tumors. Ovariectomy, treatment with MTX or G-HMAQ and with antagonist T-147 or cytotoxic antagonist T-121/B caused no changes in frequency of apoptotic alterations. Treatment with cytotoxic compounds AJ-004, T-98 or carrier [D-Lys^6]LH-RH and [D-Trp^6]LH-RH resulted in an enhancement of programmed cell death in tumors. Surgical ovariectomy did not change the number of AgNORs in tumor cells. A decrease in AgNOR count was found in all other treated groups in experiment I, with the smallest change after treatment with MTX. In experiments II and III, only therapy with T-98 and T-121/B, respectively, caused a significant reduction in AgNORs in tumor cell nuclei. The quantitative histologic data are shown in Table 1.

Table 1. Effect of treatment with MTX, G-HMAQ, [D-Lys^6]LH-RH, [D-Trp^6]LH-RH, T-147, AJ-004, T-98 and T-121/B on survival of animals, tumor weights, tumor doubling time and some histological parameters of estrogen independent MXT mouse mammary tumors

	No. of animals that died during treatment	Tumor weights (g)	Tumor doubling time (days)	No. of apoptotic cells per 1 mm^2	No. of AgNORS per nucleus
Experiment I					
(1) control	0/10	7.76 ± 0.6	1.50 ± 0.02	90.7 ± 10.1	7.2 ± 0.2
(2) MTX	1/10	2.96 ± 0.7**	1.77 ± 0.09**	108.3 ± 8.4	6.6 ± 0.2*
(3) [D-Lys^6]LH-RH	0/10	4.24 ± 1.0*	1.74 ± 0.08**	171.0 ± 14.0**	5.9 ± 0.2**
(4) AJ-004	0/10	3.43 ± 0.6**	1.76 ± 0.05**	212.7 ± 27.2**^{b}	6.3 ± 0.2**
(5) [D-Trp^6]LH-RH	0/10	4.45 ± 0.8*	1.69 ± 0.06**	179.1 ± 20.5**	6.1 ± 0.2**
(6) ovariectomy	1/5	7.65 ± 2.0	1.53 ± 0.04	128.5 ± 5.3	6.8 ± 0.3
Experiment II					
(1) control	1/10	8.17 ± 0.7	1.48 ± 0.03	158.6 ± 20.3	7.3 ± 0.2
(2) G-HMAQ	3/10	7.15 ± 2.1	1.56 ± 0.10	184.7 ± 15.8	7.0 ± 0.2
(3) [D-Lys^6]LH-RH	0/10	4.74 ± 0.7	1.62 ± 0.04	229.2 ± 14.8	6.8 ± 0.2
(4) T-98	0/10	3.37 ± 0.5**	1.76 ± 0.06**^{ac}	295.3 ± 32.3**^{ac}	6.3 ± 0.1**^{bc}
(5) ovariectomy	2/5	8.84 ± 0.8	1.41 ± 0.04	216.7 ± 47.3	7.4 ± 0.5
Experment III					
(1) control	2/10	6.75 ± 0.5	1.42 ± 0.02	136.9 ± 26.6	7.3 ± 0.1
(2) G-HMAQ	3/10	6.16 ± 0.8	1.45 ± 0.04	129.5 ± 25.1	7.0 ± 0.2
(3) T-147	1/10	5.27 ± 0.6	1.48 ± 0.04	147.7 ± 23.5	7.1 ± 0.2
(4) T-121/B	0/10	4.20 ± 0.6*^{a}	1.58 ± 0.04**^{ac}	148.8 ± 17.3	6.5 ± 0.1**^{bd}
(5) ovarictomy	1/5	7.06 ± 1.0	1.45 ± 0.07	148.7 ± 24.3	7.3 ± 0.2

Values are means ± SE. * $p < 0.05$, ** $p < 0.01$ compared with control groups. ^{a} $p < 0.05$; ^{b} $p < 0.01$ compared with the G-HMAQ or MTX groups, respectively; ^{c} $p < 0.05$; ^{d} $p < 0.01$ compared with carrier groups.

Serum LH and GH level, ovarian and uterine weights

Serum LH levels were significantly changed only after ovariectomy in experiments II and III. Serum GH levels did not change significantly after the various treatments. Ovarian weights were reduced to various extents in all groups treated with LH-RH analogs. Uterine weights showed no evaluable changes.

Receptor assays

In all control groups, [D-Trp^6]LH-RH was bound to one class of saturable, specific non-cooperative binding sites with low affinity ($K_d = 10.23 \pm 4.46 \times 10^{-9}$ M, average value of three experiments) and high capacity ($B_{max} = 2.7 \pm 0.9$ pM/mg of membrane protein, average of three experiments). Chronic treatment with two LH-RH analogs containing cytotoxic radicals (AJ-004 and T-121/B) produced significant down-regulation of membrane receptors for [D-Trp^6]LH-RH on MXT mammary tumor cell membranes. In contrast, T-98 did not produce a decrease in the number of these receptors. These results will be described in detail elsewhere.[25,28]

Discussion

Even in patients with estrogen-dependent tumors, estrogen ablation as therapy for breast cancer has disadvantages due to its inability to kill hormone-sensitive cells which survive in a resting state upon hormone deprivation.[4] These cells continue proliferating in a later stage with loss of their hormonal dependency. In view of this concept, steroid ablation may not be the best form of a long-term therapy for breast cancer and may actually accelerate the production of unresponsive cells.[29] However, it was recently detected that ovariectomy[30] or therapy with LH-RH analogs[7,8,20] not only inhibit cell proliferation but also enhance programmed cell death (apoptosis) in mammary tumors.

Because of the inadequacy of anti-estrogen therapy, the possibility has been raised of combining hormonal treatment with cytotoxic drugs in therapy of mammary cancer. The results of treatment using various combinations are variable, depending on age group or receptor status of patients investigated.[31–33].

A new class of drugs developed for the therapy of hormone-dependent prostate cancers was based on cytotoxic estrogens like estracyte (estramustine).[34–36] Nitrogen mustard compounds were chemically coupled to carrier estrogens for enhancing selectivity and cytotoxicity on estrogen receptor positive cells.[34–36] In order to take advantage of the presence of LH-RH receptors on various tumors and possible direct effects of LH-RH analogs on cancer cells, we designed, synthesized and developed a novel class of antitumor peptides based on LH-RH agonists and antagonists linked to various cytotoxic radicals.[3,21] Our early compounds contained nitrogen mustard or metal complexes.[22,23] One of the compounds used in the present study contained MTX as the cytotoxic radical,[24,25] because of its efficacy as an anticancer agent in clinical use, its chemical stability and suitability for coupling to amino acids.[37] MTX inhibits dihydrofolic acid reductase and interferes with DNA synthesis, repair and cellular replication.[37] Anthraquinone derivatives including the anthracycline antibiotic Adriamycin and Novantrone (mitoxantrone dihydro-chloride), an anthracenedione with some structural similarities to Adriamycin, are also widely used as bioreductive intercalating antineoplastic agents.[38] These compounds bind to DNA and inhibit RNA and DNA synthesis. Although derivatives of 2-(hydroxymethyl)anthraquinone show less cytotoxic activity than Adriamycin, Novantrone or mitomycin C,[39] they are stable and suitable for coupling to peptides. In order to attach this anthraquinone derivative to a free amino group in the peptide, glutaric acid was used as a spacer in G-HMAQ.[24,25] All three analogs were previously found to bind to LH-RH receptors on breast cancer cells and to inhibit [^{3}H]thymidine incorporation into DNA in these cells.[24,25] *In vitro* studies on the mechanism of action of these new cytotoxic peptides are in progress in our laboratory.

The cytotoxic LH-RH analogs AJ-004, T-98 and T-121/B caused a significant inhibition of tumor growth in our study. MTX alone also had an antitumor effect in experiment I which can be explained by the well known fact that this cytotoxic agent has a strong inhibitory effect on highly proliferative, fast growing tumors. In contrast, G-HMAQ alone had no significant effect on tumor growth and was toxic. Treatment with the carrier agonist [D-Lys^6]LH-RH resulted in a slight inhibition of tumor growth, but the cytotoxic analogs based on this agonist linked to cytotoxic radical MTX or G-HMAQ were more effective than the carrier in both experiments. The carrier antagonist T-147 had little effect on tumor

growth, but T-121/B containing two G-HMAQ radicals caused a significant inhibition. The endocrine effects of the LH-RH analogs were well-documented by the decrease in ovarian weights after treatment with [D-Lys^6]LH-RH or [D-Trp^6]LH-RH but antagonist T-147 had a smaller endocrine effect probably due to the small dose used. However, endocrine changes caused by treatment with cytotoxic LH-RH analogs have to be investigated in detail in future experiments.

The exact mechanism of action of our new compounds still remains to be elucidated. *In vitro* studies on the affinity of the AJ-004 conjugate for dihydrofolate reductase were not carried out in this study, but it is known that compounds combined to the gamma carboxyl group of MTX do not change its activity.[37] LH-RH analogs carrying cytotoxic radicals retain their endocrine activity, as shown by their biological activity *in vitro*, as well as the decreased ovarian weights in the animals treated with AJ-004 or T-98. The significant enhancement of apoptosis in tumors after treatment with these compounds suggest that the tumor inhibitory effect of these cytotoxic analogs may be manifested, at least partly, by LH-RH analog-like action. Programmed cell death (apoptosis) is an active, energy-dependent process which can be activated by endocrine manipulations in hormone-dependent normal tissues or tumors.[7,8,29] However, results of treatment with T-121/B imply more than a simple endocrine action in inhibition of tumor growth, as ovarian weights and apoptotic alterations remained unchanged in the group treated with this cytotoxic LH-RH antagonist. The number of interphase AgNORs shows a close relationship with proliferation rate of cells, and so can reflect the degree of malignancy or prognosis of tumors.[40] Both cytotoxic analogs T-98 and T-121/B were significantly more active in reducing AgNORs in tumor cells than the cytotoxic radicals or carrier analogs alone, while AJ-004 and MTX had similar effect on cell proliferation as shown by AgNOR data. Coupling G-HMAQ to LH-RH analogs decreased the toxicity of the cytotoxic agent, since no animals died in groups treated with T-98 or T-121/B while three mice died in the two groups treated with G-HMAQ alone. Since the antitumor action may selectively be exerted to a greater degree on the neoplasm having cell membrane receptors for LH-RH, the peripheral toxicity of these analogs would be greatly reduced, compared with free chemotherapeutic agents. This aspect certainly needs further toxicologic investigations.

Conclusions

Three LH-RH analogs linked to cytotoxic radicals were able to inhibit growth of estrogen-independent MXT mouse mammary tumor. The carrier molecules retained their hormonal activity, however the antitumor effect of two cytotoxic analogs was superior to that obtained by treatment with the cytotoxic agent or the carrier alone. The targeted use of cytotoxic compounds might reduce the peripheral toxicity of the drugs. In addition to breast and prostate cancers, such compounds could be considered for treatment of ovarian, endometrial and pancreatic cancer. Further development should lead to more powerful analogs. The availability of compounds based on cytotoxic radicals linked to hormonal analogs of LH-RH that can be targeted to certain cancers possessing receptors for those peptides, and therefore more selective for killing cancer cells, could be of practical therapeutic importance.

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