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Receptors for luteinizing hormone releasing hormone (LHRH) expressed in human non-Hodgkin's lymphomas can be targeted for therapy with the cytotoxic LHRH analogue AN-207

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Abstract

We determined by immunohistochemistry the presence of LHRH receptors in surgical specimens of human non-Hodgkin's lymphomas (NHL) and investigated the expression of LHRH receptors in two human NHL cell lines, RL and HT by RT-PCR, Western blot and radioligand binding studies. In *in vivo* experiments with nude mice bearing tumours of these NHL cell lines, the efficacy of cytotoxic LHRH analogue AN-207 and its cytotoxic radical AN-201 was examined. LHRH receptors were detected in 94.1% of the human NHL specimens and in both NHL cell lines. AN-207 significantly ($P < 0.01$) inhibited the growth of RL and HT tumours, while the non-targeted AN-201 had no effects. Blockade of the LHRH receptors with an excess of LHRH agonist Decapeptyl suppressed the antitumour effects of AN-207. Our findings indicate that LHRH receptors expressed in a high percentage of human NHL specimens can be used for effective targeted therapy with the cytotoxic LHRH analogue AN-207.

Keywords: Targeted chemotherapy; Non-Hodgkin's lymphoma; LHRH receptor; Cytotoxic LHRH analogue AN-207

1. Introduction

Non-Hodgkin's lymphoma (NHL) is the most frequently diagnosed haematological malignancy and the sixth most common cancer in the United States with an estimated incidence of about 56,000 new cases and 21,000 deaths in 2005 [1]. Depending on the subtype of the tumour, the treatment of choice can include surgical intervention, conventional chemotherapy, radiotherapy or a combination of these three therapeutic modalities. However, patients with advanced disease face a dismal prognosis with a 5-year survival rate of only 26% [2], emphasizing the need for novel therapeutic approaches for NHL.

The elucidation of molecular characteristics of cancer cells prompted the development of new classes of drugs known as targeted therapeutics. These include inactivators of genes or gene products involved in oncogenesis, antibodies against tumour surface structures and conjugates consisting of tumour specific ligands linked to toxins, radionuclides or chemotherapeutic agents [3–9]. Thus, the monoclonal anti-CD20 antibody rituximab and the radiolabeled anti-CD20 antibodies Zevalin and Bexxar were approved by the US Food and Drug Administration (FDA) for therapy of NHL and showed promising results in clinical trials [10,11].

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Targeted chemotherapy can be based on peptide hormone carriers which deliver cytotoxic agents directly to neoplastic cells expressing the corresponding receptors [7,8]. Because LHRH receptors are present in breast, ovarian, endometrial and prostate cancers [12,13], we synthesized a targeted cytotoxic LHRH analogue, AN-207, which consists of the LHRH agonist [D-Lys^6] linked to a highly potent derivative of doxorubicin (DOX), 2-pyrrolino-DOX (AN-201) [14]. AN-207 binds with high affinity to LHRH receptors [15] and effectively inhibits the growth of various experimental LHRH receptor positive tumours *in vivo* [7,8]. In non-malignant tissues, LHRH receptors seem to be limited to organs regulated by the pituitary–gonadal axis [16]. However, binding sites for LHRH were detected in human tumour specimens and cancer cell lines originating from liver, larynx, pancreas, colon, kidney, skin and brain [17–23].

Thus, in the current study we examined the expression of LHRH receptors in human NHL specimens as well as in two human NHL cell lines and evaluated the antitumour activity of cytotoxic LHRH analogue AN-207 *in vivo* in nude mice bearing xenografts of these cell lines.

2. Materials and methods

2.1. Immunohistochemistry

Tissues of 17 human NHL specimens of B-cell origin were prepared for immunohistochemical staining as described earlier [24]. Slides were incubated with an antibody for LHRH receptors (clone A9E4, Novocastra, UK) for 4 h (1:10 dilution in antibody diluent (DAKO)). The reaction was stopped with 100 μl PBS buffer per slide. After washing in 1400 μl PBS buffer for 7 min, the slides were incubated with 120 μl EnVision HRP antimouse antibody (DAKO) for 30 min. After washing as above, the staining reaction was performed with 120 μl/slide DAB solution (DAKO; 1:50 dilution in substrate buffer) for 10 min. The reaction was stopped with 100 μl PBS buffer for 20 min, followed by washing with 1400 μl PBS buffer for 7 min, the slides were then washed 3 times every 2 h with PBS buffer. Finally, the slides were rinsed in water, counterstained with Harris' hematoxylin, covered with a glass slide and examined by light microscopy.

2.2. Peptides and cytotoxic agents

Cytotoxic LHRH analogue AN-207 was synthesized in our laboratory by coupling one molecule of 2-pyrrolino-DOX-14-*O*-hemiglutarate to the ε-amino-group of carrier peptide [D-Lys^6] LHRH [14]. Cytotoxic radical AN-201 was prepared as described [14]. For intravenous (i.v.) injection, the compounds were dissolved in 20 μl of 0.01N aqueous acetic acid and diluted with 5% (w/v) aqueous D-annitol solution (Sigma, St. Louis, MO).

2.3. Cell lines and cell culture

RL and HT human NHL cell lines were obtained from American Type Culture Collection (ATCC). RL is a mature, Ebstein–Barr–Virus (EBV) negative, CD20+, B-cell line derived from a diffuse large-cell lymphoma and carries a chromosomal marker t(14;18) (q32;q21). HT is an EBV-negative, CD20+, B-cell line, derived from a diffuse mixed small- and large-cell lymphoma. RL and HT cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Sigma Chemical Co.), 50 μg/ml penicillin G, 50 μg/ml gentamicin and 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY, USA).

2.4. Animals

Five- to six-week-old female athymic nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (NCI, Bethesda, MD, USA). The animals were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12-h light/12-h dark schedule and were fed autoclaved chow and water *ad libitum*.

2.5. Experimental protocol

Tumours resulting after 3–5 weeks subcutaneous (s.c.) injection of NHL cancer cells in donor animals were aseptically dissected and mechanically minced. Pieces of tumour tissue, about 3 mm^3, were transplanted s.c. into the experimental animals by a trocar needle. Tumour volume (length × width × height × 0.5236) and body weight were measured weekly. At the end of each experiment, mice were sacrificed under anesthesia and necropsy was performed. Tumours were excised, weighed, snap frozen and stored at −70 °C.

All experiments were in accordance with the institutional guidelines for the welfare of experimental animals and the NIH Guide for Care and Use of Laboratory animals.

2.5.1. Experiment 1

Animals bearing RL tumours were divided into three groups with an average tumour size of 110–120 mm^3. Mice received the following treatment as single i.v. injections into the jugular vein on day 1: Group 1, control, vehicle solution (5% mannitol) (11 mice); group 2, cytotoxic LHRH analogue AN-207 at a dose of 200 nmol/kg (12 mice); and group 3, cytotoxic radical AN-201 at a dose of 200 nmol/kg (11 mice). The experiment was terminated on day 22.

2.5.2. Experiment 2

Mice with HT tumours were assigned to six groups when tumours had reached a size of approximately 80 mm^3. The animals received the following treatment on day 1 and day 15: Group 1, control, vehicle solution (11 mice); group 2, AN-207 at 200 nmol/kg (11 mice); group 3, AN-201 at 200 nmol/kg (11 mice); group 4, the carrier [D-Lys^6] LHRH at 200 nmol/kg (5 mice); group 5, unconjugated mixture of the cytotoxic radical AN-201 and the carrier [D-Lys^6] LHRH at 200 nmol/kg (5 mice); and group 6, 200 μg of the LHRH analogue Triptorelin (Decapeptyl) i.v. 15 min prior to the i.v. injection of cytotoxic analogue AN-207 at 200 nmol/kg (5 mice). The experiment was terminated on day 29.

2.6. Toxicity

Toxicity was evaluated on the bases of number of deaths, total leukocyte count (WBC) and bodyweight. WBC was determined with the Unopette microcollection kit (Becton–Dickinson, Franklin Lakes, NJ) before, 7 days after each drug administration and at the end of the experiments. Body weights were measured weekly.

2.7. RNA extraction and reverse transcription (RT)-PCR

RNA was isolated from approximately 100 mg of tumour tissue according to the protocol for TRI-Reagent® (Sigma–Aldrich), following the manufacturer's instructions. The RT reaction was performed with the iScript™ cDNA synthesis kit from Bio-Rad (Hercules, CA) according to the manufacturer's instructions. Five hundred nanogram RNA was transcribed into cDNA in a final volume of 10 μl. All PCR were carried out in an Applied Biosystems PCR system 2700 (Applied Biosystems, Norwalk, Connecticut). For the amplification of the cDNA, gene-specific primers for the LHRH-receptor were used as described [25]. About 10 μl of each PCR product was loaded on a 1.8% agarose gel and subjected to electrophoresis. The gel was stained with ethidium bromide and analyzed using Kodak 1D 3.6 imaging analysis software (Kodak, Rochester, NY). A total RNA negative control, which received only water in the RT reaction, was used in each PCR to rule out genomic DNA contamination. DU-145 human prostate cancer cell line was used as a positive control for mRNA of LHRH receptors in the PCR.

2.8. Western immunoblot analysis

For immunodetection of LHRH receptors, an extraction of membrane protein from RL and HT tumour samples was performed as previously reported [25]. The presence of LHRH receptor protein was then demonstrated by Western blotting using a goat polyclonal antihuman LHRH receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described [25].

2.9. LHRH receptor binding studies

Receptors for LHRH on RL and HT tumours from the experimental groups were characterised by ligand competition assay. Preparation of tumour membrane fractions and receptor binding studies of LHRH were performed as described [15,26]. The LIGAND-PC computerised curve-fitting program of Munson and Rodbard [27] was used to determine the type of receptor binding, dissociation constant (K_d) and maximal binding capacity of the receptors (B_{max}).

2.10. Statistical analysis

Data are expressed as means ± SE. Results were compared by one-way ANOVA and Bonferoni test was carried out for posthoc comparisons. $P < 0.05$ was considered significant.

3. Results

3.1. Immunohistochemistry of human NHL specimens

A total of 17 human NHL specimens were evaluated for LHRH receptor expression by immunohistochemistry. Positive staining for LHRH receptors was found in 16 of 17 samples examined (94.1%). Surrounding non-malignant tissue, e.g., connective tissue showed no or only marginal LHRH receptor expression. (Table 1 and Fig. 1).

3.2. Inhibition of tumour growth in experimental models

In experiment 1, a single administration of AN-207 at 200 nmol/kg significantly ($P < 0.01$) inhibited the growth of RL human NHL compared to control and AN-201 treated animals. Tumour volume in the AN-207 group was reduced by 71.5% and tumour weight by 74.7%. Tumour doubling time was significantly ($P < 0.05$) prolonged compared to the control- and the AN-201 group. Equimolar doses of the cytotoxic radical AN-201 had no significant effects on any tumour growth characteristics (Fig. 2 and Table 2).

In experiment 2, the administration of AN-207 at a dose of 200 nmol/kg on day 1 and day 15 significantly ($P < 0.01$) inhibited the growth of HT human NHL xenografts, compared to control- and AN-201 treated animals. AN-207 significantly ($P < 0.001$) decreased tumour volume and weight by 82.5% and 79.6%,

Table 1
Immunohistochemical detection of LHRH receptors in surgically removed specimens of human non-Hodgkin's lymphoma

	NHL	LHRH-R
1	Follicle center lymphoma (mixed small and large cell), grade II	+
2	Follicle center lymphoma (large cell), grade III	+
3	Follicle center lymphoma (large cell), grade III	+
4	Follicle center lymphoma (small cell), grade I	+
5	Follicle center lymphoma (mixed small and large cell), grade II	+
6	Follicle center lymphoma (small cell), grade I	+
7	Follicle center lymphoma (mixed small and large cell), grade II	+
8	Follicle center lymphoma (mixed small and large cell), grade II	+
9	Follicle center lymphoma (large cell), grade III	–
10	Follicle center lymphoma (large cell), grade III	+
11	Follicle center lymphoma (small cell), grade I	+
12	Follicle center lymphoma (mixed small and large cell), grade II	+
13	Diffuse large B cell lymphoma (immunoblastic subtype)	+
14	Diffuse large B cell lymphoma (anaplastic subtype)	+
15	Diffuse large B cell lymphoma (centroblastic subtype)	+
16	Diffuse large B cell lymphoma (immunoblastic subtype)	+
17	Diffuse large B cell lymphoma (centroblastic subtype)	+

–, no expression; +, LHRH receptor expression.

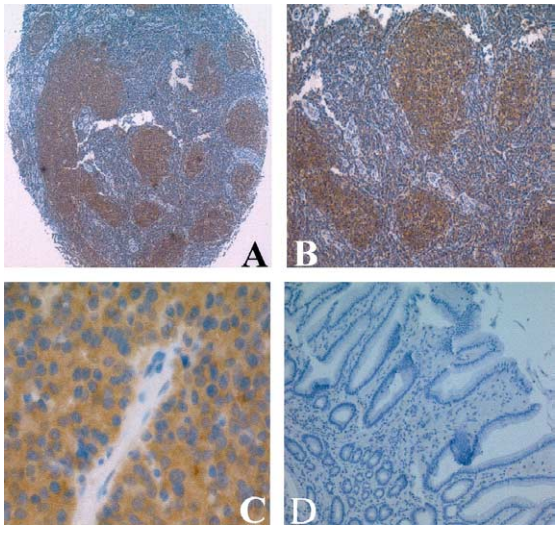

Fig. 1. Expression of LHRH receptors in surgically removed specimens of human non-Hodgkin's lymphoma: the tissue was stained by immunohistochemistry methods using a monoclonal antibody for LHRH receptors (clone A9E4, Novocastra). Follicular lymphoma Grade II with expression of LHRH receptors in pseudofollicular aggregates of neoplastic lymphoid cells. (A) 20× magnification; (B) 111× magnification; (C) positive control, pituitary gland (anterior lobe). Intense staining of LHRH receptor expressing cells. In the center a capillary with unstained endothelial cell; (D) negative control, colon; no specific staining.

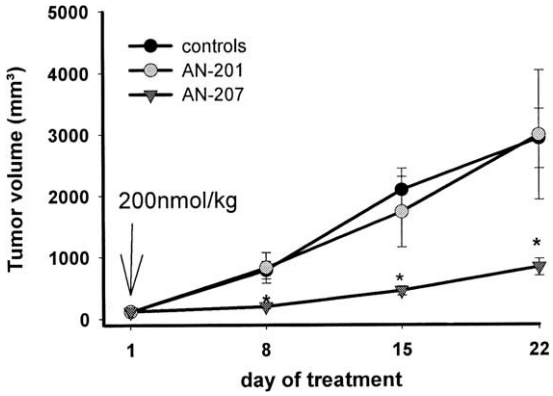

Fig. 2. Effects of targeted cytotoxic LHRH analogue AN-207 and its radical AN-201 on the growth of xenografts of RL human NHL. (Arrows indicate treatment; $^{*}P < 0.01$ *vs.* controls and AN-201.)

respectively. Tumour doubling time was significantly ($P < 0.05$) extended from 5.7 to 12.1 days. Equimolar doses of AN-201, the carrier [D-Lys^6] LHRH or the mixture of AN-201 and [D-Lys^6] LHRH had no significant effects on any parameters of tumour growth. The effect of AN-207 was abolished by injecting 200 μg of the LHRH agonist Decapeptyl 15 min prior to the administration of AN-207 (Fig. 3 and Table 2).

3.3. Toxicity

In all treatment groups, except for animals injected with the carrier alone, a slight, non significant loss of body weight was observed 8 days after treatment, which ranged from 0.4% to 8.9% and normalised eight days later. In both experiments, AN-207 did not significantly affect the WBC at any time. However, AN-201 significantly ($P < 0.01$) suppressed the WBC on day 8 compared to controls and mice treated with AN-207. In experiment 2, the number the WBC in the AN-201 group was within normal ranges before the second injection. However, the administration of the cytotoxic radical on day 15 significantly ($P < 0.05$) suppressed the WBC until the experiment was terminated on day 29. In experiment 1, one mouse died on day 3 and one on day 7 in the AN-201 group. One mouse died on day 15 in the control group. In experiment 2, one mouse died on day 4 and one on day 8 after the injection of AN-201.

3.4. Expression of mRNA for LHRH-receptor in tumours of human NHL cell lines

mRNA for LHRH-receptor was found in RL and HT human NHL xenografts by RT-PCR analyses. The PCR products were of the expected size of 319 bp. No PCR products were amplified from the negative controls, ruling out the possibility of genomic contamination (Fig. 4A).

Table 2
Effects of therapy with cytotoxic LHRH analogue AN-207, the cytotoxic radical AN-201, the carrier [D-Lys^6]LHRH, an unconjugated mixture of AN-201 and [D-Lys^6]LHRH, and AN-207 after blockade of the receptors with Decapeptyl on the growth of human non-Hodgkin's RL and HT lymphomas xenografted in nude mice

Treatment	Number of mice per group	Tumour volume (mm^3)		Tumour doubling time (days)	Tumour weight (mg) (% inhibition)
		Initial	Final (% inhibition)		
Experiment 1					
RL					
Control	11	116.9 ± 16.7	2921.9 ± 484.8	4.9 ± 0.3	3536.6 ± 519.7
AN-207	12	117.5 ± 19.4	831.3 ± 137.2** (71.5)	9.2 ± 1.1*	894.6 ± 119.4** (74.7)
AN-201	11	114.3 ± 19.6	2980.0 ± 648.0 (−2.0)	5.3 ± 0.6	3235.0 ± 558.9 (8.5)
Experiment 2					
HT					
Control	11	78.3 ± 14.8	3113.1 ± 426.5	5.7 ± 0.6	3244.7 ± 289.6
AN-207	11	80.5 ± 10.5	543.6 ± 68.1** (82.5)	12.1* ± 1.5	663.0 ± 55.9** (79.6)
AN-201	11	81.4 ± 14.4	2562.3 ± 397.7 (17.7)	6.0 ± 0.4	2771.4 ± 332.4 (14.6)
Carrier	5	82.3 ± 16.9	2451.7 ± 616.9 (21.2)	6.0 ± 0.2	2427.0 ± 528.6 (25.2)
Mixture	5	84.4 ± 17.9	2519.8 ± 625.4 (19.0)	6.1 ± 0.6	2587.1 ± 495.0 (20.3)
Blockade	5	84.0 ± 19.5	2226.6 ± 808.9 (28.5)	6.4 ± 0.2	2227.0 ± 689.4 (31.4)

* $P < 0.05$ *vs.* controls and AN-201.
** $P < 0.01$ *vs.* controls and AN-201.

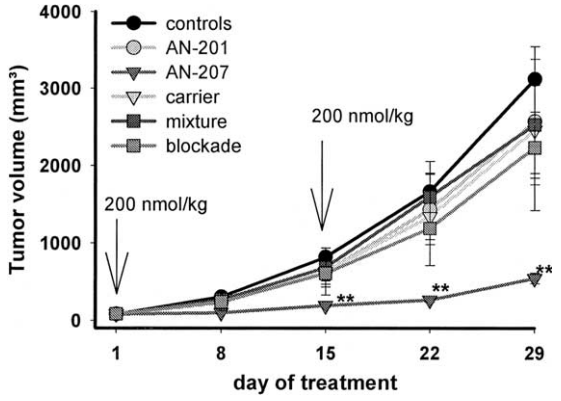

Fig. 3. Effects of targeted cytotoxic LHRH analogue AN-207, the cytotoxic radical AN-201, the carrier [D-Lys^6] LHRH, an unconjugated mixture of AN-201 and [D-Lys^6] LHRH, and AN-207 after blockade of the LHRH receptors with Decapeptyl on the growth of HT human NHL. (Arrows indicate treatment; $^*P < 0.05$ *vs.* controls, $^{**}P < 0.01$ *vs.* controls and AN-201.)

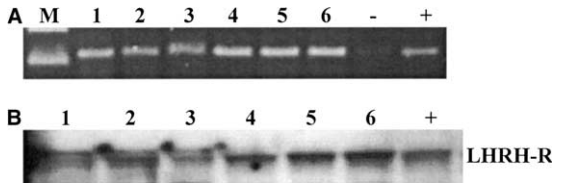

Fig. 4. (A) Expression of mRNA for LHRH receptors in RL and HT human NHL xenografts as revealed by reverse transcriptase (RT) PCR. Lane M: 100 base-pair ladder, lanes 1–3: RL, lanes 4–6: HT; lane −: RNA negative control; lane+: positive control (DU-145 human prostate cancer); (B) Expression of LHRH receptor protein in RL and HT human NHL xenografts as revealed by Western blot analysis: Lane +: positive control (ES-2 human ovarian cancer); lanes 1–3: RL; lanes 4–6: HT.

3.5. Expression of LHRH-receptor protein

The presence of LHRH receptor protein in untreated RL and HT tumour tissues was evaluated by Western blotting using specific antibodies. A specific band at 64 kDa was found in all investigated tumour samples. (Fig. 4B).

3.6. LHRH-receptor binding studies

In membranes of RL and HT human NHL tumours from the control group, receptor analyses revealed a single class of specific, high affinity binding sites for LHRH. Mean K_d values were 4.41 ± 0.2 nM in RL tumours and 6.19 ± 0.1 nM in HT tumours. Mean maximal binding capacities (B_{max}) were 294.2 ± 17.9 fmol/mg membrane protein in RL; and 496.8 ± 8.2 fmol/mg membrane protein in HT tumours.

4. Discussion

Management of advanced stages of NHL remains a challenge and the prognosis for patients with systemic disease is poor [2]. Consequently, new therapeutic approaches for NHL have to be explored. Targeted therapy represents a modern treatment modality developed to achieve higher efficacy and increased tissue selectivity. Treatment with the monoclonal anti-CD20 antibody rituximab or the radiolabeled anti-CD20 antibodies Zevalin and Bexxar yielded promising clinical results and prolonged the overall survival of patients with NHL [10,11]. However, monoclonal antibodies are large molecules and it can be assumed that smaller ligands such as peptide hormones can penetrate more easily into

neoplastic tissue [28]. Since receptors for peptide hormones are found on a wide variety of cancers, several targeted hormone analogues were synthesized in our laboratory including the cytotoxic LHRH analogue AN-207. AN-207 consists of [D-Lys^6] LHRH covalently linked to superactive derivative of DOX, 2-pyrrolino-DOX (AN-201) and shows enhanced antitumour efficacy in different experimental models of LHRH receptor-positive human cancers [7,8].

The discovery of LHRH receptors in several malignancies originating from organs, which are not part of the reproductive system, raised our interest and prompted us to evaluate the LHRH receptor status in human NHL. In order to provide a rationale for the possible use of cytotoxic LHRH analogues in the treatment of NHL, we investigated specimens of surgically removed human NHL specimens. Immunohistochemical analysis showed positive staining for LHRH receptors in 94.1% of the samples, suggesting that a high percentage of NHL appears to receptors for LHRH. In surrounding non-malignant tissue no or only marginal LHRH receptor expression was found. In addition, in two human NHL cell lines, RL and HT, we detected high affinity binding sites for LHRH, the LHRH receptor protein as well as the corresponding mRNA by RT-PCR, Western blot analysis and radioligand binding assays. These results are in agreement with previous findings demonstrating the presence of specific, high affinity binding sites for LH-RH in surgical specimens of human cancers and various human cancer cell lines. Our results are also in good agreement with previous observations on the binding affinity and capacity of receptors for LH-RH expressed on the pituitary. Cytotoxic LHRH analogue AN-207 significantly inhibited the growth of RL and HT tumours xenografted into nude mice, while the cytotoxic radical AN-201 had no significant effects. In the second experiment, we injected additional animals bearing HT tumours with an unconjugated mixture of AN-201 and the carrier peptide, [D-Lys^6] LHRH, or the carrier alone. As these treatment regimens did not affect tumour growth, it can be assumed that the high antitumour efficacy of AN-207 cannot be attributed to its components but rather to the ability of the carrier, [D-Lys^6] LHRH, to deliver AN-201 to neoplastic cells. This targeting concept is further supported by the fact that a blockade of LHRH receptors by Decapeptyl nullified the antitumour activity of the targeted cytotoxic analogue AN-207.

The toxicity of targeted cytotoxic LHRH analogue AN-207 and its cytotoxic radical AN-201 was compared with respect to mortality, body weight loss and WBC. No deaths occurred after treatment with AN-207, but four animals died after the injection of AN-201. All treatment regimens, except the carrier alone, caused a slight, not significant loss of body weight eight days after the injection, which was transient and normalized in the following week. Myelotoxicity is considered the most serious side effect and the dose-limiting factor in chemotherapy. In this study, AN-207 did not significantly decrease the WBC at any time, while AN-201 significantly lowered the number of leucocytes in all experiments indicating that the targeting of AN-207 to tumoural LHRH receptors can reduce the toxic side effects of chemotherapy. Potential toxicity of AN-207 to the LH-producing cells of the anterior pituitary was examined in previous experiments in rats and mice. In both studies, the cytotoxic LHRH analogue only transiently affected the pituitary function at the maximal tolerated doses [29,30].

The current study demonstrates for the first time that LHRH receptors are expressed in a very high percentage of human NHL specimens. Our work also shows that targeted cytotoxic LHRH analogue AN-207 can powerfully inhibit the growth of experimental human NHL *in vivo*. These findings suggest that targeted therapy with cytotoxic LHRH analogues could be a promising new modality for the treatment of advanced NHL.

Conflict of interest statement

None declared.

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