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h-R3

Treatment of Head and Neck Cancer
Treatment of Epithelial Tumors
Anti-EGFR MAb

TheraCIM

Humanized IgG_1 monoclonal antibody to epidermal growth factor receptor (EGF-R)

EN: 280998

Abstract

Epidermal growth factor receptor (EGFR) overexpression has been associated with alterations in cell cycle progression, increased invasive capacity, enhanced angiogenesis and decreased apoptosis of tumor cells. h-R3 is a genetically engineered humanized monoclonal antibody that recognizes an epitope located in the extracellular domain of human EGFR. The antibody blocks EGF binding to the receptor and inhibits its intrinsic tyrosine kinase activity. In preclinical experiments, h-R3 demonstrated remarkable antiproliferative, proapoptotic and antiangiogenic effects. No significant signs of toxicity attributable to h-R3 were observed in animal studies. Phase I/II clinical studies in patients with advanced stage epithelial tumors or squamous cell carcinomas of the head and neck have demonstrated the efficacy and safety of single and multiple doses of the antibody in combination with radiotherapy.

Description

h-R3 is a humanized monoclonal antibody (MAb) (IgG_1 isotype) that was obtained by transplanting the complementarity determining regions (CDR) of the murine MAb ior egf/r3 (IgG_{2a}) to a human framework, assisted by computer modeling. The light and heavy chains (REI and Eu, respectively) were selected as human immunoglobulin frameworks for CDR-grafting based on their high homology with the corresponding sequences of murine MAb ior egf/r3. CDR-grafting dramatically reduced h-R3 binding capability and then the antibody was reshaped. A variant in which 3 murine residues were retained (Ser 75, Thr 76 and Thr 93) exhibited a similar capacity to inhibit the binding of EGF to its receptor as compared with the original antibody (Fig. 1). The expression vectors bearing the initial versions of h-R3 heavy chain variable region (VH) and kappa light chain variable region (VK) domains were cotransfected into NSO myeloma cells. Afterwards, mycophenolic acid resistant clones were selected and screened by enzyme linked immunosorbent assay (ELISA) (1).

Apart from other anti-epidermal growth factor receptor (EGFR) antibodies, the parental antibody of h-R3 (murine MAb ior egf/r3) was generated by fusing the murine myeloma cells SP2/Ag14 with splenocytes from Balb/c mice immunized with a purified human placenta fraction enriched in EGFR, and not with EGFR purified from cultured cells (2).

Introduction

The aberrant signal transduction pathway plays a key role in the development of cancer, and receptor tyrosine kinases (RTK) such as erbB (EGFR), neu (Her2), kit (stem cell factor receptor) and met (HGF receptor) have been studied as potential targets for anticancer therapy (3-5).

The proto-oncogenic properties of EGFR have been known since the early 1980s, when it was initially cloned and found to be the homologue to the v-*erb*B oncogene (6-8). Elevated levels of EGFR are associated with malignant transformation of squamous cells (9, 10) and it is overexpressed in a wide variety of human tumors, including non-small cell lung cancer (11), head and neck (12), breast (13), brain tumors (14), bladder cancer (15), hepatocarcinomas (16), esophageal tumors (17), as well as gastric (18) and colorectal adenocarcinomas (19).

EGFR signal transduction pathways have been correlated with various processes that contribute to the development of malignancy, such as cell cycle progression, inhibition of apoptosis, angiogenesis, tumor cell motility and metastasis (20). EGFR overexpression has also

T. Crombet Ramos*, A. Casacó Parada, N. Iznaga Escobar. Center of Molecular Immunology, Clinical Immunology Department, P.O. Box 15040, Havana 11600, Cuba. *Correspondence.

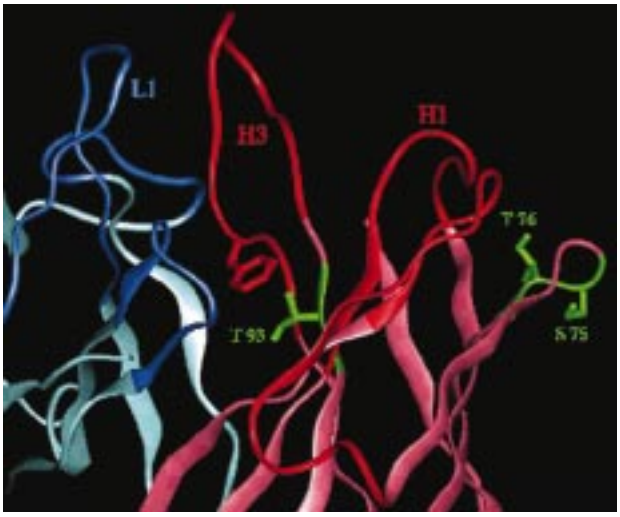

Fig. 1. Model of the variable region of murine MAb ior egf/r3. The binding site is at the top. The VL and VH frameworks are light blue and pink, respectively and their corresponding CDRs are marked in dark blue and red. Side chains of residues Ser 75, Thr 76 and Thr 93 are shown in green.

been associated with chemo- and radioresistance (20, 21).

To date, several EGFR antagonists have been investigated in the clinical setting, including antibodies (IMC-C225, ABX EGF, EMD-72000), small-molecule tyrosine kinase inhibitors (gefitinib, erlotinib hydrochloride), antisense oligonucleotides and cancer vaccines (22-25).

h-R3 is a new genetically engineered humanized antibody that recognizes an epitope located in the extracellular domain of human EGFR, with high affinity and specificity, blocking growth-factor binding and receptor activation (1).

Murine ior egf/r3, the parental antibody of h-R3, inhibits the activation of the receptor associated tyrosine kinase by its natural ligand (2) and blocks the proliferation of a variety of cultured tumor cell lines which express EGFR. ^{99m}Tc-Labeled ior egf/r3 was used for radioimmunodiagnosis of epithelial tumors in 148 patients. An overall sensitivity and specificity of 84.1% and 100%, respectively, were obtained in the *in vivo* detection of epithelial neoplasms of all localization (26). In addition, 58 patients were included in 4 therapeutic clinical trials with murine ior egf/r3 antibody doses ranging from 160-3600 mg (cumulative dose). In these trials, 4 patients developed anaphylactic reactions after several cycles of treatment, probably related to the appearance of human anti-mouse antibodies (HAMA response). There was evidence of long-lasting disease stabilizations when the antibody was used as monotherapy at repeated doses in advanced non-small cell lung cancer patients and in high-grade glioma patients. In order to decrease the toxicity and to improve the immunological effector functions of murine ior egf/r3, the antibody was humanized (27, 28). Humanized h-R3 has a molecular weight of 150 KDa, a dissociation constant (K_d) in the order of 1 nM and exhibits a G0 glycosilation pattern.

Pharmacological Actions

h-R3 immunohistochemical recognition was evaluated in small skin biopsy samples obtained from NMRI nu/nu, C57Bl/6 and OF-1 mice, Sprague-Dawley rats, Hartley guinea pigs, New Zealand rabbits, vervet monkeys and cynomolgus monkeys and normal subjects. h-R3 showed strong recognition of EGFR in human samples as well as in cynomolgus monkeys, New Zealand rabbits and OF-1 mice biopsies, whereas vervet monkeys, Sprague-Dawley rats and C57Bl/6 mice skins showed a moderate staining. No staining was observed in the skin samples from Hartley guinea pigs and NMRI nu/nu mice (manuscript in preparation).

h-R3 recognition of human tumors was also evaluated by immunohistochemistry. The antibody staining was classified as strong positive (a strong complete membrane staining in more than 80% of the tumor cells) in 3 of 4 (75%) non-small cell lung cancer samples, in 6 of 10 (60%) breast carcinoma samples and in 10 of 10 (100%) biopsies from squamous cell carcinomas of the head and neck (SCCHN).

The immunogenicity of h-R3 after the humanization process was evaluated in 2 vervet monkeys that were immunized with either murine (ior egf/r3) or the humanized MAb (h-R3). h-R3 was at least 8-fold less immunogenic in vervet monkeys than the murine variant (1).

Biodistribution of h-R3 in comparison with ior egf/r3 was evaluated in nude mice xenografted with A431 epidermoid carcinoma cell line. Twenty-one athymic female NMRI nu/nu mice were injected intravenously with 10 μg/100 μCi of ^{99m}Tc-labeled MAb. Immunoreactivity of the ^{99m}Tc-labeled MAbs was measured by ELISA on A431 cell line and the immunoreactive fractions determined by the Lindmo method. Four hours after the injection, significant accumulation was found in tumor: 6.14 ± 2.50% injected dose/g (ID/g) and 5.06 ± 2.61% ID/g for the murine and humanized MAbs, respectively (29).

The growth-inhibitory potential of h-R3 was tested *in vitro* in two-dimensional (monolayer) and three-dimensional (spheroid) cultures in an established vulvar squamous carcinoma EGFR overexpressing cell line (A431). This cell line is a good model to evaluate the activity of EGFR antagonists due to its high dependence on EGFR activity for *in vitro* and *in vivo* growth. Three-dimensional structures are considered to resemble the spatial structure found in actual solid tumors (30).

h-R3 exerted a maximum antiproliferative activity of 40% when used to treat A431 cells *in vitro* for 48 h. This growth-inhibitory effect was similar in either monolayer or spheroid cultures, although it was achieved at a lower concentration (50 μg/ml) in spheroid cultures. Propidium iodide staining was carried out to characterize the cell cycle profile of A431 cells treated with h-R3. The flow

cytometry analysis revealed a G_1 arrest accompanied by a decrease in the S phase in cells exposed to the antibody. Cell death detection ELISA kit was used to study the proapoptotic effect of h-R3 in A431 cell cultures. Using this method, a weak proapoptotic effect (2-fold) was detected *in vitro* after 48 h of treatment with h-R3 in spheroid cultures but not in two-dimensional cultures. This, in addition to the absence of an apparent hypodiploid peak in the flow cytometry analysis (representative of apoptosis) suggests that h-R3, at least *in vitro*, acts primarily as a cytostatic and not as a cytotoxic agent in the A431 model (31).

In addition to its effect on cell growth, the capacity of h-R3 to downregulate a relevant proangiogenic factor, *i.e.*, vascular endothelial growth factor (VEGF), was studied at the protein and RNA level, as an antiangiogenic surrogate effect. A commercially available human VEGF kit was used to quantify the VEGF level in the conditioned medium from monolayer cultures of A431 treated with h-R3. VEGF Northern blotting was performed by using either *in vitro* cultured cells or liquid nitrogen-frozen tumor tissue (A431) from mice treated with the antibody. The effect of h-R3 on VEGF protein levels was pronounced (~40%) and dose-dependent at 100 μg/ml. This paralleled a dose-dependent downregulation of VEGF mRNA to a maximum of 50% at 100 μg/ml (31).

Several *in vivo* experiments were carried out in SCID mice xenografted with the vulvar carcinoma cell line A431 and treated with several doses of h-R3 or ior egf/r3 (murine counterpart). In a short-term experiment, each treatment group received 4 intraperitoneal (i.p) injections of either PBS or the indicated MAb (1 mg/injection) every 48 h. Tumors were then removed for histological or Northern blot analysis. In an independent experiment, mice bearing established tumors received i.p injections of 1 mg (high-dose groups) or 0.25 mg (low-dose groups) of either h-R3 or ior egf/r3 every 48 h per 2 weeks. In both experiments, h-R3 and ior egf/r3 showed a similar antitumor effect on A431 tumor xenografts, which was significant after only 4 doses (short-term experiment). The extent of this antitumor effect was further studied at 2 different doses during the long-term intervention experiment. After 8 injections of either antibody, most tumors showed marked responses and all mice treated with 1 mg of h-R3 or ior egf/r3 showed complete remissions of the tumors by day 22 (31).

Immunohistochemical analysis of tumor sections was used to evaluate differences in apoptosis, proliferation and vascular density between control and h-R3 or ior egf/r3 treated samples. Blood vessel staining revealed a significant decrease in vascularity in the treated samples. Furthermore, a high degree of apoptosis was seen in the anti-EGFR treated tumor specimens and an antiproliferative effect of both h-R3 and ior egf/r3 antibodies was also evident *in vivo*; staining for Ki67 nuclear antigen – a marker of cell proliferation – showed a significant decrease in mitotic activity of treated *versus* control tumors (31).

Pharmacokinetics

For the pharmacokinetic study in mice, groups of 5 Balb/c mice were injected i.p with ^{188}Re-labeled h-R3 (50 μg/100 μCi) in a total volume of 0.1 ml. Blood samples were collected at various time intervals. The radioactivity content of the mice blood expressed as percentage of injected dose per gram of tissue (% ID/g of tissue) was plotted against the time of blood drawing in a linear plot. Blood time-activity curves were best fit to a monoexponential equation with a correlation coefficient of 0.98 ± 0.02 and a half-life ($t_{1/2}$) of 14.27 ± 1.32 h (mean ± SD).

Thirty female Wistar rats received 1 mg h-R3/200 μCi ^{99m}Tc dose in a total volume of 0.3 ml by i.v. bolus infusion. Following injection, blood samples were collected and the radioactivity in serum was determined by counting in an automatic gamma counter. The following pharmacokinetic parameters were obtained: $t_{1/2}$ = 13.89 ± 1.0 h; AUC = 1020.27 ± 57.3 μCi·h/ml; volume of distribution = 66.4 ± 6.1 ml/kg; volume of distribution at steady state = 76.25 ± 3.7 ml/kg; clearance = 5.7 ± 0.4 ml/h.

The pharmacokinetic profile of h-R3 was characterized in rabbits after i.v. administration of 3 dose levels (1, 6.72 and 10.33 mg). Antibody concentration was quantified using two validated methods (ELISA and a radioiodine method). Plasma concentration-time curves were best fit to a biexponential equation. A biphasic decline in plasma concentration was observed for all 3 doses. The dominant terminal disposition was characterized with a mean half-life of 27.4 ± 3.4 h, 41.5 ± 10.2 h and 52.4 ± 9.2 h for doses of 1, 6.72 and 10.33 mg, respectively. The volumes of distribution during the terminal phase were estimated to be 213.3 ± 57.1 ml, 177.8 ± 62.0 ml and 127.1 ± 40.7 ml for the respective doses and clearance values were 5.5 ± 1.7, 3.12 ± 1.55 and 1.68 ± 0.51.

Pharmacokinetic studies were run in 9 male vervet monkeys that received i.v. infusions of h-R3 obtained by 2 different fermentation technologies. The 2 groups of animals received a trace dose of ^{125}I-labeled h-R3 (160-184 μCi/kg) obtained by hollow fiber or stirred tank, in coadministration with a cold MAb dose of 10 mg/kg. Plasma concentration *versus* time curves were best fit to biexpotential equations. The dominant terminal disposition was characterized with a mean half-life of 7.78 ± 0.31 days for h-R3 produced by stirred tank fermentation and 7.92 ± 0.97 days for h-R3 produced by hollow fiber technology. The volumes of distribution during the terminal phase and clearance values were estimated to be 824.9 ± 61.5 and 939.6 ± 323.6 ml and 13.4 ± 1.1 and 13.1 ± 2.9 ml/day for the stirred tank and hollow fiber products, respectively.

Toxicology

Several studies were carried out in order to assess the toxicity profile and safety of h-R3 in different animal species.

Two different single dose toxicity studies were conducted in Sprague Dawley rats to determine the acute toxicity resulting from a single administration of h-R3. The MAb dose was escalated up to 10 times the intended dose for clinical trials. In both experiments, there were no significant signs of toxicity attributable to the Mab dose.

Two 14-day repeated dose toxicity studies were conducted in Sprague Dawley rats to evaluate the local and systemic toxicity resulting from 1 daily injection for 14 days, using doses equivalent to 10 times the maximum projected dose for the phase I trial. Analysis of all data, including clinical, hematology, clinical chemistry, gross necropsy and limited histopathology, revealed no treatment-related systemic toxicity. With regard to local toxicity, there was a limited inflammatory response at the site of injection, which was similar in frequency and severity for both test and control groups. Lymphoid follicular activity measured by numbers (not size) of proliferating germinal (B-cell) follicles in available sections of cervical lymph nodes and spleen were similar between control and treatment groups, indicating that h-R3 did not elicit a significant antigenic effect over the normal reactivity expected at these locations for young rodents.

Finally, a 14-day repeated dose toxicity study was conducted in vervet monkeys to evaluate the safety profile of h-R3 in nonhuman primates. Eighteen monkeys were treated daily for 14 consecutive days. Monkeys were distributed into 3 experimental groups with 3 animals of each sex per group. Group I received saline i.v. and served as controls, group II received 2.85 mg/kg i.v. of h-R3 MAb (about 1 time the maximum human dose) and group III received 11.4 mg/kg i.v. of the h-R3 MAb (about 4 times the maximum human dose). During the study there were no deaths, pathological clinical signs or variations in the corporal weight curves. The electroneurophysiological and hematological chemistry results showed no drug-related alterations. Signs of hematomas, hemorrhages and inflammation were observed at the site of injection in all animals. The electrocardiography study showed that 1 female monkey, from the highest dose group, shifted its cardiac axis from +60° to +120°; this finding could be interpreted as a right ventricular elongation in relation to the relative high daily dose. Overall, these results indicate that h-R3 administered daily at doses up to 11.4 mg/kg i.v. for 14 consecutive days to vervet monkeys did not produce significant toxicity (manuscript in preparation).

Clinical Studies

A phase I clinical trial was conducted in patients with advanced epithelial-derived tumors to study the safety, pharmacokinetics, biodistribution and immunogenicity of h-R3. Twelve patients were included in 4 treatment cohorts and received a single dose of h-R3 (50, 100, 200 or 400 mg i.v.). Patients were not eligible for any further treatment. Safety evaluation was made according to the World Health Organization toxicity criteria (33).

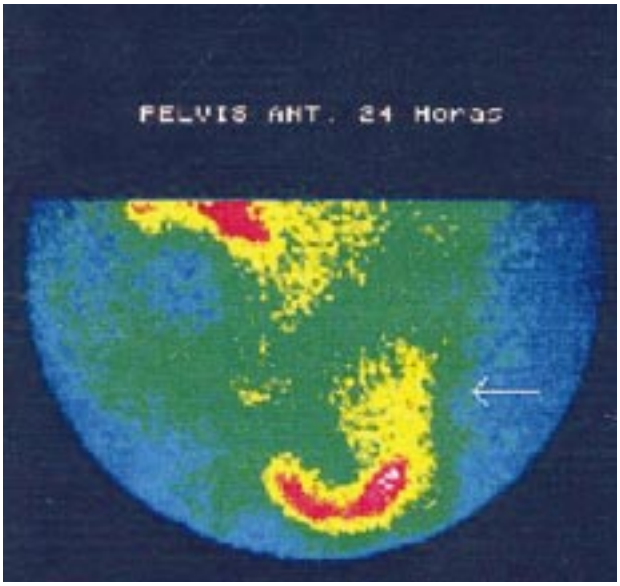

Fig. 2. Positive h-R3 uptake in an ovarian primary tumor.

After treatment, no evidence of serious adverse events were observed. Seven of 12 patients (58.3%) developed mild or moderate adverse reactions consisting mainly of tremors, fever, vomiting, nausea, dry mouth, asthenia, hypertension and flushing. In contrast to other EGFR antagonists, no acneiform rash was detected after h-R3 (33).

A 3-mg dose of the total antibody dose was labeled with ^{99m}Tc and pooled with the rest of the unlabeled MAb to evaluate the *in vivo* biodistribution of h-R3. Anterior and posterior whole body images were then obtained with a gamma camera at 10 min and at 1, 3, 5 and 24 h after MAb infusion. Total counts were computed for the selected organs at different time intervals. Then, organ count values were converted to activity, corrected for decay and expressed as percentage of the total administered activity. The whole body images showed h-R3 accumulation in the liver, heart, kidneys, urinary bladder and spleen through all time intervals. The liver had a statistically significant higher accumulation in comparison with the rest of the body for the 4 doses. Positive tumor images were obtained after injection of ^{99m}Tc-labeled h-R3. Figure 2 shows positive tumor uptake in a patient bearing an ovarian primary tumor (33).

Pharmacokinetics were evaluated after h-R3 infusion at the same dose levels (50, 100, 200 or 400 mg). Samples were collected at various time intervals. Plasma disappearance curves of the humanized anti-EGFR MAb were best fit by a biexponential equation and elimination half-lives ($t_{1/2\beta}$) were 62.91, 82.60, 302.95 and 304.51 h for the 50-, 100-, 200- and 400-mg doses, respectively. The AUC was 45 458, 145 931, 573 612 and 635 275 ng/ml·min and the C_{max} was 27 790, 36 612, 52 713, 57 117 ng/ml for the doses of 50, 100, 200 and 400 mg, respectively. The apparent volume of the central

compartment (V_c) was 2321.96, 2823.67, 4279.71 and 7173.99 ml and the total clearance (Cl) was 1.08, 0.67, 0.34 and 0.76 ml/h/kg for the doses of 50, 100, 200 and 400 mg, respectively (33).

Blood samples were collected at 0 and 7 days and then monthly up to 6 months to evaluate the immunogenicity of h-R3. A qualitative direct ELISA was performed by first coating high binding ELISA plates with ior egf/r3 (10 μg/ml) overnight at 4 °C in order to detect antibodies that react with the murine ior egf/r3 idiotype. None of the patients in the 4 dose groups had detectable anti-idiotypic antibodies after 1 infusion of h-R3 (33).

A total of 24 patients with histologically confirmed advanced stage (unresectable) SCCHN were included in a phase I/II clinical trial designed to evaluate the safety and preliminary activity of multiple doses of h-R3 in combination with radiotherapy. At the time of inclusion, all patients were eligible for radiation therapy, 20 (83%) of whom had either T3 or T4 and 11 (46%) were either T4 or N3 at presentation. Initially, 12 patients received 6 weekly i.v. infusions of h-R3 of 50, 100, 200 or 400 mg. Total cumulative MAb doses were 300, 600, 1200 or 2400 mg. Patients also received either 60 or 66 Gy radiation depending on response from Cobalt 60 sources, at 2 Gy given for 5 days over 6-7 weeks. Two patients interrupted radiation and h-R3 therapy within the first weeks and 2 additional subjects were recruited to complete response evaluation. Then, 10 new patients were included at the highest dose levels (200 and 400 mg) to evaluate the relationship between antitumor response and serum antibody levels (34, 35).

Toxicity was graded according to the National Cancer Institute's Common Toxicity Criteria (NCI-CTC) version 2.0 and the Radiation Therapy Oncology Group (RTOG) morbidity scoring system. The combination of h-R3 and external beam irradiation was very well tolerated. Eighteen of 24 patients developed h-R3 related adverse events. The most common toxicities were fever, hypotension, tremors, myalgia and headache. These events were classified as mild or moderate. One patient developed a grade 3 adverse event consisting of somnolence after the first dose. After 6 doses, h-R3 did not provoke acneiform rash or folliculitis and radiotherapy-related toxicity was not exacerbated after addition of the antibody. The most frequent radiation-associated toxicities were mucositis, dermatitis and dysphagia. Anti-idiotypic response was detected in 1 patient who achieved a complete response after treatment with the highest dose (400 mg) (34, 35).

For patients receiving doses of 200 mg or 400 mg, mean minimum and maximum serum steady-state concentrations ranged from 19.20-75.61 μg/ml and 39.02-147.12 μg/ml, respectively. Respective trough levels for the two doses were approximately of 34.6 μg/ml and 81.9 μg/ml (35).

Clinical response was classified as complete remission, partial remission, stable disease and progressive disease according to the Response Evaluation Criteria in Solid Tumors (RECIST). In the first trial set, 6 of 12 SCCHN patients achieved complete response after h-R3 and radiotherapy. Two additional patients achieved partial response. One of these subjects was disease free after surgical resection of the residual primary tumor. The best therapeutic responses were obtained at higher doses. Overall survival significantly increased after doses of 200 or 400 mg in comparison with the lower doses (p=0.03 by log rank test). At a median follow-up of 45.2 months, the median survival for patients in the 50-mg and 100-mg treatment groups was 8.60 months, while the median survival of patients in the 200-mg and 400-mg groups was 44.30 months. The 3-year overall survival was 16.7% for patients in the 50- and 100-mg treatment groups, and 66.7% for patients treated with 200 mg and 400 mg doses (34, 35).

In the last 10 patients treated with 200 or 400 mg, 9 patients exhibited objective responses, of which 5 were complete (35). Follow-up is ongoing. In a parallel study conducted in Canada with h-R3, 70% of the patients bearing locally advanced SCCHN achieved complete response after doses of 100 and 200 mg plus radiotherapy (70 Gy) without concurrent chemotherapy (36).

Prior to inclusion in the trial, a small biopsy on the primary tumor was taken under anesthesia to evaluate EGFR expression. Another sample was taken from the residual tumor tissue at week 3 (on therapy) to assess the antiproliferative and antiangiogenic activity of h-R3 plus radiotherapy. Preliminary studies of tumor specimens showed that the antitumor response could be attributed in part to direct antiproliferative effects and the suppression of tumor angiogenesis (34, 35).

Summary

h-R3 is a promising new antagonist of the EGFR oncogene, which mediates its antineoplastic effects via signal transduction inhibition, which might directly or indirectly affect cell proliferation, angiogenesis and cell survival. A multicenter, randomized, double-blind phase II clinical trial is ongoing in patients with advanced head and neck carcinomas who are included in 2 treatment groups: radiation plus h-R3 or radiation plus placebo. Another phase I/II trial is ongoing in high-grade glioma patients. In this trial, h-R3 is administered in combination with external beam irradiation after surgery excision of the brain tumor.

Source

Center of Molecular Immunology, Havana (CU); YM Biosciences Inc. Mississauga, Ontario (CA).

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