Development of Tumor Targeting Magnetic Nanoparticles for Cancer Therapy

Robert Ivkov*, Sally J. DeNardo**, Laird A. Miers**, Arutselvan Natarajan**, Allan R. Foreman*, Cordula Gruettner***, Grete N. Adamson**, and Gerald L. DeNardo**

*1Triton BioSystems, Inc., 200 Turnpike Rd., Chelmsford, MA 01824, rivkov@tritonbiosystems.com
**2Radiodiagnosis and Therapy, Molecular Cancer Institute, University of California, Davis, Sacramento, CA
***Micromod Partikelteknologie GmbH, Rostock, Germany

ABSTRACT

Magnetic nanoparticles will heat when activated by an alternating magnetic field (AMF). Systemic delivery of magnetic nanoparticles conjugated to a monoclonal antibody (MAb) offers potential for selective and minimally invasive cancer treatment by heating the micro-environments of cells containing such particles. ^{111}In-Chimeric L6 (Ch L6) antibody was conjugated to carboxylated polyethylene glycol (PEG-COOH) on dextran-coated iron oxide 20-nm particles to yield bioprobes. Pharmaco-kinetics, histopathology, and AMF/bioprobe therapy were done using intravenous doses (20μg/2.2mg ChL6/ bioprobe) with 50μg ChL6 in athymic mice bearing human breast tumor, HBT3477, xenografts. A 153 kHz AMF was applied 72 hr post injection for therapy using several AMF amplitudes. Significant therapeutic response was demonstrated. Toxicity was observed only at extreme AMF conditions, and was due to nonspecific heating resulting from the AMF.

Keywords: Monoclonal antibodies (MAb), thermoablation, cancer, magnetic nanoparticles

1 INTRODUCTION

Evidence has long suggested that thermotherapy can provide an alternative therapeutic modality for cancer when standard therapies have failed [1-4]. Nevertheless, few clinically relevant methods have been developed to provide such options. Much of this failure results from the absence of technology that is capable to specifically deliver sufficient energy to heat the cancer tissue while maintaining temperatures tolerable to normal tissues. Attempts to develop such technology involve ultrasound, laser, IR radiation, microwave, radio frequency, and alternating magnetic fields (AMF). Catheters and probes, as well as external transducers, have been used in attempts to deliver thermal therapy more selectively. Treatments that use radio frequency and microwaves have suffered from the limitation that the intervening tissues are heated because the energy source is also the heat source.

Because magnetic nanoparticles generate heat in response to an AMF, they may enable selective thermal ablation of cancer cells. Systemic delivery of magnetic nanoparticles attached to antitumor monoclonal antibodies (MAb), or bioprobes, can target the extravascular cancer cells selectively, enabling remote activation by an external AMF to induce heating of the cancer cell bound bioprobes.

The degree to which this approach is practical depends upon (a) the ability to systemically deliver bioprobes to the cancer in sufficient concentrations to achieve thermoablation when AMF is applied and (b) a combination of bioprobes and AMF characteristics that result in extremely rapid heating of the specific microenvironment while maintaining temperatures tolerable to normal tissues.

Reported herein are development of ^{111}In-MAb-conjugated iron oxide nanoparticles (Figure 1) as previously described [5] and therapeutic responses resulting from the combination of AMF with the systemically delivered ^{111}In-bioprobes.

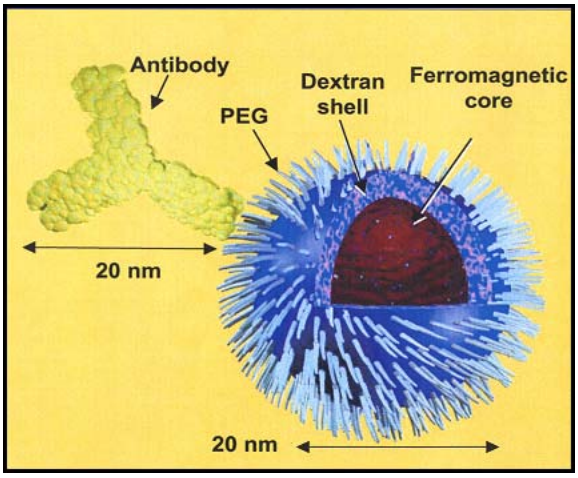

Figure 1: Schematic of a bioprobes.

2 MATERIALS AND METHODS

Carrier-free ^{111}In (MDS Nordion, Ontario, Canada) was purchased as chloride in 0.05 M HCl. Chimeric L6 (ChL6), human-mouse antibody chimera (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA), reacts with an integral membrane glycoprotein highly expressed on human breast, colon, ovary, and lung carcinomas [6-8]. ChL6 was specified as greater than 95% pure monomeric IgG by polyacrylamide gel electrophoresis.

A high gradient magnetic field (HGMF) column separator and nanomag®-D-spio 20-nm nanoparticles with an iron oxide dextran matrix and polyethylene glycol (PEG) COOH surface groups in 0.1M MES buffer, pH 6.3, 17 mg/ml, were obtained from micromod Partikeltechnologie, GmbH (Rostock, Germany). MES (2-(4-morpholino)ethane-sulphonic acid), EDC (1-ethyl-3- (3-dimethylaminopropyl)-carbodiimide HCl), NHS (N-hydroxysuccinimide), Glycine, 2IT (2-iminothiolane), PBS (Sigma Chemical Co., St. Louis, MO) and 3400 MWCO dialysis modules (Pierce, Rockford, IL.) were purchased.

2.1 Radiolabeling and Conjugation of ChL6 with BAD (2-[*p*-(bromoacetamido) benzyl]-DOTA

The immunoconjugate, 2IT-BAD-ChL6, was prepared by conjugating 2-[*p*- (bromoacetamido) benzyl]-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid DOTA (BAD) to ChL6 via 2-iminothiolane (2IT) as previously described [9].

Purified ^{111}In-2IT-BAD-ChL6 was evaluated by cellulose acetate electrophoresis (CAE), molecular sieving high performance liquid chromatography (HPLC, SEC 3000) and radioimmunoassay (RIA) using HBT 3477 human breast cancer cells [10-13]. ^{125}I-ChL6, lightly labeled and previously shown to be indistinguishable from ChL6, was assayed in parallel as a reference standard. HPLC and CAE indicated that 100% and 97%, respectively, of ^{111}In-ChL6 was in monomeric form. The absolute binding in the live cell assays was 70% or more, and 100% relative to the ^{125}I-ChL6 reference standard.

2.2 Conjugation of ^{111}In-DOTA-2IT-ChL6 with nanoparticles

Radiolabeled ^{111}In-DOTA-2IT-ChL6 was conjugated with 20-nm nanoparticles via amide linkage to the PEG-COOH coating under conditions selected, as previously described [5], to retain the immunoreactivity of the final ^{111}In-ChL6 bioprobes. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 24.0 mg, and N-hydroxysuccinimide (NHS), 48 mg in 10 ml of 0.1M MES buffer were mixed with 200mg/12ml of spio beads. This suspension was incubated for 1 hour at RT with continuous mixing, placed into 3400 MWCO dialysis bags (each 12 ml) and dialyzed against 4 liter of saline for 1 hour. ^{111}In-DOTA-2IT-ChL6 ($1.3x10^{16}$ molecules; 4.6mCi/3.2mg/1.4 mL of saline) was transferred into 10 ml of 0.1 M MES buffer, mixed with the activated nanoparticle suspension and incubated 1 hour at RT with continuous mixing (reaction ratio was $6.5x10^{13}$ molecules of MAb/mg of 20 nm spio beads). These conjugated ^{111}In-DOTA-2IT-ChL6-D-spio beads (20nm), 32 ml, were again placed into 4 dialysis bags (3400 MWCO) (each 8.0 ml), and dialyzed against 4 liter of saline at RT for 1 hour. The dialyzed product was mixed with 4.0 ml of 25mM glycine and mixed for 15 minutes to quench remaining active sites on the particle surface. The conjugated suspension was purified with the HGMF column separator using saline as both washing buffer and final eluent. The final suspension was collected from the magnetic column after removing it from the magnetic field. This suspension was brown with specific activity of 1.0-2.0 mCi/110mg/20ml (^{111}In-DOTA-2IT-ChL6-D-spio beads). The final product was assayed with multiple CEA strips for electrophoresis of 11 and 45 minutes and the immunoreactivity was evaluated in live cell assay as described above.

2.3 Mouse Studies

Female (7-9 week old), athymic Balb/c nu/nu mice (Harlan Sprague Dawley, Inc., Frederick, MD), were maintained according to University of California animal care guidelines on a normal diet *ad libitum* and under pathogen-free conditions. HBT 3477 cells were harvested in log phase; 3.0×10^6 cells were injected subcutaneously on the right side of the abdomen of each mouse. All studies were initiated 2-3 weeks after implantation, when the mean tumor volume was 125±44 mm^3. Studies were performed using 20-30 μCi ^{111}In-ChL6 bioprobes injected i.v. into a lateral tail vein. ^{111}In ChL6 bioprobe doses, 20-30uCi (20μg/2.2mg ChL6/ bioprobe), were injected iv with 50 μg ChL6 in 200 μl saline. Cohorts of mice were sacrificed 48 hours after bioprobe injection and PK data obtained to determine if tumor uptake warranted therapy. Blood and organ activity results have been reported elsewhere [5].

Bioprobe/AMF therapy was performed in 32 mice, each bearing one tumor, randomized into 3 groups of 8 or 12 mice**.** In therapy studies, mice were treated with AMF 3 days after bioprobe injection. At that time, each mouse was anesthetized by injecting 0.02 ml i.p. per gram body weight of an anesthetic solution prepared by dissolving 0.5 g 2,2,2-tribromoethanol in 1.0 ml warm *tert*-amyl alcohol then diluting the solution with 40 ml distilled water and filtering through a 0.2 μm filter.

After the mouse was anesthetized, four fiber optic temperature probes (FISO, Inc., Quebec, Canada) were placed to monitor tissue and core temperatures. One was inserted sc proximal to the lower spine by inserting a 16 ga x 11/2 in. hypodermic needle at the base of the tail, and threading the fiber optic probe through the needle under the skin. This procedure was repeated for a second probe placed sc near the tumor. A third probe was taped onto the skin of a hind limb using wound dressing, and a fourth probe was inserted one cm into the rectum. After the probes were in place, the mouse was wrapped lightly in absorbent paper and inserted into a 50 ml centrifuge tube from which the bottom had been removed. The tube and mouse were inserted into the felt-lined AMF coil, so that the tumor was positioned in a 1-cm high amplitude region of the induction coil. Once the mouse was in place and the parameters programmed into the controls, the AMF generator was turned on. AMF was applied in the coil with

amplitudes of 103 (n=12), 79 (n=8), or 55 (n=12) kA/m, as described below. AMF parameters, i.e. amplitude, duration and nature of exposure, were selected to minimize non-specific tissue heating due to the production of eddy currents, yet provide sufficient energy to activate the bioprobes in the region of the tumor to create localized heating. The AMF system was designed to confine high amplitude AMF in a one-centimeter wide band in the tumor region. Data used for the choice of parameters was obtained in an earlier study [14], which also contains a detailed description of AMF equipment and methods. After exposure, each mouse was left in the coil until the core (rectal) temperature began to fall, and then all probes were removed. The mouse was removed from the coil and centrifuge tube and placed on a warm recovery pad. When the righting reflex returned, the mouse was returned to its cage.

The results from a previously conducted study to determine parameters for safe application of high amplitude AMF [11] were used to select AMF levels for the bioprobe treatment herein. Response was evaluated for groups of mice receiving no treatment and cohorts treated at each of 3 magnetic field (energy) levels. These 3 groups of mice were followed for tumor growth and body weight 3 times per week for 12 weeks. Tumors were measured with calipers in 3 orthogonal directions and the volume was calculated using the formula for a hemiellipsoid [12]. Tumor effects were analyzed by Wilcoxin Rank Sum comparison of time to quintupling tumor volume in each AMF treatment group. Twenty-five additional control mice in 3 groups were followed in the same manner described above; one group of 5 mice received AMF but no bioprobes; one group of 15 mice received ^{111}In ChL6 bioprobes but no AMF; and a third group of 15 mice received no treatment.

3 RESULTS

The final ^{111}In-ChL6-bioprobe products were brownish suspensions without precipitate. The concentration of the beads was estimated by UV/visible spectrophotometry at a wavelength of 492nm using unconjugated beads as a reference, and protein concentration was estimated at a wavelength of 280 nm. Specific activity of the ^{111}In-DOTA-2IT-ChL6 bioprobes was 7-20 uCi/ ChL6 (4.4×10^{13} molecules)/164µL/mg of nanoparticles. The CAE of the preparations at 11 and 45 min revealed 94-98% and 91-97% monomeric final product at 11 and 45 minutes, respectively. ^{111}In-ChL6 bioprobe products bound in vitro to HBT 3477, 50-70% relative to the ^{111}In-ChL6 standard [13], except that the bioprobe control, produced without glycine quenching, bound only 20% relative to the standard

3.1 Pharmacokinetics

Liver and spleen uptake at 20 ± 2 and 13 ± 5 %ID/gm, respectively, at 48 hr was twice the uptake previously observed for ^{111}In-ChL6 [15]. A tumor uptake of 14 ± 4 %ID/gm of the ^{111}In-ChL6 bioprobes, however, was not different than that previously reported for ^{111}In-ChL6.

3.2 Toxicity

After slight weight loss associated with the procedure, the average weights of treated and untreated mice were equivalent throughout the 84-day trial. Death occurred acutely (within 24 hrs) in 4 of 12 mice which had been treated at 103 kA/m. Acute erythematic skin changes were also noted in mice treated at this level. Toxicity was not observed in the other groups.

3.3 Efficacy

Initial tumor volume was defined as the volume on the day before treatment. Mean tumor volume for any given day after treatment was calculated from actual measurements on that day, or from values derived from linear interpolation if that day fell between actual measurements. Normal tumor growth was evaluated for mice receiving no treatment. Tumor effect was analyzed by Wilcoxin Rank Sum comparison of the doubling, tripling, and quintupling time for individual tumors in each test group compared with individual tumors in the untreated control group. Tumors in all of the groups receiving bioprobes and AMF had a statistically decreased tumor growth rate with significantly higher mean time to double, triple, or quintuple tumor volume than those for untreated control tumors (Figure 2). Mean time to doubling demonstrated response as well but statistically was not as robust. Many tumors showed regression but no tumors had complete regression. Mean tumor temperature at the highest AMF conditions was 42 ± 2 °C.

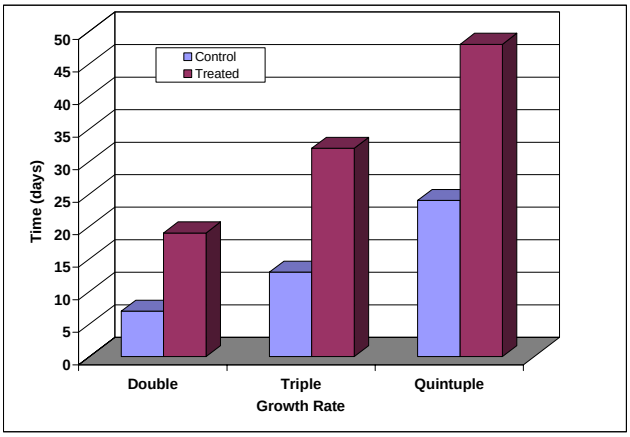

Figure 2: Efficacy is reflected by a decreased tumor growth rate (regression) in all of the treated groups. A statistically significant increase in mean time to double, triple, or quintuple tumor volume, with $P = 0.0013$, for all treated tumors compared with untreated tumors was observed.

4 DISCUSSION

This study confirms immunotargeting of bioprobes to human breast cancer tumors in vivo using ^{111}In-MAb linked to the nanoparticles to produce bioprobes. The radioactive tracer provided a means to assure that adequate tumor delivery had occurred prior to giving AMF to achieve thermal ablative therapy. Thus, thermomagnetic bioprobes, systemically delivered to metastatic tumor with delivery monitored by radioimmunoimaging may provide a safe and effective approach to develop AMF thermal ablation.

Prior PK studies with ^{111}In-ChL6 in athymic mice bearing HBT3477 xenografts demonstrated excellent tumor targeting [15] that was successfully translated into breast and prostate cancer imaging and therapy protocols for patients [16, 17]. Therefore, this model was considered an appropriate tool for initial in vivo bioprobe studies. Since the nanoparticles were assembled with dextran coating on the iron oxide magnetic core and a brush border of stabilizing PEG polymer on the surface, covalent conjugation of 1-2 MAb per nanoparticle could be achieved using carbodiimide activation of the carboxyl ends of the PEG brush border (Figure 1). This chemistry provided both immunoreactive MAb and a stable link to the nanoparticle. ^{111}In-DOTA conjugated to the MAb prior to bioprobe linkage facilitated assurance of tumor binding of the ^{111}In-MAb bioprobes in vivo. Furthermore, by providing in vivo pharmacokinetics prior to AMF and thereby assuring tumor uptake, the effects of variations in AMF could be evaluated and the time for application of AMF could be selected.

Compared to previously published nanoparticle studies [18, 19], the ^{111}In-ChL6 bioprobes remained substantially longer in the circulation and had substantially higher tumor uptake. The time that the ^{111}In-ChL6 bioprobes remained in circulation provided ample opportunity for these 20 nm particles to exit the blood and access the cancer cells. This study also demonstrated that MAb targeted nanoparticles can be produced, purified and characterized, and targeted to cancer cells in vivo in sufficient amounts to cause cancer cell necrosis from thermal ablation after externally applied AMF.

New bioprobes with nanoparticles having enhanced thermal magnetic properties are under development. While a consideration in the development of bioprobe therapy, iron toxicity is unlikely. Clinical experience with parental iron preparations suggests iron doses and forms, considered integral to this thermal ablative therapy, should be well tolerated.

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