Construction and Preclinical Evaluation of $^{211}$At Labeled Anti-mesothelin Antibodies as Potential Targeted Alpha Therapy Drugs

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ABSTRACT

Targeted alpha therapy (TAT) is a promising tumor therapy that can specifically transport $\alpha$ particle to the vicinity of tumor cells while the normal cells are only slightly irradiated. Mesothelin is a highly promising molecular signature for many types of solid tumors including malignant mesothelioma, pancreatic cancer, ovarian cancer and lung adenocarcinoma etc., while the expression in normal human tissues are limited, thus making mesothelin a promising antigen for TAT. Previously we developed a theoretical model that could predict and optimize in vitro screening of potential TAT drugs. The aim of the study is construction and preclinical evaluation of $^{211}$At labeled anti-mesothelin antibodies as potential TAT drugs. Mesothelin expression of two tumor cell lines were confirmed by flow cytometry, and their radiosensitivities were also evaluated. We used two kinds of anti-mesothelin antibodies, ET210–6 and ET210–28, to construct TAT drugs. Then, radiochemical purity, stability in vitro, affinity of the conjugates and mesothelin expression level were assessed. The specific killing of mesothelin-positive cancer cells treated by $^{211}$At-ET210–28 and $^{211}$At-ET210–6 were studied via Cell Counting Kit-8 assay and colony formation assay. $^{211}$At-ET210–28 and $^{211}$At-ET210–6 revealed excellent affinity and stability in both phosphate buffer saline and fetal bovine serum environment. Radiolabeled antibody conjugates bound specifically to mesothelin-positive cells in vitro. Both $^{211}$At-ET210–28 and $^{211}$At-ET210–6 could specifically kill mesothelin-positive cells with negligible damages to mesothelin-negative cells. Our findings provide initial proof-of-concept for the potential use of $^{211}$At labeled ET210–28/ET210–6 anti-mesothelin antibody in specific killings of mesothelin-positive tumor cells.

Keywords: mesothelin; targeted alpha therapy; $^{211}$At; preclinical evaluation

INTRODUCTION

Targeted alpha therapy (TAT) is a very promising therapy for cancer [1]. The mechanism of TAT is using a carrier (usually monoclonal antibodies) that can specifically recognize cancer cells and transport $\alpha$ particle emitters to the vicinity of cancer cells. This mechanism allows the specific delivery of high dose radiation to cancer cells while the normal cells receive few dose, so it can greatly reduce its toxic side effects while eliminating the cancer cells [1]. Since $\alpha$ particles have the characteristics of high ray energy and short range, TAT is very effective for tumors that have undergone micrometastases and tumor cells are dispersed throughout the body [2]. In fact, metastasis is responsible for as much as 90% of cancer-associated mortality [3]. Moreover, the biological effect of $\alpha$ particle is independent of tissue oxygenation, which is essential for $\beta$ particle and photon to effectively kill tumor cells [4]. Due to these excellent characters of $\alpha$ particles, TAT is ideal for curing micrometastases [5].

For TAT, selecting an appropriate antigen as the target is very important. Mesothelin (MSLN) may serve as a promising antigen of TAT. Mesothelin was first discovered by Kai Chang et al. more than two decades ago [6, 7]. The full-length human mesothelin gene encodes
a 71-kDa precursor protein [8]. The mesothelin precursor is cleaved into two products, a 40-kDa membrane-bound protein termed mesothelin and a 31-kDa shed fragment called megakaryocyte-potentiating factor (MPF) that is released from the cell [9]. Mesothelin is highly expressed in many cancers, including malignant mesothelioma, pancreatic cancer, ovarian cancer and lung adenocarcinoma [7, 10–12]. The expression is usually homogeneously distributed on the cell-surface, and cytoplasmic expression is low. Furthermore, the expression of mesothelin in normal human tissues is limited to the pleura, pericardium and peritoneum [6, 7]. As mesothelin is expressed only on dispensable tissues, the therapeutic risk of nonspecific toxicity by TAT is decreased [13]. Comparing to mesothelin, some other wide used target molecular have certain drawbacks. For instance, expression level of HER2 on many tumors, such as osteosarcoma and glioblastoma, is too low to be effectively recognized by trastuzumab [14].

Although there are about 100 radionuclides that decay by the emission of α particles, only a few have physical half-lives that are compatible with endoradiotherapeutic applications [15]. Three of them got the most attention: 213Bi, 211At and 225Ac [16–18]. Compared with other α particle emitters, 211At is generally considered to be a more versatile radionuclide for endoradiotherapy [19, 20], taking account of half-life, toxicity of daughter nuclide, security consideration and convenience of detection. Every 211At decay results in an alpha emission with 7.2 hours half-life, either by direct alpha decay to 207Bi or indirectly via the rapid decay of its daughter, 211Po. The half-life of 211Bi is only 46 minutes, which is too short for drug preparation and clinical application. For 225Ac, its free daughter radionuclides after decay in circulation raise concern about the potential toxicity [18]. The 7.2 hours half-life of 211At is relative long for radiation delivered to target site. So, 211At-labeled constructs could be used even when the targeting molecule does not gain immediate access to tumor cells. Additionally, its daughter, 211Po, emits γ rays that allow photon counting of samples and external imaging for biodistribution studies [18].

In vitro experiments are the essential step for drug screening before animal and clinical experiment. To predict and optimize in vitro screening of potential TAT drugs, we previously developed a theoretical model [21]. This model could be used to optimize in vitro experiments design. In that study, ET210–6, a kind of anti-mesothelin antibody, was used to construct TAT drug.

In this study, we chose 211At as the α particle emitter and conjugated it with ET210–6 and ET210–28 to form radio-probes targeting mesothelin. ET210–6 and ET210–28 are antibodies from human source expressing high mesothelin affinity. The basic structures of ET210–6 and ET210–28 are the same, and ET210–28 has stronger affinity to mesothelin than ET210–6. Compared with murine antibodies, human-source antibodies avoid development of human anti-mouse antibodies (HAMA), which may lead to clearance of the antibody and adverse events that are sometimes fatal [22]. We performed experiments to evaluate the radiolabeled antibodies as a potential TAT drug for the mesothelin-positive tumor cells.

**MATERIALS AND METHODS**

**Radionuclide**

211At was produced by the 209Bi (α, 2n) 211At nuclear reaction on the CS-30 cyclotron accelerator in Sichuan University, detailed procedures as reported elsewhere [23]. 131I was bought from Chengdu Gaotong Isotope Co., Ltd. (CNNC).

**Cell lines**

MDA-MB-231 and MDA-MB-231-mesothelin cell lines were used in this study. The MDA-MB-231 cell line was purchased from American type culture collection (ATCC) and verified through short tandem repeat (STR) assay. MDA-MB-231-mesothelin cell line is stably transfected with full length human mesothelin cDNA as reported previously [24]. Both cell lines are mesenchymal phenotype and kind gifts from Eureka Therapeutics (USA). MDA-MB-231-mesothelin and MDA-MB-231 were cultured with Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin with or without 1 μg/mL puromycin respectively. Cells were all cultured at 37 °C in a humidified incubator with 5% CO2.

**Antibodies**

Mesothelin-specific antibody ET210–28 and ET210–6 were intact human IgG1 antibodies, which are kind gifts provided by Eureka Therapeutics (USA).

**Flow cytometry**

Cells were harvested and suspended in cold phosphate buffer saline (PBS) at a concentration of 1 × 10⁶/mL. The cells were incubated with 10 μg/mL antibody for 30 minutes at 37 °C, then washed three times by cold PBS, and centrifuged. After incubation with FITC-labeled goat anti-human immunoglobulin G (IgG, 1:500 dilution, Beyotime) for 30 minutes at 37 °C, the cells were washed three times by cold PBS. For the negative controls, cells were incubated with only secondary antibody. The cells were analyzed using an LSRFortessa™ cell analyzer (BD Biosciences) and mean fluorescence intensities were processed.

**Radiolabeling**

Because the production capacity of 211At and mesothelin-specific antibody was insufficient, 211At labeled antibodies were used for receptor saturation and binding affinity assay. 131I labeled antibodies were used for other studies such as in vitro stability and CCK-8 assay etc.

As for 211I labeling, chloramine-T method was adopted as reported previously [25]. Briefly, in a 1.5 mL vial, 20 μg ET210–28 antibody at the concentration of 1.9 mg/mL was added with Na131I (40 μCi, 3 μL). Then 1 μL chloramine-T (114 mg/mL), freshly prepared in water, was added. The reaction lasted for 30 minutes at room temperature. Then it was terminated by adding 10 μL of Na2S2O5 (11.4 mg/mL, freshly prepared in water).

As for 211At labeling, direct procedure was adopted according to a previous study [26]. The reagent m-MeATE (TRC, Canada) was dissolved in chloroform at a concentration of 1.3 mM. A sample of 8 μL was taken from the stock solution, and the chloroform was evaporated. The dry residue of the reagent was then dissolved in dimethyl sulfoxide at a concentration of 1.3 mM. ET210–28, ET210–6 and IgG control were prepared in 0.2 M sodium carbonate buffer (pH 8.5) at a concentration of 1.3 mg/mL. A 7.5-fold molar excess of m-MeATE over the antibody was used in the conjugation. The conjugation reaction proceeded for 30 minutes during gentle agitation at room temperature.
The conjugate fraction was diluted with 0.2 M acetate buffer (pH 5.5) and finally isolated by Amicon Ultra-0.5 (Millipore).

$^{211}$At was prepared as a dry residue from a chloroform preparation after distillation. Then it reacted with N-iodosuccinimide (NIS) immediately for about 1 minute before labeling with a NIS stock solution at a concentration of 30 μg/mL in methanol:1% acetic acid. After that, the mixture was incubated with 40 μg prepared protein (antibodies or IgG) for 1 minute at room temperature. The reaction was terminated by adding 122 μL sodium acetate buffer and 1.2 μL sodium ascorbate (20 mg/mL).

Radiolabeled antibodies were then purified by size-exclusion chromatography using a PD-10 column (GE Healthcare) with PBS as the mobile phase. The labeling efficiency and radiochemical purity were determined using instant thin-layer chromatography (ITLC) and methanol precipitation. The developing solvent, which was used in the ITLC analysis, was acetone: water = 3: 1. Purified protein solutions were filtered through a 0.45 μm filter (Millipore) to keep sterile.

Radiochemical purity and stability

$^{211}$At-labeled ET210–6, ET210–28 and IgG were mixed with PBS or FBS for the stability test. The admixture was incubated at room temperature. Radiochemical purity was determined by methanol precipitation. In vitro stability was evaluated at various time points (0, 20 and 48 hours). Methanol precipitation was performed in triplicate. For a 1.5 mL tube, 100 μL of 1% bovine serum albumin in PBS, 5 μL purified radiotracer and 500 μL methanol were added. 5 minutes later, the tubes were centrifuged. Radioactivity in the protein-bound pellet was measured by a gamma counter.

Receptor and binding affinity

$^{211}$I-ET210–28 was used to determine its cellular binding affinity with MDA-MB-231-mesothelin cells. Cells were harvested and resuspended in binding buffer. For each tube, 2 × 10^5 cells at the volume of 500 μL were added, followed by the addition of $^{211}$I-ET210–28 (from 8 to 4000 ng/mL final concentration). After 30 minutes of incubation, cells were washed with PBS, then collected and counted with an automated gamma counter (PerkinElmer, USA). The number of binding sites per cell of mesothelin and the affinity of labeled antibodies were determined by a Scatchard plot of the data [27]. All the tubes used in this assay were precoated by PBS containing 1% bull serum albumin (BSA) for 1 hour to reduce nonspecific binding.

In vitro colony formation assay

For γ ray irradiation, both cell lines were dissociated with Accutase (Invitrogen), then washed with medium and resuspended at an appropriate concentration, and then immediately irradiated at room temperature with $^{60}$Co γ ray of 1, 2, 4 and 6 Gy at a dose rate of 1.1 Gy/min (College of Chemistry and Molecular Engineering, Peking University).

Colony formation assay was performed immediately after irradiation by plating cells into triplicate 6-wells plates as described previously [28]. In brief, cells were trypsinized and re-suspended at an appropriate concentration, and then cells were inoculated into each well of 6-well plates in triplicate. After 14 days, cells were fixed with 4% paraformaldehyde and then stained with 1% crystal violet, and colonies containing more than 50 cells were counted.

For α particle irradiation, MDA-MB-231-mesothelin cells were harvested and re-suspended at the concentration of 250 cells/mL. After addition of different dose of $^{211}$At-labeled antibodies or IgG, colony formation assay was performed. Its procedures were the same as γ ray irradiation.

Cell counting kit-8 (CCK-8) assay

24 hours before the radiolabeling, MDA-MB-231-mesothelin cells were seeded in a 96-well plate at the concentration of 3000 cells/well. Different doses of purified proteins (from 0.02 to 500 kBq/mL) were added to different wells. After 10 days of incubation, every well was added with 10 μL CCK-8 agent. The plate was incubated in 37 °C for 2 hours. The absorbance value of each well at a wavelength of 450 nm (OD450) was determined using a microplate reader.

RESULTS

Mesothelin expression of cell lines

By using flow cytometry assays, the expression levels of mesothelin were evaluated in MDA-MB-231-mesothelin and MDA-MB-231 cell lines. Compared with MDA-MB-231 cells, which showed no binding signal, MDA-MB-231-mesothelin cells displayed significantly enhanced fluorescence (Fig. 1A-B). The result confirmed the high expression of mesothelin for MDA-MB-231-mesothelin cells and nearly no mesothelin expression for MDA-MB-231 cells.

Radiochemical purity and stability

For the in vitro stability test, radiochemical purities of $^{211}$At-labeled ET210–28 and ET210–6 mixed with PBS or FBS at different time points were measured (Fig. 2A). The radiochemical purity decreased slightly from 0 to 48 hours. And the stability did not show significant differences in PBS and FBS. In vitro stability test demonstrated that the $^{211}$At-labeled proteins were stable when incubated in PBS or FBS until 48 hours without an obvious decrease in their radiochemical purity.

Receptor and binding affinity

Using the Scatchard analysis, we got the binding affinity of ET210–28 and mesothelin expression level of MDA-MB-231-mesothelin cell line. The binding affinity of ET210–6 was 1.25 nM [21]. As shown in Fig. 3B, the dissociation constant ($K_d$) of ET210–28 was 0.251 nM and mesothelin expression level was 4.37 × 10^5.

Specific cytotoxicity to mesothelin-positive cancer cells of $^{211}$At-labeled antibodies

MDA-MB-231-mesothelin cells were treated with different dose of $^{211}$At-labeled ET210–28, ET210–6 and IgG, the viabilities of cells were measured by CCK-8 assay 10 days post inoculation (Fig. 3A). Both $^{211}$At-ET210–28 and $^{211}$At-ET210–6 showed high cytotoxicity compared with IgG control. $^{211}$At-ET210–28 had revealed ability of cell-killing at the dose of 0.27 kBq/mL (corresponding survival fraction was 83.74 ± 10.64%). At the dose of 0.82 kBq/mL, the survival fraction of cells treated by $^{211}$At-ET210–28 was as low as 9.83 ± 1.83%. $^{211}$At-ET210–6 also almost killed cells efficiently (survival fraction = 3.76 ± 0.98%) at the dose of 7.41 kBq/mL. $^{211}$At-ET210–28 expressed higher cytotoxicity than $^{211}$At-ET210–6.
Fig. 1. A representative flow cytometry comparing the binding of human anti-mesothelin antibody ET210–28 to two different human cell lines. Results indicated that MDA-MB-231 cell (A, B) was mesothelin-negative, and MDA-MB-231-mesothelin cell (C, D) was mesothelin positive.

Fig. 2. Properties of labeled antibodies. A. In vitro radiochemical purity and stability of labeled ET210–28, ET210–6 and IgG. B. Binding affinity of labeled ET210–28 and binding sites of MDA-MB-231-mesothelin cell, which were analyzed by Scatchard plot.

Fig. 3B showed that, as another proof of cancer cell killing effectiveness, after treatment with the same dose (0.80 kBq/mL) of $^{211}$At-ET210–28, the survival fraction of MDA-MB-231-mesothelin was much lower than that of MDA-MB-231 (1.94 ± 0.20% vs. 97.32 ± 10.61%).

The colony formation assay of MDA-MB-231-mesothelin cells treated by escalating dose of $^{211}$At-ET210–28 and $^{211}$At-ET210–6 also indicated the specificity (Fig. 4A). The survival fraction for $^{211}$At-ET210–28 and $^{211}$At-ET210–6 were much lower than $^{211}$At-IgG. Fig. 4B-D showed the typical pictures of colony formation assay.
**Fig. 3.** CCK-8 assays for labeled antibodies. A. Survival fraction of MDA-MB-231-mesothelin cell after incubated with different dose of $^{211}$At-labeled ET210–28, ET210–6 and IgG control. B. Survival fraction of MDA-MB-231-mesothelin cell and MDA-MB-231 cell after incubated with 800 Bq/mL dose $^{211}$At-labeled ET210–28 (***, $P < 0.001$).

**Fig. 4.** Colony formation assay of MDA-MB-231-mesothelin cell. A. Survival fraction of MDA-MB-231-mesothelin cells after incubation with $^{211}$At-labeled ET210–28 and IgG. Representative colony formation assay image of MDA-MB-231-mesothelin cells after incubation with different dose of $^{211}$At-labeled IgG control (B), ET210–6 (C), ET210–28 (D).
DISCUSSION
In the present study, the construction and preclinical evaluation of $^{211}$At labeled ET210–6 and ET210–28 anti-mesothelin antibodies as potential TAT drugs were investigated. According to the experimental results, the conjugates revealed high affinity to mesothelin antigen, long-term stability and specific cytotoxicity to mesothelin-positive cells. We also determined the effective dose interval for $^{211}$At-ET210–28 and $^{211}$At-ET210–6 in which tumor cells were significantly suppressed while the damage to normal cells was negligible. Therefore, $^{211}$At-labeled ET210–6 and ET210–28 are hopeful to serve as probes for specific killing of mesothelin-positive cancer cells.

More and more TAT studies have been carried out in recent years [29]. For instance, Weihao Liu et al. [23] labeled a novel small molecule fusion peptide, VP2, with $^{211}$At through a one-step method and the TAT conjugate had a high affinity for tumor cells expressing vasoactive intestinal peptide receptors (VIPRs); Charles Zhu et al. [30] presented a transmission dosimetry design for alpha particles using A549 lung carcinoma cells, an external alpha particle emitting source (radium 223; Ra-223) and a Timepix pixelated semiconductor detector. As for mesothelin, Urs B. Hagemann et al. used $^{223}$Ra to label BAY 2287411 and did the preclinical evaluation [31]. However, the 18.7 days half-life of $^{223}$Ra is too long, and the free daughter radionuclides of $^{223}$Ra also raises concern about the potential toxicity, just like $^{211}$Ac.

The flow cytometry results confirmed that MDA-MB-231-mesothelin cell line highly expressed mesothelin while MDA-MB-231 cell line almost did not express mesothelin (Fig. 1). Experimental results showed that the differences of survival fractions of MDA-MB-231-mesothelin and MDA-MB-231 were striking, when treated with the same dose of $^{211}$At-labeled antibodies (Fig. 3). It means that $^{211}$At-labeled antibodies we constructed could kill mesothelin-positive cells efficiently and did little damage to mesothelin-negative cells. This is especially important for TAT.

Besides, radiolabeled antibodies still maintained high radiochemical purity 48 hours later after radiolabeling. Dehalogenation may impair radiotracer function due to both durable retention of nuclide in normal tissues and loss of internalized tracer from tumor [32]. The reliable stability of radiolabeled antibodies guaranteed further potential application in vivo for realizing low normal background and high accumulation of the positive tumor.

One important principle of TAT is that TAT medicine should kill tumor cells as much as possible while damage normal tissues as slightly as possible. For effective TAT drugs, there should be a dose interval in which tumor was effectively killed and the toxicity to normal tissues is negligible. According to the result of CCK-8 assay, we could determine the meaningful dose interval for $^{211}$At-ET210–28 and $^{211}$At-ET210–6 were 0.82 ∼ 22.22 kBq/mL and 2.47 ∼ 22.22 kBq/mL, respectively. According the model we built previously, the effective dose interval for $^{211}$At-ET210–28 was wider than that for $^{211}$At-ET210–6 resulting mainly due to more outstanding mesothelin affinity [21].

As mentioned above, one important principle of TAT is that TAT drug should kill tumor cells as much as possible while damage normal tissues as slightly as possible. For patients treated with TAT, the most desirable result is that tumor cells are inhibited by high doses of radiation, and normal tissues are excitatory by low doses of radiation. As Fig. 3A shown, when treated with $^{211}$At-IgG at 0.09, 0.27 and 0.82 kBq/mL, the survival of MDA-MB-231-mesothelin were 104.03 ± 3.87%, 103.91 ± 5.13% and 103.22 ± 3.92%, suggesting that MDA-MB-231-mesothelin cells shown radiation hormesis in the background dose interval from 0.09 to 0.82 kBq/mL. Meanwhile the survival of MDA-MB-231-mesothelin was as low as 9.83 ± 1.83% when treated with 0.82 kBq/mL of $^{211}$At-ET210–28. This implied that when treated with appropriate dose of $^{211}$At-ET210–28 (∼0.82 kBq/mL), the tumor was significantly suppressed, while normal tissues could benefit from radiation hormesis for low background dose.

In summary, experimental results showed that $^{211}$At-ET210–28 and $^{211}$At-ET210–6 had excellent binding affinity to mesothelin and specific killings ability of mesothelin-positive tumor cells, suggesting they might be promising potential TAT drugs for the treatment of mesothelin-expressing tumors.

CONFLICT OF INTEREST STATEMENT
The authors have declared that no competing interest exists.

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