Preclinical Pharmacokinetics, Tolerability, and Pharmacodynamics of Metuzumab, a Novel CD147 Human-Mouse Chimeric and Glycoengineered Antibody
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Abstract

Metuzumab is an affinity-optimized and nonfucosylated anti-CD147 human–mouse chimeric IgG1 monoclonal antibody with enhanced antibody-dependent cellular cytotoxicity (ADCC). The purpose of this study was to characterize the pharmacokinetics, safety, and antitumor activities of metuzumab in mouse, rat, and monkey. The ADCC activity was assessed by a lactate dehydrogenase release assay. The pharmacokinetics of metuzumab were determined in Sprague–Dawley rats and in cynomolgus monkeys. Single- and repeat-dose toxicology studies of the i.v. administration of high-dose metuzumab were conducted in cynomolgus monkeys. Mice bearing human tumor xenografts were used to evaluate the antitumor efficacy of metuzumab. The ADCC potency of metuzumab was enhanced compared with the nonglycoengineered parental antibody. Metuzumab also effectively inhibited tumor growth in A549 and NCI-H520 xenograft models. In the monkey model, the total clearance of metuzumab decreased with increasing dose. The nonspecific clearance in monkeys was estimated to be 0.53 to 0.92 mL/h/kg. In single- and repeat-dose toxicity studies in cynomolgus monkeys, metuzumab did not induce any distinct or novel adverse findings and was well tolerated at all tested doses. These preclinical safety data facilitated the initiation of an ongoing clinical trial of metuzumab for the treatment of non–small cell lung cancer (NSCLC) in China. Mol Cancer Ther; 14(1); 162–73. ©2014 AACR.

Introduction

Lung cancer is the leading cause of cancer morbidity and mortality in men and women worldwide (1), and is particularly prevalent in China. In China in 2012, there were an estimated 0.59 million new lung cancer cases (0.33 and 0.26 million men and women, respectively) and 0.68 million deaths (0.38 and 0.30 million men and women, respectively) representing 25.2% of all cancer deaths in China (2). In men, lung cancer ranks as most common cancers in China in terms of incidence and mortality. The high rate of lethality of lung cancer is primarily attributed to an advanced stage of disease at the time of diagnosis. Non–small cell lung cancer (NSCLC) comprises approximately 75% to 80% of all lung cancer. Systemic chemotherapy is the current treatment for patients with NSCLC and has improved clinical outcomes; however, only approximately 15% of patients are expected to be alive at 5 years (3). Molecularly targeted anticancer drug therapies have achieved remarkable advances in NSCLC treatment over the past several years. A major target molecule in the targeted cancer therapy is EGFR. Anti-EGFR antibody (cetuximab) and small-molecule EGFR tyrosine kinase inhibitors (TKI), such as gefitinib or erlotinib, have been widely used in the treatment of NSCLC. However, only 20% to 30% of patients with EGFR-activating mutations respond to treatment with gefitinib and erlotinib. In addition, outcomes are not significantly improved compared with chemotherapy at a second-/third-line therapy (4). In addition, preclinical and clinical trials indicated that TKI drugs are ineffective in patients with EGFR VIII mutations (5, 6). Cetuximab is a chimeric mouse–human antibody that targets the EGFR extracellular domain III; it competes with activating ligands, thereby preventing subsequent signaling (7). However, a multicenter clinical trial revealed that progression-free survival was not increased in patients treated in EGFR-overexpressing, advanced stage (stage IIIIB/IV) NSCLC treated with cetuximab plus chemotherapy (8). Thus, novel therapeutic targets and improved treatment modalities for NSCLC are urgently needed, particularly for patients who are refractory to cetuximab or TKI drugs.

CD147 is a highly glycosylated transmembrane protein that belongs to the immunoglobulin superfamily and is involved in physiologic and pathologic tumor processes (9, 10). CD147 stimulates the production of membrane-type matrix metalloproteinases (11, 12), promotes cell migration, invasion, and angiogenesis (13–16), and plays an important role in tumorigenesis. Aberrant expression of CD147 has been observed in various epithelial malignancies (17, 18), including hepatocellular carcinoma, lung
cancer, breast cancer, and carcinomas of the pancreas, prostate, and bladder. Importantly, the levels of CD147 overexpression have been implicated in poor patient prognosis for many tumor types (17, 19–22). Thus, CD147 is a potential therapeutic target in cancer. We previously generated a CD147 monoclonal antibody (mAb), HAb18, and developed a 131I-labeled HAb18 F(ab′)2 (named Licartin) to treat liver cancer. Licartin has been shown to be effective in the treatment of anti-liver cancer, supporting the development of anti-CD147 antibodies as targeted therapeutics. Clinical trial results showed that Licartin is safe and active for patients with primary HCC (23), and that Licartin is effective in the prevention of post-orthotopic liver transplantation (OLT) tumor recurrence in patients with advanced HCC, and Licartin significantly decreased the tumor recurrence rate by 30.4% and increased the survival rate by 20.6% (24). However, the inclusion of radioactive 131I restricts the general clinical application of Licartin. Although safe and tolerable, its immunogenicity results in the development of human–anti-mouse antibodies (HAMA) in a fraction of treated patients (unpublished data). Such limitations have prompted efforts to improve the efficacy and tolerability of HAb18 mAb through antibody engineering.

Accordingly, we recently developed a CD147 mAb, metuzumab, an antibody-dependent cellular cytotoxicity (ADCC)–optimized recombinant human and mouse chimeric with a glycoengineered IgG1 isotype and developed by humanizing and affinity optimizing of the HAb18 mAb (25). Metuzumab is expressed in a fucosyltransferase-deficient CHO cell line, which generates a homogeneously afucosylated mAb with increased affinity for FcγRIIA and enhanced ADCC activity.

In this study, we demonstrated that metuzumab is highly effective against lung cancer cells in vitro and in vivo. In ADCC assays using lung cancer cell lines that express CD147, metuzumab was generally more potent than cHAb18 (fucosylated), which was used as positive control. We also demonstrated that metuzumab inhibits the growth of human tumor xenografts in SCID mice. In addition, in good laboratory practice of drug (GLP)-compliant tissue cross-reactivity studies, metuzumab and cHAb18 exhibited similar staining patterns in full rat, human, and non-human primate tissue panel, and metuzumab exhibited increased specificity and sensitivity in multiple human cancer types. Finally, we evaluated the preclinical pharmacokinetics and safety of metuzumab in preclinical studies. Taken together, our results demonstrate that metuzumab exhibits potent antitumor activity in preclinical models. A phase I clinical study of metuzumab in NSCLC is in progress.

Materials and Methods

Cell lines and cell culture

The human lung cancer cell lines A549 and NCI-H520 were obtained from the ATCC. After expansion, these cell lines were deposited into the National Platform of Experimental Cell Resources for Sci-Tech. All cell lines were subjected to STR tests and authenticated before utilization for researches. The cell lines were maintained in high-glucose DMEM supplemented with 10% FBS at 37°C and 5% CO2 in a humidified incubator.

Metuzumab

Metuzumab is a recombinant, glycoengineered human/mouse chimeric IgG1 mAb directed against the extracellular C2 domain of CD147 in human cancer. Glycoengineering of metuzumab was expressed in a fucosyltransferase-deficient CHO cell line, which is generated by using magnified ADCC through glycosylation engineering technology (MAGE 1.5; Eureka Therapeutics). The chimeric antibody is encoded from the variable region cDNAs of the murine anti-human CD147 mAb and the cDNAs of human kappa and gamma 1 constant regions. Metuzumab is produced by cell culture in bioreactors, using serum-free media at Pacific Meinuoke Biopharmaceutical Company. After purification, mAb was resolved in histidine/histidine hydrochloride buffer solution (pH 7.2) containing polysorbate 80 and sucrose, vialized under aseptic conditions, and formulated as a colorless, sterile product without any preservatives.

Analysis of N-linked oligosaccharides

The oligosaccharide and monosaccharide profiles of the antibodies were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and raw data provided by Eureka Therapeutics.

Flow cytometry assay

Levels of CD147 expression on the lung cancer cells were determined by flow cytometry using a FITC-labeled anti-CD147 primary antibody (BD Pharmingen) and a mouse IgG1 mAb as an isotype control. Relative antigen expression is reported as median fluorescence intensity (MFI).

Surface plasmon resonance measurements

The affinity (Kd) for the binding of human and murine FcRs and human CD147 antigen to IgG mAbs were measured on a multi-SPR array system (ProteOn XPR36, Bio-Rad) and data analysis was performed using ProteOn Manager Software (Bio-Rad). The detailed protocol can be found in the Supplementary Materials and Methods section.

ADCC assays

In vitro ADCC assays were performed as previously described (26). In vitro ADCC assays were performed with human lung cancer cell lines as targets and human peripheral blood mononuclear cells (PBMC), NK cells, or murine PBMCs (mPBMC), murine NK cells, and macrophages/monocytes were used as effector cells. The effector cell at an effector/target ratio of 30:1 (5:1 when NKs or macrophages/monocytes were used as effectors) was generated in a ratio of 30:1 (5:1 when NKs or macrophages/monocytes were used as effectors). A brief description is present in the Supplementary Materials and Methods.

Antitumor efficacy studies in mice

The efficacy studies in mice were conducted in accordance with Institutional Animal Care and Use Committee approved protocols. Inhibitions of tumor growth in vivo were conducted in SCID lung cancer xenograft models. Animals were housed in specific pathogen-free (SPF) condition. For the localized subcutaneous xenograft mouse model, 1 × 106 NCI-H520 or A549 cells, suspended in Hank’s balanced salt solution were implanted in the right dorsal flank of male SCID mice (6–8 week old). When tumors reached a mean volume of 100 mm3, mice were randomized into treatment cohorts (n = 10 mice per group). Seven days after tumor cell injection, mice were randomized into treatment cohorts (n = 10 mice per group). Mice in different treatment received a single dose of metuzumab, ranging from 2 mg/kg to 30 mg/kg, chemical drugs (2 mg/kg cis-platinum plus 100 mg/kg gemcitabine) or saline (control group) via tail vein, respectively.
Antibody injections were administered twice weekly and chemical drug injections once weekly. Tumor size was measured twice each week for the duration of the study using calipers. Tumor volume was determined using the following formula: \( V = \frac{4}{3} \pi \frac{a^3}{3} \), where \( a \) is the length of the longest diameter and \( b \) is the length of the shortest diameter. Antitumor activity was assessed by calculating inhibition ratio of tumor volume \((IR_V)\) based on medians by using following formula: 
\[
\frac{[1-\text{average} (T_{\text{treatment}} (\text{day } x) - T_{\text{treatment}} (\text{day } 0))] / \text{average} (T_{\text{control}} (\text{day } x) - T_{\text{control}} (\text{day } 0))] \times 100\%.
\]

In disseminated SCID mouse models, 5.0 \( \times \) 10^5 cells were injected i.v., and then mice were randomized into different treatment groups (10 mice per group). After 10 days for tumor cell inoculation, metuzumab, chemical drugs (2 mg/kg cis-platinum plus 100 mg/kg gemcitabine) or saline (control group) were administrated i.v. once weekly until mice death, respectively. Median overall survival (OS) was defined as the experimental day when 50% or more of animals in the group were sacrificed. Survival time data were represented using Kaplan–Meier curves and differences in median survival were compared with the pairwise log-rank test.

**Tissue cross-reactivity studies**

Tissue cross-reactivity of metuzumab was evaluated in formalin-fixed paraffin-embedded (FFPE) tissue sections from a selected panel of cynomolagus monkey, Rhesus monkey, rat, and human tissues. Human lung cancer tissue arrays (Chaoying Biotechnology Co.) were included as a positive control. The detailed protocol can be found in the Supplementary Materials and Methods.

**Rat tissue distribution and excretion studies**

For rat tissue distribution studies, four groups of rats (n = 6 per group, 3 male and 3 female) were administrated a single i.v. dose of 10 mg/kg 125I-labeled metuzumab at 123.78 Bq/µg via the tail vein. 125I-labeled metuzumab was radiolabeled and prepared using the modified chloramine-T method with a specific activity of 123 Bq/µg. The purity of the 125I-labeled metuzumab was determined to be 97.8% ± 0.5% by high-performance liquid chromatography. The rats in the four groups were sacrificed by decapitation at 2, 72, 360, and 600 hours after the administration, respectively. The blood samples were collected into heparinized tubes, and the plasma was then harvested by centrifugation at 10,000 rpm for 10 minutes. The supernatant and the resultant TCA precipitate were counted in an automatic gamma counter, respectively. Composite serum radioactivity was constructed for pharmacokinetic analysis. For cynomolagus monkey pharmacokinetic study, 4 groups of cynomolagus monkeys (n = 4 per group) were used. Three of the groups were administered a single i.v. injection of metuzumab at a dose of 1.5, or 25 mg/kg. The fourth group was a multiple-dose group in which the monkeys received 4 consecutive i.v. injections of metuzumab at a dose of 5 mg/kg once per week. Whole blood samples were drawn from the femoral veins on the opposite side of the injection sites using a puncture needle immediately before administration and at 10 and 40 minutes; 1, 2, 4, 8, 10, and 24 hours, and 2, 4, 6, 7, 8, 10, 12, and 15 days in the single-dose groups and at the first and fourth doses in the multiple-dose group. In the multiple-dose group, samples were also collected at 0, 1, 1.5, 2, 4, 8, 12, and 24 hours on the fourth day after the final administration of metuzumab. Blood samples were immediately collected into heparinized tubes, and the plasma was then harvested by centrifugation at 1,000 \( \times \) g for 4°C for 10 minutes. The plasma was separated and stored at –80°C until further analysis.

Pharmacokinetic modeling and the estimation of pharmacokinetic parameters were performed by noncompartmental analysis (WinNonlin, version 5; Pharsight Corporation). The following variables were calculated and derived directly from the data using this model: AUC (the area under the serum curve), \( V_{ss} \) (volume of distribution at steady state), \( C_{max} \) (maximum serum concentration), \( t_{1/2} \) (terminal half-life), and \( t_{max} \) (the time of \( C_{max} \)). \( C_{last} \) is the last measured concentration time point. The clearance (CI = dose/AUC) rate was also evaluated.

**Repeat-dose toxicity studies/13-week repeat-dose toxicity study with 4-week recovery**

A 13-week repeat-dose toxicity study with a 4-week recovery period was conducted to evaluate the safety of metuzumab. Sprague–Dawley rats and monkeys were assigned to 4 groups (30 rats or 4 monkeys per group). The animals were administered twice once-weekly doses of 20, 60, or 200 mg/kg (rat only) or 10, 50, or 200 mg/kg (monkeys only) of metuzumab by short i.v. infusion. The control group was infused with normal saline. Repeat-dose toxicity was evaluated on the basis of mortality, were anesthetized by intraperitoneal injection with pellitobarbital natrium. The abdomens of the rats were then opened and the bile ducts were cannulated with a PE-10 cannula to facilitate the collection of bile samples. Bile samples were collected at 0, 2, 4, 8, 12, and 24 hours after the rats received a single i.v. injection at 10 mg/kg [123.7 Bq/µg]. Bile samples were stored at –20°C until analysis after measuring the bile volume.

**Pharmacokinetic study**

The pharmacokinetic study in Sprague–Dawley rat and in cynomolagus monkey was approved by the Institutional Animal Care and Use Committee at National Institutes for Food and Drug control, National Center for Safety Evaluation of Drug. Sprague–Dawley rat received a single i.v. dose of 10 mg/kg of metuzumab and 125I-labeled Metuzumab mix (1:1) via the tail vein (n = 6/ group, 3 male and 3 female). A total of 0.5 mL of serum was collected via tail vein from each animal at the following time points: 2, 4, 8, and 24 hours; and 2, 3, 4, 5, 7, 9, 14, 20, and 26 days after the administration of metuzumab. After addition of 400 µL of trichloroacetic acid solution, the samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant and the resultant TCA precipitate was counted in an automatic gamma counter, respectively. Composite serum radioactivity–time profiles were constructed for pharmacokinetic analysis. For cynomolagus monkey pharmacokinetic study, 4 groups of cynomolagus monkeys (n = 4 per group) were used. Three of the groups were administered a single i.v. injection of metuzumab at a dose of 1, 5, or 25 mg/kg. The fourth group was a multiple-dose group in which the monkeys received 4 consecutive i.v. injections of metuzumab at a dose of 5 mg/kg once per week. Whole blood samples were drawn from the femoral veins on the opposite side of the injection sites using a puncture needle immediately before administration and at 10 and 40 minutes; 1, 2, 4, 8, 10, and 24 hours, and 2, 4, 6, 7, 8, 10, 12, and 15 days in the single-dose groups and at the first and fourth doses in the multiple-dose group. In the multiple-dose group, samples were also collected at 0, 1, 1.5, 2, 4, 8, 12, and 24 hours on the fourth day after the final administration of metuzumab. Blood samples were immediately collected into heparinized tubes, and the plasma was then harvested by centrifugation at 1,000 \( \times \) g and 4°C for 10 minutes. The plasma was separated and stored at –80°C until further analysis.

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clinical observations, body weight, food consumption, ophthalmology, clinical pathology (including hematology, chemistry, CD3+ /CD4− and CD3+/CD8+ T cells, and bone marrow cells), and/or macroscopic and microscopic pathologies.

Statistical analysis
Tumor growth rates were calculated by linear regression of the tumor volume versus time plots for individual tumors for the period of 1 to 28 days. Student t test was used to determine statistical significance between two groups. One-way ANOVA was used to compare multiple groups. Analysis was performed using Prism version 5. P values of <0.05 were considered statistically significant. Descriptive statistics are provided for the pharmacokinetics, toxicity, and efficacy data. Continuous data are summarized as the mean values ± SD. Differences in numeric variables between groups were assessed by two-sided t test or one-way ANOVA. P < 0.05 was considered statistically significant.

Results
Metuzumab exhibits increased affinity for human FcRIIIα, resulting in enhanced ADCC
The humanized and affinity-optimized anti-CD147 mAb, cHAb18, was expressed in a fucosyltransferase-deficient CHO-k1 cell line (named MAGE1.5) that generates homogenously fucosylated mAbs (Eureka Therapeutics). The fucosylated form of mAb is called metuzumab. Previous studies have demonstrated that Fc receptor-mediated ADCC depends significantly on the patterns of Fc glycosylation and that a fucose-negative antibody exhibits enhanced binding affinity to FcRIIIα, with significantly increased binding to NK cells, which promote ADCC efficacy (27). The glycans profile of the antibodies was determined by HPAEC-PAD (28, 29; Fig. 1A; see Supplementary Materials and Methods for more details); in cHAb18, fucosylated IgG secreted from the wild-type CHO cells, the core fucose was determined and G0F, G1F, G1T glycoforms were the most prominent forms; however, metuzumab exhibits homogeneous glycosylation with a single major N-glycan and generates a Man5 glycoform, but no fucose or xylose was detected.

Furthermore, the binding affinity profile of FcγRs to metuzumab and cHAb18, were determined by SPR (Fig. 1B, and see Supplementary Materials and Methods for more details). Compared with the cHAb18, metuzumab demonstrates increased affinity for human FcRIIIα (CD16a) (176, 177; Kd from 211 to 20 nm), but decreased binding to human FcRIIIB/c (CD32b/c), an inhibitory FcγR (Kd from 1.48 nm to 650 nm). Binding to human FcRIIa (CD64), the high-affinity activating FcγR, and FcRIIa (CD32a) is not substantially modified.

Both metuzumab and cHAb18 Fc domains bind similarly to mouse FcγR (CD64) and this consistency with the substantial change observed with human FcγR. As with human FcγR Ib/ c, the metuzumab Fc domain imparts reduced binding to mouse FcγR Ib/ c (CD32), the mouse inhibitory FcγR. Both metuzumab and cHAb18 Fc domain bind similarly to mouse FcγRII (CD16), a low-affinity activating FcγR that is distributed on murine NK and mononuclear phagocytes (30, 31). Binding to the metuzumab Fc domain to murine FcγR IV (CD16-2) is increased and is consistent to its increased binding to human FcγRIIa (Kd from 3.14 to 33 nm; Fig. 1B). FcγRIV is another low-affinity activating FcγR that is homologous to human FcγRIIa (CD16a) in sequence, and is distributed on murine myeloid cells (30, 31).

To confirm that removal of fucose does not affect CD147 binding, we performed antibody binding to the CD147-expressing A549 cell analysis by FACS (Fig. 1C). Metuzumab and cHAb18 exhibited similar CD147-binding ability. The calculated affinities of antibodies by SPR analysis for metuzumab and cHAb18 (Kd, 0.429 nmol/L and 0.475 nmol/L, respectively) were equal (Fig. 1D). These results indicated that lack of fucose does not affect CD147 binding.

Antitumor activity of metuzumab
To assess whether metuzumab has altered effector functions, a series of ADCC assays were conducted (Fig. 2A–D). The ADCC assays revealed that metuzumab increased in vitro ADCC. The ability to mediate ADCC improved 6.3-, and 2.5 -fold (EC50, n = 6) in NCI-H520 and A549 cell lines when human PBMC were used as effectors (Fig. 2A). When purified NK cells from human PBMC were used as effectors, metuzumab was approximately 10.2-, and 20-fold (EC50, n = 6) more potent than cHAb18 at inducing ADCC in NCI-H520 and A549 cell lines, respectively (Fig. 2B).

Metuzumab also exhibited superior in vivo efficacy when the NCI-H520 and A549 cell lines were used in a tumor xenograft model. To determine the dose response, metuzumab was administrated at 2, 10, and 30 mg/kg via tail vein (twice weekly) when the tumors reached a volume of 100 mm3 (see Supplementary Materials and Methods for full details). Metuzumab elicited significant dose-dependent tumor growth inhibition as determined by the tumor doubling time and tumor volume in NCI-H520 and A549 xenograft model.

In the A549 cell model, an i.v. dose of 10–30 mg/kg metuzumab and the chemical drug were effective in prolong tumor doubling time. The tumor doubling time increased to 6.88 ± 0.57, 10.16 ± 0.85, and 10.24 ± 0.86 days compared with that in control group (5.01 ± 0.41 days). Treatment with 2 mg/kg group (5.87 ± 0.38 days) failed to prolong tumor doubling time. In NCI-520 cell model, the tumor doubling time increased to 5.03 ± 1.90, 5.16 ± 0.92, 5.27 ± 1.45, and 4.08 ± 1.06 days in the 2–30 mg/kg metuzumab and chemical drug groups compared with that in control group (2.46 ± 1.22, P < 0.05).

Tumor growth rate was also calculated. all of treatment groups displayed significantly inhibited tumor growth compared with the control treatment group (P < 0.01; Fig. 2C and Supplementary Fig. S1). In the NCI-H520 models, the injection of 2–30 mg/kg metuzumab and chemical drug resulted in a significant inhibition in the IRV (48.85%, 49.53%, 53.34%, 57.75% for the chemical drug, 2 mg/kg, 10 mg/kg, and 30 mg/kg metuzumab groups, respectively; P < 0.001). Our results revealed a trend of dose-dependent trend of antitumor effect among the three metuzumab treatment groups (P = 0.5699), and the antitumor effects observed in the metuzumab treatment groups were equal or a slight superior than those observed in the group treated with the chemical drug group alone. In A549 models, each treatment group showed significant tumor growth inhibition compared with the control treatment group. The tumor volume inhibition rates were 14.78%, 26.52%, 50.64%, and 51.06% in 2, 10, and 30 mg/kg metuzumab and chemical drug group (P < 0.01). The antitumor efficacy of 2 and 10 mg/kg metuzumab was lower than that of the chemical drugs treatment, and the efficacy of the 30 mg/kg metuzumab treatment was equivalent to that of the chemical drugs treatment.
Therapeutic antibodies are often used in combination with chemical drugs. We also evaluated the tumor growth induced by treatment with 30 mg/kg metuzumab (twice weekly) in combination with chemical drugs (2 mg/kg cis-platinum plus 100 mg/kg gemicitabine, once weekly) in subcutaneous SCID NSCLC models (NCI-H520 and A549). The combination of metuzumab with chemical drug enhanced the suppression of tumor growth in both subcutaneous xenograft models compared with treatment with metuzumab or chemical drugs alone. The IRc50 was 61.06% and 63.1% in the NCI-H520 and A549 cell models, respectively. The results indicated that the combination of metuzumab with chemical drugs enhanced the antitumor efficacy compared with mAb or chemical drug alone in preclinical models of human NSCLC.

The antitumor efficacy of metuzumab was further tested with NCI-H520 and A549 cell xenografts in an intravenous disseminated tumor model. Survival time was the primary endpoint. As shown in Fig. 2D, Kaplan–Meier survival analyses revealed that...
administration of metuzumab in NCI-H520 and A549 models extended the median survival time compared with the control group (43 days in NCI-H520 and 56 days in A549). The median time for the 2, 10, and 30 mg/kg injections were 63, 66, and 80 days for the NCI-H520 models and 72, 86.5, and 90 days for the A549 models, respectively. The addition of chemical drugs elicited a minor effect in both of the disease models; the mean survival time was 51 days and 62 days in the NCI-H520 and A549 cell models, respectively. All of above, studies on the in vitro and in vivo antitumor activity of metuzumab confirms that metuzumab can

Figure 2. In vitro and in vivo antitumor efficacy of metuzumab. Effector functions of metuzumab in in vitro ADCC assays. The ability of metuzumab and cHAb18 mAb mediated in vitro ADCC by human PBMCs (HPBMCs; A), purified NK cells (hNK; B) were measured using LDH release assay. CD147-expressing A549 and/or NCI-H520 cells were used as target cells in a 30:1 effector/target ratio (5:1 when NK cells were used as effectors). The results are shown as the mean values ± SD and are representative of six independent experiments. C, tumor growth inhibition with metuzumab in SCID mice bearing NCI-H520 (left) and A549 (right) human lung cancer cell line xenografts. Mice were treated with 4 weekly intraperitoneal doses of metuzumab, chemical drug (cisplatin plus gemcitabine), or metuzumab combined with chemical drug once the tumor volume reached 100 mm³. Tumor size was measured by calipers. D, Kaplan–Meier overall survival curves for each treatment group. Age-matched SCID mice were injected with 5.0 × 10⁵ NCI-H520 (left) or A549 cells (right; resuspended in PBS) via the tail vein. At 10 days after tumor cell injection, mice were treated with a single dose of metuzumab, chemical drugs (cisplatin plus gemcitabine), or saline. In the disseminated tumor model, survival time was used as endpoint.
enhance effector functions and can be developed as a potential therapeutic antibody.

Mouse FcγRI, a homolog of human FcγRIIIa, is not expressed on murine NK cells (32), and the antitumor efficacy of metuzumab in murine system predominantly depends on the Fc-mediated engagement of macrophages/monocytes and neutrophils. NK cell, macrophages/monocytes from human or murine PBMCs were separated by FACS. We demonstrated that human PMBCs can induce more significant ADCC activity than mPMBCs (Supplementary Fig. S2A). In addition, macrophages/monocytes were the major reason for the ADCC activity in murine PBMCs (Supplementary Fig. S2B). Together, these results show that metuzumab has increased affinity for human FcγRIIIa, which results in enhanced ADCC.

Tissue cross-reactivity studies
To identify potential models for toxicology studies, tissue cross-reactivity of metuzumab was evaluated in FFPE sections from a selected panel of humans, Sprague–Dawley rat, cynomolgus monkey, and Rhesus monkey tissues (See Supplementary Materials and Methods for full details). Tissue cross-reactivity studies in different species (Supplementary Table S1) showed similar staining patterns in vast majority tissue samples from humans, SD rat, cynomolgus monkey, and Rhesus monkey. Some staining was observed in human gastric fundic epithelium and renal tubular epithelial tissue and in the Rhesus monkey gastric fundic epithelium. These results indicated that the cynomolgus monkey and rat can be chosen for the nonclinical safety program for metuzumab.

In addition, metuzumab tissue-specific analysis was subsequently assessed in human cancer tissue arrays. In summary, metuzumab specifically bound to tumor tissues (Fig. 3 and Supplementary Table S2), including lung cancer (60/68), ovarian cancer (9/12), stomach cancer (18/23), esophagus cancer (30/32), breast cancer (36/40), cervical cancer (17/20), liver cancer (17/20), and colon cancer (41/50). The staining pattern of the reference mAb HAb18 was similar to that of metuzumab in cancer tissues, suggesting that the staining detected is specific for CD147.

**Pharmacokinetic study of metuzumab in Sprague–Dawley Rats and cynomolgus monkeys**
Pharmacokinetic analysis after a single of i.v. injection of metuzumab at 10 mg/kg was investigated in Sprague–Dawley rats (Fig. 4A; Table 1). The concentration–time curve revealed that after i.v. administration, metuzumab exhibited a rapid maximum plasma concentration (T_max) ranging from 0 to 10 minutes after injection. The distribution t_{1/2} was 162.8 ± 46.6 hours, with an AUC_{0–24h} of 12,377.6 ± 1,942.6 h/μg/mL. Single-dose pharmacokinetics were further investigated in groups of 2 male and 2 female cynomolgus monkeys that received a single i.v. injection of 1.0, 5.0, or 25 mg/kg metuzumab. The pharmacokinetic profiles are shown in Fig. 4B, and pharmacokinetic parameters are summarized in Table 1. As the dose increase from 1 to 25 mg/kg, AUC_{inf} increased in a greater than dose-dependent manner. The mean AUC_{inf} values were 1,094.68, 6,888.93, and 46,698.49 in the 1, 5, or 25 mg/kg mAb-injected groups, respectively. The ratio of the three groups was 1:5:25, while the corresponding ratio of AUC_{(0–960)} was approximately 1:6.3:42.6. Total clearance (Cl) decreased with increased dosage; the mean Cl values in the 1, 5, or 25 mg/kg treated groups were 0.92, 0.7, and 0.53 mL/h/kg, respectively. The mean V_{ss} values were 140.5, 153.03, and 103.38 mL/kg in the 1, 5, or 25 mg/kg metuzumab-treated groups. The maximum serum concentration (C_{max}) increased almost dose dependently from 22.33 ± 1.45 mg/mL after a single 1 mg/kg dose to 727.34 ± 80.51 mg/mL following the infusion of 25 mg/kg. The t_{1/2} at β phase (t_{1/2β}) were 213.47 ± 13.99, 337.08 ± 39.54, and 274.71 ± 51.75 hours at dose of 1, 5, or 25 mg/kg, respectively. Thus, within the examined dose range, the pharmacokinetic behavior of metuzumab fit a nonlinear profile.

**Figure 3.** Tumor-specific analysis of metuzumab in human multitype cancer tissues.
To evaluate the systemic exposure of metuzumab after repeated dosing, we conducted a study of the multiple i.v. administration of metuzumab study for 4-week (once weekly administration) period at a dosage of 5 mg/kg, the pharmacokinetic results showed that the metuzumab concentrations at the same time points following injection at the fourth administration were higher than that at first administration (Fig. 4C, Table 1). The C_{max} increased from 121.17 ± 20.64 μg/mL (first) to 141.70 ± 17.06 μg/mL (fourth), and the AUC also increased from 4,467.08 ± 986.03 μg·h/mL (first) to 5,277.09 ± 1,241.46 μg·h/mL (fourth). However, these differences were not significant (P > 0.05). These results indicate that metuzumab did not exhibit a tendency to accumulate in the cynomolgus monkeys. The pharmacokinetic results also indicated that the concentration–time curves of metuzumab exhibited no apparent gender differences in multiphase elimination profiles (Fig. 4D).

### Tissue distribution and excretion of 125I-metuzumab in rats

The individual tissue distribution in TCA-precipitated peptide of 125I-metuzumab peptide in the rats was summarized in Fig. 5A (see Supplementary Materials and Methods for full details). 125I-metuzumab underwent a rapid and widespread distribution in tissues throughout the whole body during the experiment. After 2 hours of injection, the highest radioactivity value was in blood (96.2 μg/mL), followed by the heart, lung, liver, and kidney. The heart, lung, and kidney absorbed relative more 125I-metuzumab at an earlier time compared with other tissues/organs. However, the radioactivity concentration in these tissues decreased rapidly in contrast with the count values found in the liver, which were sustained up to 72 hours. The radioactivity concentration in the adipose, muscle, and thymus were extremely low. Radioactivity accumulated at low levels in the brain tissue throughout the experiment, suggested that metuzumab did not efficiently cross the blood–brain barrier. The counts were increased in the jejunum at 360 hours after injection, which suggested that metuzumab is at least partially excreted (Fig. 5B).

A time course of the excreted radioactivity derived from 125I-metuzumab in urine, feces, and bile are illustrated in Fig. 5C and D. At 24 hours after the injection of 125I-metuzumab, the excretory rates of radioactivity into the bile, urine, and feces were 1.35 ± 0.20 ± 0.20, and 12.10 ± 5.73%, respectively. At 816 hours, the excretory rates in the urine and feces were 70.5 ± 9.7 and 10.1 ± 2.2%, respectively, and the total excretory rate was 80.6 ± 9.6%. These results suggested that the urinary excretion represents the dominant route of elimination, whereas the excretion of metuzumab via the feces and bile is minimal (see Supplementary Materials and Methods for full details).

### Repeat-dose toxicity studies

To support phase I clinical studies, a 13-week repeat-dose toxicity study with 4-week recovery was conducted to evaluate the safety of metuzumab in cynomolgus monkeys. Metuzumab was well tolerated in all animals, and no Metuzumab-related adverse effects on mortality, clinical observations, body weight, food consumption, local irritation, ophthalmology, heart rate, ECGs, clinical pathology were observed during the study.

In the 13-week repeat-dose toxicity study in cynomolgus monkey, no significant differences in hematology (Supplementary Table S3), urine, bone marrow cell counts, and CD3+/CD4+, CD3+/CD8+ lymphocyte counts (Supplementary Table S4) were found. The blood clinical chemistry results showed that AST and creatine kinase (CK) levels increased in a time- and dose-
dependent manner (Supplementary Table S5). Increased AST levels were observed in 1 female and 3 male monkeys of the 10 mg/kg group, 3 female and 2 male monkeys of the 50 mg/kg group, and 3 female and 3 male monkeys of the 200 mg/kg group at week 13; however, the majority of animals recovered at the end of study, except 1 monkey in 200 mg/kg group. Increased CK levels were observed in 3 male monkeys of the 10 mg/kg group, 1 female and 1 male monkeys of the 50 mg/kg group, and 2 female and 2 male monkeys of the 200 mg/kg group at the last injection. However, at the end of study, only 1 female monkey in the 50 mg/kg group, and a female monkey in the 200 mg/kg group exhibited abnormal in CK values. No macroscopic or microscopic morphologic changes were associated with metuzumab exposure. Additional modeling was conducted in rats, and no undesirable side effects were detected (Supplementary Table S3, S5, and S6). The no observed adverse effect level (NOAEL) was estimated to be 200 mg/kg.

The toxicokinetic analysis of metuzumab administration revealed that serum concentration of metuzumab increased in a dose-dependent manner. The $C_{\text{max}}$ of the last injection was significantly higher than that of the first administration in the 200 mg/kg group. After multiple doses, the exposure AUC\textsubscript{inf}–168h accumulated up to 1.9 ± 0.7-fold, 1.58 ± 0.35-fold, and 1.63 ± 0.44-fold (dosing period 13/dosing period 1) in the 10, 50, and 200 mg/kg group, respectively.

### Discussion

CD147 is a validated target for cancer therapy. The advantages of CD147 as a mAb target include tumor cell-specific expression, which were associated with tumor prognosis. Although Licartin, an 131I-labeled anti-CD147 mAb HAB18 F(ab')\textsubscript{2}, is an effective and marketed agent for the treatment of CD147-positive liver cancer and the prevention of post-OLT tumor recurrence, radioisotopes are hazardous and must be handled with extreme care. These limitations have promoted efforts to improve the effectiveness and tolerability of HAB18 mAb through antibody engineering.

ADCC represents a major mechanism underlying therapeutic antibody-mediated tumor cell killing. The ADCC activity of therapeutic mAb is dependent on the interaction between Fc region of mAb and the Fcγ receptors on the surface of immune effectors cells, which is affected by the antibody isotype, Fc region and glycosylation, and polymorphism Fcγ receptors (33). ADCC efficacy of IgG antibodies is significantly dependent on Fc glycosylation patterns (34). The absence of fucose on N297 of mAbs can result in conformational changes in the Fc region and increase ADCC by increasing the affinity between the Fc region and Fcγ receptors (27, 35–38).

Here, we report the development of metuzumab, a novel, human/mouse chimeric anti-CD147 IgG1 mAb. We produced metuzumab in MAGE 1.5 (CHO) cells. Metuzumab exhibits homogeneous glycosylation with a single major N-glycan but no detectable fucose or xylose (Fig. 1A). The fucose deficiency of metuzumab can result in great affinity increase to FcγRs, and can enhance ADCC activity. To determine the therapeutic potential of metuzumab, we assessed its pharmacokinetics, tissue distribution, safety in mouse, rat, and cynomolgus monkey, and antitumor activity in xenograft models.

Compared with the original antibody, metuzumab maintains the affinity and specificity (Fig. 1C, D and Fig. 4); however, specifically glycosylation modifications in Fc enhance its interaction with human FcγRIIIa, and promote ADCC activity in vitro and in vivo. The engineered Fc domain of metuzumab imparts 10-fold increase in affinity to the human low-affinity activating FcγR, FcγRIIIa (CD16a), and murine FcγRI, and decreased affinity for the inhibitory FcγR, FcγRIb/c (CD32b; Fig. 1B), and consequently, greatly enhanced ADCC effector function. In this study, we evaluated the ADCC activity of the metuzumab and chHAb18 mAb in NCI-H520 and A549 cell lines. With every cell line tested, the activity of metuzumab was greatly enhanced over the fucosylated mAb, chHAb18 (Fig. 2A). Nonetheless, FcγRIIIa (CD16a) is not only FcγR expressed on NK cells, and is also expressed on macrophages and DCs (39, 40); furthermore, NK cells are a key effector population for ADCC elicited by human IgG1 mAb and have a major contribution in the ADCC activity mediated by human PBMCs (39). Our data also implicated that NK cells are important mediators of the antitumor activity of metuzumab (Fig. 2B).

When tested in vivo in SCID human NSCLC xenograft models, metuzumab induced potent tumor growth inhibition. Metuzumab exhibited significant antitumor activity in both the A549 and the NCI-520 human lung cancer cell xenograft models, in which human A549 or NCI-H520 lung tumor-bearing mice were treated with metuzumab and chemical drugs, and we observed a significant delay in tumor progression measured by tumor doubling time and tumor size (Fig. 2C). The results of the assays with A549 and NCI-H520 xenograft models also indicate a trend of increased antitumor activity of metuzumab with increasing levels of antigen expression (Supplementary Fig. S3). MIs were 87.38 ± 12.31 in A549, and 378.55 ± 23.56 in NCI-H520, respectively).

In the A549 and NCI-H520 xenograft models, metuzumab does not produce a satisfactory treatment response; the tumor volume inhibition rates in both models were less than 60%, even in 30 mg/kg/dose. Notably, murine FcγRI, a homolog of human FcγRIIIa, is a low-affinity activating FcγR and is not expressed on mouse NK cells (32). Thus, the antitumor efficacy of mAb in

### Table 1. Pharmacokinetic parameters estimated by noncompartmental model analysis following single and multiple i.v. injection of metuzumab in rats and cynomolgus monkeys

| Parameter | Rat | Single i.v. injection | Cynomolgus monkeys | Multiple i.v. injection |
|-----------|-----|-----------------------|---------------------|------------------------|
|           | 10 mg/kg | 1.0 mg/kg | 5.0 mg/kg | 25.0 mg/kg | 5.0 mg/kg once a week |
| $T_{\text{max}}$ (h) | 0.17 ± 0.01 | 0.55 ± 0.25 | 0.42 ± 0.29 | 0.55 ± 0.25 | 0.17 ± 0.00 |
| $C_{\text{max}}$ (µg/mL) | 175.9 ± 32.9 | 22.3 ± 1.45 | 126.12 ± 16.2 | 727.34 ± 80.51 | 141.70 ± 17.06 |
| AUC\textsubscript{inf} (µg·h/mL) | 12.377.6 ± 1.942.6 | 1.094.68 ± 188.13 | 6,888.93 ± 928.86 | 46,688.49 ± 9,987.55 | 8,703.14 ± 2,286.63 |
| $V_{d}$ (µL/kg) | 156.8 ± 20.1 | 140.50 ± 21.22 | 153.01 ± 23.26 | 101.38 ± 21.6 | 145.02 ± 30.90 |
| $t_{1/2}$ (h) | 162.8 ± 46.6 | 213.47 ± 13.99 | 337.08 ± 39.54 | 274.71 ± 51.75 | 260.52 ± 26.09 |
| $C_{\text{l}}$ (µL/h/kg) | 0.78 ± 0.19 | 0.92 ± 0.17 | 0.70 ± 0.11 | 0.53 ± 0.11 | 0.57 ± 0.13 |

Abbreviation: $C_{\text{l}}$, total body clearance of drug from serum.
murine system predominantly depends on the Fc-mediated engagement of macrophages/monocytes and neutrophils (32). We compared the ADCC activity of metuzumab in vitro when human PMBCs or murine PMBCs were used as effector cells, and we demonstrated that human PMBCs can induce more significant ADCC activity than mPMBCs (Supplementary Fig. S2A). In addition, in human blood, NK cells account for almost all ADCC activity (30); however, macrophages/monocytes play a role in the induction of ADCC activity in murine PBMCs (Supplementary Fig. S2B). Thus, the activity of metuzumab in the mouse tumor models does not fully reflect the in vivo antitumor potency. Therefore, differences in the distribution and usage of FcγRs between mice and humans may contribute to some of the differences observed between in vitro and in vivo assays. Overall, the single-agent activities of metuzumab in the NSCLC xenograft models are superior to those of conventional chemotherapeutics. Although metuzumab combined with chemotherapy resulted in the most dramatic antitumor effects, preclinical evidence of the pharmacokinetics and safety of metuzumab in combination with chemical drugs is needed.

To evaluate the therapeutic potential of metuzumab, we assessed its pharmacokinetics in rat and monkeys. Following the i.v. administration of a single dose of metuzumab, the pharmacokinetic data suggested that the antibody is a rapid absorption and elimination in rats and in monkeys. The nonlinear pharmacokinetic data implied that the antibody is a rapid absorption and elimination in rats and in monkeys. In monkeys, C_{max} and AUC values were proportional to the

Figure 5.
The distribution of precipitated radioactivity (AUC_{0-600h}) in tissues and cumulative excretion ratio of 125I-metuzumab after a single i.v. administration in Sprague-Dawley rats. A, the distribution of precipitated radioactivity (AUC_{0-600h}) in tissues/organs after i.v. administration of 10 mg/kg (123.7 Bq/μg) 125I-metuzumab in rats (n = 6 per group). B, the tissues/organs distribution of precipitated radioactivity at 2, 72, 360, and 600 hours after i.v. 10 mg/kg 125I-metuzumab in rats (mean values ± SD, n = 6). The data were expressed as μg equivalent per gram weight of tissues or mL of fluid. C, cumulative excretion ratio of 125I-metuzumab in bile after a single i.v. administration to Sprague-Dawley rats (n = 6). D, the accumulative total radioactivity recovered from urine, feces, and urine plus feces during the examined time (at 600 hours urine and feces, and 24 hours for bile) after a single i.v. administration of 10 mg/kg (123.7 Bq/μg) 125I-metuzumab. The data are the mean ± SD of the results for 6 rats.
administered dose in the range from 1–25 mg/kg. The estimated nonspecific clearance of metuzumab was 12.72 ml/day/kg and was approximately two-fold greater than what is typically observed from a human IgG1.

The repeat administrations revealed that metuzumab exhibited a slight tendency to accumulate in the monkeys after successive i.v. administration over 13 weeks with an interval of 1 week between doses. The TCA-perceptible radioactivity was widely distributed to most of the tissues/organs examined and sharply decreased with time after a single i.v. administration in rats. The immunohistochernical staining results of CD147 in multiple types of tumor tissue indicated that metuzumab recognized and specifically bound CD147. Excretion via urinary and feces represented the dominant route of elimination following i.v. administration; conversely, bile radioactivity was low.

The safety and toxicity of metuzumab had been evaluated in rats and in cynomolgus monkeys, including both single- and repeat-dose evaluations. The results demonstrated that metuzumab was well tolerated by both rats and monkeys (Supplementary Table S3–S6). No mortalities, significantly adverse reactions, or toxicities were observed in the treated animals. No changes in blood temperature, food consumption, platelet counts were observed. No respiratory, cardiovascular, ophthalmology, or central nervous system impairments were noted in any of treated animals. The NOAEL was estimated to be 200 mg/kg in rats and monkeys. Transient increases in AST or CK levels were found in some monkeys, although no macroscopic or microscopic pathologic changes were associated with antibody. Notably, CD147 is an erythrocyte receptor for PfRH5 and essential for erythrocyte invasion (41, 42). Metuzumab, as an anti-CD147 mAb, may induce adverse side effects in liver and blood compartments; however, toxicity study results showed that it was well tolerated in rat and monkey models. Some clinical trials also suggested that anti-CD147 therapy appears to be a safe and effective treatment of cancer (23, 24, 43–45). Overall, careful attention should be focused on monitoring blood, liver, and muscle function impairments in clinical trials.

In conclusion, the glycoengineered CD147 mAb metuzumab has greatly enhanced ADCC effector function and is active in vitro against multitype lung cancer cell lines. The pharmacokinetics of metuzumab was nonlinear in mouse and monkey. The results from this preclinical study suggest that metuzumab is safe and acceptably tolerated and exhibits potential efficacy in tumor growth inhibition in mouse xenograft models. Phase I/II clinical trials evaluating metuzumab alone or combined with chemotherapeutics in treating Chinese patients with NSCLC are currently in progress.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Chen, Z. Zhang, Q. Sun, M. Huhe, L. Mi Development of methodology: Z. Zhang, Y. Zhang, Q. Sun, F. Feng, M. Huhe, L. Mi Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Zhang, Y. Zhang, Q. Sun, F. Feng, M. Huhe Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Zhang, Y. Zhang, Q. Sun, M. Huhe, L. Mi Writing, review, and/or revision of the manuscript: Z. Chen, Z. Zhang, F. Feng Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Zhang, Q. Sun, L. Mi Study supervision: Y. Zhang, F. Feng, L. Mi

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