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ORIGINAL ARTICLE

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Correlation of pharmacokinetics with the antitumor activity of Cetuximab in nude mice bearing the GEO human colon carcinoma xenograft

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Abstract *Purpose*: The epidermal growth factor receptor (EGFR), a protein tyrosine kinase expressed in many types of human cancers including colon and breast, has been strongly associated with tumor progression. Cetuximab, an IgG1 anti-EGFR chimeric mouse/human monoclonal antibody, has been proven to be effective in the treatment of advanced colon cancer. To date, there has not been a study to systematically evaluate the pharmacokinetics (PK) of Cetuximab in a preclinical model and to further explore any correlation of drug exposure between animal models and cancer patients. In the present study, we characterized the PK of Cetuximab in nude mice at efficacious dose levels and further compared the preclinical optimal dose and active plasma drug concentration with those determined in clinical studies. *Experimental design*: The antitumor activity of Cetuximab was evaluated using the GEO human colon carcinoma xenografts implanted subcutaneously in nude mice. The drug was administered ip every 3 days for five total injections (inj) (q3dx5) at dose levels ranging from 1 mg/inj to 0.04 mg/inj. The plasma PK of Cetuximab was determined at dose levels of 1.0, 0.25, and 0.04 mg/inj with a single bolus iv or ip administration in nude mice. The tumoral PK of Cetuximab was determined at dose levels of 0.25, and 0.04 mg/inj with a single bolus ip administration in nude mice bearing GEO tumor xenografts. The plasma and tumoral levels of Cetuximab were quantitated by an ELISA assay. *Results*: Cetuximab demonstrated a dose-dependent antitumor activity at dose levels of 0.25, 0.1, and 0.04 mg/inj, with a statistically significant tumor growth delay (in reaching a tumor target size of 1 gm) of 18 days ($P < 0.001$), 12.3 days ($P < 0.01$), and 10 days ($P < 0.01$) for 0.25, 0.1, and 0.04 mg/inj, respectively. A separate study employing the same treatment schedule showed that Cetuximab was equally active at dose levels ranging from 0.25 mg/inj to 1 mg/inj. Therefore, dose levels of Cetuximab from 1 mg/inj to 0.04 mg/inj can be considered to be within the efficacious range, while dose levels of 0.25 mg/inj or higher appeared to be optimal for the antitumor activity of Cetuximab in the GEO tumor model. When Cetuximab was given iv to mice, the elimination half life ($t_{1/2}$) was 39.6, 37.8, and 42.2 h for doses of 1.0, 0.25, and 0.04 mg/inj, respectively, suggesting a similar disposition kinetics of Cetuximab within this dose range. The volume of distribution (V_d) ranged from 0.062 l/kg to 0.070 l/kg, suggesting that Cetuximab is primarily confined to the plasma compartment with limited peripheral tissue distribution. Clearance (CL) was similar and no apparent PK saturation was observed across the dose ranging from 0.04 mg/inj to 1.0 mg/inj. When mice were administered with a single bolus ip administration at doses of 1, 0.25, and 0.04 mg/inj, the maximum plasma concentration (C_{max}) was 407.6, 66.4, and 16.5 μg/ml. The area under the curve of plasma drug concentration (AUC) was 19212.4, 3182.4, and 534.5 μg/ml h, for 1.0, 0.25, and 0.04 mg/inj, respectively. The average steady state plasma concentration ($C_{ss\ avg}$) for the multiple dosing schedule was estimated to be 73.1 μg/ml at 0.25 mg/inj and

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was considered as an active plasma drug concentration. The maximum tumoral concentration of Cetuximab was 2.6 and 0.53 ng/mg-tumor while the tumoral drug exposure was 112.6 and 18.3 ng/mg h for 0.25 and 0.04 mg/inj, respectively. The EGFR was estimated to be nearly completely occupied by Cetuximab at the optimal dose of 0.25 mg/inj. *Conclusion*: In the present study, we compared the preclinical optimal dose and the corresponding active plasma concentration determined in mice with those being observed in cancer patients, i.e. 65–100 μg/ml. The preclinical optimal dose of 0.25 mg/inj was significantly lower than the current clinical dose. However, the active plasma concentration at 0.25 mg/inj is within the range of the active drug concentrations in cancer patients treated with Cetuximab under the current optimal dosing regimen. It appears that the active plasma drug concentration determined in preclinical model predicts better than the optimal preclinical dose for the clinical development of antibody drugs.

Keywords Pharmacokinetics · Pharmacodynamics · Biomarker · EGFR · Cetuximab (Erbitux · C-225)

Introduction

The epidermal growth factor receptor EGFR is a 170-kda plasma membrane glycoprotein composed of an extracellular ligand-binding domain, a transmembrane lipophilic segment, and an intracellular protein kinase domain with a regulatory carboxyl terminal segment [1]. Upon binding of ligand, EGFR receptors dimerize, which results in high affinity ligand binding, activation of the intrinsic protein kinase activity, and tyrosine autophosphorylation of the intracellular domain [1]. The activation of the EGFR kinase initiates a cascade of intracellular signal transduction events which are not only critical for cell proliferation, but also drive other processes including angiogenesis, metastasis, and the inhibition of apoptosis [2–7]. Since EGFR pathways are commonly deregulated in human epithelial tumors, therapeutic agents directed at the EGFR, such as monoclonal antibody against extracellular ligand-binding domain, represent a promising and important group of molecularly targeted therapies that are in various stages of preclinical and clinical development [8–10]. After extensive proof of principal studies demonstrating therapeutic benefit with mouse monoclonal antibodies [11–15], a chimeric human–mouse antibody, Cetuximab (IMC-225, Erbitux) containing the human IgG1 constant region, has recently been developed for clinical use in order to avoid human immunoresponse to mouse antibody that can interfere with the therapeutic efficacy with repeated administrations [7, 16, 17]. Cetuximab binds to the EGFR with a greater affinity than the EGFR natural ligands and is able to block the ligand-induced autophosphorylation and activation of the EGFR kinase in cells in vitro [17, 18]. It also induces dimerization and internalization of the EGFR, which prevents further receptor binding and activation by the ligands [11–13, 18, 19]. Cetuximab has demonstrated inhibitory potency on the growth of tumor cells in vitro [7, 20–24] as well as human carcinoma xenografts in vivo [17, 18, 20, 25, 26]. In 2004, Cetuximab was approved by FDA for the treatment of advanced colon cancer in combination with CPT-11.

When the PK of Cetuximab was initially evaluated, three consecutive phase I clinical trials had been carried out, in which Cetuximab was administered with three different dosing regimens including (1) a single iv infusion; (2) weekly multiple infusion for 4 weeks, (3) weekly multiple infusion in combination with Cisplatin [27]. All were open-label, dose escalation studies (5, 20, 50, and 100 mg/m^2). Cetuximab was additionally escalated to 200 and 400 mg/m^2 in the combination study with Cisplatin. The maximum tolerated dose (MTD) was not reached in any of these studies. The plasma concentrations of Cetuximab at 5 mg/m^2 were generally below the detection limit, therefore, could not be analyzed. Non-compartmental analysis of data for dose levels of 20, 50, and 100 mg/m^2 showed that the systemic clearance (CL) decreased with increasing dose, whereas the V_d at steady state remained relatively constant and approximately equivalent to the plasma volume. The clearance values among the 20, 50, and 100 mg/m^2 dose groups were statistically different, suggesting that Cetuximab exhibits a non-linear PK within this dose range. At dose levels of 200 and 400 mg/m^2, Cetuximab achieved a complete saturation of CL. Mean circulating plasma levels of Cetuximab were sustained above 29.9 μg/ml beyond the initial dose for patients treated with multiple doses at 200 and 400 mg/m^2 . Plots of concentration versus time at 400 mg/m^2 of Cetuximab are linear over the first 96 h after infusion, indicating a zero-order elimination. An estimated $C_{ss\ avg}$ of 56 μg/ml was achieved for approximately 7 days in patients treated at 400 mg/m^2 . The estimated $t_{1/2}$ of Cetuximab is about 7 days for both 200 mg/m^2 and 400 mg/m^2 dose levels. On the basis of a complete saturation of CL and a likely saturation of the EGFR at dose levels of 200 and 400 mg/m^2, the recommended dosing regimen for subsequent phase II/III trials is a 400 mg/m^2 loading dose, followed by a 250 mg/m^2 weekly maintenance dose.

For many molecularly targeted agents, the appropriate dose continues to be defined by traditional means. For agents such as ZD1839, OSI-774, investigators were able to define an MTD associated with dose limiting toxicity. The dose chosen for further studies was then selected at a lower but tolerable dose, which has evidence of biological or clinical activity [28–30]. However, there remain agents which are associated with minimal toxicities in the clinical setting, such as Cetuximab. In these cases the decision regarding the optimal efficacious dose needs to be based on other criteria or instances where defining the optimal efficacious dose may be warranted in addition to definition of the MTD. In general, Cetuximab appears to be well

tolerated both alone and in combination with cytotoxic agents in clinical studies, so the MTD has not been reached in any of clinical studies in contrast to other small molecule signal transduction inhibitors. The current dosing regimen, a 400 mg/m^2 loading dose followed by a 250 mg/m^2 weekly maintenance dose, was recommended based on the clinical observation of nonlinear PK of Cetuximab in the dose range of 200–400 mg/m^2 being associated with a complete saturation of CL [27, 31]. However, there has not been enough experimental evidence associated with any clinical study proving the complete inhibitory status of the EGFR under the drug exposure associated with the current clinical dosing regimen. Considering the complexity of tumor biology and human metabolism, it might be over-simplified to equate the saturation of CL to the saturation of the EGFR in tumor. Therefore, there remains a strong need for preclinical studies to predict the optimal efficacious dose or drug exposure for future clinical development of antibody drugs. We proposed a PK/pharmacodynamics (PD) study in an animal model to assess the optimal efficacious dose and the associated drug exposure. Taking advantage of the current status of Cetuximab, we further compared the preclinical optimal dose and drug exposure with those being determined in cancer patients. To the best of our knowledge, this is the first study to systematically characterize the PK parameters of Cetuximab at the efficacious dose range in an animal model and to further correlate the preclinical PK data with those determined in cancer patients. We believe that this study forms the basis for future studies to predict the optimal dose and regimen in humans, at least for antibody drugs, utilizing the PK/PD data determined in preclinical models.

Materials and methods

Chemical reagents

Complete protease inhibitor tablets were from Roche Diagnostics (Indianapolis, IN, USA). Unless otherwise specified, all other chemicals and reagents were from Sigma (St. Louis, MO, USA). Sterile buffers and solutions were obtained from GIBCO/BRL (Carlsbad, CA, USA). Sterile tissue culture were was obtained from Fisher Scientific Co. (Hanover Park, IL, USA).

Animals

Female nude mice, 5–6 weeks of age, were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN, USA), and maintained in an ammonia-free environment in a defined and pathogen-free colony. Animals were quarantined for approximately 3 weeks prior to their use for tumor propagation and drug efficacy testing. They were given food and water ad libitum. All studies were performed in accordance with Bristol-Myers Squibb (BMS) and the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Drug formulation and administration

Cetuximab (lot#: 00C01178) was supplied by ImClone Systems, Inc. (New York, NY, USA) at a concentration of 2 mg/ml in a buffer consisting of 10 mM sodium phosphate and 145 mM sodium chloride at pH 7. For all PK/PD and in vivo efficacy studies requiring lower concentrations of Cetuximab, the stock solution was diluted with sterile phosphate buffered saline (PBS) pH 7.4. Cetuximab was administered intraperitoneally (ip) or intravenously (iv) at a constant volume of 0.5 ml/inj (e.g. 1 mg/0.5 ml/mouse, 0.5 mg/0.5 ml/mouse etc.).

In vivo antitumor testing

Tumors were propagated in nude mice as subcutaneous (sc) transplants using tumor fragments obtained from donor mice. Tumor passage occurred approximately every 2 to 4 weeks. Tumors were then allowed to grow to the pre-determined size range (usually between 100 mg–200 mg, tumors outside the range were excluded) and animals were evenly distributed to various treatment and control groups. Treated animals were checked daily for treatment related toxicity/mortality. Each group of animals was weighed before the initiation of treatment (Wt1) and then again following the last treatment dose (Wt2). The difference in body weight (Wt2–Wt1) provides a measure of treatment-related toxicity. Tumor response was determined by measurement of tumors with a caliper twice a week, until the tumors reached a predetermined target size of 1 g or became necrotic. Tumor weights (mg) were estimated from the formula:

$$\text{Tumor weight} = \frac{\text{length} \times \text{width}^2}{2} \quad (1)$$

Antitumor activity was determined in terms of primary tumor growth inhibition. This was determined in two ways: (A) calculating the relative median tumor weight (MTW) of the treated (T) and the control (C) mice at various time points (effects were expressed as %T/C). (b) calculating the tumor growth delay (T–C value), defined as the difference in time (days) required for the treated tumors (T) to reach a predetermined target size compared to those of the control group (C). Data were evaluated statistically using Gehan's Generalized Wilcoxon test for comparisons of time to reach tumor target size [32]. Statistical significance was declared at $P < 0.05$. Antitumor activity was defined as a continuous MTW %T/C $\leq$ 50% for at least 1 tumor volume doubling time (TVDT) any time after the start of treatment, where TVDT = median time (days) for

control tumors to reach target size—median time (days) for control tumors to reach half the target size. In addition, treatment groups had to be accompanied by a statistically significant tumor growth delay (T–C value, $P < 0.05$) to be termed active. Treated animals were checked daily for treatment related toxicity/mortality. When death occurred, the day of death was recorded. Treated mice dying prior to having their tumors reach target size were considered to have died from drug toxicity. No control mice died bearing tumors less than target size. Treatment groups with more than one death caused by drug toxicity were considered to have had excessively toxic treatments and their data were not included in the evaluation of a compound's antitumor efficacy.

Pharmacokinetic analysis

To characterize the PK of Cetuximab, nude mice ($n = 3$ per time points) were bled by cardiac puncture, following a single iv or ip administration at dose levels of 0.04, 0.25, and 1 mg/inj, at the time points of 0, 0.05, 0.17, 0.5, 1, 3, 6 24, 48, and 72 h. The blood was immediately centrifuged and the plasma was pooled and frozen at −80°C until analysis by an ELISA assay [33]. Briefly, the ELISA assay employed a recombinant human EGFR (extracellular domain) adsorbed onto a microtiter plate to capture Cetuximab in 10% mouse plasma. The captured Cetuximab was then detected using a peroxidase-conjugated affinipure rabbit anti-human IgG Fc fragment. The assay has a calibration range of 0.1–6 ng/ml (1–60 ng/ml in 100% mouse plasma). The lower limit of quantification is 1 ng/ml. Pharmacokinetic data analysis was performed by the noncompartmental method using Kinetica (v4.0.2, InnaPhase Corporation, Philadelphia, PA, USA). C_{max} and the time reaching C_{max} (T_{max}) were determined by visually inspecting the profiles of plasma level of drug vs. time. $T_{1/2}$ was calculated as 0.693 divided by the slope obtained by log-linear regression of the terminal phase of drug plasma profiles. AUC was estimated by the trapezoidal rule. Other pharmacokinetic parameters, including CL, V_d, and $C_{ss\ avg}$ with multiple ip dosing, were calculated by as the following equations:

$$\mathrm{CL} = \frac{D_{iv}}{\mathrm{AUC}_{iv}} \tag{2}$$

$$V_d = \frac{D_{iv}}{C_{0iv}} \tag{3}$$

$$C_{ss\,avg} = \frac{D_{ip}/t}{\mathrm{CL}/F_{ip}} \tag{4}$$

where D is the dose administered, C_0 is the estimated initial plasma concentration by extrapolating drug concentration to time zero after iv bolus administration, and t is the drug dosing interval.

Determination of tumoral levels of Cetuximab

To determine the PK of Cetuximab in tumor, nude mice bearing human GEO tumor ($n = 3$ per time point), were administered ip with a single bolus injection at dose levels of 0.25 and 0.04 mg/inj. Tumors were surgically removed and immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Frozen tumors were then lyzed in ice-cold TTG lysis buffer (1% Triton X-100, 5% glycerol, 20 mM Tris pH 7.7, 1 mM EDTA, 0.15 M NaCl, 1 mM sodium orthovanadate, 40 μM ammonium molybdate and 2% complete protease inhibitor). The total protein concentration of tumor tissue lysate was determined using the MicroBCA method (Pierce, Rockford, IL, USA). The tumor lysate was analyzed with the same ELISA assay mentioned above in order to quantitate the tumoral level of Cetuximab [33]. The values of tumoral C_{max} and T_{max} of Cetuximab were determined by visually inspecting the profile of plasma levels of drug versus time. AUC was estimated by the trapezoidal rule.

Estimation of the EGFR occupancy

It has been known that Cetuximab directly competes the extracellular binding site of the EGFR with its natural ligands [17, 18]. A competitive inhibition was thus assumed for the inhibition of the EGFR kinase by Cetuximab.

Where E stands for the EGFR kinase, S represents natural ligands, and I stands for Cetuximab. Upon reaching the equilibrium, the Michaelis constant K_m can be expressed as the following:

$$\begin{array}{c} E \\ I\downarrow\uparrow \\ EI \end{array} + S \rightleftharpoons {}^{E}ES \tag{5}$$

$$K_m = \frac{[E][S]}{[ES]} \tag{6}$$

The drug dissociation constant K_i can be expressed by Eq. 7:

$$K_i = \frac{[E][I]}{[EI]} \tag{7}$$

The EGFR occupancy can be estimated by the following equation:

$$\mathrm{Occupancy}(\%) = \frac{[EI]}{[ES] + [E]} \tag{8}$$

Equation 8 can be further expressed by substituting Eqs. 6 and 7 as the following:

$$\mathrm{Occupancy}(\%) = \frac{[I]}{K_i + [S] \times K_i/K_m + [I]}. \tag{9}$$

It has been reported that the tissue levels of the EGFR kinase natural ligands are at least 100 fold less than [I] and also K_m is several fold greater than K_i [17, 27, 34, 35]. Therefore, the contribution of [S] × (K_i/K_m) can be neglected and Eq. 9 can be further simplified into Eq. 10:

$$\text{Occupancy}(\%) = \frac{[I]}{K_i + [I]} \quad (10)$$

The EGFR occupancy was estimated using Eq. 10 in this study.

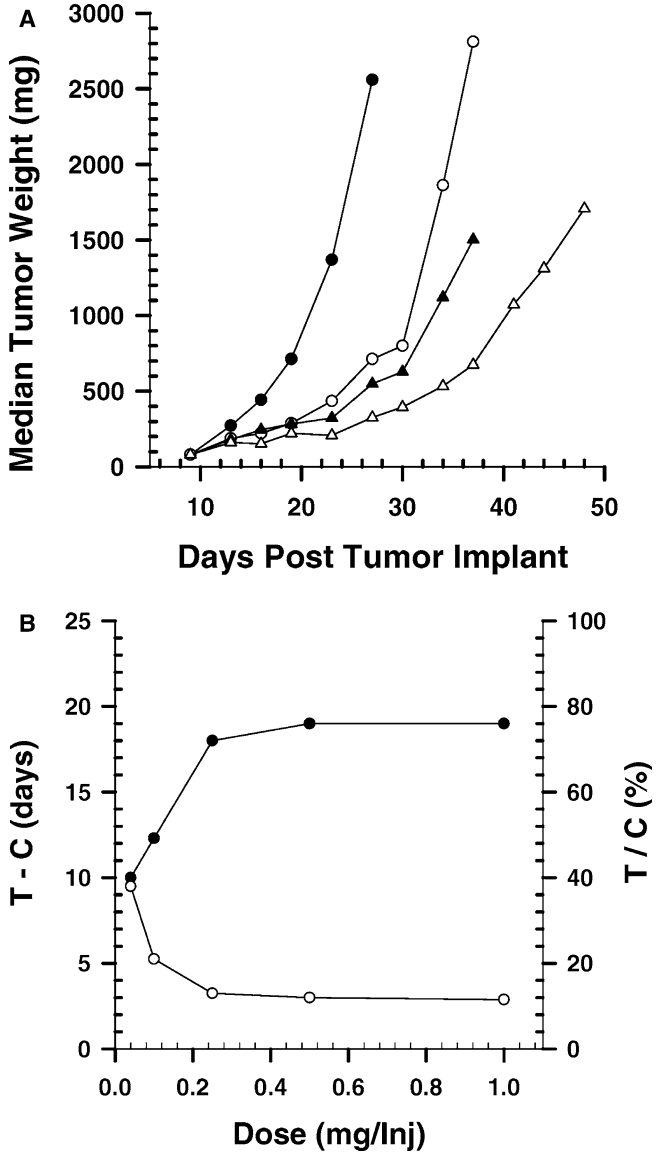

Fig. 1 **A, B** In vivo antitumor activity of cetuximab in nude mice bearing the GEO human colon carcinoma. Mice bearing the GEO human colon tumors were treated at the indicated dosage level when tumors reached ~100–200 mg. Cetuximab was administered ip every three days for five injections (q3dx5). Each symbol represents the median tumor weight of a group of eight mice. **A** Plot of median tumor weight versus time: ● control, △ 0.25 mg/inj, ▲ 0.1 mg/inj, ○ 0.04 mg/inj; **b** Plot of antitumor activity of Cetuximab versus dose: ● T - C at tumor size of 1 g, ○ T / C % at the end of treatment

Results

Antitumor activity of Cetuximab

Previous studies have shown that Cetuximab effectively inhibits GEO human colon carcinoma tumor growth in nude mice [20, 36]. This study was designed to define the optimal efficacious dose of Cetuximab in the same tumor model system. Cetuximab was administered ip on day 11 post-tumor implant (tumor weight of 100–200 mg) using an every 3 days × 5 schedule (q3dx5) at dose levels of 0.25, 0.1, and 0.04 mg/inj. All three dose levels were active and achieved a continuous MTW %T/C ≤ 50% for at least 1 TVDT after the start of treatment (Fig. 1a, 1b). In addition, all three dose levels were associated with a statistically significant delay (T – C) in reaching a tumor target weight of 1 gm, corresponding to 18 days ($P < 0.001$), 12.3 days ($P < 0.01$), and 10 days ($P < 0.01$) for the 0.25, 0.1, and 0.04 mg/inj dose levels, respectively, indicating a dose dependent inhibitory activity of Cetuximab (Fig. 1b). More precise statistical analysis also showed that the 18-day tumor growth delay obtained by the 0.25 mg/inj (q3dx5) dose level was significantly different from the growth delay achieved by the 0.1 and 0.04 mg/inj treatment groups ($P < 0.05$).

A separate study employing the same treatment schedule showed that Cetuximab was equally active at dose levels ranging from 0.25 mg/inj to 1 mg/inj (Fig. 1B). Taking all data together, Cetuximab dose levels from 1 mg/inj to 0.04 mg/inj can be considered to be within the efficacious range while dose levels of 0.25 mg/inj or higher appeared to be optimal for the antitumor activity of Cetuximab in the GEO human colon carcinoma tumor model. Essentially, our data are consistent with those published previously using the same tumor xenograft model [20, 36].

Pharmacokinetics of Cetuximab

An exploratory PK study was conducted for Cetuximab in GEO tumor bearing nude mice following a single bolus ip administration at efficacious dose levels of 1.0, 0.25, 0.1, and 0.04 mg/inj. The mouse blood was sampled at 6 and 24 h and the plasma level of Cetuximab was quantitated by an ELISA assay [33]. The concentrations of Cetuximab achieved in plasma appeared to be dose proportional and were 153.6, 36.3, 11.2, and 3.9 μg/ml, respectively, at 6 h post-treatment for dose levels of 1, 0.25, 0.1, and 0.04 mg/inj. At 24 h post-treatment, the plasma concentrations of Cetuximab were 17.7, 3.1, 1.5, and 0.3 μg/ml, respectively. To fully characterize the relationship between PK and the antitumor activity of Cetuximab, a detailed PK study was conducted in nude mice with a single bolus ip administration at 1, 0.25, and 0.04 mg/inj. The mouse blood was collected at 0, 1, 3, 6, 24, 48, and 72 h and the plasma levels of Cetuximab were determined according to the

same protocol of ELISA validated in the exploratory PK study (Fig. 2). The plasma profiles were analyzed using the software Kinetica, the PK parameters were derived and listed in Table 1. The T_{max} was 3 h at all three doses, indicating a similar absorption kinetics for Cetuximab from the mouse peritoneal cavity to the blood stream. The values of C_{max}were 407.6, 66.4, and 16.5 μg/ml and the AUC was 19212.4, 3182.4, and 534.5 μg/ml h, for dose levels of 1.0, 0.25, and 0.04 mg/inj, respectively. It appeared that drug exposure was dose proportional, which is consistent with our first pilot PK study.

The disposition of Cetuximab was characterized in nude mice following a single bolus iv administration of Cetuximab at dose levels of 1.0, 0.25, and 0.04 mg/inj. The plasma levels of Cetuximab were determined according to the same protocol of ELISA. The plasma profiles were analyzed using the software Kinetica, the PK parameters were derived and also listed in Table 1. Cetuximab exhibited a mono-exponential decay when given iv at doses ranging from 1.0 mg/inj to 0.04 mg/inj (Fig. 3). The $t_{1/2}$ was 39.6 h for 1.0 mg/inj, 37.8 h for 0.25 mg/inj, and 42.2 h for 0.04 mg/inj, respectively, which is not statistically different, suggesting a similar disposition kinetics of Cetuximab within this dose range. The V_d was determined to be 0.062–0.070 l/kg for dose levels ranging from 0.04 mg/inj to 1.0 mg/inj, suggesting that Cetuximab is primarily confined to the plasma compartment with limited peripheral tissue distribution.

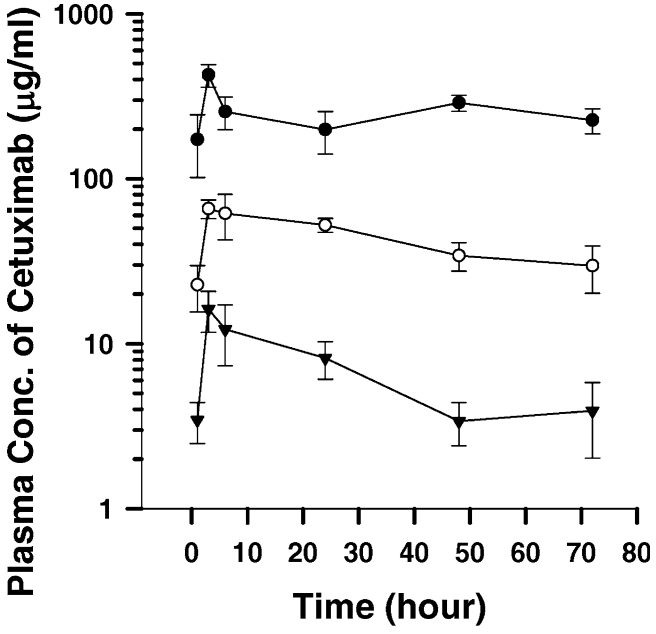

Fig. 2 Plasma pharmacokinetics of cetuximab in the GEO tumor-bearing mice following a single ip administration at 1.0, 0.25, and 0.04 mg/inj. Nude mice, following a single bolus ip administration, were bled by cardiac puncture at 1, 3, 6, 24, 48, and 72 h. The blood was immediately centrifuged, the plasma was pooled and frozen at −80°C until ELISA analysis. Each point represents the mean (±SD) for three observations. ● 1.0 mg/inj, ○ 0.25 mg/inj, ▲ 0.04 mg/inj

Table 1 Summary of pharmacokinetic parameters of cetuximab in mice following a single iv or ip administration at dose levels of 1.0, 0.25 and 0.04 mg/inj

	Plasma	Plasma	Plasma
Dose (mg/inj)	1.0	0.25	0.04
C_{max} (μg/ml)	407.6	66.4	16.5
T_{max} (h)	3.0	3.0	3.0
$AUC_{0\text{-}72\ hr}$ (μg/ml h)	19212.4	3182.4	534.5
IV administration	Plasma	Plasma	Plasma
Dose (mg/inj)	1.0	0.25	0.04
Clearance (ml/min/kg)	0.052	0.055	0.048
$t_{1/2}$ (h)	39.6	37.8	42.2
V_d (l/kg)	0.063	0.070	0.062
$AUC_{0\text{-}48\ hr}$ (μg/ml hr)	16313.6	3828.9	693.8
IP administration	Tumor	Tumor	Tumor
Dose (mg/inj)	N/A	0.25	0.04
C_{max} (ng/mg tumor)	N/A	2.6	0.53
T_{max} (h)	N/A	24	24
$AUC_{0\text{-}72\ hr}$ (ng/mg hr)	N/A	112.6	18.3

The CL was similar and no apparent saturation was observed across doses ranging from 0.04 mg/inj to 1.0 mg/inj.

Estimation of the $C_{ss\ avg}$ with a multiple dosing schedule for Cetuximab

Since Cetuximab does not bind to the mouse EGFR, its distribution is likely confined in the plasma compartment. Therefore, a one-compartment model with the first-order absorption kinetics was chosen for PK modeling. The PK model was simultaneously fitted to the PK data in nude mice administered a single bolus dose of Cetuximab iv or ip at 1.0, 0.25, and 0.4 mg/inj. The absorption constant (k_a) and elimination constant (k_d) were estimated to be 0.44 ± 0.09 and $0.017 \pm 0.002\ h^{-1}$, respectively, the V_d was 0.094 ± 0.003 l/kg. With those defined PK parameters and an assumption of no change of PK parameters for Cetuximab with multiple dosing schedule at dose levels ≤ 1.0 mg/inj, the plasma profile was therefore simulated for the schedule of q3dx5 ip for the dose levels of 1.0, 0.25, and 0.04 mg/inj (Fig. 4). Since Cetuximab had a long half-life in plasma, an accumulated plasma profile was observed as we expected for the in vivo efficacy study when using multiple dosing schedules. The value of $C_{ss\ avg}$ of Cetuximab was estimated to be 336.3, 73.1, and 11.9 μg/ml for dose levels of 1.0, 0.25, and 0.04 mg/inj, respectively.

Estimation of the EGFR occupancy by Cetuximab in tumor

The levels of Cetuximab in GEO tumor were also quantitated in order to estimate the EGFR occupancy by Cetuximab in vivo. Upon administration of Cetuximab to tumor bearing mice, tumors were surgically removed and lyzed in ice-cold TTG lysis buffer (1% Triton

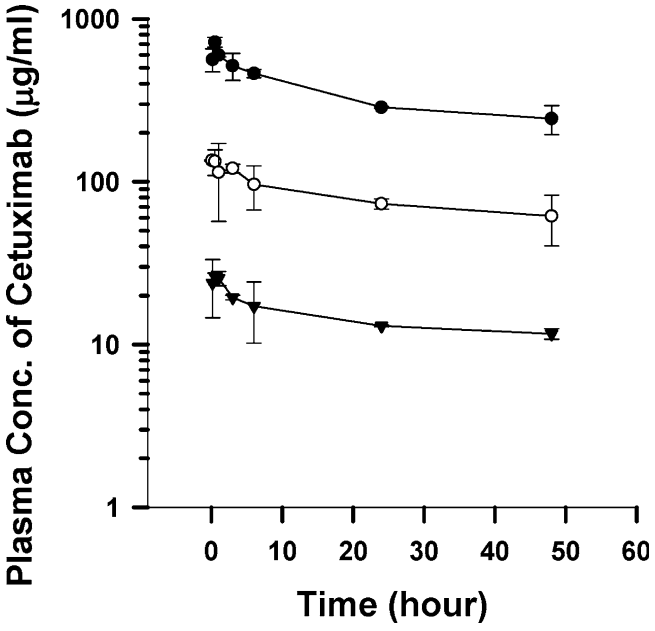

Fig. 3 Plasma pharmacokinetics of cetuximab in nude mice following a single iv administration at 1.0, 0.25, and 0.04 mg/inj. Nude mice, following a single bolus iv administration, were bled by cardiac puncture at 0.5, 1, 3, 6, 24, and 48 h. The blood was immediately centrifuged, the plasma was pooled and frozen at −80°C until ELISA analysis. Each point represents the mean (±SD) for three observations. ● 1.0 mg/inj, ○ 0.25 mg/inj, ▲ 0.04 mg/inj

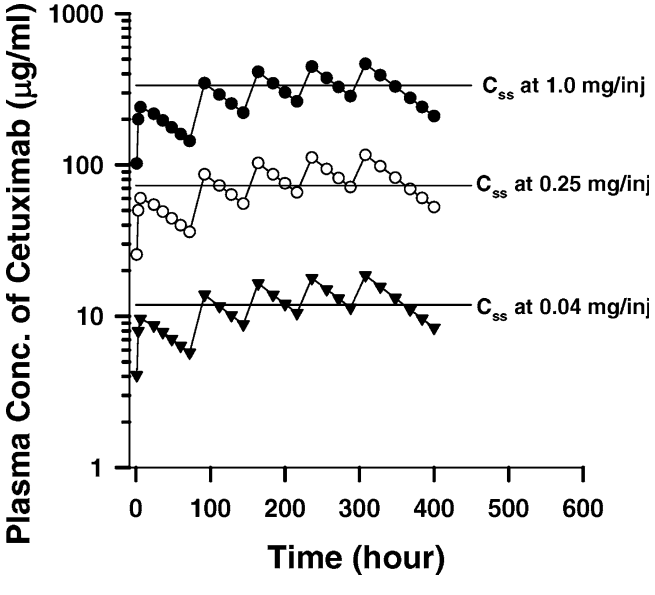

Fig. 4 Simulation of plasma levels of cetuximab in mice with a dosing schedule of q3dx5 at 1.0, 0.25, and 0.04 mg/inj. The pharmacokinetic data were simultaneously fitted using a one-compartment model with first-order absorption kinetics. The plasma profile was simulated based on an assumption of linear pharmacokinetics for cetuximab with multiple administrations at dose range of 1.0–0.04 mg/inj. ● 1.0 mg/inj, ○ 0.25 mg/inj, ▲ 0.04 mg/inj

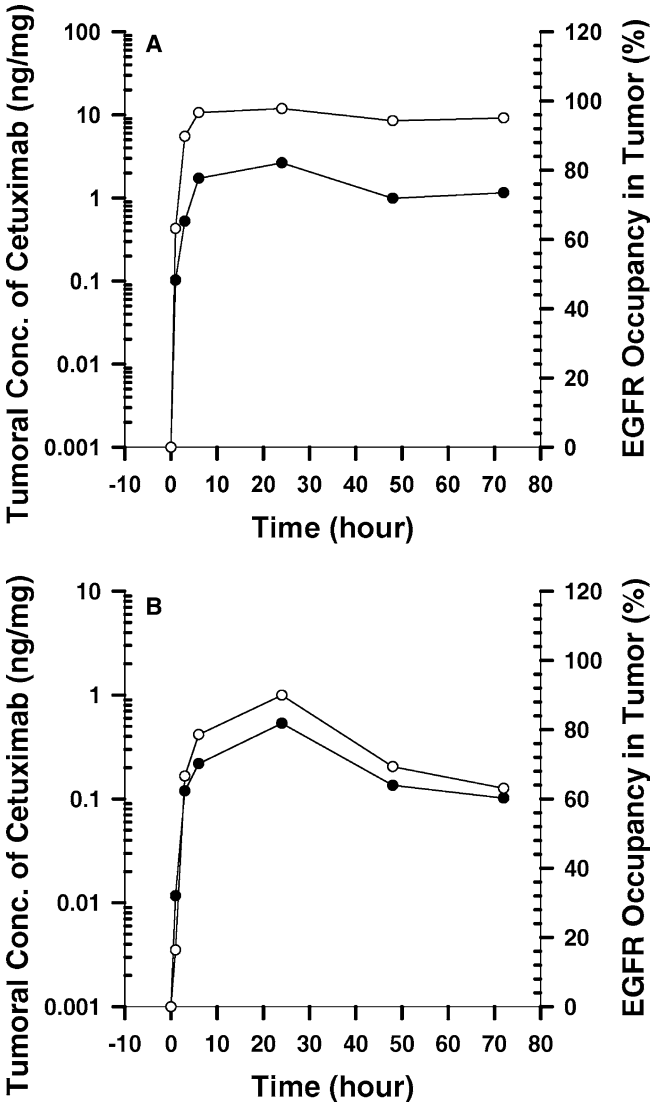

Fig. 5 A, B Tumoral pharmacokinetics of cetuximab in the GEO tumor-bearing mice following a single ip administration at 0.25 and 0.04 mg/inj. Following a single ip administration, the tumor was surgically removed at 1, 3, 6, 24, 48, and 72 h and immediately frozen at −80°C until ELISA analysis. Each point represents the mean (±SD) for three observations. **A** 0.25 mg/inj: ● tumoral concentration of cetuximab, ○ % EGFR occupancy; **b** 0.04 mg/inj: ● tumoral concentration of cetuximab, ○ % EGFR occupancy

X-100, 5% glycerol, 20 mM Tris pH 7.7, 1 mM EDTA, 0.15 M NaCl, 1 mM sodium orthovanadate, 40 μM ammonium molybdate and 2% Complete protease inhibitor). The tumor lysate was isolated by centrifugation and analyzed by the ELISA assay in order to quantitate tumoral concentrations of Cetuximab. It was determined that the concentrations of Cetuximab in tumor lysate was 50–100-fold less than those in plasma (Fig. 5a and 5b). This could suggest a low tumor tissue distribution of Cetuximab. However, a previous study with ^{111} In-labeled mouse version of Cetuximab demonstrated that, when given to nude mice bearing subcutaneous xenografts, the total level of Cetuximab was several fold higher in tumor than in blood [37].

Therefore, the tumoral concentrations of Cetuximab measured in our study are less likely to represent the total levels of Cetuximab in tumor. We suspected that our tumor lysis buffer was unable to dissociate Cetuximab from its binding to receptor and therefore Cetuximab in the tumor lysate represented unbound or free drug. We thus conducted a separate study which had confirmed our hypothesis (data not shown). Therefore, the levels of Cetuximab in tumor lysate measured in this study likely represented the tumoral levels of unbound Cetuximab after an equilibrium was reached among the receptor, drug, and natural ligands. The EGFR occupancy was estimated using the Eq. 10 and was found to correlate with the tumoral levels of Cetuximab in a dose dependent manner. At 0.25 mg/inj, the EGFR in tumor was nearly 100% occupied by Cetuximab between 7 h and 72 h. However, at 0.04 mg/inj, the maximal occupancy of the EGFR by Cetuximab was 84% at 24 h, while the occupancy gradually decreased between 24 h and 72 h and remained 62% at 72 h. In general, the time course of the estimated EGFR occupancy correlated with both plasma and tumoral levels of Cetuximab. The estimated EGFR occupancy is essentially consistent with our efficacy study in which 0.25 mg/inj was found to be optimal, i.e. with almost 100% of the EGFR occupancy, while 0.04 mg/inj was sub-optimal with less degree of the receptor occupancy.

Discussion

For antibody drugs such as Cetuximab with minimal toxicities, the optimal efficacious dose may not be characterized by traditional means defining MTD [28–30]. The current dosing regimen for Cetuximab, a 400 mg/m^2 loading dose followed by a 250 mg/m^2 weekly maintenance dose, was recommended based on the clinical observation of nonlinear PK in the dose range of 200–400 mg/m^2 being associated with a complete saturation of CL [27, 31] in human. It is believed that Cetuximab, once administered in the systemic circulation in human, will bind to the EGFR in a large number of tissues, such as skin and liver, followed by receptor internalization of the antibody receptor complex, which represents the major route of clearance for Cetuximab. Therefore, saturation of CL was assumed to equate to saturation of the EGFR in body tissues in clinical studies of Cetuximab. However, there has not been adequate experimental evidence to equate saturation of CL to the saturation of the target in tumor by Cetuximab. Moreover, the functionally activated EGFR, the predominant driving force for cell proliferation in Cetuximab responsive tumors, could be a portion of the total EGFR. This would further imply that the dose required to saturate CL or the total EGFR in tissues could be different from the one required to inhibit the activity of the functional EGFR in tumor. Even though the current clinical dosing regimen appeared to be acceptable in terms of efficacy and tolerable side effects, it may still not be economically optimal if it is much greater than the one required just to inhibit the functional EGFR. Cost effectiveness should always be one of factors being fconsidered together with efficacy and toxicity when patient compliance has an impact on the marketing of a drug. We are making this argument here by no means to tackle the current clinical dosing regimen of Cetuximab, but we do intend to advocate that more preclinical PK/PD studies be carried out to lay a solid foundation for defining the optimal efficacious dose or drug exposure for drug clinical development. It is our hope and also our eventual goal that the optimal clinical dose for targeted therapy could also be both biologically and economically optimal. The drug discovery, clinical development, and marketing of the drug should be harmonized to a certain extent at an early stage.

In a phase I study of Cetuximab in which cancer patients were treated with multiple weekly dosing at 200 mg/m^2, the mean circulating plasma level of Cetuximab (from 3 patients) was sustained above 30 μg/ml beyond the initial dose. At multiple weekly dose of 400 mg/m^2, the $C_{ss\ avg}$ (from 4 patients) was estimated to be 56 μg/ml [27]. In a separate phase I study employing the schedule of 400 mg/m^2 loading dose followed by a 250 mg/m^2 weekly maintenance dose, the mean peak and trough plasma levels of Cetuximab (from 3 patients) were estimated to be 192 and 75 μg/ml, respectively while the $C_{ss\ avg}$ was estimated to be around 100 μg/ml [38]. The PK data are generally in agreement considering the size of cohort from two separate studies. Based on the PK data from those two studies, we estimated that the $C_{ss\ avg}$ of Cetuximab in cancer patients is probably within the range of 56–100 μg/ml under the current clinical dosing regimen.

Our preclinical study has demonstrated a dose dependent tumor growth inhibition in mice bearing GEO human colon tumor xenografts at dose levels ranging from 0.04 mg/inj to 0.25 mg/inj. Since it is also observed that Cetuximab is about equally active within a dose range of 0.25–1.0 mg/inj in the same xenograft model, 0.25 mg/inj is thus recommended as a preclinical optimal dose. This is consistent with the estimated status of the receptor occupancy in this study, i.e. the EGFR is nearly 100% occupied by Cetuximab at 0.25 mg/inj. In line with the observation 0.04 mg/inj is a sub-optimal dose with a lesser degree of the EGFR occupancy. The preclinical optimal efficacious dose of 0.25 mg/inj, if scaled up based on the formula recommended by Freireich et al. [39] with an assumption of a 70 kg average body weight of patients, is equivalent to a clinical dose of 37.5 mg/m^2, which is well below the current clinical dose of Cetuximab. This probably can be explained by the different disposition kinetics of Cetuximab between mouse and human. Since Cetuximab only binds to the human but not to the mouse EGFR, a significant amount of Cetuximab, when given to cancer patients, will be required to saturate the EGFR in a large number of tissues, such as skin and liver, followed by receptor

internalization of the antibody receptor complex. Therefore, it is likely that the discrepancy of the optimal efficacious dose between preclinical mouse model and cancer patients is due to different routes of drug clearance, which significantly confounds the prediction of the optimal clinical dose based on the preclinical data.

However, it became encouraging when the active plasma concentration of Cetuximab was used as a link between our study and clinical studies. Given the fact that the plasma drug concentration is a convoluted outcome of drug absorption and disposition and is directly correlated with target inhibition, we hypothesized that the active plasma concentration is more likely comparable between mice and human for Cetuximab which has minimal plasma protein binding. The estimated $C_{ss\ avg}$ is 73.1 μg/ml for Cetuximab in our study at the optimal preclinical dose of 0.25 mg/inj, and this $C_{ss\ avg}$ is therefore considered as an optimal efficacious plasma concentration. Since no further tumor growth inhibition can be achieved at any dose higher than 0.25 mg/inj in our study, it is likely that a complete in vivo inhibition of the active EGFR in GEO tumor would be achieved at the plasma concentrations of Cetuximab ≥73 μg/ml. The estimated $C_{ss\ avg}$ of Cetuximab in patients is within the range of 56–100 μg/ml under the current clinical dosing regimen, our preclinical $C_{ss\ avg}$ value is therefore fairly comparable to active drug concentrations achieved in cancer patients. We further speculate that the EGFR function in tumors is probably either nearly or completely blocked by Cetuximab in cancer patients under the current clinical dosing regimen.

Overall, our study demonstrates that the active plasma drug concentration determined in the preclinical model has more predicting power compared to the preclinical optimal dose when used to predict the clinical outcome for Cetuximab. The clinical dose that achieves the active plasma drug concentration should consequently be considered as the optimal dose in drug escalation study in clinics. However, we would view this study as one of the successful cases to predict the clinical scenario for an antibody drug, but do not intend to generalize the current model due to the complexity of biological system, especially tumor heterogeneity and tumor drug resistance. More detailed studies are warranted to investigate the PD biomarkers for Cetuximab, for example, the inhibitory status of target function in tumor, the subsequent inhibition of signaling pathway, and tumor cell cycle arrest induced by drug targeting. Implementing those PD biomarkers into a PD model coupled with a PK model, we could be closer than ever before to predicting the optimal efficacious dose for biological drugs. There is no doubt that the established preclinical PK/PD model will have a positive impact on the clinical development of molecularly targeted agents, especially small molecule kinase inhibitors, so that patients could fully benefit from therapeutic efficacy while suffering minimal side effects at the optimal biological dose instead of the MTD.

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