Supplemental data for Lammerts van Bueren et al.

Pharmacokinetic analyses. A mono-exponential curve-fit was used to determine elimination half-life of 2F8 pharmacokinetics in mice after a single intraperitoneal dose. For the monkey studies a different approach was chosen, since mono- or bi-exponential curve-fitting appeared to be inappropriate. First, we estimated pharmacokinetic parameters AUC(0-∞), Kel and CL by noncompartmental analysis using using WinNonlin Professional v3.1 software (Pharsight Corporation, Mountain View, CA, USA). The area under the curve (AUC; serum drug concentration versus time plot) from time zero to infinity and clearance (CL). AUC(0-∞) was calculated with the formula $AUC(0\text{-}\infty) = AUC(0\text{-}t) + [\,Ct/Kel\,]$ where Ct is serum concentration at time 't' and Kel terminal elimination rate constant. CL, the apparent volume of the central compartment cleared of test item per unit time and per unit body weight, was calculated with the formula: $CL = [Dose/AUC(0\text{-}\infty)]$

Pharmacokinetic model. A pharmacokinetic model was developed to describe the proposed mechanisms ruling the dose-dependency of the antibody clearance in monkeys. As a basis we adopted an open two-compartment model which is well established for describing the pharmacokinetic behavior of IgG (20, 21). This model, depicted in figure 1, represents a rapid mixing of intravenously infused IgG in the plasma compartment, from which it is redistributed by approximately 50% into a second compartment. Elimination occurs from the central plasma compartment by a non-saturable mechanism. The physiological basis of the redistribution is the extravasation into the interstitial space via paracellular and transcellular movement of antibody and return to plasma via the lymphatic system. Elimination corresponds to fluid-phase uptake and degradation throughout the body, likely mainly by endothelial cells. This model is characterized by linear pharmacokinetics and is probably applicable on therapeutic antibodies for which target binding is negligible.

A second route of clearance, which is saturable, was introduced for describing the non-linear clearance behavior. As shown in figure 1, a binding compartment was added which represents EGFr expressed on cell membranes and which has a certain binding maximum at full saturation (Bmax). In the model, bound antibody is eliminated with a certain rate constant (k_{deg}), which represents internalization and degradation. In fact, antibody will be transferred from plasma to the interstitial space surrounding the EGFr-expressing cells before it can bind to the receptors. However, it is difficult to make a detailed and realistic model for the antibody transfer from plasma to the local interstitial compartment and back since there are too many unknowns. For example, the volume of the local interstitial compartment is largely unknown, diffusion coefficients are unknown. Therefore, it was chosen to base the binding process primarily on the experimentally determined equilibrium binding curve for 2F8 to EGFr-expressing cells in vitro, which had an EC50 of 0.5 µg/ml. An arbitrary rate constant for binding and dissociation (k_b = -0.069 hour^{-1}) is introduced to account for the fact that the transfer through the interstitial space slows down equilibration. In the resulting model Bmax and k_{deg} are the principal parameters determining the additional clearance.

Changes in the amounts of antibody (mg) present in the central plasma compartment and interstitial space and bound to EGFr were calculated by numerical integration using the following equations:

Δ A-pl = (T-inf - T-pi + T-ip - T-el) $\cdot$ Δt

Δ A-int = (T-pi - T-ip - T-b) $\cdot$ Δt

Δ A-b = (T-b - T-deg) $\cdot$ Δt

The antibody transfers per interval (mg/Δt) were calculated as:

T-el = A-pl $\cdot$ (exp (FCR $\cdot$ Δt) – 1)

T-pi = A-pl $\cdot$ (exp (k_{ip} $\cdot$ Δt) – 1)

T-ip = A-int $\cdot$ (exp (k_{pi} $\cdot$ Δt) – 1)

T-deg = A-b $\cdot$ (exp (k_{deg} $\cdot$ Δt) − 1)

T-b = (1-exp(k_b $\cdot$ Δt)) $\cdot$ (A-b$_{eq}$ − A-b$_{actual}$),

in which A-b$_{eq}$ = Bmax / (1+ (EC50 / C-int) ^{HillSlope})

A four-parameter logistic equation was used to describe the concentration-dependent saturation

of binding after full equilibration. The concentration for half-maximal binding (EC50) and the

hill slope of 0.5 µg/ml and 0.8, respectively, were derived from experimental data for 2F8

binding to EGFr-expressing cells in vitro. The concentration in the interstitial space was

approximated by assuming an arbitrarily chosen volume of 80 ml/kg. An arbitrary rate constant

for binding (k_b) was introduced to obtain a slowed establishment of equilibrium conditions.

Negative values for T-b indicate dissociation.

After equal redistribution of the mAb over V-pl and V-int, the elimination half-life (T1/2$_{el}$)

relates to FCR as: $T_{\frac{1}{2}el}$ = [ln(2)/(*FCR*/2)·24] (days).

As an alternative route for mAb binding and degradation, a clearance route from the central

compartment was described with the Michalis-Menten equation, characterized by a maximum

rate of elimination (Vmax) and a mAb concentration for half-maximum elimination (Km). The

antibody transfer (mg/Δt) via this route was calculated as: T-mm = (Vmax $\cdot$ A-pl) **/** (Km + A-

pl)

Calculations were performed in an Excel (Microsoft) worksheet with time steps (Δt) ranging

from 0.1 to 0.5 hours. Model parameters were optimized by minimizing the 1/Y^2-weighted

sum-of-squares using the Excel's Solver add-in.

Table I. Nomenclature used in the pharmacokinetic model

 Model parameters

BW   body weight, (kg)

Dose   antibody infused over a certain period of time (mg.hour^{-1}),

V-pl   volume central compartment (ml.kg^{-1})

V-int   volume interstitial compartment (ml.kg^{-1})

FCR   plasma clearance as fraction of intravascular pool per hour (hour^{-1})

k_{pi} rate constant for transfer from plasma to interstitial space (hour^{-1})

k_{ip} rate constant for transfer from interstitial space to plasma (hour^{-1})

Bmax   binding max, amount of HuMax-EGFr that binds at saturating conc.

            (mg.kg^{-1})

EC50   concentration of half-maximal binding (µg.ml^{-1})

K_b rate constant for binding to and dissociation from EGFr (hour^{-1})

k_{deg} rate constant for elimination by EGFr internalization and degradation

            (hour^{-1})

Antibody quantities and concentrations

A-pl (t)   amount of antibody in central compartment (plasma) (mg)

C-pl (t)   concentration of antibody in plasma (µg.ml^{-1})

A-int (t)   amount of antibody in total interstitial space (mg)

A-b (t)   amount of antibody bound to EGFr (mg)

Antibody transfer per time interval

T-inf (Δt)   Infused antibody (mg.hour^{-1})

T-el (Δt)   mAb elimination from plasma (mg.hour^{-1})

T-pi (Δt)   extravasated from plasma into interstitium (mg.hour^{-1})

T-ip (Δt)   returned from interstitium to plasma (mg.hour^{-1})

T-b (Δt)   binding to or dissociation from receptor (mg.hour^{-1})

T-deg (Δt)   eliminated via receptor internalization and degradation (mg.hour^{-1})

Monkey pharmacokinetics studies. Serial blood samples were drawn from 2F8-treated cynomolgus monkeys for the determination of mAb serum concentrations and for the determination of antibody formation against 2F8 (primate anti-human antibodies [PAHA]). Plasma concentrations of 2F8 and PAHA levels were determined by ELISA.

Mouse pharmacokinetics study. Plasma clearance of 2F8 was studied in an experiment with three mice that received a single i.p. injection of 2F8 at a dose of 4 mg/kg, to which an equal amount of human IgG1 anti-KLH control antibody was admixed for comparison. Blood samples were taken at predetermined intervals over a period of 3 weeks. Plasma concentrations of 2F8 and anti-KLH were determined by ELISA.

PAHA formation. Measurement of PAHA was performed on monkey serum samples obtained pre-trial, prior to dosing and on days 8, 15, 21, 31 and 36. Briefly, 96-well Microlon ELISA plates (Greiner, Germany) were coated with Fab' fragments of 2F8. Cynomolgus monkey serum was added in 1:10 - 1:400 dilutions. PAHA were detected with peroxidase-conjugated goat anti-human IgG (Jackson, West Grace, PA) and developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, Mannheim, Germany). Negative control samples of normal cynomolgus monkey serum gave a titer of 1:200. Cut point of the assay was set at 1:400 dilution of the samples.

ELISA to determine 2F8 concentration. 2F8 concentrations were determined by ELISA as previously described by Bleeker et al. (19). Briefly, purified EGFr (Sigma, St Louis, MO) was

coated onto ELISA plates (Greiner). After blocking plates with ELISA buffer (PBS

supplemented with 0.05% Tween-20 and 2% chicken serum), samples were incubated at room

temperature for 1 h. Plates were incubated with peroxidase-labeled goat anti-human IgG Fc-

specific immunoglobulin (Jackson) and developed with ABTS (Roche). Absorbance was

measured in a microplate reader (Biotek, Winooski, VT) at 405 nm. 2F8 plasma concentrations

were calculated with KC4 software using a non-linear 4 parameter logistic curve fit and

OD405nm measurements of a serial diluted 2F8 reference. Limit of detection (background plus

2 standard deviations) and limit of quantification of the ELISA was 1.6 ng/ml 2F8 (< 20% CV).

Assay precision was 5.1% CV for 6 replicates of 5 ng/ml 2F8 spiked monkey serum samples.