An Enzyme-Linked Immunosorbent Assay for Therapeutic Drug Monitoring of Cetuximab

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Abstract: Cetuximab is an anti–epidermal growth factor receptor monoclonal antibody used in the treatment of colorectal and head and neck cancers. Part of the interindividual differences in response may be explained by interindividual variability in pharmacokinetics. An assay measuring cetuximab serum concentrations is therefore needed. An enzyme-linked immunosorbent assay was developed using microtiter plates sensitized with a recombinant human epidermal growth factor receptor extracellular domain. Lower and upper limits of quantitation and limit of detection were determined. Eight standard calibrators (SCs) and 3 quality controls (QCs), that is, 0.75, 7.5, and 15 mg/L, were tested on 5 occasions on 1 day and on 5 occasions on different days. Trough and peak serum concentrations of cetuximab were measured in 15 patients with metastatic colorectal cancer and 1 patient with undetermined neoplasia undergoing cetuximab-based chemotherapy. Cetuximab concentrations were described using a 2-compartment population pharmacokinetic model. Imprecision and accuracy of SC and QC were ≤20%, except for the 0 and 0.1 mg/L SC concentrations (≤20%). The limit of detection was 0.012 mg/L. Lower and upper limits of quantitation were 0.75 and 15 mg/L, respectively. A total number of 198 blood samples were available from the 16 patients. Median (range) trough and peak concentrations during the treatment were 49.6 (5.8–105.4) and 177.2 (97.5–235) mg/L, respectively. This method is rapid, accurate, and reproducible and may be useful for pharmacokinetic and pharmacokinetic-pharmacodynamic studies, as well as in therapeutic drug monitoring of cetuximab.

Key Words: cetuximab, enzyme-linked immunosorbent assay, pharmacokinetics

(*Ther Drug Monit* 2009;31:597–601)

INTRODUCTION

Cetuximab is a 170 KDa human–mouse chimeric immunoglobulin (Ig) G1κ monoclonal antibody (mAb), selectively directed against the extracellular domain III of the epidermal growth factor receptor (EGFR), also known as HER1 or ErbB1.[1] EGFR is a tyrosine kinase receptor triggering a complex intracellular signaling network, including the mitogen-activated protein kinases, phosphatidylinositol-3 kinase, and the signal transducer and activator of transcription pathways.[2] EGFR is constitutively expressed in many epithelial tissues and is overexpressed in many malignancies such as colorectal, head and neck, and lung cancers. The consequences of EGFR overexpression in tumor cells are increased cellular proliferation, angiogenesis, metastatic capacity,[2] and poorer prognosis. Cetuximab binds to EGFR with high affinity and antagonizes endogenous ligands; it is therefore a potent inhibitor of EGFR-mediated signaling pathways in tumor cells. This mAb inhibits tumor growth efficiently and has a synergistic antitumor activity with conventional anticancer drugs[3] or radiation therapy.[4] A major cetuximab side effect is a cutaneous acneiform-like rash, due to high EGFR expression in the epidermis.

Since its approval by Food and Drug Administration for cancer treatment in 2004, cetuximab (Erbitux) became widely used in the treatment of colorectal and head and neck cancers. However, cetuximab pharmacokinetics and concentration–effect relationship remain poorly understood. Phase I and II studies[5–9] reported cetuximab trough and peak concentrations of 0–108 mg/L[5] and 130–298 mg/L,[5,6] respectively. Elimination half-life was estimated to be between 40[7] and 168 hours.[8,9] Initial studies reported relationships both between cetuximab cutaneous toxicity and therapeutic effect and between cetuximab concentrations and cutaneous toxicity.[3,10] More recently, Fracasso et al[11] reported an influence of cetuximab concentrations on both skin toxicity and treatment efficacy. These data provide indirect evidence for the potential interest of therapeutic drug monitoring of cetuximab, based on the measurement of its serum concentrations in treated patients. Additional studies are nevertheless required to improve our knowledge of concentration–effect relationship. Most cetuximab pharmacokinetic studies are based on enzyme-linked immunosorbent assays (ELISA) but neither the techniques used nor their validation criteria and results were described.

We report here the development and validation of an ELISA measuring serum cetuximab concentrations in treated patients.

PATIENTS, MATERIALS, AND METHODS

Reagents

Microtiter Nunc Maxisorp 96-well plates were purchased from Fischer Scientific Labosi (Elancourt, France).

Received for publication January 23, 2009; accepted June 17, 2009.

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Supported by the Canceropôle Grand Ouest.

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Bovine serum albumin (BSA), Tween 20, anti-human IgG Fc peroxidase-conjugated goat $F(ab')_2$, and FAST orthophenylenediamine dihydrochloride tablet sets (peroxidase substrate) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Phosphate-buffered saline (PBS) was purchased from BMD (Marne la Vallée, France). Pooled human serum was purchased from the local blood transfusion center (EFS Centre-Atlantique, Tours, France). The capture antigen used was a 100 kDa recombinant form of the extracellular region of human EGFR (EGFR-ECD 404GS mutant)[12] produced in the human cell line HEK293 and purified from the culture medium by Tag affinity chromatography. Cetuximab (buffered saline 2 mg/mL solutions) was obtained from the manufacturer (Merck Lipha Santé, Lyon, France).

Reagent Preparation

Stock solutions of 2 mg/mL cetuximab were stored at +4°C. Calibrator solutions and quality controls (QCs) were prepared in pooled normal human serum and stored at −20°C until use. Concentrations of the standard calibrators (SCs) were 0, 0.1, 0.25, 0.5, 1, 5, 10, 20, and 30 mg/L. The QC concentrations for assay validation were 0.75, 7.5, and 15 mg/L. Samples, SC, and QC were extemporaneously diluted 1:100 in PBS containing 1% BSA (PBS-BSA).

Assay Procedure

Microtiter 96-well plates were sensitized by incubating 100 μL of a 1.25 mg/L solution of EGFR-ECD 404SG in coating buffer (1 N carbonate–bicarbonate buffer, pH 9.6) overnight at +4°C. The plates were then washed 4 times with PBS containing 0.05% Tween 20. The remaining protein-binding sites were blocked by 2 hours incubation at ambient temperature with 200 μL blocking buffer (PBS-BSA). Plates were washed another time like above and 100 μL of 1:100 diluted standards, QC, or unknown samples were added in the different wells of the plate. After incubation of the plates for 1 hour at ambient temperature and another washing round, 100 μL of a solution of the goat anti-human IgG peroxidase-conjugated $F(ab')_2$ was added to each well. After 1 hour at room temperature followed by washings, 100 μL of orthophenylenediamine dihydrochloride substrate was added to each well and the color reaction was stopped by adding 50 μL of 4 N sulfuric acid per well. Reading was performed at 2 wavelengths (492 and 620 nm), using an iEMS ELISA plate reader (Labsystems). Indeed, the absorbance at 492 nm wavelength corresponds to the background signal related notably to the plates and was subtracted from the absorbance at 620 nm. Nonspecific background optical density was determined by performing the assay using well coated with carbonate buffer alone and subtracted from absorbance test for each determination. SCs, QC, and unknown samples were run in duplicates, and the mean was calculated. Results were rejected if the difference between the 2 duplicates was $> 20\%$. Cetuximab serum concentrations were extrapolated from the standard curve, obtained from the standards using a 4-parameter logistic curve-fitting program (Genesis; Labsystems).

Prestudy Validation

Each SC was tested on 5 occasions on the same day (intraday) and on 5 occasions on separate days (interday). Intraday and interday precisions were determined as coefficients of variation (CV%). Intraday and interday accuracies were determined as bias (%) from actual concentrations. The limit of detection (LOD) was calculated using 28 cetuximab-free sera (obtained from patients who had never received cetuximab) and was defined as the observed mean concentration plus 2 SDs. SCs and QC performances were determined by the lowest and highest SC for which the intra- and interday precision and bias are both $\leq 20\%$ and the sum of the 2 is $\leq 30\%$. Lower and upper limits of quantitation (LLOQ, respectively) were determined by the low and high QC values.

Specificity was studied by analyzing 5 serum samples containing rheumatoid factor and 5 serum samples containing high IgG levels. Interactions with other mAbs were tested by assaying serum samples spiked with high concentrations (150–250 mg/L) of bevacizumab (3 samples), infliximab (2 samples) and rituximab (2 samples).

To detect matrix effects, calibration curve was prepared in PBS-BSA, and each QC control was prepared both in PBS-BSA and in human serum. These samples were tested 5 times. Measured concentrations were compared in terms of precision and bias.

Because serum samples could be kept at room temperature within 1 day, at +4°C within 2 weeks, and kept several months at −20°C, stability was assessed by repeatedly assaying at days 0, 1, 3, 7, 10, 14, and 17 the 7.5 mg/L QC kept at room temperature and +4°C, and after 1, 2, 3, and 4 freeze–thaw cycles. In stock solutions, the IgG concentration is regularly verified by nephelometry.

Because peak cetuximab concentrations are expected to be outside of the quantification limits, we also tested the reproducibility of the dilution. This was done by performing straight 1:10 and 1:100 dilutions of 50, 200, and 300 mg/L cetuximab-spiked serum samples, respectively, with normal human serum. These diluted samples were assayed 5 times on the same plate and on the same day.

Detection of Monovalently Bound Cetuximab

Cetuximab is a bivalent molecule and is able to bind 2 molecules of EGFR. Because shedding of EGFR in serum (sEGFR) can be detected in healthy subjects and patients with colorectal cancer,[13] 3 different cetuximab species may be present in the serum during cetuximab treatment: free cetuximab and cetuximab bound to either 1 or 2 sEGFR molecules. Among these species, only free cetuximab and cetuximab monovalently bound to a molecule of sEGFR are biologically active. We therefore tested our ELISA by assaying serum samples containing different cetuximab to EGFR-ECD 404GS molar ratios. Cetuximab 5.10^{-8} mol/L (8.45 mg/L) was mixed with increasing EGFR-ECD 404GS concentrations in the following cetuximab to EGFR-ECD 404GS ratios: 1:0, 1:0.5, 1:1, and 1:2 and incubated for 1 hour at room temperature. Each ratio was assayed on 5 occasions on the same day.

Patients and Pharmacokinetic Analysis

Patients and Blood Samples

As part of routine monitoring of patients treated for colorectal cancer, blood samples were collected to measure cetuximab serum concentrations. Sixteen patients were treated

by chemotherapy including weekly infusions of cetuximab, in which 15 were patients with metastatic colorectal cancer and 1 was an undetermined patient with neoplasia. The first infusion (400 mg/m^2) was administered over 2 hours and the following ones (250 mg/m^2) were administered over 1 hour. All the patients were treated with irinotecan (180 mg/m^2 every 2 weeks). Trough samples were collected just before the infusion, and peak samples were collected 2 hours (T2h) after the end of the infusion to estimate individual pharmacokinetic parameters, which were compared with the literature.[6,8,9,11] Individual results were sent to the prescriber within the framework of a routine therapeutic drug monitoring service and were discussed during clinical interdisciplinary rounds. The samples were not drawn specifically for this study, which was done retrospectively.

Pharmacokinetic Modeling

Cetuximab serum concentrations were used to estimate its pharmacokinetic parameters by a population approach (WinNonMix 2.0.1; Pharsight Corporation). One- and 2-compartment models, with first-order distribution and elimination constants and with nonlinear Michaelis–Menten elimination, were tested. The interindividual (η) and residual (ε) variabilities of the pharmacokinetic parameters were described using an exponential and an additive model, respectively. Estimation of typical values of pharmacokinetic parameters and of random parameters used the conditional first-order estimation (FOCE) method.[13]

RESULTS

ELISA Assay Parameters

Calibration Curve and Limit of Detection

Four standard curves obtained on separate occasions are shown on Figure 1. Mean (SD) concentration obtained for blank sera was 0.003 (0.0043) mg/L. The LOD of our assay was therefore 0.012 mg/L. Among the 28 blank sera assayed, 5 had nonspecific absorbances exceeding the LOD (corresponding to cetuximab concentrations between 0.02 and 0.095 mg/L).

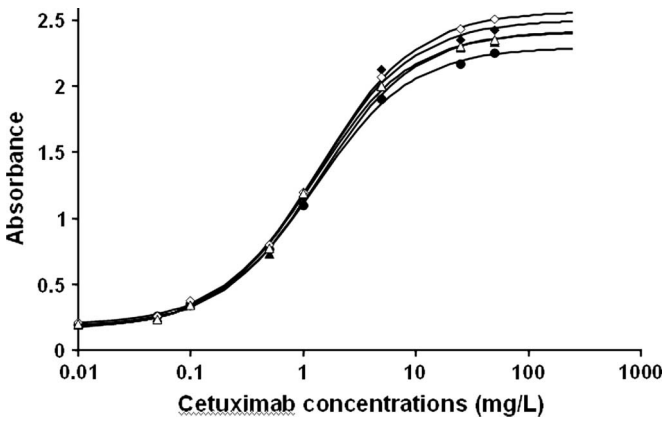

FIGURE 1. Four calibration curves of the ELISA. Absorbance reading is performed at 492 and 620 nm. Absorbance at 492 nm was subtracted to that at 620 nm. Resulting absorbance is plotted against cetuximab serum concentrations.

Imprecision and Accuracy of the Assay

For the intrarun and interrun reproducibility, imprecision and accuracy of all SC and QC were ≤20%, except for 0 and 0.1 mg/L (Table 1). Because both imprecision and bias for low and high QCs were ≤15% (Table 1), LLOQ and upper limit of quantitation were 0.75 and 75 mg/L, respectively.

Matrix Effect

For low, medium, and high QC prepared in PBS-BSA, the mean (CV, bias) was 0.72 (2.0%, −3.4%), 8.1 (14.1%, +8.7%), and 16.6 mg/L (10.2%, 10.4%), respectively. For 1 QC prepared in human serum, these values were 0.71 (7.6%, −5.7%), 6.3 (10.0%, −15.9%), and 12.7 mg/L (14.4%, −15.4). For medium QC, concentrations in PBS-BSA were significantly lower than those in human serum (Mann–Whitney test, $P = 0.02$).

Specificity and Interferences

Among the 5 rheumatoid factor–containing sera obtained from patients untreated with cetuximab, none had

TABLE 1. Results of QC Analysis

Spiked Concentration	Average Measured Concentration	Imprecision (CV%)	Inaccuracy (bias, %)	Absolute Bias + CV
Intrabatch				
0	0.005	21.1	—	—
0.1	0.09	4.1	−14.6	18.7
0.25	0.21	4.4	−14.2	18.6
0.5	0.52	2.7	3.4	6.1
1	1.0	1.1	4.7	5.9
5	5.3	1.9	5.7	7.6
10	8.8	3.0	−11.8	14.8
20	22.8	9.9	13.8	23.6
30	28.9	6.8	−3.8	10.6
0.75	0.79	4.0	5.3	9.3
7.5	7.0	8.9	−6.5	15.4
15	14.3	5.1	−4.8	9.8
Interbatch				
0	0.007	26.3	—	—
0.1	0.08	8.9	−22.8	31.7
0.25	0.24	8.9	−5.2	14.1
0.5	0.55	5.8	10.8	16.6
1	1.0	6.5	1.0	7.6
5	4.7	4.9	−6.2	11.1
10	10.5	5.3	5.2	10.5
20	22.3	4.5	11.6	16.1
30	26.8	5.7	−10.5	16.2
0.75	0.73	7.9	−2.6	10.5
7.5	7.4	14.1	−1.4	15.5
15	14.2	8.5	−5.1	13.6

Each SC and QC sample was tested on 5 occasions on the same day (intrarun) and on 5 occasions on 5 separate days (interrun).
CV percentage = (SD of estimate/mean) × 100.

an absorbance corresponding to a concentration above LOD. Among the 5 sera with high IgG concentrations obtained from patients untreated with cetuximab, one had an absorbance corresponding to a concentration above LOD (0.024 mg/L) but that is below LLOQ. Among the infliximab-, bevacizumab-, and rituximab-spiked sera tested, none had an absorbance corresponding to a concentration exceeding LOD.

Stability

For QC kept at room temperature and at +4°C, concentrations of cetuximab fluctuated around 7.5 mg/L in solutions and no trend of increase or decrease in concentrations could be observed; mean concentration across time was 7.8 and 8.1 mg/L, respectively, and concentration (bias) at day 17 was 9.1 (+21%) and 8.7 mg/L (+16%), respectively.

Dilution Reproducibility

Mean concentration obtained by assaying five 50 mg/L cetuximab-spiked samples after 1:10 dilution in normal human serum on the same day was 51.5 mg/L. Corresponding CV and inaccuracy were 4.5% and +3.1%, respectively. After a 1:100 dilution in normal human serum on the same day, the mean concentrations (CV, bias) obtained by assaying five 200 and 300 mg/L cetuximab-spiked samples were 205.3 (2.6%, +2.6%) and 291.9 mg/L (5.5%, +2.7%).

Detection of Free and Monovalently Bound Cetuximab

Mean cetuximab concentrations (SD) were 8.26 (0.58), 6.57 (0.31), 5.13 (0.38), and 3.78 (0.08) mg/L for 1:0, 1:0.5, 1:1, and 1:2 cetuximab to EGFR-ECD 404SG ratios, respectively.

Concentrations in Cetuximab-Treated Patients

A total number of 198 blood samples were available from 16 patients (Table 2). All the blood samples collected before the beginning of cetuximab treatment were below LOD. All other sera had cetuximab concentrations above LLOQ. Median (range) trough and T2h concentrations during the treatment were 49.6 (5.8–105.4) and 177.2 (97.5–235) mg/L, respectively.

Cetuximab concentrations were best described with a 2-compartment pharmacokinetic model with first-order distribution and elimination constants (Fig. 2). Michaelis–Menten elimination could not be described using our data.

TABLE 2. Summary of Patients Characteristics

	Number	Median (Range)
Pathology		
Colorectal cancer	15	
Undetermined neoplasia	1	
Demographic characteristics		
Age (yr)		68 (34–78)
Weight (kg)		69 (38–95)
Sex (women/men)	3/13	
Blood samples		
Trough concentrations (mg/L)	96	46.7 (5.8–105.4)
Peak concentrations (mg/L)	102	174.4 (97.5–235.0)

DISCUSSION

Relationships between cetuximab concentrations and therapeutic effects and between concentration and adverse side effects need to be studied to understand the sources of the interindividual variability in cetuximab effects. This knowledge should allow optimizing the use of this biopharmaceutical in patients. Such studies require an accurate, reproducible, and validated assay to measure cetuximab serum concentrations in treated patients. Previous studies used 2 different approaches: ELISA[6,9,11] or BiaCore technology.[5,8] Because they are rapid, inexpensive, sensitive, and reliably reproducible techniques, ELISA techniques are most commonly used. However, only 2 studies report technical details on the ELISA assay used.[9,11] The most complete description is given by Fracasso et al,[11] who report that the antigen used was

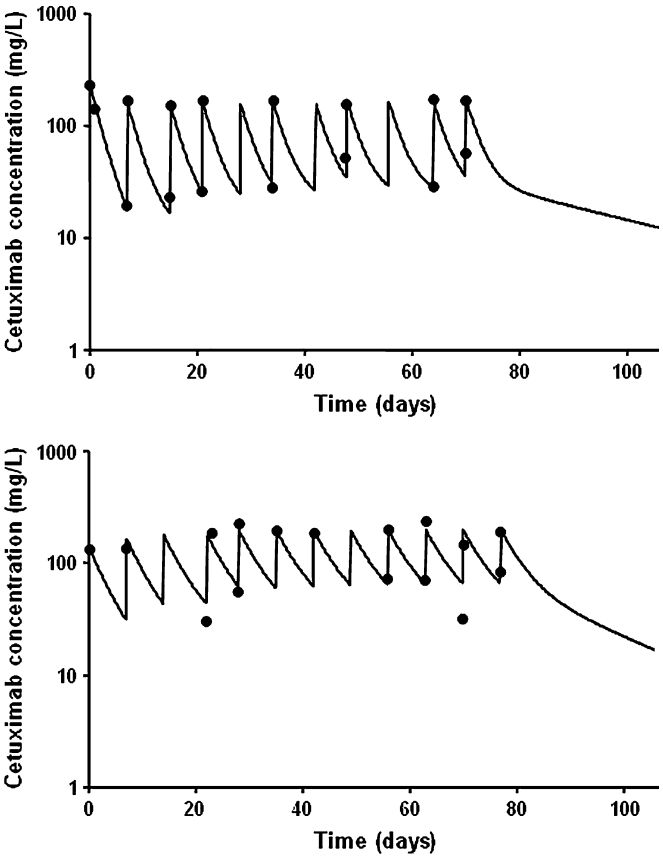

FIGURE 2. Observed (●) and model-predicted (lines) cetuximab concentrations in 2 representative subjects: patient 8 (upper panel) and patient 11 (lower panel). Estimated parameters of patient 8 were central volume of distribution: 3.6 L, peripheral volume of distribution: 5.5 L, systemic clearance: 0.77 L/d, and distribution clearance: 0.21 L/d Estimated parameters of patient 11 were central volume distribution: 2.5 L, peripheral volume of distribution: 7.8 L, systemic clearance: 0.98 L/d, and distribution clearance: 0.37 L/d, respectively.

a recombinant form of the human EGFR-ECD and that the LLOQ was 0.475 mg/L. Intra- and interrun variabilities were 5.4% and 4.6%, respectively, and the inaccuracy of the controls did not exceed 3.7%. However, this study provides no information on LOD, specificity, interferences, and dilution-induced inaccuracy or imprecision.

The ELISA described in the present study is rapid and simple. Because the validation parameters meet the criterion of acceptability (Table 2), this assay can be considered as accurate and reproducible when cetuximab concentrations are between 0.75 and 15 mg/L, imprecision and inaccuracy being $< 15\%$. The LLOQ is 0.75 mg/L, a value that is far lower than that of trough serum concentration expected in patients treated with the recommended regimen (between 30 and 100 mg/L in most of the studies).[5–9] Sample dilution does not decrease precision significantly. The observer underestimation of QC concentrations in human serum when the calibrators are prepared in PBS-BSA suggests that there may be a matrix effect, which justifies the use of human serum for standards and QC when cetuximab concentrations are realized in human serum.

Among the 28 blank sera and the 13 pretreatment sera assayed, 5 blank sera displayed nonspecific antibody binding exceeding LOD (corresponding to cetuximab concentrations between 0.02 and 0.095 mg/L), but all these values are well below LLOQ. Nevertheless, serum samples should be obtained from all patients before their first cetuximab infusion to determine the extent of nonspecific binding to EGFR-ECD.

The presence of bevacizumab, rituximab, or infliximab (other IgG1 mAbs) in the serum did not interfere with our assay, which can therefore be considered as highly specific of cetuximab. This lack of interference with other humanized or chimeric mAbs was mandatory because patients may receive other mAbs during associated or overlapping treatments. For example, patients with colorectal cancer may be successively treated with bevacizumab and cetuximab and the 2 mAbs may be present in their serum simultaneously.

As a bivalent molecule, cetuximab is expected to bind 1 or 2 EGFR molecules. Biologically active cetuximab species are free cetuximab and cetuximab with 1 bound EGFR molecule. We used our assay to measure cetuximab concentrations in solutions containing cetuximab mixed with increasing amounts of EGFR-ECD. We found that cetuximab was still detected despite of the added EGFR-ECD. Measured cetuximab concentration for the 1:2 cetuximab to EGFR-ECD 404GS ratio was 2.2 times lower than that for the 1:0 ratio. EGFR bound to cetuximab might therefore be displaced by coated EGFR. A fraction of cetuximab bound to 2 EGFR molecules may therefore be detected by our assay.

In the present study, cetuximab concentrations were assessed in 15 patients with metastatic colorectal cancer and 1 patient with undetermined neoplasia. Mean trough and T2h concentrations were 49.6 and 177.2 mg/L, respectively. These results are close to those reported from previous studies.[5–9] The 2-compartment model gave a satisfactory description of cetuximab concentrations (Fig. 2). The present pharmacokinetic modeling was performed on a small number of patients and did not pretend to describe cetuximab pharmacokinetics but rather to show that our assay is suitable in "real life" studies of cetuximab. We used our assay and this pharmacokinetic model to describe the pharmacokinetics of cetuximab in a patient with end-stage kidney disease under hemodialysis.[14]

CONCLUSIONS

We developed a sensitive, reproducible, and accurate immunoassay to measure cetuximab serum concentrations in cetuximab-treated patients. This assay can be easily and reliably applied to both pharmacokinetic and concentration–effect relationship studies of cetuximab.

ACKNOWLEDGMENTS

The authors thank Me Anne-Claire Duveau for her excellent technical assistance and Prof K. Dane Wittrup, Massachusetts Institute of Technology, Cambridge, MA, for providing us EGFR-ECD 404GS complementary DNA.

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