Characterization of ABT-806, a Humanized Tumor-Specific Anti-EGFR Monoclonal Antibody

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Abstract

Despite clinical efficacy, current approved agents targeting EGFR are associated with on-target toxicities as a consequence of disrupting normal EGFR function. MAb 806 is a novel EGFR antibody that selectively targets a tumor-selective epitope suggesting that a mAb 806-based therapeutic would retain antitumor activity without the on-target toxicities associated with EGFR inhibition. To enable clinical development, a humanized variant of mAb 806 designated ABT-806 was generated and is currently in phase 1 trials. We describe the characterization of binding and functional properties of ABT-806 compared with the clinically validated anti-EGFR antibody cetuximab. ABT-806 binds the functional properties of ABT-806 compared with the clinically validated anti-EGFR antibody cetuximab. ABT-806 binds the mutant EGFRvIII with high affinity and, relative to cetuximab, exhibits increased potency against glioblastoma multiforme cell line and patient-derived xenografts expressing this form of the receptor. ABT-806 also inhibits the growth of squamous cell carcinoma xenograft models expressing high levels of wild-type EGFR, associated with inhibition of EGFR signaling, although higher doses of ABT-806 than cetuximab are required for similar activity. ABT-806 enhances in vivo potency of standard-of-care therapies used to treat glioblastoma multiforme and head and neck squamous cell carcinoma. An indium-labeled version of ABT-806, [111In]-ABT-806, used to investigate the relationship between dose and receptor occupancy, revealed greater receptor occupancy at lower doses in an EGFRvIII-expressing model and significant uptake in an orthotopic model. Collectively, these results suggest that ABT-806 may have antitumor activity superior to cetuximab in EGFRvIII-expressing tumors, and similar activity to cetuximab in tumors highly overexpressing wild-type EGFR with reduced toxicity. Mol Cancer Ther; 14(5); 1141–51. ©2015 AACR.

Introduction

EGFR is expressed in a majority of human carcinomas and its frequent overexpression, activation, and mutations in tumors are often associated with aggressive cancer phenotypes (1). Two major classes of EGFR-targeting agents have been developed including tyrosine kinase inhibitors, such as gefitinib and erlotinib, which competitively bind to the ATP pocket of EGFR (2), and mAbs, including cetuximab and panitumumab, that bind domain III of EGFR and inhibit ligand binding (3, 4). Despite the success of this class of EGFR antagonists, they are associated with on-target toxicity, most notably a severe papulopustular rash thought to be the consequence of disruption of normal EGFR function in skin (5).

MAb 806 is a novel anti-EGFR antibody that selectively targets a unique epitope of the EGFR which is largely inaccessible when EGFR is expressed at normal physiologic levels (6, 7). The targeted epitope is accessible in tumors with wild-type EGFR amplification or in tumors that express EGFRvIII, the most common deletion mutant of EGFR that lacks the ligand-binding domain of exons 2–7 and retains constitutive kinase activity (8–10). Thus mAb806 has tumor-specific binding properties and would not be expected to elicit the side effects, including skin rash, observed with other cetuximab-like EGFR targeting (mAbs) associated with targeting of normal tissues. Consistent with these expectations, in a phase I trial, a radiolabeled chimeric version of mAb806 (ch806) demonstrated tumor uptake in multiple tumor types with minimal evidence of normal tissue uptake (11).

To reduce the risk of immunogenicity and enable clinical development, we humanized mAb 806 to generate ABT-806, a recombinant IgG1/k mAb. In a phase I study in patients with advanced solid tumors, ABT-806 was well tolerated, with a very low level of cutaneous toxicity and linear pharmacokinetics indicating the absence of extensive EGFR binding in normal tissues (12).

Here, we characterize the binding and functional characteristics of ABT-806 including assessment of pharmacodynamic tumor changes in total EGFR tyrosine phosphorylation in response to ABT-806. We compared the activity of ABT-806 with cetuximab against different human tumor xenografts, including patient-derived xenograft (PDX) models, and correlated outcomes with EGFR expression and genotype. Because its safety profile suggests that ABT-806 may be more amenable to higher dosing and combination with chemotherapy than are other EGFR-directed therapies, combinations with standard-of-care (SOC) therapy...
were also investigated. The limited normal tissue-binding properties of ABT-806 also permitted evaluation of $^{[111}\text{In}]$ABT-806 tumor uptake in mouse xenograft models to validate its use as a novel molecular imaging technique for patient selection and to investigate the relationship between ABT-806 dose and receptor occupancy.

**Materials and Methods**

**Antibodies and reagents**

The soluble ECDs of EGFR were expressed from transiently transfected HEK293 cells as secreted proteins (inclusive of signaling peptide 1–24 that is cleaved during secretion) with a C-terminal LE5RGFP-Myc-NMHTG-6His and purified by Ni-IMAC and SEC. Details of EGFR protein forms (based on numbering from accession# NP_005219.2) are as follows: wild-type EGFR comprised the entire extracellular binding domain (amino acids 1–645); EGRFrVIII contained an in-frame deletion generating a truncated protein with a novel glycine (residues 1–29 and G-298–645); EGFR 1-501 (the first 1-525 amino acids inclusive 24 amino acid leader); and a double mutant EGFR$(^{271}\text{A,C283A})$ (containing EGFR 1-645, C295A, C307A) replacing cysteine at 271 and 283 in the processed protein with alanine (8). ABT-806 was produced by transient transfection of HEK-293-6E cells; cells were grown in Freestyle 293 medium (Invitrogen) and transfected with plasmids encoding the light chain and heavy chain of the ABT-806 construct. Antibodies (Thermo Scientific) and reagents (including leucovorin (Bedford Laboratories), cisplatin (Bedford Laboratories), Cetuximab (Bristol-Meyer Squibb), 5-FU (GeneraMedix Inc), temozolomide (Merck & Co.), and human IgG (Innovative Research)) were purchased.

**Cell culture**

The human tumor cell lines A431 (human vulvar squamous carcinoma), U87MG (human glioma), and U87MGde2-7 (engineered from U87MG to overexpress EGRFrVIII) utilized in previous published work with mAb806 and ch806 were provided by the Ludwig Institute for Cancer Research (Melbourne, Victoria, Australia) in 2010 (9, 13). SCC15 cells, human head and neck squamous cell carcinoma (HNSCC), were acquired from the ATCC in 2002. All cell lines were expanded in culture upon receipt and cryopreserved to provide cells at similar stage passages for all subsequent experiments. Cell lines were not authenticated in the 6 months before use; however, their EGFR expression levels were confirmed by both FACS and Western blot analysis. A431 cells were maintained in DMEM/F12 (1:1; Life Technologies) supplemented with 10% FBS (Life Technologies) and GlutaMAX (Life Technologies). U87MG and U87MGde2-7 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS and sodium pyruvate (Life Technologies). U87MGde2-7 cells were maintained under selection with 0.4 mg/mL Geneticin (Life Technologies). SCC15 cells were maintained in RPMI-1640 (Life Sciences) supplemented with 10% FBS.

**FACS analysis**

Cells were harvested from flasks when approximately 80% confluent using Cell Dissociation Buffer (Life Technologies). Cells were washed once in PBS/1% FBS (FACS buffer) then resuspended at $2.5 \times 10^6$ cells/mL in FACS buffer. Of note, 100 µL of cells/well were added to a round bottom 96-well plate. Of note, 10 µL of antibody prepared at 10× concentration (titrated down in half-log increments from 100 mmol/L) was added, and the plate was incubated at 4°C for 1 hour. Wells were washed twice with FACS buffer then resuspended in 50 µL of secondary antibody (Alexa Fluor 488, Life Technologies) diluted in 250 µL FACS buffer. The plate was incubated at 4°C for 1 hour then washed twice with FACS buffer. Cells were then resuspended in 100 µL of PBS/1% formaldehyde and analyzed on a Becton Dickinson LSRII flow cytometer. Data were analyzed using WinList flow cytometry analysis software (Verity Software House).

**Binding ELISA**

Flat-bottomed, 96-well, high-binding plates (Costar) were coated with 1 µg/mL of mouse anti-His antibody (Life Technologies) overnight at 4°C. Plates were washed three times with PBS and blocked with 250 µL of SuperBlock (Thermo Scientific Pierce) for 2 hour at room temperature. Plates were washed and 100 µL of His-tagged human wild-type EGFR, EGFR 1-501, EGFRrVIII, or EGFR$(^{271}\text{A,C283A})$ were plated at 2 µg/mL for 1 hour at room temperature with shaking. After three washes, ABT-806 and cetuximab were added at 50 and 3 µg/mL, respectively, and 1:3 serial dilutions were prepared. Test antibody was incubated in a 100-µL final volume for 1 hour at room temperature with shaking. Following three washes, a secondary goat anti-human IgG-HRP antibody (Thermo Scientific Pierce) was added at 100 µL/well and incubated for 1 hour at room temperature with shaking. Plates were washed three times and developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Scientific Pierce). The reaction was stopped with 1N phosphoric acid and OD$_{450}$ signal was measured using a SpectraMax (Molecular Devices).

**Surface plasmon resonance of antibodies**

A Biacore T100 surface plasmon resonance instrument (Biacore Life Sciences) was used to measure binding kinetics of recombinant soluble EGFR (wild-type, EGFRrVIII, EGFR 1-501, EGFR$(^{271}\text{A,C283A})$) protein forms (analytes) binding to anti-EGFR mAbs (ligands). The assay format was Fc-based capture via immobilized anti-human (Fc) antibody (Thermo Scientific Pierce). A standard amine coupling protocol was used to immobilize the capture reagents via primary amines to the carboxymethyl (CM) dextran surface of CM5 sensor chips according to the manufacturer's instructions (Biacore Life Sciences). For binding kinetics measurements, the assay buffer was HBS-EP+ (Biacore Life Sciences): 10 mmol/L Hepes (pH 7.5), 150 mmol/L NaCl, 3 mmol/L EDTA, 0.05% P20. During the assay, all measurements were referenced against the capture surface alone. Each assay cycle consisted of the following steps: (i) ligand capture; (ii) analyte injection over both reference and test surface, 240 µL at 80 µL/minute; and (iii) regeneration of capture surface with low pH glycine. For kinetic determinations, analyte injections were randomized 3-fold dilution series from 3 mmol/L to 12.35 mmol/L. Buffer only injections were included for secondary referencing. Data were processed and fit to a 1:1 binding model using Biacore T100 Evaluation Software to determine the binding kinetic rate constants, $k_{	ext{on}}$ (on-rate) and $k_{	ext{off}}$ (off-rate), and the $K_D$ (equilibrium dissociation constant, also referred to as "affinity").

**In vivo studies**

All animal studies were reviewed and approved by AbbVie's Lake County Institutional Animal Care and Use Committee. Animal studies were conducted in an AAALAC accredited program.

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and veterinary care was available to ensure appropriate animal care. Mice were group-housed 10 per cage with food and water available ad libitum and were acclimated to the animal facilities for a period of at least one week before initiation of experiments.

Tumor cells were mixed with 50% Matrigel (BD Biosciences) and injected s.c. into the flank of 6- to 8-week-old female mice (Charles River Laboratories) as described: SCC15 cells were injected at 1 × 10^6 cells per mouse into SCID Beige mice; A431 cells were injected at 3 × 10^6 cells per mouse into Nu/Nu mice; U87MGdE 2–7 cells were injected at 3 × 10^6 cells per mouse into Nu/Nu mice. For the SN0199 PDX model (Jackson Laboratory), 3 to 5 mm^3 passage 3 (P3) tumor fragments were s.c. trochar implanted in the right rear flank of NSG mice.

Tumors were allowed to grow to a predetermined size, at which time mice were allocated by tumor volume into study groups (n = 10 mice/group) so that the mean tumor volumes of the groups were statistically similar. Mice were then entered into the dosing phase of the study (described below). The PDX tumors were randomly assigned into study groups (n = 7–9 mice/group) when the individual tumors reached approximately 250 mm^3 volume. Tumor volumes were recorded two to three times per week based on tumor growth rate. Tumor volumes were estimated by the formula V = (L × W^2)/2, where V is the volume (mm^3), L is the tumor length (mm), and W is the tumor width (mm), measured at right angles using a digital caliper. The effect of a treatment on tumor growth inhibition (TGI) was determined as %TGI = 1 – (mean tumor volume of treatment group/tumor volume of treatment control group) × 100. Data were analyzed using the Student t test for % TGI values [Excel (Windows XP), Microsoft Corporation]. The tumor growth delay method for efficacy analysis was performed using data achieved with a 60-day study endpoint or a 1,500 mm^3 tumor endpoint for calculating time (days) to tumor endpoint (14). A log-rank test with a two-tailed statistical analysis with a 95% confidence interval was performed with GraphPad Prism 6.0 to determine the significance of comparisons between treatment groups for the time to endpoint (TTE) tumor responses and for plotting Kaplan–Meier survival curves.

Western blot analysis

Cell lysates (10 μg) were resolved by SDS-PAGE using 4% to 12% Bis-Tris midi gels (Invitrogen, catalog no. WG1402BX10) and transferred to nitrocellulose membranes using an iBlot Dry Transfer System (Invitrogen, catalog number IB3010-01). Anti-phosphotyrosine blots were blocked with 3% milk/PBS for 30 minutes, washed three times with PBS-T, and then incubated overnight with anti-phosphotyrosine 4G10 biotin conjugate (Millipore, catalog number16-103; 1:1000) at 4°C. Blots were then washed twice with water then incubated with 1:1000 streptavidin-HRP (KPL, catalog number 474-3000) for 1 hour at room temperature, washed twice in water, once in PBS-T, and then incubated for 3.5 hours in water (changing water several times). Blots were treated with Super Signal West Dura Extended Dura- tion chemiluminescent substrate (Thermo Scientific, product number 34076) and were visualized by scanning using an LAS-4000. Total EGFR blots were blocked with 5% milk/TBS-T for 1 hour and incubated overnight with rabbit anti-EGFR (Lifespan Biosciences, catalog number LS-C6625; 1:2000). Blots were then washed three times with PBS-T for 5 minutes and then incubated with donkey anti-rabbit IgG-HRP secondary antibody (The Jackson Laboratory, catalog number 711-0152; 1:2000) for 1 hour at room temperature. Blots were then washed three times with PBS-T for 5 minutes, then treated with chemiluminescent substrate (Thermo Scientific) and were visualized by scanning using an LAS-4000.

IHC

IHC analysis of EGFR, phospho-EGFR, phospho-H3, and activated caspase-3 levels in tumors from human IgG-, cetuximab-, or ABT-806-treated mice was evaluated. Briefly, 5 μm paraffin sections were deparaffinized and rehydrated. Antigen retrieval was performed, endogenous peroxidase and nonspecific protein-binding sites were blocked [Peroxidase Blocking Reagent (Dako); Background Sniper (Biocare)] and subsequently incubated in primary antibodies (1 hour at room temperature) and detected with EnVision+ polymer for mouse or rabbit with DAB as chromogen (Dako). Primary antibodies used included: EGFR mAb (Life Technologies; catalog number AHR5062), phospho-EGFR pAb (Cell Signaling Technology; catalog number 2234); rabbit anti-human mouse and rat phospho-histone H3 pAb proliferation marker (Cell Signaling Technology; catalog number 9701); rabbit anti-human/multi-species, and activated caspase-3 pAb apoptosis marker (Cell Signaling Technology; 9661). IHC results were obtained by semiquantitative assessment of the percent positive cells within viable areas across treatment groups at various time points. Data represent four samples per treatment group, per time point. Data were analyzed using ANOVA/Fisher PLSD analysis (Statview, SAS Institute).

DTPA conjugation

Cyclic diethylenetriamine pentaacetic acid dihydroxide (cDTPA) was used as bifunctional chelator for preparation of DTPA-ABT-806. ABT-806 was transferred into 0.1 mol/L sodium bicarbonate (pH 8.2) by size-exclusion chromatography and ultrafiltration. A 10-fold molar excess of DTPA was added to the antibody and incubated for 1 hour (15). Unbound DTPA was removed by size-exclusion chromatography and ultrafiltration and the DTPA-antibody was transferred into 0.1 mol/L sodium acetate (pH 7.2). The resultant DTPA antibody was aliquoted and stored at –80°C for radiolabeling. All DTPA-antibody conjugates contained an average of two to three DTPA groups per antibody. Cetuximab, ABT-806, and human polyclonal IgG (hIgG) were used as controls and were conjugated to DTPA in parallel with ABT-806 using the same procedure. For DTPA-ABT-806 and cetuximab, the ability to retain a similar affinity as the parental antibody was verified by FACS-based cell binding assays before use in imaging experiments.

Radiolabeling

For mouse imaging studies, 1 mg of DTPA-antibody (0.4 mL) was mixed with 40 mCi 111InCl3 (20–100 μL, MDS Nordion). The reaction vial was swirled for 10 seconds and incubated for 30 minutes before analytical testing. The labeling efficiency and radiochemical purity were determined by both radio-size exclusion–high-performance liquid chromatography and instant thin layer chromatography with 100 mmol/L. acetate buffer/20 mM EDTA as the mobile phase. Labeling reactions produced between 95% and 99% incorporation of 111In into DTPA-antibody, and no postlabeling purification was needed.

SPECT/CT imaging

A SPECT scanner with a built-in CT dedicated to small animal imaging was used (nanoSPECT/CT, BioScan). The nanoSPECT...
Table 1. Binding affinity of ABT-806 and cetuximab to recombinant EGFR

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<th>ABT-806</th>
<th>Cetuximab</th>
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<tr>
<td>EGFR</td>
<td>0.96 ± 0.34</td>
<td>0.10 ± 0.000</td>
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<tr>
<td>EGFRvIII</td>
<td>0.09 ± 0.04</td>
<td>0.07 ± 0.006</td>
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<tr>
<td>EGFR</td>
<td>0.12 ± 0.04</td>
<td>0.03 ± 0.006</td>
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<tr>
<td>EGFR</td>
<td>0.66 ± 0.14</td>
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Surface plasmon resonance (binding constants)*

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<th></th>
<th>ABT-806</th>
<th>Cetuximab</th>
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<tbody>
<tr>
<td>EGFR</td>
<td>7.0E-03</td>
<td>4.1E-05</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>4.5E-03</td>
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<tr>
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<td>3.6E-03</td>
<td>8.3E-05</td>
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*EC50 values derived from ELISA in which EGFR ECD was captured on the plate via a His tag. Values are averages of three experiments ±SD.

contains four detectors; each fitted with a tungsten collimator and a nine-pin aperture for increased spatial resolution (<1 mm) and detection sensitivity. Tumors were size matched using calipers to ensure comparable tumor sizes within each experimental group of mice. After injection of [111In]antibody (2 mCi/50 μg), mice were imaged under isoflurane (2%) anesthesia with CT and SPECT sequentially. CT and SPECT images were reconstructed, coregistered, and the tumor biodistribution of the [111In]antibody was measured using InVivoScope software (Bioscan Inc.). All images were quantified as percentage of injected dose per cm³ (%ID/cc) or percentage of injected dose per cm² of the maximum voxel (%IDmax/cc) within the tumor.

Results

Characterization ABT-806–binding properties

A series of binding assays was performed to characterize the binding of ABT-806 to recombinant EGFR. In an ELISA format, ABT-806 exhibits higher binding to EGFRvIII and EGFR<sub>C277A,C285A</sub>, a point mutant known to expose the cryptic 860 epitope, relative to truncated wild-type EGFR (1-501) and full-length wild-type EGFR ECD (1-621). In contrast, cetuximab displays tight binding to all forms of recombinant EGFR (Table 1).

Affinity (K<sub>d</sub>) measurements using Biacore analysis confirm this differential binding (Table 1). Cetuximab exhibits nearly equivalent high-affinity binding to all four EGFR forms (~2 nmol/L K<sub>d</sub>) in Biacore, whereas ABT-806 shows differential binding kinetics for EGFRvIII, EGFR<sub>C277A,C285A</sub>, and EGFR-truncated ECD (1-501) and no observable binding to full-length EGFR ECD (1-621).

ABT-806 shows highest affinity for EGFRvIII (18 nmol/L K<sub>d</sub>) followed by EGFR<sub>C277A,C285A</sub> (170 nmol/L K<sub>d</sub>) and EGFR1-501 (16 nmol/L K<sub>d</sub>). The high affinity of ABT-806 for EGFRvIII is due to enhanced on-rates and off-rates compared with EGFR<sub>C277A,C285A</sub> and EGFR1-501, while these latter two forms differ in off-rates only. These observations are consistent with the EGFRvIII having unique epitope exposure (Table 1). The increased ABT-806 binding observed in the ELISA format compared with the Biacore format is most likely due to an antibody avidity effect. Biacore measures intrinsic 1:1 binding of recombinant soluble EGFR forms to antibody without additional avidity type interactions and surface effects.

FACS was used to compare the binding of ABT-806 and cetuximab to A431 cells that express amplified wild-type EGFR (16). Nonsaturable binding of ABT-806 to A431 cells was observed even at high concentrations (1.3 μmol/L; Fig. 1A). In contrast, cetuximab binding was saturated at single digit nmol/L concentrations (Fig. 1A). This binding analysis was extended to a panel of human tumor cell lines expressing wild-type EGFR. In all cases, ABT-806 binding was consistently lower than cetuximab indicating that, at least in vitro, the majority of cell surface wild-type EGFR receptors remain unbound (Fig. 1B and data not shown). In contrast, ABT-806 effectively bound EGFRvIII-expressing U87MGde2-2 cells similar to the binding observed for cetuximab (Fig. 1C). Both ABT-806 and cetuximab displayed limited binding to parental U87MG cells which express low levels of wild-type EGFR (Fig. 1D).

ABT-806 in vivo potency in EGFRvIII-expressing glioblastoma multiforme tumors

The binding characteristics of ABT-806 suggest that it may be effective against tumors expressing EGFRvIII. Because glioblastoma multiforme (GBM) has a high frequency of EGFRvIII expression, the activity ABT-806 was compared with cetuximab in the U87MGde2-7 GBM model. This tumor model was engineered to express EGFRvIII as cell lines do not maintain expression of endogenous EGFRvIII (17, 18). Growth of U87MGde2-7 tumors was significantly inhibited by ABT-806 treatment (Fig. 2A). In contrast, cetuximab showed only minimal activity in this model even at the 40 mg/kg high dose level tested (Fig. 2A).

These studies were extended to the evaluation of ABT-806 activity against the EGFRvIII-positive PDX model SN0199. GBM PDX models like SN0199, unlike standard cell line xenograft models, can maintain expression of endogenous EGFRvIII and thus may be more clinically relevant than standard cell line-derived xenografts (17, 19, 20). Because of the variable growth rate of the implanted PDX tumors, these studies were performed with an accrual design with data presented as a Kaplan–Meier plot. A 10 mg/kg dose of cetuximab was selected as the comparator since the exposures achieved with this dose would be expected to exceed those achievable in patients (21). In contrast, ABT-806 was assessed at doses of both 10 and 40 mg/kg (dosed three times a week for 2 weeks) because reduced normal tissue binding of ABT-806 compared with cetuximab has been demonstrated thereby allowing higher tolerated doses in patients. As shown in Fig. 2B, ABT-806 treatment resulted in significant tumor
inhibition with all mice surviving beyond the end of the observation period. A second PDX GBM model (SN0207) expressing low levels of wild-type EGFR was unresponsive to either ABT-806 or cetuximab treatment (data not shown).

To investigate whether TGI of the SN0199 model was associated with an inhibition of EGFR signaling, tumor-bearing mice were treated with ABT-806 (40 mg/kg) or cetuximab (10 mg/kg) twice 3 days apart and tumors harvested 72 hours after the final dose. Total and pEGFR status in the SN0199 tumor was assessed by Western blot analysis (Fig. 2C). ABT-806, but not cetuximab, reduced levels of pEGFR consistent with the potency of ABT-806 in EGFRvIII tumor models.

**ABT-806 in vivo potency in squamous cell carcinoma tumor models expressing wild-type EGFR**

ABT-806–binding properties and published results with mAb 806 and ch806 suggested that ABT-806 may also be effective against tumor cells overexpressing wild-type EGFR (6, 13, 16). Because EGFR is frequently overexpressed in squamous tumors, the antitumor efficacy of ABT-806 was compared with cetuximab in the A431 squamous xenograft model that expresses amplified EGFR (22). Despite poor in vitro binding and an inability to inhibit signaling of wild-type EGFR-expressing cells (data not shown), ABT-806 dosed at 10 mg/kg three times per week for 2 weeks exhibited comparable activity to cetuximab dosed in an equivalent manner (Fig. 3A). At higher doses (40 mg/kg), cetuximab was more effective at inhibiting tumor growth than was ABT-806 dosed (87% TGI for cetuximab and 58% TGI for ABT-806 on day 30; Fig. 3B). These results suggest that higher exposures of ABT-806 relative to cetuximab may be required to achieve similar efficacy and are consistent with the higher affinity binding of cetuximab to wild-type EGFR (Table 1).

ABT-806 was also highly effective in a HNSCC xenograft model SCC15 that overexpresses wild-type EGFR (Fig. 3C). ABT-806 treatment resulted in tumor regressions although higher dosing was required to show activity comparable with that seen with cetuximab (Fig. 3C).

**ABT-806 downregulates wild-type EGFR and pEGFR in vivo**

To investigate the mechanism of ABT-806–mediated TGI in wild-type EGFR-expressing tumors, SCC15 tumor-bearing mice were treated with a single dose of ABT-806 or cetuximab and tumors harvested over a time course. IHC analysis of sections derived from tumors harvested over 120 hour demonstrated that ABT-806 mediated time-dependent reductions in the level of pEGFR and total EGFR (Fig. 4A). Consistent with the antitumor effects observed in Fig. 3C, a more pronounced effect was observed following cetuximab treatment. Representative IHC images of tumor sections collected at the 120-hour time point are shown in Fig. 4B. Similar levels of human IgG staining were observed in tumors treated with either ABT-806 or cetuximab (data not shown). Both ABT-806 and cetuximab-treated samples also demonstrated reduced downstream EGFR signaling and cell proliferation, as measured by phospho-histone H3, and increased apoptosis as measured by caspase-3 cleavage (Fig. 4C).

**ABT-806 combinations with chemotherapy**

The safety profile of ABT-806 makes it an attractive candidate for combination with chemotherapy in the treatment of EGFR-positive malignancies. To test this premise, ABT-806 in
combination with different chemotherapy agents was assessed in both wild-type EGFR and EGFRvIII-expressing tumor models.

Cetuximab is FDA-approved for use in combination with cisplatin and 5-FU in recurrent or metastatic HNSCC, so these combinations with ABT-806 were evaluated in the wild-type EGFR-expressing HNSCC tumor model SCC15 (23). Because ABT-806 was highly potent as monotherapy in this model (Fig. 2C), it was dosed at a suboptimal level (10 mg/kg) to assess its ability to enhance activity of SOC therapy. The combination of ABT-806 and cisplatin (Fig. 5A) or ABT-806 and 5-FU (Fig. 5B) was more effective in inhibiting SCC15 tumor growth than were any of the agents tested individually. Increased antitumor activity was also observed when ABT-806 was combined with both cisplatin and 5-FU (Fig. 5C).

The ability of ABT-806 to combine with SOC chemotherapy was also evaluated in the EGFRvIII-expressing U87MGde2-7 xenograft model. The current SOC therapy for newly diagnosed glioblastoma following surgical debulking is radiation therapy (RT) in combination with temozolomide (24, 25). ABT-806 was dosed at 10 mg/kg in this model to enable evaluation of combination effects. Addition of ABT-806 to RT resulted in a modest

Figure 2. ABT-806 is more potent than cetuximab at inhibiting EGFRvIII tumor growth and pEGFR expression. The in vivo potencies of ABT-806 and cetuximab, both dosed at 40 mg/kg in a QD, 3X/week x 2-week format, were evaluated in U87MGde2-7 tumor-bearing mice (A). ABT-806 and cetuximab, both dosed in a 3X/week x 2 week format, were evaluated in a EGFRvIII-expressing PDX tumor model. The SN0199 tumor model was performed with an accrual design and data are presented as a Kaplan-Meier plot, with a tumor size of 1,500 mm$^3$ defined as the end point (B). Finally, total and pEGFR status in SN0199 tumors were assessed following treatment of mice with ABT-806 at 40 mg/kg and cetuximab at 10 mg/kg. Antibodies were administered Q3D x2, with tumors harvested 72 hour after the final dose (C).

Figure 3. Antitumor activity of ABT-806 in wild-type EGFR-expressing squamous cell carcinoma xenograft models. The in vivo potency of ABT-806 and cetuximab was evaluated in mice implanted with A431 cells (A and B) or SCC15 cells (C). Antibodies were dosed QD, 3X/week x 2 weeks i.p. at 10 mg/kg (A) and 40 mg/kg (B) with all treatments well tolerated.
increase in TGI (data not shown); however, the addition of ABT-806 to temozolomide produced pronounced increase in TGI (Fig. 5D).

Specific tumor uptake and receptor occupancy measurements using $^{111}$In-ABT-806
ABT-806 was labeled with $^{111}$Indium to evaluate ABT-806 tumor uptake and to establish an ABT-806 dose-versus-receptor occupancy relationship. Mice bearing U87MGde2-7 xenografts were predosed with unlabeled ABT-806 at 0, 10, 20, 40, and 80 mg/kg. One hour later, $^{111}$In-ABT-806 was injected into each animal. Mice were imaged 72 hours postinjection and tumor uptake was quantified. Unlabeled ABT-806 pretreatment dose dependently inhibited $^{111}$In-ABT-806 tumor uptake as shown in representative SPECT/CT images (Fig. 6A). The %ID$_{max}$/cc was plotted against the amount of predosed unlabeled ABT-806, revealing significant inhibition of uptake even with a single dose of 10 mg/kg (Fig. 6B). The %ID$_{max}$/cc was selected for this calculation to avoid inclusion of necrotic areas of the tumor void of target antigen. A parallel imaging experiment was also performed to establish a cetuximab dose-versus-receptor occupancy relationship using the U87MGde2-7 model. In comparison with the experiment with ABT-806, higher levels of unlabeled cetuximab pretreatment were required to inhibit $^{111}$In-cetuximab tumor uptake (Fig. 6C).

To evaluate tumor uptake in a wild-type EGFR-positive model, similar experiments were performed using mice bearing A431 xenografts. Tumor uptake of $^{111}$In-ABT-806 was dose dependently inhibited by pretreatment with unlabeled ABT-806 (Fig. 6D and E). Similarly tumor uptake of $^{111}$In-cetuximab was dose dependently inhibited by pretreatment with unlabeled cetuximab (Fig. 6F). These results demonstrate that a higher dose of ABT-806 would be needed (Fig. 6E, extrapolated dotted line) to reach the similar level of inhibition to that of cetuximab in this wild-type EGFR-positive tumor model (Fig. 6F).

The blood brain barrier (BBB) is a challenge to antibody therapy of GBM, potentially restricting access to tumors located within the brain. To evaluate the ability of ABT-806 to penetrate the BBB, mice were injected intracranially with U87MGde2-7 cells and following tumor development dosed intravenously with $^{111}$In-ABT-806. Tumor uptake of the radiolabeled antibody, measured by SPECT/CT imaging was observable at 4 hour with maximal uptake observed at 120 hour post $^{111}$In-ABT-806 dose (Fig. 6G). In contrast, there was no significant uptake of $^{111}$In-ABT-806 in mice that received sham tumor implants (Fig. 6G). These result demonstrates efficient $^{111}$In ABT-806 uptake in this glioma model when grown orthotopically and is consistent with the utility of ABT-806 treatment in the GBM setting.

Discussion
ABT-806 binds to EGFRvIII with high affinity and exhibits potent antitumor activity both against glioblastoma cell line and PDX models that express this form of the receptor. PDX models may more accurately recapitulate the cellular heterogeneity, architectural and molecular characteristics of the primary human tumor compared with standard cell line-passaged xenograft models (26). PDX models are particularly relevant for assessing EGFRvIII-expressing tumors because standard GBM tumor cell lines show loss of EGFRvIII expression during passage in cell culture (17–19). In contrast with ABT-806, cetuximab binds to EGFRvIII with similar affinity but does not inhibit signaling and showed little or no activity in the EGFRvIII-expressing xenograft and PDX models. The different epitopes that these antibodies recognize underlie their distinct mechanisms of action and consequent efficacies against human xenograft tumors overexpressing EGFRvIII. Previous studies suggest that binding to the ABT-806 epitope blocks receptor dimerization and subsequent activation (8). In contrast, cetuximab exerts its antitumor activity, at least in part, by binding to its epitope on domain III of the EGFR.
ABT-806 augments the effect of SOC in SCC15 HNSCC tumor-bearing mice (A–C). ABT-806 was administered (i.p. at 10 mg/kg (QD, 3X/week x 2 weeks); ABT-806 was dosed in combination with cisplatin at 5 mg/kg (i.v., QD x 2; A), in combination with 5-FU at 50 mg/kg/d (i.p., QD 2 days on/5 days off, 2 cycles; B) and in combination with cisplatin at 5 mg/kg (i.v., QD x 1 and 5-FU at 25 mg/kg (i.p., QD x 2; C). In B and C, 5-FU was co-dosed on the same schedule with leucovorin i.p. at 30 and 15 mg/kg, respectively. Against U87MGde2-7 tumor-bearing mice, ABT-806 was combined with temozolomide at 5 mg/kg (PO, QD x 5; D).

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preventing ligand binding (27). Because EGFRvIII is constitutively active, cetuximab does not directly block signaling from this receptor. In support of this conclusion ABT-806, but not cetuximab, blocked EGFRvIII phosphorylation in vitro and in vivo.

EGFR is overexpressed in approximately 50% of primary GBM patients (28) and the high prevalence of EGFRvIII in GBM (25%–35%) is well established (29). GBM patients generally have a very poor prognosis, making EGFR an attractive therapeutic target in this indication. The EGFR expression profile in GBM suggests that targeting both wild-type EGFR and EGFRvIII-expressing tumors in an appropriately stratified GBM patient population may provide clinical benefit particularly when combined with other therapies. Toward this end, ABT-806 synergizes with SOC temozolomide chemotherapy in the U87MGde2-7 tumor model. The ability of ABT-806 to cross the BBB and specifically target glioblastoma in patients is supported by the selective imaging of 

\[^{111}\text{In}\]ATBT-806 in a GBM orthotopic mouse model. Phase I clinical observations with both radiolabeled ch806 and ABT-806 also indicate excellent uptake in GBM patients demonstrating that this antibody can effectively cross the BBB or that the BBB is sufficiently comprised in these patients to allow antibody uptake (11, 30).

In contrast with its high-affinity binding to EGFRvIII, ABT-806 displays lower affinity binding to wild-type EGFR with nonsaturable binding to cells overexpressing wild-type EGFR even at high concentrations. Despite its low binding to wild-type EGFR, ABT-806 is efficacious in the wild-type EGFR-expressing A431 and SCC15 squamous carcinoma xenograft models. The reason for efficacy against wild-type EGFR-expressing tumor models in vivo despite low binding in vitro remains unknown but may be explained by an increased prevalence of the ABT-806 epitope in vivo. Although antibody effector functions may contribute to the antitumor activity, ABT-806 is also effective at inhibiting EGFR phosphorylation and downstream signaling in vivo, consistent with an antitumor effect by inhibition of EGFR signaling.

Cetuximab was more potent than ABT-806 at inhibiting tumor growth and downstream signaling in these EGFR wild-type in vivo models. An important consideration when extrapolating these preclinical results to human patients is that reduced normal tissue binding of ABT-806 compared with cetuximab has been demonstrated thereby allowing higher tolerated doses and subsequent higher plasma levels of ABT-806 in patients compared with cetuximab. This outcome is supported by phase I studies in which ABT-806 dosed up to 24 mg/kg was well tolerated, with none of the characteristic EGFR-inhibitor skin toxicity observed (12, 30). These results suggest that for tumors with high levels of EGFR expression, ABT-806 may achieve similar potency to cetuximab with reduced toxicity. ABT-806 was ineffective in several
cetuximab-responsive models with lower levels of EGFR (data not shown) suggesting that this therapeutic may not be optimal for treatment of tumors with low EGFR expression.

EGFR overexpression has been well documented in tumors with squamous histology suggesting that patients with tumors such as HNSCC where other EGFR therapeutics have proven successful may be well suited for ABT-806 therapy. ABT-806 may provide additional clinical benefit in these indications because its clean toxicity profile suggests that it may be better tolerated than other EGFR antagonists when combined with chemotherapy in patients. Results showing additive effects of ABT-806 and SOC chemotherapy regimens in the SCC15 HNSCC tumor model support this strategy.

The tumor specificity of ABT-806 provides a unique opportunity to develop a clinical imaging agent for the identification and characterization of EGFR-expressing malignancies where other EGFR antibodies may be limited by their normal tissue binding properties. Specific tumor targeting of [111In]ABT-806 of wild-type EGFR and EGFRvIII-expressing tumors implanted into mice has been demonstrated and used to investigate the relationship between ABT-806 and cetuximab dose and receptor occupancy. In the EGFRvIII-expressing xenograft model, a single dose of 10 mg/kg ABT-806 or 28 mg/kg of cetuximab achieved 50% receptor occupancy. These results are consistent with the high-affinity binding of ABT-806 to the EGFRvIII form of the receptor and the potent antitumor activity of ABT-806 observed in the U87MGde2-7 xenograft model. In contrast, ABT-806 required a dose approximately twice that of cetuximab to achieve similar receptor occupancy in the A431 wild-type EGFR-expressing tumor model. Extrapolation of these results to patients predicts that a higher dose of ABT-806 may be required to achieve similar receptor occupancy and efficacy as cetuximab in wild-type tumors.

Figure 6. Tumor uptake of [111In]ABT-806 and [111In]cetuximab in U87MGde2-7 and A431 tumor-bearing mice. SPECT/CT images of U87MGde2-7 (A) or A431 (D) tumor-bearing mice predosed with unlabeled ABT-806 before the injection of [111In]ABT-806. Images at 72-hour after [111In]ABT-806 injection. Dose-dependent inhibition of [111In]ABT-806 uptake in U87MGde2-7 (B) or A431 (E) tumors by pretreatment with unlabeled ABT-806. Dose-dependent inhibition of [111In]cetuximab uptake in U87MGde2-7 (C) or A431 (F) tumors by pretreatment with unlabeled cetuximab. SPECT/CT images (G) and quantification of tumor uptake (H) of [111In]ABT-806 in mice with intracranial U87MGde2-7.
EGFR-expressing tumors. Higher tolerated doses of ABT-806 in patients have been demonstrated in phase I trials supporting the feasibility of this approach in patients with tumors expressing wild-type EGFR (30). ABT-806 represents an attractive clinical candidate and it is currently under investigation in phase II trials. Furthermore, its safety profile and functional characteristics, including internalization into tumor cells, support its use to deliver a cytotoxic payload to cancer cells.

Disclosure of Potential Conflicts of Interest

N.C. Goodwin is the Vice President of Champions Oncology, Inc. A.M. Scott reports receiving commercial research support from AbbVie and has ownership interest (including patents) in the Ludwig Institute for Cancer Research. No potential conflicts of interest were disclosed by the other authors.

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Characterization of ABT-806, a Humanized Tumor-Specific Anti-EGFR Monoclonal Antibody


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