The Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitor AG1478 Increases the Formation of Inactive Untethered EGFR Dimers

**IMPLICATIONS FOR COMBINATION THERAPY WITH MONOCLONAL ANTIBODY 806**

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The epidermal growth factor receptor (EGFR) has at least two fundamental conformations: an inactive tethered conformation and an active untethered, ligand-bound “back-to-back” dimer, which may be part of an oligomeric complex. Monoclonal antibody (mAb) 806 is an EGFR-specific antibody that only binds a transitional form of the receptor after it untethers but before forming the back-to-back, ligated, active oligomer. We have shown that AG1478, a tyrosine kinase inhibitor of the EGFR, synergistically inhibits the growth of tumors overexpressing EGFR when used in combination with mAb 806 but the mechanism for this was not elucidated (Johns, T. G., Luwor, R. B., Murone, C., Walker, F., Weinstock, J., Vitali, A. A., Perera, R. M., Jungbluth, A. A., el elucidated (Johns, T. G., Luwor, R. B., Murone, C., Walker, F., Weinstock, J., Vitali, A. A., Perera, R. M., Jungbluth, A. A., el elucidated (Johns, T. G., Luwor, R. B., Murone, C., Walker, F., Weinstock, J., Vitali, A. A., Perera, R. M., Jungbluth, A. A.,

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2 The abbreviations used are: EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; wt, wild type; PBS, phosphate-buffered saline; IP, immunoprecipitation; FACS, fluorescence-activated cell sorting; ANOVA, analysis of variance; DK, dead kinase.
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Monoclonal antibodies mAb 806 (IgG2b) and mAb 528 (IgG2a) were produced in the Biological Production Facility (Ludwig Institute of Cancer Research, Melbourne, Australia) as previously described (24, 25). Tyrphostin AG1478 (4-(3-chloroanilino-6,7-dimethoxyquinazoline) mesylate, \(M_c, 411.1\)) was manufactured by the Institute of Drug Technology (IDT, Melbourne, Australia) and solubilized in Me\(_2\)SO (stock 50 mM) (1).

Immunoprecipitation and Immunoblotting for the EGFR—Cells were grown in media containing AG1478 (2 \(\mu\)M) or an equivalent amount of vehicle (Me\(_2\)SO, 1:25,000 v/v). In those experiments where we aimed to determine the durability of the AG1478 effect, the AG1478 media was then discarded, and cells were washed twice with ice-cold PBS before being followed in pre-warmed media without AG1478. After treatment, cells were then lysed before being subjected to immunoprecipitation (IP) and immunoblotted for the EGFR at 4 °C, as previously described in detail (24). In brief, cells were lysed with lysis buffer (1% Triton X-100, 30 mM HEPES, 150 mM NaCl, 500 nM aprotinin, 1 mM PMSF, pH 7.4) for 20 min, clarified by centrifugation at 14,000 g for 30 min, immunoprecipitated with the relevant antibodies at a final concentration of 5 \(\mu\)g/ml for 60 min, and captured by Sepharose-A beads overnight. Samples were then eluted with 2X NuPAGE SDS Sample Buffer (Invitrogen), resolved on Immobilon-P transfer membrane (Millipore), and then probed with the relevant antibodies before detection by chemiluminescence radiography on a Storm 804 PhosphorImager (Amerham Biosciences). Results were then quantified using Image-QuanT TL Image Analysis Software (Version 2005) (34).

Flow Cytometric Analysis (FACS) Analysis—Where indicated, cells were incubated with AG1478 (2–20 \(\mu\)M) or vehicle for 20 min or 24 h prior to FACS. Cells were then washed twice with ice-cold PBS, probed with the relevant antibodies for 1 h at 4 °C, and then bound antibody was detected using
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an fluorescein isothiocyanate-labeled goat anti-mouse antibody (Calbiochem). Cells were read on a BD Biosciences FACScan (CellQuestPro, Version 4.0.2).

Chemical Cross-linking of EGFR in Intact Cells—Cells were treated with AG1478 (2 μM) or vehicle for 20 min or 24 h. EGFR dimers were then covalently cross-linked using membrane-impermeable bis(sulfosuccinimidyl) suberate (Pierce) (35, 36) as per the manufacturer’s instructions. In brief, cells were incubated with bis(sulfosuccinimidyl) suberate (1 mM) at 4°C for 30 min. Cells were then lysed, subjected to IP with the relevant antibodies, and immunoblotted for EGFR.

35S Pulse-Chase—A431 cells were labeled for 5 min with 100 μCi of Trans35S-Label (ICN Biomedicals, Irvine, CA) in Dulbecco’s modified Eagle’s medium without methionine/cysteine, supplemented with 10% dialyzed fetal calf serum. Cells were washed twice in PBS before being chased in media containing either AG1478 (2 μM) or vehicle. At selected time points, cells were lysed and subjected to IP with the relevant antibodies. Samples were then resolved by SDS-PAGE and detected by autoradiography.

Biotinylation and Endoglycosidase-H Digestion of A431 Cells—A431 cells were treated with AG1478 (2 μM) or vehicle for 20 min or 24 h. Cells were then biotinylated with the Amersham Biosciences Protein Biotinylation Module as per the manufacturer’s instructions. In brief, 20 μl of biotinylation agent in bicarbonate buffer (pH 8.6) was added at 4°C for 30 min. Cells were then lysed, subjected to IP with mAb 806, and immunoblotted with Streptavidin-horseradish peroxidase for surface EGFR. Prior to immunoblotting, some precipitates were eluted to endoglycosidase-H digestion as previously described (37).

Subcutaneous Xenograft Model—A431 cells (3 × 10⁶) in 100 μl of PBS were inoculated subcutaneously into both flanks of 4- to 6-week-old, female nude mice (Animal Research Centre, Perth, Australia). All studies were conducted using established tumor models as previously reported (25). Treatment commenced once tumors had reached the mean volume indicated in the appropriate figure legend. Tumor volume in mm³ was determined using the formula (length × width²)/2, where length was the longest axis and width was the perpendicular measurement. Data are expressed as mean tumor volume ± S.E. for each treatment group. All data were analyzed for significance by one-sided Student’s t test where p < 0.05 was considered statistically significant. Where multiple groups were involved, data were analyzed by ANOVA, and if appropriate, post-hoc testing with Student’s t test was undertaken. Some data were also analyzed using survival analysis, with p < 0.05 by log rank testing considered significant. This research project was approved by the Animal Ethics Committee of the Austin Hospital.

RESULTS

AG1478 Increases mAb 806 Binding to Cells Overexpressing wt EGFR—The ability of AG1478 to influence mAb 806 binding to the EGFR on A431 cells was examined initially by determining the amount of receptor captured by mAb 806 IP in the presence and absence of AG1478. Cells were grown in media containing AG1478 (2 μM) or vehicle for periods ranging from 20 min to 3 days before being lysed, subjected to IP with the relevant antibodies, and immunoblotted for EGFR. AG1478 was able to increase mAb 806 reactivity as reflected by the increased amount of EGFR immunoprecipitated in the AG1478-treated groups (Fig. 1). The addition of AG1478 for 20 min rapidly increased mAb 806 reactivity; the mean increase was 181% above control (Fig. 1, A and C, lower panels). This was a relatively selective effect on mAb 806 binding as mAb 528 reactivity increased by only 25% with similar treatment (Fig. 1, A and C, upper panels). Where cells were exposed to AG1478 for 24 h or greater, IP was performed on samples containing equal amounts of total cellular protein to control for the cytostatic effects of AG1478 on A431 cells. AG1478 treatment for 24 h increased mAb 806 reactivity by 121%, at 2 days the increase was 242%, and at 3 days it was 449% (Fig. 1, B and D, lower panels). Again, this increase was not a reflection of a total increase in EGFR, because IP with mAb 528 showed no increase in total EGFR at identical time points of AG1478 treatment (Fig. 1, B and D, upper panels).

The effect of AG1478 on mAb 806 binding was examined in other cell lines overexpressing the wt EGFR. All cell lines were treated with 2 μM AG1478 for 20 min (Fig. 2A) or 24 h (Fig. 2B). The parental U87MG cells, which does not overexpress the wt EGFR or its ligands, show no mAb 806 reactivity (24) and were
not rendered mAb 806-positive by treatment with AG1478 (Fig. 2, A and B). The derivative cell line U87MG.wtEGFR, which has been transfected to overexpress wt EGFR, was mAb 806-positive at baseline, and this reactivity was increased by 96% with 20 min AG1478 (Fig. 2, A and C, left panel) and by 209% with 24 h of AG1478 (Fig. 2, B and C, right panel). Similar results were seen with another cell line transfected with wt EGFR, 293T.wt (Fig. 2, A and B). There was a 139% increase in mAb 806 reactivity with 20 min of AG1478 (Fig. 2, A and D, left panel) and a 145% increase with 24 h of AG1478 (Fig. 2, B and D, right panel). Thus, the effect of AG1478 on mAb 806 reactivity was not limited to A431 cells but was observed in several cell lines.

The durability of this effect was examined by treating A431 cells with AG1478 for 20 min, withdrawing the media containing AG1478 and replacing it with media devoid of AG1478. At subsequent time points, cells were lysed and mAb 806 reactivity was determined by IP (Fig. 3). mAb 806 reactivity increased with 20 min of AG1478, and this effect persisted for at least 4 h following AG1478 withdrawal (Fig. 3).

**AG1478 Increases mAb 806 Binding to Cell Surface EGFR**—The experiments above showed an increase in mAb 806 binding to total cellular EGFR, but they do not distinguish between mAb 806 reactivity at the cell surface and that within the cell. This is important, because we have previously shown that mAb 806 recognizes the substantial intracellular pool of high mannosyl EGFR in A431 and U87MG.wtEGFR cells (9). To determine what effect AG1478 has on mAb 806 reactivity at the cell surface, A431 cells were treated with AG1478 (2 μM) or vehicle; cell surface proteins were then biotinylated before IP with mAb 806 and immunoblotted with streptavidin-horseradish peroxidase. There was a 71% mean increase in mAb 806 after 20-min exposure to AG1478, and this was still apparent after 60 min (Fig. 4A).

Assessment of mAb 806 binding to the cell surface in the absence or presence of AG1478 was also analyzed by FACS. BaF/3.wt cells were employed in these experiments because of their ease of use with FACS analysis. As previously reported (28), these cells were transfected to overexpress EGFR and bind mAb 806; they do not express EGFR ligands. There was a reproducible increase in surface mAb 806 reactivity at both the 20-min (Fig. 4B) and 24-h (Fig. 4C) incubation with AG1478. With 20 min of AG1478 (Fig. 4B), the median fluorescence increased from 4.1 (vehicle-treated) to 6.8 (AG1478-treated). Similar results were seen with 24 h of AG1478 (Fig. 4C), where the median fluorescence increased from 4.0 (vehicle-treated) to 7.4 (AG1478-treated). Thus, the increase in mAb 806 surface binding induced by AG1478 is reproducible using two different techniques in several cell lines.

Finally, Fabs of mAb 806 were also analyzed by FACS using BaF/3.wt and A431 cells in the presence or absence of AG1478. Although the mAb 806 Fab bound robustly to both cell lines, there was no increase in binding seen following 20 min of treat-
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**FIGURE 4.** AG1478 increases mAb 806 reactivity at the cell surface. (A), A431 cells were treated with AG1478 (2 μM) for the times shown then cell surface-biotinylated, lysed, subjected to IP with mAb 806, and immunoblotted with streptavidin-horseradish peroxidase. (B) and (C), BaF3.wt cells were stained with an irrelevant antibody (dark gray, solid), mAb 528 for total EGFR (light gray, solid), mAb 806 after treatment with vehicle only (dashed line, hollow), and mAb 806 after AG1478 treatment (solid line, hollow) for 20 min (B) or 24 h (C). All experiments were repeated three times.

AG1478 Alters the Glycosylation of EGFR—We have previously reported that A431 cells misdirect a low level of high mannose EGFR from the endoplasmic reticulum to the cell surface and that these high mannose forms are readily bound by mAb 806 (9). We further confirmed this previous work by treating A431 cells overnight with swainsonine and monensin, two agents that increase the high mannose form of the EGFR. As expected both agents dramatically increased the amount of mAb 806-reactive EGFR expressed in A431 cells as determined by IP and subsequent immunoblotting (Fig. 5A).

**FIGURE 5.** AG1478 increases the amount of high mannose EGFR. (A), A431 cells were treated overnight with swainsonine or monensin, subjected to IP with mAb 806, and immunoblotted for EGFR. (B), A431 cells were treated with AG1478 (2 μM) for 20 min or 24 h before being cell surface-biotinylated, subjected to IP with mAb 806, sham (left panel)- or endoglycosidase-H (right panel)-digested, and then immunoblotted with streptavidin-horseradish peroxidase. The EGFR runs as a doublet as previously reported. The appearance of a new band in lane 8 after endoglycosidase-H digestion is indicated by an arrow. (C) and (D), treatment with 20 min (C) or 1 day (D) of AG1478 in U87MG.Δ2–7 (top row) and U87MG.DK cells (bottom row) was followed by lysis, IP with mAb 806, and immunoblotting for EGFR. All experiments were repeated at least twice.

To investigate the contribution of high mannose forms of EGFR to the increase in mAb 806 reactivity at the cell surface, A431 cells were incubated with AG1478, cell surface-biotinylated, lysed, and subjected to IP with mAb 806. Samples then underwent endoglycosidase-H digestion, a process that specifically removes mannose residues from glycoproteins, or sham digestion. In samples that underwent a sham digestion (Fig. 5B, left panel), the precipitated EGFR migrated as two bands, an observation that has previously been reported (9). In samples that underwent endoglycosidase-H digestion, samples treated for 20 min (lane 6) and 24 h (lane 8) with AG1478 migrated more quickly than the relevant vehicle-treated samples (lanes 5 and 7, respectively), indicative of an increase in the high mannose content of these samples. More importantly, the sample treated with 24 h of AG1478 (lane 8) migrated more quickly...
then the sample treated with 20 min of AG1478 (lane 6) and was associated with the appearance of a new band at 130 kDa (lane 8, arrow) (Fig. 5B, right panel). This suggests that 24 h of AG1478 induces substantially more high mannose forms of EGFR than 20 min of AG1478, with the new band representing a population of receptors whose side chains were wholly composed of mannose sugars and that were subsequently reduced to the naked peptide backbone following endoglycosidase-H treatment (38). Furthermore, it is clear that these high mannose iso-forms of EGFR traffic to the cell surface as evidenced by their successful biotinylation.

To confirm that 24 h of AG1478 increases the number of high mannose receptor forms, we treated U87MG.Δ2–7 cells with AG1478 (2 μM) or vehicle for 20 min (Fig. 5C) or 24 h (Fig. 5D). U87MG.Δ2–7 cells overexpress the de2–7 EGFR-mutated receptor, and approximately half of these are known to be high mannose iso-forms; as a result, the receptor migrates as two distinct bands, with the upper and slower moving band representing the fully glycosylated receptor and the lower, faster moving band representing the high mannose form of the receptor (9). In the vehicle control groups (Fig. 5, C and D), the receptor is equally distributed between the upper and lower bands. With 20 min of AG1478 (Fig. 5C), there is neither an increase in total mAb806 reactivity nor a change in the proportion of protein between the two bands. With 24 h of AG1478 (Fig. 5D), there is a slight increase in total mAb 806 reactivity (mean increase of 19%), which is associated with a change in the proportion of mature versus high mannose receptors. The intensity of the upper (mature) band is reduced by 25%, whereas the intensity of the lower (high mannose band) is increased by 25%.

To investigate whether this effect on EGFR glycosylation requires the physical binding of AG1478 to EGFR, we investigated the effect of AG1478 on U87MG.DK cells. Like U87MG.Δ2–7 cells, U87MG.DK cells express the de2–7 EGFR mutation. However, this de2–7 EGFR contains a single point mutation in the kinase domain, which prevents ATP and AG1478 from binding to the receptor and therefore is a dead kinase (DK) (16, 36). When U87MG.DK cells are treated with 20 min (Fig. 5C) or 24 h of AG1478 (Fig. 5D), there is neither an increase in total mAb 806 reactivity nor a change in the proportion of protein within each band. We therefore conclude that the effect of AG1478 on EGFR glycosylation requires a specific interaction between AG1478 and the kinase domain of EGFR.

AG1478 Disrupts the Post-translational Processing of EGFR—The AG1478-induced increase in high mannose EGFR suggests that it may influence the biosynthesis of the receptor. Therefore, A431 cells were pulse-labeled with [35S]methionine/cysteine for 5 min, chased at 37 °C in media containing AG1478 (2 μM) or vehicle, then lysed for IP with relevant antibodies. In the vehicle group, a significant amount of mAb 806-reactive EGFR was present at 0 min, which then progressively diminished at subsequent time points (Fig. 6, A (upper right panel) and B). At the end of the 4-h chase, mean mAb 806 reactivity had only decreased by 11% as compared with the 56% decline seen in the vehicle group. mAb 528 reactivity was still lowest at the start of the chase but only increased for the first 2 h before reaching a plateau (Fig. 6A, lower right panel). This suggests that AG1478 disrupts the post-translational glycosylation of the EGFR, resulting in retention of increased amounts of high mannose EGFR. Taken together, the above data show that AG1478 specifically alters the biosynthesis of EGFR so that the immature, high mannose forms of EGFR are not processed into mature glycoproteins but are instead trafficked to the cell surface where they contribute to increased mAb 806 binding.

AG1478 Increases mAb 806-reactive EGFR Dimers—The effects of AG1478 on EGFR biosynthesis with the subsequent accumulation of high mannose EGFR at the cell surface require at least a 24-h incubation with AG1478. Clearly, this is not the mechanism that causes the rapid and large rise in mAb 806 reactivity seen with short incubations (such as 20 min) of...
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AG1478. The rapidity of the increase argues for a separate and conformationally based mechanism at this time point. It has previously been reported that AG1478 reversibly induces the formation of inactive EGFR dimers in A431 cells (36). We therefore investigated whether these AG1478-induced dimers might preferentially expose the mAb 806 epitope and thereby increase its reactivity. A431 cells in the vehicle-treated group were found to spontaneously contain a small amount of mAb 806-reactive dimers (Fig. 7A). Because visualization of dimers and trimers requires longer radiographic exposures, the monomer bands are overexposed, and therefore differences in intensity cannot be observed. Both the 20-min and 24-h incubation with AG1478 reproducibly increased the amount of dimer precipitated by mAb 806. Because the mAb 806 epitope is not accessible in the ligand stabilized back-to-back dimer (23), these dimers must be of an alternative conformation.

To confirm that these mAb 806 dimers are not the back-to-back dimer, we repeated the cross-linking experiments in 293T.ΔCR1 cells. 293T.ΔCR1 lack the dimerization/activation arm in the CR1 domain and are therefore unable to form the back-to-back dimer (33). Nevertheless, untreated 293T.ΔCR1 cells were found to express mAb 806-reactive dimers (Fig. 7B), and AG1478 induced a marked increase in these dimers after 20-min incubation (Fig. 7B) and a more modest increase after 24-h incubation (Fig. 7B). Thus we confirm that AG1478 increases the level of an alternate dimer, which is recognized by mAb 806.

The AG1478-induced Increase in mAb 806 Reactivity Is Not the Result of Kinase Inhibition—Because AG1478 effectively inactivates the kinase domain of the EGFR, we sought to determine if mAb 806 reactivity correlates with kinase activity in the absence of AG1478. BaF/3 cells expressing wt EGFR with different degrees of kinase activity (BaF/3.wt >> BaF/3.V741G > BaF/3.K721R) (32) were analyzed by FACS for mAb 806 reactivity in the absence of AG1478. mAb 806 reactivity was expressed as the ratio of the median fluorescence of mAb 806 to the median fluorescence of an irrelevant antibody. As seen in Table 1, there was no increase in mAb 806 reactivity in either of the kinase-impaired cell lines (BaF/3.V741G or BaF/3.K721R) compared with the cell line expressing functional EGFR (BaF/3.wt). Thus, it is unlikely that AG1478 inactivation of the EGFR kinase produces the receptor conformation changes that cause increased mAb 806 binding. Similarly, when mAb 806 reactivity was compared between cell lines overexpressing the mutated de2–7 EGFR, there was no increase in mAb 806 reactivity in the U87MG.DK cell line, which has an inactivating mutation in the kinase domain of de2–7 EGFR (16), when compared with U87MG Δ2–7, which has a functional and constitutively active form of de2–7 EGFR (15, 16) after 20 min of AG1478 treatment (data not shown).

Treatment of A431 Xenografts with mAb 806 and AG1478 Using Rationally Designed Schedules—Because AG1478 increases mAb 806 reactivity by two distinct mechanisms (a conformational effect and a change in EGFR glycosylation), we conducted three concurrent in vivo experiments to explore the relative contribution that each mechanism makes toward the therapeutic synergy between AG1478 and mAb 806 (1). Mice bearing A431 xenografts were treated with PBS, single agent AG1478, single agent mAb 806, or three different combinations of AG1478 and mAb 806. One group received mAb 806 before AG1478 (Fig. 8A), another concurrently with AG1478 (Fig. 8B), and one group received mAb 806 after AG1478 (Fig. 8C). The different pharmacokinetic profiles of AG1478 and mAb 806 after intraperitoneal injection (AG1478 is completely cleared by 24 h (1), whereas the level of mAb 806 declines slowly over 4–5 days (24)) means that the group receiving mAb 806 before AG1478 will mainly benefit from the effects of AG1478 on EGFR conformation, the group receiving mAb 806 concurrently with AG1478 will benefit from both the conformational and glycosylation effects, and the group receiving mAb 806 after AG1478 will benefit mainly from the effect on EGFR glycosylation. These treatment schedules were designed to investigate how AG1478 enhances mAb 806 activity rather than to achieve maximal tumor inhibition.

In the group receiving mAb 806 before AG1478 (Fig. 8A), all of the treatment groups were significantly smaller than the PBS control group on day 24, when the PBS control group was culled for ethical considerations (p = 0.009 by ANOVA with post-hoc testing showing p < 0.05 for all groups compared with control). By day 26, the combination group was also significantly smaller than both of the single agent treatment groups (p = 0.009 by ANOVA with post-hoc testing showing p < 0.05 for all groups compared with control).

Similar results were seen in the group receiving mAb 806 concurrently with AG1478 (Fig. 8B). All the treatment groups

TABLE 1

| Cell line | Median fluorescence | mAb 806 | mAb 806/irrelevant mAb (ratio) |
|-----------|---------------------|---------|-------------------------------|
| BaF/3.wt  | 1.6                 | 2.0     | 1.3                           |
| BaF/3.V741G | 2.4               | 2.6     | 1.1                           |
| BaF/3.K721R | 1.7               | 1.8     | 1.1                           |
were significantly smaller than PBS control group on day 24
\( (p = 0.0003 \text{ by ANOVA with post-hoc testing showing } p < 0.05 \text{ for all groups compared with control}) \). By day 26, the combination group was also significantly smaller than the single agent treatment groups \( (p = 0.002 \text{ by ANOVA with post-hoc testing showing } p < 0.05 \text{ for all groups compared with control}) \).

Results were slightly different in the group receiving mAb 806 after AG1478 (Fig. 8C). Although all the treatment groups were significantly smaller than the PBS control group on day 24 \( (p = 0.001 \text{ by ANOVA with post-hoc testing showing } p < 0.05 \text{ for all groups compared with control}) \), there was no significant benefit to combination therapy compared with single agent therapy on further follow-up. A confirmatory experiment again showed that combination treatment with AG1478 in this schedule was not better than mAb 806 alone (data not shown).

Hence, we found that there is enhancement of tumor inhibition when A1478 and mAb 806 are combined in such as way as to allow AG1478 to favorably alter EGFR conformation (Fig. 8, A and B), whereas there is no added benefit when the AG1478 and mAb 806 combination only allows AG1478 to influence mAb 806 binding through effects on glycosylation (Fig. 8C).

Mice bearing A431 xenografts were then treated with an optimized treatment schedule (Fig. 8D) based on the preceding series of experiments. Single agent AG1478 was given on days 7–11, mAb 806 was given on days 8 and 10, and the combination group received both agents concurrently. All of the treatment groups were significantly smaller than the PBS control on day 21 when the control group was culled for ethical considerations \( (p = 0.002 \text{ by ANOVA with post-hoc testing showing } p < 0.05 \text{ for all groups compared with control}) \). By day 24, the combination group was significantly smaller than the single agent groups \( (p = 0.002 \text{ by ANOVA with post-hoc testing showing } p < 0.05 \text{ for all groups compared with control}) \).

Data from survival analysis (Fig. 8E) confirmed the differences seen in the growth curve (Fig. 8E). Using a combined end point of death or tumor size of 1500 mm\(^3\), log rank analysis showed significant differences across the four treatment groups \( (p = 0.0026) \). Post-hoc analysis showed that the combination group survived longer than all the other groups \( (p < 0.002) \). No complete regressions were seen in any group.

**DISCUSSION**

All in vivo studies (1, 39–41) and in vitro studies (39, 40), except one (42), have shown that combination therapy with cetuximab and a TKI (either gefitinib or erlotinib) results in superior tumor inhibition. Combination treatment results in greater growth inhibition in vivo (1, 39–41) with enhanced apoptosis (39, 40) and superior inhibition of phosphorylated EGFR (39, 40), MAPK (39, 40), Akt (39, 40), cell proliferation (39–41), and vascularization (39). The reason postulated for this increased efficacy is that cetuximab and TKIs have different...
modes of action, and in combination they result in improved EGFR inhibition. Matar, et al. (39) showed that, although a common set of genes were affected when cells were treated with cetuximab or a TKI, there clearly were also genes that were differentially affected.

We have previously demonstrated synergy between mAb 806 and AG1478 (1). The interaction of AG1478 and mAb 806 appeared more complicated than just a more comprehensive inhibition of EGFR, though, with data showing that AG1478 increased mAb 806 binding to cells overexpressing EGFR. Our current study shows that AG1478 increases binding of mAb 806 to the surface of cells through two distinct mechanisms: an immediate conformational change in the EGFR and a longer term increase in cell surface under-glycosylated EGFR. AG1478 disrupts the post-translational glycosylation of EGFR leading to increased retention of high mannose forms of EGFR, some of which are then trafficked to the cell surface. The requirement for 24 h of AG1478 treatment to obtain this effect is in keeping with the 20 h required for the complete post-translation processing of EGFR in A431 cells (38). The fact that this effect is seen with the U87MG.D2–7 cell line, but not in the U87MG.DK cell line, where AG1478 cannot bind to the kinase domain of the receptor, demonstrates that this effect requires a direct interaction between AG1478 and EGFR.D2–7.

Our data on the immediate effects of AG1478 on the conformation of EGFR support and extend a growing body of literature about the diversity of conformations that EGFR can assume. Several groups have shown that some, if not most, unligated and inactive EGFRs spontaneously exist in preformed dimeric or oligomeric forms in cells overexpressing EGFR (7, 43–46). Two groups have shown that the treatment of EGFR-expressing cells with quinazoline compounds such as AG1478 can induce the formation of further inactive EGFR dimers that sequester ligand (36, 47). The precise conformation of these AG1478-induced dimers was unclear then, although the ability of AG1478 to bind to the kinase domain was shown to be crucial for this effect (36). The unique conformational specificity of mAb 806 now demonstrates that these dimers are not the back-to-back dimer but rather some alternate conformation. Their presence in cells expressing the ΔCR1 EGFR, which is incapable

FIGURE 9. Model of EGFR activation and its inhibition by AG1478 and mAb 806. A, we propose that, as the EGFR untethers, it forms the inactive alternate dimer described in this report. B, addition of AG1478 accelerates EGFR untethering but locks the receptor in the alternate dimer, thus reducing signaling. Dissociation of AG1478 allows the EGFR to adopt the back-to-back dimer and participate in signaling. C, binding of mAb 806 to the alternative dimer locks the EGFR into this confirmation even if the AG1478 dissociates, thus reducing signaling more effectively.
of forming the back-to-back dimer, convincingly supports this concept. We postulate that this alternate inactive dimer is composed of two untethered EGFR monomers stabilized by both intracellular and extracellular interactions. Interestingly, the bivalent nature of mAb 806 may further stabilize this conformation, because we could not detect the AG1478-induced changes using Fab fragments. Previous reports have shown that the formation of unligated EGFR dimers requires the intra-cytoplasmic domain of EGFR (46) and that AG1478-induced dimers require the presence of a normal kinase domain (36). Our data show that deletion of amino acids 6–273 in de2–7 EGFR prevents a short incubation of AG1478 from increasing mAb 806 reactivity, suggesting that this extracellular region is also crucial in stabilizing the alternative dimer.

Combining the insights gained into EGFR conformation above and knowledge about the mechanisms of AG1478, we speculate that combination therapy with AG1478 and 806 does not only increase the binding of mAb 806, and therefore its activity, but also potentiates the activity of AG1478. Fig. 9A shows the activation of the EGFR taking into account our observation of an alternate EGFR dimer. We propose this alternate dimer is an important intermediate form in the transition of the tethered, inactive receptor to the active, ligated, back-to-back dimer. The function of ligand binding remains unclear, but it would seem a likely role is to promote the transformation from inactive dimer to the active back-to-back dimer (28, 45). If correct, EGFR ligands may have very little effect on receptor untethering; rather it is the binding of ATP, or ATP-mimetics such as AG1478, to the kinase domain that regulates untethering.

In this model, the addition of AG1478 would not only competitively inactivate the kinase domain but would also trap EGFR in these inactive dimers (Fig. 9B). It is quite likely that ligands for the EGFR would also be trapped in these dimers, based on previous experimental observations that AG1478-induced dimers act as a ligand trap (36, 47). However, these AG1478-induced dimers do not undergo normal internalization and degradation (48) but are trapped on the cell surface, where they can readily signal again when AG1478 eventually dissociates from the receptor (Fig. 9B).

We therefore postulate that another way in which combining AG1478 with mAb 806 may increase efficacy is by mAb 806 binding to the enlarged pool of alternate dimers induced by AG1478 and trapped on the cell surface. Binding by mAb 806 would prevent them from undergoing the final transformation to the active back-to-back dimer or higher order oligomeric states (7) when AG1478 dissociates (Fig. 9C). It may also enhance the endocytosis and degradation of these AG1478-induced dimers, because mAbs against EGFR can activate alternate pathways of receptor degradation (49).

Based on the in vitro data, we explored whether both effects of AG1478 on mAb 806 were equally important in vivo. We designed schedules of administration that pharmacokinetically favored the conformational effect, the glycosylation effect or both effects in vivo. The results suggest that the conformational effect of AG1478 was the more biologically relevant with respect to anti-tumor efficacy in vivo. Indeed, the schedule that de-emphasized the conformational effects of AG1478 in favor of the glycosylation effects (Fig. 8C, where mAb 806 was given after AG1478) showed no improvement of combination therapy over mAb 806 alone in two independent animal experiments. These observations may potentially be of value in obtaining optimal growth inhibition in humans.

In conclusion, the unique specificity of mAb 806 has provided further insights into the complexity of EGFR biology. We have used it to further characterize the structure of the alternative dimer first detected by Arteaga (36) and Lichtner (47) and to suggest a role for it in the activation of the receptor. We have also shown that the interaction of AG1478 and mAb 806 is more complicated than just an additive effect, with AG1478 definitely influencing the conformation and biosynthesis of mAb 806. It is possible that mAb 806 may also favorably modulate the activities of AG1478 as proposed in Fig. 9. Lastly, this knowledge may potentially be of use in designing better schedules of administration in vivo, and we believe that the complex interactions between TKIs and mAbs against EGFR merit further investigation as the understanding gained may profoundly affect the use of these EGFR-specific agents clinically.

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