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Acridine Yellow G Blocks Glioblastoma Growth via Dual Inhibition of Epidermal Growth Factor Receptor and Protein Kinase C Kinases

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Background: PTEN deletion renders glioblastomas resistant to epidermal growth factor receptor (EGFR) inhibitors.

Results: Acridine yellow G inhibits both EGFR and PKCs, resulting in cell growth repression, cell cycle arrest in the G1 phase, and shrinkage of brain tumors.

Conclusion: Acridine yellow G is a potent anti-tumor agent for malignant gliomas.

Significance: Combinatorial inhibition of EGFR and PKCs provides the proof of concept for treating glioblastomas.

Amplification of the epidermal growth factor receptor (EGFR), frequently expressed as a constitutively active deletion mutant (EGFRvIII), occurs commonly in glioblastoma multiformes (GBM). However, blockade of EGFR is therapeutically disappointing for gliomas with PTEN deletion. To search for small molecules treating this aggressive cancer, we have established a cell-based screening and successfully identified acridine yellow G that preferentially blocks cell proliferation of the most malignant U87MG/EGFRvIII cells over the less malignant U87MG/PTEN cells. Oral administration of this compound markedly diminishes the brain tumor volumes in both subcutaneous and intracranial models. It directly inhibits EGFR and PKCs, resulting in a blockade of mammalian target of rapamycin (mTOR) signaling and cell cycle arrest in the G1 phase, which leads to activation of apoptosis in the tumors. Hence, combinatorial inhibition of EGFR and PKCs might provide proof of concept in developing therapeutic agents for treating malignant glioma and other human cancers.

Glioblastomas are the most malignant human brain tumors. They are highly aggressive, infiltrative, and destructive. Approximately 40–50% of glioblastomas show amplification of the EGFR gene locus (1) and about half of these tumors express a mutant receptor (EGFRvIII) that is constitutively active due to an in-frame deletion of exons 2 to 7 within the extracellular ligand-binding domain (2, 3). The EGFR is a receptor tyrosine kinase that regulates the fundamental processes of cell growth and differentiation. Overexpression of EGFR or its ligands is reported in various epithelial tumors and triggers interest in EGFR as a potential target for cancer therapy (4, 5). Numerous human cancers can be therapeutically treated with EGFR tyrosine kinase inhibitors and EGFR-specific monoclonal antibodies. These tumors include non-small cell lung cancer (6, 7), colorectal carcinoma (8), and glioblastoma (9, 10).

EGFR signals through a complex network of intermediates including phosphatidylinositol 3-kinase (PI3K)/Akt, MAPK, and phospholipase C-γ1. The kinase mammalian target of rapamycin (mTOR) is a critical target of EGFR signaling, linking growth factor abundance to cell growth and proliferation. PTEN serves as a negative regulator of the PI3K pathway by removing the third phosphate from the inositol ring of the second messenger phosphatidylinositol 1,4,5-trisphosphate. PTEN inactivation results in accumulation of phosphatidylinositol 1,4,5-trisphosphate levels and persistent signaling through Akt (11). It is worth noting that mutation of the gene encoding the tumor suppressor PTEN drives activation of PI3K and Akt in an EGFR-independent manner and may confer resistance to upstream inhibition of EGFR (12). Because EGFR is implicated as a driving oncogene in malignant glioma, inhibition of EGFR signaling would be expected to present an effective therapeutic strategy. Nonetheless, initial results with EGFR inhibitors in glioblastoma have been disappointing as most patients do not respond. Less than 10% of patients with amplified, mutated, and activated EGFR and wild-type PTEN show a response to EGFR inhibitors (13). Subsequent analysis demonstrates that patients with EGFR-driven tumors that carry PTEN mutations do not respond to EGFR inhibitor therapy. Loss of
PTEN is highly correlated with treatment failure. In fact, it has been shown that coexpression of EGFRvIII and PTEN strikingly predicts treatment responses (10).

It is very difficult to treat glioblastoma due to several complicating factors: tumor cells are resistant to conventional therapies, the brain is susceptible to damage due to conventional therapy, the brain has a very limited capacity to repair itself, and many drugs cannot cross the blood-brain barrier to act on the tumor. Surgical resection together with post-surgical radiation increases survival rate and, more recently, radiotherapy plus concomitant and adjuvant temozolomide significantly improve survival of GBM patients without reduction in quality of life (14). However, the infiltrative and aggressive nature of GBM renders current treatments, such as surgical resection, radiation, and chemotherapy, relatively ineffective. Novel and effective therapeutic interventions for GBM are urgently needed.

Recently, Mishel and co-workers (15) demonstrated that the mTOR inhibitor rapamycin enhances the sensitivity of PTEN-deficient tumor cells to the EGFR kinase inhibitor erlotinib in U87MG and SF295 glioblastoma cells expressing EGFR, EGFRvIII, and PTEN in relevant combinations, and the combination therapy promotes cell death in PTEN-deficient tumor cells. Nonetheless, clinical trials with the combined therapy reveal disappointing results (16). In this study, we report the identify and characterize one family of compounds: the acridine tricycle heteroatomic compound, which selectively blocks proliferation of PTEN-deficient U87MG/EGFRvIII cells. Acridine yellow G potently inhibits EGFR and PKC activation in a dose-dependent manner, leading to mTOR signaling inactivation. Oral administration of this compound evidentially decreases the tumor volumes in both subcutaneous and intracranial models and elongates the life span of brain tumor inoculated nude mice. It also displays a potent antitumor effect against human lung cancers. Moreover, it significantly decreases cell proliferation and enhances apoptosis in tumors. Chronic treatment with this compound exhibits undetectable toxicity in animals. Therefore, these data suggest that this compound is a safe and effective therapeutic agent for treating aggressive gliomas and other human cancers.

**EXPERIMENTAL PROCEDURES**

**Cells, Reagents, and Mice**—Human glioblastoma cell line U87MG was stably transfected with vector control, PTEN, EGFRvIII, EGFRvIII/PTEN, EGFR, and EGFR/PTEN, and the stable transfected cells were maintained in DMEM with 10% FBS and 1 × penicillin/streptomycin/glutamine supplemented with various selected antibiotics. For PTEN, 400 μg/mL of G418 was included, for wild-type EGFR, 0.7 μg/mL of puromycin was added. For EGFRvIII, 150 μg/mL of hygromycin was employed. All antibodies and the BrdU incorporation assay kit were from Cell Signaling. Go 6967 and Ro 32-0432 were from Tocris Biosciences. Bisindolylmaleimide I (BIM I) and phorbol 12-myristate 13-acetate (PMA) were from EMD. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma. The Histo-SP AEC kit was obtained from Invitrogen. Nude mice (nu/nu), 5–6 weeks of age, were obtained from the NCI, National Institutes of Health. Mice were housed with a maximum of 6 per cage and fed autoclaved chow and water with 12-h light and dark cycles. All efforts were made to minimize discomfort to the animals. The animals required physical restraint for injection of tumor cells and delivery of drugs, and measurement of tumor size with a caliper (hand-held) and all procedures were done with approval by the Institutional Animal Care and Use Committee of Emory University.

**In Vitro Proliferation Assay**—Three thousand cells were cultured in a 96-well plate. The next day, the medium was replaced with fresh medium containing different concentrations of compounds or vehicle controls. Cells were incubated at 37 °C for the indicated times. The cell proliferation was monitored by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide/BrdU incorporation assay according to the manufacturer’s protocols.

**Subcutaneous and Intracranial Xenograft Model**—Cells (4 × 10⁶) in 100 μL of serum-free DMEM were inoculated subcutaneously into 5–6-week-old female nude mice. Treatment commenced once tumors had reached a mean volume indicated in the appropriate figure legend. The mice were treated with vehicle control or C2 (50 and 100 mg/kg) administrated by oral gavage once a day for 3 weeks. Tumor volume in mm³ was determined using the formula (length × width²)/2, where length was the longest axis and width being the measurement at right angles to the length. For the intracranial model, mice were placed in the stereotaxic instrument and cells (1 × 10⁵) were stereotactically inoculated into the right striatum, 3 mm below the dural surface on day 0. On day 7, the mice were examined with MRI to confirm tumor formation. Ten days after drug treatment, mice from each group were analyzed by MRI again for tumor volumes.

**Flow Cytometric Analysis**—Cells were treated with or without acridine yellow G for the indicated times, then centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol. Tubes containing the cell pellets were stored at −20 °C for at least 24 h. After this, the cells were centrifuged at 3000 × g for 10 min and the supernatant was discarded. Cells were then washed with 5 ml of PBS and incubated with propidium iodide (20 μg/ml) for 30 min and RNase A (20 μg/ml) in PBS for 45 min. The samples were analyzed on a Coulter Elite flow cytometer.

**Depletion of PKCα and PKCβ by siRNA**—Cells were transfected with 20 nm PKCa siRNA (Qiagen)/PKCB siRNA (sc-29450, Santa Cruz Biotechnology, Santa Cruz, CA) using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Four independent siRNAs directed against PKCa (A1, A2) were used along with control scrambled siRNAs (C): A1, AAGGCTTCCAGTGCAAGTCT; A2, AAGAGCTGCACATGAATTTGAT; C, AATTGCCGAAAAGTGCACGT.

**Magnetic Resonance Imaging (MRI)**—Magnetic resonance imaging scans were carried out on a 3T MRI scanner (Siemens Tim/Trio) using a volume wrist coil. Animals were anesthetized by intraperitoneal injection of the mixture of ketamine/xylazine (95:5, mg/kg). Images were collected in the axial direction, i.e. from the head to tail, covering the entire brain. The MRI protocol included pre-contrast T₁ weighted spin echo and T₂ weighted fast spin echo sequences using a field of view of 25 × 64 mm, matrix of 192 × 256, and slice thickness of 1 mm (no gap). Image acquisition parameters were: TR/TE = 560/14 ms with flip angle of 70 for T₁, weighted spin echo imaging and

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**Acridine Yellow G Suppresses Glioblastoma**

FBS and 10% FBS. Cells, Reagents, and Mice—Human glioblastoma cell line U87MG was stably transfected with vector control, PTEN, EGFRvIII, EGFRvIII/PTEN, EGFR, and EGFR/PTEN, and the stable transfected cells were maintained in DMEM with 10% FBS and 1 × penicillin/streptomycin/glutamine supplemented with various selected antibiotics. For PTEN, 400 μg/mL of G418 was included, for wild-type EGFR, 0.7 μg/mL of puromycin was added. For EGFRvIII, 150 μg/mL of hygromycin was employed. All antibodies and the BrdU incorporation assay kit were from Cell Signaling. Go 6967 and Ro 32-0432 were from Tocris Biosciences. Bisindolylmaleimide I (BIM I) and phorbol 12-myristate 13-acetate (PMA) were from EMD. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma. The Histo-SP AEC kit was obtained from Invitrogen. Nude mice (nu/nu), 5–6 weeks of age, were obtained from the NCI, National Institutes of Health. Mice were housed with a maximum of 6 per cage and fed autoclaved chow and water.
Acridine Yellow G Suppresses Glioblastoma

In Vitro Kinase Inhibitory Profile and EGFR/mTOR Kinase Assay—Acridine yellow G was subjected to the Invitrogen SelectScreen™ Kinase Profiling Panel analysis using Z'-LYTETM substrates. The screening was conducted following the manufacturer’s protocol. The EGFR kinase assay was performed according to guidelines from the protein-tyrosine kinase assay kit (Sigma). The mTOR kinase assay was performed according to guidelines from the K-ELISA mTOR activity kit (Calbiochem). Both kinase assays were performed in triplicate.

Statistics Analysis—Data are presented as mean ± S.E. of three or four independent experiments. Statistical evaluation was carried out by Student’s t test or one-way analysis of variance. Data were considered statistically significant when \( p < 0.05 \). All statistical analysis was performed by program Prism (GraphPad Software, La Jolla, CA).

RESULTS

Screening for Compounds Selectively Blocking U87MG/EGFRvIII—PTEN-deficient GBMs are resistant to EGFR inhibitor therapy (10). To search for pharmacological agents to treat these malignant cancers, we developed a cell-based screening assay to identify small molecules that effectively block GBM cells with EGFRvIII amplification and PTEN deletion but only weakly inhibit less malignant cells with normal copies of EGFR and PTEN. The positive hits that strongly blocked cell proliferation were picked for the cytotoxicity assay on non-cancerous HEK293 cells and mouse embryonic fibroblast cells. Only compounds that exhibited negligible toxicity on these cells were selected.

Intracranial tumors were identified by pre-contrast T2 weighted fast spin echo MRI. The tumor volume was obtained using the region of interest analysis. Tumor margins that define the regions of interest were traced manually on each image slice based on the contrast enhancement where the signal increase was more than 30% of the background brain tissue signals. The tumor volume (V) of each animal was then calculated by summing the products of each signal enhanced area and thickness of its slice.

In Vitro Kinase Inhibitory Profile and EGFR/mTOR Kinase Assay—Acridine yellow G was subjected to the Invitrogen SelectScreen™ Kinase Profiling Panel analysis using Z'-LYTETM substrates. The screening was conducted following the manufacturer’s protocol. The EGFR kinase assay was performed according to guidelines from the protein-tyrosine kinase assay kit (Sigma). The mTOR kinase assay was performed according to guidelines from the K-ELISA mTOR activity kit (Calbiochem). Both kinase assays were performed in triplicate.

Statistics Analysis—Data are presented as mean ± S.E. of three or four independent experiments. Statistical evaluation was carried out by Student’s t test or one-way analysis of variance. Data were considered statistically significant when \( p < 0.05 \). All statistical analysis was performed by program Prism (GraphPad Software, La Jolla, CA).

RESULTS

Screening for Compounds Selectively Blocking U87MG/EGFRvIII—PTEN-deficient GBMs are resistant to EGFR inhibitor therapy (10). To search for pharmacological agents to treat
blue O and methylene blue. Quantitative analysis showed that 1 μM C2 demonstrated more than 70% inhibition on U87MG/EGFRvIII cells, but toluidine blue O and methylene blue displayed about 50% activity. On the other hand, these two compounds barely blocked U87MG/PTEN cell growth, whereas C2 exhibited weak inhibitory activity as compared with the control (Fig. 2). Toluidine blue O and methylene blue both contain a thiazine in the middle ring with the nitrogen atom on the transposition to the diamino groups. These two compounds possessed a much weaker inhibitory effect on cell proliferation than C2, suggesting that the middle ring plays a critical role in the anti-proliferative activity of C2.

To gain insight into the structural moiety that is essential for the anti-proliferation activity, we conducted a SAR study by analyzing a panel of acridine derivatives with various substituent groups on the diamino groups or in the ring system. Methylation of the middle nitrogen atom alone (compound 1) almost completely abolished the anti-proliferative activity of C2 in both U87MG/EGFRvIII and U87MG/PTEN cells, indicating that the middle nitrogen atom cannot tolerate any chemical modification. Furthermore, methylation of the carbon atom in the middle ring (compound 7) also markedly decreased the anti-proliferative activity, underscoring that the middle ring has to remain intact. However, demethylation of C2 in the two side rings (compound 9, called proflavine, and compound 6, called acridine orange) did not significantly alter the anti-proliferative effect on U87MG/EGFRvIII cells, but they both appear to display a more potent inhibitory effect on U87MG/PTEN cells. It is worth noting that oxidizing the middle carbon in compound 12 completely stripped the anti-proliferative activity, emphasizing the middle ring plays a critical role in the anti-proliferative activity of C2. Notably, switching methyl
groups with amino groups such as in compound 2 and compound 4 did not significantly alter the activity of C2 on U87MG/EGFRvIII cells, although its selectivity toward U87MG/EGFRvIII cells versus U87MG/PTEN cells was abrogated. Remarkably, addition of a bulky group to the diamino groups (compound 3 and 5) almost totally sabotaged the anti-proliferative activity of C2, but addition of a small chloroacetyl (compound 8) barely affected the activity of C2. Therefore, the diamino groups can tolerate covalent modification by a functional group with less steric hindrance. However, demethylation of C2 allowed its diamino groups to accommodate a larger aryl group (compound 10 and 11) without profoundly sacrificing its inhibitory activity (Fig. 2). Together, SAR analysis demonstrated that the middle heteroatom ring is critical for the anti-proliferative activity. Once the middle ring was changed into a thiazine ring or is methylated on either nitrogen or carbon atom, the anti-proliferative effect was significantly decreased. Nonetheless, the two amino groups on the side rings could be modified by small alkyl groups or by switching methyl group positions without substantially changing the activity. However, bulky group addition to the amines significantly disrupted the inhibitory activity. The effects of the series of acridine compounds on cell proliferation were also confirmed by the BrdU incorporation assay (supplemental Fig. 1A).

**Acridine Yellow G Inhibits EGFR, PKC, and mTOR Signaling**—mTOR, a crucial mediator of PI3K signaling, is a compelling molecular target in GBM patients, and p-mTOR is a robust biomarker for the anti-proliferative activity of EGFR inhibitors (17, 18). Inhibition of mTOR promotes response to the EGFR inhibitor in PTEN-deficient and PTEN-intact GBM cells (15). To explore whether C2 exerts its anti-proliferative action through targeting EGFR or mTOR kinase, we conducted an in vitro kinase assay using the purified active kinases in the presence of various concentrations of C2. At low concentrations, C2 had no effect on either kinase. At 5–10 μM, EGFR was markedly inhibited by C2 with an IC_{50} of ~7.5 μM, although mTOR was not affected at all (Fig. 3A). The control inhibitor wortmannin robustly blocked mTOR kinase (data not shown). Thus, C2 directly and selectively inhibits EGFR but not mTOR kinase. To further search for the molecular targets of C2, we conducted in vitro kinase profile screening (Invitrogen, SelectScreen™). Interestingly, C2 potently blocked PKC kinases. A titration assay showed that C2 inhibited the PKC activity via its p38, δ, and γ isoforms more potently than the other two family isoforms: novel and atypical PKCs. The values of IC_{50} for PKCα, PKCβ, PKCδ, and PKCγ were: 4935.2, 5021.7, 8539.8, and 9598.1 nM, respectively (Fig. 3B). These findings fit well with a previous report that the acridine derivatives inhibit PKC kinases (19).

To explore whether C2 affects the EGFR/P13K/mTOR signaling cascades in intact cells, we treated U87MG/EGFRvIII and U87MG/PTEN cells with various concentrations of C2 and monitored the activation of EGFR and its downstream effectors by immunoblotting. We found that EGFRvIII tyrosine phosphorylation was prominently blocked by C2 in a concentration-dependent manner (Fig. 3C, left top panel). We made a similar observation in mTOR activation and its numerous downstream effects including 4E-BP1, p70S6K, and the substrate rpS6 of p70S6K, indicating that C2 indeed exerts the inhibitory effect on the signal pathways that are essential for cell proliferation (Fig. 3C, left panel, sixth to tenth lanes). By contrast, Akt and ERK1/2 phosphorylation were not altered at all (Fig. 3C, left panel, third and fifth lanes). As expected, PKCs were strongly inhibited, which was demonstrated by both p-PKC-pan and p-PKCa/β antibodies (Fig. 3C, left panel, 12th and 13th lanes). Nonetheless, we barely detected EGFR phosphorylation in U87MG/PTEN cells due to its low expression levels (data not shown). mTOR activation and phosphorylation of p70S6K and substrate rpS6 were decreased by C2 at about 2–5 μM, in contrast to 4E-BP1 phosphorylation, which was not changed in U87MG/PTEN cells (Fig. 3C, right panel, fifth to ninth lanes). Interestingly, Akt phosphorylation was decreased by C2, whereas ERK1/2 phosphorylation remained unchanged (Fig. 3C, right panel, first and third lanes). Hence, restoring PTEN couples the C2 EGFR inhibition to Akt inactivation in U87MG/PTEN cells. Notably, PKC phosphorylation signals were much stronger in U87MG/PTEN cells than U87MG/EGFRvIII cells. At higher concentrations, PKC activation was also robustly blocked by C2 (Fig. 3C, right panel, 10th and 11th lanes).

To quantitatively analyze the selectivity of C2, we determined the IC_{50} values on U87MG/EGFR, U87MG/EGFR/PTEN, U87MG/EGFRvIII, U87MG/EGFRvIII/PTEN, U87MG, and U87MG/PTEN cells. As shown in Fig. 3D, U87MG/EGFRvIII cells were the most sensitive to C2 among the 6 isogenic U87MG cell lines. The titration assay revealed that the IC_{50} for C2 on U87MG/EGFRvIII was around 0.2 μM, and 0.75–2.5 μM for the other 5 cell lines. Therefore, the differential EGFR, mTOR, and PKC signaling inhibition responses to C2 in U87MG/EGFRvIII and U87MG/PTEN cells might provide insight into the molecular mechanism why C2 exerts a more potent anti-proliferative effect on U87MG/EGFRvIII cells versus U87MG/PTEN cells.

**Acridine Yellow G Arrests Cell Cycle at G_{1} Phase**—Recent studies demonstrate that PKCα plays a pivotal role in coupling EGFR to mTOR independent of Akt in gliomas, and PKC signals downstream of PTEN (17). Moreover, we have observed a much stronger PKC activity in U87MG/PTEN cells than U87MG/EGFRvIII cells (Fig. 3). These findings indicate that PKCs are up-regulated in PTEN-intact cells compared with PTEN-deficient cells. To examine this possibility, we monitored the PKC expression profiles in 6 isogenic U87MG cell lines, and found that PKCα-γ, β, and δ isoforms were all markedly up-regulated in PTEN-intact cells than the PTEN-deficient counterparts. In addition, the PKCs were also robustly phosphorylated in PTEN-intact cells (Fig. 4A). To assess the effect of C2 on the cell cycle, we incubated the vehicle and 1 μM C2 with isogenic U87MG cell lines for 24 h. Flow cytometry analysis revealed that C2 treatment led to G_{1} arrest irrespective of PTEN or EGFRvIII status. Interestingly, cotreatment with PMA, a potent PKC activator, diminished the G_{1} phase arrest effect by C2. Notably, PMA treatment alone greatly decreased the G_{1} phase in both U87MG and U87MG/PTEN cells, whereas it barely exhibited any effect on the G_{1} phase in EGFR-overexpressed U87MG/EGFRvIII and U87MG/EGFRvIII/PTEN cells (Fig. 4B). The effect by PMA on C2 is similar to a previously
reported finding that activation of PKC by PMA abolishes the EGFR inhibitor effect of erlotinib on G1 phase arrest (17).

Because C2 targets both PKCs and EGFR, it remains unknown which molecular partner accounts for the G1 cell cycle arrest effect. To address this question, we treated the above 4 cell lines with BIM, Go6976, Ro 32-0432 (PKC inhibitors), erlotinib (EGFR inhibitor), or a mixture with both types of compounds for 24 h. Flow cytometry analysis showed that PKC inhibitors had almost no effect on the cell cycles of the 4 cell lines, whereas erlotinib dramatically arrested the cell cycle at the G1 phase. A combination of both compounds did not further escalate the inhibition (Fig. 4C). Immunoblotting analysis of these samples showed that the phosphorylation of PKCs was blocked by the inhibitors, but they had no effect on rpS6 phosphorylation by themselves in U87MG/EGFRVIII. However, C2 potently inhibited EGFR, PKCs, and rpS6 phosphorylation. Erlotinib selectively blocked EGFR phosphorylation and slightly decreased rpS6 phosphorylation but it did not affect phosphorylation of PKCs. Remarkably, a mixture of erlotinib and BIM I greatly blocked p-rpS6, an inhibitory effect comparable with C2 (supplemental Fig. S1B, left, 4th panel and bottom panel). Akt phosphorylation was slightly reduced by the PKC inhibitors, addition of EGFR inhibitors further elevated the inhibitory effect in U87MG/EGFRVIII cells (supplemental Fig. S1B, left, 3rd panel). Interestingly, in U87MG/PTEN cells, p-Akt signaling was robustly blocked by erlotinib, and almost eradicated when erlotinib was mixed with PKC inhibitors. Moreover, rpS6 phosphorylation was reduced by the mixture of PKC and EGFR inhibitors, which also occurred to C2-treated sample (supplemental Fig. S1B, right, 2nd and 4th panels). Surprisingly PKC phosphorylation, blocked by each of the individual PKC inhibitors, was up-regulated when the erlotinib and PKC inhibitors mixture was employed (supplemental Fig. S1B, right, 3rd panel). The underlining molecular mechanism for this phenomenon remained unknown. Thus, a combination of EGFR inhibitor and PKC inhibitors only partially mimics the biological effects by C2, which can dually block both EGFR and PKC.

To further examine the contribution of PKCs in this event, we selectively depleted PKCα and PKCβI by their specific siRNAs in U87MG/EGFRVIII and U87MG/EGFRVIII/PTEN cells, followed by incubation with 1 μM C2. Flow cytometry analysis showed that knocking down PKCα or PKCβI did not affect the C2-induced G1 phase arrest in either cell line (Fig. 4D). The depletion of PKCα and PKCβI was confirmed by immunoblotting (supplemental Fig. S1C). Therefore, these data suggest that C2 inhibition of EGFR, but not PKCs, might account for its G1 phase cell cycle arrest effect.

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Oral Administration of C2 Decreases Subcutaneous Tumor Volumes in Nude Mice—To explore whether the C2 compound can block GBM cell proliferation in vivo, we inoculated U87MG/EGFRVIII cells in nude mice. After treatment with either vehicle or drugs (via oral gavage, 50 or 100 mg/kg; n = 11–12) once a day for 21 consecutive days, tumors found in vehicle-treated mice were much larger than those found in mice injected with C2 (Fig. 5A). The average tumor weight on the final day of therapy (day 28 after inoculation) was: 2.93 g for vehicle; 2.04 g for C2 (50 mg/kg), and 1.52 g for C2 (100 mg/kg) (**, p < 0.01) (Fig. 5B). Extending the same experimental paradigm into human lung cancer cells (supplemental Fig. S2). C2 displayed a dose-dependent anti-proliferative effect on lung cancer cells with IC_{50} around 0.75–5 μM for most of the tested lung cancer cells. Oral administration of C2 also elicited a dose-dependent inhibitory activity on lung cancer growth in subcutaneous models. At 100 mg/kg, C2 treatment profoundly reduced tumor weight (0.75 ± 0.05 g) versus the vehicle group (1.97 ± 0.55 g).

To determine the molecular events in the drug-treated tumors, we examined the major signaling effectors’ phosphorylation status in the tumors by immunoblotting, and found that p-EGFR was prominently decreased by C2 (Fig. 5, C and E, **p < 0.01). As expected, p-PKC and its downstream mTOR signaling machinery p-p70S6K, p-rpS6, and p-4E-BP1 were all markedly reduced by C2. In contrast, p-Akt 473 was not significantly inhibited, although one sample from the 50 mg/kg group exhibited a slightly decreased p-Akt signal (Fig. 5C). We also monitored the signaling profiles by immunohistochemistry staining of the tumor sections. Compared with the robust p-EGFR, p-rpS6, and p-PKC signals in vehicle-treated tumors, C2 potently reduced these signals (Fig. 5D). Notably, Ki67 staining was also substantially diminished in C2-treated tumors compared with control tumors (Fig. 5F), indicating that this compound strongly blocks cancer cell proliferation in vivo. To test whether this compound inhibits cell proliferation by increasing apoptosis in tumors, we performed anti-active caspase-3 immunofluorescent staining on the tumor sections, and found that the C2 elicited demonstrable apoptosis, whereas negligible apoptosis was observed in vehicle-treated samples (Fig. 5G). These findings were also confirmed by TUNEL staining (supplemental Fig. S3A), suggesting that C2 blocks tumor growth in vivo by inhibiting cell proliferation and triggering apoptosis. Hence, C2 inhibits EGFR, mTOR, and PKC signaling and significantly blocks tumor growth in animals.

Therapeutic Efficacy of C2 in Intracranial Xenograft Model—To further assess the therapeutic potential of C2 on the aggressive gliomas, we employed the intracranial xenograft model.

FIGURE 3. Acridine yellow G blocks EGFR/PKC/mTOR signaling pathways. A, in vitro kinase assay. Acridine yellow G inhibited EGFR kinase activity but had no effect on mTOR kinase activity in vitro. Purified human EGFR kinase or rat mTOR kinase were incubated with different concentrations of acridine yellow G on ice for 20 min and the mixture was added to the respective substrate-coated wells. The phosphorylated substrates were detected by anti-phospho-tyrosine or anti-phospho-p70S6K 389 for EGFR kinase and mTOR kinase, respectively. The kinase assays were performed in triplicate. Data were expressed as mean ± S.E.

B, in vitro PKC kinase assay. Acridine yellow G inhibited different PKC kinases in a dose-dependent manner. Acridine yellow G was subjected to the SelectScreen Kinase Profiling Panel analysis using Z’LYTE substrates. The kinase activities of PKCα, PKCβI, and PKCγ were totally suppressed by 10 μM acridine yellow G. C, acridine yellow G suppresses EGFR, mTOR, and PKC signaling pathways in GBM cells. U87MG/EGFRVIII and U87MG/PTEN cells were treated with different concentrations of acridine yellow G for 6 h and the cells were lysed. The lysates were analyzed by immunoblotting (IB) with various antibodies as indicated. EGFR tyrosine phosphorylation and numerous mTOR downstream effectors’ activation were blocked by acridine in a dose-dependent manner. Acridine yellow G also suppressed PKC phosphorylation. D, cell proliferation assay. Acridine yellow G preferentially blocked the cell proliferation of U87MG/EGFRVIII cells in a dose-dependent manner. The IC_{50} is about 0.25 μM. Data were expressed as mean ± S.E.
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FIGURE 4. Acridine yellow G arrests cell cycle at G1 phase. A, the expression profiles and phosphorylation of PKCs in U87MG glioblastoma cells expressing EGFR, EGFRvIII, and PTEN in all relevant combinations. Cells that overexpress PTEN showed higher PKC protein expression levels as well as stronger phosphorylation than the counterpart without PTEN. B, acridine yellow G arrests the cell cycle at the G1 phase. U87MG, U87MG/PTEN, U87MG/EGFR, and U87MG/EGFRvIII cells were treated with acridine yellow G (1 μM) for 24 h. Flow cytometric analysis showed that acridine yellow G induced cell cycle arrest at G1 in the 4 tested isogenic U87MG cell lines. C, flow cytometry analysis of the effect of PKC inhibitors in combination with EGFR inhibitor erlotinib. Four different U87MG isogenic cell lines were incubated with 100 nM of different PKC inhibitors in the presence or absence of erlotinib (10 μM) for 24 h. The treated cells were analyzed by flow cytometry. PKC inhibitor had a negligible effect on cell cycle profiles. D, knocking down the PKCs had no effect on the acridine yellow G1 phase arrest effect. PKCα and PKCβ were depleted by its specific siRNA in U87MG/EGFRvIII and U87MG/EGFRvIII/PTEN cells. After 24 h, the siRNA-treated cells were further treated with 1 μM acridine for another 24 h, followed by flow cytometry analysis.
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We implanted 10⁵ cells of U87MG/EGFRvIII cells stereotactically in the brain of athymic nude mice and monitored tumor growth by MRI on day 7. After confirming tumor formation in brains, each group of animals (n = 12) was immediately treated with vehicle control (0.5% methylcellulose), C2 (50 and 100 mg/kg) once a day via oral gavage. Ten days after drug treatment, the mice were subjected to MRI analysis again. Immediately after, the animals were sacrificed and corresponding sections of the brains were analyzed by immunoblotting and H&E staining. Both MRI and pathology results evidenced smaller tumors in mice treated with C2 (Fig. 6A and supplemental Fig. S3C). Remarkably, C2 demonstrated a dose-dependent inhibitory effect on tumor growth. It exhibited an ~40 and 70% inhibitory effect on brain tumor volumes at doses of 50 and 100 mg/kg, respectively (Fig. 6B). TUNEL assay also showed that C2 elicited evident apoptosis in the brain tumors (supplemental Fig. S3B). Next, we examined the signaling cascades in these samples. Similar to what we observed in the tumor samples (subcutaneously), C2 strongly blocked p-EGFR, p-4E-BP1, p-p70S6K, p- rpS6, and p-PKC in the tumor sections (intracranial) as compared with the vehicle controls. Once again, p-Akt was not greatly diminished in most of the drug-treated samples (Fig. 6C). Immunohistochemistry staining of these tumor sections demonstrated that p-EGFR, p-PKC, and p-rpS6 were dramatically repressed by C2 treatment; in contrast, p-Akt in drug-treated tissues remained comparable with that in the vehicle-treated group (Fig. 6D). As expected, Ki67 staining and active caspase-3 immunofluorescent staining of tumor samples (intracranially) also exhibited similar patterns as displayed in tumor samples (subcutaneously) (Fig. 6, E and F). Animal survival studies were performed in 3 groups of mice implanted with these highly tumorigenic U87MG/EGFRvIII xenografts. All the animals that underwent vehicle treatment were dead by 18–25 days after tumor implantation (median survival, 21 days). A significant increase in survival was found in animals that were treated with C2 at 50 mg/kg (median survival, 24 days) and C2 at 100 mg/kg (median survival, 25 days), compared with animals that underwent vehicle control (p < 0.05) (Fig. 6G). This was consistent with the decreased expression of p-EGFR in the C2-treated group (Fig. 6H, **, p < 0.01). Although C2 elongated the lifespan of the tumor bearing mice, however, the effect was not dramatic. To rule out unanticipated neurotoxicity or neuronal damage caused by C2 that might account for this modest efficacy, we performed histological analysis (anti-caspase-3 and TUNEL staining) on the adjacent neural tissues of animals receiving C2 and vehicle. Compared with the vehicle group, C2 exerted no demonstrable neurotoxicity or neuronal damage (Fig. 7A). In vitro, C2 (1 μM) did not induce demonstrable apoptosis in the U87 isogenic cell lines; nevertheless, at higher doses C2 induced clear apoptosis in both U87MF/EGFRvIII and U87MG/PTEN cells (Fig. 7, B and C). Therefore, oral administration of C2 blocks EGFR/PKC/mTOR signalings in gliomas and shrinks the brain tumors, enhancing animal survival.

DISCUSSION

In this report we have identified and characterized acridine yellow G, which demonstrates selective anti-proliferative activity against PTEN-deficient U87MG glioblastoma cells that overexpress EGFRvIII versus PTEN stably transfected U87MG cells. Strikingly, oral administration of this compound displayed a dose-dependent inhibitory effect against the tumor growth of U87MG/EGFRvIII in both subcutaneous and intracranial models, which might result from its anti-proliferative and apoptosis-inducing effects. In vitro, we did not detect demonstrable apoptosis in C2-treated cells at least at a dose of 1 μM. However, when the concentrations were elevated to 5 or 10 μM, we observed evident apoptosis in both U87MF/EGFRvIII and U87MG/PTEN cells (Fig. 7). In vivo, the apoptosis was prominent in tumors (subcutaneously and intracranially) after oral administration of C2 at 50 or 100 mg/kg. There are several possibilities to explain this phenomena: 1) in vitro, a 1 μM dose is not high enough to trigger apoptosis, although it is potent enough to elicit cell cycle arrest and inhibits EGFR and PKCs; 2) in vivo, at doses of 50 or 100 mg/kg, the real drug exposure for C2 is more than 1 μM in tumor samples, hence, the apoptosis-inducing effect is evident in the tumors; 3) it is possible that C2 can be metabolized after oral administration, and changed into modified derivatives. For instance, some 9-position modified acridine elicited apoptosis in numerous human cancers (20, 21).

It is worth noting that C2 also significantly elongated the survival of mice in the intracranial xenograft model. Accordingly, the tumor volumes in drug-treated mouse brains were substantially decreased compared with the vehicle group. In both the subcutaneous and intracranial tumor models, C2 was administrated via oral injection, suggesting that it might be orally bioactive. C2 markedly decreased the tumor growth, supporting that this compound itself or its metabolites might be able to penetrate the brain-blood barrier. This is consistent with previous reports that acridine derivatives as pharmacotherapeutics for prior disease or tumors could pass the blood-brain barrier (22, 23). Moreover, C2 exhibited much less toxicity against non-cancerous HUVEC cells and immortalized mouse embryonic fibroblast cells than GBM cells up to 10 μM (supplemental Fig. S4). In contrast, it exerted anti-proliferative

FIGURE 5. Acridine yellow G blocks subcutaneous tumor growth of U87MG/EGFRvIII cells. A and B, acridine yellow G significantly inhibits the growth of U87MG/EGFRvIII xenograft tumors. U87MG/EGFRvIII cells were inoculated subcutaneously in nude mice, and after the tumors formed, the nude mice were treated orally with vehicle or acridine yellow G at doses of 50 and 100 mg/kg, respectively. Control mice were treated with vehicle (0.5% methylcellulose). The drug and vehicle control were administrated via oral gavage. Data represent mean ± S.E. (*, p < 0.05; **, p < 0.01, n = 11–12/group). C and D, acridine yellow G inhibits EGFR, mTOR, and PKC signalings in vivo. C, the tumor tissue lysates from vehicle or drug-treated samples were analyzed by immunoblotting (IB) with the indicated antibodies. EGFR tyrosine phosphorylation and numerous mTOR downstream effectors’ activation were blocked by C2. Moreover, C2 also inhibited PKC phosphorylation but had no effect on Akt signaling. D, immunohistochemistry for p-EGFR (Y1068, p-Akt (S473), p-PKC (pan), and p-rpS6 (S235/236) on tumor sections derived from animals treated with or without acridine yellow G. Brown represents positive staining (arrows). Bar represents 100 μm. E, quantification of p-EGFR expression level versus the total EGFR in subcutaneous tumor. Data represent mean ± S.E. (***, p < 0.001). F and G, acridine yellow G inhibits tumor growth in vivo. Ki67 (F) and active caspase-3 (G) staining were conducted on tumor sections derived from animals treated with or without acridine yellow G. Bar represents 50 μm.
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activity with IC\textsubscript{50} values of 0.2–2.5 μM on malignant U87MG/EGFRvIII and other U87MG isogenic cells, indicating that it specifically targets malignant cancers but not non-cancerous normal cells. This selectivity indicates that we have achieved the primary goal of our counterscreening to ensure low toxicity and few side effects for drug recipients. This specificity could possibly be the reason for the observed non-toxicity in animals chronically treated with 100 mg/kg of C2 for 1 month. Both pathological examination and complete blood count analysis support that acridine yellow G is safe (supplemental Fig. S5 and Table 1). Moreover, body weight and animal behaviors were not changed upon drug administration (data not shown), underscoring that this compound does not trigger demonstrable side effects.

FIGURE 6. Acridine yellow G suppresses intracranial tumor growth of U87MG/EGFRvIII cells. A and B, acridine yellow G suppresses the growth of U87MG/EGFRvIII xenograft tumors in brain. A, MRI scans of individual mice 14 days after intracranial implantation of 10\textsuperscript{5} tumor cells. The presence of a glioma is detected through the bright areas (red arrows) by contrast enhancement from the MRI contrast agent (Gd-DTPA). U87MG/EGFRvIII brain tumor growth in brains of mice was reduced by orally administrated acridine yellow G. Note the small tumors in acridine yellow G-treated mice and the large tumor in vehicle-treated animals. B, quantitative analysis of intracranial tumor volume in mice treated with or without the compound. Acridine yellow G significantly suppressed tumor growth compared with the control group. Data represent mean ± S.E. (*, \( p < 0.05 \), \( n = 12 \)/group). C and D, acridine yellow G represses EGFR/PKC/mTOR signalings in brain tumors. C, Western blot (IB) analysis of intracranial tumor lysates from the animals treated with acridine yellow G (50 and 100 mg/kg) or vehicle for 15–20 days. EGFR tyrosine phosphorylation and numerous mTOR downstream effectors’ activation were blocked by C2. D, immunohistochemistry for p-EGFR (Y1068), p-Akt (S473), p-PKC (pan), and p- rpS6 (S235/236) in tumor sections derived from animals treated with or without acridine yellow G. Brown represents positively stained cells (arrows). Bar represents 100 μm. E and F, acridine yellow G inhibits intracranial tumor growth. Ki67 (E) and active-caspase-3 (F) staining were conducted on tumor sections derived from animals treated with or without acridine yellow G. Bar represents 50 μm.

FIGURE 7. Acridine yellow G displays no neurotoxicity in vivo. A, acridine yellow G exhibits no neurotoxicity. Histological analysis as well as anti-caspase-3 and TUNEL staining were carried out on the adjacent neural tissues of intracranial model animals receiving C2 and vehicle. Compared with the vehicle treatment group, C2 exerts no neurotoxicity or neuronal damage. B, acridine yellow G does not induce apoptosis at 1 μM. U87MG, U87MG/PTEN, U87MG/EGFR, and U87MG/EGFRvIII cells were treated with C2 (1 μM) for the indicated times. The cell lysates were analyzed by immunoblotting (IB) with the indicated antibodies. C, acridine yellow G triggers apoptosis at higher doses. U87MG/PTEN and U87MG/EGFRvIII cells were treated with the indicated doses of C2 for 24 h. A high dose of C2 (5 and 10 μM) induced clear apoptosis in cells.

Acridine Yellow G Suppresses Glioblastoma

Acridine Yellow G displays no neurotoxicity in vivo. A, acridine yellow G exhibits no neurotoxicity. Histological analysis as well as anti-caspase-3 and TUNEL staining were carried out on the adjacent neural tissues of intracranial model animals receiving C2 and vehicle. Compared with the vehicle treatment group, C2 exerts no neurotoxicity or neuronal damage. B, acridine yellow G does not induce apoptosis at 1 μM. U87MG, U87MG/PTEN, U87MG/EGFR, and U87MG/EGFRvIII cells were treated with C2 (1 μM) for the indicated times. The cell lysates were analyzed by immunoblotting (IB) with the indicated antibodies. C, acridine yellow G triggers apoptosis at higher doses. U87MG/PTEN and U87MG/EGFRvIII cells were treated with the indicated doses of C2 for 24 h. A high dose of C2 (5 and 10 μM) induced clear apoptosis in cells.
PKC appears to be critical in regulating many aspects of gliomas. Higher doses of PKC up-regulate cell proliferation and invasion (24), decrease apoptosis (25), and reduce susceptibility to radiation (26). Malignant human gliomas have high PKC activity that correlates with their rapid growth rate. Growth suppression of gliomas can be achieved with PKC inhibition (27, 28). PKC is a serine–thrreonine kinase that is activated by association with diacylglycerol and Ca\textsuperscript{2+} as well as phosphorylation by PKD1. Upon EGFR activation, phospholipase C–γ is recruited via its Src homology 2 domain to the phosphorylated receptor where it is activated and cleaves phosphatidylinositol 4,5-trisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. Production of diacylglycerol leads to PKC activation and phosphorylation of downstream targets that include RAF1 and GSK3β (29, 30). Using a highly selective PKC inhibitor (BIM I), Fan et al. (17) demonstrated that decreased tumor cell viability was observed regardless of the EGFR or PTEN protein abundance status, suggesting that PKC inhibitors may provide alternatives for patients with mutant PTEN who are refractory to EGFR monotherapy. BIM I, a competitive inhibitor for the ATP-binding site of PKC, inhibits PKC with IC\textsubscript{50} of 10 nm. Nonetheless, the cell death effects in GBM cells took place at 1–10 μM, a concentration 100–1,000-fold higher than necessary to block PKCs (17). Presumably, the inhibitory effect by BIM I on GBM cell proliferation might not only be attributed by inhibition of PKCs, because at such a high concentration BIM I also antagonizes other kinases including GS3 etc. Using non-biased irrational screening, surprisingly, we found that acridine yellow G dually blocks both EGFR and PKCs, and exerts prominent anti-proliferative activity in U87MG/EGFRvIII versus U87MG/PTEN cells (Figs. 3 and 4). To explore whether a combination of PKC inhibitors and an EGFR inhibitor might simulate the anti-proliferative effect by C2 on GBM cells, we employed the EGFR inhibitor erlotinib and three PKC inhibitors: BIM I, Go6976 (IC\textsubscript{50} 7.9 nm), and Ro 32-0432 (IC\textsubscript{50} 9.3 nm). For PKC inhibitors alone, we did not observe any cell growth inhibitory effect for 10, 100, and 1000 nm doses in either cell line, although 10 μM erlotinib exhibited ~30% inhibition in both types of cells. The combination of PKC inhibitors with erlotinib slightly decreased cell proliferation compared with erlotinib alone; in contrast, C2 (1 μM) dramatically blocked GBM cell proliferation (supplemental Fig. S6). Thus, a combination of two different inhibitors for EGFR and PKCs only partially mimic the biological effects of the single agent that possesses the dual EGFR and PKC inhibitory activities.

The EGFR inhibitor erlotinib arrested the cell cycle at the G\textsubscript{1} phase in PTEN\textsuperscript{wt} cells and had no effect on PTEN\textsuperscript{mt} cells. Treatment with the PKC inhibitor BIM induced cell cycle arrest at the G\textsubscript{1} phase in PTEN\textsuperscript{wt} cells and at the G\textsubscript{2} phase in PTEN\textsuperscript{mt} cells (17). However, C2 arrested the cell cycle at the G\textsubscript{1} phase regardless of PTEN genotype (Fig. 4). To examine the role of PKC in the C2-mediated cell cycle arrest, we treated U87MG cell lines with the PKC activator PMA alone or in combination with C2 and found that PMA treatment decreased G\textsubscript{1} phase accumulation in U87MG and U87MG/PTEN cells. However, this effect was not observed in EGFRvIII overexpressed U87MG cells. Hence, overexpression of EGFRvIII renders the cells more resistant to PMA-induced cell cycle profile alteration. Nonetheless, PMA co-treatment reduced the G\textsubscript{1} phase arrest by C2 in all of the tested cell lines (Fig. 4). This effect is consistent with the reported phenomenon that PMA or over-expression of active PKC fragments decreases the G\textsubscript{1} phase arrest by erlotinib (17). However, depletion of PKCs did not abolish the G\textsubscript{1} phase arrest effect by C2, indicating that PKC inhibition by C2 might not account for its G\textsubscript{1} phase arrest effect. On the other hand, DNA intercalators including doxorubicin and amsacrine (an acridine derivative) usually arrest the cell cycle at the G\textsubscript{2} phase. Although C2 might also act as a DNA intercalator like many other acridine family members, it does not arrest the cell cycle at the G\textsubscript{2} phase, suggesting that EGFR inhibition by C2 might provide an explanation for its G\textsubscript{1} phase arrest effect.

Interestingly, our immunoblotting analysis reveals that PKCs are selectively up-regulated in PTEN overexpressed cells. In addition, the phosphorylation levels in PKCs were highly escalated (Fig. 4). It remains unknown how overexpression of PTEN in U87MG cells up-regulates PKC proteins and their phosphorylation levels. A previous study (31) shows that TGFβ downregulates the tumor suppressor PTEN via PKCβ in pancreatic cancer cells. However, in gliomas, PKCα appears to act downstream of PTEN (17). Thus, the cross-talk between PKC and PTEN might be complicated and cell type dependent. Nonetheless, up-regulation of PKC protein levels in U87MG/PTEN cells might overwhelm C2 and its anti-proliferative effect, which might account for the molecular mechanism explaining U87MG/PTEN cells’ decreased sensitivity to C2 versus U87MG/EGFRvIII cells. p-rpS6 is an important marker for cell proliferation (17). The level of p-rpS6 was significantly suppressed in cells treated with C2, which can be mimicked by a combined treatment with the PKC inhibitor (BIM I) and erlotinib. However, PKC inhibitor alone exerted negligible inhibition on p-rpS6 (supplemental Fig. S1B, left panel). Erlotinib alone barely inhibited p-rpS6 in PTEN-deleted U87MG/EGFRvIII cells. In U87MG/PTEN cells, the inhibition of p-rpS6 by C2 or erlotinib treatment was not as strong as in U87MG/EGFRvIII cells. These observations are consistent with the robust anti-proliferative effect on EGFRvIII cells compared with PTEN cells by C2 (Figs. 1 and 3D).

Acridine yellow G, also called 3,6-diamino-2,7-dimethylacridine, belonging to the nitrogen heterocyclic backbone chemical family, is a yellow dye with strong bluish-violet fluorescence. Acridine and related derivatives bind to DNA and RNA due to their abilities to intercalate, and are known carcinogens. Acridine causes mutations in incorporating into the bacterial DNA, and in doing so creating an additional base on the opposite strand (32). Although intercalation may lead to a propensity for frameshift mutagenesis in repetitive sequences, it is far from proven what has been shown in bacteria and bacteriophage into mammalian cells. Simple intercalators do not seem to have other major mutagenic effects (33). Acridine-based compounds can be used as antibacterial, anti-malarial, and anti-tumor agents. For instance, quinacrine has been used as an anti-malarial drug and as an antibiotic. It is used to treat giardiasis, a protozoal infection of the intestinal tract, and certain types of lupus erythematosus, an inflammatory disease that affects the joints, tendons, and other connective tissues and organs. Amsa-
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crine, another FDA-approved anti-neoplastic agent that is effective for treating acute leukemias and lymphomas, is a 9-amino-substituted acridine derivative. Amsacrine binds to DNA both externally and through intercalation. Rapidly dividing cancer cells are two to four times more sensitive to amsacrine than are resting cells. Amsacrine appears to cleave DNA by inducing double stranded breaks, and also targets and inhibits topoisomerase II (34, 35). Interestingly, amsacrine exhibits a dose-dependent anti-proliferative effect on U87MG/EGFRvIII and U87MG/PTEN cells. Nonetheless, its anti-cancer effect is much weaker than C2. C2 inhibited more than 70% of EGFRvIII cells at 1 \( \mu \)M, whereas amsacrine blocked about 60% cell proliferation at 8 \( \mu \)M (supplemental Fig. S7). Taken together, our studies support that C2 is a promising hit. Optimization of this compound by improving its potency through medicinal chemistry modification might warrant a novel anticancer drug for malignant human cancers.

Acknowledgment—We thank M. Otten Reed for proofreading the manuscript.

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Supplementary figure legends

Supplementary Figure 1. The effect of PKC inhibitors and EGFR inhibitor on EGFR/PKC/mTOR signaling.

(A) BrdU incorporation assay. A structure-activity-relationship (SAR) study was carried on U87MG/EGFRvIII cells and U87MG/PTEN cells. Numerous tricyclic compounds were selected and analyzed on these two cell lines for their anti-proliferative activity. U87MG/EGFRvIII cells and U87MG/PTEN cells were treated with different compounds (1 μM) having similar structures for 96 h. Then cell proliferation were examined using BrdU incorporation assay. Data were expressed as mean ± s.e.m. (B) The mixture of PKC inhibitors and EGFR inhibitor partially imitate the biologic activities on cell signaling by Acridine Yellow G. U87MG/EGFRvIII and U87MG/PTEN cells were treated with either individual PKC inhibitor (100 nM each), EGFR inhibitor erlotinib (10 μM) or their combination for 24 h. The cell lysates were analyzed by immunoblotting with indicated antibodies. Acridine Yellow G decreased EGFR, PKC and rpS6 phosphorylation, but it had no effect on Akt phosphorylation, whereas the inhibitor mixture completely blocked p-EGFR, decreased p-Akt and p-rpS6. Surprisingly, they had not effect on p-PKC. (C) Immunoblotting analysis confirmation of the depletion of PKCalpha and PKCbeta1 by the siRNAs in U87MG/EGFRvIII.

Supplementary Figure 2. Acridine Yellow G suppresses human lung cancer cell growth both in vitro and in vivo. (A) In vitro Cell proliferation assay. Acridine Yellow G significantly inhibits the proliferation of human lung cancer cells (H292G, H522, H1975, H460, H1792, H1299, H385 and H226B) in a dose-dependent manner. Cells treated with indicated dose of Acridine Yellow G for 96 h followed by SRB assay. (B, C) Acridine Yellow G significantly inhibits the growth of H460 xenograft tumors. The lung cancer cells were subcutaneously inoculated in nude mice. After the tumors were formed, the mice were treated with vehicle control or Acridine Yellow G for 14 days (once daily). Data represent mean ± s.e.m. (*P< 0.05, n = 5/group)

Supplementary Figure 3. Acridine Yellow G induces subcutaneous and intracranial tumor cells apoptosis in vivo. (A, B) TUNEL staining on tumor sections (subcutaneous and intracranial) derived from animals treated with or without Acridine Yellow G. Acridine Yellow G potently triggered apoptosis in the tumor samples, whereas the vehicle had negligible effect. Bar represents 50 μM. (C) Acridine Yellow G inhibits intracranial tumor growth. After treatment with Acridine Yellow G, the brain sections were stained with H&E.

Supplementary Figure 4. Acridine Yellow G exhibits modest cytotoxicity to non-cancerous cells. (A) LDH assay. Acridine Yellow G exhibits little toxicity in MEF and HUVEC cells compared with U87MG and U87MG/EGFRvIII groups using LDH assay. MEF, HUVEC and GBM cells (5 ×10^3) were plated in a 96-well plate. The cells were treated with various concentrations of Acridine Yellow G for 1 day. The released LDH in the medium was monitored by an LDH assay. (B) MTT assay. MEF, HUVEC and GBM cells (5×10^3) were plated in a 96-well plate. The cells were treated with various concentrations of Acridine Yellow G for 1 day. The cell survival was examined by an MTT assay. Cell viabilities of MEF and HUVEC cells were affected much less than U87MG and U87MG/EGFRvIII cancer cells. Data represent mean ± s.e.m.

Supplementary Figure 5. Histological examination of major organs from mice treated with Acridine Yellow G. C57BL/6J mice (2-3 months old) were orally administrated with 100 mg/kg of acridine Yellow G for 30 days. At the end of the experiment, the mice were perfused with 4% paraformaldehyde/PBS and major organs were collected and sectioned, followed by H & E staining. There are no significant changes in the organs derived from Acridine Yellow G-treated group compared with vehicle treatment group.
Supplementary Figure 6. Cell proliferation effect by PKC inhibitors or combined with EGFR inhibitor in U87MG/EGFRvIII and U87MG/PTEN cells. (A) In vitro cell proliferation assay. Cell proliferation of U87MG/EGFRvIII and U87MG/PTEN cannot be inhibited significantly by PKC inhibitors Go6967 and Ro32. Three thousand of both cells were plated in a 96-well plate, followed by treatment with various concentrations of PKC inhibitors for 96 h. MTT assay was conducted to assess cell proliferation. (B) The mixture of PKC inhibitors with EGFR inhibitor weakly displays an additive effect on cell proliferation. Combination treatment with PKC inhibitors (Go6967, Ro32 and BIM 1) at lower concentration (100 nM) with EGFR inhibitor Erlotinib (10 μM) exerted modest anti-proliferative effect in U87MG/EGFRvIII and U87MG/PTEN cells compared with erlotinib alone. (C) PKC inhibitor BIM inhibits U87MG/EGFRvIII and U87MG/PTEN cells in a concentration-dependent manner, but this anti-proliferative effect occurred at higher dose. Data represent mean ± s.e.m.

Supplementary Figure 7. Effect of Amsacrine on U87MG/EGFRvIII and U87MG/PTEN cells. Cells were treated for 96 h by different concentrations of Amsacrine. Cell proliferation was analyzed by MTT assay. Amsacrine exhibited a dose-dependent anti-proliferative effect on U87MG/EGFRvIII and U87MG/PTEN cells. Nonetheless, its anti-cancer effect is much weaker than C2. Data represent mean ± s.e.m.

Supplementary Table 1. Complete blood count of vehicle and C2 treated mice. C57BL/6J mice were orally treated with C2 (25, 50, and 100 mg/kg) for 30 days and the blood was collected and analyzed by complete blood count (CBC). The parameters of blood chemistry and biochemistry in C2 treated animals are with normal ranges compared with vehicle treated control mice.
Supplementary figure 1

(A) Cell proliferation (% of Control)

(B) U87MG/EGFRvIII

Vehicle

C2 (1 μM)

BIM (100 nM)

Go (100 nM)

Ro (100 nM)

BIM (100 nM) + Er (10 μM)

Go (100 nM) + Er (10 μM)

Ro (100 nM) + Er (10 μM)

Er (10 μM)

IB: anti-EGFR
IB: anti-p-EGFR (Y1068)
IB: anti-p-Akt (S473)
IB: anti-p-PKC (pan)
IB: anti-p-rpS6 (S235/236)

(C) U87MG/PTEN

Vehicle

C2 (1 μM)

BIM (100 nM)

Go (100 nM)

Ro (100 nM)

BIM (100 nM) + Er (10 μM)

Go (100 nM) + Er (10 μM)

Ro (100 nM) + Er (10 μM)

Er (10 μM)

IB: anti-EGFR
IB: anti-p-EGFR (Y1068)
IB: anti-p-Akt (S473)
IB: anti-p-Akt (S473)
IB: anti-p-PKC (pan)
IB: anti-p-PKC (pan)
IB: anti-p-rpS6 (S235/236)
IB: anti-p-rpS6 (S235/236)
Supplementary figure 4

(A) LDH assay of C2 cytotoxicity

(B) MTT assay of C2 cytotoxicity
Supplementary figure 7

Cell proliferation (% of Control)

- U87-EGFRvIII
- U87-PTEN

Amsacrine (μM)

DMSO  C2 (1μM)  1  2  4  8  16
Complete blood count of vehicle and C2 treated mice.

C57BL/6J mice were orally treated with C2 (25, 50, and 100 mg/kg) for 30 days and the blood was collected and counted.

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<th>Parameter</th>
<th>Vehicle</th>
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<td>2.58±1.02</td>
<td>3.185±1.14</td>
<td>2.275±0.91</td>
<td>3.08±0.10</td>
<td>0.9-9.3</td>
</tr>
<tr>
<td>Lymphocytes (% in WBC)</td>
<td>80.40±3.92</td>
<td>50.19±3.75</td>
<td>81.785±3.37</td>
<td>64.12±14.45</td>
<td>55.8-91.6</td>
</tr>
<tr>
<td>Monocytes ($\times 10^3/\mu l$)</td>
<td>0.04±0.01</td>
<td>0.325±0.05</td>
<td>0.155±0.09</td>
<td>0.265±0.08</td>
<td>0.0-0.4</td>
</tr>
<tr>
<td>Monocytes (% in WBC)</td>
<td>1.20±0.19</td>
<td>5.35±0.75</td>
<td>5.145±1.06</td>
<td>5.355±0.54</td>
<td>0.0-7.5</td>
</tr>
<tr>
<td>Eosinophils ($\times 10^3/\mu l$)</td>
<td>0.13±0.01</td>
<td>0.575±0.05</td>
<td>0.115±0.14</td>
<td>0.265±0.08</td>
<td>0.0-0.2</td>
</tr>
<tr>
<td>Eosinophils (% in WBC)</td>
<td>5.70±3.19</td>
<td>9.41±1.92</td>
<td>0.58±0.13</td>
<td>3.345±4.62</td>
<td>0.0-3.9</td>
</tr>
<tr>
<td>Basophils ($\times 10^3/\mu l$)</td>
<td>0.02±0.01</td>
<td>0.195±0.08</td>
<td>0.005±0.01</td>
<td>0.095±0.12</td>
<td>0.0-0.2</td>
</tr>
<tr>
<td>Basophils (% in WBC)</td>
<td>0.80±0.07</td>
<td>3.08±0.31</td>
<td>0.125±0.08</td>
<td>1.68±2.12</td>
<td>0.0-2.0</td>
</tr>
<tr>
<td>RBC ($\times 10^6/\mu l$)</td>
<td>10.80±0.66</td>
<td>7.48±0.45</td>
<td>8.12±0.23</td>
<td>7.46±0.07</td>
<td>6.36-9.42</td>
</tr>
</tbody>
</table>