Small Molecule Therapeutics

The AMPK Inhibitor Compound C Is a Potent AMPK-Independent Antiglioma Agent

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Abstract

AMP-activated protein kinase (AMPK) is an evolutionarily conserved energy sensor important for cell growth, proliferation, survival, and metabolic regulation. Active AMPK inhibits biosynthetic enzymes like mTOR and acetyl CoA carboxylase (required for protein and lipid synthesis, respectively) to ensure that cells maintain essential nutrients and energy during metabolic crisis. Despite our knowledge about this incredibly important kinase, no specific chemical inhibitors are available to examine its function. However, one small molecule known as compound C (also called dorsomorphin) has been widely used in cell-based, biochemical, and in vitro assays as a selective AMPK inhibitor. In nearly all these reports including a recent study in glioma, the biochemical and cellular effects of compound C have been attributed to its inhibitory action toward AMPK. While examining the status of AMPK activation in human gliomas, we observed that glioblastomas express copious amount of active AMPK. Compound C effectively reduced glioma viability in vitro both by inhibiting proliferation and inducing cell death. As expected, compound C inhibited AMPK; however, all the antiproliferative effects of this compound were AMPK independent. Instead, compound C killed glioma cells by multiple mechanisms, including activation of the calpain/cathepsin pathway, inhibition of AKT, mTORC1/C2, cell-cycle block at G2–M, and induction of necroptosis and autophagy. Importantly, normal astrocytes were significantly less susceptible to compound C. In summary, compound C is an extremely potent antglioma agent but we suggest that caution should be taken in interpreting results when this compound is used as an AMPK inhibitor. Mol Cancer Ther; 13(3); 1–10. ©2014 AACR.

Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine kinase and a molecular hub for cellular metabolic control. It is a heterotrimer of catalytic \( \alpha \), and regulatory \( \beta \) and \( \gamma \) subunits. Mammals express two \( \alpha \) (\( \alpha_1, \alpha_2 \)), two \( \beta \) (\( \beta_1, \beta_2 \)), and three \( \gamma \) subunits (\( \gamma_1, \gamma_2 \) and \( \gamma_3 \)) in a tissue-specific manner (1–4). Decreasing energy (ATP) levels increase cellular AMP:ATP ratio, resulting in increased AMP binding to AMPK with consequent phosphorylation and activation of AMPK \( \alpha \) subunits. Full activation of AMPK requires specific phosphorylation of the \( \alpha \) subunit at Thr172 by upstream kinases—LKB1, CAMKK\( \beta \), and probably other kinases (1, 5, 6). AMPK activation is crucially important for restoring the intra-cellular energy balance via AMPK-dependent inhibition of energy-consuming biosynthetic processes and the activation of reactions that produce ATP.

Because AMPK inhibits biosynthetic pathways through its inhibition of mTOR and acetyl CoA carboxylase (ACC), many studies correlate pharmacologic AMPK activation by two indirect AMPK activators (AICAR and metformin) with reduced cancer-cell proliferation (7–11).

Compound C (6-[4-(2-Piperidin-1-yloxy) phenyl]-3-pyridin-4-ylpyrazolo[1,5-alpyrimidine) is the only available agent that is used as a cell-permeable AMPK inhibitor. It has been used to rescue the antiproliferative actions of AICAR and metformin (12, 13), although the effect of compound C alone on cell proliferation is not well documented. Surprisingly, this compound (also known as dorsomorphin) is also used as a selective inhibitor of the bone morphogenetic protein pathway (14, 15). Indeed, in an exhaustive study of kinase specificities of inhibitors, compound C was found to inhibit a number of kinases other than AMPK (16, 17).

Despite the existing controversy about its selectivity, compound C is still being used as an AMPK inhibitor. In fact, in a recent study, compound C was used as an AMPK inhibitor in vitro and in vivo to effectively reduce proliferation and growth of astrocytic tumors (18). To address the controversy and definitively determine whether there is a molecular link between pharmacologic AMPK inhibition by compound C and cell proliferation, we conducted a

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pharmacogenetic study. We demonstrate that compound C is a potent cytotoxic agent that inhibits glioma proliferation in vitro through multiple mechanisms independent of AMPK. Although our findings highlight the effectiveness of compound C as an antiglioma agent, they also warrant the development of specific pharmacologic AMPK inhibitors to investigate the function of physiologically active AMPK in cancer.

Materials and Methods

Cell culture

T98G, A172, and U87 cells were obtained from the American Type Culture Collection (ATCC) in 2012, expanded, and frozen down in several aliquots. Each aliquot was thawed and used for no more than 6 months. ATCC uses Promega PowerPlex system to authenticate their cell lines. These cell lines were not reauthenticated by our laboratory. All glioma cells and normal astrocytes were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS). Human primary glioblastoma spheres were established at the Ohio State University under an institutional review board–approved protocol according to the NIH guidelines. Cells were maintained in DMEM/F12 supplemented with B27, EGF (10 ng/mL), bFGF (basic fibroblast growth factor; 10 ng/mL), GlutaMAX, and heparin (5 mg/mL). For proliferation and viability analysis, direct counting using Trypan blue method and also a fluorescence-based method (CellTiter-Fluor; Promega) were used. Drugs were added 24 hours postseeding and cell viability was determined at indicated times.

Reagents

The following reagents were used at doses indicated and as described in the text and figure legends. M7GTP Sepharose (GE Healthcare), Protein Agarose (Millipore), 3MA, DMSO (dimethyl sulfoxide; Sigma), PI/RNase (BD Biosciences), ZVAD (Promega), ALLN, and compound C (EMD Chemicals).

shRNA and lentivirus

The AMPKβ1 shRNA clone (TRCN0000004770) and the nontarget hairpin were purchased from the Lenti-shRNA Library Core, Cincinnati Children’s Hospital Medical Center (CCHMC). The 293T cells used to generate the shRNA lentivirus supernatant were cultured in DMEM with 10% FBS. Briefly, 293T cells were cotransfected with pLKO.1 (transfer vector), Δ8.9, and VSVG vectors by FuGENE HD (Roche) according to the manufacturer’s instructions. The viral supernatants were collected every 24 hours for three days after the initial medium change, 16 hours posttransfection. For Lentiviral infection, cells were infected overnight with viral particles in the presence of 8 μg/mL polybrene and antibiotic selection was started 48 hours post infection. Stable clones and cell populations were selected in puromycin (1 μg/mL for A172 or U87 and 2 μg/mL for T98G) and gene knockdown was assayed by immunoblotting.

Transfection

Glioma cells were transfected with pBABE-puro (control) and pBABE-puro Myr-Akt plasmids using jetPRIME transfection reagent (Polyplus-transfection) following the manufacturer’s instructions.

Immunoblot analysis and CAP binding assay

Western blot analysis was carried out following standard methods. Glioma cells were lysed with RIPA lysis buffer (20 mmol/L Tris, 10 mmol/L EGTA, 40 mmol/L β-glycerophosphate, 1% NP40, 2.5 mmol/L MgCl₂, 2 mmol/L orthovanadate, 1 mmol/L PMSF, 1 mmol/L DTT, and protease inhibitor cocktail). For CAP binding assay, glioma cells were washed with cold PBS and lysed in NL buffer [50 mmol/L HEPES-KOH (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 0.1 mmol/L GDP, 2 mmol/L Na₃VO₄, protease inhibitor cocktail, 1 mmol/L EDTA, 10 mmol/L β-glycerophosphate, and 50 mmol/L NaF]. Protein lysates were incubated with 20 μL of (1:1) slurry of m7GDP-agarose at 4°C for 1 hour, washed four times with the lysis buffer, resuspended in Laemmi Sample Buffer, boiled, and resolved by SDS-PAGE. The following antibodies (all from Cell Signaling Technology) were used—phospho AMPK Thr172, AMPK, AMPK β1/β2, phospho ACC Ser79, ACC, phospho S6 Ser235/236, phospho 4EBP1 Thr37/46, 4EBP1, mTOR, phospho Akt Ser473, phospho Akt Thr308, Akt, phospho Erk1/2 Thr202/Tyr204, PARP, LC3, P62, actin, and tubulin. Detection was performed using anti-rabbit or anti-mouse horseradish peroxidase (HRP)–linked secondary antibodies (Cell Signaling Technology) followed by chemiluminescence (Millipore).

Cell-cycle analysis

1.5 × 10⁵ glioma cells (control) treated with compound C (24 hours) were trypsinized, washed with cold PBS, fixed with 70% cold ethanol for 1 hour, and stained with propidium iodide (PI-RNase solution; BD Biosciences) for 15 minutes in the dark. Cell-cycle analysis was done in a FACScan analyzer (BD Biosciences).

Apoptosis/necrosis assay

Simultaneous detection of both apoptotic and necrotic cells in a single assay was done with the Apoptosis and Necrosis Quantification Kit (Biotium). Quantitation Kit features Annexin V labeled with fluorescein isothiocyanate (FITC) for staining of apoptotic cells with green fluorescence and ethidium homodimer III (EthD-III), a highly positively charged nucleic acid probe, which stains necrotic cells and late apoptotic cells with red fluorescence. A total of 3 × 10⁵ glioma cells (control) and treated with compound C (72 hours) were harvested via trypsinization and washed with PBS. Assay protocol was as per manufacturer’s instructions. Staining was followed by flow cytometry acquisition (FACScan analyzer; BD Biosciences) of the cells to measure fluorescence in FITC and propidium iodide channels. Data were analyzed by BD FACSDiva software.
Colony formation assay
Minimal cell numbers required for colony formation by three types of glioma cells were optimized. Accordingly, $2 \times 10^5$ A172 cells or $1 \times 10^5$ U87 cells or $0.5 \times 10^5$ T98G were seeded onto six-well culture plates in triplicates. The following day, cells were treated with compound C with indicated doses. After a change of fresh medium 72 hours later, the cells were allowed to form colonies for 14 days in the absence of the drug. Following removal of the medium, the wells were rinsed twice with PBS and 0.05% methylene blue solution (prepared in 50% methanol) was added to each well. Plates were incubated for 30 minutes at room temperature to facilitate staining of the colonies. After three rinses with distilled water, the plates were dried, photographed, and colonies were counted.

Cell migration assay
Glioma cells were grown to confluence and a uniform scratch was made using a 200-mL pipet tip. Floating cells were discarded by changing the existing medium with fresh growth medium containing either DMSO (vehicle control) or compound C. The same scratch area was photographed with a digital camera attached to a Nikon microscope. The distance between the edges of the cells migrating from two sides was measured by the ImageJ software.

Statistical analysis
Student t test was used to calculate statistical significance with $P < 0.05$ representing a statistically significant difference.

Results
Compound C inhibits AMPK activity and proliferation of human glioma cells
Cancer cells in solid tumors including gliomas undergo sweeping metabolic reprogramming during the process of tumorigenesis (2). Because AMPK is a key regulator of cellular energy metabolism and is activated during metabolic stress to increase cell survival, we examined if pharmacologic AMPK inhibition blocks glioma cell proliferation. To test this, we measured glioma cell viability in the absence or presence of the AMPK inhibitor compound C (Fig. 1A). Compound C inhibited AMPK kinase activity in a dose-dependent manner in multiple glioma cells as observed by reduced phosphorylation of the canonical
AMPK substrate ACC (Fig. 1B). Compound C potently inhibited proliferation of established glioma cell lines T98G, A172, and U87 (Fig. 1C) as well as U87 cells over-expressing the oncogene EGFR and its variant form EGFR-vIII (not shown). It also significantly inhibited proliferation of a pediatric glioblastoma cell line (not shown). In fact, the inhibitory effect of compound C on cell proliferation was considerably more than that of the AMPK activators AICAR and metformin (not shown). Prolonged exposure to compound C killed nearly 100% of glioma cell lines (Supplementary Fig. S1A). Compound C also potently killed primary glioblastoma spheres established from freshly resected glioma tissue (Fig. 1D). Importantly, the effect of compound C on normal astrocytes was significantly less than that on glioma cells (Fig. 1E). In colony formation assays, 5 μmol/L compound C significantly prevented formation of T98G, A172, and U87 colonies (Supplementary Fig. S1B–S1E). On the basis of the potent cytotoxic effect of this agent on glioma cells, it is not surprising that in contrast with previous studies, compound C did not reverse the antiproliferative effects of AICAR and metformin (Supplementary Fig. S1F). Collectively, our results demonstrate that compound C is an effective anti-glioma agent with considerably less toxicity toward normal cells.

**Compound C inhibits glioma proliferation independent of AMPK**

We next questioned if compound C exerts anti-glioma action by inhibiting AMPK. To examine this, we reduced AMPK activity by genetic means. The regulatory β sub-units of AMPK play an obligatory role in the stability of the catalytic α subunits and AMPK complex formation. While examining the expression of AMPK subunits in glioma cells, we observed that the regulatory β subunit is expressed at 80% to 90% higher levels than the β2 subunit (Fig. 2A). Indeed, shRNA-mediated knockdown of the β1 subunit reduced 80% to 90% of basal AMPK activity and phosphorylated ACC levels (Fig. 2B). To examine if compound C requires AMPK to suppress proliferation, we treated control (nt; nontarget) and AMPKβ1shRNA glioma cells with 10 μmol/L of compound C. Surprisingly, compound C inhibited proliferation regardless of AMPK (Fig. 2C) and in fact, AMPK-silenced glioma cells were more sensitive to the antiproliferative effects of compound C. These genetic data clearly indicate that AMPK inhibition is not a mechanism by which compound C inhibits glioma proliferation.

**Compound C inhibits glioma proliferation by multiple mechanisms**

As compound C inhibited proliferation independent of AMPK, we sought to determine its mechanism/s of inhibition. Signaling through the mTOR kinase pathway is crucial for proliferation and growth of cancer cells, including gliomas (19, 20). The mTORC1 complex (containing the mTOR partner raptor) mediates its downstream effects through phosphorylation of S6 kinase1 and the protein translation-initiation factor binding protein 4EBP1 (20, 21). S6K1 in turn phosphorylates the ribosomal protein S6 (22). Treatment of glioma cells with compound C significantly inhibited S6 and 4EBP1 phosphorylation (Fig. 3A), indicating that compound C is a potent mTORC1 inhibitor. Dephosphorylated 4EBP1 sequesters eIF4E with eIF4G, thus inhibiting cap-dependent protein translation (22). We immunoprecipitated eIF4E from glioma cells treated with Compound C or DMSO (control) and examined the amount of 4EBP1 bound to eIF4E. Consistent with our immunoblot results, we observed significant amount of 4EBP1 bound to eIF4E in compound C-treated cells (Fig. 3B).

Akt phosphorylates and inhibits TSC2 to enhance signaling through mTORC1 (10). Thus, while the mTORC1 complex functions downstream of Akt, the mTORC2 complex (containing the mTOR partner rictor) phosphorylates and activates Akt, thus operating upstream of Akt. We examined if compound C has any effect on mTORC2 as well. Indeed, compound C strongly inhibited Akt phosphorylation at serine 473, the mTORC2 target site (Fig. 3C). Because Akt phosphorylation by phosphoinositide 3-kinase (PI3K) is crucial for glioma proliferation, growth, and survival, we examined if the PI3K site on Akt is also affected by compound C. Compound C robustly inhibited PI3K-mediated Akt phosphorylation at threonine 308 (Fig. 3C). It, however, did not inhibit MAP–ERK kinase (MEK) activity toward Erk as Erk1/2 phosphorylation was not reduced by compound C (not shown).
To examine the mechanism of cell death, we examined if compound C caused apoptosis. Consistent with other studies (23, 24), compound C induced apoptosis as evident from caspase-dependent cleavage of PARP in compound C–treated glioma cells (Fig. 3D). However, the extent of apoptosis varied among cell lines. Another protective mechanism that allows cancer cell survival is autophagy—a mechanism that can become destructive if extensive and unchecked (25). Because Akt and mTORC1 inhibit autophagy, and compound C inhibited both kinases, we examined if autophagy is activated by compound C in glioma cells. Compound C significantly enhanced autophagy as shown by increased conversion of the microtubule-associated light chain 3 protein LC3A/I to LC3A/I in glioma cells (Fig. 3E). To confirm this result, we transfected glioma cells with plasmid encoding LC3-EGFP before treating with compound C. Induction of autophagy changes the diffuse cytoplasmic localization of LC3 to distinct autophagic structures (also called puncta; refs. 25, 26). Consistent with our immunoblot results, compound C caused redistribution of LC3 to numerous autophagic puncta (Supplementary Fig. S2A). Autophagy is also associated with decreased abundance of autophagic substrates, including p62. As expected, compound C reduced p62 protein levels in glioma cells (Supplementary Fig. S2B). To explore if necrosis is also involved in compound C’s action and to more accurately quantify cell death, we examined both apoptosis and necrosis by a flow cytometry–based assay in which apoptotic cells are detected by FITC-Annexin V reactivity, whereas necrotic cells are detected by ethidium homodimer III. We observed that compound C induced cell death by both necrosis and apoptosis (Fig. 3F). Taken together, our results show that compound C blocks glioma cell proliferation by multiple mechanisms.

All antiglioma effects of compound C are independent of AMPK

We next sought to determine whether some or all the above effects of compound C are AMPK independent.
Compound C reduced S6 and 4EBP1 phosphorylation similarly in control and AMPKβ1 knockdown cells (Fig. 4A). Importantly, AMPK silencing did not block eIF4E binding to 4EBP1 (cap binding assay) in compound C–treated glioma cells (Fig. 4B), suggesting that AMPK is not involved in compound C’s mTORC1 inhibition. Inhibition of Akt phosphorylation by compound C both at serine 473 and threonine 308 was also similar in glioma cells expressing nontarget or β1 shRNA (Fig. 4C), suggesting AMPK-independent inhibitory effects of this agent on mTORC2 and PI3K, respectively. In fact, compound C–induced autophagy, apoptosis, and necrosis also occurred similarly in control and AMPK-silenced glioma cells (Fig. 4D–F and Supplementary Fig. S2C).

We then tested if pharmacologic inhibition of autophagy or apoptosis could block compound C’s inhibitory action on cell viability. As expected, the autophagy inhibitor 3MA and the pan-caspase inhibitor ZVAD reduced autophagy and apoptosis, respectively (Supplementary Fig. S2D and S2E). However, neither 3MA nor ZVAD was sufficient to rescue glioma cells treated with compound C (Supplementary Fig. S2F). While ZVAD treatment alone showed an expected increase in cell viability, 3MA alone caused considerable cell death, suggesting that either blocking basal autophagy is detrimental to glioma cell survival or 3MA itself is toxic to the glioma cells that we tested (Supplementary Fig. S2F).

Because compound C inhibited Akt phosphorylation, we explored whether expression of constitutively active Akt could block the antiviability effect of compound C. Transfection of glioma cells with myristylated Akt increased total Akt, phosphorylated Akt, and phosphorylation of the Akt substrate glycogen synthase kinase beta (GSK3β; Supplementary Fig. S3A). However, compound C still killed glioma cells that expressed constitutively active Akt (Supplementary Fig. S3B). Collectively, our results suggest that compound C is a potent antiglioma agent that exerts multiple pleiotropic...
actions to reduce glioma viability in vitro, independent of AMPK.

**Compound C inhibits glioma cell migration independent of AMPK**

AMPK has been shown to play a role in the migration of normal as well as cancer cells (27–29). In these studies, AMPK function was examined by using either compound C alone or in conjunction with AMPK silencing RNA. Because of the many AMPK-independent effects of compound C that we observed in this study, we questioned whether inhibition of cell migration by compound C is also AMPK-independent. Compound C strongly inhibited migration of T98G (Fig. 5A), A172, and U87 glioma cells (data not shown). The effect was, however, not dose dependent. The inhibition of migration was similar at 1 and 2.5 \( \mu \text{mol/L} \) (not shown) as was with 5 and 10 \( \mu \text{mol/L} \) (Fig. 5A). Although compound C had little inhibitory effect on AMPK inhibition at 1 and 2.5 \( \mu \text{mol/L} \) (not shown), it still inhibited migration, suggesting that its effect is likely to be AMPK independent. To definitively examine if AMPK inhibition was required for this process, we used AMPK–\( \beta_1 \)-silenced T98G cells. Compound C inhibited migration of control (nt shRNA) and AMPK–\( \beta_1 \)-silenced T98G cells similarly at all time points studied (Fig. 5B), clearly indicating that the inhibitory effects of compound C on cell migration is an AMPK-independent effect.

**Compound C blocks glioma cell cycle at G2–M independent of AMPK**

We have shown that compound C affects cell viability by inducing apoptosis and necrosis. To directly examine whether compound C affects cell proliferation, we analyzed the glioma cell cycle. Glioma cells were treated with compound C or DMSO (control) for 24 hours and cell-cycle analysis was conducted by flow cytometry. Compound C did not have any effect on the G0–G1 stage (Supplementary Fig. S4A) or S phase (Supplementary Fig. S4B). However, compound C caused a consistent accumulation of all three glioma cells at G2–M (Fig. 6A–C). To examine if compound C requires AMPK to induce cell-cycle arrest, we treated control or AMPK knockdown cells with compound C and analyzed cell cycle. AMPK silencing did not alter G0–G1, or S phase (Supplementary Fig. S4C and S4D), but consistent with AMPK’s role in mitosis (30–35), AMPK knockdown cells showed a tendency toward G2–M accumulation. However, compound C caused G2–M accumulation of glioma cells similarly in control and AMPK knockdown cells (Fig. 6D–H). Collectively, our findings demonstrate that compound C exerts cell-cycle arrest in glioma cells independent of AMPK.

**Inhibition of calpain/cathepsin-mediated cell-death pathway partially rescues viability of compound C-treated glioma cells**

Although the regulating mechanisms are still unclear, necrosis is an important mechanism of death in eukaryotic cells. Necrosis is induced by various stimuli, including drugs like Smac mimetics and TNF-\( \alpha \) inhibitors. Recent studies have shown that activation of calpain and cathepsin proteases are involved in necrotic cell death. Because apoptosis and autophagy inhibitors failed to protect glioma cells from compound C, and because we observed significant necrosis in compound C–treated glioma cells, we examined whether calpain and cathepsin inhibitors rescue compound C–treated glioma cells. We used ALLN, a cell-permeable inhibitor of calpain I, calpain II, cathepsin B, and cathepsin L at nanomolar concentrations. As shown in Fig. 7, although compound C alone reduced cell viability to about 17%, nearly 50% of the cells were alive when cells were treated with compound C in combination with ALLN. When combined with ZVAD, ALLN did not increase the viability of compound C–treated cells. This partial rescue by ALLN indicates that activation of a calpain/cathepsin-mediated pathway is an important mechanism by which compound C induces glioma cell death.
Discussion

The role of physiologically active AMPK in cancer remains unknown. Because of AMPK’s inhibitory effects on biosynthetic pathways and its effects on increasing insulin sensitivity (a desirable effect for the treatment of type II diabetes), efforts by both academia and industry are biased toward developing AMPK activators. As a result, unfortunately, compound C remains the only small-molecule AMPK inhibitor that has been widely used to study AMPK signaling and various aspects of cell physiology, including proliferation, survival, and migration. The use of compound C continues despite reports that it inhibits other kinases with a lower \( K_m \) than AMPK (17). Indeed, compound C alone is significantly cytotoxic (23), and the use of this compound to examine AMPK functions is not recommended (16).

To clearly establish that the cellular effects of compound C are AMPK independent, we conducted a pharmacogenetic study in glioma cells. Our results firmly demonstrate that all cellular effects of compound C are AMPK independent. However, compound C also proved to be one of the most potent antiglioma agents among all the chemotherapy agents that we tested. These agents include mTOR kinase inhibitors, PI3K inhibitors, DNA alkylating, and microtubule-disrupting agents. The primary mechanism/s by which compound C kills cancer cells varies, which likely depends on the type of cancer and the associated mutations in such cells. Others have shown that compound C induces protective autophagy in U251 human glioma cells through AMPK-independent inhibition of the Akt/mTOR pathway (36). However, in another study (37), compound C inhibited proliferation of colorectal carcinoma cells through induction apoptosis and autophagy. Depending on the degree and the duration, autophagy can be protective or destructive to cells. Unlike the results of Vucicevic and colleagues, (36) autophagy inhibitors (chloroquine and 3MA) failed to rescue...
glioma cells from the antiproliferative effects of compound C, suggesting that autophagy induction by this agent is not protective. Our results are in contrast with the results of Vucicevic and colleagues, and akin to the results of Yang and colleagues (37).

Compound C also induced caspase 3–mediated apoptosis in the glioma cells that we studied, and this effect was AMPK-independent. This effect was also echoed in breast cancer cells in which compound C treatment independent of AMPK, led to ceramide production and redistribution of Bax from the cytoplasm to the mitochondria (23). However, blocking apoptosis by the pan-caspase inhibitor ZVAD failed to protect glioma cells of compound C’s effects, suggesting that induction of apoptosis is only one of the many mechanisms by which compound C kills glioma cells, and that blocking apoptosis alone is not sufficient to block compound C’s antiproliferative effects.

In at least three studies, compound C has been used to examine the role of AMPK in cell migration (27–29). In these studies, although compound C did inhibit cell migration, it was not examined whether this effect was AMPK dependent. Our studies undoubtedly demonstrate that this effect of compound C does not require AMPK. In fact, it inhibited glioma cell migration at low doses (1 and 2.5 mmol/L) that are insufficient to inhibit AMPK kinase activity. In most pharmacologic studies, two indirect AMPK activators AICAR and metformin have been used to inhibit cancer cell proliferation and compound C was shown to reverse the effects of AICAR and metformin (12, 13). We found that compound C alone is an extremely potent antiproliferative agent and clearly does not reverse the effects of AICAR and metformin. Despite all the pleiotropic effects of compound C, it is still being used as an AMPK inhibitor to examine cellular functions of AMPK in vitro and even in vivo (18). In this report (18), compound C was used as an AMPK inhibitor, and at 10 μmol/L, it inhibited proliferation and glioma formation of human U87MG glioma cells in vivo. These compound C studies were conducted to corroborate the genetic findings that AMPK activity is required for cancer cell proliferation. However, it was not examined whether compound C exerts the same antiproliferative effects in AMPK-silenced cells.

Our pharmacogenetic study clearly shows that although compound C is a potent antglioma agent, AMPK is absolutely not required for its antiproliferative effects.

The cell-cycle arrest of compound C–treated glioma cells at G2–M is in line with that observed for colorectal cancer cells (37) and is interesting in the context of glioma therapy. The DNA-alkylating agent temozolomide is routinely used in standard-of-care glioma therapy. The G2-M arrest by compound C is particularly attractive to temozolomide therapy as the integrity of the G2-M checkpoint is a key determinant of temozolomide cytotoxicity of glioblastoma cells (38). Interestingly, although DNA fragmentation is a hallmark of apoptotic cells that can appear in the sub-G1 fraction, we did not observe significant increase in the sub-G1 fraction of compound C–treated cells. Apoptotic cells in which DNA degradation is terminated at 50- to 300-kb fragments and does not proceed to internucleosomal-size fragments may not be identified as the sub-G1 cells as they are also weakly labeled in the TUNEL assay (39). Alternatively, some cells may undergo apoptosis without significant DNA digestion (40).

We finally show a novel mechanism of compound C–mediated cell death. We observed that pharmacologic inhibition of the calpain/cathepsin pathway partially blocked compound C–induced death of glioma cells. This rescue, although partial, was significant. Therefore, activation of this pathway is an important mechanism of cell death caused by compound C. In summary, our results demonstrate that compound C is a potent antiglioma agent that kills cancer cells by multiple mechanisms, all of which are independent of AMPK.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: B. Dasgupta
Development of methodology: X. Liu, B. Dasgupta
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Liu, R.R. Chhipa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Dasgupta
Writing, review, and/or revision of the manuscript: R.R. Chhipa, B. Dasgupta
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Liu, I. Nakano, B. Dasgupta
Study supervision: B. Dasgupta

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