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Research Paper

# Pharmacological inhibition of lipid droplet formation enhances the effectiveness of curcumin in glioblastoma

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ABSTRACT

Increased lipid droplet number and fatty acid synthesis allow glioblastoma multiforme, the most common and aggressive type of brain cancer, to withstand accelerated metabolic rates and resist therapeutic treatments. Lipid droplets are postulated to sequester hydrophobic therapeutic agents, thereby reducing drug effectiveness. We hypothesized that the inhibition of lipid droplet accumulation in glioblastoma cells using pyrrolidine-2, a cytoplasmic phospholipase A2 alpha inhibitor, can sensitize cancer cells to the killing effect of curcumin, a promising anticancer agent isolated from the turmeric spice. We observed that curcumin localized in the lipid droplets of human U251N glioblastoma cells. Reduction of lipid droplet number using pyrrolidine-2 drastically enhanced the therapeutic effect of curcumin in both 2D and 3D glioblastoma cell models. The mode of cell death involved was found to be mediated by caspase-3. Comparatively, the current clinical chemotherapeutic standard, temozolomide, was significantly less effective in inducing glioblastoma cell death. Together, our results suggest that the inhibition of lipid droplet accumulation is an effective way to enhance the chemotherapeutic effect of curcumin against glioblastoma multiforme.



## 1. Introduction

In cancers such as glioblastoma multiforme, enhanced fatty acid storage and synthesis provide the necessary resources for survival and rapid proliferation [1]. Although otherwise highly heterogeneous, most cancer types present a lipogenic phenotype characterized by the upregulation of key enzymes and transcriptional factors controlling lipid metabolism (e.g. Akt, fatty acid synthase, hypoxia-inducible factor 1-alpha, sterol regulatory element binding proteins, acetyl-CoA carboxylase alpha), and a boost in *de novo* lipogenesis [2–5]. In solid malignancies, the hypoxic conditions found at the core of the tumors induce adaptive pathways aimed at maintaining lipid synthesis, homeostatic pH and cell survival [6]. The resulting metabolic changes correlate with poor prognosis, poor treatment response and recurrence in diseases such as breast, liver and brain cancer [7–10]. Emerging therapeutic approaches have thus targeted lipid synthesis to counter the effects of metabolic reprogramming in cancer cells [11,12].

Aside from their increased *de novo* fatty acid synthesis, cancer cells are also characterized by their increased number of lipid droplets compared to normal tissues [13,14]. Lipid droplets are dynamic organelles that support cells with metabolic fuel, membrane biosynthesis, inflammatory intermediates and signaling mediators [15–17] . Although they can be found in almost all cell types, their increased biogenesis in neoplastic and inflammatory conditions defines them as targets for therapeutic intervention [18]. Aside from fueling the accelerated metabolism of cancer cells, lipid droplets also harbor numerous proteins and transcription factors critical to lipid metabolism and related pathways [19]. The hydrophobic core found in lipid droplets provides a favorable compartment to attract and sequester lipophilic proteins and compounds, fat-soluble vitamins, and even environmental pollutants [20]. As such, lipid droplets can sequester lipophilic drugs and prevent them from reaching their targets, thus decreasing drug effectiveness [21,22]. Therefore, it seems that inhibition by pharmacological or genetic means of enzymes necessary for lipid droplet formation could provide a way to reduce drug sequestration and improve drug effects. We tested this concept in glioblastoma cells treated with curcumin in combination with pyrrolidine-2, and inhibitor of cytosolic phospholipase A2 alpha (cPLA2α). Curcumin, a plant-derived polyphenol isolated from

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the turmeric spice, has been shown to be a safe, potent and effective anticancer agent both *in vitro* and *in vivo* [23]. It was found to affect multiple targets, leading to the inhibition of inflammation and cell proliferation [24]. Curcumin has a broad range of effects that are advantageous against multifactorial diseases such as cancer, and clinical trials have suggested biological activity in patients with colorectal and pancreatic cancer [25,26]. Despite being well-tolerated in humans, its poor bioavailability has limited prospects of broader clinical applications [26,27]. Given its lipophilic properties, curcumin has been found to localize in lipid membranes and lipid droplets, thereby decreasing its availability at drug targets. Pyrrolidine-2, also known as pyrrophenone, is a potent and reversible inhibitor of cPLA2α, a key enzyme in the processes of arachidonic acid release, eicosanoid synthesis and lipid droplet formation [28,29]. We hypothesize that pre-treatment of cancer cells with pyrrolidine-2 will enhance the cell killing effect of curcumin by reducing its sequestration in lipid droplets.

Glioblastoma multiforme is a highly aggressive and drug-resistant type of brain cancer which currently lacks effective treatments [30,31]. Temozolomide, a DNA methylating agent, is the first-line drug used in concomitant and adjuvant radiochemotherapy against glioblastoma [32–34]. However, a large subset of patients is resistant to temozolomide due to the expression of the $O^6$-methylguanine-DNA methyltransferase gene (*MGMT*), a DNA repair protein, and there is an urgent need for alternative mono- and combination therapies [35]. We have investigated the susceptibility of human glioblastoma monolayer (2D) and spheroid (3D) cultures to curcumin and temozolomide used either individually or in combination with pyrrolidine-2. We also compared the effect of pyrrolidine-2 to that of buthionine sulfoximine (BSO), an irreversible inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis, found to be effective as a sensitizing agent in patient-derived neuroblastoma cell lines [36,37].

We further monitored the mode of cell death induced by these treatments using a newly developed, dimerization-based biosensor which provides a highly sensitive method to visualize and quantify caspase-dependent apoptotic activity in living cells [38]. The cysteine protease caspase-3 is a main executioner caspase in the process of caspase-dependent apoptosis [39,40]. It was shown to be strongly activated in response to curcumin treatment in different cell lines [41–43]. By investigating the activity of caspase-3 in cancer cells in response to chemotherapeutics and sensitizers, we can reveal drug efficacy and underlying mechanisms of cell death.

The objective of these studies was to investigate the sensitization of human glioblastoma cells in 2D and 3D cultures to caspase-3-mediated cell death induced by curcumin. We used the pharmacological agent pyrrolidine-2 to manipulate lipid droplet number in glioblastoma cells and assessed caspase-3 activity in living cells using a recently developed biosensor. The results indicate a significant increase in caspase-3-mediated cell death induced by curcumin when lipid droplet formation is reduced.

## 2. Materials and methods

### 2.1. Materials

Curcumin (Sigma–Aldrich, Canada), pyrrolidine-2 (Calbiochem, United States), temozolomide (Sigma–Aldrich, Canada), buthionine sulfoximine (Sigma–Aldrich, Canada), staurosporine (Sigma–Aldrich, Canada), dimethyl sulfoxide (Sigma–Aldrich, Canada), Nile Red (Sigma–Aldrich, Canada), paraformaldehyde (Sigma–Aldrich, Canada), BODIPY 493/503 (Invitrogen, Canada), Hoechst 33342 (Sigma–Aldrich, Canada) and propidium iodide (Sigma–Aldrich, Canada) were used as received.

**Table 1**
Construct of Plasmid 1 and Plasmid 2, which form the caspase-3 biosensor.

| Name | Gene | GenBank accession numbers | Addgene plasmid ID |
|---|---|---|---|
| $GA^{NES}$-DEVD-$B^{NLS}$ | XhoI-GA-NES-KpnI-DEVD-B-NLS-HindIII | KF976777 | 50842 |
| $RA^{NLS}$ | XhoI- RA-NLS-HindIII | KF976778 | 50843 |

### 2.2. Cell culture

The U251N human glioblastoma cell line was originally obtained from the American Type Culture Collection. Unless otherwise specified, U251N cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Canada) containing 10% (v/v) fetal bovine serum (Invitrogen, Canada), 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Canada), and 1% non-essential amino acids. Cells were incubated at 37 °C with 5% $CO_2$.

### 2.3. Spheroid preparation

Spheroid cultures were prepared using a protocol adapted from the previously established liquid overlay system: confluent U251N monolayer cell cultures were detached using 0.05% trypsin–EDTA (Invitrogen, Canada), and seeded at 5,000 cells per well in 96-well plates pre-coated with 2% agarose (Invitrogen, Canada) in serum-deprived DMEM [44]. Spheroids were seeded and maintained in complete DMEM medium for four days before drug treatments. Spheroids expressing the caspase-3 biosensor were prepared following the same method, using transfected cells.

### 2.4. Cell treatment

Confluent monolayer cell cultures were detached using 0.05% trypsin–EDTA, seeded in 24-well or 96-well cell culture plates (Sarstedt, Canada) at 50,000 or 10,000 cells per well, respectively, and treated after 24 h. For 24 h dose–response experiments, drugs were administered in the following concentrations: curcumin (Cur; 5–100 μM); pyrrolidine-2 (Pyr-2; 1–5 μM). For 72 h dose–response experiments, curcumin was added at concentrations 5–30 μM. For pyrrolidine-2 (3 μM) and buthionine sulfoximine (BSO; 5 mM) pre-treatments, cells were treated for 24 h, after which the medium was refreshed. To establish the time course of drug effects in spheroid cultures, treatments were maintained for 24 h or 72 h. BSO stocks (200 mM) were prepared fresh in purified water. Stock solutions of temozolomide, curcumin, staurosporine and pyrrolidine-2 were prepared in dimethyl sulfoxide (DMSO), and added to cells for a final DMSO concentration <0.5%. Vehicle controls were included in each experiment.

### 2.5. Lipid droplet labeling, imaging and quantification

After treatment with curcumin (10–30 μM) for 24 h, the media were refreshed, and cells were incubated with Nile Red (2 μM; 10 min). Nile red is a commonly used fluorescent label for lipid droplets [45,46], but it can also bind to hydrophobic protein domains and be employed to probe hydrophobic pockets in purified native proteins [47]. Labeled cells were washed with phosphate buffered saline (PBS), and imaged using a fluorescence microscope (Leica, Canada). To quantify the number of lipid droplets per cell, U251N cells were fixed with paraformaldehyde (4%; 15 min) following treatment, labeled with BODIPY 493/503 (2 μM; 10 min), and then imaged using a fluorescence microscope.