Chemically Modified Variants of Fenofibrate with Antiglioblastoma Potential

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Abstract

Anticancer effects of a common lipid-lowering drug, fenofibrate, have been described in the literature for a quite some time; however, fenofibrate has not been used as a direct anticancer therapy. We have previously reported that fenofibrate in its unprocessed form (ester) accumulates in the mitochondria, inhibits mitochondrial respiration, and triggers a severe energy deficit and extensive glioblastoma cell death. However, fenofibrate does not cross the blood brain barrier and is quickly processed by blood and tissue esterases to form the PPARα agonist fenofibric acid, which is practically ineffective in triggering cancer cell death. To address these issues, we have made several chemical modifications in fenofibrate structure to increase its stability, water solubility, tissue penetration, and ultimately anticancer potential. Our data show that, in comparison to fenofibrate, four new compounds designated here as PP1, PP2, PP3, and PP4 have improved anticancer activity in vitro. Like fenofibrate, the compounds block mitochondrial respiration and trigger massive glioblastoma cell death in vitro. In addition, one of the lead compounds, PP1, has improved water solubility and is significantly more stable when exposed to human blood in comparison to fenofibrate. Importantly, mice bearing large intracranial glioblastoma tumors demonstrated extensive areas of tumor cell death within the tumor mass following oral administration of PP1, and the treated mice did not show any major signs of distress, and accumulated PP1 at therapeutically relevant concentrations in several tissues, including brain and intracranial tumors.

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Introduction
Glioblastomas are highly lethal brain tumors for which therapeutic options are limited. Rapidly growing and highly invasive glioblastoma cells rely on both glycolysis and mitochondrial respiration to generate sufficient amounts of ATP and intermediate metabolites (anaplerosis). Interfering with these pathways may be a promising therapeutic strategy to induce “metabolic catastrophe” in these practically incurable brain neoplasms. We have demonstrated that tumor cells of neuroectodermal origin, including melanoma, medulloblastoma, and glioblastoma, are highly sensitive to the metabolic drug fenofibrate (FF) [1–11]. FF is routinely used as a lipid-lowering drug through the ability of its metabolite, fenofibric acid (FA), to activate peroxisome proliferator activated receptor alpha (PPARα) [12]. Although activation of PPARα may explain some of the observed anticancer effects, glioblastoma cells treated with PPARα siRNA retain sensitivity to FF, indicating a PPARα-independent mechanism of its anticancer action. Indeed, our previously published data demonstrate that unprocessed FF (ester) accumulates in mitochondrial membranes, with evidence that mitochondrial FF triggers a severe and immediate inhibition of mitochondrial respiration. This leads to a severe decline in intracellular ATP, and apoptotic tumor cell death [11]. We also reported that FF is promptly processed to FA by blood and tissue esterases, and FA is much less effective in triggering tumor cell death, and that both FF and FA do not cross the blood brain tumor barrier (BBTB) [4]. To address these issues, which are hampering development of more effective FF-based antitumoral therapies, we have made several chemical modifications to improve FF stability, water solubility, tissue penetration, and ultimately antiglioblastoma efficacy. Our data show that, in comparison to FF, four compounds, designated as PP1, PP2, PP3, and PP4, have improved cytotoxicity against glioblastoma cells in vitro; similar to FF, they block mitochondrial respiration. In addition, PP1 is significantly more stable when exposed to human blood and has improved water solubility. We have also demonstrated that mice treated with PP1 (oral administration) accumulated PP1 at therapeutically relevant concentrations in several tissues including intracranial glioblastoma tumors and survived the treatment without any major signs of distress. Importantly, mice with large intracranial glioblastomas demonstrated extensive areas of necrosis, which were detected exclusively in PP1-treated mice, further supporting a strong antiglioblastoma potential of this new metabolic compound.

Materials and Methods

Chemical Procedures to Prepare PP Compounds

All starting materials were reagent grade purchased from Sigma-Aldrich or Ark Pharm. 1H-NMR spectra were recorded on Varian Mercury Plus 400-MHz instrument in CDCl₃ or DMSO-d₆, with the solvent chemical shifts as an internal standard. All computed molecular descriptors were generated by Chemaxon MarvinSketch version 18.8.0.

Preparation of 2-[4-(4-Chlorobenzoyl)phenoxy]-2-methyl-1-(4-methylpiperazin-1-yl)propan-1-one (PP2). Dichloromethane (30 ml) of fenofibric chloride (1 mmol; prepared as described above for PP1 preparation) was slowly added in stirring water (5 ml) solution of sodium carbonate (216 mg; 2 mmol) with tetrahydrofuran (10 ml) and of 1-methylpiperazine (0.13 ml; 120 mg; 1.2 mmol). Resulting reaction mixture was stirred at room temperature for 1 hour. Additional water (30 ml) was added, and the organic layer was separated; washed with water (3×10 ml), 5% hydrochloric acid (3×10 ml), 10% sodium carbonate (3×10 ml), and water (3×10 ml) and dried over anhydrous sodium carbonate. After evaporation, oily residue was crystalized from dichloromethane/hexane (11:4) to give pure product (640 mg; 85% yield). 1H-NMR (CDCl₃) δ 7.74 (2H, d, J = 8.8 Hz), 7.71 (2H, d, J = 8.8 Hz), 7.45 (2H, d, J = 8.8 Hz), 6.92 (2H, d, J = 8.8 Hz), 3.78 (2H, t, J = 4.8 Hz), 3.53 (2H, t, J = 4.8 Hz), 3.6 (1H, broad s), 3.17 (3H, s), and 1.71 (6H, s) ppm. 13C-NMR (CDCl₃) δ 194.1, 173.5, 159.3, 138.3, 136.1, 132.2, 131.1, 130.3, 128.5, 115.6, 81.4, 60.3, 52.8, 36.7, and 25.7 ppm.

Preparation of 2-[4-(4-Chlorobenzoyl)phenoxy]-N-(2-hydroxyethyl)-N,2-dimethylpropanamide (PP1). Dichloromethane (20 ml) suspension of FA (318.75 mg; 1 mmol) and oxalyl chloride (0.25 mL; 380.1 mg; 3 mmol), and one drop of N,N-dimethylformamide were stirred at room temperature for 5 minutes. Solvent was evaporated under reduced pressure. White solid residue was resolved in in dichloromethane (10 ml) and again evaporated to the solid residue. This solid residue was dissolved in dichloromethane (20 ml), and at room temperature with stirring, dichloromethane (10 ml) solution of 2-(methylamino)ethanol (0.24 ml; 225 mg; 3 mmol) was gradually added. Reaction mixture was stirred at room temperature. Dichloromethane reaction mixture was washed with water (3×15 ml), 5% hydrochloric acid (3×15 ml), water (3×15 ml), 10% sodium carbonate (3×15 ml), and finally water (3×15 ml) and dried over anhydrous sodium carbonate. Solvent was evaporated under reduced pressure to result in a viscous pale-yellow liquid (390 mg). Product was crystallized from dichloromethane/hexane (30 ml; 1:4) at room temperature by slow solvent evaporation to 1/5 original volume, and formed crystals were washed with ice-cold hexane. Isolated yield 340 mg (90%).
After the solvent was evaporated, the solid residue was washed with hexane (3×5 ml) and dried in a vacuum under reduced pressure to give 350 mg (71%) of pure product. Selected signals for $^1$H-NMR (CDCl$_3$) $\delta$ 7.71 (2H, d, $J$ = 8.8 Hz), 7.67 (2H, d, $J$ = 8.8 Hz), 3.17 (3H, s), and 1.66 (6H, s) ppm.

Preparation of 2-[4-(4-Chlorobenzoyl)phenoxy]-2-methyl-1-[4-(morpholin-4-yl)piperidin-1-yl]propan-1-one (PP4) FA chloride prepared from FA (160 mg; 0.5 mmol) and oxalyl chloride (0.25 mL; 380.1 mg; 3 mmol) as described for preparation of PP1 was dissolved in dichloromethane (30 ml) and mixed with tetrahydrofuran (10 ml) solution of 4-morpholinopiperidine (100 mg; 0.6 mmol), and water (10 ml) solution of sodium carbonate (106 mg; 1 mmol). After drying over anhydrous sodium carbonate, the solvent was evaporated to give the pure oil product (200 mg; 85%) that was extensively washed with 5% hydrochloric acid (5×20 ml), water (20 ml) was added, and the organic layer was separated and the resulting mixture was stirred at room temperature for 1 hour. Water (10 ml) solution of sodium carbonate (106 mg; 1 mmol). The solution of 4-morpholinopiperidine (100 mg; 0.6 mmol), and water (10 ml) was mixed with the corresponding volumes of DMSO (vehicle control; final concentration 0.1%). GBM12 cells were routinely propagated in the subcutaneous tissue of nude mice and isolated from the tumor tissue for short-term cultures as previously described [11] and according to IACUC protocol #3444 5 (LSUHSC, New Orleans, LA).

Evaluation of Metabolic Parameters

Metabolic responses of human glioblastoma cells were evaluated with Extracellular Flux Analyzer XFe24 (Agilent Technologies). During the day prior to each assay, the cells were plated at 4×10^4 cells/well in Agilent Seahorse 24-well XF cell culture microplates with growth-supporting media and incubated overnight. At the time of measurement, growth media were replaced with serum-free XF assay medium (Seahorse XF Base Medium supplemented with 1 mM pyruvate, 2 mM glutamine, and 5.5 mM glucose), and cartridges equipped with oxygen-sensitive and pH-sensitive fluorescence probes (Seahorse) were placed above the cells. The oxygen consumption rate (OCR; indicative of mitochondrial respiration) and extracellular acidification rate (ECAR; indicative of glycolysis) were evaluated after injecting the PP compounds (all used at 25 μM), FF (50 μM), or DMSO (0.1%; vehicle control) followed by injections of metabolic toxins, including oligomycin (inhibitor of ATP synthase; 0.5 μM), carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; uncoupling factor; 0.5 μM), rotenone (inhibitor of mitochondrial complex I; 0.3 μM), and antimycin A (inhibitor of mitochondrial complex III; 0.3 μM).

PPAR Luciferase Assay

The PPAR transcriptional activity was determined by utilizing the JstkJpGL3 reporter plasmid, which contains a firefly luciferase gene driven by the PPAR responsive element (PPRE), which consists of three copies of the J site from the apo-AII gene promoter. Together with JstkJpGL3 plasmid HepG2 cells were transfected with pSV40-GLuc (New England Biolabs., Ipswich, MA) control plasmid expressing Gaussia luciferase under the control of the constitutive SV40 early promoter, used to normalize for efficiency of transfection. Twenty-four hours after transfection, the cells were incubated with glutathione (30 μM), FF, PP1, PP2, PP3, and PP4 (all 25 μM) for an additional 24 hours. The luciferase activity was detected with the Dual-Luciferase reporter assay system (Promega, Madison, WI), and the resulting luminescence was measured with Synergy 2 microplate reader (BioTek, Winooski, VT).

Cell Death Assays

Cell death was evaluated by assays based on cell membrane integrity. We used either the trypan blue exclusion test [15] or the GUAVA easyCyte 8HT flow cytometer with ViaCount reagent (Millipore) and Guava/ViaCount software for data analysis. Briefly, the cells were plated at 1×10^4 cells/cm² in 24-well plates in growth medium. After 24 hours, the medium was replaced by fresh growth medium containing PP compounds, FF, or 0.1% DMSO (vehicle control) and further incubated for the amount of time specified for each experiment. The cells were then harvested with 0.05% trypsin/EDTA, centrifuged, resuspended in PBS, and counted in a hemocytometer with trypan blue (0.4%, 1:1) or incubated with the ViaCount reagent (1:10; 5 minutes at room temperature) before cell

Detection of PP Compounds by High-Performance Liquid Chromatography (HPLC)

All HPLC data were obtained from the Agilent 1100 apparatus equipped with a line degasser, binary pump (high pressure mixer), autosampler, column thermostat, and Diode Array Detector (Agilent Technologies, Santa Clara, CA). The analytical column (3 μm, 4.6x150 mm; Ocyt Silane C8; YMC America, Inc.), solvent A–50 mM acetic acid in water, and solvent B–acetonitrile with isocratic flow were used to detect and quantify PP compounds in culture media and in cells, tissues, and body fluids. The flow rate was set to 1 ml/min, column temperature was 20 °C, and the sample volume was 5 μl. Diode Array Detector wavelength was set to 285 nm.

Sample Preparation. Blood, cell culture media, and cellular and tissue lysates were deproteinized by adding 150 μl of acetonitrile to 150 μl of sample, mixed well, and centrifuged (15,000g; 5 minutes). The lysates were sonicated on ice and centrifuged (15,000g; 5 minutes). Finally, 150 μl of the supernatant was mixed with the equal volume of acetonitrile, filtered through 0.22-μm centrifuge filter (Sigma), and analyzed by HPLC.

Cell Culture

We have used two human glioblastoma cell lines, LN-229 (ATCC# CRL-2611) and U-87MG (ATCC# HTB14); GBM12, which are patient-derived human glioblastoma cells [13,14], and the mouse glioblastoma cell line GL261-Red-FLuc (Bioware Brite; PerkinElmer# BW134246). All cell lines were maintained as semiconfluent monolayer cultures in DMEM (1 g/l glucose; with sodium pyruvate and L-glutamine) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. The cells were treated with PP compounds at different doses ranging from 5 to 50 μM. In addition, the cells were treated with FF (Sigma Aldrich, St. Louis, MO) at concentrations ranging from 10 to 50 μM. Control cultures were treated with the corresponding volumes of DMSO (vehicle control; final concentration 0.1%). GBM12 cells were routinely propagated in the subcutaneous tissue of nude mice and isolated from the tumor tissue for short-term cultures as previously described [11] and according to IACUC protocol #3444 5 (LSUHSC, New Orleans, LA).
viability was assessed by the Guava/ViaCount according to the manufacturer’s recommendations.

**Animal Studies**

All described procedures involving experimental animals were performed in accordance with the IACUC protocol #3444 at LSUHSC, New Orleans.

**Intracranial Tumor Growth.** C57BL/6NHsd mice, 11-12 weeks of age (Envigo), were anesthetized with 4% isoflurane and secured in a stereotactic head frame (Harvard Apparatus, Holliston, MA). GL-261-Red-Fluc cells (1×10^5 cells suspended in 2 μl of PBS) were injected into the brain parenchyma (coordinates: 3 mm anterior to bregma; 1.5 mm lateral to sagittal suture; 3 mm down from surface) through a burr hole in the skull using a 10-μl Hamilton syringe. Biophotonic images of the skull were captured using a Xenogen IVIS 200 imaging system (Palo Alto, CA) 2 weeks after initial cell implantation (Figure 6C). Prior to imaging, each mouse received an intraperitoneal injection of 100 μl of D-luciferin (30 mg/ml solution; PerkinElmer, Waltham, MA) and was anesthetized by isoflurane inhalation. The resulting images were evaluated, and luminescence measurements from equivalent regions of interest encompassing the entire skull were collected using Living Image 4.1 software (Xenogen).

**Treatment.** C57BL mice bearing well established intracranial mouse glioblastoma tumors (GL-261) were treated with PP1 (50 mg/ kg/day, dissolved in 2% DMSO) administered by oral gavage. Control mice were treated with equal volume of the solvent. Following 14 days of daily drug or solvent administration, the animals were euthanized according to the standard ethically accepted procedure, and the following organs/body fluids were collected: blood, liver, kidneys, spleen, heart, intact brain, and intracranial tumors. These tissues were subjected to sample preparation for the HPLC analysis (PP1 tissue content) and for the routine pathological evaluation.

**Pathological Evaluations.** After harvesting the brain and tumors, liver, spleen, kidneys, heart, and lungs, the tissues were placed in 10% buffered formalin for 24 hours, processed, and embedded into paraffin blocks. Sections were cut at 4 μm in thickness, placed in electromagnetically charged slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin for routine histopathological examination.

**Statistical Analysis**

Data were analyzed by a Student’s t test corrected for multiple comparisons using Bonferroni-Dunn method. The differences between control and experimental groups were considered significant and marked with an asterisk (*) for P values lower or equal .05.

**Results**

**Synthesis of New FF-Based Compounds for Glioblastoma Therapy**

We have modified certain specific physicochemical properties of FF to improve its resistance to blood and tissue esterases, water solubility, tissue uptake, and ultimately anticancer activity. As a result, we have generated 26 new compounds, which have been designated here as PP compounds. They all contain a structural motif outlined in Figure 1A. Specifically, we have replaced the ester group that is present in FF with different amide groups. This is because amide hydrolysis rate under physiological conditions is substantially slower than esters [16,17]. In contrast, esters such as FF are readily cleaved by hydrolytic reactions catalyzed by acids, bases, metal ions, and hydrolytic proteins such as human serum albumin [18], and more specifically by blood and tissue esterases [4,19–22]. Unless designed with specific functional groups, amide bonds are rarely cleaved by chemical hydrolysis under physiological conditions, and their chemical cleavage requires harsh conditions such as high temperature in combination with the presence of strong acids or bases [16]. In addition to higher resistance to the hydrolyses, tertiary amides have higher water solubility as compared to esters and to primary or secondary amides.

Based on our computational and biological studies, we had initially selected four compounds (PP1-PP4) for further analyses (Figure 1A). Preparation procedures for these compounds are outlined in Figure 1B and described in the Methods section. There are two feasible methods that are commonly used in organic synthesis for preparation of amides from carboxylic acid: a) through acid chlorides and b) by activating carboxylic acid with carbodiimides [23]. FA was used as a primary substrate and was converted into the corresponding acid chloride with oxalyl chloride. This chloride was coupled with secondary amine in basic media resulting in preparation of the PP compounds. Isolated yields were good to excellent, and the products (PP1-PP4) were purified by extraction and crystallization.

Computed physicochemical properties of the four preselected PP compounds are presented in Table 1. Using computational methods for the estimation of physicochemical properties of potential new lead compounds is well established in medicinal chemistry [24,25]. If we compare our computed descriptors to one obtained from lead compounds with anticancer activity [25], our amides PP1, PP2, and PP4 are well in the desired range (Table 1). Molecular weight should be around 380, ClogP around 3.7, PSA around 80, and HBA around 5. On the other hand, PP3 was designed to substantially increase water solubility, and it is a saccharide derivative that was perfectly reflected on its estimated physicochemical properties. It is well hydrated (5 hydrogen bond donors and 16 hydrogen bond acceptors) in water media, and it is hydrophilic (low ClogP); however, it has a large polar surface area that might decrease its cell membrane permeability [26].

**In Vitro Anticancer Effects of PP Compounds Compared to FF**

Since FA was used as a primary substrate for the proposed chemical modifications (Figure 1) and FA has only marginal anticancer properties in comparison to unprocessed FF [11], we have first tested to see if the new compounds (PP1-PP4) are indeed cytotoxic. We used two human glioblastoma cell lines (LN-229 and U87MG), patient-derived glioblastoma cells (GBM12), and a mouse glioblastoma cell line (GL-261) in this evaluation. Results in Figure 2A demonstrate changes in the percentage of cell death in LN-229 cells cultured in 10% FBS ± PP1, PP2, PP3, and PP4. All compounds were used at 5, 10, 25, and 50 μM, and the cells were treated for 24, 48, 72, and 96 hours. The control cultures were treated either with an equal volume of the vehicle (DMSO), or with 25 and 50 μM FF (positive control). In DMSO-treated cultures, the average cell death varied from 6% ±1.4% to 7% ±1.2% (Figure 2A). In the presence of 50 μM FF (single dose), LN-229 cells demonstrated 24% ±4% cell death at 48 hours; 90% ±1% at 72 hours, and 99% ±1.2% at 96 hours. In contrast, 25 μM FF was not cytotoxic; however, the treated cells demonstrated a significant growth arrest (Figure 2B).

Next, we compared FF data to PP1, PP2, PP3, and PP4. Similar to FF, all four compounds showed no cytotoxic effects during the first 48 hours following the treatment at all concentrations (Figure 2A). At
72- and 96-hour time points, PP1, PP2, and PP4 triggered extensive cell death at both 25 and 50 μM. This strong cytotoxicity was also observed at 10 μM PP1 (92%±2% cell death) and at 5 μM PP1 (74%±3% cell death); all four compounds were cytostatic at 5 μM (Figure 2, C-F), and FF was cytostatic at 25 μM (Figure 2B). In contrast to PP1, PP2, and PP4, PP3 demonstrated kinetics of growth retardation and cytotoxicity similar to FF (Figure 2, A and B). Like FF, PP3 was cytotoxic only at 50 μM and demonstrated cytostatic activity at 10 μM (Figure 2, A and B). We also observed extensive accumulation of peroxisomes in cells treated with PP3, which resembled morphology of LN-229 cells treated with 50 μM FF (not shown). This is also consistent with the data showing that PP3 does not repress PPREs (Figure 4C). Therefore, the anticancer activity of PP3 could be different from PP1, PP2, and PP4 and more similar to FF. However, PP3 dissolved in DMSO was unstable and needed to be prepared fresh for each experiment (data not shown).

On the basis of these initial findings, we have selected PP1 for additional experiments and confirmed its high in vitro cytotoxicity in...
another human glioblastoma cell line, U87MG (Figure 3A); in patient-derived glioblastoma cells (GBM12) [13,27] (Figure 3B); and in the mouse glioblastoma cell line GL261 (Figure 3C).

Physiochemical and Metabolic Effects of PP Compounds Compared to FF

The main purpose for the described chemical modifications was to generate new compounds, which in comparison to FF are more stable, more resistant to blood and tissue esterases, better soluble in water, and possibly more effective in penetrating BBB. Our data show that, in comparison to FF, PP1 was significantly more resistant to blood esterases (Figure 4A). In this experiment, 50 μM of PP1 and 50 μM of FF were incubated with human blood at 37°C at indicated time points. Following 48-hour incubation, almost all FF was converted to FA (Figure 4A; right panel). In contrast, nearly 25 μM of PP1 was still detected at the 48-hour time point (Figure 4A; left panel). In addition, we also demonstrate that, in comparison to FF, PP1 is much more soluble in water (Figure 4B), further supporting its potential as a new anticancer drug.

We have also demonstrated that PP1, PP2, and PP4 attenuated PPREs (Figure 4C). This unexpected finding indicates that these
three new compounds may have a different effect on tumor cells in comparison to FF, which following its conversion to FA becomes a potent agonist of PPAR\(\alpha\). In contrast, PP3 does not share this PPAR-inhibitory activity, and its action on glioblastoma tumor cells could be more similar to FF (see also Figure 2, A and B).

Since FF anticancer effects are mediated mainly by the inhibition of mitochondrial respiration [11], we used an Extracellular Flux Analyzer (XF24, Seahorse Biosciences) to measure real-time OCR (indicative of mitochondrial respiration) and ECAR (indicative of glycolytic activity) in LN229 human glioblastoma cells treated with the PP1 at 25 \(\mu\)M concentration. The cells treated with DMSO (vehicle) or with 25 \(\mu\)M FF were used as a background control and positive control, respectively. These metabolic parameters were measured in monolayer cultures after sequential injections of the following metabolic toxins: oligomycin [inhibitor of complex V (ATP synthase)], FCCP (mitochondrial uncoupling factor); rotenone [inhibitor of mitochondrial complex I (NADH dehydrogenase)], and antimycin A (inhibitor of mitochondrial complex III). In a typical “mitochondrial stress” experiment (Figure 5), addition of FF and PP1 resulted in a dramatic decrease of OCR values and the corresponding increases in ECAR. Similar results were obtained for PP2, PP3, and PP4 (not shown). OCR and ECAR values in the control (DMSO) were practically unaffected (blue plot). In DMSO-treated controls, addition of oligomycin inhibited OCR, addition of FCCP resulted in a maximal increase of OCR values, and finally addition of rotenone completely blocked the oxygen consumption, as expected (Figure 5, A and B). Conversely, cells treated with either FF or PP1 demonstrated repressed oxygen consumption, accompanied by increased ECAR values (glycolysis) throughout the experiment. Other relevant metabolic parameters calculated from this experiment confirmed extremely low ATP production and almost none existing spare respiratory capacity in the cells treated with either with FF or with PP compounds (Figure 5A).

**PP1 Tissue Distribution and Toxicity Evaluated in Large Intracranial Glioblastomas**

We used a syngeneic mouse glioblastoma model in which GL-261-luc cells (PerkinElmer Inc.) were injected into the brain parenchyma of C57BL/6 mice. Following 2 weeks of a continuous tumor growth, mice with well-established, large intracranial tumors were selected using biophotonic imaging (Xenogen IVIS 200) (Figure 6C). We have selected mice with large tumors mostly because, in glioblastoma patients, the tumors are detected late usually and often are too big or located in brain areas, which prevent successful surgical resection. Control mice were treated with DMSO (vehicle), and experimental mice were treated with the PP1 at 25-75 mg/kg/day, administered by oral gavage. Following 14 days of drug administration, mice were euthanized and the following organs were collected: blood, liver, kidneys, spleen, heart, intact brain, and intracranial tumors. Both tumor-free and tumor-bearing mice were used for HPLC-based measurement of PP1 tissue distribution (Figure 6A) and for tissue toxicity analyses (Figure 6, B, D and E). Results in Figure 6A demonstrate that PP1 accumulates in all tissues examined, and importantly, an average concentration of the compound found in
intracranial tumors was 5.8 ± 0.7 μM. According to our in vitro data (Figure 2), this concentration could be therapeutically relevant. The highest PP1 accumulation was found in the liver (10.3 ± 4.3 μM) and in the kidney (8.8 ± 4.7). In the blood, spleen, heart, and intact brains, the levels were 3.05 ± 0.2, 7.6 ± 3.2, 6.1 ± 2.4, and 4.9 ± 3.4 μM, respectively. Importantly, both tumor-free and tumor-bearing mice treated with PP1 did not show any major signs of toxicity to PP1 and maintained their body weight during the course of the treatment (Figure 6B). However, histological evaluation of the PP1-treated mice demonstrated inflammation and focal necrotic areas in the liver and an enlarged spleen with accumulation of hemosiderin (Figure 6D). In contrast, heart, kidney, and brain tissue showed no signs of pathology (Figure 6D).

Results in Figure 6C demonstrate four examples of intracranial tumors selected for the treatment. In this experiment, the tumors were allowed to grow for 14 days before treatment. Subsequently, mice with large intracranial tumors were selected using the biophotonic imaging and treated either with DMSO (vehicle) or with PP1 (50 mg/kg/day; oral administration). Although our data do not show obvious signs of the tumor regression following the treatment, most likely because tumors were already too big for a successful intervention, we detected large areas of tumor cell death found exclusively in mice treated with PP1, indicating a positive effect of the treatment (Figure 6E). In contrast, DMSO-treated mice had only small and sparse areas of necrosis (Figure 6E). In this experiment, both control and PP1-treated mice were euthanized at the same time when mice started to demonstrate signs of distress. Importantly, large necrotic areas found exclusively in PP1-treated animals indicate a promising therapeutic potential of this new metabolic drug.

**Discussion**

Glial tumors account for nearly 50% of all adult primary intracranial neoplasms, among which glioblastoma is the most aggressive and practically incurable [28,29]. A large variety of different genetic and epigenetic modifications have been found in glioblastomas, among which p53 mutations, EGF receptor amplification, and PTEN mutations are most common [30]. However, gene therapy, molecular and immunological approaches targeting these molecules and their pathways, as well as recently tested antibodies against immune
checkpoint inhibitors [31] have yet to produce improvements in patient outcomes. In addition to the introduction of the personalized medicine approach to target these specific pathways in glioblastoma patients [32–34], metabolic methods including calorie restriction and ketogenic diet are surprisingly effective as supplemental therapies for glioblastoma patients [35–37]. In addition, interesting anticancer effects of lipid-lowering drugs, fibrates, and statins have also been reported [3,8,38–42]. A 10-year all-cause mortality study involving 7722 patients treated with different fibrates revealed that the use of these metabolic compounds was associated with a significantly lower total mortality and reduced probability of death from cancer [43]. In cell culture and in animal studies, various members of the fibrate family demonstrated a broad range of anticancer activities [1–3,10,41,42,44–47]. These multiple reports encouraged clinical trials in which chronic administration of low doses of FF was tested along with chemotherapeutic agents, minimizing their toxicity and acute side effects in patients with recurrent brain tumors and leukemia [48,49]. Although these beneficial anticancer effects of FF

Figure 5. Metabolic effects of PP compounds compared to FF. Metabolic responses of LN-229 human glioblastoma cells were evaluated with Extracellular Flux Analyzer XF24 (Seahorse Biosciences, North Billerica, MA). Prior each assay, the cells were plated at $4 \times 10^4$ cells/well in 24-well plates in growth supporting media. At the time of measurement, growth media were replaced with serum-free XF medium (Seahorse) in cartridges equipped with oxygen-sensitive and pH-sensitive fluorescent probes. The OCR (indicative of mitochondrial respiration) and ECAR (indicative of glycolysis) were evaluated after injecting the following metabolic toxins: oligomycin, FCCP, rotenone, and antimycin A. Immediate metabolic effects were determined by comparing OCR and ECAR values in cells preincubated with DMSO (vehicle) or with PP1 and FF; all used at 25 μM. (A) Representative experiment in which real OCR (pmol/min) and ECAR (mP/ min) values are shown. Other relevant metabolic parameters calculated from this experiment include ATP production, spare respiratory capacity, basal OCR levels, and proton leak. (B) Average OCR and ECAR data (% change over basal OCR and ECAR values) from three independent experiments repeated five times ($n = 15$). Data represent average values ± SD. * indicates PP1 values significantly different from DMSO.
are still suspected to rely on PPAR-dependent mechanism/s, we have recently demonstrated that brain tumor cells retain sensitivity to FF in the presence of PPARα antagonists or PPARα siRNA [1,11]. This strongly suggests that FF may have additional PPARα-independent activity. Other groups also demonstrated that FF could have PPAR-independent cellular effects including PPAR-independent activation of GDF15 [50], effects of FF on cell membrane fluidity [51], and the FF-induced inhibition of mitochondrial respiration in isolated cardiac and liver mitochondria [52,53]. Therefore, a growing line of evidence supports the interaction of unprocessed FF (ester) with biological membranes, which could be a reason for the observed strong anticancer activity of this lipid-lowering drug. We have demonstrated previously that unprocessed FF (ester) accumulates in mitochondrial membranes [11]. As a consequence, the affected glioblastoma cells underwent immediate impairment of mitochondrial respiration and compensatory attempt of increasing glycolysis, which initially supported cell survival. However, prolonged (48 hours) exposure to FF depleted intracellular ATP and activated the AMPK-mTOR autophagy axis in glioblastoma cells. Although autophagy gave the affected cells an additional 12-24 hours of survival, it was ultimately followed by a massive tumor cell death [11].

In spite of these promising results, FF does not cross the blood-brain barrier (BBB) [4] and is quickly converted to FA by blood and tissue esterases (Figure 4A and [4]). We have made several chemical modifications in FF structure to address these problems. From the initial 26 new compounds, four with the most promising anticancer properties were evaluated in this study (Figure 1 and Table 1). These chemical modifications were designed to improve the compounds' stability, water solubility, tissue penetration, and ultimately anticancer potential. One of the lead compounds, PP1, is highly effective in triggered extensive glioblastoma cell death in vitro (Figures 2 and 3) and, similar to FF, induces immediate repression of mitochondrial respiration (Figure 5). In difference to FF, PP1 is much more stable when exposed to human blood (Figure 4A) and is significantly better soluble in water (Figure 4B). For animal studies, we have selected mice with large tumors. This is because in patients glioblastomas are detected late usually and often are too big for surgery or are located in brain areas, which prevent safe surgical resection. Although PP1-
treated mice did not show obvious signs of tumor regression, most likely because the selected tumors were too big for a successful treatment, large areas of tumor cell death found exclusively in mice treated with PP1 are highly encouraging. This is very important since brain tumors are particularly difficult to treat due to distinct anatomical and physiological traits of neural tissue and vasculature, BBTB. Although BBTB is more permeable than BBB, it still represents the major obstacle preventing anticancer agents from reaching therapeutically relevant concentrations. The only FDA-approved chemotherapy drug against glioblastoma which can cross BBB is temozolomide; however glioblastoma cells quickly develop temozolomide resistance, and recurrent tumors are practically incurable [13].

Our new metabolic compound, PP1, can be detected both in intact brain and in the brain tumors at μM concentrations following its oral administration. This is a very promising feature for glioblastoma therapy; however, we still do not understand why PP1 triggers extensive tumor cell death within the tumor mass but not in tumor-free normal brain. Although we do not have a definitive answer at this point, our previous data show that 50 μM FF was significantly less toxic to normal human astrocytes (NHA) compared to glioblastomas [11]. This is in spite of the immediate mitochondrial responses to FF, which are similar in both glioblastoma cells and in NHA [11]. This apparent discrepancy indicates that the observed low sensitivity of NHA to FF may involve mechanisms, which are not directly linked to FF-induced or PP1-induced inhibition of mitochondrial respiration. Further experiments are required to address this significant issue and, more specifically, to explain the difference between normal brain cells and brain tumor cells in responding to this new class of metabolic drugs.

Conclusions

We have evaluated the antiglioblastoma effects of four new compounds, which are modifications of a common lipid-lowering drug, FF. These four chemical modifications were developed specifically to increase the compounds’ resistance to blood and tissue esterases, water solubility, brain tissue penetration, and ultimately drug, FF. These four chemical modifications were developed specifically, to explain the difference between normal brain cells and brain tumors compared to FF may involve mechanisms, which are not directly linked to FF-induced or PP1-induced inhibition of mitochondrial respiration. Further studies are required to address this significant issue and, more specifically, to explain the difference between normal brain cells and brain tumor cells in responding to this new class of metabolic drugs.

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