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Fenofibrate as an anti-cancer treatment: an *in vitro* study on glioblastoma cells at various oxygen levels and on normal astrocytes.

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Abstract

Fenofibrate, an active pharmaceutical ingredient (API) of the fibrate class approved by the U.S. Food and Drug Administration (FDA) for the treatment of hypercholesterolemia and hyperlipidemia, has emerged as a promising candidate for repurposing in cancer. Indeed, beyond its lipid-lowering effects, fenofibrate has demonstrated antitumor properties, including antiproliferative and cytotoxic effects normoxia (21% O₂) in preclinical models of GBM. In addition, fenofibrate may inhibit hypoxia-inducible factor-1 alpha (HIF-1 α) and reduce the expression of hypoxia-inducible genes, potentially overcoming hypoxia-induced chemoresistance. These findings, combined with its established clinical safety profile, support further investigation of fenofibrate as a potential therapeutic strategy in GBM.

On this basis, physico-chemical characterization of fenofibrate was assessed to determine its ability to cross the blood-brain barrier. We determined *in vitro* on GBM cell lines U251-MG,

U87-MG and GL261, the efficacy of fenofibrate in decreasing GBM cell viability and proliferation under normoxia (21% O₂) and hypoxia (1% and 0.2% O₂). We investigated the kinetics of its internalization by HPLC. In addition, the safety of fenofibrate was evaluated on non-tumoral brain cells. Dunn's post-hoc test was used after a significant Kruskal-Wallis.

Being practically insoluble in water, fenofibrate was dissolved in dimethyl sulfoxide (DMSO) for studies. Primary astrocytes showed no signs of toxicity following treatment with fenofibrate. The effect of fenofibrate on GBM cell lines was studied at various time points and exhibited a cell type, time- and concentration-dependent cytotoxicity with an LC₅₀ of 25, 43.7 and 49.6 μM for U251-MG, U87-MG and GL261 cells, respectively. Interestingly, fenofibrate uptake was confirmed, 12.9 ± 5.7% and 14.2 ± 6.6% of fenofibrate was found in U251-MG and U87-MG cells, respectively. Intracellular concentrations of fenofibrate increased over time and no precipitation was observed. In hypoxia (1% and 0.2% of O₂), the cytotoxic effect of fenofibrate was still present, albeit decreased. Furthermore, fenofibrate induced a cytostatic effect on U251-MG and U87-MG under both normoxia (21% O₂) and hypoxia (1% and 0.2% O₂), reducing their proliferation. Under the experimental conditions tested, namely *in vitro* studies, fenofibrate appeared more efficient compared with temozolomide, the standard treatment for GBM.

This study demonstrated the cytotoxic and cytostatic effect of fenofibrate on GBM cells. These results indicate that fenofibrate may be a therapeutic alternative for GBM treatment.

Keywords

Glioblastoma, hypoxia, fenofibrate, cytotoxicity, cytostaticity

Background

Glioblastoma (GBM) is a malignant and aggressive brain tumor with an overall survival rate of less than 15 months [1]. The standard treatment, consisting of surgery, combined with chemotherapy, based on temozolomide (TMZ), and radiotherapy [2], has limited efficacy and one key player for resistance is hypoxia. Hypoxia is defined as a decrease in oxygen pressure leading to the transcription of genes that causes various changes such as the activation of angiogenesis [3], multidrug-resistance genes through increased expression of efflux pumps (ATP Binding Cassettes) [4,5]. The blood-brain barrier (BBB) is also involved in the multidrug resistance to treatment [6]. The development of new therapies remains necessary to deliver drugs within tumor cells with lower sensitivity to hypoxia.

Fenofibrate, an agonist of peroxisome proliferator-activated receptor α (PPAR α), is a lipophilic active pharmaceutical ingredient (API) belonging to the fibrate class and is currently approved by the U.S. Food and Drug Administration (FDA) as a lipid-lowering agent for the treatment of hypercholesterolemia and hyperlipidemia [7,8]. Beyond its established clinical use in metabolic disorders, accumulating evidence suggests that fenofibrate may induce various effects that extend to cancer therapy [9]. In the context of glioblastoma (GBM), several preclinical studies have reported multiple antitumor effects. *In vitro* studies performed in normoxia have shown that fenofibrate induces a decrease in tumor proliferation in multiple GBM cell lines such as U87-MG and LN229 through cell cycle arrest and apoptosis [10,11]. *In vivo* studies have confirmed the anticancer effect of fenofibrate through the inhibition of tumor growth in a U87-MG model [12,13]. Moreover, the degradation of hypoxia-inducible factor-1 alpha (HIF-1 α) on GBM cell lines U251-MG and U87-MG following fenofibrate treatment make it a perfect candidate for GBM therapy [14].

Given its regulatory approval, and emerging antitumor properties, fenofibrate represents an attractive candidate for therapeutic repurposing in GBM. In this study, we specifically investigated its potential to overcome chemoresistance and evaluated its anticancer effects on three GBM cell lines in normoxia (21% O₂) and hypoxia (1% and 0.2% O₂), thereby modeling the oxygen heterogeneity characteristic of the tumor microenvironment. To date, investigations have primarily focused on the modulation of HIF-1 α expression with no study examining the impact of fenofibrate on GBM cell viability in hypoxia (1% and 0.2% O₂). Furthermore, we assessed the safety of fenofibrate on healthy brain cells, such as astrocytes, to anticipate its repurposing and provide additional insight into its therapeutic window. Collectively, our findings extend previous knowledge and further support the potential of fenofibrate as a therapeutic strategy in glioblastoma.

Methods

Thermodynamic solubility

The thermodynamic solubility at pH 7.4 of fenofibrate (Cayman Chemical, Ann Arbor, Michigan, USA) was determined according to a miniaturized shake-flask method [15]. Phosphate buffer solution (10 mM, pH 7.4, ionic strength 150 mM) was prepared from Na₂HPO₄, KH₂PO₄, and KCl (Merck, Steinheim, Germany). 990 μ L of buffer were put into a 5 mL glass tube. 10 μ L of 50 mM fenofibrate solution in DMSO (Fisher Scientific, Geel, Belgium) were added to buffer. Then, glass tubes were shaken by inversion at room temperature for 24 hours. Tube contents were centrifuged at 12 225 \times g for 10 minutes. 100 μ L of supernatant were mixed with 100 μ L acetonitrile (Prolabo VWR International, Fontenay-sous-Bois, France) in a Greiner UV microplate. The solubility determination was realized by spectrophotometry (Infinite M200, Tecan, Männedorf, Switzerland) at 290 nm. The calibration curve was obtained with standard fenofibrate solutions in a 50:50 (v/v) mixture of

buffer with acetonitrile / phosphate buffer solution. Calibration curves were linear with $R^2 > 0.99$.

Blood-brain barrier assays (PAMPA-BBB)

The PAMPA-BBB experiments were conducted using the PAMPA Explorer Kit (Pion Inc., Billerica, Massachusetts, USA) according to manufacturer's protocol. Fenofibrate was first dissolved in DMSO at 20 mM. Each stock solution was then 1/200 (v/v) diluted in Prisma HT buffer pH 7.4 (pION). 200 μ L of these solutions were added to the donor plates. Each membrane filter of the acceptor plate (P/N 110243) was coated with 5 μ L of BBB-1 Lipid (P/N 110672). Each well of the acceptor plates was filled with 200 μ L of Brain Sink Buffer (P/N 110674), or Acceptor Sink Buffer (P/N 110139). In both experiments, after incubation at room temperature for 4 hours, the sandwich was separated, and the amounts of drug in both donor and acceptor compartments were measured through UV-visible spectra by the microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). The permeability value (P_e) was calculated by the PAMPA Explorer software version 3.7 (pION). Corticosterone and theophylline were used as references for high and low permeability, respectively.

Cell Lines

The murine GBM cell line GL261 (NCI-DCTD (Division of Cancer Treatment and Diagnosis), Repository) and two human GBM-derived cell lines, U251-MG cells (Cellosaurus CVCL_0021) purchased from National Cancer Institute (NCI, Bethesda, MD, USA) and U87-MG cells (Cellosaurus CVCL_0022) obtained from the American Type Culture Collection (ATCC, LGC standards, Molsheim, France), were used. U251-MG and U87-MG cell lines were cultured in DMEM 1 g/L of glucose (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 10% fetal bovine serum (FBS) (Grosseron, Couëron, France), 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin. GL261 cells were cultured in

RPMI (Roswell Park Memorial Institute, Sigma-Aldrich, Saint-Quentin Fallavier, France) medium supplemented similarly to DMEM 1 g/L of glucose. The cell lines were maintained in culture at 37°C with 5% of CO₂ and 95% humidity.

Primary Culture of Astrocytes

Cerebral cortices were isolated from neonatal (1-3 days old) mice (Swiss, CURB) and dissociated after carefully removing the meninges. A cell suspension was obtained and seeded in 6-well plate. The culture was maintained in DMEM 4.5 g/L of glucose (Sigma-Aldrich, Saint-Quentin Fallavier, France) supplemented with 10% FBS, 10% horse serum (Eurobio, Courtaboeuf, France), 2mM L-glutamine at 37°C with 5% of CO₂ and 95% humidity until confluence (12 days) and then 5% FBS, 5% horse serum were used. The astrocytic identity of the primary cultures was confirmed by immunocytochemistry using glial fibrillary acidic protein (GFAP), a specific astrocyte marker.

Hypoxia

Hypoxic workstations (IN VIVO 2 500, Baker Ruskinn, Alliance Bio Expertise, Guipry, France) were employed for hypoxia studies, set at 1% or 0.2% O₂, 5% CO₂ at 37°C in humidified atmosphere. Cell lines and primary culture of astrocytes were cultured under normoxia (21% O₂) or hypoxia (1% and/or 0.2% O₂) 4h before treatment with increasing concentrations of fenofibrate and maintained under these conditions until the end of experiments.

Quantification of intracellular and extracellular fenofibrate

U251-MG and U87-MG cells were incubated for 1, 3 or 24 hours at 37°C with 40 µM of fenofibrate after dilution in DMSO and medium. After treatment, the supernatant was collected and centrifuged at 13 500 rpm for 5 min at 4°C to remove precipitated fenofibrate and cellular debris. The cells were rinsed with cold Phosphate-buffered saline (PBS)

(Eurobio) and with an extraction buffer containing 0.1% Triton X-100 (Sigma-Aldrich, Saint-Quentin Fallavier, France). The lysate was centrifuged at 13 500 rpm for 10 min at 4 °C. Then, both supernatants were collected and methanol (Prolabo VWR International) was added to precipitate the proteins. The sample was vortexed and centrifuged at 13 500 rpm for 5 min at 4°C. Then, the supernatant was filtered through 0.2 µm regenerated cellulose syringe filters (Minisart® Syringe Filter, Sartorius, Goettingen, Germany) before injection.

Fenofibrate concentrations were determined by HPLC [16]. The HPLC system consists in an Agilent® 1290 Infinity binary pump, an Agilent 1290 Infinity autosampler, and an Agilent 1260 Infinity diode-array detector (Agilent technologies, Santa Clara, CA, USA). A reversed phase column C18 (5 µm, 2.1 × 50 mm, Restek® Ultra) was used as the analytical column. The mobile phase was composed of acetonitrile containing 0.1% (v/v) formic acid (Prolabo VWR International, Fontenay-sous-Bois, France) (A) and water containing 0.1% (v/v) formic acid (B). Detection wavelength (λ), flow rate, total run time (T), gradient, injection volume, concentration range, and retention time (Rt) used to analyze fenofibrate are listed in Table 1. The column temperature was 40 °C. Linearity was good within the concentration ranges studied with a correlation coefficient higher than 0.99.

Table 1: HPLC methods used in the present work.

API	λ (nm)	Flow (mL/min)	T (min)	Gradient T (min)	A (%)	Vinj (µL)	Rt (min)	Concentration Range (µg/mL)
Fenofibrate	290	0.6	3.5	0	60	3.0	2.11	0.25 - 50
				1.5	68			
				2.2	68			
				2.3	100			

Cell viability

The viability of cell lines and primary culture of astrocytes was measured by a crystal violet assay. Fenofibrate, fenofibric acid and TMZ were diluted in DMSO and medium to obtain various concentrations (25, 40 and 50 mM). Cells were then incubated for 72 hours at 37°C with various concentrations of fenofibrate, fenofibric acid and/or TMZ after dilution of the stock and standard solutions in medium by a factor of 1:100, v/v. After treatment, the cells were rinsed with cold PBS and fixed with a solution of crystal violet (0.1%) (Sigma-Aldrich, Saint-Quentin Fallavier, France) diluted in ethanol and PBS. The cells were then rinsed three times with deionized water. After complete drying, an acetic acid solution diluted in water 1/10 (v/v) was used to release crystal violet contained in the cells. The optical density of the solution was determined using a spectrophotometer (SPARK® reader, Tecan, Männedorf, Switzerland) at a wavelength of 600 nm. From the cell viability values, a biological parameter was calculated: a chemoresistance index for hypoxia was calculated as the ratio of cell viability (%) in hypoxia (1% and 0.2% O₂) / cell viability (%) in normoxia (21% O₂).

Colony formation assay

The cell proliferation was measured by a clonogenic assay. Fenofibrate was diluted in DMSO and medium to obtain various concentrations (25, 40 and 50 mM). Cells were plated in 6-well plates (500 cells/mL), exposed to various concentrations of fenofibrate after dilution of the standard solutions in medium by a factor of 1:100, v/v. The cells were kept in the incubator or the hypoxic chamber for duration of 10-18 days. Colonies were then stained with a solution of crystal violet (0.1%) diluted in ethanol and PBS. Only colonies with more than 50 cells were counted manually using ImageJ® software.

Cell death study

All flow cytometry experiments were performed on a Gallios™ flow cytometer (Beckman Coulter SAS, Villepinte, France) from the US PLATON platform of the Normandy University - Caen. Apoptosis was assessed using the Annexin V-FITC/Propidium Iodide (PI) kit (Beckman Coulter SAS, Villepinte, France). For sample preparation, the culture supernatant was first collected, and adherent cells were washed with PBS and harvested by trypsinization. Cells were then centrifuged ($300 \times g$, 5 min) and the pellet resuspended in binding buffer and kept on ice. Cells were stained with 1 μL Annexin V-FITC and 5 μL PI, incubated for 15 minutes on ice in the dark, and analyzed by flow cytometry after addition of 400 μL binding buffer. This method allows the identification of four populations: viable cells (Annexin V $-$ /PI $-$), early apoptotic cells (Annexin V $+$ /PI $-$), late apoptotic cells (Annexin V $+$ /PI $+$), and necrotic cells (Annexin V $-$ /PI $+$).

Microscopy

The images were acquired on a fluorescence microscope (Leica DM IL LED, Leica microsystems, Wetzlar, Germany) with MetaMorph® Software.

Statistical analysis

To calculate the LC_{50} , the relative cell viability values obtained for each condition were plotted in GraphPad Prism. The data were then fitted using a nonlinear regression model (quadratic polynomial), and the resulting equation was used to determine the concentration corresponding to 50% viability. The correlation was determined with results of $R^2 > 0.93$ in normoxic conditions and hypoxic conditions.

The data were analyzed using GraphPad® Prism statistical software (version 10.4.1, GraphPad® Software, La Jolla, CA, USA) and are presented as mean \pm standard deviations (SD). Dunn's post-hoc test was used after a significant Kruskal-Wallis.

Results

1. Physico-chemical characterization of fenofibrate

According to the European Pharmacopeia (Ph. Eur., 11th ed., monograph 01/2017:1322), fenofibrate is practically insoluble in water which can be related to its high log P value of 5.24 [17,18] (Table 2). The solubility of fenofibrate in Hanks' Balanced Salt Solution (HBSS) was evaluated at 3.3 $\mu\text{g/mL}$ [19] and the API exhibits also a low solubility, lower than 1 mg/mL (0.8 μM) in phosphate buffer. According to the manufacturer's technical data sheet (Cayman Chemical, 2022), fenofibrate exhibits a solubility of 15 mg/mL in DMSO. Due to its relatively high solubility in DMSO, this solvent is frequently used for dissolution in *in vitro* assays. Moreover, the results of a PAMPA assay revealed that fenofibrate was not able of crossing the BBB *via* passive diffusion ($\text{Pe} < 1 \text{ nm/s}$).

Table 2: Properties of fenofibrate.

Molecular weight	360.83 g/mol
LogP	5.24
Thermodynamic solubility at pH 7.4	0.8 μM
PAMPA blood-brain barrier permeability assay at pH 7.4: Pe	0.45 nm/s

2. Biological evaluation of fenofibrate

a. *In vitro* cytotoxicity of fenofibrate on glioblastoma cell lines in normoxia (21% O₂)

The cytotoxicity of fenofibrate was assessed on various GBM cell lines: U251-MG, U87-MG and GL261 cells to mimic a broader view of the biological mechanisms and tumor heterogeneity of GBM. (Fig. 1).

A time-dependent effect was observed across all cell lines. After 24 hours of treatment, there was no noticeable change in cell morphology and the cell density appeared unaffected by fenofibrate. However, after 48 hours, some cells exhibited rounding shapes and a slight decrease in cell density was observed (additional file 1).

As illustrated in Figure 1A., in normoxia (21% O₂), a dose dependent response to the drug was observed across all cell lines after treatment for 72 hours. The U251-MG cells showed a notable sensitivity to fenofibrate since there was a significant decrease in cell density at 25 μM. At 40 μM of fenofibrate, the cells exhibited a rounded morphology, a hallmark of cellular stress linked to early processes of cell death. In contrast, the U87-MG cell line displayed greater resistance to the treatment than U251-MG cells with a decrease in cell density becoming apparent only at 40 μM of fenofibrate. The GL261 cells were the most resistant, with a number of cells still being high even at 50 μM.

Quantitatively, the LC₅₀ for U251-MG cells was reached at 25 μM after 72 hours (Fig. 1B). At 50 μM, fenofibrate induced a significant decrease of cell viability ($p = 0.0029$, Kruskal–Wallis with Dunn’s post-hoc). For U87-MG and GL261 cells, the LC₅₀ was reached at 43.7 μM and 49.6 respectively, indicating a greater resistance of these two cell lines to fenofibrate.

To compare the efficacy of fenofibrate with that of the reference drug used to treat GBM, we evaluated the cytotoxicity of TMZ on human GBM cell lines, U251-MG and U87-MG, after 72h of exposure (Fig. 2A). In normoxia (21% O₂), TMZ induced a slight decrease in cell viability for both cell lines across all concentrations (20 to 30%) (Fig. 2A). There was no difference between U251-MG and U87-MG cells. In response to TMZ, LC₅₀ were higher than 100 μM for both cell lines.

Thus, we compared the difference between the effect of fenofibrate and TMZ on human GBM cell lines after 72h of exposure (Fig. 2B). At 25 μM, U251-MG cells were more sensitive to

fenofibrate than U87-MG whereas resistance to TMZ were similar between both cell lines. Fenofibrate appeared more cytotoxic than TMZ but only on U251-MG. At 50 μM , the effect of fenofibrate was enhanced, with both cell lines having a decrease of their viability. Again, the sensitivity differed between U251-MG and U87-MG, while no difference was observed for TMZ. Moreover, the cytotoxicity of TMZ was not evidenced even at 50 μM , indicating a great resistance of both cell lines to this conventional treatment under the experimental conditions tested, namely *in vitro* studies. Finally, to evaluate potential synergistic effects, cells were co-treated with fenofibrate (25 μM) and TMZ (25 μM) for 72 hours (additional file 2). The observed viability of cells treated with FF + TMZ was around 50%. The expected additive effect of the combination (CI) was calculated using the multiplicative model: $\text{Expected CI} = \text{Viability (FF)} \times \text{Viability (TMZ)} / 100 = 46.25\% \times 80.00\% / 100 = 37.00\%$. Therefore, the observed viability was higher than the expected additive effect, indicating that the combination did not produce synergistic cytotoxicity.

b. *In vitro* cytotoxicity of fenofibrate on glioblastoma cell lines in hypoxia (1% and 0.2% O_2)

We then sought to investigate the cytotoxicity of fenofibrate in hypoxia (Fig. 3) by conducting similar experiment under two hypoxic conditions, 1% and 0.2% of O_2 .

In 1% O_2 , 72 hours after treatment, the density of U251-MG cells decreased at 25 μM , and the cells exhibited rounded shape morphology at 40 μM (Fig. 3A). At 50 μM of fenofibrate, all cells were dead or undergoing significant cell death ($p = 0.0177$, Kruskal–Wallis with Dunn's post-hoc). For U87-MG cells, the cytotoxic effect of fenofibrate appeared at 50 μM , accompanied by phenotypic changes in the cells. For GL261 cells, the cell monolayer remained intact and fenofibrate cytotoxicity was not observed.

In 0.2% O₂, 72 hours after treatment, U251-MG and U87-MG cells were affected by hypoxia, as their morphology changed for all conditions (Fig. 3B). Thus, the cytotoxicity of fenofibrate was masked by hypoxia (0.2% O₂). However, fenofibrate toxicity appeared at 25 µM for U251-MG as more cells became rounded. At 50 µM, fenofibrate induced a significant decrease of cell viability ($p = 0.0109$, Kruskal–Wallis with Dunn’s post-hoc). For U87-MG, the change was subtler and no difference could be observed even at 50 µM of fenofibrate. Finally, for GL261 cells, hypoxia (0.2% O₂) appeared less toxic and the cell density remained stable at all fenofibrate concentrations.

In response to fenofibrate in hypoxia (1% and 0.2% O₂), a chemoresistance occurred resulting in an increase in LC₅₀ of all GBM cell lines (Fig. 3C). For U251-MG cells, the LC₅₀ was higher than 100 µM of fenofibrate in hypoxia (1% O₂) and was at 36 µM of fenofibrate in hypoxia (0.2% O₂). For GL261 cells, the LC₅₀ was 51.4 µM and 56.0 µM in hypoxia (1% and 0.2% O₂, respectively). The treatment response profile became similar between the two cell lines. U87-MG cells exhibited the greatest hypoxia-induced chemoresistance, with a LC₅₀ higher than 100 µM in both hypoxia (1% and 0.2% O₂) conditions.

A chemoresistance index was calculated at 25 µM of fenofibrate. For both hypoxia (1% and 0.2% O₂) conditions, the values were similar for U87-MG and GL261 cells (Table 3). U251-MG cells displayed the highest index, between 1.5 and 1.9. The cells were very sensitive to the treatment in normoxia (21% O₂), and twice less sensitive in hypoxia (1% and 0.2% O₂). U87-MG and GL261 exhibited a similar index at around 1, both cell lines appeared to be as resistant in hypoxia (1% and 0.2% O₂) as in normoxia (21% O₂). For 50 µM of fenofibrate, the difference became more pronounced between U87-MG and GL261 cells. U87-MG cells became more sensitive to the treatment in normoxia (21% O₂) than in hypoxia (1% and 0.2% O₂) while GL261 cells remained equally responsive to fenofibrate in normoxia (21% O₂) and hypoxia (1% and 0.2% O₂) with an index close to 1. The chemoresistance index in hypoxia

(1% and 0.2% O₂) also increased for U251-MG cells. This cell line became more than 2 times more sensitive to treatment in normoxia (21% O₂) compared to hypoxia (1% and 0.2% O₂) as the concentration of fenofibrate increased.

Table 3: Hypoxia-induced chemoresistance after fenofibrate treatment on GBM cell lines.

Cell line	Condition	Chemoresistance index for 25 µM of fenofibrate	Chemoresistance index for 50 µM of fenofibrate
Hypoxia 1% O ₂ /	U251-MG	1.9 ± 0.6 *	2.6 ± 1.3
Normoxia 21% O ₂	U87-MG	1.1 ± 0.5	2.1 ± 0.8 *
	GL261	1.2 ± 0.2	1.1 ± 0.4
Hypoxia 0.2% O ₂ /	U251-MG	1.5 ± 0.5	2.0 ± 0.8
Normoxia 21% O ₂	U87-MG	1.2 ± 0.3	1.5 ± 0.1
	GL261	1.2 ± 0.4	1.1 ± 0.3

Chemoresistance index in hypoxia (1% and 0.2% O₂) for U251-MG, U87-MG and GL261 cells after 72h of exposure to 25 and 50 µM of fenofibrate. * p<0.05, Hypoxia (1% and 0.2% O₂) vs Normoxia (21% O₂) (Mann-Whitney test)

c. *In vitro* cytostatic effect of fenofibrate on glioblastoma cell lines

Since fenofibrate has also been reported to induce cell cycle arrest, we determined how fenofibrate affects the ability of GBM cells to proliferate in normoxia (21% O₂) and hypoxia (1% and 0.2% O₂) (Fig. 4A). Clonogenic assays showed a sensitivity of U251-MG and U87-MG to fenofibrate in normoxia (21% O₂) within the same range of concentration used previously. The number of U251-MG colonies significantly decreased to 13% at 50 µM, compared to the vehicle condition (1% DMSO) (p = 0.0134, Kruskal–Wallis with Dunn’s post-hoc). The number of U87-MG colonies also decreased as the concentration of fenofibrate

increased, dropping significantly to 13% of colonies at 50 μM ($p = 0.0050$, Kruskal–Wallis with Dunn’s post-hoc). However, no changes in colony number were observed on GL261 cells relative to the vehicle condition (1% DMSO). Interestingly, in hypoxia (1% and 0.2% O_2), fenofibrate induced a cytostatic effect on both human GBM cell lines (Fig. 4B). The number of U251-MG colonies in hypoxia (1% O_2) reduced by half at 25 μM compared to the vehicle condition (1% DMSO). At 50 μM , fenofibrate induced a significant decrease of the number of U251-MG colonies ($p = 0.0181$, Kruskal–Wallis with Dunn’s post-hoc). In hypoxia (0.2% O_2), no colonies were detected at any concentration. Similarly, in both hypoxia (1% and 0.2% O_2) conditions, the number of U87-MG colonies reached 0% at 25 and 50 μM of fenofibrate. In contrast to the cytotoxicity study, these cells did not display a chemoresistance to fenofibrate.

d. Evaluation of fenofibrate uptake by glioblastoma cell lines

Another experiment was performed to evaluate the uptake of fenofibrate by the cells after dissolution in DMSO in normoxia (21% O_2) (Fig. 5). The amount of fenofibrate quantified in the medium after 3 hours of incubation with the cells represented $91.8 \pm 27.1\%$ and $86.8 \pm 23.9\%$ of the quantity deposited respectively for U251-MG and U87-MG cells (Fig. 5A). The amount of internalized fenofibrate in the cells was also assessed, and represented $12.9 \pm 5.7\%$ and $14.2 \pm 6.6\%$ of the quantity deposited respectively for U251-MG and U87-MG cells. To further investigate the cellular uptake of fenofibrate over time, cells were exposed to fenofibrate for either 1 or 24 hours (Fig. 5B). The amount of internalized fenofibrate increased with time, while its concentration in the surrounding medium decreased accordingly and this is similar for both cells lines. The sum of intracellular and extracellular fenofibrate consistently approached 100%, indicating minimal loss due to precipitation or degradation.

e. *In vitro* cytotoxicity of fenofibrate on glioblastoma cell lines with medium change

Since we reported that fenofibrate entered the cells after 3 hours of treatment (Fig. 5), we performed the same cell viability assay, with an additional medium change 3 hours after treatment to assess the time-dependent cytotoxicity of fenofibrate (Fig. 6). Notably, under all conditions (normoxia (21% O₂) and hypoxia (1% and 0.2% O₂)), no decrease in cell viability was observed for any of the GBM cell lines. As illustrated in this figure, fenofibrate cytotoxicity disappeared when exposure was less than 3 hours.

f. *In vitro* cytotoxicity of fenofibrate on astrocytes

All of the previous results indicate a toxicity of fenofibrate on various GBM cell lines. For potential application in GBM treatment, it is necessary to ensure that fenofibrate was safe on astrocytes, as GBM cells are considered as malignant astrocytes (Fig. 7). Therefore, fenofibrate was tested on primary culture of astrocytes in normoxia (21% O₂) (Fig. 7A, 7B) and hypoxia (1% O₂) (Fig. 7C, 7D). The results showed that fenofibrate did not induce any toxicity to astrocytes under both conditions.

Discussion

Recently, fenofibrate, a hypolipidemic drug, has been identified as an API that should be interestingly repurposed for its antitumor proprieties [9]. Nevertheless, questions remain about its efficacy on different types of GBM cells and subjected to a microenvironment promoting tumor resistance (TME). Thus, the present study aimed to evaluate the effects of fenofibrate not only on cell viability but also on cell proliferation under both normoxia (21% O₂) and hypoxia (1% and 0.2% O₂) on various GBM cell lines.

Fenofibrate proved its efficiency to induce cell death in multiple cancers *in vitro* and *in vivo*. For instance, through cell cycle arrest, apoptosis or autophagy, fenofibrate was able to target multiple cellular pathways to inhibit tumor growth [10,20,14]. Our study assessed its impact on both human and murine GBM cancer cell lines. The efficacy of fenofibrate at 25 μM and

50 μM in reducing clone formation and cell viability has been previously reported [10,20]. We therefore tested fenofibrate at both 25 μM and 50 μM , and included an intermediate concentration of 40 μM to further refine the dose–response effect. Fenofibrate was effective in inducing cell death and inhibiting colony formation. The investigation of fenofibrate’s effect proved to be useful as the sensitivity to the treatment varied depending on the cell type and oxygen level, U251-MG cells exhibited greater sensitivity to the treatment when compared to U87-MG and GL261 cells. While fenofibrate reduced human cell proliferation, its limited effect on murine cells *in vitro* suggests that the response may depend on the genetic background of the cells. Moreover, treatment with fenofibrate for 24, 48 or 72 hours showed different effects on human U251-MG, U87-MG and murine GL261 cells, notably a time- and concentration-dependent cytotoxicity. These observations underscore the necessity of evaluating cytotoxicity with varying exposure time, culture conditions, cellular models and the choice on *in vivo* model.

In our study, fenofibrate induced a cytotoxic effect on GBM cell lines. In the literature, the anticancer role of fenofibrate has been attributed to both PPAR α -dependent and PPAR α -independent mechanisms. We only performed one mechanistic study to determine which pathways were responsible for the cytotoxicity observed in our cell lines which is a limitation of the study. Annexin V-FITC/PI staining showed that fenofibrate at the highest concentration (50 μM) primarily induced necrosis rather than apoptosis, with many cells still appearing viable in this assay (additional file 3). This suggests that additional forms of cell death may be involved, consistent with crystal violet results showing more extensive cell loss. Based on previous studies, we can hypothesize the involvement of PPAR α -independent pathways, such as the accumulation of fenofibrate in mitochondrial membranes, leading to ATP depletion and an energy catastrophe. Fenofibrate is then responsible for the reduction of mitochondrial respiration (inhibition of complex I of the electron transport chain) *via* AMPK activation,

mTOR inhibition and autophagy induction, as well as the reduction of glycolysis caused by a decreased glucose uptake and inhibition of the GLUT4 glucose transporter [13]. Another PPAR α -independent mechanism may also alter cellular energy levels by targeting ketogenesis and inducing growth arrest [21]. Fenofibrate is additionally known for its anti-inflammatory properties, which involve inhibition of several signaling pathways, including NF- κ B/RelA, Interleukine-1, Interleukine-6, and Toll-like receptor 4 [22].

The standard treatment for GBM is based on TMZ [2]. The effectiveness of temozolomide is reduced by several factors that induce treatment resistance, notably chemoresistance leading to recurrence. Resistance to TMZ is associated with the MGMT gene, which is responsible for repairing DNA strand breaks induced by alkylating agents such as TMZ [23]. Although TMZ has been used for its anticancer property *in vitro*, it requires high concentrations that exceed clinically relevant concentrations, ranging from 100 μ M to 4000 μ M [24]. In comparison, the conventional treatment is based on a daily dose of 150 to 200 mg / m² of body-surface area [2] which corresponds to low plasma concentrations at the level of micromolars. Therefore, in this study, we used a dose range below 100 μ M and the resulting cytotoxic effect appeared to be low. Furthermore, under the experimental conditions tested, namely *in vitro* studies, and at clinically achievable concentrations of 25 or 50 μ M, the results demonstrated a greater efficacy of fenofibrate with lower cell viability compared to TMZ. These findings underscore the potential of fenofibrate as a treatment for GBM. Moreover, our results show that the MGMT profile can confer resistance to TMZ, especially in the context of a non-functional p53 status [25]. However, it is not the only factor involved. MGMT expression also plays a role: although all the cell lines used here are MGMT-methylated, the level of MGMT protein expression still has an impact [26]. When MGMT protein is absent, the cells are sensitive to treatment. Differences in MGMT expression between cell lines can therefore lead to differences in treatment response. In our case, both U87 and U251 show very low MGMT

expression, which explains why the effect is similar in the two lines. Nonetheless, chemoresistance is still observed, indicating that another factor must be contributing.

To date, no study has provided evidence of a cytotoxicity of fenofibrate in hypoxia (1% and 0.2% O₂) on GBM cell lines. However, the ability of fenofibrate to overcome the resistance induced by the TME by inhibiting HIF-1 α expression in GBM cells has been reported [27,14]. Our data demonstrated its cytotoxicity on various GBM cell lines under hypoxia (1% and 0.2% O₂). Fenofibrate induced a cytotoxic effect under hypoxic conditions, and its impact on cell survival is demonstrated here for the first time. Previous studies had only shown that fenofibrate inhibits the expression of HIF-1 α and carbonic anhydrase IX via the AMPK/HO-1/SIRT1 pathway [14] and its antiangiogenic properties [21]. Therefore, based on that study, we can hypothesize that inhibition of these signaling pathways reduces the chemoresistance of these tumor cells, thereby promoting fenofibrate-induced cell death. While a chemoresistance was observed, the dose dependent effect of fenofibrate remained significant, particularly for U251-MG. Indeed, sensitivity to hypoxia (1% and 0.2% O₂) varies depending on the cell line [28].

Tumor cells are characterized by a high degree of plasticity and can shift from oxidative phosphorylation to glycolytic metabolism depending on oxygen availability [29], a phenomenon that is further amplified under hypoxia. In addition, the study of Basilotta *et al.* demonstrated that certain GBM subtypes rely on fatty acid oxidation (FAO), a process enhanced in hypoxia and associated with increased energy production in tumor cells in addition to glycolysis. Since fenofibrate also increases FAO, one could hypothesize that it might promote energy gain and support cell survival under hypoxia, which could explain the chemoresistance observed [30]. Moreover, HIF-1 α stabilization under hypoxic conditions drives the expression of genes that promote glycolysis and adapt cells to low oxygen. PPAR α activation, on the other hand, can reprogram metabolism toward fatty acid oxidation,

potentially reducing the cell's reliance on glycolysis and creating a metabolic shift that may influence tumor survival under hypoxia [14]. Nevertheless, fenofibrate also induces a depletion of ATP through other signaling pathways, which ultimately counteracts FAO, suggesting that it would be valuable to modulate the specific pathways activated by fenofibrate [13]. Therefore, in addition to targeting glycolysis, as demonstrated in many studies [31], it is important to consider mechanisms linked to oxidative phosphorylation and other energy-metabolism processes. Indeed, among the glioblastoma cell lines we studied, some preferentially rely on one pathway or the other depending on their metabolic profile, which contributes to differences in treatment response [32,33,28]. Finally, fenofibrate, which modulates fatty acid metabolism by promoting β -oxidation, could be further optimized when combined with a detailed metabolic characterization of the tumor using molecular imaging, integrating general metabolism (FDG-PET), hypoxia (FMISO-PET), and ideally FAO-PET. In our human glioblastoma models, we have already performed two PET studies combining FMISO and FDG [34,35], although FAO-PET imaging has not yet been explored.

Alterations in fatty acid metabolism in cancer have been associated with decreased apoptosis and drug resistance, highlighting the need to identify molecules that target key metabolic proteins to enhance the efficacy of anticancer therapies. Fenofibrate induces metabolic reprogramming by decreasing glucose utilization and mitochondrial respiration in cancer cells and promoting the use and oxidation of fatty acids for their energy demands [36]. Overall, the role of PPAR α modulators remains highly debated, with some studies reporting a dose-dependent effect: low concentrations may promote tumor cell proliferation, while higher concentrations induce tumor cell death [37]. The role of PPAR α in therapeutic resistance has been investigated, showing that activation can induce chemo- and radioresistance through fatty acid-related pathways [38]. Some studies have therefore aimed to modulate PPAR α

activation by targeting fatty acid oxidation (FAO) pathways as a strategy to influence tumor metabolism and improve therapeutic outcomes [39].

The effect of fenofibrate varied depending on the cell type. Previous studies have suggested that GBM cell lines exhibit different phenotypes: U87-MG and GL261 have an energetic phenotype that favors oxidative phosphorylation, whereas U251-MG cells prioritize a glycolytic pathway [32,33,28]. One of the effects of fenofibrate is the inhibition of the mitochondrial respiration, leading to a decline in energy metabolism and transient glycolysis [40]. Furthermore, PPAR α activation is responsible for inhibiting glycolysis, further decreasing ATP production [41,42]. These opposing effects on glucose mechanisms could impair cell survival and may also explain the differences observed on our GBM cell lines. Interestingly, our results showed that healthy astrocytes were protected from fenofibrate-induced toxicity, potentially due to their enhanced ability to metabolize fenofibrate more efficiently than tumor cells [13]. Accordingly, our additional study demonstrated that fenofibrate was more cytotoxic than its active metabolite, fenofibric acid (additional file 4).

Thus, for further preclinical studies, an alternative to the oral route of administration should be considered to prevent hydrolysis of fenofibrate. To treat GBM, fenofibrate should be administered via a parenteral route. Although it is a BCS class II drug with high permeability, its low solubility limits oral bioavailability [43,44], and its rapid conversion to fenofibric acid in the bloodstream [45] further restricts brain exposure. Multiple studies have reported very low BBB permeability for both fenofibrate and fenofibric acid [46,21,47]. Consistently, our PAMPA assay indicated that fenofibrate cannot cross the BBB via passive diffusion ($P_e < 1$ nm/s). However, while PAMPA reflects passive membrane permeability, it does not account for the full complexity of BBB transport, which should be considered when interpreting these results. Additionally, fenofibrate was dissolved in DMSO to perform *in vitro* studies and overcome its aqueous insolubility. Extracellular and intracellular quantification revealed

100% recovery of fenofibrate after 1, 3 or 24 hours with increased uptake over time. In this study, we did not distinguish between subcellular localizations, which may have led to an overestimation of our measurements, especially when compared with the study of Grabacka *et al.*, where fenofibrate was shown to partition between membrane and cytosolic fractions [21]. Nevertheless, following the medium change, fenofibrate exhibited no cytotoxic effect. These findings highlight the importance of allowing sufficient time for cellular uptake and the necessity for a new strategy to deliver fenofibrate directly into tumor bulk and prevent a time-dependent onset of its effect.

Translation implications and future directions

Although DMSO is commonly used in *in vitro* studies, its toxicity and membrane penetration restrict its *in vivo* application [48]. These findings underscore potential translational strategies to enhance the delivery of fenofibrate in glioblastoma. Collectively, they emphasize the necessity of further development of appropriate formulation strategies, such as drug delivery systems. Some of these strategies already demonstrated their efficiency and safety for other drugs and pathologies [49–51] and could be applied to fenofibrate. Due to its lipophilic nature and poor aqueous solubility, fenofibrate could benefit from encapsulation in lipid-based nanocarriers, such as oil-in-water nanoemulsions, which have been successfully used for other anticancer agents to improve bioavailability and cellular uptake [16,50]. Additionally, intratumoral administration could provide a means to bypass the blood–brain barrier entirely, allowing direct delivery of the drug to the tumor site and potentially increasing local efficacy [52]. While these approaches were not explored in the present study, they represent promising avenues for future preclinical investigations aimed at optimizing fenofibrate's therapeutic potential in glioblastoma.

Conclusions

The development of a new strategy for the treatment of GBM faces several obstacles. Hypoxia (1% and 0.2% O₂) participates to the poor prognosis of solid tumors. In this context, fenofibrate has shown many advantages in overcoming these challenges. Its benefits stem from its cytotoxic and cytostatic effect in normoxia (21% O₂) and hypoxia (1% and 0.2% O₂) as well as its distinct metabolic pathway, which differently affects various cell types. However, its administration must be carefully determined and optimized to successfully target tumor cells and maintain therapeutic efficiency.

List of abbreviations

AMPK: AMP-activated protein kinase

API: active pharmaceutical ingredient

ATCC: American Type Culture Collection

BBB: blood-brain barrier

CI: combination index

DCTD: Division of Cancer Treatment and Diagnosis

DMSO: dimethyl sulfoxide

FAO: Fatty acid oxidation

FBS: fetal bovine serum

FDA: Food and Drug Administration

GBM: glioblastoma

GFAP: glial fibrillary acidic protein

GLUT4: insulin-regulated glucose transporter 4

HO-1: Heme-oxygenase 1

HIF-1 α : hypoxia-inducible factor-1 alpha

MGMT: O⁶-methylguanine-DNA methyltransferase

mTOR: Mammalian target of rapamycin

NF- κ B: nuclear factor-kappa B

PBS: Phosphate-buffered saline

PI: Propidium Iodide

PPAR: peroxisome proliferator-activated receptor

RPMI: Roswell Park Memorial Institute

SIRT1: Sirtuin 1

TMZ: temozolomide

Declarations

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, A-C.G., A.M-F., S.V.; methodology, Y.C., A-C.G., S.C., A.M-F., S.V.; formal analysis, Y.C., S.C., A-C.G.; investigation, Y.C., S.C., A-C.G., O.B.H.H., I.B.; writing—original draft preparation, Y.C., A-C.G., S.C., A.M-F., S.V.; writing—review and editing, Y.C., A-C.G., S.C., A.M-F., S.V.; supervision, A-C.G., A.M-F., S.V.; project administration, A-C.G., A.M-F., S.V.; funding acquisition, Y.C., A-C.G., A.M-F., S.V.. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1: Fenofibrate exerts a cytotoxic effect on GBM cell lines in normoxia (21% O₂).

Impact of fenofibrate on U251-MG, U87-MG and GL261 cells morphology in normoxia (21% O₂). A. Representative images of cells after 72 h of exposure to increasing concentrations of fenofibrate in normoxia (21% O₂). Scale bar = 100 μm. B. Viability of

U251-MG, U87-MG and GL261 cells after 72h of exposure to increasing concentrations of fenofibrate (n=4 independent biological repeats), data are expressed as the mean±SD. ** p<0.01, vs vehicle condition (1% DMSO) for each cell line (Dunn's post-hoc test after a significant Kruskal-Wallis).

Figure 2: Fenofibrate induces greater cytotoxicity than temozolomide in GBM cell lines under normoxia (21% O₂).

Impact of temozolomide (TMZ) on U251-MG and U87-MG cells in normoxia (21% O₂) and comparison with fenofibrate (FF). A. Viability of U251-MG and U87-MG cells after 72h of exposure to increasing concentrations of temozolomide (n=3 independent biological repeats), data are expressed as the mean±SD. B. Comparison of U251-MG, U87-MG cell responses to fenofibrate and temozolomide at 25 µM and 50 µM, data are expressed as the mean±SD.

Figure 3: GBM cell lines demonstrate hypoxia-induced chemoresistance after fenofibrate treatment.

Impact of fenofibrate on U251-MG, U87-MG and GL261 cells morphology in hypoxia (1% and 0.2% O₂). A. Representative images of cells after 72 h of exposure to increasing concentrations of fenofibrate in hypoxia (1% O₂). Scale bar = 100 µm. B. Representative images of cells after 72 h of exposure to increasing concentrations of fenofibrate in hypoxia (0.2% O₂). Scale bar = 100 µm. C. Viability of U251-MG, U87-MG and GL261 cells after 72h of exposure to increasing concentrations of fenofibrate (n=4 independent biological repeats), data are expressed as the mean±SD. * p<0.05, vs vehicle condition (1% DMSO) for each cell line (Dunn's post-hoc test after a significant Kruskal-Wallis).

Figure 4: Fenofibrate induces a cytostatic effect on U251-MG and U87-MG cells but not on GL261 cells.

The colony-formation assay was performed to determine the effects of increasing concentrations of fenofibrate (25 and 50 μM) on U251-MG and U87-MG cells in normoxia (21% O_2) and hypoxia (1% and 0.2% O_2). (A) Effect of fenofibrate on U251-MG cells, U87-MG and GL261 cells in normoxia (21% O_2) (B) Effect of fenofibrate on U251-MG cells, U87-MG and GL261 cells in hypoxia (1% and 0.2% O_2). Data are expressed as mean \pm SD (n=4 independent biological repeats for U251-MG and n=3, 4 independent biological repeats for U87-MG and GL261). * $p < 0.05$ ** $p < 0.01$, vs vehicle condition (1% DMSO) for each cell line (Dunn's post-hoc test after a significant Kruskal-Wallis).

Figure 5: Over 10% of fenofibrate is taken up by the cells

A. Intracellular and extracellular fenofibrate quantification in U251-MG and U87-MG cells after 3h of exposure to 40 μM of fenofibrate (n=5), data are expressed as the mean \pm SD. B. Intracellular and extracellular fenofibrate quantification in U251-MG and U87-MG cells after 1, 3 and 24h of exposure to 40 μM of fenofibrate (n=3 independent biological repeats for 1 and 24h), data are expressed as the mean \pm SD.

Figure 6: Fenofibrate does not induce a cytotoxic effect after a medium change at 3h of treatment.

Viability of U251-MG, U87-MG, and GL261 cells after 3h of continuous exposure to increasing concentrations of fenofibrate (n = 4 independent biological repeats). Cell viability assessed 72 hours after treatment. Data are expressed as mean \pm SD.

Figure 7: Fenofibrate is safe on astrocytes.

Impact of fenofibrate on the morphology of primary culture of astrocytes in normoxia (21% O_2). A. Representative images after 72 h of exposure to increasing concentrations of fenofibrate in normoxia (21% O_2). Scale bar = 100 μm . B. Cell viability after 72h of exposure to increasing concentrations of fenofibrate in normoxia (21% O_2) (n=4 independent biological

repeats) data are expressed as the mean \pm SD. C. Representative images after 72 h of exposure to increasing concentrations of fenofibrate in hypoxia (1% O₂). Scale bar = 100 μ m. D. Cell viability after 72h of exposure to increasing concentrations of fenofibrate in hypoxia (1% O₂) (n=4 independent biological repeats) data are expressed as the mean \pm SD.

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