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FK506 confers chemosensitivity to anticancer drugs in glioblastoma multiforme cells by decreasing the expression of the multiple resistance-associated protein-1

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive of brain tumors and is extremely insensitive to anticancer drugs. Studies have attributed the ABC transporter Mrp1 (ABCC1, multiple-drug resistance protein 1) with conferring chemoresistance in this tumor by extrusion of a wide spectrum of anticancer drugs. Therefore it is crucial to search for and investigate inhibitors of Mrp1 activity in GBM cells, particularly those that could be safe as chemosensitizers to anticancer drugs in clinical studies. We find that in primary cultured or T98G GBM cells exposed to therapeutic plasma concentrations of FK506 (tacrolimus), the expression of Mrp1 was decreased in a dose-dependent manner. The activity of this transporter, measured by CFDA fluorescent substrate extrusion, decreased significantly in primary cultured GBM cells on exposure to FK506 at concentrations of 15 ng/ml. When GBM cells were exposed to anticancer drugs vincristine, etoposide or taxol, cell viability was not affected. However when the anticancer drugs were assayed in combination with FK506, cell viability was significantly decreased by as much as 50% in GBM primary culture. We conclude that FK506 could be a valuable tool for chemosensitization of GBM cells, offering a possible improvement to the current poor therapy available for high-grade human gliomas.

1. Introduction

Cancer is one of the most frequently occurring diseases worldwide. Despite treatment advances and early diagnosis, mortality rates continue to be extremely high, particularly for high-grade brain tumors [1]. The most aggressive form of primary brain cancers is glioblastoma multiforme (GBM) [2]. Life expectancy is only 9–12 months [3,4]. The current standard protocol for treatment of patients with GBM involves surgical removal of the tumor, followed by a course of chemo- and/or radiotherapy [2]. However, the infiltrative nature of this tumor and restricted surgical removal because of probable tissue damage, lead to recurrence and considerably limit life expectancy [2]. Further, GMB cells are extremely chemoresistant [3]. To date, chemotherapy has not made a positive contribution to life expectancy of GMB patients.

The classic phenomenon of multi-drug resistance (MDR) is essentially due to the expression of proteins from the ABC transporter family (ATP-binding cassette) [5]. These proteins function as ATP-dependent pumps for removal of diverse metabolites and xenobiotics from the cell, including anticancer drugs [6]. The discovery and design of "chemosensitizer" molecules that are able to inhibit the activity of resistance transporters and thus increase the effectiveness of antitumor agents is currently of great clinical relevance. The greatest progress in development of these chemosensitizers has been the production of inhibitors that are highly selective for P-glycoprotein (P-gp, coded by ABCB1 gene), classically recognized as conferring resistance in several human tumors [7]. However, there are few advances in the development of chemosensitizers for other transporters that confer MDR in human tumors, such as those overexpressing members of the ABCC subfamily of transporters. Our studies in primary cultures of glioblastoma multiforme have shown an unusually low expression of the P-gp transporter, whilst immunolocalization of the multi-drug resistance associated protein-1 (Mrp1, ABCC1 gene) was noteworthy in tumoral tissue that co-localized with proliferation markers such as Ki67 [8]. Likewise, other studies confirm a preferential Mrp1 content in high-grade gliomas [9–12]. Therefore, inhibitors of Mrp1 activity could be valuable tools for sensitization of GBM cells to chemotherapy.

It has been previously reported that the classical immunosuppressant FK506 (tacrolimus) has been linked to inhibition of

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multiple drug resistance in cancer cells by a mechanism that implies inhibition of P-gp [13–15] and ABCg2 [16] activity. However, high concentrations were required for these effects. In this study, we demonstrate that FK506 at therapeutic concentrations increases chemosensitivity to anticancer drugs in Mrp1-abundant GBM cells. This effect was due to a significant decrease in the expression of Mrp1 in these cells.

2. Materials and methods

2.1. Cell cultures and drugs

The human glioblastoma multiforme T98G cell line was acquired from ATCC Company (ATCC CRL-1690). The cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, at 37 °C in an atmosphere humidified with 5% CO_2. The SVG p12 cell line derived from human fetal glial cells was purchase from ATCC Company (ATCC CRL-8621). The cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 1× MEM vitamins, 1× non-essential amino acids, 1% penicillin/streptomycin, at 37 °C in an atmosphere humidified with 5% CO_2. Antitumor drugs and MK571 were from Tocris Bioscience.

2.2. Brain tumors

Tissue samples were obtained from resection procedures in brain tumor patients by therapeutic indication at the Department of Neurosurgery, Institute of Neurosurgery Asenjo, Santiago, Chile. All procedures were carried out with the approval of the Bioethics Committee of the Universidad Austral de Chile. Part of each sample was introduced immediately into *RNAlater* solution (Sigma) or was used for primary culture. Further assays were carried out following histological confirmation of grade IV glioma [4].

2.3. Primary culture of glioblastoma multiforme

The resected tissues were washed twice with D-Hank's solution under aseptic conditions. After clearing out of visible blood vessel and necrotic tissue, the glioma tissue was processed for primary culture as described previously [8]. The cells were grown in DMEM supplemented with 20% fetal bovine serum and 100 units/ml of penicillin/streptomycin and incubated at 37 °C in a humidified atmosphere and 5% CO_2. The purity of cells and their identity were assessed by glial fibrillary acidic protein (GFAP) antigen staining and tumoral proliferation marker Ki67 [8].

2.4. Reverse transcription and PCR

Total cellular RNA was isolated using Trizol® Isolation Reagent (Invitrogen). First strand cDNA was synthesized from 1 μg of total RNA using 50 U of MMLV Reverse Transcriptase (Invitrogen) and oligo $(dT)_{18}$ according to manufacturers' recommendations. PCR amplifications were carried out in a Personal Thermal Cycler (Eppendorf) using 1 μl of cDNA, 1× PCR buffer, 1.5 mM Mg^{2+}, 0.4 mM dNTP's, 1.25 U Taq DNA polymerase (Invitrogen) and 0.5 μM of gene-specific oligonucleotides [17]. PCR amplifications were performed for 30 cycles, each of which consisted of denaturing at 92 °C for 60 s, annealing to 55 °C for 45 s and extension to 72 °C for 30 s. The PCR products were separated by 2% agarose gel electrophoresis. EtBr fluorescence intensities were quantified by densitometry analysis using a Molecular Analyst Software (Bio-Rad). The relative abundance of transcripts was estimated by the ratio to β-actin amplification.

2.5. Western blot analysis

Total protein extracts from T98G cells or primary cultures of brain tumors were obtained in 10 mM Tris–HCl buffer, 2% SDS, 10% glycerol, 1 mM PMSF and protease inhibitors (Complete, Roche). Aliquots (50 μg) were fractionated in 10% SDS–PAGE followed by transfer to nitrocellulose membranes (BioRad). The upper fraction of the membranes were incubated with primary antibodies anti Mrp1 (sc-18835), anti P-gp (sc-55510) and anti ABCg2 (sc-58222) from Santa Cruz Biotechnology at 1:1000 dilutions for 1 h and the lower fraction of membranes with an anti β-actin antisera (sc-47778, Santa Cruz Biotech) at 1:5000 dilution. After washing, the blots were further incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (DAKO) at 1:5000 dilution for 1 h at RT. The immune detections were revealed using the chemiluminescent reagent ECL-plus (Amersham Pharmacia).

2.6. Mrp1 functional assays

The cells (1×10^5) were pre-incubated in serum-free medium DMEM for 6 h at 37 °C in 24-well plates and further incubated with 7, 15 or 30 ng/ml of FK506 for 0, 8 or 24 h. Also, cells were treated with 20 μM MK571 (inhibitor of Mrp1 activity) for 24 h or transfected 48 h previous to functional assay with a plasmid coding a siRNA interfering with the expression of Mrp1 [8]. The cells were then loaded with 500 nM CFDA for 15 min. After that, they were washed three times with 1× PBS and incubated for 15 min in serum-free DMEM medium. Next, cells were washed three times with ice-cold PBS and lysed in PBS containing 0.4% Triton X-100. The fluorescence in cell extracts was measured with a spectrofluorimeter (Perkin–Elmer LS-55) at excitation 488 nm and emission 530 nm [8].

2.7. [^{3}H]vincristine incorporation assays

The cells (2×10^5) were preincubated in serum-free medium DMEM for 6 h at 37 °C in 24 well plates and further incubated with 15 ng/ml of FK506 or 20 μM MK571 for 24 h. Also, the cells were transfected 48 h previous to incorporation assay with a plasmid coding a siRNA interfering with the expression of Mrp1 [8]. The cells were then exposed for 15 min in serum-free DMEM medium containing 100 nM vincristine obtained by mixing of non-radioactive and tritiated drug. Cells were washed three times with ice-cold PBS and lysed in 0.1 M NaOH. Radioactivity in cell extracts will be determined by scintillation counting and normalized to cellular protein content [11].

2.8. Cell viability assays

1.0×10^4 cells per well were cultured in 96-well plates for 24 h and then exposed to FK506 (15 ng/ml), anticancer drugs vincristine (100 nM), etoposide (2 μM) or taxol (0.1 μM) or anticancer drugs in combination with FK506 (15 ng/ml) for 24 h. Cells were then incubated with 5 mg/ml MTT reagent (thiazol blue tetrazolium) in culture medium for 1 h and formazan crystals were lysed using 100 μl DMSO. The absorbance at 550 nm was read using a microplate reader. Absorbance values are expressed as a percentage relative to control cells without treatment.

2.9. Immunofluorescence

T98G cells grown on circular coverslips at semi-confluence were washed with 0.1 M phosphate buffer (pH 7.4), fixed with Histochoice (Sigma) and permeabilized using 0.3% Triton X-100 in 1× PBS. Preparations were blocked with 1% BSA and incubated with monoclonal antibody anti-Mrp1 (sc-18835, Santa Cruz Biotechnology). A secondary antibody tagged with Alexa fluor 488 was also

used (Molecular Probes). The subcellular distribution of Mrp1 was visualized using a confocal microscope (Olympus Fluoview 1000).

2.10. Statistical analysis

Analyses were carried out on raw data using the Peritz' *F* multiple means comparison. Student's *t*-test was applied for unpaired data and $P < 0.01$ was considered statistically significant.

3. Results

To determine the effects of FK506 on the expression of ABC transporters, T98G cells were exposed to FK506 at concentrations in the physiological range seen in plasma of patients undergoing immunosuppressive therapy [17,18]. After 24 h exposure of T98G cells to FK506, the transcript levels of Mrp1 were shown to have decreased with increasing concentrations of FK506 (7–30 ng/ml; Fig. 1A). In contrast, the transcript levels of ABCg2 and P-gp were not affected under these conditions. Likewise, the Mrp1 content in total protein extracts decreased upon exposure to FK506, with the greatest effect reached at a dose of 15 ng/ml, a decrease by up to 80% that was sustained at 30 ng/ml of FK506 (Fig. 2A). No changes were observed in ABCg2 or P-gp protein contents (Fig. 2A). Further, the effect of FK506 on the expression of Mrp1 was evaluated by immunofluorescence detection in T98G cells. In control cells the expression of Mrp1 was localized within the plasma membrane and the cytoplasm. Exposing cells to increasing concentrations of FK506 showed no observed signal at the plasma membrane and the total fluorescence was decreased (Fig. 2B).

As there was only a limited quantity of tumor samples, primary cultured GBM cells were exposed to a single concentration of FK506 (15 ng/ml) for 24 h. In primary cultures of GBM cells, treatment with FK506 decreased the mRNA content and protein levels of Mrp1, by 54% and 85%, respectively, whilst ABCg2 and P-gp expression was not effected (Figs. 1B and 2).

To probe the functional consequence of a decreased expression of Mrp1 in FK506-treated cells, we evaluated the activity of this transporter by measuring the intracellular accumulation of the fluorescent substrate CFDA. Treatment with FK506 for 24 h significantly decreased Mrp1 activity in T98G and primary cultured GBM cells (Fig. 3A). The particular effect of FK506 on Mrp1 was comparable to the increased accumulation of the substrate CFDA when the cells were exposed to the inhibitor of Mrp1 activity MK571 or when knocking down its expression using siRNA (Fig. 3A). In contrast, FK506 did not alter the accumulation of CFDA in the non-tumoral glial cell line SVG p12 (Fig. 3C), which is not chemoresistant because we found a notable decrease in viability of cells when exposed to vincristine, etoposide and taxol (Fig. 4). Notably, the decreased activity of Mrp1 in GBM cells was observed after 8 h of treatments using FK506 (Fig. 3B).

Because our essential goal is to optimize chemotherapy against GBM, we evaluated FK506 as a chemosensitizer of GBM cells to antitumor drugs. Treatment of T98G cells with FK506 for 24 h increased the intracellular accumulation of ^{3}H-vincristine (Table 1). Similarly, knocking down the expression of Mrp1 or using an inhibitor of Mrp1 activity increases the intracellular accumulation of ^{3}H-vincristine (Table 1). We then evaluated the effect on cell viability on exposure to anticancer drugs alone or in conjunction with FK506. The primary cultured or T98G GBM cells were

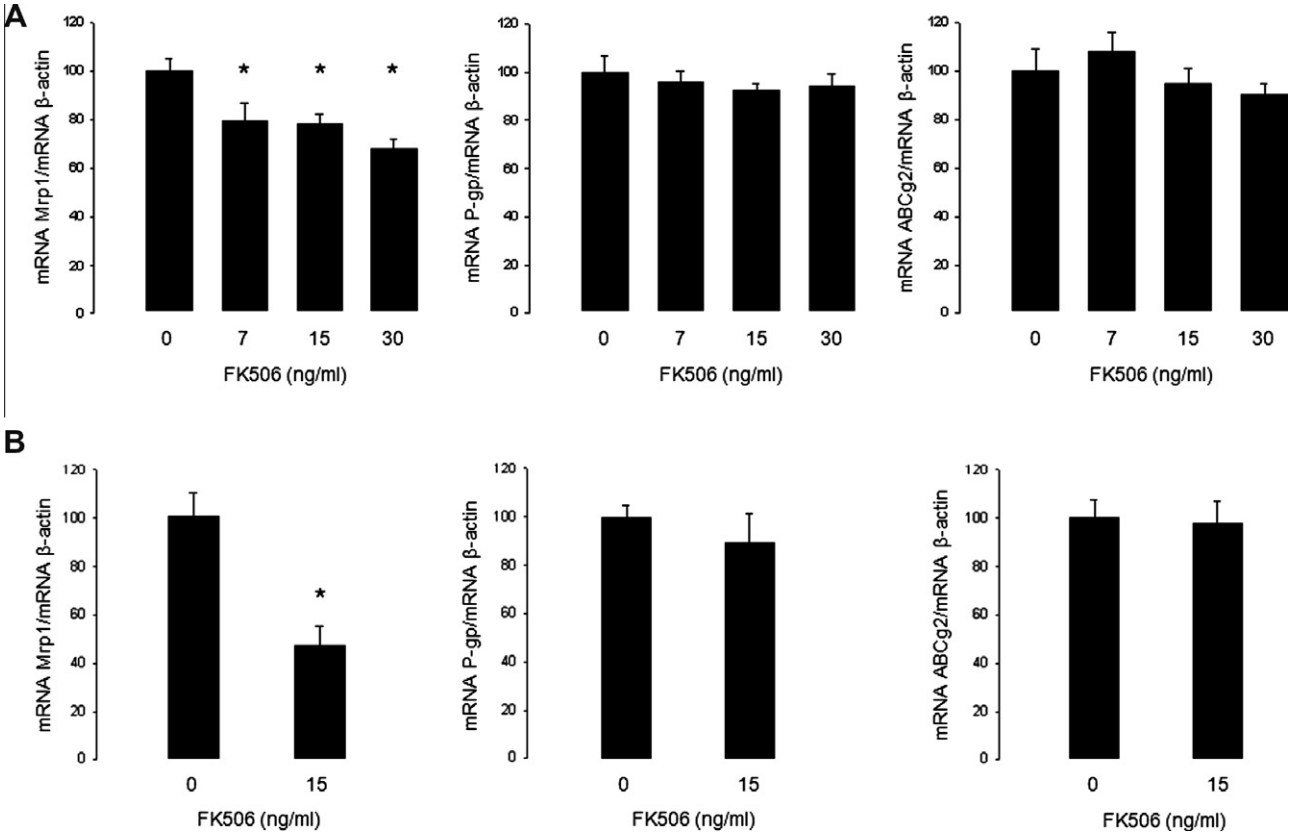
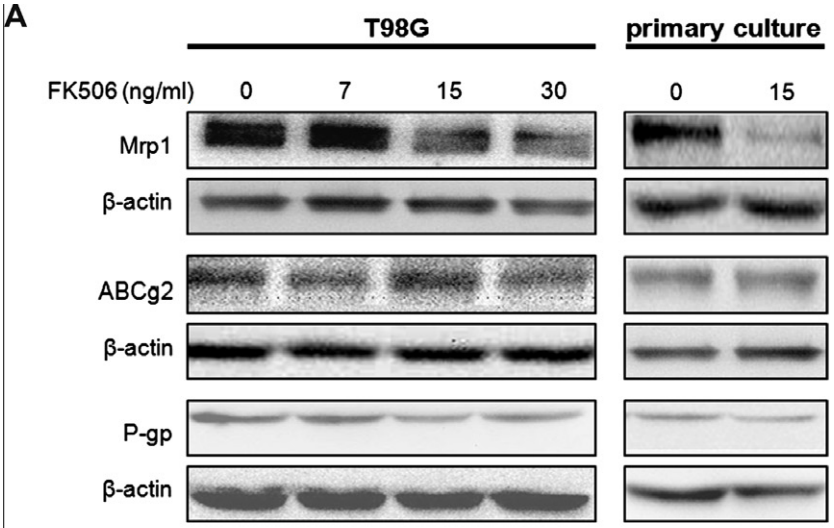


Fig. 1. Effect of FK506 on mRNA levels of ABC genes in GBM cells. T98G cells (A) or primary cultured GBM cells (B) were exposed for 24 h to FK506 at the concentrations indicated below each graph. Total cellular RNA was isolated and transcript levels of the Mrp1, P-gp and ABCg2 coding genes were amplified by RT-PCR (see Section 2). The graphs depict the ratios between amplification products from the ABC gene versus β-actin. The ratios obtained under conditions of FK506 0 ng/ml were normalized to 100. Means ± S.D. ($n = 9$). $^{*}P < 0.01$ versus FK506 0 ng/ml.



	T98G				primary culture	
FK506 (ng/ml)	0	7	15	30	0	15
Mrp1	1	0.89 ± 0.10	0.22 ± 0.09*	0.24 ± 0.08*	1	0.15 ± 0.05*
ABCg2	1	0.96 ± 0.08	0.98 ± 0.08	0.92 ± 0.09	1	1.03 ± 0.09
P-gp	1	0.98 ± 0.11	0.95 ± 0.05	0.98 ± 0.05	1	0.95 ± 0.05

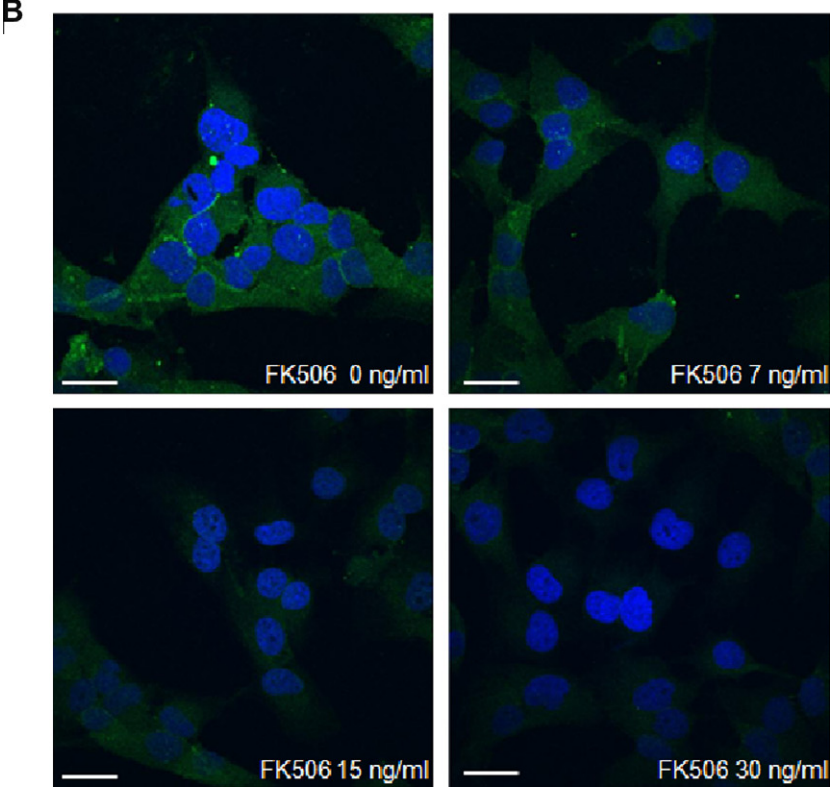


Fig. 2. FK506 decreases Mrp1 protein content in GBM cells. (A) Representative Western blot analysis of Mrp1, ABCg2 and P-gp protein content in T98G and primary cultured GBM cells exposed to FK506 for 24 h. The immune signal intensities for Mrp1 (170 kDa), ABCg2 (130 kDa) and P-gp (190 kDa) were divided by β-actin signal intensities. The ratios of chemiluminescence intensities in FK506 0 ng/ml were normalized to 1. The table represents the means ± S.D. ($n = 3$). *$P \leqslant 0.01$ versus FK506 0 ng/ml. (B) Immunofluorescent detection of Mrp1 protein (green) in T98G cells exposed for 24 h to increasing concentrations of FK506. Nuclear staining using DAPI is shown in blue. Magnification bar 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

chemoresistant to vincristine, etoposide and taxol (Fig. 4). However, when the antitumoral drugs were applied to cells pretreated with FK506, cell viability decreased by up to 34%, 32% and 42% for each drug in T98G and by 38%, 40% and 52% for each drug in primary cultured GBM cells (Fig. 4).

4. Discussion

Several mechanisms have been described to explain how tumor cells are refractory to anticancer agents [19]. Of most importance, with major clinical impact, is the overexpression of some members

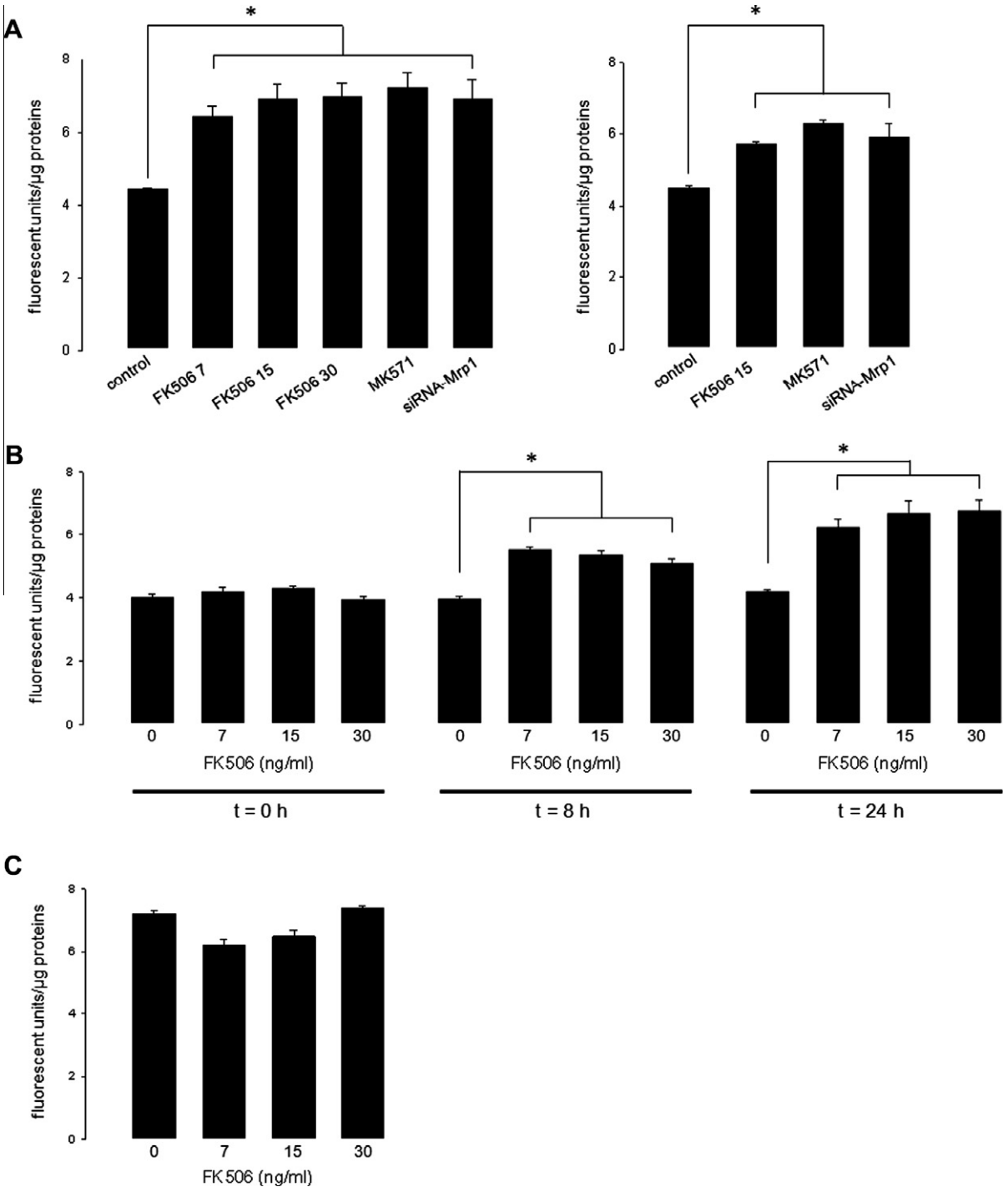


Fig. 3. Mrp1 activity in GBM cells. (A) The intracellular accumulation of fluorescent CFDA was used as an indicator of Mrp1 activity in T98G (left) and primary cultured GBM (right) cells. The effect of FK506 on Mrp1 activity was evaluated in cells exposed to the drug for 24 h. MK571 is a selective inhibitor of Mrp1 activity. siRNA-Mrp1 knocked down the expression of Mrp1. The graphs represent the means ± S.D. $^*P < 0.01$ versus control, $n = 9$. (B) The activity of Mrp1 was evaluated in T98G cells exposed to FK506 for 0, 8 or 24 h. The graphs depict the means ± S.D. of intracellular accumulation of the substrate CFDA. $^*P < 0.01$ versus control, $n = 7$. (C) The effect on Mrp1 activity in SVG p12 cells was evaluated following a 24 h exposure to FK506. The graphs depict the means ± S.D. of intracellular accumulation of the substrate CFDA. $n = 6$.

of the ABC (ATP-binding cassette) superfamily of transporters. Particular overexpression of Mrp1 has been associated with intrinsic chemoresistance of glioblastoma multiforme cells [8,11]. In addition, the key role of Mrp1 activity in conferring chemoresistance to glioblastoma cell lines T98G and G44 was recently demonstrated by us. The refractoriness of these cells to anticancer drugs was overcome by the combined use of antiproliferative drugs with the Mrp1 inhibitors MK571 or probenecid, decreasing cell viability by up to 70–80% [8]. The main finding of this work has been the identification of FK506 as a chemosensitizer to GBM cells, down-regulating Mrp1 expression with an efficiency that may permit use of FK506 as a coadjuvant at doses known to be safe in clinical studies.

FK506, a macrolide lactone widely used as an immunosuppressant in transplant patients, has been previously described as a modulator of the activity of some ABC proteins such as P-gp and

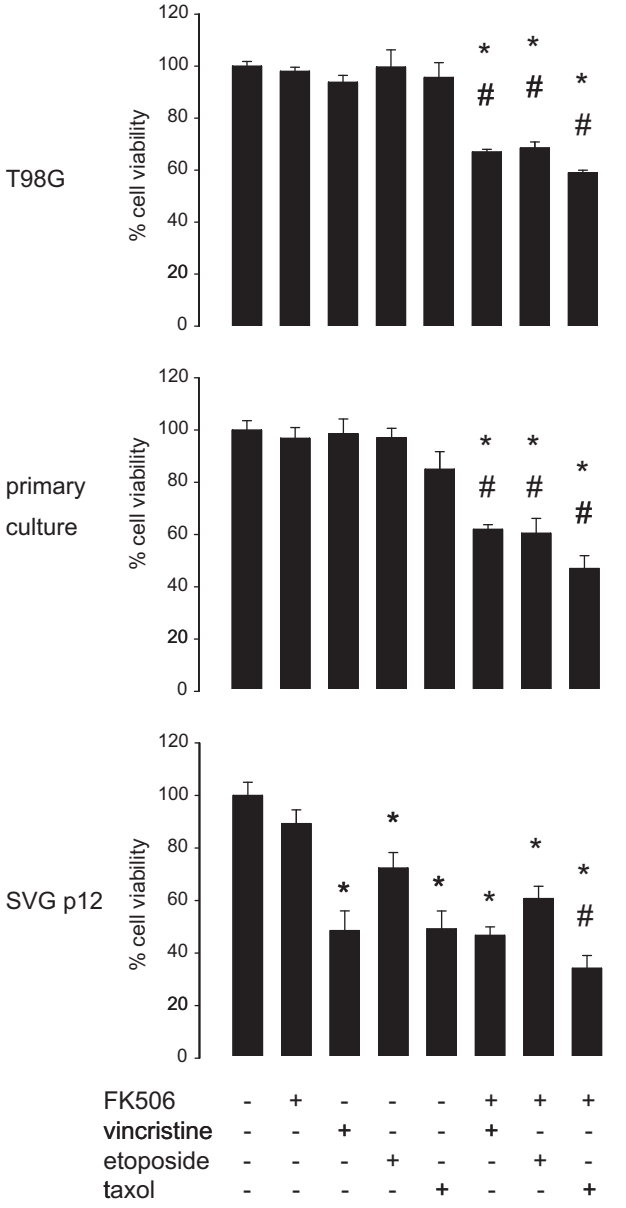


Fig. 4. Chemosensitization of GBM cells by FK506. GBM and non-tumor glial SVG p12 cells were exposed to anticancer drugs alone or in conjunction with FK506 (15 ng/ml) for 24 h. The graphs represent the means ± S.D. of cell viability assays. Cell viability of controls without drugs was normalized to 100%. $^{*}P < 0.01$ versus control without drugs. $^{\#}P < 0.01$ versus anticancer drugs alone. $n = 12$.

Table 1
Intracellular ^{3}H-vincristine accumulation in T98G cells.

	Basal (cpm/μg protein)	Treatment 24 h (cpm/μg protein)	Induced accumulation (cpm/μg protein)
MK571	447.8 ± 6.5	1055.1 ± 14.2*	607.3 ± 7.3
FK506	400.4 ± 5.8	700.4 ± 6.9*	300.0 ± 4.2
siRNA Mrp1	378.9 ± 4.5	668.1 ± 6.5*	289.2 ± 5.1

The values represent the means ± S.D. of incorporation assays carried out in triplicates for each condition. MK571 is an inhibitor of Mrp1 activity used at 20 μM. The cells were exposed to 15 ng/ml FK506.
* Indicates statistical differences with respect to basal values, $P < 0.01$ ($n = 3$).

ABCg2, although the concentrations required to reverse chemoresistance are substantially higher than those that are immunosuppressive by approximately 1000- and 100-fold, respectively [16,20]. Most pharmacokinetic studies of FK506, when administered alone or in association with other drugs in transplant patients, have shown that blood tacromilus concentration is close to values of 30 ng/ml, although the average C_{min} and C_{max} values may range from 6 to 26 and from 14 to 38 ng/ml, respectively [17,18,21–23]. Based on this information we decided to use a treatment study range of 7–30 ng/ml FK506. It is important to note that in high-grade brain cancer, such as glioblastoma multiforme, the greatest resistance comes from MDR transporters expressed in glioma because the blood brain barrier (BBB) becomes more permeable through break-up of the tight junctions between constituent endothelial cells [24]. Notably, treatment of cells with FK506 at concentrations similar to those seen in plasma of patients resulted in a 50% of effectiveness compared to the total inhibition on the extrusion of [^{3}H]vincristine achieved by MK571 (Table 1), the benchmark inhibitor for blocking Mrp1-mediated drug transport [25].

Worth noting, is that the mechanism by which FK506 sensitizes GBM cells is mediated by decreasing the expression of the ABCC1 gene. This could be a valuable tool when administered to patients as a chemosensitizer for a short period of time. We see no inhibition of Mrp1 activity over short periods of exposure. In accordance with our finding, Pawarode et al. [26] also show that therapeutic concentrations of tacromilus were not able to inhibit Mrp1 activity in cells that were artificially transformed to overexpress this protein. The intracellular pathways involved in inhibition of Mrp1 expression by FK506 have still to be investigated [27–29]. Other compounds that downregulate Mrp1 expression or those that block the activity of this transporter [8,30–33] have been assayed in cancer cells *in vitro*. However, their clinical use is, so far, limited by adverse effects or effects that have not yet been evaluated. To our knowledge, this is the first study that shows chemosensitization of GBM cells with a molecule that seems to be safe in humans. CBT-1 [34] and biricodar [35] are drugs which are characterized by their dual inhibition of P-gp and Mrp1 activity and which are currently undergoing clinical trials.

Current standard treatment of glioblastoma multiforme is with temozolomide after resection of the tumor. However, the observed beneficial effects on progression-free survival rates remain poor, at less than 20% in these patients [36]. The low effectiveness of temozolomide has been attributed to the phenomenon known as "specific resistance" due to the high activity of O^{6}-methyguanine-DNA-methyltransferase (MGMT) [37]. The standard regime of procarbazine, lomustine and vincristine (PCV) in cohorts of patients suffering recurrent high-grade glioma has not shown to improve survival rates, nor was better than treatment with temozolomide [38]. The intrinsic property of GBM to be refractory to anticancer drugs was evident when we exposed cells to vincristine, etoposide and taxol, to no effect on cell viability. However, cell viability was considerably decreased when cells were pretreated with FK506. We propose that chemosensitization using FK506 may be a plausible tool for the treatment of GBM with anticancer drug substrates for the Mrp1 transporter, such as those used in our *in vitro* assays.

Acknowledgments

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