Carcinogenesis vol.34 no.1 pp.48–57, 2013
doi:10.1093/carcin/bgs328
Advance Access publication October 18, 2012

Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents

Massimo Nabissi[1,†], Maria Beatrice Morelli[1,†], Matteo Santoni[2] and Giorgio Santoni[1,*]

[1]Department of Experimental Medicine, School of Pharmacy, University of Camerino, Camerino, MC 62032, Italy and [2] Department of Medical Oncology, Polytechnic University of Marche, Ancona AN 60126, Italy

*To whom correspondence should be addressed. Department of Experimental Medicine, School of Pharmacy, University of Camerino, Via Madonna delle Carceri 9, Camerino, MC 62032, Italy. Tel: +39 0737 403312/403319; Fax: +39 0737 403325;
Email: giorgio.santoni@unicam.it

The aggressive behavior of Glioblastoma multiforme (GBM) is mainly due to high invasiveness and proliferation rate as well as to high resistance to standard chemotherapy. Several chemotherapeutic agents like temozolomide (TMZ), carmustine (BCNU) or doxorubicin (DOXO) have been employed for treatment of GBM, but they display limited efficacy. Therefore, it is important to identify new treatment modalities to improve therapeutic effects and enhance GBM chemosensitivity. Recently, activation of the transient receptor potential vanilloid type 2 (TRPV2) has been found to inhibit human GBM cell proliferation and overcome BCNU resistance of GBM cells. Herein, we evaluated the involvement of cannabidiol (CBD)-induced TRPV2 activation, in the modulation of glioma cell chemosensitivity to TMZ, BCNU and DOXO. We found that CBD increases TRPV2 expression and activity. CBD by triggering TRPV2-dependent Ca^{2+} influx increases drug uptake and synergizes with cytotoxic agents to induce apoptosis of glioma cells, whereas no effects were observed in normal human astrocytes. Moreover, as the pore region of transient receptor potential (TRP) channels is critical for ion channel permeation, we demonstrated that deletion of TRPV2 poredomain inhibits CBD-induced Ca^{2+} influx, drug uptake and cytotoxic effects. Overall, we demonstrated that co-administration of cytotoxic agents together with the TRPV2 agonist CBD increases drug uptake and parallelly potentiates cytotoxic activity in human glioma cells.

Introduction

Glioblastoma multiforme (GBM) is the most frequent class of malignant primary brain tumors, and it is one of the most devastating cancers with a median survival of approximately 12–15 months (1). The aggressive behavior of GBM is mainly due to its high invasiveness and proliferation rate as well as to high resistance to standard chemotherapy and radiotherapy (2). Several chemotherapeutic agents such as temozolomide (TMZ), carmustine (BCNU) and doxorubicin (DOXO) are used in the clinical management of GMB, but they display limited efficacy. Therefore, it is important to identify new treatment modalities, as well as to modify existing therapies to improve their therapeutic effects without increasing their toxicity (3). A promising approach to achieve these goals is to enhance glioma chemosensitivity to existing treatment regimens.

Abbreviations: BCNU, c1,3-bis(2-chloroethyl)-1-nitrosurea; CBD, cannabidiol; cDNA, complementary DNA; CPS, capsaicin; DOXO, doxorubicin; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; GBM, Glioblastoma multiforme; MFI, mean fluorescence intensity; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NHA, normal human astrocyte; RR, ruthenium red; TMZ, temozolomide; Δ^9-THC, Δ9-tetrahydrocannabinol; TRP, transient receptor potential; TRPV2, transient receptor potential vanilloid type 2.

[†]These authors equally contributed to this work.

Recent data have described the functional role of some transient receptor potential (TRP) cation channels in the regulation of GBM cell growth and progression (4,5). In this regard, we have previously reported that triggering of TRP vanilloid-1 (TRPV1) by the synthetic agonist capsaicin (CPS) induces p38 mitogen-activated protein kinase-dependent apoptosis of glioma cells (6). In addition, we have recently demonstrated that TRP vanilloid-2 (TRPV2), a paralogue of TRPV1, inhibits GBM cells proliferation and overcomes the resistance of GBM cells to BCNU treatment (7).

TRP channels are pore-forming transmembrane proteins that allow ions and xenobiotics to permeate biological membranes (8). Pore domain plays a crucial role in determining the TRP channel permeation and selectivity properties. Recent experiments clearly demonstrate that the GM(L)GD sequence motif in the putative TRPV1/2/3/4 pore region determines Ca^{2+} and Mg^{2+} permeation properties of these channel (8,9). A selective introduction of charged molecules into specific target cells by permeation through TRPV channels has been demonstrated (10–14). Treatment with the synthetic TRPV1 agonist CPS by opening the TRPV1 channels permits the selective introduction into TRPV1-expressing pain-sensing neurons of a charged membrane-impermeant lidocaine derivative (QX-314) with a molecular mass of 263 Da, and of FMI-43 dye, with molecular mass of 452 Da (11). Moreover, it has been reported that the entry of CPS analogs capsaicinoids into sensing neurons requires TRPV1 pore domain, and this process is enhanced by protein kinase C (PKC) and protein kinase A (PKA) activation or oxidative covalent modification (15).

Selective introduction of antiproliferative and cytotoxic molecules by permeation into different types of cancer cells expressing TRP channels has been recently suggested (14). Among these channels, we evaluated the potential role of TRPV2 in the permeation of cytotoxic drugs into GBM cells.

TRPV2 is a non-selective cation channel showing Ca^{2+} permeability. Structurally, it is composed of six transmembrane domains, a putative pore-loop region, a cytoplasmic amino terminus with three ankyrin-repeat domains and a cytoplasmic carboxy terminus (16).

Functional studies have revealed that TRPV2 responds to noxious heat with an activation threshold of >52°C, as well as to changes in osmolarity and membrane stretch (16). TRPV2 channel is triggered by agonists such as Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) (17,18). Many studies have suggested that activation of TRPV2 by growth factors causes PI-3K-dependent and independent channel translocation to the plasma membrane (19,20). Cannabinoids have been receiving a growing attention for the treatment of GBM because they can promote glioma cell death, both *in vitro* and *in vivo* (21,22). Among the cannabinoid compounds, CBD, a cannabinoid that lacks the unwanted psychotropic liability of THC (22,23), has been investigated as antitumoral agent. Moreover, CBD was found to exert anti-inflammatory, antioxidant and neuroprotective effects (24). *In vivo*, CBD enhances the inhibitory effects of Δ^9-THC on human GBM cells survival and proliferation (25), and its combination with TMZ plus Δ^9-THC reduces the growth of glioma xenografts (26). *In vitro*, CBD inhibits migration (27) and induces apoptosis in human glioma cells (28,29). The aim of this study was to evaluate the involvement of TRPV2 channel activation in the sensitization of GBM cells to chemotherapeutic agents, such as BCNU, TMZ or DOXO. Furthermore, we analyzed the influence of TRPV2 pore region in drug uptake and chemosensitivity in human glioma cells.

Materials and methods

Cell lines

The U87MG glioma cell line (American Type Culture Collection; LGC Promochem, Teddington, UK) was cultured as previously published (7). MZC

primary glioblastoma cells were prepared from enzymatic digestion of the bioptic sample surgically removed from a patient with grade IV GBM, who gave informed consent to the study, and isolated as described previously (7). Normal human astrocytes (NHA) (Cambrex, Berkshire, UK) were grown in Astrocyte Growth Media System (Cambrex) and used within 10 passages to avoid biological responsiveness and function deterioration.

Reagents

c1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU), TMZ and DOXO hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO). CBD, ruthenium red (RR) and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Tocris-Bioscience (Bristol, UK).

Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmbH, Milan, IT), and complementary DNA (cDNA) was synthesized using the high-capacity cDNA archive kit (Life Technology, Milan, IT) according to the manufacturer's instructions. Duplex real-time PCR for *TRPV2* was performed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hèrcules, CA) as previously published (7). All samples were assayed in triplicate in the same plate. Measurement of β-actin levels was used to normalize messenger RNA (mRNA) contents, and *TRPV2* levels were calculated by the $2^{-\Delta\Delta Ct}$ method and expressed as relative fold compared with the corresponding control.

Plasmid constructs

The plasmid encoding human *TRPV2* (pCMV-TRPV2) was constructed by introducing in frame *TRPV2* cDNA in BglII/HindIII pCMV-FSR (Genlantis, San Diego, CA) cloning sites, as previously published (7). Deletion of pore domain DNA sequence in *TRPV2* cDNA was obtained by amplifying 10 ng of pCMV-TRPV2 with the primer pair: forward: 5′-ctggccttccaggagcagctgc 3′ and reverse: 5′-ggcattggggcctgtaggagc 3′ by PCR using *Pfu* DNA polymerase (Stratagene, Milan, IT). Pore domain–deleted *TRPV2* construct (pCMV-ΔpTRPV2) was sequenced before its use in transfection experiments.

Cell transfections

siGENOME SMARTpools for TRPV2 (*siTRPV2*) consisting of four RNA duplex targeting *TRPV2* gene and a siCONTROL non-targeting small interfering RNA (siGLO) with at least four mismatches to any human gene used as negative control were purchased from Dharmacon (Lafayette, CO). For transfection experiments, U87MG and MZC glioma cells were plated at the density of $3 \times 10^4/cm^2$, and after overnight incubation, 50 nM of *siTRPV2* or siGLO and 1 μg/ml of pCMV-*TRPV2*, pCMV-*TRPV2*Δp or pCMV (empty vector) were added to the wells, following METAFECTENE Easy (Biontex Laboratories GmbH, Martinsried/Planegg, Germany) transfection protocol.

Western blot analysis

Glioma cells were lysed in lysis buffer as described previously (7). Plasma membrane and cytosol glioma cell fractions were isolated using the Subcellular Protein Fractionation kit (Thermo Scientific, Rockford, IL), according to the manufacturer's directions. Twenty microgram of lysates were separated on 7% sodium dodecyl sulfate–polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare Europe GmbH, Milan, IT) and blotted with the specific mouse anti-β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or the goat anti-TRPV2 antibody (1:200; Santa Cruz Biotechnology) followed by the respective horseradish peroxidase-conjugated secondary antibody as previously published (7). Anti-TRPV2 antibody specificity was evaluated as described previously (17). Furthermore, the blots were incubated with the secondary antibody. Immunostaining was revealed by enhanced chemiluminescence Western Blotting analysis system (GE Healthcare). Densitometric analysis was performed by ChemiDoc using the Quantity One software (Bio-Rad).

Calcium mobilization assay

For calcium influx analysis, cells were resuspended in calcium- and magnesium-free phosphate-buffered saline/glucose medium supplemented with 7 μmol/l FLUO 3-AM (Invitrogen, Milan, IT) and 1 μg/ml Pluronic F-127 (Molecular Probes Molecular, Milan, IT) and incubated in the dark for 30 min at 37°C and 5% CO_2. After washing, cells were resuspended in calcium- and magnesium-free phosphate-buffered saline/glucose medium containing 2 mmol/l Ca^{2+} warmed at 37°C and were stimulated with different doses of CBD or were pretreated for 30 min with RR and then treated with CBD. FLUO 3-AM fluorescence was measured every 10 s on the flow cytometer at 525 nm on the green channel (FACScan cytofluorimeter; BD Pharmingen, San Diego, CA) using the CellQuest software (BD Pharmingen). The number of events acquired was $\approx 3 \times 10^3$. $[Ca^{2+}]_i$ was determined before and after the addition of various concentrations of test compounds. To convert fluorescence values into $[Ca^{2+}]_i$, a calibration procedure was performed for each experiment.

The following equation was used to determine $[Ca^{2+}]$ free:

$$\left[Ca^{2+}\right]_{free} = K_d\left[F - F_{min}\right] \div \left[F_{max} - F\right],$$

where K_d is the constant of FLUO-3 and corresponds to 400 nM, F_{min} and F_{max} are the fluorescence intensities of FLUO-3 without or with maximal $[Ca^{2+}]$ and F is the sample mean fluorescence. Unstimulated cells were analyzed to establish baseline fluorescence levels. Dose-response curves were designed by plotting the maximum $[Ca^{2+}]_i$ values for each CBD and RR dose. EC_{50} value was determined as the concentration of CBD required to produce half-maximal increases in $[Ca^{2+}]_i$. IC_{50} value was determined as concentration exerting an half-maximal inhibition of agonist induced increases of $[Ca^{2+}]_i$.

MTT assay

The cell viability was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. About 3×10^3 cells/cm^2 were plated in a 96-well plate and then treated with different drugs for up to 3 days. Four replicates were used for each treatment. At the indicated time, 0.8 mg/ml of MTT was added to the media and incubated for 3 h. Supernatants were then discarded, and colored formazan crystals, dissolved with 100 μl/well of dimethyl sulfoxide, were read by an enzyme-linked immunosorbent assay reader (BioTek Instruments, Bad Friedrichshall, Germany). Vehicle data were omitted when no effects were observed respect to untreated cells.

Soft agar assay

Colony formation was assessed in 35 mm well plates containing two layers of soft agar. Five hundred microliter underlayers of 0.6% soft agarose (sea plaque agarose diluted in medium) were prepared in each well. The top layer was made by adding 3×10^3 cells/well into a mixture of two-thirds of 0.4% soft agarose and one-third medium, in a total volume of 500 μl. For drug treatment study, the cell layer was composed of tumor cells, mixture of two-thirds of 0.4% soft agarose and one-third medium containing BCNU, TMZ, DOXO and CBD. The plates were incubated at 37°C in a 5% CO_2 for 15 days. The number of colonies was measured under the Olympus IX71 microscope.

Apoptosis assay

Phosphatidylserine exposure on U87MG and MZC glioma cells was detected by annexin V staining and cytofluorimetric analysis. Briefly, 3×10^4 cells/cm^2 were treated with different doses of CBD for 1 day or with a combination of CBD 10 μM and chemotherapeutic drugs for 6 h. After treatment, cells were stained with 5 μl of annexin V-fluorescein isothiocyanate for 10 min at room temperature and washed once with binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$).

Doxorubicin uptake

Transfected MZC cells were pretreated with RR or EGTA for 30 min, and then CBD was added for 30 min. For each treatment, incubation with DOXO 5 μM was performed for additional 2 h. Samples were analyzed by a FACScan cytofluorimeter using the CellQuest software, and fluorescence intensity was expressed in arbitrary units on logarithmic scale. For microscopical analysis, cells were treated with DOXO 5 microM. Cell imaging was performed with Olympus BX51 Microscope.

Statistical analysis

The statistical significance was determined by Student's *t*-test and by Bonferroni test (one-way analysis of variance); *P*-values <0.01 were considered significant. IC_{50} values reported in this study were determined by computing the antilog of $logIC_{50}$ values. Standard errors for IC_{50} value were not determined; as such values are not directly correlated with $logIC_{50}$ standard errors. Graphs and statistical analysis for EC_{50} and IC_{50} were prepared using Prism 5.0a (Graph Pad).

Results

CBD acts as selective TRPV2 agonist in glioma cell lines

Previous reports have demonstrated the ability of CBD to induce calcium influx in HEK293 cells transiently expressing the human TRPV2 channel (EC_{50}: 31.7 μM (18)). Here, we analyzed the ability of CBD to induce calcium influx in U87MG cells that express TRPV2, by calcium mobilization assay. As shown in Supplementary Figure 1A, available at *Carcinogenesis* Online, the CBD induced increase of calcium influx in U87MG cells was concentration dependent, with an EC_{50} value of 22.2 μM. This effect was inhibited by pre-incubation with the specific TRP channel blocker RR, which displayed an IC_{50} value of 3.25 μM when tested in the presence of 25 μM

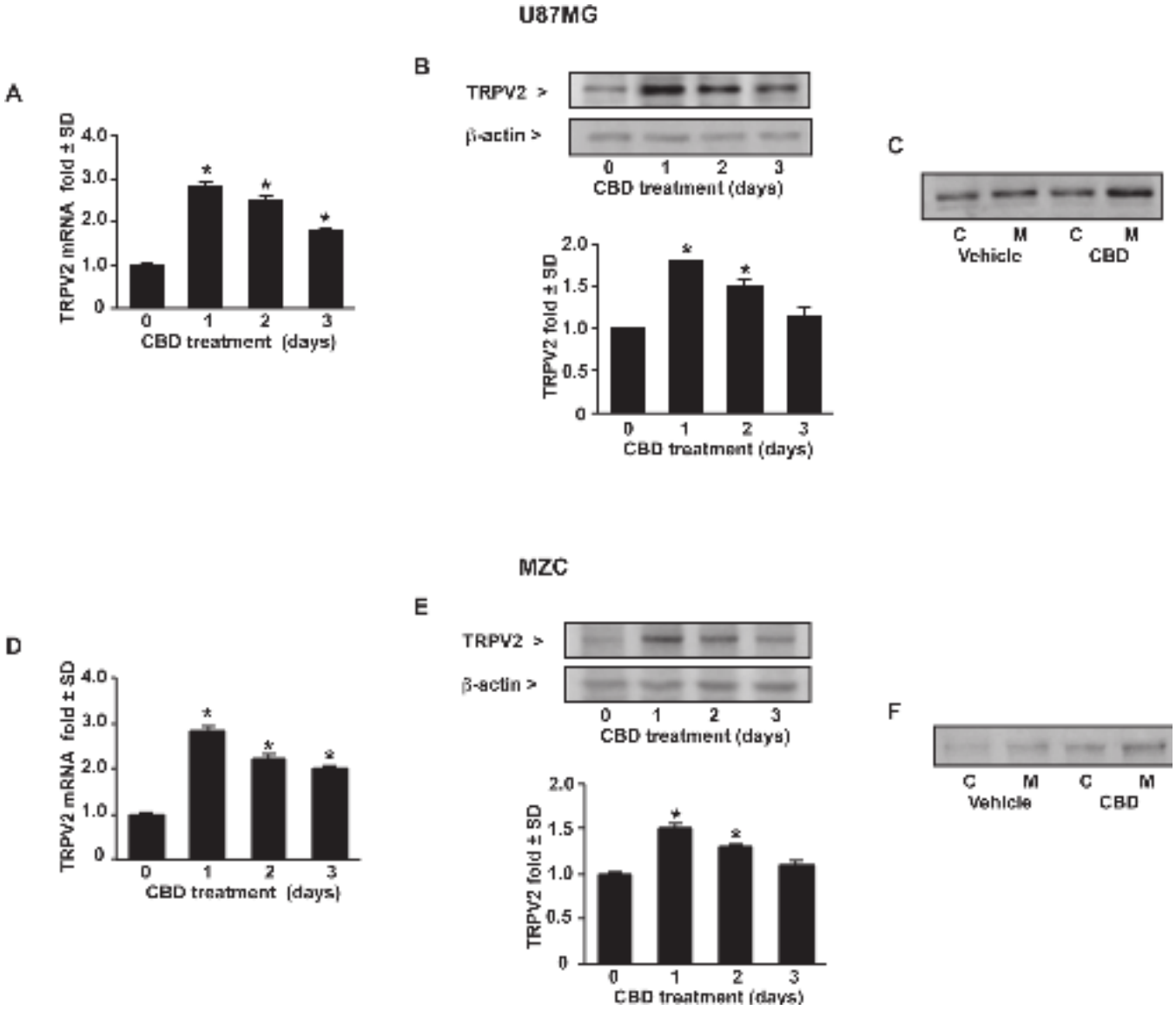

Fig. 1. CBD induces TRPV2 expression in glioma cell lines. *TRPV2* mRNA levels were evaluated by duplex quantitative real-time PCR in U87MG (**A**) and MZC (**D**) cells, after incubation with CBD (10 μM), up to 3 days. Relative TRPV2 expression normalized to β-*actin* mRNA levels was calculated considering day 0 of incubation as calibrator. TRPV2 protein levels were evaluated by western blot analysis in U87MG (**B**) and MZC (**E**) cells after incubation with CBD (10 μM), as above. The relative TRPV2 protein levels were determined using β-actin protein levels as loading control. *$P < 0.01$ versus day 0. All the data shown are the mean ± SD of at least three separate experiments. Immunoblots are representative of one out of three experiments performed with similar results. Representative immunoblots of TRPV2 protein expression in plasma membrane, M, and cytosolic, C, fractions of U87MG (**C**) and MZC (**F**) cells treated with CBD (10 μM) were shown. Twenty micrograms of total protein in membrane extracts and cytoplasmic fractions were loaded on the gel. No reactivity was observed blotting with the secondary antibody alone, with normal goat IgG and with the anti-TRPV2 antibody pre-adsorbed with its blocking peptide (data not shown).

CBD (Supplementary Figure 1B, available at *Carcinogenesis* Online). To confirm the selective role of CBD as TRPV2 agonist, we used a *TRPV2*-silenced U87MG ($U87MG_{siTRPV2}$) and a transiently *TRPV2*-transfected MZC (MZC_{TRPV2}) cell line models (Supplementary Figure 1C and G, available at *Carcinogenesis* Online) (7).

Because TRPV2 is localized in plasma membrane (M) and in cytoplasm (C) (17), we isolated M and C fractions from transfected U87MG and MZC lines first, and TRPV2 localization was determined by western blot analysis. As shown, we found that in $U87MG_{siTRPV2}$, lower TRPV2 protein levels were expressed with respect to $U87MG_{siGLO}$ (Supplementary Figure 1C and D, available at *Carcinogenesis* Online). In MZC_{TRPV2}, a TRPV2 level increase was found with respect to MZC_{EMPTY} (Supplementary Figure 1G and H, available at *Carcinogenesis* Online). In both cell line models, TRPV2 was detected mainly in M fraction (Supplementary Figure 1D and H, available at *Carcinogenesis* Online). Subsequently, we found that basal calcium levels were reduced in $U87MG_{siTRPV2}$ cells with respect to $U87MG_{siGLO}$ cells (Supplementary Figure 1E and F, available at *Carcinogenesis* Online), whereas increased basal calcium levels were observed in MZC_{TRPV2} with respect to MZC_{EMPTY} cells (Supplementary Figure 1I and L, available at *Carcinogenesis* Online). In addition, treatment with 25 μM CBD increased calcium influx in $U87MG_{siGLO}$ cells (Supplementary Figure 1E, available at *Carcinogenesis* Online) and in MZC_{TRPV2}-transfected cells (Supplementary Figure 1L, available at *Carcinogenesis* Online). Finally, 10 μM RR inhibited the CBD-mediated effects in $U87MG_{siGLO}$ and MZC_{TRPV2} cells (Supplementary Figure 1E and L, available at *Carcinogenesis* Online). To verify any putative effect of transfection in U87MG and MZC cell lines calcium influx responses, transfected and untransfected cell lines were analyzed by calcium influx assay. Results showed that no differences

in calcium influx were found between untransfected U87MG and $U87MG_{siGLO}$ control cells and between untransfected MZC and transfected MZC_{EMPTY} control cells (data not shown).

Overall, our results demonstrate that CBD acts as selective TRPV2 channel agonist in human GBM cells, by enhancing calcium influx.

CBD affects the viability of glioma cells in a time- and dose-dependent manner

Herein, we evaluated the ability of CBD treatment at different doses (1–50 μM) and times (1 to 3 days) to affect the viability and apoptosis of U87MG and MZC cells. The results obtained indicate that CBD at dose >25 μM reduced the viability of both glioma cells, in a

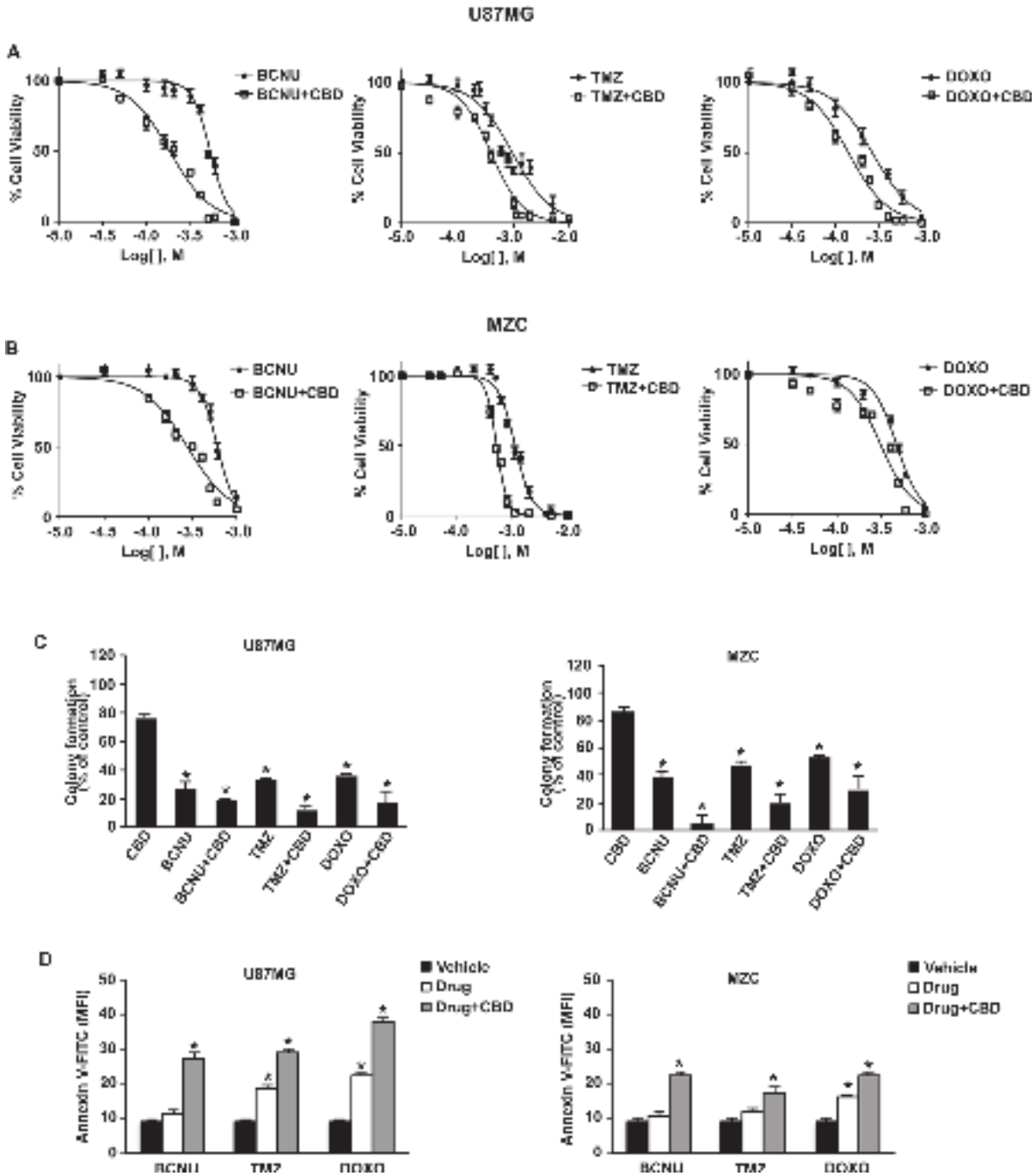

Fig. 2. CBD potentiates TRPV2-dependent glioma cell chemosensitivity. U87MG (**A**) and MZC (**B**) cells were cultured for 1 day with different doses of BCNU, TMZ or DOXO alone or in combination with CBD (10 μM). Cell viability was determined by MTT assay. Data shown are expressed as mean ± SE of three separate experiments. (**C**) U87MG and MZC cells were grown in soft agar for 14 days and treated with vehicle, CBD (10 μM) alone or in combination with BCNU (200 μM), TMZ (400 μM) or DOXO (200 μM). Soft agar colony counts were done by three independent investigators microscopically visualizing individual colonies in 10 random fields. Data shown are the mean ± SD of the percentage of colonies with respect to vehicle obtained in three separate experiments. *$P < 0.01$ versus vehicle. (**D**) MFI values of annexin V-fluorescein isothiocyanate staining in U87MG and MZC glioma cells, treated for 6 h with chemotherapeutic drugs [BCNU (200 μM), TMZ (400 μM) and DOXO (200 μM)] alone or in combination with CBD (10 μM), were determined by immunofluorescence and fluorescence-activated cell sorting analysis. Data shown are the mean ± SD of three separate experiments. *$P < 0.01$, BCNU/TMZ/DOXO versus vehicle and BCNU/TMZ/DOXO plus CBD versus BCNU/TMZ/DOXO, respectively.

time-dependent manner starting from day 1 (U87MG, IC_{50} = 30.2 µM; MZC, IC_{50} = 33.2 µM, calculated at day 1), by inducing apoptotic cell death, whereas doses of CBD <10 µM did not exert significant cytotoxic and apoptotic effects (Supplementary Figure 2, available at *Carcinogenesis* Online). Thus, in order to determine the biological activity of CBD at non-toxic and neuroprotective dose (23), we decided to use CBD at 10 µM dose in all the subsequent experiments.

CBD induces TRPV2 mRNA and protein expression and promotes its membrane localization in glioma cells

The role of CBD as modulator of gene transcription has been described previously in different human cancer cell lines (30,31). Therefore, in order to determine the ability of CBD (10 µM) to affect TRPV2 expression, the *TRPV2* mRNA and protein levels were assessed by duplex quantitative real-time PCR and western blot analysis, respectively, in CBD-treated glioma cell lines. We found that CBD rapidly increased the expression of *TRPV2* transcript (Figure 1A and 1D) and protein (Figure 1B and 1E) both in U87MG and MZC cells in a time-dependent manner, peaking at day 1. Moreover, in accordance with previous evidence (20), we found that also in U87MG and MZC cells, TRPV2 localizes in the cytosol and in plasma membrane. Moreover, we found that CBD treatments mainly increase TRPV2 localization at the plasma membrane (Figure 1C and 1F).

Overall, these results indicate that CBD increases TRPV2 expression, both at mRNA and protein levels in glioma cell lines.

CBD potentiates the cytotoxicity of chemotherapeutic agents in a TRPV2-dependent manner

We have previously reported that over-expression of TRPV2 by gene transfection in MZC cells increases the sensitivity to BCNU-induced cytotoxic effects (7). Because of the enhancing effects of CBD on TRPV2 expression, we evaluated whether CBD (10 µM) co-administered with different doses of BCNU (10^{-5} – 10^{-3} M), TMZ (10^{-5} – 10^{-2} M) and DOXO (10^{-5} – 10^{-3} M) was able to potentiate their cytotoxic effects in glioma cells. The dose-response (Figure 2) and time course analysis (Figure 3A and 3B) revealed that in U87MG cells co-administration of CBD markedly reduced the IC_{50} values of BCNU, TMZ or DOXO with respect to treatment with the chemotherapeutic agent

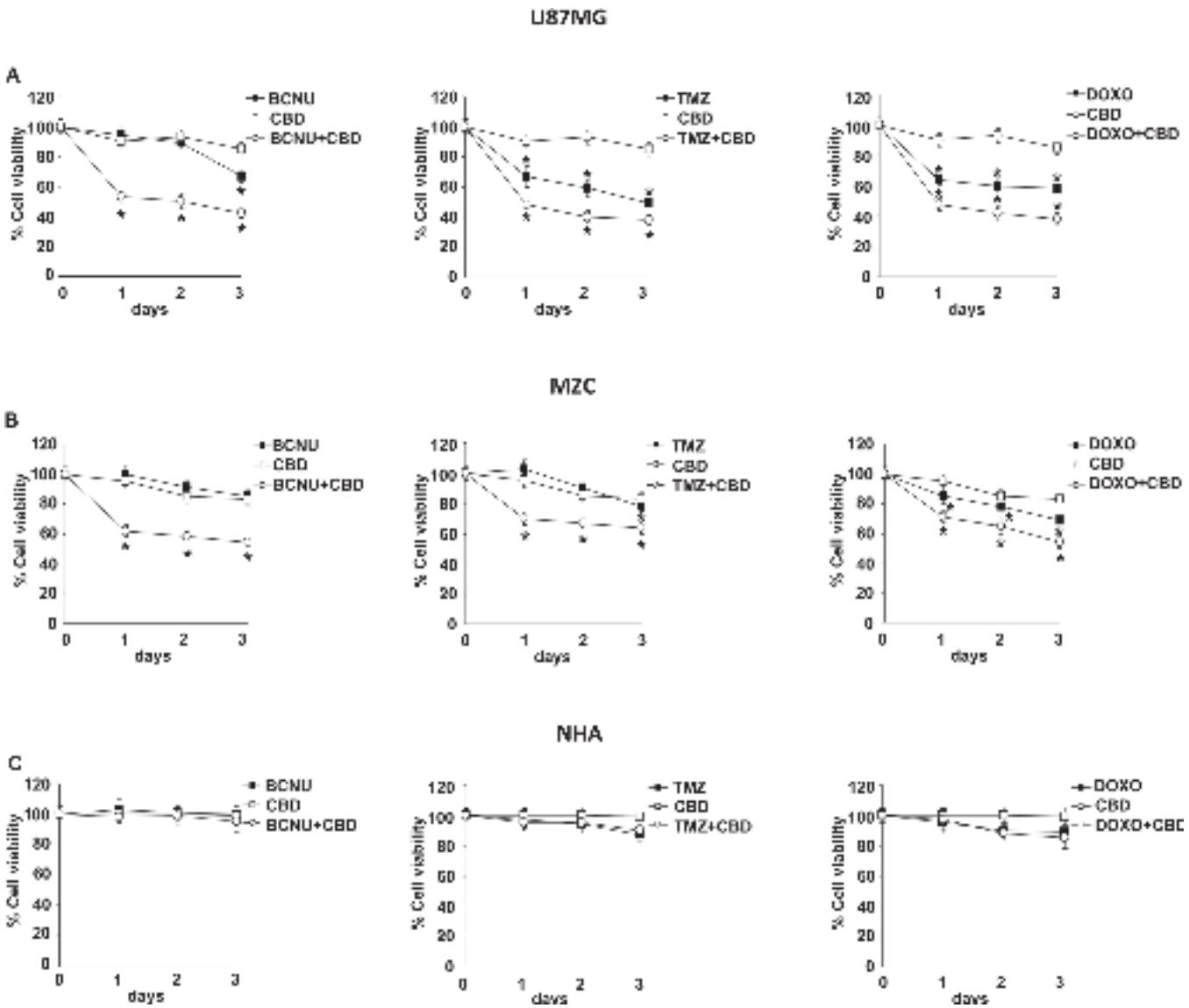

Fig. 3. CBD decreases viability in U87MG and MZC cell lines up to 3 days. U87MG (**A**), MZC (**B**) and NHA (**C**) were cultured for up to 3 days with TMZ (400 µM), BCNU (200 µM) and DOXO (200 µM) alone or in combination with CBD (10 µM). Cell viability was determined by MTT assay. Data shown are the mean ± SD of three separate experiments. *$P < 0.01$ BCNU/TMZ/DOXO versus vehicle, BCNU plus CBD versus BCNU, TMZ plus CBD versus TMZ and DOXO plus CBD versus DOXO. Vehicle data were omitted because no effects were observed with respect to untreated cells.

alone (drug versus drug + CBD: BCNU: 528 versus 183 μM; TMZ: 968 versus 397 μM; DOXO: 262 versus 142 μM) (Figure 2A). Similarly, in MZC cells, the co-administration of CBD with BCNU, TMZ and DOXO also reduced the IC_{50} values (drug versus drug + CBD: BCNU: 607 versus 275 μM; TMZ: 1113 versus 515 μM; DOXO: 473 versus 312 μM) (Figure 2B). In addition, CBD used in combination with BCNU (200 μM), TMZ (400 μM) and DOXO (200 μM) reduced the number of formed colonies evaluated by soft agar assay (Figure 2C). Moreover, co-administration of CBD with TMZ, BCNU and DOXO significantly enhanced the pro-apoptotic effects of drugs used alone on both U87MG and MZC cells as evaluated at 6 h after treatments by annexin V-fluorescein isothiocyanate staining and cytofluorimetric analysis (Figure 2D). These data strongly suggest that CBD treatments result in the potentiation of BCNU-, TMZ- or DOXO-induced pro-apoptotic effects.

Previously, it was demonstrated that alkylating agents did not affect NHAs survival, indicating a diversity of chemotherapeutic responses in NHA cells with respect to glioma cells (32–34). So, to ascribe a potential glioma-selective toxicity of CBD plus chemotherapeutic agents, we evaluated these treatments in NHA cells by MTT assay. As shown, no significant cytotoxic effects were observed in NHA (Figure 3C) with respect to U87MG (Figure 3A) and MZC (Figure 3B) cell lines, suggesting that NHA cells were refractory to CBD modulation.

We also observed that the CBD-mediated effects were TRP dependent, as shown by the ability of the specific TRP antagonist RR (10 μM) to completely abrogate the adjuvant effect induced by CBD (Figure 4A and 4B). No major effects were found by using the RR antagonist alone. To evaluate more directly the specific role of TRPV2 channel, we determined the effects of CBD in combination with the chemotherapeutic agents in $U87MG_{siTRPV2}$- and MZC_{TRPV2}-transfected cells. We found that knock-down of *TRPV2* gene completely reverted the CBD-induced potentiation of BCNU-, TMZ- or DOXO-mediated cytotoxic effects in $U87MG_{siTRPV2}$ cells (Figure 4C), whereas the co-administration of CBD with BCNU, TMZ or DOXO in MZC_{TRPV2} cells enhanced their cytotoxic effects that were reverted by RR administration (Figure 4D). No differences were found between U87MG and $U87MG_{siGLO}$ and between untransfected and transfected MZC_{EMPTY} control cells (data not shown).

CBD increases DOXO uptake in a TRPV2-dependent manner

Selective introduction of xenobiotics into target cells by permeation through TRP channels has been reported (35). DOXO, a naturally occurring fluorescent anthracycline (36) with relatively small molecular weight (MW: 580), may represent a good tracer to study drug influx via TRP channels or large pore cation channels, allowing the analysis of the cellular trafficking of other small molecular weight chemotherapeutics drugs, including BCNU (MW: 214) and TMZ (MW: 194).

Therefore, we investigated whether the TRPV2-dependent potentiation of cytotoxic effects could be the result of TRPV2 activation induced by CBD and increased drug uptake into glioma cells. Thus, MZC_{EMPTY}- and MZC_{TRPV2}-transfected cells were exposed for 2 h to spontaneously fluorescent DOXO, and fluorescent emission was evaluated by immunofluorescence and fluorescence-activated cell sorting analysis. We observed a marked DOXO uptake in MZC_{TRPV2} cells, but not in MZC_{EMPTY} cells (Figure 5A).

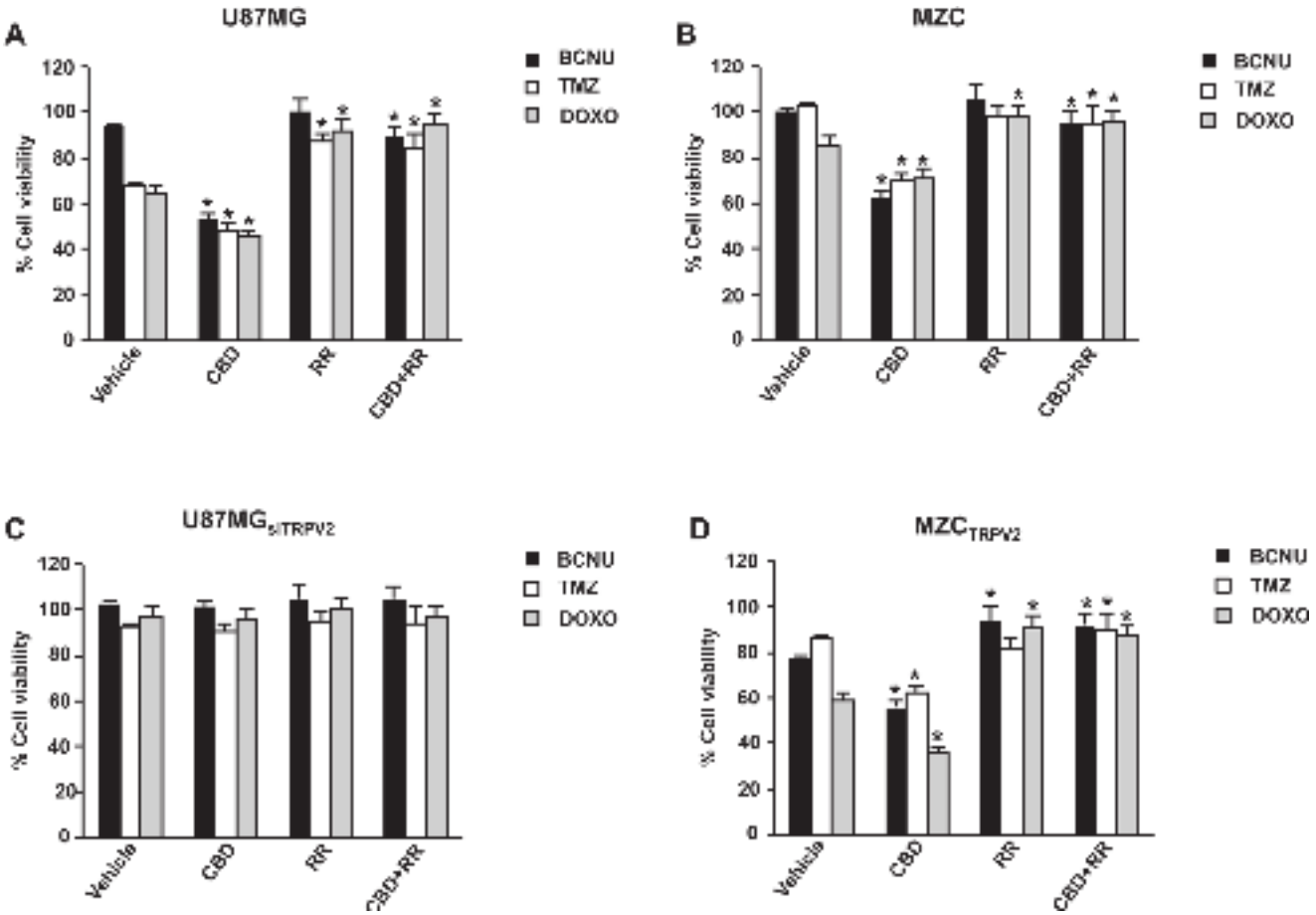

Fig. 4. CBD effects are TRPV2 dependent. U87MG (**A**), MZC (**B**), U87MGsiTRPV2 (**C**) and MZC_{TRPV2} (**D**) were treated for 1 day with BCNU (200 μM), TMZ (400 μM) or DOXO (200 μM) alone or in combination with CBD (10 μM) and the pore blocker RR (10 μM), and cell viability was measured by MTT assay. Data shown are the mean ± SD of three separate experiments. *$P < 0.01$ BCNU/TMZ/DOXO plus CBD/RR versus BCNU/TMZ/DOXO alone and BCNU/TMZ/DOXO plus CBD plus RR versus BCNU/TMZ/DOXO plus CBD.

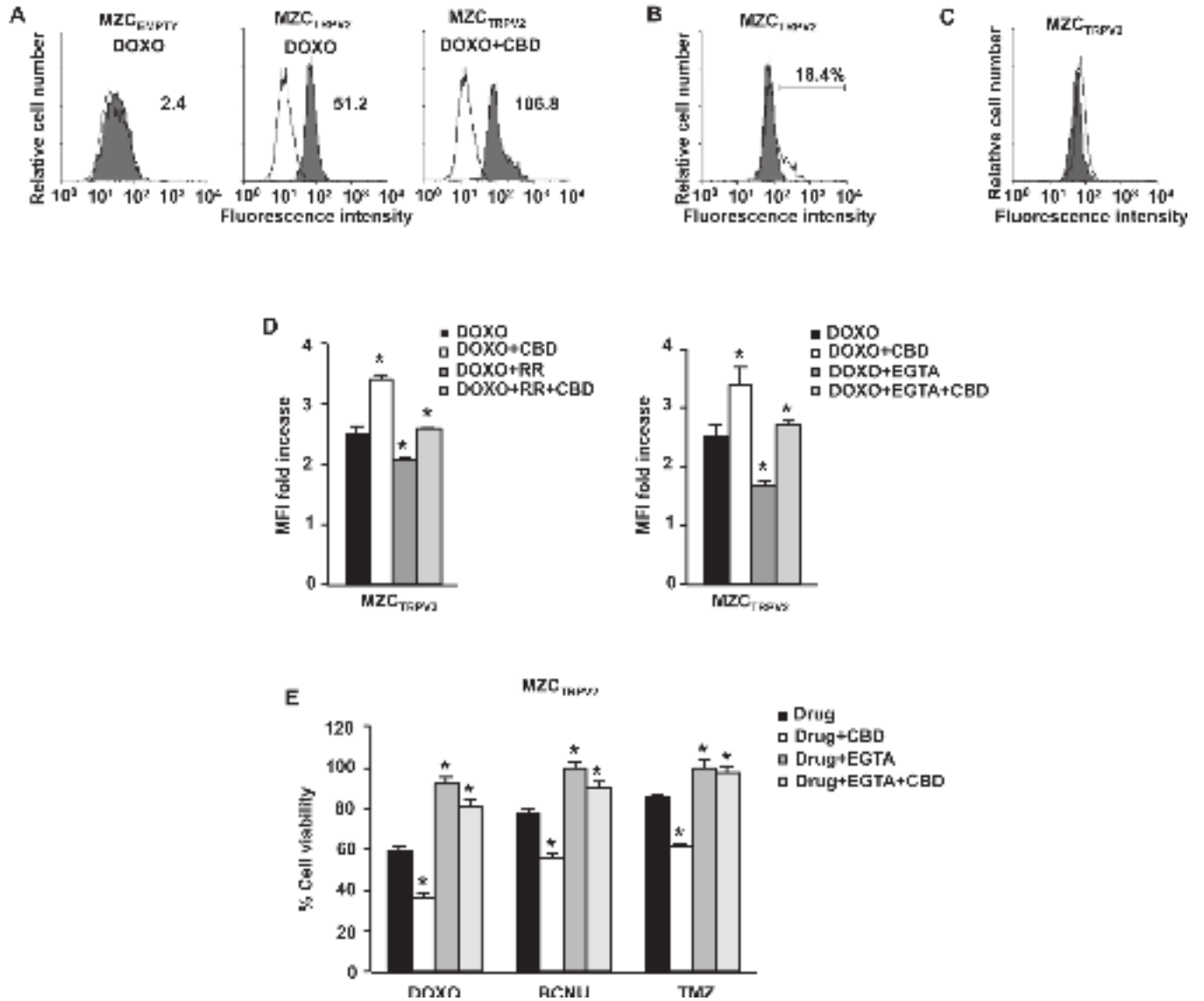

Fig. 5. TRPV2 regulates DOXO uptake. (**A**) Cytofluorimetric profile of MZC_{EMPTY} and MZC_{TRPV2} cells untreated (Control, white area) or after 2 h of exposure to 5 μM DOXO alone or DOXO plus CBD (10 μM) (Uptake, gray area). The data shown are representative of three separate experiments and are expressed as MFI by subtracting the MFI of untreated to that of treated cells. (**B**) MZC_{TRPV2} cells were treated with DOXO or CBD plus DOXO as above described and the uptake was evaluated by cytofluorimetric analysis. Numbers indicate the percentage of positive cells in CBD plus DOXO-treated cells (white area) with respect to DOXO-treated cells (gray area). (**C**) MZC_{TRPV2} cells were treated with 5 μM DOXO for 2 h, and the uptake was evaluated by cytofluorimetric analysis (white area); then, the culture medium was refreshed, and DOXO retention was evaluated after 24 h (gray area). (**D**) MZC_{TRPV2} cells were pretreated with 10 μM RR, 10 μM CBD and 1 mM EGTA, incubated with DOXO as above described and the uptake was evaluated by cytofluorimetric analysis. Data shown are the MFI fold increase of each treatment with respect to the MFI of the untreated cells and correspond to the mean ± SD of three separate experiments. *$P < 0.01$ DOXO plus CBD, DOXO plus RR and DOXO plus EGTA, DOXO plus EGTA plus CBD versus DOXO; DOXO plus RR plus CBD versus DOXO plus CBD; DOXO plus EGTA plus CBD versus DOXO plus CBD. (**E**) MZC_{TRPV2} cells were treated for 1 day with DOXO (200 μM), TMZ (400 μM), BCNU (200 μM) alone or in combination with CBD (10 μM) and the Ca^{2+} chelator, EGTA (1 mM). Cell viability was measured by MTT assay. Data shown are the mean ± SD of three separate experiments. *$P < 0.01$ DOXO plus CBD, DOXO plus EGTA and DOXO plus EGTA plus CBD versus DOXO alone and DOXO plus EGTA and DOXO plus EGTA plus CBD versus DOXO plus CBD.

No major differences were found by comparing the DOXO uptake in untransfected and MZC_{EMPTY}-transfected cells (data not shown). We also evaluated the effect of CBD in DOXO-treated MZC_{TRPV2} cells. We found a strong increase of red fluorescence with respect to untreated MZC_{TRPV2} cells (Figure 5A). Moreover, fluorescence-activated cell sorting analysis indicated that CBD can also increase the number of DOXO-positive cells in MZC_{TRPV2}, compared with cells treated with DOXO alone (Figure 5B). In addition, after drug removal followed by 24 h culture, we found that DOXO was still retained (Figure 5C). Finally, the pore-specific antagonist RR (10 μM) completely reverted the increase of red mean fluorescence intensity (MFI) induced by CBD in MZC_{TRPV2} cells (Figure 5D).

In order to analyze the role of Ca^{2+} influx in CBD-induced TRPV2-mediated increase in DOXO uptake and pro-apoptotic effects, MZC_{TRPV2} cells were incubated in the presence of the Ca^{2+} chelator EGTA (1 mM) alone or in combination with CBD. We found that EGTA completely inhibited the CBD induced increase of DOXO uptake (Figure 5D) and cytotoxicity (Figure 5E) in MZC_{TRPV2} cells. Similar findings were obtained in MZC_{TRPV2} in response to the combined treatment of CBD with TMZ or BCNU (Figure 5E). In addition, a reduced effect of drugs in combination with EGTA was found in MZC_{TRPV2}, indicating that Ca^{2+} depletion influences alkylating effects, as shown previously (37,38).

These results suggest that CBD by activating TRPV2 channels significantly enhances drug influx and retention in glioma cells.

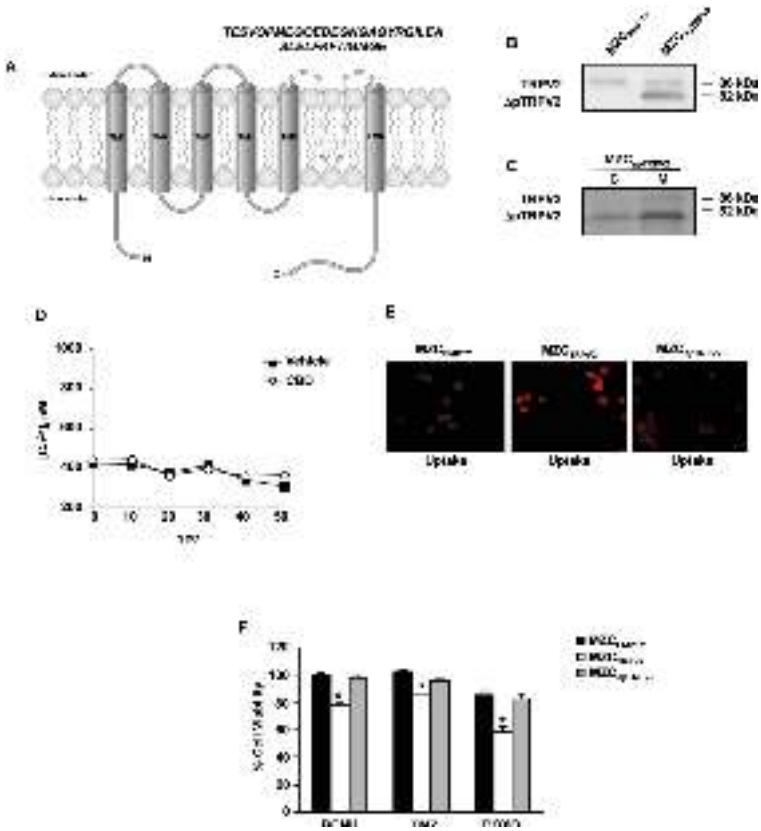

Fig. 6. TRPV2 pore domain deletion regulates DOXO uptake and reverts CBD-mediated effects. (**A**) Schematic representation of TRPV2 protein with the deleted pore region (dotted line). The corresponding pore amino acid sequence is shown. (**B**) Lysates from MZC_{EMPTY} and $MZC_{\Delta pTRPV2}$ cells were analyzed by immunoblot for TRPV2. Immunoblot is representative of three separate experiments with similar results. (**C**) Representative immunoblot of TRPV2 protein expression in cytosolic, C, and plasma membrane, M, fractions of $MZC_{\Delta pTRPV2}$ cells was shown. Twenty micrograms of total protein in membrane extracts and cytoplasmic fractions were loaded on the gel. (**D**) MZCΔpTRPV2cells were treated with CBD (25 μM) and the calcium influx was measured by monitoring fluo 3-AM. Data are representative of at least three independent experiments. (**E**) Representative images of MZC_{EMPTY}, MZC_{TRPV2} and $MZC_{\Delta pTRPV2}$ cells treated with DOXO (5 μM) for 2 h. The uptake was evaluated by fluorescent microscopy analysis (magnification ×40, bar 20 μm). (**F**) Cell viability analysis was performed on MZC_{EMPTY}, MZC_{TRPV2} and $MZC_{\Delta pTRPV2}$ cells treated for 1 day with BCNU (200 μM), TMZ (400 μM) and DOXO (200 μM). Data shown are the mean ± SD of three separate experiments. *$P < 0.01$ BCNU/TMZ/DOXO in MZC_{TRPV2} versus BCNU/TMZ/DOXO in MZC_{EMPTY}.

TRPV2 pore domain regulates CBD-mediated TRPV2-induced drug uptake and cytotoxicity in glioma cells.

Pore structure plays a crucial role in determining the TRP channel permeation and selectively properties of TRPV channels (8). However, little is known about the structure of human TRPV2 channel, and the structural organization of its cation-selective pore is still poorly understood (39).

In order to evaluate the role of the TRPV2 pore domain in the CBD-induced effects, we generated a construct expressing a TRPV2 protein ($pCMV_{\Delta pTRPV2}$) lacking the complete pore domain region (572–609 aa) (Figure 6A). As evaluated by western blot analysis, transfection of $pCMV_{\Delta pTRPV2}$ in MZC cells ($MZC_{\Delta pTRPV2}$) led to the expression of a short TRPV2 protein of about 82 kD (Figure 6B) both in the cytosol and in plasma membrane (Figure 6C). Deletion of the pore domain in $MZC_{\Delta pTRPV2}$ cells was accompanied by the failure of CBD to increase $[Ca^{2+}]_i$ influx (Figure 6D), by reduction of DOXO uptake (Figure 6E) and complete abrogation of the TRPV2-dependent potentiation of cytotoxic activity (Figure 6F), as compared with MZC_{TRPV2} cells. These data confirmed previous observations in HEK293 cells transfected with h*TRPV2* (20), showing that the pore domain of TRPV2 channel is involved in TRPV2-dependent effects, including Ca^{2+} influx. Moreover, we demonstrated that TRPV2 pore domain regulates drug uptake with a consequent potentiation of BCNU, TMZ and DOXO cytotoxic activity in glioma cells.

Discussion

Poor chemosensitivity and development of chemoresistance remain the major obstacles to successful chemotherapy of GBM (1). Standard therapies consist of surgery followed by radiotherapy and chemotherapy with alkylating agents, including BCNU and TMZ that readily cross the blood–brain barrier (1). However, despite the ability of these chemotherapeutic agents to achieve therapeutic concentrations in the brain, GBM are often resistant to these agents (3). Thus, the identification of the mechanisms responsible for the chemoresistance operating in human GBM and the development of novel approaches to overcome resistance represent the major focus of ongoing research (3,4).

Herein, we provide evidence that treatment with the TRPV2 agonist CBD, by enhancing TRPV2 expression and activation, increases the chemosensitivity of human GBM cells to the cytotoxic effects of the chemotherapeutic agents BCNU, TMZ and DOXO.

Firstly, as evaluated by calcium mobilization assay, we found that CBD increases the $[Ca^{2+}]_i$ influx in TRPV2-expressing U87MG cells and that this effect was completely abrogated by treatment with the TRP channel antagonist RR. In addition, over-expression of TRPV2 channel in MZC cells resulted in marked enhancement of the CBD-induced $[Ca^{2+}]i$ influx, whereas knock-down of *TRPV2* completely inhibited the CBD-mediated effects in U87MG, demonstrating that CBD represents a selective TRPV2 agonist in human glioma cells.

We have previously reported that reduction or complete loss of TRPV2 expression is associated with GBM progression and high

resistance of U87MG and MZC glioma cell lines to BCNU (7). Herein, we demonstrate that CBD reduces the chemoresistance of glioma cells to TMZ, BCNU or DOXO, by increasing TRPV2 expression both at mRNA and protein levels. Indeed, a marked reduction of drug IC_{50} values and numbers of colonies formed in soft agar associated with an increased number of apoptotic cells was evidenced in CBD plus chemotherapeutic-treated glioma cells. These effects were TRP dependent, as evaluated by the ability of the TRP channel antagonist RR to completely revert the CBD-mediated effects. Moreover, we directly demonstrated the involvement of the TRPV2 channel in the CBD-mediated promotion of drug cytotoxic effects by either silencing or over-expressing the *TRPV2* gene in U87MG and MZC cells, respectively. Knock-down of *TRPV2* expression by small interfering RNA completely inhibited the CBD-enhancing effects on TMZ-, BCNU- or DOXO-induced cytotoxicity in U87MG cells, whereas over-expression of *TRPV2* enhanced the CBD-mediated potentiation of chemotherapeutic drug-mediated cytotoxicity in MZC cells.

CBD has been found to promote apoptotic cell death and reduce growth of glioma xenografts, *in vitro* and *in vivo,* respectively (25,26). However, because we use CBD at no cytotoxic and pro-apoptotic doses, it is presently unclear how CBD can enhance glioma cell chemosensitivity. Herein, we could suppose that CBD, by inducing TRPV2 channel activation, results in an increased drugs permeation into glioma cells.

In fact, despite great advances in the TRP channel field during the past decade, only a limited number of studies have reported the functional characterization of cation permeation and pore properties, or the description of TRP channel pore structures (8,39). Permeation through TRP channels and other large pore cation channels, such as P2X7 adenosine triphosphate-gated channels, has been recently suggested as a new pharmacological tool to selectively introduce chemotherapeutic drugs into cancer cells with the intent/in order to increase their antiproliferative and cytotoxic effects (14).

Effective chemotherapy requires reasonably high levels of drug molecules accumulated into the cancer cells and long drug exposure times (40). Thus, by using the natural red fluorescent DOXO, we found that its uptake markedly increases only in TRPV2-over-expressing MZC_{TRPV2} glioma cells. Moreover, treatment of MZC_{TRPV2} cells with CBD, by activating TRPV2 channels, strongly promotes drug uptake as shown by enhanced fluorescence intensity and increased number of DOXO-positive cells. In addition, the CBD-mediated effects were completely inhibited by EGTA, suggesting a direct role of Ca^{2+} in regulating CBD-induced TRPV2-mediated DOXO uptake and drug cytotoxicity.

The pore diameter of TRPV channels changes from 5.4 Å of TRPV6 to >6.8 Å of TRPV1 (9,39), and channel activation by specific agonists may allow the flux of large monovalent cations (8). Thus, we hypothesized that activation of TRPV2 channel by CBD may cause a conformational change of the pore helix structure, favoring intracellular DOXO uptake, through the TRPV pore domain (9,39). Thus, the idea that DOXO permeates directly through TRPV2 pore channel opened by CBD suggests a possible exploitation of the TRPV2 channel for the delivery of DOXO or other chemotherapeutic agents into glioma cells by the usage of TRPV2 agonists. In the same view, short-term experiments in N1E-115 neuroblastoma cells transfected with TRPV1 and exposed to CPS in combination with low concentrations of DOXO have indicated that this chemotherapeutic agent accumulates only in TRPV1-transfected cells. In addition, DOXO entry requires the exposure to CPS, suggesting that the drug permeates directly through the activated TRPV1 channels (14). Similar experiments have been also conducted in basal-like breast cancer cells that express TRPM8 using agonists for TRPM8 in combination with DOXO (14).

The pore region of TRP cation channels plays a crucial role for cation permeation and selectivity (39). It has been previously reported that transfection of TRPV2 channel in HEK293 cells increases Ca^{2+} influx, and that $[Ca^{2+}]_i$ overload induces cell death (22). Moreover, mutations in the TRPV2 pore-forming domain reducing the extracellular Ca^{2+} influx abrogates the TRPV2-induced cytotoxic effects without affecting TRPV2 channel trafficking on the membrane (22).

Our findings also provide evidence indicating that TRPV2 pore deletion abolishes CBD-induced Ca^{2+} permeation, inhibits DOXO uptake, completely reverts the CBD-induced potentiation of TMZ, BCNU or DOXO cytotoxicity and increases the chemoresistance of glioma cells. Because TMZ (MW: 194) and BCNU (MW: 214) have molecular weight lower than DOXO (MW: 580), it is conceivable that TRPV2 channels can also mediate their uptake in glioma cells.

In conclusion, these data give strong biological evidences that could open the development of new effective pharmaceutical approaches for the treatment of GBM. The combination of CBD with alkylating agents, by increasing drug uptake and cytotoxic activity, allows to maintain high antineoplastic effects but at lower chemotherapeutic doses, thus reducing drug side effects.

Moreover, the ability of CBD to enhance drug-exerted cytotoxic activity might be not only restricted to glioma cells expressing high TRPV2 levels. Because TRPV2 is also expressed in low-grade gliomas (7), the properties of CBD, as its ability to increase TRPV2 expression and TRPV2-dependent alkylating drug effects, could be important in lower gliomas therapies too. In addition, it has been demonstrated that TRPV2 expression and/or activation was able to promote *in vitro* and *in vivo* GSCs differentiation and to inhibit their proliferation (17), suggesting that CBD could also be effective in reducing the proliferation of the GSC subpopulations present in GBMs (41). Because drugs selectivity for glioma cells is an important clinical issue, we also investigated the CBD and its chemotherapeutics effects in NHA cells. The results showed that NHA were refractory to CBD chemotherapeutics modulation, giving an additional interest in the use of CBD. Finally, the known CBD multiple pharmacological actions in the central nervous system and in the periphery (analgesic/anti-inflammatory, antioxidant anti-ischemic (24)) and the absence of side effects on normal brain cells, strongly support CBD use in GBM therapies.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding

Associazione Italiana Ricerca sul Cancro (AIRC) Regional Grant 2009–2011 (6353) and Fondazione Italiana Ricerca sul Cancro (FIRC) National Grant 2011–2013 (11095).

Conflict of Interest Statement: The authors declare no competing financial interests.

References

1. Nieder,C. *et al.* (2006) Therapeutic options for recurrent high-grade glioma in adult patients: recent advances. *Crit. Rev. Oncol. Hematol.*, **60**, 181–193.
2. Wong,M.L. *et al.* (2007) Targeting malignant glioma survival signalling to improve clinical outcomes. *J. Clin. Neurosci.*, **14**, 301–308.
3. Van Meir,E.G. *et al.* (2010) Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA. Cancer J. Clin.*, **60**, 166–193.
4. Santoni,G. *et al.* (2011). New insight on the role of transient receptor potential (TRP) channels in driven gliomagenesis pathways. In Ghosh,A. (ed.) *Glioma - Exploring Its Biology and Practical Relevance.* InTech, Rijeka, Croatia, pp. 163–188.
5. Santoni,G. *et al.* (2011) New deals on the transcriptional and post-transcriptional regulation of TRP channel target genes during the angiogenesis of glioma. *J. Exp. Integr. Med.*, **1**, 221–234.
6. Amantini,C. *et al.* (2007) Capsaicin-induced apoptosis of glioma cells is mediated by TRPV1 vanilloid receptor and requires p38 MAPK activation. *J. Neurochem.*, **102**, 977–990.
7. Nabissi,M. *et al.* (2010) TRPV2 channel negatively controls glioma cell proliferation and resistance to Fas-induced apoptosis in ERK-dependent manner. *Carcinogenesis*, **31**, 794–803.
8. Owsianik,G. *et al.* (2006) Permeation and selectivity of TRP channels. *Annu. Rev. Physiol.*, **68**, 685–717.

9. Voets,T. *et al.* (2004) Outer pore architecture of a Ca2+-selective TRP channel. *J. Biol. Chem.*, **279**, 15223–15230.
10. Santoni,G. *et al.* (2011) TRPV channels in tumor growth and progression. *Adv. Exp. Med. Biol.*, **704**, 947–967.
11. Meyers,J.R. *et al.* (2003) Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. *J. Neurosci.*, **23**, 4054–4065.
12. Binshtok,A.M. *et al.* (2007) Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature*, **449**, 607–610.
13. Gerner,P. *et al.* (2008) Capsaicin combined with local anesthetics preferentially prolongs sensory/nociceptive block in rat sciatic nerve. *Anesthesiology*, **109**, 872–878.
14. Santoni,G. *et al.* (2011) TRP channels and cancer: new targets for diagnosis and chemotherapy. *Endocr. Metab. Immune Disord. Drug Targets*, **11**, 54–67.
15. Li,H. *et al.* (2011) Activity-dependent targeting of TRPV1 with a pore-permeating capsaicin analog. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 8497–8502.
16. Caterina,M.J. *et al.* (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*, **398**, 436–441.
17. Morelli,M.B. *et al.* (2012) The transient receptor potential vanilloid-2 cation channel impairs glioblastoma stem-like cell proliferation and promotes differentiation. *Int. J. Cancer*, **131**, E1067–E1077.
18. Qin,N. *et al.* (2008) TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons. *J. Neurosci.*, **28**, 6231–6238.
19. Kanzaki,M. *et al.* (1999) Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. *Nat. Cell Biol.*, **1**, 165–170.
20. Penna,A. *et al.* (2006) PI3-kinase promotes TRPV2 activity independently of channel translocation to the plasma membrane. *Cell Calcium*, **39**, 495–507.
21. Sarfaraz,S. *et al.* (2008) Cannabinoids for cancer treatment: progress and promise. *Cancer Res.*, **68**, 339–342.
22. Velasco,G. *et al.* (2007) Cannabinoids and gliomas. *Mol. Neurobiol.*, **36**, 60–67.
23. Mechoulam,R. *et al.* (2007) Cannabidiol–recent advances. *Chem. Biodivers.*, **4**, 1678–1692.
24. Izzo,A.A. *et al.* (2009) Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol. Sci.*, **30**, 515–527.
25. Marcu,J.P. *et al.* (2010) Cannabidiol enhances the inhibitory effects of delta9-tetrahydrocannabinol on human glioblastoma cell proliferation and survival. *Mol. Cancer Ther.*, **9**, 180–189.
26. Torres,S. *et al.* (2011) A combined preclinical therapy of cannabinoids and temozolomide against glioma. *Mol. Cancer Ther.*, **10**, 90–103.
27. Vaccani,A. *et al.* (2005) Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism. *Br. J. Pharmacol.*, **144**, 1032–1036.
28. Massi,P. *et al.* (2004) Antitumor effects of cannabidiol, a nonpsychoactive cannabinoid, on human glioma cell lines. *J. Pharmacol. Exp. Ther.*, **308**, 838–845.
29. Massi,P. *et al.* (2006) The non-psychoactive cannabidiol triggers caspase activation and oxidative stress in human glioma cells. *Cell. Mol. Life Sci.*, **63**, 2057–2066.
30. McAllister,S.D. *et al.* (2007) Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells. *Mol. Cancer Ther.*, **6**, 2921–2927.
31. Holland,M.L. *et al.* (2006) The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochem. Pharmacol.*, **71**, 1146–1154.
32. Sato,Y. *et al.* (2009) Diversity of DNA damage response of astrocytes and glioblastoma cell lines with various p53 status to treatment with etoposide and temozolomide. *Cancer Biol. Ther.*, **8**, 452–457.
33. Short,S.C. *et al.* (2011) Rad51 inhibition is an effective means of targeting DNA repair in glioma models and CD133+ tumor-derived cells. *Neuro. Oncol.*, **13**, 487–499.
34. Chakravarti,A. *et al.* (2006) Temozolomide-mediated radiation enhancement in glioblastoma: a report on underlying mechanisms. *Clin. Cancer Res.*, **12**, 4738–4746.
35. Karasawa,T. *et al.* (2008) TRPV4 enhances the cellular uptake of aminoglycoside antibiotics. *J. Cell. Sci.*, **121**, 2871–2879.
36. Eramo,A. *et al.* (2006) Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ.*, **13**, 1238–1241.
37. Cho,S.Y. *et al.* (2012) Doxorubicin induces the persistent activation of intracellular transglutaminase 2 that protects from cell death. *Mol. Cells*, **33**, 235–241.
38. Aoyama,M. *et al.* (1998) Attenuation of drug-stimulated topoisomerase II-DNA cleavable complex formation in wild-type HL-60 cells treated with an intracellular calcium buffer is correlated with decreased cytotoxicity and site-specific hypophosphorylation of topoisomerase IIalpha. *Biochem. J.*, **336 (Pt 3)**, 727–733.
39. Voets,T. *et al.* (2003) The pore of TRP channels: trivial or neglected? *Cell Calcium*, **33**, 299–302.
40. Millenbaugh,N.J. *et al.* (2000) A pharmacodynamic analysis method to determine the relative importance of drug concentration and treatment time on effect. *Cancer Chemother. Pharmacol.,* **45**, 265–272.
41. Singh,S.K. *et al.* (2004) Identification of human brain tumour initiating cells. *Nature*, **432**, 396–401.

Received May 5, 2012; revised October 4, 2012; accepted October 14, 2012