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Short Communication

FTY720 overcomes radio-resistance of human U87G glioblastoma cells expressing an irradiation-induced SA-β-beta-gal senescence biomarker

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ABSTRACT

Radiation therapy is a major treatment for most malignant tumors, and development of new therapeutic strategies is a key challenge to overcome tumor radio-resistance. In this regard, constitutive activation of the phosphatidylinositol 3-kinase (PI3K) survival pathway and tumor cell senescence are two events critically involved in the chemo-resistance and radio-resistance of glioblastomas. In this study we demonstrated that the immunosuppressant FTY720, a PP2A activator, down regulated the PI3K-Akt pathway through inhibition of PP2A-dependant Akt-phosphorylation (ser473), and inhibited the survival and proliferation of human radio-resistant U87G and SF763 glioblastoma cell lines. Interestingly, FTY720 also inhibited the proliferation of U87G after X-irradiation and in contrast to SF763, induced acidic β-gal, a well-characterized senescence marker. Altogether our results indicate that FTY720 could be clinically evaluated as a potential powerful therapeutic tool against human PI3K-dependent and radio-resistant glioblastoma that express the SA-β-beta-gal senescence biomarker.

KEYWORDS: radio-resistance, glioma, FTY720

INTRODUCTION

Glioblastomas are common primary adult tumors of the central nervous system and usual treatments such as surgery, radiotherapy and/or chemotherapy are inefficient in maintaining patient median survival of more than one year [1, 2]. In glioblastoma, phosphatidylinositol 3-kinase (PI3K) signaling is frequently activated by the loss of the tumor suppressor lipid phosphatase PTEN [3] Challenges in targeting the PI3K pathway in cancer have been intensively discussed [4, 5]. Importantly, a constitutive activity of the PI3K-Akt pathway and induction of cellular senescence are often found associated with resistance to cancer therapies [6, 7, 8].

Protein phosphatase-2A (PP2A) is a major family of ser/thr protein phosphatases comprising multiple holoenzymes that are made of a core dimer composed of a catalytic (C) subunit and a structural (A) subunit, in association with a third variable regulatory (B) subunit that determines substrate specificity, subcellular localization and enzymatic activity of a defined holoenzyme [9]. PP2A is a tumor suppressor frequently inhibited in human tumors and it represents a drugable target against cancer [10]. Interestingly, the trimeric ABαC PP2A holoenzyme named $PP2A_1$ can inactivate Akt, and the inhibition of $PP2A_1$ activates tumor survival pathways associated with cancer progression [11].

The structural sphingolipid analog FTY720 is a synthetic analog of ISP-I, an immunosuppressive metabolite of *Isaria sinclairii* [12]. Initially developed to prevent human kidney transplant rejection, FTY720 has been approved by FDA for multiple sclerosis [13] and has also been shown to exert

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anti-cancer activity in various preclinical models via activation of PP2A [14, 15]. In addition, the promising role of FTY720 in brain tumor therapy is partly due to its lipophilic nature that allows FTY720 to cross the blood-brain barrier and to accumulate at high levels in brain tissue and cerebrospinal fluid [16, 17].

In this study we tested the potential anti-tumor properties of FTY720 in radio-resistant U87G and SF763 glioblastoma cell lines. We found that FTY720 counteracted the PI3K survival pathways in both cell lines and also exhibited toxicity against radiation-mediated senescence of U87G cells.

MATERIALS AND METHODS

Cells

Three cell lines were used: a) Two glioblastomas U87G and SF763 (kindly gifted by Prof. Marie Dutreix, Curie Institute, Orsay), and b) a primary dermal human fibroblast (DHF, Tebu). All the cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, without any antibiotic and incubated at 37 ºC in a humidified atmosphere of 5% CO_2.

Pharmacological agents

PI3K LY 29402 inhibitor and AKT A6730 inhibitor were from Sigma and FTY720 from Euromedex.

Radiation treatments

Irradiation

Cells were seeded at different densities of 10^3 to 4 x 10^4 cells per well in microplates and 24 hours later the cell cultures were irradiated with an X-ray irradiator (Faxitron 43855F) at a dose rate of 492 Gy/min. The effect of radiation was evaluated as the measurement of cell density by determining the number of seeding cells.

Measurement of cell density

Cell density was assessed by a cell DNA content assay using To-Pro-3 (Molecular Probes) as described by the manufacturer. Initially the cells were washed once with PBS quickly, fixed via 4% paraformaldehyde and permeabilized via 0.5% Triton X-100 in PBS for 10 min. The cells were then washed and labeled with To-Pro-3 at 1:800 for 1 hour at room temperature and again washed 3 times with PBS. Microplates were scanned on a laser IR scanner (Odyssey, Licor Biosciences) and the data acquired using Odyssey software. Raw data was normalized per number of seeding cells, and the cell density values were expressed as % control cultures.

Cytotoxicity assays

A total of 3,000 cells were incubated for 24 hours with different concentrations of pharmacological agents. Cell cytotoxicity was analyzed by a colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (called MTT) for adherent cells as described by the manufacturer (Sigma).

Senescence-associated β-galactosidase (SA-β-gal) assay

Senescence-associated β-galactosidase staining was performed as described previously [18]. U87G and SF763 glioblastoma cells were pre-incubated for 16 hours with 0 and 5 nM Okadaic acid (OA), respectively and a total of 5,000 irradiated (2Gy) or non-irradiated cells were incubated for a period of 4 days with FTY720 (5 μM). After that the cells were washed in PBS prior to fixing in 2% formaldehyde/0.2% glutaraldehyde at room temperature for 5 min. and incubated at 37 °C with a fresh staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl b-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$] as described previously [18].

Western blot analysis

Exponentially growing cells (10^5 cells) were seeded overnight in 24-well culture cell plates, in sub confluent monolayer, prior to pharmacological treatments. For extract preparation, cells were rinsed in cold PBS, scraped, pelletized and lysed in RIPA buffer (89900) supplemented with a cocktail of Halt protease and Phosphatase inhibitors (78442), Thermo Scientific, according to the manufacturer instructions and finally sonicated for 2 min. at 50% pulse.

The protein concentration in each sample extract was quantified using Bio-Protein assay, Bio-Rad Laboratories. Lanes were loaded with the material corresponding to 20-40 μg of cell protein extract. The following primary antibodies were used: anti-Phospho-AKT (Ser 473) (D9E) and anti-AKT (pan) (C67E7) from Cell signaling Technology, and

anti-HP1γ (2MOD-1G6) from Euromedex. In addition goat Peroxydase-labeled anti-rabbit IgG or horse Peroxydase-labeled anti-mouse IgG from VECTOR Laboratories were used as secondary antibodies.

Immunoreactive proteins were revealed using PIERCE ECL western blotting substrate, the MY ECL IMAGER, Thermo Scientific, and the software ImageJ 1.45s, National Institutes of Health, USA (http://imagej.nih.gov/ij).

RESULTS AND DISCUSSION

Specific targeting of the signaling pathways involved in survival and/or radio resistance is a major challenge in improving clinical results in human radio-resistant glioblastomas. Previous studies have established that the PI3K/Akt signaling is a major cell survival pathway for radio-resistance of glioblastoma cells [19, 20]. In the present case we studied human malignant radio-resistant glioblastomas U87G and SF763 cell lines for: (i) the status of Akt basal activation and the toxic effects of pharmacological down-modulation of Akt activity (ii) the impact of FTY720 cellular treatment on Akt activity, survival and proliferation of control and radio-resistant irradiated glioblastoma cells.

A constitutively active PI3K/Akt survival pathway in U87G and SF763 cells

We first examined the cytotoxic effect of two specific pharmacological inhibitors of the PI3K survival pathway using U87G and SF763 glioblastoma cell lines and normal non-transformed dermal human fibroblasts (DHF). We found that, in contrast to non-transformed DHF fibroblasts, a 24 hr treatment with increasing concentrations of Ly 294002, a PI3K inhibitor [21], or A6730, an Akt/PKB inhibitor [22], tested in tumor cells resulted in a dose-dependent reduction of cell viability in the two glioblastoma cell lines (see Fig. 1A left panel, IC_{50} inhibition rates). In addition, western blot analyses showed that Akt-phosphorylation (ser473) is constitutively expressed in U87G and SF763 glioblastomas but is barely detectable in non-transformed DHF cells (Fig. 1A right panel). Together, these results indicated that a constitutive activation of PI3K/Akt-PKB pathway with phosphorylated Akt (ser-473) is required for the survival of U87G and SF763 glioblastoma cell lines.

Effect of X-Irradation and PP2A pharmacologic activation on senescence-associated β galactosidase induction in U87G and SF763 glioblastoma cells

Treatment with ionizing radiation can promote accelerated senescence and radio-resistance in some tumor cells [23]. It was also hypothesized that senescence may represent a mechanism involved in radio-resistance of tumor cells [24]. Interestingly, a functional role of PP2A in the suppression of oncogene-induced senescence of melanocytes has already been documented [20]. It has also been shown that metabolically active senescent cells expressed lysosomal β-galactosidase activity at pH 6.0 [25]. In this regard, as illustrated in Fig. 2, we observed the induction of acidic β-gal, a senescent-like phenotype in U87G cells, either with PP2A pharmacological inhibition by Okadaic acid (5 days with 5 nM OA) or by X-irradiation (2Gy) as recently reported by others [26]. Induction of acidic β-gal was not observed in SF763 cells (data not shown). Moreover, FTY720 treatment of U87G cells combined with irradiation or PP2A inhibition (OA treatment) strongly reduced the emergence of U87G senescent cells expressing SA-β-gal senescent marker, (Fig. 2 lower panel). Basically the treatment of both glioblastoma cells, U87G and SF763, with increasing concentrations of FTY720 for 24 hrs resulted in a dose-dependent reduction of the cell viability (Fig. 1B left panel). Consistently, with a targeting of PP2A by FTY720, the effect on cell viability was also correlated by FTY720 inhibition of AKT phosphorylation (ser473) in the U87G and SF763 glioblastomas (Fig. 1B right panel).

Altogether, these results indicated that FTY720 counteracted a critical constitutively active PI3K/Akt survival pathway in U87G and SF763 glioblastomas by modulating PP2A activity and could overpass radio-resistance of irradiated U87G cells that expressed βeta-gal senescence marker.

Combinatorial effects of X-ray irradiation with FTY720 on growth of human glioblastoma cell lines

As shown in Fig. 3A (left panel) the two gliobastoma cell lines are resistant to X-ray radiation at 2 Gy. However we found that at very high irradiation rates (10 Gy) the number of SF763 cells decreased by 85%, whereas the number of U87G cells decreased only by 60%. Interestingly,

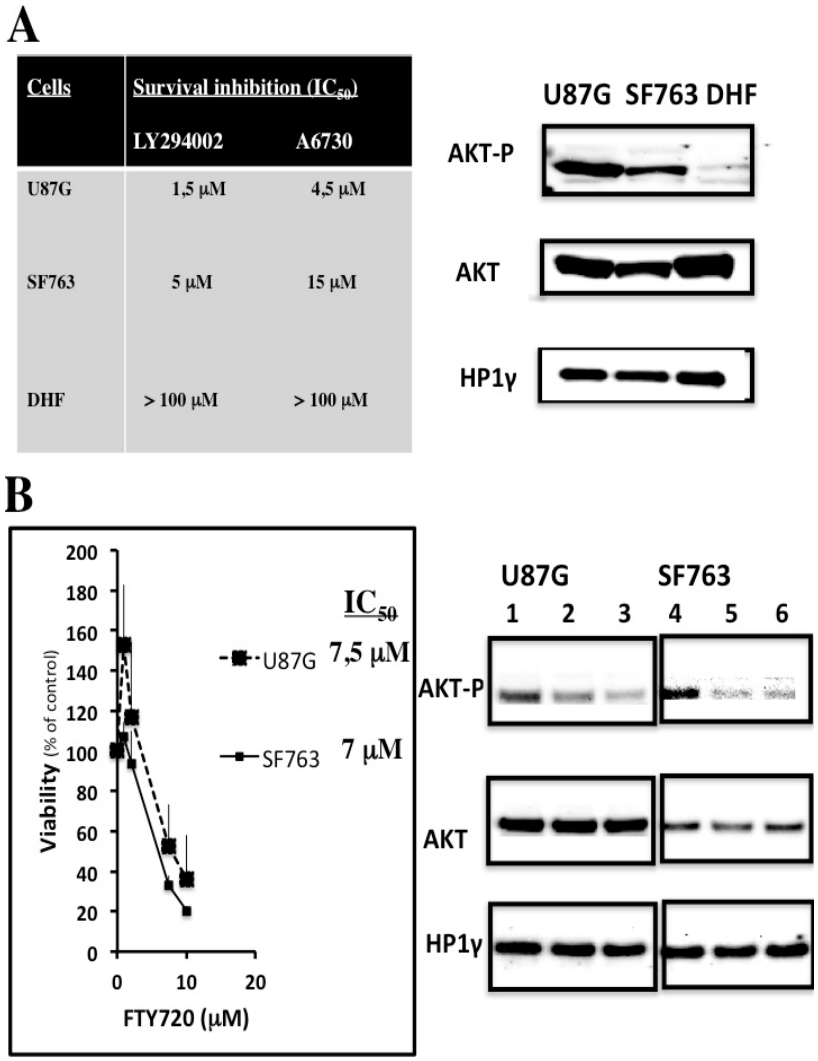

Fig. 1. FTY720 inhibits a constitutively active PI3K/Akt survival pathway in U87G and SF763 cells.
A: Left panel: IC_{50} survival of U87G, SF763 and DHF cells treated for 24 hr with Ly294002 (0-100 µM), a PI3K pharmacological inhibitor, and A6730 (0-100 µM), an Akt pharmacological inhibitor; cell viability was assessed by MTT test (n = 3). Right panel: Western blot analysis of Akt phosphorylation status from extracts of U87G, SF763 and DHF; membrane was probed successively with mAb to phosphorylated Akt (pSer473), mAb to total Akt and mAb to HP1γ (internal control).
B: Left panel: Cytotoxicity of FTY720 on U87G and SF763. Cells were treated for 24 hr with various concentrations of FTY720 (0-10 µM), and cell viability was assessed by MTT test (n = 3).
Right panel: FTY720 treatment decreased Akt phosphorylation in U87G and SF763. Western blot analysis was performed as described in A right panel. Cells were treated with 50 µM of FTY720 and cell extracts were analyzed at the different set points of FTY720 treatment 0, 30 and 60 mn, lanes (1-2-3) and (4-5-6) respectively.

the survival of irradiated mice bearing tumors induced by SF763 xenografts was also higher than that in irradiated mice containing tumors induced by U87G xenografts (data not shown). These observations indicate that U87G is more radio resistant than SF763 cells.

Furthermore, as illustrated in Fig. 3B, treatment with increasing concentrations of FTY720 for 72 hrs resulted in a dose-dependent reduction of cell density in both U87G and SF763 glioblastoma cell lines, and a dose of 7.5 µM of FTY720 efficiently killed U87G and SF763 cells. In addition, when cells

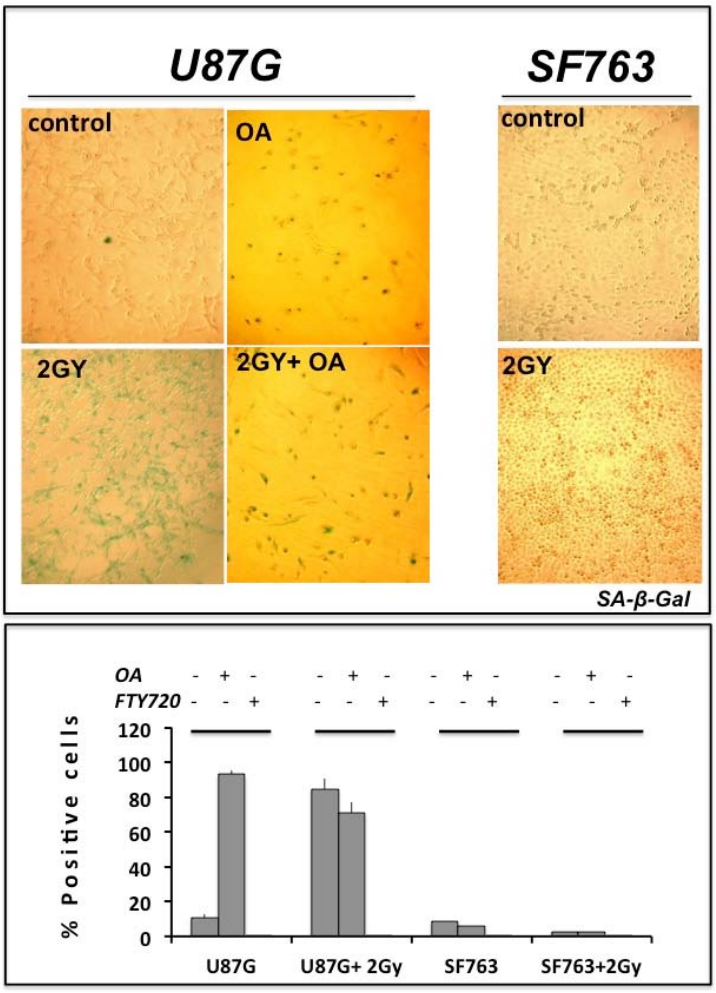

Fig. 2. Effect of X-Irradation and pharmacological treatments with OA and FTY720 on SA-β-gal activation in U87G and SF763 glioblastoma cells.
Upper panel: Staining of cells expressing SA-β-gal senescent marker in U87G and SF763 cell lines: control-untreated, 2GY - cells irradiated with 2Gy, OA - cells pre-treated with 5 nM OA, 2Gy+OA - cells pre-treated with OA for 4 days and irradiated with 2Gy. SA-β-gal staining remained negative in SF763 cells following OA treatment (not shown).
Lower panel: Histogram showing the percent of positive blue stained SA-β-gal cell in U87G and SF763 cells following different treatment combinations including 5 nM OA, 5 μM FTY720 and irradiation. At least 150 U87G and SF763 cells were counted in three independent experiments to determine the percentage of positive blue SA-β-gal staining shown in the histogram.

were irradiated (2 Gy) and cultured 4 days prior to FTY720 treatment, a dose-dependent reduction of cell density was observed, similar to non-irradiated cells but more pronounced for U87G cells (Fig. 3C). These results could suggest that the senescence propensity of radio-resistant U87G glioblastoma cells cannot antagonize FTY720-mediated growth effects.

In conclusion, in the present study the potential anti-tumor effect of FTY720 on radio-resistant glioblastomas was determined in U87G cells that became senescent following exposure to X-ray radiation (2gy) or OA treatment. We found that FTY720-mediated inhibition of the PP2A/PI3K pathway correlates with inhibition of survival in radio-resistance induced U87G senescent cells.

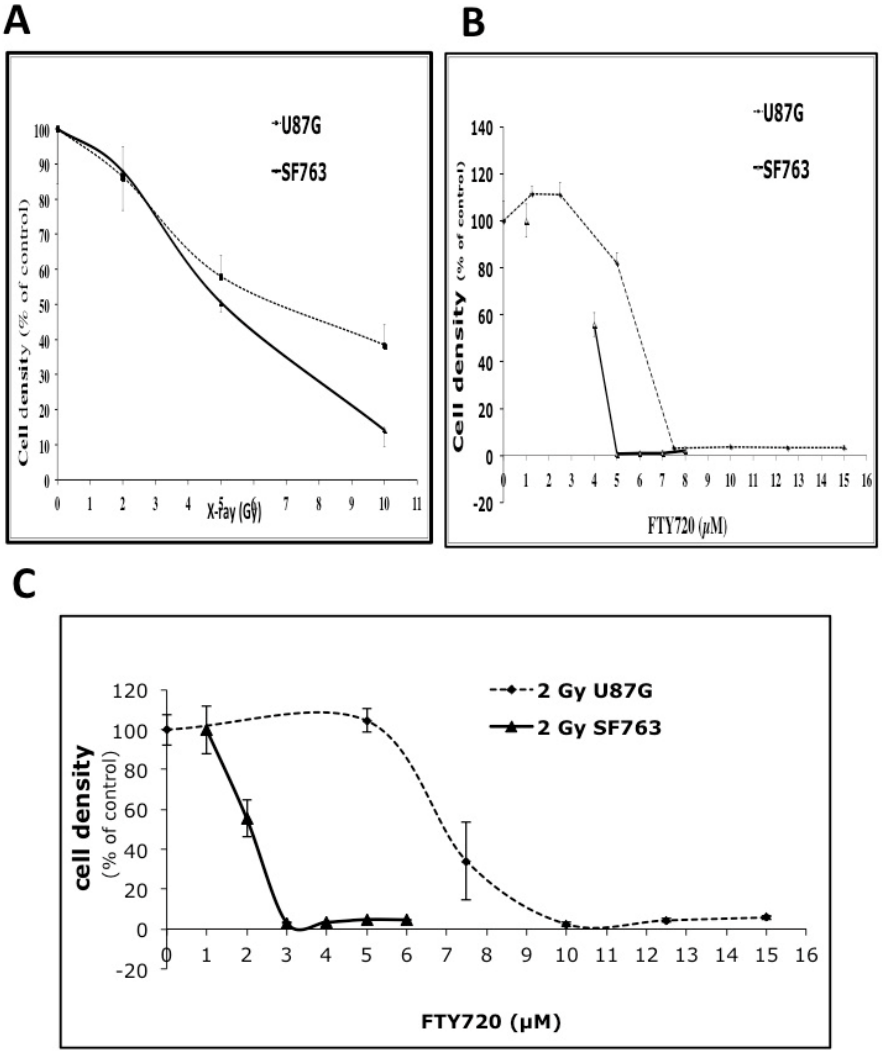

Fig. 3. Effect of X-ray irradiation and FTY720 treatments on glioblastoma cell growth.
Growth curves of U87G and SF763 cells treated by X-ray radiation or FTY720.
The curves relayed to cell densities, expressed as a percentage of the control. **A:** the cell densities were recorded on days 5 and 6 (SD is shown (n = 12)). **B:** The cell densities were recorded according to the FTY720 concentration as indicated at 72 hours after drug treatment, SD is shown (n = 6). **C:** Growth of U87G and SF763 cell lines after combined X-ray irradiation and FTY720 treatments. The cell densities were recorded in culture of X-irradiated cells (2Gy), cultured for 4 days and then treated for 24 hr by FTY720 (0-15 μM), (n = 4).

CONCLUSION

In this study we found that the constitutively activated PI3K/Akt survival pathway of two human glioblastomas U87G and SF763 cell lines, used as cellular models of radio-resistant glioblastomas, is inactivated by the immunosuppressant FTY720, a PP2A-Akt phosphatase activator, already approved by FDA for multiple sclerosis. Importantly, for FTY720 clinical evaluation of malignant glioblastomas, we demonstrated that FTY720 inhibited survival of the two irradiated radio-resistant cell lines including highly radio-resistant and radio-senescent U87G cells.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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