

## FTY720 (Fingolimod) Sensitizes Prostate Cancer Cells to Radiotherapy by Inhibition of Sphingosine Kinase-1

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### Abstract

Radiotherapy is widely used as a radical treatment for prostate cancer, but curative treatments are elusive for poorly differentiated tumors where survival is just 15% at 15 years. Dose escalation improves local response rates but is limited by tolerance in normal tissues. A sphingosine analogue, FTY720 (fingolimod), a drug currently in phase III studies for treatment of multiple sclerosis, has been found to be a potent apoptosis inducer in prostate cancer cells. Using *in vitro* and *in vivo* approaches, we analyzed the impact of FTY720 on sphingolipid metabolism in hormone-refractory metastatic prostate cancer cells and evaluated its potential as a radiosensitizer on cell lines and prostate tumor xenografts. In prostate cancer cell lines, FTY720 acted as a sphingosine kinase 1 (SphK1) inhibitor that induced prostate cancer cell apoptosis in a manner independent of sphingosine-1-phosphate receptors. In contrast,  $\gamma$  irradiation did not affect SphK1 activity in prostate cancer cells yet synergized with FTY720 to inhibit SphK1. In mice bearing orthotopic or s.c. prostate cancer tumors, we show that FTY720 dramatically increased radiotherapeutic sensitivity, reducing tumor growth and metastasis without toxic side effects. Our findings suggest that low, well-tolerated doses of FTY720 could offer significant improvement to the clinical treatment of prostate cancer. *Cancer Res*; 70(21); 8651–61. ©2010 AACR.

### Introduction

In the western world prostate cancer is now the most commonly diagnosed noncutaneous cancer in men and is the second leading cause of cancer-related death (1). In the United States the lifetime probability of developing prostate cancer is 1 in 6, and it is estimated that 192,280 new cases of prostate cancer were diagnosed during 2009, and there were 27,360 deaths.

The management of prostate cancer is complex, but for the majority of patients radiotherapy remains a definitive treatment for early-stage disease. However, up to 85% of patients with poor histologic subtypes of localized prostate cancer relapse (2). In radiotherapy research the current emphasis is on computerized planning to deliver increased dosage schedules. There is an opportunity to approach the

subject of dose intensification from a different angle, and this is through the use of radiosensitizer treatment.

Prostate cancer cell radioresistance has been linked with sustained activation of sphingosine kinase-1 (SphK1; ref. 3). SphK1 is a lipid converting enzyme responsible for the conversion of sphingosine into sphingosine-1-phosphate (S1P). S1P, and sphingosine and its precursor ceramide are lipid second messengers. In response to various stimuli ceramide and sphingosine mediate cell death, whereas S1P abrogates apoptosis and mediates cell proliferation and migration (4).

The SphK1/S1P pathway contributes to cancer progression and leads to increased cell proliferation (5), impairment of apoptosis (6), and oncogenic transformation (7). SphK1 is a tumor-associated enzyme: high levels of SphK1 expression have been shown in various human tumor tissues (8, 9). Furthermore high levels of SphK1 expression and activity are associated with a poor prognosis in breast cancer (10), glioma (11), and gastric cancer (12).

These findings have highlighted the potential of SphK1 as a therapy target. A sphingosine analogue, dimethylsphingosine, was the first reported SphK inhibitor to induce prostate cancer cell apoptosis (3). These findings were further confirmed by ourselves (13), showing a new selective SphK inhibitor, SKI-II (first reported by French et al.; ref. 9), to have proapoptotic properties in prostate cancer cells. Work from our group in prostate cancer indicates that inhibition of the SphK1/S1P pathway has a synergistic effect with chemotherapy and has the potential to act as a molecular target for cancer therapy (14–16).

FTY720 is a sphingosine analogue and a potent immunosuppressive drug that induces lymphopenia via an inhibition

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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of lymphocytes' egress from lymphoid organs through its antagonist function on the lymphocytes' S1P receptors (17, 18). Recently the results of phase III trials have become available, showing significant potential of oral FTY720 (fingolimod) in multiple sclerosis patients (19, 20).

Surprisingly, at higher doses FTY720 has been shown to be a potent apoptosis inducer in prostate, liver, and bladder cancer cell lines (21–23). In a mouse model of melanoma FTY720 inhibited tumor growth and metastasis (24) without causing detectable toxicity in vital organs. Direct mitochondria damage (25), activation of caspases (21), and dephosphorylation of Akt (26) have all been proposed as potential mechanisms for FTY720-induced apoptosis. The effects of FTY720 were reported to be both S1P receptor dependent (24) and independent (27). In prostate cancer cells FTY720 has been shown to be a potent apoptosis inducer (21), whereas normal prostate cells exhibited resistance to the drug. FTY720 has been shown to inhibit prostate cancer cell invasion via downregulation of GTP-bound active form of RhoA (28), and administration of FTY720 at 10 mg/kg/day reduced the growth of prostate CWR22R xenografts in castrated nude mice (29).

In this current study we identified a novel mechanism of FTY720-induced prostate cancer cell apoptosis. Here we report that FTY720 can act as a SphK1 inhibitor *in vitro* and *in vivo* and that SphK1 inhibition is critical for FTY720-induced apoptosis. Sublethal concentrations of FTY720 act as a radiosensitizer in cell lines and in implanted tumors, reducing tumor metastasis.

Our preliminary data point to the need for clinical testing to establish whether FTY720 therapy might provide an advantage in terms of increased local tumor control to patients with prostate cancer treated with radiotherapy.

## Materials and Methods

### Cell lines

PC-3; 22Rv1, and DU145 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, and LNCaP-C4-2B cells were from Viomed; PC-3M are a highly metastatic derivative of PC-3 cells as previously described (30). Cells were cultured between passages 4 and 30 in RPMI 1640 containing 10% fetal bovine serum. Cell lines were routinely verified by morphology and growth curve analysis and were routinely screened for mycoplasma infection (MP0035 Lookout, Sigma). SphK1-overexpressing cell models have been described previously (13). All experiments were conducted in the absence of serum at 50% confluence and then treated as indicated in the figure legends.

### Reagents

Culture medium, serum, antibiotics and Syto 13 were obtained from Invitrogen. *Escherichia coli* diacylglycerol kinase, octyl-D-glucopyranoside, and SKI-II compound were from Merck. [ $\gamma$ - $^{32}$ P]-ATP was purchased from Perkin-Elmer, and silica gel 60 high-performance TLC plates were from VWR. FTY720 was obtained from Novartis. Annexin V-FITC was from BD. All other chemicals were from Sigma Aldrich.

$\gamma$ -Irradiation was performed using the IBL 637 irradiator (Cis-Bio International) using a Cesium 137  $\gamma$ -ray source (total activity ~222 TBq) at the dose rate of 1 Gy/minute.

### siRNA transfection

SphK1 and S1P<sub>1-5</sub> were targeted using siRNA as previously described (13, 31), along with relevant control siRNA. Knockdown was assessed by real-time quantitative reverse transcription-PCR (qRT-PCR) as previously described (16).

### Cell viability, flow cytometry, and staining of apoptotic nuclei

Cell viability, flow cytometry, and staining of apoptotic nuclei were measured as previously described using the MTT assay (13), Annexin V-FITC/propidium iodide (PI) staining, and Syto13/PI staining (14).

### Sphingosine kinase-1 assay and mass measurements of ceramide, sphingosine 1-phosphate, and sphingosine

Sphingosine kinase-1 assay and mass measurements of ceramide, sphingosine 1-phosphate, and sphingosine were performed as described previously (14, 32).

### Real-time qRT-PCR

Real-time qRT-PCR was performed as described previously (16).

### Caspase-3/7 activity

Caspase-3/7 activity was measured as previously described (14).

### Western blot analysis

Western blot analysis was performed as described previously (16).

### Animal study

Animal study was performed as previously described (13). Briefly, intraprostatic and s.c. human prostate cancer xenografts were established in NMRI/Nu (*nu/nu*) 7-week-old male mice by surgical orthotopic implantation or s.c. injection of  $1 \times 10^6$  PC-3/GFP cells. Three weeks after implantation, mice were randomized into different groups and treated for two weeks with i.p. injections of PBS (control), 2.5 mg/kg/day FTY720, 5 sessions of  $\gamma$ -irradiation (4 Gy) every 3rd day, or  $\gamma$ -irradiation combined with FTY720. Two days after the last treatment, all mice were euthanized with carbon dioxide asphyxiation for direct internal imaging. Tumor area ( $a$ ) and the small diameter ( $d$ ) were used to assess tumor volume ( $v$ ) using the formula  $v = a \times d \times 2/3$ . Primary tumors were then removed, saved, and routinely processed for H&E histology to confirm the nature of the disease or were processed for sphingolipid analyses. Blood counts were determined as previously described (33).

### Data representation and statistical analysis

The statistical significance of differences between the means of two groups was evaluated by unpaired two-sided Student's  $t$  test, and the overall significance of multiple groups was evaluated by ANOVA. Calculations were performed using Graphpad Prism Software. Representative images are presented for the experiments carried out with fluorescence.

## Results

### Sphingosine analogue FTY720 induces apoptosis in prostate cancer cells by inhibition of SphK1

FTY720 induces dose-dependent loss of cell viability in metastatic prostate cancer PC-3 cells (Fig. 1A), achieving an  $IC_{50}$  at ~30 hours of exposure at 5  $\mu\text{mol/L}$  dose. This loss of cell viability was preceded by an activation of caspase 3 and/or 7 (~2.5 fold at 6 hours; Fig. 1B) and was followed by an increase in apoptotic cells ( $51 \pm 5\%$  at 48 hours; Fig. 1C). A similar decrease in cell viability (Fig. 1D) and an increase in apoptosis and caspase-3 activation (not shown) were observed in DU145 metastatic prostate cancer cells, in agreement with previous reports (21, 34).

FTY720 is a sphingosine analogue and was previously shown to inhibit enzymatic activity of recombinant SphK1 (35). Here we report that FTY720 induced a rapid inhibition of SphK1 enzymatic activity in PC-3 and DU145 cells ( $-28 \pm 5\%$  and  $-40 \pm 5\%$  in PC-3 and DU145 cells, respectively, at 6 hours; Fig. 2). This inhibition was followed by a decrease in intracellular SIP ( $-18 \pm 6\%$  and  $-31 \pm 5\%$  in PC-3 and DU145 cells, respectively, at 6 hours) and a more delayed increase in intracellular ceramide ( $51 \pm 10\%$  and  $58 \pm 5\%$  in PC-3 and DU145 cells, respectively, at 24 hours). Of note, qRT-PCR analysis showed that treatment with FTY720 did not induce significant changes in the expression of the *SphK1* gene during first 24 hours (Supplementary Fig. S1).

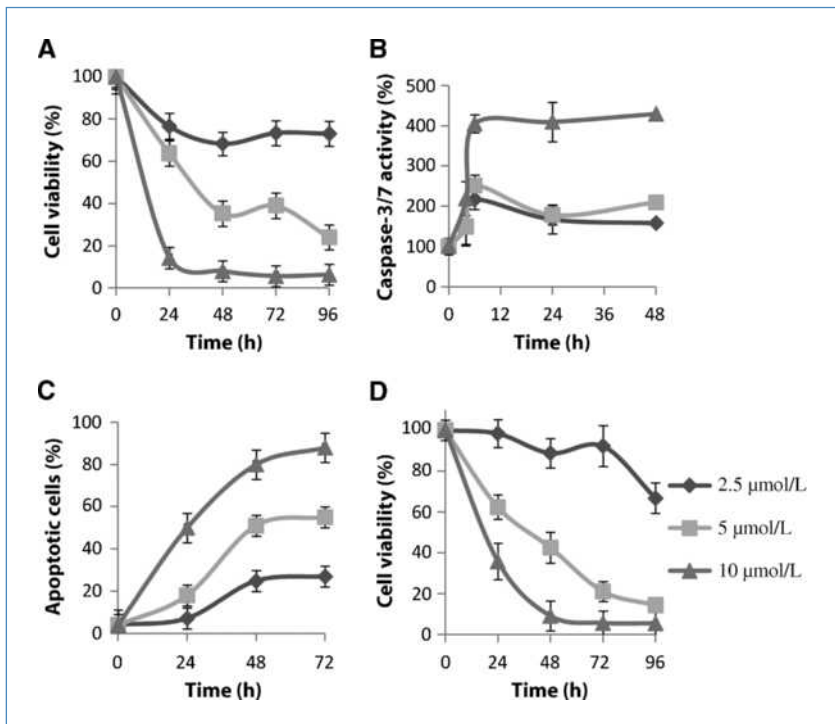
Overexpression of SphK1 in PC-3 cells rendered them less sensitive to FTY720 ( $65 \pm 8\%$  versus  $35 \pm 6\%$  of loss of cell

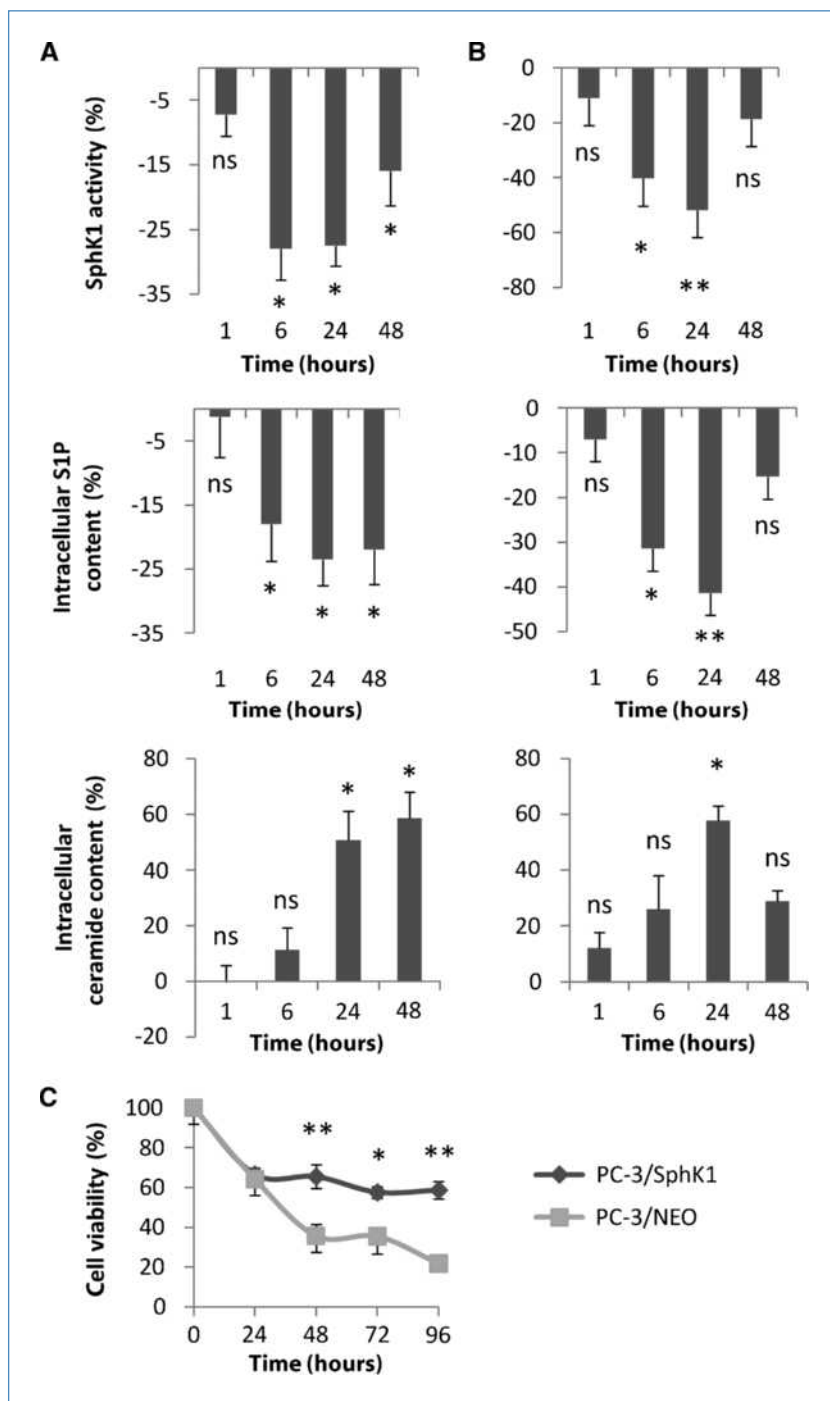
viability in PC-3/NEO versus PC-3/SphK1 cells, respectively; Fig. 2C). Conversely, the silencing SIP1 to SIP5 receptors either individually or altogether did not alter FTY720-induced PC-3 cell death (Supplementary Fig. S2), suggesting a receptor-independent mode of action. Treatment with FTY720 also decreased the levels of intracellular phospho-Akt, which, however, was downstream of FTY720-induced SphK1 downregulation because SphK1 overexpression restored the levels of phospho-Akt in FTY720-treated cells and the SphK inhibitor SKI-II could per se reduce the levels of phospho-Akt (Supplementary Fig. S3). As shown in Fig. 2, FTY720 treatment induced only a late modest increase in intracellular ceramide. Pretreatment of PC-3 cells with fumonisin B1 (inhibitor of *de novo* ceramide synthesis), GW4869 (neutral sphingomyelinase inhibitor), or imipramine (acid sphingomyelinase inhibitor) could not abrogate FTY720-induced apoptosis, suggesting that ceramide accumulation was a consequence of SphK1 inhibition (Supplementary Fig. S4).

### $\gamma$ -Irradiation induces prostate cancer cell death independently of SphK1

When treated with a single dose of  $\gamma$ -irradiation both PC-3 and DU145 cells were sensitive to 10 Gy treatment, achieving a 40% loss of cell viability at ~72 hours and 48 hours for PC-3 and DU145 cells, respectively (Fig. 3A). In both cell lines a further increase in  $\gamma$ -irradiation dose to 20 Gy did not lead to a significant increase in cell death (data not shown). On the contrary, both cell lines exhibited a partial resistance when

**Figure 1.** FTY720 induces prostate cancer cell apoptosis. PC-3 (A–C) and DU145 (D) cells were treated with FTY720 at indicated concentrations for the indicated times. A, viability of PC-3 cells was quantified by MTT reduction assay. B, caspase-3/7 activity was measured using luminescent caspase-GLO substrate. C, number of apoptotic cells was quantified by flow cytometry after staining with Annexin V/PI. D, cell viability of DU145 cells. Points, mean of four independent experiments performed in triplicate; bars, SE.





**Figure 2.** Sphingosine analogue FTY720 induces *in vitro* inhibition of SphK1, a decrease in intracellular S1P, and SphK1 overexpression reverts FTY720-induced prostate cancer cell apoptosis. PC-3 (A) and DU145 (B) cells were treated with 5  $\mu\text{mol/L}$  FTY720 for the indicated times and SphK1 activity (top), intracellular S1P (middle), and intracellular ceramide (bottom) were measured as described in Materials and Methods. C, cell viability of PC-3 cells stably overexpressing SphK1 (PC-3/SphK1) or empty vector (PC-3/NEO) as described previously (13), and treated with 5  $\mu\text{mol/L}$  FTY720 for indicated time. Columns, mean of four independent experiments performed in duplicate; points, mean of three independent experiments performed in triplicate; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; ns, not significant ( $P > 0.05$ ).

treated with lower doses of  $\gamma$ -irradiation (only ~20% loss after 120-hour treatment with 5 Gy in both cell lines; Fig. 3A).

In contrast to FTY720, treatment of prostate cancer cells with  $\gamma$ -irradiation did not result in SphK1 inhibition. Conversely in DU145 cells,  $\gamma$ -irradiation induced rather an upregulation of SphK1 activity ( $43 \pm 7\%$  and  $45 \pm 6\%$  by 5 Gy and 10 Gy, respectively,  $P < 0.05$ ; Fig. 3B), which, however, was not mediated by SphK1 transcription (Fig. 3C).

### SphK1 inhibition by FTY720 sensitizes prostate cancer cells to $\gamma$ -irradiation

In line with previous reports showing that SphK1 inhibition by dimethylsphingosine or SKI-II could respectively sensitize prostate cancer cell models to  $\gamma$ -irradiation (3) and chemotherapy (17, 18), here we report that 1 to 5  $\mu\text{mol/L}$  FTY720 successfully sensitized PC-3 and DU145 cells to  $\gamma$ -irradiation (Fig. 4A and B). Fluorescent activated cell sorter analysis

(Fig. 4C) and fluorescent microscopy (Supplementary Fig. S5) revealed that this radiosensitization was mediated by an induction of apoptosis. Isobologram analysis revealed that in both cell lines FTY720 and  $\gamma$ -irradiation act in synergy (Supplementary Fig. S6). Similarly to FTY720, the SphK inhibitor SKI-II (Fig. 4D) and SphK1 siRNA, but not SphK2 siRNA (Supplementary Fig. S7), sensitized PC-3 and DU145 (not shown) prostate cancer cells to  $\gamma$ -irradiation.

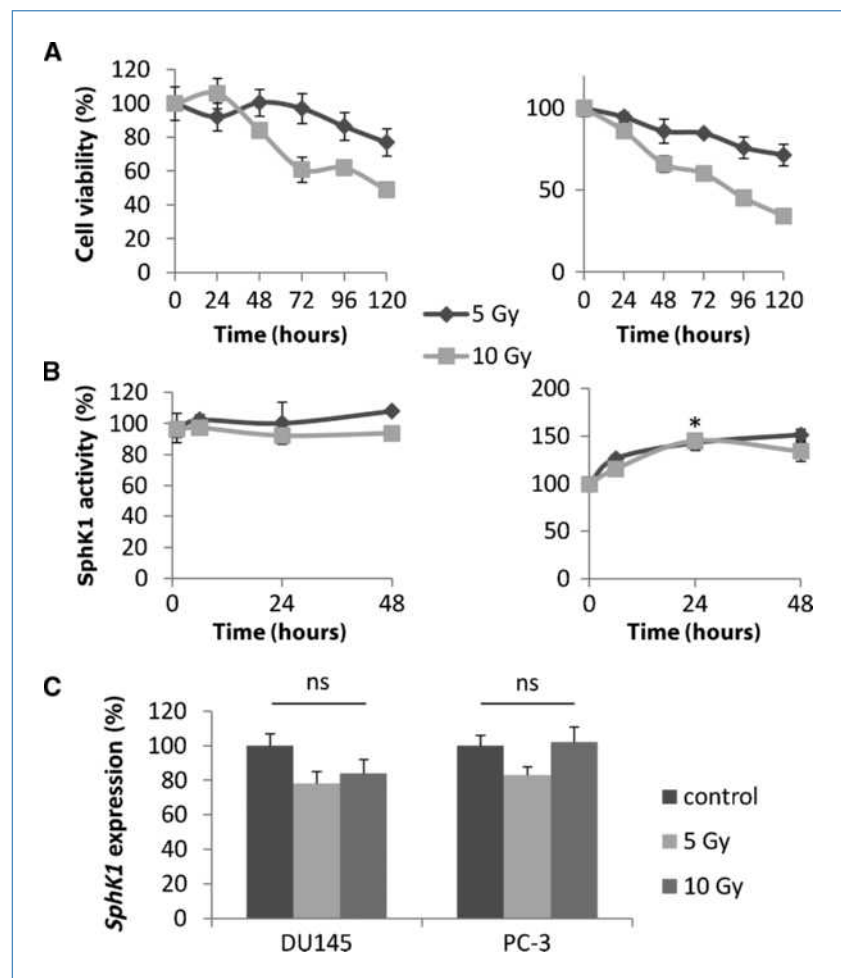
Figure 5 shows that the addition of FTY720 to PC-3 cells subjected to  $\gamma$ -irradiation significantly exacerbated the inhibition of SphK1 activity ( $-46.0 \pm 5.7\%$  versus  $0.2 \pm 6.5\%$ ,  $P = 0.0178$ ), comparable with the levels in cells treated with FTY720 alone ( $-27.5 \pm 3.1\%$ ; Fig. 5A). Similarly, the addition of FTY720 decreased the levels of intracellular S1P in irradiated PC-3 cells (Fig. 5A). There was no significant difference between the levels of intracellular ceramide in cells subjected to treatments alone or in combination (Fig. 5A).

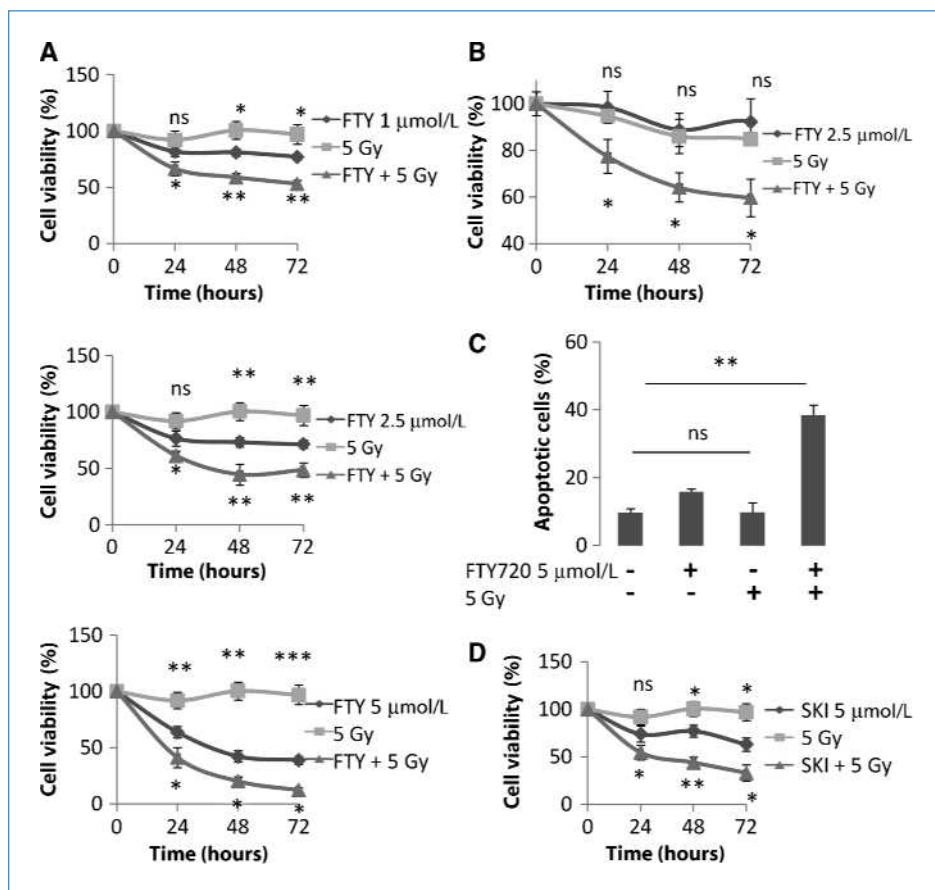
In DU145,  $\gamma$ -irradiation induced an increase in both SphK1 activity and intracellular S1P ( $43.0 \pm 6.1\%$  and  $36.3 \pm 3.6\%$ , respectively), which was completely reversed by the addition of FTY720 ( $-16.6 \pm 4.8\%$  and  $-19.7 \pm 8.2\%$ ; Fig. 5B). In contrast to PC-3 cells, combination of FTY720 and  $\gamma$ -irradiation slightly increased intracellular ceramide (Fig. 5B).

Our data show that in contrast to a modest increase in ceramide, 6 hours of treatment with FTY720 induced a significant, transient increase in intracellular sphingosine (Supplementary Fig. S8A). Furthermore, although  $\gamma$ -irradiation did not influence sphingosine production, it acted in synergy with FTY720 (Supplementary Fig. S8A). We then verified the potential of proapoptotic sphingolipids as radiosensitizers. Supplementary Figure S8B shows that both sphingosine and to a smaller extent ceramide could sensitize PC-3 cells to  $\gamma$ -irradiation. Although extracellular ceramide acted as a radiosensitizer (Supplementary Fig. S8B), blocking the generation of intracellular ceramide had no effect on PC-3 cell survival (Supplementary Fig. S9).

Similarly to blocking PC-3 cell apoptosis induced by FTY720 alone (Fig. 2), SphK1 overexpression abrogated FTY720-induced radiosensitization (Supplementary Fig. S10A). This correlated with a significant decrease in SphK1 inhibition induced by FTY720 in PC-3 cells overexpressing SphK1 (Supplementary Fig. S10B). Similarly to SphK1 overexpression, the addition of extracellular S1P partially blocked PC-3 cell death after treatment with either FTY720 or FTY720 together with  $\gamma$ -irradiation (Supplementary Fig. S11). Although FTY720 alone induced a rapid increase in caspase-3/7 activity (Fig. 1B),

**Figure 3.**  $\gamma$ -Irradiation induces PC-3 and DU145 cell death independently of SphK1. PC-3 and DU145 cells were irradiated once with 5 or 10 Gy and were left to grow for the indicated times. A, cell viability of PC-3 (left) and DU145 (right) cells was assessed by MTT assay. B, SphK1 activity of PC-3 (left) and DU145 (right) cells treated with 5 and 10 Gy. C, relative expression of the *SphK1* gene in PC-3 and DU145 cells treated with 5 and 10 Gy for 24 hours was analyzed using qRT-PCR. Points, columns, mean of three independent experiments performed in triplicate; bars, SE; \*,  $P < 0.05$ ; ns, not significant ( $P > 0.05$ ).





**Figure 4.** FTY720 sensitizes PC-3 and DU145 cells to  $\gamma$ -irradiation. Cells were pretreated for 1 hour with or without FTY720 or SKI-II at indicated concentrations and exposed to 5 Gy  $\gamma$ -irradiation. Cells were then cultured for the indicated time in presence of FTY720 or SKI-II. Cell viability of PC-3 (A, D) and DU145 (B) cells was measured by MTT assay. C, number of apoptotic PC-3 cells after 48 hours of incubation was assessed by flow cytometry after Annexin V/PI staining. Columns and points, mean of three independent experiments performed in triplicate; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant ( $P > 0.05$ ). Top  $P$  values, FTY720 alone versus irradiation; lower  $P$  values, FTY720 alone versus combined treatment.

combined treatment with FTY720 and  $\gamma$ -irradiation did not result in a further activation of executioner caspases (Supplementary Fig. S12A). Moreover, addition of a pan-caspase inhibitor, Z-vad.fmk, did not result in a significant protection from FTY720- or combination treatment-induced cell death (Supplementary Fig. S12B). Finally, to verify the universality of FTY720-induced radiosensitization, we treated androgen-sensitive metastatic LNCaP-C4-2B, PC-3M, and nonmetastatic 22Rv1 prostate cancer cells with FTY720 with or without  $\gamma$ -irradiation (Supplementary Fig. S13). Our data show that FTY720 acted as a radiosensitizer in all three cell lines.

#### FTY720 radiosensitizes human fluorescent PC-3 tumors established in nude mice

Subcutaneous and orthotopic PC-3 tumors were inoculated in nude mice and grown for three weeks. These animals were then treated for two weeks with i.p. injections of PBS (control), 2.5 mg/kg/day FTY720, 5 sessions of  $\gamma$ -irradiation (4 Gy) every 3rd day, or  $\gamma$ -irradiation combined with FTY720.

After 5 weeks s.c. tumors in nontreated animals reached  $2,190 \pm 388 \text{ mm}^3$ , whereas in animals treated with FTY720 alone their volume was  $1,355 \pm 149 \text{ mm}^3$  ( $P = 0.0043$ ).  $\gamma$ -Irradiation alone diminished the tumor volume to  $870 \pm 30 \text{ mm}^3$  ( $P < 0.0001$  versus control;  $P = 0.0007$  versus FTY720), whereas combined treatment led to the most signif-

icant reduction in tumor volume at  $390 \pm 70 \text{ mm}^3$  ( $P < 0.0001$  versus  $\gamma$ -irradiation; ANOVA;  $P < 0.0001$ ; Fig. 6A).

Orthotopic PC-3 tumors showed a similar response to the treatments:  $469 \pm 41 \text{ mg}$ ,  $281 \pm 26 \text{ mg}$ ,  $185 \pm 13 \text{ mg}$ , and  $34 \pm 12 \text{ mg}$  in mice treated with control, FTY720,  $\gamma$ -irradiation, or combined treatment respectively ( $P < 0.0001$ , ANOVA;  $P < 0.0001$ ,  $t$ -test  $\gamma$ -irradiation versus combined treatment; Fig. 6B).

Figure 6C shows that although  $\gamma$ -irradiation alone did not significantly affect the levels of tumor SphK1 activity ( $100 \pm 5.0$  versus  $89.5 \pm 7.9$ , not significant); in animals receiving control or  $\gamma$ -irradiation, respectively), treatment with FTY720 or a combination of FTY720 with  $\gamma$ -irradiation significantly reduced SphK1 activity ( $76.3 \pm 6.6$  versus  $56.2 \pm 9.4$  in animals treated with FTY720 or a combination of FTY720 with  $\gamma$ -irradiation, respectively). Levels of tumor S1P were lower in animals treated with combination therapy than in animals receiving  $\gamma$ -irradiation alone. The difference between intracellular ceramide levels in all four groups did not reach significance (Fig. 6C).

The effect of combination therapy on primary tumor growth was paralleled by a significant reduction of distant metastasis (adrenal, liver, and lungs, excluding aortic lymph nodes as being a primary site for prostate cancer metastasis; positive in all animals) with 63% (5 of 8) of animals free of distant metastases (compared with 100% in both untreated

and FTY-treated groups, and 25% in irradiated animals, with 2 of 8 metastases-free; Fig. 6D).

Fluorescent imaging revealed that FTY720 alone did not affect the number of secondary tumors ( $3.33 \pm 0.44$  versus  $3.14 \pm 0.54$  metastases/mouse in control and FTY720 groups, respectively,  $P$  not significant), whereas  $\gamma$ -irradiation significantly reduced the number of distant metastases ( $1.43 \pm 0.29$  metastases/mouse). Combination treatment further reduced the number of distant tumors ( $0.55 \pm 0.23$  metastases/mouse,  $P = 0.0366$  in comparison with  $\gamma$ -irradiation alone; Fig. 6D).

#### FTY720 and $\gamma$ -irradiation reduce WBC counts in nude mice

In nude mice a single 2.5 mg/kg dose of FTY720 induced a rapid reduction of leukocyte blood counts which within 3 days normalized to control levels (Supplementary Fig. S14A). To identify the impact of prolonged FTY720 treatment on blood cell counts, blood was analyzed at the time of sacrifice (two days after the last treatment with FTY720). Mice treated with FTY720 alone had a moderate  $\sim 30\%$  reduction in total circulating leukocytes and lymphocytes, and no changes were observed in peripheral macrophages and granulocytes (Supplementary Fig. S14B), as reported previously (36, 37).

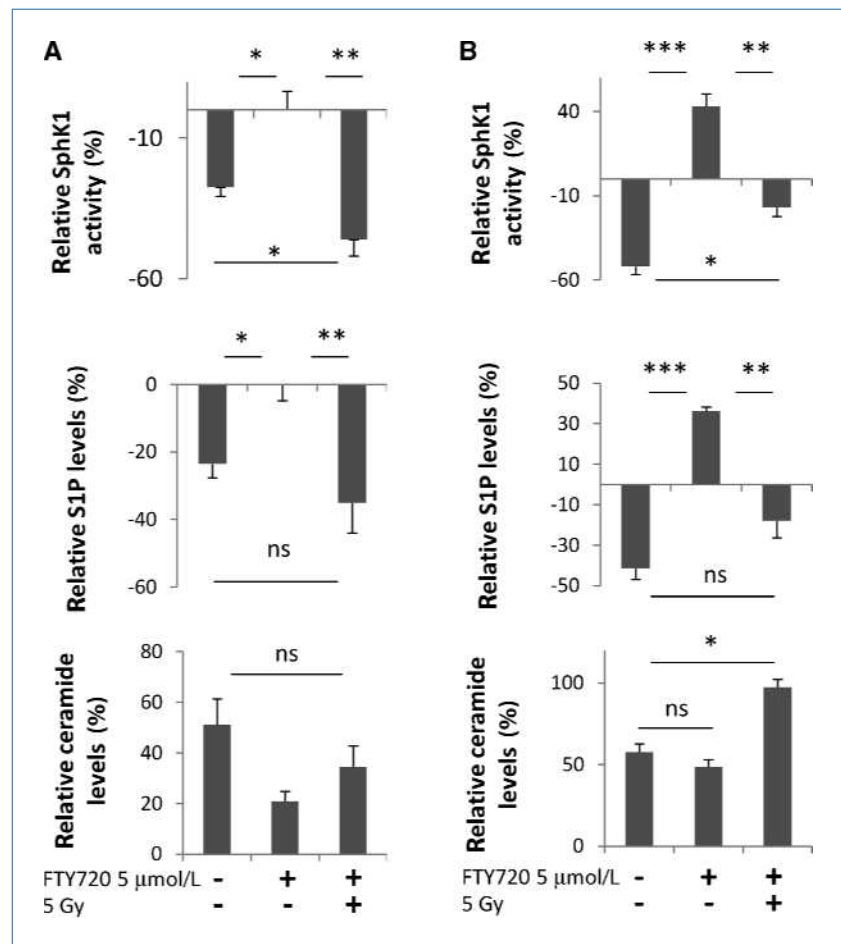
## Discussion

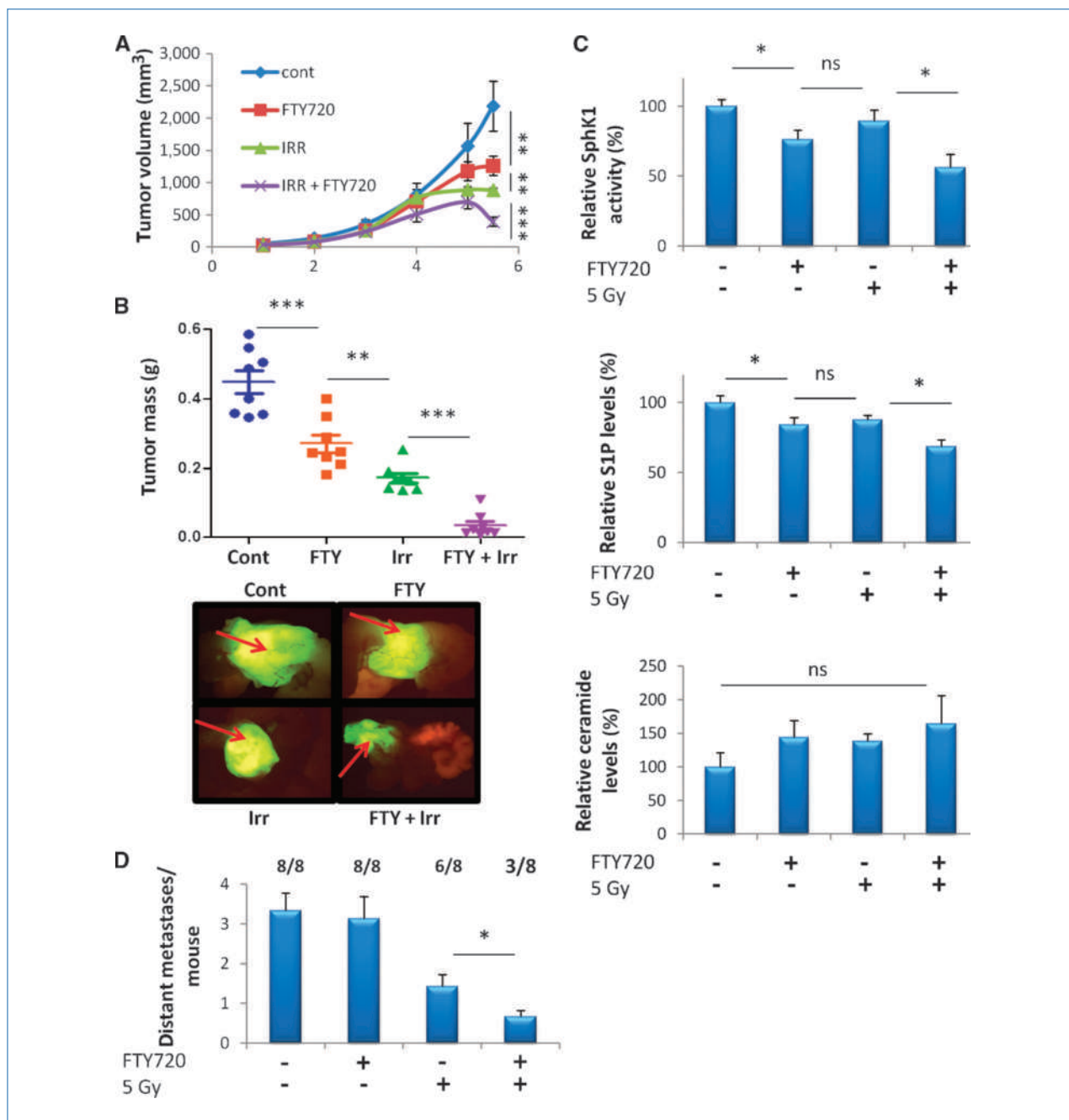
In this current study we provide compelling evidence that FTY720 is a SphK1 inhibitor and a potent radiosensitizer of human prostate cancer both *in vitro* and *in vivo*.

Several lines of evidence suggest that FTY720 has an anti-proliferative potential in prostate cancer cells. FTY720 was previously reported to induce apoptosis in DU145 cells (21, 34). Here we show for the first time that FTY720 is a strong inducer of apoptosis in hormone-refractory, metastatic prostate cancer PC-3 cells. FTY720-induced apoptosis in PC-3 cells was dose dependent and was preceded by upregulation of caspase 3 and 7 activity (Fig. 1).

FTY720 has been previously reported to inhibit SphK1 *in situ* (35). In our study we show for the first time that FTY720 has a capacity to inhibit SphK1 enzymatic activity *in vitro* (Fig. 2) and that this inhibition is not related to a reduction in SphK1 expression (Supplementary Fig. S1A). SphK1 inhibition seems to be a crucial step in FTY720-induced prostate cancer apoptosis, because SphK1 overexpression exerts a cytoprotective effect (Fig. 2). This cytoprotection is partial, however, which indicates the existence of parallel, SphK1-independent mechanisms of

**Figure 5.** FTY720 radiosensitizes PC-3 and DU145 cells by SphK1 inhibition. PC-3 (A) and DU145 (B) cells were pretreated for 1 hour with 5  $\mu\text{mol/L}$  FTY720 and exposed to 5 Gy  $\gamma$ -irradiation. Cells were incubated for 24 hours, and SphK1 activity (top), S1P (middle), and ceramide content (bottom) were measured. Columns, mean of three independent experiments performed in triplicate; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant ( $P > 0.05$ ).





**Figure 6.** FTY720 radiosensitizes human fluorescent PC-3 tumors established in nude mice. Three weeks after tumor implantation, mice were randomized into four groups and subjected to daily i.p. injections of 2.5 mg/kg FTY720 (total dose 35 mg/kg), 4 Gy radiotherapy every 3 days (total dose 20 Gy), a combination of these treatments, or a sham treatment by i.p. injections. A, volume of s.c. tumor measured with a caliper at weekly intervals. B, tumor mass of excised primary green fluorescent protein (GFP)-labeled tumor (top) and representative fluorescent primary prostate tumors at the time of autopsy from groups treated with sham (Cont), FTY720,  $\gamma$ -irradiation (Irr), and a combination of FTY720 and  $\gamma$ -irradiation (bottom). Red arrows, primary orthotopic GFP tumors. C, SphK1 activity (top), and S1P (middle) and ceramide (bottom) levels were measured in tissue extracts obtained from primary tumors. D, number of distant metastases (lungs, liver, pancreas, mesenteric and kidney) per animal. Columns, points, means of 8 animals; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant ( $P > 0.05$ ).

FTY720-induced prostate cancer apoptosis. We verified the involvement of several previously suggested mechanisms of FTY720-induced cancer cell apoptosis in our system. In contrast to La Montagne et al. (24), knockdown of S1P re-

ceptors showed that FTY720-induced cell death was S1P receptor independent, suggesting an intracellular mode of action (Supplementary Fig. S2). Similarly to Azuma et al. (26), we found that FTY720 induced a downregulation of



Akt phosphorylation (Supplementary Fig. S3). This effect was SphK1 dependent, however, because it could be mimicked by SphK1 inhibition and restored by SphK1 overexpression. Finally, our data show that FTY720-mediated ceramide accumulation was not responsible for FTY720-induced apoptosis because pretreatment of prostate cancer cells with inhibitors of sphingomyelinases and *de novo* ceramide synthesis pathway did not abrogate FTY720-induced apoptosis (Supplementary Fig. S4). Overall our data suggest a SphK1-mediated S1PR-independent mechanism of prostate cancer cell apoptosis induced by FTY720.

Sphingolipids have previously been shown to play a role in prostate cancer radioresistance. Radioresistant prostate cancer cell lines are deficient in the generation of proapoptotic ceramide and sphingosine, and the addition or increased production of these lipids is radiosensitizing (3, 38, 39). In contrast to completely radioresistant, androgen-sensitive LNCaP prostate cancer cells, both DU145 and PC-3 hormone-refractory metastatic prostate cancer cells respond to  $\gamma$ -irradiation in a dose-dependent manner. With this in mind, one of the major aims of our study was to investigate possible therapeutic dose intensification. In contrast to several DNA-damaging therapies that were previously shown to downregulate SphK1 activity (13, 40, 41), here we report that in both PC-3 and DU145 metastatic prostate cancer cell lines  $\gamma$ -irradiation-induced apoptosis was not associated with SphK1 inhibition (Fig. 3) or an increase in intracellular ceramide (Fig. 5). Interestingly in DU145 cells, irradiation even induced a slight activation of SphK1 (but not through transcription), an effect observed in several cancer cell lines treated with DNA-damaging agents (13, 42). We therefore conclude that in metastatic prostate cancer cells  $\gamma$ -irradiation does not regulate the SphK1/S1P pathway, which suggests that its external manipulation may provide benefit in terms of dose intensification.

We have shown that SK1 inhibition leads to a promotion of the effects of cytotoxic chemotherapy on cell lines (13, 14, 16). Although metastatic prostate cancer cells are partially responsive to  $\gamma$ -irradiation, sustained SphK1 activity may provide an "escape from apoptosis" for these cells. We hypothesized that SphK1 inhibition may, in addition to its effects on chemotherapy, serve as potential radiosensitizer. Here we show for the first time that FTY720 can sensitize PC-3, DU145, LNCaP-C4-2B, PC-3M, and 22Rv1 prostate cancer cells to irradiation by apoptosis induction in a dose-dependent fashion (Fig. 4, and Supplementary Figs. S5, S6, and S13). Our data suggest that FTY720-mediated radiosensitization is mediated by SphK1 inhibition (Fig. 5) and is similar to the effects that can be achieved by the SphK inhibitor SKI-II or SphK1 siRNA (Fig. 4, Supplementary Fig. S7). Conversely, the radiosensitization can be blocked by SphK1 overexpression (Supplementary Fig. S10) or extracellular SIP (Supplementary Fig. S11). Interestingly, although FTY720 induced only a small increase in ceramide (Fig. 5), it induced a significant transient increase in intracellular sphingosine (Supplementary Fig. S8A) acting in synergy with  $\gamma$ -irradiation. As

previously reported (3), both sphingosine and ceramide can act as radiosensitizers (Supplementary Fig. S8B), but blocking the generation of intracellular ceramide has no effect on PC-3 cell survival (Supplementary Fig. S9), confirming that its low intracellular levels are not sufficient to induce prostate cancer cell death. Our data show that FTY720-induced radiosensitization is caspase independent (Supplementary Fig. S12), suggesting that the depletion of prosurvival signaling (e.g., Akt, SphK1/S1P) may be the major mechanism of FTY720-induced apoptosis/radiosensitization.

This implies that although  $\gamma$ -irradiation can induce prostate cancer cell death, its effect can be potentiated by the downregulation of SphK1 activity. These data correlate with our previous findings on SphK1 downregulation potentiating the effects of docetaxel chemotherapy in prostate cancer (14, 16). These results are also in line with our previous findings in which SphK1 inhibition by dimethylsphingosine was shown to sensitize completely radioresistant androgen-sensitive LNCaP prostate cancer cells to  $\gamma$ -irradiation (3).

The efficacy of FTY720 in animal cancer models including prostate cancer was shown previously (29, 43). Here we show for the first time that FTY720 can radiosensitize hormone-refractory metastatic human prostate cancer tumors established in nude mice. Our *in vivo* data show that treatment with FTY720 alone decreased the size of both s.c. and orthotopic tumors (Fig. 6). Furthermore, cotreatment with FTY720 resulted in a further reduction in both orthotopic and s.c. tumors treated with  $\gamma$ -irradiation. In our models the efficacy of FTY720 was correlated to a decrease in tumor SphK1 activity and SIP levels (Fig. 6C).

At the selected dose (2.5 mg/kg/day), treatment with FTY720 alone had only a moderate effect on primary tumor growth (Fig. 6). In contrast to previous data showing the ability of FTY720 to block cell invasion in prostate cancer three-dimensional cell cultures (28), in our model treatment with FTY720 alone did not influence the number of distant metastases. Surprisingly  $\gamma$ -irradiation alone led to a significant reduction in the number of distant metastases, which was further enhanced by cotreatment with FTY720, suggesting a synergetic mechanism of action between FTY720 and  $\gamma$ -irradiation.

In accordance with previous studies, prolonged treatment with 2.5 mg/kg/day FTY720 led to a moderate lymphopenia (Supplementary Fig. S14B). The major reported complication of FTY720 is a transient decrease in the heart rate due to a "first dose" effect, and no toxic effects had been observed in healthy volunteers treated for 7 consecutive days with 5 mg/day FTY720 (44, 45). At the used dose (2.5 mg/kg/day) we did not observe any toxicity or gross abnormalities (like weight loss or i.p. inflammation) in animals treated with FTY720. It is worth noting that until recently no clinically tested SphK1 inhibitors were available. Conversely, recent data from phase III trials show clinical safety and significant potential of oral FTY720 (fingolimod) in multiple sclerosis patients (19, 20).

In conclusion, here we show for the first time that FTY720 induces prostate cancer apoptosis and radiosensitization through SphK1 inhibition. Our data draw attention to the potential of FTY720 as an adjuvant treatment for local tumor control in prostate cancer patients treated with radical radiotherapy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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