RESEARCH ARTICLE

Bortezomib Enhances the Antitumor Effects of Interferon-β Gene Transfer on Melanoma Cells

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Abstract: We evaluated the cytotoxic effects of the combination of bortezomib (BTZ) and interferon-β (IFNβ) gene lipofection on cultured melanoma cells. Four canine mucosal (Ak, Br, Bk and Ol) and two human dermal (A375 and SB2) melanoma cell lines were assayed. BTZ sub-pharmacological concentrations (5 nM) enhanced the cytotoxic effects of IFNβ transgene expression on melanoma cells monolayers and spheroids. The combination was also more effective than the single treatments when assayed for clonogenic survival and cell migration. The combined treatment produced a significant raise of apoptosis evidenced by DNA fragmentation as compared to either BTZ or IFNβ gene lipofection single treatments. Furthermore, BTZ significantly increased the intracellular reactive oxygen species (ROS) generation induced by IFNβ gene transfer in melanoma cells, an effect that was reversed by the addition of the ROS inhibitor N-acetyl-L-cystein. The present work encourages further studies about the potential of the combination of interferon gene transfer with proteasome inhibitors as a new combined therapy for malignant melanoma, both in veterinary and/or human clinical settings.

1. INTRODUCTION

Malignant melanoma is a fast growing form of skin cancer with increasing global incidence [1]. Whereas most melanomas diagnosed at an early stage can often be treated with surgery alone, a proportion of these cancers may present a loco-regional or a systemic disease recurrence, conferring poor prognosis [2]. Clinically, canine malignant melanoma is like human melanoma. Both diseases share comparable chemo-resistance, radio-resistance, metastatic phenotypes and site selectivity, and poorly react to treatments with conventional biological response modifiers [3-5].

The Food and Drug Administration (FDA) approved an adjuvant therapy with systemic IFNα-2b for patients with stage IIb or III resected melanoma that improves disease free survival (3.8 vs. 2.8 years) [6]. Nevertheless, the overall survival benefit of this treatment is rather small [7]. Interferon-β (IFNβ) displays antitumor effects against melanoma that are frequently more potent than those produced by IFNα [8,9]. IFN α/β antitumor effects appear as a combination of direct antiproliferative action and indirect immune modulation and angiogenesis inhibition [10]. Delivery of a gene encoding IFN provides an optional approach for IFN-based therapy for melanoma that allows a continuous exposure to IFN protein released by both tumor and non-tumor cells [11].

Bortezomib (BTZ) is the first FDA-approved proteasome inhibitor for cancer treatment. In addition to its well established clinical efficacy in patients with multiple myeloma and mantle cell lymphoma, bortezomib has been used in many other cancers including: lung cancer, breast cancer, prostate cancer, pancreatic cancer, head and neck cancer, and colon cancer [12]. BTZ was not significantly effective for melanoma patients. In a clinical trial only 22% of the patients displayed objective responses to BTZ, while systemic toxicity limited the assay of higher doses [13]. BTZ is not usually sufficient for apoptosis induction of melanoma cells, and

many *in vitro* studies demonstrated that its combination with other antineoplastic showed a strong antitumor activity [12]. For instances, BTZ synergistically interacted with IFNα gene expression on the reversion of antiapoptotic proteins over-expression, promoting the apoptosis induction in bladder carcinoma cells [14]. In a murine melanoma model, encouraging results were also obtained when BTZ was combined with temozolomide [15].

Tumor spheroids are heterogeneous cellular aggregates with multiple tridimensional intercellular connections, higher levels of anti-apoptotic proteins and internal regions with a low cell proliferation rate due to a lesser accessibility of oxygen and nutrients. Compared to monolayers, spheroids based assays are superior indicators of the responses to the treatments for solid tumors [16-18].

We have previously reported that IFNβ gene lipofection and expression displays a bystander effect and inhibits cell adhesion and migration in human and canine melanoma cells [19,20]. But in two canine melanoma cell lines (*Ol* and *Br*) cIFNβ gene expression was not able of inducing a cytotoxic effect and in two human melanoma cell lines (*A375* and *M8*), a significant multicellular resistance was observed in cells cultured as spheroids.

In this context we explored the potential of the combination of IFNβ with sub-pharmacological amounts of BTZ to increase the antitumor activity of IFNβ gene transfer.

2. MATERIALS AND METHODS

2.1. Cell Lines

Cultured cells derived from four surgically excised oral (*Bk*, *Br*, *Ol*) and ocular (*Ak*) canine melanomas were obtained as described [19]. The three canine melanomas and *A375* and *SB2* human cutaneous melanoma [21] were cultured as monolayers and spheroids as described [19]. Briefly, multicellular spheroids were generated by adding cell suspensions to pre-coated 96-well plates. Pre-coating was performed by using the liquid overlay technique: agar (Sigma-Aldrich, Argentina) was diluted to 1.5 % (w/v) with serum-free medium and coated onto each well, preventing cell adhesion [19].

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2.2. Plasmids

Plasmids carrying the *Escherichia coli* β-galactosidase gene (psCMV-βgal) [22], human IFNβ gene (psCMV-hIFNβ) [23] or canine IFNβ gene (psCMV-cIFNβ) [24] under CMV enhancer/promoter control, were amplified in *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA), grown in LB medium containing 100 mg/ml neomycin and purified by ion-exchange chromatography (Qiagen, Valencia, CA). Plasmid DNA for injection was resuspended to a final concentration of 2.0 mg/ml in sterile PBS.

2.3. Liposome Preparation and *in vitro* Lipofection

DC-Chol (3β[N-(N',N'-dimethylaminoethane)-carbamoyl cholesterol) and DMRIE (1,2-dimyristyl oxypropyl-3-dimethyl-hydroxyethylammonium bromide) were synthesized and kindly provided by BioSidus (Argentina). DOPE (1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine) was purchased from Sigma. Liposomes were prepared at lipid/co-lipid molar ratios of 3:2 (DC-Chol:DOPE) or 1:1 (DMRIE:DOPE) by sonication as described [25, 26]. Optimal lipid mixtures were determined for every cell lines.

In most experiments, cells were seeded into 12-well plates at a density of $3\text{-}6 \times 10^4$ cells/cm^2 and were allowed to adhere overnight. Monolayers were exposed to lipoplexes (0.5 μg plasmid DNA/cm^2 and 1 μl liposome/cm^2) from 2 to 5 h in a serum-free medium. Then the lipofection medium was replaced with fresh complete medium. The human and canine cells were respectively lipofected with psCMV-hIFNβ and psCMV-cIFNβ.

2.4. β-galactosidase Staining Assay

Lipofection rates were checked 24 h after lipofection by β-galactosidase staining with 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-GAL, Sigma) to ensure that they were comparable in different experiments and counted using an inverted phase contrast microscope [19].

2.5. Cell Growth Assay

Cells were seeded onto 96-well plates at $1\text{-}5 \times 10^4$ cells/well 24h after lipofection. After additional 24 h incubation at 37°C, cells were treated with bortezomib (BTZ, Janssen Cilag Farmacéutica, Argentina). After 4 days as monolayers (ML) or after 11 days as spheroids (SP), cell the medium was removed and the culture was washed with phosphate-buffered saline (PBS). Then, 100 μl/well of the assay buffer (0.1 M sodium acetate, 0.1% Triton-X-100, 2 mg/ml p-nitrophenyl phosphate; Sigma) was added and incubated for 60 (ML) or 90 min (SP) at 37°C. Following incubation, 10 μl of 1 N NaOH was poured to each well, and the absorbance was read at 405 nm in a microplate analyzer [19]. Data were normalized as a percentage of the value of the corresponding untreated cells.

2.6. Clonogenic Survival Assay

Twenty-four hours after lipofection, cells were treated with BTZ (3 or 5 nM) for 24 hours. Then, cells were washed with PBS, trypsinized to form a single-cell suspension, counted and cell number equalized for each experimental condition. Then, cells were seeded at low density ($1\text{-}3 \times 10^2$ cells/well) on 6-well plate in duplicate and incubated 10 days for colonies growth. Colonies were washed with PBS, fixed with methanol, and stained with crystal violet [27]. Data were normalized as a percentage of the value of untreated non-lipofected cells.

2.7. Cell Migration Assay

Cell migration was determined by wound healing assay on 6-well plates [28]. Twenty-four hours after lipofection, cells were treated with BTZ (5 nM) for 1 hour. Cells were then washed with PBS, and a single wound was created to each well and healing was evaluated 17 h later as described [20]. Briefly, cells detached by the wound were removed by washing twice with serum-free medium and cultures were then supplemented with complete medium, serum-free medium or conditioned medium. Immediately or 17 h after incubation at 37°C, photographs were taken in an inverted phase contrast microscope (Nikon Eclipse TE2000-S, Japan). Gap distances were measured on 4 visual fields using Image-Pro Plus software and distance changes were calculated as follows: change = (average gap distance at time 0h) - (average gap distance at time 17h). After photography, cell number was estimated by using the MTS Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. During this assay there were no significant differences in cell number between control- or βgal-, and IFNβ-treated cells. Relative cell migration was calculated by dividing the change in the gap distance of treated cells by that of the untreated control cells in each experiment.

2.8. Cell Cycle Analysis and Quantification of DNA Fragmentation

DNA fragmentation was measured by propidium iodide staining as previously described [29]. Twenty-four hours after lipofection, cells were treated with BTZ (5 nM) for 24 hours. Then, cells were washed, trypsinized, fixed in 70% (v/v) ethanol at -20 °C for 1 h, treated with RNase, stained with 10 mg/ml propidium iodide for 30 min, and analyzed by fluorescence flow cytometry. Cells displaying a hypodipoid content of DNA indicative of DNA fragmentation were scored as apoptotic.

2.9. Measurement of Cellular Reactive Oxygen Species (ROS) Production

Twenty-four hours after lipofection, cells were treated (or not) with BTZ (5 nM) for 18 hours. Then, cells were trypsinized, washed with PBS and incubated with 5 mM H$_2$DCF-DA (Sigma) in PBS for 20 min at 37 °C. The cells were then washed with PBS, resuspended in complete medium and analyzed by fluorescence flow cytometry using a 485-nm excitation beam and a 538-nm band pass filter. The mean fluorescence intensity was quantified using WinMDI software. Data were normalized as a percentage of the value of the untreated non-lipofected control cells.

2.10. Statistical Analysis

Data were represented as the means ± standard error of the mean (SEM) from three or more independent experiments. Results were analyzed using two-way ANOVA followed by Tukey's test. P values of <0.05 were considered statistically significant.

3. RESULTS

3.1. IFNβ Gene Expression Diminished Bortezomib IC$_{50}$ in Melanoma Cells Monolayers

As a first step in the study of possible advantageous antitumor interactions between IFNβ and the proteasome inhibitor bortezomib (BTZ), we determined the BTZ 50% inhibitory concentrations (IC$_{50}$) after IFNβ gene lipofection in six melanoma cell lines cultured as monolayers. BTZ/IC$_{50}$ was lower than 10nM in all control unlipofected cell lines (Fig. **1A**, Table **1**). While canine melanoma *Ak*, *Bk* and *Br* were more sensitive to BTZ (IC$_{50} \leq 5.5$ nM), canine *Ol* and human *SB2* and *A375* melanoma cells were less sensitive (IC$_{50} \geq 6$ nM). IFNβ gene lipofection induced a significant decrease of BTZ/IC$_{50}$ in 5 out of 6 evaluated cell lines as compared to βgal-lipofected cells. Even though *Ol* cells were resistant to IFNβ gene lipofection alone, this treatment sensitized them to BTZ. In *Br* cells this effect was not detected, probably due to the low lipofection rate
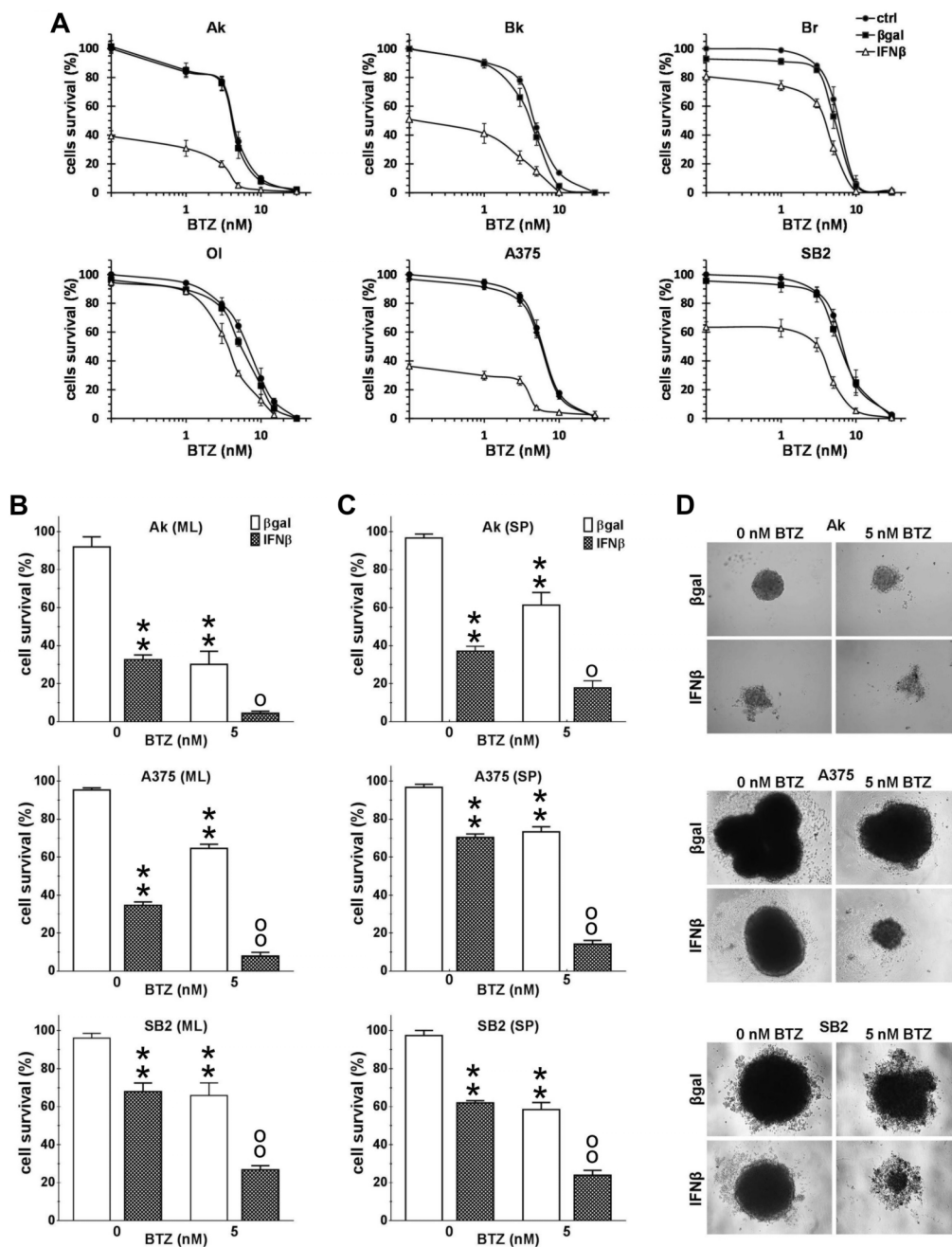
Fig. (1). Effect of IFNβ lipofection on the sensitivity of melanoma cells to bortezomib -BTZ- (A). *In vitro* dose-response curves of BTZ for control non-lipofected (ctrl), β-galactosidase (βgal)-lipofected, IFNβ-lipofected melanoma monolayers. Cytotoxic effects of IFNβ lipofection plus treatment with BTZ on monolayers -ML- (B) and spheroids -SP- (C).Transiently β-gal or IFNβ-lipofected melanoma cells were cultured as ML or SP, and exposed to BTZ (5 nM). Cell growth was quantified by APH, as described in Materials and methods. (D) Representative images of the spheroids treated as described in (**C**) at 100X magnification.
***p≤0.001; **p≤0.01 with respect to βgal; °°p≤0.01, °p≤0.05 with respect to βgal, IFNβ and βgal plus BTZ.

Table 1.   IC50s (nM) for bortezomib (BTZ) and plasmid lipofection rates (PLR).

IC50s	Ak	Bk	Br	Ol	A375	SB2
Control	4.44 ± 0.20	5.33 ± 0.29	5.33 ± 0.58	7.06 ± 0.76	6.41 ± 0.07	7.04 ± 0.46
βgal	4.22 ± 0.31	4.30 ± 0.27	5.10 ± 0.29	5.98 ± 0.55	6.08 ± 0.16	6.76 ± 0.66
IFNβ	2.78 ± 0.42*	2.47 ± 0.15**	4.34 ± 0.17	3.44 ± 0.28*	3.64 ± 0.24***	4.55 ± 0.35*
PLR	28.0 ± 1.90	5.00 ± 0.90	2.40 ± 0.30	8.50 ± 0 .80	7.50 ± 1.00	1.70 ± 0.30

IC50s values were derived from the dose-response curves displayed in Fig. **2A**. PLR were determined as described in Materials and methods. ***p≤0.001;**p≤0.01*p≤0.05 with respect to βgal and unlipofected control cells.

(2.4 ± 0.3 %) and the particular low sensitivity to IFNβ gene lipofection alone. Nevertheless, this effect was significant in *SB2* cells that displayed an even lower lipofection rate (1.70 ± 0.30%).

3.2. Bortezomib Sub-pharmacological Concentrations Enhanced the Cytotoxic Effects of IFNβ Gene Expression on Melanoma Cells Monolayers and Spheroids

The effect of the combination of IFNβ gene lipofection and 5 nM BTZ (half of the pharmacologically relevant BTZ concentration) in monolayers and spheroids was assayed in one highly (*Ak*) and two lowly (*A375* and *SB2*) BTZ-sensitive cell lines. The treatment with BTZ alone displayed a significant cytotoxic effect in the three cell lines in both spatial configurations and this effect was enhanced by IFNβ gene lipofection (Fig. **1B, C** and **D**).

While in monolayers the extent combined cytotoxic effects were *Ak>A375>SB2*, in spheroids they were *A375>Ak>SB2*. In all the cases the combined effects were much higher than a simple addition.

Previous results showed that lipofection with IFNβ gene lacking the N-terminal secretion signal (signal-free interferon, hIFNβsf) is cytotoxic for *SB2* monolayers and spheroids. Conversely, A375 monolayers and spheroids were not sensitive to hIFNβsf gene lipofection [20]. When the cytotoxic effect of combining hIFNβsf gene lipofection and 5 nM BTZ was assayed in *SB2* monolayers and spheroids, the effect was similar to that obtained with unmodified hIFNβ. However, in *A375* this enhancement effect was absent (data not shown).

3.3. Bortezomib Enhanced the Effects of IFNβ Gene Expression on Melanoma Cells Clonogenic Survival

A clonogenic assay was performed to study long term cells survival after the combined treatment. Concentrations of 3 and 5 nM BTZ were used for the more (*Ak*, *Bk* and *Br*) and less sensitive cell lines (*Ol*, *A375* and *SB2*) respectively. While BTZ alone diminished the clonogenic survival in the six evaluated cell lines, IFNβ gene lipofection alone did so in five (*Br* excluded) (Fig. **2A** and **B**). In accordance with the cytotoxicity assays (Fig. **1**), the combined were more effective than the single treatments with the exception of *Br* cell line.

3.4. Bortezomib Increased the Effects of IFNβ Gene Expression on Melanoma Cells Migration

The initial step of tumor metastasis is a process of invasive tumor cell migrating in basement membrane, which implicates cell adhesion and migration as well as proteolysis of the extracellular matrix [30]. We have previously reported that IFNβ gene lipofection inhibits cell adhesion and migration through the induction of reactive oxygen species (ROS) [19].

By using the wound healing assay we evaluated the effects of the combined treatments on *Ol* cell monolayers. BTZ (5nM) significantly increased the inhibitory effect of IFNβ gene lipofection on *Ol* cells, even though in a limited extent (about 14%) (Fig. **2C** and **D**).
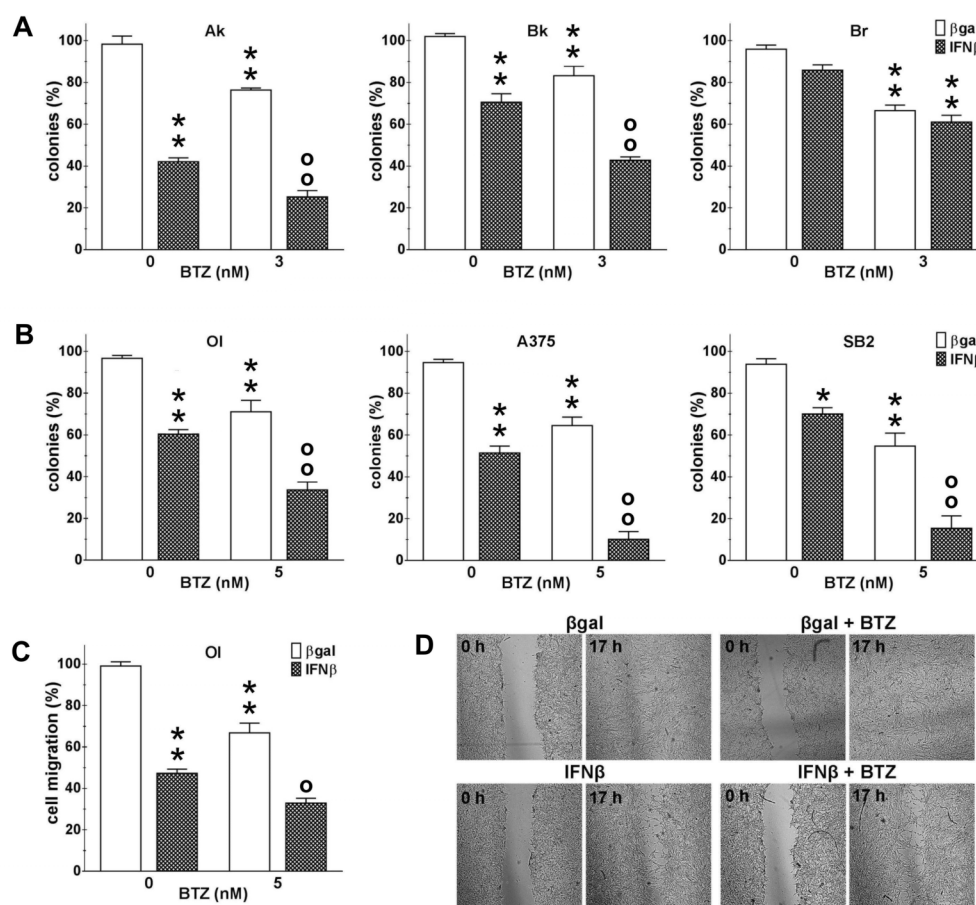

Fig. (2). Effect of the IFNβ gene plus BTZ combined treatment on clonogenic survival A, B) and cell migration (C). (A, B) BTZ was added 24 h post-lipofection at 3 nM (A) or 5 nM (B) concentrations. Then cells were harvested and assayed as described in Materials and methods. *p≤0.05; **p≤0.01 with respect to βgal; °°p≤0.01 with respect to βgal, IFNβ and βgal plus BTZ. (C) Cell migration was determined by wound healing assay, as described in Materials and methods. **p≤0.01 with respect to βgal; °p≤0.05 with respect to βgal, IFNβ and βgal plus BTZ.

3.5. Combined (but not Single) Treatments of Bortezomib and IFNβ Gene Transfer Induced Melanoma Cells Apoptosis

To evaluate the apoptosis induction by the combined BTZ plus IFNβ gene transfer treatment in the three slightly sensitive cell lines (*Ol*, *A375* and *SB2*), post treatment DNA fragmentation was measured. The levels of DNA fragmentation observed in the three cell lines treated with 5nM BTZ or IFNβ gene lipofection alone were not significantly different from the levels observed in cells treated with βgal gene. However, the combined treatment produced a significant increase of DNA fragmentation in *A375* and *SB2* (Fig. **3A**). In *Ol* cells, this increase was significant when compared to βgal-lipofected cells, but not compared to βgal + BTZ. In *A375* and *SB2* cell lines, no significant differences in the cell cycle were found after any single o combined treatments (data not shown). In *Ol* cell line the single treatment with BTZ induced a G2/M arrest that was partially reversed by the combination with IFNβ gene transfer (Fig. **3B**). As it was reported [31], BTZ induces a G2/M arrest in colon cancer cells through the ATM-CHK1 kinase phosphorylation induced by the ROS increase.

3.6. Bortezomib Substantially Enhanced the Intracellular ROS Generation Induced by IFNβ Gene Transfer in Melanoma Cells

Many laboratories reported a BTZ induced ROS overproduction that induces apoptosis in diverse tumor varieties [32,33]. Furthermore, we have previously demonstrated that IFNβ gene expression triggers a significant increase of ROS intracellular levels in *A375* and *SB2* cell lines [19]. The H_2DCF-DA probe was used to evaluate the effects of the combined treatment on intracellular ROS levels 18 h after the BTZ addition. In *Ol* and *SB2*, both single treatments increased intracellular ROS, and the combined treatment induced a significant additional increase (Fig. **3C**). On the other hand in *A375*, the combined treatment induced a significant ROS increase as compared to βgal-lipofected cells, but not as compared to IFNβ-lipofected cells in the absence of BTZ. Taking into account that 24 h after the BTZ addition A375 display a high percentage of

apoptotic cells, this behavior could be due to the chosen late time (17 h) for ROS measurement. As final result, in 2 out of 3 cell lines BTZ increased ROS levels.

3.7. The Antioxidant L-NAC Reversed the Enhanced Effect of the Combined Treatment in Melanoma Cells

To ascertain the role of ROS production on the cytotoxicity displayed by the BTZ plus IFNβ gene combination, the effects of the antioxidant N-acetyl-L-cystein (LNAC) addition were evaluated in *A375* and *SB2* cell lines.

Though IFNβ gene transfer produced a significant intracellular ROS increase (Fig. **3C**), the LNAC (2mM) addition 48 h after lipofection did not affect the efficacy of this single treatment in both cell lines growing as monolayers or spheroids (Fig. **4A** and **B**). Besides in the presence of LNAC, BTZ kept a portion of its cytotoxic efficacy but it was not able of enhancing the effect of IFNβ-lipofection. Then after LNAC addition, the effect of the combined treatment did not significantly differ from the effect of the single IFNβ-lipofection. On the other hand LNAC reduced about 50% the percentage of apoptotic cells induced by the combined treatment (Fig. **4C**). As expected, LNAC blocked the ROS levels increase triggered by the combined treatment (Fig. **4D**). Therefore intracellular ROS generation would be a critical event for the enhanced effects of the combination of IFNβ gene transfer and BTZ.

4. DISCUSSION

Previous results from our laboratory showed that IFNβ lipofection significantly diminished BTZ IC_{50} in *M8* human melanoma cell line [23]. In the present report we bring new data indicating that such behavior is shared by most of the assayed melanoma cell lines (5 out of 6).

BTZ enhanced the antitumor effects induced by IFNβ gene expression on cell survival (Fig. **1B** and **C**), clonogenic survival (Fig. **2A** and **B**), cell migration (Fig. **2C**) and apoptosis induction
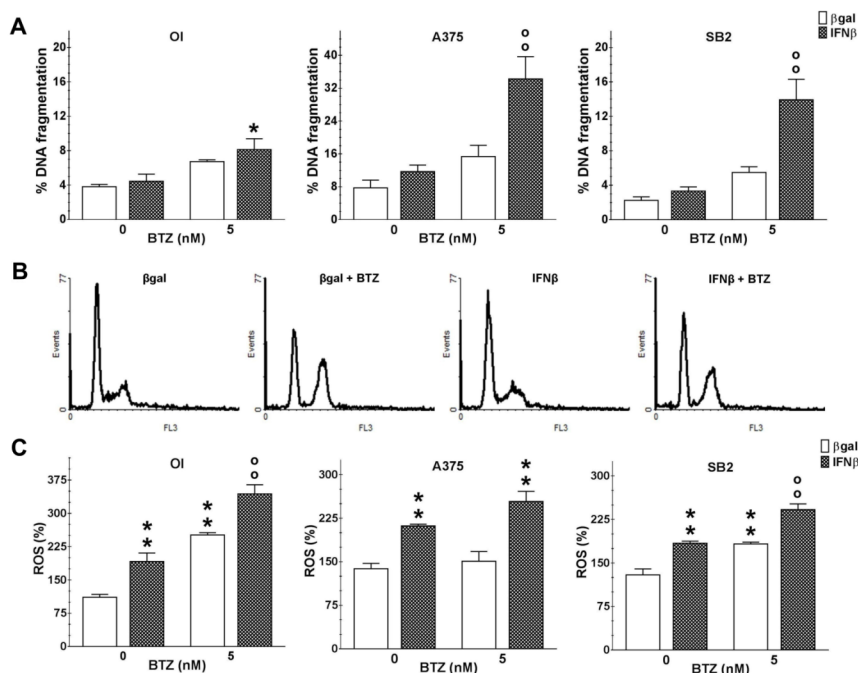
Fig. (3). Effect of the IFNβ gene plus BTZ combined treatment on apoptosis (A), cell cycle (B) and intracellular ROS levels (C). (A, B) Twenty-four hours after lipofection, cells were treated with 5 nM BTZ for 24 hours. DNA fragmentation and cell cycle alterations were evaluated by flow cytometry after propidium iodide staining, as described in Materials and methods. (B) *Ol* cell cycle analysis. (C) Twenty-four hours after lipofection, cells were treated with BTZ (5 nM) for 18 hours. DCF dye was used to measure reactive oxygen species (ROS) production, as described in Materials and methods. *p≤0.05; **p≤0.01 with respect to βgal; °°p≤0.01 with respect to βgal, IFNβ and βgal plus BTZ.
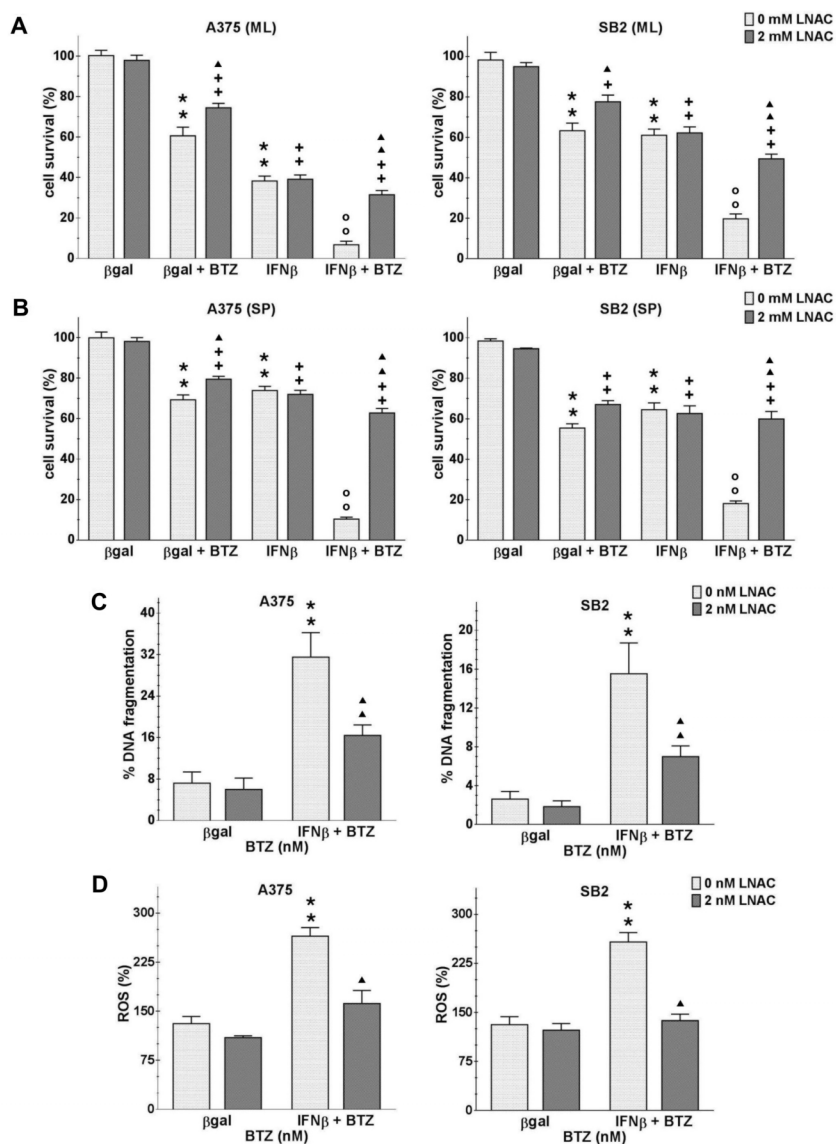
Fig. (4). Effects of N-acetyl-L-cysteine (LNAC) on IFN-βgene plus BTZ mediated cytotoxicity (A, B), apoptosis (C) and ROS generation (D). Monolayers (A) and spheroids (B) cell survival was determined as described in Materials and methods. (C) DNA fragmentation was quantified by flow cytometry after propidium iodide staining as described in Materials and methods. (D) DCF dye was used to measure reactive oxygen species (ROS) production, as described in Materials and methods. LNAC (2 mM) was always added 40 min before BTZ. **$p \leq 0.01$ with respect to βgal; °°$p \leq 0.01$ with respect to βgal; IFNβ and βgal plus BTZ; ++$p \leq 0.01$; +$p \leq 0.05$ with respect to βgal plus LNAC; ▲▲$p \leq 0.01$; ▲$p \leq 0.05$ with respect the equivalent treatment without LNAC.

(Fig. **3A**). The combined treatment was cytotoxic on cells growing both as monolayers or spheroids (Fig. **1B** and **C**), indicating that this effect should be observed also *in vivo*. The fact that this behavior was also found in very early canine melanoma cell lines (passages #3 to #7) strengthens this assumption (data not shown).

Beyond higher intercellular connections, the spheroids 3D configuration is related to higher expression of anti-apoptotic proteins and the presence of slow growing areas because of low oxygen and nutrient levels [18,34]. This drives to some degree of multicellular resistance.

Due to the re-growth resistance effect found after an early fast destruction of tumor cells both *in vivo* [27] and *in vitro* [17], the long term outcomes of the treatment often differ from short term cytotoxic effects. Clonogenic survival studies are useful for to evaluate these long term consequences and confirmed that the combination was more effective than the individual treatments (Fig. **2A** and **B**). Together with the cell migration inhibition (Fig. **2C**), this BTZ

enhancement of the IFNβ of cytotoxic (Fig. **1**) and anti-clonogenic (Fig. **2**) effects suggests a clinical potential not only to reduce tumor burden but also to inhibit its metastatic spread. Moreover, the addition of recombinant hIFNβ (1,000 to 10,000 IU/ml) enhanced the cytotoxic effects of BTZ (5 nM) in *A375* and *SB2* monolayers, but in a lesser extent than hIFNβ gene transfer (data not shown). Thus, this outcome suggests a useful interaction between BTZ and rhIFNβ that would deserve to be also explored.

Even though BTZ antitumor properties were linked to NFκB inactivation [12], we could not detect NFκB nuclear labeling in untreated *A375* and *SB2* melanoma cells. In agreement, it was reported that BTZ is effective on cells with unaltered NFκB pathway [12] and that BTZ does not decrease NFκB DNA binding or transcriptional activity in diverse melanoma cell lines (*A375* included) [35].

The enhancement of the BTZ plus IFNβ combined treatment depended on the intracellular ROS generation. In the presence of LNAC antioxidant BTZ kept part of its cytotoxic effect while it

was not able to increase the IFNβ cytotoxic effect on melanoma monolayers and spheroids (Fig. **4A** and **B**). Furthermore, LNAC abrogated the apoptosis enhancement of the combined treatment (Fig. **4C**). A synergic effect between BTZ and histone deacetylases that depends on ROS was previously reported [36]. The expression of IFNβ induced an intracellular ROS increase (Fig. **3C**) that can damage proteins by oxidation. Damaged proteins are usually degraded by a complex of the 26 S proteasome and an IFNβ induced subunit [37]. Then, 26 S proteasome inhibition by BTZ could increase cell sensitivity to apoptosis because of damaged proteins accumulation. Congruently, it was reported that BTZ sensitizes melanoma cells to apoptosis induced by agents that produce endoplasmic reticulum stress [38], and interferons, particularly IFNα expression after adenoviral gene transfer, triggers endoplasmic reticulum mediated apoptosis [39].

On the other hand, BTZ alone was highly cytotoxic in canine melanoma cells monolayers and spheroids. While BTZ IC_{50}s ranged from 2.7 to 7.1 nM in five canine melanoma cell lines (Table **1** and data not shown), IC_{50}s ranging from 3.5 to 5.6 nM in nine canine melanoma cell lines were recently reported [40]. In addition, BTZ alone inhibited canine melanoma tumor growth in a xenogeneic murine model [40]. Canine melanoma cell lines cell appeared as more sensitive to BTZ than the human equivalents.

The present work encourages further studies about the potential of the combination of interferon gene transfer with proteasome inhibitors as a new combined therapy for malignant melanoma both in veterinary and/or human clinical settings.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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