Journal of Cancer Research and Clinical Oncology
https://doi.org/10.1007/s00432-022-04129-4

ORIGINAL ARTICLE – CANCER RESEARCH

Synergistic antitumor effect of a penicillin derivative combined with thapsigargin in melanoma cells

Yanina Bellizzi[1] · Patricia G. Cornier[2] · Carina M. L. Delpiccolo[2] · Ernesto G. Mata[2] · Viviana Blank[1] · Leonor P. Roguin[1]

Received: 8 March 2022 / Accepted: 8 June 2022

Abstract

Purpose To investigate the effect of TAP7f, a penicillin derivative previously characterized as a potent antitumor agent that promotes ER stress and apoptosis, in combination with thapsigargin, an ER stress inducer, on melanoma cells.

Methods The synergistic antiproliferative effect of TAP7f in combination with thapsigargin was studied in vitro in murine B16-F0 melanoma cells, and in human A375 and SB2 melanoma cells. In vivo assays were performed with C57BL/6J mice challenged with B16-F0 cells. Immunofluorescence and Western blot assays were carried out to characterize the induction of ER stress and apoptosis. Necrotic tumor areas and the potential toxicity of the combined therapy were examined by histological analysis of tissue sections after hematoxylin-eosin staining.

Results In vitro, the combination of TAP7f with thapsigargin synergistically inhibited the proliferation of murine B16-F0, and human A375 and SB2 melanoma cells. When non-inhibitory doses of each drug were simultaneously administered to C57BL/6J mice challenged with B16-F0 cells, a 50% reduction in tumor volumes was obtained in the combined group. An apoptotic response characterized by higher expression levels of Baxenhanced PARP-1 cleavage and the presence of active caspase 3 was observed in tumors from the combined treatment. In addition, higher expression levels of GADD153/CHOP and ATF4 were found in tumors of mice treated with both drugs with respect to each drug used alone, indicating the induction of an ER stress response. No signs of tissue toxicity were observed in histological sections of different organs extracted from mice receiving the combination.

Conclusion The synergistic and effective antitumor action of TAP7f in combination with thapsigargin could be considered as a potential therapeutic strategy for melanoma treatment.

Keywords Triazolylpeptidyl penicillin · Thapsigargin · ER stress · Synergistic antitumor action · Melanoma cells

Introduction

Melanoma is recognized as a very aggressive and deadly type of skin cancer (McKean and Amaria 2018; Ascierto et al. 2018). Following surgical resection, different therapeutic alternatives, including immune checkpoint inhibitors and small inhibitors targeting activated signaling proteins, such as BRAF and MEK, have improved the outcome of the disease (Rughani et al. 2013; Azijli et al. 2014; Testori et al. 2019). Despite the continuous progress made in melanoma treatment in the last years, the appearance of adverse effects, the lack of response of some patients and the resistance to current treatments remains a challenge to solve (Sharma et al. 2017; Hamid et al. 2013; Nowicki et al. 2018). In this context, the development of new strategies, adjuvant treatments and/or the combination of medications

✉ Leonor P. Roguin
rvroguin@qb.ffyb.uba.ar

[1] Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Laboratorio de Oncología y Transducción de Señales, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Junín 956, C1113AAD Buenos Aires, Argentina

[2] Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Instituto de Química Rosario (CONICET-UNR), Suipacha 531, 2000 Rosario, Argentina

Published online: 25 June 2022

will undoubtedly help the implementation of alternatives with greater therapeutic benefits.

The endoplasmic reticulum (ER) is an intracellular organelle that plays an important role in the synthesis, folding and modification of proteins, and is also a cytosolic reservoir of calcium (Schröder and Kaufman 2005; Szegezdi et al. 2006; Wang and Kaufman 2014; Oakes and Papa 2015; Almanza et al. 2019). Different insults, including hypoxia, oxidative stress, nutrient deprivation, and drug treatments, may alter the homeostasis of the ER leading to ER stress and the consequent activation of an unfolded protein response (UPR). This response, which tries to restore ER homeostasis, is activated by three main sensor proteins of the ER membrane: the protein kinase RNA-like ER kinase (PERK), inositol-requiring kinase 1 (IRE 1), and the activating transcription factor 6 (ATF6). These sensors are maintained in an inactive form by interacting with the GRP78/BIP chaperone. When ER homeostasis is altered, GRP78/BIP is released and binds to unfolded or misfolded proteins, and activates the UPR which leads to lower protein translation, higher protein degradation and also increased synthesis of chaperones that promote protein folding. However, if the damage is too severe or the UPR fails, the ER stress response may result in apoptotic cell death (Schröder and Kaufman 2005; Szegezdi et al. 2006; Wang and Kaufman 2014; Wang et al. 2018). In this context, ER stress inducers have been considered as potential agents in cancer therapy.

We have previously identified a triazolylpeptidyl penicillin derivative, named TAP7f, as a potent and selective pro-apoptotic compound (Cornier et al. 2014). The in vivo antitumor efficacy of this derivative was demonstrated in a murine melanoma model (Blank et al. 2018). It was also found that TAP7f inhibits the adhesion, migration and invasion of melanoma cells and diminishes melanoma lung metastasis in vivo (Barrionuevo et al. 2020). In addition, it was shown that TAP7f triggers an apoptotic cell death through induction of ER stress and activation of p38, JNK and PI3K-I/Akt pathways (Bellizzi et al. 2022). Based on the ability of TAP7f to promote ER stress, in this work we decided to evaluate the in vitro and in vivo antitumor effect of the penicillin derivative in combination with thapsigargin, a well-known inhibitor of the Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA), a P-type ATPase bound to the ER membranes which pumps Ca^{2+} ions from the cytosol into the ER (Winther et al. 2010; Sehgal et al. 2017). After testing the combined effect in vitro of both drugs, we found that the combination of TAP7f and thapsigargin was synergistically cytotoxic and efficiently reduced the growth of murine B16-F0, and human A375 and SB2 melanoma cells. We also demonstrated the in vivo effectiveness of the combination in a B16-F0 murine melanoma model established in C57BL/6J mice. The in vivo synergistic action of both drugs improved the therapeutic efficacy and allowed lowering of the dose of each individual drug, reducing potential toxicity. Based on these findings, we propose the combined therapy with TAP7f and thapsigargin as a novel therapeutic strategy with potential clinical usefulness for melanoma treatment.

Materials and methods

Reagents and antibodies

TAP7f was synthesized as described in previous work (Cornier et al. 2014). A 100 mM stock solution of the penicillin derivative was prepared in dimethyl sulfoxide (DMSO) and stored at − 70 °C. The stock solution was diluted 1/10 in ethanol and then used at different concentrations in the indicated culture medium. All the experiments were performed with a final concentration of 20 µl vehicle/ml of medium. Thapsigargin was obtained from Sigma-Aldrich (MO, USA). Monoclonal anti-calnexin (610,523) and anti-GRP78/BIP (610,978) antibodies were from Becton Dickinson (New Jersey, USA). Polyclonal antibodies against the proliferating cell nuclear antigen (PCNA) (sc-7907), PARP-1(sc-7150), Bax (sc-7480), and monoclonal antibodies anti-GADD153/CHOP (sc-7351), anti-ATF4 (sc-390063) and anti-actin (sc-8432) were obtained from Santa Cruz Biotechnology (CA, USA). Polyclonal anti-active caspase-3 antibody was from Cell Signaling Technology (#9661; Danvers, Massachusetts, USA). Secondary anti-rabbit IgG (sc-2004) or anti-mouse IgG (sc-2005) horseradish peroxidase antibodies and secondary anti-rabbit IgG-FITC (sc-2359) were obtained from Santa Cruz Biotechnology (CA, USA). Polyclonal secondary anti-mouse IgG-Alexa Fluor 488 (A11029) was from Thermo Fisher (Buenos Aires, Argentina). Monoclonal Anti-tubulin (ab-7291) was obtained from Abcam (Cambridge, UK).

Cell culture conditions

Melanoma cell lines containing different genomic characteristics (BRAF/NRAS mutated or wild type) were employed. Murine melanoma cells B16-F0 (BRAF/NRAS wild type, ATCC CRL-6322, RRID:CVCL_0604) were grown in RPMI-1640 (Gibco BRL) supplemented with 10% FBS (Natocor, Argentina), 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/ml streptomycin (Sigma-Aldrich, MO, USA) in a humidified atmosphere of 5% CO_2 at 37 ºC. NRAS-mutated human melanoma cell line SB2 (RRID:CVCL_0516) was kindly provided by Dr.Osvaldo Podhajcer (Laboratorio de Terapia Molecular y Celular, Fundación Instituto Leloir, CONICET, Buenos Aires, Argentina). Human melanoma cells SB2 and BRAF-mutated A375 (ATCC CRL-1619, RRID:CVCL_0132) were grown in DMEM F-12 medium (Gibco BRL) with 10% (v/v) FBS, 2 mM L-glutamine, 50

U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich, MO, USA), in a humidified atmosphere of 5% CO_2 at 37 ºC.

Proliferation assay

Proliferation assay was performed as described previously (Cornier et al. 2014; Blank et al. 2018). Briefly, B16-F0, A375 or SB2 cells were incubated in 96-well microplates at a density of 1×10^4 cells/well for 72 h at 37 ºC in the presence of different concentrations of vehicle, TAP7f or thapsigargin, alone or in combination, in a total volume of 0.2 ml of the corresponding culture medium. Cell number was evaluated by colorimetric determination of the levels of the ubiquitous lysosomal enzyme hexosaminidase (Landergren 1984). Absorbances were measured at 405 nm with a microplate reader Biotrack II (Amersham Biosciences, USA). The percentage of cell growth inhibition corresponding to each treatment, expressed as fraction affected (Fa), was analyzed according to the median-effect equation of Chou and Talalay method, and combination index (CI) were calculated by using CompuSyn software (Chou 2010; Ashton 2015). CI values of < 1 indicates synergy, > 1.0 indicates antagonism, and values $= 1$ indicates addition.

In vivo assays: tumor growth and toxicity studies

All experiments were performed in accordance with the National Institute of Health Guide for the Care and the Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (CICUAL) of the School of Pharmacy and Biochemistry, University of Buenos Aires. Female C57BL/6J mice, obtained from the Animal Care Facility of the School of Exact and Natural Sciences, University of Buenos Aires, were housed under controlled conditions, with food and water available ad libitum, and were used for experiment at 8–10 weeks old (approximately 20–25 g). To study the effect of TAP7f and thapsigargin on tumor growth, B16-F0 cells (1×10^5) diluted in 200 µl of RPMI were injected subcutaneously in the right flank of each mouse. Following 12 days of cell inoculation, mice were divided into four groups: group I (control) received 0.2 ml of vehicle (70% v/v polyethylene glycol 400 in PBS); group II received 0,3 mg/kg of thapsigargin; group III received 4 mg/kg of TAP7f; and group IV received the combination of both compounds intraperitoneally for 8 days. Animals were daily monitored for their overall health status, and their body weights were registered weekly throughout the course of the study. Tumor sizes were measured with a caliper on alternate days, and tumor volumes were calculated using the following formula: $V = (D \times d^2)/2$, where D is the larger diameter and d is the smaller. At the end of the study, mice were anesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg), and tumors were excised, weighed, and measured. To explore if the combination of TAP7f and thapsigargin could induce some systemic toxicity, different organs from treated and non-treated mice were removed, fixed in formaldehyde 10% in PBS 0.1 M, pH 7.4 for 24 h, then dehydrated and included in paraffin. Cuts of 5 µm were made using a microtome (Leica RM2125, Leica,Wetzlar, Germany) and mounted on 2% xylane-coated slides. Sections were then stained with hematoxylin–eosin for histological analysis and examined with an inverted microscope Eclipse TE2000 (Nikon, Minato, Tokyo, Japan).

Western blot assays

The expression of some proteins related to apoptosis or ER-stress was determined in B16-F0 tumor lysates from mice treated with 4 mg/kg of TAP7f, 0.3 mg/kg of thapsigargin, the combination of both compounds or vehicle. At the end of in vivo assays, tumors were excised and lysed in a buffer solution containing 10% glycerol, 0.5% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml trypsin inhibitor, 1 µg/ml leupeptin, 10 mmol/l $Na_4P_2O_7$, 10 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l EDTA, 1 mmol/l PMSF, 150 mmol/l NaCl, and 50 mmol/l Tris, pH 7.4. Clear lysates supernatants were obtained by centrifugation and aliquots containing 100 µg of protein were resuspended in 0.063 M Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 5% 2-mercaptoethanol, submitted to SDS-PAGE and then transferred onto PVDF membranes (GE Healthcare, Piscataway, NY, USA) for 30 min at 100 V in 25 mM Tris, 195 mM glycine, 20% methanol, pH 8.2. To reduce non-specific binding, membranes were incubated for 1 h at room temperature in 10 mM Tris–HCl, 130 mM NaCl and 0.05% Tween 20, pH 7.4, (TBS-T), containing 5% non-fat milk. Membranes were then incubated overnight at 4 °C with the specific primary antibody, washed with TBS-T and incubated with the corresponding secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Immunoreactive proteins were visualized using the Pierce® ECL Plus detection system (Thermo Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Band intensity was quantified by using a densitometer (Gel Pro Analyzer 4.0). Mouse anti-tubulin or rabbit anti-actin antibodies were used to confirm equal protein loading.

Immunohistochemistry

Tumors excised from mice treated with 4 mg/kg of TAP7f, 0.3 mg/kg of thapsigargin, the combination of both compounds or vehicle were fixed in 10% formaldehyde neutral buffer solution, embedded in paraffin and sectioned before immunohistochemical analysis. Antigen retrieval was performed by treating tissue sections in 10 mM sodium citrate buffer, pH 6.0, for 50 min at 92 °C. Slides were then blocked

for 1 h with 5% bovine serum albumin (BSA) and then incubated with antibodies against PCNA or active caspase-3 overnight at 4 °C. After rinsing, sections were incubated for 1 h with goat anti-rabbit IgG-FITC or goat anti-mouse IgG-Alexa488 secondary antibodies and examined with an Olympus BX50 epifluorescence microscope provided with a Cool-Snap digital camera. Fluorescence intensity was quantified using Image J software.

Statistical analysis

All values are expressed as mean ± S.E.M. p value less than 0.05 was considered statistically significant. Analyses were performed using GraphPad Prism (version 6.01, GraphPad Software, La Jolla, California, USA). Statistical analyses were performed by one-way ANOVA followed by Dunnett's multiple comparison tests (comparing all groups to a control group) or Tuckey's multiple comparison tests (comparing all groups).

Results

Synergistic action in vitro of TAP7f and thapsigargin on melanoma cell growth

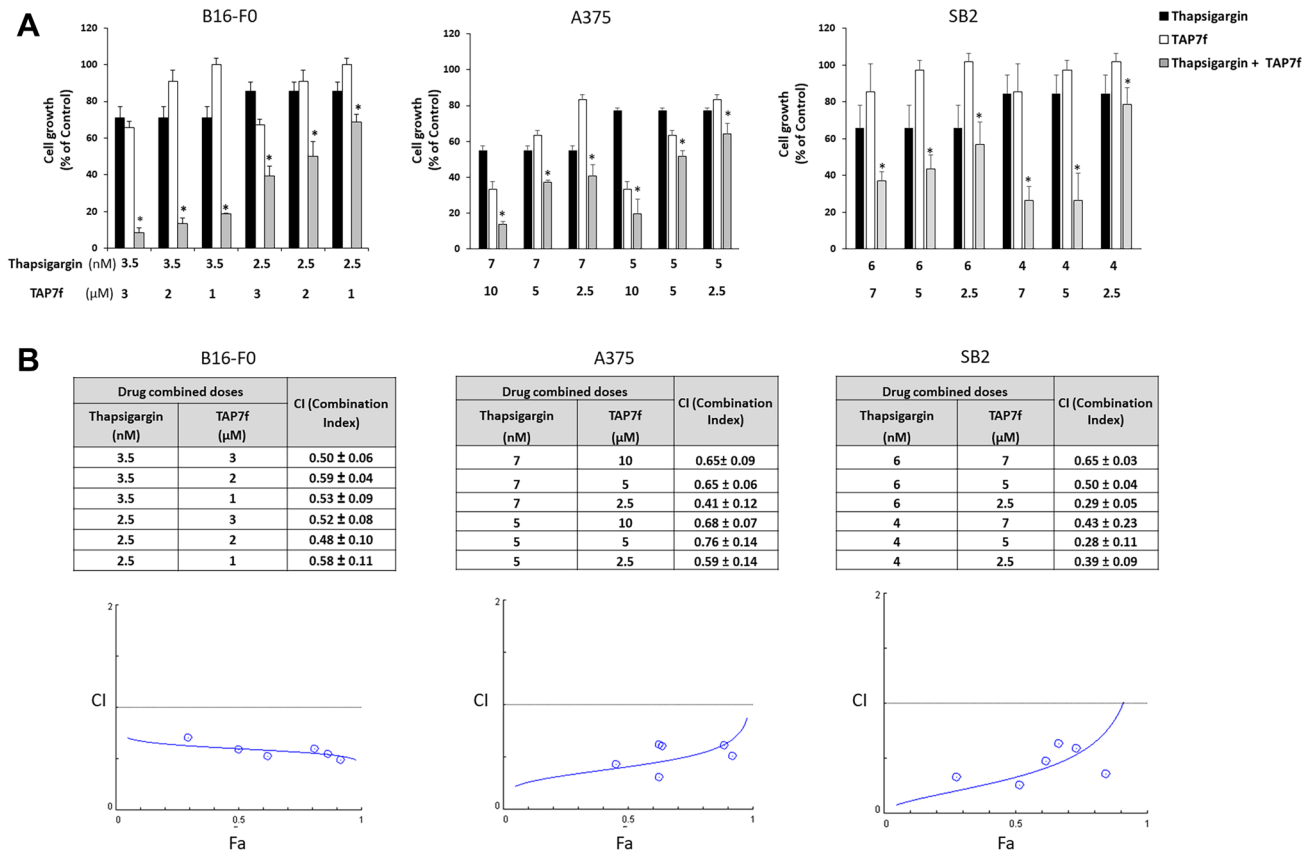
We have previously demonstrated that TAP7f exerts a potent antiproliferative activity in murine B16-F0 melanoma cells ($IC_{50} = 3 \pm 1$ µM) (Cornier et al. 2014; Blank et al. 2018) and A375 human melanoma cells ($IC_{50} = 10 \pm 1$ µM) (Barrionuevo et al. 2020). We also showed that activation of ER stress contributes to the apoptotic cell death induced by the penicillin derivative in these cell lines (Bellizzi et al. 2022). On this basis, we hypothesized that a more potent antitumor action (additive or synergistic effect) could be achieved by combining TAP7f with an ER stress inducer, such as thapsigargin. To this end, the effect of the combined therapy was examined in B16-F0, A375 and also in SB2 cells, an NRAS-mutant human melanoma cell line (Lopez et al. 2009). The cytotoxic potency of thapsigargin on these cell lines was first determined from dose–response curves. Thapsigargin inhibited cell growth in a dose-dependent manner, being IC_{50} values of 5.0 ± 1 nM, 7.0 ± 2 nM and 6.7 ± 1 nM for B16-F0, A375 and SB2 cells, respectively ($n = 3$) (Supplementary Fig. 1). An IC_{50} value of 8.6 ± 3 µM was also determined for TAP7f in SB2 cells (data not shown). Then, the effect of the combined drugs or each drug alone was evaluated on each melanoma cell line by using TAP7f or thapsigargin concentrations that individually inhibit cell proliferation at values close to 50% or less. As shown in Fig. 1A, cell growth was significantly reduced for all combinations tested compared to each drug separately in the three melanoma cell lines studied. To evaluate whether the effect of the combined compounds represents an additive or synergistic action, the combination index (CI) was calculated by using CompuSyn software (Fig. 1B). CI values < 1 were obtained for all the combinations regardless the fraction affected (Fa) value, indicating that TAP7f and thapsigargin synergistically inhibited B16-F0, A375 and SB2 cell growth.

Synergistic action in vivo of TAP7f and thapsigargin on B16-F0 melanoma growth

In previous work, we studied the in vivo action of TAP7f in a syngeneic C57BL/6J mouse melanoma model and showed that B16-F0 tumor volume was reduced approximately 70% and 50% at doses of 20 and 10 mg/kg, respectively (Blank et al. 2018). No effect was observed at 1 mg/kg of TAP7f. To explore the antitumor potency of TAP7f in combination with thapsigargin in vivo, we decided to use suboptimal doses of the two drugs in the B16-F0 melanoma model. Therefore, the penicillin derivative was first assayed at a dose of 4 mg/kg and also 20 mg/kg as a positive control. No tumor growth inhibition was observed at 4 mg/kg of TAP7f, whereas results obtained at 20 mg/kg were consistent with those previously reported at 21 days post-inoculation (Supplementary Fig. 2A) (Blank et al. 2018). The in vivo cytotoxic activity of thapsigargin was then evaluated at doses of 0.3 mg/kg and 0.5 mg/kg (Supplementary Fig. 2B). Tumor growth was reduced by ~ 50% at 0.5 mg/kg, but no significant difference was found at 0.3 mg/kg. Based on these findings, 4 mg/kg of TAP7f and 0.3 mg/kg of thapsigargin were then employed to examine the effect of the combination. As shown in Fig. 2A, a reduction of approximately 50% was observed for tumor volumes of the combined group with respect to mice injected with vehicle (control) or each drug alone. In addition, tumor weight diminished ~50% for the combination compared to control mice or the other groups of treated mice (Fig. 2B). Body weights of all mice groups were similar throughout the treatment (data not shown).

In vivo mechanism of action of TAP7f and thapsigargin

The proliferation rate of B16-F0 tumor cells was determined by evaluating the levels of the proliferating cell nuclear antigen (PCNA) in tumors from control mice and mice treated either with each drug alone or a combination of both (Fig. 3A). A significant decrease in the expression levels of PCNA was detected by immunohistochemistry in tumors from mice treated with both TAP7f and thapsigargin. Hematoxylin–eosin staining of tumors sections was also performed to examine histologically necrotic areas, which correspond to non-viable tissue without indicating the mechanism of cell death. As shown in Fig. 3B, whereas no necrotic area was observed in control tumors or tumors



B16-F0

Drug combined doses		CI (Combination Index)
Thapsigargin (nM)	TAP7f (μM)	
3.5	3	0.50 ± 0.06
3.5	2	0.59 ± 0.04
3.5	1	0.53 ± 0.09
2.5	3	0.52 ± 0.08
2.5	2	0.48 ± 0.10
2.5	1	0.58 ± 0.11

A375

Drug combined doses		CI (Combination Index)
Thapsigargin (nM)	TAP7f (μM)	
7	10	0.65± 0.09
7	5	0.65 ± 0.06
7	2.5	0.41 ± 0.12
5	10	0.68 ± 0.07
5	5	0.76 ± 0.14
5	2.5	0.59 ± 0.14

SB2

Drug combined doses		CI (Combination Index)
Thapsigargin (nM)	TAP7f (μM)	
6	7	0.65 ± 0.03
6	5	0.50 ± 0.04
6	2.5	0.29 ± 0.05
4	7	0.43 ± 0.23
4	5	0.28 ± 0.11
4	2.5	0.39 ± 0.09

Fig. 1 In vitro antitumor effect of TAP7f and thapsigargin. **A** B16-F0, A375 or SB2 cells (1×10^4) were treated with different concentrations of TAP7f, thapsigargin or the combination of both drugs for 72 h. Results are expressed as the percentage of cell growth relative to control and represent the mean ± S.E.M. of three different experiments. Statistical analyses were performed by one-way ANOVA followed by Tuckey post-hoc test, $^*p < 0.05$, significantly different from cells treated with each drug. **B** Cell growth inhibition was analysed by CompuSyn software (Chou–Talalay's method) and combination index values (CI) were calculated (mean values ± S.E.M. of three different experiments). CI plots for different combinations are shown as a function of the fraction affected (Fa). Values of CI < 1 indicate a synergistic action

from mice treated with TAP7f or thapsigargin alone, a large necrotic region was detected in tumors from mice treated with the combination.

We then examined by western blot assays the expression levels of apoptotic-related proteins, such as Bax and PARP-1. A significant increase in the expression of the pro-apoptotic protein Bax was found in tumors lysates from mice treated with the combined formulation (12-fold increase) or with thapsigargin alone (sixfold increase) (Fig. 4A). The cleavage of PARP-1, a polymerase that plays a key role in the repair of DNA damage (Rodríguez-Hernández et al. 2006; Hassa and Hottiger 2008), was significantly higher in mice treated with the combination with respect to mice treated with each individual drug (Fig. 4A). In addition, immunofluorescence assays performed to detect active caspase 3, showed an increment of this caspase in tumor slices from mice exposed to the combined treatment (Fig. 4B).

Since we have previously demonstrated that ER stress is involved in the apoptotic cell death induced in vitro by TAP7f in B16-F0 melanoma cells (Bellizzi et al. 2022), we decided to explore whether TAP7f in vivo alone or in combination with thapsigargin could activate ER stress. For comparative purposes, the expression levels of different ER stress-related proteins were first evaluated by Western blot assays performed with cell lysates from tumors removed at the end of the treatment from mice treated with 20 mg/kg of TAP7f. As shown in Fig. 5, levels of ATF4, GADD153/CHOP, calnexin and GRP78/BIP increased between 2.0 and 2.5-fold with respect to the amounts found in tumors from non-treated mice (control). We then examined the expression levels of ATF4 and GADD153/CHOP in tumors from mice treated with suboptimal doses of each drug (TAP7f: 4 mg/kg, thapsigargin: 0.3 mg/kg) or with the combination of both. ATF4 and GADD153/CHOP expression levels increased ~ eightfold and 13-fold, respectively, in tumors from mice treated with the combination (Fig. 6). Although a slight increase in the expression levels of GADD153/CHOP and ATF4 was observed in

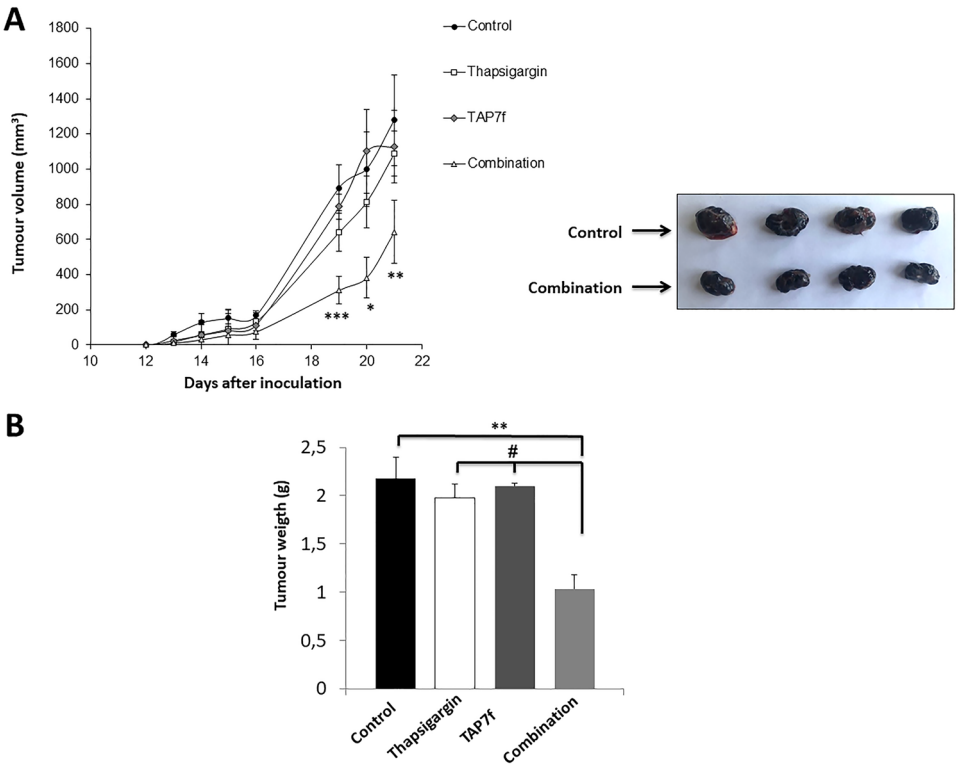


Fig. 2 In vivo antitumor effect of the combination of TAP7f and thapsigargin on B16-F0 melanoma growth. B16-F0 cells (1×10^5) were injected subcutaneously in the right flank of each C57BL/6 mouse and 12 days after cell inoculation, mice received vehicle ($n = 7$), thapsigargin (0.3 mg/kg, $n = 6$), TAP7f (4 mg/kg, $n = 6$) or the combination of both ($n = 6$) via i.p. for 8 days. **A** Tumor sizes were measured with a caliper and tumor volume was calculated by the formula ($D \times d^2$)/2. Results represent mean values ± S.E.M. Statistical analyses were performed by one-way ANOVA followed by Dunnet post-hoc test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ respect to control. Image of control tumors and tumors treated with the combination at the end of the treatment (right panel). **B** Weights of tumors extracted from the different experimental groups. Tuckey multiple comparison test was applied after one-way ANOVA, $**p < 0.01$ respect to the control group, $\#p < 0.05$ respect to groups treated only with TAP7f or thapsigargin

TAP7f-treated tumors, these changes were not significant, whereas approximately a fourfold increase was observed in tumors from mice treated with thapsigargin. Thus, it was concluded that in vivo treatment with the combination of TAP7f and thapsigargin was more effective in inducing ER stress than treatment with each drug separately.

To assess the potential toxicity of the combined therapy, we decided to examine whether the drug combination could affect other mice tissues in addition to the tumor. To this end, histological sections of different organs, including spleen, brain, heart, liver, intestine, lung and kidney, were stained with hematoxylin–eosin and analyzed by optical microscopy. As shown in Fig. 7, the tissues studied in the group treated with both TAP7f and thapsigargin showed morphological characteristics comparable to the tissues of the non-treated mice (control), with no signs of necrosis, edema or any other type of toxicity. In addition, the body weights of the mice treated with the two drugs were similar to the control group throughout the treatment (data not shown).

Discussion

To overcome the adverse effects of chemotherapeutic drugs or the acquired resistance to treatment, the combination of drugs emerges as a promising therapeutic alternative. In this sense, instead of having a drug that modulates one individual target, the association of drugs tries to slow tumor growth by simultaneously recognizing different targets (Jia et al. 2009; Chen et al. 2015; Foucquier and Guedj 2015). In this work, the antitumor effect of TAP7f, a triazolylpeptidyl penicillin derivative previously characterized in our laboratory, was explored in combination with thapsigargin both in vitro and in vivo on melanoma growth. Since we previously demonstrated that TAP7f induced an ER stress response (Bellizzi et al. 2022), we hypothesized that the combination with thapsigargin, a classical ER stress inducer, could improve the antitumor effect either by adding their effects or by inducing a synergistic action. We found that the simultaneous

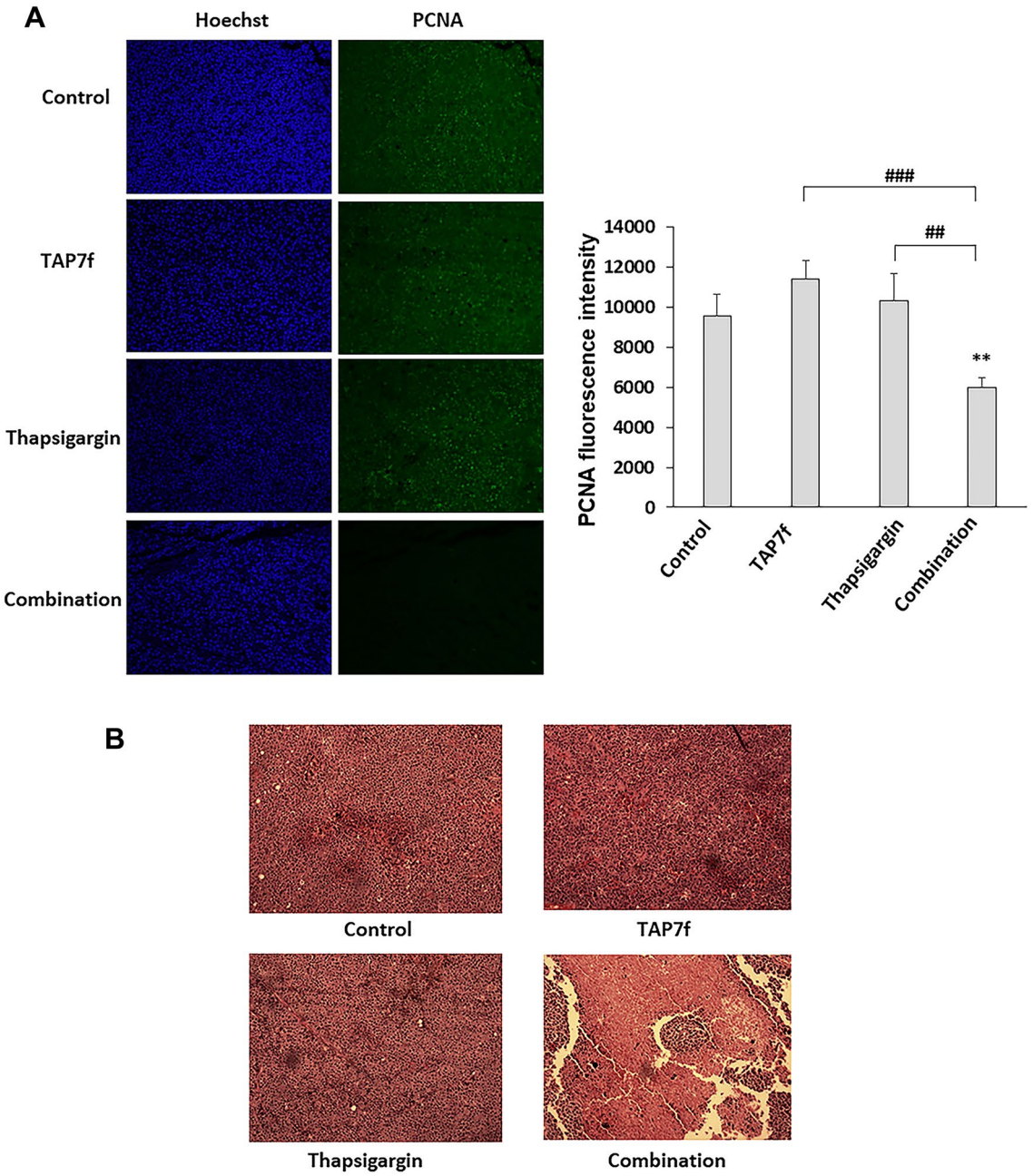
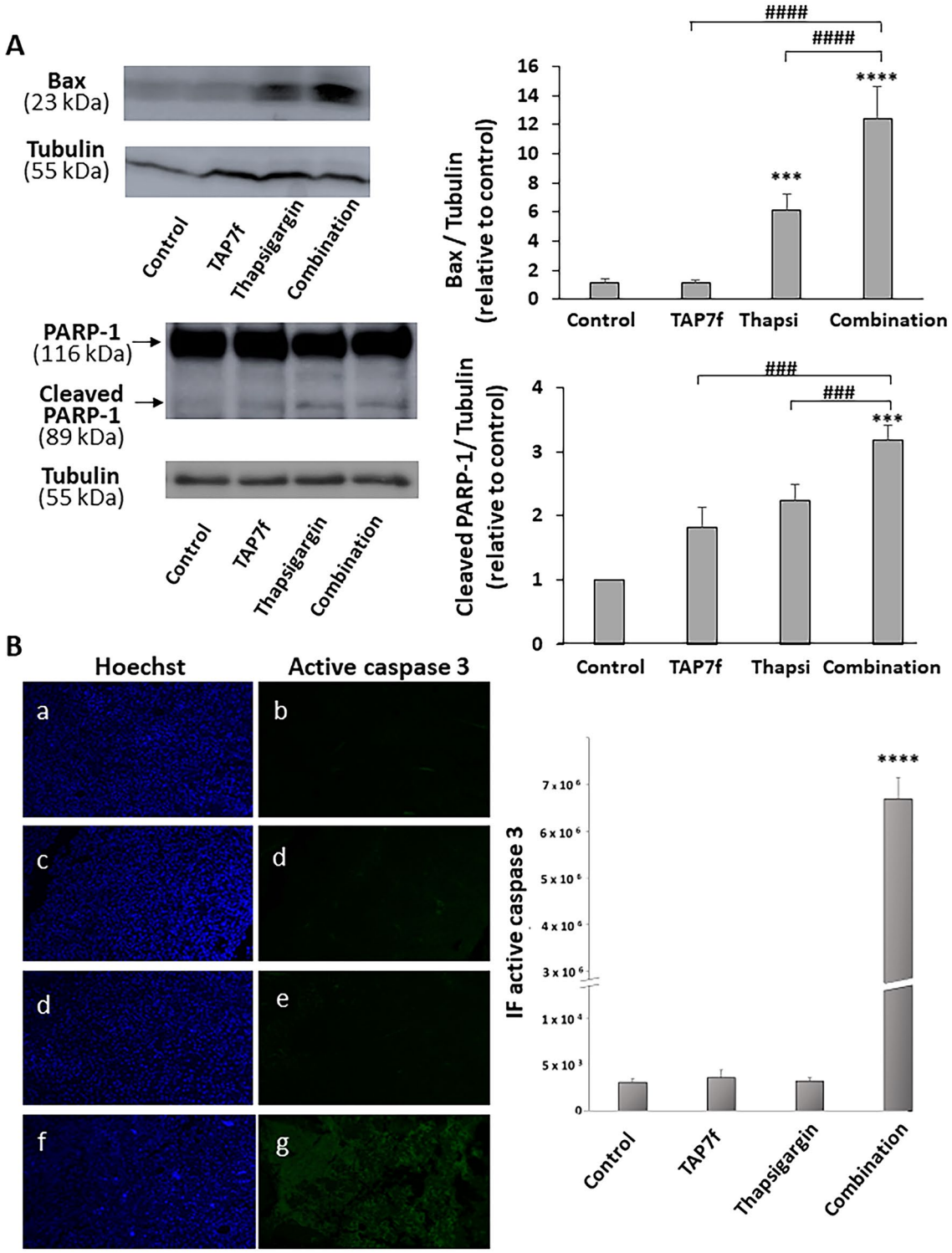


Fig. 3 Proliferation rate and histological characteristics of B16-F0 melanoma tumors. Tumors from mice injected with vehicle (control), 4 mg/kg of TAP7f, 0.3 mg/kg of thapsigargin or the two drugs were fixed in 10% formaldehyde in PBS and embedded in paraffin. **A** Tumor sections of 5 µm were incubated with an anti-PCNA antibody and then with a secondary antibody labeled with FITC. Nuclei were stained with Höechst 33,258 2 µM. Magnification: 200X (left panel). Fluorescence intensity was analysed by Image J software (right panel). Tuckey multiple comparison test was applied after one-way ANOVA, $^{**}p < 0.01$ significantly different from control; $\#\#p < 0.01$, $\#\#\#p < 0.001$, significantly different from mice treated with thapsigargin or TAP7f, respectively. **B** Tumor slices of 5 µm were stained with hematoxylin–eosin and observed with an inverted microscope. Magnification: 200X

in vitro administration of both drugs synergistically inhibited cell proliferation of murine B16-F0, and human A375 and SB2 melanoma cells, as demonstrated by Combination Index (CI) values < 1. Thus, this therapy could be an option for melanoma cell lines containing different genomic characteristics (BRAF/NRAS mutated or wild type). A synergistic effect of the two drugs means that the therapeutic effect of the combination is greater than the sum of effects caused by each individual drug (Sucher 2014). As a well-known ER stress inducer, thapsigargin



inhibited the SERCA pump and increase cytosolic Ca^{2+} concentrations by blocking the reabsorption of Ca^{2+} from the cytosol to the ER (Winther et al. 2010; Sehgal et al. 2017). Although TAP7f also induces ER stress, its particular target has not yet been identified. However, the behavior observed for the combination suggests that both drugs would be recognizing different intracellular targets. The analyses of a variety of examples from synergistic drug combinations reported in the literature revealed the complexity of these interactions since multiple targets can reside in the same or related pathways (Jia et al. 2009; Chen et al. 2015). In this sense, the specific mode of action

◂**Fig. 4** In vivo induction of apoptosis after treatment with TAP7f and thapsigargin. **A** Bax or cleaved PARP-1 levels were determined in control mice or mice treated with 4 mg/kg of TAP7f, 0.3 mg/kg of thapsigargin or the combination of both. Tumor lysates were processed for Western blot analysis as described in "Materials and methods". Results from one representative experiment are shown (left panel). Data quantification was performed by densitometric analysis (right panel). Results are expressed as mean ± S.E.M of three different experiments. Statistical analyses were performed by one-way ANOVA followed by Tuckey post-hoc test, $^{***}p < 0.001$, $^{****}p < 0.0001$, significantly different from control; $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, significantly different from tumors treated with the combination. **B** Active caspase-3 was examined by immunohistochemistry in non-treated tumors (control) and tumors of mice treated with TAP7f (4 mg/kg), thapsigargin (0.3 mg/kg) or the combination of both drugs. Magnification: 200X. Nuclei were stained with Höescht 33,258. Fluorescence intensity was analysed by Image J software (right panel). ANOVA followed by Tuckey post-hoc test, $^{****}p < 0.0001$ respect to the control group. Tumors from the control group (a, b), tumors from TAP7f-treated mice (c, d), tumors from thapsigargin-treated mice (d, e), tumors from the combined group (f, g)

of TAP7f/thapsigargin combination, two drugs that might be interacting with different targets of a related pathway could be explained by anti-counteractive, complementary or facilitating actions (Jia et al. 2009). Regardless of the mechanisms of action, which have been fully elucidated for only a few combinations of drugs, the advantage of combination therapies lies in greater efficacy with reduced doses of each drug, less toxicity or reduction of unwanted actions, and even delay in the development of drug resistance (Foucquier and Guedj 2015). Based on these features, the effect of drug combinations has been explored for the treatment of various diseases, including cancer, hypertension, asthma, etc. (Foucquier and Guedj 2015). In particular, for melanoma treatment, Gowda et al. (2017) reported that the combination of Celecoxib and Plumbagin, two anticancer drugs of limited therapeutic potential due to their toxic side effects, synergistically decreased melanoma cell proliferation by inhibition of cyclooxygenase-2 and STAT3, leading to decreased levels of key cyclins for tumor cell survival. Margue et al. (2019), after examining various kinase inhibitors involved in cell cycle regulation and inhibitors targeting different signaling pathways, identified synergistic combinations of some kinase inhibitors with BRAF inhibitors that resulted to be effective in killing melanoma cells. The combination of Brequinar sodium with doxorubicin synergistically suppressed the growth of melanoma cells both in vitro and in vivo (Dorosamy et al. 2019). It has also been reported that resveratrol, by promoting gap junction intercellular communications, can synergistically enhance the killing effect of thymidine kinase/ganciclovir suicide gene system in melanoma B16 cells (Chen et al. 2020).

We also showed that TAP7f combined in vivo with thapsigargin inhibited tumor cell proliferation, as revealed by decreased expression levels of PCNA, a key marker of cellular replication (Rodríguez-Hernández et al. 2006; Hassa and Hottiger 2008). Hematoxylin–eosin staining of tumor sections treated with the combination allowed us to detect areas defined histologically as necrotic, indicating non-viable cells regardless of the death mechanism (Blank et al. 2018; Chiarante et al. 2020). In addition, the in vivo administration of both drugs induced a more potent apoptotic response than each of the drugs separately. Thus, compared to TAP7f or thapsigargin monotherapy, the combination led to higher increases in Bax and active caspase 3 levels, and also greater PARP-1 cleavage. Although we have previously reported the in vivo apoptotic effect of TAP7f on B16-F0 melanoma tumors treated with 20 mg/kg of TAP7f (Blank et al. 2018), apoptosis-related proteins had not been examined at lower concentrations. Results herein obtained showed that 4 mg/kg of TAP7f induced a slight apoptotic response in vivo, evidenced by PARP-1 cleavage, which did not lead to a significant variation of tumor growth.

Regarding thapsigargin, its role as a compound capable of inducing ER stress and apoptosis in a variety of tumor cell lines has been earlier reported (He et al. 2002; Yamaguchi et al. 2004; Huang et al. 2004; Wang et al. 2016; Wu et al. 2019). Particularly, in several human melanoma cells, Chen et al. (2007) showed that thapsigargin induced ER stress and up-regulated cell-surface expression of TRAIL-R2, leading to enhanced TRAIL-induced apoptosis. Under our experimental conditions, we showed that 0.3 mg/kg of thapsigargin induced an apoptotic effect, supported by the increment of Bax and a slight increase of PARP-1 fragment levels in B16-F0 tumors, without significantly affecting tumor cell proliferation. In brief, although suboptimal doses of TAP7f or thapsigargin monotherapy can induce a mild apoptotic effect, this response is not sufficient to affect tumor growth.

The involvement of ER stress signaling mediators in the antitumor action exerted by the combination was then confirmed by Western blot assays of tumor lysates from treated-mice. The UPR promotes the activation of three ER membrane sensors that initiate signaling pathways in an attempt to relieve stress. Among them, PERK activates a complex cascade of effectors, including an increase in the expression of the transcription factor ATF4, which upregulates GADD153/CHOP, a protein that plays an important role in the ER stress-induced apoptotic response (Schröder and Kaufman 2005; Szegezdi et al. 2006; Wang and Kaufman 2014; Wang et al. 2018). Our results showed that although thapsigargin monotherapy with 0.3 mg/kg did not reduce tumor size, it induced ~ fourfold increment in the expression levels of GADD153/CHOP and ATF4. However, the combined therapy increased ~ 8 to 13-fold the levels of both ER stress-related proteins, confirming the synergistic effect of the administration of the two drugs. In a comparable way, some in vitro studies have shown that thapsigargin

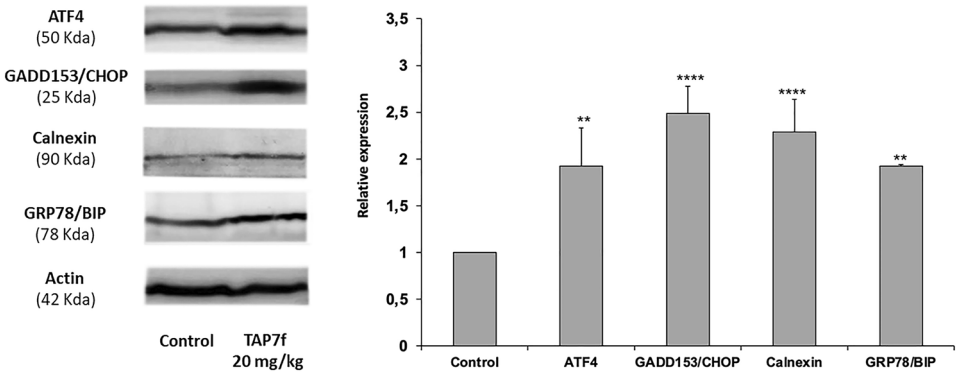


Fig. 5 Contribution of ER stress to the antitumor effect induced in vivo by TAP7f. Non-treated tumors (control) and tumors from mice treated with 20 mg/kg of TAP7f were excised and lysed as describe in "Materials and methods". 100 µg of tumor lysates were then submitted to Western blot assays and membranes were incubated with antibodies against ATF4, GADD153/CHOP, Calnexin, GRP78/BIP and actin. Results from one representative experiment are shown (left panel). Data quantification was performed by densitometric analysis of three independent experiments (right panel). Statistical analyses were performed by one-way ANOVA followed by Dunnet post-hoc test. $^{**}p < 0.01$, $^{****}p < 0.0001$

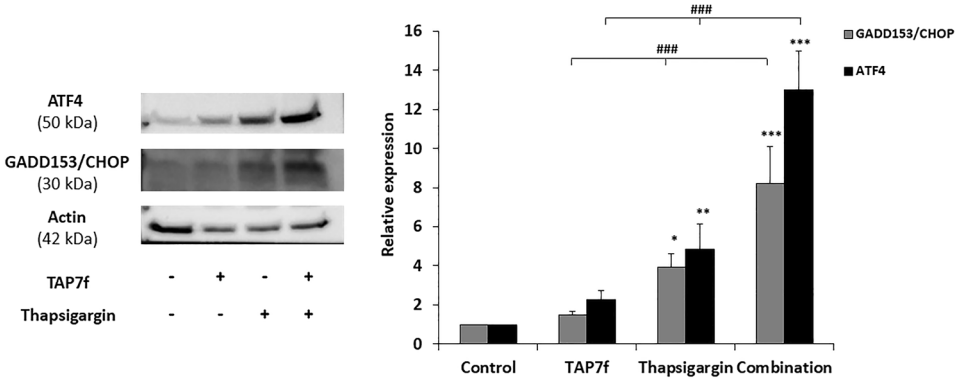


Fig. 6 In vivo expression of ER stress proteins after treatment with TAP7f and thapsigargin. Tumor lysates from non-treated mice (control) and mice treated with TAP7f (4 mg/kg), thapsigargin (0.3 mg/kg) or the combination of both drugs were submitted to Western blot assays and membranes were incubated with antibodies against ATF4, GADD153/CHOP and actin. Results from one representative experiment are shown (left panel). Data quantification was performed by densitometric analysis of three independent experiments (right panel). Statistical analyses were performed by one-way ANOVA followed by Tuckey post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from control; $^{\#\#\#}p < 0.001$, significantly different from mice treated with TAP7f or thapsigargin

can activate UPR, as determined by high levels of the ER chaperone GRP78/Bip, at concentrations that do not lead to cell death (Rutkowski et al. 2006; Ghosh et al. 2015). On the other hand, considering that thapsigargin has emerged as a remarkable antitumor lead compound, it is interesting to mention that thapsigargin-derived prodrugs are currently in clinical trials for the treatment of different cancer types, such as hepatocellular carcinoma, prostate cancer, and also to target the blood vessels of cancer cells (Christensen et al. 2009; Andersen et al. 2015; Doan et al. 2015).

In summary, we demonstrated that the combined therapy with TAP7f and thapsigargin was effective in inhibiting melanoma cell proliferation in vitro and reduced B16-F0 melanoma growth in vivo. The synergistic action exerted by the combination achieved a better antitumor effect compared to monotherapy, with the additional advantage of using suboptimal doses of each drug. We also showed that the induction of ER stress is involved in the apoptotic cell death induced by the administration of the two drugs. Therefore, the improved antitumor potency of TAP7f combined with

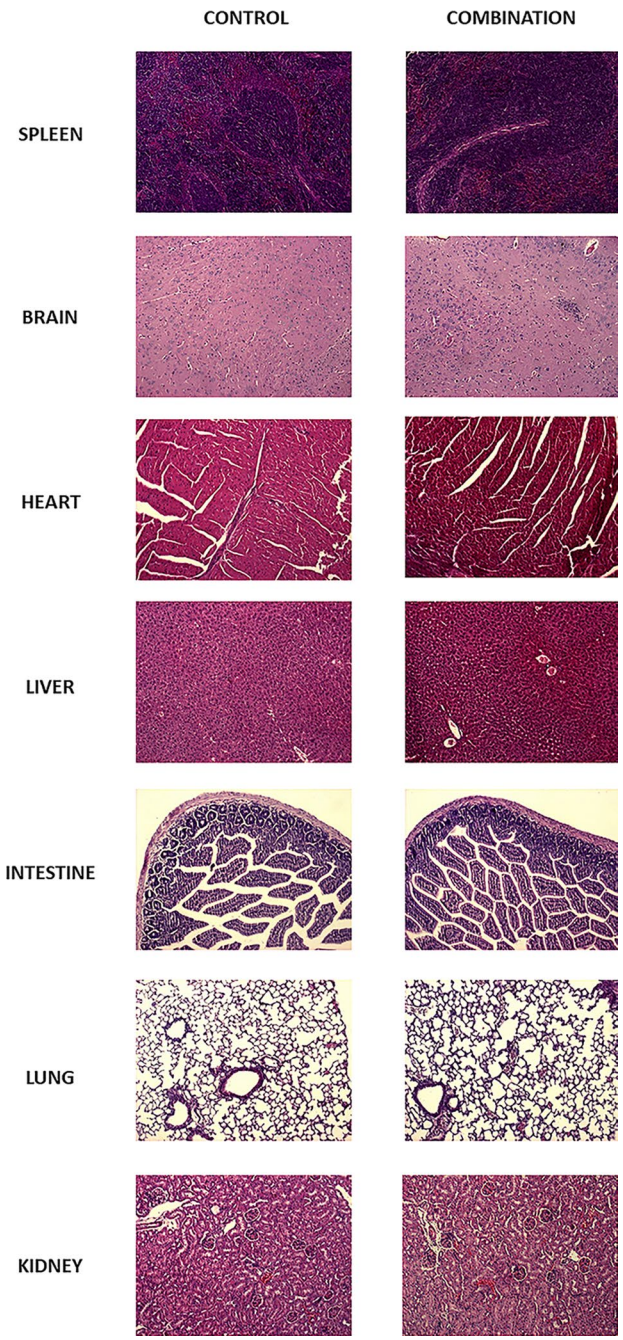


Fig. 7 Evaluation of toxicity of the combined treatment. Different tissues extracted at the end of the treatment from mice treated with vehicle (control) or with the combination were fixed in 10% formaldehyde in PBS and embedded in paraffin. Sections of 5 µm were then stained with hematoxylin–eosin. Magnification: 200X

thapsigargin might be considered a promising therapeutic strategy for the treatment of melanoma.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00432-022-04129-4.

Author contributions YB, VB and LR: conceived and designed the experiments. YB and VB: performed proliferations assays, western blots, immunohistochemistry assays. LPR and VB: performed in vivo assays. PC, CD and EM: synthesis and purification of TAP7f. YB, VB and LP: analyzed the data: YB and VB: figure preparation. LR wrote the first draft of the manuscript. All authors revised and approved the final manuscript before submission.

Funding This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 0154: "Propiedades y mecanismo de acción de nuevos agentes antitumorales: péptidos quiméricos del IFN alfa, ftalocianinas de Zn(II) y derivados sintéticos de penicilinas"; PUE 2016: "Contribución al proceso de desarrollo del medicamento. Anti-infecciosos y anti-cancerígenos"), Universidad de Buenos Aires (Programación Científica 2018–2020, UBACYT 20020170100041BA: "Mecanismos de acción in vitro e in vivo de novedosos agentes antitumorales. Implementación de estrategias terapéuticas innovadoras") and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2017–1278: "Mecanismo de acción antitumoral de péptidos quiméricos del IFN-α2b y compuestos peptidomiméticos. Efecto del tratamiento combinado con hipertermia magnética"; PICT 2017–2694: "Diseño de nuevas metodologías para la generación de scaffolds cíclicos y heterocíclicos sintética y biológicamente atractivos. Los productos naturales como fuente de inspiración"), Argentina.

Data availability All data supporting the findings of this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

References

Almanza A, Carlesso A, Chintha C, Creedican S, Doultsinos D, Leuzzi B et al (2019) Endoplasmic reticulum stress signaling—from basic mechanisms to clinical applications. FEBS J 286:241–278. https://doi.org/10.1111/febs.14608

Andersen TB, Lopez CQ, Manczak T, Martinez K, Simonsen HT (2015) Thapsigargin–from *Thapsia* L. to mipsagargin. Molecules 20:6113–6127. https://doi.org/10.3390/molecules20046113

Ascierto PA, Flaherty K, Goff S (2018) Emerging strategies in systemic therapy for the treatment of melanoma. Am Soc Clin Oncol Educ Book 38:751–758. https://doi.org/10.1200/EDBK_199047

Ashton JC (2015) Drug combination studies and their synergy quantification using the Chou-Talalay method -letter. Cancer Res 75:2400. https://doi.org/10.1158/0008-5472.CAN-14-3763

Azijli K, Stelloo E, Peters GJ, Vde AJ (2014) New developments in the treatment of metastatic melanoma: immune checkpoint inhibitors and targeted therapies. Anticancer Res 34:1493–1505

Barrionuevo E, Cayrol F, Cremaschi GA, Cornier PG, Boggián DB, Delpiccolo CML, Mata EG, Roguin LP, Blank VC (2020) A penicillin derivative exerts an anti-metastatic activity in melanoma cells through the downregulation of integrin αvβ3 and Wnt/β-catenin pathway. Front Pharmacol 11:127. https://doi.org/10.3389/fphar.2020.00127

Bellizzi Y, AnselmiRelats JM, Cornier PG, Delpiccolo CML, Mata EG, Cayrol F, Cremaschi GA, Blank VC, Roguin LP (2022) Contribution of endoplasmic reticulum stress, MAPK and PI3K/Akt pathways to the apoptotic death induced by a penicillin derivative

in melanoma cells. Apoptosis 27:34–48. https://doi.org/10.1007/s10495-021-01697-7

Blank V, Bellizzi Y, Zotta E, Cornier PG, Delpiccolo CML, Boggián DB, Mata EG, Roguin LP (2018) A novel penicillin derivative induces antitumor effect in melanoma cells. Anticancer Drugs 29:416–428. https://doi.org/10.1097/CAD.0000000000000611

Chen LH, Jiang CC, Kiejda KA, Wang YF, Thorne RF, Zhang XD, Hersey P (2007) Thapsigargin sensitizes human melanoma cells to TRAIL-induced apoptosis by up-regulation of TRAIL-R2 through the unfolded protein response. Carcinogenesis 28:2328–2336. https://doi.org/10.1093/carcin/bgm173

Chen D, Liu X, Yang Y, Yang H, Pen L (2015) Systematic synergy modeling: understanding drug synergy from a systems biology perspective. BMC Syst Biol 9:56. https://doi.org/10.1186/s12918-015-0202-y

Chen Y, Li H, Zhang G, Wu Y, Xiao J, Liu J, Qiu P, Liu X, Sun L, Du B, Tan Y (2020) Synergistic inhibitory effect of resveratrol and TK/GCV therapy on melanoma cells. J Cancer Res Clin Oncol 146:1489–1499. https://doi.org/10.1007/s00432-020-03203-z

Chiarante N, Duhalde Vega M, Valli F, Zotta E, Daghero H, Basika T, Bollati-Fogolin M, García Vior MC, Marino J, Roguin LP (2020) In vivo photodynamic therapy with a lipophilic zinc(II) phthalocyanine inhibits colorectal cancer and induces a Th1/CD8 antitumor immune response. Lasers Surg Med 53:344–358. https://doi.org/10.1002/lsm.23284

Chou TC (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70:440–446. https://doi.org/10.1158/0008-5472.CAN-09-1947

Christensen SB, Skytte DM, Denmeade SR, Dionne C, Møller JV, Nissen P, Isaacs JT (2009) A Trojan horse in drug development: targeting of thapsigargins towards prostate cancer cells. Anticancer Agents Med Chem 9:276–294. https://doi.org/10.2174/1871520610909030276

Cornier PG, Delpiccolo CML, Mascali F, Boggián DB, Mata EG, Cárdenas M, Blank V, Roguin LP (2014) In vitro anticancer activity and SAR studies of triazolyl aminoacyl(peptidyl) penicillins. Med Chem Commun 5:214–218. https://doi.org/10.1039/C3MD00332A

Doan NT, Paulsen ES, Sehgal P, Møller JV, Nissen P, Denmeade SR, Isaacs JT, Dionne CA, Christensen SB (2015) Targeting thapsigargin towards tumors. Steroids 97:2–7. https://doi.org/10.1016/j.steroids.2014.07.009

Dorasamy MS, Ab A, Nellore K, Wong PF (2019) Synergistic inhibition of melanoma xenografts by brequinar sodium and doxorubicin. Biomed Pharmacother 110:29–36. https://doi.org/10.1016/j.biopha.2018.11.010

Foucquier J, Guedj M (2015) Analysis of drug combinations: current methodological landscape. Pharmacol Res Perspect 3:e00149. https://doi.org/10.1002/prp2.149

Ghosh S, Adhikary A, Chakraborty S, Bhattacharjee P, Mazumder M, Putatunda S, Gorain M, Chakraborty A, Kundu GC, Das T, Sen PC (2015) Cross-talk between endoplasmic reticulum (ER) stress and the MEK/ERK pathway potentiates apoptosis in human triple negative breast carcinoma cells: role of a dihydropyrimidone, nifetepimine. J B Chem 290:3936–3949. https://doi.org/10.1074/jbc.M114.594028

Gowda R, Sharma A, Robertson GP (2017) Synergistic inhibitory effects of Celecoxib and Plumbagin on melanoma tumor growth. Cancer Lett 385:243–250. https://doi.org/10.1016/j.canlet.2016.10.016

Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS et al (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 369:134–144. https://doi.org/10.1056/NEJMoa1305133

Hassa PO, Hottiger MO (2008) The Diverse biological roles of mammalian PARPs, a small but powerful family of poly-ADP-ribose polymerases. Front Biosci 13:3046–3082. https://doi.org/10.2741/2909

He Q, Lee DI, Rong R, Yu M, Luo X, Klein M, El-Deiry WS, Huang Y, Hussain A, Sheikh MS (2002) Endoplasmic reticulum calcium pool depletion-induced apoptosis is coupled with activation of the death receptor 5 pathway. Oncogene 21:2623–2633. https://doi.org/10.1038/sj.onc.1205345

Huang L, Xu J, Li K, Zheng MH, Kumta SM (2004) Thapsigargin potentiates TRAIL-induced apoptosis in giant cell tumor of bone. Bone 34:971–981. https://doi.org/10.1016/j.bone.2004.02.005

Jia J, Zhu F, Ma X, Cao Z, Cao ZW, Li Y, Li YX, Chen YZ (2009) Mechanisms of drug combinations: interaction and network perspectives. Nat Rev Drug Discov 8:111–128. https://doi.org/10.1038/nrd2683

Landegren UJ (1984) Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. J Immunol Methods 67:379–388. https://doi.org/10.1016/0022-1759(84)90477-0

Lopez MV, Viale DL, Cafferata EGA, Bravo AI, Carbone C, Gould D, Chernajovsky Y, Podhajcer OL (2009) Tumor associated stromal cells play a critical role on the outcome of the oncolytic efficacy of conditionally replicative adenoviruses. PLoS ONE 4:e5119. https://doi.org/10.1371/journal.pone.0005119

Margue C, Philippidou D, Kozar I, Cesi G, Felten P, Kulms D, Letellier E, Haan C, Kreis SJ (2019) Kinase inhibitor library screening identifies synergistic drug combinations effective in sensitive and resistant melanoma cells. Exp Clin Cancer Res 38:56. https://doi.org/10.1186/s13046-019-1038-x

McKean MA, Amaria RN (2018) Multidisciplinary treatment strategies in high-risk resectable melanoma: role of adjuvant and neoadjuvant therapy. Rev Cancer Treat Rev 70:144–153. https://doi.org/10.1016/j.ctrv.2018.08.011

Nowicki TS, Hu-Lieskovan S, Ribas A (2018) Mechanisms of resistance to PD-1 and PD-L1 blockade. Cancer J 24:47–53. https://doi.org/10.1097/PPO.0000000000000303

Oakes S, Papa FR (2015) The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol 10:173–194. https://doi.org/10.1146/annurev-pathol-012513-104649

Rodríguez-Hernández Á, Brea-Calvo G, Fernández-Ayala DJM, Cordero M, Navas P, Sánchez-Alcázar JA (2006) Nuclear caspase-3 and capase-7 activation, and Poly(ADP-Ribose) polymerase cleavage are early events in camptothecin-induced apoptosis. Apoptosis 11:131–139. https://doi.org/10.1007/s10495-005-3276-y

Rughani MG, Gupta A, Middleton MR (2013) New treatment approaches in melanoma: current research and clinical prospects. Ther Adv Med Oncol 5:73–80. https://doi.org/10.1177/1758834012463260

Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Akha AAS, Raden D, Kaufman RJ (2006) Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. PLoS Biol 4(11):e374. https://doi.org/10.1371/journal.pbio.0040374

Schröder M, Kaufman RJ (2005) ER stress and the unfolded protein response. Mutat Res 569:29–63. https://doi.org/10.1016/j.mrfmmm.2004.06.056

Sehgal P, Szalai P, Olesen C, Praetorius HA, Nissen P, Christensen SB, Engedal N, Møller JV (2017) Inhibition of the sarco/endoplasmic reticulum (ER) Ca2+-ATPase by thapsigargin analogs induces cell death via ER Ca2+ depletion and the unfolded protein response. J Biol Chem 292:19656–19673. https://doi.org/10.1074/jbc.M117.796920

Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A (2017) Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 168:707–723. https://doi.org/10.1016/j.cell.2017.01.017

Sucher NJ (2014) Searching for synergy in silico, in vitro and in vivo. Synergy 1:30–43

Szegezdi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 7:880–888. https://doi.org/10.1038/sj.embor.7400779

Testori AAE, Ribero S, Indini A, Mandalà M (2019) Adjuvant treatment of melanoma: recent developments and future perspectives. Am J Clin Dermatol 20:817–827. https://doi.org/10.1007/s40257-019-00456-4

Wang M, Kaufman RJ (2014) The impact of the endoplasmic reticulum protein-folding environment on cancer development. Nat Rev Cancer 14:581–597. https://doi.org/10.1038/nrc3800

Wang C, Li T, Tang S, Zhao D, Zhang C, Zhang S, Deng S, Zhou Y, Xiao X (2016) Thapsigargin induces apoptosis when autophagy is inhibited in HepG2 cells and both processes are regulated by ROS-dependent pathway. Environ Toxicol Pharmacol 41:167–179. https://doi.org/10.1016/j.etap.2015.11.020

Wang M, Law ME, Castellano RK, Law BK (2018) The unfolded protein response as a target for anticancer therapeutics. Crit Rev Oncol Hematol 127:66–79. https://doi.org/10.1016/j.critrevonc.2018.05.003

Winther AML, Liu H, Sonntag Y, Olesen C, le Maire M, Soehoel H, Olsen CE, Christensen S, Nissen P, Møller J (2010) Critical roles of hydrophobicity and orientation of side chains for inactivation of sarcoplasmic reticulum Ca2+-ATPase with thapsigargin and thapsigargin analogs. J Biol Chem 285:28883–28892. https://doi.org/10.1074/jbc.M110.136242

Wu L, Huang X, Kuang Y, Xing Z, Deng X, Luo Z (2019) Thapsigargin induces apoptosis in adrenocortical carcinoma by activating endoplasmic reticulum stress and the JNK signaling pathway: an in vitro and in vivo study. Drug Des Devel Ther 13:2787–2798. https://doi.org/10.2147/DDDT.S209947

Yamaguchi H, Wang HJ (2004) CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 279:45495–45502. https://doi.org/10.1074/jbc.M406933200