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Original article

Palladacycle (BPC) antitumour activity against resistant and metastatic cell lines: The relationship with cytosolic calcium mobilisation and cathepsin B activity

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ABSTRACT

The search for new compounds that induce p53-independent apoptosis is the focus of many studies in cancer biology because these compounds could be more specific and would overcome chemotherapy resistance. In this study, we evaluated the *in vitro* antitumour activity of a Biphosphinic Palladacycle Complex (BPC) and extended preclinical studies to an *in vivo* model. Saos-2 cells, a p53-null human osteosarcoma drug-resistant cell line, were treated with BPC in the presence or absence of a cathepsin B inhibitor and a calcium chelator (CA074 and BAPTA-AM, respectively), and several parameters related to apoptosis were evaluated. Preclinical studies were performed with mice that were intravenously inoculated with murine melanoma B16F10-Nex2 cells and treated intraperitoneally (i.p.) with BPC (8 mg/kg/day) for ten consecutive days, when lung metastatic nodules were counted. *In vitro* data show that BPC induces cell death in Saos-2 cells mainly by apoptosis, which was accompanied by the effector caspase-3 activation. These events are most likely related to Bax translocation and increased cytosolic calcium mobilisation, mainly from intracellular compartments. Lysosomal Membrane Permeabilisation (LMP) was also observed after 12 h of BPC exposure. Interestingly, BAPTA-AM and CA074 significantly decreased BPC cytotoxicity, suggesting that both calcium and cathepsin B are required for BPC antitumour activity. *In vivo* studies demonstrated that BPC protects mice against murine metastatic melanoma. In conclusion, BPC complex is an effective anticancer compound against metastatic murine melanoma. This complex is cytotoxic to the drug-resistant osteosarcoma Saos-2 human tumour cells by inducing apoptosis triggered by calcium signalling and a lysosomal-dependent pathway.

1. Introduction

Calcium is a signal transducing ion that is important for cell life. Alterations in frequency and/or amplitude of calcium due to a wide variety of agents and conditions can lead to cell death by numerous mechanisms [1–3]. Thus, it is essential to understand the pathways by which calcium regulates cell life and death to facilitate drug development for diseases involving abnormal calcium signalling, such as heart failure, diabetes, Alzheimer's disease and cancer [4–8]. In relation to calcium signalling in cancer, there are multiple important cytotoxic agents that disrupt calcium homeostasis and

Abbreviations: BPC, Biphosphinic Palladacycle Complex; MTT, Methyl Thiazol Tetrazolium; IMDM, Iscove's Modified Dulbecco's Medium; DMSO, Dimethyl Sulfoxide; PI, propidium iodide; FITC, fluorescein isothiocyanate; LMP, Lysosomal Membrane Permeabilisation; AO, acridine orange; GFP, green fluorescent protein; TAP, thapsigargin; FCCP, *Carbonyl cyanide* 4-(trifluoromethoxy)phenylhydrazone; RFU, relative fluorescence units; FBS, foetal bovine serum; IC_{50}, half maximum inhibitory concentration; ER, endoplasmic reticulum.

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cause apoptosis. One such agent is cisplatin, a platinum-based anticancer drug that causes an IP_3R-dependent cytosolic calcium increase with calpain activation prior to the induction of apoptosis [9–11].

The involvement of Bcl-2 proapoptotic and antiapoptotic family members, such as Bax, on the modulation of calcium mobilisation dependent cell death is also expected because these proteins regulate the release of cytochrome *c* from the mitochondria into the cytosol, where other mediators of apoptosis are activated. Bax, a pro-apoptotic molecule, is required for calcium elevation in response to staurosporine (STS) [12,13], and reconstitution of Bax in a prostate cancer cell line augments cytosolic calcium and restores mitochondrial uptake. Activation of Bax appears to involve subcellular translocation and dimerisation because a substantial portion of Bax is monomeric and found either in the cytosol or loosely attached to membranes in viable cells. Following death stimulus, cytosolic and monomeric Bax translocates to the mitochondria, where Bax becomes an integral membrane protein and cross-links as a homodimer [14,15].

Recently, Bax translocation has also been involved in lysosome membrane permeabilisation (LMP). According to the literature, STS-exposed cells have a punctate distribution of Bax, which is in part co-localised to the lysosomes, suggesting that when inserted into the lysosome membrane, Bax is an LMP mediator that promotes the release of lysosome enzymes into the cytosol, where the enzymes trigger cell death [16,17]. These findings are supported by studies that show that lysosomotropic agents result in the rapid permeabilisation of the lysosome membrane, leading to apoptosis [18–20]. Kroemer and Jäätelä, 2005 [21], and that demonstrate that lysosomal hydrolases contribute to cell death when released into the cytosol and to cancer progression when released into the extracellular space.

Considering all of these aspects, a new class of palladium compounds based on cisplatin structural and pharmacological properties were developed and synthesised beginning in 1980. Although the first synthesised compounds exhibited little stability in biological systems, the use of ligands later solved this problem [22,23], resulting in a new class of organometallic compounds. One complex, called Biphosphinic Palladacycle Complex (BPC), was produced from the cyclisation of Pd by cyclometallation reactions [24]. This palladium complex is a chiral cyclopalladate derived from N,N-dimethyl-1-phenethylamine and the coordinating ligand 1,1′-bis(diphenylphosphine)ferrocene that exhibits lethal effects on human leukaemia cells and is ineffective against normal human lymphocytes [25,26]. Interestingly, parts of the BPC cytotoxic effects on leukaemia cells were dependent on LMP and cathepsin B activity. BPC has been shown to be a lysosomotropic agent, demonstrating an ability to lodge into acidic compartments, such as lysosomes [25]. Other important studies with palladium complexes were also reported. Carrera et al. [27] demonstrated that organometallic palladium complexes having a water-soluble iminophosphorane ligand are cytotoxic to various human cancer cell lines such as acute lymphoblastic leukaemia and human prostate cancer cells.

Although several palladium compounds are cytotoxic to many cancer cell lines in *in vitro* models [24–27], further studies should be conducted to prove the *in vivo* efficacy of these compounds.

In this study, we evaluated the BPC cytotoxicity mechanism in an *in vitro* model using an osteosarcoma cell line, Saos-2, which is resistant to most commercially available chemotherapy drugs and is a p-53 null cell line [28,29]. We have also evaluated, for the first time, the *in vivo* activity of BPC using a very well established metastatic melanoma murine model. Our results demonstrated that BPC is cytotoxic against Saos-2 tumour cells *in vitro* and that its antitumour potential is also verified *in vivo*.

2. Material and methods

2.1. Biphosphinic Palladacycle Complex 1:2 (BPC)

BPC was synthesised from cyclopalladated compounds derivative from *S*(−) enantiomers of *N*,*N*-dimethyl-1-phenethylamine and the ligand 1,1′-bis(diphenylphosphine)ferrocene (dppf), as previously described in Bincoletto et al. 2005 [24] (Fig. 1). The compound, which is under pre-clinical investigation [30], was diluted in ethanol (Sigma Aldrich) and then in culture medium (ethanol final concentration of 0.1%) for *in vitro* and *in vivo* experiments.

2.2. Saos-2 cell line

The Saos-2 cell line was derived from the primary osteosarcoma of an 11-year-old Caucasian girl in 1973 by Fogh et al. [31]. The cells were originally obtained from the Italian Interlab Cell Line Collection (ICLC) cell bank and generously donated by Dr. Célia Regina Nogueira from the Clinical Medical Department, Botucatu School of Medicine, São Paulo State University (UNESP), Botucatu, SP, Brazil. Saos-2 cells were cultured in a monolayer in IMDM medium (Gibco®, USA) supplemented with 20% foetal calf serum, 100 UI/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37 °C in 5% CO^2. Cells were trypsinised every 5 days using 0.01% trypsin-1 mM EDTA (Gibco®, USA).

2.3. Cytotoxicity evaluation using MTT and Trypan Blue exclusion assays

To assess cell viability, control and BPC treated (24 h) Saos-2 cells were trypsinised and resuspended in equal volumes of medium and trypan blue (0.05% solution) and were counted using a haemocytometer chamber. Cell viability was also measured using a standard methyl-thiazol-tetrazolium (MTT) assay, as previously described by Mosmmann, 1983 [32]. Briefly, 5×10^4 viable cells were seeded into 96-well flat plates (Corning, USA) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% foetal calf serum and incubated with different concentrations of

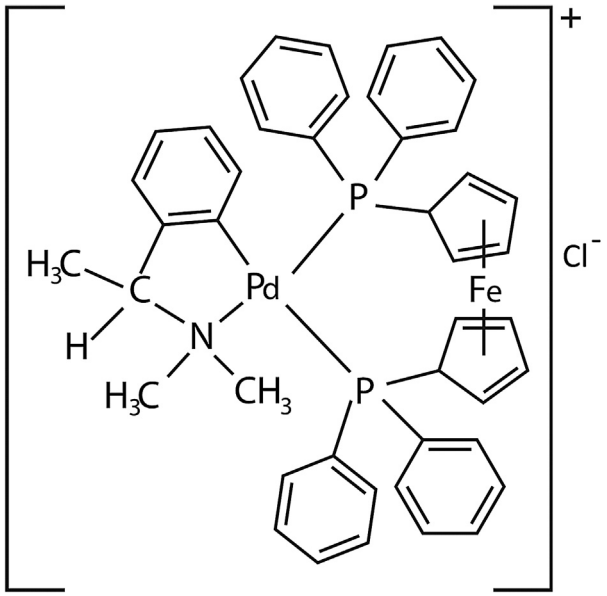

Fig. 1. Structural representation of the ionic palladacycle [Pd(C^2, N–*S*(−) dmpa)(dppf)] Cl − Biphosphinic Palladacycle Complex (BPC).

BPC for 24 h. Then, 10 μl of MTT (5 mg/ml/well) was added and incubated for 4 h. After that, 100 μl of Dimethyl Sulfoxide (DMSO) (Merck, Germany) was added to each well to solubilise the formazan crystals. Absorbance was measured at 560 nm in FlexStation® 3 Multi-Mode Benchtop Reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

2.4. Detection of apoptosis by flow-cytometry

Saos-2 cells treated with BPC for 24 h were stained with fluorescein isothiocyanate (FITC)-conjugated to annexin V and propidium iodide (PI) according to the manufacturer's instructions (Annexin V/FITC Apoptosis Detection Kit, BD Pharmingen, CA, USA). The population of annexin V^-/PI^- viable cells and annexin V^+/PI^+ apoptotic or necrotic cells was evaluated by flow cytometry. Data were collected in an FACS Calibur (Becton–Dickinson, Mountain View, CA, U.S.A.) and were analysed with CellQuest software (Becton–Dickinson).

2.5. Caspase-3 activity

Active caspase-3 was evaluated in Saos-2 cells treated with BPC for 24 h using flow cytometric analysis of the endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 according to the manufacturer's instructions (Cell Signaling, MA, U.S.A.). Briefly, after treatment, Saos-2 cells were washed with PBS and fixed in 2% paraformaldehyde in PBS (v/v) for 30 min. Cells were then permeabilised in PBS containing 0.01% saponin and 1% BSA. Afterwards, 10 μL of cleaved caspase-3 (Asp175) Alexa Fluor 488-conjugated antibody was added, and the cells were incubated in the dark at room temperature for 1 h. Cells were resuspended in 400 μL of PBS and analysed (10^5 events were collected per sample) in a FACS Calibur Flow cytometer (Beckton Dickinson, CA, USA) using the CellQuest software (Becton Dickinson).

2.6. Lysosomal Membrane Permeabilisation (LMP) evaluated by laser scanning confocal microscopy

To observe the lysosomotropic properties of BPC, we used the acridine orange (AO) relocation method, as previously described [33–35]. The ligand 1,1′-bis(diphenylphosphino)ferrocene (20 μM) and the BPC analogous, [Pd($R_{(+)}$ C^2,N-dmpa) (dppe)]Cl (RE) (20 μM), which has an ethane instead of a ferrocene [36] (Fig. 1 – supplementary material) were also evaluated to explore if LMP is an event produced only by the complex studied here (BPC) or can be induced by other agents. AO, a metachromatic fluorophore, accumulates mainly in the acidic vacuolar apparatus, preferentially in lysosomes. When excited by blue light (relocation method), AO shows red or green fluorescence at high (lysosomal) or low (nuclear and cytosolic) concentrations, respectively. Rupture of initially AO-loaded lysosomes may be monitored as an increase in cytoplasmic diffuse green fluorescence or as a decrease in granular red fluorescence [37,38]. For imaging, Saos-2 cells were grown on coverslips and labelled *in vivo* with 5 μg/ml AO in IMDM medium without serum for 15 min at 37 °C in 5% CO^2. Then, the cells were washed with IMDM medium and exposed to 20 μM BPC for 12 h at 37 °C in 5% CO_2. The fluorescent signals of AO were taken with a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany).

2.7. Bax translocation

Saos-2 cells were transiently transfected with GFP-Bax 12–24 h before experiments were performed. All transfections were performed using FuGene® HD (Roche, USA) according to the manufacturer's protocol. Images of GFP-Bax transfected cells were collected every 30 min using a high resolution fluorescence microscope (Nikon TE 300, Nikon, Osaka, Japan) coupled to a CCD camera (CoolSnap-Roper Scientific Inc., Princeton Instruments, Princeton, NJ, USA). Images were acquired by BioIP software (Anderson Eng., USA). The 488 nm line of the Argon laser was used for the excitation of GFP-Bax, and the emission fluorescence was long-pass filtered at 500 nm. To follow Bax movements, cells were placed under the scope and treated with BPC. All studies were carried out at 37 °C.

2.8. Ca^{2+} measurements of Saos-2 cells exposed to BPC

To evaluate Ca^{2+} handling, Saos-2 cells were plated on black 96 well plates for 48 h in IMDM medium supplemented with 20% FBS and were dried at 5% CO_2 and 37 °C until the experiment. Then, the culture medium was removed and 50 μL of a buffer containing FLUO-4 NW (Molecular Probes, USA) was added to each well. The plate was incubated for 30 min. The Ca^{2+} levels were assessed by a FlexStation 3 multi-mode benchtop reader® (Molecular Devices, Sunnyvale, CA, USA).

To verify the origin of the released Ca^{2+}, cells were incubated in Thapsigargin (TAP) (2 μM), carbonyl cyanide p-Trifluormetoxifenil-hydrazone (FCCP) (5 μM) or in a Ca^{2+} free buffer and pre-treated for 15 min with bafilomycin (1 μM) to deplete endoplasmic reticulum, mitochondrial, external and lysosomal calcium stores, respectively, and then exposed to BPC. Lysosomal calcium stores were also evaluated in Saos-2 cells after treatment with BPC for 12 h and then exposed to bafilomycin (1 μM). All incubations were performed in fluorescence buffer with the following composition (mM): NaCl 138; KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, NaHCO3 15 and glucose 5.5 (pH 7.4).

We observed the response signal after Ca^{2+} depletion of the ER, mitochondria, lysosomes and extracellular Ca^{2+}. Data are expressed as an increase in the amount of relative fluorescence units (RFU). Using the SoftMax Pro software 5.3, we converted the expressed data to a percentage and then performed the statistical analysis.

2.9. In vivo experiments to evaluate the antitumour activity of BPC in a model of experimental tumour metastasis

For this experiment, C57Bl/6 male mice (8 weeks old) were purchased from CEDEME (Centro de Desenvolvimento de Modelos Experimentais, EPM/UNIFESP, São Paulo, Brasil) and were maintained in a sterilised environment with food and water *ad libitum* in a 12 h light/dark cycle. This experiment was conducted and approved by the Animal Experimentation Ethics Committee of UNIFESP under protocol 0316/06.

For this experiment, a clone of B16F10-Nex2, a murine melanoma sub-line (established at the Experimental Oncology Unit, Federal University of São Paulo - UNIFESP), was used as previously described [39]. This cell line was maintained in RPMI 1640 medium (Gibco®) supplemented with 10 mM HEPES (Sigma–Aldrich), 24 mM sodium bicarbonate, 40 mg/L gentamicin (Hipolabor, MG, Brazil), pH 7.2, and 10% foetal bovine serum (FBS). C57Bl/6 mice were injected intravenously in the tail vein with 5×10^5 B16F10-Nex2 viable cells in 100 μL of RPMI medium. Twenty-four hours after cell inoculation, BPC (8 mg/kg) was administered (i.p.) to the mice for 10 consecutive days. The control group was treated with vehicle ethanol (final concentration of 0.1%). Twenty-four hours after the last dose of BPC, mice were sacrificed by cervical dislocation, the lungs were collected, and the pulmonary nodules were counted using an inverted microscope.

2.10. Statistical analysis

Data for each assay of three independent experiments run in triplicate were analysed statistically by one-way ANOVA. Data are

shown as the mean ± S.D. Multiple comparisons among group mean differences were verified with the *Tukey* or *Dunnett* post-test. Student's *t* test was also used to analyse data when pertinent. Differences were considered significant when the *P*-value was less than 0.05. The results were expressed as a percentage of the controls, and the computer software package "Origin" was used to determine the IC_{50} values (the concentration that exhibits a 50% inhibitory effect on the evaluated parameter).

3. Results

3.1. BPC-Saos-2 cells cytotoxicity and apoptosis induction

The number of living Saos-2 cells after 24 h incubation with BPC was measured with an MTT reduction test and a trypan blue exclusion assay endpoint screening (Fig. 2A and B, respectively). The half maximum inhibitory concentration (IC_{50}) values obtained from theses assays for BPC in Saos-2 cells were 17.24 μM and 22.74 μM, respectively. Thus, the concentration of 20 μM was selected to determine whether BPC causes cell death by apoptosis, which was evaluated in Saos-2 cells by staining with annexin V-FITC/PI (Fig. 2C). BPC significantly increased the percentage of apoptotic cells (comprising 34.65% ± 7.5 of cells), necrotic cells (12.21% ± 2.0) and secondary necrotic cells (12.89% ± 1.5), suggesting that more than one signalling pathway might be activated by BPC in this cellular model.

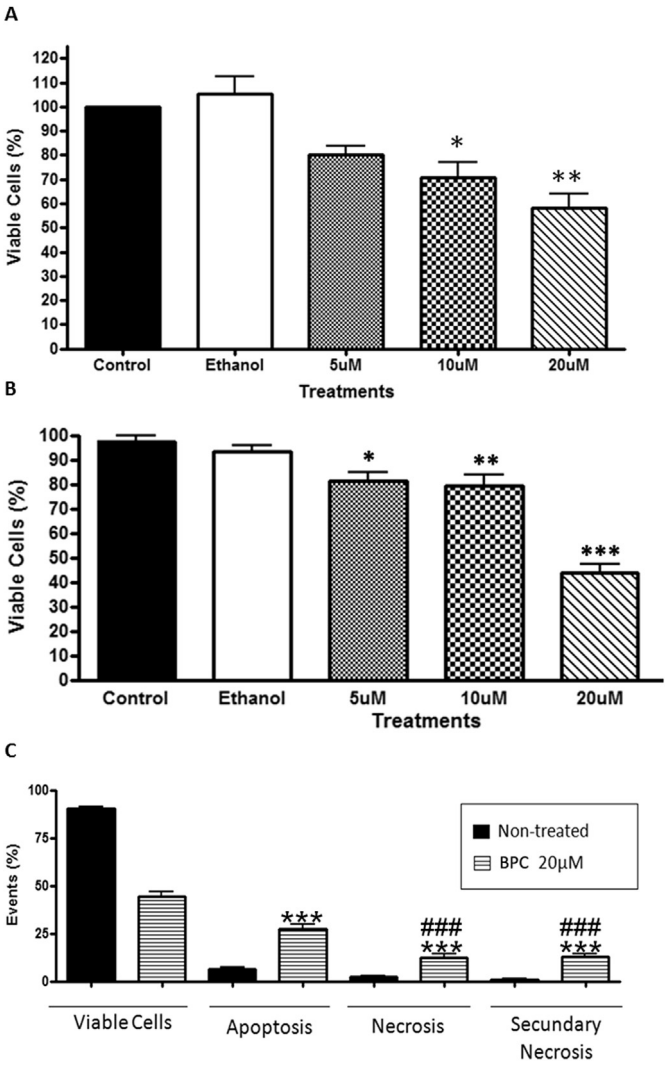

Fig. 2. Viability of Saos-2 cells after incubation with 20 μM of BPC for 24 h. Cell viability was evaluated by an MTT reduction test screening (A) and trypan blue exclusion assay (B). Each column bar represents the means ± S.E.M. of three independent experiments in replicates. Saos-2 cells' BPC IC_{50} was 17.24 μM and 22.74 μM for the MTT reduction test and the trypan blue exclusion assay, respectively. (C). Quantification of apoptosis/necrosis using fluorescence staining with Annexin-V-FITC and propidium iodide (PI) in Saos-2 cells incubated with 20 μM BPC for 24 h. One way ANOVA followed by *Tukey's* post-test. $^{*}p < 0.05$ compared to control group; $^{**}p < 0.01$ compared to control group; $^{***}p < 0.001$ compared to control group (B) or viable cells (C); $^{\#\#\#}p < 0.001$ compared to BPC treated apoptotic cells.

3.2. Cytosolic calcium dependent Saos-2 cells death induced by BPC

Calcium is an important second messenger with important roles in cell death and survival. The equilibrium between calcium release and calcium uptake by cell calcium stores is essential to deciding the fate of the cell [1]. To observe the role of calcium in BPC-induced cytotoxicity in Saos-2 cells, cytosolic calcium was measured. Our results demonstrated that BPC increased the cytosolic calcium levels by approximately 30% in relation to the basal control, as expressed by RFU (Fig. 3A). We next evaluated the role of extracellular calcium influx, mitochondria, ER and lysosomes in BPC-induced Ca^{2+} homeostasis alterations using a calcium free solution, FCCP, TAP and bafilomycin, respectively. The results suggest that the calcium is mainly supplied by the mitochondria, followed by the endoplasmic reticulum (ER) and the extracellular medium. As lysosome calcium was not released in a significant way in this experimental condition, minutes after BPC addition, we also evaluated the participation of lysosomal calcium stores after 12 h of BPC exposure. After 12 h of incubation, cytosolic calcium mobilisation after bafilomycin addition was significantly reduced in BPC treated cells when compared to BPC non-treated cells (Fig. 3B). These findings suggest that mobilisation of calcium from lysosome occurs in a function of time, which is probably dependent on LMP, observed after 12 h of BPC incubation.

The importance of calcium for BPC-cytotoxicity was also demonstrated by a trypan blue exclusion assay of Saos-2 cells pre-treated for 30 min with BAPTA-AM (a calcium chelator) before BPC incubation (20 μM) for 24 h. The results of the trypan blue assay suggest that BPC cytotoxicity is significantly reduced after treatment with BAPTA-AM (Fig. 3C), demonstrating a central role for calcium in the BPC-cytotoxicity response of Saos-2 cells.

3.3. BPC-induced GFP-Bax translocation and caspase-3 activation

Bax is a pro-apoptotic protein that is translocated to the mitochondrial membrane and contributes to the release of many pro-apoptotic factors with caspase-3 activation and apoptosis. Therefore, we evaluated the role of Bax in GFP-Bax transfected Saos-2 cells treated with BPC. We observed a GFP-Bax discrete punctuation in a time-dependent manner beginning 3 h after BPC incubation, with a maximum notable punctuation observed after 6 of exposure (Fig. 4A). This event most likely contributes to the activated caspase-3 observed in Saos-2 cells (Fig. 4B), denoting by the presence of a large endogenous fragments (17/19 kDa) of caspase-3 due to aspartic acid 175 adjacent cleavages.

3.4. LMP and cathepsin B involvement in BPC Saos-2-induced cell death

The disruption of lysosomal membrane integrity allows for the release of proteases into the cytosol, such as cathepsin B, where these proteins contribute to cell death through several mechanisms [40]. As shown in Fig. 5A, BPC-untreated control cells (quadrants A, B and C) show low green fluorescence, indicating a lower concentration of the dye AO in the cytoplasm and nucleus (A);

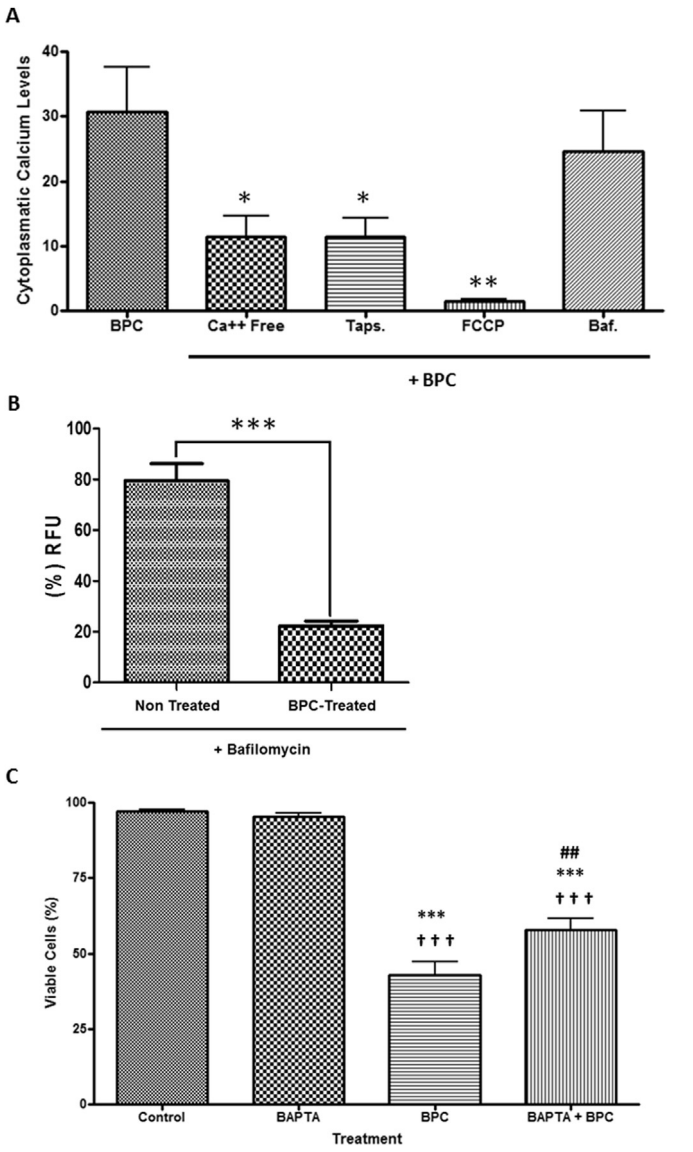

Fig. 3. (A) Cytosolic calcium levels. The first column bar represents the cytosolic calcium levels in normal buffer. The histogram represents the quantification of Ca^{2+} levels induced by BPC (20 μM) in medium without Ca^{2+}. The following histograms respectively represent the group pre-treated with TAP [2 μM] (depletion of stocks of ER Ca^{2+}); FCCP [5 μM] (depletion of mitochondrial Ca^{2+}) and bafilomycin [1 μM] (depletion of lysosomal Ca^{2+}) with subsequent addition of BPC (20 μM). One way ANOVA followed by *Dunnett* post-test. $^{*}p < 0.05$ compared to BPC group. $^{**}p < 0.01$ compared to BPC group. (B) Calcium mobilisation from lysosomal stores after addition of bafilomycin (1 μM) in 12 h of BPC-treated and non-treated Saos-2 cells. Unpaired two-tailed Student's *t*-test. $^{***}p < 0.0001$ when compared with control untreated cells. (C) The treatment of Saos-2 cells with BAPTA-AM to analyse the involvement of calcium in BPC Saos-2-induced cell death. Calcium chelation increases the amount of Saos-2 viable cells, indicating that this ion has an involvement in BPC induced cell death. Data obtained from three independent experiments in triplicate. $^{***}p < 0.001$ compared to control group; $^{\dagger\dagger\dagger}p < 0.001$ compared to BAPTA-AM group; $^{\#\#}p < 0.01$ compared to BPC group. One way ANOVA followed by *Tukey's* post-test.

compartments marked with red fluorescence that are morphologically intact and well defined within the cells (B); and the absence of the co-localisation of green and red fluorescence, which indicates the integrity of the structure of the compartments' acids (C). However, Saos-2 cells pre-incubated with BPC for a period of 12 h (quadrant D, E and F) show an increase in green fluorescence due to the release of AO marker dye into the cytosol (D); an absence of intact lysosomes, with morphologically altered structures expressing red fluorescence (E); and a clear co-localisation between the fluorescence expressed in red and green, which strongly suggests that no AO marker was leaking after incubation with 20 μM of BPC (F).

As expected, the 1,1′-bis(diphenylphosphino)ferrocene and the analogous of BPC (RE) did no induce LMP after 12 h of incubation, which strongly suggest that LMP is a specific event started by the BPC molecule studied here (Fig. 2, Supplementary material).

As LMP permits the release of proteases into the cytosol, thus allowing for cleavage of various proteins, cathepsin B involvement in BPC Saos-2 induced cell death was also studied. For this experiment, the cathepsin B activity was inhibited by CA074, a classical pharmacological cathepsin B inhibitor. The trypan blue exclusion assay showed that Saos-2 cells co-treated with CA074 and BPC exhibited a significant decrease in cell death compared to the BPC-treated group, suggesting a crucial role of this lysosomal protease in the BPC cell death mechanism (Fig. 5B). These data suggest that besides cytosolic calcium, cathepsin B also has an important role in the execution of BPC apoptosis and necrotic cell death induction.

3.5. *In vivo antitumour activity of BPC using a melanoma resistant/metastatic tumour cells (B16F10-Nex2)*

Novel compounds cytotoxic to tumour cells *in vitro* must be tested in preclinical animal models to determine the availability and effectiveness of these compounds. The *in vitro* results presented here have shown the excellent antitumour properties of BPC in Saos-2 cells. In the absence of a syngeneic murine model for osteosarcoma, we analysed the antitumour activity of BPC using a murine metastatic melanoma model. For this purpose, C57Bl/6 mice were injected e.v. with melanoma resistant/metastatic tumour cells (B16F10-Nex2) and were treated (i.p.) with BPC (8 mg/kg/day) for 10 consecutive days. Twenty-four hours after the last dose of BPC, the mice were sacrificed, and the B16F10-Nex2 lung nodules were analysed (Fig. 6A) and counted (Fig. 6B). From these results, we showed that BPC treatment resulted in significant protection, as revealed by a decreased number of lung metastases in treated mice, suggesting that BPC is also active *in vivo*.

4. Discussion

Palladacycle complexes have exhibited important antitumour properties both *in vitro* and *in vivo* [24,36,41–44]. One complex, BPC, has also been shown to inhibit cathepsin B activity [24]. To further deepen the understanding of the molecular antitumour action of BPC, we evaluated the effectiveness of this complex against tumour cells using *in vitro* and *in vivo* systems. These studies demonstrated that BPC is effective *in vivo* against a specific isogenic melanoma tumour model and shows Saos-2 cell cytotoxicity, which is an osteosarcoma cell line that presents high chemotherapy resistance properties [45] due to a p53 gene expression impairment [28,46] and ABC protein expression [47]. Part of the BPC cytotoxicity to Saos-2 cells was caused by the cytosolic calcium mobilisation, LMP and a pro-apoptotic protein Bax translocation with caspase-3 activation. The importance of calcium mobilisation and LMP to the BPC Saos-2 cells cytotoxicity was confirmed when these cells were pre-incubated with BAPTA-AM, a cytosolic calcium chelator, or CA074, a cathepsin B inhibitor, prior to BPC exposure. In both situations, BPC antitumour activity was significantly decreased.

Because calcium is an important intracellular second messenger involved in apoptosis and necrosis cell death mechanisms [3,48] and is involved in the BPC cytotoxicity to Saos-2 cells, we depleted the intracellular calcium stores with FCCP, TAP and bafilomycin A1 to evaluate the roles of the mitochondria, ER and lysosomes,

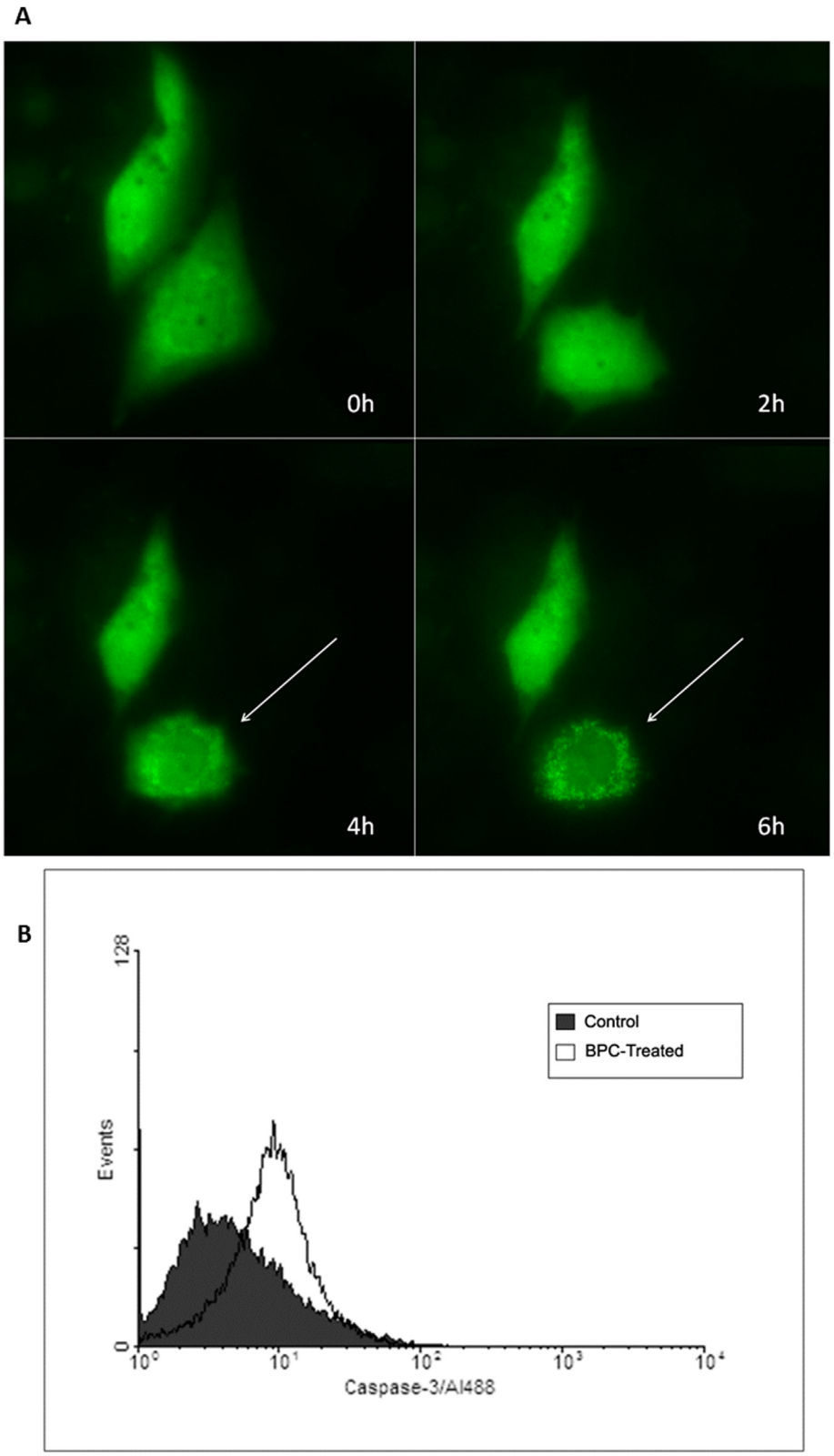

Fig. 4. (A) Analysis of qualitative temporal translocation of GFP-Bax in Saos-2 cells exposed to BPC (20 μM) for 6 h. Note that in the period that precedes the addition of BPC (0 h), the cells present a partner of homogenous green GFP-Bax protein fluorescence due to diffuse Bax protein presence in the cytosol. However, Saos-2 cells treated with BPC for 3 h (central picture) demonstrated some aspects of GFP-Bax protein aggregates, suggesting Bax translocation, which was strongly evidenced in the last quadrant of Saos-2 cells exposed to BPC for 6 h. Representative picture of the three independent experiments. (B) Presence of the inactivated form of caspase-3 (untreated cells – filled) and cleaved caspase-3 (unfilled) in Saos-2 cells exposed to BPC for 24 h. Graph deviation to the right in the treated group (unfilled) represents the presence of activated caspase-3. The image is representative of three independent experiments performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

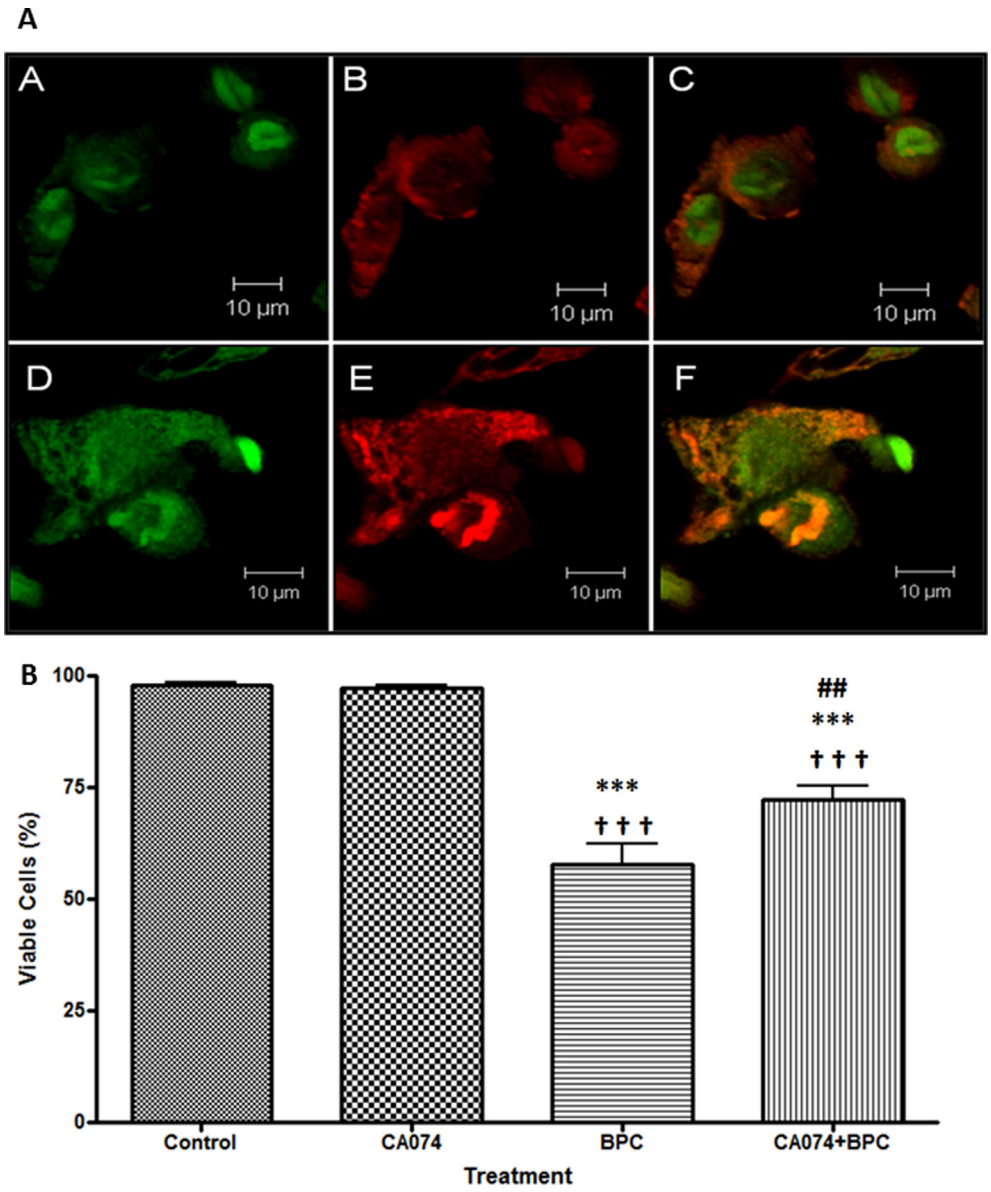

Fig. 5. (A) Evaluation of Saos-2-BPC (20 µM)-induced LMP. A decrease in lysosome (red) and an increase in cytosolic (green) fluorescence's of AO-stained cells reflecting LMP. Untreated cells (A, B and C) show (A) low green fluorescence due to the well-defined lysosomes membranes, (B) intact lysosomes within most cells and (C) the absence of co-localisation between red and green fluorescence's. BPC treated Saos-2 cells (D, E and F) show (D) increased green fluorescence due to the AO released from lysosomes into the cytosol, (E) the absence of intact lysosomes, expressed by an intense red fluorescence, and (F) a clear co-localisation between green and red fluorescence, which strongly suggests a Lysosomal Membrane Permeabilisation. Representative data obtained from three independent experiments. (B) Inhibition of Cathepsin B by CA074 to analyse the importance of this enzyme in Saos-2 cells death induced by BPC. The figure shows that Cathepsin B inhibition increases the amount of Saos-2 viable cells, indicating that it has an involvement in BPC induced cell death. $^{***}P < 0.001$ compared to control group; $^{\dagger\dagger\dagger}p < 0.001$ compared to CA074 group; $^{\#\#}p < 0.01$ compared to BPC group. Analysis performed by one way ANOVA followed by *Tukey's* post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively, in BPC antitumour properties. Under these experimental conditions, we observed a significant reduction of BPC cytosolic calcium mobilisation, mainly in Saos-2 cells previous exposed to FCCP, denoting an important mitochondrial calcium role in the palladacycle activity against Saos-2 cells. Notably, although the lysosome calcium depletion did not prevent the BPC induced cytosolic calcium mobilisation immediately following BPC administration, a reduced lysosomal calcium mobilisation was observed in Saos-2 cells treated with BPC for 12 h in relation to control non-treated Saos-2 cells when bafilomycin was added, which strongly suggests that calcium from lysosome is depleted by BPC in function of time and that this ion is released mainly due to LMP induced by BPC.

The main apoptotic role of calcium is interaction with Bcl-2 family proteins, such as Bax and Bid [1,2,49–55], thus inducing apoptosis by the mitochondrial pathway. Because we observed that BPC mobilises calcium mainly from the mitochondria, we evaluated Bax participation in BPC induced cell death. A pattern of Bax translocation was observed in Saos-2 cells exposed to BPC, which is usually in the mitochondria, although Bax can also translocate to the lysosomal membrane [16,17]. Bax promotes mitochondrial or Lysosomal Membrane Permeabilisation, leading to the release of pro-apoptotic mitochondrial factors or hydrolase from lysosomes that culminate in the activation of effector caspases, such as caspase-3, which was also activated by BPC in this study.

Another factor in BPC cytotoxicity was the Saos-2 cell LMP. As tumour cells exhibit alterations that confer resistance to LMP by overexpressing the heat shock protein Hsp70 [56], the ability of BPC to cause this event in Saos-2 cells is of great interest because a

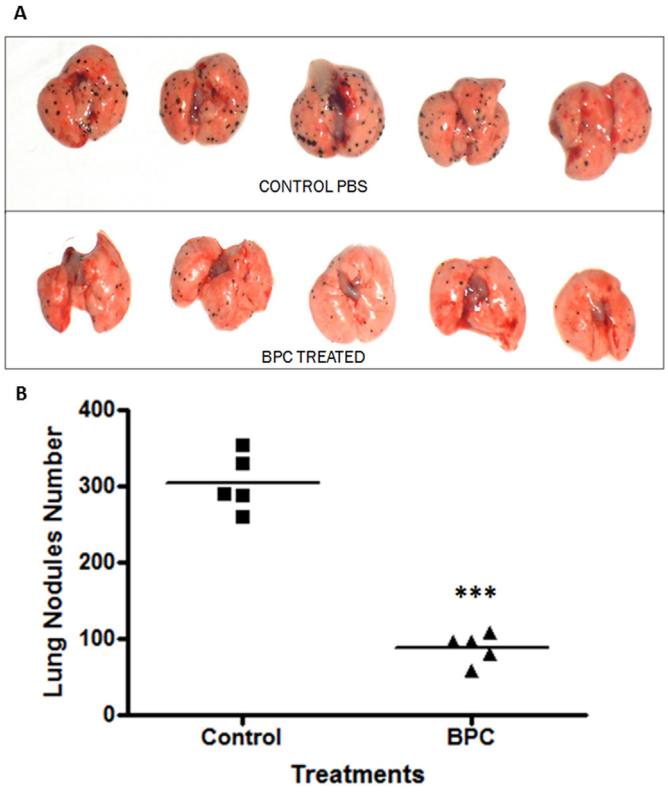

Fig. 6. Inhibitory effects of BPC (8 mg/kg/day) on pulmonary metastasis in C57Bl/6 mice intravenously injected with B16F10-Nex2 melanoma cell (10^5) BPC was administered intraperitoneally (i.p.) for 10 consecutive days. Mice were killed on day 11 after tumour cell challenge, and lungs were fixed in formaldehyde (10%). (A) Photographs of lungs showing the metastatic colonies in the BPC-treated group and untreated control group. (B) Quantification of pulmonary metastatic nodules counted using an inverted microscope. Note that the 10 consecutive days of BPC treatment significantly protected the mice from malignant B16F10-Nex2 cells lung colonisation. Analysis performed by paired *Student's t test*. $^{***}P < 0.0009$ compared to control (PBS-treated group).

compound with lysosomotropic characteristics promotes the release of lysosomal proteases to the cytosol. Once in the cytosol, these proteases participate in cell death by either apoptosis or necrosis [57,58]. In accordance with these findings, previous results reported by our group demonstrated the essential role of cathepsin B in BPC cytotoxicity to leukaemia cells [25,26]. These last authors described a reduction of caspase-3 and caspase-6 activity when a palladacycle complex BPC was pre-incubated with CA074, indicating that cathepsin B triggers leukaemia cells death and suggesting a direct relationship between cathepsin B and BPC activity.

Cathepsin B is a lysosomal cysteine protease in normal cells and tissues. In malignant tumours and premalignant lesions, the expression of cathepsin B is up regulated and the enzyme is secreted and becomes associated with the cell surface [59,60]. Once released in the extracellular space, cathepsin B contributes to metastatic potential by facilitating cell migration and invasiveness. However, when released from lysosomes to the cytosol, cathepsin B indirectly induces cell death [60]. As our previous study demonstrated that BPC inhibits cathepsin B activity *in vitro* [24] and here we showed that cathepsin B inhibition by CA074 reduced Saos-2 BPC-induced cell death, we hypothesise that when in extracellular site, inhibition of cathepsin B activity by BPC reduces the metastatic properties of tumour cells, but when inside the cell, BPC become concentrated in lysosomes due to ion trapping [25], which induces LMP, favouring the release of cathepsin B to the cytosol. Cathepsin B once in the cytosol participates in the induction of cell death observed in this study.

Based on the BPC effectiveness found *in vitro*, we have evaluated BPC activity *in vivo* using a preclinical experimental model of murine metastatic melanoma cell colonisation [61,41]. The *in vivo* results showed that tumours grew faster in the control group and slower in the BPC-treated group. The lung tumour nodules in the BPC-treated group were significantly smaller than those in the control non-treated group. These results can be explained by the inhibitory properties of BPC on cathepsin B extracellular activity [24] because cathepsin B is highly expressed in B16F10 Nex-2 cells and is commonly involved in tumour cell shedding of the extracellular matrix during the metastatic process. However, new studies are ongoing to elucidate the molecular events involved in the BPC activity *in vivo* in metastatic processes.

5. Conclusions

The results presented in this work suggest that BPC has an ability *in vitro* to induce p53-independent cell death in the human osteosarcoma Saos-2 cell line and also decreases the ability of melanoma cells to colonise in the lung in BPC-treated mice. Cytosolic calcium mobilisation and LMP are essential events involved in the molecular BPC antitumour activity *in vitro*. In conclusion, because BPC has demonstrated a great antitumour potential in preclinical studies, further studies and clinical trials are necessary to better elucidate the *in vivo* anticancer potential of BPC.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.073.

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