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Cytotoxic and toxicological effects of phthalimide derivatives on tumor and normal murine cells

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ABSTRACT

Eleven phthalimide derivatives were evaluated with regards to their antiproliferative activity on tumor and normal cells and possible toxic effects. Cytotoxic analyses were performed against murine tumors (Sarcoma 180 and B-16/F-10 cells) and peripheral blood mononuclear cells (PBMC) using MTT and Alamar Blue assays. Following, the investigation of cytotoxicity was executed by flow cytometry analysis and antitumoral and toxicological potential by *in vivo* techniques. The molecules 3b, 3c, 4 and 5 revealed *in vitro* cytotoxicity against Sarcoma 180, B-16/F-10 and PBMC. Since compound 4 was the most effective derivative, it was chosen to detail the mechanism of action after 24, 48 and 72 h exposure (22.5 and 45 μM). Sarcoma 180 cells treated with compound 4 showed membrane disruption, DNA fragmentation and mitochondrial depolarization in a time- and dose-dependent way. Compounds 3c, 4 and 5 (50 mg/kg/day) did not inhibit *in vivo* tumor growth. Compound 4-treated animals exhibited an increase in total leukocytes, lymphocytes and spleen relative weight, a decreasing in neutrophils and hyperplasia of spleen white pulp. Treated animals presented reversible histological changes. Molecule 4 had *in vitro* antiproliferative action possibly triggered by apoptosis, reversible toxic effects on kidneys, spleen and livers and exhibited immunostimulant properties that can be explored to attack neoplasic cells.

Key words: cytotoxicity, histological alterations, murine cells, phthalimide derivatives, sarcoma 180.

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INTRODUCTION

Cancer is an extremely common disease, being the second leading cause of death, surpassed by cardiovascular disorders only (Jemal et al. 2005). Resistance to cytotoxic agents, narrow therapeutic windows and high degree of genomic heterogeneity in the human cancer patient population (even among those with histologically indistinguishable disease) and tumour-derived cell lines are factors with crucial role on the variable clinical response to the treatment of the malignancies, which have generated immense difficulties to oncologists, biologists and pharmacologists to develop new anticancer drugs (Weinstein 2012, Kamb 2005).

In the fight against cancer, search for new chemical entities (NCEs) with chemotherapeutic properties is really worthy and numerous methods have been utilized to acquire compounds, including isolation from plants and animals and the use of synthetic and combinatorial chemistry and molecular modeling (Srivastava et al. 2005, Leite et al. 2007, Ferreira et al. 2013a, b, Ruchelman et al. 2013). Phthalimides, a classical protecting group for amines with two carbonyl groups bound to a secondary amine or ammonia, have been extensively exploited to synthesize molecules with multifaceted pharmacological effects, such as anti-inflammatory (Machado et al. 2005, Mazzoccoli et al. 2012, Leite et a. 2014), antimycobacterial (Akgün et al. 2012), analgesic (Andrade et al. 2012) and anticonvulsant (Palencia et al. 2011). One of the most known phthalimide derivatives is thalidomide (Thl) and its analogues, multi-target compounds that can acts as immunomodulators (Pessoa et al. 2010), angiogenesis inhibitors (D'Amato et al. 1994, Dredge et al. 2005, Badamtseren et al. 2011) and against human multiple myeloma (Kagoya et al. 2012), oral squamous cell carcinoma (Yang et al. 2011) and prostate cancer (Nabhan and Petrylak 2012).

Antitumor drugs presenting reduced toxicity have been designed by linking them to small peptides or amino acids (Trouet et al. 2001). Conjugated amino acids in such compounds can serve as substrate for enzymes that are produced by tumor and stroma cells (Leite et al. 2007). Findings have demonstrated that specific sequences of amino acids would act on tumor molecules (Arap et al. 2001). These enzymatic cascade activations such as aminopeptidases are important for cancer progression (angiogenesis and metastasis) and present a functional interplay between malignant and nonmalignant cells in the tumor microenvironment (Guzman-Rojas et al. 2011).

We designed a set of phthalimides in which bioisosterism and molecular hybridization were explored, and identified thiosemicarbazones with anticancer and immunomodulatory activity against Sarcoma 180 (S180) tumor (Pessoa et al. 2010). However, the mechanism of cell death activated by these compounds remains unclear. Thus, in this work, we evaluated some *N*-phthaloyl amino acid derivatives with regards to *in vitro* and *in vivo* antiproliferative activity on tumor and normal cells in order to investigate their mechanism of action and their toxic effects.

MATERIALS AND METHODS

Chemicals

For the design concept of the series, the molecular hybridization approach was applied through the inspiration of structural features of the prototypes thalidomide (of which phthalimide is an essential pharmacophoric fragment) and -isantins scaffold and thiosemicarbazones (Figure 1). The synthesis and structural elucidation of the tested compounds were previously published (Pessoa et al. 2010), and they are found to be stable at environment temperature and in dimethylsulfoxide (DMSO, Sigma Aldrich) [thin-layer chromatography (TLC) control]. Doxorubicin (Dox), 5-Fluorouracil (5-FU) and Alamar Blue™ (Resazurin) were purchased from Sigma Aldrich Co. (St Louis, MO, USA). All molecules were diluted in DMSO to a final concentration of 5 mg/mL (stock solution).

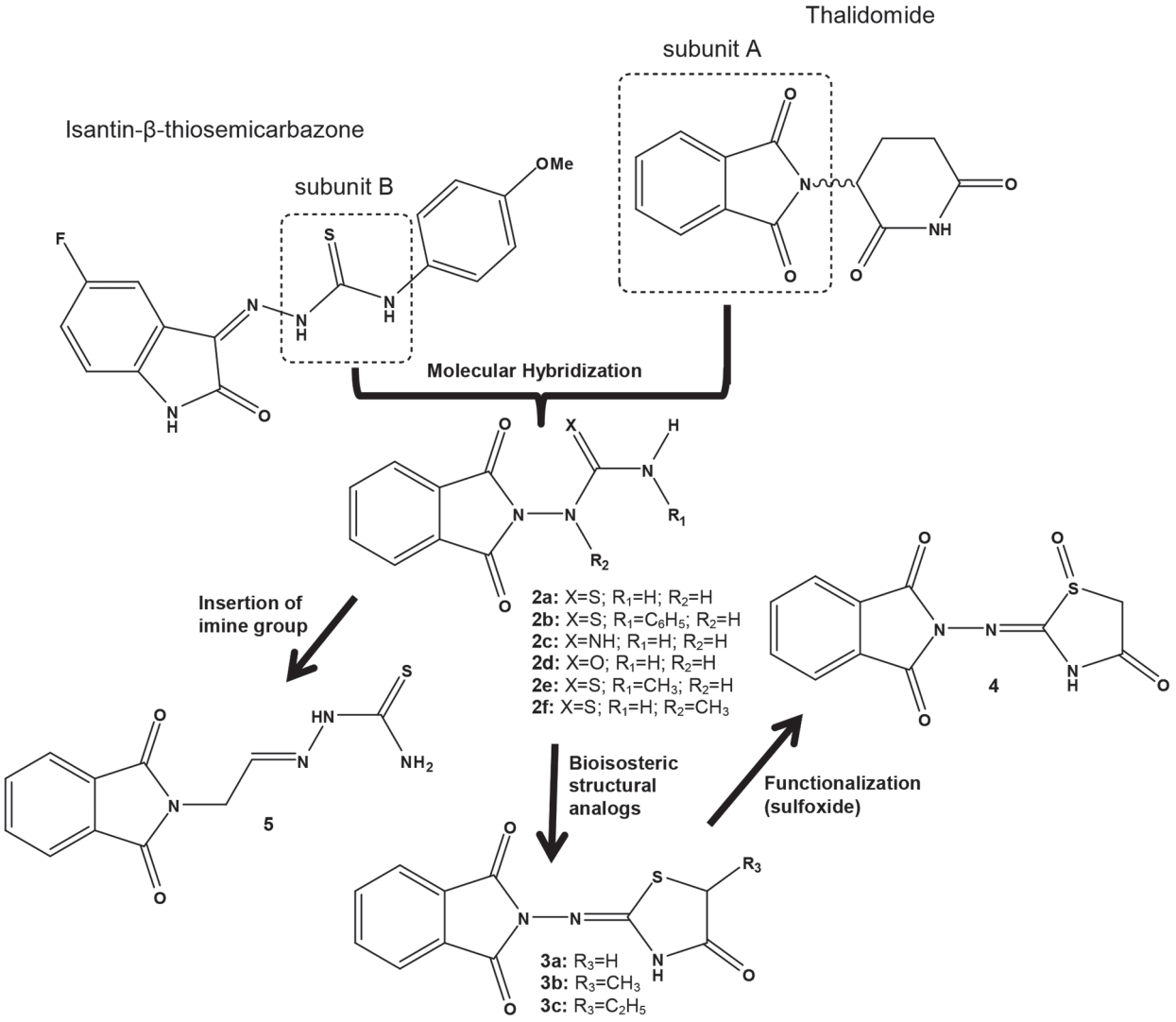

Figure 1 - Our design concept of antitumor and immunomodulatory drugs exploring the molecular hybridization of the prototypes Thalidomide and Isantin-β-thiosemicarbazone.

Animal Facilities

Adult Swiss mice (*Mus musculus* Linnaeus, 1758) were obtained from the animal facilities of Universidade Federal of Piauí (UFPI), Teresina, Piauí, Brazil. They have been kept in well-ventilated cages (Alesco, São Paulo) under standard conditions of light (12h with alternative day and night cycles) and temperature (22 ± 1°C) and were maintained with access to commercial rodent stock diet (Nutrilabor, São Paulo, Brazil) and water *ad libitum*. The investigational protocols were approved by the local Ethical Committee on Animal Research (Process No. 102/2007) and are in accordance with the national (*Colégio Brasileiro de Experimentação Animal* - COBEA) and international standard (EEC Directive 1986) on the care and use of experimental laboratory animals.

Maintenance of Tumor and Normal Cells

Sarcoma 180 (S180) tumor cells were kept in the peritoneal cavities of Swiss mice in the Laboratory of Experimental Oncology of the Federal University

of Ceará since the mid 1980s and a tumor sample was recently donated to our laboratory at UFPI.

Heparinized blood was collected from healthy mice by the orbital plexus, and peripheral blood mononuclear cells (PBMC) were isolated by a standard method of density-gradient centrifugation over Ficoll-Hypaque.

Cell cultures of B-16/F10 (murine melanoma cell line), S180 cells and PBMC were performed in RPMI 1640 medium, supplemented with 20% fetal bovine serum, 2mM glutamine, 100 U/mL penincillin and 100μg/mL streptomycin, at 37°C with 5% CO_2.

Cytotoxicity Assays

The cytotoxic properties of the synthetic molecules (2a, 2b, 2c, 2d, 2e, 2f, 3a, 3b, 3c, 4 and 5) were assessed after 72h exposure using B-16, S180 and peripheral blood mononuclear cells. Quantification of cell proliferation was determined spectrophotometrically using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter). Control groups (negative and positive) received the same amount of DMSO. Dox (0.01 - 8.6μM) and 5-FU (0.7 - 192.2μM) were used as positive controls and Thl (0.3 - 300μM) as core molecule.

B-16/F-10 cell line

The cytotoxicity against B-16/F10 was determined by MTT assay (Mosmann 1983). This is an extremely well characterized method that analyzes the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2*H-tetrazolium bromide (MTT) to a purple formazan product by a pool of cellular mitochondrial and cytosolic enzymes such as succinate dehydrogenase bound to the inner mitochondrial membrane of mammalian mitochondria. This method revolutionized cell-based drug screening by offering a high-throughput screening colorimetric assay, whereas it simplified sample processing which did not require radioisotope but it is sensitive enough for miniaturization into 96-well plate formats and have been used in the search for NCEs with antiproliferative action (Alley et al. 1988, Berridge et al. 1996, Gonzalez and Tarloff 2001, Santos et al. 2010, Ferreira et al. 2013a, Leite et al. 2014).

Briefly, cells were plated in 96-well plates (0.7 x 10^5 cells/well) and incubated to allow cell adhesion. Twenty-four hours later, compounds were added to each well (0.3 - 300μM). After 72h of incubation, the supernatant was replaced by fresh medium containing 10 % MTT, the formazan product was dissolved in DMSO and absorbance was measured at 595nm.

Primary culture of Sarcoma 180 cells

The Alamar Blue Assay™ evaluated cell proliferation after 72 h of incubation. The Alamar Blue Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. This technique has been extensively used to assess different cell types in toxicological, environmental, antimicrobial and cytotoxic susceptibility tests. It presents higher sensibility when compared to other cytotoxicity assays, since smaller amount of cells and steps are required converting it into a suitable method to evaluate proliferation in primary cultures of normal and tumor cells from mice, rats and humans (Al-Nasiry et al. 2007, Rampersad et al. 2012, Schoonen et al. 2012, Ferreira et al. 2013a).

Ascite-bearing female mice between 7 and 9 days postinoculation were sacrificed by cervical dislocation and a suspension of S180 cells was harvested from the intraperitoneal cavity under aseptic conditions. The suspension was centrifuged at 500 X g for 5 min to obtain a cell pellet and washed three times with RPMI medium. Cell concentration was adjusted to 0.5 x 10^6 cells/mL in supplemented RPMI 1640 medium, plated in a 96-well plate and incubated with increasing concentrations of the molecules (0.3 - 300μM). Eight hours before the end

of the incubation, 10μL of stock solution (0.312mg/mL) of Alamar Blue were added to each well. The absorbance was measured at 570 nm and 595nm using a multiplate reader and the drug effect was quantified as the percentage of the control (Ferreira et al. 2011).

Primary culture of normal cells

In order to investigate the selectivity of the molecules against normal proliferating cells, the Alamar Blue Assay™ was also performed with murine PBMC. PBMC were washed and resuspended (3 x 10^5 cells/mL) in supplemented RPMI 1640 medium. Phytohemagglutinin (4%) was added at the beginning of culture to promote cell proliferation (Ferreira et al. 2013a). PBMC were then plated in 96-well plates (3 x 10^5 cells/well in 100 μL of medium). After 24h, compounds (0.3 - 300 μM) dissolved in DMSO were added to each well, and the cells were incubated for 72h. Twenty-four hours before the end of the incubation, 10 μL of stock solution of Alamar Blue were added to each well. Quantification of cell proliferation was performed as described above.

Flow Cytometry Analysis

Compound 4 was added to the S180 cell cultures (0.5 x 10^6 cells/mL) to obtain final concentrations of 22.5 and 45 μM. Dox (0.6 μM) and 5-FU (60 μM) were used as positive controls. Aliquots were removed from cultures after 24, 48 and 72 h of incubation and analyzed with regards to membrane integrity, DNA fragmentation and mitochondrial transmembrane potential by flow cytometry (Guava Express Plus software, Guava EasyCyte Mine™). Five thousand events were evaluated per experiment and cellular debris was omitted from the analysis.

Cell membrane integrity

Membrane integrity of S180-treated or untreated cells was evaluated by the exclusion of propidium iodide (50 μg/mL). Fluorescence was measured and cell morphology, granularity and membrane integrity were determined (Darzynkiewicz et al. 1992).

DNA fragmentation

S180 cells were incubated at 25°C for 30 min, in the dark, in a lysis solution containing 0.1% citrate, 0.1% Triton X-100 and propidium iodide (50μg/mL) Fluorescence was measured and DNA fragmentation was analyzed (Nicoletti et al. 1991).

Mitochondrial transmembrane potential

Mitocondrial transmembrane potential was determined by the retention of rhodamine 123 dye in S180 cells. Aliquots removed from wells were incubated with 200μL of 1μg/mL rhodamine 123 for 15 min and centrifuged at 2000 rpm/5min. Subsequently, cells were harvested and incubated in phosphate-buffered saline (PBS) solution at 25°C for 30 min in the dark. Fluorescence was measured and percentage of mitochondrial depolarization was determined (Cury-Boaventura et al. 2003).

In vivo Studies

Sixty healthy Swiss male mice weighing between 23 and 26g were subcutaneously implanted with nine-day-old Sarcoma 180 ascites tumor cells (2 x 10^6 cells/0.5 mL) into the left hind groin of the mice. On the next day, they were randomly divided into six groups (n=10 each) and the molecules (3c, 4 and 5) dissolved in DMSO 4% were administered intraperitoneally for 7 days at the dose of 50 mg/kg/day (Pessoa et al. 2010). Negative and positive controls received DMSO 4%, 5-FU (25 mg/kg/day) and Thalidomide (50 mg/kg/day), respectively. On day 8, mice were sacrificed by cervical dislocation and their organs (kidneys, spleens and livers) and tumors were dissected, grossly examined for size, color changes and hemorrhage, weighed and preserved in 10% formaldehyde solution. The inhibition ratio of tumor growth (%) was calculated by the following formula: inhibition ratio (%) = [(A-B)/A] x 100, where A is the average tumor weight of the negative control, and B is the tumor weight of the treated group. To examine morphological changes by light microscopy (Olympus, Tokyo, Japan), small pieces of organs and

tumors were processed, embedded in paraffin and 3-5μm thick sections were prepared and stained with hematoxylin-eosin (Magalhães et al. 2010).

Additional six groups (n = 10 animals/group) were treated in equal conditions (doses and exposure time), and on 8th day a blood sampling was collected from each animal via retrorbital plexus (Waynforth 1980) under halothane 3% anesthetic inhalational (Fluothane, Zeneca, São Paulo) using sterile tubes and heparinize pipettes. To determine hematological parameters, an automated blood cell counter (Coulter Counter T-530, Coulter Counter Eletronics, USA) was used to measure red blood cells (RBC), platelets, white blood cells (WBC), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and the change in size distribution of erythrocyte (Red cell Distribution Width - RDW) (Ferreira et al. 2007).

Statistical Analysis

For cytotoxicity assays, the IC_{50} values and their 95% confidence intervals were obtained by nonlinear regression using the Graphpad program (Intuitive Software for Science, San Diego, CA). In order to determine differences between groups, data (means ± S.E.M) were compared by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test ($P < 0.05$). All *in vitro* studies were carried out in triplicate represented by independent biological evaluations.

RESULTS

Cytotoxicity

Most of *N*-phthaloyl derivatives exhibited absence or low cytotoxicity for B-16 line and on S180 and PBMC cultures after 72h exposure (Table I).

Only compounds 3b, 3c 4 and 5 revealed antiproliferative potential against murine tumor cells (Table I), with IC_{50} values of 119.7 μM (compound 4), 163.8 μM (3c), 195.2 μM (5) and 270.8 μM (3b) for B-16/F-10 cells and 47.6 μM (4), 88.5 μM (5) and 124.8 μM (3c) on *ex-vivo* S180 cells. Similarly, compound 4 was the most active on PBMC, showing IC_{50} of 45.8 μM, followed by compounds 3b (103.3 μM), 3c (123.1 μM) and 5 (172.3 μM).

TABLE I
Cytotoxic activity of *N*-phthaloyl amino acid derivatives determined by MTT assay (B-16 cell line) or Alamar blue technique (primary culture of S180 and PBMC).

Cell Line	CI_{50} [μg/mL(μM)]													
	Dox	5-FU	Thl	2a	2b	2c	2d	2e	2f	3a	3b	3c	4	5
B-16/ F10	0.03 (0.06) 0.02 - 0.04	0.3 (2.3) 0.2 - 0.4	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	55.3 (270.8) 51.2 - 62.8	47.4 (163.8) 32.7 - 70.0	33.2 (119.7) 20.1 - 51.5	51.2 (195.2) 49.7 -54.4
S180	1.9 (3.2) 1.42 - 2.42	0.8 (6.2) 0.4 - 1.2	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	36.1 (124.8) 30.3 - 43.0	13.2 (47.6) 12.1 - 14.2	23.2 (88.5) 17.1 -27.9
PBMC	0.9 (1.8) 0.52 - 1.80	1.4 (10.7) 0.9 - 2.1	90.7 (351.3) 83.6 - 98.6	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	>100 (> 300)	26.6 (130.3) 19.6 - 30.9	35.6 (123.1) 30.1 - 42.1	12.7 (45.8) 11.3 - 14.5	45.2 (172.3) 34.9 -58.3

Data are presented as IC_{50} values and 95% confidence intervals for murine melanoma (B-16/F10) and primary culture of Sarcoma 180 (S180) and polymorphic blood mononuclear cells (PBMC). Thl – Thalidomide was used as basis molecule. Doxorubicin (Dox) and 5-Fluorouracil (5-FU) were used as positive controls. Experiments were performed in triplicate.

B-16/F-10 line, S180 and PBMC were also susceptible to the antiproliferative action of Dox (0.06, 3.2 and 1.8 μM) and 5-FU (2.3, 6.2 and 10.7 μM), respectively. On the other hand, Thl revealed low *in vitro* cytotoxic effects against PBMC and no activity on tumor cells.

Membrane Disruption, DNA Fragmentation and Mitochondrial Depolarization

As described in Table I, compound 4 was the most potent derivative upon S180 cells, being chosen for further detailed studies at the concentrations of IC_{50} (45 μM) and $IC_{50}/2$ (22.5 μM) to understand its mechanism of action. Afterwards, flow cytometry analysis were performed with Sarcoma cells after 24, 48 and 72h of incubation to notice whether these derivatives would alter the membrane integrity, mitochondrial potential or cause DNA fragmentation.

Compound 4 caused a decrease in cell viability, modifying the membrane integrity after 24h (45 μM, 84.7 ± 1.9 %), 48h (45 μM, 71.3 ± 1.8 %) and 72h [22.5 μM (70.4 ± 2.1 %), 45 μM (56.8 ± 3.3 %)] in comparison to negative control (93.4 ± 3.2, 90.7 ± 2.8 and 86. 1 ± 1.5 %, respectively) (Figure 2A, $P < 0.05$). Doxorubicin (87.4, 75.2 and 58.4.%) and 5-FU (86.3, 80.7 and 51.9 %) also caused viability reduction in a time-dependent way ($P < 0.05$).

DNA size was quantified and sub-diploid pattern was considered fragmented. Both concentrations (22.5 and 45 μM) produced statistically significant amount of sub-G1 cells in a time- and dose-dependent manner after 48h (16.3 ± 1.3 and 30.4 ± 3.0 %) and 72h (22.1 ± 0.7 and 45.9 ± 2.2 %) of incubation in comparison with the control group (6.5 ± 0.8 and 8.1 ± 1.9 %, respectively). Meanwhile, only the highest concentration tested was able to induce DNA fragmentation after 24h exposure (12.7 ± 0.8 %) (Figure 2B, $P < 0.05$). Standard drugs used (Dox: 30.2, 45.5 and 50.6 %; 5-FU: 21.7, 33.1 and 42.4 %) caused DNA breaks in all periods examined (24, 48 and 72h, respectively) ($P < 0.05$).

S180 treated cells showed significant mitochondrial depolarization after 48h (15.7 ± 1.8%) and 72h (21.2 ± 1.6 %, Figure 2C) at the highest concentration tested. Dox (14.9, 32.2 and 44.6 %) and 5-FU (15.5, 19.7 and 35.1 %) alter mitochondrial polarization in all times studied when compared to negative control (8.2, 9.3 and 10.0 after 24, 48 and 72h exposure, respectively) ($P < 0.05$).

In vivo Assessment

The aim of the *in vivo* investigation was to determine the antitumor and toxicological effects of synthetic *N*-phthaloyl amino acids derivatives. The inhibition rates are described in Table II. Compounds 3c, 4 and 5 were not able to interfere on tumor growth of mice bearing Sarcoma 180 cells after 7 days of treatment, since they presented respective tumor inhibition rates of 10.5 ± 7.6, 29.7 ± 13.2 and 20.1 ± 8.9 % that were not statistically different ($P > 0.05$) when compared to the tumor mass in the negative control (2.1 ± 0.2 g). Meanwhile, 5-FU and Thl caused tumor growth diminution of 83.9 % (0.3 ± 0.1 g) and 53.5 % (0.9 ± 0.2 g), respectively ($P < 0.05$).

For the toxicological assessments, blood and key organs were collected from treated and untreated mice to identify probable toxic systemic effects of the phthalimide derivatives. Firstly, a complete hematological investigation was performed and alterations were not noticed in the blood cell profile in compound 3b and 5-treated animals after 7 days of administration ($P > 0.05$, Table III), however, animals treated with compound 4 showed an increase in total leukocytes (8.2 ± 0.4 x $10^3/\mu L$) and lymphocytes (73.7 ± 1.3 for 80.6 ± 1.3 %) and decrease of neutrophils (19.4 ± 0.8 for 14.2 ± 0.6 %) ($P < 0.05$). On the other hand, 5-FU-treated animals presented an intensive leukopenia (3.5 ± 0.3 x $10^3/\mu L$) in comparison with negative group (5.7 ± 0.7 x $10^3/\mu L$), lymphocyte and monocyte reduction and augment of segmented cells ($P < 0.05$).

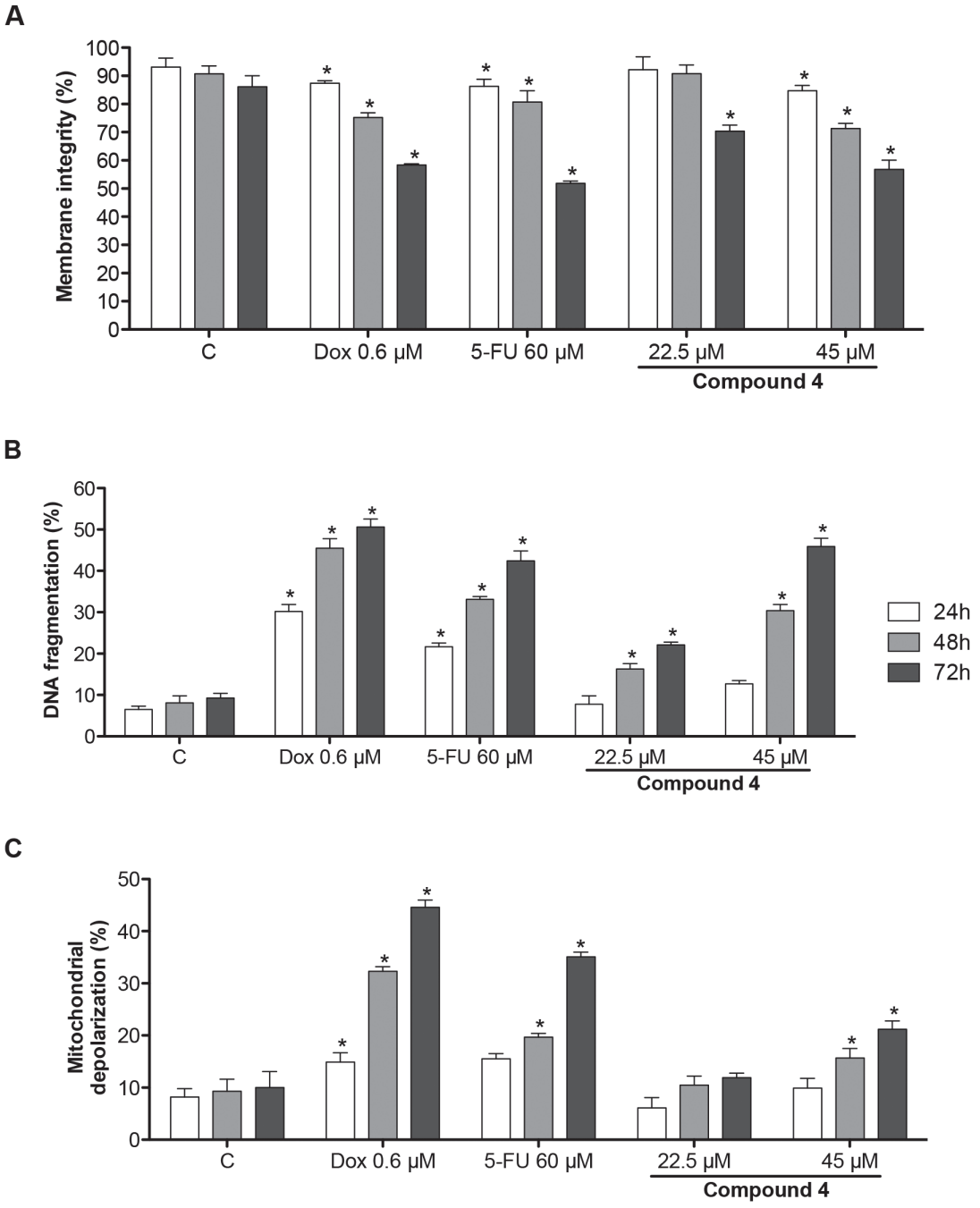

Figure 2 - Flow cytometry analysis of the *N*-phthaloyl derivative effects on primary culture of Sarcoma 180 cells after 24, 48 and 72 h of incubation. A – Membrane integrity determined by exclusion of propidium iodide (50 µg/mL); B – Internucleosomal DNA fragmentation evaluated by nuclear fluorescence using citrate, Triton X-100 and 50 µg/mL propidium iodide; C – Mitochondrial depolarization quantified using rhodamine 123. Negative control (C) was treated with the vehicle used for diluting the tested substance. Doxorubicin (Dox, 0.6 µM) and 5-Fluorouracil (5-FU, 60 µM) were used as positive controls. Results are expressed as mean ± standard error of measurement (S.E.M) from three independent experiments. * $P < 0.05$ compared to control by ANOVA followed by Student Newman-Keuls test.

TABLE II
Effects of Thalidomide and *N*-phthaloyl amino acid derivatives on mice transplanted with Sarcoma 180 cells after 7 days of treatment by intraperitoneal route.

Treatment	Dose (mg/kg/day)	Animal weight (g)	Liver	Kidney	Spleen	Tumor (g)	Tumor Inhibition (%)
			g/100g body weight				
Control	–	31.4 ± 1.3	5.3 ± 0.1	1.4 ± 0.1	0.5 ± 0.1	2.1 ± 0.2	–
5-FU	25	24.6 ± 0.4 *	4.9 ± 0.3	1.3 ± 0.1	0.2 ± 0.1 *	0.3 ± 0.1 *	83.9 ± 2.3 *
Thl	50	29.0 ± 0.9	4.9 ± 0.1	1.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.2 *	53.5 ± 7.2 *
3c	50	29.4 ± 0.8	5.2 ± 0.2	1.5 ± 0.1	0.5 ± 0.1	1.9 ± 0.2	10.5 ± 7.6
4	50	30.3 ± 0.8	5.1 ± 0.2	1.4 ± 0.1	0.8 ± 0.1 *	1.5 ± 0.3	29.7 ± 13.2
5	50	29.8 ± 1.8	5.5 ± 0.1	1.3 ± 0.1	0.4 ± 0.1	1.7 ± 0.2	20.1 ± 8.9

Data are means ± S.E.M., n=10 animals/group, treated for seven days. Thl – Thalidomide was used as basis molecule and 5-Fluorouracil (5-FU) as positive controls. * $P < 0.05$ compared to control by ANOVA followed by Student Newman-Keuls test.

TABLE III
Effects of Thalidomide and *N*-phthaloyl amino acid derivatives on hematological parameters in peripheral blood of mice transplanted with Sarcoma 180 cells and treated during 7 days by intraperitoneal route.

Hematological parameters	Groups					
	Control	Thl	5-FU	Compound 3b	Compound 4	Compound 5
		50 mg/kg	25 mg/kg		50 mg/kg	
Erythrocytes (x 10^6/μL)	7.8 ± 0.2	8.1 ± 0.5	7.5 ± 0.3	8.0 ± 0.4	7.9 ± 0.3	7.7 ± 0.6
Hematocrit (%)	44.5 ± 1.2	43.7 ± 2.5	41.8 ± 1.0	42.0 ± 1.7	45.0 ± 0.7	44.9 ± 0.9
Hemoglobin (g/dL)	15.1 ± 0.7	16.9 ± 1.1	13.8 ± 1.2	15.2 ± 1.5	16.3 ± 1.4	17.4 ± 1.5
MCV (fL)	55.9 ± 1.7	53.5 ± 0.9	52.9 ± 1.7	56.0 ± 0.8	54.7 ± 0.5	55.1 ± 0.4
MCH (pg)	18.0 ± 1.3	17.5 ± 0.2	16.3 ± 0.5	18.3 ± 0.9	17.3 ± 0.4	16.9 ± 1.0
MCHC (g/dL)	31.9 ± 0.8	29.6 ± 0.6	29.0 ± 0.3	32.3 ± 2.8	30.3 ± 0.8	29.7 ± 1.1
Platelets (μL)	1.920 ± 58.1	1.800 ± 67.8	1.678 ± 43.6	1.772 ± 72.5	1.861 ± 49.7	1.903 ± 53.6
Total leukocytes (x 10^3/μL)	5.7 ± 0.7	6.3 ± 1.7	3.5 ± 0.3 *	5.6 ± 1.6	7.7 ± 0.4 *	6.1 ± 1.5
Segmented (%)	19.4 ± 0.8	17.8 ± 1.2	47.3 ± 1.8 *	19.0 ± 0.8	14.2 ± 0.6 *	18.0 ± 0.7
Lymphocytes (%)	73.7 ± 1.3	76.3 ± 1.1	49.8 ± 2.2 *	75.0 ± 2.0	80.6 ± 1.3 *	75.8 ± 1.3
Monocytes (%)	6.8 ± 0.8	5.7 ± 0.7	2.9 ± 0.3 *	5.9 ± 1.4	5.2 ± 0.9	6.0 ± 0.4
Eosinophils (%)	0.1 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
RDW (%)	15.3 ± 1.0	13.9 ± +1.9	17.2 ± 1.5	17.3 ± 2.1	17.3 ± 2.1	16.2 ± 0.9

Data are means ± S.E.M., n=10 animals/group. MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; RDW, Red cell Distribution Width. Thl – Thalidomide was used as basis molecule and 5-Fluorouracil (5-FU) as positive controls. * $P < 0.05$ compared to control by ANOVA followed by Student Newman-Keuls test.

Histopathological evaluation of tumors from control mice showed groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei and binucleation. In addition, mitosis, muscle invasion and several degrees of cellular and nuclear pleomorphism were seen (Figure 3A). In the tumors extirpated from animals treated with 5-FU, Thl and compounds 3c, 4 and 5, areas of coagulative necrosis were observed (Figures 3B-3F), but these areas were much more extensive in 5-FU and Thl-treated animals.

Intraperitoneal treatment with *N*-phthaloyl derivatives was not able to change body weight gain of experimental groups when compared to control group ($P > 0.05$). Neither mortality nor morbidity was recorded during the experiment (Table II). There were no statistical differences in relative liver and kidneys weights despite spleens having shown an increase in compound 4-treated mice (0.8 ± 0.1 g) compared to DMSO group (0.5 ± 0.1 g) ($P < 0.05$). Spleens removed from control mice showed normal and preserved white pulp, and

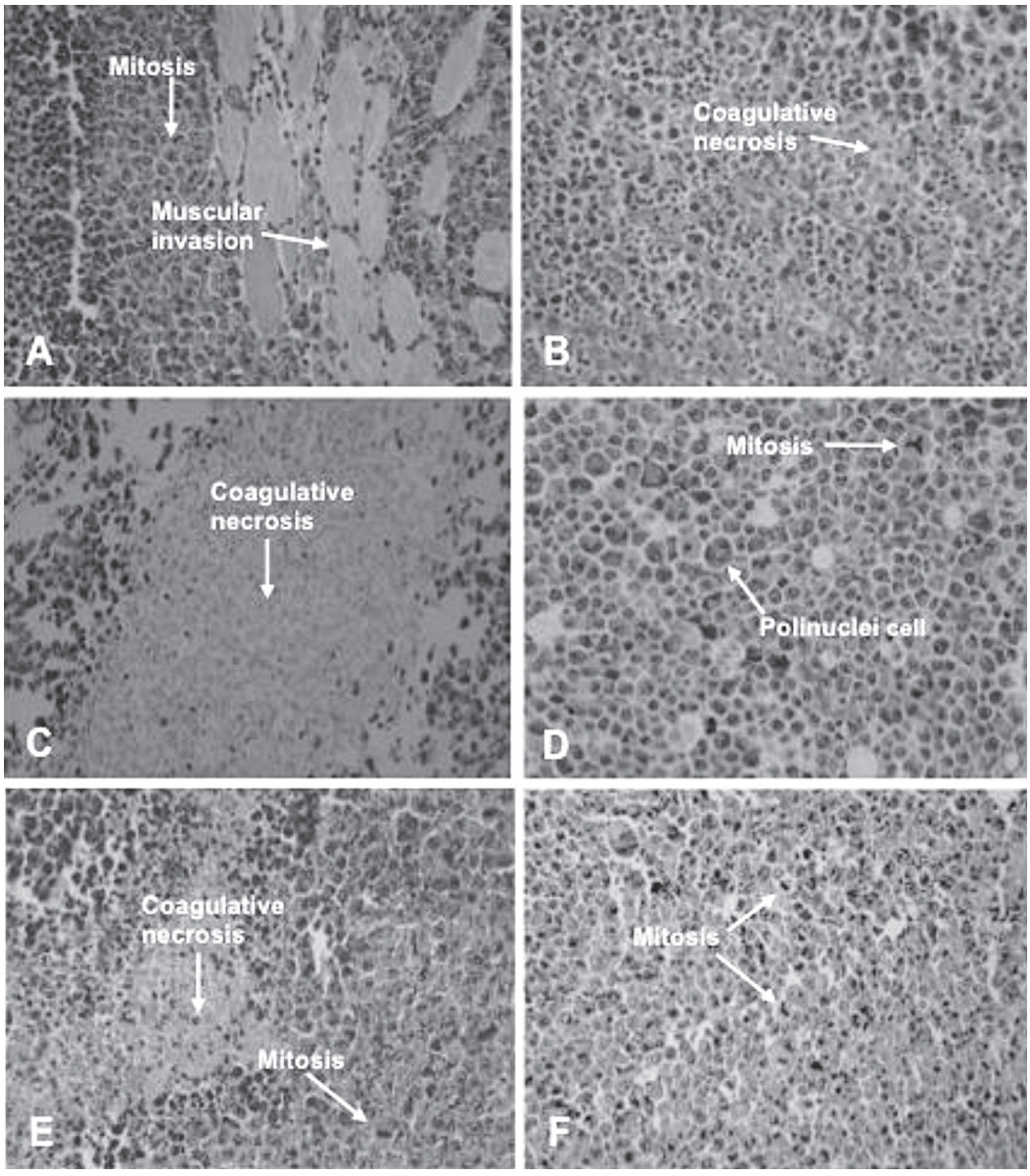

Figure 3 - Histology of Sarcoma 180 tumors removed from untreated or N-phthaloyl amino acid derivatives-treated mice with 50 mg/kg/day after 7 days. A – Control (4 % DMSO); B – Thalidomide; C – 5-Fluorouracil (25 mg/kg/day); D – Compound 3c; E – Compound 4; F – Compound 5. Hematoxylin-eosin staining. Magnification, x 400.

megakaryocytes with hyperlobulated nuclei without morphological alterations (Figure 4A). Pigments of hemosiderin and disorganization of white and red pulps were more present in treated animals (Figures 4B-4F). Meanwhile, compound 4-treated animals presented hyperplasia of white pulp, 5-FU caused white pulp atrophy with reducing in relative spleen weight (0.2 ± 0.1 g) ($P < 0.05$).

Kidneys of treated and untreated animals showed glomerular and tubular hemorrhage, hydropic degeneration of tubular epithelium and hyaline cylinders (Figures 5A-5F). Intense cell swelling in the proximal tubule epithelial was detected in animals treated with 5-FU and compounds 3c and 4 (Figures 5C-5F). In addition, 5-FU- and compound 5-treated animals also showed inflammatory cells

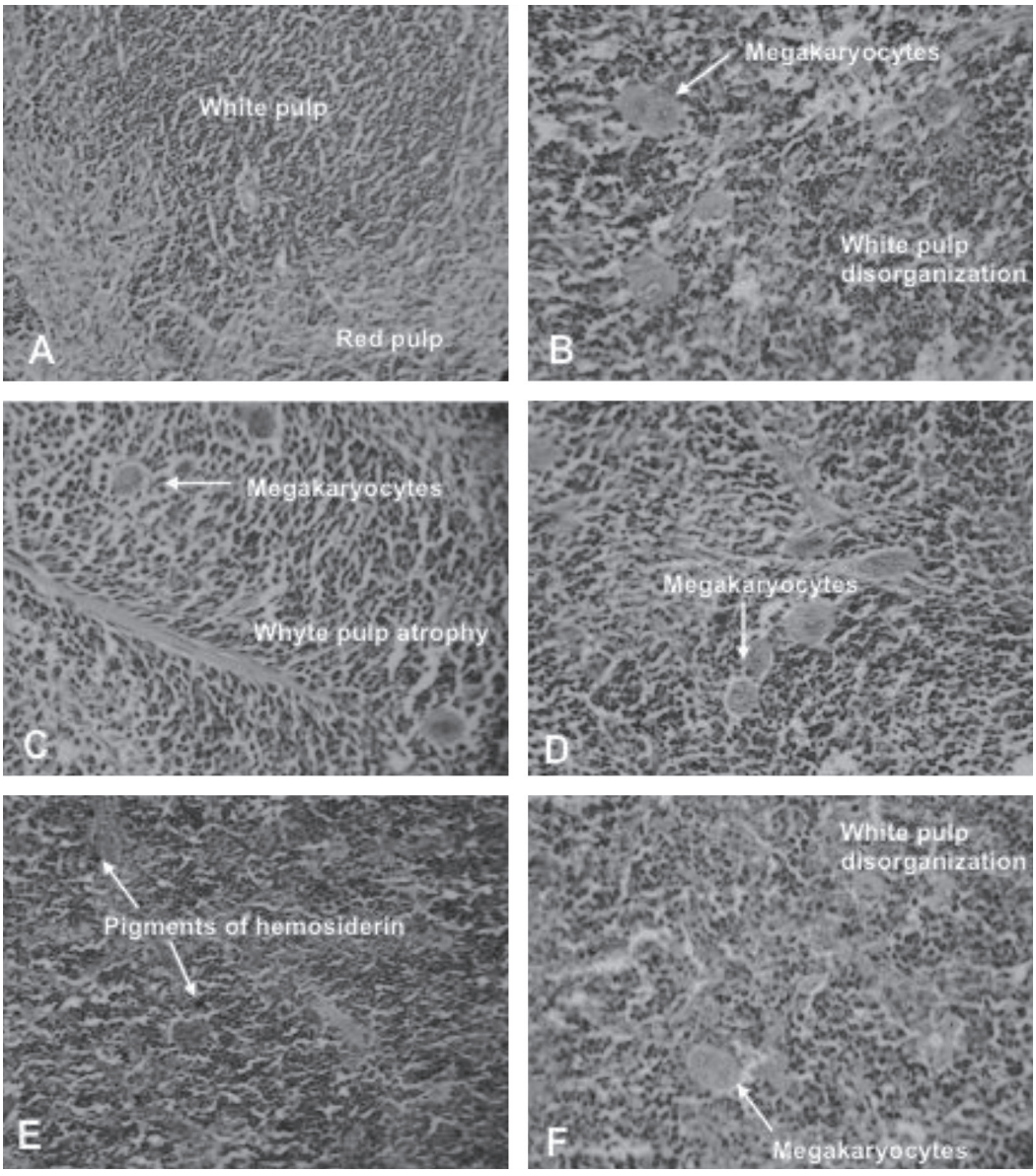

Figure 4 - Histology of spleens removed from untreated or *N*-phthaloyl amino acid derivatives-treated mice with 50 mg/kg/day after 7 days. A – Control (4 % DMSO); B – Thalidomide; C – 5-Fluorouracil (25 mg/kg/day); D – Compound 3c; E – Compound 4; F – Compound 5. Hematoxylin-eosin staining. Magnification, x 400.

and focal nephrotoxic necrosis that led to interstitial tissue damages (Figures 5C and 5F).

Liver findings included Kupffer cell hyperplasia, portal and centrolobular venous congestion and ballooning degeneration of hepatocytes in all groups. Hydropic degeneration and Kupffer cell hyperplasia were intense in 5-FU- and phthalimide-treated mice (Figures 6A-6F). Once again, the presence of leucocytes close to focal necrosis of hepatocytes in compound 5-treated animals indicates inflammation (Figure 6F).

DISCUSSION

The search for new phthalimides has gained more attention over the last decades, since these molecules have revealed promising anti-

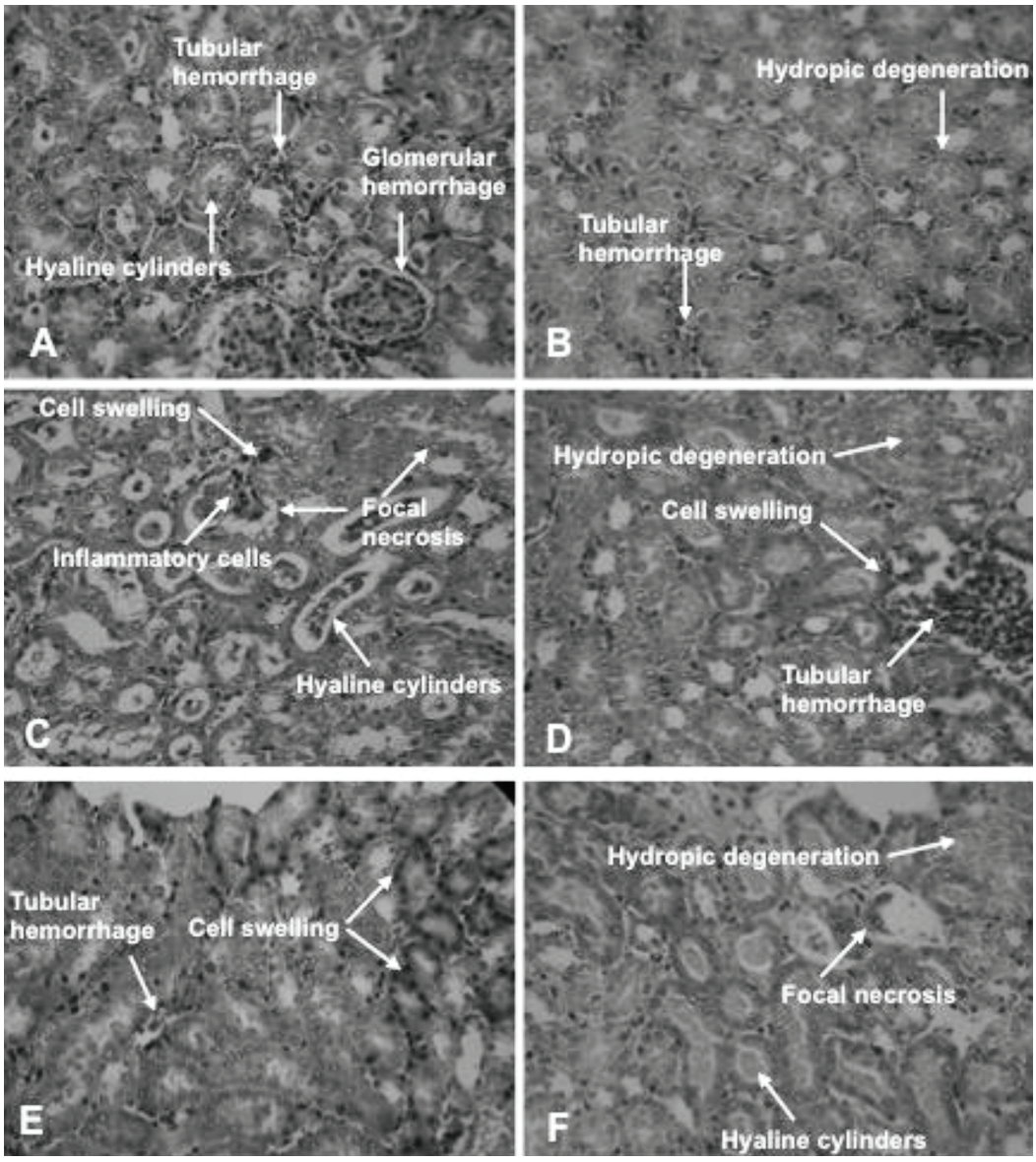

Figure 5 - Histology of kidneys removed from untreated or *N*-phthaloyl amino acid derivatives-treated mice with 50 mg/kg/day after 7 days. A – Control (4 % DMSO); B – Thalidomide; C – 5-Fluorouracil (25 mg/kg/day); D – Compound 3c; E – Compound 4; F – Compound 5. Hematoxylin-eosin staining. Magnification, x 400.

inflammatory, immunomodulatory, anti-angiogenic and tumor growth inhibition effects (D'Amato et al. 1994, Du et al. 2005, Machado et al. 2005, Noguchi et al. 2005, Pessoa et al. 2010, Nabhan and Petrylak 2012, Ruchelman et al. 2013, Leite et al. 2014). In this work, we evaluated 11 phthalimide derivatives on murine cells and their *in vivo* antitumoral and deleterious properties.

Murine tumor cells are very exploited as preclinical tools in research for new antitumor entities (Magalhães et al. 2010, Ferreira et al. 2011). Initially, we tested all molecules against cancer cells (B-16 and S180) using *in vitro* protocols. Only compounds 3b, 3c, 4 and 5 showed antiproliferative activity after 72h exposure, of which molecule 4 revealed to be the most active.

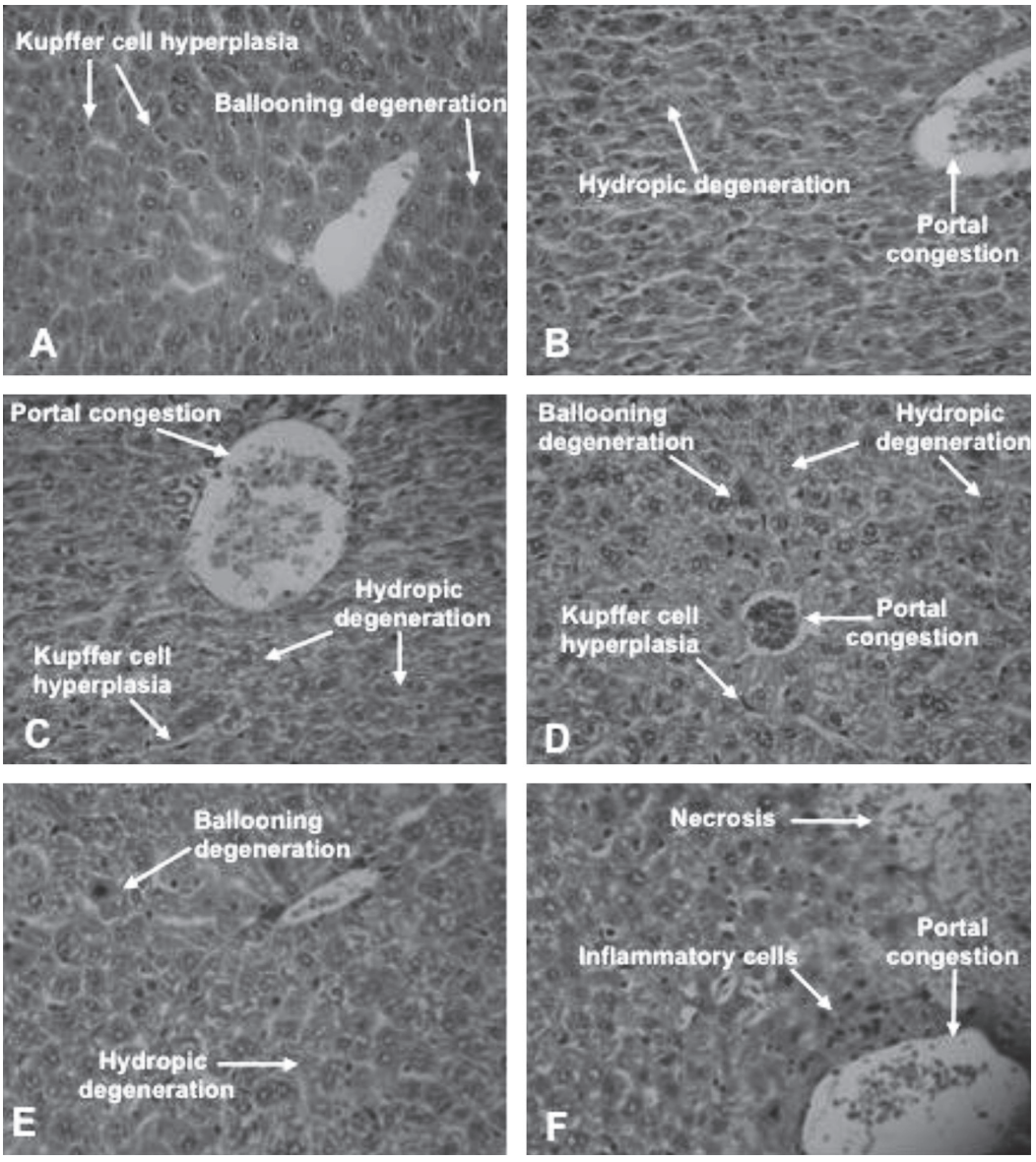

Figure 6 - Histology of livers removed from untreated or *N*-phthaloyl amino acid derivatives-treated mice with 50 mg/kg/day after 7 days. A – Control (4 % DMSO); B – Thalidomide; C – 5-Fluorouracil (25 mg/kg/day); D – Compound 3c; E – Compound 4; F – Compound 5. Hematoxylin-eosin staining. Magnification, x 400.

Compounds of this series are phthaloyl derivatives bearing a thiosemicarbazide, aminoguanidine, semicarbazide or a thiazolyl moiety. Compounds 3a, 3b, 3c and 4 have the most closed structures, whereas they possess a thiazolyl ring system and a carbonyl group at position 4. Compound 4, the less lipophilic of the series, is the only one that possesses a carbonyl group at position 4 and a thiocarbonyl group. Morever, compound 3a was inactive, since the only difference between 3a and 4 is the thiocarbonyl group presented in the molecule 4. Previously, it was demonstrated that the majority of these compounds do not have cytotoxic activity on tumor cells, though compound 4 was the strongest substance, inhibiting the multiplying of human histological tissues, as leukemia (HL-60,

8 μM), colon (HCT-8, 31.6 μM), nervous system (SF-295, 10 μM) and melanoma (MDA/MB-435, 16 μM) cancer lines (Pessoa et al. 2010).

In fact, many works relate the potentiality of phthaloyl derivates against distinct histological types of tumors, such as lenalidomide and pomalidomide against Namalwa lymphoma cell line (Ruchelman et al. 2013), thalidomide sulfur analogs with antimitotic, apoptotic and necrotic activities against Ehrlich carcinoma (Zahran et al. 2008) and *N*-phthaloyl organogallium(III) complexes with cytotoxic action on anaplastic thyroid (8505C) and colon (DLD-1) carcinoma (Gómez-Ruiz et al. 2009). So, extensive preclinical studies and clinical trials, both as a single agent and in combination, have confirmed the benefit of thalidomide and phenylphthalimide analogues in various malignancies (Du et al. 2005, Lee et al. 2006, Ochalski et al. 2011), especially for multiple myeloma and myelodysplastic syndromes, where thalidomide has already become a part of standard therapy for the treatment of patients with relapsed and refractory multiple myeloma (Richardson et al. 2002, Kagoya et al. 2012). Thalidomide was observed to significantly potentiate the antiproliferative activity of the simvastatin and lovastatin and improves their proapoptotic effect. In combination with thalidomide, statins inhibited cell migration, decreased VEGF (Vascular Endothelial Growth Factor) production and MMP-9 (Matrix Metalloproteinase-9) in multiple myeloma cells in a more effective way when compared to the statins separately and augmented activation of the caspase-3, -8 and -9, specially after use of p38 MAP kinase inhibitor, indicating that p38 inhibitors together with the combination of simvastatin and thalidomide can be used in the treatment of multiple myeloma (Slawinska-Brych et al. 2013).

The antimyeloma potential of thalidomide and immunomodulatory derivatives of thalidomide (IMiDs) may be associated with antiangiogenic effects. The thalidomide, when incorporated in poly-lactide-co-glycolide (PLGA) implants in an animal model for Ehrlich tumor, showed antiangiogenic activity in the model of chorioallantoic membrane (CAM) by reducing blood vessels. So, it is possible that these antiangiogenic effects of the IMiDs depend on the inhibition of endothelial cell growth rather than on cytotoxic mechanisms (Bauer et al. 1998, Badamtseren et al. 2011, Pereira et al. 2013) which may explain, at least in part, the *in vitro* weak cytotoxic activities of some *N*-phthaloyl derivatives.

With regards to the chemotherapeutic prospective, it is important to determine if the compounds show damaging effects in normal dividing cells such as proliferating leukocytes. Herein, data indicate a narrow selectivity of the derivatives towards cancer cells, since IC_{50} values were very similar for normal cells. Despite this low discrimination between neoplasic and normal tissues, a direct membrane injury is an implausible possibility, since even high concentrations (200 μg/mL) were not able to lyse mice erythrocytes (Pessoa et al. 2010), suggesting that cytotoxicity was not related to lytic properties or membrane instability, being probably caused by a more specific pathway. Absence of selectivity was also found with Dox and 5-FU, whereas both molecules also inhibited white blood cell proliferation. Nonetheless, Dox and 5-FU are anticancer agents currently used to treat various types of human cancers, such as breast, leukemias, bladder, stomach, head and neck (Gonen and Assaraf 2012, Kizek et al. 2012).

Despite the molecular mechanism of the antitumor effect of phthalimide derivatives still being unclear, tumor cell proliferation inhibitions by such analogues has being reported against several tumor cell types, probably due to an enhanced susceptibility to apoptosis via DNA damage and G_0/G_1 cell cycle arrest (Sleijfer et al. 2004, Dredge et al. 2005, Slawinska-Brych et al. 2013). Herein, we noted that the derivative 4 caused, in a time- and concentration-dependent manner, *in vitro* DNA fragmentation and mitochondrial depolarization, typical findings of cell death activated by apoptosis also seen in Dox- and 5-FU-treated sarcoma cells.

Cell apoptosis is characterized by distinct biochemical and morphological features, such as plasma membrane blebbing, cell shrinkage and changes in mitochondrial membrane permeability, which leads to the release of intermembrane space mitochondrial proteins (especially cytochrome *c*), caspase triggering and nuclear translocation of a caspase-activated DNase, resulting in internucleosomal DNA cleavage (Krysko et al. 2008).

With increasing in time and concentration, compound 4 caused membrane disruption, probably indicating an existence of secondary necrosis or late apoptosis, when apoptotic cells do not maintain ATP production or control oxidative stress due to the mitochondrial failure (Marinho-Filho et al. 2010). Therefore, depending on the concentration used, many different processes may be influenced and/or altered (Czyz et al. 2005).

It has been demonstrated that inhibition of angiogenesis and tumor growth by thalidomide or analogs requires metabolic activation (Bauer et al. 1998, Noguchi et al. 2005, Badamtseren et al. 2011, Pereira et al. 2013). In a similar way, Pessoa et al. (2010) also suggest that a prior metabolic activation might can be necessary to produce one or more active metabolites from *N*-phthaloyl amino acids derivatives, explaining their antiproliferative activity only in *vivo* models. Then, it is also possible that the metabolizing of the molecules (3c, 4 and 5) also explains, at least in part, their absence of *in vivo* antitumor action, since it is very known that most of the medicines undergo hepatic enzymatic reactions to generate metabolites of increasing polarity to be eliminated from the body (Kumar et al. 2004). Liver and kidneys are the main organs in biotransformation and detoxification, supposedly being responsible for producing and eliminating active, inactive and/or inert substances. The liver is often the primary site of exposure to toxins, and hepatic injury occurs frequently as seen here after treatment with phthalimide analogues. Regardless, the liver possesses great adaptive and regeneration ability. Even when hepatocelular necrosis is present, whether adjacent conjunctive tissue is preserved, the regeneration is almost complete (Ramaiah 2007). So, despite points of focal necrosis, the hepatic and renal alterations observed are considered reversible.

Although compound 4 has failed to avoid tumor expansion, it caused enhancement of blood peripheral lymphocytes and spleen enlargement, a clear finding suggestive of immunostimulant action of this molecule. Indeed, immunomodulatory drugs based on thalidomide, mainly amino-substituted thalidomide analogs, have been reported to be superior candidates as antitumor agents (Bartlett et al. 2004). Despite thalidomide not leanding to spleen morphological alterations in this work, it is known that some phthalimide analogues act as costimulators, increasing the response of T-lymphocytes to T-cell-receptor-mediated stimulation, the production of interleukin-2 and interferon-γ as well as the number of natural killer cells (Haslett et al. 1998, Pessoa et al. 2010, Pan and Lentzsch 2012). On the other hand, most chemotherapy drugs are immunosuppressive and have negative side effects (Gonen and Assaraf 2012), which explain the leucocyte suppression, and hypoplasia of the splenic white pulp and small lymphoid aggregates in 5-FU-treated mice, findings which display the importance about enhancement of host defenses as an alternative to the traditional cancer cytotoxic chemotherapy since it involves minor side effects.

CONCLUSIONS

Phthalimide derivatives 3c, 4 and 5 possess *in vitro* cytotoxic action possibly triggered by apoptosis, reversible toxic effects and the analogue 4 exhibited immunostimulant properties that can be explored to attack neoplasic cells. These discoveries stimulate further optimization studies aiming to improving the antitumor properties of this prototype.

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RESUMO

Onze derivados da ftalimida foram avaliados quanto a sua atividade antiproliferativa em células tumorais e normais e possíveis efeitos tóxicos. Avaliou-se a citotoxicidade contra tumores murinos (células de Sarcoma 180 e B-16/F-10) e células mononucleares do sangue perférico (CMSP) usando os ensaios de MTT e Alamar Blue. Em seguida, a investigação de citotoxicidade foi executada por citometria de fluxo e potencial antitumoral e toxicológico por meio de métodos in vivo. As moléculas 3b, 3c, 4 e 5 revelaram citotoxicidade in vitro contra Sarcoma 180, B-16/F-10 e PBMC. Uma vez que o composto 4 foi o derivado mais efetivo, ele foi escolhido para detalhar o mecanismo de ação após 24, 48 e 72h de exposição (22.5 e 45 μM). Células de Sarcoma 180 tratadas com o composto 4 mostraram desintegração de membrana, fragmentação de DNA e despolarização mitocondrial de maneira tempo e concentracão dependente. Os compostos 3c, 4 e 5 (50 mg/kg/dia) não inibiu o crescimento tumoral in vivo. Os animals tratados com o composto 4 exibiram aumento do total de leucócitos, linfócitos e no peso relativo do baço, diminuição de neutrófilos e hiperplasia da polpa branca esplênica. Os animais tratados apresentaram alterações histológicas reversíveis. A molécula 4 tem ação antiproliferativa in vitro provavelmente por ativação de apoptose, efeitos tóxicos reversíveis e exibiu propriedades imunoestimulantes que podem ser exploradas para atacar células neoplásicas.

Palavras-chave: alterações histológicas, células murinas, citotoxicidade, derivados da ftalimida, sarcoma 180.

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