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Synthesis and Preliminary Evaluation of Selected 2-Aryl-5(6)-nitro-1*H*-benzimidazole Derivatives as Potential Anticancer Agents

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In this study we report the synthesis and preliminary evaluation of a series of six 2-aryl-5(6)-nitro-1*H*-benzimidazole derivatives (**1**-**6**) as potential anticancer agents. Cytotoxicity was evaluated against seven human neoplastic cell lines using the MTT assay. Compound **6** [2-(4-chloro-3-nitrophenyl)-5(6)-nitro-1*H*-benzimidazole] was the most active of the series, showing an IC_{50} of 28 nM against the A549 cell line. This compound displayed a selective *in vitro* cytotoxic activity index (>700) in non neoplastic HACAT cells (IC_{50} = 22.2 μM). Compounds **3** and **6** induce arrest in the S phase of the cell cycle, and compounds **1**-**6** induce apoptosis in the K562 cell line. Compound **6** induces poly (ADP-ribose) polymerase (PARP) inhibition activity as a potential mechanism of action. These results suggest that compound **6** could be a potent anticancer agent. Compound **3** displayed the best inhibitory activity against PARP with an IC_{50} value of 0.05 μM, compared to the activity shown by the positive control 3-aminobenzamide (IC_{50} = 28.5 μM).

Key words: Anticancer, Benzimidazole, Nitrocompounds, Cytotoxicity, PARP

INTRODUCTION

Cancer is a leading cause of death worldwide and had accounted for 7.9 million deaths (approximately 13% of all deaths) in 2007. Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year (WHO, 2009). Current treatments have many limitations such as side effect of the drugs as well as drug resistance. Hence, the identification and synthesis of novel, efficient and less toxic anticancer agents remains an important and challenging task for cancer treatment. In medicinal chemistry the benzimidazole nucleus is the key building block for a variety of biologically important molecules with different therapeutic properties, such as anti-parasitic, antimicrobial, antiviral, anti-helminthic, anti-hypertensive, analgesic, anti-inflammatory, anti-ulcer, anti-allergic (Özden et al., 2005) and antitumor activity (Bielawski et al., 2002; Ghodousi et al., 2004). Studies showed that, 5-phenylterbenzimidazole is a cytotoxic agent against the RPMI 8402 cell line (Kim et al., 1997). A series of 2-aryl-5(6)-substituted-1*H*-benzimidazole derivatives were synthesized which showed cytotoxicity against human cancer cell lines and 5-nitro-2-(4-methoxyphenyl)-1*H*-benzimidazole was chosen as the lead compound (Min et al., 2005). Bendamustine (TREANDA®) contains benzimidazole, which has been approved by FDA for the treatment of leukemia. In addition, 1-substituted-2-methyl-5-nitrobenzimidazoles have been reported as antitumoral compounds (Ramla et al., 2006).

It is reported that the cytotoxicity of nitro compounds against hypoxic cells is attributed to the reduction of the nitro group, yielding several reactive metabolites including the nitro anion ($R\text{-}NO_2^-$), hydro-

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nitroxide (R-HNO˙), and amino cation free radical (R-NH_2^+) (Helsby et al., 2003). In addition, novel targets against cancer have been discovered. The poly (ADP-ribose) polymerases (PARP) are a family of nuclear enzymes. PARP1 is involved in the regulation of cellular processes such as DNA repair, gene transcription, cell cycle progression, cell death, chromatin function and genomic stability (Jagtap and Szabó, 2005; Tentori and Graziani, 2005; Moree et al., 2008). Because of the role of PARP in DNA repair, many PARP-1 inhibitors have been developed in order to enhance the efficacy of chemotherapy and radiotherapy. These inhibitors interact with the nicotinamide binding domain of the enzyme (Curtin et al., 2004; White et al., 2004; Ashworth, 2008).

In this work, we designed a series of novel 2-aryl-5(6)-nitro-1*H*-benzimidazole derivatives taking into consideration the physicochemical properties of compounds that were predicted using Lipinski's Rule (Rule of five, Ro5) that is widely accepted for predicting the permeability behavior of compounds in biological membranes. We also predicted biological activity of the compounds *in silico* using the computational program PASS (Institute of Biomedical Chemistry of Russian Academy of Medical Sciences). The novelty of this study was the addition of an extra nitro group in the phenyl ring of the 2-arylbenzimidazole core. Since previous reports showed that 2-(4-methoxyphenyl)-5-nitrobenzimidazole possessed a strong cytotoxic action, we decided to exchange the substituent of the phenyl ring (methoxy) and replace it with another nitro group. It is well known that the nitro group can be used in the chemotherapy of hypoxic cells. In these series, we changed the position of the extra-nitro group in the phenyl ring, with the aim to improve anticancer activity, and determine the correct pattern of the dinitro-substitution.

We report the synthesis and screening of antiproliferative activities such cytotoxicity, PARP inhibition activity, cell cycle effect and apoptosis induction of a series of novel 2-aryl-5(6)-nitro-1*H*-benzimidazole derivatives as potential chemotherapeutic agents (Table I).

MATERIALS AND METHODS

Chemicals

Melting points were determined on an EZ-Melt MPA120 automated melting point device from Stanford Research Systems and were used uncorrected. Reactions were monitored by TLC on 0.2 mm precoated silica gel 60 F254 plates (Merck). ^{1}H-NMR spectra were recorded on a Varian INOVA 400 (400 MHz). Chemical shifts are given in ppm relative to tetramethylsilane (TMS, $\delta = 0$) in DMSO-*d6* and *J* values are given in Hz. The following abbreviations are used: s, singlet; d, doublet; q, quartet; dd, doublet of doublet; t, triplet; m, multiplet; bs, broad signal. EIMS were recorded on a JEOL JMS-700 spectrometer (JEOL Ltd.). Predictive values for antineoplastic activities were investigated using the chemistry software server PASS (http://195.178.207.233/PASS/). All starting materials were commercially available from Sigma-Aldrich and used without purification.

Chemistry

Synthesis of compounds 1-4

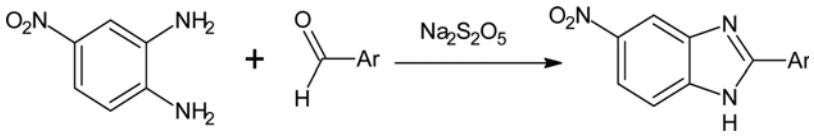
Four benzimidazole derivatives (**1-4**) were synthesized by the reaction of 4-nitro-1,2-phenylenediamine with appropriate aromatic substituted aldehydes and sodium metabisulfite under microwave irradiation (Scheme 1), using a method previously reported by our laboratory (Navarrete-Vázquez et al., 2006).

Synthesis of compounds 5 and 6

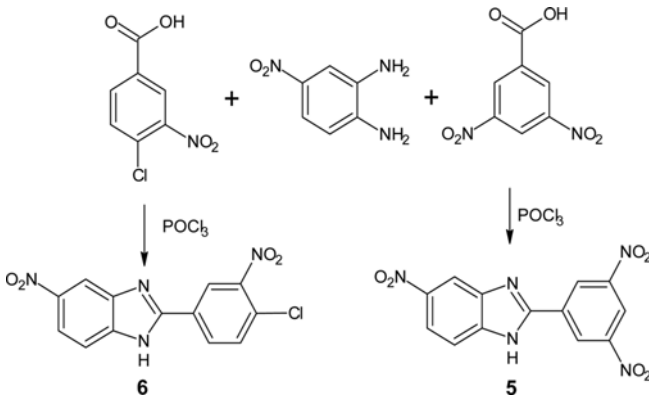
Compounds **5** and **6** were obtained using a classical method. A mixture of 4-nitro-1,2-phenylenediamine (0.0313 mol), 1.01 equivalents of 3,5-dinitrobenzoic acid or 4-chloro-3-nitrobenzaldehyde and 2.0 equivalents of phosphorous oxychloride was heated to reflux for 30 h (Scheme 2), the reaction was stopped by neutralizing with ammonium hydroxide. The precipitate was collected by filtration. Purification was done by chromatography on a silica gel using chloroform: methanol (95:5) to elute and then dried. Some physicochemical properties are listed in Table I. The structure of the pure compounds (**1-6**) was established by spectroscopic and spectrometric data.

5(6)-Nitro-2-phenyl-1*H*-benzimidazole (1)

Yield 0.97 g (63%) of brown solid, mp 147-149ºC. ^{1}H-NMR (400 MHz, DMSO-d_6) 7.60 (m, 3H, H-3', H-4', H-5), 7.75 (bs, 1H, H-7, $J = 7.8$ Hz), 8.12 (dd, 1H, H-6, $J = 2.0, 2.0, 8.8$ Hz), 8.21 (dd, 2H, H-2', H-6', $J = 1.6, 8.0$



Scheme 1. Chemical synthesis of 5(6)-nitro-1*H*-benzimidazole derivatives **1-4**



Scheme 2. Preparation of compounds **5** and **6**

Hz), 8.50 (bs, 1H, H-4, *J* = 8.5 Hz). EIMS: *m/z* (% rel. int.) 239 (M+, 100), 223 (2), 193 (30), 166 (20).

5(6)-Nitro-2-(2-Nitrophenyl)-1*H*-benzimidazole (2)
Yield 1.43 g (78%) of yellow crystals, mp 130-132 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) 7.81 (d, 1H, H-7, *J* = 8.8 Hz), 7.85 (dd, 1H, H-5', *J* = 1.6, 1.2, 8.0 Hz), 7.92 (ddd, 1H, H-4', *J* = 1.6, 1.6, 7.6 Hz), 8.01 (dd, 1H, H-3', *J* = 1.6, 7.6 Hz), 8.13 (dd, 1H, H-6', *J* = 1.2, 8.0 Hz), 8.17 (dd, 1H, H-6, *J* = 2.4, 8.8 Hz), 8.53 (d, 1H, H-4, *J* = 2.4 Hz). EIMS: *m/z* (% rel. int.) 284 (M+, 100), 268 (80), 238 (5), 192 (20).

5(6)-Nitro-2-(3-Nitrophenyl)-1*H*-benzimidazole (3)
Yield 1.16 g (63%) of Brown solid, mp 281-283°C. ^{1}H-NMR (400 MHz, DMSO-d_6) 7.79 (d, 1H, H-7, *J* = 8.8 Hz), 7.87 (d, 1H, H-5', *J* = 8.0 Hz), 8.13 (dd, 1H, H-6, *J* = 2.0, 8.8 Hz), 8.37 (dd, 1H, H-6', *J* = 2.8, 8.0 Hz), 8.49 (bs, 1H, H-2'), 8.60 (dd, 1H, H-4', *J* = 2.8, 8.0 Hz), 8.99 (d, 1H, H-4, *J* = 2.0 Hz). EIMS: *m/z* (% rel. int.) 284 (M+, 100), 238 (30), 192 (17).

5(6)-Nitro-2-(4-Nitrophenyl)-1*H*-benzimidazole (4)
Yield 0.93 g (51%) of brown crystals, mp 267°C. ^{1}H-NMR (400 MHz, DMSO-d_6) 7.40 (m, 4H, H-2', H-3', H-4 , H-5'), 7.85 (d, 1H, H-7, *J* = 8.4 Hz), 8.01 (dd, 1H, H-3', *J* = 1.6, 7.6 Hz), 8.06 (d, 1H, H-6, *J* = 8.5 Hz), 8.46 (s, 1H, H-4). EIMS: *m/z* (% rel. int.) 284 (M+, 100), 268 (4), 238 (25), 192 (25).

2-(3,5-Dinitrophenyl)-5(6)-nitro-1*H*-benzimidazole (5)
Yield 1.39 g (60%) of brown crystals, mp 189-190°C. ^{1}H-NMR (400 MHz, DMSO-d_6) 7.84 (d, 1H, H-7, *J* = 8.0 Hz), 8.16 (dd, 1H, H-6, *J* = 2.0, 8.0 Hz), 8.55 (s, 1H, N-H), 8.90 (d, 1H, H-4, *J* = 2.0 Hz), 9.17 (d, 1H, H-4', *J* = 2.4 Hz), 9.32 (d, 2H, H-2', H-6', *J* = 2.4 Hz). EIMS: *m/z* (% rel. int.) 329 (M+, 4), 283 (4), 237 (3), 192 (11).

2-(4-Chloro-3-nitrophenyl)-5(6)-nitro-1*H*-benzimidazole (6)
Yield 1.95 g (95%) of brown solid, mp 285-286°C. ^{1}H-NMR (400 MHz, DMSO-d_6) 7.73 (d, 1H, H-5', *J* = 8.8 Hz), 7.94 (d, 1H, H-7, *J* = 8.4 Hz), 8.08 (dd, 1H, H-6', *J* = 2.0, 8.8 Hz), 8.37 (dd, 1H, H-6, *J* = 2.0, 8.4 Hz), 8.42 (d, 1H, H-2', *J* = 2.4 Hz), 8.75 (d, 1H, H-4, *J* = 2.4 Hz). EIMS: *m/z* (% rel. int.) 318 (M+, 10), 283 (100), 267 (50).

Biological assays

The biological assays were performed using the MTT assay (Monks et al., 1991) against the following human cell lines: A549 (lung, carcinoma), K562 (bone marrow, chronic myelogenous leukemia), KB (HeLa contaminant), HL60 (peripheral blood, acute promyelocytic leukemia), MDA231 (breast, adenocarcinoma), MCF7 (breast, adenocarcinoma) and HT29 (colon, adenocarcinoma). HACAT cells were used as a non neoplastic control. Cell cycle arrest and apoptosis induction were evaluated in K6562 cells. The PARP inhibition activity was evaluated using the PARP Universal Colorimetric Assay Kit (R&D Systems) (Zhu et al., 2008).

Cell culture

A549, K562, HL60, KB, MDA-231, MCF-7 and HACAT cell lines were obtained from the National Cancer Institute of México (INCAN). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 1% l-glutamine, at 37°C in a water-jacketed CO_2 incubator.

In vitro cytotoxicity assay

For cytotoxicity assays, the activity of synthesized compounds on each cell line was determined by the MTT assay. Cells were seeded in DMEM containing 10% FBS into wells of 96-well ELISA-type plates and exposed to a range of drug concentrations (0.01, 0.1, 1.0, 10.0 and 100.0 μM) for 48 h at 37°C. The initial seeding densities ranged from 2×10^4 cells/well. Cell viability was assessed by Trypan blue dye exclusion at the beginning of each experiment and was always higher than 95%. At the end of the drug exposure period, the cells were incubated in the medium and MTT (0.5 mg/mL, 100 μL) was added. Then, the plates with MTT were incubated in the dark for 4 h. After that, the medium was removed and the water-insoluble MTT-formazan crystals were dissolved in DMSO and the absorbance was determined in an ELISA microtiter plate reader at 570 nm with a reference filter of 630 nm. Viability was defined as the ratio (ex-

pressed as a percentage) of absorbance of treated cells to untreated cells. Drug concentration dependence and time-dependence of cell death were calculated as a toxic effect (TE): TE = [DC]/[Cell] control, where [Cell] control is cell concentration, in control wells (cells incubated for the same time length with no compound) and [DC] is the dead cell concentration. [DC] is calculated as [Cell] control [Cell] final, were [Cell] final, is the live cell concentration in wells exposed to the drug. Cell concentration was measured using a calibration curve made for all cell lines tested using MTT staining. The cytotoxic effect (IC_{50}) of the tested compounds was estimated as $100 \times (T - T_0)/T_0 = -50$, where T_0 and T are optical densities of the test well at time zero (when the compound is added) and after exposure to test compound. In this study, we use Carboplatin as the positive control, and the cell line HACAT (immortalized fibroblasts) as the non cancer cell line.

In vitro PARP inhibition assay

PARP Universal Colorimetric Assay Kit (Catalog Number 4672-096-K, R&D Systems) measures the incorporation of biotinylated poly (ADP-ribose) onto histone proteins in a 96-well plate. Briefly, we added 25 μL of 1 × PARP cocktail to each well. and prepared an activity control (PARP-HSA enzyme without inhibitors, 100% of activity) and a negative control without PARP to determine background absorbance. Then, we added 10 μL of the inhibitor of interest to the wells as well as 1 unit/well of PARP-HSA enzyme (the final reaction volume were 50 μL). The plate was incubated at room temperature for 90 min. For detection, the plate was washed 4 times with 1 × PBS (200 μL/well), and 50 μL of diluted strep-HRP (horseradish-peroxidase) was added to each well and incubated for 20 min at room temperature. The plate was then washed with 1 × PBS, and 50 μL of TACS-Sapphire (a HRP substrate that generate a soluble blue color) was added to each well and incubated for 30 min, after which absorbance was determined at 630 nm.

Cell cycle arrest and apoptosis induction

K6562 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 1% l-glutamine, at 37°C in a water-jacketed CO_2 incubator. Cells were seeded in ELISA-type 6-well plates and exposed to the following concentrations of compounds **1**-**6**: 0.1 μM for cell cycle and 100 μM for apoptosis, over a period of 12 h at 37°C. The initial seeding densities ranged from 1×10^6 cells/well. Cell viability was assessed by Trypan blue dye exclusion at the beginning of each experiment and was always higher than 95%. At the end of the drug exposure period, the cells were collected in 12 × 75 Falcon tubes and centrifuged at 1500 rpm, 4°C for 5 min, after 1 mL of cold PBS was added to the cell pellet. Then cells were fixed by adding 4 mL of −20°C absolute ethanol. The cells were stored for 72 h at −20°C in this fixation buffer until ready for analysis. For staining, at the end of 72 h, the pellet was washed in 1 mL of PBS containing: 0.1% (v/v) of Triton X-100, 10 μg/mL of propidium iodide and 5 units (k unitz) of DNase-free RNase and incubated at 37°C for 30 min. Finally, samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer and the ModFit LT V.3.0 program was used for analysis.

RESULTS AND DISCUSSION

Synthesis and in *silico* activity

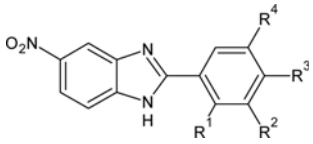
We designed a series of six compounds which were 2-arylbenzimidazole derivatives and predicted their biological activity *in silico*. In this series, we changed the position of the nitro group in the phenyl ring, with the goal to improve the activities described above. For this purpose, the chemistry software server PASS (prediction of activity spectra for substances) was used; the values obtained for antineoplastic activities are shown in Table I. Results of prediction are presented as the probability *Pa* for the compounds activity (Stepanchikova et al., 2003). For the designed compounds **1**-**6**, *Pa* values were > 0.8; then, these compounds are very likely to reveal antineoplastic activity.

MTT assay

Biological results reported in Table II showed that compound **2** was the most potent agent against the HL60 cell line (IC_{50} = 4.9 μM), and showed low cytotoxicity against the HACAT cell line (IC_{50} = 15.2 μM). Compound **3** also displayed strong potency against HT29 cells (IC_{50} = 6.4 μM). However, this compound was cytotoxic against the non cancer cell line HACAT. The features of these compounds are the presence of a nitro group in the phenyl ring at positions 2 and 3, respectively. Compound **1** was active against cell lines MDA231 (IC_{50} = 1.3 μM) and A549 (IC_{50} = 2.2 μM), but this compound was less active against the HT29 and KB cell lines than HACAT (non cancer cell line). Interestingly, compound **1** did not bear the nitro group in the phenyl ring.

Compound **4** (with nitro group at *para*-position of the phenyl ring), was the most active agent against the HL60 cell line, showing an IC_{50} of 0.9 μM, and good cytotoxic activity against MCF7, MDA231, A549 and HT29 cell lines. This compound was the most

Table I. Physicochemical properties, antineoplastic activity predicted by PASS, and PARP inhibition activity of compounds **1-6**



Compound	R^1	R^2	R^3	R^4	Antineoplastic Pa value*	Mp (°C)	Unoptimized yield (%)	Inhibition of PARP activity IC_{50} (μM)
1	-H	-H	-H	-H	0.855	147 - 149	63	2.18
2	$-NO_2$	-H	-H	-H	0.799	130 - 132	78	0.08
3	-H	$-NO_2$	-H	-H	0.843	281 - 283	63	0.05
4	-H	-H	$-NO_2$	-H	0.857	267 (dec)	51	1.27
5	-H	NO_2	-H	$-NO_2$	0.834	189 - 190	60	40.97
6	-H	NO_2	Cl	-H	0.793	285 - 286	95	38.59
3-ab								28.54

*Pa: Prediction activity using PASS program.

Table II. *In vitro* cytotoxic activity of synthesized 2-aryl-5(6)-nitro-1*H*-benzimidazole derivatives **1-6** against the eight cell lines used in this study

Compound	IC_{50} (μM)[a]							
	K562	HL60	MCF7	MDA231	A549	HT29	KB	HACAT
1	10.1 ± 2.3	> 100	> 100	1.3 ± 0.2	2.2 ± 0.1	8.9 ± 0.2	7.1 ± 0.4	9.9 ± 2.4
2	> 100	4.9 ± 0.4	74.5 ± 3.8	11.0 ± 0.2	8.6 ± 0.5	4.8 ± 1.2	18.1 ± 1.1	15.2 ± 1.2
3	9.2 ± 0.2	22.2 ± 1.2	50.4 ± 0.6	12.0 ± 1.0	9.3 ± 0.2	7.0 ± 1.2	29.4 ± 1.4	6.4 ± 2.6
4	> 100	0.9 ± 0.4	2.4 ± 0.4	1.2 ± 0.5	2.8 ± 0.3	9.7 ± 3.1	11.2 ± 1.2	0.39 ± 0.2
5	2.4 ± 0.2	1.1 ± 0.5	0.6 ± 0.2	0.4 ± 0.1	0.8 ± 0.1	0.4 ± 0.3	2.67 ± 0.2	1.2 ± 0.8
6	4.9 ± 0.9	2.1 ± 0.7	8.0 ± 0.2	4.0 ± 0.1	0.028 ± 0.1	1.8 ± 0.3	3.0 ± 0.7	22.2 ± 2.1
Carboplatin	1.5 ± 0.2	0.3 ± 0.1	8.9 ± 0.1	2.6 ± 0.4	0.05 ± 0.0	0.9 ± 0.1	4.1 ± 0.7	0.6 ± 0.1

[a]Data are presented as the mean ± S.D. of six independent series of experiments with five to six repetitions in each. Statistic analysis was performed on the Student's t-test. The Dunnet test with 95% confidence was applied to compare the means. Statistic analyses were done using the Prisma V3.0 software program.

cytotoxic agent against HACAT non cancer cell line.

Compound **5** displayed strong cytotoxic activity against all cancer cell lines tested: K562 (IC_{50} = 2.4 μM), HL60 (IC_{50} = 1.1 μM), MCF7 (IC_{50} = 0.6 μM), MDA231 (IC_{50} = 0.4 μM), A549 (IC_{50} = 0.8 μM), HT29 (IC_{50} = 0.4 μM) and KB (IC_{50} = 2.7 μM). Unfortunately, this compound was also cytotoxic against the non cancer cell line HACAT. This compound was as active as Carboplatin.

Compound **6** was the most active derivative, with IC_{50} value of 0.028 μM against A549 cell line and the activity was similar to that of Carboplatin in cancer cell lines. Interestingly, this compound was the least cytotoxic agent against the non-cancer cell line HACAT,

Table III. Selectivity Index (SI) of synthesized derivatives **1-6**

Compound	SI[b]						
	K562	HL60	MCF7	MDA231	A549	HT29	KB
1	1.0	ND	ND	7.6	4.5	1.1	1.4
2	ND	3.1	0.2	1.4	1.8	3.2	0.8
3	0.7	0.3	0.1	0.5	0.7	0.9	0.2
4	ND	0.4	0.2	0.3	0.1	0.04	0.03
5	0.5	1.1	2.0	3.0	1.5	3.0	0.4
6	4.5	10.6	2.8	5.6	740.0	12.3	7.4
Carboplatin	0.4	2.0	0.1	0.2	12.0	0.7	0.1

[b] SI = IC_{50} HACAT / IC_{50} Cell line.

with a selective index > 700 (Table III). This compound has a chloro atom at position 4 and a nitro group at position 3 of the phenyl ring. The nanomolar activity of this compound makes it a suitable lead for further *in vivo* research.

PARP inhibition

Furthermore, in order to identify a potential mechanism of action, we assayed the activity of this novel series of benzimidazoles as PARP inhibitors. To initiate this study, we considered a series of compounds developed by other research groups which explore the interaction of many structures with the nicotinamide binding domain of PARP-1. There are similarities between the structures of the series developed in our study and other inhibitors that have been reported and designed as nicotinamide mimics (Zhu et al., 2008). For this reason, we proposed that our compounds could act as nicotinamide mimics. Our results obtained were consistent with recent studies which reported a new class of potent benzimidazole PARP inhibitors (Zhu et al., 2008). In this assay, 3-aminobenzamide (**3-ab**) was used as a control inhibitor.

From this screening effort, we identified that compounds **1-6** inhibited the PARP activity in a concentration-dependent manner (Fig. 1).

Under our experimental conditions, the data obtained indicated that synthesized compounds have similar or even better activity than **3-ab** (Table I). Compounds **1-4** showed excellent inhibitory PARP activity. Compound **3** displayed the best inhibitory activity against PARP with an IC_{50} value of 0.05 μM, compared with the activity of the positive control **3-ab** (IC_{50} = 28.54 μM).

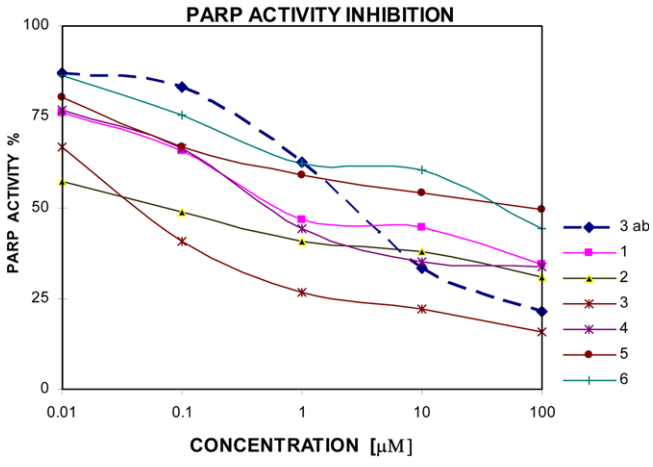


Fig. 1. *In vitro* PARP inhibition activity of the compounds **1-6**. The results represent the mean of two independent experiments by triplicate. Measurement was made using the PARP Universal Colorimetric Assay Kit, from R&D Systems. In this assay the compounds interact directly with the enzyme.

Cell cycle and apoptosis

We were interested in understanding the mechanism by which these compounds induce cell death. In order to study whether compounds could inhibit cell proliferation by cell cycle arrest or cell cycle-mediated apoptosis, we performed DNA quantification in the K562 cell line, searching for an alteration in cell cycle and apoptosis using the Sub-G1 method, in which after DNA fragmentation, there are small fragments of DNA that are eluted following a washing step in either PBS or a specific phosphate-citrate buffer. This means that after staining with a quantitative DNA-binding dye, cells that have lost DNA will take up less stain and appear to the left of the G1 peak. We used a Becton Dickinson FACSCalibur flow cytometer. Interestingly, we found that only compounds **3** and **6** induce arrest in S phase of the cell cycle, and these compounds have a nitro group in position 3 of the phenyl ring. Compounds **1**, **2**, **4**, and **5** did not affect the cell cycle of K562 cells (Fig. 2). The advantage of this method was its rapid ability to detect cumulative apoptosis as well as being applicable to all cell types. Results showed that compounds **1** - **6** induced apoptosis in K562 cells at a concentration of 100 μM before 12 h of exposure (Fig. 3).

Analyzing the cell cycle and apoptosis results for compounds **3** and **6**, our studies suggested that growth inhibition could be mediated through a DNA replication defect followed by the cell cycle arrest and finally leading to apoptosis. It is reported that the arrest in the S phase of the cell cycle is associated to topoisomerase I poisons mediated by ATR (mediated ataxia telangiectasia-Rad-3-related kinase) which modulates the response of cells to S phase-associated DNA double-stranded breaks induced by topoisomerase poisons (Cliby et al., 2002). However, the assay for correlate topo I poisoning activity with cytotoxicity was not realized in our study, it is a possibility that compounds **3** and **6** work with a similar mechanism of action.

In conclusion, a series of six novel 2-aryl-5(6)-nitro-1*H*-benzimidazoles derivatives were synthesized, characterized and subjected to preliminary evaluation as anticancer agents against seven human cancer cell lines of various histological origins. Cytotoxicity, induction of cell cycle arrest, induction of apoptosis and PARP inhibition activity were assayed in these lines. Compound **5** had two nitro groups at position 3 and 5 of the phenyl ring and displayed strong cytotoxic activity. Toxicity of these types of compounds increased when the compound had a chlorine atom in posi-

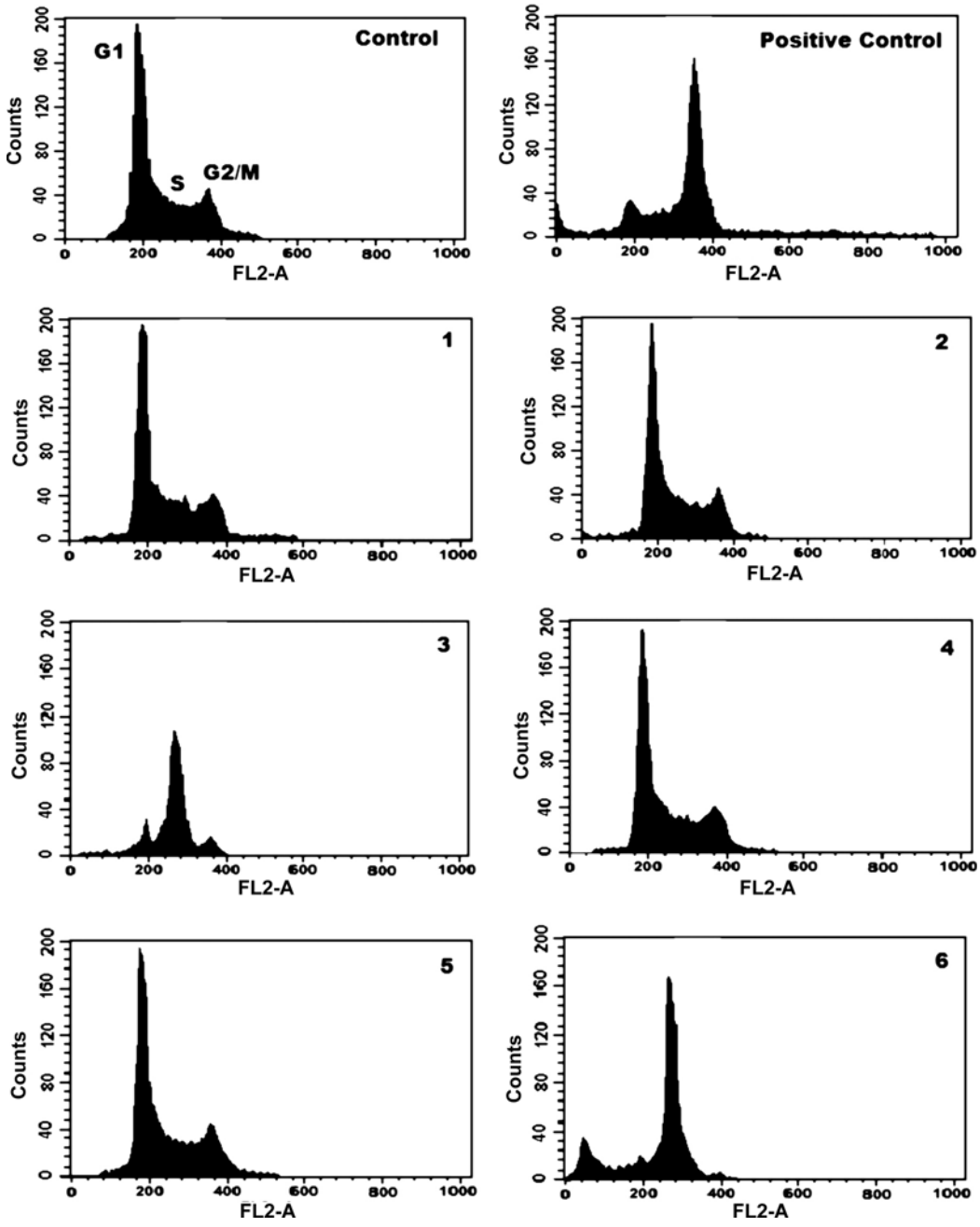


Fig. 2. Compounds **3** and **6** induce S phase arrest in K562 cells. Leukemic cells were exposed with compounds **1 - 6** at 0.1 μM for 12 h. Cells without treatment were the control and cells treated with vincristine (induce G2/M phase arrest) were the positive control.

tion 3 of the phenyl ring. Compound **6** was the most cytotoxic agent against all cancer cell lines tested, but interestingly displayed less cytotoxicity against the cell line HACAT which is non-neoplastic. Compounds **5** and **6** displayed similar activity to carboplatin which was used a positive control. Compounds which bear a nitro group at position 3 of the phenyl ring (**3**, **6**) induce arrest in the S phase of the cell cycle in K562 cells. These compounds are good candidates as anti-cancer agents. These results will direct the focus of our future research program to evaluate the dual activity of these compounds alone or in addition with alkylating agents or chemotherapy in an *in vivo* tumor model.

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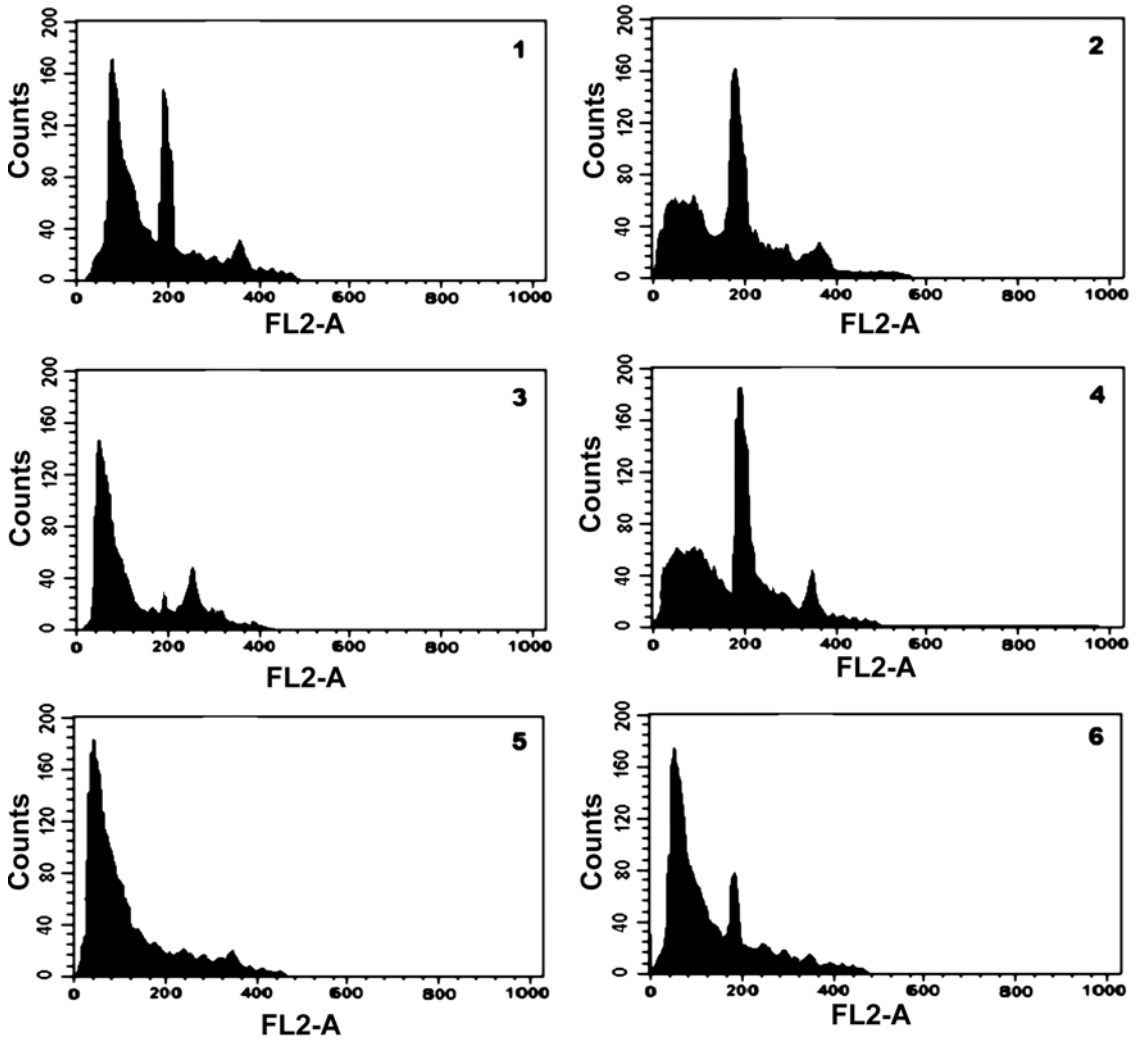

Fig. 3. Compounds **1 - 6** induce apoptosis in K562 cells. Leukemic cells were exposed with compounds (100 μM) for 12 h.

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