Bioorganic & Medicinal Chemistry 19 (2011) 6285–6291

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Evaluation of apoptotic effect of cyclic imide derivatives on murine B16F10 melanoma cells

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ARTICLE INFO

Article history:
Received 28 June 2011
Revised 29 August 2011
Accepted 6 September 2011
Available online 10 September 2011

Keywords:
Cyclic imide derivatives
Cytotoxicity
Apoptotic effect
Murine B16F10 melanoma Cells

ABSTRACT

Cyclic imides are a large class of compounds obtained by organic synthesis including several sub-classes (succinimides maleimide, glutarimide, phthalimides naphtalimides, and its derivatives). Recently, some cyclic imide derivatives have shown important results as potential antitumor agents, as a Mitonafide and Amonafide. Based on this fact, we have studied antitumoral properties of nine cyclic imide derivatives, four of which are unpublished compounds, against Murine Melanoma Cells (B16F10). Initially, the MTT assay was used to select the compound with the best cytotoxic potential. After this selection, the compound 2-benzyl-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**4**), which showed the best cytotoxic effects, was evaluated by flow cytometry, and a significant increase was observed in the proportion of cells in the subG0/G1, S and G2/M phases accompanied by a significant decrease in the G0/G1 phases. Then the mechanism involved on the death route (necrosis or apoptosis) was evaluated the by bromide and acridine orange method and by an Annexin V-FITC Apoptosis Detection kit. These results confirm that the percentage of B16F10 cells observed in the sub G0/G1 phase were undergoing apoptosis. The biological effects observed in the current study for the cyclic imide derivatives suggested promising applications, especially for the prototype compound **4**.

1. Introduction

Malignant melanoma is the most invasive and deadly form of skin cancer and its incidence is expected to rise over the next two decades.[1–3] At present, there are no effective therapies for advanced melanoma.[4,5] So the search for new drugs that are able to predict individual responses to chemotherapy has been the subject of research by several authors.[6–8] The increasing global incidence of malignant melanoma combined with the poor prognosis and low survival rates of patients requires the development of new chemotherapeutic strategies.

Cyclic imides are a large class of compounds obtained by organic synthesis including several subclasses (succinimides maleimide, glutarimides, phthalimides naphtalimides, and its derivatives), they are electrically neutral, hydrophobic compounds which can easily pass through cell membranes and reach several intracellular targets.[9] Several biological effects suggest a potential pharmaceutical use of cyclic imides, such as antinociceptive,[10] anti-inflammatory,[8] antimicrobial,[11,12] and antitumor,[13,14] apparently related to the size and characteristic of the substituents at the imide ring, which can modify the steric properties of these compounds, potentially altering their activity.[11]

The cytotoxic properties of several substituted cyclic imides are well documented. For example, substituted naphthalimides containing *N*-(2,2-dimethylaminoethyl) chain best represented by Mitonafide and Amonafide have been shown to possess significant anticancer activity.[15,16] Both Amonafide and Mitonafide entered the clinical trial stages for the use as anticancer chemotherapeutics but failed as a consequence of unexpected side effects. These observations warranted structural optimization by keeping the key naphthalimide moiety intact while adding appropriate functional groups that could help reduce systemic toxicity. Concerning their mode of drug action multiple mechanisms have been observed including DNA intercalation, topoisomerase inhibition, photoactivation or induction of lysosomalmembrane permeabilization and apoptosis.[17–22] Within this context the synthesis of new cyclic imide derivatives has been featured in studies to evaluate the cytotoxic potential in order to optimize the activity increasing the bioavailability of the same.

Recently, some cyclic imides derivatives have shown important results as potential antitumor agents against HeLa, A549, P388, HL-60, MCF-7, HCT-8, and A375 cancer cell lines in vitro with IC_{50} values around 10^{-6} and 10^{-5} M.[20,23,24] Sulphonamide derivatives

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doi:10.1016/j.bmc.2011.09.008

are active against several human tumor cell lines, in vitro and in vivo, and do not exhibit toxicity in mice.[25,26] Sulfonyl-hydrazones were able to eliminate tumors in 20–80% of leukemia-bearing mice, after six days of treatment[27] and showed selective inhibition of a phosphoinositide-3-kinase that is overexpressed in 30% of tumors.[28]

In view of these data, this study proposed a synthesis of sulphonamide and sulphonyl-hydrazone naphthalimide derivatives and the evaluation of their cytotoxic properties.

2. Results and discussion

2.1. Chemistry[29,30]

The new compounds, sulfonamide **10**, sulfonyl-hydrazides **11**–**12** and sulfonyl-hydrazones **13**–**16**, were synthesized from previously synthesized analogs **3**–**6** (Scheme 1). In the first step, the preparation of cyclic imides **3**–**6** by reaction between the 1,8-naphthalimide **1** or 4-chloro-1,8-naphthalimide **2** and the appropriate amines (aniline, benzylamine or phenylethylamine) was carried out by reflux in ethanol or acetic acid. In the next step, the cyclic imide derivatives **4**–**6** were added over 6 equiv of chlorosulfonic acid in an ice bath. The mixture was heated to 50 °C for approximately 10–15 min until the evolution of HCl ceased. The mixtures were poured in cold water and the sulfonyl chlorides **7**–**9** were precipitated. The sulfonamide **10** was obtained by condensation of chloride **7** with pyrrolidine. The novel sulfonyl-hydrazides **11**–**12** were obtained by condensation of chlorides **8**–**9** with different amines. For the novel sulfonyl-hydrazones **13**–**16**, the chlorides **8**–**9** were condensed with hydrazine hydrate, followed by condensation with different benzaldehydes.

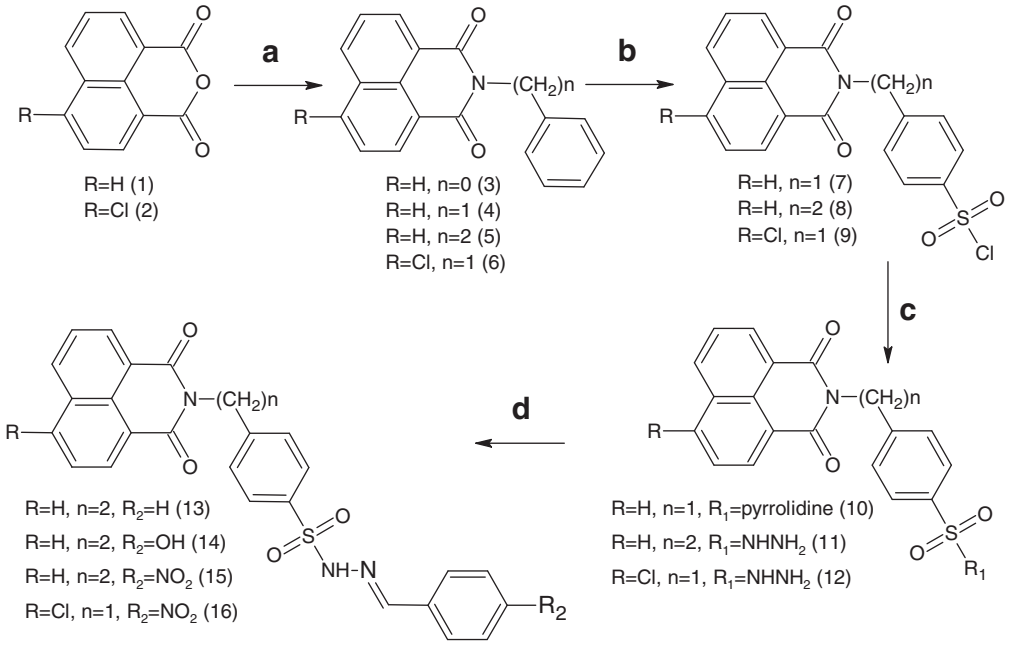
The structures of the unpublished compounds **13**–**16** were confirmed by chemical identification data: ^{1}H NMR, ^{13}C NMR, IR and elemental analysis. The known compounds were compared with literature melting points. The intermediate reaction compounds (chlorides **7**–**9** and hydrazides **11**–**12**) were characterized for IR, melting point and their respective sulfonyl-hydrazides and sulfonyl-hydrazones.

2.2. Biological evaluation

2.2.1. Cytotoxic effect

The cytotoxicity of different concentrations of cyclic imide derivatives against murine B16F10 melanoma cells was measured MTT assay.[31] The cell viability percentage and IC_{50} (50% inhibitory concentration) was assessed for the cyclic imides (**3**–**6**), one sulfonamide (**10**), and four sulfonyl-hydrazones (**13**–**16**) (Fig. 1 and Table 1, respectively). The intermediates sulfonyl chlorides (**7**–**9**) and sulfonyl-hydrazide (**12**–**13**) were not evaluated because of their instability; considering this fact, the purpose of this study was to precisely evaluate the cytotoxic effects of cyclic imides in relation to sulfonamides and sulfonyl-hydrazone derivatives. After incubation of B16F10 cells with increasing concentrations (0.1–100 μM) of the cyclic imides for 24 h, compounds **3**, **4**, **5**, **13**, **14**, **15**, and **16** reduced the viable cells in a concentration-dependent manner when compared to the control (not treated cells) (Fig. 1). Among the evaluated compounds, compound **4** had the best result with an IC_{50} of 77.75 ± 1.3 μM and is therefore chosen for further testing (Table 1, Fig. 2). Cyclic imides **6** and **10** showed a non-significant effect at the tested concentration. Unfortunately when compared to control drug, Taxol with an IC_{50} of 19.14 ± 1.5 μM, results do not seem significant, but as guidance to assist the synthesis of new derivatives with better bioavailability. A total of 100 μM was chosen as maximal concentration because much higher doses do not normally reach the blood plasma[8,32] and we are looking for compounds with potential activity (low IC_{50}) which could eventually be used in the future as a prototype in directing the synthesis of new drugs for chemotherapy with very little to no side effects.

Previous studies have demonstrated the antitumor activity of compounds derived from imides.[33–35] Cechinel Filho et al., 1994, suggested that this activity can be attributed to the hydrophobic nature of compounds and their electrically neutral potential, which would facilitate the entry through the cell membrane.[12] The current results confirm the importance of cyclic imide derivatives to the cytotoxic activity, since all the compounds evaluated caused a reduction in cell viability. It can be observed that the insertion of bulky groups (benzenesulfonyl choride, benzenesulfonohydrazide)



Scheme 1. Reagents and conditions: (a) EtOH/AcOH, reflux, 1–6 h., aniline (**3**), benzylamine (**4**, **6**), phenylethylamine (**5**); (b) $HClSO_3$, 0–50 °C; (c) MeOH, pyrrolidine (**10**), hydrazine hydrate (**11**, **12**), at 0 °C, rt, 0.5 h, ca. 0 °C; (d) EtOH, 2 or 3 drops HCl conc., rt, 1–3 h, benzaldehydes (**13**), hydroxybenzaldehyde (**14**), nitrobenzaldehyde (**15**, **16**).

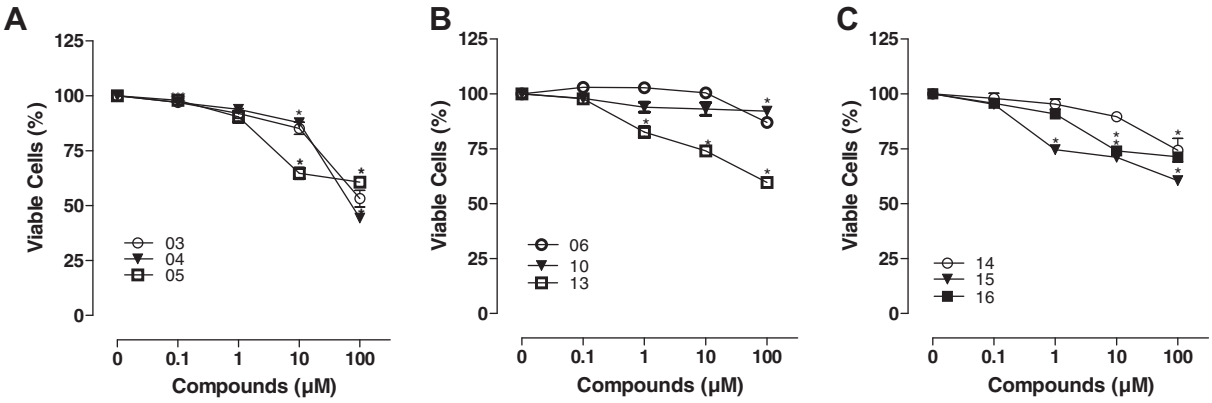


Figure 1. Cytoxicity of cyclic imides against murine B16F10 melanoma cells, the compounds (0.1–100 μM) were incubated with the cells (5×10^4) for 24 h. The cell viability was monitored through MTT assay. Optical density of control groups was taken as 100% of cell viability. Cell viability was checked at the beginning of the experiment by Trypan Blue exclusion. The results are the mean ± SEM of at least 3 independent experiments. *p <0.001 compared to control groups, using ANOVA followed by Bonferroni's t-test

Table 1
IC_{50} values of the cyclic imide derivatives and Taxol for cell viability on B16F10 cell

Compound	IC_{50} (μM)
3	103.90 (±2.8)
4	77.75 (±1.3)
5	108.20 (±8.2)
6	>300
10	>300
13	116.40 (±7.3)
14	271.00 (±7.3)
15	115.00 (±9.4)
16	201.70(±12.1)
Taxol	19.14 (±1.5)

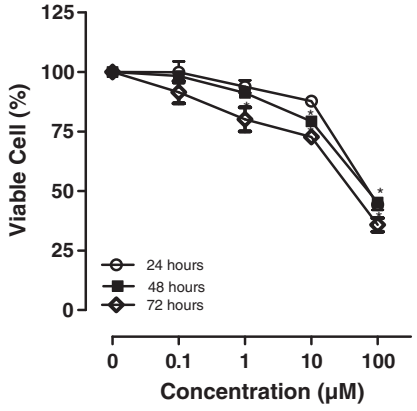


Figure 2. Toxicity of compound **4** against melanoma cells (B16F10). The B16F10 cells were incubated in the presence of different concentrations of compound **4** (0.1–100 μM), and at different times (24, 48 and 72 h), in order to calculate the IC_{50}%. Cell viability was monitored through MTT assay. Optical density of control groups was taken as 100% of cell viability. Cell viability was checked at the beginning of the experiment by Trypan Blue exclusion. The results are the mean ± SEM of at least 3 independent experiments. *p <0.001 compared to control groups, using ANOVA followed by Bonferroni's t-test.

discourage the cytotoxic activity of the evaluated compounds, since the most interesting activities were shown by compounds **3**, **4**, and **5.** This fact can be explained by the change of hydrophobic and steric parameters that are possibly involved in the interaction of these compounds with a possible target site.

Others studies have reported that the cytotoxic effect may be related with the size and characteristics of the substituent groups of the imide ring, which may modify the electronic and steric properties of these compounds, thus altering their potential activity. Regarding the effect of substitution in the imide ring, molecules **3**, **4**, and **5** could also be rated among the most active, since the substitution in the imide ring with chlorine in compound **5** reduced this compound's cytotoxicity percentage when compared to non-substituted compounds **3** and **4**.

The antitumor activity mechanism of cyclic imides remains to be fully clarified. In order to gain a better insight into the cytotoxicity mechanism, we selected compound **4**, the most active, to evaluate its effect on cell proliferation, cell cycle progression, and apoptosis.

First, the cytotoxic effect of compound **4** was analyzed at different times (24, 48 and 72 h) with different concentrations (0.1–100 μM). As shown in Figure 2, compound **4** induced cytotoxic effects on B16F10 in a concentration- and time-dependent manner. The IC_{50} was 77.75 ± 1.3 μM, 69.62 ± 2.3 μM and 38.99 ± 3.0 μM at 24, 48 and 72 h, respectively.

The effect of compound **4** (IC_{50} = 77.75 ± 1.3 μM) on the cell cycle progression of B16F10 cells at 24 h was studied by DNA content analysis through flow cytometry (Fig. 3, Panels A and B). The data listed in Table 2 were obtained by separation of cell cycle phases by setting adjacent cursors without deconvolution of overlapping G0/G1, S and G2/M phases. In comparison with the control group (without treatment), treated cells showed a significant increase in the proportion of cells in the subG0/G1, S and G2/M phases accompanied by a significant decrease in the G0/G1 phase (Table 2).

Several studies show that cell cycle disorders are often associated with neoplastic growth, so the knowledge of these mechanisms plays a key role in the development of effective therapies against cancer. The cell cycle arrest activates several mechanisms to repair DNA,[36,37] however, if DNA repair does not occur properly, p53 can induce apoptosis.[38–40] In the presence of genomic damage, a cell three choices: repair the damage, go into apoptosis, or die by necrosis. If the damage is extensive enough to deplete the concentration of ATP or inactivate the caspases, apoptosis becomes impossible and the cell dies by necrosis. If the damage is more moderate, the cell can induce p53 protein and repair the damage during the cell cycle arrest, or deliberately induce apoptosis.

In some situations, the cell can initiate an apoptosis process and then, with ATP depletion and inactivation of caspases, progress into necrosis.[37,41]

To elucidate the death mechanism of B16F10 cells induced by compound **4**, we investigated if these compounds could induce apoptosis by two different methods: ethidium bromide and acridine orange, and Annexin V-FITC. In the ethidium bromide and

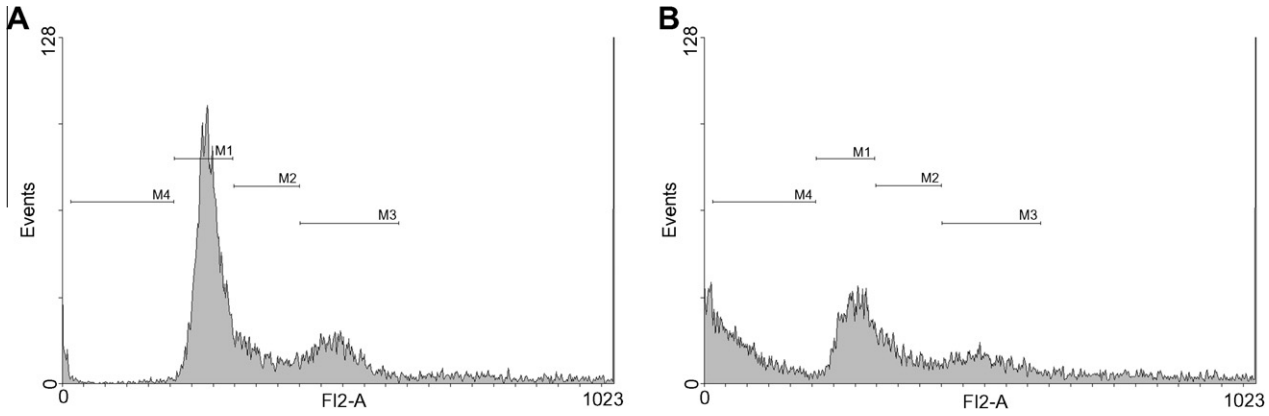


Figure 3. Effect of compound **4** on the cell cycle of melanoma cells (B16F10). The B16F10 cells were incubated in the presence or absence of 77.75 ± 1.3 μM compound **4** and the percentage of cells in each phase of cell cycle was examined through DNA content. Panel A–B: The phases of cell cycles are well represented: cell in apoptosis (Sub-G0/G1), cells in the G0/G1 phase (M1), cells in the S phase (M2), and cells in G2/M phase (M3). Panel A: Cells treated for 24 h in the absence of cycle imides derivatives. Panel B: Cells treated for 24 h in the presence of cycle imide. This figure is representative of 3 independent experiments.

Table 2
Effect of compound **4** (77.75 ± 1.3 μM), on the cell cycle of B16F10 melanoma cells

	G0/G1 (%)	S (%)	G2/M (%)	Sub-G0/G1 (Apoptosis) (%)
Control	62.40	19.79	17.81	1.29
Compound **4**	47.47*	31.05*	21.48*	18.76*

* p <0.001 compared to control groups, using ANOVA followed by Bonferroni's t-test.

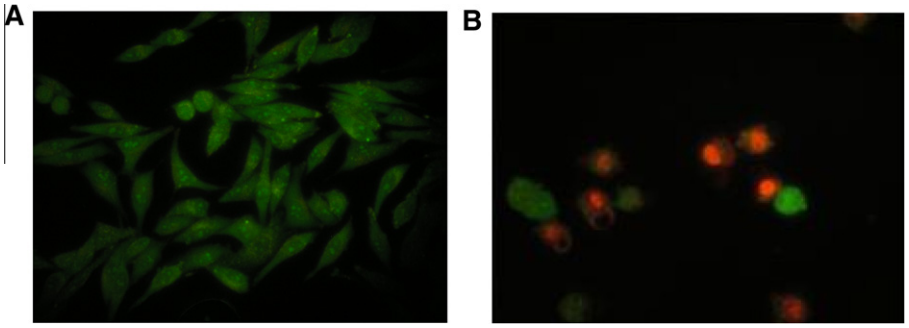


Figure 4. Induction of apoptosis by compound **4** on melanoma cells (B16F10). Panel A: Cells treated for 24 h in the absence of cycle imide. Panel B: Cells treated for 24 h in the presence of compound **4** (77.75 ± 1.3 μM). The apoptosis was determined by analysis of ethidium bromide and acridine orange by a fluorescence microscope. The figure is representative of 3 independent experiments.

acridine orange method, the viable cells exhibited a green fluorescence (acridine orange staining) whereas apoptotic cells exhibited an orange-red nuclear fluorescence (ethidium bromide staining) by intercalation of ethidium bromide into DNA damage in apoptotic cells.[42] Compound **4** (77.75 ± 1.3 μM) was incubated with B16F10 cells for 24 h and the results show a significant increase of apoptotic cells (Fig. 4, Panel B) when compared with the control (Fig. 4, Panel A).

Cells in early apoptosis still have their intact membranes therefore have the green core, but not uniformly stained, chromatin condensation occurring in them, cleavage of DNA and/or nuclear fragmentation, these are no longer stuck and its morphology was changed, since cells in late apoptosis show chromatin condensation and orange areas in the nucleus, because in the final stages of the process have lost membrane integrity and ethidium bromide on the predominant acridine orange (Fig. 4, Panel A). In the control group we can observe living cells with nuclei well formed and adhered to the blade.

Imide-induced apoptosis was confirmed by externalization of phosphatidylserine, which is detected by the Annexin V-FITC

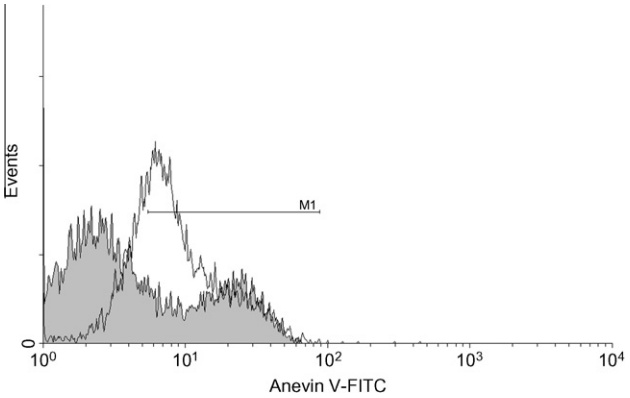


Figure 5. Induction of apoptosis by compound **4** on melanoma cells (B16F10). The cells were treated for 24 h in the absence and presence of compound **4** (77.75 ± 1.3 μM). The apoptosis was determined by analysis of tagging cells with Annexin V-FITC by flow cytometry. This figure is representative of 3 independent experiments.

Table 3
Effect of compound **4** (77.75 ± 1.3 μM), on the apoptosis in B16F10 melanoma cells

Compound	Percentage (%) annexin positive
Control	36.70
Compound **4**	69.27*

* p <0.001 compared to control groups, using ANOVA followed by Bonferroni's t-test.

method.[43] As shown in Figure 5 and Table 3, compound **4**, after 12-h incubation, induces cell death via an apoptosis pathway, showing a significant increase in the percentage of annexin V positive cells when compared with the control group. These results confirm that the percentage of B16F10 cells observed in sub G0/G1 phase are undergoing apoptosis (Fig. 3) and are compatible with the results obtained in the assessment of cell viability by MTT (Fig. 2) and with ethidium bromide/acridine orange (Fig. 4) methods.

3. Conclusion

In general, new anticancer drugs are expected to induce cell death through apoptosis. In the carcinogenesis process, the cell cycle is deregulated and apoptosis is suppressed; these are the minimal conditions for neoplastic progression. The biological effects observed in the present study for the cyclic imide derivatives suggested promising applications. We observed that compound **4** causes cell death via apoptosis. However, further studies are needed to determine the mechanism of apoptosis induced by this compound.

4. Experimental section

4.1. Synthetic methods

All solvents and reagents were purchased from Merck and Sigma-Aldrich. The purity of these compounds was determined by TLC by using several solvent systems of different polarity. Infrared spectra were determined with a Perkin Elmer 16PC spectrophotometer (Perkin Elmer, Wellesley, MA, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-200F spectrometer (Rheinstetten, Germany at 200 or 400 MHz and 50 or 100 MHz, respectively). $CDCl_3$ and DMSO were used as solvents with tetramethylsilane (TMS) as the internal standard; chemical shifts (δ) were in parts per million. For the CHN analysis, a PERKIN ELMER 2400 (Boston, MA, USA) CHN elemental analyzer was used. In the thin layer chromatography, aluminum sheets with silica gel 60 F-254 and 0.2 mm thickness were utilized.

The sulfonamide **10**, as well as the precursors **4**, **6**, **7**, and **9**, were recently published by our research group.[30]

4.1.1. 2-Phenyl-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (3)

1,8-Naphthalic anhydride **1** (5.00 g, 25.20 mmol) was added to a solution of aniline (2.3 ml, 25.2 mmol) in acetic acid. The mixture was refluxed for 3 h. The product was precipitated in cold water and recrystallized in ethanol. Yield: 72%. Mp 183.3–183.6 °C (Lit: 185 °C).[44] IR (KBr) 1693 and 1651 [ν N(C=O)$_2$], 1346 (ν –CN), 759 (ν Ar.) cm^{-1}.

4.1.2. 2-(2-Phenylethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (5)

1,8-Naphthalic anhydride **1** (3.00 g, 15.00 mmol) was added to a solution of phenylethylamine (3.80 ml, 30.00 mmol) in ethanol. The mixture was refluxed for 6 h. The crystal was formed on cooling of the solution. The solid was filtered through a Buchner funnel and washed twice with 20 ml of cold ethanol. Yield: 70%. Mp: 150.7–151.8 °C.[45] IR (KBr) 1690 and 1652 [ν N(C=O)$_2$], 1343 (ν –CN), 767 (ν Ar.) cm^{-1}.

4.1.3. 4-[2-(1,3-Dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)ethyl]benzenesulphonyl chloride (8)

Compound **5** (2.0 g, 6.6 mmol) was slowly added to cold chlorosulfonic acid (2.73 ml, 4.0 mmol). The reaction was carried out as described for compound **7**. Yield: 70%. Mp: 267.9–269.0 °C. IR (KBr): 1695 and 1655 [ν N(C=O)$_2$], 1344 and 1173 (ν –SO$_2$), 1232 (ν –CN), 776 (ν Ar.) cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.88–2.92 (t, 2H, J = 7.9 Hz, CH_2CH_2–Ph), 4.17–4.21 (t, 2H, J = 7.9 Hz, CH_2CH_2-Ph); 7.22–7.25 (d, 2H, J = 9.8 Hz, ArH), 7.54–7.56 (m, 2H, ArH); 7.79–7.81 (d, 2H, J = 9.8 Hz, ArH); 8.38–8.40 (d, 2H, J = 9.8 Hz, ArH); 8.42–8.43 (d, 2H, J = 9.8 Hz, ArH). ^{13}C NMR (200 MHz, DMSO-d_6) δ: 33.91 (1C, NCH_2CH_2), 41.58 (1C, NCH_2CH_2); 109.99 (2C, Ar), 122.53 (2C, Ar), 126.43 (2C, Ar), 127.85 (1C, Ar), 128.72 (2C, Ar), 131.39 (1C, Ar), 131.90 (2C, Ar), 135.02 (2C, Ar), 140.13 (1C, Ar), 146.52 (1C, C–S); 163.89 (2C, C=O). Anal. Calcd for $C_{20}H_{14}ClNO_4S$: C, 60.08; H, 3.53; Cl, 8.87; N, 3.50; O, 16.01; S, 8.02. Found: C, 60.01; H, 3.72; Cl, 8.52; N, 3.60; O, 15.99; S, 8.00. Unpublished compound.

4.1.4. 4-[2-(1,3-Dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)ethyl]benzenesulfonohydrazide (11)

In a solution of sulfonyl chloride **8** (400 mg, 1.00 mmol) in 30 ml of methanol, 2 equiv of hydrazine hydrate (70 μL, 2.00 mmol) were slowly added at approximately 0 °C. The product was filtered and washed twice with 20 mL of cold methanol. Yield: 88%. Mp: 179.0–180.5 °C. IR (KBr) 3414 (ν –NH–), 1689 and 1659 [ν N(C=O)$_2$], 1341 and 1112 (ν –SO$_2$), 780 (ν Ar.) cm^{-1}. Unpublished compound. This compound was characterized by their correspond sulfonyl-hydrazones **13**, **14** and **15**.

4.1.5. 4-[(6-Chloro-1,3-dioxo-1*H*-benzo[*de*]isoquinolina-2(3*H*)-yl)methyl]benzenesulfonohydrazide (12)

In a solution of sulfonyl chloride **9** (400 mg, 0.95 mmol) in 30 ml of methanol, 2 equiv of hydrazine hydrate (66.7 μL, 1.90 mmol) were slowly added at approximately 0 °C. The reaction was carried out as described for compound **11**. Yield: 78%. Mp: 146.0–148.7 °C. IR (KBr): 3414 (ν NH), 1700–1657 [ν N(C=O)$_2$], 1589 (ν NH$_2$), 1341 and 1111 (ν SO$_2$), 1235 (ν C–N), 618 (ν Ar) cm^{-1}. Unpublished compound. This compound was characterized by the correspond sulfonyl-hydrazone **16**.

4.1.6. 4-[2-(1,3-Dioxo-1*H*-benzo[de]isoquinoline-2(3H)-yl)benzyl]-*N*′-[(1E)-phenylmethylene]benzenesulphonyl hydrazone (13)

Sulfonyl-hydrazide **11** (400 mg, 1.00 mmol) was added to a solution of benzaldehyde (103 μL, 1.00 mmol) and 2 drops of hydrochloride acid concentrate in 20 ml of ethanol. The product was filtered and washed twice with 20 mL of cold ethanol. Yield: 43%. Mp: 177.4–179.0 °C. IR (KBr): 3226 (ν NH), 1697 and 1656 [ν N(C=O)$_2$], 1341 and 1160 (ν SO$_2$), 777 (ν ArH) cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.97–2.99 (t, 2H, J = 8.0 Hz, CH_2–Ph), 4.24–4.27 (t, 2H, J = 8.0 Hz, CH_2–N); 7.37–7.39 (m, 2H, ArH); 7.49–7.55 (d, 4H, J = 10.0 Hz, ArH), 7.78–7.82 (m, 4H, ArH); 7.89 (s, 1H, CH=N); 8.30 (s, 1H, ArH), 7.40–7.46 (d, 4H, J = 10.0 Hz, ArH), 11.47 (s, 1H, NH–N=C). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 34.16 (1C, NCH_2CH_2), 41.09 (1C, NCH_2CH_2); 122.40 (2C, Ar), 127.00 (2C, Ar), 127.40 (2C, Ar), 128.13 (2C, Ar), 128.20 (1C, Ar), 128.71 (2C, Ar), 129.71 (1C, Ar), 130.53 (1C, Ar), 131.36 (2C, Ar), 131.60 (1C, Ar), 133.08 (1C, Ar), 134.19 (2C, Ar), 136.35 (2C, Ar), 144.89 (1C Ar), 147.89 (1C, C=N); 164.06 (2C, C=O). Anal. Calcd for $C_{27}H_{21}N_3O_4S$: C, 67.07; H, 4.38; N, 8.69; S, 6.63. Found: C, 67.01; H, 4.35; N, 8.59; S, 6.51. Unpublished compound.

4.1.7. 4-[2-(1,3-Dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-*N*'-[(1*E*)-(4-hydroxyphenyl)methylene]benzenesulfonyl hydrazone (14)

Sulfonyl-hydrazide **11** (400 mg, 1.00 mmol) was added to a solution of *p*-hydroxybenzaldehyde (100 μL, 1.00 mmol) and 2 drops of hydrochloride acid concentrate in 20 ml of ethanol. The reaction was carried out as described for compound **13**. Yield: 77%. Mp: 163.6–165.8 °C. IR (KBr): 3186(ν NH), 1701 and 1652 [ν N(C=O)$_2$], 1596 (ν Ph–OH), 1353 and 1164 (ν –SO$_2$–), 780 (Ar.) cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.31–2.51 (t, 2H, J = 8.2 Hz, –CH–Ph), 5.13 (t, 2H, J = 8.2 Hz, –CH$_2$–N–C=O), 6.42–6.61 (m, 4H, Ar), 7.46–7.53 (m, 6H, ArH.), 7.91–8.03 (m, 6H, ArH, –N=CH– and –OH), 11.55 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 33.38 (1C, NCH$_2$CH$_2$), 44.51 (1C, NCH$_2$CH$_2$); 121.83 (2C, Ar), 124.57 (2C, Ar), 125.71 (2C, Ar), 127.05 (2C, Ar), 127.64 (1C, Ar), 127.97 (1C, Ar), 128.48 (1C, Ar), 129.38 (2C, Ar), 131.22 (2C, Ar), 134.35 (1C, Ar), 137.23 (2C, Ar), 139.33 (2C, Ar), 144.32, (1C, Ar), 147.56 (1C, C=N), 159.44 (1C, OH–C Ar), 163.22 (2C, C=O). Anal. Calcd for $C_{27}H_{21}N_3O_5S$: C, 64.92; H, 4.24; N, 8.41; S, 6.42. Found: C, 64.93; H, 4.29; N, 8.49; S, 6.31. Unpublished compound.

4.1.8. 4-[2-(1,3-Dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)-*N*'-[(1*E*)-(4-nitrophenyl)methylene]benzenesulfonyl hydrazone (15)

Sulfonyl-hydrazide **11** (400 mg, 1.00 mmol) was added to a solution of *p*-nitrobenzaldehyde (151 μg, 1.00 mmol) and 2 drops of hydrochloride acid concentrate in 20 ml of ethanol. The reaction was carried out as described for compound **13**. Yield: 67%. Mp: 193.7–195.0 °C. IR (KBr): 3189(ν NH), 1697 and 1654 [ν N(C=O)$_2$], 1517 and 1341 (ν –NO$_2$), 1341 and 1162 (ν SO$_2$), 776 (Ar.) cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.98–3.02 (t, 2H, J = 8.0 Hz, –CH$_2$–Ph), 4.21–4.26 (t, 2H, J = 8.0 Hz, –CH$_2$–N–C=O–), 7.20–7.22 (d, 2H, J = 10.0 Hz, ArH.), 7.49–7.53 (m, 2H, ArH), 7.76–7,86 (m, 4H, ArH), 7.99 (s, 1H, –N=C–), 8.18–8.21 (d, 2H, J = 10.0 Hz,ArH), 8.33 (m, 4H, ArH.), 11.93 (s, 1H, NH). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 33.55 (1C, NCH$_2$CH$_2$), 41.91 (1C, NCH$_2$CH$_2$); 121.93 (2C, Ar), 122.04 (2C, Ar), 124.27 (2C, Ar), 126.07 (2C, Ar), 127.43 (2C, Ar), 127.51 (1C, Ar), 127.96 (1C, Ar), 128.37 (2C, Ar), 129.97 (1C, Ar), 131.42 (2C, Ar), 134.69 (1C, Ar), 137.04 (2C, Ar), 140.00 (1C, Ar), 145.02 (1C, C=N), 148.10 (1C, NO$_2$–C Ar), 163.57 (2C, C=O). Anal. Calcd for $C_{27}H_{20}N_4O_6S$: C, 61.36; H, 3.81; N, 10.60; S, 6.07. Found: C, 61.49; H, 3.85; N, 10.65; S, 6.22. Unpublished compound.

4.1.9. 4-[(6-Chloro-1,3-dioxo-1*H*-benzo[*de*]isoquinoline-2(3*H*)-yl)methyl]-*N*'-[(1*E*)-(4-nitrophenyl)methylene]benzenesulfonyl hydrazone (16)

Sulfonyl-hydrazide **12** (400 mg, 0.96 mmol) was added to a solution of *p*-nitrobenzaldehyde (145 μg, 0.96 mmol) and 2 drops of hydrochloride acid concentrate in 20 ml of ethanol. The reaction was carried out as described for compound **13**. Yield: 60%. Mp: 204.4–206.8 °C. IR (KBr): 3435 (ν NH), 1696 and 1657 [ν N(C=O)$_2$], 1341 and 1166 (ν SO$_2$), 848 (ν ArH) cm^{-1}. ^{1}H NMR (200 MHz, DMSO-d_6) δ 4.19 (s, 2H, CH$_2$), 7.55 (s, 1H, N=CH), 7.60–7.53 (m, 3H, ArH), 7.83–7.73 (m, 4H, ArH), 7.97 (s, 1H, ArH); 8.43–8.12 (m, 5H, ArH); 11.09 (s, 1H, NH). ^{13}C NMR (50 MHz, DMSO-d_6) δ 33.76 (1C, CH$_2$); 121.74 (2C, Ar), 123.03 (2C, Ar), 124.56 (1C, Ar), 126.43 (1C, Ar), 127.71 (1C, Ar), 128.27 (2C, Ar), 128.70 (2C, Ar), 128.95 (2C, Ar), 129.15 (1C Ar), 130.69 (1C, Ar), 131.53 (1C, Ar), 132.23 (1C, Ar), 138.23 (1C Ar), 140.02 (1C Ar), 140.37 (1C, N–CH$_2$–Ar), 145.07 (1C, –Cl–C Ar); 146.50 (1C, C=N); 148.39 (1C, NO$_2$–C Ar); 163.15 (1C, C=O); 163.43 (1C, C=O). Anal. Calcd for $C_{26}H_{17}ClN_4O_6S$: C, 56.89; H, 3.12; Cl, 6.46; N, 10.21; O, 17.49; S, 5.84. Found: C, 56.88; H, 3.10; Cl, 6.22; N, 10.25; O, 17.42; S, 5.91. Unpublished compound.

4.2. Cell culture and viability assay (MTT assay)[31]

Murine B16F10 melanoma cells (ATCC, Manassas, VA) maintained in Dulbecco's modified Eagle's medium were cultured in DMEM (GIBCO, São Paulo, SP, Brazil) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES, pH 7.4 at 37 °C in a 5% CO_2 humidified atmosphere in plastic culture flasks. Test compounds were added to cells in a maximum volume of 20 μl. For compounds soluble in DMSO, the same volume of the solvent was added to control wells. Treatments with the imides at indicated concentrations were carried out until 12, 24, 48 and 72 h. Cell viability was assessed by using MTT (3-(4,5-dimethiazol-zyl)-2-5-diphenyltetrazolium bromide, Sigma Chemical Co., St. Louis, MO, USA) assay.

4.3. Cell cycle analysis

To assess cell cycle arrest, a PI/RNase solution kit (Immunostep, Salamanca, Spain) was used. Cells (1×10^6 cells/well) were incubated with vehicle or cyclic imide **4** at 77.75 ± 1.3 μM. After 24 h of incubation, cells were harvested and cell cycle analysis was assessed according to the kit protocol. Briefly, cells were washed with PBS buffer, fixed with 70% ethanol, washed with PBS buffer supplemented with 2% bovine albumin and stained with 500 μl PI/RNase solution. Analysis was performed by flow cytometry (FACSCantoII™, Becton Dickinson Immunocytometry Systems). The data were analyzed by WinMID software.

4.4. Analysis of the apoptotic effects with ethidium bromide and acridine orange[42]

For determination of apoptotic death with ethidium bromide and acridine orange, 1×10^6 cells/well were incubated with cyclic imide **4** at 77.75 ± 1.3 μM. After 24 h, the coverslips covering the bottom of the plate were removed, washed with PBS and covered with a solution of ethidium bromide and acridine orange (1:1) at 1% concentration. Cells were analyzed by a fluorescence microscope (Olympus BX41).

4.5. Analysis of the apoptotic effects with annexin[43]

For determination of apoptotic death, an Annexin V-FITC Apoptosis Detection kit was used according to the manufacturer's instructions. 1×10^6 cells/well were incubated with cyclic imide **4** at 77.75 ± 1.3 μM. After 12 h of incubation, cells were harvested, washed with PBS buffer, annexin buffer (1:10) and double-stained with Annexin V-FITC solution. After incubation, 300 μl of annexin buffer was added and fluorescence was analyzed by flow cytometry. Analyses were performed by flow cytometry (FACSCanto™, Becton Dickinson Immunocytometry Systems). The data were analyzed by WinMID software.

Acknowledgments

This research was supported by grants and scholarship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and CAPES (Coordenação de Pessoal de Nível Superior).

References and notes

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