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Research Article

Antiproliferative Activity Evaluation of a Series of *N*-1,3-Benzothiazol-2-ylbenzamides as Novel Apoptosis Inducers

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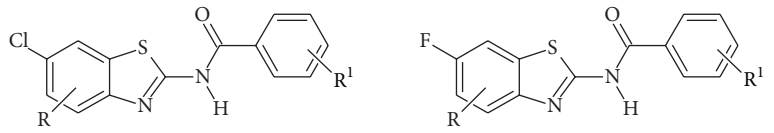
A series of *N*-1,3-benzothiazol-2-ylbenzamide derivatives were studied for their antiproliferative activity on human liver hepatocellular carcinoma (HepG2) and human breast cancer (MCF-7) cell lines. Most of them were found to show a prominent inhibitory effect on cell growth. Among the most active compounds, **1k** emerged for its proapoptotic effect that is particularly evident towards MCF-7 cancer cell lines.

1. Introduction

Cancer, a group of diseases characterized by uncontrolled growth of abnormal cells, is one of the major worldwide health problems. It is a lethal disease and one of the primary causes of death standing next to the cardiovascular diseases. Currently, studies for the identification of potent, safe, and selective anticancer drugs are at the forefront. Although the tumor-specific action of most anticancer drugs has been attributed to their debilitating effects on actively proliferating cells, several studies conversely suggest that anticancer agents induce apoptosis, a physiological mode of cell death in higher eukaryotes [1–3]. Apoptosis has been recognized as a basic component in the pathogenesis of cancer. It may be considered a defense mechanism to remove potentially dangerous cells, such as tumor cells. Its deregulation may be conceivably involved in the pathogenesis of cancer. Many antitumor compounds induce the apoptotic process in tumor cells [4]; thus, development of new drugs that can effectively trigger apoptosis in tumor cells may be envisaged. A number of benzothiazole containing compounds have exhibited interesting biological activities such as antimicrobial and antitumor activities [5–10]. Indeed, the benzothiazole nucleus constitutes an important scaffold for drugs and attracts continuous interest for further molecular exploration to find new useful anticancer agents [11–14]. Anticancer activity on human carcinoma cell lines has been reported for a benzothiazole linked to a phthalimide moiety [15]; moreover, benzothiazole-2-thiol derivatives have been proposed as novel apoptosis inducers [16]. A novel benzothiazole derivative has been shown to induce apoptosis via the mitochondrial apoptosis pathway in vitro with antitumor activity in solid malignancies [17]. Furthermore, it has been reported that antimicrobials often induce cytotoxicity via apoptotic mechanism, thus making themselves candidates as new tools for anticancer therapy. In the last decade, our research group was interested in the design, synthesis, and biological evaluation of novel benzothiazole derivatives as antimicrobial agents [8, 10, 18]. Thus, to further investigate the panel of activity for this class of compounds, we decided

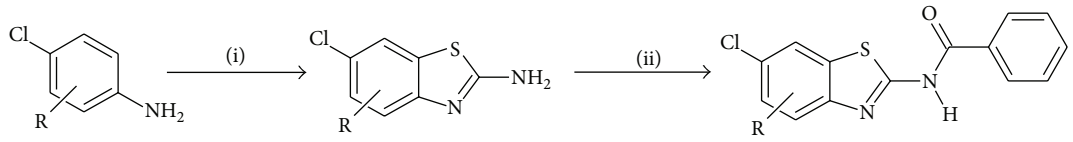


1 **2**

R: 4-F or 5-F
R^1: H or difluoro

R: 4-Cl or 5-Cl
R^1: H or difluoro

Figure 1: Structures of compounds **1** and **2**.



3m,**n** **4m**,**n** **1m**,**n**

m: R = 5-F
n: R = 4-F

Scheme 1: Synthesis of compounds **1m**,**n**. Reagents and conditions: (i) Br_2, KSCN, AcOH, 30–35°C; (ii) benzoyl chloride, Et_3N, dioxane, 50–60°C.

to study some of them as potential anticancer agents. Based on the results obtained by Wang et al. [16] on a series of benzothiazole derivatives that exhibited antiproliferative activity against the human hepatocellular carcinoma cell line, HepG2, and the human mammary carcinoma cell line, MCF-7, we selected a small series of compounds among the previously synthesized benzothiazoles [18] for biological evaluation as potential apoptosis inducers. The selected compounds belong to two small series (**1** and **2**, Figure 1). Then, in order to improve structure-activity relationship (SAR) studies on this class of compounds, three additional analogues (**1m**,**n** and **2a**) were synthesized and evaluated, too.

2. Results and Discussion

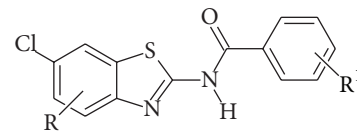
Nineteen compounds belonging to two small series (**1a**–**n** and **2a**–**e**, Figure 1, Table 1) were synthesized and evaluated for their potential antiproliferative and proapoptotic activities as described below.

2.1. Chemistry. Compounds **1a**–**l** and **2b**–**e** were previously reported by this group [18]. Compounds **1m**,**n** and **2a** were prepared as reported in Schemes 1 and 2, respectively. The appropriate aniline was reacted with bromine and potassium thiocyanate to give the corresponding 2-aminobenzothiazole which was reacted with benzoyl chloride to give the final compound. Novel benzothiazoles were fully characterized by IR, both ^1H and ^{13}C NMR spectra, and GC-MS analysis.

2.2. Biological Results. With the aim to develop novel tumor growth inhibitors and apoptosis inducers as potential anticancer agents, two small series of *N*-1,3-benzothiazol-2-ylbenzamide derivatives (**1a**–**n** and **2a**–**e**, Figure 1, Table 2) were investigated. Thus, the cell growth inhibitory activities in cultures of HepG2 and MCF-7 cells were evaluated by means of MTT assay. In order to select compounds with high activity, a concentration of 10 μM was chosen for the assays, which also meets the properties of low solubility of the tested compounds. Amongst the two cancer cell lines under

Table 1: Structures of compounds **1a**–**n** and **2a**–**e**.

Compound	R	R^1
1a	4-F	2,3-F_2
1b	4-F	2,4-F_2
1c	4-F	2,5-F_2
1d	4-F	2,6-F_2
1e	4-F	3,4-F_2
1f	4-F	3,5-F_2
1g	5-F	2,3-F_2
1h	5-F	2,4-F_2
1i	5-F	2,5-F_2
1j	5-F	2,6-F_2
1k	5-F	3,4-F_2
1l	5-F	3,5-F_2
1m	5-F	—
1n	4-F	—
2a	4-Cl	—
2b	4-Cl	2,4-F_2
2c	4-Cl	2,5-F_2
2d	4-Cl	3,4-F_2
2e	4-Cl	3,5-F_2



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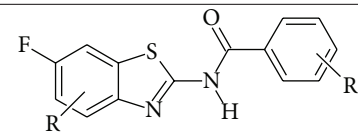

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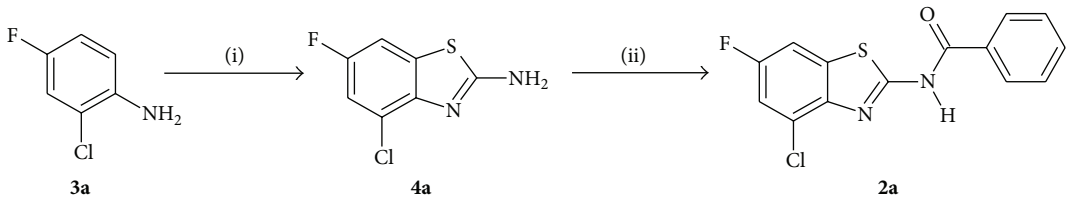

Scheme 2: Synthesis of compounds **2a**. Reagents and conditions: (i) Br_2, KSCN, AcOH, 30–35°C; (ii) benzoyl chloride, Et_3N, dioxane, 50–60°C.

Table 2: Antiproliferative activity (% inhibition, MTT assay) of the target compounds **1a–n** and **2a–e** over HepG2 and MCF-7 cells.

	HepG2[a] Inhibition % (10 μM)	MCF-7[a] Inhibition % (10 μM)
1a	34 ± 3	20 ± 1
1b	70 ± 1	24 ± 2
1c	52 ± 3	26 ± 15
1d	0 ± 10	0 ± 2
1e	45 ± 2	39 ± 1
1f	80 ± 2	56 ± 2
1g	36 ± 1	40 ± 1
1h	64 ± 1	22 ± 2
1i	76 ± 1	46 ± 1
1j	39 ± 2	31 ± 8
1k	64 ± 2	64 ± 6
1l	46 ± 7	41 ± 8
1m	33 ± 4	10 ± 5
1n	32 ± 5	25 ± 12
2a	5 ± 3	14 ± 5
2b	23 ± 15	28 ± 26
2c	58 ± 5	35 ± 14
2d	58 ± 3	54 ± 24
2e	20 ± 8	40 ± 10

[a]Data are presented as the means ± SD of three independent experiments.

evaluation, the highest antiproliferative activity values were found for the HepG2 one. In particular, three compounds out of nineteen (**1b**,**f**,**i**) showed inhibition values of at least 70% towards this cell line. Since all these compounds belong to series **1**, conceivably the presence of the chlorine atom at position 6 of the benzothiazole nucleus should be crucial for the antiproliferative activity. On the contrary, none of the compounds bearing a fluorine atom at the same position (series **2**) showed considerable antiproliferative activity. All tested compounds showed low antiproliferative activity towards MCF-7 cell line, except for compounds **1f**,**k** and **2d** with an inhibition value of more than 50%. As it is known, the antitumor efficacy of a chemotherapeutic agent could be related to its apoptosis inducing activity. Hence, we examined whether the most active compounds of the series could induce apoptosis; thus, we selected compounds showing an inhibition value higher than 50% towards HepG2

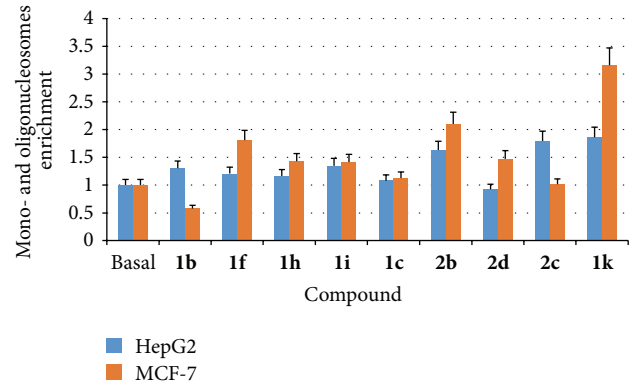

Figure 2: ELISA detection of mono- and oligonucleosomes enrichment after apoptosis.

and/or MCF-7 cell lines (**1b**,**c**,**f**,**h**,**i**,**k** and **2c**,**d**). In addition, compound **2b** was also tested because of its questionable inhibition values (i.e., high SD). The proapoptotic activity of compounds was performed by measuring the specific enrichment of mono- and oligonucleosomes after treatment (Figure 2). Results on HepG2 cell line suggest a proapoptotic effect for compounds **1k** and **2b**,**c** showing about 2-fold mono- and oligonucleosomes enrichment higher than the basal one. A clear-cut apoptotic effect on MCF-7 cell line was shown by compound **1k**, followed by **2b** and **1f**. No substantial proapoptotic effect was observed when the other molecules were tested, conceivably suggesting a mechanism of cell growth inhibition not related to apoptosis. The most promising compounds of the series seem to be **1f**,**i**,**k**, being the first two quite selective for HepG2, while compound **1k** shows interesting activity on both cell lines conceivably acting as proapoptotic inducer.

3. Conclusions

Two series of *N*-1,3-benzothiazol-2-ylbenzamides were evaluated for their antiproliferative activity in vitro. The higher growth inhibition activity in MTT assay was found towards HepG2 cell line, being **1f**,**i**,**k** the most interesting compounds. In particular, **1k** which showed antiproliferative activity on MCF-7 comparable to that of HepG2 cell line may conceivably act via proapoptotic mechanism as demonstrated by means of ELISA detection of mono- and oligonucleosomes enrichment. Further studies will be required to gain better inside into the mechanism of their antiproliferative activity.

4. Experimental

4.1. General Experimental Details. Chemicals were purchased from Sigma-Aldrich or Lancaster. Yields refer to purified products and were not optimized. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Only spectra for compounds not previously described are given. Compounds **4a**,**m**,**n** and **2b**–**e** were prepared as previously described [18, 19]. Melting points were determined on a Gallenkamp apparatus in open glass capillary tubes and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer (Norwalk, CT) Spectrum One FT spectrophotometer and band positions are given in reciprocal centimeters (cm^{-1}). ^{1}H NMR spectra were recorded on a Varian VX Mercury spectrometer operating at 300 MHz using $CDCl_3$ and DMSO-d_6 (where indicated) as solvents. Chemical shifts are reported in parts per million (ppm) relative to the residual nondeuterated solvent resonance: $CDCl_3$, δ 7.26 and DMSO-d_6, δ 2.48. J values are given in Hz. GC-MS was performed on a Hewlett-Packard 6890-5973 MSD at low resolution. Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 60, 0.040–0.063 mm, Merck, Darmstadt, Germany) as previously reported [20–22]. TLC analyses were performed on precoated silica gel on aluminum sheets (Kieselgel 60 F_{254}, Merck).

*4.1.1. N-(6-Chloro-5-fluoro-1,3-benzothiazol-2-yl)benzamide (**1m**).* A mixture of **4m** (1.0 g, 5.0 mmol) and triethylamine (0.50 g, 5.0 mmol) in dry dioxane (50 mL) was stirred for 30 min at 50–60°C. A solution of benzoyl chloride (0.70 g, 5.0 mmol) in dry dioxane (50 mL) was added dropwise. The mixture was stirred for 2 h and then poured into crushed ice. The resulting solid, so separated, was collected by filtration and washed with 1% potassium bicarbonate aqueous solution giving 0.50 g (33%) of **1m** as a yellow solid: mp 246–248°C; GC-MS (70 eV, electron impact) m/z (%) 306 (M^+, 16), and 105 (100); IR (KBr): 3218 (NH), 1675 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.55 (t, J = 7.4 Hz, 2H, Ar), 7.60–7.70 (m, 1H, Ar), 7.81 (d, J = 9.9 Hz, 1H, Ar), 8.08–8.16 (m, 2H, Ar), 8.30 (d, J = 7.4 Hz, 1H, Ar), and 13.07 ppm (br s, 1H, N*H*, exch D_2O); ^{13}C NMR (DMSO-d_6): δ 108.4 (1C), 115.9 (1C), 123.7 (2C), 129.1 (2C), 129.2 (2C), 132.4 (1C), 133.7 (1C), 149.0 (1C), 158.4 (1C), 162.5 (1C), and 166.9 (1C).

*4.1.2. N-(6-Chloro-4-fluoro-1,3-benzothiazol-2-yl)benzamide (**1n**).* Prepared as reported above for **1m** starting from **4n**. Yield: 66%; brown solid: mp 226-227°C; GC-MS (70 eV, electron impact) m/z (%) 306 (M^+, 36), 105 (100); IR (KBr): 3241 (NH), 1679 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.40–7.80 (m, 4H, Ar), 7.62–7.70 (m, 1H, Ar), 7.96–8.10 (m, 1H, Ar), 8.12–8.22 (m, 1H, Ar), and 13.2 ppm (br s, 1H, N*H*); ^{13}C NMR (DMSO-d_6): δ 113.6 (1C), 118.5 (1C), 128.1 (1C), 128.2 (1C), 129.1 (3C), 132.1 (1C), 133.8 (1C), 136.3 (1C), 136.8 (1C), 152.3 (1C), 160.9 (1C), and 166.8 (1C).

*4.1.3. N-(4-Chloro-6-fluoro-1,3-benzothiazol-2-yl)benzamide (**2a**).* Prepared as reported above for **1m** starting from **4a**. Yield: 35%; slightly yellowish solid: mp 208-209°C; GC-MS (70 eV, electron impact) m/z (%) 306 (M^+, 16), 105 (100); IR (KBr): 3252 (NH), 1670 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.42–7.70 (m, 5H, Ar), 7.87–7.95 (m, 1H, Ar), 8.05–8.15 (m, 1H, Ar), and 13.1 ppm (br s, 1H, N*H*); ^{13}C NMR (DMSO-d_6): δ 108.2 (1C), 115.5 (1C), 125.4 (1C), 129.2 (4C), 132.1 (1C), 133.8 (1C), 134.5 (1C), 143.2 (1C), 157.0 (1C), 160.5 (1C), and 166.9 (1C).

4.2. Biology

4.2.1. MTT Assay for Cell Viability. Cell viability was measured using the MTT assay [23, 24]. The cell lines (human breast adenocarcinoma cell lines MCF-7 and human hepatocellular carcinoma cell lines HepG2) were obtained from the ITCC (Genova, Italy). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 10 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine in a 5% CO_2 atmosphere at 37°C. Cells were seeded at a density of $1–5 \cdot 10^4$ cells/well into 96-well flat bottom culture plates containing test compound (100 μM final concentration), in a final volume of 100 μL. Test compounds were dissolved in DMSO (1% final concentration; DMSO carrier had no effect on cell proliferation). Control wells lacked inhibitor. After 48 h of incubation at 37°C in a 5% CO_2 atmosphere, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL stock solution) was added to a final concentration of 0.5 mg/mL. To control for background absorbance, six wells of cells were lysed by adding Triton X-100 (0.1% v/v final concentration) immediately prior to the addition of MTT reagent. After incubation under the same conditions for further 3-4 h, the culture medium was removed, the insoluble product dissolved by the addition of 100 μL of solvent (50% DMSO, 50% EtOH v/v), and the absorbance of the well was measured at 570 nm using a PERKIN–ELMER Victor V^3 plate reader. Cell growth inhibition was then calculated using

$$V\% = \frac{A - A_b}{A_c - A_b} \times 100, \quad (1)$$

where V% is the percentage of cell viability, A is the absorbance of treated cultures, A_b is the absorbance of background control, and A_c is the absorbance of control cultures.

4.2.2. Detection of Mono- and Oligonucleosomes Enrichment. The MCF-7 and HepG2 cells were seeded into 96-well plates in the absence and presence of known concentrations (10 μM) of a panel of molecules (Table 1), added to a final volume of 200 μL/well of standard growth medium, and incubated at 37°C for 48 h. Afterwards, the supernatant was removed carefully and the adherent cells were lysed directly with 200 μL of Lysis buffer, incubated for 30 min at 15–25°C. After incubation time the lysate was centrifuged at 200 ×g for 10 min and 20 μL of culture supernatants after centrifugation and treatment was transferred into the streptavidin coated MP of Cell Death Detection ELISA kit (Roche, Basel, Switzerland) to determine the specific enrichment of mono- and oligonucleosomes after inducing cell death according to the manufacturer's protocol. The optical density was measured at 405 nm and 490 nm wavelengths using Multiskan Ascent (Thermo Fischer, Scoresby, Australia).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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