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Short communication

Cell cycle disruption and apoptotic activity of 3-aminothiazolo[3,2-*a*] benzimidazole-2-carbonitrile and its homologues

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ABSTRACT

3-Aminothiazolo[3,2-*a*]benzimidazole-2-carbonitrile (**2**) was prepared and upon hydrolysis using concentrated sulfuric acid or phosphoric acid resulted in the corresponding 3-aminothiazolo[3,2-*a*] benzimidazole-2-carboxamide derivative (**3**). Cyclization of the **2** using acetic anhydride or formic acid gave the corresponding pyrimido[4′,5′:4,5]thiazolo[3,2-*a*]benzimidazol-4(3H)-one (**5**) in good yields. Acetylation of **2** with acetic anhydride in pyridine afforded *N*-acetylaminothiazolo[3,2-*a*]benzimidazole-2-carbonitrile (**6**). *In vitro* antiproliferative activities of synthesized compounds were investigated at The National Cancer Institute (NCI), USA, according to their applied protocol. Compound **6** revealed significant antiproliferative activity, however, weak activity was shown by the other derivatives. Cell cycle disruption and apoptotic activity of **6** were studied, interestingly, **6** has the ability to arrest G2/M phase and it can induce apoptosis in time dependant manner.

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1. Introduction

Unlimited and uncontrolled cell proliferation is characteristic of tumor cells [1,2]. Disruption of cell cycle has a crucial role in cancer progression [3], as a result of this carcinogenesis can be controlled by agent which have an effect of cell proliferation. Thus various natural and synthetic agents are gaining widespread attention due to their cell cycle regulation and modulation activity [4–17]. In fact, all the suspected contributory factors for oncogenesis and mutagens, such as viruses and inherited predisposing factors – have been shown to impair G1 checkpoint function. Consequently, more than half of all human cancer cells with impaired G1 checkpoint function rely on the G2 checkpoint to survive against the DNA damage which most cytotoxic cancer treatments cause. The G2 cell cycle checkpoint is rarely used by normal cells, which makes a cell cycle G2 checkpoint abrogation strategy attractive against cancer [18,19]. From a medicinal chemistry point of view, synthetic small molecule modulators of the G2/M checkpoint are of particular interest. Several clinically important anticancer compounds, such as vinca alkaloids and taxanes, which act as prominent inhibitors of G2/M transition, can be an effective for controlling some cancer types. Nonetheless, all of these compounds disrupt tubulin directly and they have complex chemical structures that restrict chemical modification. In addition, several prominent disrupters of tubulin, such as nocodazole and colchicine, lack antitumor efficacy. Therefore, novel chemical structures that block G2/M phase transition are valuable as pharmacological probes and as lead structures for future therapeutic agents [20]. The antitumor activity of thiazolo[3,4-*a*]benzimidazoles on human T-lymphoblastic CEM leukemia cells has been reported [21–24]. According to these reports thiazolobenzoimidazole derivatives exert their antitumor effect by activating the programmed cell death pathway (apoptosis). Thiazolo[3,4-*a*]benzimidazoles were also shown to effect the P-glycoprotein mechanism in the HL60R cells, thus suggesting that they are not suitable substrates for the multidrug transporter. In

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addition, they caused more apoptosis in drug resistant cells [25]. The mechanisms of this selective activity in multidrug resistant (MDR) cells are not yet understood; however, this property may be useful in the treatment of MDR malignancies to selectively kill Pgp-expressing neoplastic cells only while preserving Pgp-negative normal cells [26].

This work presents the synthesis of some thiazolo[3,2-*a*]benzimidazole derivatives, *in vitro* apoptotic effect, cell cycle regulation and modulation activities.

2. Results and discussion

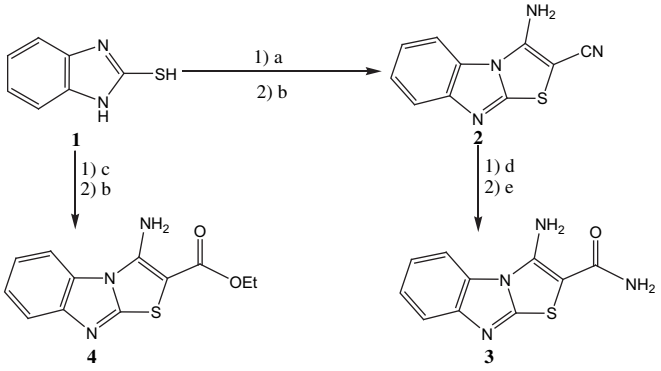
2.1. Chemistry

The synthesis and reactions of the 3-aminothiazolo[3,2-*a*]benzimidazole-2-carbonitrile (**2**) was achieved as previously reported by Sarhan et al. [27,28] from 2-mercaptobenzimidazole (**1**). Applying the same procedures but instead of bromomalononitrile, the ethyl bromocyanoacetate was reacted with 2-mercaptobenzimidazole (**1**) in ethanol in the presence of potassium hydroxide. This step was followed by refluxing the resulting precipitate in absolute ethanol and anhydrous sodium acetate to give the corresponding 3-aminothiazolo[3,2-*a*]benzimidazole-2-ethoxycarbonyl (**4**) as colorless crystals in 41% yield. Upon hydrolysis of **2** using concentrated sulfuric acid at room temperature or heating with phosphoric acid at 70 °C, the 3-aminothiazolo[3,2-*a*]benzimidazole-2-carboxamide (**3**) was obtained as colorless crystals in 50 and 56% yield, respectively [29], Scheme 1.

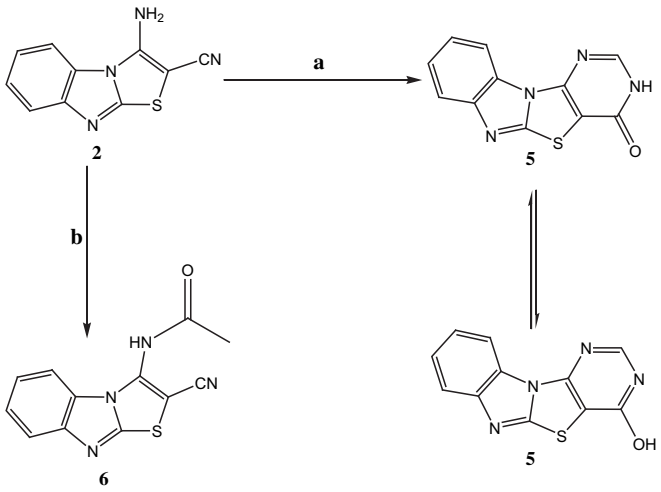
Refluxing of 3-aminothiazolo[3,2-*a*]benzimidazole-2-carbonitrile (**2**) in a mixture of formic acid, formamide and DMF (1:1:1) afforded the corresponding pyrimido[4′,5′:4,5]-benzimidazole-4(3H)-one (**5**) as colorless crystals in 38% yield [30,31]. Compound **5** could be also prepared upon refluxing of compound **2** in formic acid only in 31% yield but along with the desired product, an undesired benzimidazolyl-2-thioacetic acid was obtained in 36% yield. Treatment of **2** with acetic anhydride in pyridine (1:1) at 50–60 °C afforded 3-*N*-acetylaminothiazolo[3,2-*a*]benzimidazole-2-carbonitrile (**6**) as white crystals in 55% yield [32], as shown in Scheme 2. Structures of these synthesized compounds were verified using spectral and elemental analyses and were consistent with the assigned structures. The new compound **4** was identified and characterized in a similar manner.

2.2. In vitro antiproliferative activity

In vitro antiproliferative activity of the synthesized compounds was investigated according to the NCI recommendations. In this protocol, each cell line is inoculated and preincubated on a microtiter plate. Test agents are then added at a single concentration (10^{-4} M) and the culture is incubated for 48 h. Results for each test agent are reported as the percent of growth of the treated cells when compared to the untreated control cells [33,34]. According to our results, among the synthesized compounds, only compound **6** revealed significant antiproliferative activity, however, week activities were shown by other derivatives. The mode of action of the antiproliferative activity of **6**, such as the cell cycle disruption and apoptotic activity of this compound were investigated.



Scheme 1. Reported synthesis of compounds **2**–**4**. Reagents and conditions (a) BrCH(CN)$_2$/KOH, EtOH; (b) AcONa/EtOH, reflux; (c) BrCH(CN)CO$_2$Et/KOH, EtOH; (d) H_2SO_4, RT and (e) H_3PO_4, 70 °C.



Scheme 2. Synthesis of compounds **5** and **6**. Reagents and conditions (a) $HCONH_2$/HCO_2H/DMF, reflux, 3 h, 100 °C and (b) Ac_2O, pyridine, 50–60 °C, 2 h.

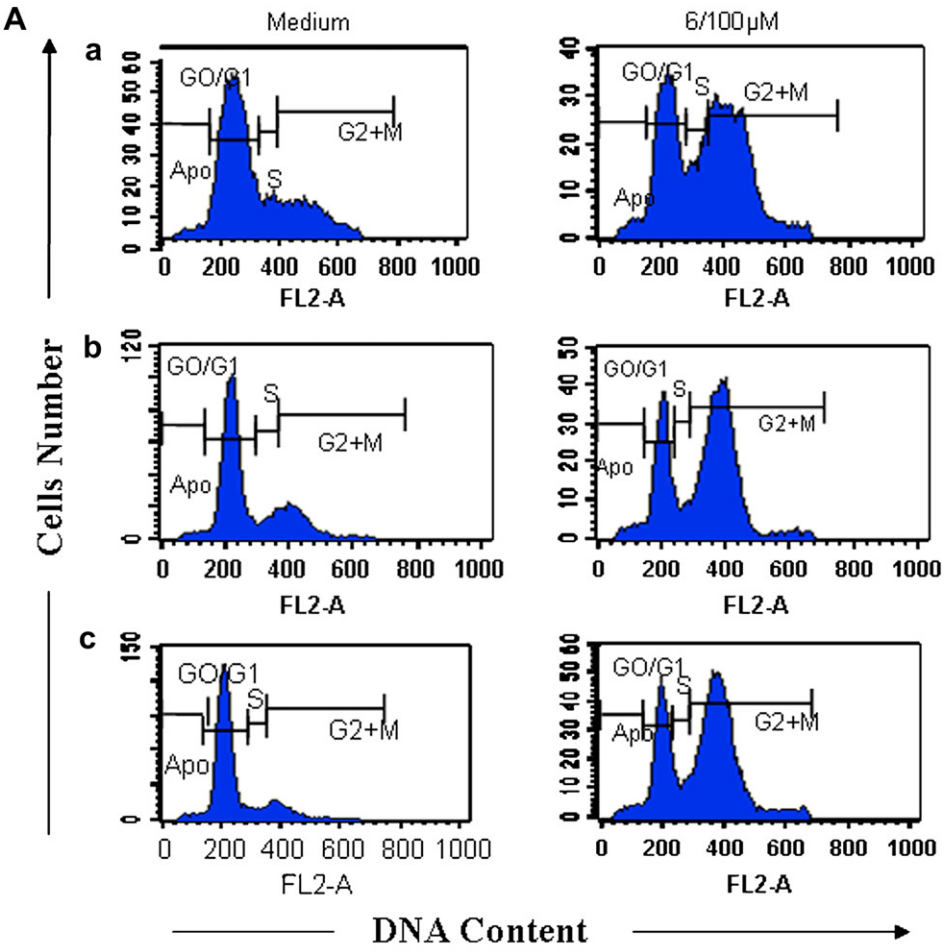
To gain insight into the mechanism of antiproliferation, the effect on cell cycle distribution was investigated by fluorescence-activated cell sorting (FACS) analysis. MDA-MB-231 cells were exposed to 100 μM of compound **6** for 24, 48 and 72 h which resulted in the accumulation of G2/M phase, from 25.71 to 42, 46.22 and 45.79, respectively. This was also accompanied by compensatory decrease in G_1 phase cells (Fig. 1A and B). Compound **6** caused a time dependant G2/M phase arrest, since the majority of accumulation effect was detected in the first 24 h exposure time. Compound **6** only had minor effect on S phase. These results suggested that compound **6** inhibited the cellular proliferation *via* G2/M phase arrest in a time dependant manner.

2.3. Apoptotic effect of compound *6*

The apoptotic effect of compound **6** was evaluated using annexin V staining and a time dependent relationship was found (Fig. 2A and B). The apoptotic population increased from 9.54% in the control group to 23.77%, 54.59% and 80% increased after 24, 48 and 72 h exposure time using 100 μM of compound **6** for incubation. The cytotoxic effect of compound **6** is not correlated to the increase of necrotic cell population.

3. Conclusions

Synthesis of 3-aminothiazolo[3,2-*a*]benzimidazole-2-carbonitrile and its derivatives starting from 2-mercaptobenzimidazole are reported in this manuscript. *In vitro* antiproliferative activity of these compounds demonstrated that compound **6** possesses notable activity in G2/M phase arrest and induction of apoptosis in a time dependant manner. These results suggest that compound **6** has the potential to be developed into a useful cell cycle specific blocking agent. For this reason, further investigations are in



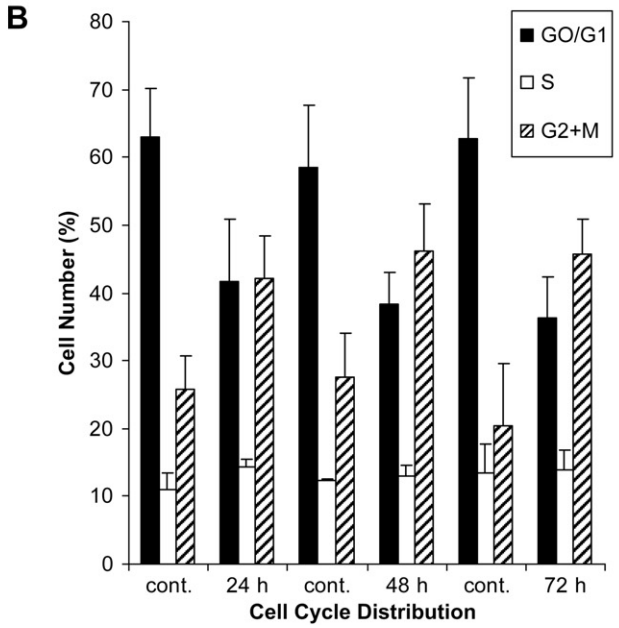


Fig. 1. (A) Time dependant effect of compound **6** on cell cycle distribution. MDA-MB-231 cells were treated with 100 μM of compound **6** and analyzed at, 24 (**a**), 48 (**b**) and 72 h (**c**) by DNA flow cytometry. (B) Histograms show the number of cells per channel (vertical axis) vs DNA content (horizontal axis). The values indicate the percentage of cells in the relevant phases of the cell cycle. The data shown was representative of three independent experiments with similar findings.

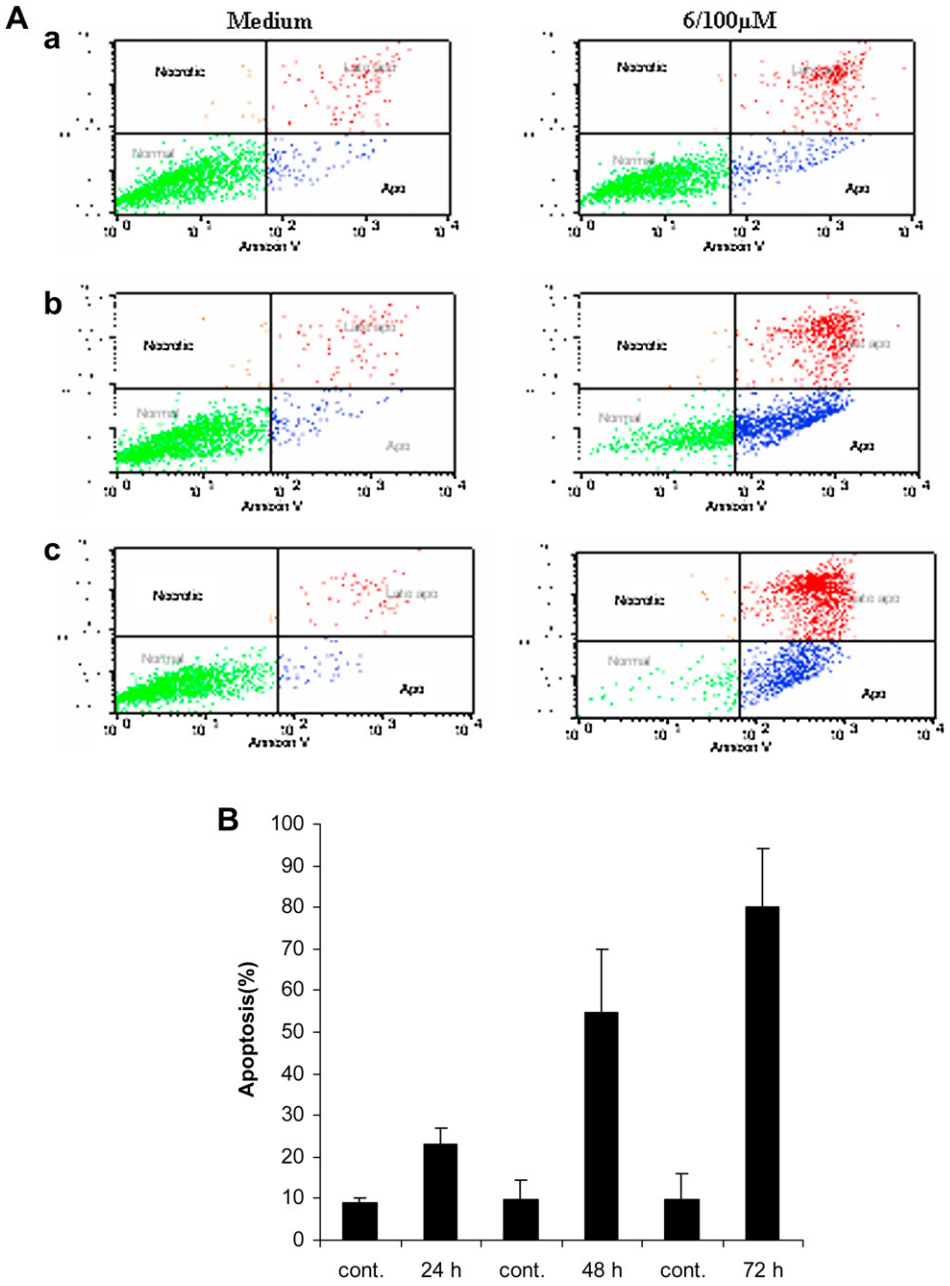


Fig. 2. (A) Time dependant apoptotic response of MDA-MB-231 cells. MDA-MB-231 cells were treated with 100 μM of compound **6** and analyzed at, 24 (**a**), 48 (**b**) and 72 h (**c**) by flow cytometry. Cells were harvested and incubated with annexin V-FITC and PI as described in Section 4.8. 10,000 Cells were analyzed per determination. Dot plots show annexin V-FITC binding on the *X* axis and PI staining on the *Y* axis. Dots represent cells as follows: *lower left quadrant*, normal cells (FITC−/PI−); *lower right quadrant*, apoptotic cells (FITC+/PI−); *upper right quadrant*, late apoptotic cells (FITC+/PI+), *upper left quadrant* (FITC−/PI+) necrotic cells. The results shown here are representative of three experiments. (B) Histograms show time dependant apoptotic response of MDA-MB-231 cells treated by compound **6**.

progress for the cell cycle and apoptotic activity of compound **6** in addition to structural modulation of the 3-aminothiazolo[3,2-*a*] benzimidazole-2-carbonitrile moiety.

4. Experimental section

4.1. General methods

All melting points were determined using a kofler hot – stage. IR Spectra were measured on a Perkin-Elmer FT spectrometer 1710 by KBr techniques. ^{1}H NMR Spectra were recorded at room temperature on a Varian EM-390, 90 MHz Spectrometer or on a Jeol LA 400 MHz FT-NMR spectrometer (Central Lab. Spectral Unit, Assiut University, Egypt). Chemical shifts are given in δ ppm values, relative to the tetramethylsilane (TMS) as internal standard, *J* values are given in Hz. $CDCl_3$ was used as a deuterated solvent unless otherwise stated. MS Spectra were obtained using a JEOL JMS-600 mass spectrometer at ionization energy of 70 eV. Elemental analyses were carried out at the Micro analytical laboratory of the Chemistry Department (Assiut University, Egypt).

Cell culture MDA-MB-231 cells were cultured in RPMI 1640 medium supplemented with 100 ml/l fetal bovine serum with addition of 100 U/ml penicillin, 100 U/ml streptomycin. Cells in suspension (500 μL) were added to each 60 mm culture dishes and incubate for 24 h at 37 °C in a humidified atmosphere of 50 ml/l CO_2 in air. Cells were treated with the most active five members of

the NCI antitumor screening program. Compound **6** is the only active member which induced apoptosis by disruption of cell cycle.

4.2. Synthesis of 3-aminothiazolo[3,2-a]benzimidazole-2-carbnitrile (**2**)

A mixture of 2-mercaptobenzimidazole (**1**, 1.5 g, 1.0 mmol) and KOH (1.1 mmol) was stirred in ethanol (25 ml) at room temperature until complete solubility occurred. The solution is then filtered off and stirred in an ice bath for further 10 min. A solution of freshly prepared bromomalononitrile (1.1 mM) in ethanol was added drop wise in an ice bath and stirring was continued for further 15 min. The formed precipitate was filtered, washed with water (3×) followed by dilute ethanol wash and finally dried. The precipitate was then refluxed in absolute ethanol in the presence of fused sodium acetate for 3 h. The resulting precipitate was collected by filtration, washed with water (3×) followed by crystallization from pyridine or dilute acetic acid to give the corresponding 3-aminothiazolo[3,2-*a*]benzimidazole-2-carbnitrile (**2**) as colorless crystals in 50% yield. Analytical data of compound **2** was in satisfactory agreement with the previously published literature [31,32].

4.3. Synthesis of ethyl 3-aminothiazolo[3,2-a]benzimidazole-2-carboxylate (**4**)

4 Was prepared in a similar manner as the synthesis of compound **2**. Instead of bromomalononitrile a solution of ethyl bromocyanoacetate in absolute ethanol was used. After the reaction was complete, the resulting precipitate was collected and crystallized from ethanol to give the expected ester **4** as colorless needles crystals in 41% yield, mp. 228–230 °C.

IR (KBr) $\nu = 3380$s, 3292s, 3216s, 3148, 1672s, 1632s, 1608s, 1540s, 1496s, 1440s, 1300s, 752s cm^{-1}.

^1H NMR(400 MHz, $CDCl_3$+ trifluoroacetic acid(TFA)): 8(s, 2H, NH_2), 7.7(m, 1H, aromatic-H), 7.4–7.1 (m, 3H, aromatic-H), 4.3(q, $J = 7.5$, 14.5, 2H, CH_3CH_2), 1.4(t, $J = 12.5$, 3H, CH_3CH_2).

^{13}C NMR (75 MHz, $CDCl_3$ + TFA): 170.28 (CO), 161.69 (C-9a), 152.21 (C-3), 133.99 (C-4a, C-8a), 125.63 (C-6, C-7), 122.66 (C-5, C-8), 117.82, 113.93 (C-2, CN), 62.48 (CH_2), 14.20 (CH_3).

MS (EI 70 eV) *m*/*z* (%): 261 (M^+, 100), 215 (79), 187 (9), 161 (17), 145 (25), 134 (11), 118 (10), 102 (10), 90 (19), 76 (5), 63 (7).

4.4. Synthesis of 3-aminothiazolo[3,2-a]benzimidazole-2-carboxamide (**3**)

Thizolo[3,2-*a*]benzimidazole derivative **2** (2.14 g, 0.1 mol) was dissolved in phosphoric acid 85% (20 ml) and stirred at 60–70 °C for 30 min. The reaction mixture was diluted with ice/cold water and neutralized with ammonium hydroxide. The resulting precipitate was collected by filtration, dried and recrystallized from ethanol to give the corresponding amide derivative **3** as silvery crystals, yield 1.3 g (56%), mp. 249–25 °C.

IR (KBr) $\nu = 3448$s, 3363s, 3196s, 1656s, 1604s, 1543s, 1483s, 1449s, 1206s, 750s cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): 8.2–8.1 (m, 1H, aromatic-H), 7.5 (m, 3H, 1H, aromatic-H and 2H, $CONH_2$), 7.4–7.1 (m, 4H, 2H, aromatic-H and 2H, NH_2).

^{13}C NMR (75 MHz, $CDCl_3$ + TFA): 167.35 (CO), 150.43 (C-9a), 145.30 (C-3), 135.61 (C-4a, C-8a), 126.71, 126.73 (C-5, C-8), 129.54 (C-6, C-7), 115.43, 112.72 (C-2, CN).

EI-MS *m*/*z* (%) = 232 (M^+, 100), 215 (52), 187 (2), 161 (13), 145 (27), 134 (7), 118 (11), 102 (5), 90 (11), 76 (6), 63 (5).

Anal. calcd. for $C_{10}H_8N_4OS$: C 51.7, H 3.5, N 24.1, S 13.8, found: C 51.5, H 3.4, N 24.0, S 13.7.

4.5. Synthesis of pyrimido[4′,5′:4,5]thiazolo[3,2-a]benzimidazol-4(3H)-one (**5**)

Thizolo[3,2-*a*]benzimidazole derivative **2** (2.14 g, 0.1 mol) was dissolved and refluxed in formic acid, formamide and DMF (30 ml) 10 ml of each at 100 °C for 12 h. The hot reaction mixture was then cooled, diluted with water and the formed precipitate was collected by filtration. The product was further purified by crystallization from acetic acid to give the corresponding pyrimidone as white crystals in 38% yield. Analytical data of compounds **5** was in satisfactory agreement with the previously published data in the literature [30].

4.6. Synthesis of N-acetyl aminothiazolo[3,2-a]benzimidazole-2-carbonitrile (**6**)

Compound **2** (1.0 g, 4.6 mM) was stirred in a mixture of Ac_2O/pyridine 1:1 (20 ml) at 50–60 °C for 2 h. The reaction was worked up and crystallized from ethanol following the method that described in the literature to give **6** as white crystals in 55% yield, mp. 247–249 °C [29].

IR (KBr), $\nu = 3220$ m, 2220s, 1686s, 1617s, 1594s, 1489s, 1451s, 1217s, 754s, 741s cm^{-1}. ^1H NMR (90 MHz, TFA) $\delta = 7.8$–8.8 (m, 4H, aromatic-H), 3.0 (s, 3H, CH_3).

EI-MS *m*/*z* (%) = 256 (M^+, 18), 240 (1), 214 (100), 187 (11), 165 (1), 160 (2), 149 (3), 134 (4), 118 (5), 95 (2), 90 (60), 77 (3), 70 (5), 63 (3), 55 (4), 45 (2).

Anal. calcd. for $C_{12}H_8N_4OS$: C 56.2, H 3.1, N 21.9, S 12.5, found: C 56.4, H 3.2, N 21.8, S 12.3.

4.7. Flow cytometric analysis of cellular DNA content

2×10^6 Cells were fixed in 1 ml ethanol (70%) for 60 min at room temperature. Harvested cells were resuspended in 1 ml Na citrate (50 mM) containing 250 μg RNase A and incubated at 50 °C for 60 min. Next, cells were resuspended in the same buffer containing 4 μg propidium iodine (PI) and incubated for 30 min before being analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). The percentage of cells in various cell cycle phases was determined by using Cell Quest Pro software (Becton Dickinson).

4.8. Measurement of annexin V binding by flow cytometry

It has been shown that loss of phospholipids asymmetry of the plasma membrane is an early event of apoptosis [35,36]. Annexin V binds to negatively charged phospholipids, like phosphatidylserine. During apoptosis, the cells react with annexin V once chromatin condenses but before the plasma membrane loses its ability to exclude propidium iodide (PI). Hence, by staining cells with a combination of fluorescein isothiocyanate (FITC) annexin V and PI it is possible to detect non-apoptotic live cells, early apoptotic cells and late apoptotic or necrotic cells [37,38]. Annexin-V staining was performed using the Vybrant Apoptosis Assay Kit# 2 (Molecular Probe) following the manufacturer's recommendations. Annexin-V stained cells were analyzed by flow cytometry, measuring the fluorescence emission at 530 and less than 575 nm.

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