Full Paper

Immunomodulatory and Anticancer Activities of Some Novel 2-Substituted-6-bromo-3-methylthiazolo[3,2-*a*]benzimidazole Derivatives

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Ethyl 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **2** was prepared by the ambient temperature bromination of ethyl 3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **1**. The acid hydrazide **4** was obtained by the reaction of ester **2** with hydrazine hydrate. Treatment of compound **4** with benzaldehyde or 2-thiophenaldehyde yielded the corresponding hydrazones **6a** and **6b**, respectively, while the reaction of acid hydrazide **4** with ethoxymethylene malononitrile (**7a**) or with ethyl ethoxymethylene cyanoacetate (**7b**) in refluxing ethanol afforded pyrazole derivatives **9a** and **9b**, respectively. Taken together, from the biological investigations compounds **9a** and **9b** were the most significant inhibitors of LPS-stimulated NO generation from Raw murine macrophage 264.7, and, as another result, compounds **2** and **4** had a weak radical scavenging activity against DPPH radicals. Moreover, **2**, **4**, and **9a** had a concomitant strong cytotoxicity against both colon carcinoma cells (HCT-116) and hepatocellular carcinoma cells (Hep-G2) while **9b** showed specific cytotoxicity only against colon carcinoma cells.

Keywords: Anticancer / Immunomodulatory / Pyrazole / Thiazolo[3,2-*a*]benzimidazole

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Introduction

Thiazolo[3,2-*a*]benzimidazole derivatives have a wide range of biological and pharmacological activities with therapeutic potential [1, 2]. These compounds are used for treatment of cancer [3] and bone diseases [4] and treatment of metabotropic glutamate receptor type 1 (mGlu1)-related diseases (epilepsy, inhibition of nerve cell death, Parkinson's disease, migraine headache, cerebral infarction, neurogenic pain, and anxiety disorder) [5–7]. Among those derivatives, 6-amino-*N*-cyclohexyl-*N*,3-dimethyl-thiazolo[3,2-*a*]-benzimidazole-2-carboxamide (YM-298198) has been reported as a potent, selective, and non-competitive mGluR1 antagonist [1, 2] and 3-(4-chlorophenyl)thiazolo[3,2-*a*]benzimidazole-2-acetic acid or tilomisole (WY-18,251) has been used in the treatment of cancer [8] and rheumatoid arthritis [9] beside its benefits as both anti-inflammatory [10] and immunomodulatory agent [11]. In addition, several pyrazole derivatives are of significant pharmaceutical importance as anticancer agents [12, 13].

Enlightened by these findings and in continuation of our interest in the chemical and biological properties of thiazolo[3,2-*a*]benzimidazole derivatives [14, 15] as well as in the course of our previous studies on the synthesis of biologically active heterocycles [16–19], we present in this work a novel attempt to synthesize a sequence of compounds starting from the key precursor ethyl 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **2** and to investigate their anti-inflammatory, immunomodulatory, and anticancer activities.

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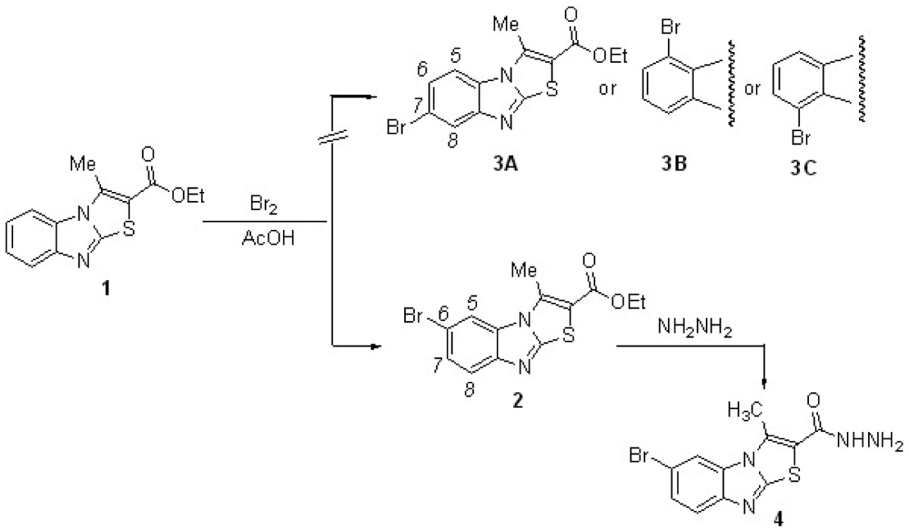


Scheme 1. Synthesis of compounds **2** and **4**

Results and discussion

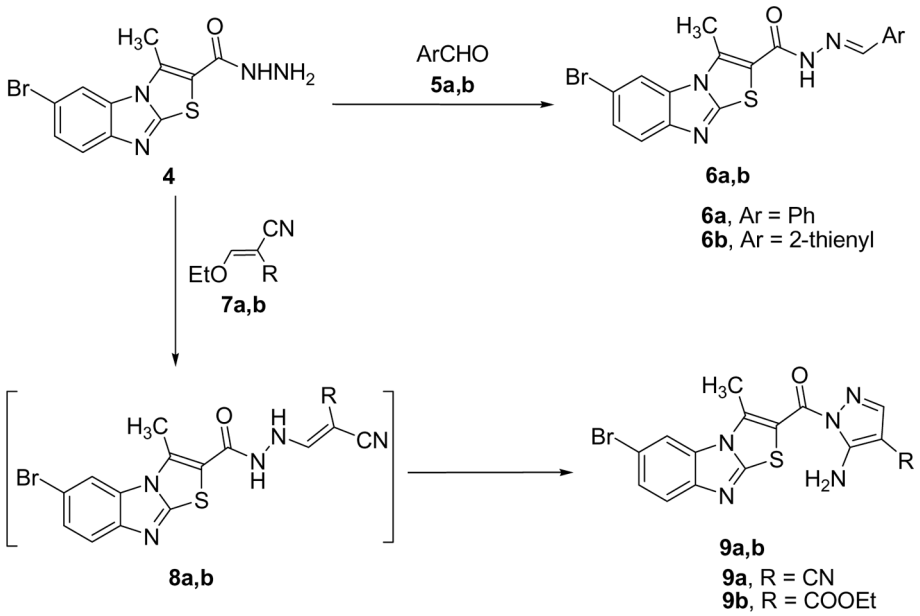
Ethyl 3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **1** was prepared according to literature methods [20]. Ethyl 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **2**, which has not been reported hitherto, was prepared by bromination of compound **1**. Thus, the ester **1** reacts with bromine in acetic acid at room temperature to give one isolable product, which was analyzed correctly for $C_{13}H_{11}BrN_2O_2S$ (Scheme 1), however structures **3A-3C** seemed also possible (Scheme 1).

The ^{1}H-MR spectrum of the isolated product revealed the characteristic triplet and quartet signals due to the ethoxy function of the ester group at δ = 1.33 and δ = 4.34. It also showed the singlet signal of the methyl function in position 3 at δ = 3.07 in addition to the pattern of a 1,2,4-substituted benzene in the aromatic region which is represented as one duplet of duplet signal and two duplets at δ = 7.54 (dd, *J* = 8.7, 1.8 Hz, 1H, ArH), 7.64 (d, *J* = 8.4 Hz, 1H, ArH) and at δ = 8.18 (d, *J* = 1.5 Hz, 1H, ArH). These findings exclude the possibilities of bromination at C_5 or C_8 (1,2,3-substituted benzene pattern is represent as three duplets of duplet signals), which correspond to structures **3B** and **3C**, respectively, but this is not enough to exclude structure **3A** where possibly bromination may take place at C_7. Recently, we synthesized and confirmed the structure of 1-(6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazol-2-yl)ethanone [21] on the basis of its single crystal X-ray diffraction as structure analogue for compound **2**. However, in the ^{1}H-NMR spectrum of the reaction product the signals due to 1,2,4-substituted benzene system appeared in the aromatic region, which are completely similar to those of 1-(6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazol-2-yl)ethanone [21] this provided a firm support to the assigned structure **2** and easily ruled out the other structure **3A**.

Next, the reaction of 6-bromo-3-methylthiazolo[3,2-*a*]benzimidazole-2-carboxylic acid ethyl ester **2** with hydrazine hydrate in refluxing ethanol gave the hydrazide **4**. Its IR spectrum revealed the appearance of three absorption bands at 3305, 3220, and 3150 cm^{-1} due to NH_2 and NH functions in addition to the carbonyl absorption band at 1631 cm^{-1}. Its mass spectrum showed a peak corresponding to its molecular ion at *m/z* = 325 [M$^+$] (Scheme 1).

Furthermore, the treatment of compound **4** with benzaldehyde **5a** or 2-thiophenaldehyde **5b**, in refluxing ethanol yielded the corresponding hydrazones **6a** and **6b**, respectively (Scheme 2). The structures of these hydrazones were established on the basis of their spectral data. For example, the IR spectrum of **6a** revealed a band of NH absorption at 3149 cm^{-1} in addition to a band of carbonyl function at 1646 cm^{-1}, whereas its ^{1}H-NMR spectrum revealed the appearance of a signal due to the -CH=N- proton at δ = 8.11 and a D_2O-exchangeable signal due to NH function at δ = 11.87.

On the other hand, the reaction of hydrazide **4** with ethoxymethylene malononitrile **7a** or with ethyl ethoxymethylene cyanoacetate **7b** in ethanol afforded pyrazole derivatives **9a** and **9b**, respectively (Scheme 2). The structures of reaction products were established on the basis of their spectral data. For example, the IR spectrum of **9a** revealed a bands of the amino group at 3290 and 3160 cm^{-1} in addition to absorption bands at 2228 and



Scheme 2. Synthesis of compounds **6a**, **b** and **9a**, **b**.

1678 cm^{-1} corresponding to nitrile and carbonyl functions, respectively, while its ^{1}H-NMR showed a singlet signal of pyrazole proton at δ = 8.04 in addition to D_2O-exchangeable broad singlet at δ = 8.17 of the NH_2 group. Its mass spectrum showed a peak corresponding to its molecular ion at *m/z* = 401 (cf. Experimental, Section 3).

Macrophages are the first line of defense in innate immunity against microbial infection. Phagocytes engulf and kill microorganisms and present antigens for triggering adaptive immune responses [22]. During phagocytosis, macrophages secrete preformed granule constituents and newly synthesized products that play a critical role in tissue repair [22]. Accordingly, the induction of macrophage proliferation is crucial in the assessment of the innate immunity.

To investigate the possible effect of the tested compounds on the growth of Raw 264.7 cells, macrophages were incubated with different concentrations of the compounds for 24 h. The treatment with compounds depicted in Schemes 1 and 2 (**2**, **4**, **6a**, **6b**, **9a**, and **9b**) revealed a variable effect on the macrophage proliferation, as shown in Fig. 1a. Here, **6a** and **9a** are shown as insignificant growth stimulants (P>0.05), **2**, **4**, **6b**, and **9b** were significant dose-dependant growth stimulators (P < 0.05 at doses 50 and 100 μg/mL), except for **2**, with the highest growth induction at 50 μg/mL, which then was decreased with the increase of the dose. The treatment with M-CSF, which was used as a reference inducer of macrophage proliferation, induced the macrophage proliferation to 1.78 ± 0.14-fold of the control.

These overall macrophage proliferative activity of most of the tested compounds needs to be investigated whether it is specific for macrophages only or towards other immune cells as well. Thus, we tested the effect of these compounds on the proliferation of T-lymphocytes (1301 cell line). Our findings indicated that **2**, **4**, and **6a** were strong dose-dependant inducer of T-lymphocytes growth at doses of 25–100 μg/mL, and they seem to have low slope of the growth curve with the increase of doses (Fig. 1b). Compound **9a** had no effect on T-lymphocytes growth (Fig. 1b). On the other hand, **6a**, and **9a** were cytotoxic against T-lymphocytes and their calculated IC_{50} values were 94.2 and 88.2 μg/mL, respectively.

Our results suggested that the induced macrophages and lymphocytes proliferation is due to the skeleton of 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole itself and that the substitution with phenylmethylene in the hydrazone derivative **6a** and with carbonitrile in the pyrazole derivative **9a** led to the depression in the proliferation of macrophage and the cytotoxicity against lymphocytes. On the other hand, the substitution with thienylmethylene in the hydrazone derivative **6b** resulted in a dramatic enhancement in the proliferation of both types of immune cells.

In inflammation many mediators are involved including cytokine secretions, and inflammation mediators like nitric oxide (NO) [23]. NO is a highly reactive free radical and it can form a number of oxidation products such as NO_2, NO_2^-, N_2O_3, and S-nitrosothiols [22]. In inflammatory diseases, NO is produced in large quantities by the

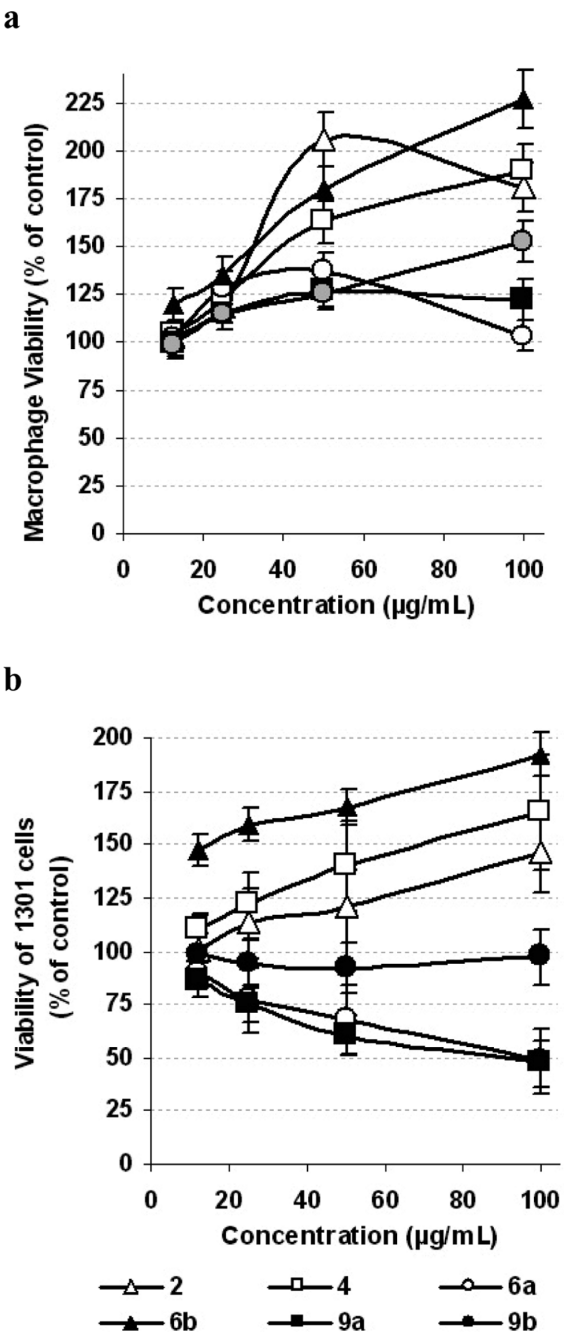


Figure 1. The effect of the treatment with different doses of the synthesized compounds in Scheme 1 and Scheme 2 on the proliferation of Raw macrophages 264.7 (a) and 1301 T-lymphocytes (b), as measured after 24 h of treatment by MTT assay.
The results are represented as the percentage of control cells (Mean ± S.D.; n = 4).

action of inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) [24], leading to inflammation and persistent pain. Subsequently, finding of new anti-inflammatory agents represents a real need to help the patients of inflammatory diseases.

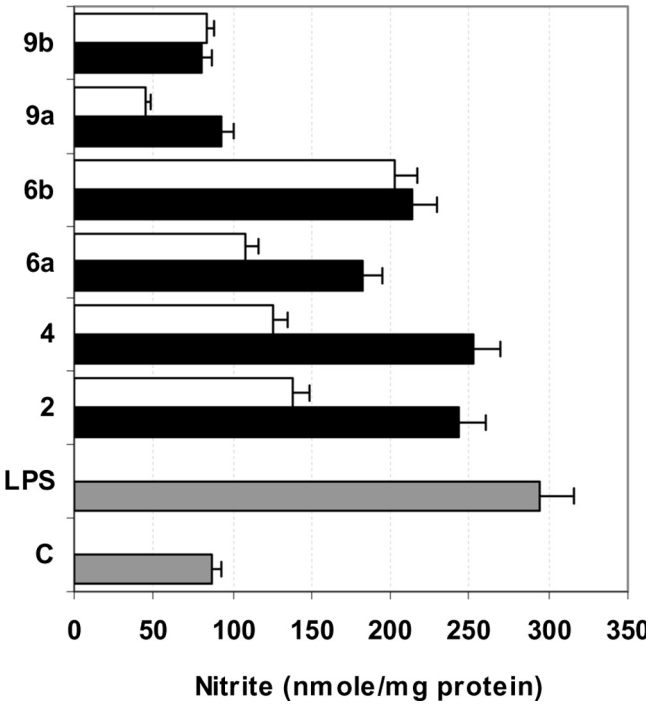


Figure 2. Anti-inflammatory activity of two doses (12.5 µg/mL as black bars, and 25 µg/mL as white bars) of the synthesized compounds on the LPS-stimulated NO generation from macrophages, in comparison with LPS-treated and untreated macrophages (grey bars).
The nitrite concentration, as an index of NO generation, was measured by Griess assay (Mean ± S.D.; n = 4).

As a mimic *in-vitro* model of inflammation, macrophages were stimulated by incubation with the bacterial LPS, which activates the macrophage functions and the release of inflammatory mediators including enhancement of iNOS, the generation of NO, and the secretion of the pro-inflammatory cytokines. Since most of the cellularly generated NO is converted immediately into nitrites, Raw 264.7 were stimulated with LPS and the nitrite level was measured in cell culture supernatants before and after treatment with different compounds (12.5 and 25 µg/mL) to investigate their possible anti-inflammatory activity as indicated from the inhibition of the LPS-stimulated NO generation.

The results revealed that **9a** and **9b** were the most significant inhibitors of LPS-stimulated NO generation in both tested doses with inhibition percentage ranged from 56.0 to 88.2% ($P < 0.05$) (Fig. 2), which suggest a potential anti-inflammatory activities of those compounds. Additionally, compounds **2**, **4**, and **6a** were also strong inhibitors of the generated NO, at the high dose (25 µg/mL), which may be due to the skeleton of 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole itself. Our findings suggested that the pyrazole substitution in the

derivatives (**9a** and **9b**) of 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole led to moderate anti-inflammatory activity as indicated from the NO inhibition, and that substitution with carbonitrile in the pyrazole derivative **9a** was more effective in the NO inhibition. The treatment with L-NMMA (N^G-monomethyl-L-arginine), as a reference NO inhibitor, resulted in a significant inhibition of the NO production with IC_{50} value of 7.69 μg/mL.

The inhibition of the generated NO by the tested compounds may be due to a direct scavenging capacity of NO, an inhibition of iNOS pathway, and / or a modulation of other factors in the NO cascade such as transcriptional factors. To investigate the radical scavenging activity of those compounds, we submitted them to a DPPH assay, which revealed that all of the compounds had no radical scavenging activity at the tested concentrations >100 μg/mL, except for compounds **2** and **4** with calculated SC_{50} values of 78.2 and 88.0 μg/mL, respectively, which are relatively weak antioxidant activities compared with the reference antioxidant; ascorbic acid (SC_{50} = 8.6 μg/ml). These findings suggested that the NO inhibition was not due to direct scavenging of NO, but rather due to other molecular pathways.

Exploring the cytotoxic effect of the tested compounds on cancer cell lines, Hep-G2 and HCT-116 cells were treated with and without doses of the compounds, and submitted to the MTT assay, a metabolic cytotoxicity assay. The experiment showed that compounds **6a** and **6b** had no effect on the growth of both types of cells, while compounds **2**, **4**, and **9a** had a concomitant cytotoxic effect on both types of cells as indicated by their IC_{50} values as noted in Fig. 3, in addition to compound **9b** a weak cytotoxic compound against HCT-116 cells. As shown in Fig. 3, the most remarkable cytotoxic compounds against Hep-G2 cells were **9a**>**4**, and against HCT-116 were **9a** > **2**. This suggests that **9a** is a non-specific anticancer compound but with lower cytotoxicity than the reference anticancer agent (paclitaxel), which was found to have an IC_{50} value of 710 nM against Hep-G2 cells and 825 nM against HCT-116 cells, respectively.

These findings suggested that substitution with carbonitrile in the pyrazole derivative **9a** of 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole led to strong cytotoxicity against both types of solid tumor cells Hep-G2 and HCT-116 as indicated from the low IC_{50} values 18.4 and 12.5 μg/mL, respectively, and that the hydrazide derivative **4** was more cytotoxic (IC_{50} = 21.2 μg/mL) against Hep-G2 cells than the original ester **2** (IC_{50} = 53.2 μg/mL).

In conclusion, we have investigated the reactivity and synthetic potency of ethyl 6-bromo-3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **2** as reactive precursors in the synthesis of a sequence of derivatives as promising potent biologically active agents. Taken together from the biological investigations, **9a** and **9b** were the most significant inhibitors of LPS-stimulated NO generation, and **2** and **4** had a weak radical scavenging activity against DPPH (1,1-diphenyl-2-picryl-hydrazyl) radicals. Moreover, **2**, **4**, and **9a** had a concomitant strong cytotoxicity against colon carcinoma and hepatocellular carcinoma cells and **9b** had a specific cytotoxicity against colon carcinoma cells only.

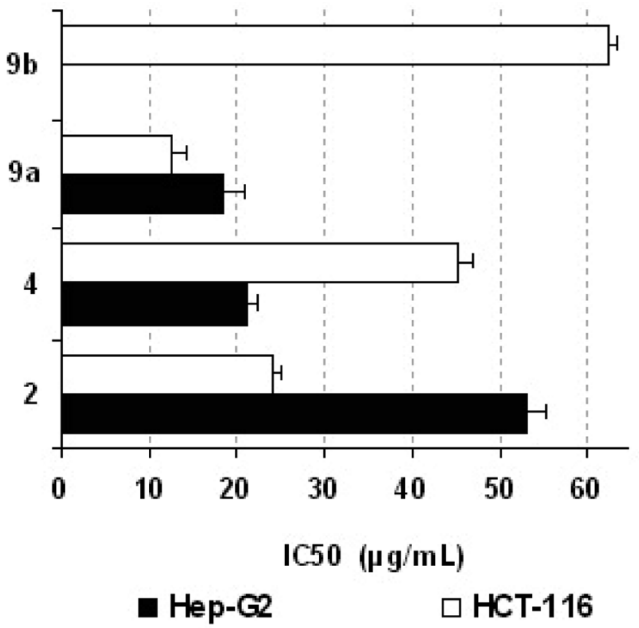


Figure 3. The IC_{50} values of the cytotoxic compounds against Hep G2 cells (black bars) and HCT-116 cell (white bars), as measured after 24 h of treatment by MTT assay.
The results are represented as mean ± S.D.; n = 4.

The authors have declared no conflict of interest.

Experimental

Melting points were measured with a Gallenkamp apparatus (Weiss-Gallenkamp, London, UK) and are uncorrected. IR spectra were recorded on Shimadzu FT-IR 8101 PC infrared spectrophotometer (Shimadzu, Tokyo, Japan). The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer (Varian Inc., Palo Alto, CA, USA). ^{1}H-spectra were run at 300 MHz in deuterated dimethylsulphoxide (DMSO-d_6). Chemical shifts are quoted in δ [ppm] and were related to that of the solvents. Mass spectra were measured on a GCMS-QP1000 EX spectrometer (Shimadzu) at 70 eV. Elemental analyses were carried out at the Microanalytical center of Cairo University. Follow up of the reactions and checking the compound purities were made by TLC on silica gel-precoated aluminum sheets (Type 60 F254; Merck, Darmstadt, Germany). Ethyl 3-methyl-1,3-thiazolo[3,2-*a*]benzimidazole-2-carboxylate **1** [20] was prepared by the reported method.

Chemistry

*Ethyl 6-bromo-3-methyl-1,3-thiazolo[3,2-a]benzimidazole-2-carboxylate **2***

A solution of ester **1** (2.6 g, 10 mmol) in glacial acetic acid (50 mL) was stirred at room temperature while a bromine solution (1.6 g, 10 mmol) in glacial acetic acid (10 mL) was added drop-wise over a period of 30 min. The mixture was stirred at room temperature for further 30 min. The partially oily reaction mixture was poured into crushed ice. The solid was filtered off, washed with water and dried. Recrystallization from EtOH afforded compound **2** as white needles.

Yield: 68%; m.p.: 203-205°C; IR (KBr) ν_{max}/cm^{-1}: 1707 (C=O), 1597 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 1.33 (t, J = 7.2 Hz, 3H, CH_3), 3.07 (s, 3H, CH_3), 4.34 (q, J = 7.2 Hz, 2H, CH_2), 7.54 (dd, J = 8.7, 1.8 Hz, 1H, ArH of C_7), 7.64 (d, J = 8.4 Hz, 1H, ArH of C_8), 8.18 (d, J = 1.5 Hz, 1H, ArH of C_5); MS m/z (%): 342 [M$^+$ + 3] (63.4), 341 [M$^+$ + 2] (14.98), 340 [M$^+$ + 1] (82.1), 339 [M$^+$] (16.77), 338 (100), 312 (40.04), 310 (42.35). Anal. calcd. for $C_{13}H_{11}BrN_2O_2S$ (339.21): C, 46.03; H, 3.27; N, 8.26; S, 9.45. Found: C, 45.88; H, 3.32; N, 8.44; S, 9.37.

*6-Bromo-3-methyl-1,3-thiazolo[3,2-a]benzimidazole-2-carboxylic acid hydrazide **4***

A mixture of compound **2** (3.39 g, 10 mmol) and hydrazine hydrate (0.6 mL, 99%) in 100 mL of absolute ethanol was refluxed for 5 h. The separated white solid was filtered off and recrystallized from EtOH / DMF to give the title compound **4**.

Yield: 74%; m.p.: 304-306°C; IR (KBr) ν_{max}/cm^{-1}: 3305, 3220, 3150 (NH, NH_2), 1631 (C=O), 1583 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 2.98 (s, 3H, CH_3), 4.61 (br. s, 1H, NH_2, D_2O exchangeable), 7.52 (dd, J = 8.7, 1.8 Hz, 1H, ArH of C_7), 7.67 (d, J = 8.7 Hz, 1H, ArH of C_8), 8.19 (d, J = 1.5 Hz, 1H, ArH of C_5), 9.71 (br. s, 1H, NH, D_2O exchangeable); MS m/z (%): 328 [M$^+$ + 3] (2.94), 327 [M$^+$ + 2] (5.84), 326 [M$^+$ + 1] (100), 325 [M$^+$] (97.4), 324 (70.09), 295 (84.49), 292 (83.09), 223 (36.33), 221 (42.75). Anal. calcd. for $C_{11}H_9BrN_4OS$ (325.18): C, 40.63; H, 2.79; N, 17.23; S, 9.86. Found: C, 40.46; H, 2.77; N, 17.04; S, 9.68.

*Reaction of 6-bromo-3-methylthiazolo[3,2-a]benzimidazole-2-carboxylic acid hydrazide (**4**) with benzaldehyde (**5a**) and thiophenaldehyde (**5b**), ethoxymethylene malononitrile (**7a**) and ethyl ethoxymethylene cyanoacetate (**7b**)*

A mixture of the hydrazide **4** (0.33 g, 1 mmol) and benzaldehyde (**5a**) or 2-thiophenaldehyde (**5b**), and ethoxymethylene malononitrile (**7a**) or ethyl ethoxymethylene cyanoacetate (**7b**) (1 mmol) in ethanol (50 mL) was refluxed for 4 h. The formed solid product was collected by filtration, washed with ethanol and dried. Recrystallization from the proper solvent afforded the corresponding hydrazones **6a**–**6b** and pyrazole derivatives **9a**–**9b**, respectively.

*6-Bromo-3-methyl-N′-[1-phenylmethylene]-1,3-thiazolo[3,2-a]benzimidazole-2-carbohydrazide **6a***

White powder; yield: 60%, m.p.: >300°C; IR (KBr) ν_{max}/cm^{-1}: 3149 (NH), 1646 (C=O), 1548 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 3.18 (s, 3H, CH_3), 7.47-7.57 (m, 5H, ArH), 7.67 (dd, J = 8.7, 1.8 Hz, 1H, ArH of C_7), 7.79 (d, J = 7.2 Hz, 1H, ArH of C_8), 8.11 (s, 1H, -N=CH-), 8.25 (d, J = 1.2 Hz, 1H, ArH of C_5), 11.87 (s, 1H, NH, D_2O exchangeable); MS m/z (%): 416 [M$^+$ + 3] (4.9), 415 [M$^+$ + 2] (19.6), 414 [M$^+$ + 1] (100), 413 [M$^+$] (26.5), 412 (50.3), 292 (90.8), 223 (63.6). Anal. calcd. for $C_{18}H_{13}BrN_4OS$ (413.29): C, 52.31; H, 3.17; N, 13.56; S, 7.76. Found: C, 52.00; H, 3.35; N, 13.72; S, 7.55.

*6-Bromo-3-methyl-N′-[1-thien-2-ylmethylene]-1,3-thiazolo[3,2-a]benzimidazole-2-carbohydrazide **6b***

Greenish yellow crystals; yield: 68%, m.p.: >300°C; IR (KBr) ν_{max}/cm^{-1}: 3131 (NH), 1642 (C=O), 1547 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 3.19 (s, 3H, CH_3), 7.15-7.73 (m, 5H, ArH), 8.23 (d, J = 1.5 Hz, 1H, ArH of C_5), 8.27 (s, 1H, -N=CH-), 11.99 (s, 1H, NH, D_2O exchangeable); MS m/z (%): 422 [M$^+$ + 3] (8.4), 421 [M$^+$ + 2] (16.1), 420 [M$^+$ + 1] (58.8), 419 [M$^+$] (10.5), 309 (24.1), 295 (100), 221 (47.0), 70 (15.8). Anal. calcd. for $C_{16}H_{11}BrN_4OS_2$ (419.32): C, 45.83; H, 2.64; N, 13.36; S, 15.29. Found: C, 46.05; H, 2.65; N, 13.59; S, 15.57.

*5-Amino-1-[(6-bromo-3-methyl-1,3-thiazolo[3,2-a]benzimidazol-2-yl)carbonyl]-1H-pyrazole-4-carbonitrile **9a***

White powder; yield: 52%, m.p.: >300°C; IR (KBr) ν_{max}/cm^{-1}: 3290, 3160 (NH_2), 2228 (C=N), 1678 (C=O), 1626 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 3.24 (s, 3H, CH_3), 7.55 (dd, J = 8.4, 1.8 Hz, 1H, ArH of C_7), 7.66 (d, J = 8.7 Hz, 1H, ArH of C_8), 8.04 (s, 1H, pyrazole), 8.17 (br. s, 2H, NH_2, D_2O exchangeable), 8.28 (d, J = 1.5 Hz, 1H, ArH of C_5); MS m/z (%): 403 [M$^+$ + 2] (12.88), 402 [M$^+$ + 1] (23.62), 401 [M$^+$] (35.03), 400 (47.74), 292 (100), 232 (55.61), 220 (65.79). Anal. Calcd. for $C_{15}H_9BrN_6OS$ (401.24): C, 44.90; H, 2.26; N, 20.95; S, 7.99. Found: C, 44.68; H, 2.11; N, 21.17; S, 7.75.

*Ethyl 5-amino-1-[(6-bromo-3-methyl[1,3]thiazolo[3,2-a]benzimidazol-2-yl)carbonyl]-1H-pyrazole-4-carboxylate **9b***

White powder; yield: 50%, m.p.: >300°C; IR (KBr) ν_{max}/cm^{-1}: 3262 (NH_2), 1703, 1671 (2 C=O), 1616 (C=N); ^{1}H-NMR (DMSO-d_6) δ: 1.26 (t, J = 7.2 Hz, 3H, CH_3), 3.09 (s, 3H, CH_3), 4.15 (q, J = 7.2 Hz, 2H, CH_2), 7.58 (dd, J = 8.7, 1.8 Hz, 1H, ArH of C_7), 7.66 (d, J = 8.4 Hz, 1H, ArH of C_8), 8.04 (s, 1H, pyrazole), 8.17 (br. s, 2H, NH_2, D_2O exchangeable), 8.22 (d, J = 1.5 Hz, 1H, ArH of C_5); MS m/z (%): 450 [M$^+$ + 2] (54.29), 449 [M$^+$ + 1] (43.44), 448 [M$^+$] (35.49), 447 (86.37), 295 (100), 292 (54.5), 155 (24.7). Anal. calcd. for $C_{17}H_{14}BrN_5O_3S$ (448.29): C, 45.55; H, 3.15; N, 15.62; S, 7.15. Found: C, 45.81; H, 3.32; N, 15.35; S, 7.37.

Cell culture

Several human cell lines were used to test the anticancer activity including: lymphoblastic leukemia (1301 cells, a generous gift from The Training Center of DakoCytomation, Elly, UK), hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116), and Raw murine macrophage (RAW 264.7) (ATCC, VA, USA). Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), except RAW 264.7 cells, which were grown in RPMI-1640 at 37°C in humidified air containing 5% CO_2. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin sulphate, and 250 ng/mL amphotericin B. Monolayer cells were harvested by trypsin / EDTA treatment, except for RAW 264.7 cells, which were collected by gentle scraping. The tested compounds were dissolved in dimethyl sulphoxide (DMSO, 99.9%, HPLC grade) and diluted 1000-fold in the assays. In all the cellular experiments, results were compared with DMSO-treated cells. Compound dilutions were tested

before assays for endotoxin using Pyrogent1 Ultra gel clot assay, and they were found endotoxin free. All experiments were repeated four times, unless mentioned, and the data were represented as (mean ± S.D.). Except mentioned, cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark), and all chemicals were from Sigma (USA), unless mentioned otherwise.

Proliferation of immune cells

Macrophages and lymphocytes are essential immune cells. Raw macrophage 264.7 and 1301 cells (T-lymphocytes) were relevant cell lines to study the effect of the synthesized compounds on the immune cells proliferation, which was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [25]. The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5×10^4 cells/well) were incubated with various concentrations of the compounds at 370C in a FBS-free medium, before being submitted to the MTT assay. The absorbance was measured with an ELISA reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. Treatment of macrophage with 1000 U/mL recombinant macrophage colony-stimulating factor (M-CSF, Pierce, USA) was used as positive control.

Nitrite assay

Raw macrophage 264.7 is known as sensitive cell line that can be stimulated by bacterial lipopolysaccharides (LPS) to produce different inflammatory mediators and the stimulated cells are commonly used as a mimic *in-vitro* model of inflammation [26, 27]. The accumulation of nitrite, an indicator of nitric oxide (NO) synthesis, was measured by Griess reagent [26]. Raw 264.7 were grown in phenol red-free RPMI-1640 containing 10% FBS. Cells were incubated for 24 h with LPS (1μg/mL) in presence or absence of different compounds (12.5 and 25 μg/mL). 50 μL of cell culture supernatant were mixed with 50 μL of Griess reagent and incubated for 10 min. The absorbance was measured spectrophotometrically at 550 nm. A standard curve was blotted using serial concentrations of sodium nitrite. Different concentrations of a nonisoform-specific NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA) was used as a reference inhibitor of NO production. The nitrite content was normalized to the cellular protein content as measured by bicinchoninic acid assay [28].

Cytotoxicity assay

Hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) are solid tumor cell lines, which are commonly used to study the anticancer activity against epithelial cell carcinomas [29–30]. The cytotoxicity was estimated by MTT assay against both cell lines, since MTT assay can accurately measure metabolic activity of living cells via MTT reaction with mitochondrial dehydrogenases [25]. The relative cell viability was expressed as the mean percentage of viable cells as compared to the respective DMSO-treated cells (control). The half maximal growth inhibitory concentration IC_{50} values were calculated from the line equation of the dose–dependent curve of each compound. The results were compared with the anti-proliferative activity of paclitaxel, a reference anticancer drug.

Antioxidant assay

The antioxidant capacities of the tested compounds were studied through their scavenging activity against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals [26, 31]. The bleaching of DPPH was monitored at an absorbance of 515 nm. The percentage of DPPH bleaching utilized for SC_{50} (half maximal scavenging concentration) was calculated as follows: 0% is the absorbance of DPPH with solvents (ethanol) and 100% is the absorbance of DPPH with an efficient scavenger (10 mM ascorbic acid, AA). The results were compared with ascorbic acid activity, as a reference antioxidant.

Statistical analysis

Data were statistically analyzed using IBM computer supplied with Statistical Package for Social Scientists (SPSS) 10.00 for windows (SPSS Inc., Chicago, USA). The student's unpaired t-test as well as the one-way analysis of variance (ANOVA) test was used to detect the statistical significance. p value more than 0.05 was considered insignificant.

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