European Journal of Medicinal Chemistry 44 (2009) 4547–4556

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

New pyridone, thioxopyridine, pyrazolopyridine and pyridine derivatives that modulate inflammatory mediators in stimulated RAW 264.7 murine macrophage

Nehal A. Hamdy [a,*], Amira M. Gamal-Eldeen [b,*]

[a] Applied Organic Chemistry Department, National Research Centre, Dokki, Cairo, Egypt
[b] Cancer Biology Laboratory, Center of Excellence for Advanced Sciences, National Research Center, Dokki 12622, Cairo, Egypt

ARTICLE INFO

Article history:
Received 21 December 2008
Received in revised form
6 June 2009
Accepted 20 June 2009
Available online 27 June 2009

Keywords:
Pyridine
Pyridone
Thioxopyridine
Pyrazolopyridine
Anti-inflammatory
Cycloxygenase-2
5-Lipoxygenase

ABSTRACT

The reaction of 2-acetyl-5,6,7,8-tetrahydronaphthalene **1** with some aldehydes was conducted in the presence of ethyl cyanoacetate and ammonium acetate, yielded the cyanopyridones **2a**–**c**, which react with phosphorous pentasulphide to afford the corresponding thioxopyridine derivatives **3a**–**c**, respectively. Compounds **2a**,**b** were converted to 2-chloropyridine derivatives **4a**,**b** by heating with phosphorous oxychloride and phosphorous pentachloride, which were fused with hydrazine hydrate and benzyl amine to afford the corresponding pyrazolopyridine **5a**,**b** and cyanopyridine derivatives **6a**,**b** respectively. Compounds **2a**,**b** also afforded 3-cyanopyridinyl oxy acetic acid ethyl ester **7a**,**b** by reaction with ethyl bromoacetate in dry acetone in the presence of anhydrous potassium carbonate, which upon condensation with hydrazine hydrate gave the corresponding acid hydrazide **8a**,**b** and with benzyl amine gave the corresponding acetamide **9a**,**b**. We investigated the effect of those new compounds on the macrophage growth, macrophage binding affinity to fluorescein isothiocyanate-conjugated bacterial lipoopolysaccharide (FITC-LPS), phagocytosis of FITC-zymosan, and radical scavenging affinity against $OH^{\bullet}$, $ROO^{\bullet}$, and $O_2^{-\bullet}$, in addition to their influence of the inflammatory mediators [nitric oxide (NO), tumor necrosis factor-α (TNF-α), prostaglandin E-2 (PGE-2), cycloxygenase-2 (COX-2), and 5-lipoxygenase (5-LO)] in LPS-stimulated macrophages. The findings revealed that the derivatives **2b**, **3b**, **5a**, **7b, 9a** and **9b** can be recognized as promising multi-potent anti-inflammatory agents.

© 2009 Elsevier Masson SAS. All rights reserved.

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 to 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 beneficial 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 anti-inflammatory molecules [4]. Prostaglandin E2 [5], transforming growth factor-h [6], and reactive oxygen and nitrogen intermediates [3] are among those molecules with a dual role in both promoting and suppressing inflammation. 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 [7–9].

The phagocytosis of apoptotic cells also promotes an anti-inflammatory response, such as enhancing the production of anti-inflammatory mediators [10–12]. However, if inflammation resolution is dysregulated, cellular response changes to the pattern of chronic inflammation. 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

Abbreviations: COX-2, cycloxygenase-2; FITC, fluorescein isothiocyanate; 5-LO, 5-lipoxygenase; LPS, bacterial lipopolysaccharide; NO, nitric oxide; $O_2^{-\bullet}$, superoxide radicals; $OH^{\bullet}$, hydroxyl radicals; ORAC, oxygen radical absorbance capacity; PGE-2, prostaglandin E-2; $ROO^{\bullet}$, peroxyl radicals; TNF-α, tumor necrosis factor-α.

* Corresponding author. Tel.: +20106053903; fax: +20233370931.
E-mail addresses: drnehalhamdy63@hotmail.com (N.A. Hamdy), aeldeen7@yahoo.com (A.M. Gamal-Eldeen).

0223-5234/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2009.06.023

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 [14]. 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 anti-inflammatory agents represents a concrete strategy in fighting not only different inflammatory diseases but also cancer.

Synthesis of the pyridine ring system and its derivatives occupy an important place in the realm of synthetic organic chemistry, due to their therapeutic and pharmacological properties [14–16]. They have emerged as integral backbones of over 7000 existing drugs [17,18]. The pyridine ring is also an integral part of anticancer and anti-inflammatory agents [19,20]. Tetralins (tetrahydronaphthalene derivatives) are of increasing interest since many of these compounds play a vital role in the biological activities, because of their biological potentialities; for examples as potent agonists for D_2-type receptors [21], as a treatment of Alzheimer's disease [22], cardiovascular diseases [23], and as a preventer of dopamine-induced cell death [24]. On the other hand, cyanopyridone and cyanopyridine derivatives have promising antimicrobial [25] and anticancer activities [26]. The interest in 3-cyano-2(1*H*)-pyridone and their derivatives is due to their wide range of practical uses as medicinal compounds [27]. Recently, new pyridine carbonitriles were reported as anti-inflammatory agents [28], and pyrazolopyridine derivatives have been recently reported as anti-tumor agents [29]. In this work, we synthesized some new heterocyclic compounds containing pyridone, thioxopyridine, halogenated pyridine carbonitriles, pyrazolopyridine and pyridine derivatives and we investigated their role in the modulation of various inflammatory mediators.

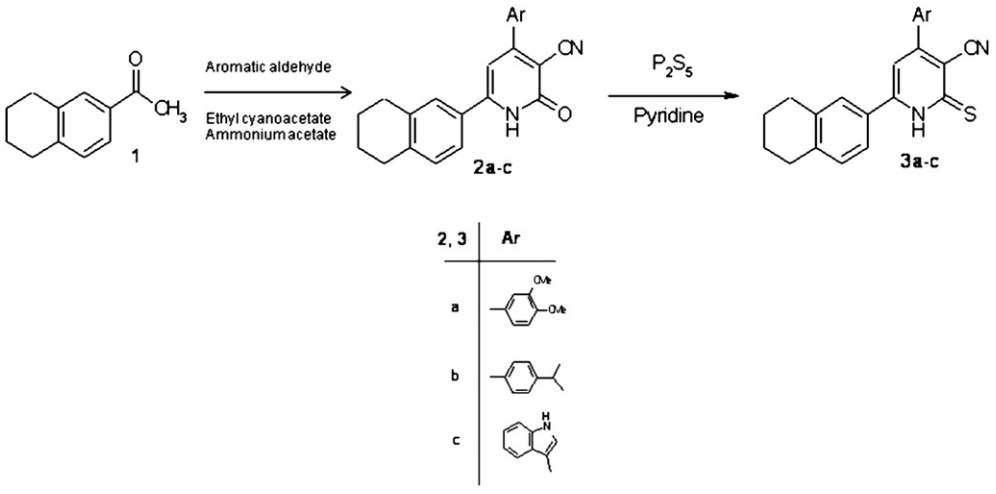
2. Results and discussion

The required 2-acetyl-5,6,7,8-tetrahydronaphthalene **1** was prepared following the literature method [30]. Cyano condensation reaction of compound **1** with the appropriate aromatic aldehydes and ethyl cyanoacetate in the presence of excess ammonium acetate in n-butanol gave the corresponding cyanopyridones **2a–c** in one pot reaction. The structure of the isolated products was confirmed on the basis of their elemental analysis and spectral data. For example their IR spectrum exhibited absorption bands at 3420, 3424, 3306 respectively due to NH groups, 2219, 2219, 2217 respectively due to CN groups and 1638, 1630, 1643 respectively due to C=O groups.

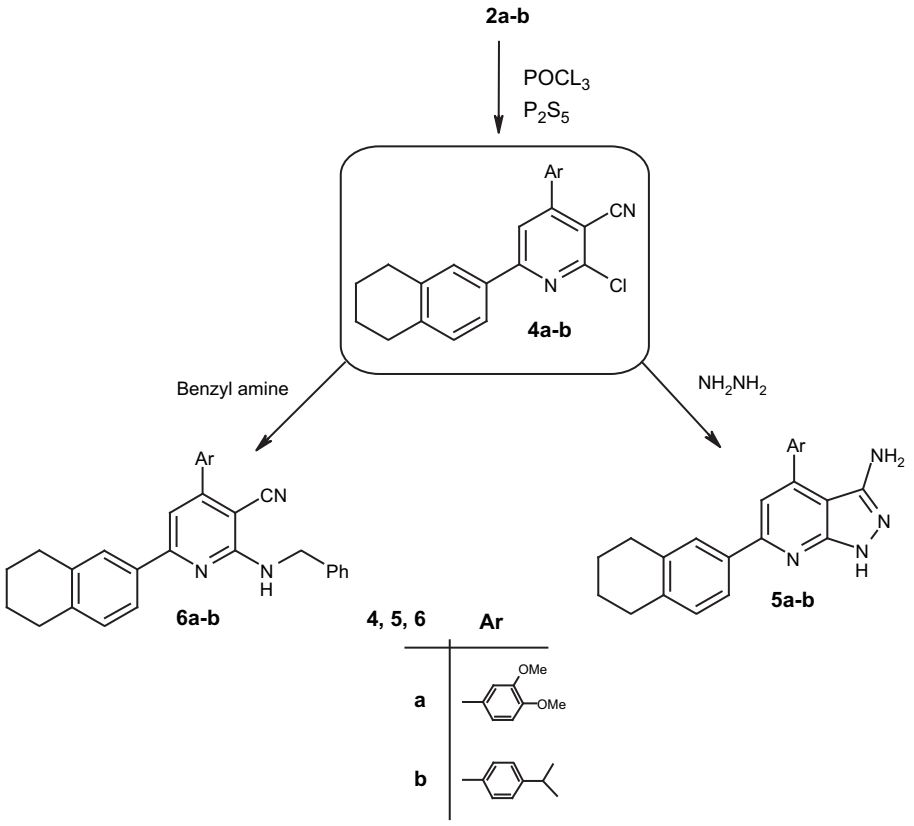
Their ^{1}H NMR spectrum showed singlet signals (D_2O exchangeable) at δ 11.2, 11.2, 12.3 respectively due to NH protons in addition to an aromatic multiplet in the region δ 6.7–8.1, whereas their mass spectrum showed a peak corresponding to its molecular ion at *m*/*z* 386.3, 368.2, 365.3 (M^+) respectively. Heating a mixture of the obtained cyanopyridone derivatives **2a–c** with phosphorous pentasulphide in dry pyridine afforded the corresponding thioxopyridine **3a–c.** The structures of the latter compounds were confirmed on the basis of their elemental analysis and spectral data. For example their IR spectrum revealed absorption bands at 1202, 1209, 1200 respectively due to C=S groups and disappearance of C=O absorption bands also their mass spectrum showed a peaks corresponding to their molecular ion at *m*/*z* 402.3 (M^+), 384.4 (M^+), 380 ($M^+ - 1$) respectively (Scheme 1).

On the other hand, heating compounds **2a**,**b** with phosphorous pentachloride and phosphorous oxychloride afforded 2-chloropyridines **4a**,**b**, which upon fused with hydrazine hydrate afforded pyrazolopyridine derivatives **5a**,**b** (Scheme 2). The structures of the latter compounds were confirmed on the basis of their elemental analysis and spectral data. The mass spectrum showed the expected molecular ion peaks M^+ at *m*/*z* 400 (100%), 382 (100%) respectively. The IR spectrum displaying bands at 3453, 3294, 3194 and 3453, 3294, 3194 for NH_2, NH respectively, C=N at 1596, 1585 respectively also the IR showed the disappearance of band of CN. Also when compounds **4a**,**b** fused with benzyl amine afforded the corresponding cyanopyridine derivatives **6a**,**b** (Scheme 2). The structure of the latter products were established on the basis of the appearance of an NH absorption band in the region of 3360–3431 cm^{-1} and a band of CN function in the region of 2205, 2202 cm^{-1} respectively in their IR spectra, whereas their ^{1}H NMR spectra revealed the appearance of a doublet signal due to $-CH_2$ in the region 4.7 and a signal (NH, D_2O exchangeable) in the region 11.2 (cf. Experimental section).

Moreover, treatment of compounds **2a**,**b** with ethyl bromoacetate and anhydrous potassium carbonate in dry acetone afforded, 3-Cyanopyridinyl oxy acetic acid ethyl esters **7a**,**b** respectively (Scheme 3), where their structures were confirmed by the mass



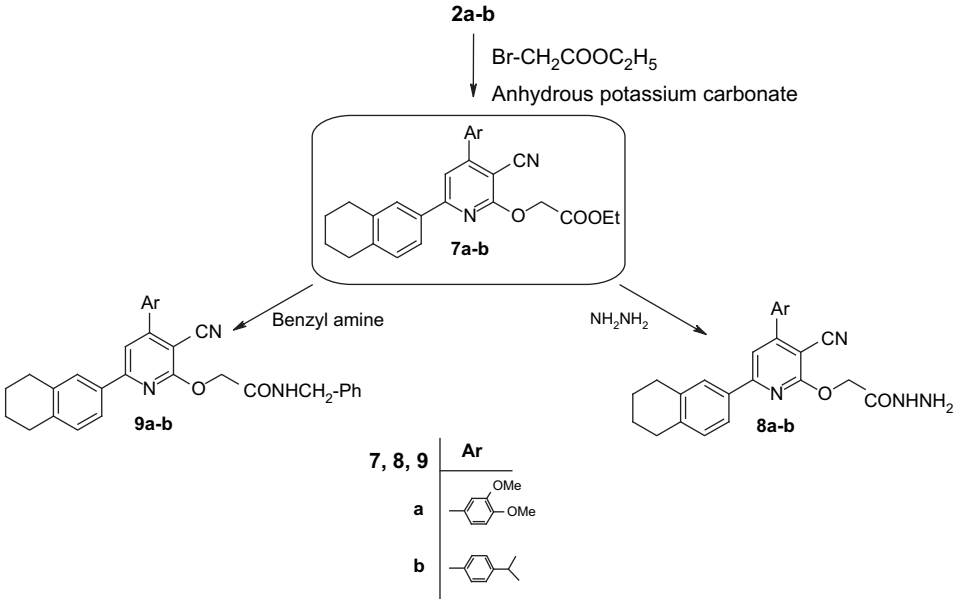
Scheme 1.



Scheme 2.

spectrum containing a peak due to the corresponding molecular ion *m*/*z* 472.28, 454.26 (M^+) respectively. The IR spectrum showed absorption bands at 1745, 1749 respectively due to C═O of ester groups, also their ^{1}H NMR spectrum showed the protons of ethyl ester group.

The reaction of **7a**,**b** with hydrazine hydrate in refluxing ethanol gave the hydrazides **8a**,**b**. Their IR spectrum showed the appearance of three absorption bands due to NH_2, NH functions in addition to the carbonyl absorptions and CN bands. Also their ^{1}H NMR spectra revealed the appearance of a signal due (NH_2, D_2O exchangeable) in the region 4.3, 4.27 respectively and in the region 9.4 for NH, while treatment of **7a**,**b** with benzyl amine gave the corresponding amides **9a**,**b**. Their structure were supported by their correct analytical data and IR spectrum which showed the



Scheme 3.

appearance of the absorption bands due to NH, CN, C=O. Also their ^{1}H NMR revealed the appearance of a signal at 8.7 due to (NH_2, D_2O exchangeable).

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 [31]. The rate of macrophages growth represents a controlling key in that defense system. The incubation of macrophages with the compounds of Scheme 1 (**2a–c** and **3a–c**) for 24 h indicated that only **2b** and **3b** significantly induced the cell growth up to 4-folds of the control growth (Fig. 1A), while the rest of compounds were not cytotoxic ($IC_{50} > 100$ μM), except **3a**, which possessed moderate cytotoxicity (IC_{50} 29.4 μM). On the other hand, all compounds of Scheme 2 (**4a**,**b**, **5a**,**b**, and **6a**,**b**) and Scheme 3 (**7a**,**b**, **8a**,**b**, and **9a**,**b**) had no cytotoxic effect on macrophage as indicated from there IC_{50} values (50 to >100 μM) (Fig. 1B and C), except compounds **4b**, **6a**, **8a**, and **9b**, which possessed dramatic cytotoxicity to macrophage with IC_{50} values of 8.4, 4.9, 3.1, and 9.9 μM, respectively, in addition to compounds **7a** and **8b**, which had moderate cytotoxic effect with IC_{50} values of 26.2 and 27.7 μM, respectively (Fig. 1B and C).

These results revealed that neither of the pyridine ring nor the tetrahydronaphthalene part is directly responsible for modulation of the macrophage growth, but the substitution with isopropyl phenyl in the pyridine ring of the cyanopyridone (**2b**) and of the thioxopyridine (**3b**) led to a high induction of macrophage growth. In contrast, the substitution with isopropyl phenyl in the chloropyridine (**4b**) and acetamide (**9b**) derivatives led to a remarkable cytotoxicity to macrophages. Similarly, the substitution with dimethoxy phenyl group in the cyanopyridine (**6a**) and the hydrazide derivatives (**8a**) resulted in a cytotoxic effect on macrophages. Due to the results of the viability assay, 20 μM of

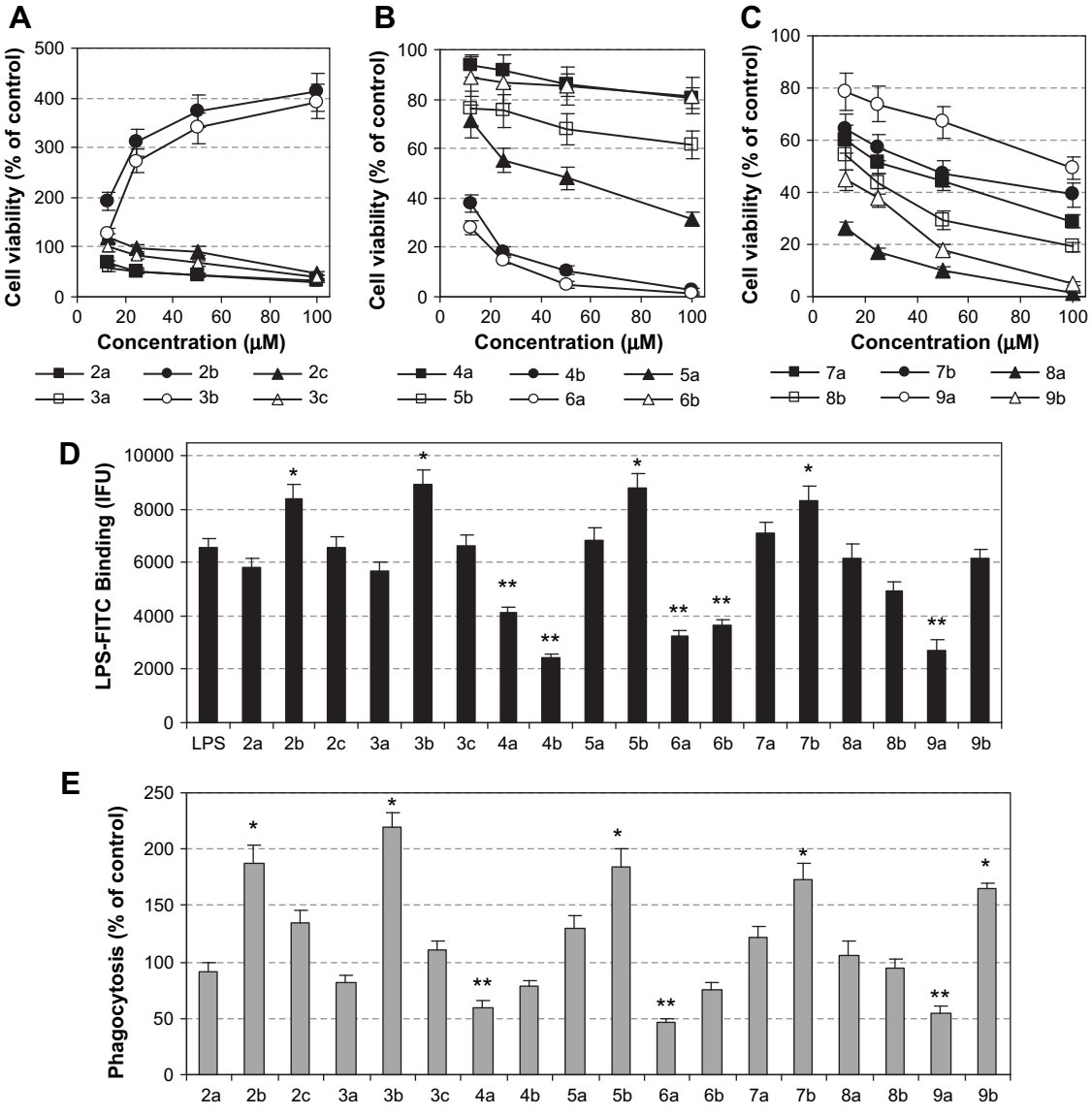


Fig. 1. The effect of different doses of the synthesized compounds of Scheme 1 (A), Scheme 2 (B), and Scheme 3 (C) on the viability of macrophages was measured by MTT assay. Macrophage viability was compared with the induced proliferation by 1000 U/ml M-CSF (178% of control). The results are represented as the percentage of control untreated cells (Mean ± SD, $n = 4$). The effect of treatment with different compounds on the FITC-LPS-binding affinity to RAW 264.7 cells (D) in comparison with untreated and FITC-LPS treated cells. The mean parentage of the binding affinity is compared with FITC-LPS treated cells. Phagocytic activity of RAW 264.7 cells against FITC-zymosan (E) was measured fluorometrically after treatment with different compounds. The results were compared to the phagocytic activity of the control untreated cells (represented as 100% activity). The significant induction (*) and the significant inhibition (**) are indicated upon the bars ($P < 0.05$).

non-cytotoxic compounds ($IC_{50} > 100$ μM) and 20% of the IC_{50} value of the cytotoxic compounds were used for the rest of cellular experiments.

During phagocytosis, macrophages secrete preformed granule constituents and newly synthesized products that play a critical role in inflammation and tissue repair [31]. Accordingly, phagocytic activity and macrophage reactivity against bacterial epitopes are crucial in the assessment of macrophage function. Macrophage defense against pathogens includes cytokine secretions like tumor necrosis factor-α (TNF-α), and inflammation mediators like nitric oxide (NO). In phagocytes, NO is produced in large quantities by the action of inducible NO synthetase (iNOS), in addition to 5-lipoxygenase (5-LO) and cyclooxygenase-2 (COX-2) [32].

We examined the effect of the synthesized compounds on some of macrophage functions including LPS-binding affinity and phagocytosis. Fluorometric analysis of LPS-binding affinity to macrophages was investigated using LPS-FITC (Fig. 1D). The analysis indicated that compounds **2b**, **3b**, **5b**, and **7b** significantly enhanced the macrophages binding affinity to LPS ($P < 0.05$), while compounds **4a**, **4b**, **6a**, **6b**, and **9a** significantly depressed the macrophages binding affinity to LPS ($P < 0.05$). In phagocytosis experiment, compounds **2b**, **2c**, **3b**, **5b**, **7b**, and **9b** significantly induced the phagocytic activity of macrophages, while compounds **4a**, **6a**, and **9a** significantly inhibited phagocytosis. The findings of LPS-binding and phagocytosis experiments revealed that the substitution with isopropyl phenyl group in the pyridine ring, in many compounds, is responsible for the enhancement of the macrophage binding affinity to LPS and the macrophage phagocytic activity. In contrast, it seems that the substitution with dimethoxy phenyl group is responsible for the inhibition of both of LPS-binding affinity and phagocytosis.

TNF-α is produced mainly by activated macrophages, and by tumor cells [33]. In inflammation, TNF-α plays a critical role in tissue destruction, tissue remodeling and damage recovery, and in maintaining the reversibility of microenvironments and stimulating the cellular changes [34]. TNF-α may initiate an inflammatory cascade consisting of other inflammatory cytokines, chemokines, growth factors, and endothelial adhesion factors, recruiting a variety of activated cells at the site of tissue damage [34]. TNF-α has both anticancer and procancer actions, where high-dose administration of TNF-α might destruct tumor vasculature and have necrotic effects in tumors [34]. In contrast, TNF-α has been found to be required in chemical carcinogen-elicited skin carcinogenesis [35]. In addition, TNF-α can induce DNA damage, inhibit DNA repair [36], and act as a growth factor for tumor cells [37]. Accordingly, the inhibition of stimulated TNF-α is an important target in prevention of cancer and in counteracting of inflammatory diseases.

Surprisingly, the treatment of macrophages with compounds **4a**, **4b**, **6a**, **6b**, and **8b**, without LPS, led to highly significant increase ($P < 0.05$) in the nitrites, as an index of NO generation, and in TNF-α secretion (Table 1). It is clear that [**R**-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-nicotinonitrile}part of the chloropyridine (**4a** and **4b**) and the cyanopyridine (**6a** and **6b**) derivatives, whatever is the substitution of **R**, led to an induction in the secretion of TNF-α and in the generation of NO, which may be due to an induced iNOS expression. It can be noticed also that those derivatives additionally depressed the macrophages binding affinity to LPS and that **4a**, and **6a** inhibited phagocytosis.

In LPS pre-treated macrophages, the nitrites and TNF-α level, in the macrophage culture supernatants, were significantly generated compared with control level in untreated cells (Table 1). The co-treatment of LPS-stimulated macrophages with compounds **2a**, **2b**, **3b**, **5a**, **5b**, **7b**, **8a**, **9a**, and **9b** potentially inhibited the NO generation ($P < 0.05$), while **4a**, **4b**, **6a**, **6b**, and **8b** led to further additional generation of NO more that they generate alone. On the other hand, the co-treatment of LPS pre-treated macrophages with compounds **2b**, **3b**, **5b**, **7b**, **9a**, and **9b** significantly reduced the secreted stimulated TNF-α ($P < 0.05$), as shown in Table 1.

Arachidonic acids are the substrate for COX-2 to produce prostaglandins. Interestingly, arachidonic acids can be converted

Table 1
Effect of different compounds on the levels of nitrites, TNF-α and PGE-2 in LPS-stimulated and un-stimulated macrophages.

Sample	Nitrites[a] (nmol/mg protein)		TNF-α[a] (pg/mg protein)		PGE2[a] (pg/mg protein)	
LPS	−	+	−	+	−	+
Control	8.26 ± 2.58	573.29 ± 44.15[b]	84.50 ± 11.20	5800.08 ± 650.18 [b]	36.50 ± 4.50	3150.00 ± 136.5[b]
2a	14.80 ± 3.85	355.78 ± 41.15[c]	69.56 ± 9.33	3860.25 ± 51.77	30.35 ± 5.33	3120.45 ± 127.88
2b	8.51 ± 1.25	32.55 ± 4.61[c]	91.67 ± 13.13	1255.60 ± 98.16[c]	40.13 ± 5.74	2105.30 ± 157.67[c]
2c	16.25 ± 3.46	505.75 ± 18.97	88.44 ± 15.11	5100.15 ± 184.98	32.63 ± 6.74	3210.30 ± 197.52
3a[d]	8.58 ± 2.22	380.12 ± 66.67	79.18 ± 9.55	4652.40 ± 159.94	28.66 ± 3.36	3309.30 ± 270.20
3b	12.66 ± 3.44	25.58 ± 3.61[c]	101.5 ± 22.71	1458.16 ± 171.36[c]	41.84 ± 5.16	1843.42 ± 164.25[c]
3c	15.50 ± 4.06	480.05 ± 21.84	75.40 ± 9.87	4925.35 ± 211.09	49.82 ± 4.50	3005.30 ± 198.85
4a	512.35 ± 45.77[b]	766.85 ± 62.34	810.75 ± 85.55[b]	5716.66 ± 204.08	38.33 ± 7.60	3325.87 ± 310.29
4b[d]	439.13 ± 38.48[b]	611.68 ± 59.12	350.05 ± 27.46[b]	5110.40 ± 261.22	45.39 ± 6.10	2870.25 ± 231.86
5a	10.53 ± 1.94	145.67 ± 19.78[c]	94.88 ± 11.03	4185.85 ± 194.87	28.60 ± 3.68	3415.23 ± 332.08
5b	5.62 ± 1.12	62.42 ± 8.91[c]	105.60 ± 13.05	1925.78 ± 184.54[c]	32.21 ± 7.46	2974.23 ± 305.40
6a[d]	720.91 ± 68.41[b]	915.90 ± 95.22	2460.46 ± 222.23[b]	5520.55 ± 405.08	31.29 ± 9.10	3010.66 ± 280.27
6b	614.63 ± 77.28[b]	922.50 ± 86.21	1890.16 ± 136.84[b]	6325.08 ± 344.16	48.30 ± 4.87	3222.55 ± 194.28
7a[d]	2.65 ± 0.95	388.40 ± 40.58	66.57 ± 8.41	3945.11 ± 256.56	22.40 ± 4.84	2954.2 ± 273.2
7b[d]	3.19 ± 1.02	110.54 ± 13.47[c]	72.45 ± 6.52	2810.66 ± 177.08[c]	50.54 ± 8.81	2870.50 ± 274.05
8a	1.34 ± 0.57	78.54 ± 9.88[c]	124.68 ± 11.79	4924.22 ± 117.23	25.60 ± 4.38	3111.94 ± 265.57
8b[d]	440.24 ± 37.57[b]	596.40 ± 47.86	1364.75 ± 108.99[b]	5754.50 ± 242.37	43.60 ± 7.51	3405.50 ± 389.77
9a	26.65 ± 5.03	155.25 ± 18.76[c]	116.8 ± 14.52	854.16 ± 73.73[c]	53.10 ± 6.31	2018.96 ± 140.20[c]
9b[d]	10.19 ± 2.31	32.75 ± 4.82[c]	69.65 ± 5.19	615.33 ± 53.77[c]	48.15 ± 7.64	3260.20 ± 280.42

[a] The nitrites, as an index for NO generation was used by Griess assay, while TNF-α and PGE-2 were measured using commercial ELISA kits.
[b] Significantly different from control untreated macrophages ($P < 0.05$).
[c] Significantly different from LPS-stimulated macrophages ($P < 0.05$).
[d] These compounds are toxic, so 20% of their IC_{50} values were used for macrophage treatment, while the rest of the compounds were not toxic ($IC_{50} > 100$ μM), so 20 μM of each compounds were used for macrophage treatment.

by another enzyme lipoxygenases (LO) to leukotrienes, which were suggested to be another link between inflammation and cancer [32]. The prostaglandins (PG) are biologically active derivatives of arachidonic acid and other polyunsaturated fatty acids that are released from membrane phospholipids by phospholipase A2 [32]. PGE2 plays a role both in normal physiology and in pathology [38]. The biological actions include inflammation, pain, tumorigenesis, vascular regulation, neuronal functions, female reproduction, gastric mucosal protection, and kidney function [38]. Measurement of PGE-2 by commercial kit, revealed that neither of the compounds led to a significant change in PGE-2 level in macrophages ($P > 0.05$), when treated in the absence of LPS (Table 1). However, the treatment with LPS resulted in a dramatic significant increase in PGE-2 level compared to untreated cells and only the co-treatment with compounds **2b**, **3b**, and **9a** led to a significant inhibition in this stimulated secretion of PGE-2 ($P < 0.05$), as shown in Table 1.

LOs, including 5-LO, mediate oxidation of potent carcinogens such as benzidine, o-dianisidine, and others; this activation can be blocked by adding the LO inhibitors, which products have inhibited the development and progression of human cancers [39]. In comparison with normal tissues, significantly elevated levels of LO metabolites have been found in several types of cancer cells [39]. LO-mediated products elicit diverse biological activities needed for neoplastic cell growth, the activation of growth factor and transcription factor, the induction of oncogene, stimulation of tumor cell adhesion, and the regulation of apoptotic cell death [39]. Agents that block LO activity may be effective in preventing cancer. LO inhibitors have prevented carcinogen-induced lung adenomas and rat mammary gland cancers [39]. Pharmacological agents that specifically inhibit the LO-mediated signaling pathways are now commercially available to treat inflammatory diseases such as asthma, arthritis, and psoriasis [39].

COX-2 expression may be induced by a wide range of stimuli, including lipopolysaccharide, proinflammatory cytokines, such as IL-1 and TNF, and growth factors, such as epidermal growth factor [32]. COX-2 is also overexpressed in various types of cancer and involved in cellular proliferation; antiapoptotic activity, angiogenesis, and an increase of metastasis [32]. The products of COX-2 enzyme are prostaglandins, which are key mediators of inflammation. Various nonsteroidal anti-inflammatory drugs inhibit COX-2 activity, which reduce the risk of several cancers.

Using immunoblotting analysis, COX-2 and 5-LO enzymes were assessed in macrophage lysates. The treatment of macrophage with all of the compounds alone resulted in an unchangeable level of 5-LO, while COX-2 level was significantly induced ($P < 0.05$) by **4a**, **6a**, **6b**, and **8b** (Table 2). In LPS-stimulated macrophages, COX-2 and 5-LO were remarkable enhanced ($P < 0.05$), as shown in Table 2. The treatment of LPS-stimulated macrophages with compounds **2b**, **3b**, **3c**, **5a**, **5b**, **7b**, and **9b** significantly depressed the stimulated synthesis of COX-2 ($P < 0.05$), similarly, compounds **2b**, **3b**, **3c**, **5b**, **7b**, and **9b** were recognized as potential inhibitors of 5-LO ($P < 0.05$), as shown in Table 2.

During inflammation, different types of reactive oxygen and nitrogen species are released. Scavenging of those species represents a defense mechanism against everlasting inflammation in inflammatory diseases and in cancer. For this reason we screened the radical scavenging activity of the synthesized compounds revealed that **3b**, **3c**, **5b**, **6a**, **7a**, **8a**, **9a**, and **9b** were strong non specific scavengers of different types of radicals ($OH^{\bullet}$, $ROO^{\bullet}$, and $O_2^{-\bullet}$), as indicated from their high ORAC values against $OH^{\bullet}$ and $ROO^{\bullet}$ and low SC_{50} values against $O_2^{-\bullet}$ (Table 3). On the other hand, **5a** was a specific strong scavenger of $OH^{\bullet}$, in comparison to trolox activity, and **7b** was a relative specific scavenger of $ROO^{\bullet}$ (Table 3).

Table 2
Effect of different compounds on the levels of COX-2 and 5-LO in LPS-stimulated and un-stimulated macrophages.

Sample	COX-2,[a] % of relative intensity		5-LO,[a] % of relative intensity	
LPS	−	+	−	+
Control	16.80 ± 2.40	87.40 ± 5.30[b]	21.10 ± 3.10	93.60 ± 9.10[b]
2a	14.60 ± 2.40	76.30 ± 5.60	15.80 ± 3.10	92.10 ± 8.40
2b	21.40 ± 1.20	42.70 ± 4.80[c]	20.30 ± 2.40	25.10 ± 3.40[c]
2c	16.30 ± 1.80	82.40 ± 6.40	23.50 ± 1.80	82.30 ± 7.70
3a[d]	12.30 ± 1.70	72.80 ± 6.60	18.30 ± 2.20	85.40 ± 7.50
3b	18.20 ± 1.40	36.70 ± 5.40[c]	14.20 ± 2.20	31.60 ± 5.20[c]
3c	28.10 ± 3.30	59.80 ± 4.40[c]	23.80 ± 3.20	69.40 ± 6.20[c]
4a	36.60 ± 4.30[b]	88.70 ± 5.20	25.30 ± 3.40	84.50 ± 5.40
4b[d]	28.70 ± 2.50	80.10 ± 8.30	29.50 ± 1.60	94.60 ± 5.90
5a	15.70 ± 2.60	63.70 ± 8.20[c]	24.00 ± 3.20	81.80 ± 6.50
5b	22.60 ± 3.40	28.10 ± 4.20[c]	18.10 ± 2.30	43.60 ± 4.80[c]
6a[d]	46.30 ± 6.40[b]	99.20 ± 2.60	30.20 ± 3.80	94.70 ± 4.40
6b	53.20 ± 6.30[b]	97.30 ± 4.20	29.80 ± 3.60	88.70 ± 4.50
7a[d]	27.5 ± 2.60	68.70 ± 6.70	21.90 ± 3.30	79.60 ± 8.40
7b[d]	18.70 ± 3.20	31.30 ± 4.30[c]	11.90 ± 2.60	38.70 ± 6.20[c]
8a	10.20 ± 2.80	71.30 ± 5.80	13.80 ± 2.60	83.20 ± 4.80
8b[d]	63.10 ± 7.80[b]	92.60 ± 4.50	33.12 ± 4.60	91.80 ± 7.60
9a	23.70 ± 3.30	73.30 ± 3.40	18.90 ± 2.80	77.40 ± 9.10
9b[d]	20.60 ± 3.20	20.10 ± 5.20[c]	21.70 ± 2.40	61.70 ± 8.60[c]

[a] The change in COX-2 and 5-LO content were assayed by dot immunoblotting.
[b] Significantly different from control untreated macrophages ($P < 0.05$).
[c] Significantly different from LPS-stimulated macrophages ($P < 0.05$).
[d] These compounds are toxic, so 20% of their IC_{50} values were used for macrophage treatment, while the rest of the compounds were not toxic ($IC_{50} > 100$ μM), so 20 μM of each compounds were used macrophage treatment.

Table 3
Radical scavenging activity of different compounds against $OH^{\bullet}$ and $ROO^{\bullet}$ as measured by ORAC assay, and against $O2^{-\bullet}$ as measured by xanthine/xanthine oxidase assay.

Compound[a]	ORAC-$OH^{\bullet}$(units)[b]	ORAC-$ROO^{\bullet}$(units)[b]	X/XO ($O2^{-\bullet}$) SC_{50} (μg/ml)[c]
2a	0.54	0.41	>100
2b	0.92	0.75	85.40
2c	0.21	0.35	78.51
3a	0.85	0.90	65.42
3b	5.41	4.32	10.15
3c	4.2	6.13	4.22
4a	0.35	0.61	87.87
4b	1.1	0.92	>100
5a	5.85	1.92	>100
5b	2.28	2.45	21.88
6a	3.54	2.95	38.43
6b	1.01	1.34	77.56
7a	2.47	2.01	55.68
7b	1.05	2.36	81.42
8a	5.84	5.12	8.67
8b	0.99	0.81	42.47
9a	2.31	3.56	19.89
9b	3.44	2.22	24.62

[a] In ORAC assay, only 1 μM of each compound was used.
[b] 1.0 ORAC unit equals the net protection of β-phycoerythrin produced by 1.0 μM Trolox.
[c] SC_{50}: half-maximal scavenging concentration was calculated using dose dependant curve of each compound.

3. Conclusion

Collectively, the cyanopyridone **2b** induced the macrophage growth, macrophages binding affinity to LPS, and phagocytic activity, and it inhibited LPS-stimulated NO, TNF-α, PGE-2, 5-LO, and COX-2. Other cyanopyridones **2a** and **2c** were only NO inhibitor and phagocytosis induced, respectively. The thioxopyridine **3b** induced macrophage growth, macrophages binding affinity to LPS,

and phagocytic activity, and inhibited the LPS-stimulated NO, TNF-α, PGE-2, 5-LO, and COX-2 and it was a strong radical scavenger. The other thioxopyridine **3c** was a strong COX-2 and 5-LO inhibitor and radical scavenger. The 2-chloropyridines **4a**,**b** were found to be significant inducer of NO, and TNF-α. Compound **4a** inhibited and phagocytosis the macrophages binding affinity to LPS and induced COX-2, while **4b** was cytotoxic to macrophages and inhibited the macrophages binding affinity to LPS. The pyrazolopyridine **5a** inhibited the LPS-stimulated NO and COX-2 and it was specific scavenger of OH•, while the other pyrazolopyridine **5b** induced phagocytosis and macrophages binding affinity to LPS and it inhibited the LPS-stimulated NO, TNF-α, COX-2 and 5-LO and it was a strong radical scavenger.

The cyanopyridine derivatives **6a**,**b** were found to be significant inducers of NO, TNF-α, and COX-2 and inhibitors of macrophages binding affinity to LPS. The cyanopyridine **6a**, regardless its high antioxidant activity, was cytotoxic to macrophages and it inhibited phagocytosis. Although the 3-Cyanopyridinyl oxy acetic acid ethyl ester **7a** showed only radical scavenging affinity, **7b** showed a multi-potent agent, since it induced phagocytosis and macrophages binding affinity to LPS, inhibited the LPS-stimulated NO, TNF-α, 5-LO, and COX-2, and it was a specific scavenger of ROO•. Despite the cytotoxic effect of the hydrazide derivative **8a** to macrophage, it was strong antioxidant and NO inhibitor. However, the hydrazide derivative **8b** was an inducer of NO, TNF-α, and COX-2. The acetamide derivative **9a** inhibited phagocytosis, the macrophages binding affinity to LPS, NO, TNF-α, and PGE-2 and had high antioxidant activity. On the other hand, the acetamide derivative **9b** was cytotoxic to macrophage, induced the phagocytic activity, inhibited NO and TNF-α, COX-2, and 5-LO and had a strong antioxidant activity. Taken together, the derivatives cyanopyridone **2b**, thioxopyridine **3b**, pyrazolopyridine **5a**, 3-cyanopyridinyl oxy acetic acid ethyl ester **7b** and acetamide **9a** and **9b** can be recognized as promising multi-potent anti-inflammatory agents, with considering the cytotoxicity of **9b**.

4. Experimental section

4.1. Chemistry

All melting points are uncorrected and measured using Electro-thermal IA 9100 apparatus (Shimadzu, Tokyo, Japan). IR spectra were recorded as potassium bromide pellets on a Perkin–Elmer 1650 spectrophotometer (Perkin–Elmer, Norwalk, CT, USA). ^{1}H NMR was determined on a Jeol-Ex-300 NMR spectrometer (JEOL, Tokyo, Japan) and chemical shifts were expressed as part per million (ppm) (δ values) against TMS as internal reference. Mass spectra were recorded on VG 2AM-3F mass spectrometer (Thermo electron corporation, USA). Microanalyses were operated using Mario El Mentar apparatus, Organic Microanalysis Unit, and the results were within the accepted range (±0.20) of the calculated values. Follow up of the reactions and checking the purity of the compounds was made by TLC on silica gel-precoated aluminum sheets (Type 60 F254, Merck, Darmstadt, Germany). Compounds **1** were prepared according to a reported method [30].

4.1.1. General procedure for the synthesis of compounds (**2a**–**c**)

A mixture of 1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethanone (**1**) (0.01 mol), aromatic aldehydes namely: 3,4-dimethyloxybenzaldehyde, *p*-isopropyl benzaldehyde, 3-indolaldehyde (0.01 mol), ethyl cyanoacetate (0.01 mol) and ammonium acetate (0.08 mol) in n-butanol (40 mL) was refluxed for 3 h. The obtained precipitate was filtered off, washed successively with ethanol and finally recrystallized from ethanol/DMF to afford the corresponding derivatives **2a**–**c** as yellow crystals, respectively.

*4.1.1.1. 4-(3,4-Dimethoxy-phenyl)-2-oxo-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-dihydro-pyridine-3-carbonitrile (**2a**).* Yield (85%); mp 268–270 °C; IR (KBr) (ν, cm^{-1}): 3420 (NH), 2933 (CH alicyclic), 2219 (CN), 1628 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.7 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.7 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 3.8 (s, 6H, OCH$_3$), 6.7–7.9 (m, 7H Ar-H) and 11.2 (s, 1H, NH D$_2$O exchangeable); MS *m*/*z* (%): 386.3 (M$^+$, 100%). Anal. calcd. for $C_{24}H_{22}N_2O_3$: C, 74.59; H, 5.74; N, 7.25. Found: C, 74.48; H, 5.82; N, 7.30.

*4.1.1.2. 4-(4-Isopropyl-phenyl)-2-oxo-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-dihydro-pyridine-3-carbonitrile (**2b**).* Yield (86%); mp 308–310 °C; IR (KBr) (ν, cm^{-1}): 3424 (NH), 2934 (CH alicyclic), 2219 (CN), 1630 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.25 (d, *J* = 6.85 Hz, 6H, 2CH$_3$), 1.75 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.8 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 3.1 (m, 1H, CH), 7.43 (d, *J* = 6.85 Hz, 2H, Ar-H), 7.65 (d, *J* = 6.85 Hz, 2H, Ar-H), 7.9–8.0(m, 4H Ar-H) and 11.2 (s, 1H, NH D$_2$O exchangeable); MS *m*/*z* (%): 368.2 (M$^+$, 100%). Anal. calcd. for $C_{25}H_{24}N_2O$: C, 81.49; H, 6.57; N, 7.60. Found: C, 81.38; H, 6.60; N, 7.65.

*4.1.1.3. 4-(1H-Indol-3-yl)-2-oxo-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-1,2-dihydro-pyridine-3-carbonitrile (**2c**).* Yield (83%); mp 320–321 °C; IR (KBr) (ν, cm^{-1}): 3306 (NH), 2929 (CH alicyclic), 2217 (CN), 1643 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.7 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.8 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 6.85–8.1 (m, 9H Ar-H), 11.97 (s, 1H, NH D$_2$O exchangeable) and 12.30 (s, 1H, NH D$_2$O exchangeable); MS *m*/*z* (%): 365.3 (M$^+$, 100%). Anal. calcd. for $C_{24}H_{19}N_3O$: C, 78.88; H, 5.24; N, 11.50. Found: C, 78.75; H, 5.34; N, 11.55.

4.1.2. General procedure for the synthesis of compounds (**3a**–**c**)

A mixture of compound **2** (0.01 mol) and P_2S_5 (2 g) in pyridine (10 mL) was refluxed for 6 h. The product poured into ice-cold dilute HCl. The separated solid was filtered off and recrystallized from appropriate solvent to afford the corresponding thione derivatives **3a**–**c**, respectively.

*4.1.2.1. 4-(3,4-Dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-2-thioxo-1,2-dihydro-pyridine-3-carbonitrile(**3a**).* This compound was obtained as orange crystals and recrystallized from $CHCl_3$/petroleum ether. Yield (70%); mp 218–220 °C; IR (KBr) (ν, cm^{-1}): 3420 (NH), 2934 (CH alicyclic), 2243 (CN), 1591 (C═N) and 1202 (C═S); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.78 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 3.8 (s, 6H, OCH$_3$), 7.1–7.9 (m, 7H Ar-H) and 8.8 (s, 1H, NH D$_2$O exchangeable); MS *m*/*z* (%): 402.3 (M$^+$, 100%). Anal. calcd. for $C_{24}H_{22}N_2O_2S$: C, 71.61; H, 5.51; N, 6.96; S, 7.97. Found: C, 71.53; H, 5.62; N, 6.72; 7.84.

*4.1.2.2. 4-(4-Isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-2-thioxo-1,2-dihydro-pyridine-3-carbonitrile (**3b**).* This compound was obtained as orange crystals and recrystallized from benzene. Yield (72%); mp 244–246 °C; IR (KBr) (ν, cm^{-1}): 3444 (NH), 2928 (CH alicyclic), 2230 (CN), 1595 (C═N) and 1209 (C═S); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.25 (d, *J* = 6.9 Hz, 6H, 2CH$_3$), 1.75 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.78 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 3.2 (m, 1H, CH), 7.08 (s, 1H, Ar-H), 7.2 (d, *J* = 8.45 Hz, 2H, Ar-H), 7.44 (d, *J* = 8.45 Hz, 2H, Ar-H), 7.6–7.67(m, 3H Ar-H) and 8.0 (s, 1H, NH D$_2$O exchangeable); MS *m*/*z* (%): 384.4 (M$^+$, 100%). Anal. calcd. for $C_{25}H_{24}N_2S$: C, 78.09; H, 6.29; N, 7.28; S, 8.34. Found: C, 78.15; H, 6.21; N, 7.34; S, 8.29.

*4.1.2.3. 4-[4-(1H-Indol-3-yl)-phenyl]-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-2-thioxo-1,2-dihydro-pyridine-3-carbonitrile (**3c**).* This compound was obtained as yellow crystals and recrystallized from $CHCl_3$. Yield (68%); mp 273–275 °C; IR (KBr) (ν, cm^{-1}): 3445 (NH), 3248 (NH), 2923 (CH alicyclic), 2215 (CN), 1596 (C=N) and 1200 (C=S); ^1H NMR (DMSO-d_6): δ (ppm) 1.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.78 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.1–8.3 (m, 9H Ar-H), 12.0 (s, 1H, NH D_2O exchangeable) and 13.7 (s, 1H, NH D_2O exchangeable); MS *m/z* (%): 380 (M^+-1, 100%), 381 (M^+, 49%). Anal. calcd. for $C_{24}H_{19}N_3O$: C, 78.74; H, 5.07; N, 9.18; S, 7.01. Found: C, 78.62; H, 5.15; N, 9.22; S, 6.93.

*4.1.3. General procedure for the synthesis of compounds (**4a**,**b**)*

A suspension of compound **2a** or **2b** (0.01 mol), PCl_5 (0.5 g) and $POCl_3$ (5 ml) was heated on a water bath for 3 h. The reaction mixture was poured gradually into ice-cold water and neutralized by dilute ammonia solution. The separated solid was filtered off and recrystallized from ethanol/DMF to afford the corresponding derivatives **4a**,**b** as yellow crystals, respectively.

*4.1.3.1. 2-Chloro-4-(3,4-dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-nicotinonitrile (**4a**).* Yield (64%); mp 172–174 °C; IR (KBr) (ν, cm^{-1}): 2930 (CH alicyclic), 2224 (CN), 1576 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.78 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.86 (s, 6H, 2 $^{\circ}CH_3$), 7.15–8.12 (m, 7H Ar-H); MS *m/z* (%): 406 (M^+, Cl^{37}, 33.79), 404 (M^+, Cl^{35}, 100). Anal. calcd. for $C_{24}H_{21}ClN_2O_2$: C, 71.19; H, 5.23; Cl, 8.76; N, 6.92. Found: C, 71.32; H, 5.15; Cl, 8.84; N, 6.87.

*4.1.3.2. 2-Chloro-4-(4-isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-nicotinonitrile (**4b**).* Yield (60%); mp 182–184 °C; IR (KBr) (ν, cm^{-1}): 2930 (CH alicyclic), 2223 (CN), 1581 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.25 (d, $J = 6.7$ Hz, 6H, $2CH_3$), 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.78 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.0 (m, 1H, CH), 7.19 (d, $J = 8.1$ Hz, 1H, Ar-H), 7.47 (d, $J = 6.6$ Hz, 2H, Ar-H), 7.70 (d, $J = 6.6$ Hz, 2H, Ar-H), 7.9 (m, 2H Ar-H), 8.13 (s, 1H, CH-pyridine ring); MS *m/z* (%):388 (M^+, Cl^{37}, 34), 386 (M^+, Cl^{35}, 100). Anal. calcd. for $C_{25}H_{23}ClN_2$: C, 77.61; H, 5.99; Cl, 9.16; N, 7.24. Found: C, 77.69; H, 5.89; Cl, 9.21; N, 7.10.

*4.1.4. General procedure for the synthesis of compounds (**5a**,**b**) and (**6a**,**b**)*

Compound **2a** or **2b** (0.01 mol) was fused with hydrazine hydrate (1 ml) or benzyl amine (1.2 ml) over sand bath for 3 h. The reaction mixture poured into ice-cold dilute hydrochloric acid and the separated solid was filtered off and recrystallized from ethanol to afford the corresponding derivatives **5a**,**b** or **6a**,**b**, respectively.

*4.1.4.1. 4-(3,4-Dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-ylamine (**5a**).* Yield (69%); mp 142–144 °C; IR (KBr) (ν, cm^{-1}): 3444 (NH), 3351 (NH), 3192 (NH), 2927 (CH alicyclic), 1596 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.8 (s, 6H, OCH_3), 5.6 (s, 2H, NH_2 D_2O exchangeable), 7.1–7.8 (m, 7H Ar-H), 12.0 (s, 1H, NH D_2O exchangeable); MS *m/z* (%): 400 (M^+, 100). Anal. calcd. for $C_{24}H_{24}N_4O_2$: C, 71.98; H, 6.04; N, 13.99. Found: C, 72.03; H, 6.00; N, 13.88.

*4.1.4.2. 4-(4-Isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-1H-pyrazolo[3,4-b]pyridin-3-ylamine (**5b**).* Yield (65%); mp 190–192 °C; IR (KBr) (ν, cm^{-1}): 3453 (NH), 3294 (NH), 3194 (NH), 2925 (CH alicyclic), 1585 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.27 (d, $J = 6.9$ Hz, 6H, $2CH_3$), 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.0 (m, 1H, CH), 5.6 (s, 2H, NH_2 D_2O exchangeable), 7.12–7.88 (m, 8H Ar-H), 12.3 (s, 1H, NH D_2O exchangeable); MS *m/z* (%):382 (M^+, 100). Anal. calcd. for $C_{25}H_{26}N_4$: C, 78.50; H, 6.85; N, 14.65. Found: C, 78.62; H, 6.79; N, 14.56.

*4.1.4.3. 2-Benzylamino-4-(3,4-dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-nicotinonitrile (**6a**).* Yield (70%); mp 160–162 °C; IR (KBr) (ν, cm^{-1}): 3360 (NH), 2930 (CH alicyclic), 2205 (CN), 1581 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.7 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.7 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.8 (s, 6H, OCH_3), 4.7 (d, $J = 5.4$ Hz, 2H, CH_2), 7.1–7.75 (m, 12H Ar-H) and 11.2 (s, 1H, NH D_2O exchangeable); MS *m/z* (%): 474.3 ($M^+ - 1$, 100%), 475.3 (M^+, 87.5%). Anal. calcd. for $C_{31}H_{29}N_3O_2$: C, 78.29; H, 6.15; N, 8.84. Found: C, 78.33; H, 6.04; N, 8.72.

*4.1.4.4. 2-Benzylamino-4-(4-isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-nicotinonitrile (**6b**).* Yield (68%); mp 132–134 °C; IR (KBr) (ν, cm^{-1}): 3431 (NH), 2929 (CH alicyclic), 2202 (CN), 1579 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.23 (d, $J = 6.85$ Hz, 6H, $2CH_3$), 1.7 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.7 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.0 (m, 1H, CH), 4.7 (d, $J = 5.75$ Hz, 2H, CH_2), 7.38 (d, $J = 8$ Hz, 2H, Ar-H), 7.56 (d, $J = 8$ Hz, 2H, Ar-H), 7.9–8.1 (m, 9H Ar-H) and 11.2 (s, 1H, NH D_2O exchangeable); MS *m/z* (%): 456.35 ($M^+ - 1$, 100%), 457.3 (M^+, 85.74%). Anal. calcd. for $C_{31}H_{29}N_3$: C, 83.99; H, 6.83; N, 9.18. Found: C, 84.08; H, 6.79; N, 9.22.

*4.1.5. General procedure for the synthesis of compounds (**7a**,**b**)*

A mixture of compound **2a** or **2b** (0.01 mol), ethyl bromoacetate (0.01 mol), anhydrous potassium carbonate (0.04 mol) in dry acetone (30 mL) was refluxed for 20 h. After cooling, water was added to the mixture and the formed solid was filtered off, recrystallized from appropriate solvent to afford the corresponding derivatives **7a**,**b**, respectively.

*4.1.5.1. [3-Cyano-4-(3,4-dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-pyridin-2-yloxy]-acetic acid ethyl ester (**7a**).* This compound was obtained as white crystals and recrystallized from ethanol. Yield (69%); mp 154–156 °C; IR (KBr) (ν, cm^{-1}): 2936 (CH alicyclic), 2214 (CN), 1745 (C=O) and 1590 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.2 (t, 3H, CH_3), 1.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.85 (s, 6H, 2 $^{\circ}CH_3$), 4.2 (q, 2H, $COOCH_2$), 5.12 (s, 2H, OCH_2COO), 7.1–7.8 (m, 7H Ar-H); MS *m/z* (%): 472.28 (M^+, 100%). Anal. calcd. for $C_{28}H_{28}N_2O_5$: C, 71.17; H, 5.97; N, 5.93. Found: C, 71.25; H, 5.85; N, 5.87.

*4.1.5.2. [3-Cyano-4-(4-isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-pyridin-2-yloxy]-acetic acid ethyl ester (**7b**).* This compound was obtained as white crystals and recrystallized from ethanol/DMF. Yield (71%); mp 168–170 °C; 2931 (CH alicyclic), 2219 (CN), 1749 (C=O) and 1590 (C=N); ^1H NMR (DMSO-d_6): δ (ppm) 1.5 (m, 9H, $CH_3$of ethyl group + $2CH_3$), 2.08 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.09 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.3 (m, 1H, CH), 4.52 (q, 2H, $COOCH_2$), 5.45 (s, 2H, OCH_2COO), 7.5 (d, $J = 12$ Hz, 1H, Ar-H), 7.79 (d, $J = 9$ Hz, 2H, Ar-H), 8.02 (d, $J = 9$ Hz, 2H, Ar-H), 8.13 (s, 1H, pyridine-H), 8.18–8.2 (m, 2H Ar-H); MS *m/z* (%): 454.26 (M^+, 100%). Anal. calcd. for $C_{25}H_{24}N_2O$: C, 81.49; H, 6.57; N, 7.60. Found: C, 81.38; H, 6.60; N, 7.65.

*4.1.6. General procedure for the synthesis of compounds (**8a**,**b**)*

A solution of compound **7a** or **7b** (0.01 mol) in ethanol (50 ml) and hydrazine hydrate (0.01 mol) was refluxed for 6 h. The separated solid after cooling was recrystallized from appropriate solvent to afford the corresponding hydrazides **8a**,**b**, respectively.

*4.1.6.1. [3-Cyano-4-(3,4-dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-pyridin-2-yloxy]-acetic acid hydrazide (**8a**).* This compound was obtained as white crystals and recrystallized from AcOH. Yield (70%); mp 234–236 °C; IR (KBr) (ν, cm^{-1}): 3442-3329 (NH_2,NH), 2929 (CH alicyclic), 2220 (CN), 1676 (C═O) and 1589 (C═N); ^1H NMR (DMSO-d_6): δ (ppm) 1.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.8 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.86 (s, 6H, 2 $^{\circ}CH_3$), 4.3 (s, 2H, NH_2 D_2O exchangeable), 4.9 (s, 2H, OCH_2), 7.15–7.91 (m, 7H Ar-H) and 9.4 (s, H, NH D_2O exchangeable); MS *m/z* (%): 458.3(M^+, 10.70%), 82.9 (100%). Anal. calcd. for $C_{26}H_{26}N_4O_4$: C, 68.11; H, 5.72; N, 12.22. Found: C, 68.22; H, 5.68; N, 12.20.

*4.1.6.2. [3-Cyano-4-(4-isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-pyridin-2-yloxy]-acetic acid hydrazide (**8b**).* This compound was obtained as white crystals and recrystallized from ethanol, Yield (76%); mp 190–191 °C; IR (KBr) (ν, cm^{-1}): 3440-3277 (NH_2,NH), 2919 (CH alicyclic), 2215 (CN), 1665 (C═O) and 1593 (C═N); ^1H NMR (DMSO-d_6): δ (ppm) 1.27 (d, $J = 6.9$ Hz, 6H, $2CH_3$), 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.0 (m, 1H, CH), 4.27 (s, 2H, NH_2 D_2O exchangeable), 4.98 (s, 2H, OCH_2), 7.38 (d, $J = 8.4$ Hz, 1H, Ar-H), 7.47 (d, $J = 8.4$ Hz, 2H, Ar-H), 7.6–7.9 (m, 5H Ar-H) and 9.4 (s, 1H, NH D_2O exchangeable); MS *m/z* (%): 440.14 (M^+, 55%), 368.22 ($M^+ - C_2H_5N_2O$, 100%). Anal. calcd. for $C_{27}H_{28}N_4O_2$: C, 73.61; H, 6.41; N, 12.72. Found: C, 73.61; H, 6.41; N, 12.72.

*4.1.7. General procedure for the synthesis of compounds (**9a**,**b**)*

A solution of compound **7a** or **7b** (0.01 mol) in ethanol and benzyl amine (0.02 mol) was refluxed for 6 h. The cooled solution was poured into ice-cold dilute hydrochloric acid. The separated solid was filtered off and recrystallized from an appropriate solvent to afford the corresponding derivatives **9a**,**b**, respectively.

4.1.7.1. N*-Benzyl-2-[3-cyano-4-(3,4-dimethoxy-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-pyridin-2-yloxy]-acetamide (**9a**).* This compound was obtained as white crystals and recrystallized from AcOH. Yield (73%); mp 198–200 °C; IR (KBr) (ν, cm^{-1}): 3279 (NH), 2924.5 (CH alicyclic), 2215 (CN), 1648.5 (C═O) and 1589 (C═N); ^1H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.85 (s, 6H, OCH_3), 4.33 (d, $J = 6$ Hz, 2H, N–CH_2), 5.0 (s, 2H, OCH_2), 7.1–7.79 (m, 12H Ar-H) and 8.7 (s, 1H, NH, D_2O exchangeable); MS *m/z* (%): 533 (M^+, 3%), 387 (M^+ – ph-$CH_2NHCOCH$, 100%). Anal. calcd. for $C_{33}H_{31}N_3O_4$: C, 74.28; H, 5.86; N, 7.87. Found: C, 74.35; H, 5.72; N, 7.99.

4.1.7.2. N*-Benzyl-2-[3-cyano-4-(4-isopropyl-phenyl)-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-pyridin-2-yloxy]-acetamide (**9b**).* This compound was obtained as white crystals and recrystallized from ethanol. Yield (80%); mp 188–189 °C; IR (KBr) (ν, cm^{-1}): 3294.6 (NH), 2922.5 (CH alicyclic), 2218 (CN), 1647.5 (C═O) and 1590 (C═N); ^1H NMR (DMSO-d_6): δ (ppm) 1.26 (d, $J = 7.2$ Hz, 6H, $2CH_3$), 1.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.0 (m, 1H, CH), 4.33 (d, $J = 5.7$ Hz, 2H, N-CH_2), 5.0 (s, 2H, OCH_2), 7.09–7.78 (m, 12H, Ar-H), 7.93 (s, 1H, pyridine-H), and 8.7 (s, 1H, NH D_2O exchangeable); MS *m/z* (%): 515 (M^+, 5%), 369.17($M^+ - C_9H_9NO$, 100%). Anal. calcd. for $C_{34}H_{33}N_3O_2$: C, 79.19; H, 6.45; N, 8.15. Found: C, 79.00; H, 6.56; N, 8.08.

4.2. Effect of synthesized compounds on inflammatory mediators

4.2.1. Cell culture

Murine raw macrophage cell line (RAW 264.7, ATCC, USA) was routinely cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin sodium, 100 units/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained sub-confluent at 37 °C in humidified air containing 5% CO_2. 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 were tested for endotoxin, using Pyrogent® Ultra gel clot assay, and they were found endotoxin-free. After the viability assay, for the rest of cellular experiments, 20 μM of non-cytotoxic compounds ($IC_{50} > 100$ μM) and 20% of the IC_{50} value of the cytotoxic compounds were used. All experiments were repeated four times, unless mentioned, and the data were represented as mean values. Cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark). Chemicals were purchased from Sigma Aldrich (VA, USA), except mentioned.

4.2.2. Viability assay

The effect of different compounds on the viability of RAW 264.7 cells was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. 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; acidic isopropanol [40]. RAW 264.7 (5×10^4 cells/well) were incubated for 24 h) with various concentrations 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 U/ml recombinant macrophage colony-stimulating factor (M-CSF, Pierce, USA) was used as positive control.

4.2.3. Macrophage binding to bacterial lipopolysaccharide

The binding of fluorescein isothiocyanate-conjugated bacterial lipopolysaccharides (LPS-FITC) to RAW 264.7 cells was monitored according to [41]. In brief, cells (5×10^5) were pre-incubated in the absence or presence of different compounds for 15 min at 37 °C in RPMI-1640 containing 10% FBS (as a source of CD14 and LPS-binding protein to induce binding) before incubated for 1 h with FITC-conjugated LPS (200ng/ml). After washing with phosphate buffered saline (PBS), the binding of FITC-LPS was analyzed by microplate fluorometer (FluoStarOptima, BMG Labtechnologies, NC, USA). The fluorescence was measured at excitation λ of 485 nm and an emission λ of 530 nm, and median fluorescence intensity, international fluorescence unit (IFU), was determined.

4.2.4. Phagocytosis assay

We examined the phagocytic activity of macrophages using FITC-conjugated zymosan according to [42]. RAW 264.7 cells were plated in a cell density of 1×10^5 cells/well in phenol red-free RPMI-1640 and incubated for 24 h at 37 °C in humidified air containing 5% CO_2. Cells were incubated for 1 h with compounds. Twenty microlitres of FITC-zymosan (Molecular Probes, USA) were added and re-incubated for 1 h. After washing with medium for three times, 100 μl of triton X-100 (10%) were added, and vigorously shacked. The fluorescence was measured at excitation λ of 485 nm and an emission λ of 530 nm.

4.2.5. Nitrite assay

The accumulation of nitrite, an indicator of nitric oxide (NO) synthesis, was measured by Griess reagent [43]. RAW 264.7 were grown in phenol red-free RPMI-1640 containing 10% FBS. In the first experiment, cells were incubated for 24 h with compounds without LPS, while in the second experiment; cells were incubated for 24 h with LPS (1 μg/ml) in the presence or absence of different

compounds. Fifty microlitres 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. The nitrite content was normalized to the cellular protein content as measured by bicinchoninic acid assay [44].

4.2.6. 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 μg/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., MA, USA) and (Cayman Chemical, MI, USA), respectively, according to the manufacturer protocols.

4.2.7. Estimation of COX-2 and 5-LO by dot immunoblotting

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 μg/ml) in the presence or absence of different compounds. Lysates, extracted from macrophages, were used for the assessment of COX-2 and 5-LO. Briefly, 20 μg of isolated soluble proteins were applied at dot blotting set using nitrocellulose membranes. Membranes were blocked and then incubated with rabbit anti-mouse COX-2 (# ab6665) or rabbit anti-mouse 5-LO (# ab59341) and β-actin proteins (# ab1801, Abcam, Cambridge, UK).

After being washed, bound antibody was detected using goat anti-rabbit antibody linked to horseradish peroxidase (# ab6721, Abcam, Cambridge, UK) and bound complexes were detected using O-phenylenediamine dihydrochloride (OPD). The percentage of the relative enhanced color intensity was normalized to dot intensity with β-actin control. The dot photographing and analysis was performed using gel documentation system (Biometra, Goettingen, Germany).

4.2.8. Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) of tested compounds against peroxyl, and hydroxyl ($OH^{\bullet}$) radicals, was investigated by ORAC assay [45] as modified at [46]. β-Phycoerythrin was used as a radical-sensitive fluorescent indicator protein, 2,2,-azobis-(2-amidinopropane) dihydrochloride (AAPH) was used as a peroxyl radical ($ROO^{\bullet}$) initiator, and a mixture of H_2O_2–$CuSO_4$ was used as a hydroxyl radical generator. A final concentration of each compound (1 μM) was used, and the reaction was initiated by addition of one of the radical generators. The decay of β-phycoerythrin fluorescence was measured kinetically, at 37 °C for 2 h using a microplate fluorescence reader. One ORAC unit equals the net protection of β-phycoerythrin produced by 1.0 μM Trolox, a known standard antioxidant.

4.2.9. Scavenging of superoxide anion radicals

Superoxide anion radicals ($O_2^{-\bullet}$) were generated by the oxidation of 50 μM hypoxanthine to uric acid by 12 mU xanthine oxidase (XO), in a non cellular assay. Scavenging of $O_2^{-\bullet}$ was quantified by a method based on [47] and modified by [48], which depends on the reduction rate of nitroblue tetrazolium to dark-blue formazan. The half-maximal scavenging concentration SC_{50} was calculated from serial dilutions of each compound, and superoxide dismutase was used as a standard inhibitor.

4.2.10. Statistical analysis

Data were statistically analyzed using 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 followed by the Tukey post hoc test was used to detect the statistical significance. *p* value more than 0.05 was considered insignificant.

Acknowledgements

This work was supported by the Center of Excellence for Advanced Sciences, National Research Center, Dokki, Cairo, Egypt.

References

[1] M. Philip, D.A. Rowley, H. Schreiber, Semin. Cancer Biol. 14 (2004) 433–439.
[2] L.M. Coussens, Z. Werb, Nature 420 (2002) 860–867.
[3] C. Nathan, Nature 420 (2002) 846–852.
[4] M.C. Maiuri, G. Tajana, T. Iuvone, Am. J. Pathol. 165 (2004) 115–126.
[5] B.D. Levy, C.B. Clish, B. Schmidt, K. Gronert, C.N. Serhan, Nat. Immunol. 2 (2001) 612–619.
[6] J. Hodge-Dufour, M.W. Marino, M.R. Horton, Proc. Natl. Acad. Sci. USA 95 (1998) 13806–13811.
[7] J. Savill, A.H. Wyllie, J.E. Henson, M.J. Walport, P.M. Henson, C. Haslett, J. Clin. Invest. 83 (1989) 865–875.
[8] J. Savill, V.A. Fadok, Nature 407 (2000) 784–788.
[9] J. Savill, I. Dransfield, C. Gregory, C. Haslett, Nat. Rev. Immunol. 2 (2002) 965–975.
[10] V.A. Fadok, D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, P.M. Henson, J. Clin. Invest. 101 (1998) 890–898.
[11] P.P. McDonald, V.A. Fadok, D. Bratton, P.M. Henson, J. Immunol. 163 (1999) 6164–6172.
[12] M.L.N. Huynh, V.A. Fadok, P.M. Henson, J. Clin. Invest. 109 (2002) 41–50.
[13] M. Macarthur, G.L. Hold, E.M. El-Omar, Am. J. Physiol. Gastrointest. Liver Physiol. 286 (2004) G515–G520.
[14] Henry G.D., Tetrahedron 60 (2004) 6043.
[15] M.C. Bagley, K. Chapaneri, D.W. Dale, X. Xiong, J. Bower, J. Org. Chem. 70 (2005) 1389–1396.
[16] T.L. Gilchrist, J. Chem. Soc., Perkin Trans. (2001) 2491–2497.
[17] A.H. Li, S. Moro, N. Forsyth, N. Melman, X.-D. Ji, K.A. Jacobsen, J. Med. Chem. 42 (1999) 706–721.
[18] B. Vacher, B. Bonnand, F. Funes, N. Jubault, W. Koek, M.-B. Assie, C. Cosi, M. Kleven, J. Med. Chem. 42 (1999) 1648–1655.
[19] J.K. Son, L.X. Zhao, A. Basnet, P. Thapa, R. Karki, Y. Na, Y. Jahng, T.C. Jeong, B.S. Jeong, C.S. Lee, E.S. Lee, Eur. J. Med. Chem. 43 (2008) 675–682.
[20] A.G. Amr, M.M. Abdulla, Bioorg. Med. Chem. 14 (2006) 4341–4352.
[21] A.K. Dutta, X. Fei, M.E.A. Reith, Bioorg. Med. Chem. Lett. 12 (2002) 619–622.
[22] P. Taddei, A. Torreggiani, G. Fini, Biopolymers 67 (2002) 289–293.
[23] H. Hatori, B. Sato, I. Sato, T. Shibata, Y. Tsurumi, K. Sakamoto, S. Takase, H. Veda, M. Hino, T. Fujii, J. Antibiot. 57 (2004) 264–270.
[24] J.H. Song, A.J. Slot, R.W.J. Ryan, G.M. Ross, Neuropharmacology 46 (2004) 984–993.
[25] A.G. Hammam, N.A. Abdel Hafez, W.H. Midura, M.Z. Mikolajczyk, Z. Naturforsch. 55 (2000) 417–423.
[26] M. Abo-Ghalia, M.M.Z. Abdulla, A.E. Amr, Z. Naturforsch. 58 (2003) 903–912.
[27] P. Dorigo, R.M. Gaion, P. Beiiuco, D. Fraccarollo, I. Maragno, G. Bombieri, F. Benetollo, L. Mosti, F. Orsini, J. Med. Chem. 36 (1993) 2475–2484.
[28] M.M. Ismail, Y.A. Ammar, H.S. El-Zahaby, S.I. Eisa, E.S. Barakat, Arch. Pharm. (Weinheim) 340 (2007) 476–482.
[29] Z. Lu, G.R. Ott, R. Anand, R.Q. Liu, M.B. Covington, K. Vaddi, M. Qian, R.C. Newton, D.D. Christ, J. Trzaskos, J.J. Duan, Bioorg. Med. Chem. Lett. 18 (2008) 1958–1962.
[30] N.L. Allinger, E.S. Jones, J. Org. Chem. 27 (1962) 70–73.
[31] M. Girotti, J.H. Evans, D. Burke, C.C. Leslie, J. Biol. Chem. 279 (2004) 19113–19121.
[32] H. Lu, W. Ouyang, C. Huang, Mol. Cancer Res. 4 (2006) 221–233.
[33] R.M. Locksley, N. Killeen, M.J. Lenardo, Cell 104 (2001) 487–501.
[34] F. Balkwill, Cytokine Growth Factor Rev. 13 (2002) 135–141.
[35] C. Huang, J. Li, W.Y. Ma, Z. Dong, J. Biol. Chem. 274 (1999) 29672–29676.
[36] M. Jaiswal, N.F. LaRusso, L.J. Burgart, G.J. Gores, Cancer Res. 60 (2000) 184–190.
[37] S. Wu, C.M. Boyer, R.S. Whitaker, Cancer Res. 53 (1993) 1939–1944.
[38] B. Samuelsson, R. Morgenstern, P.J. Jakobsson, Pharmacol. Rev. 59 (2007) 207–224.
[39] V.E. Steele, C.A. Holmes, E.T. Hawk, L. Kopelovich, R.A. Lubet, J.A. Crowell, C.C. Sigman, G.J. Kelloff, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 467–483.
[40] M.B. Hansen, S.E. Nielsen, K. Berg, J. Immunol. Methods 119 (1989) 203–210.
[41] J. Carracedo, R. Ramirez, A. Martin-Malo, M. Rodriguez, P. Aljama, Nephrol. Dial. Transplant. 17 (2002) 428–434.
[42] K.I. Kim, K.S. Shin, W.J. Jun, B.S. Hong, D.H. Shin, Biosci. Biotechnol. Biochem. 65 (2001) 2369–2377.
[43] C. Gerhäuser, K. Klimo, E. Heiss, I. Neumann, A. Gamal-Eldeen, Mutat. Res. 523–524 (2003) 163–172.
[44] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, Anal. Biochem. 150 (1985) 76–85.
[45] G. Cao, R.L. Prior, Methods Enzymol. 299 (1999) 50–62.
[46] A. Gamal-Eldeen, S. Kawashty, L. Ibrahim, M. Shabana, S. Negoumy, J. Nat. Remedies 4 (2004) 81–96.
[47] H. Ukeda, S. Maeda, T. Ishii, M. Sawamura, Anal. Biochem. 251 (1997) 206–209.
[48] C. Gerhäuser, A. Alt, E. Heiss, A. Gamal-Eldeen, K. Klimo, et al., Mol. Cancer Ther. 1 (2002) 959–969.