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Original article

Synthesis of [1,2,4]triazolo[1,5-*a*]pyridines of potential PGE_2 inhibitory properties

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Anti-inflammatory
PGE_2
Anti-tumor

ABSTRACT

A variety of 5-amino-6,8-dicyano-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate containing compounds **3a–i**, **5a–c** were prepared via reaction of arylidenemalononitriles **1a–c**, **4a** and **4b** with 2-[(substituted amino)thiocarbonyl]cyanoacetohydrazides **2a–d** in refluxing ethanol in the presence of triethylamine. Anti-inflammatory activity screening of the synthesized compounds (at a dose of 50 mg/kg body weight) utilizing in vivo acute carrageenan-induced paw oedema standard method in rats exhibited that the prepared heterocycles possess considerable pharmacological properties especially, **3f**, **3h**, **5b** and **5c** which reveal remarkable activities relative to indomethacin (which was used as a reference standard at a dose of 10 mg/kg body weight). PGE_2 inhibitory properties of the highly promising synthesized anti-inflammatory active agents (**3f**, **3h**, **5b** and **5c**) were determined by PGE_2 assay kit technique, which reveal remarkable activity coinciding greatly with the observed anti-inflammatory properties. Anti-tumor activity screening of **3b** and **3e**, as representative examples of the synthesized compounds, at a dose of 10 μM utilizing 59 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney exhibited that the tested compounds reflect mild or no activity at all against most of the used human tumor cell lines. However, compound **3e** reveals considerable anti-tumor properties against leukemia CCRF-CEM and HL-60(TB) cell line.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are mainly used in the treatment of pain and inflammation related to a large variety of pathologies [1,2]. Their mechanism of action involves inhibition of production of prostaglandins by the enzyme cyclo-oxygenase (COX) [3]. Prostaglandins are among the most important mediators of inflammation. They promote blood vessel dilation and vascular permeability, causing the typical redness, heat and swelling phenomena involved in inflammation. Moreover, they promote pain transmission from nociceptors to the brain by increasing the sensitivity of the nerve endings. However, prostaglandins also play a cytoprotective role in the gastrointestinal tract and they are necessary for normal platelet aggregation and renal function. Therefore, a reduction in the production of circulating prostaglandins leads to numerous side effects, the most important being gastrointestinal ulcers [4]. The double nature (i.e. inflammation reduction vs. gastrolesivity) of the effects caused by early NSAIDs acting as COX inhibitors was explained by the discovery of two isoforms of the COX enzyme [5,6]. One isoform (COX-1) is constitutive and regularly expressed producing prostaglandins involved in the cytoprotection of the gastrointestinal tract. The other isoform (COX-2) is associated with inflammatory states and is generally absent from all tissues, unless induced by inflammation mediators. Therefore, the inducible isoform COX-2 constitutes the real target for anti-inflammatory drugs. Several lines of evidence suggest that selective COX-2 inhibitors may also provide an opportunity for both cancer prevention and therapy [7]. Furthermore, promising in vitro data also indicate that treatment with selective COX-2 inhibitors may reduce the risk of Alzheimer's [8,9] and Parkinson's diseases [10,11] and may also be effective in the treatment of asthma [12].

In the present work, it is intended to investigate synthesis of [1,2,4]triazolo[1,5-*a*]pyridine containing compounds adopting facile synthetic approaches and utilizing easily accessible starting chemicals. The anti-inflammatory properties of the synthesized heterocycles will be screened. The prostaglandin (PGE_2) inhibitory activity for the most promising prepared anti-inflammatory active agents will be determined. Additionally, anti-tumor properties of representative examples of the prepared heterocycles will be investigated against various human tumor cell lines. The interest for construction of this heterocyclic nucleus is attributed to the

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biological and pharmacological properties associated with its structure. Where many publications reported their efficiency as adenosine (A_{2a}) receptor antagonist [13–18], ALK5 receptor modulator activity [19] beside anti-implantation properties (pregnancy-terminating activity) [20]. Other reports describe their ability to inhibit secretion of gastric acid so, useful for treating gastrointestinal disorders [21], in addition to their anti-tumor [22], antibacterial [23,24], antifungal [25] and herbicidal [26–28] properties.

2. Results and discussion

2.1. Chemistry

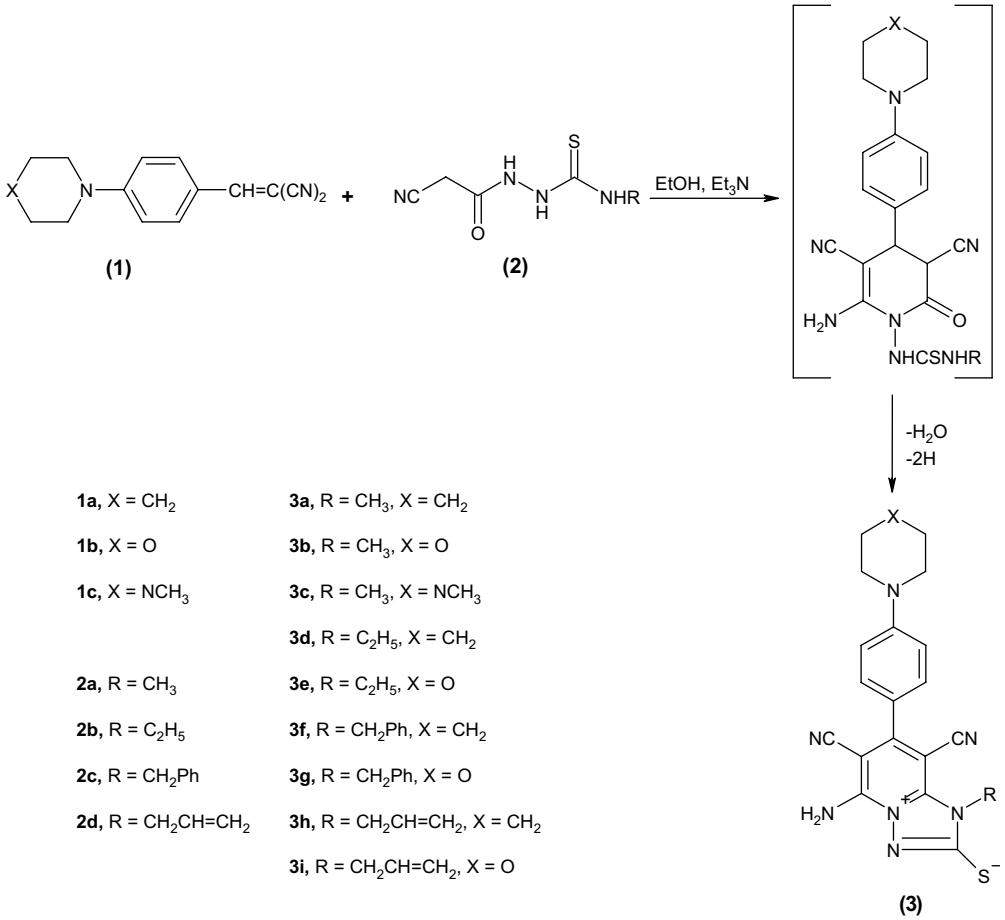
Reaction of arylidenemalononitriles **1a–c** with 2-[(substituted amino)thiocarbonyl]cyanoacetohydrazides **2a–d** in refluxing ethanol in the presence of triethylamine as a basic catalyst, afforded directly 5-amino-6,8-dicyano-1,7-disubstituted-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolates **3a–i**. The structure of **3** was established through spectroscopic (IR, ^{1}H NMR) as well as elemental analyses data. The IR spectra of **3a–i** reveal the presence of amino stretching vibration bands at $\nu = 3350–3130\ cm^{-1}$ besides the nitrile stretching vibration band at $\nu = 2225–2220\ cm^{-1}$. ^{1}H NMR spectra of **3a–i** add a sharp evidence for the presumed structure, exhibiting only one D_2O exchangeable amino signal at $\delta = 9.00–9.44$, excluding any other assumed pyridine intermediate isolation. The alicyclic-amino moiety signals (1.59–1.61, 3.26–3.37 "assignable for piperidinyl **3a**, **3d**, **3f**, **3h**"; 3.21–3.29, 3.74–3.77 "assignable for morpholinyl **3b**, **3e**, **3g**, **3i**" and 2.27, 2.53 "assignable for piperazinyl **3c**") are also well recognized (Scheme 1).

The reaction was assumed to proceed via active methylene nucleophilic attack of **2** at the β-carbon of α,β-unsaturated dinitrile system of **1** followed by cyclization, dehydration with subsequent dehydrogenation giving finally **3**. Similarly, reaction of bis(ylidenemalononitrile) containing compounds **4a**, **4b** with **2a**, **2b** under similar reaction conditions, proceeded smoothly affording the corresponding bis([1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolates) **5a–c** (Scheme 2).

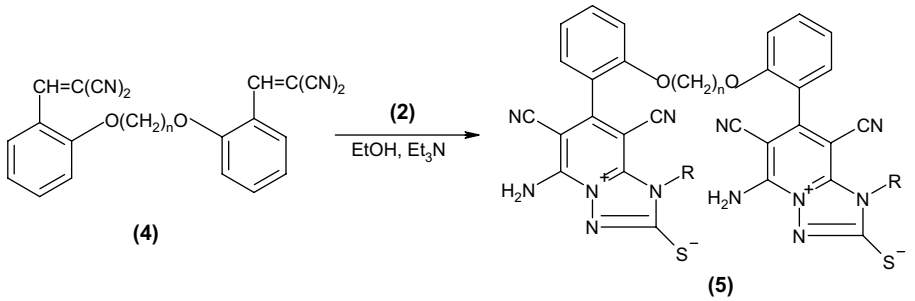
2.2. Anti-inflammatory activity

Anti-inflammatory activity of the synthesized compounds **3a–i**, **5a–c** (at a dose of 50 mg/kg body weight) was determined in vivo by the acute carrageenan-induced paw oedema standard method in rats [29–32]. The anti-inflammatory properties were recorded at successive time intervals (1, 2, 3 and 4 h) and compared with that of indomethacin (at a dose of 10 mg/kg body weight) which was used as a reference standard. From the obtained results (Table 1), it has been noticed that many of the tested compounds exhibit considerable anti-inflammatory properties, especially **3f**, **3h**, **5b** and **5c** which reveal remarkable activities with potency (% oedema inhibition of the tested compounds relative to % oedema inhibition of indomethacin) 99.4, 101.0, 110.9 and 114.0, respectively.

Structure–activity relationships based on the obtained results indicated that, the type of the alicyclic-amino moiety attached to the phenyl group oriented at the 7-position of heterocyclic nucleus seems to be a controlling factor in developing the total pharmacological properties. Utilization of piperidinyl function seems more favorable than the case of using morpholinyl residue for



Scheme 1.



4a, n = 2 **5a**, R = CH_3, n = 2

4b, n = 3 **5b**, R = CH_3, n = 3

5c, R = C_2H_5, n = 2

Scheme 2.

constructing an anti-inflammatory active agent as exhibited in pairs **3a**, **3b** (potency, 75.4, 67.0, respectively), **3d**, **3e** (potency, 90.6, 81.1, respectively), **3f**, **3g** (potency, 99.4, 58.0, respectively) and **3h**, **3i** (potency, 101.0, 89.4, respectively). Alternatively, adoption of piperazinyl function as an alicyclic-amino residue is associated with decrease in the gained anti-inflammatory properties as exhibited in compounds **3a**, **3b** and **3c** (potency, 75.4, 67.0, 54.0, respectively).

It is also obvious that, the length of carbon chain attached to the 1-position of heterocyclic nucleus is an effective factor governing the observed pharmacological properties. The allyl (3 carbon chain length) function seems more enhancing element for developing an anti-inflammatory active agent than ethyl (2 carbon chain length) and methyl (one carbon chain length) residues as exhibited in compounds **3a**, **3d**, **3h** (potency, 75.4, 90.6, 101.0, respectively), **3b**, **3e**, **3i** (potency, 67.0, 81.1, 89.4, respectively) and **5a**, **5c** (potency, 86.4, 114.0, respectively). It is also noticed that, construction of compounds possessing two pharmacologically active moieties (i.e. two triazolopyridinyl systems) is considered an effective tool for developing an enhanced pharmacological active agent as exhibited in compounds **5a**–**c**.

2.3. PGE_2 inhibitory properties

PGE_2 inhibitory properties of the most promising prepared anti-inflammatory active agents (**3f**, **3h**, **5b** and **5c**) were determined using the previously described 6-day air pouch standard method in rats [33] via a commercial PGE_2 assay kit (R&D Systems, Inc., Minneapolis, USA). From the obtained results (Table 2), it has been noticed that all the tested compounds effectively reduce PGE_2 level.

Table 1
Anti-inflammatory activity of the tested compounds using acute carrageenan-induced paw oedema in rats.

Compound	Mean swelling volume "ml" (% inhibition of oedema)				Potency[c]
	1 h	2 h	3 h	4 h	
Control	0.207 ± 0.101[b] (00.0)	0.332 ± 0.146[b] (00.0)	0.520 ± 0.134[b] (00.0)	0.705 ± 0.134[b] (00.0)	–
Indomethacin	0.090 ± 0.081[a] (56.5)	0.158 ± 0.102[a] (52.4)	0.180 ± 0.132[a] (65.4)	0.227 ± 0.170[a] (67.8)	100.0
3a	0.202 ± 0.047[b] (2.4)	0.298 ± 0.133 (10.2)	0.353 ± 0.067[b] (32.1)	0.345 ± 0.127[a] (51.1)	75.4
3b	0.203 ± 0.123[b] (1.9)	0.275 ± 0.109 (17.2)	0.303 ± 0.086[a] (41.7)	0.385 ± 0.143[a] (45.4)	67.0
3c	0.172 ± 0.063 (16.9)	0.287 ± 0.170 (13.6)	0.322 ± 0.168[a] (38.1)	0.447 ± 0.175[a,b] (36.6)	54.0
3d	0.142 ± 0.123 (31.4)	0.110 ± 0.104[a] (66.9)	0.230 ± 0.110[a] (55.8)	0.272 ± 0.121[a] (61.4)	90.6
3e	0.118 ± 0.100 (43.0)	0.232 ± 0.158 (30.1)	0.322 ± 0.169[a] (38.1)	0.317 ± 0.125[a] (55.0)	81.1
3f	0.192 ± 0.116 (7.2)	0.325 ± 0.112[b] (2.1)	0.313 ± 0.144[a] (39.8)	0.230 ± 0.124[a] (67.4)	99.4
3g	0.207 ± 0.122[b] (00.0)	0.328 ± 0.180[b] (1.2)	0.485 ± 0.196[b] (6.7)	0.428 ± 0.157[a,b] (39.3)	58.0
3h	0.160 ± 0.062 (22.7)	0.307 ± 0.105 (7.5)	0.338 ± 0.128[a] (35.0)	0.222 ± 0.129[a] (68.5)	101.0
3i	0.188 ± 0.063 (9.2)	0.268 ± 0.131 (19.3)	0.387 ± 0.242[b] (25.6)	0.278 ± 0.190[a] (60.6)	89.4
5a	0.097 ± 0.096[a] (53.1)	0.293 ± 0.120 (11.7)	0.370 ± 0.167[b] (28.8)	0.292 ± 0.250[a] (58.6)	86.4
5b	0.190 ± 0.063 (8.2)	0.257 ± 0.114 (22.6)	0.307 ± 0.102[a] (41.0)	0.175 ± 0.105[a] (75.2)	110.9
5c	0.188 ± 0.067 (9.2)	0.232 ± 0.084 (30.1)	0.312 ± 0.108[a] (40.0)	0.160 ± 0.066[a] (77.3)	114.0

[a] Statistically significant from the control at $p < 0.05$.
[b] Statistically significant from indomethacin at $p < 0.05$.
[c] Potency was expressed as % oedema inhibition of the tested compounds relative to % oedema inhibition of indomethacin "reference standard" at 4 h effect.

It has also been noticed that the PGE_2 inhibitory properties of the tested compounds coincide greatly with our described anti-inflammatory properties. In other words, compound **5c**, which exhibits the highest promising anti-inflammatory properties also reveals the highest inhibitory level of PGE_2 (87.67 pg/ml) comparable with indomethacin (98.33 pg/ml) which was used as a reference standard in this study. Similarly, compounds **3f**, **3h** and **5b** reveal considerable PGE_2 inhibitory activities (102.33, 94.50, 92.17 pg/ml, respectively).

2.4. Anti-tumor activity

Anti-tumor activity screening of **3b** and **3e**, as representative examples of the synthesized compounds adopted by developmental therapeutics program of National Cancer Institute (Bethesida, Maryland, USA), at a dose of 10 μM utilizing 59 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney was carried out according to the previously reported standard procedure [34–37]. The obtained results represent percentage growth of the tumor cell lines treated with compounds under investigation relative to control cell experiments.

From the observed data it has been noticed that, the tested compounds reflect mild or no activity at all against most of the used human tumor cell lines. However, compound **3e** exhibits considerable anti-tumor properties against leukemia CCRF-CEM and HL-60(TB) cell lines (percentage growth of the tumor cell lines are 43.55 and 42.85, respectively), considering cell line growth inhibition with >50% at a concentration of 10 μM usually seems a noticeable activity.

Finally, it could be concluded that the constructed ring system, [1,2,4]triazolo[1,5-*a*]pyridine, could be a hint for developing good promising pharmacological agent, especially as anti-inflammatory properties. However, the variety of attached functional residues must be considered during designing of these hint targets.

3. Experimental

Melting points are uncorrected and recorded on an Electrothermal 9100 digital melting point apparatus. IR spectra (KBr) were recorded on Bruker Vector 22 and Jasco FT/IR plus 460 spectrophotometers. ^{1}H NMR spectra were recorded on a Varian MERCURY 300 (300 MHz) in DMSO-d_6. The starting compounds **1a–c** [38,39], **2a–d** [40,41] and **4a**, **4b** [42] were prepared according to the previously reported procedures.

3.1. Synthesis of 5-amino-6,8-dicyano-1,7-disubstituted-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolates **3a–i** (general procedure)

A mixture of equimolar amounts of **1a–c** and **2a–d** (10 mmol) in absolute ethanol (25 ml) containing triethylamine (11 mmol) was boiled under reflux for the appropriate time. The separated solid while refluxing was collected, washed with ethanol and crystallized from a suitable solvent affording **3a–i** as yellow crystals.

Table 2
PGE_2 inhibitory activity of the tested compounds.

Compound	Concentration of PGE_2 (pg/ml)
Control	533.33 ± 19.26[b]
Indomethacin	98.33 ± 3.33[a]
3f	102.33 ± 4.74[a]
3h	94.50 ± 3.63[a]
5b	92.17 ± 2.86[a]
5c	87.67 ± 5.55[a]

Results are means of 6 experiments ± standard error.
[a] Statistically significant from the control at $p < 0.05$.
[b] Statistically significant from indomethacin at $p < 0.05$.

3.1.1. 5-Amino-6,8-dicyano-1-methyl-7-[4-(1-piperidinyl)phenyl]-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolate (**3a**)

Reaction time 5 h, crystallizes from *N*,*N*-dimethylformamide–methanol mixture as 1:1 v/v, mp 318–320 °C, yield 69%. IR: ν_{max}/cm^{-1} 3330, 3138 (NH_2), 2222 (C≡N), 1648, 1595 (C=N, C=C). ^{1}H NMR: δ 1.61 (br. s, 6H, piperidinyl 3 CH_2), 3.33 (br. s, 4H, piperidinyl 2 NCH_2), 3.90 (s, 3H, CH_3), 7.08 (d, 2H, arom. H, $J = 9.0$ Hz), 7.41 (d, 2H, arom. H, $J = 9.0$ Hz), 9.32 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{20}H_{19}N_7S$ (389.471): C, 61.67; H, 4.92; N, 25.18. Found: C, 61.87; H, 5.03; N, 25.27.

3.1.2. 5-Amino-6,8-dicyano-1-methyl-7-[4-(4-morpholinyl)phenyl]-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolate (**3b**)

Reaction time 5 h, crystallizes from *N*,*N*-dimethylformamide–methanol mixture as 1:2 v/v, mp 320–322 °C, yield 69%. IR: ν_{max}/cm^{-1} 3350, 3276, 3241, 3179 (NH_2), 2225 (C≡N), 1641, 1591 (C=N, C=C). ^{1}H NMR: δ 3.29 (t, 4H, morpholinyl 2 NCH_2, $J = 4.8$ Hz), 3.76 (t, 4H, morpholinyl 2 OCH_2, $J = 4.8$ Hz), 3.90 (s, 3H, CH_3), 7.12 (d, 2H, arom. H, $J = 9.0$ Hz), 7.44 (d, 2H, arom. H, $J = 8.7$ Hz), 9.34 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{19}H_{17}N_7OS$ (391.451): C, 58.29; H, 4.38; N, 25.05. Found: C, 58.43; H, 4.46; N, 25.21.

3.1.3. 5-Amino-6,8-dicyano-1-methyl-7-[4-(4-methylpiperazinyl)phenyl]-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolate (**3c**)

Reaction time 5 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 1:1 v/v, mp 308–310 °C, yield 67%. IR: ν_{max}/cm^{-1} 3332, 3177 (NH_2), 2220 (C≡N), 1644, 1597 (C=N, C=C). ^{1}H NMR: δ 2.27 (s, 3H, piperazinyl NCH_3), 2.53 (br s, 4H, piperazinyl 2 H_3CNCH_2), 3.31 (br s, 4H, piperazinyl 2 $ArNCH_2$), 3.89 (s, 3H, CH_3), 7.11 (d, 2H, arom. H, $J = 8.7$ Hz), 7.42 (d, 2H, arom. H, $J = 8.7$ Hz), 9.00 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{20}H_{20}N_8S$ (404.494): C, 59.38; H, 4.98; N, 27.71. Found: C, 59.57; H, 5.15; N, 27.91.

3.1.4. 5-Amino-6,8-dicyano-1-ethyl-7-[4-(1-piperidinyl)phenyl]-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolate (**3d**)

Reaction time 5 h, crystallizes from n-butanol, mp 292–294 °C, yield 62%. IR: ν_{max}/cm^{-1} 3264 (NH_2), 2220 (C≡N), 1634, 1588 (C=N, C=C). ^{1}H NMR: δ 1.33 (t, 3H, CH_3, $J = 6.9$ Hz), 1.61 (br s, 6H, piperidinyl 3 CH_2), 3.37 (m, 4H, piperidinyl 2 NCH_2), 4.49 (q, 2H, CH_3CH_2, $J = 6.9$ Hz), 7.08 (d, 2H, arom. H, $J = 9.0$ Hz), 7.42 (d, 2H, arom. H, $J = 8.7$ Hz), 9.33 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{21}H_{21}N_7S$ (403.501): C, 62.51; H, 5.25; N, 24.30. Found: C, 62.45; H, 5.18; N, 24.49.

3.1.5. 5-Amino-6,8-dicyano-1-ethyl-7-[4-(4-morpholinyl)phenyl]-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolate (**3e**)

Reaction time 5 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 1:1 v/v, mp 305–307 °C, yield 62%. IR: ν_{max}/cm^{-1} 3332, 3133 (NH_2), 2220 (C≡N), 1648, 1594 (C=N, C=C). ^{1}H NMR: δ 1.33 (t, 3H, CH_3, $J = 6.9$ Hz), 3.24 (t, 4H, morpholinyl 2 NCH_2, $J = 4.8$ Hz), 3.77 (t, 4H, morpholinyl 2 OCH_2, $J = 4.8$ Hz), 4.49 (q, 2H, CH_3CH_2, $J = 6.9$ Hz), 7.12 (d, 2H, arom. H, $J = 9.3$ Hz), 7.45 (d, 2H, arom. H, $J = 8.7$ Hz), 9.36 (s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{20}H_{19}N_7OS$ (405.471): C, 59.24; H, 4.72; N, 24.18. Found: C, 59.35; H, 4.82; N, 24.40.

3.1.6. 5-Amino-1-benzyl-6,8-dicyano-7-[4-(1-piperidinyl)phenyl]-1H-[1,2,4]triazolo[1,5-a]pyridin-4-ium-2-thiolate (**3f**)

Reaction time 6 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 4:1 v/v, mp 297–299 °C, yield 54%. IR: ν_{max}/cm^{-1} 3274 (NH_2), 2220 (C≡N), 1637, 1592 (C=N, C=C). ^{1}H NMR: δ 1.59 (br s, 6H, piperidinyl 3 CH_2), 3.26 (br s, 4H, piperidinyl 2 NCH_2), 5.74

(s, 2H, NCH_2Ph), 7.02–7.35 (m, 9H, arom. H), 9.40 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{26}H_{23}N_7S$ (465.561): C, 67.07; H, 4.98; N, 21.06. Found: C, 66.90; H, 4.86; N, 21.24.

*3.1.7. 5-Amino-1-benzyl-6,8-dicyano-7-[4-(4-morpholinyl)phenyl]-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate (**3g**)*

Reaction time 6 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 4:1 v/v, mp 283–285 °C, yield 54%. IR: ν_{max}/cm^{-1} 3301, 3252 (NH_2), 2221 (C≡N), 1642, 1595 (C═N, C═C). ^1H NMR: δ 3.21 (t, 4H, morpholinyl 2 NCH_2, $J = 5.1$ Hz), 3.74 (t, 4H, morpholinyl 2 OCH_2, $J = 5.1$ Hz), 5.74 (s, 2H, NCH_2Ph), 7.07–7.38 (m, 9H, arom. H), 9.44 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{25}H_{21}N_7OS$ (467.541): C, 64.22; H, 4.53; N, 20.97. Found: C, 63.99; H, 4.39; N, 21.17.

*3.1.8. 5-Amino-6,8-dicyano-7-[4-(1-piperidinyl)phenyl]-1-(prop-2-en-1-yl)-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate (**3h**)*

Reaction time 5 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 1:1 v/v, mp 276–277 °C, yield 58%. IR: ν_{max}/cm^{-1} 3333, 3271, 3130 (NH_2), 2223 (C≡N), 1643, 1595 (C═N, C═C). ^1H NMR: δ 1.61 (br s, 6H, piperidinyl 3 CH_2), 3.28 (br s, 4H, piperidinyl 2 NCH_2), 5.04-5.24 (m, 4H, CH_2═$CHCH_2$ + NCH_2CH═CH_2), 5.91–6.03 (m, 1H, NCH_2CH═CH_2), 7.07 (d, 2H, arom. H, $J = 9.0$ Hz), 7.38 (d, 2H, arom. H, $J = 8.7$ Hz), 9.35 (br s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{22}H_{21}N_7S$ (415.511): C, 63.59; H, 5.09; N, 23.60. Found: C, 63.50; H, 4.98; N, 23.43.

*3.1.9. 5-Amino-6,8-dicyano-7-[4-(4-morpholinyl)phenyl]-1-(prop-2-en-1-yl)-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate (**3i**)*

Reaction time 5 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 1:1 v/v, mp 282–283 °C, yield 53%. IR: ν_{max}/cm^{-1} 3330, 3271, 3131 (NH_2), 2223 (C≡N), 1645, 1597 (C═N, C═C). ^1H NMR: δ 3.29 (t, 4H, morpholinyl 2 NCH_2, $J = 4.8$ Hz), 3.76 (t, 4H, morpholinyl 2 OCH_2, $J = 4.8$ Hz), 5.04–5.24 (m, 4H, CH_2═$CHCH_2$ + NCH_2CH═CH_2), 5.91–6.04 (m, 1H, NCH_2CH═CH_2), 7.11 (d, 2H, arom. H, $J = 9.0$ Hz), 7.42 (d, 2H, arom. H, $J = 8.7$ Hz), 9.38 (s, 2H, D_2O exchangeable NH_2). Anal. Calcd. for $C_{21}H_{19}N_7OS$ (417.481): C, 60.41; H, 4.59; N, 23.49. Found: C, 60.29; H, 4.53; N, 23.25.

*3.2. Synthesis of [alkanediylbis(oxy-2,1-phenylene)]bis(5-amino-6,8-dicyano-1-substituted-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolates) **5a–c** (general procedure)*

A mixture of **4a**, **4b** (5 mmol) and the corresponding **2a**, **2b** (10 mmol) in absolute ethanol (25 ml) containing triethylamine (11 mmol) was boiled under reflux for the appropriate time. The separated solid while refluxing was collected, washed with ethanol and crystallized from a suitable solvent affording **5a–c** as yellow crystals.

*3.2.1. 7,7′-[1,2-Ethanediylbis(oxy-2,1-phenylene)]bis(5-amino-6,8-dicyano-1-methyl-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate) (**5a**)*

Reaction time 16 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 2:1 v/v, mp 341–343 °C, yield 54%. IR: ν_{max}/cm^{-1} 3291, 3186 (NH_2), 2226 (C≡N), 1642, 1603 (C═N, C═C). ^1H NMR: δ 3.79 (s, 3H, NCH_3), 3.81 (s, 3H, NCH_3), 4.39 (br. s, 4H, 2 OCH_2), 7.11–7.48 (m, 8H, arom. H), 9.40 (s, 4H, D_2O exchangeable 2 NH_2). Anal. Calcd. for $C_{32}H_{22}N_{12}O_2S_2$ (670.72): C, 57.30; H, 3.31; N, 25.06. Found: C, 57.11; H, 3.11; N, 25.20.

*3.2.2. 7,7′-[1,3-Propanediylbis(oxy-2,1-phenylene)]bis(5-amino-6,8-dicyano-1-methyl-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate) (**5b**)*

Reaction time 10 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 1:1 v/v, mp 302–304 °C, yield 53%. IR: ν_{max}/cm^{-1} 3291, 3187 (NH_2), 2228 (C≡N), 1638, 1601 (C═N, C═C). ^1H NMR: δ 2.09 (quintet, 2H, OCH_2CH_2, $J = 6.0$ Hz), 3.82 (s, 6H, 2 NCH_3), 4.17 (br s, 4H, 2 OCH_2), 7.15–7.55 (m, 8H, arom. H), 9.43 (s, 4H, D_2O exchangeable 2 NH_2). Anal. Calcd. for $C_{33}H_{24}N_{12}O_2S_2$ (684.74): C, 57.88; H, 3.53; N, 24.55. Found: C, 57.99; H, 3.57; N, 24.78.

*3.2.3. 7,7′-[1,2-Ethanediylbis(oxy-2,1-phenylene)]bis(5-amino-6,8-dicyano-1-ethyl-1*H*-[1,2,4]triazolo[1,5-*a*]pyridin-4-ium-2-thiolate) (**5c**)*

Reaction time 24 h, crystallizes from *N*,*N*-dimethylformamide–water mixture as 1:1 v/v, mp 308–310 °C, yield 49%. IR: ν_{max}/cm^{-1} 3270, 3177 (NH_2), 2226 (C≡N), 1634, 1600 (C═N, C═C). ^1H NMR: δ 1.21 (t, 3H, NCH_2CH_3, $J = 7.5$ Hz), 1.26 (t, 3H, NCH_2CH_3, $J = 7.2$ Hz), 4.29-4.45 (m, 8H, 2 NCH_2 + 2 OCH_2), 7.11–7.49 (m, 8H, arom. H), 9.42 (br s, 4H, D_2O exchangeable 2 NH_2). Anal. Calcd. for $C_{34}H_{26}N_{12}O_2S_2$ (698.77): C, 58.44; H, 3.75; N, 24.06. Found: C, 58.64; H, 3.94; N, 24.22.

3.3. Anti-inflammatory activity screening

Anti-inflammatory activity screening for the prepared compounds was determined in vivo by the acute carrageenan-induced paw oedema standard method in rats [29–32]. Wister albino rats of either sex (pregnant female animals were excluded) weighing 160–190 g were divided into 14 groups of 6 animals each. Administration of indomethacin (reference standard at a dose of 10 mg/kg body weight) and the tested compounds (**3a–i** and **5a–c**) dissolved in DMSO, at a dose of 50 mg/kg (body weight) was given intraperitoneally 1 h before induction of inflammation. The control group was given DMSO only. Carrageenan paw oedema was induced by subcutaneous injection of 1% solution of carrageenan in saline (0.1 ml/rat) into the right hind paw of rats. Paw volumes were measured volumetrically after successive time intervals (1, 2, 3 and 4 h) with plethysmometer 7140 (UGO BASILE, Italy) and compared with the initial hind paw volume of each rat for determining the oedema volume. Data were collected, checked, revised and analyzed. Quantitative variables from normal distribution were expressed as means ± SE "standard error". The significant difference between groups was tested by using one-way ANOVA followed by post hoc test and the chosen level of significance was $p < 0.05$.

The anti-inflammatory activity was expressed as percentage inhibition of oedema volume in treated animals in comparison with the control group (Table 1).

$$\%\text{Inhibition of oedema} = \frac{V_c - V_t}{V_c} \times 100$$

where V_c and V_t are the volumes of oedema for the control and drug-treated animal groups, respectively.

Potency of the tested compounds was calculated relative to indomethacin "reference standard" treated group according to the following equation.

Potency = (% Oedema inhibition of tested compound treated group)/(% Oedema inhibition of indomethacin treated group).

3.4. Measurement of PGE_2 level

Measurement of PGE_2 level was determined by the previously described 6-day air pouch standard method in rats [33]. Male albino rats weighing 200–250 g were divided into 6 groups of 6 animals each. The air pouch was induced as follows, on the first day of the experiment; 20 ml of air was injected subcutaneously at the back of each rat. Two days later, another 10 ml of air was injected at the same site. On the fifth day after the first injection, a further 10 ml of air was injected into the pouch. Then, 24 h later and before injecting the pouch with carrageenan (2 ml of 1% solution in saline),

four groups of animals were treated orally with the tested compounds (**3f**, **3h**, **5b** and **5c**) "at a dose of 50 mg/kg body weight", one group with indomethacin (reference standard) "at a dose of 10 mg/kg body weight" suspended in saline solution by the aid of few drops of Tween 80 and the last group with sterile saline (control group). All injections were given under light ether anaesthesia. Six hours after the carrageenan injection, animals were lightly anaesthetised with ether and the contents of the pouch were aspirated using a Pasteur pipette and transferred into graduated plastic tube kept in ice. The bulk of the exudates was frozen and stored at −20 °C until required for PGE_2 assay. PGE_2 was measured by an ELISA (Beckman Biomek™ 1000 automated laboratory workstation apparatus) technique using PGE_2 assay kit supplied by R&D Systems Inc., Minneapolis, USA, according to the manufacturer's specifications.

3.5. Anti-tumor activity screening

Anti-tumor activity screening for the synthesized compounds (**3b**, **3e**) at a dose of 10 μM utilizing 59 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney was carried out using adriamycin as a reference standard according to the previously reported standard procedure [34–37]. The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated in 96-well-microtiter plates in 100 μl at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental tested compounds. After 24 h, two plates of each cell lines are fixed in situ with trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of tested compound addition (time zero, T_z). Experimental tested compounds are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of the tested compound addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Aliquots of 100 μl of the tested compound dilutions are added to the appropriate microtiter wells already containing 100 μl of medium, resulting in the required final concentrations.

Following the tested compound addition, the plates are incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate readed at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 μl of 80% TCA (final concentration, 16% TCA).

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