Design, Synthesis and Biological Evaluation of New Thieno[2,3-d]pyrimidines as Anti-inflammatory Agents

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Abstract: Background: Long term use of NSAIDS is mainly accompanied by major health implications such as gastrointestinal erosions, ulcerations and nephrotoxicity. These side effects arise from local irritation by the carboxylic acid moiety, that is common to most of NSAIDs (topical effect), in addition to decreased cytoprotective prostaglandin production. Therefore, in the medicinal chemistry research area, there is an ongoing need for the discovery of new, potent and safer anti-inflammatory lead compounds devoid of the irritant carboxylic acid moiety.

Methods: A series of new 3-substituted-2-thioxo-thieno[2,3-d]pyrimidine derivatives were synthesized through reacting the starting 3-amino-2-thioxo-thieno[2,3-d]pyrimidines with different aromatic aldehydes. The structure of all newly synthesized compounds was confirmed with spectral and elemental analyses. The synthesized thieno[2,3-d]pyrimidines were investigated for in vivo anti-inflammatory activity, using the carrageenan induced paw edema test. The possible anti-inflammatory mechanism was also evaluated by determining the concentration of prostaglandin E2 (PGE2) in blood serum using a rat specific PGE2 ELISA kit.

Results: All test compounds could significantly reduce carrageenan induced paw edema comparable to diclofenac sodium as a potent anti-inflammatory drug. Moreover, they could decrease the concentration of PGE2 in blood serum. Interestingly, compound 4c exhibited the most potent in vivo anti-inflammatory activity with protection of 35%, 36% and 42% against carrageenan-induced paw edema after 1h, 2h and 3h, representing 92%, 86% and 88% respectively of diclofenac activity. It also decreased the concentration of PGE2 in blood serum to 19 pg/mL which is comparable to diclofenac with PGE2 concentration of 12 pg/mL. Moreover, Compounds 4f, 4a, 4i and 4e exerted significant anti-inflammatory activity after 4h, representing 71%, 69%, 63% and 61% respectively of diclofenac activity. Furthermore, they significantly decreased the concentration of PGE2 in blood serum.

Conclusion: These thienopyrimidines may be used as good candidates for the search of promising, potent and safe anti-inflammatory leads for being free from acidic functions.

Keywords: Anti-inflammatory activity, synthesis, 2-Thioxo-thieno[2,3-d]pyrimidines, prostaglandin E2 (PGE2).

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDS) are important class of widely used therapeutics for the treatment of inflammation and pain [1, 2]. The pharmacological effects of NSAIDS are due to the inhibition of a membrane enzyme called cyclooxygenase (COX), which is involved in the prostaglandin biosynthesis [3, 4]. 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. Two isoforms of COX are found; COX-1, which is constitutive, regularly expressed and involved in the production of cytoprotective prostaglandins, mainly having a physiological role in kidneys and stomach and is responsible for maintaining homeostasis (gastric and renal integrity) [5], while COX-2 is involved in the production of
prostaglandins mediating pain such as prostaglandin E2 (PGE2) and associated with inflammatory states [6, 7]. Long term use of NSAIDS is mainly accompanied by major health implications such as gastrointestinal erosions, ulcerations and nephrotoxicity [8, 9]. These side effects arise from local irritation by the carboxylic acid moiety, that is common to most of NSAIDs (topical effect), in addition to decreased cytoprotective prostaglandin production. Therefore, in the medicinal chemistry research area, there is an ongoing need for the discovery of new, potent and safer anti-inflammatory lead compounds devoid of the irritant carboxylic acid moiety. The thieno[2,3-d]pyrimidine system is considered today as a relevant pharmacophore, due to its diverse biological activities, and particularly, it was found to be an integral part of numerous potent anti-inflammatory agents [10-17] (Fig. 1). One of the most interesting characteristics of these compounds is their non-acidic structure. Certain thieno[2,3-d]pyrimidines were originally prepared as bioisosters of potent anti-inflammatory quinazolines [18-20] including Proquazone (Biarison®) (Fig. 2) [21] which is a marketed NSAID. It was found that a wide structure variations of thieno[2,3-d]pyrimidines, possessing an interesting anti-inflammatory activity, has been synthesized. The structure variations include substituted thieno, or cyclohexyl fused thieno moiety, a mercapto group or another substitution on pyrimidine C-2, amino group or substituted amino on pyrimidine N-3. The presence of the pyrimidine-4-one was the structural constant. We have designed and synthesized some new 3-substituted-2-thioxo-cycloalkylthieno [2,3-d]pyrimidines. Two elements in the target compounds were varied, while the 2-thioxo group and the pyrimidin-4-one were reserved:

1) The N3- substituents in pyrimidine.

2) The size of cycloalkyl ring fused to the thiophene ring to substantiate the possible effects of such variations on the in vivo anti-inflammatory activity using the carrageenan induced rat paw edema test and compared to diclofenac sodium as a potent anti-inflammatory drug. The possible anti-inflammatory mechanism was also evaluated by determining the concentration of PGE2 in blood serum, using rat specific PGE2 ELISA kit, which reflects the effect of our synthesized compounds on inhibition of COX-2 enzyme.

Fig. (1). Structures of potent anti-inflammatory thienopyrimidines (A, B, C and D) and the designed thienopyrimidines (3a,b and 4a-j).
MATERIALS AND METHODS

Chemistry

Melting points were obtained on a Griffin apparatus and were uncorrected. Microanalyses for C, H and N were carried out at the Microanalytical center, Cairo University. IR spectra were recorded on a Shimadzu 435 spectrometer, Faculty of Pharmacy, Cairo University, Cairo, Egypt using KBr discs. 1H NMR spectra were performed on joel NMR FXQ-300 MHz and Bruker 400 MHz spectrophotometers. 13C NMR were carried out on Bruker 100 MHz spectrophotometer using TMS as the internal standard. Mass spectra were recorded on a GCMP-QP1000 EX Mass spectrometer. Progress of the reactions were monitored by TLC using precoated aluminum sheet silica gel MERCK 60F 254 and was visualized by UV lamp.

General Procedure for the Preparation of ethyl-2-(((methylthio)carbonothioyl)amino)thiophene-3-carboxylate (2a,b)

To a vigorously stirred solution of 2-amino-3-carbethoxy-4,5-substituted thiophene (1) (0.02 mol) in dimethyl sulphoxide (10 mL) at room temperature, carbon disulphide 1.98 g (0.026 mol) and aqueous sodium hydroxide 1.2 mL (20 mol solution) were added simultaneously over 30 min. Stirring was continued for a further 30 min. Dimethyl sulphone 2.5 g (0.02 mol) was added dropwise to the reaction mixture with stirring at 5-10 °C; it was further stirred for 3 h and poured into ice-water; the solid obtained was filtered, dried and recrystallized from ethanol.

**Ethyl 2-(((methylthio)carbonothioyl)amino)-5,6,7,8-tetrahydro -4H-cyclohepta[b]thiophene-3-carboxylate** (2a)

mp 165-167 °C (ethanol); yield 61%; IR (KBr) νmax: 3170 (NH), 1654 (C=O), 1232 (C=S) cm⁻¹; 1H NMR (CDCl₃): δ 1.39-1.44 (t, 3H, J= 7.2 Hz , CH₂CH₃), 1.63-1.71 (m, 4H, 2CH₂), 2.73 (s, 3H, SCH₃), 2.75-2.83 (t, 2H, J= 5.4 Hz, CH₂), 3.05-3.08 (t, 2H, J= 5.4 Hz, CH₂), 4.36-4.43(q, 2H, J= 7.2 Hz , CH₂CH₃) and 12.93 (s, 1H, NH, D₂O exchangeable) ppm; MS [m/z, %]: 329 [M⁺, 6.61]. Anal. Calcd for C₁₄H₁₉NO₂S₃ (329.50): C, 51.03; H, 5.81; N, 4.25. Found: C, 51.17; H, 5.89; N, 4.37.

**Ethyl 2-(((methylthio)carbonothioyl)amino)-4,5,6,7,8,9-hexahydro-cycloocta[b]thiophene-3-carboxylate** (2b)

mp 190-192 °C (ether); yield 54%; IR (KBr) νmax: 3120 (NH), 1654 (C=O), 1234 (C=S) cm⁻¹; 1H NMR (DMSO-d₆): δ 1.25-1.30 (m, 2H, CH₂), 1.32-1.34 (t, 3H, J= 7.2 Hz , CH₂CH₃), 1.35-1.43 (m, 2H, CH₂), 1.55-1.65 (m, 4H, 2CH₂), 2.54 (s, 3H, SCH₃), 2.70-2.80 (m, 2H, CH₂), 2.82-2.86 (m, 2H, CH₂), 4.23-4.32 (q, 2H, J= 7.2 Hz , CH₂CH₃) and 11.76 (s, 1H, NH, D₂O exchangeable) ppm; MS [m/z, %]: 329 [M⁺, 6.61]. Anal. Calcd for C₁₅H₂₁NO₂S₃ (343.53): C, 52.44; H, 6.16; N, 4.08. Found: C, 52.71; H, 6.24; N, 4.13.

General Procedure for the Preparation of 3-amino-2-thioxo-thieno[2,3-d]pyrimidin-4-ones (3a,b)

A mixture of 2a,b (0.01 mol) and hydrazine hydrate (4.3 g, 0.01 mol, 99%) in absolute ethanol (30 mL) was heated under reflux on a water bath until the methylmercaptan evolution ceases (8 h). The separated solid after cooling was filtered, dried and recrystallized from ethanol.

**3-Amino-2-thioxo-thieno[2,3-d]pyrimidin-4(5H)-one** (3a)

mp 280-282 °C (ethanol); yield 55%; IR (KBr) νmax: 3147, 3118 (NH₂, NH), 1670 (C=O), 1220 (C=S) cm⁻¹; 1H NMR (DMSO-d₆): δ 1.55-1.70 (m, 2H, CH₂), 1.80-1.85 (m, 2H, CH₂), 2.71-2.73 (m, 2H, CH₂), 3.15-3.21 (m, 2H, CH₂), 5.26 (s, 2H, NH₂, D₂O exchangeable) and 5.65 (s, 1H, NH, D₂O exchangeable) ppm; MS [m/z, %]: 267 [M⁺, 100]. Anal. Calcd for C₁₁H₁₃N₃O₂ (267.37): C, 49.41; H, 4.90; N, 15.72. Found: C, 49.62; H, 4.98; N, 15.89.
3-Amino-2-thioxo-2,3,5,6,7,8,9,10-octahydrocycloocta[4,5]thieno[2,3-d]pyrimidin-4(1H)-one (3b)

mp 280-282 °C (ethanol); yield 50 %; IR (KBr) vmax: 3199, 3174 (NH, NH), 1676 (C=O), 1255 (C=S) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.24-1.32 (m, 2H, CH₂), 1.35-1.48 (m, 2H, CH₂), 1.55-1.61 (m, 4H, 2CH₂), 2.85-2.95 (m, 2H, CH₂), 5.69 (s, 2H, NH₂, D₂O exchangeable) and 12.21 (s, 1H, NH, D₂O exchangeable) ppm; MS [m/z, %]: 281 [M+, 4.25]. Anal. Calcd for C₁₂H₁₅N₃OS₂ (281.40): C, 51.22; H, 5.37; N, 14.93. Found: C, 51.40; H, 5.43; N, 15.09.

General Procedure for the Preparation of 3-substituted-2-thioxo-thieno[2,3-d]pyrimidin-4-ones (4a-j)

A mixture of 3a,b (0.001 mol) and the selected aromatic aldehyde (0.001 mol) in dimethylformamide (5 mL) containing glacial acetic acid (0.2 mL) was refluxed for 24 h. The mixture was cooled, filtered and the precipitate was recrystallized from the appropriate solvent.

3-(Benzylideneamino)-2-thioxo-2,3,6,7,8,9-hexahydro-1H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4(5H)-one (4a)

mp 188-190 °C (ethanol); yield 54 %; IR (KBr) vmax: 3338 (NH), 1681 (C=O), 1161 (C=S) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.50-1.70 (m, 4H, 2CH₂), 1.85-1.95 (m, 2H, CH₂), 2.80-2.95 (m, 4H, 2CH₂), 6.02 (s, 1H, NH, D₂O exchangeable), 7.32-7.40 (t, 1H, ArH), 7.65-7.69 (t, 2H, ArH), 7.99 (d, 2H, ArH) and 8.15 (s, 1H, N=CH) ppm; MS [m/z, %]: 355 [M+, 23.17]. Anal. Calcd for C₁₈H₁₇N₃O₃S₂ (355.48): C, 60.82; H, 4.82; N, 11.82. Found: C, 60.97; H, 4.85; N, 11.98.

3-((2-Nitrobenzylidene)amino)-2-thioxo-2,3,6,7,8,9-hexahydro-1H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4(5H)-one (4c)

mp 250-252 °C (ethanol); yield 70 %; IR (KBr) vmax: 3323 (NH), 1658 (C=O), 1527, 1350 (NO₂), 1242 (C=S) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.60-1.70 (m, 4H, 2CH₂), 1.85-1.90 (m, 2H, CH₂), 2.85-2.90 (m, 4H, 2CH₂), 6.68-6.73 (t, 1H, J= 8.1 Hz, ArH), 6.93 (d, 1H, J= 8.1 Hz, ArH), 7.03 (s, 1H, NH, D₂O exchangeable), 7.29-7.34 (t, 1H, J= 8.1 Hz, ArH), 7.42 (d, 1H, J= 8.1 Hz, ArH) and 7.95 (s, 1H, N=CH) ppm; ¹³C NMR (DMSO-d₆): δ 20.41, 26.67, 28.98, 31.77, 34.33, 118.29, 124.70, 125.36, 128.34, 130.97, 131.93, 134.43, 135.08, 135.72, 136.29, 151.46, 169.53, 172.50 ppm; MS [m/z, %]: 400 [M+, 22.76]. Anal. Calcd for C₁₈H₁₆N₄O₃S₂ (400.47): C, 53.98; H, 4.03; N, 13.99. Found: C, 54.14; H, 4.11; N, 14.18.

3-((2-Nitrobenzylidene)amino)-2-thioxo-2,3,5,6,7,8,9,10-octahydro-cycloocta[4,5]thieno[2,3-d]pyrimidin-4(1H)-one (4e)

mp 280-282 °C (benzene); yield 60 %; IR (KBr) vmax: 3294 (NH), 1680 (C=O), 1541, 1319 (NO₂), 1240 (C=S) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.25-1.40 (m, 2H, CH₂), 1.45-1.50 (m, 2H, CH₂), 1.55-1.65 (m, 4H, 2CH₂), 2.70-2.85 (m, 2H, CH₂), 2.95-3.05 (m, 2H, CH₂), 5.65 (s, 1H, NH, D₂O exchangeable) 7.32 (d, 1H, ArH), 7.40-7.45 (t, 1H, ArH), 7.62 (d, 1H, ArH), 7.81-7.84 (t, 1H, ArH) and 8.05 (s, 1H, N=CH) ppm; MS [m/z, %]: 414 [M⁺, 5.56]. Anal. Calcd for C₁₉H₁₈N₄O₃S₂ (414.50): C, 55.05; H, 4.38; N, 13.52. Found: C, 55.21; H, 4.50; N, 13.78.

3-((4-Bromobenzylidene)amino)-2-thioxo-2,3,5,6,7,8,9,10-octahydro-cycloocta[4,5]thieno[2,3-d]pyrimidin-4(1H)-one (4f)

mp 250-252 °C (ethanol); yield 68 %; IR (KBr) vmax: 3327 (NH), 1687 (C=O), 1190 (C=S) cm⁻¹; ¹H NMR (DMSO-d₆): δ 1.50-1.65 (m, 4H, 2CH₂), 1.80-1.90 (m, 2H, CH₂), 2.70-2.90 (m, 4H, 2CH₂), 5.67 (s, 1H, NH, D₂O exchangeable) and 12.21 (s, 1H, NH, D₂O exchangeable) ppm; ¹³C NMR (DMSO-d₆): δ 24.88, 25.72, 25.95, 26.73, 30.19, 31.90, 118.54, 127.74, 128.90, 129.39, 130.11, 133.22, 135.11, 154.01, 157.50, 161.50, 178.20 ppm; MS [m/z, %]: 369 [M⁺, 16.33]. Anal. Calcd for C₁₀H₁₉N₃O₂S₂ (369.50): C, 61.76; H, 5.18; N, 11.37. Found: C, 61.88; H, 5.24; N, 11.52.
5.65 (s, 1H, NH, D$_2$O exchangeable), 7.24 (d, 2H, ArH), 7.61 (d, 2H, ArH) and 8.68 (s, 1H, N=CH) ppm; MS [m/z, %]: 435 [(M+2)$^+$, 9.02], 433 [M$^+$, 20.77]. Anal. Calcd for C$_{18}$H$_{16}$BrN$_3$OS$_2$ (434.37): C, 49.77; H, 3.71; N, 9.67. Found: C, 49.93; H, 3.72; N, 9.84.

3-((4-Bromobenzylidene)amino)-2-thioxo-2,3,5,6, 7,8,9,10-octahydro- cycloocta[4,5]thieno[2,3-d]pyrimidin-4(1H)-one (4f)

mp 280-282 °C (acetonitrile); yield 60 %; IR (KBr) $v_{\text{max}}$: 3320 (NH), 1683 (C=O), 1238 (C=S) cm$^{-1}$; $^1$H NMR (DMSO-d$_6$): δ 1.17-1.55 (m, 8H, 4CH$_2$), 2.80-2.95 (m, 4H, 2CH$_2$), 5.69 (s, 1H, NH, D$_2$O exchangeable), 7.30 (d, 2H, ArH), 7.61 (d, 2H, ArH) and 8.45 (s, 1H, N=CH) ppm; MS [m/z, %]: 447 [(M-H$^+$, 0.24]. Anal. Calcd for C$_{19}$H$_{18}$BrN$_3$OS$_2$ (448.40): C, 50.89; H, 4.05; N, 9.37. Found: C, 51.08; H, 4.09; N, 9.44.

3-((4-Chlorobenzylidene)amino)-2-thioxo-2,3,6, 7,8,9-hexahydro-1H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4(5H)-one (4g)

mp 272-274 °C (ethanol); yield 80 %; IR (KBr) $v_{\text{max}}$: 3369 (NH), 1685 (C=O), 1242 (C=S) cm$^{-1}$; $^1$H NMR (DMSO-d$_6$): δ 1.60-1.70 (m, 4H, 2CH$_2$), 1.85-1.95 (m, 2H, CH$_2$), 2.85-2.95 (m, 4H, 2CH$_2$), 7.45 (s, 1H, NH, D$_2$O exchangeable), 7.70 (d, 2H, ArH), 7.95 (s, 1H, N=CH) and 7.99 (d, 2H, ArH) ppm; MS [m/z, %]: 391 [(M+2)$^+$, 12.99], 389 [M$^+$, 100]. Anal. Calcd for C$_{18}$H$_{16}$ClN$_3$OS$_2$ (389.92): C, 55.45; H, 4.14; N, 10.78. Found: C, 55.62; H, 4.49; N, 10.91.

Pharmacological Activity

Animals

Adult male albino Wister rats weighing 150-200 g were used in the present study. Rats were purchased from the Modern Veterinary Office for Laboratory Animals (Cairo, Egypt). Rats were kept under constant laboratory conditions and were allowed free access to food and water throughout the period of investigation. Animals were divided into different experimental groups; 6 rats for each with food and water ad libitum. All experiments were conducted between 16:00 and 18:00 h to ensure animal activity and to eliminate circadian influence on animal behavior. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Faculty of Pharmacy, Cairo University, Cairo, Egypt (serial number of the protocol: OC 1322 in 31/3/2015).
Preparation of the Test Compounds and the Standard Drug

Test compounds and diclofenac sodium, used as a reference anti-inflammatory drug, were suspended in a 1% sodium carboxymethylcellulose (CMC) aqueous solution. Therapies were administered orally to rats in a dose level of 10 mg/kg. Control rats received appropriate volumes of the dosing vehicle. Carrageenan (Sigma Aldrich, St. Louis, Missouri, USA) was freshly prepared as suspension (1% w/v in 0.9% saline, injected as 0.1 mL/rat).

In vivo Anti-inflammatory Activity

The anti-inflammatory activity of the newly synthesized thienopyrimidines was evaluated in vivo using the carrageenan induced paw edema test [22]. Suspensions of test compounds and diclofenac in CMC solution (0.5% w/v in water) were administered orally in a dose level of (10 mg/kg). Control animals were similarly treated with CMC solution (0.5% w/v in water). After 30 min, 0.1 mL of freshly prepared 1% carrageenan solution in normal saline was injected into the sub plantar region of the right hind paw of rats according to the method of Winter et al. An equal volume of saline was injected into the left hind paw of each rat. The right paw thickness was measured using the mercury displacement technique with the help of a plethymograph directly before and after 1, 2, 3, 4 h following carrageenan injection. The anti-inflammatory activity of test compounds and diclofenac was calculated as percentage decrease in edema thickness induced by carrageenan using the following formula:

\[
\% \text{ of edema inhibition} = \frac{(V_R - V_L)_{control} - (V_R - V_L)_{treated}}{(V_R - V_L)_{control}} \times 100
\]

Where \(V_R\) represents the mean right paw thickness and \(V_L\) represents the mean left paw thickness. \((V_R - V_L)_{control}\) represents the mean increase in paw thickness in the control group of rats. \((V_R - V_L)_{treated}\) represents the mean increase in paw thickness in rats treated with test compounds.

In vitro Suppression of PGE\(_2\) Biosynthesis

For determining the content of PGE\(_2\) in blood serum, all rats were bled from eyes, and the blood was put in Eppendorf tubes and centrifuged for 10 min at 3000 r/min, then the supernatant was collected to determine the content of PGE\(_2\) by using a PGE\(_2\) rat specific ELISA kit (R & D System GmbH, Wiesbaden, Germany) through adopting the kit instructions [23] and using diclofenac as a standard. PGE\(_2\), presenting in a serum sample, competes with horseradish peroxidase (HRP)-labeled PGE\(_2\) for a limited number of binding sites on a mouse monoclonal antibody. 200 \(\mu\)L of calibrator diluent was added to the non-specific binding (NSB) wells. 150 \(\mu\)L of calibrator diluent was added to the zero standard (B0) wells, then 150 \(\mu\)L of standard, control, or sample was added to the remaining wells. 50 \(\mu\)L of the primary antibody solution was added to each well (excluding the NSB wells). Then they were incubated for 1 h at room temperature. 50 \(\mu\)L of PGE\(_2\) conjugate was added to each well. They were incubated for 2 h at room temperature on the shaker. Each well was washed four times, with wash buffer (400 \muL). 200 \(\mu\)L of substrate solution was added to each well which was incubated for 30 minutes at room temperature. Then, 100 \(\mu\)L of stop solution was added to each well. The optical density of each well was measured within 30 minutes, using a microplate reader set to 450 nm. A standard curve was created by reducing the data and using computer software. Then, the concentration of PGE\(_2\), corresponding to the mean absorbance, was calculated from the standard curve.

Statistical Analysis

The data used in statistical analyses were obtained from six animals for each group. Data were collected, tabulated and expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test were used for comparison of means of different groups. Differences between groups were considered statistically significant at a level of \(p < 0.05\).

RESULTS AND DISCUSSION

Chemistry

The synthetic route to the target thieno[2,3-d]pyrimidines is outlined in Scheme 1 and the o-amino esters of thiophene 1a,b were selected as our primary starting materials for this series of reactions, and were prepared via two steps method according to the reported procedure [24]. Compounds 1a,b were reacted with carbon disulfide...
and sodium hydroxide to give the sodium salt of dithiocarbamate, followed by methylation with dimethyl sulfate to give compounds 2a,b. The $^1$H NMR spectra of 2a and 2b showed a singlet signal at $\delta$ 2.73 and 2.54 ppm, corresponding to SCH$_3$ protons. In addition, NH proton appeared as an exchangeable singlet signal at $\delta$ 12.93 and 11.76 ppm respectively.

Reacting compounds 2a,b with hydrazine hydrate in ethanol afforded the thiosemicarbazides, which undergo internal cyclization to yield 3-amino-2-thioxo-thieno[2,3-d]pyrimidines 3a,b. The $^1$H NMR spectra of 3a and 3b showed two exchangeable singlet signals at $\delta$ 5.26, 5.69 and $\delta$ 5.65, 12.21 ppm, corresponding to NH$_2$ and NH protons. Further evidence was obtained from the IR spectra that showed absorption bands at 3147-3118 and 3199-3174 cm$^{-1}$, corresponding to NH and NH$_2$ groups, while the C=O group appeared as an absorption band at 1670 and 1676 cm$^{-1}$, respectively.

The 3-substituted-2-thioxo-thieno[2,3-d]pyrimidine derivatives 4a-j were obtained through the reaction of 3a,b with the appropriate aromatic aldehyde in dry dimethylformamide in the presence of glacial acetic acid.

The $^1$H NMR spectra of the products 4a-j revealed the presence of NH proton exchangeable signals resonating at the range of $\delta$ 5.65-8.05 ppm, in addition to the expected signals corresponding to different N-substituted groups, which were indicative for the success of the reaction.
Anti-inflammatory Activity

The in vivo anti-inflammatory activity of all newly synthesized thieno[2,3-d]pyrimdines was evaluated using carrageenan induced paw edema test in rats. Test compounds and reference drug diclofenac sodium were administered orally at a dose level of 10 mg/kg; 30 minutes before carrageenan injection at the right hind paw of Albino male rats. The thickness of both paws was measured at different times of 1, 2, 3 and 4 h after carrageenan injection. The anti-inflammatory activity of the test compounds and diclofenac sodium was calculated as the percentage decrease in edema thickness induced by carrageenan and was determined using the following formula:

\[
\text{% of edema inhibition} = \left( \frac{(V_R - V_L)_{\text{control}} - (V_R - V_L)_{\text{treated}}}{(V_R - V_L)_{\text{control}}} \right) \times 100
\]

Where \(V_R\) represents the mean right paw thickness and \(V_L\) represents the mean left paw thickness. \((V_R - V_L)_{\text{control}}\) represents the mean increase in paw thickness in the control group of rats. \((V_R - V_L)_{\text{treated}}\) represents the mean increase in paw thickness in rats treated with the test compounds. The results are listed in Table 1, showing the percentage of edema inhibition induced by carrageenan for test compounds and diclofenac sodium versus time in h. The obtained in vivo results indicated that all of the test compounds revealed significant (p < 0.05) inhibition against carrageenan induced paw edema in rats and the maximum anti-inflammatory activity was obtained after 4 h. Diclofenac sodium showed protection percentages of 38%, 42%, 48% and 51% against carrageenan induced paw edema after 1h, 2h, 3h and 4h respectively. Compound 4c exhibited the most potent remarkable anti-inflammatory activity with protection of 35%, 36% and 42% against carrageenan-induced paw edema after 1h, 2h and 3h, representing 92%, 86% and 88%, respectively of diclofenac activity. Compounds 4f, 4a, 4i and 4e exerted significant anti-inflammatory activity with protection of 36%, 35%, 32% and 31% against carrageenan-induced paw edema after 4h, representing 71%, 69%, 63%

Table 1. The anti-inflammatory activity of synthesized thienopyrimidines in a dose level of 10 mg/kg at different times compared to diclofenac.

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<tr>
<th>Compound</th>
<th>% Of edema inhibition (% mean ± SD)</th>
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<tr>
<td></td>
<td>1h</td>
</tr>
<tr>
<td>3a</td>
<td>26±1.41*</td>
</tr>
<tr>
<td>3b</td>
<td>26±1.41*</td>
</tr>
<tr>
<td>4a</td>
<td>28±1.01*</td>
</tr>
<tr>
<td>4b</td>
<td>23±1.32*</td>
</tr>
<tr>
<td>4c</td>
<td>35±1.41*</td>
</tr>
<tr>
<td>4d</td>
<td>23±1.31*</td>
</tr>
<tr>
<td>4e</td>
<td>22±1.42*</td>
</tr>
<tr>
<td>4f</td>
<td>25±1.29*</td>
</tr>
<tr>
<td>4g</td>
<td>24±1.22*</td>
</tr>
<tr>
<td>4h</td>
<td>21±1.25*</td>
</tr>
<tr>
<td>4i</td>
<td>28±1.01*</td>
</tr>
<tr>
<td>4j</td>
<td>18±1.44*</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>38±1.22*</td>
</tr>
<tr>
<td>Control</td>
<td>2±0.21</td>
</tr>
</tbody>
</table>

Bold values indicate the most active compounds.

*Significantly different from control group at P < 0.05.

Note. one way ANOVA test was applied to determine the significance of the difference between the control group and rats treated with the test compounds (n = 6).
and 61%, respectively of diclofenac activity. Compounds 3b, 3a and 4b showed moderate activity with protection of 30% and 28% against carrageenan induced paw edema after 4h, representing 59% and 55%, respectively of diclofenac activity. Compounds 4g, 4h, 4j and 4d showed variable anti-inflammatory activity, ranging from 21% to 27% that represents from 41% to 53% of diclofenac activity.

It was found that 3-amino-2-thioxo-thienopyrimidines 3a and 3b showed moderate anti-inflammatory activity. Introduction of 2-nitrobenzylideneamino group (4c) resulted in a marked increase in the anti-inflammatory activity compared to the 3-amino derivative, on the other hand, compounds bearing 4-bromobenzylideneamino group (4e and 4f), benzylideneamino group (4a), 4-flourobenzylideneamino group (4i) showed significant anti-inflammatory activity. Substitution of N-3 of pyrimdine with 4-chlorobenzylideneamino group in compounds 4g and 4h is associated with a decrease in the anti-inflammatory activity. Results in Table 1 also showed that the thienopyrimidines (4c, 4e, 4a and 4i) with cycloheptyl moiety fused to the thiophene ring remarkably exerted more potent anti-inflammatory activity than the thienopyrimidines with cyclooctyl moiety.

Content of PGE2 in Blood Serum

In an attempt to explore the mechanism of anti-inflammatory effect produced by the newly synthesized compounds, in vitro assay for PGE2 was estimated by using a PGE2 rat specific ELISA kit (R & D System GmbH, Wiesbaden, Germany) through adopting the kit instructions and using diclofenac as a standard. The concentration of PGE2 in serum for all test compounds as well as diclofenac after 4 h was determined as picograms of antigen per milliliter of protein (pg/mL) (Table 2). It was found that all thienopyrimidines significantly (p < 0.05) decreased the concentration of PGE2 in serum. It is obvious that compound 4c which exhibited the most potent in vivo anti-inflammatory activity also remarkably decreased the concentration of PGE2 in serum to 19 pg/mL comparable to diclofenac, which decreased the concentration of PGE2 in serum to 26 pg/mL. Furthermore, compounds 4a, 4f and 4i exerting significant in vivo anti-inflammatory activity decreased PGE2 concentration in serum to 23, 21 and 22 pg/mL, respectively. Compounds 4d, 4h and 4j showed PGE2 concentration: 22 and 21 pg/mL. Moreover, compounds 3a and 3b, that showed moderate in vivo anti-inflammatory activity, decreased PGE2 concentration in serum to 26 and 25 pg/mL, respectively, and compounds 4b, 4e and 4g exhibited moderate effect on the production of PGE2.

Table 2. Effect of synthesized thienopyrimidines in a dose level of 10 mg/ kg on the content of PGE2 in blood serum after 4h compared to diclofenac.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PGE2 (pg/mL) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>26±3.21*</td>
</tr>
<tr>
<td>3b</td>
<td>25±2.31*</td>
</tr>
<tr>
<td>4a</td>
<td>23±2.13*</td>
</tr>
<tr>
<td>4b</td>
<td>28±2.93*</td>
</tr>
<tr>
<td>4c</td>
<td>19±2.56*</td>
</tr>
<tr>
<td>4d</td>
<td>22±3.22*</td>
</tr>
<tr>
<td>4e</td>
<td>25±2.65*</td>
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<tr>
<td>4f</td>
<td>21±3.27*</td>
</tr>
<tr>
<td>4g</td>
<td>29±3.15*</td>
</tr>
<tr>
<td>4h</td>
<td>22±3.41*</td>
</tr>
<tr>
<td>4i</td>
<td>22±2.42*</td>
</tr>
<tr>
<td>4j</td>
<td>21±3.21*</td>
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<tr>
<td>Diclofen</td>
<td>12±1.74*</td>
</tr>
<tr>
<td>Control</td>
<td>45±2.22</td>
</tr>
</tbody>
</table>

Bold values indicate the most active compounds.
*Significantly different from control group at P < 0.05.

Note. one way ANOVA test was applied to determine the significance of the difference between the control group and rats treated with the test compounds (n = 6).

CONCLUSION

A series of new 3-substituted-2-thioxothieno[2,3-d]pyrimidine derivatives were synthesized and confirmed with spectral and elemental analyses. Most of the synthesized compounds showed significant in vivo anti-inflammatory activity. Moreover, the newly synthesized thienopyrimidines remarkably decreased the concentration of PGE2 in serum. Compound 4c exhibited the
most potent *in vivo* anti-inflammatory activity after 1h, 2h and 3h, representing 92%, 86% and 88%, respectively, of diclofenac activity. It also, decreased the concentration of PGE2 in serum to 19 pg/mL comparable to diclofenac. Further, compounds 4a, 4f and 4i, exerting significant *in vivo* anti-inflammatory activity, showed significant decrease in PGE2 concentration in serum. So the anti-inflammatory activity of these compounds may be attributed to the suppression of PGE2 biosynthesis. Thus, these thienopyrimidines may be used as good candidates for the search of promising, potent and safe anti-inflammatory leads for being free from acidic functions.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest regarding the publication of this paper.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


[23] Prostaglandin E2 Assay kit (Catalog No. KGE004B), R & D System GmbH, Wiesbaden, Germany.