

Pharmacological activities of some triazinopyrazolothieno pyrimidine derivatives

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Triazinopyrazolothieno pyrimidine derivatives **1–5** were evaluated for their anti-inflammatory, analgesic and anti-cancer activities and acute toxicity. Anti-inflammatory activity of the compounds was studied using the carrageenan test. All tested compounds showed analgesic activity, 3-methoxycarbonyl-4,6-dimethyl-8-[(*N*-methylindolyl)methyl]pyrimido [5',4':4,5]thieno [3',2'-3,4]pyrazolo [5,1-*c*]triazine (**4**) showed activity comparable to that of diclofenac. Compounds **1–5** were also screened for anticancer activity on a human lung cancer cell line (A549) and a human prostate cancer cell line (DU145) using the MTT micro-cultured tetrazolium assay method. Compound **4** showed also significant anticancer activity against both cancer cell lines, comparable to that of doxorubicin. The most active compounds were tested for their acute toxicity and median lethal doses were evaluated.

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Investigations of some triazinopyrazolothieno pyrimidine derivatives and their biological activities have gained great importance in recent years. Pyrazolopyrimidine derivatives showed the most pronounced activity against *Mycobacterium tuberculosis* comparable to that of the first line anti-tubercular drug isoniazid (**1**). Pyrimidine compounds showed an indirect anti-angiogenic effect through suppression of the secretion of VEGF and TGF- β 1 from prostate cancer cells (**2**) and have, among others, been evaluated as cGMP phosphodiesterase inhibitors, but also as kinase inhibitors and potential anti-cancer agents (**3**). Pyrimidine derivatives were also found to have anti-inflammatory activity in BALB/c mice with locally induced edema (**4**). Pyridopyrimidine derivatives were recognized for their anticonvulsant and antidepressant activities but showed no neurotoxicity (**5**). Some of pyrimidine derivatives showed *in vitro* antiplasmodial activity against chloroquine-resistant (CQR) (K1) and drug-sensitive CQS (3D7) strains of *Plasmodium falciparum* (**6**). Also, pyrimidine derivatives showed good analgesic activity when compared to the standard

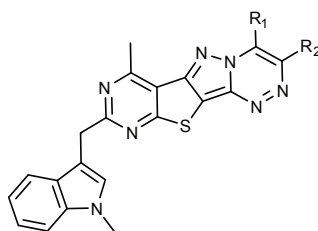
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drug pentazocine (7). Thioxopyrimidine derivatives showed good antifungal activity when screened *in vitro* against *Candida albicans*, *Penicillium* ssp. and *Aspergillus niger*, using the agar well disk diffusion method and using amphotericin-B as standard drug (8). Pyrazolopyrimidine and pyrimidine derivatives showed good antifungal activity when screened against *C. albicans*, *Aspergillus fumigatus*, *Geotrichum candidum*, *Syncephalastrum racemosum*. Also, pyrimidine derivatives exhibited significant activity against bacteria and the results were comparable with the standard control (9). New, poly-fused ring pyrazolo-thieno-pyrimidine derivatives showed good anti-parkinsonian, hypoglycemic and antimicrobial activities (10). Moreover, some new polycyclic triazolopyrazolo-pyridazine derivatives showed pharmacological activity (11). Herein, some triazinopyrazolothieno pyrimidine derivatives were tested for their acute toxicity, anti-inflammatory, analgesic and anticancer activities.

EXPERIMENTAL

Chemistry

Triazinopyrazolothieno pyrimidine derivatives 1–5 (Fig. 1) were synthesized according to the reported methods (10). 3-Acetyl-4,6-dimethyl-8-[(*N*-methylindolyl)methyl]pyrimido [5',4':4,5]thieno[3',2'-3,4]pyrazolo[5,1-*c*]triazine (1), 3,6-dimethyl-8-[(*N*-methylindolyl)methyl]pyrimido[5',4':4,5]thieno[3',2'-3,4]pyrazolo[5,1-*c*]triazin-4-ol (2), 6-methyl-8-[(*N*-methylindolyl)methyl]pyrimido[5',4':4,5]thieno[3',2'-3,4]pyrazolo[5,1-*c*]triazin-3-carbonitrile-4-ol (3), 3-methoxycarbonyl-4,6-dimethyl-8-[(*N*-methylindolyl)methyl]pyrimido[5',4':4,5]thieno[3',2'-3,4]pyrazolo[5,1-*c*]triazine(4) and 4-amino-6-methyl-8-[(*N*-methylindolyl)methyl]pyrimido[5',4':4,5]thieno[3',2'-3,4]pyrazolo[5,1-*c*]triazine-3-carbonitrile (5) were afforded by the reaction of the 3-diazopyrazolothienopyrimidine derivative with the active reagent, namely, acetyl acetone, ethyl acetoacetate, ethyl cyanoacetate, di-



Compd.	R ₂	R ₁
1	-COCH ₃	-CH ₃
2	-CH ₃	-OH
3	-CN	-OH
4	-COOCH ₃	-CH ₃
5	-CN	-NH ₂

Fig. 1. Structures of compounds 1–5.

ethyl malonate and/or malononitrile, respectively, in the presence of sodium acetate as basic medium. Compounds 1–5 were evaluated for anti-inflammatory, analgesic and anticancer activities.

Animals

One hundred and fifty-six male and female Swiss albino mice, five to six weeks old and weighing 16–18 g, were obtained from the animal house of Taibah University (Madinah Munawara, KSA) in collaboration with King Saudi University (Riyadh, KSA). Experiments were approved by the Animal Ethical Committee of Taibah University. The animals were maintained according to the accepted standards of animal care.

Anti-inflammatory evaluation

Tested triazinopyrazolothieno pyrimidine derivatives 1–5 were dissolved in 0.5 % carboxymethyl cellulose (CMC) solution and administered intraperitoneally (*i.p.*). Ninety-six mice were divided into sixteen groups, each group consisting of six animals. Edema was induced in the right hind paw of all mice by subcutaneous injection of 0.1 mL of 1.0 % (*m/V*) carrageenan in 10 % formal saline (0.85 % NaCl in 10 % formalin). The first group was kept as a control and was given the respective volume of the solvent (0.5 mL 10 % formal saline). Groups 2–15 were orally administered aqueous suspension of the tested compounds in doses of 5 mg kg⁻¹ 1 h before carrageenan injection. The last group was administered diclofenac sodium (reference drug) in a dose of 5 mg kg⁻¹, orally as aqueous suspension. The right paw volume was measured using a digital plethysmometer, directly before and 1, 2 and 3 h after administration of the tested compounds (11, 12).

Analgesic activity

Sixty mice were divided into 10 groups, one group was kept as a control (received saline), the second group received the vehicle (gum acacia), and the third received diclofenac sodium as a reference drug, whereas the other groups received compounds 1–5 *via* subcutaneous administration. The mice were dropped gently into a dry glass beaker of 1 dm³ capacity maintained at 55 to 55.5 °C (13). Normal reaction time in seconds for all animals was determined at time intervals of 30, 60 and 90 min. The reaction time is defined as the time that elapses from the moment when the mouse reaches the hot beaker till the animal licks its feet or jumps out of the beaker (dose 5 mg kg⁻¹). Relative potencies *versus* diclofenac sodium were determined.

Anticancer activity

Test compounds 1–5 were screened for anticancer activity on a human lung cancer cell line (A549) and a human prostate cancer cell line (DU145) using the MTT micro-cultured tetrazolium assay method (14). Doxorubicin was used as the reference drug. Cells were harvested from the logarithmic phase cultures and re-suspended in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum. Cell counts were adjusted and equal numbers of cells were plated in each well of 96-well culture plates and allowed to grow overnight at 37 °C in the presence of 5 % CO₂. The cells were treated with test sub-

stances at various concentrations for 72 hours. In vehicle control culture wells, a maximum of 0.5 % DMSO saturated with CO₂ was added. Fresh culture medium supplemented with test substances was renewed every 24 hours. Thereafter, 0.5 mg mL⁻¹ of MTT reagent was added to each well and the micro plate was incubated for further 4 hours at 37 °C in the presence of 5 % CO₂. Finally, the cells were solubilized by adding a solubilizing solution and allowed to incubate at 37 °C overnight. After complete solubilization of formazan crystals, the absorbance was read at 540 nm in a micro plate reader (Bio-Rad, USA). The results obtained from quadruplicate wells were used to calculate IC₅₀ of the test compounds.

Acute toxicity

Median lethal doses (*LD*₅₀) of the most active compounds **2** and **4** were determined in mice (15). Groups of male mice, each of six animals, were injected *i.p.* with graded doses of each of the test compounds. The percentage of mortality in each group of animals was determined 24 h after injection. Computation of *LD*₅₀ was processed by a graphical method.

Statistical analysis

Assay results are shown as the mean ± standard error of the mean (SEM). Statistical analyses were carried out using the Sigma Plot software (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to assess the presence of significant differences. Differences were considered statistically significant at *p* ≤ 0.05.

RESULTS AND DISCUSSION

Anti-inflammatory activity

Anti-inflammatory studies after 1, 2 and 3 h showed the same trend of activity of the five triazinopyrazolothieno pyrimidine compounds **1–5** but also revealed different activity

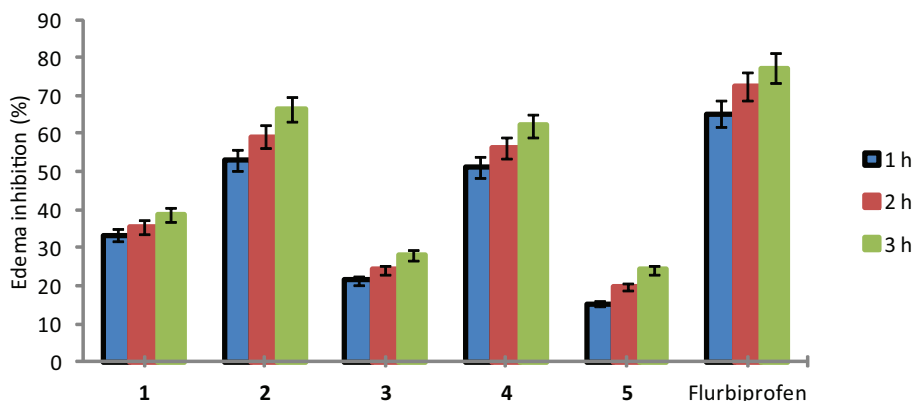


Fig. 2. Anti-inflammatory activity of compounds **1–5** (dose 5 mg kg⁻¹ b.m, *p.o.*). Mean ± SEM, *n* = 6.

of each of them. After 1 h, compounds **3** and **5** showed weak anti-inflammatory activity (21.4 ± 1.3 and 15.2 ± 1.1 %, resp.), compound **1** showed moderate anti-inflammatory activity (33.2 ± 1.3 %) and compounds **2** and **4** showed strong anti-inflammatory activity (53.1 ± 1.2 and 51.2 ± 1.4 %) in comparison with flurbiprofen (65.3 ± 1.3 %). After 2 h, compounds **3** and **5** still showed weak anti-inflammatory activity (24.2 ± 1.2 and 19.6 ± 1.2 %, resp.), compound **1** moderate activity (35.4 ± 1.3 %) but compounds **2** and **4** possessed strong anti-inflammatory activity (59.2 ± 1.5 and 56.3 ± 1.4 %) compared to the standard drug (72.6 ± 1.1 %). Accordingly, after 3 h compounds **3** and **5** decreased paw edema by 28.2 ± 1.2 and 24.3 ± 1.1 %, compound **1** by 38.7 ± 1.1 %, while compounds **2** and **4** showed the most pronounced reduction of paw inflammation (66.5 ± 1.3 and 62.1 ± 1.2 %) in comparison with the standard drug flurbiprofen (77.3 ± 1.4 %) (Fig. 2).

Analgesic activity

Activity of all compounds was normalized relative to the standard drug diclofenac sodium with arbitrary unit activity (1.00 ± 0.02) and expressed as a respective ratio. Compounds **1–5** differed in analgesic activity although the same trend of activity increasing with time was observed for each of them (Fig. 3). After 30 min, compounds **3** and **5** exerted weak (0.35 ± 0.01 and 0.21 ± 0.01), compounds **1** and **2** moderate (0.61 ± 0.01 and 0.62 ± 0.02) and compound **4** strong analgesic activity (0.82 ± 0.03) After 60 min, compounds **3** and **5** still showed weak (0.39 ± 0.02 and 0.25 ± 0.01), compounds **1** and **2** moderate (both 0.65 ± 0.01) and compound **4** strong analgesic activity (0.83 ± 0.03). After 90 min, compounds **3** and **5** showed weak (0.43 ± 0.02 and 0.31 ± 0.03), compounds **1** and **2** moderate (0.69 ± 0.02 and 0.67 ± 0.03), while compound **4** showed strong analgesic activity (0.88 ± 0.01). After 120 min, compound **5** still showed weak (0.34 ± 0.02), compound **3** moderate (0.46 ± 0.02), compounds **1** and **2** strong analgesic activity (0.73 ± 0.03 and 0.71 ± 0.01), and compound **4** even higher analgesic activity (0.92 ± 0.01) comparable to that of diclofenac.

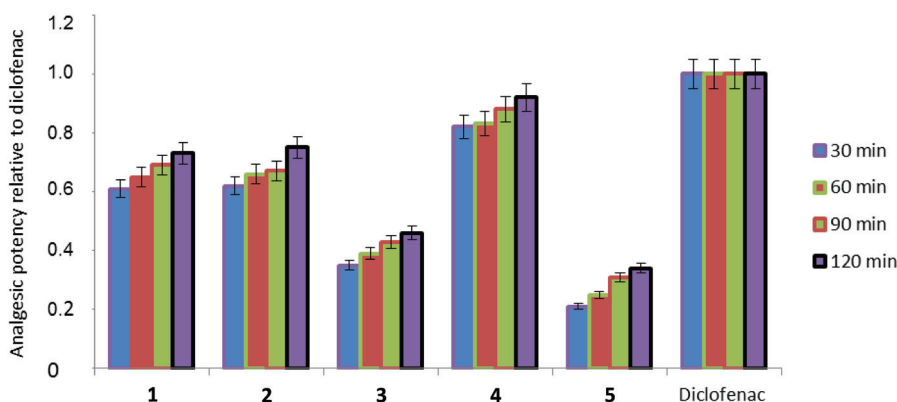


Fig. 3. Analgesic activity of the tested compounds **1–5** relative to diclofenac (dose $5 \text{ mg kg}^{-1} \text{ b.m.}$, *p.o.*). Mean \pm SEM, $n = 6$. All results were significantly different ($p \leq 0.05$) from the standard (diclofenac) and normal control (10 % formal saline) (Tukey's *post-hoc* test).

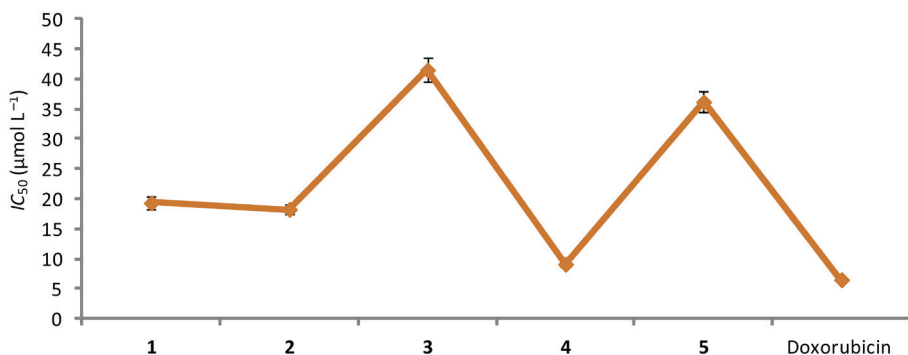


Fig. 4. IC_{50} values of compounds 1–5 against the human lung cancer cell line (A549). Mean \pm SEM, $n = 6$.

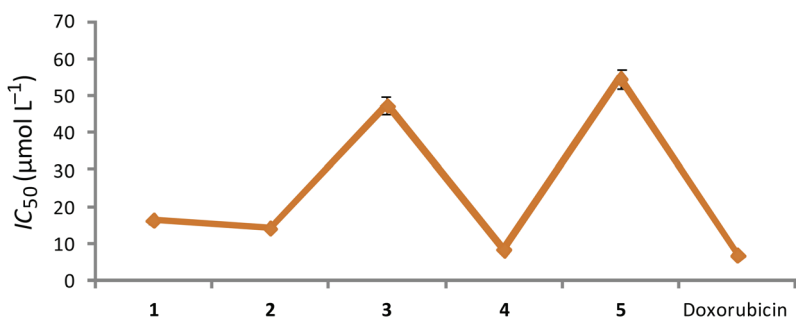


Fig. 5. IC_{50} values of compounds 1–5 against the human prostate cancer cell line (DU145). Mean \pm SEM, $n = 6$.

Anticancer activity

Some of the tested compounds showed significant anticancer activity against the human lung cancer cell line (A549) and the human prostate cancer cell line (DU145) (Figs. 4 and 5).

It is evident from Fig. 4 that compound 4 showed the highest activity against the human lung cancer cell line with IC_{50} of $9.11 \mu\text{mol L}^{-1}$, compounds 1 and 2 were found to be moderately effective (19.34 and $18.26 \mu\text{mol L}^{-1}$) whereas compounds 3 and 5 were the least effective (41.51 and $36.19 \mu\text{mol L}^{-1}$) in comparison with the standard drug doxorubicin ($6.52 \mu\text{mol L}^{-1}$).

It is clear from Fig. 5 that the highest activity against the human prostate cancer cell line with the IC_{50} value of $8.42 \mu\text{mol L}^{-1}$ was shown by compound 4, whereas compounds 1 and 2 were found to be moderately effective (16.28 and $14.17 \mu\text{mol L}^{-1}$) and compounds 3 and 5 weakly effective (47.25 and $54.61 \mu\text{mol L}^{-1}$) in comparison with doxorubicin ($6.87 \mu\text{mol L}^{-1}$).

Table I. Acute toxicity of selected compounds

Compound	LD_{50} ($\mu\text{mol kg}^{-1}$) ^a
2	4.57 \pm 0.03
4	5.68 \pm 0.03
Flurbiprofen	5.32 \pm 0.06

^a Values represent the mean \pm SEM, $n = 6$.

Structure activity relationship

Compounds **2** and **4** showed the highest anti-inflammatory activity. This is probably due to the presence of hydroxyl-triazinopyrazolo moiety in compound **2** and methoxycarbonyl-triazinopyrazolo moiety in compound **4**. The latter might also be responsible for the highest analgesic and anticancer activity of compound **4**.

Acute toxicity

The most active compounds were tested for acute toxicity. LD_{50} of compounds **2** and **4** was 4.57 and 5.68 $\mu\text{mol kg}^{-1}$ *bm*, resp., whereas that of flurbiprofen was 5.32 $\mu\text{mol kg}^{-1}$ (*i.p.*) (Table I). These LD_{50} values indicate comparable safety of compounds **2** and **4** to that of flurbiprofen.

CONCLUSIONS

In the present investigation, five triazinopyrazolothieno pyrimidine derivatives **1–5** were screened for their anti-inflammatory, analgesic and anticancer activity. Compound **4** with an ester functional group showed the strongest anti-inflammatory and analgesic activity, comparable to that of diclofenac. The same compounds exerted the highest antiproliferative activity against the human lung cancer cell line (A549) and the human prostate cancer cell line (DU145), similar to that of the standard drug doxorubicin. The other tested compounds exhibited low or moderate activities.

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