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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of some new 2,6-disubstituted-3(2*H*)-pyridazinone derivatives and investigation of their analgesic, anti-inflammatory and antimicrobial activities

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Abstract In this study, 12 new 3(2*H*)-pyridazinone derivatives carrying 4-substituted phenylpiperazinylethyl moiety on lactam nitrogen were synthesized and their chemical structures were confirmed by ^{1}H-NMR, mass, and elemental analysis. Analgesic and anti-inflammatory activities of the synthesized compounds were evaluated in mice. Among the synthesized compounds, compound **9c** showed the best analgesic and anti-inflammatory activities without causing any gastric effect in stomachs of tested animals. In addition, the synthesized compounds were screened for their antibacterial and antifungal activities against some pathogenic strains.

Keywords 3(2*H*)-pyridazinone · Analgesic activity · Anti-inflammatory activity · Antimicrobial activity

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are important therapeutic agents for the treatment of pain, fever, and inflammation associated with a number of pathological conditions. Especially, NSAIDs are the first choice in the treatment of rheumatic disorders and other degenerative inflammatory joint diseases. The main mechanism of action of these drugs is the inhibition of cyclooxygenase enzyme (COX) which is catalyzing the conversion of arachidonic acid to the prostaglandins (PGs) mediating both inflammation response and physiologic homeostasis. Accordingly, chronic use of NSAIDs is often accompanied by side effects such as gastrointestinal lesions, bleeding, and nephrotoxicity (Williams and Lemke, 2008).

In the early 1990s, two isoforms of the COX enzyme, known as COX-1 and COX-2, have been identified (Masferrer *et al.*, 1992). Subsequent studies showed that COX-1 is a constitutive enzyme and synthesizes PGs mediating normal homeostasis in the gastrointestinal tract, kidneys, and platelets, whereas COX-2, mostly inducible, is related to the production of PGs mediating inflammation, pain, and fever (Dannhardt and Laufer, 2000). In the meantime, classical NSAIDs were determined to inhibit both isoforms (Dannhardt and Lauferm, 2000). These findings have led to the development of selective COX-2 inhibitors to improve the therapeutic potency and to reduce the classical side effects coupled with the use of NSAIDs (Penning *et al.*, 1997; Prasit *et al.*, 1999). However, selective COX-2 inhibitors have been reported to be associated with serious cardiovascular side effects (Dogne' *et al.*, 2005). Therefore, the search for new analgesic and anti-inflammatory agents devoid of side effects continues to be an active area of research in medicinal chemistry.

On the other hand, despite many significant progresses in antimicrobial therapy, the existing antimicrobial drugs (antibacterial and antifungal) have become less effective or ineffective due to the development of multidrug-resistant pathogens (i.e., microbial isolates such as bacteria and fungi), which is a serious health problem (Perea and Patterson, 2002; Grare *et al.*, 2007).

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As a result, there is an increasing need to design new antibacterial and antifungal agents with better activity profile and lower toxicity.

Many compounds carrying 3(2*H*)-pyridazinone have been reported to be associated with analgesic and anti-inflammatory activities (Takaya *et al.*, 1979; Şüküroğlu *et al.*, 2005; Şüküroğlu *et al.*, 2006; Gökçe *et al.*, 2009; Abouzid *et al.*, 2010), as well as antimicrobial activity (Sonmez *et al.*, 2006; Doğruer *et al.*, 2008). In addition, it has been reported that compounds bearing arylpiperaziny lalkyl moiety on lactam nitrogen of pyridazinone ring have analgesic and anti-inflammatory activities (Rohet *et al.*, 1996; Dal Piaz *et al.*, 2003; Giovanni *et al.*, 2003).

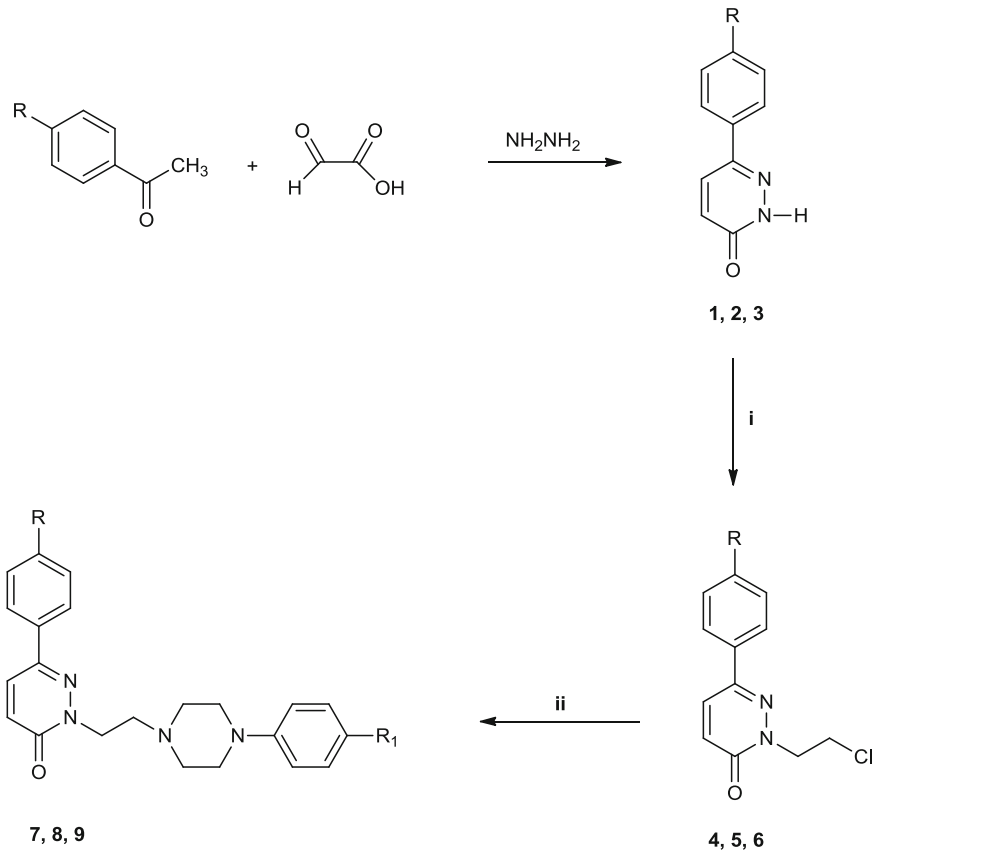
Consequently, we synthesized 12 new pyridazinone derivatives carrying 4-substituted phenylpiperazinylethyl moiety at position 2 to investigate their analgesic, anti-inflammatory, and antimicrobial activities.

Results and discussion

Chemistry

In this study, 12 new 2,6-disubstituted-3(2*H*)-pyridazinone derivatives were synthesized as shown in the Scheme 1. 6-Phenyl-3(2*H*)-pyridazinone (**1**), 6-(4-methoxyphenyl)-3(2*H*)-pyridazinone (**2**), and 6-(4-chlorophenyl)-3(2*H*)-pyridazinone (**3**) used as starting compounds were synthesized by the reaction of glyoxylic acid, appropriate acetophenones and hydrazine hydrate. Subsequently, the treatment of compounds **1–3** with 1-bromo-2-chloroethane in the presence of potassium carbonate in anhydrous DMF gave the compounds 2-(2-chloroethyl)-6-phenyl-3(2*H*)-pyridazinone (**4**), 2-(2-chloroethyl)-6-(4-methoxyphenyl)-3(2*H*)-pyridazinone (**5**), and 2-(2-chloroethyl)-6-(4-chlorophenyl)-3(2*H*)-pyridazinone (**6**). The synthesis of

Scheme 1 Synthetic route of the title compounds



R = H R_1 = H **(7a)**, Cl **(7b)**, F **(7c)**, OCH_3 **(7d)**

R = -OCH_3 R_1 = H **(8a)**, C l **(8b)**, F **(8c)**, OCH_3 **(8d)**

R = -Cl R_1 = H **(9a)**, Cl **(9b)**, F **(9c)**, OCH_3 **(9d)**

i = Bromochloroethane, K_2CO_3 / DMF ii = NaI, K_2CO_3, p-substituted phenylpiperazine derivatives/ acetonitrile

compounds **1–3** (Coates and McKillop, 1993) and **4** (Yamada *et al.*, 1983) was previously reported. However, compounds **5** and **6** were prepared for the first time in this study.

Finally, new title compounds (**7a–d**, **8a–d,** and **9a–d**) were prepared by the reaction of 2-(2-chloroethyl)-6-(4-non-substituted/methoxy/chlorophenyl)-3(2*H*)-pyridazinones, NaI with appropriate phenylpiperazine derivatives in the presence of potassium carbonate in CH_3CN. The chemical structures of the newly synthesized compounds were elucidated by ^{1}H-NMR, mass, and elemental analysis. The ^{1}H-NMR, mass spectra, and elemental analysis data of the compounds are in agreement with the proposed structures.

In vivo analgesic and anti-inflammatory activities

Analgesic and anti-inflammatory activities of the title compounds were determined by *p*-benzoquinone-induced writhing test and carrageenan-induced hind paw edema test, respectively (Okun *et al.*, 1963; Kasahara *et al.*, 1985). In addition, all the synthesized compounds were also evaluated in terms of their gastric effects. As shown in Table 1, among the synthesized compounds, **9b** and **9c** were found more active than ASA. The best activity was obtained with compound **9c**. Moreover, compounds **8b** and **8c** exhibited analgesic activity almost equal to that of acetylsalicylic acid (ASA). In addition, while compounds **9b** and **9c** did not cause any gastric lesion and bleeding in stomachs of tested animals, compounds **8b** and **8c** caused damage to gastric mucosa to some extent at 100 mg/kg dose. The rest of the compounds were less active than ASA.

On the other hand, anti-inflammatory activities of compounds **8b**, **9b**, and **9c** demonstrated parallel results with their corresponding analgesic activities. However, except for compounds **8b**, **9b,** and **9c** other compounds did not have any significant anti-inflammatory activity. When the chemical structures of the active compounds are taken into consideration, compounds **9b** and **9c** having *p*-chloro and *p*-fluoro substituents on phenyl ring of phenylpiperazine moiety at the side chain, respectively, are 6-(4-chlorophenyl)-3(2*H*)-pyridazinone derivative. However, compounds **8b** and **8c** having same substituents at same position are 6-(4-methoxyphenyl)-3(2*H*)-pyridazinone's derivatives.

These results will give some idea about further research on these molecules.

Antimicrobial activity

The synthesized compounds were tested for their antibacterial activity against 4 gram-positive bacteria (*Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA, isolate), *Enterococcus faecalis*, and *E. faecalis* isolate), 4 gram-negative bacteria (*Escherichia coli*, *E. coli* producing extended spectrum β-lactamase, *Pseudomonas aeruginosa*, and *P. aeruginosa* isolate), and for their antifungal activity against *Candida albicans* and *Candida krusei* by microdilution method (Clinical and Laboratory Standards Insti-

Table 1 Analgesic and anti-inflammatory activities of the synthesized compounds

Compound	Dose (mg/kg)	Analgesic activity (inhibition of writhing %)	Gastric ulcerogenic effect	Anti-inflammatory activity (inhibition of edema %)			
				90 min	180 min	270 min	360 min
7a	100	10.7	0/6	–	–	6.4	3.1
7b	100	23.3	0/6	16.2	14.9	14.8	12.1
7c	100	16.8	0/6	10.6	26.3**	29.9**	19.5
7d	100	8.6	0/6	–	–	–	–
8a	100	18.4	0/6	11.9	12.6	–	–
8b	100	44.4***	1/6	16.9	19.8	27.5**	26.2**
8c	100	47.6***	2/6	16.2	15.5	22.9*	18.3
8d	100	5.6	0/6	–	–	13.6	7.9
9a	100	20.1	0/6	–	1.4	7.4	–
9b	100	53.2***	0/6	20.6	29.7***	31.0***	28.2**
9c	100	57.5***	0/6	29.5**	28.3**	35.3***	28.8**
9d	100	30.2**	0/6	4.7	12.3	18.5	15.5
ASA	100	45.9***	4/6	–	–	–	–
Indomethacin	10	–	–	25.3**	36.9***	37.6***	39.4***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significant from control

SEM standard error mean

tute, 2006, 2008). Ampicillin, ofloxacin, fluconazole, and amphotericin B were used as references.

As shown in the Table 2, the synthesized compounds showed antimicrobial activity with MIC values in the range of 32–128 μg/mL. Regarding the antibacterial activity, all the compounds were found active at 64 μg/mL concentrations against all the tested gram bacteria except *S. aureus* isolate (MIC 128 μg/mL). However, the antifungal activity of the compounds was observed against both *C. albicans* and *C. krusei* at 32 μg/mL concentration. According to the antimicrobial activity results, fungi can be said to be more sensitive to title compounds rather than gram bacteria.

As a result, these compounds might be relatively good candidates to develop better antifungal agents against *C. albicans* and *C. krusei.*

Experimental

All the chemicals used for the synthesis of the compounds were purchased from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). Melting points of the compounds were recorded on an Electrothermal-9200 digital melting points apparatus (Southent, Great Britain) and the values are uncorrected. Thin-layer chromatography (TLC) was performed on Merck $60F_{254}$ plates. Reactions were monitored by TLC on silica gel with detection by UV light (254 nm). The ^{1}H-NMR spectra (400 mHz) was recorded employing a Varian Mercury 400 mHz FT spectrometer (Varian Inc., Palo Alto, CA, USA), in DMSO-d_6 at Faculty of Pharmacy, Ankara University, Ankara, Turkey. The mass spectra were recorded on a Micromass LCT Premier XE (Waters, Milford, MA, USA) LC–MS spectrometer using an positive electrospray ion source (ESI+) at Faculty of Pharmacy, Gazi University, Ankara, Turkey. Elemental analysis was performed on a Leco 932 CHNS instrument (St. Joseph, MI, USA) at Faculty of Pharmacy, Ankara University, Ankara, Turkey, and the results were within ±0.4 % of the theoretic values.

The synthesis of compounds **1–3** (Coates and McKillop, 1993) and **4** (Yamada *et al.*, 1983) was previously reported. However, 2-(2-chloroethyl)-6-(4-methoxy)-3(2*H*)-pyridazinone (**5**) and 2-(2-chloroethyl)-6-(4-chlorophenyl)-3(2*H*)-pyridazinone (**6**) were prepared for the first time in this study.

Synthesis of 6-(4-nonsubstituted/methoxy/chlorophenyl)-3(2*H*)-pyridazinones (**1**, **2**, **3**)

Glyoxylic acid (0.05 mol) and (0.15 mol) acetophenone derivative (nonsubstituted/4-methoxy/4-chloroacetophenone derivatives) were heated at 100–105 °C for 2 h. At the end of this period, the reaction mixture was cooled to 40 °C and then, 20 mL water and 5 mL ammonium hydroxide solution (25 %) were added to the reaction mixture until the medium pH became 8. Then, reaction mixture was extracted with dichloromethane (4 × 25 mL), then hydrazine hydrate

Table 2 Antimicrobial activity of the synthesized compounds (MICs, μg/mL)

Compounds	*S. aureus* ATCC 29213	*S. aureus* isolate (MRSA)	*E. faecalis* ATCC 29212	*E. faecalis* isolate	*E. coli* ATCC 25922	*E. coli* isolate (ESBL)	*P. aeruginosa* ATCC 27853	*P. aeruginosa* isolate	*C. albicans* ATCC 10231	*C. krusei* ATCC 6258
7a	64	128	64	64	64	64	64	64	32	32
7b	64	128	64	64	64	64	64	64	32	32
7c	64	128	64	64	64	64	64	64	32	32
7d	64	128	64	64	64	64	64	64	32	32
8a	64	128	64	64	64	64	64	64	32	32
8b	64	128	64	64	64	64	64	64	32	32
8c	64	128	64	64	64	64	64	64	32	32
8d	64	128	64	64	64	64	64	64	32	32
9a	64	128	64	64	64	64	64	64	32	32
9b	64	128	64	64	64	64	64	64	32	32
9c	64	128	64	64	64	64	64	64	32	32
9d	64	128	64	64	64	64	64	64	32	32
Ampicillin	2	32	2	4	8	64	–	–	–	–
Ofloxacin	0.5	2	1	2	0.12	16	1	32	–	–
Fluconazole	–	–	–	–	–	–	–	–	1	64
Amphotericin B	–	–	–	–	–	–	–	–	<0.25	0.5

(0.05 mol) was added to the separated aqueous layer and the reaction mixture was refluxed for 2 h. After the completion of the reaction, reaction mixture was cooled to room temperature. The resulting precipitate was filtered to give compounds **1**, **2**, and **3**.

Synthesis of 2-(2-chloroethyl)-6-(4-nonsubstituted/methoxy/chlorophenyl)-3(2*H*)-pyridazinones (**4**, **5**, **6**)

Appropriate 3(2*H*)-pyridazinone derivative (0.003 mol), anhydrous potassium carbonate (0.009 mol), and 1-bromo-2-chloroethane (0.009 mol) in 7 mL of anhydrous DMF were stirred at room temperature for 2.5 h. At the end of this period, the reaction mixture was poured into ice water and the resulting precipitate was filtered to give compounds **4**, **5**, and **6**. These compounds were used to synthesize title compounds without further purification.

General procedure for the synthesis of title compounds (**7a–d**, **8a–d** and **9a–d**)

2-(2-Chloroethyl)-6-(4-nonsubstituted/methoxy/chlorophenyl)-3(2*H*)-pyridazinone derivative (1.7 mmol) and NaI (1.8 mmol) in 30 mL of CH_3CN were refluxed for 30 min and cooled room temperature; then, anhydrous potassium carbonate (3.4 mmol) and appropriate phenylpiperazine derivatives (3.4 mmol) were added. The resulting mixture was refluxed for 2.5 h. At the end of this period, the reaction mixture was evaporated to dryness and treated with cold water; then, the forming precipitate was crystallized from an appropriate solvent.

*2-[2-(4-Phenylpiperazin-1-yl)ethyl]-6-phenylpyridazin-3(2H)-one (**7a**)*

Yield 68 %, m.p.: 139 °C. Recrystallized from *n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.00 (d, $J = 9.6$ Hz, 1H), 7.86 (d, 2H), 7.48–7.41 (m, 3H), 7.15 (t, 2H), 7.01 (d, $J = 9.6$ Hz, 1H), 6.86 (d, 2H), 6.71 (t, 1H), 4.27 (t, 2H), 3.03 (t, 4H), 2.75 (t, 2H), 2.57 (t, 4H). MS ESI(+) *m/e* 361 (M+H, 100). Anal. ($C_{22}H_{24}N_4O$): C, H, N calc. 73.31, 6.71, 15.54 found 73.24, 6.72, 15.52.

*2-[2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl]-6-phenylpyridazin-3(2H)-one (**7b**)*

Yield 72 %, m.p.: 126 °C. Recrystallized from ethanol/water. ^{1}H-NMR (DMSO-d_6) δ: 8.00 (d, $J = 9.6$ Hz, 1H), 7.86 (d, 2H), 7.50–7.38 (m, 3H), 7.16 (d, 2H), 7.01 (d, $J = 9.6$ Hz, 1H), 6.87 (d, 2H), 4.27 (t, 2H), 3.03 (t, 4H), 2.74 (t, 2H), 2.56 (t, 4H). MS ESI(+) *m/e* 395 (M+H, 100). Anal. ($C_{23}H_{24}ClN_3O$): C, H, N calc. 66.91, 5.87, 14.19 found 66.88, 6.01, 14.23.

*2-[2-[4-(4-Fluorophenyl)piperazin-1-yl]ethyl]-6-phenylpyridazin-3(2H)-one (**7c**)*

Yield 53 %, m.p.: 102 °C. Recrystallized from acetone/*n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.00 (d, $J = 9.6$ Hz, 1H), 7.86 (d, 2H), 7.48–7.41 (m, 3H), 7.02–6.85 (m, 5H), 4.27 (t, 2H), 2.98 (t, 4H), 2.74 (t, 2H), 2.57 (t, 4H). MS ESI(+) *m/e* 379 (M+H, 100). Anal. ($C_{22}H_{23}FN_4O$): C, H, N calc. 69.82, 6.13, 14.80 found 69.62, 6.24, 14.76.

*2-[2-[4-(4-Methoxyphenyl)piperazin-1-yl]ethyl]-6-phenylpyridazin-3(2H)-one (**7d**)*

Yield 67 %, m.p.: 117 °C. Recrystallized from acetone/*n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.01 (d, $J = 9.6$ Hz, 1H), 7.86 (d, 2H), 7.48–7.41 (m, 3H), 7.01 (d, $J = 9.6$ Hz, 1H), 6.82 (d, 2H), 6.75 (d, 2H), 4.27 (t, 2H), 3.63 (s, 3H), 2.92 (t, 4H), 2.74 (t, 2H), 2.57 (t, 4H). MS ESI(+) *m/e* 391 (M+H, 100). Anal. ($C_{23}H_{26}N_4O_2$): C, H, N calc. 70.75, 6.71, 14.35 found 70.48, 6.57, 14.25.

*2-[2-(4-Phenylpiperazin-1-yl)ethyl]-6-(4-methoxyphenyl)pyridazin-3(2H)-one (**8a**)*

Yield 87 %, m.p.: 98 °C. Recrystallized from *n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 7.95 (d, $J = 9.6$ Hz, 1H), 7.80 (d, 2H), 7.15 (t, 2H), 7.00 (d, 2H), 6.97 (d, $J = 9.6$ Hz, 1H), 6.85 (d, 2H), 6.71 (t, 1H), 4.24 (t, 2H), 3.76 (s, 3H), 3.03 (t, 4H), 2.73 (t, 2H), 2.56 (t, 4H). MS ESI(+) *m/e* 391 (M+H, 100). Anal. ($C_{23}H_{26}N_4O_2$): C, H, N calc. 70.75, 6.71, 14.35 found 70.69, 6.64, 14.28.

*2-[2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl]-6-(4-methoxyphenyl)pyridazin-3(2H)-one (**8b**)*

Yield 60 %, m.p.: 118 °C. Recrystallized from ethanol/water. ^{1}H-NMR (DMSO-d_6) δ: 7.93 (d, $J = 9.6$ Hz, 1H), 7.78 (d, 2H), 7.14 (d, 2H), 6.98 (d, 2H), 6.96 (d, $J = 9.6$ Hz, 1H), 6.84 (d, 2H), 4.22 (t, 2H), 3.75 (s, 3H), 3.01 (t, 4H), 2.71 (t, 2H), 2.54 (t, 4H). MS ESI(+) *m/e* 425 (M+H, 100). Anal. ($C_{23}H_{25}ClN_4O_2$): C, H, N calc. 65.01, 5.93, 13.19 found 64.81, 6.01, 13.09.

*2-[2-[4-(4-Fluorophenyl)piperazin-1-yl]ethyl]-6-(4-methoxyphenyl)pyridazin-3(2H)-one (**8c**)*

Yield 33 %, m.p.: 100 °C. Recrystallized from acetone/*n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 7.99 (d, $J = 9.6$ Hz, 1H), 7.85 (d, 2H), 7.05–6.89 (m, 7H), 4.29 (t, 2H), 3.81 (s, 3H), 3.02 (t, 4H), 2.77 (t, 2H), 2.61 (t, 4H). MS ESI(+) *m/e* 409 (M+H, 100). Anal. ($C_{23}H_{25}FN_4O_2$): C, H, N calc. 67.63, 6.17, 13.72 found 67.78, 6.25, 13.83.

*2-[2-[4-(4-Methoxyphenyl)piperazin-1-yl]ethyl]-6-(4-methoxyphenyl)pyridazin-3(2H)-one (**8d**)*

Yield 86 %, m.p.: 123,5 °C. Recrystallized from *n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 7.99 (d, $J = 9.6$ Hz, 1H), 7.84 (d, 2H), 7.05–7.0 (m, 3H), 6.85 (d, 2H), 6.79 (d, 2H), 4.28 (t, 2H), 3.81 (s, 3H), 3.67 (s, 3H), 2.95 (t, 4H), 2.77 (t, 2H), 2.60 (t, 4H). MS ESI(+) *m/e* 421 (M+H, 100). Anal. ($C_{24}H_{28}N_4O_3$): C, H, N calc. 68.55, 6.71, 13.32 found 68.70, 6.62, 13.37

*2-[2-(4-Phenylpiperazin-1-yl)ethyl]-6-(4-chlorophenyl)pyridazin-3(2H)-one (**9a**)*

Yield 40 %, m.p.: 121 °C. Recrystallized from ethyl acetate/*n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.05 (d, $J = 9.6$ Hz, 1H), 7.93 (d, 2H), 7.56 (d, 2H), 7.19 (t, 2H), 7.06 (d, $J = 9.6$ Hz, 1H), 6.90 (d, 2H), 6.75 (t, 1H), 4.31 (t, 2H), 3.07 (t, 4H), 2.78 (t, 2H), 2.61 (t, 4H). MS ESI(+) *m/e* 395 (M+H, 100). Anal. ($C_{22}H_{23}ClN_4O$): C, H, N calc. 66.91, 5.87, 14.19 found 67.05, 5.732, 14.32.

*2-[2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl]-6-(4-chlorophenyl)pyridazin-3(2H)-one (**9b**)*

Yield 37 %, m.p.: 116 °C. Recrystallized from ethyl acetate/*n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.05 (d, $J = 9.6$ Hz, 1H), 7.93 (d, 2H,), 7.56 (d, 2H), 7.20 (d, 2H), 7.06 (d, $J = 9.6$ Hz, 1H), 6.91 (d, 2H), 4.31 (t, 2H), 3.07 (t, 4H), 2.78 (t, 2H), 2.56 (t, 4H). MS ESI(+) *m/e* 430 (M+H, 100). Anal. ($C_{22}H_{22}Cl_2N_4O$): C, H, N calc. 61.54, 5.16, 13.05 found 61.28, 5.158, 13.21.

*2-[2-[4-(4-Fluorophenyl)piperazin-1-yl]ethyl]-6-(4-chlorophenyl)pyridazin-3(2H)-one (**9c**)*

Yield 23 %, m.p.: 113 °C. Recrystallized from acetone/*n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.05 (d, $J = 9.6$ Hz, 1H), 7.93 (d, 2H), 7.56 (d, 2H), 7.07–6.89 (m, 5H), 6.75 (t, 1H), 4.31 (t, 2H), 3.02 (t, 4H), 2.78 (t, 2H), 2.61 (t, 4H). MS ESI(+) *m/e* 413 (M+H, 100). Anal. ($C_{22}H_{22}ClFN_4O$): C, H, N calc. 64.00, 5.37, 13.57 found 63.93, 5.51, 13.65.

*2-[2-[4-(4-Methoxyphenyl)piperazin-1-yl]ethyl]-6-(4-chlorophenyl)pyridazin-3(2H)-one (**9d**)*

Yield 59 %, m.p.: 129 °C. Recrystallized from *n*-hexane. ^{1}H-NMR (DMSO-d_6) δ: 8.05 (d, $J = 9.6$ Hz, 1H), 7.93 (d, 2H), 7.56 (d, 2H), 7.06 (d, $J = 9.6$ Hz, 1H), 6.85 (d, 2H), 6.79 (d, 2H), 4.30 (t, 2H), 3.67 (s, 3H), 2.95 (t, 4H), 2.78 (t, 2H), 2.60 (t, 4H). MS ESI(+) *m/e* 425 (M+H, 100). Anal. ($C_{23}H_{25}ClN_4O_2$): C, H, N calc. 65.01, 5.93, 13.19 found 64.76, 5.79, 13.31.

Biologic assays

Preparation of test mice for analgesic and anti-inflammatory assays

Male Swiss albino mice (20–25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey). The animals, left for 2 days for acclimatization to animal room conditions, were maintained on Standard pellet diet and water ad libitum. The food was withdrawn 1 day before the experiment, but free Access to water was allowed. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals. The experiments were confirmed by the Gazi University Faculty of Medicine Laboratory Animals Ethics Committee. The mice were randomly assigned into the groups and six animals were used in each group.

Preparation of test samples for analgesic and anti-inflammatory assays

Test samples were suspended in a mixture of distilled water and 0.5 % sodium carboxymethylcellulose (CMC) and were given orally to the test animals. The animals of the control group received the same experimental handling except that the drug treatment was replaced with appropriate volumes of the vehicle. ASA (100 mg/kg) and indomethacin (10 mg/kg) in 0.5 % CMC were used as reference drugs.

Analgesic assay (*p*-benzoquinone-induced writhing test)

Sixty minutes after the oral administration of the test samples and ASA, the mice were intraperitoneally injected with 0.1 mL/10 g body weight of 2.5 % (vlv) *p*-benzoquinone (PBQ, Merck, Darmstadt, Germany) solution in distilled water. Control animals received an appropriate volume of dosing vehicle. The mice were then kept individually for observation and the total number of abdominal contraction (writhing movements) was counted for the next 15 min, starting on the 5th min after the PBQ injection. The data represent an average of the total number of writhing movements observed. The analgesic activity was expressed as the percentage change compared to writing controls (Okun *et al.*, 1963)

$$\text{Analgesic activity (writhing inhibition \%)} = \frac{n - n'}{n} \times 100$$

n = The mean writhing count of control group

n' = The mean writhing counts of test groups

Anti-inflammatory assay (Carrageenan-induced hind paw edema test)

Sixty minutes after the oral adminstration of either test sample or dosing vehicle, each mouse was injected with freshly prepared (0.5 mg/25 μL) suspension of carrageenan (Sigma, St. Louis, Missouri, USA) in physiologic saline (154 nM NaCl) into subplantar tissue of the right hind paw. As the control, 25 μL saline solution was injected into that of the left hind paw. Paw edema was measured in every 90 min during 6 h after the induction of inflammation. The difference in footpad thickness between the right and left foot was measured with a pair of dial thickness gauge callipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed by statistical methods (Kasahara *et al.*, 1985).

Ulcerogenic effect

After *p*-benzoquinone-induced writhing test, the surviving mice were killed under deep ether anesthesia and stomachs were removed. Then, each stomach was opened through great curvature and examined under dissecting microscope for lesions or bleedings.

Statistical analysis

Data obtained from animal experiments were expressed as mean standard error (±SEM). Statistical differences between the treatments and the control were tested by ANOVA test and Student–Newman–Keuls post hoc test. A value of $p < 0.05$ was considered to be significant.

Antimicrobial assay

The in vitro minimum inhibitory concentrations (MICs) of the synthesized compounds were determined by the two-fold serial dilution technique in 96-well microtest plates according to the methods recommended by the Clinical Laboratory Standards Institute (CLSI). Standard and the isolated strains of the bacteria: *S. aureus*, ATCC 29213, methicillin-resistant *S. aureus*, *E. faecalis*, ATCC 29212, *E. faecalis* isolate, *E. coli* ATCC 35218, *E. coli*, producing extended spectrum *β*-lactamase (ES*β*L), *P. aeruginosa*, ATCC 27853 and its isolate were used to determine antibacterial activity. As for antifungal activity, *C. albicans* ATCC 10231 and *C. krusei* ATCC 6258 were used to determine antifungal activity. Clinical isolates were obtained from Gazi University Hospital, Microbiology Laboratory.

Standard powders of ampicillin, ofloxacin, fluconazole, and amphotericin B were dissolved in appropriate solvents recommended by CLSI guidelines.

Stock solutions of the tested compounds were dissolved in DMSO. Stock solutions of the tested compounds and reference drugs were diluted twofold in microplate wells. All solvents and diluents, pure microorganisms, and pure media were used in control wells.

Bacteria were subcultured in Mueller–Hinton Agar (MHA) (Merck) plates and incubated overnight at 37 °C and Candida was subcultured in Sabouraud Dextrose Agar (SDA; Merck) plates at 35 °C for 24–48 h.

Bacterial susceptibility testing was performed according to the guidelines of CLSI M100-S18 (Clinical and Laboratory Standards Institute, 2008). Mueller–Hinton Broth (MHB; Merck) was added to each microplate well. Then, the tested compounds and reference drugs were added to this medium in wells by twofold serial dilution to obtain the required concentrations of 512, 256,128…0.5 μg/mL. The bacterial suspensions used for inoculation were prepared at 10^5 CFU/mL by diluting fresh cultures at McFarland 0.5 density (10^7 CFU/mL). Suspensions of the bacteria at 10^5 CFU/mL concentrations were inoculated with a two-folddiluted solution of the compounds. A 10 μL bacteria inoculum was added to each microplate well. There were 10^4 CFU/mL bacteria in the wells after inoculation.

Fungal susceptibility testing was performed according to the guidelines of CLSI M27-A (Clinical and Laboratory Standards Institute, 2006). Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine (Sigma) buffered to pH 7 with 3-(*N*-morpholino)propanesulfonic acid (MOPS) (Sigma) was added each microplate well. Then, the tested compounds and reference drugs were added to this medium in wells by twofold serial dilution to obtain the required concentrations of 512, 256,128…0.125 μg/mL. The yeast suspensions used for inoculation were prepared at 10^4 CFU/mL by diluting fresh cultures at McFarland 0.5 density (10^6 CFU/mL). Suspensions of the yeast at 10^4 CFU/mL concentrations were inoculated to the twofold-diluted solution of the compounds. A 10 μL yeast inoculum was added to each well of the microplates. There were 10^3 CFU/mL yeast in the wells after inoculations.

Microplates were incubated at 37 °C for 24 h for antibacterial activity and at 37 °C for 48 h for antifungal activity. After incubation, the lowest concentration of the compounds that completely inhibits macroscopic growth was determined and reported as MICs. All the experiments were done in 3 parallel series.

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