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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and evaluation of analgesic, anti-inflammatory, and anticancer activities of new pyrazole-3(5)-carboxylic acid derivatives

Burcu Çalışkan · Akın Yılmaz · İlker Evren · Sevda Menevşe · Orhan Uludag · Erden Banoglu

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Abstract In this article, we synthesized a series of novel 1-benzyl-5(3)-*p*-tolyl-1*H*-pyrazole-3(5)-carboxylic acid derivatives and characterized by IR, ^{1}H NMR, and mass spectroscopy. Compounds were evaluated for their in vivo analgesic and anti-inflammatory activity using the *p*-benzoquinone-induced writhing test and the carrageenan-induced paw edema model, respectively. Out of 14 compounds tested, **7a**, **7c**, **7e**, **7f**, **7i**, **8a–b**, and **8f–g** exhibited potent analgesic and/or anti-inflammatory activity as compared to reference drugs aspirin and indomethacin. Anticancer activity of these compounds was assessed against five cancer cell lines with the MTT assay (HL-60, human promyelocytic leukemia cells; HeLa, human cervical cancer cells; Raji, human B lymphocyte cell line; MCF7, human breast adenocarcinoma cell line; MDA-MB-231, estrogen-independent human breast cancer cell line). Compounds **7a**, **8a**, and **8b** with high anti-inflammatory activity, and also **7d** and **7j** with mild anti-inflammatory activity exhibited promising anticancer activity against some selected cell lines.

Keywords Carrageenan · Hind paw edema · Benzoquinone · Writhings · Cell lines

B. Çalışkan · E. Banoglu (✉)
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Etiler, 06330 Yenimahalle, Ankara, Turkey
e-mail: banoglu@gazi.edu.tr

A. Yılmaz · S. Menevşe
Department of Medical Biology and Genetics, Faculty of Medicine, Gazi University, Etiler, 06100 Besevler, Ankara, Turkey

İ. Evren · O. Uludag
Department of Pharmacology, Faculty of Pharmacy, Gazi University, Etiler, 06330 Yenimahalle, Ankara, Turkey

Introduction

Fast and effective relief of pain and inflammation in human beings is an ongoing challenge for medical researchers. Non-steroidal anti-inflammatory drugs (NSAIDs) represent an important therapeutic class for the alleviation of pain and inflammation associated with a number of pathological conditions including arthritis, Alzheimer's disease, atherosclerosis, and cancer (Barthel and Axford-Gatley, 2010; Bhalke and Chavan, 2011; Charo and Taub, 2011; Fischer *et al.*, 2011; Heneka *et al.*, 2011). Recent epidemiological studies have shown a link between inflammation and cancer suggesting that patients with chronic inflammatory diseases could be more prone to suffering from various types of cancers such as bladder, cervical, gastric, skin, intestinal, and prostate cancers (Grossman *et al.*, 2000; Mantovani *et al.*, 2008; Muller-Decker *et al.*, 1998). An increasing body of evidence has demonstrated that certain tumors and cancer lines overexpress arachidonic acid (AA) metabolizing enzymes, i.e., 5-lipoxygenase (5-LO) and cyclooxygenases (COX-1 and COX-2) (Grossman *et al.*, 2000; Muller-Decker *et al.*, 1998; Radmark and Samuelsson, 2010; Sheng *et al.*, 1997; Wang and Dubois, 2010) and their pro-inflammatory metabolic products, i.e., leukotrienes (LT) and prostaglandins (PG). Pharmacological inhibition of 5-LO has been shown to potently suppress tumor cell growth by inducing cell cycle arrest and triggering cell death via an intrinsic apoptotic pathway (Ghosh and Myers 1997, 1998). There is also a strong evidence for COX-2 being the major form in cancer cells for production of PGE_2 which promotes cancer cell growth and survival by several mechanisms including increased proliferation and inhibition of apoptosis (Greenhough *et al.*, 2009; Grossman *et al.*, 2000; Muller-Decker *et al.*, 1998; Sheng *et al.*, 1997). In addition, microsomal prostaglandin E synthase-1 (mPGES-1), a downstream

enzyme for PGE_2 formation, has been linked to development of various types of cancers including nonsmall cell lung, colorectal, gastric, and pancreatic cancers (Nakanishi *et al.*, 2010; Radmark and Samuelsson, 2010). Although our understanding of the complicated roles of AA-derived eicosanoids in tumor microenvironment is continuously improving, there is a strong chance that the development of novel anti-inflammatory agents clearly may lead to more effective cancer chemopreventive and/or therapeutic agents.

Pyrazoles are widely used as core motifs for a large number of compounds for development of new therapeutics for inflammation and cancer (Bekhit *et al.*, 2010; Keter and Darkwa, 2011). In the last decade, we and others have reported a large series of pyrazole derivatives having promising anti-inflammatory (Banoglu *et al.*, 2005, 2004, 2003; Caliskan *et al.*, 2011; Ergun *et al.*, 2010; Nagarapu *et al.*, 2011) and anticancer activities (Ding *et al.*, 2009; Farag *et al.*, 2010; Lian *et al.*, 2009; Nitulescu *et al.*, 2010; Xia *et al.*, 2007, 2008; Zheng *et al.*, 2010, 2009), indicating the use of pyrazole motif as a powerful tool for novel medicines. Among the reported studies, pyrazole-5-carboxylate (Wei *et al.*, 2006; Zheng *et al.*, 2010), pyrazole-5-carboxamide (Ding *et al.*, 2009), and pyrazole-5-carbohydrazide (Lian *et al.*, 2009; Xia *et al.*, 2007; 2008; Zheng *et al.*, 2009) derivatives were shown to have a significant anticancer activity against A549 lung cancer cell lines by inducing apoptosis or autophagy. Therefore, with the aim of obtaining new compounds with anti-inflammatory and also with anticancer activity, we synthesized a series of novel 1-benzyl-3(5)-*p*-tolyl-1*H*-pyrazole-5(3)-carboxylic acid amides and esters and evaluated the effects of these compounds on carrageenan-induced hind paw edema and benzoquinone-induced writhing test as well as for their anticancer activity against five different cancer cell lines with the MTT assay.

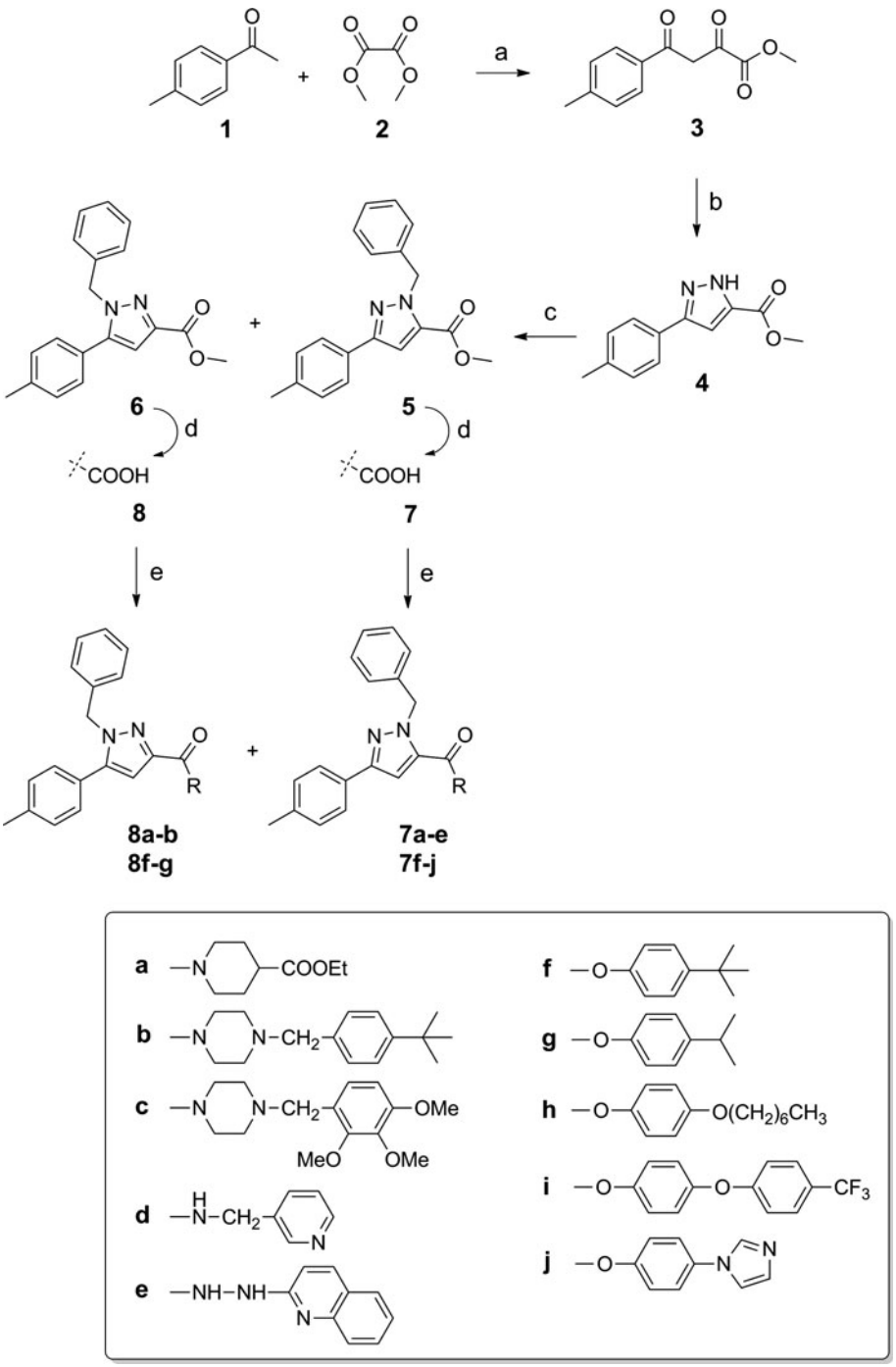
Results and discussion

Chemistry

The synthesis of the resulting amide and ester derivatives is outlined in Scheme 1. Methyl 2,4-dioxo-4-(*p*-tolyl)butanoate (**3**) was prepared by Claisen condensation of 4-methylacetophenone (**1**) with dimethyl oxalate (**2**) in the presence of sodium metoxide. Then, the starting intermediate methyl 3-(*p*-tolyl)-1*H*-pyrazole-5-carboxylate (**4**) was readily obtained by the reaction of **3** with hydrazine in acetic acid. After benzylation of **4** with benzyl chloride in the presence of K_2CO_3 in acetonitrile, the regioisomers formed were successfully separated by automated flash chromatograpy to yield methyl 1-benzyl-3-(*p*-tolyl)-1*H*-pyrazole-5-carboxylate (**5**) and methyl 1-benzyl-5-(*p*-tolyl)-1*H*-pyrazole-3-carboxylate (**6**) in 77 and 15 % yields, respectively. The benzylation process predominantly occured at a position to yield 1-benzyl-3-phenylpyrazole-5-carboxylate isomer in a ratio of 5:1. The difference between the absolute chemical shift values of methylene protons in ^{1}H NMR was a clear indication of distinguishing between both regioisomers as pointed out previously for similar derivatives (Xia *et al.*, 2007, 2008). Thus, comparison of the ^{1}H NMR spectra of both isomers showed that while the two methylene protons in 1-benzyl-3-phenyl isomer (**5**) appeared at relatively downfield around 5.8 ppm, the same protons in 1-benzyl-5-phenyl isomer (**6**) resonated at relatively upfield at about 5.4 ppm, therefore clearly distinguishing between the regioisomers. After hydrolysis of the esters to carboxylic acids (**7**, **8**), the title amide and ester derivatives were obtained by the reaction of appropriate amines and phenols using EDC as the carboxylate activator. Compounds were purified by automated flash chromatography and checked for purity with UPLC before being tested in biological experiments (purity was >97 %). The structures of all compounds were elucidated by high resolution mass spectrometry, IR, and ^{1}H NMR spectral data.

Pharmacology

Our previous findings with certain pyrazole-based compounds resulted in derivatives with potent in vivo anti-inflammatory and analgesic activity (Banoglu *et al.*, 2005, 2004, 2003). Our ongoing work also demonstrated that transformation of 1,5-diarylpyrazole-3-propanoic acid scaffold into lipophilic amides and esters acted as suppressors of LT biosynthesis in intact PMNL, and some compounds showed a potent inhibition of COX-1 along with inhibition of LT biosynthesis providing a potent anti-inflammatory potential (Caliskan *et al.*, 2011; Ergun *et al.*, 2010). Especially, amidation with *t*-butylbenzylpiperazine and ethyl piperidinecarboxylate, and esterification with *i*-propyl- or *t*-butylphenol highly contributed to the efficiency of compounds for inhibition of LT formation in intact cells (Caliskan *et al.*, 2011). In addition, combination of N1-phenyl and C5-*p*-methylphenyl substitution at a central pyrazole for amides and esters even increased the potency leading to dual inhibition of platelet COX-1 activity and LT biosynthesis for potential anti-inflammatory activity. Based on aforementioned observations and also the previous findings indicating pyrazole-5-carboxylic acid nucleus as suitable for anti-cancer activity (Wei *et al.*, 2006; Xia *et al.*, 2007, 2008; Zheng *et al.*, 2010, 2009), we prepared a novel series of amide and ester derivatives of 1-benzyl-3-(*p*-tolyl)-1*H*-pyrazole-5-carboxylic acid (**7a–j**) and also the regioisomeric counterparts bearing 1-benzyl-5-(*p*-tolyl)-1*H*-pyrazole-3-carboxylic acid core as **8a–b** and **8f–g**. Since we previously reported the positive effects of C5-*p*-methylphenyl substitution about a central pyrazole and amidation with piperazines, we kept the



Scheme 1 Synthesis of the amide and ester derivatives of 1-benzyl-5(3)-*p*-tolyl-1*H*-pyrazole-3(5)-carboxylic acids (Reagents and conditions: *a* (i) sodium methoxide, MeOH (ii) H^+; *b* $NH_2NH_2{\cdot}H_2O$, AcOH; *c* Benzyl chloride, K_2CO_3, AcCN, chromatographic separation of regioisomers 5 and 6; *d* LiOH, THF, H_2O; *e* amine/phenol, EDC, DMAP, CH_2Cl_2)

p-methylphenyl substitute pyrazole core structure constant and studied the effect of different amide and ester side chains on their analgesic, anti-inflammatory, and anticancer activities.

Analgesic activity of title compounds was screened in vivo in mice using the *p*-benzoquinone-induced writhing test (Okun *et al.*, 1963). *p*-Benzoquinone test commonly used as a fast screening model to access the activity of novel candidates as potential analgesics. In addition, quinone derivatives that were used as a chemical inducer of algesia exhibited more effective and potent activity as of acetic acid which is an another commonly used method. These models induce a characteristic and quantifiable overt pain-like behavior described as writhing response or abdominal contortions. It was demonstrated that cytokines such as TNFα (tumor necrosis factor α), IL (interleukin)-1β, and chemokines act in synergy to induce the writhing response. In fact, cyclooxygenase products and cysteinyl

Table 1 Analgesic and anti-inflammatory effects of the test compounds on *p*-benzoquinone (PBQ)-induced abdominal constriction test and carrageenan (CG)-induced hind paw edema model in mice, respectively

Test compounds	Number of writhings ± SEM (analgesic activity, %)[a]	Swelling in thickness ($\times 10^{-2}$ mm) ± SEM (inhibition of edema, %)[b]			
		90 min	180 min	270 min	360 min
Control	28.2 ± 2.8	38.0 ± 2.0	51.0 ± 3.0	75.0 ± 3.0	77.0 ± 3.0
7a	6.5 ± 2.1 (77.0%)***	28.0 ± 4.0 (26.7%)	34.0 ± 5.0 (33.3%)	34.0 ± 4.0 (54.4%)***	33.0 ± 5.0 (57.6%)***
7b	29.8 ± 4.3	44.2 ± 5.4	49.0 ± 4.0 (4.1%)	55.0 ± 3.0 (26.7%)*	53.0 ± 2.0 (30.4%)*
7c	13.3 ± 2.7 (53.0%)*	31.0 ± 7.0 (17.8%)	28.0 ± 5.0 (44.7%)*	19.0 ± 5.0 (74.4%)***	36.0 ± 5.0 (53.3%)***
7d	30.3 ± 4.6	36.0 ± 7.0 (3.7%)	43.0 ± 5.0 (16.5%)	57.0 ± 5.0 (24.4%)*	57.0 ± 8.0 (25.3%)*
7e	14.4 ± 3.5 (48.9%)*	29.0 ± 6.0 (23.0%)	39.0 ± 8.0 (24.1%)	42.0 ± 7.0 (44.4%)***	44.0 ± 6.0 (42.8%)***
7f	25.1 ± 3.6 (10.9%)	40.0 ± 6.1	33.0 ± 4.0 (35.0%)	38.0 ± 4.0 (50.0%)***	29.0 ± 3.0 (62.0%)***
7g	18.7 ± 5.8 (33.0%)	38.3 ± 4.0	39.0 ± 4.0 (24.0%)	53.0 ± 5.0 (29.0%)*	53.0 ± 6.0 (31.5%)*
7h	27.4 ± 4.4	42.5 ± 5.3	47.0 ± 5.0 (9.0%)	68.0 ± 6.0 (10.0%)	80.0 ± 6.2
7i	12.2 ± 4.2 (56.9%)*	40.0 ± 5.8	43.0 ± 5.0 (17.0%)	58.0 ± 4.0 (22.2%)	66.0 ± 4.0 (14.1%)
7j	24.0 ± 3.4 (14.9%)	35.0 ± 5.0 (6.6%)	43.0 ± 4.0 (15.4%)	61.6 ± 5.0 (19.2%)	62.0 ± 7.0 (18.8%)
8a	4.8 ± 1.7 (83.2%)***	40.0 ± 4.3	44.0 ± 4.0 (13.9%)	32.0 ± 3.0 (57.8%)***	31.0 ± 4.0 (59.8%)***
8b	11.5 ± 3.6 (59.2%)*	34.0 ± 4.0 (8.9%)	28.0 ± 8.0 (46.3%)*	31.0 ± 5.0 (58.9%)***	28.0 ± 5.0 (63.4%)***
8f	7.5 ± 3.5 (73.4%)***	28.0 ± 4.0 (26.0%)	37.0 ± 6.0 (28.5%)	34.0 ± 6.0 (54.0%)***	32.0 ± 7.0 (58.7%)***
8g	15.3 ± 1.8 (45.7%)	27.0 ± 5.0 (27.4%)	42.0 ± 8.0 (18.7%)	37.0 ± 5.0 (50.4%)***	40.0 ± 3.0 (47.8%)***
ASA	8.8 ± 2.9 (68.9%)***				
INDO		32.0 ± 4.0 (15.5%)	35.0 ± 4.0 (31.7%)	41.0 ± 3.0 (45.6%)***	42.0 ± 4.0 (45.7%)***

Data obtained from animal experiments were expressed as means ± SEM. *ASA* aspirin, 100 mg/kg (body weight); *INDO* indomethacin, 10 mg/kg (body weight); and all test drugs, 100 mg/kg (body weight) were s.c. administered to mice ($n = 6$–12) for [a] PBQ-induced writhing and [b] CG-induced paw edema tests, respectively. * Statistically significance were evaluated from the control by One-way ANOVA post hoc Dunnett's test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

leukotrienes also contribute to nociception in this model. The PBQ but not acetic acid writhing also depends on cytokines such as IL-18 and interferon (IFN)-γ, and endothelin-1. It was also shown that PBQ-induced writhing response in mice depends on spinal activation of ERK, JNK, p38, and PI3K, and microglia (Pavao-de-Souza *et al.*, 2012; Verri *et al.*, 2008). Meanwhile, a hotplate latency tests are reported to quantify antinociception mediated primarily by spinal and supraspinal mechanisms, and generally used to evaluate the analgesic activity of centrally acting substances such as narcotics (Baker *et al.*, 2002). As it is very common in the literature and also our experience in our laboratory also confirms this fact in our various demonstrations, we employed *p*-Benzoquinone-induced writhing test to evaluate the analgesic activity of title compounds.

The results shown in Table 1 indicated that the preparation of the amide derivatives of both regioisomeric 1-benzyl-3(5)-*p*-tolyl-1*H*-pyrazole-5(3)-carboxylic acids with ethyl piperidinecarboxylate resulted in compounds with potent analgesic activity (**7a**, 77 % and **8a**, 83 %) which was higher than aspirin at the same dose of 100 mg/kg. In the case of *t*-butylbenzylpiperazine amides (**7b**, **8b**), there was a discrepancy in favor of 1-benzyl-5-(*p*-tolyl)-1*H*-pyrazole-3-carboxamide core (**8b**, 59 %) since **7b** failed to induce analgesic activity. In addition, trimethoxybenzylpiperazine amide (**7c**) and the carbohydrazide derivative (**7e**) of pyrazole-5-carboxylic acid nucleus resulted in comparable activity with respect to reference aspirin with a small decrease in the inhibitory potential of writhing movements. In the case of ester analogs, while the *t*-butylphenyl ester of 1*H*-pyrazole-3-carboxylate core (**8f**) demonstrated very potent analgesic activity (73 %), the regioisomeric counterpart (**7f**) lacked the similar potency (11 %). The replacement of *t*-butyl in **8f** with *i*-propyl (**8g**) in the ester part caused a decrease in analgesic potency (45 %). Moreover, while the **7h** with a larger heptyloxyphenyl group in the ester portion was ineffective, trifluoromethylphenyloxyphenyl group in the ester portion (**7i**) restored the analgesic efficiency (57 %).

Anti-inflammatory activities of the compounds were also in good correlation with the analgesic activity results, tested by using the carrageenan-induced hind paw edema model (Table 1) (Kasahara *et al.*, 1985). As shown, the same amide derivatives (**7a**, **7c**, **7e**, **8a**, and **8b**) as well as ester derivatives (**7f**, **8f**, and **8g**) resulted in potent anti-inflammatory activity which was comparable to indomethacin. It is claimed that an edema produced by carrageenan is a biphasic event and the inhibitory effects of compounds which act on the first stage are attributable to

the inhibition of the release of chemical mediators such as histamine, serotonin, and bradykinin (Vinegar *et al.*, 1969). However, the second phase of edema might be related to the release of AA metabolites since it is potently suppressed by aspirin, indomethacin, and other COX inhibitor drugs (Vinegar *et al.*, 1969). As shown in Table 1, the most active derivatives showed a similar pattern with indometacin with an absolute gradual increase in the second phase (270 min), therefore suggesting that these compounds might exert their anti-inflammatory activities through the mechanisms involving the inhibition of the formation of AA metabolites. It should be noted that the amidation of the carboxylic acid with lipophilic amines such as ethyl piperidinecarboxylate (**7a**–**8a**), *t*-butylbenzylpiperazine (**8b**), and trimethoxybenzylpiperazine (**7c**) had a significant impact for rising the anti-inflammatory potential of either regioisomeric pyrazole-3(5)-carboxylic acids. In the case of ester derivatives, only the incorporation of small lipophilic ester parts such as *t*-butylphenyl (**7f**–**8f**) or *i*-propylphenyl (**8g**) was able to contribute to an increase in the anti-inflammatory activity. Taken together, we can conclude that 1-benzyl-3(5)-(*p*-tolyl)-1*H*-pyrazole-5(3)-carboxylic acid cores can be modified to a potent analgesic and anti-inflammatory compounds by preparing lipophilic amide (i.e. 4-*t*-butylbenzylpiperazine and ethyl piperidinecarboxylate) and ester derivatives (i.e. 4-*t*-butylphenyl) with potential for novel treatments of inflammation-related disorders as was in good agreement with our previous findings for 1,5-diarylpyrazole-3-propanoic acid amides (Caliskan *et al.*, 2011).

Since the formation of pro-inflammatory eicosanoids (i.e. PGs and LTs) are evidenced to be a visible key regulators of cell proliferation and neo-angiogenesis, the inhibitors of the pathways for their formation are being investigated as potential anti-cancer drugs (Claria and Romano, 2005; Romano and Claria, 2003). Hormone-dependent MCF-7 cells have a basal level of COX-1 and a barely detectable and transient COX-2 inducible expression, and hormone-independent MDA-MB-231 breast cancer cell lines show a low expression of COX-1 but a constitutive level of COX-2 (Liu and Rose, 1996). Moreover, overexpression of COX-2 has been reported in hematological cancer models such as Raji (Burkitt's lymphoma) (Sobolewski *et al.*, 2010; Wun *et al.*, 2004). COX-2 and 5-LO also differentially expressed in the HeLa (human cervical cancer cells) and HL-60 (a human promyelocytic leukemia) cell lines and their growth was found sensitive to NSAID treatment (Agrawal and Fentiman 2008; Chao *et al.*, 2005; Liu *et al.*, 2011; Pereg and Lishner 2005; Sobolewski *et al.*, 2010; Totzke *et al.*, 2003; Werz *et al.*, 1997). Considering that our compounds displayed promising in vivo anti-inflammatory activities presumably by inhibiting the formation of AA metabolites, we selected above cell lines which provides a suitable cellular model system to investigate the anti-cancer potential of these derivatives.

The results for each tested compounds at a 50 μM screening dose are reported as growth percentages compared with the untreated control cells after 24 and 48 h of drug exposure (Table 2). Owing to the solubility problems, compounds **7c** and **7e** were not evaluated in the MTT assay. The compound **8b** bearing *t*-butylbenzylpiperazine group in the amide part of pyrazole-3-carboxamide core displayed significant growth inhibitory activity against all cell lines (85 % for HL-60, 60 % for MDA-MB-231, 65 % for HeLA, 50 % for MCF7, and 70 % for Raji cells at 48 h). For estrogen-independent breast cancer cells (MDA-MB-231), all compounds demonstrated mild to moderate inhibitory activity on cell viability at 48 h (25–75 % inhibition), as the compound **7j**, an imidazolylphenyl ester of pyrazole-5-carboxylate, being the most efficient derivative (75 %). The efficiency of compounds against HeLa cervical cancer cells was also ranged between 20 and 70 % with **7j** (66 %) and **8b** (65 %) showing equipotent inhibitory activity which was followed by **8a** (52 %) and **7a** (50 %) having the most considerable growth inhibition at both end time points. The sensitivity of **8a** (60 %) and **8b** (70 %) was found higher to B lymphocyte cell line (Raji) among all other derivatives. For human breast adenocarcinoma MCF7 cell line, only **7j** (58 %) and **8b** (54 %) showed inhibitory potential on cell growth while others found mostly inefficient. Lastly, for HL-60 leukemia cell line, **8b** (88 %) followed by **7d** (70 %, 3-pyridinylmethyl amide derivative) displayed significant antitumor activity, especially at 24 h. HL-60 leukemia cells were insensitive to all other derivatives. Compounds **7j** and **8b** were chosen for concentration–response studies at five-concentrations on selected cell lines where their inhibitory potential was more pronounced. IC_{50} values for **8b** for HeLa and HL-60, and for **7j** for HeLa cell lines were calculated as 30.5, 24.4, and 30.4 μM, respectively.

Since lipophilicity is an important factor for molecules to cross cell membranes and the partition coefficient log *P* is a parameter describing this behavior of a drug molecule, we calculated the predicted lipophilicity (log *P*) values of the synthesized compounds using ACD/Labs 6.0 software. While the lipophilicity (log *P*) values of tested compounds were in the range of 3.15–8.62, our results indicated that compounds possessing the lipophilicity with log *P* values in the range of 3.15–5.31 (**7a**, **7d**, **7j**, **8a**, and **8b**) demonstrated better inhibitory effects on the growth of selected cancer cells. Our results showed good correlation with previously published anticancer activity of similar pyrazole derivatives in which they showed growth inhibitory effect on A549 lung cancer cells with log *P* values in the range of 3.12–4.94 (Xia *et al.*, 2007, 2008).

Table 2 Effects of the compounds on the viability of selected cancer cell lines

Compounds	MDA-MB-231		HeLa		Raji		MCF7		HL60	
	CV%		CV%		CV%		CV%		CV%	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
DMSO	100 ± 7.08	100 ± 3.71	100 ± 4.13	100 ± 3.8	100 ± 5.02	100 ± 4.26	100 ± 2.74	100 ± 5.08	100 ± 3.55	100 ± 8.41
7a	96.5 ± 2.07	64 ± 9.02***	53.5 ± 4.79***	49.9 ± 4.73***	77 ± 4.61***	97.3 ± 3.39	54.2 ± 6.68***	88.2 ± 7.43*	102.4 ± 10.2	91.7 ± 5.26
7b	74 ± 8.68***	51 ± 5.77***	84.8 ± 6.97**	60.6 ± 1.16***	100.8 ± 4.98	108.7 ± 8.32	94.4 ± 1.05**	66.6 ± 10.6***	128.5 ± 1.67	103.8 ± 2.65
7d	65.1 ± 9.76***	43.1 ± 5.78**	74.9 ± 5.16***	82.6 ± 8.58**	86.4 ± 1.82***	93.4 ± 6.96	104.7 ± 7.88	100.4 ± 9.09	31.7 ± 5.86***	41.9 ± 2.69***
7f	119 ± 8.26	62.9 ± 4.9***	83.2 ± 4.4***	81.4 ± 4.83***	94.9 ± 4.24	102.8 ± 6.11	93.2 ± 3.55**	94.9 ± 7.0	120.1 ± 7.56	111.8 ± 1.83
7g	71.9 ± 9.57***	50.2 ± 2.99***	87.2 ± 1.98	69.7 ± 5.3***	105 ± 4.27	94.2 ± 4.47	80.3 ± 9.27**	90.5 ± 2.85**	114.7 ± 8.56	125.9 ± 5.9
7h	114.6 ± 4.96	63.2 ± 12.05***	109 ± 4.41	85.3 ± 6.47**	76.3 ± 1.46***	84.3 ± 3.31***	100.5 ± 4.07	97.6 ± 9.38	118.1 ± 6.27	108.4 ± 9.85
7i	75.5 ± 3.95***	50.3 ± 2.11***	89.1 ± 8.09*	77.4 ± 6.49***	111 ± 2.84	120.9 ± 2.73	63.6 ± 2.91***	107.2 ± 9.8	114.1 ± 4.3	86.3 ± 8.34***
7j	33.1 ± 4.39***	24.4 ± 2.1***	36.7 ± 6.01***	33.9 ± 6.47***	96.5 ± 3.86	105.6 ± 3.94	67.6 ± 1.98***	52.4 ± 7.78***	107.7 ± 2.46	120.8 ± 2.58
8a	121.3 ± 5.23	71.8 ± 8.67***	66.4 ± 4.09***	47.1 ± 2.85***	56.5 ± 0.85***	41.6 ± 0.92***	77.8 ± 8.77***	94.5 ± 3.93	88.2 ± 0.95***	78.8 ± 1.59***
8b	67.2 ± 2.79***	41.1 ± 4.82***	52.5 ± 2.4***	34.5 ± 4.82***	44.4 ± 2.19***	29.6 ± 2.74***	51.4 ± 5.14***	56.7 ± 5.63***	11.9 ± 3.92***	15.5 ± 3.64***
8f	93.7 ± 4.55	70.5 ± 8.2***	117.5 ± 5.33	62.1 ± 3.32***	110.9 ± 5.72	95.3 ± 5.55**	90.9 ± 8.57	92.4 ± 4.88*	134.1 ± 5.32	69.7 ± 8.47***
8g	106.2 ± 9.35	66.2 ± 2.85***	79.6 ± 4.88**	54.6 ± 4.57***	90.1 ± 5.7***	97 ± 6.75**	79.6 ± 0.52***	82.6 ± 9.78**	120.2 ± 4.82	83.6 ± 7.8*
Cispl	94.5 ± 2.57	88.4 ± 4.78**	92.3 ± 3.41*	71.6 ± 4.32**	79.4 ± 2.29***	31 ± 0.51***	87.9 ± 5.39**	61.1 ± 6.18***	74.8 ± 0.95***	23.9 ± 1.79***

MDA-MB-231 estrogen-independent human breast cancer cells, *HeLa* human cervical cancer cells, *Raji* human B lymphocyte cancer cells, *MCF7* human breast adenocarcinoma cells, *HL-60* human promyelocytic leukemia cancer cells. Data obtained were expressed as means ± SD ($n = 5$. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$ vs control)

Conclusion

Our results clearly show that derivatization of 1-benzyl-3(5)-(*p*-tolyl)-1*H*-pyrazole-5(3)-carboxylic acid scaffolds into lipophilic amides and esters act as in vivo potent analgesic and anti-inflammatory compounds as compared to reference drugs aspirin and indomethacin, exemplified by **7a**, **7c**, **7e–f**, **8a–b**, and **8f–g**. Comparison between the regiosiomeric main pyrazole scaffolds, amide (**8a–b**), and ester (**8f–g**) derivatives of 1-benzyl-5-(*p*-tolyl)-1*H*-pyrazole-3-carboxylic acid were more preferred over the same amides (**7a–b**)/esters (**8f–g**) of 1-benzyl-3-(*p*-tolyl)-1*H*-pyrazole-5-carboxylic acid. Meanwhile, none of the other ester derivatives of pyrazole-5-carboxylic acid regioisomer were found active indicating that amide derivatization was more useful approach for this scaffold.

The results of the anti-cancer assays revealed an activity at the tested μM range linked particularly five compounds (**7a**, **7d**, **7j**, **8a**, and **8b**) in which three of them (**7a**, **8a–b**) endowed with potent in vivo anti-inflammatory activity in the second phase of carrageenan-induced hind paw edema. Interestingly, among these compounds, the only ester derivative **7j** with considerable anti-cancer activity for HeLa and MDA-MB-231 cells lacked the potency as an analgesic or anti-inflammatory agent. Although induction of cytotoxicity toward selected cancer-specific cells does not preclude the probable cytotoxicity against normal cells, the promising results obtained with the compounds bearing 1-benzyl-3(5)-(*p*-tolyl)-1*H*-pyrazole-5(3)-carboxylic acid scaffolds encourage us to develop new antitumor substances with good selectivity toward cancer cells and studies for determining the selectivity of new series of compounds are under ongoing work in our laboratory.

Materials and methods

Chemistry

^{1}H NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a Varian Mercury 400 MHz High Performance Digital FT-NMR spectrometer using tetramethylsilane as the internal standard at the NMR facility of Faculty of Pharmacy, Ankara University. All chemical shifts were recorded as δ (ppm). High resolution mass spectra data (HRMS) were collected in-house using a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) operating in ESI (+) method, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA). Melting points were determined with an SMP-II Digital Melting Point Apparatus and are uncorrected (Schorpp Geaetetechnik, Germany). IR spectra were obtained using a Perkin Elmer Spectrum 400 FTIR/FTNIR spectrometer equipped with a Universal ATR Sampling Accessory. Flash chromatography was performed with a Combiflash®Rf automated flash chromatography system with RediSep columns (Teledyne-Isco, Lincoln, NE, USA) using hexane–ethyl acetate or dichloromethane–methanol solvent gradients. The purity of the final compounds was determined to be >97 % by UPLC with UV detector. 4-(4-methylphenyl)-2,4-dioxobutanoate (**3**) was synthesized by the reaction of 4′-methylacetophenone with dimethyloxalate (Maurin *et al.*, 2004). 2-Hydrazinoquinolin was synthesized by the reaction of 2-chloroquinoline with hydrazine hydrate (Gupta *et al.*, 2007). *p*-Benzoquinone (PBQ), λ-Carrageenan (type IV) and other biochemical's used in this study were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA).

*Methyl 3-(p-tolyl)-1H-pyrazole-5-carboxylate **(4)***

To a mixture of 4-(*p*-tolyl)-2,4-dioxobutanoate (**1**) (12 mmol) in acetic acid (20 ml), hydrazine hydrate (13.2 mmol) was added and heated at reflux temperature for 40 min. The resulting solution was cooled, the precipitate was collected by filtration and washed with water. The product was used for the next step without further purification.Yield 96 %. HRMS (*m/z*): $[M + H]^+$ calcd for $C_{12}H_{13}N_2O_2$, 217.0977; found, 217.0974.

*Methyl 1-benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylate **(5)** and methyl 1-benzyl-5-(4-methylphenyl)-1H-pyrazole-3-carboxylate **(6)***

To a mixture of **4** (10 mmol) and K_2CO_3 (12 mmol) in acetonitrile (40 ml), benzyl chloride (15 mmol) was added and refluxed for 8 h. The reaction mixture was cooled to room temperature, filtered, and solvent was evaporated under reduced pressure and the regioisomers were separated by automated flash chromatography. The product **5** was obtained as white solid in 77 % yield and **6** was obtained as white solid in 15 % yield. For **5**; HRMS (*m/z*): $[M + H]^+$ calcd for $C_{19}H_{19}N_2O_2$, 307.1447; found, 307.1444. For **6**; HRMS (*m/z*): $[M + H]^+$ calcd for $C_{19}H_{19}N_2O_2$, 307.1447; found, 307.1452.

*1-Benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylic acid (7) and 1-benzyl-5-(4-methylphenyl)-1H-pyrazole-3-carboxylic acid **(8)***

Compounds **5** and **6** (5 mmol) were hydrolyzed to corresponding acids, in THF/water (1:1) with $LiOH{\cdot}H_2O$ (15 mmol) by heating at 70 °C for 2 h. The reaction mixture was poured into water and acidified with 6 N HCl, precipitate was collected by filtration and washed with water, and used in

the synthesis of amide and ester derivatives. For **7**; Yield 96 %; Mp 190–191 °C; IR (ATR): 1,686 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.38 (s, 3H), 5.80 (s, 2H), 7.22–7.29 (m, 8H), 7.73 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{18}H_{17}N_2O_2$, 293.1290; found, 293.1290. For **8**; yield 98 %; Mp 120–121°C; IR (ATR): 1,675 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.39 (s, 3H), 5.40 (s, 2H), 6.91 (s, 1H), 7.03–7.06 (m, 2H), 7.17–7.28 (m, 7H); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{18}H_{17}N_2O_2$, 293.1290; found, 293.1281.

General synthesis of amide and ester derivatives

To a mixture of the appropriate carboxylic acid derivative **7** or **8** (1 mmol) in 10 ml CH_2Cl_2, the appropriate amine or phenol derivative (1.1 mmol), 4-dimethylaminopyridine (DMAP) (0.2 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (1.1 mmol) were added, and stirred at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 and washed with 1 N HCl (3 × 20 ml), 5 % $NaHCO_3$ (3 × 20 ml), and brine. After drying over Na_2SO_4, solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography.

*Ethyl 1-{[1-benzyl-3-(4-methylphenyl)-1H-pyrazol-5-yl]-carbonyl}piperidine-4-carboxylate (**7a**)*

Prepared from ethyl piperidine-4-carboxylate. Yield 63 %; Mp 96–97 °C; IR (ATR): 2976, 1717, 1624, 1440, 1043, 702 cm^{-1}; ^1H NMR (DMSO-d_6): δ 1.06 (bs, 1H), 1.18 (t, 3H, $J = 7.2$ Hz), 1.40 (bs, 1H), 1.60 (bs, 1H), 1.84 (bs, 1H), 2.32 (s, 3H), 2.52–2.58 (m, 1H), 2.89–3.02 (m, 2H), 3.70 (bs, 1H), 4.07 (q, 2H, $J = 7.2$ Hz), 4.26 (bs, 1H), 5.42 (s, 2H), 6.93 (s, 1H), 7.18–7.32 (m, 7H), 7.72 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{26}H_{30}N_3O_3$, 432.2287; found, 432.2271.

*1-{[1-Benzyl-3-(4-methylphenyl)-1H-pyrazol-5-yl]-carbonyl}-4-(4-tert-butylbenzyl) piperazine (**7b**)*

Prepared from 1-(4-*t*-butylbenzyl)piperazine. Yield 56 %; Mp 145.2–145.5 °C; IR (ATR): 2950, 1626, 1442, 832, 749, 706 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.31 (s, 9H), 1.93 (s, 2H), 2.34 (s, 2H), 2.37 (s, 3H), 3.29 (s, 2H), 3.36 (s, 2H), 3.64 (s, 2H), 5.53 (s, 2H), 6.52 (s, 1H), 7.16–7.34 (m, 11H), 7.68 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{33}H_{39}N_4O$, 507.3124; found, 507.3127.

*1-{[1-Benzyl-3-(4-methylphenyl)-1H-pyrazol-5-yl]-carbonyl}-4-(2,3,4-trimethoxybenzyl)piperazine (**7c**)*

Prepared from 1-(2,3,4-trimethoxylbenzyl)piperazine. Yield 50 %; Mp 134–134.5 °C; IR (ATR): 2917, 1628, 1458, 821, 748 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.98 (bs, 2H), 2.37 (m, 5H), 3.28 (s, 2H), 3.36 (s, 2H), 3.63 (bs, 2H), 3.85 (s, 3H), 3.87 (s, 6H), 5.52 (s, 2H), 6.52 (s, 1H), 6.62 (d, 1H, $J = 8.0$ Hz), 6.90 (d, 1H, $J = 8.0$ Hz), 7.21 (d, 2H, $J = 8.0$ Hz), 7.24–7.28 (m, 5H), 7.69 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{32}H_{37}N_4O_4$, 541.2815; found, 541.2795.

*1-Benzyl-3-(4-methylphenyl)-N-(pyridin-3-ylmethyl)-1H-pyrazole-5-carboxamide (**7d**)*

Prepared from pyridin-3-ylmethylamine. Yield 55.7 %; Mp 172–173 °C; IR (ATR): 3035, 2935, 1647, 1522, 1428, 1281, 715 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.36 (s, 3H), 4.56 (d, 2H, $J = 6.4$ Hz), 5.82 (s, 2H), 6.37 (t, 1H), 6.79 (s, 1H), 7.19–7.29 (m, 8H), 7.52 (m, 1H), 7.67 (d, 2H, $J = 8.4$ Hz), 8.52–8.54 (m, 2H); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{24}H_{23}N_4O$, 383.1872; found, 383.1854.

*1-Benzyl-3-(4-methylphenyl)-N′-quinolin-2-yl-1H-pyrazole-5-carbohydrazide (**7e**)*

Prepared from 2-hydrazinoquinoline. Yield 25 %; Mp 231–232 °C; IR (ATR): 3252, 2909, 1654, 1618, 1509, 1429, 759, 718, 699 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.36 (s, 3H), 5.72 (s, 2H), 6.80 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.17–7.33 (m, 8H), 7.51–7.54 (m, 1H), 7.62–7.68 (m, 4H), 7.86 (d, 1H, $J = 8.4$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{27}H_{24}N_5O$, 434.1981; found, 434.1970.

*4-tert-Butylphenyl 1-benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylate (**7f**)*

Prepared from 4-*t*-butylphenol. Yield 78 %; Mp 127–127.6 °C; IR (ATR): 3134, 2958, 1725, 1439, 1247, 1201, 1066, 817 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.33 (s, 9H), 2.39 (s, 3H), 5.82 (s, 2H), 7.05 (d, 2H, $J = 8.8$ Hz), 7.23–7.34 (m, 7H), 7.42 (d, 2H, $J = 8.8$ Hz), 7.77 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{28}H_{29}N_2O_2$, 425.2229; found, 425.2209.

*4-Isopropylphenyl 1-benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylate (**7g**)*

Prepared from 4-*i*-propylphenol. Yield 79 %; Mp 96–97 °C; IR (ATR): 3127, 2949, 1735, 1504, 1241, 1194, 1046, 817 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.25 (d, 6H, $J = 6.8$ Hz), 2.39 (s, 3H), 2.93 (m, 1H), 5.82 (s, 2H), 7.05 (d, 2H, $J = 8.8$ Hz), 7.23–7.35 (m, 10H), 7.77 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{22}H_{27}N_2O_2$, 411.2073; found, 411.2059.

*4-(Heptyloxy)phenyl 1-benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylate (**7h**)*

Prepared from 4-heptyloxyphenol. Yield 78 %; Mp 91.5–92 °C; IR (ATR): 3062, 2947, 1734, 1504, 1246,

1197, 1060, 818 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.89 (t, 3H, $J = 6.8$ Hz), 1.31–1.47 (m, 8H), 1.77 (m, 2H), 2.39 (s, 3H), 3.94 (t, 2H, $J = 6.8$ Hz), 5.81 (s, 2H), 6.90 (d, 2H, $J = 8.8$ Hz), 7.03 (d, 2H, $J = 8.8$ Hz), 7.23–7.35 (m, 7H), 7.76 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{31}H_{35}N_2O_3$, 483.2648; found, 483.2632.

*4-[4-(Trifluoromethyl)phenoxy]phenyl 1-benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylate (**7i**)*

Prepared from **5** 4-[4-(trifluoromethyl)phenoxy]phenol. Yield 68 %; Mp 109–110 °C; IR (ATR): 3037, 1734, 1495, 1318, 1054, 818, 723 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.39 (s, 3H), 5.83 (s, 2H), 7.06–7.10 (m, 4H), 7.16 (d, 2H, $J = 8.8$ Hz), 7.24–7.32 (m, 7H), 7.35 (s, 1H), 7.59 (d, 2H, $J = 8.8$ Hz), 7.77 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{31}H_{24}F_3N_2O_3$, 529.1739; found, 529.1746.

*4-(1H-Imidazol-1-yl)phenyl 1-benzyl-3-(4-methylphenyl)-1H-pyrazole-5-carboxylate (**7j**)*

Prepared from 4-(1*H*-imidazol-1-yl)phenol. Yield 64 %; Mp 167.5–168 °C; IR (ATR): 3133, 2945, 1736, 1518, 1508, 1199, 1051, 808, 726 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.40 (s, 3H), 5.82 (s, 2H), 7.22–7.35 (m, 11H), 7.37 (s, 1H), 7.44 (d, 2H, $J = 8.4$ Hz), 7.78 (d, 2H, $J = 8.0$ Hz), 7.84 (s, 1H); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{27}H_{23}N_4O_2$, 435.1821; found, 435.1820.

*Ethyl 1-{[1-benzyl-5-(4-methylphenyl)-1H-pyrazol-3-yl]-carbonyl}piperidine-4-carboxylate (**8a**)*

Prepared from ethyl piperidine-4-carboxylate. Yield 79 %; Mp 98–99 °C; IR (ATR): 3131, 2923, 1720, 1624, 1486, 1164, 1043, 987, 734 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.26 (t, 3H, $J = 7.0$ Hz), 1.73–1.83 (m, 2H), 1.92 (m, 1H), 2.02 (m, 1H), 2.38 (s, 3H), 2.59 (m, 1H), 3.00 (t, 1H), 3.30 (t, 1H), 4.15 (q, 2H, $J = 7.0$ Hz), 4.57 (m, 1H), 4.68 (m, 1H), 5.32 (s, 2H), 6.69 (s, 1H), 7.04–7.06 (m, 2H), 7.23 (s, 4H), 7.24–7.30 (m, 3H); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{26}H_{30}N_3O_3$, 432.2287; found, 432.2273.

*1-{[1-benzyl-5-(4-methylphenyl)-1H-pyrazol-3-yl]-carbonyl}-4-(4-tert-butylbenzyl) piperazine (**8b**)*

Prepared from 1-(4-*t*-butylbenzyl)piperazine. Yield 49 %; Mp 125–126 °C; IR (ATR): 3022, 2958, 1621, 1483, 1255, 963, 815, 729 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.32 (s, 9H), 2.39 (s, 3H), 2.80 (bs, 2H), 3.40 (bs, 2H), 3.71 (s, 1H), 4.13 (m, 3H), 4.80 (bs, 1H), 5.28 (s, 3H), 6.77 (s, 1H), 7.00–7.02 (m, 2H), 7.18–7.29 (m, 7H), 7.46 (d, 2H, $J = 8.0$ Hz), 7.53 (d, 2H, $J = 8.0$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{33}H_{39}N_4O$, 507.3124; found, 507.3114.

*4-tert-Butylphenyl 1-benzyl-5-(4-methylphenyl)-1H-pyrazole-3-carboxylate (**8f**)*

Prepared from 4-*t*-butylphenol. Yield 67 %; Mp 119.5–120 °C; IR (ATR): 2946, 1732, 1462, 1204, 1196, 993, 807, 716 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.33 (s, 9H), 2.39 (s, 3H), 5.46 (s, 2H), 6.99 (s, 1H), 7.06–7.08 (m, 2H), 7.17 (d, 2H, $J = 8.8$ Hz), 7.19–7.29 (m, 7H), 7.42 (d, 2H, $J = 8.8$ Hz); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{28}H_{29}N_2O_2$, 425.2229; found, 425.2215.

*4-Isopropylphenyl 1-benzyl-5-(4-methylphenyl)-1H-pyrazole-3-carboxylate (**8g**)*

Prepared from 4-*i*-propylphenol. Yield 82 %; Mp 129–130 °C; IR (ATR): 3141, 2957, 1732, 1455, 1167, 990, 873, 734 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.26 (d, 6H, $J = 6.8$ Hz), 2.39 (s, 3H), 2.93 (m, 1H), 5.46 (s, 2H), 6.99 (s, 1H), 7.06–7.08 (m, 2H), 7.16 (d, 2H, $J = 8.8$ Hz), 7.20–7.30 (m, 9H); HRMS (*m/z*): $[M + H]^+$ calcd for $C_{27}H_{27}N_2O_2$, 411.2073; found, 411.2054.

Pharmacological screening

Animals

Male, Swiss albino mice (25–30 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey). They were housed in a room with controlled temperature (22 ± 1 °C), humidity (55 ± 10 %), and photoperiod (12:12 h) light–dark cycle for at least a week before being used. They were maintained on standard pellet diet and water ad libitum throughout the experiment. A minimum of six animals were used in each group. Throughout the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals under the audit of Gazi University Commission of Animal Ethics (Permission no: G.U.ET-11.091/188-17453).

Analgesic activity

Analgesic activity was evaluated using the *p*-Benzoquinone (PBQ)-induced writhing test in mice (Okun *et al.*, 1963). Thirty minutes after the subcutaneous administration of a test sample in dimethyl sulfoxide (DMSO) (100 mg/kg body weight), the mice were intraperitoneally injected with 0.1 ml/10 g body weight of 2.5 % (w/v) PBQ solution in distilled water. Control animals received an appropriate volume of dosing vehicle (DMSO). The mice were then kept individually for the observation and the total number of the abdominal contractions (writhing

movements) was counted for the following 15 min, starting 5 min after the PBQ injection. The data represent the average of the total number of writhes observed. Analgesic activity was then expressed as the percentage change from writhing controls.

Anti-inflammatory activity

Carrageenan-induced hind paw edema model was utilized for evaluation of anti-inflammatory activity (Kupeli *et al.*, 2007). Each experimental group contained seven animals minimally. Thirty minutes after the subcutaneous administration of a test sample in DMSO (100 mg/kg body weight) or dosing vehicle, each mouse was injected with freshly prepared suspension of carrageenan (0.5 mg/25 µl) in physiological saline into sub-plantar tissue of the right hind paw. As a control group, 25 µl saline was injected into the left hind paw. Paw edema was measured in 90, 180, 270, and 360 min after carrageenan injections. The difference in hind paw thickness was measured by a caliber compasses (Ozaki Co., Tokyo, Japan). Mean values of each treated groups were compared with control group and analyzed by using statistical methods.

Acute toxicity

At the end of these experiments after 48 h observation of all animals, no morbidity or mortality has been recorded.

Cell culture and viability assay

MDA-MB-231 and MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) whereas HeLa, HL60, and Raji cells were grown in RPMI (Roswell Park Memorial Institute)-1640 medium in a humidified atmosphere containing 5 % CO_2 at 37 °C. Both DMEM and RPMI-1640 medium were supplemented with 10 % fetal bovine serum (FBS), 200 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all from Hyclone Laboratories, Logan, UT, USA). Cell viability was assessed using MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide) assay (Cell Proliferation Kit I, Roche, Germany). In brief, cells were seeded in a 96-well plate at 10,000 cells per well, cultured for overnight in complete growth medium-containing 1 % FBS. Then cells treated with 50 µM of test compounds in DMSO for 24 and 48 h. At the end of the treatment times, MTT reagent at the final concentration of 0.5 mg/ml was added to each well and incubated for an additional 4 h. After formation of blue formazan crystals, medium containing MTT was discarded and DMSO was added to the wells to dissolve crystals. The absorbance of samples was measured with Spectra Max M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm. Cell treated with 10 µM cisplatin were used as a positive control.

Statistical analysis

The data were expressed as means ± SEM or ±SD. The significance of differences between the treatment and the control group of animals were determined by one-way ANOVA with Barlett test following post hoc Student–Newman–Keuls multiple comparisons test for analgesic activity and two-way ANOVA following post hoc Bonferroni test for anti-inflammatory activity. Data for anticancer activity were analyzed by one-way ANOVA. P value <0.05 was considered statistically significant.

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