Computer-Aided Discovery of Anti-Inflammatory Thiazolidinones with Dual Cyclooxygenase/Lipoxygenase Inhibition

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Received July 24, 2007

New anti-inflammatory agents possessing dual cyclooxygenase/lipoxygenase (COX/LOX) inhibition were discovered by computer-aided prediction of biological activity for 573 virtually designed chemical compounds. Prediction of biological activity was performed by PASS, and prediction results were analyzed with PharmaExpert software. Nine 2-(thiazole-2-ylamino)-5-phenylidene-4-thiazolidinone derivatives differing by the phenyl group substitution were selected for synthesis and experimental testing as potential COX/LOX inhibitors. Eight tested compounds exhibited anti-inflammatory activity in the carrageenin-induced paw edema. It was shown that seven tested compounds (77.8%) were LOX inhibitors, seven compounds were COX inhibitors (77.8%), and six tested compounds (66.7%) were dual COX/LOX inhibitors. Analysis of lipophilicity of the compounds showed a negative correlation with inhibition of edema formation. The binding modes of the most active compounds of this series (2-(thiazole-2-ylamino)-5-(*m*-chlorophenylidene)-4-thiazolidinone for COX-1 and COX-2, and 2-(thiazole-2-ylamino)-5-(*m*-nitrophenylidene)-4-thiazolidinone for 15-LOX) were proposed on the basis of docking studies.

Introduction

For many years, clinicians have treated patients by combinations of drugs with different pharmacotherapeutic actions. It is being recognized that a balanced modulation of several targets can provide a superior therapeutic effect and a favorable side effect profile compared to the action of a selective ligand.[1] Compared to drug combinations, there are several advantages associated with ligands acting on multiple targets, such as the more predictable pharmacokinetic and pharmacodynamic properties that are a consequence of the administration of a single pharmaceutical substance, as well as improved patient compliance.[2] The molecular starting point of search for dual-acting molecules is determined by rational design using a combination of pharmacophores or by screening of compound libraries of known drugs. This screening may be executed by both in vitro and in silico. A study performed by Morphy and coauthors[1] showed that it is most probable to discover molecules acting on related proteins from the same or close protein families. Study of structures of ligands acting on different targets jointly with analysis of protein similarity, size, and features of their active centers can be used to discover potential targets for dual-acting agents. We have shown previously that the prediction of biological activity spectra for substances on the basis of their structural formulas by the computer program PASS[a] (Prediction of Activity Spectra for Substances) can be used in search of dual-acting antihypertensive agents (dual angiotensin-converting enzyme/neutral endopeptidase inhibitors).[3] The current version of PASS predicts more than 3300 types of biological activity including pharmacotherapeutic effects, mechanisms of action, interaction with drug-metabolizing enzymes, side effects, and toxicity. To analyze the PASS prediction results, taking into account the mechanism-effect relationships, and to search for compounds with the desirable profiles of biological activity, PharmaExpert[4] software was developed. The current version of PharmaExpert, based on information extracted from the literature, contains about 5700 mechanism-effect relationships. The combination of PASS and PharmaExpert provides a unique possibility to search for compounds with possible multitargeted action. The purpose of the present study is to find compounds with dual cyclooxygenase/lipoxygenase (COX/LOX) inhibition as a mechanism of anti-inflammatory action.

Inflammation is a multifactorial process. It reflects the response of organism to various stimuli and is related to many disorders such as arthritis, asthma, and psoriasis, which require prolonged or repeated treatment. Cyclooxygenase (COX) and lipoxygenase (LOX) produce two groups of arachidonic acid metabolites, prostaglandins (COX products) and leucotrienes (LOX products), that play a key role in inflammation. Traditional nonsteroidal anti-inflammatory drugs (NSAIDs) act via the inhibition of the COX-1 isoenzyme or the combined inhibition of COX-1 and COX-2 isoenzymes. For example, acetylsalicylate (aspirin) is a COX-1 selective inhibitor, whereas indomethacin (Indocin) and naproxen (Naprosyn) are COX-1/COX-2 inhibitors. Because COX-1 is mainly responsible for mucus formation in the gastrointestinal (GI) tract, COX-1 inhibition is blamed for inducing GI irritation, the main undesired side effect of such drugs.[5] Another side effect of selective COX-1 inhibitors, mild bleeding diathesis, also results from the inhibition of the COX-1 catalyzed synthesis of the platelet aggregation factor, thromboxan (TXA_2).[6]

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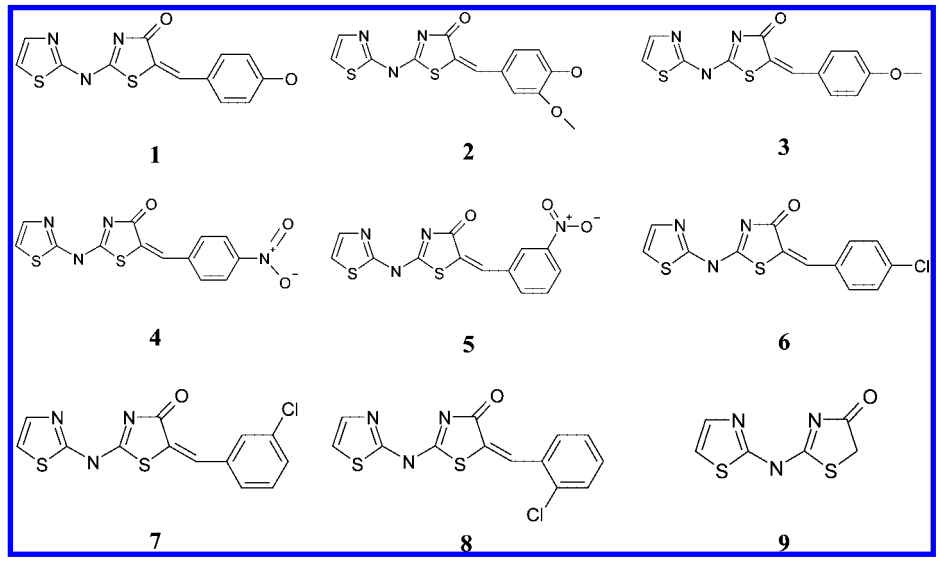
[a] Abbreviations: COX, cyclooxygenase; CPE, carrageenin-induced paw edema; LDL, low density lipoprotein; SAR, structure–activity relationships; LOX, lipoxygenase; NSAID, nonsteroidal anti-inflammatory drug; PASS, prediction of activity spectra for substances; RPTLC, reversed-phase thin-layer chromatography; TNF, tumor necrosis factor.

10.1021/jm701496h CCC: $40.75
Published on Web 02/27/2008

Scheme 1. Structure of the Tested Compounds



Because COX-2 isoenzyme was found to be overexpressed during inflammation, drug investigation was focused on selective COX-2 inhibition, hoping to prevent inflammation by sidestepping the undesired side effect of COX-1 inhibitors.[7,8] However, drugs of the new generation of NSAIDs, which selectively inhibit COX-2 isoenzyme, were associated with increased risk of myocardial infraction and cardiovascular thrombotic events. The thrombotic effect of these drugs seems to be due to the decrease of the level of the prostacyclin (PGI_2), a molecule with vasodilatory and antiaggregatory properties, synthesized via the action of COX-2. In addition, COX-2 was found to exhibit a protective role in asthma[9] and GI tract irritation.

The failure of COX-1 and COX-2 selective inhibitors evoked the concept that inflammation should be considered as a multifactorial process and all biochemical pathways should be taken into account, including the LOX pathway.

Leucotrienes and lipoxins produced via the LOX pathway also play a role in inflammation. They are associated with leucocyte activation and adhesion to vascular endothelium[10] and are involved in the pathogenesis of bronchial asthma[11] and edema formation. It is also believed that they play a role in the damage of gastric mucosa.[12–14] Enhanced 15-LOX activity has an additional undesired side effect because it enhances atheromatic plaque formation via LDL oxidation, resulting in elevated probabilities of atheromatosis[15] in patients suffering from chronic inflammatory diseases.

Compounds that combine COX-1/2 and LOX inhibition present multiple advantages because they act on the two major arachidonic acid metabolic pathways and possess a wide range of anti-inflammatory activity.[12] Because leucotrienes have their role in blood coagulation and gastric tract irritation, LOX inhibitory action seems to be able to ameliorate GI tract irritation resulting from COX-1 inhibition as well as the prothrombotic tendency resulting from COX-2 inhibition. For the reasons mentioned above, recent research has been focused on the development of dual-acting COX/LOX inhibitors. Currently, licofelone, a COX-2/5-LOX inhibitor, has been evaluated in phase III clinical trials.[16] Several derivatives containing pyrazoline, thiophene, di-*tert*-butylphenol, hydrazone, pyrrolidine, and pyrazole subunits have been found to be potent dual inhibitors.[5] Most recently, Rao and co-workers[17] tested a number of 1,3-diarylprop-2-yn-1-ones as well as rofecoxib derivatives for COX-1, COX-2, and 5,15-LOX inhibitory activity and Ziakas and co-workers[18,19] tested a number of tolfenamic and BHT derivatives for COX-1, COX-2, and LOX inhibition.

Results and Discussion

In this paper, we describe the computer-aided discovery of dual COX/LOX thiazolidinone inhibitors (Scheme 1) that were expected to offer protection against inflammation.

Computer Prediction of Biological Activity. Prediction of biological activity spectra was obtained using PASS software. The PASS prediction results for 573 chemical compounds designed in silico by the Department of Pharmaceutical Chemistry of Aristotle University were analyzed using PharmaExpert software. We took into consideration 40 known types of molecular mechanisms of anti-inflammatory activity predicted by PASS. The 573 virtually designed compounds are derivatives of thiazole/benzothiazoles and benzoisothiazoles that may be divided into several groups: (1) thiazole/benzothiazole amides (e.g., 2/3-substituted *N*-(4,5-substituted-thiazole-2-yl)acetamides/propionamides and *N*-(benzo[*d*]isothiazol-3-yl)-3-aminopropionamide)); (2) ketone derivatives of thiazoles (e.g., 1-(2-substituted 4-methyl-thiazol-5-yl)-3-substituted propane-1-one); (3) guanidine-containing derivatives of benzoisothiazole (e.g., benzo[*d*]isothiazol-3-yl)guanidine derivatives); (4) sulfonamide derivatives of thiazoles (e.g., *N*-(thiazol-2-yl)benzensoufonamide); (5) thiazolyl derivatives of coumarin; (6) Schiff bases (e.g., N-substituted benzylidene/phenylbenzylidene)benzo[*d*]thiazol/isothiazol-3-amines); (7) thiazolidinones.

Thirty-one of the designed compounds were predicted to have dual mechanism of anti-inflammatory activity acting as COX, LOX, or TNF convertase inhibitors and as prostaglandin or interleukin-1 antagonists with probability higher than 50% (Table 1). Twenty of the selected compounds were predicted to be nonspecific COX/LOX inhibitors, whereas 22 were predicted to be nonspecific COX and specific 5-LOX inhibitors. All 22 compounds predicted to be COX/5-LOX inhibitors were 2-(thiazole-2-ylamino)-5-phenylidene-4-thiazolidinone derivatives differing in the thiazolyl group substitution and in the phenyl group substitution. The nine compounds finally selected for evaluation of their biological activity had no substituent at the thiazolyl group and differed by the phenyl group substitution (Scheme 1). In addition to the positive prediction results and the fact that these compounds structurally belong to the same subgroup, the selection of the final nine compounds was

Table 1. Number of Compounds That Have >50% Probabilities of Dual Mechanisms of Anti-Inflammatory Activity

no.	P_a (max),[a] %	name[b]	number[c]
1	83.7	5-LOX inhibitor/COX inhibitor	22
2	78.3	COX inhibitor/LOX inhibitor	20
3	74.2	COX inhibitor/interleukin 1 antagonist	2
4	73.3	COX inhibitor/prostaglandin antagonist	6
5	71.3	5-LOX inhibitor/prostaglandin antagonist	4
6	69.9	5-LOX inhibitor/interleukin 1 antagonist	2
7	66.9	interleukin 1 antagonist/lipoxygenase inhibitor	2
8	65.8	LOX inhibitor/prostaglandin antagonist	4
9	54.4	prostaglandin antagonist/TNF convertase inhibitor	7
10	52.1	COX inhibitor/TNF convertase inhibitor	2

[a] P_a(max): maximal probability that was found in the PASS prediction results for each dual mechanism of anti-inflammatory activity among the 573 compounds initially designed. [b] Name: dual mechanism of anti-inflammatory activity predicted by PASS and selected by PharmaExpert. [c] Number: number of compounds exhibiting more than 50% probability to combine both activities.

Table 2. Experimentally (R_M) and Theoretically Calculated Lipophilicity Values (ClogP and logPsk)[a]

compd	R_f (±SD)	R_M (±SD)	ClogP	logPsk
1	0.782 ± 0.012	−0.556 ± 0.032	1.82	−0.393
2	0.726 ± 0.016	−0.410 ± 0.034	1.67	0.367
3	0.754 ± 0.011	−0.486 ± 0.027	2.41	0.226
4	0.838 ± 0.009	−0.713 ± 0.028	2.23	0.020
5	0.828 ± 0.009	−0.684 ± 0.026	2.23	0.144
6	0.809 ± 0.006	−0.628 ± 0.018	3.20	1.065
7	nd	nd	3.20	nd
8	nd	nd	3.20	nd
9	nd	nd	2.49	nd

[a] R_f values are the mean from 10 measurements (SD < 10%) of the coefficient in thin layer chromatography. R_M values are the mean from 10 measurements (SD < 10%). nd: not determined.

supported by the fact that these compounds were found to be potent antimicrobial agents.[21] Because inflammation is often a consequence of microbial infection, the production of agents with combined antimicrobial and anti-inflammatory action would be beneficial for treatment of infectious diseases. The selected compounds were evaluated in vitro to determine their COX-1/2 and LOX inhibitory activity and in vivo to determine their anti-inflammatory activity.

It is clear from Table 3, presenting the prediction results and the experimental data of studied compounds, that PASS successfully predicted the anti-inflammatory activity and COX and LOX inhibitory activity of eight of the nine compounds (89%). Compounds with a predicted P_a value lower than 0.300 exhibited no activity, as observed in 7 out of 9 cases (77.8%), or weak activity in 2 out of 9 cases (22.2%). Compounds with predicted P_a value greater than 0.350 exhibited activity in 28 out of 32 cases (87.5%).

Chemistry. 2-Chloro-*N*-(thiazol-2-yl)acetamide (**II**), synthesized using procedures reported earlier[21] starting from 2-aminothiazole (**I**), upon heterocyclization in the presence of ammonium thiocyanate in refluxing ethanol, efficiently produced 2-(thiazol-2-ylimino)thiazolidin-4-one (**III**).[20] The 2-thiazolylimino-5-arylidene-4-thiazolidinones (**1**–**8**) were obtained by refluxing **III** with appropriate aldehydes in buffered glacial acetic acid (Scheme 2).

Physicochemical Studies. Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolism, and excretion, we tried to experimentally determine the lipophilicity of the synthesized derivatives as R_M values from reversed-phase thin-layer chromatography (RPTLC)[22] and to compare them with the corresponding theoretically calculated ClogP values[23] in *n*-octanol buffer (Table 2).

From our results (Table 2) it can be concluded that R_M values could not be used as a successful relative measure of the overall lipophilic/hydrophilic balance of these molecules, as this is indicated by the calculated ClogP values, which express their theoretical log *P* in the standard octanol/water system ($r < 0.5$). We could attribute this to the different nature of the hydrophilic and lipophilic phases in the two systems and to the presence of the nitrogen atom in the examined compounds, which could disturb the absorption/desorption process.

Biological Results

In Vitro Experiments. COX-1 Inhibition. With the exception of compound **9**, lacking the aryliden group, all compounds inhibit COX-1 in a range from 8% to 90% when they were added to the assay mixture at 200 μM (Table 3) and act as competitive inhibitors because their inhibitory activity decreased when increased substrate concentrations were used. No inhibition was observed when arachidonic acid was added in a concentration of 100 μM, which is slightly higher than the saturating substrate concentration, showing that inhibition can be overcome by increasing substrate concentrations. The results shown in Table 3 were obtained using an arachidonic acid concentration of 1 μM. IC_{50} values were calculated for the most active compounds (Table 4).

2-(Thiazole-2-ylamino)-5-(*m*-chlorophenylidene)-4-thiazolidinone (**7**) exhibited the highest inhibitory activity (inhibition, 90%). The lower inhibitory activity of ortho- (**8**) and para- (**6**) substituted derivatives (50% and 31%, respectively) shows that the addition of the Cl substituent at the meta position of the phenyl ring is more favorable. Addition of the hydroxy group at the para position or nitro group at the meta or para position of the phenyl ring also resulted in less active compounds (*p*-OH (**1**), 62%; *p*-NO_2 (**4**), 60%; *m*-NO_2 (**5**), 25%) compared to compound **7**. High π values (π is the contribution of the group to lipophilicity) of R are correlated with higher inhibitory activity *m*-NO_2 < *m*-Cl (π-NO_2 = −0.28; π-Cl = 0.71). When a methoxy substituent was added to the meta position (**2**) of the *p*-hydroxy derivative, inhibition was even more reduced. Moreover, replacement of the *p*-hydroxy (**1**) with a *p*-methoxy (**3**) substituent diminished the inhibitory activity dramatically (8% inhibition) probably because of stereochemical interactions of the methyl group.

COX-2 Inhibition. All compounds showed no or low (0−30%) COX-2 inhibition when they were added to the assay mixture in a concentration of 200 μM. The results shown in Table 3 were obtained at an arachidonic acid substrate concentration of 0.1 μM. Increase of arachidonic acid concentration led to loss of activity, showing that the compounds are competitive inhibitors of the enzyme.

2-(Thiazole-2-ylamino)-5-(*m*-chlorophenylidene)-4-thiazolidinone (**7**) exhibited the best COX-2 inhibition (30%). Replacement of the more lipophilic (π- Cl = 0.71) meta-Cl (**7**) substituent by the less lipophilic nitro group (**5**) gave a compound with less than half-inhibitory action (12.1%). Substitution at the para (**6**) or ortho (**7**) position resulted in compounds with practically no inhibitory action (0.0−6.2%). According to the docking studies (Figure 2), the *m*-Cl substituent of compound **7** is placed in a hydrophobic area of the molecule. Consequently, substituents bringing polar or charged groups at the proximity of the hydrophobic amino acids of the area would not favor complex stability. 2-(Thiazole-2-ylamino)-4-thiazo-

Table 3. Experimental Data and Prediction Results for the Studied Compounds

	anti-inflammatory (CPE)[a] activity and COX/LOX[b] inhibitory activity				PASS prediction results of anti-inflammatory (CPE) and COX/LOX inhibitory activity (P_a values)				
		inhibition %							
compd	CPE%	COX-1	COX-2	LOX	CPE	COX	COX-1	COX-2	LOX
1	57.3 ± 3.4	62.0	0.0	44.0	0.725	0.701	0.490	0.278	0.872
2	72.7 ± 6.8	25.0	6.2	51.0	0.697	0.715	0.485	0.245	0.859
3	51.1 ± 4.2	8.0	2.5	22.4	0.729	0.748	0.577	0.289	0.868
4	66.1 ± 1.2	60.0	4.5	12.5	0.650	0.595		0.288	0.826
5	69.4 ± 2.3	25.0	12.1	76.0	0.645	0.609		0.273	0.817
6	54.2 ± 2.4	31.0	6.2	25.0	0.732	0.740	0.485	0.307	0.868
7	44.5 ± 1.8	90.0	30.4	12.0	0.718	0.736	0.442	0.296	0.857
8	62.0 ± 2.5	50.0	2.1	44.2	0.673	0.660	0.357	0.285	0.847
9		0.0	0.0		0.725	0.701	0.490	0.278	0.872

[a] Inhibitory activity on carrageenin-induced rat paw edema. The results are expressed as the mean (n = 6–10) and SD (Standard deviation) of percentage reduction of the difference in weight between the carrageenin-injected and uninjected paws following a 0.01 mmol/kg intraperitoneal injection of the test compound. Each value represents the mean of two independent experiments with 6–10 animals in each group. [b] Values are the mean of three determinations, and deviation from the mean is <10% of the mean value. Compound concentration in LOX inhibition assay was 100 μM and in COX-1 and COX-2 inhibition assays was 200 μM.

Scheme 2. Synthetic Pathway for the Preparation of Title Compounds

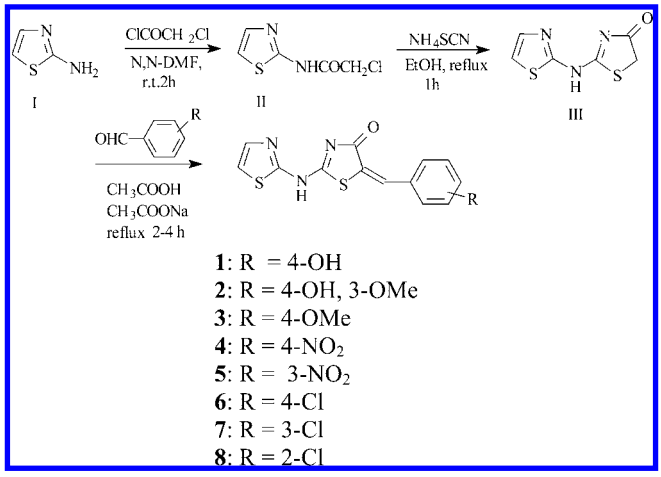


Table 4. IC_{50} Values of COX/LOX Inhibition for Studied Compounds

	IC_{50} (μM)		
compd	COX-1	COX-2	LOX
1	158.0		116.0
2			99.5
3			122.2
4	141.3		251.2
5			89.1
6			120.0
7	125.0	262.0	125.9
8			114.6
9			

lidinone (**9**) exhibited no COX-2 inhibition, leading to the conclusion that the phenylidene substituent is probably important for the inhibitory action on both COX isoenzymes.

When indomethacin at 200 μM was tested for COX-1 or COX-2 inhibitory activity at the same conditions as our compounds, it exhibited a 100% inhibition. All our compounds are less potent COX-1/2 inhibitors than indomethacin. Addition of naproxen to the reaction mixture at 200 μM caused a 60.0% inhibition of COX-2 and a 52.2% inhibition of COX-1 at a substrate concentration of 0.1 μM. All our compounds are less potent COX-2 inhibitors than naproxen, but some of them are better COX-1 inhibitors.

LOX Inhibition. Compounds were further evaluated for inhibition of soybean lipoxygenase by the UV absorbance based enzyme assay. It has been shown that inhibition of plant LOX activity by NSAIDs is qualitatively similar to the inhibition of the rat mast cell LOX and can be used as a simple screen for such activity.[24,25]

Analysis of percentage inhibition values shows that compound **5** is the most active within the set, followed by compounds **2**, **8**, and **1** (Table 3). Compound **5** with a *m*-NO_2 group demonstrates highly significant inhibition compared to the corresponding *m*-Cl substituted derivative (**7**). Low π values of R are correlated with higher inhibitory activity *m*-NO_2 > *m*-Cl (π-NO_2 = −0.28; π-Cl = 0.71). The presence of the vanillin moiety (**2**) is correlated with high inhibitory activity. Presence of a free OH group (**1**) is also beneficial for the LOX inhibitory activity, while compound **3** with a methoxy group demonstrates lower activity.

The *m*-Cl (**7**) substitution of the aryliden group favors COX inhibitory activity, whereas *m*-NO_2 (**5**) substitution has the best effect on LOX inhibitory action. The *m*-NO_2 derivative, also exhibiting COX inhibitory activity, has good anti-inflammatory action. Compound **2** (*m*-OCH_3, *p*-OH derivative), exhibiting the greatest anti-inflammatory activity, is also one of the most potent LOX inhibitors also exhibiting moderate COX-1 inhibitory action.

In Vivo Experiment. Inhibition of the Carrageenin-Induced Edema. In acute toxicity experiments, the compounds examined in vivo did not present toxic effects in ip doses up to 0.5 mmol/kg body weight of the mouse.

The in vivo anti-inflammatory effect of the tested compounds was assessed by using the functional model of carrageenin-induced mouse paw edema and is presented as the percentage of inhibition of induction of edema at the right hind paw (Table 3). Edema was measured by weight increase of the right hind paw in comparison to the uninjected left paw. Carrageenin-induced edema is a nonspecific inflammation that is highly sensitive to nonsteroidal anti-inflammatory drugs (NSAIDs), and so carrageenin has been accepted as a suitable agent for the study of new compounds with anti-inflammatory activity. As shown in Table 3, the majority of the investigated compounds induced protection against carrageenin-induced paw edema. The protection ranged up to 72.7%, while the reference drug, indomethacin, induced 47% protection at an equivalent dose. Low π values of R are correlated with higher anti-inflammatory activity; e.g., for compounds **4** and **6**, *p*-NO_2 > *p*-Cl (π-NO_2 = −0.28; π-Cl = 0.71), and for compounds **1** and **3**, *p*-OH > *p*-OCH_3).

Although based on very preliminary results, it seems that molecular hydrophilicity (low lipophilicity values, ClogP[23])

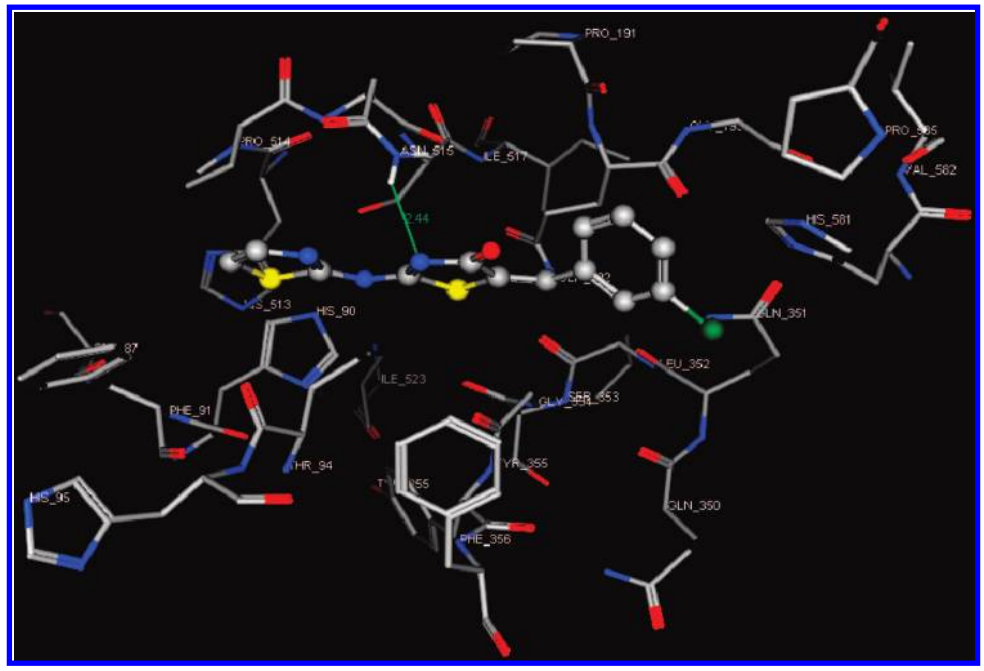


Figure 1. Docking of 2-(thiazole-2-ylamino)-5-(*m*-chloroyphenylidene)-4-thiazolidinone (**7**) in the active site of human COX-1. Green lines represents H-bonding. H-atoms are not shown for clarity.

partly affects the anti-inflammatory activity, as can be concluded from the following equation:[23]

$$\log \text{CPE\%} = -0.125(0.097)\text{ClogP} + 2.067(0.239) \quad (1)$$

No clear correlation of in vivo activity, expressed in CPE% values, and in vitro activity, expressed as COX1/2 and LOX inhibition, can be established. This is not surprising because it is commonly observed as the results of many researchers.[14] Because other mechanisms are involved at the first steps of edema formation, a straight correlation between in vivo results and LOX and COX inhibition cannot be obtained.

Docking. Docking studies were undertaken to gain insight into the binding mode of the most active compounds of this series: 2-(thiazole-2-ylamino)-5-(*m*-chlorophenylidene)-4-thiazolidinone (**7**) for COX-1 and COX-2; 2-(thiazole-2-ylamino)-5-(*m*-nitrophenylidene)-4-thiazolidinone (**5**) for 15-LOX.

The binding pockets of COX-1[25] and COX-2 were found to be similar except one amino acid residue at position 523 where COX-2 has a smaller Val residue, while COX-1 has Ile at that position. This minor difference in the binding site produces a secondary pocket extending off the primary binding site in COX-2, which is absent in COX-1. The combined volume of the primary binding site and the secondary pocket in COX-2 is about 25% larger (394 Å^3) than the volume of the COX-1 binding site (316 Å^3).[26] The various isoforms of LOX also have different volumes of binding site; 5-LOX binding site has larger volume (470 Å^3) than 15-LOX (390 Å^3).[27]

It was shown that 2-(thiazole-2-ylamino)-5-(*m*-chlorophenylidene)-4-thiazolidinone (**7**) binding within COX-1 includes a favorable H-bonding interaction between the N3 atom on the thiazole ring and the NH of the side chain of Asn515 (distance 2.438 Å). The other thiazole ring was found in proximity to the amino acids Phe91, His90, Thr94, His513, and Pro514, while the phenyl ring of the compound is found in the vicinity of the amino acids Gly354, Gln192, Gln351, Ser353, and His581, which are within a distance of 4.5 Å from the ligand (Figure 1). These results suggest that this compound docks well in the binding pocket[17] and explains its high inhibitory action. 2-(Thiazole-2-ylamino)-5-(*p*-methoxyphenylidene)-4-thiazolidinone (**3**), having the lowest inhibitory activity, lacks the favorable H-bonding interaction between the N3 atom of the thiazole ring and Asn515 (distance 3.76 Å). Moreover, the methoxy substituent of the phenyl ring is in proximity to the polar amino acid residues Gln192 and Gln 351, resulting in unfavorable stereochemical interactions.

Docking of 2-(thiazole-2-ylamino)-5-(*m*-chlorophenylidene)-4-thiazolidinone (**7**) in COX-2 shows that this compound docks well in the active site that consists of the amino acids Phe518, Ser353, Gly354, Ile517, Arg513, His90, Tyr355, and Arg120 (Figure 2). The nitrogen of the thiazole ring forms a hydrogen bond (distance 2.76 Å) with Arg120 of COX-2. The other thiazole ring is located in the vicinity of amino acids Tyr355, Val523, Leu359. The chlorophenyl ring settles in a hydrophobic cavity lined by Tyr135, Phe381, Met522, Trp387, and Phe518. The chlorophenyl ring is optimally oriented to make $\pi-\pi$ interaction with the phenyl ring of Tyr385. It can be seen that a meta substitution favors the phenyl ring optimal orientation between Tyr385 and Phe381. In contrast, para substitution (**6**) prohibits this orientation because of steric interaction with Met522. This may explain the higher activity of the *m*-Cl analogue.

It is interesting to note that compounds **5** and **7**, which are structurally very similar to one another, show strikingly different binding modes. As proved by the docking studies, the existence of a *m*-NO_2 substituent favors an orientation of compound **5** opposite that of compound **7**; i.e., the aryl ring of **5** goes where the thiazole ring of **7** settles, while the thiazole ring of **5** settles in the hydrophobic cavity where the chlorophenyl ring of **7** is located. This difference in binding mode may be attributed to the highly electronegative nature of NO_2 group which favors an orientation that enables hydrogen bonding between the $-NO_2$ group and Arg120. However, this orientation results in an unfavorable settlement of the thiazole ring in the hydrophobic cavity. Because of the different orientation, compound **5** lacks the hydrogen bonding between the thiazole ring and Arg120 and the $\pi-\pi$ interactions of the phenyl ring with Tyr385. This different binding mode appears to be the reason behind the

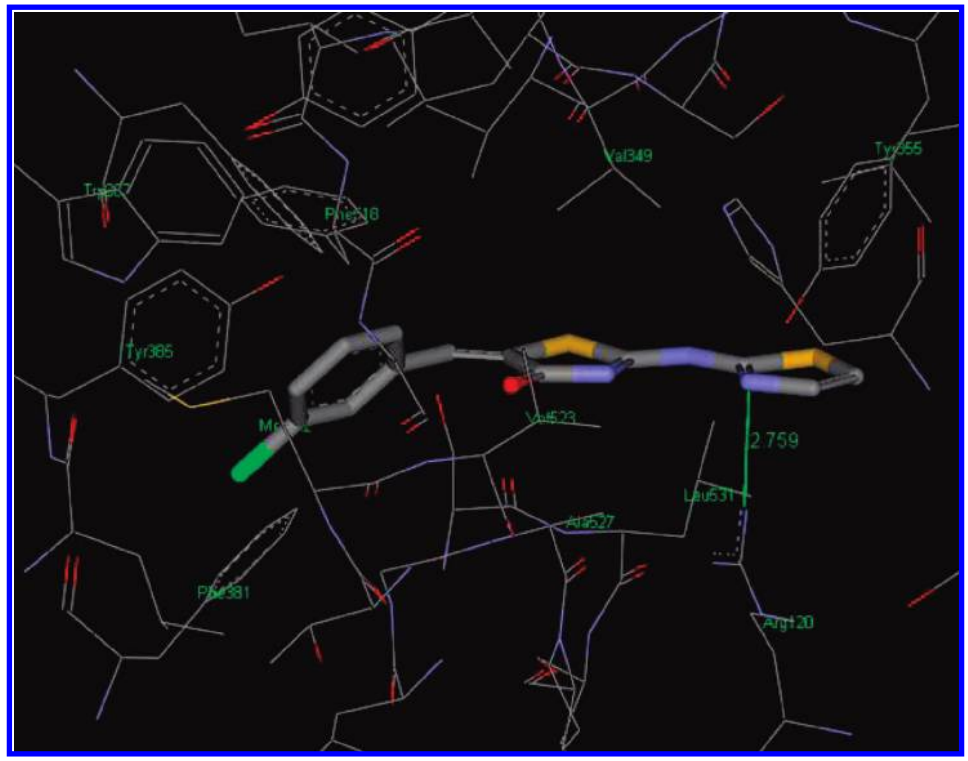


Figure 2. Docking of 2-(thiazole-2-ylamino)-5-(*m*-chloroyphenylidene)-4-thiazolidinone (**7**) in the active site of mouse COX-2. Green lines represents H-bonding. H-atoms are not shown for clarity.

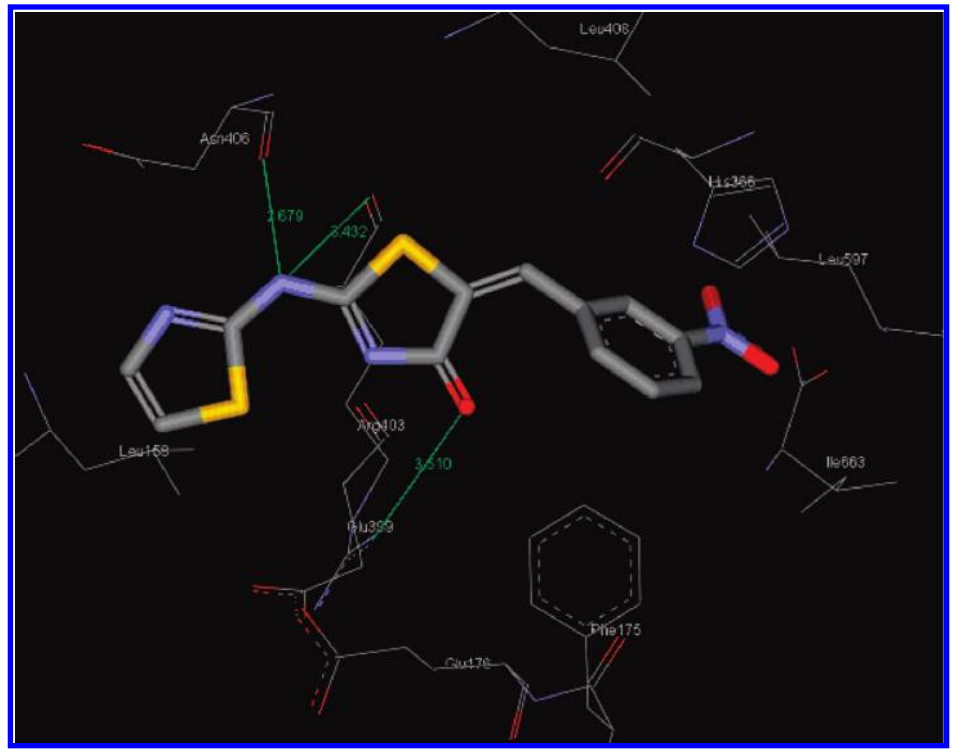


Figure 3. Docking of 2-(thiazole-2-ylamino)-5-(*m*-nitrohenylidene)-4-thiazolidinone (**5**) in the active site of rabbit 15-LOX. Green lines represents H-bonding. H-atoms are not shown for clarity.

difference in binding affinity and subsequently in activity of compounds **5** and **7**.

The binding site of 15-LOX is a boot shaped cavity consisting of amino acid residues Phe353, Ile418, Met419, and Ile593 located at the base of the binding site with the charged Arg403 residue present at the opening of the binding cavity. Docking of the most active compound, 2-(thiazole-2-ylamino)-5-(*m*-nitrohenylidene)-4-thiazolidinone (**5**), showed favorable hydrogen bonding interactions between compound **5** and Asn406 and Arg403 of LOX (Figure 3). The CO group of compound **5** is making hydrogen bond interactions with the free NH_2 of Arg 403 (distance 3.51 Å), while the amino group on the ligand is making hydrogen bond interaction with the backbone CO of this residue (distance 3.43 Å). This amino nitrogen of compound

5 is also having a hydrogen bond interaction with Asn406, contributing to additional stability (distance 2.68 Å). The nitrophenyl ring settles in a cavity marked by Phe175, His366, Leu597, and Ile663. According to docking results, compound **7** exhibits a binding mode similar to that of compound **5**. However, His336 present at the active site of LOX predominantly favors electrostatic interactions and the NO_2 group of **5** enables such an interaction better than Cl of compound **7**. Thus, the observed difference in binding affinity between compounds **5** and **7** may be due to the highly electrostatic nature of LOX active site which favors the presence of NO_2. In contrast to COX-2, where hydrophobicity plays a major role, the NO_2 derivative exhibits higher inhibition of LOX activity.

As mentioned above in the discussion of the COX 1/2 and LOX inhibitory action of the compounds, the docking results explain well the inhibitory potential of the most effective compounds.

Conclusions

Eight tested compounds exhibited anti-inflammatory activity. It was shown that 7 out of 9 (77.8%) tested compounds were LOX inhibitors, 7 out of 9 (77.8%) were COX inhibitors, and 6 (66.7%) tested compounds were dual COX/LOX inhibitors. The compounds can be considered as LOX inhibitors and less potent COX-1 inhibitors. One of the compounds exhibited COX-2 inhibitory activity as well.

Compounds **2** (*m*-OCH_3, *p*-OH derivative) and **5** (*m*-NO_2 derivative), which exhibit the highest anti-inflammatory activity, also have the best LOX inhibition. Moreover, they combine LOX inhibitory activity with moderate COX-1 inhibitory action. They inhibit effectively inflammation, acting on enzymes involved in both arachidonic acid catabolic pathways, and thus are promising anti-inflammatory agents with reduced undesired side effects. The fact that these compounds were found to be potent antimicrobial agents[21] increases the importance of discovery of their anti-inflammatory activity because inflammation is often a consequence of microbial infection and the production of agents with combined antimicrobial and anti-inflammatory action would be beneficial for treatment of infectious diseases.

The most active compounds dock well in the active site of the related enzymes. Inhibitory activity of the most potent compounds is explained mostly by hydrogen bonding and electrostatic or $\pi-\pi$ interactions within the active site of the enzymes. Hydrophilicity and steric requirements are also the most important factors in terms of SAR.

From our results, the PASS program in collaboration with the PharmaExpert software can successfully predict COX and LOX inhibitory action in a qualitative manner. Considerable activity was confirmed for P_a values greater than 0.350.

Despite IC_{50} values of the studied compounds varying from 10^{-4} to 10^{-5} mol/L for COX/LOX inhibition, anyone should take into account that they are the first known compounds among thiazolidinones with such a mechanism of anti-inflammatory action. Therefore, they may be considered as basic structures for further optimization.

Experimental Section

Materials. All used chemicals were analytical grade and commercially available. Soybean lipoxygenase, linoleic acid sodium salt, and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO). For the in vivo experiments, male and female mice (g) were used. The kit for COX activity assay was purchased from Cayman.

Computer Prediction of Biological Activity. PASS is a computer program for evaluation of general biological potential in a molecule on the basis of its structural formulas.[3,28–31] The list of predictable biological activity contains 2820 types (PASS, 2006 version) including main and side pharmacological effects (antihypertensive, hepatoprotective, anti-inflammatory, etc.), mechanisms of action (5-hydroxytryptamine agonist, cyclooxygenase inhibitor, adenosine uptake inhibitor, etc.), specific toxicities (mutagenicity, carcinogenicity, teratogenicity, etc.), and metabolic terms (CYP1A substrate, CYP3A4 inhibitor, CYP2C9 inducer, etc.).

The results of prediction are represented as a list of probable biological activity types for which the probability to be revealed (P_a) and the probability not to be revealed (P_i) are calculated. PASS estimates P_a and P_i values for each activity type independently. P_a and P_i values vary from 0 to 1.

The training set of the current version of PASS contains 781 cyclooxygenase inhibitors, while the number of lipoxygenase inhibitors is currently 679. The accuracy of prediction for these compounds calculated by the leave-one-out cross-validation procedure is 92.4% for the cyclooxygenase inhibitors and 93.4% for the lipoxygenase inhibitors.

PharmaExpert is a software package that includes a knowledge base of several thousand activity−activity relationships and provides a flexible tool for selection of prospective compounds with the desirable pharmacological profile and estimation of probable drug−drug interactions.[4] Since PharmaExpert contains the information about mechanism-effect relationships, it can be applied to search for compounds with multitargeted action. This possibility was used for selection of compounds with dual COX/LOX inhibition among 573 virtually designed molecules that could be synthesized by the Department of Pharmaceutical Chemistry of Aristotle University.

General Procedure for Synthesis of 2-Thiazolylimino-5-arylidene-4-thiazolidinones (1–8).[21] To a well-stirred solution of 2-(thiazol-2-ylimino)thiazolidin-4-one (0.8 g, 4 mM) in acetic acid (35 mL) buffered with sodium acetate (8 mM), the appropriate arylaldehyde (6 mM) was added. The solution was refluxed for 4 h and then poured into ice-cold water. The precipitate was filtered and washed with water, and the resulting crude product was purified by recrystallization from dioxane.

Physicochemical Studies.

(a) Determination of Lipophilicity as R_M Values. Reversed phase TLC (RPTLC) was performed on silica gel plates impregnated with 55% (v/v) liquid paraffin in light petroleum ether.[32] The mobile phase was a methanol/water mixture (75/25, v/v) containing 4% aqueous ammonia (27%). The plates were developed in closed chromatography tanks saturated with the mobile phase at 24 °C. Spots were detected under UV light or by iodine vapors. R_M values were determined from the corresponding R_f values (coefficient in thin layer chromatography from 10 individual measurements) using the equation $R_M = \log [(1/R_f) - 1]$ (Table 2).

(b) Determination of Lipophilicity as ClogP. Lipophilicity was theoretically calculated as ClogP values in *n*-octanol buffer by the CLOGP program of Biobyte Corp.[23]

Biological Assays. In Vitro Experiments. In the in vitro assays each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the average values.

Soybean Lipoxygenase Inhibition Study in Vitro. Lipoxygenase inhibition was evaluated as reported previously.[25] The tested compounds, dissolved in DMSO, were added to the reaction mixture at a final concentration of 100 μM and were preincubated for 4 min at 28 °C with soybean lipoxygenase at a concentration of 7×10^{-7} w/v. Enzyme reaction was initiated by the addition of sodium linolate at a final concentration of 0.1 mM. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was measured at 234 nm. Nordihydroguaretic acid, an appropriate standard inhibitor, was used as positive control (inhibition = 94.4% at 0.1 mM).

COX Inhibitor Screening Assay. The COX-1 and COX-2 activities of the compounds were measured using ovine COX-1 and human recombinant COX-2 enzymes included in the "COX Inhibitor Screening Assay" kit provided by Cayman (Cayman

Chemical Co., Ann Arbor, MI). The assay directly measures PGF_{2a} produced by $SnCl_2$ reduction of COX-derived PGH_2. The prostanoid production was quantified via enzyme immunoassay using a broadly specific antibody that binds to all the major prostaglandin compounds.[18] In an attempt to study the type of inhibition, the inhibitory activity of the compounds was tested at various concentrations of arachidonic acid (from 0.1 to 100 μM). The final estimation of % inhibition (Table 3) was performed at a substrate concentration much lower than the saturating concentration. For better visualization of compound differences in a 0–100% inhibition scale, COX-1 inhibitory activity was tested at an arachidonic acid concentration of 1 μM and COX-2 inhibitory activity was tested at an arachidonic acid concentration of 0.1 μM. The compounds were added to the reaction mixture at a final concentration of 200 μM. IC_{50} values were calculated for the most active compounds. Naproxen and indomethacin, used as positive controls, were added to the reaction mixture at the same concentration, 200 μM, as the tested compounds.

In Vivo Experiments. Inhibition of the Carrageenin-Induced Edema. Edema was induced in the right hind paw of mice (AKR) by the intradermal injection of 0.05 mL of 2% carrageenin in water. Both sexes were used, but pregnant females were excluded. Each group was composed of 6–10 animals. The animals, which have been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum prior to experimentation, but they were fasted during the experimental period. The tested compounds (0.01 mmol/kg body weight) were suspended in water with a few drops of Tween-80 and ground in a mortar before use and were given intraperitoneally simultaneously with the carrageenin injection. The mice were euthanized 3.5 h after the carrageenin injection. The difference between the weight of the injected and uninjected paws was calculated for each animal. It was compared with that in control animals (treated with water) and expressed as a percent inhibition of the edema CPE% values (Table 3). Each experiment was performed in duplicate, and the standard deviation was less than 10%.

Docking Analysis. For the docking studies we used GOLD 3.0.1[33] software running on windows based PC. The docked poses were scored using a total of seven scoring functions, Goldscore (GS),[33] ChemScore (CS),[34] PLP 1 and 2,[35] LigScore 1 and 2,[36] and PMF,[37] to find the better docking pose. Reference protein coordinates for the docking studies were taken from Protein Data Bank (PDB). The protein−ligand complexes of COX-2 (PDB code 1cx2) and 15-LOX (PDB code 1lox) were minimized up to a gradient of 0.01 kcal/(mol Å), and the hydrogens were added using the force field AMBER99 available in the software MOE. Charges on the protein were assigned using the force field AMBER99, while the charges on the ligands were assigned by using force field MMF94X available in the software MOE. In the case of COX-1 (PDB code 1prh), the protein molecule was superimposed on the COX-2 protein–ligand complex. COX-2 protein was then removed, and COX-1 was minimized up to a gradient of 0.01 kcal/(mol Å) with the ligand (SC558) of COX-2 using the force field AMBER99 available in the software MOE. The ligand binding sites of COX-1, COX-2, and 15-LOX were analyzed.

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JM701496H