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Original article

Fragment-based design, docking, synthesis, biological evaluation and structure–activity relationships of 2-benzo/benzisothiazolimino-5-aryliden-4-thiazolidinones as cycloxygenase/lipoxygenase inhibitors

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ABSTRACT

Balanced modulation of several targets is one of the current strategies for the treatment of multi-factorial diseases. Based on the knowledge of inflammation mechanisms, it was inferred that the balanced inhibition of cyclooxygenase-1/cyclooxygenase-2/lipoxygenase might be a promising approach for treatment of such a multifactorial disease state as inflammation. Detection of fragments responsible for interaction with enzyme's binding site provides the basis for designing new molecules with increased affinity and selectivity. A new chemoinformatics approach was proposed and applied to create a fragment library that was used to design novel inhibitors of cycloxygenase-1/cycloxygenase-2/lipoxygenase enzymes. Potential binding sites were elucidated by docking. Synthesis of novel compounds, and the *in vitro*/*in vivo* biological testing confirmed the results of computational studies. The benzothiazolyl moiety was proved to be of great significance for developing more potent inhibitors.

1. Introduction

The "one-molecule-one-target" concept led to the discovery of many successful drugs in the XX century and will probably lead to many more pharmaceutical agents in the future [1]. However, a balanced modulation of several targets can provide advanced therapeutic effects and a favourable side effects profile compared to the action of a selective ligand, particularly for complex diseases (cancer, diabetes, atherosclerosis, stroke, depression, and many others) [2–9].

Inflammation is a multifactorial process which reflects the response of the organism to various stimuli and is related to many disorders such as arthritis, asthma, psoriasis, etc. Non-steroidal anti-inflammatory agents (NSAIDs) [10–16] act by inhibition of cyclooxygenase (COX), which mediates the production of prostaglandins, prostacyclins and thromboxanes from arachidonic acid. Old generation of NSAIDs, mainly non-selective or selective COX-1 inhibitors, were associated with gastrointestinal problems [12], renal disorders [13] and bleeding diathesis, since COX-1 isoenzyme is needed for mucus formation and thromboxan production [14]. On the other hand, certain new generation NSAIDs, selective COX-2 inhibitors, were associated with serious cardiovascular problems due to the increased platelet aggregation [13,15,16]. Compounds that combine COX-1/2 and lipoxygenase (LOX) inhibition present multiple advantages because they act on the two major arachidonic acid metabolic pathways [15]. Since leucotrienes play a role in blood coagulation and gastric tract irritation, LOX inhibition can ameliorate GI tract irritation caused by COX-1 inhibition as well as the prothrombotic tendency resulting from COX-2 inhibition [17]. Several derivatives with pyrazoline, thiophene, di-*tert*-butylphenol, hydrazone, pyrrolidine, and pyrazole subunits have been found to be potent dual COX/LOX inhibitors [11]. More recently, some other compounds such as a number of 1,3-diarylprop-2-yn-1-ones, rofecoxib [18] as well as a number of diaryl isoselenazoles [19],

Abbreviations: MNA, multilevelneighbourhoods of atoms; FBDD, fragment-based drug design.

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novel acrylic acid [20] and nemesulide derivatives [21] were tested for COX-1, COX-2, and LOX inhibitory activity. Licofelone, a COX-2/5-LOX inhibitor, has been evaluated in phase III clinical trials [17].

In the present study PASS-based approach, appropriately modified, was proposed for rational, fragment-based design of pharmaceutical agents that simultaneously inhibit COX-1, COX-2 and LOX enzymes, involved in the inflammation process, in order to develop anti-inflammatory agents with activity superior to the earlier obtained substances [18,20,22]. FBDD can be applied for finding multi-target ligands since it enables to (1) include in the designed compounds fragments or functional groups that increase affinity to the studied targets and bioavailability, and (2) exclude fragments that increase the risk of side and toxic effects.

Selection of fragments that can be used for the design of new chemicals with the required biological activity profile is a key stage in FBDD. Currently, both experimental (X-ray diffraction, NMR) and computational methods (molecular modelling, similarity search, (Q)SAR) are applied to fragment library preparation [23]. X-ray determination of 3D structure of proteins is difficult in case of membrane-spanning proteins, such as G-protein coupled receptors that are known to be important drug targets [24]. On the other hand, NMR is heavily used for determination of 3D structure of large proteins. Molecular modelling also requires the accurate determination of 3D structure of "ligand–protein" complexes. In the methods based on maximal common substructure comparison or similarity with the structures from the training set [25,26] typically relatively large substructures are used as predefined scaffold for the search. This significantly reduces the chance to find principally new compounds without such scaffold. The main problem of QSAR application to fragment-based drug design [27–29] is associated with necessity to collect large homogeneous datasets of chemical structures with experimentally determined self-consistent quantitative values of activity [30].

It was shown earlier that, using the Bayesian approach implemented in computer program PASS (Prediction of Activity Spectra for Substances) [31,32], it is possible to identify the multi-target antihypertensive agents (dual angiotensin-converting enzyme/neutral endopeptidase inhibitors) [33] and multi-target anti-inflammatory agents (dual cyclooxygenase/lipoxygenase inhibitors) among the commercially available substances and virtually designed chemical structures [22]. Here we applied the FBDD method, based on the PASS algorithm, to the preparation of a fragment library, focused on the inhibition of COX-1, COX-2 and LOX enzymes. We modified the standard PASS algorithm, with the aim to allow for considering the mutual influence of atoms in the particular fragment (contribution of atoms) and the occurrence of entire fragment in the set of active compounds (contribution of fragment).

We prepared a fragment library focused on COX-1, COX-2 and LOX inhibition, and, also, designed and synthesized novel compounds comprising fragments of this library. All compounds were tested *in vitro* for COX-1, COX-2 and LOX inhibitory activity and *in vivo* for their anti-inflammatory activity in mice using the carrageenan paw oedema model. Structure–activity relationships for the studied multi-target anti-inflammatory agents were analyzed and compared with the predictions obtained using the proposed FBDD method.

2. Results and discussion

2.1. Computer-aided estimation of fragments' importance for COX-1, COX-2 and LOX inhibition

The earliest computer-aided methods employed decomposition of molecules into a set of fragments (RECAP) [34] around bonds, which are formed by common chemical reactions. Several methods for splitting of molecules into a set of cyclic and acyclic fragments had been developed [35–37]. In contrast to these methods our approach for splitting the whole structure into fragments is based on the probability of a chemical bond to break, which is inversely proportional to the energy of the bond formation, available from the literature [38]. The developed rules include some empirical restrictions on splitting structures into linkers and rings. Such an approach should provide splitting molecules, which are potentially synthesizable but occur rarely in the set of common chemical reactions' products.

The rules are presented below:

- The following bond types – C–N, N–N, N–O, C–C, S–S, C–O – as well as bonds between the ring atom and non-ring atom and bonds connecting two cycles can be split.
- Double and triple bonds cannot be broken.

The example of splitting of the acetylsalicylic acid structure (active pharmaceutical ingredient of Aspirin) is shown in Fig. 1.

To estimate the fragments' contribution into the COX-1, COX-2 and LOX inhibition we have taken into account the mutual influence of atoms included in the structure of the considered fragment. Such kind of influence could be estimated, using the representation of each atom included in the fragment, as a set of MNA descriptors (Multilevel Neighbourhoods of Atoms) [31].

MNA descriptors are calculated iteratively for each atom in the structure and reflect the peculiarities of its neighbourhoods. MNA descriptors of the 2nd level are currently used in PASS for biological activity prediction [31]. The probability of a chemical compound to be active (P_a) or inactive (P_i) is estimated using MNA descriptors' frequencies in the sub-sets of active and inactive compounds from the PASS training set. The accuracy of prediction is represented by Independent Accuracy of Prediction (IAP) values [31], which is calculated in leave-one-out cross validation (LOO CV) procedure for the whole PASS training set (for details see the Supplementary data).

To determine the influence of a specific atom X on a particular biological activity, the partial contribution of this atom is calculated on the basis of zero, the first and the second level's MNA descriptors, obtained for each atom positioned in the immediate vicinity of the considered atom X. Such approach allows taking into account the influence of the parts of molecule surrounding this atom (see Supplementary data).

For each atom X in the fragment under consideration and for the analyzed biological activity we calculated the probabilities of influence on the activity (P_a) and inactivity (P_i) on the basis of PASS algorithm [31].

P_a–P_i values are used for estimation of fragments' contribution into the activity:

$$C_f = \frac{\sum_j (P_a - P_i)}{AN}$$

Where *AN* is the number of atoms in the fragment.

Integral characteristic of the fragments' contribution *C* into the particular activity was calculated as a sum of *Cf* values while taking into account each occurrence of a given fragment into the set of active compounds from the training set (see Supplementary data).

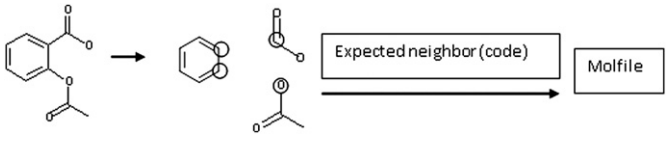


Fig. 1. An example of defragmentation of the acetylsalicylic acid structure.

The fragments, which have the highest *C*-values, are considered as the most significant for a particular activity. Some of the most significant fragments for COX-1, COX-2 and LOX inhibition are given in the appropriate columns of Table 1. They are arranged in the descending order of COX-1 *C-values*.

Fragments, which have high *C*-values for two or three enzymes simultaneously, can be selected for design, synthesis and testing of novel potent anti-inflammatory agents with a multiple mechanism of action. However organic synthesis is a complex field with many subtleties and different restraints which should be taken into consideration when chemical synthesis of certain molecules is planned [39]. Based on the results of fragment library analysis and taking into account the synthetic accessibility and SAR analysis of the designed thiazole derivatives [22], seven fragments, designated with dark grey background in Table 1, were selected to be incorporated into new anti-inflammatory candidates: benzothiazole, benzol, phenol, fluorobenzol, chlorobenzol, anisole and 2-imino-5-methylidene-1,3-thiazolidin-4-one.

During the process of structure design we took into account previous investigations concerning the interactions between COX-1/2 and thiazolidinones and LOX and thiazolidinones which were based on the molecular docking results and were confirmed by experimental studies [22]. According to these studies, the central position of 2-thiazolylimino-5-methylidene-1,3-thiazolidin-4-one plays a role in the interaction between COX-1, LOX and thiazolidinones. Therefore we decided to save central position of 2-thiazolylimino-5-methylidene-1,3-thiazolidin-4-one. Taking into account previous results of experimental and theoretical studies on thiazolidinones [22], we proposed to introduce fluoro-benzene, benzole, phenole, anisole, chloro-benzene as substituents of the **5**th (unoccupied) carbon atom of 2-thiazolylimino-5-methylidene-1,3-hiazolidin-4-one. Following the similar logic we have decided to replace thiazole by benzothiazole in *de-novo* designed molecule for comparison with the previously investigated thiazolidinones. The fragments-substituents of 2-thiazolylimino-5-methylidene-1,3-thiazolidin-4-one (benzole, phenole, anisole, chlor-benzene) were selected among other fragments with high *C*-values (for example, phenyl acetate, di-fluor benzene, acetic acid) taking into account the convenience of synthesis. The reason for linking 2-thiazolylimino-5-methylidene-1,3-thiazolidin-4-one, benzo(iso)thiazole and benzene-substituents into one molecule was based on the earlier reported data on the biological activity of thiazolidinones [22].

Since the benzothiazole fragment was among the proposed ones for incorporation, a series of twenty benzothiazolyl/benzisothiazolyl derivatives were selected from the bulk of already synthesized compounds [40,41] (Fig. 2: Compounds **1**–**6**, **8**, **9**, **11**–**20**, **22** and **23**). Three more compounds (Fig. 2: Compounds **7**, **10** and **21**) were designed and synthesized according to the method described below.

2.2. Chemistry

Compounds **7**, **10** and **21** (Fig. 2) were synthesised by the multi-step reaction protocol (Scheme 1), which we reported earlier [40–44].

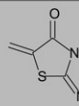
All the new compounds were characterized with respect to mps, elemental analyses and spectroscopic data (^{1}H NMR, MS and IR).

Concerning the ^{1}H NMR data, as it was shown by us earlier [41], the low field NH signals of compounds account for the amino–imino tautomeric equilibrium. It is worthwhile mentioning that in the biological systems the amino–imino equilibrium is of great importance in the binding process with the target biomolecule.

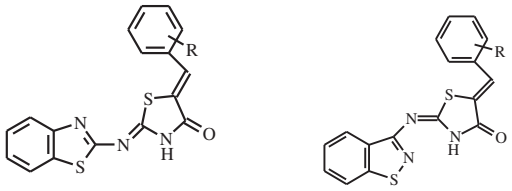
2.3. Biological results

The potential pharmacotherapeutic action of benzothiazolyl (BT) and benzisothiazolyl derivatives (BIT) was evaluated by the

Table 1
Influence of different fragments on the COX-1, COX-2 and LOX inhibition, obtained by the estimation of fragments' contribution.

Fragment	Chemical name	Final C-values		
		COX-1	COX-2	LOX
 1	phenyl acetate	100	100	--
 2	fluor-benzene	100	100	84
 3	2-imino-5-methylidene-1,3-hiazolidin-4-one	100	96	100
 4	di–fluor-benzene	100	100	-33
 5	benzothiazole	89	100	69
 6	isobutane	88	58	96
 7	benzole	85	94	79
 8	phenole	85	87	97
 9	anisole	77	95	93
 10	acetic acid	75	86	66
 11	chlor-benzene	73	100	-13
 12	pyridine	65	80	22
 13	trifluormethane	0	100	-100
 14	1,2-oxazole	-60	100	71
 15	furan	-100	62	100
 16	3-chloro-2H-pyrazole	--	--	100

* Rows, containing structures of fragments, which were selected for design of novel chemicals are marked in grey.



Benzothiazole series

1. R = 2-NO_2	**7**. R = 3-OH
2. R = 3-NO_2	**8**. R = 4-OH
3. R = 4-NO_2	**9.** R = 3-OMe, 4-OH
4. R = 2-Cl	**10.**R=3,5-OMe, 4-OH
5. R = 3-Cl	**11**. R = 4-OMe
6. R = 4-Cl	**12**. R = H

Benzisothiazole series

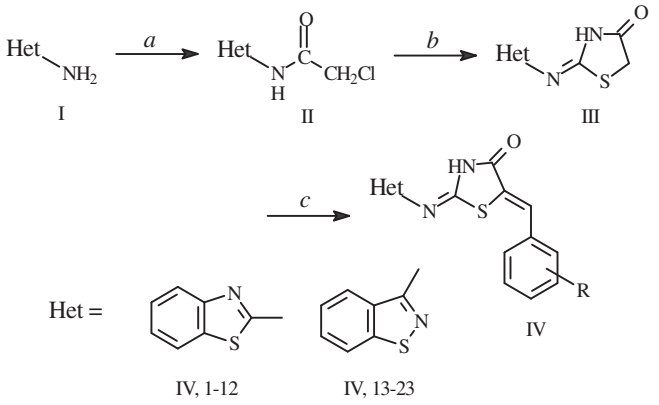
13. R = 2-NO_2	**19**. R = 4-OH
14. R = 3- NO_2	**20.**R = 3-OMe, 4-OH
15. R = 4- NO_2	**21**.R =3,5-OMe,4-OH
16. R = 2-Cl	**22.** R = H
17. R = 3-Cl	**23**.R= 4-OMe
18. R = 4-Cl	

Fig. 2. Structure of designed compounds.

in vitro study of COX-1, COX-2 and LOX inhibitory activity and by the *in vivo* study of anti-inflammatory activity using the carrageenan mouse paw oedema model (for results see Table 2) [40,41]. Previously obtained data on thiazolyl derivatives (TH) are also shown for comparison purposes [33].

2.3.1. In vitro experiments

2.3.1.1. COX-1 inhibition. With the exception of compounds **1**, **10** and **11**, all benzothiazol-2-yliminothiazolidin-4-ones showed good inhibitory activity with IC_{50} values ranging between 0.018 and 39.8 μM (Table 2). The results shown in Table 2 were obtained using an arachidonic acid concentration of 0.1 μM. 5-(2-Chloro-benzylidene)-2-(benzo[*d*]thiazol-2-ylimino) thiazolidin-4-one (**4**) exhibited the highest inhibitory activity (IC_{50} 0.018 μM). The lower inhibitory activity of derivatives substituted in position 3 (**5**) and 4 (**6**) (IC_{50} 22.4 and 0.3 μM respectively) shows that the addition of the Cl substituent at the 2-position of the phenyl ring is more favourable. In case of nitro derivatives the positions 3- and 4- are more favourable than position 2- (IC_{50}, 0.31 μM (**2**), 0.51 μM (**3**) and >200 μM (**1**), respectively). Addition of a hydroxy group at position 3 (**7**) and 4 (**8**), of the phenyl ring also resulted in less active compounds (IC_{50}, 5.6 μM and 7.9 μM, respectively). Moreover, replacement of the 4-hydroxy (**8**) with a 4-methoxy (**11**) substituent diminished the inhibitory activity dramatically (IC_{50} 200.5 μM) probably due to the stereochemical hindrance of the methyl group. The introduction of a methoxy group at positions 3 and 3,5 of compound **8** led to less active compounds (IC_{50}, 39.8 μM and 200 μM, respectively).



Scheme 1. Synthesis of designed compounds. Reagents and conditions: (*a*) $ClCOCH_2Cl$, *N*,*N*-DMF, rt., 2 h., (*b*) NH_4SCN, EtOH, reflux, 1–3 h., (*c*) RC_6H_4CHO, MeCOOH, MeCOONa, reflux, 2–4 h.

In both cases (i.e. with benzothiazole and benzisothiazole derivatives) the results confirm the findings that with increasing the overall lipophilicity the biological response is also increased. The opposite was observed for the thiazolylimino-5-aryliden-4-thiazolidinones.

It should be mentioned that benzo[*d*]thiazolylimino-5-aryliden-4-thiazolidinones exhibited more potent COX-1 inhibition than their thiazole analogues and proved to be even more potent COX-1 inhibitors than naproxen.

The influence on the activity of different scaffold types could be presented in the following order: benzothiazole >> benzisothiazole ≥ thiazole.

2.3.1.2. COX-2 inhibition. Most of compounds added to the assay mixture in a concentration of 200 μM showed COX-2 inhibition. The results shown in Table 2 were obtained with an arachidonic acid substrate concentration of 0.1 μM.

It was observed that among the benzothiazole derivatives chloro substitution is more favourable. 5-(2-Chloro-benzylidene)-2-(benzo[*d*]thiazol-2-ylimino) thiazolidin-4-one (**4**) exhibited the highest COX-2 inhibition (58.8%) followed by 3- and 4-chloro derivatives (compounds **5** and **6**, 32.0% and 20.0%, respectively).

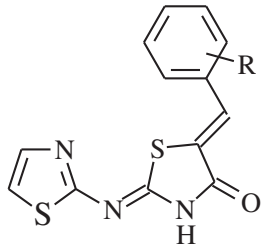
The replacement of the more lipophilic chloro substituent by the less lipophilic nitro group, leads to a decrease of COX-2 inhibitory activity in the case of 2- and 4-substituted derivatives (9.4% and 8% respectively), while 3-NO_2 derivative (compound 2, 32%) was the most active. Replacement of the 4-nitro group with 4-methoxy and 4-hydroxy-group resulted in obtaining compounds with increased activity (compounds **11** and **8**, 26.4% and 20.6% respectively), while introduction of a hydroxy-group in the 3-position led to an inactive compound (compound **7**). The same result was observed upon introduction of a methoxy group in positions 3 and 5 of *p*-hydroxy substituent (compounds **9** and **10**).

Benzisothiazole-2-ylimino-5-aryliden-4-thiazolidinones were also found to exhibit some COX-2 inhibitory activity (12.3–45.0%). In this case, 2-(benzo[*d*]isothiazol-3-ylimino)-5-(4-chloro-benzylidene) thiazolidin-4-one (**18**) exhibited the best COX-2 inhibition (45.1%), followed by 3-Cl (29%) and 2-Cl (24.5%) derivatives. Replacement of 4-Cl substituent with 4-nitro (**15**) resulted in a decrease of inhibition (14.0%), while 2-nitro derivative had the second most potent inhibitory activity after compound **18**.

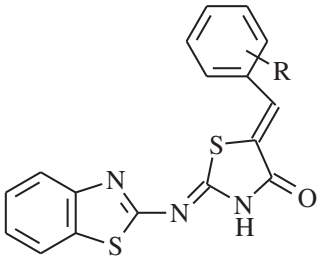
Although, no significant changes were observed within different scaffold types and within different substituents, the occurrence of slight variations in inhibitory activity of compounds with different substituents points to the influence of benzothiazole, benzisothiazole and thiazole scaffolds on the COX-2 inhibition. This influence may be relatively ordered as: benzothiazole ≈ benzisothiazole > thiazole.

2.3.1.3. LOX inhibition. All benzothiazolylthiazolidinone derivatives tested were more potent than their thiazolyl analogues. The presence of the OMe group at *o*- and *m*-positions of the molecule, favours activity (compounds **9**, **10** and **11**). The *p*-OMe derivative (**11**) is the most potent compound (IC_{50} 28.2 μM). Derivative **10**, bearing an OH group at the *p*-position and two OMe groups at the two *m*-positions, is the second more potent compound (IC_{50} 33.9 μM), while the presence of a *p*-OH and only one *m*-OMe substituent in compound **9** resulted in reduced activity (IC_{50} 35.5 μM). On the other hand the *p*-OH derivative (**8**) was even less active (IC_{50} 36.3 μM). The *m*-OH derivative (**7**) exhibited slightly higher activity than the *p*-OH analogue (IC_{50} 34.7 μM). Compared to compound **5** with an *m*-Cl group and compound **2** with an *m*-NO_2 substituent compound **7** demonstrated slightly higher inhibition. *ortho*-NO_2 (**1**) substitution has a beneficial effect on LOX inhibitory action. Interestingly, *m*- and *p*-NO_2 derivatives (**2** and **3**) as well as

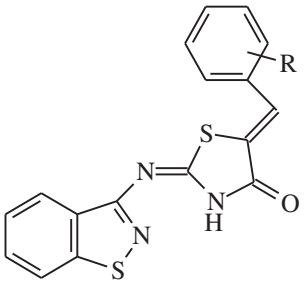
Table 2
Experimental evaluation of LOX, COX-1, and COX-2 inhibition obtained for the set of benzothiazole- (BT), benzisothiazole-(BIT) and thiazole (TH) [25] derivatives.



TH



BT



BIT

N	R	CPE[a] %	COX-1 inhibition (IC_{50}, μM)		COX-2 inhibition %		LOX inhibition (IC_{50}, μM)	
		BT	**BT**	TH[b]	**BT**	TH[b]	**BT**	TH[b]
1	2-NO_2	–	>**200**	–	**9.4**		**35.5**	–
2	3-NO_2	**40.0**	**0.31**	>200	**32.0**	12.1	**42.0**	89.1
3	4-NO_2	**57.0**	**0.51**	141.3	**8.0**	4.51	**50.1**	251.2
4	2-Cl	**71.8**	**0.018**	>200	**58.8**	2.11	**71.0**	114.6
5	3-Cl	**78.0**	**22.4**	125.0	**32.0**	30.4	**35.5**	125.9
6	4-Cl	**68.7**	**0.31**	>200	**20.0**	6.2	**50.0**	120.0
7	3-OH	–	**5.61**	–	**1.0**	–	**34.7**	–
8	4-OH	**68.7**	**7.91**	158.0	**20.6**	0.0	**36.3**	116.0
9	3-OMe, 4-OH	**72.7**	**39.8**		**6.6**	6.21	**35.5**	99.5
10	3,5-OMe, 4-OH	**36.5**	**200.0**	>200	**0.0**	–	**33.9**	122.2
11	4-OMe	**37.8**	**200.5**	>200	**26.4**	2.51	**28.2**	–
12	H	–	**4.0**	>200	**6.7**	0.0	>**100**	–
		BIT	**BIT**	TH	**BIT**	TH	**BIT**	TH
13	2-NO_2	–	**35.5**		**34.9**		**30.0**	
14	3-NO_2	**49.0**	**125.9**	>200	**0.0**	12.1	**79.4**	89.1
15	4-NO_2	**28.6**	**56.2**	141.3	**14.0**	4.5	**36.6**	251.2
16	2-Cl	**67.0**	>**200**	>200	**24.5**	2.1	**43.6**	114.6
17	3-Cl	–	>**200**	125.0	**29.0**	30.4	**100.0**	125.9
18	4-Cl	**40.3**	**52.4**	>200	**45.0**	6.2	**28.2**	120.0
19	4-OH	**69.8**	>**200**	158.0	**12.3**	0.0	**17.7**	116.0
20	3-OMe, 4-OH	**80.0**	>**200**	–	**0.0**	6.2	**33.9**	122.2
21	3,5-OMe, 4-OH	**47**	>**200**	>200	**3.0**	–	**100**	99.5
22	H	**73.5**	>**200**	>200	**0.0**	0.0	–	–
23	4-OCH_3	**37.8**	–	–	–	–	–	–
Indomethacin		47%						
Naproxen			19.5		68%			
Celecoxib			34.5		0.1 (IC_{50})			
Nordihydroguaretic acid							31.3	

Values are means of three determinations and deviation from the mean is <10% of the mean value.
[a] The percentage of inhibition of induction of oedema at the right hind paw in the carrageenan-induced paw oedema inflammation model.
[b] Reference [25].

p- and *o*-Cl derivatives (**6** and **4**) exhibited distinctively low activity with IC_{50} values ranging between 42.0 and 71.0 μM. All derivatives were more potent than non-substituted compound **12**.

Among the benzisothiazolylthiazolidinone derivatives, the best LOX inhibitory activity was observed for compound **19** (IC_{50} 17.7 μM), followed by compounds **18**, **13**, **20** and **15**. It seems that the presence of *p*-OH group in this case is beneficial for LOX inhibitory activity. The introduction of a methoxy group in *m*-position of compound **19** led to compound **20** (IC_{50} 33.9 μM) with the activity twice less than that of compound **19**. The introduction of a second methoxy group (compound **21**, IC_{50}100 μM) led to a dramatic decrease of activity, showing an opposite influence of the OMe group than to observed in benzothiazolyl derivatives. The replacement of a *p*-OH group with *p*-Cl (**18**) or *p*-NO_2 (**15**) also leads to compounds with decreased activity. Compound with an *o*-NO_2 group (**13**, IC_{50} 30 μM) demonstrates a higher inhibitory activity compared to the corresponding *o*-Cl (**16**, IC_{50} 43.6 μM) substituted derivative. In both cases it seems that the lower π values of R are correlated with a higher inhibitory activity.

Taking into account differences in affinity to LOX (IC_{50}) between benzothiazole, benzisothiazole and thiazole derivatives, the influences of benzothiazole, benzisothiazole and thiazole scaffolds on the LOX inhibition may be ordered as: benzothiazole ≈ benzisothiazole >> thiazole.

As seen from the inhibitory action of the above compounds on the three enzymes, the majority of substituents exerted a similar influence on the inhibition of COX-1 and COX-2 isoenzymes, while, in most cases, LOX inhibition was differently affected by the substituents. Thus, the *p*-OMe derivative (**11**) which is the most potent LOX inhibitor among the benzothiazolyl thiazolidinones tested is, at the same time, one of the less potent COX-1/2 inhibitors. In general, the presence of OMe substituent has the opposite

effect on COX-1/2 to that on LOX inhibition. On the other hand, the most potent COX-1 and COX-2 inhibitor (**4**) is one of the less potent LOX inhibitors. However, the *m*-NO_2 derivative, which is the second most potent COX-1/2 inhibitor also exhibited good although not the best LOX inhibitory action.

In a similar way, compound **19** which is the most potent LOX inhibitor among benzisothiazolyl derivatives, is one of the less active COX-1/2 inhibitors. However, the *p*-Cl derivative which is the second more potent LOX inhibitor also exhibits good COX-1/2 inhibitory action.

2.3.2. *In vivo experiment. Inhibition of the carrageenan-induced oedema*

The *in vivo* anti-inflammatory effect of the tested compounds was assessed using the functional model of carrageenan-induced mouse paw oedema and is presented as the percentage of inhibition of oedema induction at the right hind paw (Table 2). Oedema was measured by the weight increase of the right hind paw compared to uninjected left paw. Carrageenan-induced oedema is a nonspecific inflammation that is highly sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), therefore, carrageenan has been accepted as a suitable agent for the study of new compounds with anti-inflammatory activity. As shown in Table 2, the majority of the investigated compounds inhibited carrageenan-induced paw oedema. The protection ranged up to 80%, while the reference drug, indomethacin, induced 47% protection at an equivalent dose. As apparent from the data presented in Table 2, chloro-derivatives are the most active compounds compared to other benzothiazolyl thiazolidinones.

It seems that high π values of *R* are correlated with higher anti-inflammatory activity, e.g., for compounds **3** and **6**, *p*-Cl > *p*-NO_2 (π-$NO_2 = -0.28$, π-Cl = 0.71). The most active among *o*-, *m*- and *p*-chloro derivatives is compound **5** (*m*-derivative), indicating that the substituted position in this case is important for biological activity. Replacement of *p*-OH group (compound **8**, 68.7%) with a methoxy group led to less potent compound (**11**, 37.8%), while introduction of OCH_3 group at position *meta* of *p*-hydroxy derivative gave compound **9** with increased activity (72.7%). Introduction of the second methoxy group (position *m*) resulted in the derivative with the much lower activity (36.5%).

In case of benzisothiazole derivatives compound **20** is the most active one, being followed by compounds **22**, **19** and **16**. Here again low π values of R are correlated with lower anti-inflammatory activity, e.g., for compounds **15** and **18**, *p*-NO_2 > *p*-Cl (π-$NO_2 = -0.28$, π-Cl = 0.71) - as in the case with benzothiazolyl derivatives. Again, replacement of *p*-OH group with methoxy, as well as introduction of two methoxy groups at positions 3,5 decreased the activity, while introduction of one methoxy group led to the most potent compound (**20**).

No clear correlation between *in vivo* activity, expressed in CPE (carrageenan paw oedema) % values, and *in vitro* activity, expressed as COX-1/2 and LOX inhibition, has been established. This is not surprising because of the several different mechanisms, operating at the first step of oedema formation; of note, the lack of such correlation is reported by other authors [20].

2.4. *Influence of specific fragments onto the COX-1, COX-2 and LOX inhibition*

To estimate the statistical significance of the differences in inhibitory action of compounds containing thiazolyl, benzothiazolyl and benzisothiazolyl fragments, the Wilcoxon two tailed rank test for paired samples was carried out (XLSTAT 2010, Addinsoft, the results of which are given in Supplementary data).

Benzothiazolyl moiety strongly favoured COX-1/2 as well as LOX inhibition compared to thiazolyl moiety. The differences were statistically significant in all three cases ($N = 9$, $p = 0.022$ for COX-1, $N = 9$, $p = 0.014$ for COX-2 and $N = 8$, $p = 0.014$ for LOX).

The presence of benzisothiazolyl moiety as opposed to thiazolyl moiety improved inhibitory action towards all the three enzymes; in case of LOX the improvement was statistically significant ($N = 8$, $p = 0.021$).

Comparing the influence of benzothiazolyl to that of benzisothiazolyl moiety we conclude that statistically significant increased inhibitory activity was observed for benzothiazoles in case of COX-1 ($N = 9$, $p = 0.044$).

The application of Wilcoxon criterion to benzothiazole/thiazole derivatives clearly demonstrated that fragments' contributions estimated by PASS algorithm roughly agree with the experimental data.

Benzothiazolyl moiety was among the fragments predicted to have positive influence on all three enzymes inhibitory activities.

Benzisothiazole has not been identified as a significant fragment for inhibition of COX-1, COX-2 and LOX, which could be explained by the relative novelty of benzisothiazole derivatives (small number of compounds from this chemical class is included in the PASS training set).

To explain the influence of a particular fragment on the inhibition of COX-1, COX-2 and LOX depending on the chemical class (and therefore depending on different combinations of fragments), the splitting of the whole structure set into the sub-sets of benzothiazole, benzisothiazole and thiazole derivatives was performed. These three sets were included into the PASS training set with the activities "Lipoxygenase inhibitor, benzothiazole", "Lipoxygenase inhibitor, thiazole" and "Lipoxygenase inhibitor, benzisothiazole". Besides, the structures with IC_{50} exceeding 100 μM, were included to the PASS training set with indication of low activity, for instance, "Lipoxygenase inhibitor, thiazole, low inhibition". Similar procedures were performed for COX-1 and COX-2 enzymes. Therefore, for each enzyme and every chemical class three possible types of influence on the interaction could be predicted: "no influence", "high influence" and "low influence".

It can be inferred, that the major fragments that have a positive influence on COX-1 inhibition are: benzothiazole and 2-imino-5-methylidene-1,3-thiazolidine-4-one (Table 3). At the same time, different substituents in the benzene ring, do not significantly increase the affinity to COX-1/2 and LOX. Therefore, different substituents play only a supplementary role in COX-1 inhibition. This conclusion is in agreement with experimental data: differences in average IC_{50} values depending on the type of substituents at the same positions are less than differences between the structures with altered scaffold type (thiazole/benzothiazole).

Although there are no significant changes in average values for COX-2 (%) and LOX (IC_{50}) in thiazole derivatives depending on the type of substituent, some fragments including Cl, OH and OMe are predicted as important in almost all cases. The assessments obtained for these fragments correspond to experimental data. The cases where the assessments are not in agreement with the experimental data are marked by grey colour. For all the three enzymes there are differences in assessments between the 1,3 thiazole-4-one (fragment 5) and 2-imino-5-methylidene-1,3-thiazolidine-4-one (fragment 6).

It can be concluded therefore that the nitrogen atom in 2-imino-5-methylidene-1,3-thiazolidine-4-one (central ring of thiazole with substituents) is the essential feature for ligand binding to COX-1, COX-2 and LOX.

Due to the nature of MNA descriptors, the fragments important for COX-1, COX-2 and LOX inhibition in the benzothiazole and benzisothiazole series, appeared to be equally important for

Table 3
Fragments' contribution into the cyclooxygenase-1, cyclooxygenase-2 and lipoxygenase inhibition, depending on the chemical class where fragment occurs.[a]

	Influence on COX-1 inhibition			Influence on COX-2 inhibition			Influence on LOX inhibition		
	TH	BIT	BT	TH	BIT	BT	TH	BIT	BT
 1	No	Low	No	No	High	No	No	High	No
 2	Low	No	High	High	No	High	High	No	High
 4	Low	No	No	No	No	No	High	No	High
 5	No	No	High	No	No	No	No	No	No
 6	Low	High	High	High	High	High	High	High	High
 7	High	High	Low	High	High	High	High	High	High
 8	Low	High	High	High	High	High	High	High	High
 9	Low	High	High	High	High	High	High	High	High
 10	Low	Low	High	High	No	High	High	High	High
 11	No	Low	Low	No	No	No	High	No	High
 12	Low	Low	Low	Low	No	No	No	No	No
 13	No	High	High	High	High	High	High	High	High

[a] The influence of particular fragment on inhibition of COX-1, COX-2 and LOX depending on the specific chemical class is demonstrated in column TH (influence in classes of thiazole derivatives), BIT (influence in the class of benzisothiazole derivatives).

thiazole series. This fact demonstrates the extrapolative abilities of the proposed approach.

2.5. Results of docking experiments

Docking studies were undertaken using the automated GOLD docking program [45] in order to gain insight into the binding mode of the most active compounds and elucidate the impact of structural differences on their COX-1, COX-2 and LOX inhibitory activities. Although, only crystallographic data can fully clarify the binding mode of chemical compounds, the docking analysis still remains a helpful tool in SAR studies.

2.5.1. Docking to COX-1 active centre

The binding mode of the most active compound, 2-(benzothiazol-2-ylimino)-5-(2-chloro-benzylidene)-thiazolidin-4-one, **4**, within the binding site of COX-1 (PDB-ID: 1cqe) [46] is illustrated in Fig. 3. The compound is oriented with its 2-chloro-benzylidene moiety in proximity to the hydrophobic cavity containing the amino acids Pro585 and His581. The thiazolidinone ring is in the vicinity of

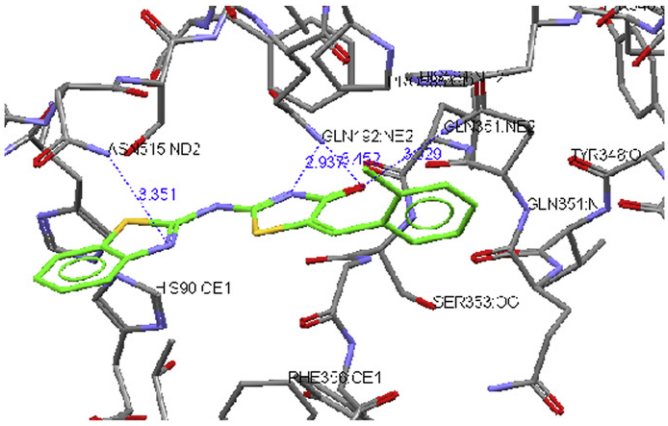


Fig. 3. Docking of 2-(benzothiazol-2-ylamino)-5-(2-chloro-benzylidene)-thiazol-4-one (**4**) into the active site of COX-1 (blue dotted line represents hydrogen bonding). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the amino acid residues Gln192, Ser353, while the benzothiazolyl moiety of the compound is oriented in the vicinity of the hydrophobic pocket comprised of the amino acids Pro514, His90, Phe91, and His513, both acting as a hydrophobic group and a hydrogen bond (Hb) acceptor. N atom of the benzothiazolyl ring is involved in hydrogen bond formation with hydrogen of the side chain of Asn515 (distance = 3.3 Å), while N atom of the thiazolidinone moiety interacts with the hydrogen atom of the NH group of the side chain of Gln192 (distance = 2.93 Å). Two more Hbs are formed between O atoms of the oxo group of thiazolidinone ring and H atoms of the NH group of the side chain of the amino acids Gln192 (distance = 3.45 Å) and Gln351(distance = 3.02 Å). The four hydrogen bonds and the hydrophobic interactions assure stable complex formation and explain the very high inhibitory activity of this compound.

Replacement of 2-Cl substituent of compound **4** with a NO_2 group in compound **1** leads to such changing of the orientation of the compound in the active site of COX-1 which do not allow the formation of any of the hydrogen bonds observed in case of compound **4**. The 2-nitro-benzylidene group of 2-(benzothiazol-2-ylimino)-5-(2-nitro-benzylidene)-thiazolidin-4-one (**1**) lies in the vicinity of the amino acids Pro585, His581, Gln192, Gln351, and Phe356, in a way similar to 2-Cl-benzylidene group of compound **4**, while the benzothiazole ring is surrounded by the amino acid His90, Thr94, Pro514 and Phe91, at a distance enabling hydrophobic interaction (distance = 2.74 Å). However, the lack of Hbs results in reduced stability thus explaining the inactivity of this compound.

However, existence of a NO_2 group at position 3 (compound **2**, Fig. 4A), has a totally different effect on the binding mode of the compound. The *m*-substituted compound lacks steric obstruction of the bulky NO_2 group thus having the ability to bend and acquire more favourable structural conformations. The orientation of the 3-NO_2 derivative now favours formation of three Hbs providing a much more stable binding, which is in accordance with the relatively high activity of the compound. Orientation of compound **2** (Fig. 4A) differs from that of compound **4**. Benzothiazole moiety is now placed in the vicinity of the amino acid residues Asn515, Thr94, Phe356. However, N atom of the benzothiazole ring also participates in hydrogen bond interaction with the hydrogen of the NH of the side chain of Asn515 amino acid (distance = 3.4 Å) in a way similar to that of compound **4**. The 4-oxo group of the thiazolidinone ring, strongly contributes to complex stabilization by forming two hydrogen bonds with hydrogen atoms offered now by the NH group of the side chain of His90 (distance = 2.5 Å) and the OH group of Ser516 (distance = 2.33 Å). On the other hand, the benzyl group of

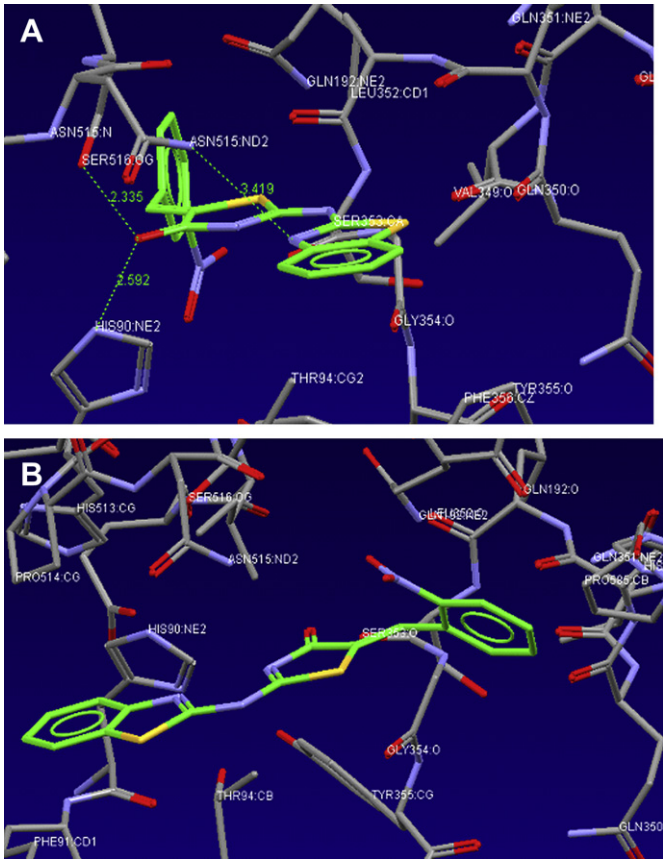


Fig. 4. A. Docking of 2-(benzothiazol-2-ylamino)-5-(3-nitro-benzylidene)-thiazol-4-one (**2**) (capped sticks) into the active site of COX-1 (green dotted line represents hydrogen bonding). B. Docking of 2-(benzothiazol-2-ylamino)-5-(2-nitro-benzylidene)-thiazol-4-one (**1**) into the active site of COX-1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the 3-nitro-benzylidene moiety, placed in immediate vicinity of the amino acids Tyr355, Gln192, Leu352 and Ser516 at the mouth of the active site, participates in hydrophobic interactions.

The number of Hbs formed, has the most crucial role in complex stabilization although hydrophobic interactions also contribute to stability. Asn515 seems to participate in Hb formation with NH group of the benzothiazole moiety of the most active derivatives in this series. Interestingly, formation of one Hb between Asn515 and the NH group of the thiazolidinone ring, was also observed in case of the most active thiazole derivative, 2-(thiazole-2-ylimino)-5-(*m*-chlorophenylidene)-4-thiazolidinone, studied previously [22]. In general, orientation of the benzothiazole derivatives favours formation of more Hbs compared to the previously tested thiazole derivatives, explaining the best activity of these compounds. However, Hbs with Arg120 and Tyr355, observed in most monoaryl or di(bi)aryl NSAIDS, like Ibuprofen (active *S*-isomer) and flubriprofen [47,48], are absent in case of benzothiazole as well as thiazol derivatives.

2.5.2. Docking to COX-2 active centre

For the docking analysis of the compounds, crystallographic data on the mouse COX-2 enzyme (PDB-ID: 3ln1) were used. Since the data on murine COX-2 have been reported in many studies [49–51], the numbers of amino acid residues in murine COX-2 analogue are shown in brackets for easy comparison with crystallographic data and docking results presented in the literature. The binding site of COX-2 (PDB-ID: 3ln1) [52] is about 23 Å (between Ser516:O^{γ} and Asp501:O); the deep cylindrical shaped pocket being more expanded at the bottom. The affinity of selective COX-2

ligands is mainly governed by the hydrophobic interactions within the very confined COX-2 binding pocket as reported by Kurumbail et al., studying the crystal structure of COX-2 bound with the selective COX-2 inhibitor SC-558 [53].

Docking of the most active *o*-Cl derivative, **4**, is shown in Fig. 5B. Compound **4** is oriented deep in the cleft of the binding site where it is stabilized in a bent (L-shaped) conformation. Hydrophobic interactions with the surrounding residues Leu345(359), Met99(113), Val102(116), Ile331(345), Ala512(527) and Leu517(531) strongly contribute to complex stabilization. Moreover, a H-bond (2.331 Å) formed between oxygen of the keto-group of the thiazolidinone ring and hydrogen of OH group of Ser516(530), a residue being acetylated by aspirin, further stabilizes the complex. L-shaped conformation and an overall orientation (very similar to that of compound 4), are also attained by the *o*-NO_2 derivative, **1**. Although, Ser516(530) is also involved in Hb formation, the presence of the bulky NO_2 group partly prevents hydrophobic interactions, thus explaining the lower activity of the compound. Unlike *ortho*-derivatives, the *meta*- and *para*-substituted analogues in this series are unable to fit deep in the hydrophobic pocket due to the steric perturbations of the *meta*- and *para*-substituents with the surrounding residues and thus attain extended conformations at the entry of the active site (Fig. 5A). In all cases, the benzo[*d*]thiazole ring is placed in the vicinity of His75(86), Tyr341(355), Ser331(345), Leu338(352), Arg499(513) and Ile503(517) residues while the keto group of thiazolidinone ring may now form a hydrogen bond with Tyr341. Moreover, Hb interactions between N atom of the benzo[*d*]thiazole moiety and hydrogens of the side chain keto and amidic bond keto groups of Gln178(192) and Leu338(352) respectively were observed with all the *meta*- and *para*-substituted compounds in both benzo[*d*]thiazole and benzo[*d*]isothiazole series. Despite such additional H-bonding, the failure of these compounds (*meta*- and *para*-substituted) to be placed deep inside the hydrophobic pocket and the lack of hydrophobic interactions lead to a formation of a less stable complex, thus exhibiting lower inhibitory action.

In the benzo[*d*]isothiazole series, all derivatives attain (L-shaped) conformation and are placed at the deep pocket of the COX-2, in the vicinity of the amino acids Met99(113), Val102(116), Ile331(345), Leu345(459), Ala512(527) and Leu517(531). Moreover, Hb interaction with Ser516(530) is formed. Close similarity in orientation between *ortho*- and *para*-substituted analogues of benzo[*d*]isothiazole series and *ortho*-substituted analogues of benzo[*d*]thiazole series was observed, supporting the particular role of lipophilic interactions within the COX-2 binding pocket as an

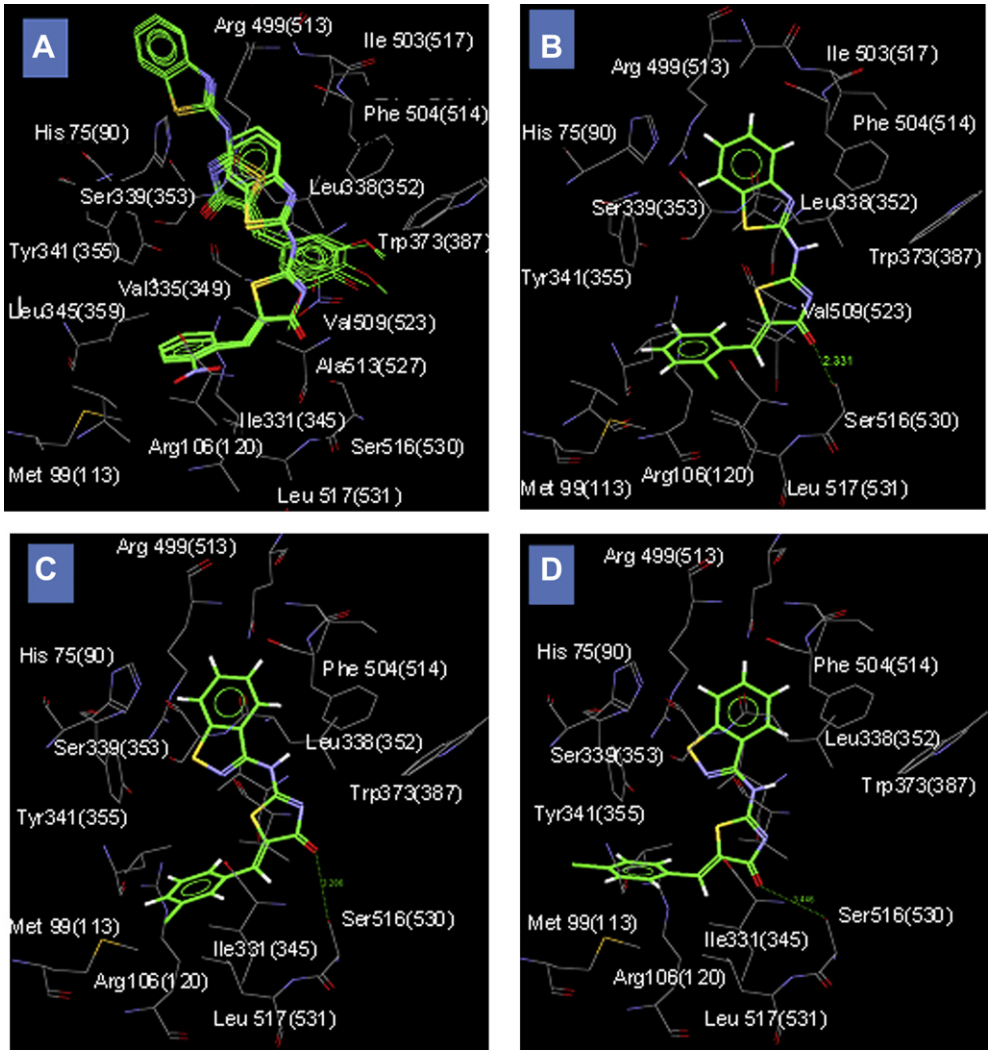


Fig. 5. Docking of selected derivatives in the COX-2 (PDB-ID: 3ln1) active site. The number of the amino acid analogue in murin COX-2 [1–3] is shown in brackets for easy comparison with other crystallographic or docking results A. Docking of benzo[*d*]thiazole derivatives. Thick lines were used for *ortho*-derivatives and thin lines were used for *meta*- and *para*-derivatives. B. Docking of compound **4**. **C**. Docking of compound **17**. D. Docking of compound **18**.

essential factor for potential inhibition of the COX-2 enzyme. In case of benzo[*d*]isothiazole derivatives, *para*-substitution seems to permit pretty stronger hydrophobic interactions compared to the *meta*-substituted derivatives thus explaining the higher activity of the former. Fig. 5D and C shows the docking of the most active *p*-Cl benzo[*d*]isothiazole derivative, **18**, and its less active *m*-Cl analogue, **17**, respectively.

The bended conformation of the benzo[*d*]thiazole/isothiazole derivatives is more close to the non-linear, tricyclic structure of the diarylheterocycle class of COX-2-selective inhibitors (e.g. celecoxib and rofecoxib). Hydrogen bond formation with Ser530 was observed in many COX-2 inhibitors, among which diclofenac and lumiracoxib. Ser530 as well as Tyr385 are placed deep in the cleft of the active centre. These amino acids are normally reached mainly by COX-2 inhibitors. Tyr-385/Ser-530 chelation of electron-rich centres was found to be crucial for acetylation of COX-2 by aspirin and was mainly associated with time-dependent, non-covalent inhibition of COX-2 by inhibitors like diclofenac [54]. The binding mode of benzo[*d*]thiazole/isothiazole derivatives to the active centre strikingly differs from that of previously studied thiazolyl derivatives which attain a more straight conformation. Although they are placed relatively deep in the active site, they form hydrogen bond interactions with Arg120 and are less potent inhibitors than their benzo[*d*]thiazole/isothiazolyl analogues. Interaction with Arg120 was observed in most carboxylic acid inhibitors, among which indomethacin, a non-selective, time-dependent, functionally irreversible NSAID [55].

2.5.3. Docking to LOX active centre

Lipoxygenases are enzymes containing a single polypeptide chain with a molecular mass of 75–80 kDa in animals and 94–104 kDa in plants [56,57] which share a great identity with each other. They all have two-domains: the small N-terminal b-barrel domain and a larger catalytic domain, containing a non-haem iron atom which participates in catalysis. The catalytic iron is ligated to three conserved histidines, one His/Asn/Ser, and the C-terminal isoleucine [58]. The highest sequence identity between LOXs is in the catalytic domain near the iron atom [59]. A high identity (63%) was observed between soybean-3 and potato 5-LOX isoenzymes, according to NCBI protein BLAST [60]. Interestingly, higher identity is observed between soybean LOX b and human 5-LOX than between human 5-LOX and potato 5-LOX enzymes.

The binding site of LOX (PDB-ID: 1lox) [52] is a deep and wide hydrophobic pocket formed mainly by the side chains of 23 amino acid residues, namely Ile172, Ile173, Phe175, Ser178, Phe353, Glu357, His361, Leu362, His366, Ile400, Arg403, and Ala404, Leu408, Val409, Ile414, Phe415, Ile418, Met419, His545, Gln548, Ile593, Val594, Gln596, and Leu597. The Leu597 located at the C-terminal is a key determinant for controlling the shape and size of the cavity. The non-haem iron is ligated to residues, His361, His366, His545, and Ile663 forming the site for the regioselective and stereospecific hydroperoxidation of polyunsaturated fatty acids.

Molecular docking of the known LOX inhibitor Nordihydroguaiaretic acid (NDGA), and compounds of both the benzo[*d*]thiazole and benzo[*d*]isothiazole series into the active site of LOX suggest that hydrophobic interactions play a major role in modulating respective LOX inhibitory activities. Docking studies showed that compounds of the benzo[*d*]isothiazole series were considerably better fitted into the active site of LOX than compounds of the benzo[*d*]thiazole series, due to the observed steric disturbances in case of the the benzo[*d*]thiazole derivatives.

Fig. 6 shows the optimal binding mode of the most potent compound **19** (LOX IC_{50} = 17.7 μM) into the active site of 15-LOX. The benzo[*d*]isothiazole moiety is placed in the hydrophobic cavity formed by the amino acid residues Phe353, Glu357, Leu358,

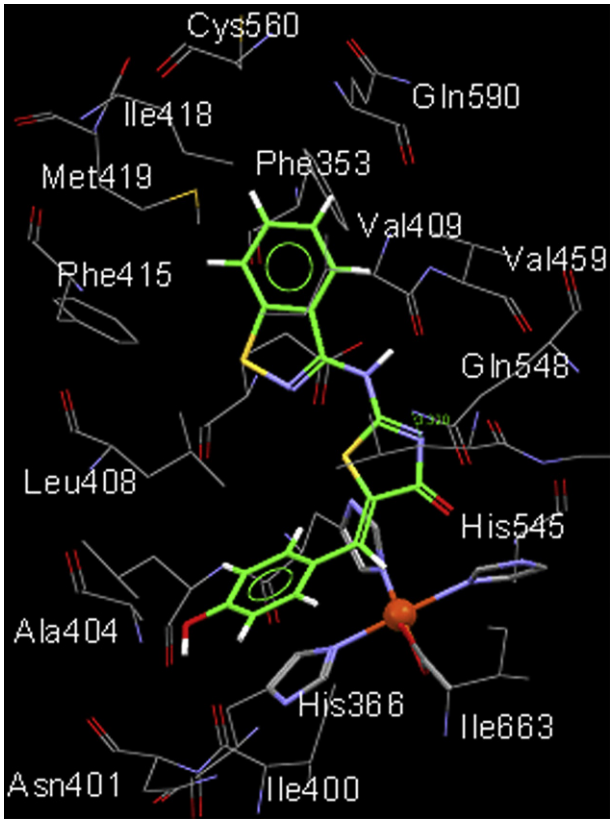


Fig. 6. Predicted binding mode of compound **19** at the active site of LOX. The ligands are shown in stick form with green coloured carbon. Hydrogens of the protein are not shown for better visualization of the binding poses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Phe415, Ile418, Met419, Ile593, Val594, and Leu597, while nitrogen of the thiazolidinone ring is involved in H-bond interaction with Gln548, an amino acid residue present in close proximity to the non-haem iron. On the other hand, the phenyl ring of the benzylidene moiety is in parallel disposition to the His366 residue (3.31 Å) exhibiting aromatic π–π interaction. The double bond C=C attached to the thiazolidinone core is placed above the non-haem iron (Fe) with Fe–CH distance of 2.78 Å, while the 4-OH substituent of the benzylidene moiety is in close proximity to His366, Leu362 and Asn401. It is obvious that, although hydrophobic interactions play a major role in complex stabilization, H-bond formation, aromatic π–π interactions and iron coordination bonds participate in stability. Compounds **13** and **18**, bearing a 2-nitro and 4-chloro group respectively, in place of the 4-OH substituent of compound **19**, exhibited an overall orientation similar to compound **19**. However, the slight differences resulting in lack of certain interactions like H-bond formation may be the reason behind comparatively lower LOX inhibitory activity of these compounds compared to compound **19**.

The predicted binding mode of the most active derivative of the benzo[*d*]thiazole series, **11** (LOX IC_{50} = 28.2 μM), into the active site of LOX is shown in Fig. 7. This compound attains a bent-shaped conformation with the benzo[*d*]thiazole moiety oriented in the hydrophobic pocket formed by the amino acid residues Phe353, Glu357, Leu358, Phe415, Ile418, Met419, Ile593, Val594, and Leu597, and the thiazolidinone ring placed in parallel (4.64 Å) to the His361 with the keto group projected towards the plane of His366. Unlike the most potent compound **19**, the 4-methoxybenzylidene group is now placed in the pocket formed by Phe175, Leu597, Arg403 and Ile663 amino acid residues, exhibiting hydrophobic interactions,

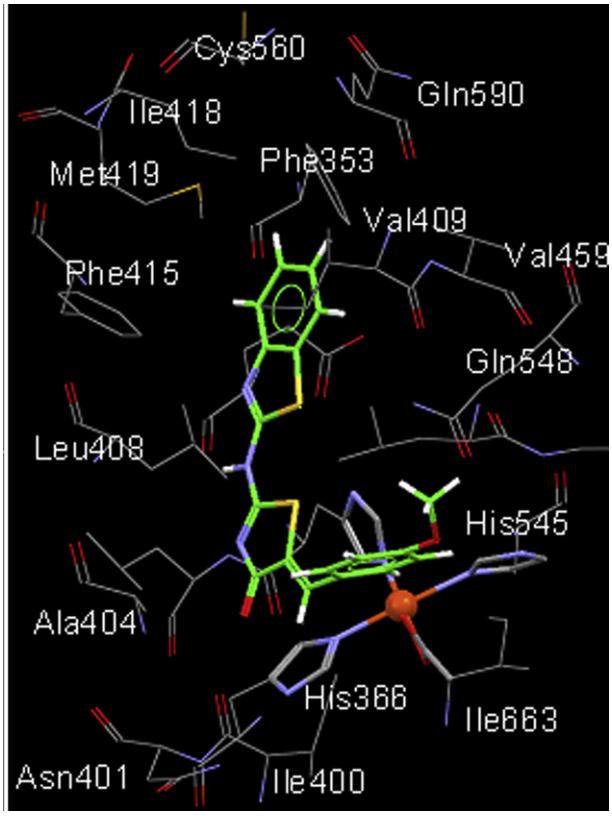


Fig. 7. Predicted binding mode of compound **11** at the active site of LOX. The ligands are shown in stick form with green coloured carbon. Hydrogens of the protein are not shown for better visualization of the binding poses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while the C=C bond of the 4-methoxybenzylidene moiety is still in close proximity to the non-haem iron. The relatively distinct orientation of this compound (**11**) compared to the most potent compound **19**, may be one of the possible reasons behind its lower LOX inhibitory activity.

The high importance of hydrophobic interactions and iron coordination in complex stabilization of LOX inhibitors has been previously reported in the literature when activity of novel acrylic acid derivatives was discussed [20]. Better contribution to hydrophobic interactions of the benzo[*d*]thiazolyl/isothiazolyl moiety compared to the thiazolyl ring may be responsible for the better activity of the benzo[*d*]thiazolyl/isothiazolyl derivatives compared to the previously studied thiazolyl compounds.

3. Conclusions

In this work we have demonstrated that, using the proposed FBDD method, one can identify the fragments important for simultaneous COX-1, COX-2 and LOX inhibition. Based on this knowledge, we designed new, more active derivatives.

Using this approach a fragment library was prepared, including about 4200 small fragments with MW less than 200 Da. Fragments with the highest positive influence on inhibition of COX-1, COX-2 and LOX enzymes were selected. Benzothiazole was considered as a fragment of great significance for the inhibition of all three enzymes and was among the fragments proposed to be incorporated into the new scaffold. Appropriate derivatives were designed, synthesized or selected from the bulk of already synthesized compounds and tested in *in vitro* and *in vivo* assays, which confirmed the computational predictions. In general the influences of benzothiazole, benzisothiazole and thiazole scaffolds on COX-1, COX-2 and LOX inhibition can be ordered as: benzothiazole ≈ benzisothiazole >> thiazole. Docking studies of the most active compounds explain the observed activity. The results of docking correspond well to the fragment-based analysis on the basis of the PASS algorithm.

Tanimoto coefficients (*Tc*) were calculated for the pairs of thiazolidinones/benzothiazolylthiazolidinones and thiazolidinones/benzisothiazolylthiazolidinones (see Supplementary data). Minimal value of *Tc* is 0.48 for *o*-Cl, *m*-Cl and *p*-Cl substituents of thiazole/benzisothiazole derivatives. Maximal *Tc*-value is 0.7 for 4-hydroxy-3,5-dimethoxyphenyl-methylidene substituents of thiazole/benzothiazole. As was shown in our earlier paper [61], if the estimated *Tc*-values do not exceed 0.7, the synthesized compounds can be considered as New Chemical Entities. Therefore, we have shown that the proposed FBDD approach provides the possibility to design the multi-target compounds with a considerable chemical novelty.

4. Experimental section

Melting points (mp °C) were determined with a Buchi 512 apparatus or with a Boetius apparatus and are uncorrected. Elemental analysis was performed on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyser, for C, H, N, and S. The values found for C, H, N, S were within ±0.4% of the theoretical ones. IR spectra, such as KBr pellets, were recorded on a JASCO FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan), while in Nujol, on a Perkin Elmer Spectrum BX. Wave numbers in the IR spectra are given in cm^{-1}. ^1H NMR spectra of the newly synthesized compounds, in DMSO-d_6 solutions, were recorded on a Bruker AC 300 instrument (Bruker, Karlsruhe, Germany) at 298 K. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard, coupling constants *J* are expressed in Hz. The progress of the reactions was monitored by thin layer chromatography with F_{254} silica-gel precoated sheets (Merck, Darmstadt, Germany). UV light was used for detection.

Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting material, reagents and solvents were purchased from Aldrich Chemie (Steinheim Germany) and from Fluka.

4.1. Materials

All chemicals were of analytical grade and commercially available. Soybean lipoxygenase 1b, 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.

4.2. Chemistry

4.2.1. General procedure for synthesis of 2-(heteroarylimino) thiazolidin-4-ones (*III*)

A solution of 2-chloro-*N*-(heteroaryl)acetamide (5 mmol) and ammonium thiocyanate (10 mmol) in 20 mL of 96% ethanol was refluxed for 1–3 h and allowed to stand overnight. The precipitate was filtered, washed with water and then recrystallised.

4.2.2. General procedure for synthesis of 2-heteroarylimino-5-benzylidene-4-thiazolidinones (*IV*)

To a well stirred solution of 2-(heteroarylimino) thiazolidin-4-one (4 mmol) in 35 mL of acetic acid, buffered with sodium acetate (9 mmol), appropriate aldehyde (6 mmol) was added. The solution was refluxed over different periods till the completion of the reaction, monitored by TLC. After cooling the reaction mixture

to the room temperature the precipitate formed, was filtered abundantly washed with water and then recrystallized.

4.2.3. 5-(3-Hydroxybenzylidene)-2-(benzo[d]thiazol-2-ylimino) thiazolidin-4-one (**7**)

Reaction time: 6 h. Yield: 42%, mp 293–294 °C (dioxane). TLC: eluent = toluene/dioxane/acetic acid 90/10/5. IR(KBr): ν = 3400 (O–H), 3129 (N–H), 1696 (C=O), 1564 (N=C) cm^{-1}. ^1H NMR (DMSO-d_6): δ = ^1H NMR (DMSO-d_6): δ = 6.95–7.56 (m, 6H, Ar, benzothiazole), 7.72 (s, 1H, Ar2), 7.96–8.05 (m, 2H, benzothiazole), 9.95 (s, 1H, OH), 12.93 (s, 1H, NH). Elemental Anal. for $C_{17}H_{11}N_3O_2S_2$ C, H, N.

4.2.4. 5-(4-Hydroxy-3,5-dimethoxybenzylidene)-2-(benzo[d] thiazol-2-ylimino)thiazolidin-4-one (**10**)

Reaction time: 4 h. Yield: 73%, m.p. 272–273 °C (dioxane). TLC: eluent = toluene/dioxane/acetic acid 90/10/5. IR(KBr): ν = 3441 (O–H), 3193 (N–H), 1698 (C=O), 1581(N=C) cm^{-1}. ^1H NMR (DMSO-d_6): δ = 3.88 (s, 3H, CH_3–O), 7.00 (d, 1H, J = 8.4 H-5′), 7.21 (d, 1H, J = 8.1 H-6′), 7.30–7.39 (m, 2H, H-2′, H-6′), 7.49 (t, 1H, J = 7.9 H-5), 7.71 (s, 1H, CH), 7.89 (d, 1H, J = 7.8 H-4), 8.01 (d, 1H, J = 8.1 H-7), 10.01 (s, 1H, OH), 12.79 (s, 1H, NH). Elemental Anal. for $C_{19}H_{15}N_3O_4S_2$ (413.48): C, H, N.

4.2.5. 2-(Benzo[d]isothiazol-3-ylimino)-5-(4-hydroxy-3,5-di methoxybenzylidene)thiazolidin-4-one (**21**)

Reaction time : 6 h. Yield: 78%, m.p.247–249°. TLC: eluent = toluene/dioxane/acetic acid 90/10/5. IR(KBr): ν = 3392 (OH), 3171 (NH), 2965, 2856 (CH_3),1706 (C=O), 1579 (C=N) cm^{-1}. ^1H NMR (DMSO-d_6): δ = 3.85 (s, 6H, 2 CH_3O), 6.98 (s, 2H, 2′+6′), 7.54 (t, 1H, J = 7.61, 6), 7.67–7.62 (m, 2H, CH+5), 8.07 (d, 1H, J = 7.91, 7) 8.16 (d, 1H, J = 8.09, 4), 9.36 (s, 1H, OH), 12.65 (s, 1H, NH). Elemental Anal. for $C_{19}H_{15}N_3O_4S_2$ (413.48): C, H, N.

4.3. Biological assays

4.3.1. 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.

4.3.1.1. Soybean lipoxygenase inhibition study in vitro. Lipoxygenase inhibition was evaluated using soybean LOX type 1b as reported previously [20,43]. sLOX-1 is the soybean isoenzyme mostly used in drug screening, although sLOX-3 has also been used [62]. Because of structural and functional similarities with mammalian LOXs, sLOX is commonly used for both mechanistic and inhibition studies and is widely accepted as a model for LOXs from other sources [63]. 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 [61]. Similarity in inhibition behaviour between soybean LOX-1 and human recombinant 5-LOX or soybean LOX-3 and human blood serum LOX has been observed in many cases [62,63].

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.

4.3.1.2. COX inhibitor screening assay [22]. 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 PGF2a produced by SnCl2 reduction of COX-derived PGH2. The prostanoids production was quantified via enzyme immunoassay using a broadly specific antibody that binds to all the major prostaglandin compounds. For better visualization of compounds' differences in a 0–100% inhibition scale and for straight comparison with results obtained previously for their thiazolyl analogues, 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. Both arachidonic acid concentrations are much lower than the saturating concentration. The compounds were added to the reaction mixture at a final concentration of 200 μM for the estimation of activity %. Different compounds' concentrations were used for the calculation of IC_{50} values for the most active compounds Naproxen was tested under the same experimental conditions to be used as a positive control.

4.3.2. In vivo experiments. Inhibition of the carrageenan-induced oedema

Oedema was induced in the right hind paw of mice (AKR) by the intradermal injection of 0.05 mL of 2% carrageenan in water. Mice of 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, ground in a mortar before use and were given intraperitoneally simultaneously with the carrageenin injection. Indomethacin was also administered under the same conditions to be used as a positive control. 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 oedema CPE% values (Table 2). Each experiment was performed in duplicate, and the standard deviation was less than 10%.

4.4. Docking analysis

The docking experiments were carried our using the GOLD [45] docking program. For docking studies, the atomic co-ordinates of COX-1 (PDB-ID: 1cqe) [46], COX-2 (PDB-ID: 3ln1) [49] and LOX (PDB-ID: 1lox) [64] were downloaded from the Brookhaven Protein Data Bank (www.rcsb.org) and prepared using *Protein Preparation Wizard* where bond orders were assigned, water and other residues except bound ligands were deleted and finally minimized using OPLS-2005 force field implemented in Schrodinger software package [65]. The bound conformations of flurbiprofen, celecoxib and ADA were used as controls in order to define the active site in COX-1, COX-2 and LOX, respectively, and optimized 3D-structures of new ligands were docked within 10 Å radius by running 20 genetic algorithm (GA) steps for each. The docked poses of geometry optimized ligands were ranked using the GoldScore (GS), and ChemScore (CS) to find the most optimal binding pose of each ligand. In the GOLD program, the default parameters: population size (100); selection-pressure (1.1); number of operations (10,000); number of islands (1); niche size (2); and operator weights for migrate (0), mutate (100) and crossover (100) were applied. Finally, the top five binding poses of each ligand were analyzed to select the best binding pose of each ligand in order to untangle the essential parameters in terms of direct- (H-bonds) and indirect

(hydrophobic) interactions governing binding disparities among the series of these compounds.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.10.029.

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