Conventional and Microwave-Assisted Synthesis of Benzimidazole Derivatives and Their *In Vitro* Inhibition of Human Cyclooxygenase

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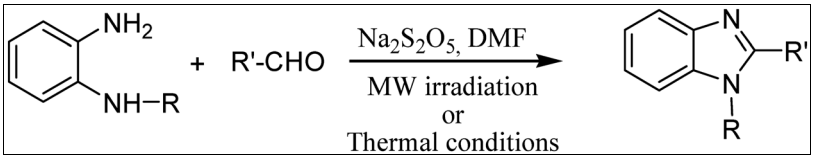

A large series of 1,2-diaryl-benzimidazole and 2-aryl-1*H*-benzimidazole derivatives were synthesized with slight differences using both microwave irradiation and conventional heating methods. Usually higher yields and time reactions reduction were obtained with the former method. All compounds were assayed for their *in vitro* ability to inhibit human cyclooxygenases, and most of them showed an encouraging inhibitory activity and isoform selectivity in the micromolar range.

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed drugs due to their high efficacy in the treatment of pain, fever, inflammation, and rheumatic disorders. The exact mechanism of action is not fully understood due to the complexity of inflammation response. However, as many symptoms of inflammation are caused by prostaglandins (PG), the efficacy of NSAIDs should be in part attributed to their ability to inhibit PG synthesis and release. This inhibition occurs through the blockage of the arachidonic acid cascade, which is catalyzed by two isoforms of the cyclooxygenase (prostaglandin G/H synthase, EC 1.14.99.1): COX-1 and COX-2 [1]. The major differences between the two iso-enzymes involve their distribution and expression. COX-1 is widely distributed in human tissues and is supposed to be encoded by a "Housekeeping Gene" which is involved in gastroprotection, platelets aggregation, renal sodium and water balance. Conversely, COX-2 is almost undetectable under normal physiological conditions, while its expression is dramatically increased by inflammatory mediators. Traditional NSAIDs are nonselective COX-inhibitors [2], which are the most representative and prescribed anti-inflammatory agents, even though their use is often associated with the occurrence of a range of hazardous side effects such as nausea, constipation, dyspepsia, gastrointestinal erosions, peptic ulcer, overt bleeding and perforation which frequently induce therapy suspension or a low compliance. Based on the assumption that COX-2 is an inducible enzyme responsible for inflammation and hyperalgesia but devoid of gastroprotective functions [3], the obvious solution to the gastrointestinal and renal toxicity of NSAIDs is the development of selective COX-2 inhibitors (COXIB) [4]. However, after repeated clinical trials, it was clear that the prolonged administration of COXIBs could enhance the incidence of cardiovascular events such as myocardial infarctions and strokes [5]. Hence, the search for novel analgesic/anti-inflammatory agents devoid of severe side effects continues to be an active area of research in medicinal chemistry.

Starting from these assumptions, the aim of our work is to propose the MW-assisted synthesis and biological evaluation of a large number of 2-monosubstituted and 1,2-disubstituted benzimidazoles as anti-inflammatory agents. The benzimidazole nucleus, in fact, overlaps with the structures of some potent traditional NSAIDs such as sulindac and indometacin (Fig. 1).

Moreover, 1,2-disubstituted benzimidazoles were reported to exhibit biologically important activities as anti-inflammatory and analgesic agents [6], and to possess higher potency than celecoxib and rofecoxib when tested as COX-2 selective inhibitors [7]. Finally, the benzimidazole moiety fulfils the minimum structural requirements for anti-inflammatory compounds: the pseudoacidic nature of its nucleus (pK_a 5.5) falls within desirable pK_a range of 5.3–7.9 for acidic NSAIDs [6].

Although some of the reported 1,2-disubstituted-benzimidazole and 2-substituted-1*H*-benzimidazole derivatives have already been synthesized by classical thermal

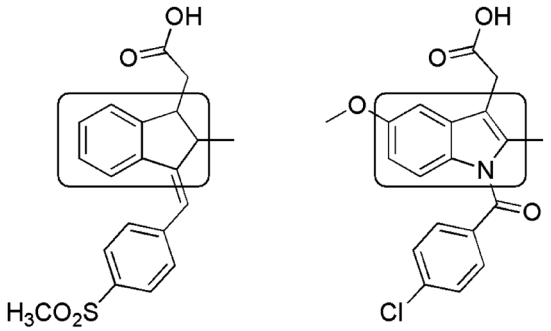


Figure 1. Indane and indole scaffolds.

methods, which required rather forcing conditions and reagents, we found out that there was no information about oxidative cyclization performed by using microwave reactors and only minimal data about household oven microwave-assisted synthesis [8]. Therefore, aiming at the synthesis of new heterocyclic systems with remarkable biological importance, we report here on the detailed synthesis by conventional and microwave-assisted methods of a large series of 1,2-diaryl-benzimidazoles and 2-aryl-1*H*-benzimidazoles.

CHEMISTRY

We synthesized molecules with mono-, bi-, and poly-substituted 2-aryl groups (**1a–42a** and **1b–42b**) and heteroaromatic ring (**43a**–**48a** and **43b–48b**) based on *N*-phenyl-benzimidazole and benzimidazole scaffolds (Table 1). The design of these derivatives explores the change in the electronic density and steric hindrance of the pharmacophoric group. For this reason, we introduced many different substituents, electron-withdrawing and electron-donor groups, (—CH_3, —OH, —F, —Cl, —Br, —OCH_3, —NMe_2, —NO_2, —SCH_3, —CF_3, —SO_2CH_3, —OCH_2Ph) at position 2′, 3′, 4′, 5′, and 6′ of the phenyl ring. In addition, we changed the aromatic group at position 2 of the benzimidazole scaffold with some heteroaromatic rings such as pyrrole, furan, thiophene, indole, benzodioxole, and naphtalene. In this study, 96 derivatives have been synthesized by the reaction of *N*-phenyl-*o*-phenylenediamine and 1,2-phenylenediamine with substituted aldehydes and sodium metabisulfite under microwave irradiation. Compounds **21a** and **21b** were synthesized using oxone as oxidizing agent from compounds **19a** and **19b** (Scheme 1).

Solid compounds were purified by chromatography, and the structure of the pure compounds was established by spectroscopic and spectrometric data. First, the reaction between 1,2-phenylenediamines and the corresponding aromatic aldehydes was carried out in 10–40 min (80–100°C) under microwave irradiation and afforded the corresponding products **1a–48a** and **1b–48b** in good yields. All reactions were performed with the minimum amounts of solvent (DMF) in vials of 10 mL, confirming that the focused microwave irradiation is a very effective technique for accelerating thermal organic reactions. The same products were also prepared by the classical thermal method, using the same reagents of microwave method in a reflux flask with about 50 mL of DMF. Classical heating afforded lower yields for almost all compounds with many difficulties in separating pure products even by liquid chromatography. The most important result of our approach is the optimization of yields and reaction times using microwave irradiation. In conclusion, we have developed a very simple, rapid, and efficient method for the preparation of 1,2-diaryl-benzimidazole and 2-aryl-1*H*-benzimidazole compounds using readily available and inexpensive reagents.

BIOLOGICAL EVALUATION

Unless otherwise specified, results shown in the text and tables are expressed as mean ± standard error of the mean (SEM) from five experiments. Significant differences between two means ($P < 0.05$ or $P < 0.01$) were determined by one-way analysis of variance (ANOVA) followed by the Dunnett's *post hoc* test. To study the possible effects of the test drugs (new compounds or reference inhibitors) on human COX isoform enzymatic activity, we evaluated the rate of *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine formation, i.e., the increase in absorbance at 600 nm per unit of time (ΔA_{600} U/min). In these experiments, the inhibitory activity of the tested drugs (new compounds and reference inhibitors) is expressed as IC_{50}, i.e., the concentration of these compounds required for a 50% reduction of the control COX isoform enzymatic activity, estimated by least-squares linear regression, using the program Origin 5.0 (Microcal Software, Northampton, MA), with X, log of tested compound molar concentration, and Y, the corresponding percentage of inhibition of control *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine production obtained with each concentration. This regression was performed using data obtained with four to six different concentrations of each tested compound that inhibited the control hCOX isoform enzymatic activity by between 20 and 80%. In addition, we calculated the corresponding COX-1 selectivity index (SI): IC_{50} (COX-2)/IC_{50} (COX-1) (Table 2).

RESULTS AND DISCUSSION

Most of the tested compounds inhibited human COX-1 and COX-2 at micromolar concentrations with selectivity index values ranging from <0.02 (**47a**) to >8.6

Table 1

Conventional and microwave-assisted synthesis conditions of benzimidazole derivatives.

			Microwave heating			Conventional heating		
Comp.	R	R′	Temperature (°C)	Time (h)	Yield (%)	Temperature (°C)	Time (h)	Yield (%)
1a [9]	Ph	Ph	100	0.1	62	80	24	51
2a [10]	Ph	2′-CH_3-Ph	100	0.1	67	80	24	47
3a [10]	Ph	3′-CH_3-Ph	100	0.1	60	80	24	50
4a [10]	Ph	4′-CH_3-Ph	100	0.1	60	80	24	43
5a [11]	Ph	2′-OH-Ph	100	0.1	73	80	24	55
6a [10]	Ph	3′-OH-Ph	100	0.1	66	80	24	61
7a [10]	Ph	4′-OH-Ph	100	0.1	99	80	24	65
8a [12]	Ph	4′-F-Ph	100	0.1	70	80	24	67
9a [10]	Ph	2′-OCH_3-Ph	100	0.1	61	80	24	44
10a [10]	Ph	3′-OCH_3-Ph	100	0.1	51	80	24	39
11a [10]	Ph	4′-OCH_3-Ph	100	0.1	90	80	24	65
12a [10]	Ph	2′-Cl-Ph	100	0.1	99	80	24	87
13a [10]	Ph	3′-Cl-Ph	100	0.1	75	80	24	67
14a [10]	Ph	4′-Cl-Ph	100	0.1	53	80	24	49
15a [13]	Ph	4′-$N(CH_3)_2$-Ph	100	0.1	80	80	24	30
16a	Ph	2′-NO_2-Ph	100	0.1	55	80	24	31
17a [10]	Ph	3′-NO_2-Ph	100	0.1	65	80	24	48
18a [10]	Ph	4′-NO_2-Ph	100	0.1	85	80	24	76
19a	Ph	4′-SCH_3-Ph	100	0.1	98	80	24	85
20a [10]	Ph	4′-CF_3-Ph	100	0.1	73	80	24	62
21a [14]	Ph	4′-SO_2CH_3-Ph	ND	ND	ND	80	24	80
22a [15]	Ph	2′-Br-Ph	100	0.1	65	80	24	60
23a [16]	Ph	3′-Br-Ph	100	0.1	86	80	24	75
24a [17]	Ph	4′-Br-Ph	100	0.1	62	80	24	36
25a	Ph	(2′-OCH_2Ph)-Ph	100	0.2	85	80	24	42
26a	Ph	(3′-OCH_2Ph)-Ph	100	0.2	60	80	24	39
27a [18]	Ph	(4′-OCH_2Ph)-Ph	100	0.2	73	80	24	33
28a	Ph	2′,3′-OH-Ph	100	0.2	69	80	24	49
29a	Ph	2′,4′-OH-Ph	100	0.2	73	80	24	48
30a	Ph	2′,5′-OH-Ph	100	0.2	68	80	24	51
31a	Ph	2′-OH-4′-OCH_3-Ph	100	0.2	71	80	24	60
32a	Ph	2′-OH-5′-Cl-Ph	100	0.2	74	80	24	65
33a [19]	Ph	2′,4′-OCH_3-Ph	100	0.2	99	80	24	79
34a	Ph	2′,5′-OCH_3-Ph	100	0.2	75	80	24	60
35a [10]	Ph	3′,4′-OCH_3-Ph	100	0.2	74	80	24	60
36a	Ph	2′-OH-5′-NO_2-Ph	100	0.3	99	80	24	75
37a	Ph	2′-OH-5′-Br-Ph	100	0.2	76	80	24	70
38a	Ph	2′,4′,5′-OCH_3-Ph	100	0.2	89	80	24	75
39a [10]	Ph	3′,4′,5′-OCH_3-Ph	100	0.2	61	80	24	36
40a	Ph	2′-OH-3′,5′-Br-Ph	100	0.2	55	80	24	39
41a	Ph	2′-OH-3′,5′-I-Ph	100	0.2	60	80	24	50
42a	Ph	4′-OH-3′,5′-I-Ph	100	0.2	99	80	24	88
43a	Ph	Pyrrol-2′-yl	80	0.4	77	80	24	65
44a [19]	Ph	Fur-2′-yl	80	0.4	56	80	24	47
45a [10]	Ph	Thiophen-2′-yl	80	0.4	85	80	24	78
46a	Ph	Indol-3′-yl	80	0.4	62	80	24	58
47a	Ph	Benzodioxol-5′-yl	80	0.4	60	80	24	46
48a [20]	Ph	Naphtalen-1′-yl	80	0.4	59	80	24	32
1b [21]	H	Ph	80	0.1	90	80	24	75
2b [22]	H	2′-CH_3-Ph	80	0.1	80	80	24	63
3b [22]	H	3′-CH_3-Ph	80	0.1	76	80	24	55
4b [20]	H	4′-CH_3-Ph	80	0.1	82	80	24	58
5b [20]	H	2′-OH-Ph	80	0.1	99	80	24	74
6b [23]	H	3′-OH-Ph	80	0.1	61	80	24	52
7b [21]	H	4′-OH-Ph	80	0.1	68	80	24	53
8b [21]	H	4′-F-Ph	80	0.1	79	80	24	59
9b [24]	H	2′-OCH_3-Ph	80	0.1	73	80	24	54
10b [24]	H	3′-OCH_3-Ph	80	0.1	62	80	24	48
11b [21]	H	4′-OCH_3-Ph	80	0.1	67	80	24	47

(*Continues*)

Table 1
(Continued)

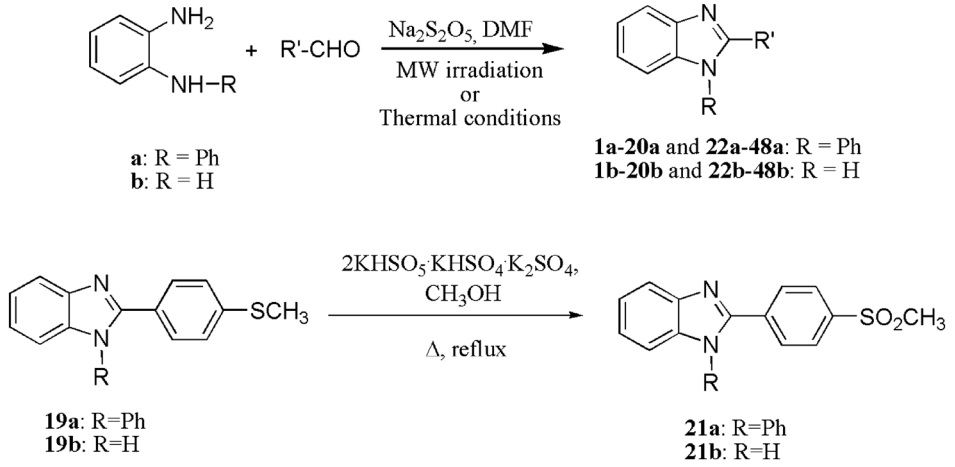
			Microwave heating			Conventional heating		
Comp.	R	R′	Temperature (°C)	Time (h)	Yield (%)	Temperature (°C)	Time (h)	Yield (%)
12b [25]	H	2′-Cl-Ph	80	0.1	69	80	24	61
13b [26]	H	3′-Cl-Ph	80	0.1	93	80	24	87
14b [21]	H	4′-Cl-Ph	80	0.1	99	80	24	85
15b [27]	H	4′-$N(CH_3)_2$-Ph	80	0.1	82	80	24	80
16b [25]	H	2′-NO_2-Ph	80	0.1	89	80	24	73
17b [27]	H	3′-NO_2-Ph	80	0.1	65	80	24	55
18b [21]	H	4′-NO_2-Ph	80	0.1	90	80	24	81
19b [28]	H	4′-SCH_3-Ph	80	0.1	99	80	24	80
20b [22]	H	4′-CF_3-Ph	80	0.1	70	80	24	47
21b [29]	H	4′-SO_2CH_3-Ph	ND	ND	ND	80	48	65
22b [30]	H	2′-Br-Ph	80	0.1	88	80	24	63
23b [27]	H	3′-Br-Ph	80	0.1	67	80	24	49
24b [21]	H	4′-Br-Ph	80	0.1	66	80	24	44
25b [8]	H	(2′-OCH_2Ph)-Ph	80	0.2	80	80	24	58
26b [24]	H	(3′-OCH_2Ph)-Ph	80	0.2	85	80	24	55
27b [31]	H	(4′-OCH_2Ph)-Ph	80	0.2	96	80	24	72
28b [32]	H	2′,3′-OH-Ph	80	0.2	87	80	24	59
29b [23]	H	2′,4′-OH-Ph	80	0.2	62	80	24	46
30b [23]	H	2′,5′-OH-Ph	80	0.2	62	80	24	44
31b [33]	H	2′-OH-4′-OCH_3-Ph	80	0.2	62	80	24	47
32b [34]	H	2′-OH-5′-Cl-Ph	80	0.2	90	80	24	75
33b [26]	H	2′,4′-OCH_3-Ph	80	0.2	87	80	24	61
34b [27]	H	2′,5′-OCH_3-Ph	80	0.2	75	80	24	59
35b [21]	H	3′,4′-OCH_3-Ph	80	0.2	94	80	24	78
36b [35]	H	2′-OH-5′-NO_2-Ph	80	0.2	99	80	24	81
37b [26]	H	2′-OH-5′-Br-Ph	80	0.2	73	80	24	56
38b [8]	H	2′,4′,5′-OCH_3-Ph	80	0.2	73	80	24	62
39b [21]	H	3′,4′,5′-OCH_3-Ph	80	0.2	63	80	24	59
40b [34]	H	2′-OH-3′,5′-Br-Ph	80	0.2	92	80	24	77
41b [36]	H	2′-OH-3′,5′-I-Ph	80	0.2	92	80	24	68
42b [37]	H	4′-OH-3′,5′-I-Ph	80	0.2	83	80	24	52
43b [38]	H	Pyrrol-2′-yl	80	0.4	95	80	24	58
44b [21]	H	Fur-2′-yl	80	0.4	54	80	24	43
45b [21]	H	Thiophen-2′-yl	80	0.4	99	80	24	70
46b [39]	H	Indol-3′-yl	80	0.4	99	80	24	73
47b [31]	H	Benzodioxol-5′-yl	80	0.4	97	80	24	68
48b [22]	H	Naphtalen-1′-yl	80	0.4	99	80	24	61

ND: not obtained with microwave irradiation.

(**22a**). With the only exception of compounds **28b** and **30b**, all the derivatives characterized by the absence of the phenyl substituent on the N1 ("**b**" series) were shown to be inactive. On the other side, compounds belonging to the "**a**" series resulted quite active and selective. In fact, thanks to the presence of the phenyl ring on the N1, the "**a**" series is almost superposable to some well-known anti-inflammatory drugs such as sulindac or indometacin.

With reference to the phenyl ring on the C2 of the benzimidazole, it is outstanding that monosubstitution endowed compounds with a greater inhibitory activity against both hCOX-1 and hCOX-2. However, if the substituent was an *ortho*-hydroxy group, disubstitution was well tolerated (**30a–32a, 28b**, **30b**) and, in the particular case in which both substituents were OH, it was possible to reach a quite good activity (11.79 ± 0.26 μ*M*) and selectivity index (>8.5) against hCOX-1 in the "**a**" series (**30a**), and to find a surprising regain of activity in the corresponding derivatives of the "**b**" series (**28b** and **30b**). Concerning about the activity against hCOX-1, electron withdrawing substituents, especially halogens (F, Br, Cl), seemed to assure good results when placed in all the positions of the phenyl ring, while the para-position could stand the presence of both electron withdrawing and releasing groups (**4a**, **11a**, **15a**, **19a**). On the contrary, the introduction of bulky benzyloxy substituents on the phenyl ring, especially in ortho (**25a**) and meta (**26a**) position, or heteroaromatic moieties directly on the C2 of the benzimidazole (**46a** and **47a**), determined a switch of selectivity toward hCOX-2. These results could be easily ascribed to the presence of a second hydrophobic pocket in the active site of hCOX-2 which is not present in hCOX-1, and to the

Scheme 1. General chemistry of the benzimidazole derivatives.



establishment of hydrogen bonds between the heteroatoms of the compounds and the active site of the enzyme that are known to contribute to hCOX-2 selectivity [6, 7]. It is also worthwhile to evaluate the MW effects on kinetic and yields enhancements. In fact, the same reactions have been carried out with slight differences under microwave and thermal conditions. The products obtained by both methodologies were found to be identical but the superiority of the former procedure in this scaffold may be ascribed to a concentration effect. The robustness of our protocol derives from the large synthesized scaffold which ranges from mono- to poly-substitution on the aromatic ring at C2 of the benzimidazole nucleus with moieties differing from size, steric hindrance, lipophilicity, and electronic properties.

EXPERIMENTAL

The compounds, synthesized with the microwave method, were obtained with a Biotage Initiator™ 2.0. The chemicals, solvents for synthesis, and spectral grade solvents were purchased from Aldrich (Italy) and used without further purification. Melting points (uncorrected) were determined automatically on an FP62 apparatus (Mettler-Toledo). ^{1}H-NMR spectra were recorded at 400 MHz on a Bruker spectrometer using DMSO-d_6 or $CDCl_3$ as solvent. Chemical shifts are expressed as δ units (parts per millions) relative to the solvent peak. Coupling constants *J* are valued in Hertz (Hz). Elemental analyses for C, H, and N were recorded on a Perkin-Elmer 240 B microanalyzer and the analytical results were within ±0.4% of the theoretical values for all compounds. All reactions were monitored by TLC performed on 0.2-mm thick silica gel plates (60 F_{254} Merck).

General procedure for the synthesis of derivatives 1a–20a, 22a–48a, 1b–20b, and 22b–48b under MW irradiation. *N*-phenyl-*o*-phenylenediamine or 1,2-phenylenediamine (1 mmol), sodium metabisulfite (1 mmol), and the corresponding aldehyde (1 mmol) were dissolved in 2 mL of *N*,*N*-dimethylformamide (DMF) in a 10-mL vial suitable for an automatic single-mode microwave reactor (2.45-GHz high-frequency microwaves, power range 0–300 W). The mixture was prestirred for 30 s and then heated by microwave irradiation for 10–40 min at 80–100°C (irradiation power reaches its maximum at the beginning of reaction, then it decreases to lower and quite constant values). The internal vial temperature was controlled by an IR sensor. After cooling with pressurized air, the reaction mixture was poured onto ice, filtered, and dried under vacuum.

General procedure for the synthesis of derivatives 1a-20a, 22a-48a, 1b-20b, and 22b-48b under thermal conditions. In a 500-mL flask, *N*-phenyl-*o*-phenylenediamine or 1,2-phenylenediamine (1 mmol), sodium metabisulfite (1 mmol), and the corresponding aldehyde (1 mmol) were dissolved in 50 mL of *N*,*N*-dimethylformamide. The mixture was refluxed for 24 h. The reaction mixture was then poured onto ice and extracted with $CHCl_3$ (3 × 50 mL). The organic layer was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by chromatography to obtain the desired benzimidazole.

General procedure for the synthesis of derivatives 21a and 21b. In a 100-mL flask, compounds **19a** or **19b** (1 mmol) was dissolved in 50 mL of methanol. The mixture was refluxed at 100°C. In a flask, oxone (3 mmol) was dissolved in the minimum amount of water. Then, the mixture with oxone was added dropwise to the flask. The mixture was refluxed for 24 h. The desired benzimidazole was filtered and dried under vacuum.

Chemical-physical data for new compounds. ***2-(2-Nitrophenyl)-1-phenyl-1H-benzo[d]imidazole (16a).*** Yellow solid, mp 180–181°C. ^{1}H-NMR (deuteriochloroform): δ 7.23–7.25 (m, 2H, Ar), 7.34–7.41 (m, 6H, Ar), 7.57–7.61 (m, 1H, Ar), 7.67–7.68 (m, 2H, Ar), 7.90–7.92 (m, 1H, Ar), 7.97–7.99 (m, 1H, Ar).

2-[4-(Methylthio)phenyl]-1-phenyl-1H-benzo[d]imidazole (19a). White solid, mp 257–258°C. ^{1}H-NMR (deuteriochloroform): δ 2.47 (s, 3H, SCH_3), 7.14–7.16 (d, J_o = 8.5 Hz, 2H, Ar), 7.16–7.24 (m, 2H, Ar), 7.32–7.34 (m, 3H, Ar), 7.48–7.52 (m, 5H, Ar), 7.85–7.87 (m, 1H, Ar).

2-[2-(Benzyloxy)phenyl]-1-phenyl-1H-benzo[d]imidazole (25a). Green solid, mp 144–145°C. ^{1}H-NMR (deuteriochloroform):

Table 2

In vitro inhibition of hCOX-1 and hCOX-2 by compounds **1a–48a** and **1b–48b.**

"a" series (N-Ph)	hCOX-1 (IC_{50})	hCOX-2 (IC_{50})	SI	"b" series (N—H)	hCOX-1 (IC_{50})	hCOX-2 (IC_{50})	SI
1a	13.34 ± 1.06 μ*M*	[a]	>7.5	**1b**	[b]	[b]	
2a	18.16 ± 1.32 μ*M*	[b]	>5.5***	**2b**	[b]	[a]	
3a	12.12 ± 1.15 μ*M**	21.93 ± 1.27 μ*M*	1.8	**3b**	[b]	[a]	
4a	9.15 ± 0.76 μ*M**	23.85 ± 1.58 μ*M*	2.6	**4b**	[b]	[b]	
5a	[c]	[c]		**5b**	[b]	[b]	
6a	[c]	[c]		**6b**	[b]	[a]	
7a	[c]	[c]		**7b**	[b]	[b]	
8a	6.57 ± 0.48 μ*M**	35.48 ± 0.14 μ*M*	5.4	**8b**	[b]	[a]	
9a	26.91 ± 2.57 μ*M*	[a]	>3.7***	**9b**	[b]	[a]	
10a	[b]	[b]		**10b**	[a]	[b]	
11a	14.63 ± 1.23 μ*M**	29.03 ± 1.46 μ*M*	2.0	**11b**	[b]	[b]	
12a	23.54 ± 1.32 μ*M**	36.52 ± 1.90 μ*M*	1.6	**12b**	[b]	[b]	
13a	8.02 ± 0.65 μ*M**	32.70 ± 1.28 μ*M*	4.1	**13b**	[b]	[b]	
14a	16.71 ± 1.38 μ*M***	19.61 ± 0.06 μ*M*	1.2	**14b**	[b]	[b]	
15a	9.04 ± 0.35 μ*M**	38.13 ± 1.72 μ*M*	4.2	**15b**	[b]	[b]	
16a	[b]	18.54 ± 1.23 μ*M*	<0.19***	**16b**	[b]	[b]	
17a	[a]	[a]		**17b**	[b]	[b]	
18a	[b]	[b]		**18b**	[b]	[b]	
19a	24.26 ± 2.10 μ*M*	39.66 ± 3.15 μ*M*	1.6	**19b**	[a]	[b]	
20a	[b]	[b]		**20b**	[a]	[b]	
21a	[b]	[a]		**21b**	[b]	[b]	
22a	11.61 ± 0.89 μ*M*	[a]	>8.6***	**22b**	[b]	[b]	
23a	11.27 ± 0.53 μ*M**	23.57 ± 2.86 μ*M*	2.1	**23b**	[b]	[b]	
24a	[a]	[b]		**24b**	[a]	[b]	
25a	[b]	24.77 ± 0.99 μ*M*	<0.25***	**25b**	[a]	[b]	
26a	[b]	19.67 ± 0.76 μ*M*	<0.20***	**26b**	[b]	[b]	
27a	[b]	[b]		**27b**	[a]	[b]	
28a	29.52 ± 2.37 μ*M*	[a]	>3.4***	**28b**	21.87 ± 0.92 μ*M*	26.89 ± 2.13 μ*M*	1.2
29a	[a]	[a]		**29b**	[a]	[b]	
30a	11.79 ± 0.26 μ*M*	[a]	>8.5***	**30b**	8.30 ± 0.76 μ*M*	17.63 ± 1.46 μ*M*	2.1
31a	27.83 ± 0.69 μ*M*	[a]	>3.6***	**31b**	[b]	[a]	
32a	28.60 ± 0.48 μ*M*	[a]	>3.5***	**32b**	[b]	[b]	
33a	[c]	[c]		**33b**	[a]	[b]	
34a	[a]	[a]		**34b**	[a]	[b]	
35a	[a]	[a]		**35b**	[b]	[b]	
36a	[b]	[a]		**36b**	[b]	[a]	
37a	[a]	[a]		**37b**	[b]	[b]	
38a	[b]	[b]		**38b**	[b]	[b]	
39a	[a]	[a]		**39b**	[a]	[b]	
40a	[a]	[a]		**40b**	[b]	[b]	
41a	[a]	[a]		**41b**	[a]	[b]	
42a	[a]	[a]		**42b**	[b]	[b]	
43a	[b]	[b]		**43b**	[b]	[b]	
44a	[c]	[c]		**44b**	[b]	[b]	
45a	23.96 ± 0.32 μ*M*	[b]	>4.2***	**45b**	[b]	[a]	
46a	[b]	3.84 ± 0.19 μ*M*	<0.04***	**46b**	[a]	[b]	
47a	[b]	2.34 ± 0.15 μ*M*	<0.02***	**47b**	[b]	[b]	
Indometacin	12.16 ± 1.16 μ*M***	35.20 ± 1.41 μ*M*	2.9				
Diclofenac	18.23 ± 1.73 μ*M*	23.62 ± 1.97 μ*M*	1.3				
FR122047	93.80 ± 6.55 n*M*	[b]	>1066***				
Nimesulide	[d]	231.40 ± 19.84 μ*M*	<0.46***				
DuP 697	22.61 ± 1.56 μ*M**	126.32 ± 7.41 n*M*	0.0056				

Each IC_{50} value is the mean ± SEM from five experiments.
[a]100 μ*M* inhibits hCOX-1 or hCOX-2 activity by ~40–45%.
[b]Inactive at 100 μM (highest concentration tested).
[c]Inactive at 25 μM (highest concentration tested).
[d]Inactive at 500 μ*M* (highest concentration tested).
Level of statistical significance:
*$P < 0.01$ *versus* the corresponding IC_{50} values obtained against hCOX-2, as determined by ANOVA/Dunnett's *post hoc* test.
**$P < 0.05$ *versus* the corresponding IC_{50} values obtained against hCOX-2, as determined by ANOVA/Dunnett's *post hoc* test.
***Value obtained under the assumption that the corresponding IC_{50} against hCOX-1 or hCOX-2 is the highest concentration tested.

δ 4.69 (s, 2H, OCH_2Ar), 6.73–6.75 (d, J_o = 8.1 Hz, 1H, Ar), 6.97–6.98 (m, 2H, Ar), 7.07–7.13 (m, 3H, Ar), 7.22–7.37 (m, 11H, Ar), 7.70–7.72 (d, J_o = 7.7 Hz, 1H, Ar), 7.92–7.94 (d, J_o = 7.4 Hz, 1H, Ar).

2-[3-(Benzyloxy)phenyl]-1-phenyl-1H-benzo[d]imidazole (26a). Grey solid, mp 120–121°C. ^{1}H-NMR (dimethyl sulfoxide d_6): δ 4.93 (s, 2H, OCH_2Ar), 6.99–7.00 (m, 1H, Ar), 7.11–7.12 (m, 1H, Ar), 7.18–7.38 (m, 13H, Ar), 7.49–7.53 (m, 3H, Ar), 7.90–7.92 (d, J_o = 7.5 Hz, 1H, Ar).

3-(1-Phenyl-1H-benzo[d]imidazol-2-yl)benzene-1,2-diol (28a). Orange solid, mp 122–123°C. ^{1}H-NMR (deuteriochloroform): δ 6.36–6.38 (m, 2H, Ar), 6.44–6.46 (d, J_o = 8.0 Hz, 1H, Ar), 6.91–7.45 (m, 6H, Ar), 7.62–7.64 (m, 3H, Ar), 7.81–7.83 (d, J_o = 8.0 Hz, 1H, Ar), 8.62 (bs, 1H, OH, D_2O exch.), 9.58 (bs, 1H, OH, D_2O exch.).

4-(1-Phenyl-1H-benzo[d]imidazol-2-yl)benzene-1,3-diol (29a). Brown solid, mp 167–168°C. ^{1}H-NMR (deuteriochloroform): 6.06–6.08 (d, J_o = 8.2 Hz, 1H, Ar), 6.48–6.71 (m, 3H, Ar), 7.04–7.42 (m, 4H, Ar), 7.57–7.60 (m, 3H, Ar), 7.76–7.78 (d, J_o = 7.6 Hz, 1H, Ar), 8.53 (bs, 1H, OH, D_2O exch.), 9.58 (bs, 1H, OH, D_2O exch.).

2-(1-Phenyl-1H-benzo[d]imidazol-2-yl)benzene-1,4-diol (30a). Grey solid, mp 91–92°C. ^{1}H-NMR (deuteriochloroform): δ 6.34 (s, 1H, Ar), 6.72–6.78 (m, 1H, Ar), 6.96–6.98 (d, J_o = 8.6 Hz, 1H, Ar), 7.07–7.09 (d, J_o = 8.3 Hz, 1H, Ar), 7.26–7.42 (m, 3H, Ar), 7.58–7.60 (m, 3H, Ar), 7.79–7.81 (d, J = 7.7 Hz, 1H, Ar), 7.98 (m, 1H, Ar), 8.51 (bs, 1H, OH, D_2O exch.), 9.49 (bs, 1H, OH, D_2O exch.).

5-Methoxy-2-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (31a). Green solid, mp 174–175°C. ^{1}H-NMR (deuteriochloroform): δ 3.78 (s, 3H, OCH_3), 6.04–6.12 (m, 1H, Ar), 6.62–6.63 (m, 1H, Ar), 6.72–6.75 (d, J_o = 8.6 Hz, 1H, Ar), 7.06–7.33 (m, 5H, Ar), 7.41–7.43 (m, 3H, Ar), 7.61–7.62 (m, 1H, Ar), 13.41 (bs, 1H, OH, D_2O exch.).

4-Chloro-2-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (32a). Yellow solid, mp 109–110°C. ^{1}H-NMR (deuteriochloroform): δ 6.75 (s, 1H, Ar), 7.03–7.43 (m, 8H, Ar), 7.66–7.67 (m, 3H, Ar), 7.82–7.84 (d, J_o = 8.2 Hz, 1H, Ar), 12.83 (bs, 1H, OH, D_2O exch.).

2-(2,5-Dimethoxyphenyl)-1-phenyl-1H-benzo[d]imidazole (34a). Green solid, mp 69–70°C. ^{1}H-NMR (deuteriochloroform): δ 3.25 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 6.65–6.67 (d, J_o = 8.3 Hz, 1H, Ar), 6.92–6.93 (m, 1H, Ar), 7.25–7.41 (m, 9H, Ar), 7.89–7.91 (d, J_o = 7.7 Hz, 1H, Ar).

4-Nitro-2-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (36a). Yellow solid, mp 179–180°C. ^{1}H-NMR (dimethyl sulfoxide d_6): δ 7.07–7.09 (d, J_o = 9.2 Hz, 2H, Ar), 7.25–7.27 (d, J_o = 7.2 Hz, 2H, Ar), 7.37–7.39 (m, 3H, Ar), 7.55–7.61 (m, 2H, Ar), 7.88–7.89 (d, J_o = 7.0 Hz, 1H, Ar), 8.11–8.12 (m, 1H, Ar), 8.17–8.20 (m, 1H, Ar), 12.05 (bs, 1H, OH, D_2O exch.).

4-Bromo-2-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (37a). Brown solid, mp 120–121°C. ^{1}H-NMR (deuteriochloroform): δ 6.88–7.43 (m, 8H, Ar), 7.66–7.68 (m, 3H, Ar), 7.82–7.83 (m, 1H, Ar), 13.36 (bs, 1H, OH, D_2O exch.).

1-Phenyl-2-(2,4,5-trimethoxyphenyl)-1H-benzo[d]imidazole (38a). Green solid, mp 104–105°C. ^{1}H-NMR (deuteriochloroform): δ 3.25 (s, 3H, OCH_3), 3.88 (s, 6H, 2 × OCH_3), 6.32 (s, 1H, Ar), 7.22–7.41 (m, 9H, Ar), 7.89–7.91 (m, 1H, Ar).

2,4-Dibromo-6-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (40a). Orange solid, mp 186–187°C. ^{1}H-NMR (deuteriochloroform): δ 6.85 (s, 1H, Ar), 6.86–7.89 (m, 11H, Ar), 13.79 (bs, 1H, OH, D_2O exch.).

2,4-Diiodo-6-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (41a). Yellow solid, mp 180–181°C. ^{1}H-NMR (deuteriochloroform): δ 7.25–7.38 (m, 9H, Ar), 7.58–7.61 (m, 1H, Ar), 7.89–7.90 (s, 1H, Ar), 12.32 (bs, 1H, OH, D_2O exch.).

2,6-Diiodo-4-(1-phenyl-1H-benzo[d]imidazol-2-yl)phenol (42a). Brown solid, mp 96–97°C. ^{1}H-NMR (deuteriochloroform): δ 6.06 (s, 1H, Ar), 6.92–6.94 (m, 1H, Ar), 7.04–7.34 (m, 4H, Ar), 7.47 (s, 1H, Ar), 7.67–7.71 (m, 2H, Ar), 8.11 (s, 1H, Ar), 8.48 (s, 1H, Ar), 14.09 (bs, 1H, OH, D_2O exch.).

1-Phenyl-2-(1H-pyrrol-2-yl)-1H-benzo[d]imidazole (43a). White solid, mp 185–186°C. ^{1}H-NMR (deuteriochloroform): δ 6.12–6.14 (m, 1H, Ar), 6.92–6.94 (m, 1H, Ar), 7.10–7.20 (m, 4H, Ar), 7.30–7.34 (m, 1H, Ar), 7.67–7.71 (m, 2H, Ar), 8.11–8.12 (m, 1H, Ar), 8.47–8.49 (m, 1H, Ar), 14.11 (bs, 1H, OH, D_2O exch.).

2-(1H-Indol-3-yl)-1-phenyl-1H-benzo[d]imidazole (46a). Grey solid, mp 223–224°C. ^{1}H-NMR (deuteriochloroform): δ 6.59–6.60 (m, 1H, Ar), 7.01–7.03 (m, 1H, Ar), 7.16–7.25 (m, 4H, Ar), 7.40–7.42 (m, 1H, Ar), 7.52–7.54 (m, 2H, Ar), 7.55–7.67 (m, 3H, Ar), 7.74–7.76 (d, J = 7.8 Hz, 1H, Ar), 8.47–8.49 (d, J = 7.0 Hz, 1H, C_2H-indole), 11.41 (bs, 1H, NH, D_2O exch.).

2-(Benzo[d][1,3]dioxol-5-yl)-1-phenyl-1H-benzo[d]imidazole (47a). Grey solid, mp 122–123°C. ^{1}H-NMR (deuteriochloroform): δ 5.96–5.97 (s, 2H, OCH_2O), 6.72–6.74 (d, J = 7.7 Hz, 1H, Ar), 7.05–7.07 (m, 2H, Ar), 7.23–7.26 (m, 2H, Ar), 7.31–7.34 (m, 3H, Ar), 7.50–7.54 (m, 3H, Ar), 7.86–7.87 (d, J = 7.3 Hz, 1H, Ar).

Preparation of microsomes from human platelets. Human platelets were isolated by centrifugation from buffy coats obtained from the Centro de Transfusión de Galicia (Santiago de Compostela, Spain) and prepared as we have previously described [40]. Briefly, buffy coat was diluted 1:1 with washing buffer of the following composition (m*M*): NaCl 120, KCl 5, trisodium citrate 12, glucose 10, sucrose 12.5 (pH 6) and then centrifuged at 400 g for 8 min in a centrifuge (Omnifuge 2.0 RS, Heraeus Sepatech, Osterade, Germany) at 25°C to obtain platelet rich plasma. The upper layer obtained in this centrifugation, containing platelet rich plasma, was gently removed and centrifuged at 850 g for 20 min at 4°C in a centrifuge (J2-MI, Beckman Instruments, Palo Alto, CA). The platelet pellet was recovered, resuspended with washing buffer, and centrifuged again at 850 g for 20 min at 4°C. To prepare human platelets microsomes, the resultant platelet pellet of the above centrifugation was resuspended in 7 mL of sodium phosphate buffer (10 m*M*, pH 7.4), sonicated at 50 W for 50 s (5 pulses of 10 s), and centrifuged at 850 g for 20 min at 4°C in a refrigerated centrifuge (J2-MI, Beckman Instruments, Palo Alto, CA). The pellet was discarded and the supernatant was subsequently centrifuged at 10,000 g for 10 min at 4°C in the same centrifuge. The pellet obtained in this centrifugation was discarded and the supernatant was finally centrifuged at 100,000 g for 1 h at 4°C in a centrifuge (J2-MI, Beckman Instruments, Palo Alto, CA). The resultant pellet containing platelet microsomes was resuspended in 1 mL of sodium phosphate buffer (50 m*M*, pH 7.4) and the protein concentration in the platelet microsome suspension (∼2 mg/mL) was measured by the method of Bradford [41] using a protein assay kit from BioRad Laboratories (Alcobendas, Spain). Platelet microsome aliquots were stored at −80°C for several days (without apparent loss of COX activity) until use.

Determination of human cyclooxygenase (hCOX) isoform activity. The biological evaluation of the test drugs on total hCOX activity (bisdioxygenase and peroxidase reactions) was investigated by measuring their effects on the oxidation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) to *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine, using araquidonic acid as common substrate for both hCOX-1 and hCOX-2, microsomal COX-2 prepared from insect cells (Sf 21 cells) infected with recombinant baculovirus containing cDNA inserts for hCOX-2 (Sigma-Aldrich Química S.A., Alcobendas, Spain) and COX-1 from human platelet microsomes (obtained as described in the above paragraph since, unlike hCOX-2, hCOX-1 is not commercially available). The formation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine (a colored compound) from *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) catalyzed by COX can be detected spectrophotometrically at 600 nm. In this study, hCOX activity was evaluated using the above spectrophotometric method following the general procedure described previously [42] with several modifications. Briefly, 0.1 mL of Tris-HCl buffer (100 m*M*, pH 8) containing 1 μ*M* hematin, 100 μ*M* TMPD, various concentrations of the test drugs (new compounds or reference inhibitors), and appropriate amounts of hCOX-1 and hCOX-2 required and adjusted to obtain in our experimental conditions the same control absorbance increase (0.08 A600 U/min) were incubated for short periods of time (3–5 min) to avoid a notable loss of COX activity at 37°C in a flat-bottom 96-well microtest plate (BD Biosciences, Franklin Lakes, NJ) placed in the dark multimode microplate reader chamber. After this incubation period, the reaction was started by adding (final concentration) 100-μ*M* arachidonic acid and the formation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine from TMPD, i.e., the increase in absorbance at 600 nm was measured at 37°C in a multimode microplate reader (Fluostar Optima, BMG Labtech GmbH, Offenburg, Germany) for 25 s, a period in which the absorbance increased linearly from the beginning.

The specific absorbance (used to obtain the results) was calculated after subtraction of the background absorbance generated in wells containing a blank solution, i.e., all components except the COX isoforms, which were replaced by a Tris-HCl buffer solution. In our experimental conditions, this background activity was practically negligible.

Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the absorbance of the reaction mixture due to nonenzymatic inhibition (e.g., for directly reacting with TMPD) was determined by adding these drugs to solutions containing only TMPD in a Tris-HCl buffer solution.

Drugs and chemicals. The drugs, vehicle, and chemicals used in the experiments were the new compounds, dimethyl sulfoxide (DMSO), hematin porcine, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), arachidonic acid sodium salt, indometacin (purchased from Sigma-Aldrich Química S.A., Alcobendas, Spain), diclofenac sodium salt, FR122047 monohydrochloride hydrate, nimesulide, and DuP 697 (purchased from Cayman Chemical, Ann Arbor, MI). Appropriate dilutions of some of the above drugs were prepared every day immediately before use in deionized water from the following concentrated stock solutions kept at −20°C: the new compounds, diclofenac, FR122047 hydrate, nimesulide, and DuP 697 (0.1 *M*) in DMSO. In addition, stock solutions of the following drugs were prepared daily before use: arachidonic acid (30 m*M*) and indometacin (10 m*M*) in ethanol; hematin (0.2 m*M*) and TMPD (40 m*M*) in DMSO. Because of the instability of some chemicals (e.g., DuP 697, arachidonic acid), the corresponding solutions of these chemicals were maintained in inert atmosphere until use. In all assays, neither deionized water (Milli-Q, Millipore Ibérica S.A., Madrid, Spain) nor appropriate dilutions of the vehicle used (DMSO) had significant pharmacological effects.

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