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Structure–activity relationship of celecoxib and rofecoxib for the membrane permeabilizing activity

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) achieve their anti-inflammatory effect by inhibiting cyclooxygenase activity. We previously suggested that in addition to cyclooxygenase-inhibition at the gastric mucosa, NSAID-induced gastric mucosal cell death is required for the formation of NSAID-induced gastric lesions in vivo. We showed that celecoxib exhibited the most potent membrane permeabilizing activity among the NSAIDs tested. In contrast, we have found that the NSAID rofecoxib has very weak membrane permeabilizing activity. To understand the membrane permeabilizing activity of coxibs in terms of their structure–activity relationship, we separated the structures of celecoxib and rofecoxib into three parts, synthesized hybrid compounds by substitution of each of the parts, and examined the membrane permeabilizing activities of these hybrids. The results suggest that the sulfonamidophenyl subgroup of celecoxib or the methanesulfonylphenyl subgroup of rofecoxib is important for their potent or weak membrane permeabilizing activity, respectively. These findings provide important information for design and synthesis of new coxibs with lower membrane permeabilizing activity.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently used classes of medicines.[1] NSAIDs are inhibitors of cyclooxygenase (COX), a protein essential for the synthesis of prostaglandins (PGs), which have a strong ability to induce inflammation. However, NSAID use is associated with gastrointestinal complications, such as gastric ulcers and bleeding. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications.[2] Thus, understanding the mechanism of NSAID-induced gastric lesions and its application to design and synthesis of new NSAIDs with reduced adverse effects on the gastric mucosa is important.

The inhibition of COX by NSAIDs was initially thought to be responsible for the adverse gastric side effects manifested by such treatment, because PGs have a strong protective effect on the gastric mucosa. Thus, after the identification of two subtypes of COX (COX-1 and COX-2), which are responsible for the majority of COX activity at the gastric mucosa and in inflammatory tissues, respectively,[3,4] selective COX-2 inhibitors (most of which are coxibs, such as celecoxib and rofecoxib) were developed as NSAIDs with reduced adverse gastric side effects.[5–7] However, due to the observation that rofecoxib was associated with an increased potential risk of cardiovascular thrombotic events,[8,9] this NSAID was withdrawn from the market. At first, this increased risk was believed to be due to the class effect of selective COX-2 inhibitors, because prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2.[10–12] However, some clinical studies showed that the potential risk of cardiovascular thrombotic events was indistinguishable between celecoxib users and classic NSAID users.[13,14] Thus, it is possible that the increased potential risk of cardiovascular thrombotic events is not due to the class effect of selective COX-2 inhibitors, but rather is a specific characteristic of rofecoxib. While mechanisms to explain this rofecoxib-specific increase in the potential risk of cardiovascular thrombotic events have been proposed,[15–17] a definitive explanation for this increase has not yet been forthcoming.

It is now believed that the inhibition of COX by NSAIDs is not the sole explanation for the adverse gastric side effects of NSAIDs, given that the increased incidence of gastric lesions and the

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decrease in PG levels induced by NSAIDs do not always occur in parallel.[18–20] We proposed that, in addition to COX-inhibition at the gastric mucosa, NSAID-induced gastric mucosal cell death is required for the formation of NSAID-induced gastric lesions in vivo.[21,22] Furthermore, we reproduced NSAID-induce cell death in cultured gastric mucosal cells in vitro[22–26] and showed that the primary target of NSAIDs for the induction of cell death is the cytoplasmic membrane. Moreover, a close relationship between membrane permeabilizing activity and cell death-inducing activity among various NSAIDs was shown.[23,25] Thus, decreasing the membrane permeabilizing activity of NSAIDs may be another strategy to synthesize safer NSAIDs for the gastric mucosa. In fact, we recently reported that screening for NSAIDs with lower membrane permeabilizing activity resulted in the identification of an interesting new NSAID, fluoro-loxoprofen, which has much lower membrane permeabilizing and gastric ulcerogenic activities compared with clinically used NSAIDs.[27–31] These results suggest that NSAIDs with lower membrane permeabilizing activity could be therapeutically beneficial. Thus, it is important to understand how the membrane permeabilizing properties of NSAIDs are affected by their structure–activity relationship.

We previously reported that celecoxib showed the most potent membrane permeabilizing and cytotoxic activities among the NSAIDs we tested.[23,25] We also reported that the cytotoxic activity of rofecoxib is much lower than that of celecoxib.[21] As these results suggested that the membrane permeabilizing activity of rofecoxib is lower than that of celecoxib, our objective here was to confirm this hypothesis.

Furthermore, to identify how the structure–activity relationship of coxibs affects their membrane permeabilizing activity, we synthesized hybrid compounds from celecoxib and rofecoxib and examined their membrane permeabilizing activities. The results suggest that the sulfonamidophenyl subgroup of celecoxib and the methanesulfonylphenyl subgroup of rofecoxib are important for determining the membrane permeabilizing activities of these NSAIDs.

2. Chemistry

The synthetic route for target compounds **3**–**5** is outlined in Scheme 1. Pyrazole compounds **3**–**5** were synthesized by the condensation of appropriate 1,3-diketones and hydrazine. The reaction of 4,4,4-trifluoro-1-*p*-tolylbutane-1,3-dione **9** with 4-methylphenylhydrazine hydrochloride **11**, 4,4,4-trifluoro-1-phenylbutane-1,3-dione **10** with **11**, or **10** with 4-sulfamoylphenylhydrazine hydrochloride **12** afforded target compounds **3**, **4** or **5**, respectively.

The synthetic route for target compounds **6**–**8** is outlined in Scheme 2. Furanone compounds **6**–**8** were synthesized by the condensation of a phenylacetic acid analog and phenacyl bromide. The reaction of **13** with **15**, **14** with **17** or **14** with **16** in the presence of triethylamine afforded the phenacyl phenylacetate products **18**, **19** or **20**, respectively. Treatment of intermediates **18**–**20** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) provided 3,4-diphenyl-2(5*H*)furanone **21** or target compounds **7** or **8**. chlorosulfonylation of **21** by the reaction with chlorosulfonic acid followed by sulfonamidation using ammonium hydroxide gave target compound **6**.

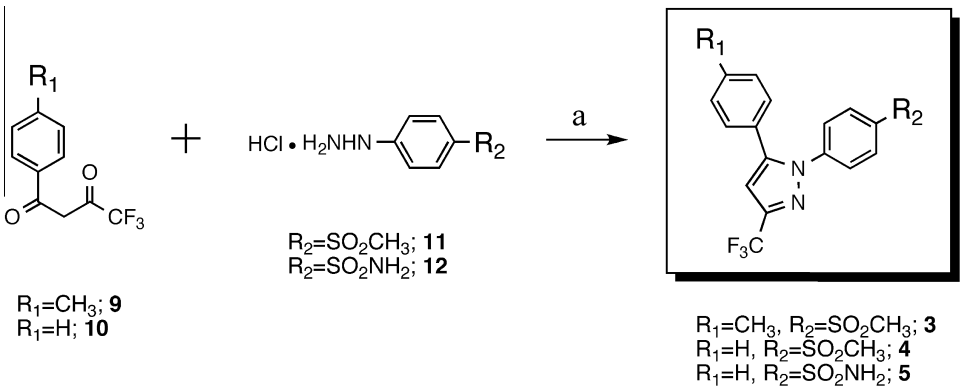
The final compounds were characterized by nuclear magnetic resonance (NMR), infrared spectroscopy (IR), high resolution mass spectra (HR-MS) and elemental analysis.

3. Results and discussion

The chemical structures of celecoxib and rofecoxib exhibit some similarities (Fig. 1) and can be divided into three parts (A–C in Table 1); part A, methylphenyl for celecoxib, phenyl for rofecoxib; part B, trifluoromethylpyrazole for celecoxib, furanone for rofecoxib; part C, sulfonamidophenyl for celecoxib, methanesulfonylphenyl for rofecoxib. Thus, in addition to celecoxib and rofecoxib, there are six possible combinations of these three parts that could be used to obtain hybrid compounds of celecoxib and rofecoxib (compounds **3**–**8** in Table 1). We synthesized these six compounds and tested their membrane permeabilizing and COX-inhibitory activities.

To begin with, we used calcein-loaded liposomes to compare the membrane permeabilizing activities of celecoxib and rofecoxib. As calcein fluorescence is very weak at high concentrations due to self-quenching, the addition of membrane-permeabilizing drugs to a medium containing calcein-loaded liposomes causes an increase in fluorescence by diluting the calcein.[25] As shown in Figure 2, celecoxib and rofecoxib increased the calcein fluorescence in a dose-dependent manner. Compared with celecoxib, however, a rofecoxib concentration about 100 times higher was required to increase the fluorescence by the same amount. Figure 2 shows that rofecoxib has a much lower membrane permeabilizing activity than celecoxib.

We next examined the membrane permeabilizing activities of the six hybrid compounds in a similar manner. As shown in Figure 3, all of the hybrid compounds increased the calcein fluorescence in a dose-dependent manner. To compare the membrane permeabilizing activity of these compounds, we used the EC_{50} (half-maximal effective concentration) index, which is defined as the concentration of each compound required for 50% of the calcein in loaded liposomes to be released (Table 2). Comparison of the EC_{50} index of **3**, **5** and **8** (compounds with one part substitution from celecoxib) showed that the membrane permeabilizing activity of **3** was much lower than that of **5** or **8**, suggesting that part

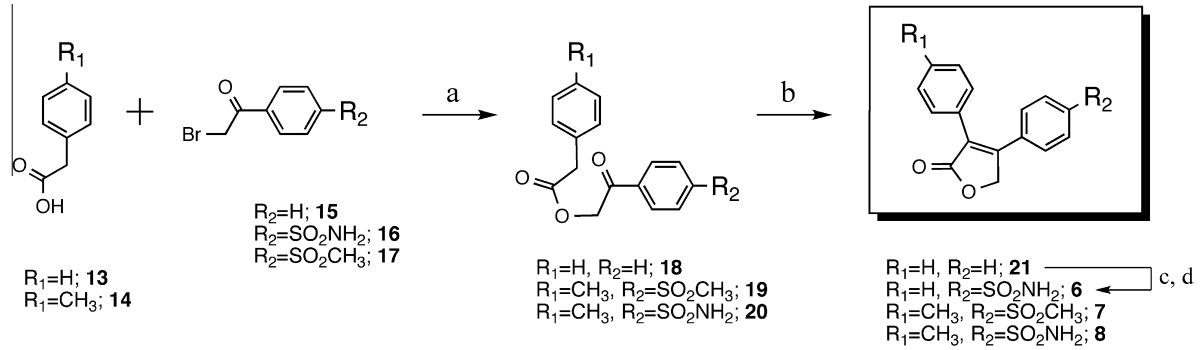


R_1=CH_3; **9**
R_1=H; **10**

R_2=SO_2CH_3; **11**
R_2=SO_2NH_2; **12**

R_1=CH_3, R_2=SO_2CH_3; **3**
R_1=H, R_2=SO_2CH_3; **4**
R_1=H, R_2=SO_2NH_2; **5**

Scheme 1. Synthesis of pyrazole compounds **3**–**5**.



Scheme 2. Synthesis of furanone compounds **6**–**8**.

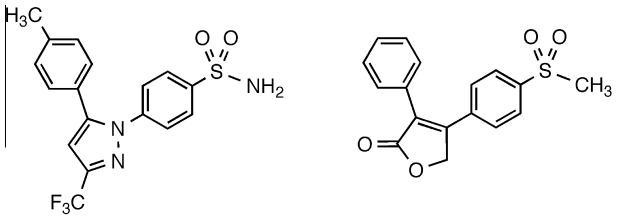
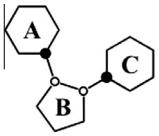
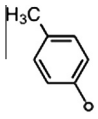
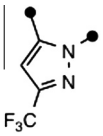
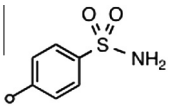
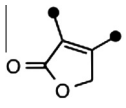
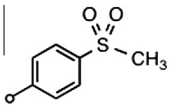
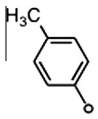
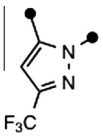
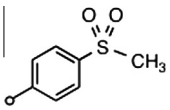
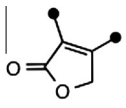
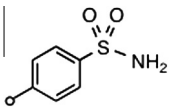
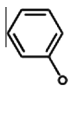
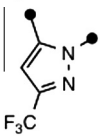
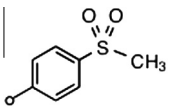
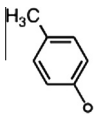
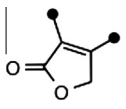
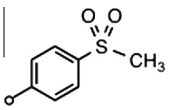
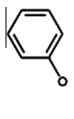
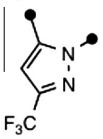
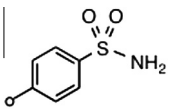
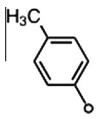
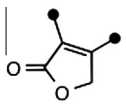
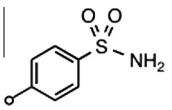


Figure 1. Structures of celecoxib and rofecoxib.

C of celecoxib (the sulfonamidophenyl subgroup) is the most important subgroup determining its high membrane permeabilizing activity. On the other hand, comparison of the EC_{50} index of **4**, **6** and **7** (compounds with one part substitution from rofecoxib) revealed that the membrane permeabilizing activity of **6** was higher than that of **4** or **7**. In this case, part C of rofecoxib (the methanesulfonylphenyl subgroup) was also seemed to be the most important subgroup determining its low membrane permeabilizing activity. Thus, part C seems to be important for determining the permeabilizing activity of these coxibs. The fundamental structural requirement underlying the ability of molecule to permeabilize membrane is a shape in which clusters of hydrophobic and hydrophilic parts are spatially organized with appropriate distance, because this structure enable the hydrophobic part to be located near the surface of membrane, resulting in perturbation of membrane structure. In the present case, because the sulfonamidophenyl subgroup is more hydrophilic than the methanesulfonylphenyl subgroup, the former but not the latter produces hydrophilic part. The methanesulfonylphenyl subgroup is hydrophobic enough to be totally buried within the lipid bilayer structure and therefore, does not affect the membrane structure drastically. On the other hand, the sulfonamidophenyl subgroup is relatively hydrophilic, which allows the compound to face membrane surface, resulting in perturbation of the membrane structure.

Table 1
Structures of celecoxib, rofecoxib and their hybrid compounds (**3**–**8**)



	A	B	C		A	B	C
Celecoxib				Rofecoxib			
3				**6**			
4				**7**			
5				**8**			

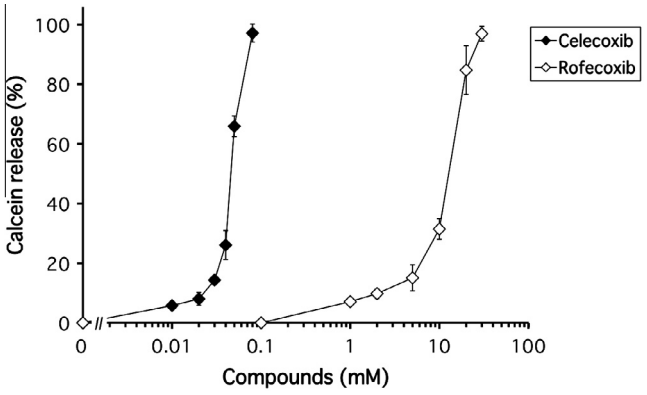


Figure 2. Membrane permeabilization by celecoxib and rofecoxib. Calcein-loaded liposomes were incubated for 10 min at 30 °C with the indicated concentration of each compound. The release of calcein from the liposomes was determined by measuring fluorescence intensity as described in the experimental section. Triton X-100 (10 μM) was used to establish the 100% level of calcein release. Values are mean ± SD (n = 3).

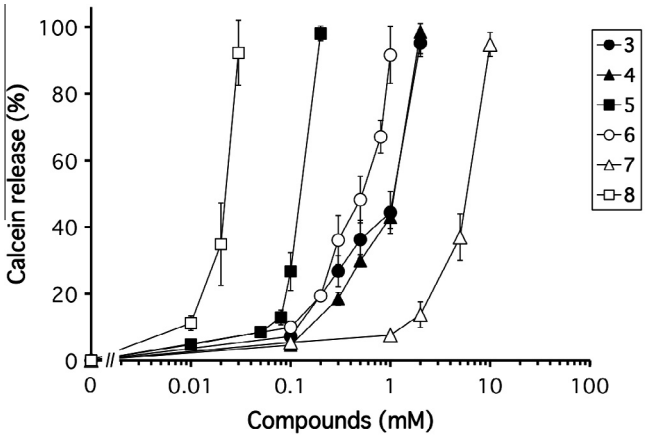


Figure 3. Membrane permeabilization by pyrazole compounds **3**–**5** and furanone compounds **6**–**8**. Experiments and data analysis were performed as described in the legend of Fig. 2. Values shown are mean ± SD (n = 3).

The results presented in Figures 2 and 3 thus provide important information for the design and synthesis of new coxibs with lower membrane permeabilizing activity.

The ionization of these compounds would not be related to their membrane permeabilizing activities. This is because pK_a values of all compounds (celecoxib, rofecoxib, **3**– **8**) are higher than 10 and membrane permeabilizing assay was performed under the conditions of pH = 6.8. Furthermore, such ionization would increase the osmotic pressure outside vesicles and thus, would not stimulate the release of calcein from vesicles.

The inhibitory effects on COX-1 and COX-2 of these compounds were compared by using the IC_{50} (half-maximal inhibitory concentration) index, which is defined as the concentration of each compound required for 50% inhibition of each enzyme. The IC_{50} values for COX-1 and COX-2 of celecoxib and rofecoxib were roughly similar to those reported previously,[32] and the IC_{50} values for COX-1 of all the hybrid compounds were relatively high (Table 2). With the exception of **4** and **6**, the IC_{50} values for COX-2 of the hybrid compounds were within the range seen for celecoxib and rofecoxib, and all the hybrid compounds (except 6) showed COX-2 selectivity (Table 2). Since both **4** and **6** are compounds with one part substitution from rofecoxib, the structure of rofecoxib rather than that of celecoxib appears to be sensitive to modification in relation to COX-2 inhibitory activity.

Table 2
Membrane permeabilizing activities and inhibitory activities on COX-1 and COX-2 of celecoxib, rofecoxib and their hybrid compounds (**3**–**8**)

Compounds	EC_{50} (mM)	IC_{50} (μM)		COX-1/COX-2	EC_{50}/IC_{50}
	Calcein release	COX-1	COX-2		
Celecoxib	0.050	117	0.07	1670	0.71
Rofecoxib	12.6	>500	0.36	>1380	35.0
3	1.44	>500	0.13	>3830	11.1
4	1.30	>500	6.84	>73	0.19
5	0.12	213	0.16	1330	0.75
6	0.48	>500	>500	—	—
7	6.23	>500	0.30	>1680	20.8
8	0.024	385	0.11	>3500	0.22

The EC_{50} value for membrane permeabilization (concentration of each compound required for 50% release of calcein) was calculated based on the data shown in Figures 2 and 3. The inhibitory effect of each compound on COX-1 and COX-2 was examined using purified ovine COX-1 and human recombinant COX-2 as described in the experimental section. The values of IC_{50} (concentration of each compound required for 50% inhibition) were estimated from the sigmoid-like dose-response curve (4-parameter logistic curve model) drawn using logistic-curve fitting software (ImageJ 1.43u; National Institutes of Health, USA), and the COX-1/COX-2 ratios of IC_{50} values were calculated. EC_{50} for membrane permeabilization/IC_{50} for COX-2 inhibition was shown. Values are mean (n = 3).

Among the six hybrid compounds, **4** and **6** can be eliminated as candidates for future clinical use based on their relatively weak inhibitory activity on COX-2 (Table 2). On the other hand, **5** and **8** can be eliminated as candidates based on their relatively potent membrane permeabilizing activity (Table 2). To further compare the potential value of these compounds, we calculated the value of the EC_{50} index for calcein release/IC_{50} index for COX-2 (Table 2). Compounds **3** and **7** appear as the most likely selections as candidates for future clinical use based on this index (Table 2). As described in the introduction section, rofecoxib was withdrawn from the market due to an observed increased potential risk for cardiovascular thrombotic events,[8,9] which may not be a drug class effect but actually something characteristic of rofecoxib alone. According to this hypothesis, it could be postulated that **3** and **7** might have fewer adverse effects associated with their use. Nevertheless, because the mechanism underlying the increased cardiovascular thrombotic events remains to be elucidated, the potential risk of these compounds has not yet been tested without a large-scale clinical study.

4. Conclusion

We here found that rofecoxib has very weak membrane permeabilizing activity compared with celecoxib. Furthermore, analysis of the membrane permeabilizing activities of hybrid compounds derived from celecoxib and rofecoxib suggested that the sulfonamidophenyl subgroup of celecoxib and the methanesulfonylphenyl subgroup of rofecoxib are important for their potent or weak membrane permeabilizing activity, respectively.

5. Experimental section

5.1. Chemistry

All solvents and reagents were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) or Wako Pure Chemical Industries (Tokyo, Japan) and used without further purification. Fourier transform IR spectra were recorded as films with NaCl plates on a JASCO FT/IR-480 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on VARIAN 400- or 500-MR spectrometer (Agilent Thechnologies Japan, Tokyo, Japan) operating at 400 MHz, in a ca. 2% solution of $CDCl_3$ or DMSO-d_6. Coupling constant (J) values are estimated in hertz (Hz) and spin multiples are given as s (singlet), d

(double), m (multiplet), and br (broad). Mass spectra were detected with an electrospray ionization time-of-flight (ESI-TOF) mass spectrometer (Bruker MicroTOF, Bruker, Bremen, Germany) in the negative mode. The progress of all reactions was monitored by thin-layer chromatography (TLC) with silica gel glass plates (60 F_{254}) (Merck Ltd, Tokyo, Japan), and spots were visualized with ultraviolet (UV) light (254 nm) and stained with 5% ethanolic phosphomolybdic acid. Column chromatography was performed using Silica gel 60 N (Kanto Chemical Co., Tokyo, Japan). Elemental analyses were performed for C, H and N (Central Service Research Center, Keio University) and were within ±0.4% of the theoretical values. Melting points (mp) were obtained using a Yanaco melting point apparatus MP-J3 (Yanaco, Kyoto, Japan) without correction. Celecoxib and rofecoxib were from LKT Laboratories Inc. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. (Tokyo, Japan).

5.1.1. General procedure for preparation of compounds 3–5

Phenylhydrazine hydrochloride (**11** or **12**) was added to a stirred solution of the dione (**9** or **10**) in ethanol (30 mL), and the mixture was refluxed for 20 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The resulting residue was dissolved in AcOEt (50 mL) and washed with brine. The organic fraction was dried over Na_2SO_4 and filtered. The filtrate was concentrated in vacuo and the residue was purified by silica gel chromatography (*n*-hexane/AcOEt, 2:1) to afford pyrazole compounds **3**–**5**.

5.1.2. 1-(4-Methanesulfonylphenyl)-5-*p*-tolyl-3-(trifluoromethyl)-1*H*-pyrazole (3)

Compound **3** was synthesized from **9** (500 mg, 2.2 mmol, 1.0 equiv) and **11** (774 mg, 3.5 mmol, 1.6 equiv). Colorless needle-like crystals (yield 35%); mp 125.1–126.1 °C; IR (film) *v*: 1160, 1325 (SO_2), 2930 (C-H), 3015 (Ar-H) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ: 2.36 (s, 3H, Ar-C*H*$_3$), 3.04 (s, 3H, SO_2CH_3), 6.72 (s, 1H, pyrazole-H4), 7.10 (d, *J* = 8.0, 2H, *p*-tolyl-H3, -H5), 7.16 (d, *J* = 8.0, 2H, *p*-tolyl-H2, -H6), 7.52 (d, *J* = 8.0, 2H, methanesulfonylphenyl-H2, -H6), 7.91 (d, *J* = 8.0, 2H, methanesulfonylphenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m*/*z*): 379.0706 ([M−H]$^-$, Calcd for $C_{18}H_{14}F_3N_2O_2S$: 379.0728). Anal. Calcd for $C_{18}H_{15}F_3N_2O_2S$: C, 56.84; H, 3.97; N, 7.36. Found: C, 56.64; H, 3.75; N, 7.20. IR and ^1H NMR spectral data for **3** were consistent with reported results.[33,34]

5.1.3. 1-(4-Methanesulfonylphenyl)-5-phenyl-3-(trifluoromethyl)-1*H*-pyrazole (4)

Compound **4** was synthesized from **10** (500 mg, 2.3 mmol, 1.0 equiv) and **11** (567 mg, 2.5 mmol, 1.6 equiv). Colorless needle-like crystals (yield 50%); mp 135.2–136.1 °C; IR (film) *v*: 1162, 1320 (SO_2), 2935 (C-H), 3020 (Ar-H) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ: 3.04 (s, 3H, SO_2CH_3), 6.77 (s, 1H, pyrazole-H4), 7.22–7.23 (m, 2H, phenyl-H2, -H6), 7.35–7.41 (m, 3H, phenyl-H3, -H4, -H5), 7.51 (d, *J* = 8.5, 2H, methanesulfonylphenyl-H2, -H6), 7.91 (d, *J* = 8.5, 2H, methanesulfonylphenyl-H3, -H5); ^{13}C NMR ($CDCl_3$, 500 MHz) δ: 44.28, 106.6, 125.5, 128.4, 128.7, 129.0, 129.5, 139.8, 143.2, 144.0, 144.3, 145.1; HR-ESI-TOF/MS (negative, *m*/*z*): 365.0550 ([M−H]$^-$, Calcd for $C_{17}H_{12}F_3N_2O_2S$: 365.0572). Anal. Calcd for $C_{17}H_{13}F_3N_2O_2S$: C, 55.73; H, 3.58; N, 7.65. Found: C, 55.58; H, 3.60; N, 7.44.

5.1.3.1. 1-(4-Sulfonamidophenyl)-5-phenyl-3-(trifluoromethyl)-1*H*-pyrazole (5).

Compound **5** was synthesized from **10** (500 mg, 2.3 mmol, 1.0 equiv) and **12** (560 mg, 3.5 mmol, 1.6 equiv). Colorless needle-like crystals (yield 66%); mp 164.1–165.2 °C; IR (film) *v*: 1165, 1325 (SO_2), 3025 (Ar-H), 3680 (N-H) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ: 4.92 (br s, 2H, N*H*$_2$), 6.75 (s, 1H, pyrazole-H4), 7.22–7.24 (m, 2H, phenyl-H2, -H6), 7.32–7.40 (m, 3H, phenyl-H3, -H4, -H5), 7.45 (d, *J* = 8.5, 2H, 4-sulfonamidophenyl-H2, -H6), 7.88 (d, *J* = 8.5, 2H, 4-sulfonamidophenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m*/*z*): 368.0622 ([M−H]$^-$, Calcd for $C_{16}H_{12}F_3N_2O_2S$: 368.0681). Anal. Calcd for $C_{16}H_{13}F_3N_2O_2S$: C, 52.17; H, 3.56; N, 11.41. Found: C, 52.12; H, 3.45; N, 11.28. ^1H NMR spectral data for **5** were consistent with reported results.[35,36]

5.1.4. General procedure for preparation of compounds 21, 7 and 8

To a stirred solution of phenylacetic acid (**13** or **14**) and triethylamine in dry CH_3CN, phenacyl bromide (**15**–**17**) in dry CH_3CN was added dropwise at room temperature. The reaction mixture was stirred for 1 h and was concentrated in vacuo. The resulting residue was re-dissolved in AcOEt (50 mL) and washed with 1 M HCl (20 mL). The organic fraction was dried over Na_2SO_4 and filtered. The filtrate was evaporated under reduced pressure to give crude product (**18**–**20**) that was used in the next step without further purification.

DBU (1.0 equiv) in dry CH_3CN (2 mL) was added dropwise to a stirred solution of the crude intermediate (**18**–**20**, 1.0 equiv) in dry CH_3CN (8 mL) at 0 °C. After stirring at 0 °C for 15 min, the mixture was poured into dilute HCl solution and the product was extracted with AcOEt. Evaporation of the solvent and purification of the residue by silica gel chromatography (n-hexane/AcOEt, 2:1) yielded the furanone compounds **21**, **7** or **8**.

5.1.4.1. 3-Phenyl-4-(4-sulfonamidophenyl)-2(5*H*)-furanone (6).

Compound **6** was prepared by chlorosulfonylation in chloroform and sulfonamide formation using ammonium hydroxide in ethanol of **21** that was obtained from phenylacetic acid (**13**, 0.5 g, 3.7 mmol, 1.0 equiv) and 4-sulfonamidophenacyl bromide (**15**, 1.03 g, 3.7 mmol, 1.0 equiv) via the intermediate **18** by the method described previously.[36] Colorless needle-like crystals (yield 22%, three steps); mp 248.2–249.5 °C; IR (film) *v*: 1145, 1320 (SO_2), 1740 (C=O), 3030 (Ar-H), 3230 (N-H) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ: 5.42 (s, 2H, C*H*$_2$), 7.35–7.53 (m, 5H, phenyl-H), 7.42 (br s, 2H, NH_2), 7.52 (d, *J* = 8.6, 2H, 4-sulfonamidophenyl-H2, -H6), 7.85 (d, *J* = 8.6, 2H, 4-sulfonamidophenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m*/*z*): 314.0495 ([M−H]$^-$, Calcd for $C_{16}H_{12}NO_4S$: 314.0487). Anal. Calcd for $C_{16}H_{13}NO_4S$: C, 60.94; H, 4.16; N, 4.44. Found: C, 61.01; H, 4.02; N, 4.30. IR and ^1H NMR spectral data for **6** were consistent with reported results.[37,38]

5.1.4.2. 3-(4-Methylphenyl)-4-(4-methanesulfonylphenyl)-2(5*H*)-furanone (7).

Compound **7** was synthesized via the intermediate **19** from 4-methylphenylacetic acid (**14**, 0.5 g, 3.7 mmol, 1.0 equiv) and 4-methanesulfonylphenacyl bromide (17, 1.03 g, 3.7 mmol, 1.0 equiv). Yellow needle-like crystals (yield 67%, two steps); mp 174.5–175.4 °C; IR (film) *v*: 1150, 1320 (SO_2), 1750 (C=O), 3040 (Ar-H), 2930 (C-H) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ: 2.38 (s, 3H, Ar-C*H*$_3$), 3.07 (s, 3H, SO_2CH_3), 5.17 (s, 2H, C*H*$_2$), 7.20 (d, *J* = 8.1, 2H, *p*-tolyl-H3, -H5), 7.28 (d, *J* = 8.2, 2H, *p*-tolyl-H2, -H6), 7.52 (dd, *J* = 6.8, 2.0, 2H, methanesulfonylphenyl-H2, -H6), 7.92 (dd, *J* = 6.8, 2.0, 2H, methanesulfonylphenyl-H3, -H5); HR-ESI-TOF/MS (negative, *m*/*z*): 327.0718 ([M−H]$^-$, Calcd for $C_{18}H_{15}O_4S$: 327.0691). Anal. Calcd for $C_{18}H_{16}O_4S$: C, 65.84; H, 4.91. Found: C, 66.12; H, 5.00. IR and ^1H NMR spectral data for **7** were consistent with reported results.[39]

5.1.4.3. 3-(4-Methylphenyl)-4-(4-sulfonamidophenyl)-2(5*H*)-furanone (8).

Compound **8** was synthesized via the intermediate **20** from 4-methylphenylacetic acid (14, 0.5 g, 3.7 mmol, 1.0 equiv) and 4-sulfonamidophenacyl bromide (16, 1.03 g, 3.7 mmol, 1.0 equiv). Yellow needle-like crystals (yield 67%, two steps); mp 218.5–219.2 °C; IR (film) *v*: 1140, 1325 (SO_2), 1735 (C=O), 3025 (Ar-H), 3220 (N-H), 2925 (C-H)cm^{-1}; ^1H NMR (DMSO-d_6,

400 MHz) δ: 2.32 (s, 3H, Ar-CH_3), 3.32 (s, 3H, SO_2CH_3), 5.36 (s, 2H, CH_2), 7.22 (d, J = 8.0, 2H, p-tolyl-H3, -H5), 7.24 (d, J = 8.0, 2H, p-tolyl-H2, -H6), 7.44 (brs, 2H, NH_2), 7.54 (d, J = 8.8, 2H, 4-sulfonamidophenyl-H2, -H6), 7.80 (d, J = 8.8, 2H, 4-sulfonamidophenyl-H3, -H5); HR-ESI-TOF/MS (negative, m/z): 328.0694 ([M−H]$^-$, Calcd for $C_{17}H_{14}NO_4S$: 328.0644). Anal. Calcd for $C_{17}H_{15}NO_4S$: C, 61.99; H, 4.59; N, 4.25. Found: C, 62.05; H, 4.68; N, 4.42.

5.2. Membrane permeability assay

Permeabilization of calcein-loaded liposomes was assayed as described previously,[25] with some modifications. Liposomes were prepared using the reversed-phase evaporation method. Egg phosphatidylcholine (PC) (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, dissolved in 1.5 mL of diethyl ether and added to 1 mL of 100 mM calcein-NaOH (pH 7.4). The mixture was then sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed and the resulting suspension of liposomes was centrifuged and washed twice with fresh buffer A (10 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4) (pH 6.8) containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 mL buffer A. A 30 μL aliquot of this suspension was diluted with buffer A to 20 mL and the diluted suspension was then incubated at 30 °C for 10 min in the presence of each compound. The release of calcein from liposomes was determined by measuring the fluorescence intensity at 520 nm (excitation at 490 nm). The EC_{50} value was estimated from non-linear regression plots with the average of triplicate experiments for each compound; Triton X-100 (10 μM) was used to establish the 100% level of calcein release.

5.3. COX-inhibition assay

The inhibitory effect of each compound on COX-1 and COX-2 activity was examined using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA), including purified ovine COX-1 and human recombinant COX-2 according to the manufacturer's procedures.

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Supplementary data

Supplementary data (^{1}H NMR spectra of final new compounds for **4** and **8**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.032. These data include MOL files and InChiKeys of the most important compounds described in this article.

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