Supporting information:

Propyphenazone-based Analogs as Prodrugs and Selective Cyclooxygenase-2 Inhibitors.

Mohamed F. Radwan[a-b], Kevin N. Dalby[c,*] and Tamer S. Kaoud[b-c,*]

[a]Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt
[b]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Kingdome of Saudi Arabia
[c]Division of Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712, United States

[*]Corresponding authors: Kevin N. Dalby or Tamer S. Kaoud

107 West Dean Keaton, BME, The University of Texas at Austin, TX 78712, USA. E mail: kinases@me.com or tkaood1@utexas.edu.

Supplementary Figures

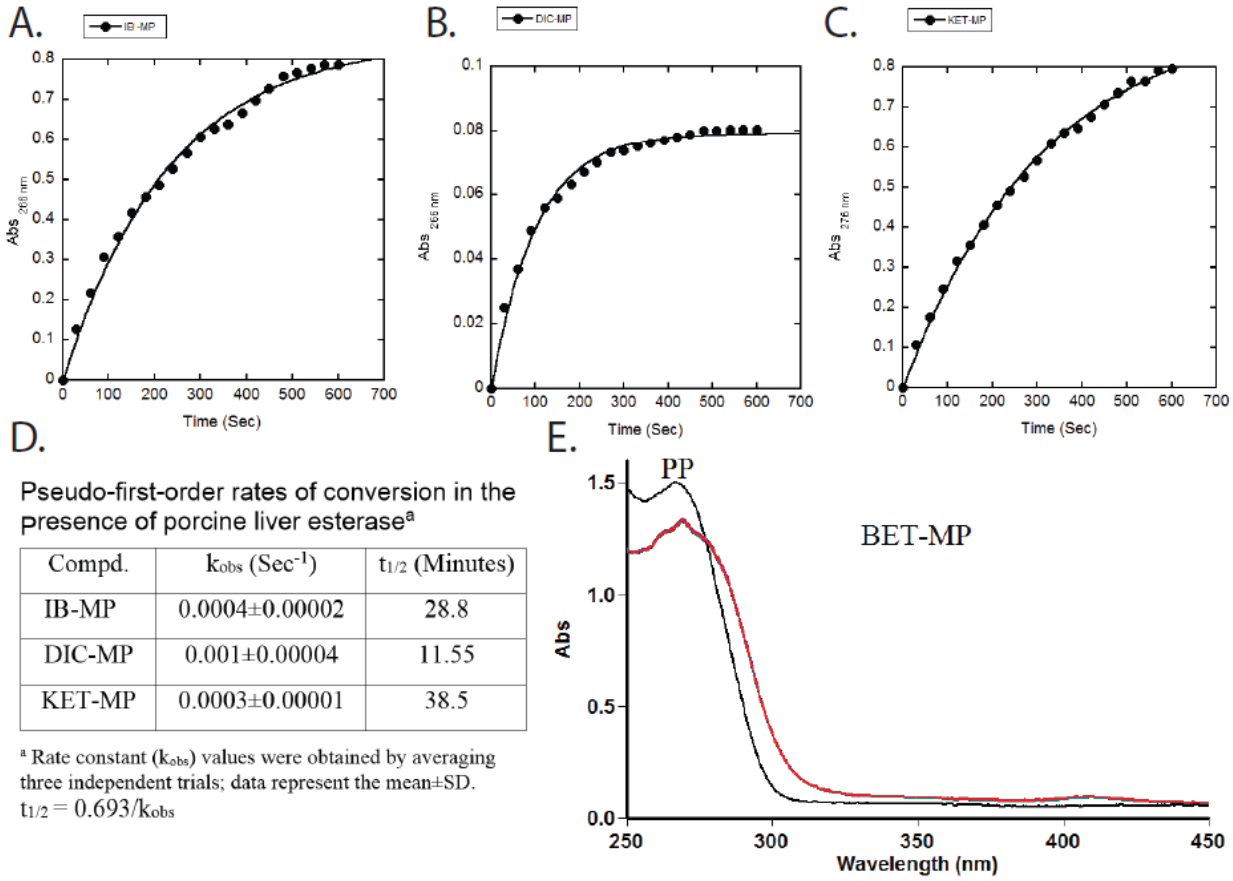

Figure S1: Pseudo-first-order kinetic measurements of the rates of conversion in the presence of porcine liver esterase (PLE) were performed using UV–Vis absorption spectroscopy for IB-MP (A), DIC-MP (B) and KET-MP (C). Pseudo-first-order rates of release of each prodrug in the presence of porcine liver esterase is calculated and shown in table (D). Absorption spectra of BET-MP (E) in the presence of porcine liver esterase (PLE) monitored every 4 minutes for 100 min. The red line represents initial absorbance spectrum before addition of PLE. One labeled line represents the absorbance of an authentic sample of propyphenazone (PP).

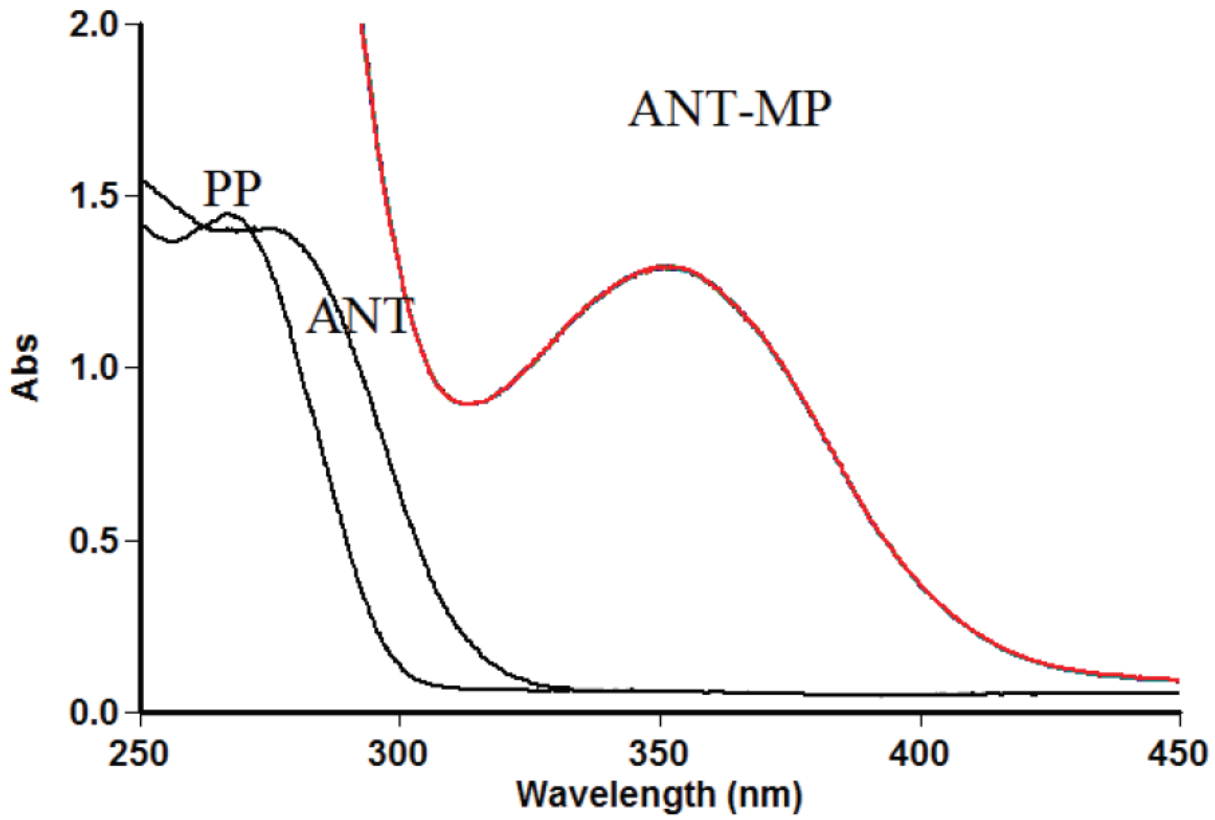

Figure S2. Absorption spectra of ANT-MP in the presence of porcine liver esterase (PLE) monitored every 4 minutes for 100 min. The red line represents initial absorbance spectrum before addition of PLE. Two labeled lines depict the absorption spectra of authentic samples of propyphenazone (PP) and 4-aminoantipyrine (ANT).

Experimental Section

Materials.

Melting points (m.p.) were determined on digital electro-thermal melting point apparatus (BI, Barnstead, UK) and were uncorrected. Follow up the reactions progress was came out using TLC technique utilizing silica gel F254 pre-coated plates 20 x 20 cm, layer thickness 0.2 mm (E. Merck, Darmstadt, Germany). The *in vitro* COX selectivity tests were carried on Perkin-Elmer (Waltham, MA) VICTOR3V 1420 multilabel counter plate reader (Excitation filter F540 and emission filter F572). COX fluorescent inhibitor screening assay kits were purchased from Cayman Chemical Company, Ann Arbor, MI, USA. Computer program Prism was used for studying the statistical analyses. Values of $p < 0.05$ or $p < 0.01$ (significant difference) were used as the limit for statistical significance.

Animals

Either male sprague dawely rats, weighing 200-250 g (for the analgesic assay) or male swiss albino mice, weighing 20-25 g (for the anti-inflammatory assay), were obtained from King Fahad Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. All animal treatments were approved by the local Biomedical Ethics Committee, Faculty of Pharmacy, King Abdulaziz University. Animals were acclimatized in the animal facility with controlled temperature (23 ± 2 ◦C), humidity (55 ± 10%), and photoperiod (12:12 h) for 7 days before experiments. A minimum of 4 animals was used in each group.

Statistical analysis

All data are expressed as means ± SE. Multiple group comparisons were carried out using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for post hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. All statistical analyses were

performed using GraphPad InStat software, version 3.05 (GraphPad Software, Inc., La Jolla, CA, USA).

Chemistry.

5-(bromomethyl)-4-isopropyl-1-methyl-2-phenyl-1H-pyrazol-3(2H)-one (BMP).

A solution of bromine solution (6.95 g, 44 mmol) in CH_2Cl_2 (5 ml) was added drop wise to an ice-cooled stirred solution of propyphenazone (10 g, 43.5 mmol) in a mixture of CH_2Cl_2: dioxane (4: 1, v/v) (25 ml) and the reaction mixture was left while stirring for 1 hour. Then 30 ml 10% cold aqueous sodium carbonate solution was added with vigorous shaking. The organic phase was separated and dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum at 40 °C until the volume was about 10 ml. The reaction mixture was cooled to room temperature, and about 15 ml diethyl ether was added. This solution was left to stand in stoppered flask at room temperature in dark. The colorless crystals formed were separated and washed with cold diethyl ether. (yield: 94%, m.p. 90 ^oC, reported yield 86%, m.p. 96 ^oC). *MS data* for $C_{14}H_{17}$ Br N_2O (309.2) m/z: M^+ 309 (100%); 311 (98%); MS2 of 309: 215 (100%); 230 (28%); 201 (9%); 267 (4%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.1 (d, 6H, -CH($\underline{CH_3}$)$_2$); 2.5-2.65 (m, 1H, -$\underline{CH}$(CH_3)$_2$); 3.2 (s, 3H, N-CH_3); 4.02 (s, 2H, -$\underline{CH_2}$-Br); 7.0-7.2 (m,1H, *p*-phenyl-H; 7.4-7.3 (m, 4H, *o*- & *m*-phenyl-H).

5-(Hydroxymethyl)-4-isopropyl-1-methyl-2-phenyl-1H-pyrazol-3(2H)-one (HPP).

Bromopropyphenazone (3.0 g, 9.7 mmol) was heated with water (100 ml) for 30 minutes and allowed to attain room temperature. The precipitated solid was filtered, washed, and recrystallized from dried diethyl ether (yield: 96%, m.p. 125 ^oC, reported yield, m.p. 00 ^oC). M+H (247), *MS data for* $C_{14}H_{18}$ N_2 O_2. (246.3) m/z: 217 (100%), 175 (100%), 205 (41%), 187 (23%), 159 (19%), 229 (7%). ^{1}H- NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.1 (d, 6H, -CH($\underline{CH_3}$)$_2$),

2.5-2.6 (m, 1H, -CH$(CH_3)_2$), 3.2 (s, 3H, N-CH_3), 3.2 (b, 1H, -OH), 4.3 (s, 2H, -CH_2-OH), 7.0-7.1 (m,1H, p-phenyl-H), 7.3-7.4 (m, 4H, o- & m-phenyl-H).

(4-isopropyl-2-methyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-3-yl)methyl 2-(4-isobutylphenyl)propanoate (IB-MP).

A mixture of bromoprpyphenazone (0.50 g, 1.6 mmol), ibuprofen (0.33 g, 1.6 mmol) and anhydrous K_2CO_3 (1.0 g) in acetone (15 ml) was heated under refluxed with stirring for 1 h. After being cooled to room temperature, the reaction mixture was evaporated under vacuum at 30 ºC, and the remaining residue was treated with CH_2Cl_2 (50 ml). The organic layer was separated, washed with H_2O, dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum at 35^{o}C. The remained pale yellow viscous mass was further dried in a vacuum dessicator (over anhydrous $CaCl_2$) (yield, 94%).^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.87 (d, 6H, -CH_2-CH$(CH_3)_2$), 1.27 (d, 6H, -C-CH$(CH_3)_2$, 1.55 (d, 3H, -CH-CH_3), 1.8-1.9 (m, 1H, -CH_2-CH$(CH_3)_2$), 2.45 (d, 2H, -CH_2-CH$(CH_3)_2$), 2.78 (s, 3H, N-CH_3), 2.87-2.95 (m, 1H, -C-CH$(CH_3)_2$), 5.6 (d, 2H, O-CH_2-C), 7.11 (d, 2H, phenyl-H), 7.21 (d, 1H, phenyl-H), 7.26-7.29 (m, 2H, phenyl-H), 7.42-7.44 (m, 4H, phenyl-H). ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 15.1 (CH_3), 20.9, 20.9 (CH_3), 22.1, 22.1 (CH_3), 29.9 (CH), 36.1 (CH), 44.8 (CH_3), 55.0 (CH), 44.5 (CH_2), 65.7 (CH_2), 119.3, 147.6 (pyrazolone C), 123.4, 126.1, 126.9, 126.9, 128.7, 128.7 (Ar C), 126.1, 129.2, 129.2, 136.4, 136.4, 140.2 (Ar C), 164.1 (CO), 173.7 (CO). MS (ESI) m/z $C_{27}H_{34}N_2O_3$ calculated M+1: 435.57 *found* 435.951

[(4-isopropyl-2-methyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-3-yl)methyl 2-(2-((2,6-dichlorophenyl)amino)phenyl)acetate](DIC-MP).

A mixture of bromoprpyphenazone (0.50 g, 1.6 mmol), diclofenac (0.48 g, 1.6 mmol) and

anhydrous K_2CO_3 (1.0 g) in acetone (15 ml) was heated under refluxed with stirring for 1 h. Working up the reaction mixture was carried out as described under compound **3**. The yellowish brown solid was further dried in vacuum desiccator over $CaCl_2$, overnight, in dark (m. p. 104 ^oC, yield, 89%).^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.33 (d, 6H, -CH($\underline{CH_3}$)$_2$), 1.66 (d, 1H, -$\underline{CH}$(CH_3)$_2$), 2.90-2.93 (m, 1H, -C-$\underline{CH}$(CH_3)$_2$), 2.91 (s, 3H, N-$\underline{CH_3}$), 3.81 (s, 2H, -CO-$\underline{CH_2}$-C), 4.59 (s, 1H, C-$\underline{NH}$-C), 5.14 (s, 2H, -C-$\underline{CH_2}$-O), 6.59 (d, 1H, phenyl-H), 6.99 (d, 1H, phenyl-H), 7.01-7.04 (m, 2H, phenyl-H), 7.27-7.29 (m, 4H, phenyl-H), 7.42-7.47 (m, 4H, phenyl-H). ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 21.0, 21.0 (CH_3), 24.3 (CH), 36.3 (CH_3), 38.1 (CH_2), 55.6 (CH_2), 118.7, 147.1 (pyrazolone C), 118.7, 122.1, 122.1, 128.6, 128.6, 129.3 (Ar C), 123.1, 128.8, 128.8, 130.7, 137.4, 137.4 (Ar C), 122.1, 123.7, 123.7, 128.8, 128.8, 134.3 (Ar C), 164.0 (CO), 171.3 (CO). MS (ESI) m/z $C_{28}H_{27}Cl_2N_3O_3$ calculated M+1 : 524.44 found 524.654.

[(4-isopropyl-2-methyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-3-yl)methyl 2-(4-benzoylphenyl)propanoate](KET-MP).

A mixture of bromoprpyphenazone (0.50 g, 1.6 mmol), ketoprofen (0.41 g, 1.6 mmol) and anhydrous K_2CO_3 (1.0 g) in acetone (15 ml) was heated under refluxed with stirring for 1 h. Working up the reaction mixture was carried out as described under compound **3**. The yellow viscous mass was further dried in vacuum desiccator over $CaCl_2$, overnight, in dark (yield, 86%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.26 (d, 6H, -CH($\underline{CH_3}$)$_2$), 1.62 (d, 3H, CH-$\underline{CH_3}$), 2.692.71 (m, 1H, -$\underline{CH}$(CH_3)$_2$), 2.87 (s, 3H, N-$\underline{CH_3}$), 3.9-3.92 (m, 1H, $\underline{CH}$-CH_3), 5.05 (s, 2H, C-$\underline{CH_2}$-O), 7.27-7.29 (m, 2H, phenyl-Hs), 7.34-7.35 (m, 2H, phenyl-Hs), 7.42-7.51 (ddd, 5H, phenyl-Hs), 7.56-7.62 (dd, 2H, phenyl-Hs), 7.70 (d, 1H, phenyl-H), 7.79 (d, 1H, phenyl-H). ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 17.9 (CH_3), 20.9, 20.9 (CH_3), 24.2 (CH), 36.3 (CH_3), 45.0 (CH),

65.6 (CH_2), 119.6, 137.9 (pyrazolone C), 123.4, 126.2, 126.2, 128.2, 128.2, 134.3 (Ar C 128.2, 128.4, 128.4, 132.4, 134.3, 134.3 (Ar C), 128.4, 128.4, 130.3, 130.3, 132.4, 138.4 (Ar C),), 164.0 (CO), 172.7 (CO), 196.0 (CO). MS (ESI) m/z $C_{30}H_{30}N_2O_4$ calculated M+1 : 483.2 found 483.600

[3-((Methyl(2-(pyridine-2-yl)ethylamino)methyl)-2-methyl-4-isopropyl-1-phenyl-3-pyrazolin-5-one] (BET-MP).

A mixture of bromoprpyphenazone (0.50 g, 1.6 mmol), betahistine (0.22 g, 1.6 mmol) and anhydrous K_2CO_3 (1.0 g) in acetone (15 ml) was heated under refluxed with stirring for 1 h. Working up the reaction mixture was carried out as described under compound **3**. The pale brown viscous mass was dried in vacuum desiccator over $CaCl_2$, overnight, in dark (yield, 92%). ^{1}H-NMR (CD_3OD, 400 MHz) δ (ppm): 1.28 (d, 6H, -CH($\underline{CH_3}$)$_2$), 2.36 (s, 3H,s, N-$\underline{CH_3}$), 2.44-2.46 (m, 1H, -$\underline{CH}$(CH_3)$_2$), 2.81 (s, 2H, -$\underline{CH_2}$-), 2.86 (s, 4H, 2-$\underline{CH_2}$-), 3.5 (s, 3H, N-$\underline{CH_3}$), 7.10-7.13 (m, 1H, phenyl-H), 7.22-7.40 (m, 2H, pyridine-H), 7.48-7.50 (m, 4H, phenyl-H), 7.55-7.60 (m, 1H, pyridine-H), 7.61-7.63 (s, 1H, pyridine-H). ^{13}C-NMR (CD_3OD, 600 MHz) δ (ppm): 21.3, 21.3 (CH_3), 33.7 (CH), 35.4 (CH_3), 40.4 (CH_3), 53.9 (CH_2), 55.6 (CH_2), 57.6 (CH_2), 115.8, 134.9 (pyrazolone C), 123.3, 126.9, 143.5, 148.6, 153.6 (pyridine C), 126.8, 127.9, 127.9, 130.2, 130.2, 133.8 (Ar C) 164.4 (CO). MS (ESI) m/z $C_{22}H_{28}N_4O$ calculated M+1: 364.48 found 364.513.

[5-(((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)amino)methyl)-4-isopropyl-1-methyl-2-phenyl-1H-pyrazol-3(2H)-one](ANT-MP).

A mixture of bromoprpyphenazone (0.50 g, 1.6 mmol), 4-aminoantipyrine (0.32 g, 1.6 mmol) and anhydrous K_2CO_3 (1.0 g) in acetone (15 ml) was heated under refluxed with stirring for 1 h. Working up the reaction mixture was carried out as described under compound **3**. The brown viscous mass was further dried in vacuum desiccator over $CaCl_2$, overnight, in dark (yield, 92%).

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.37 (d, 6h, -CH($\underline{CH_3}$)$_2$), 2.0 (s, 3H, C-$\underline{CH_3}$), 2.88-2.90 (m, 1H, -$\underline{CH}$-(CH_3)), 2.94 (s, 6H, 2 -N-$\underline{CH_3}$), 4.36 (s, 2H, -$\underline{CH_2}$-), 7.27-7.33 (m, 2H, 2 phenyl *p*-CH), 7.46-7.51 (m, 8H, 2 phenyl-H). ^{13}C-NMR ($CDCl_3$, 500 MHz) δ (ppm): 10.2 (CH_3), 21.1, 21.1 (CH_3), 24.2 (CH), 35.5 (CH_3), 36.4 (CH_3), 46.7 (CH_2), 117.0, 134.4 (pyrazolone C), 117.7, 133.9 (pyrazolone C), 119.3, 123.0, 123.0, 128.7, 128.7, 134.7 (Ar C), 123.0, 123.3, 123.3, 126.4, 126.4, 133.9 (Ar C), 162.8 (CO), 164.4 (CO). MS (ESI) m/z $C_{25}H_{29}N_5O_2$ calculated M+1: 431.23 found 432.050

Analgesic activity

The analgesic activity of the newly synthesized compounds was evaluated using the acetic acid induced abdominal constriction method (Writhing effect).[1, 2] All doses were calculated according to the method described previously by Paget et al.[3] Rats were divided into 12 groups as shown in tables 3 and 4. They were food deprived for 12 hours before the experiment. The drugs were suspended in carboxymethylcelluloe (0.5%, CMC) as previously reported[2] and administered orally 1 hour before the intraperitoneal administration of acetic acid (10 ml/kg) that freshly prepared with 1% concentration. After 3 minutes of acetic acid administration, the writhes number has been recorded for each animal during 20 minutes period. The average number of writhes for each group treated with a drug has been compared to the control group. Percentage protection from writhes has been calculated for each group to express the degree of analgesia that caused by each drug.

Anti-inflammatory activity

The potential anti-inflammatory activity of the tested compounds was evaluated using carrageenan-induced paw edema model as described previously by Winter et al.[4, 5] Each mouse was injected into the subplantar tissue of the right hind paw with a freshly prepared suspension

of carrageenan (0.5 mg/25 μL) in saline, 30 minutes after the subcutaneous administration of each drug (50 mg/kg body weight) or dosing vehicle. Swelling (volume) of the injected paw of each animal was measured at 1, 2, 3 and 4 hours by a mercury plethysmometer (Model 520, IITC, Life Sciences, USA).

Inhibition of purified COX-1 and COX-2 activity

Purified COX-1 (ovine) or COX-2 (human recombinant) was incubated with different concentrations of each compound and the enzyme activity was assayed *in vitro* using a fluorescent inhibitor screening assay kit (catalog number 700100, Cayman Chemical, Ann Arbor, MI, USA) following the procedure suggested by the manufacturer. Briefly, in 96-well plate, 10 μL of various concentrations of each tested compound (to produce final concentrations of 0 - 160 μM in 5% DMSO), either pre-treated for 1 hour at room temperature with 3.00 U of porcine liver esterase or not, were added to 150 μL of the assay buffer (100 mM Tris-HCl, pH 8.0) that mixed with either COX-1 or COX-2 (10 μL) enzyme in the presence of Heme (10μL) and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) (10 μL) as the fluorometric substrate. After incubation for 20 minutes at room temperature, the reactions were initiated by addition of 10 μL arachidonic acid solution and then incubated for 2 min at room temperature. Fluorescence of resorufin that is produced by the reaction between PGG2 and the fluorometric substrate, were analyzed. DuP-697 (selective COX-2 inhibitor) and SC-560 (selective COX-1 inhibitor) were used as reference compounds. The measured fluorescence intensity is proportional to the amount of produced resorufin, which is proportional to the amount of PGG2 present in each well during the reaction. Percent inhibition was calculated by comparison to the 100% maximum activity in absence of any inhibitor. IC50 (μM) which corresponds to the concentration of the inhibitor that causes 50% inhibition of COX-1 or COX-2 activity was calculated from the dose response curve of inhibition

(triplicate determinations). Dose-response curves for data conforming to inhibition were fitted to:

$$V_0 = V' - \left(V' \frac{i}{i + (IC_{50})} \right) + V_\infty$$

Where; V_0 is the observed rate; i is the concentration of inhibitor I; V' is the observed rate in the absence of inhibitor; V_∞ is the observed rate constant at saturating inhibitor, I; *IC50* is the concentration that leads to half the maximal change in V_0.

UV-Vis spectroscopy

Following Perez et al protocol,[6] 1 ml of 50 µM solution of each compound in 25 mM HEPES buffer and 5% DMSO (pH 7.5) was treated with 2.00 U of porcine liver esterase and absorption spectra were collected on a PerkinElmer Lambda 25 UV-vis spectrophotometer every 4 minutes over 100 minutes at room temperature. The change in absorption was recorded at λ_{max} of 266 nm for IB-MB, 266 nm for DIC-MP and 276 nm for KET-MP and termed Amax, The pseudo-first-order rate constant (k_{obs}) and the half life for each prodrug hydrolysis by esterase were calculated.

Molecular modeling

The docking study was performed using Surflex docking program impeded in SYBYL-X v.20 molecular modeling package (Sybyl-X).[7] The crystal structure of COX II used in the study (Pdb Code: 3OLU)[8] was selected based on certain criteria to get the most suitable conditions for Surflex to predict the binding model that is closest to reality. Indeed, Surflex binding predictability is affected by the ligand of the crystal structure without ignoring features of the active site as determined by Surflex probes. The COX II crystal structure that employed in this study contains glyceryl ester of arachidonic acid (AG), a non-acidic (ester) ligand in which Arg-120 interacts with 2'-hydroxy group of the glyceryl moiety *via* a hydrogen bond but not as ionic salt bridge as in case of the free acid.[9] It is analogous to our compound which is devoid of

carboxylic acid groups. The second advantage of having AG as a ligand, is its long hydrophobic tail that composed of 20 carbon atoms. The end of this hydrocarbon chain (the ω carbon) reaches the bottom of the active site which is characterized by strong hydrophobic environment. This is advantageous to our docking study because the modeled compound is significantly bulkier and its structure is characterized by having two hydrophobic terminals on both sides of the compound.

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