MOLECULAR BASIS FOR CYCLOOXYGENASE INHIBITION BY THE NON-STEROIDAL ANTI-FLAMMATORY DRUG, NAPROXEN

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Running Title: Determinants of COX Inhibition by Naproxen

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Supplemental Information

Suppl. A) Detailed Synthesis and Characterization of naproxen analogs

2-(6-methoxynaphthalen-2-yl)butanoic acid. To a flask charged with 10 mL of THF and purged with argon was added magnesium ribbon (242 mg, 10 mmol) and a crystal of iodine. To this solution was added 2-bromo-6-methoxynapthalene (2.0 g, 8.4 mmol) in 10 mL of THF. An off brown color appeared, and the reaction was allowed to reflux for 1 hour. The reaction was then allowed to cool, and methyl 2-bromobutyrate (3.0 g, 1.9 mL, 16.8 mmol) was added. Following 2 hours of reflux, the reaction was quenched with 1M HCl, then extracted with ethyl ether (3x), washed with brine, dried with $MgSO_4$ and concentrated under reduced pressure. The crude mixture was suspended in 10 mL of a 3M KOH solution in MeOH, held at reflux for 2 hours, cooled, and then quenched with water. The reaction mixture was extracted with ethyl ether (3x). The aqueous layer was then acidified with 1 M HCl, extracted into ethyl ether (3x), washed with brine, dried with $MgSO_4$, and concentrated under reduced pressure to give crude product. The product was purified using HPLC on a C18 column (1.1 g, 54%) to give pure racemic product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (t, 3H), 2.17 (q, 2H), 3.59 (t, 1H), 3.91 (s, 3H), 7.11 (d, J = 4.7 Hz, 1H), 7.15 (d, J = 2.4 Hz, 1H), 7.41 (dd, J = 1.5, 8.4 Hz, 1H), 7.68 (s, 2H), 7.71 (s, 1H); MS *m/z*: 243 $(M - H)^+$.

***(S)*-methyl 2-(6-hydroxynaphthalen-2-yl)propanoate.** To (*S*)-naproxen (3 g, 13 mmol) in acetic acid (20 mL) was added 48% HBr (11.2 g, 7.5 mL, 138 mmol) at 0 °C (1). Following 3 h of reflux, water (40 mL) was added to precipitate out the product which was isolated by filtration. To the crude product was added methanol (35 mL) and TMSCl (1.7 g, 2.0 mL, 15.7 mmol), and the mixture was stirred at room temperature for 2 hours (2). The solvent was removed *in vacuo* resulting in a tan solid (2.8 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.59 (d, 3H), 3.70 (s, 3H), 3.89 (q, 1H), 6.85 (s, 1H), 7.06 (d, J = 2.5, 1H), 7.08 (s, 1H), 7.37 (dd, J = 1.8, 8.5 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.65 (m, 2H): MS *m/z*: 231 $(M + H)^+$.

(*S*)-methyl 2-(6-ethoxynaphthalen-2-yl)propanoate. Potassium hydroxide (920 mg, 16.4 mmol) in 5 mL of methanol was added to a round bottom flask. (*S*)-methyl 2-(6-hydroxynaphthalen-2-yl)propanoate (2.5 g, 10.9 mmol) in DMF (30 mL) was then added to the flask. The reaction was then allowed to stir at room temperature for 30 minutes. Iodoethane (3.4 g, 1.7 mL, 21.8 mmol) was added which was allowed to stir at room temperature for 3 hours. The reaction mixture was quenched with water and extracted with CH_2Cl_2 (3x), the organic layer was then dried over $MgSO_4$, filtered, and finally concentrated *in vacuo*. Purification using 3:1 hexanes/ethyl acetate afforded the desired product as a yellow solid (1.5 g, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (t, 3H), 1.55 (d, 3H), 3.62 (s, 3H), 3.82 (q, 1H), 4.06 (q, 2H), 7.06 (d,

J = 2.5, 1H), 7.08 (s, 1H), 7.37 (dd, *J* = 1.8, 8.5 Hz, 1H), 7.63 (m, 2H), 7.66 (d, *J* = 1.8, 1H); MS *m/z*: 259 $(M + H)^+$.

2-(6-ethoxynaphthalen-2-yl)propanoic acid. To (*S*)-methyl 2-(6-ethoxynaphthalen-2-yl)propanoate (500 mg, 1.9 mmol) was added 10 mL of a 3M KOH solution in MeOH. The reaction was held at reflux for 2 hours, then cooled and quenched with water. The reaction mixture was extracted with ethyl ether (3x). The aqueous layer was then acidified with 1 M HCl and then extracted into ethyl ether (3x), washed with brine, dried with $MgSO_4$, and concentrated under reduced pressure to give pure racemic product as an off-white solid (400 mg, 86 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (t, 3H), 1.58 (d, 3H), 3.84 (q, 1H), 4.11 (q, 2H), 7.06 (d, *J* = 2.5, 1H), 7.08 (s, 1H), 7.37 (dd, *J* = 1.8, 8.5 Hz, 1H), 7.63 (m, 2H), 7.66 (d, *J* = 1.8, 1H); MS *m/z*: 243 $(M - H)^+$.

(*S*)-methyl 2-(6-(trifluoromethylsulfonyloxy)naphthalen-2-yl)propanoate.
To **(***S*)-naproxen (3 g, 13 mmol) in acetic acid (20 mL) was added 48% HBr (11.2 g, 7.5 mL, 138 mmol) at 0 ^{o}C.(1) Following 3 h of reflux, water (40 mL) was added to precipitate out the product which was isolated by filtration. To the crude product was added methanol (35 mL) and TMSCl (1.7 g, 2.0 mL, 15.7 mmol), and the mixture was stirred at room temperature for 2 hours (2). The solvent was removed *in vacuo* resulting in a tan solid, which was dissolved in CH_2Cl_2 (20mL). Following the addition of triethylamine (2.6 g, 3.6 mL, 26.1 mmol) at 0 ^{o}C, trifluoromethanesulfonic anhydride (2.8 g, 2.7 mL, 15.7 mmol) was added dropwise, and the mixture was warmed to room temperature and allowed to stir for 1 hour. The mixture was then diluted with diethyl ether, quenched with 1 M HCl, and washed with saturated sodium bicarbonate and brine. The organic layer was dried over $MgSO_4$ and then concentrated, resulting in the desired product (4.5 g, 95 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.57 (d, 3H), 3.65 (s, 3H), 3.89 (q, 1H), 7.34 (dd, *J* = 4, 8 Hz, 1H), 7.52 (dd, *J* = 4, 8 Hz, 1H), 7.70 (d, *J* = 4 Hz, 1H), 7.77 (s, 1H), 7.81 (d, *J* = 8 Hz, 1H), 7.86 (d, *J* = 8 Hz, 1H); MS *m/z*: 363 $(M + H)^+$.

(*S*)-methyl 2-(6-vinylnaphthalen-2-yl)propanoate. (*S*)-methyl 2-(6-(trifluoromethyl sulfonyloxy)naphthalen-2-yl)propanoate (3 g, 8.3 mmol) and potassium vinyltrifluoroborate (1.7 g, 12.5 mmol) were added to EtOH (75 mL) along with 1 M of cesium carbonate (15 mL). To the mixture was added tetrakis(triphenylphosphine) palladium (480 mg, 0.42 mmol) and triethylamine (1.7 g, 2.3 mL, 17 mmol) and the mixture was heated to 50 ^{o}C for 16 hours. The reaction was cooled, water (75 mL) was added and the resulting mixture was stirred at room temperature for 30 minutes. The reaction mixture was filtered resulting in a brown solid (1.8 g, 90 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.59 (d, 3H), 3.67 (s, 3H), 3.88 (q, 1H), 5.33 (d, *J* = 12 Hz, 1H), 5.86 (d, *J* = 12 Hz, 1H), 6.87 (q, *J* = 4, 12 Hz, 1 H), 7.42 (dd, *J* = 4, 8 Hz, 1H), 7.63 (dd, *J* = 4, 8 Hz, 1H), 7.71 (d, *J* = 12 Hz, 1H), 7.77 (m, 2H); MS *m/z*: 241 $(M + H)^+$.

(*S*)-methyl 2-(6-ethylnaphthalen-2-yl)propanoate. To a round bottom flask purged with argon was added THF (25 mL), palladium(II) acetate (37 mg, 0.17 mmol), and tri-*tert*-butyl phosphine (67 mg, 81 mL, 0.33 mmol). This was brought to reflux and allowed to stir for 30 minutes. The reaction was then cooled and (*S*)-methyl 2-(6-vinyl naphthalen-2-yl)propanoate (1.5 g, 6.2 mmol) and formic acid (1.9 g, 1.6 mL, 42 mmol) were added to the reaction mixture. The mixture was again brought to reflux for 30 minutes, then cooled to room temperature, and allowed to stir for 12 hours. The reaction mixture was filtered through a bed of Celite and then concentrated *in vacuo*. The crude residue was purified using flash chromatography using 10:1 hexanes:ethyl acetate resulting in pure product (750 mg, 50 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (t, 3H), 1.58 (d, 3H), 2.79 (q, 2H), 3.65 (s, 3H), 3.90 (q, 1H), 7.32 (dd, *J* = 4, 8 Hz, 1H), 7.40 (dd, *J* = 4, 8 Hz, 1H), 7.60 (s, 1H), 7.75 (m, 3H); MS *m/z*: 243 $(M + H)^+$.

2-(6-ethylnaphthalen-2-yl)propanoic acid. To (*S*)-methyl 2-(6-ethylnaphthalen-2-yl)propanoate (500 mg, 2.1 mmol) was added 10 mL of a 3M KOH solution in MeOH. The reaction was held at reflux for 2 hours, then cooled and quenched with water. The reaction mixture was extracted with ethyl ether (3x).

The aqueous layer was then acidified with 1 M HCl and then extracted into ethyl ether (3x), washed with brine, dried with $MgSO_4$, and concentrated under reduced pressure to give pure racemic product (450 mg, 94 %). The enantiomers were separated by chiral HPLC using a Chiralpak AD column (35°C) which was eluted with 90% hexanes/0.05% TFA (A) and 10% ethanol/0.05% TFA (B). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (t, 3H), 1.59 (d, 3H), 2.80 (q, 2H), 3.89 (q, 1H), 7.34 (dd, *J* = 4, 8 Hz, 1H), 7.41 (dd, *J* = 4, 8 Hz, 1H), 7.59 (s, 1H), 7.72 (s, 2H), 7.75 (d, *J* = 8 Hz); MS *m/z*: 227 $(M - H)^+$.

(*S*)-methyl 2-(6-(triisopropylsilylthio)naphthalen-2-yl)propanoate. To a solution of (*S*)-methyl 2-(6-(trifluoromethylsulfonyloxy)naphthalen-2-yl)propanoate (3 g, 8.3 mmol) in benzene (20 mL) was added $Pd(Ph_3)_4$ (0.95 g, 0.8 mmol) and sodium triisopropylsilanethiolate (3) (2.1 g, 9.9 mmol) dissolved in THF (10 mL). The solution was refluxed for four hours, then quenched with water and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography using 10:1 hexane:ethyl acetate to give pure product (2.9 g, 87 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.10 (d, 18 H), 1.25 (m, 3H), 3.65 (s, 3H), 3.86 (q, 1H), 7.31 (m, 2H), 7.41 (dd, *J* = 4, 8 Hz, 1H), 7.54 (dd, *J* = 4, 8 Hz, 1H), 7.67 (m, 2H); MS *m/z*: 403 $(M + H)^+$

(*S*)-methyl 2-(6-(methylthio)naphthalen-2-yl)propanoate. To 15 mL of THF was added (*S*)-methyl 2-(6-(triisopropylsilylthio)naphthalen-2-yl)propanoate (2.5 g, 6.2 mmol) followed by tetrabutylammonium fluoride (3.3 g, 3.6 mL, 12.4 mmol). The mixture was allowed to stir at room temperature for 2 hours. Methyl iodide (2.6 g, 1.2 mL, 18.6 mmol) was then added, and the resulting mixture was stirred for an additional 2 hours at room temperature. The reaction mixture was extracted with ethyl ether (3x), dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by flash chromatography using 7:1 hexane:ethyl acetate to give pure product (1.2 g, 75 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ; 1.58 (d, 3H), 2.57 (s, 3H), 3.67 (s, 3H), 3.87 (q, 1H), 7.37 (dd, *J* = 4, 8 Hz, 1H), 7.42 (dd, *J* = 4, 8 Hz, 1H), 7.58 (s, 1H), 7.67 (s, 2H), 7.70 (d, *J* = 8Hz, 1H); MS *m/z*: 261 $(M + H)^+$

2-(6-(methylthio)naphthalen-2-yl)propanoic acid. To (*S*)-methyl 2-(6-(methylthio)naphthalen-2-yl)propanoate (500 mg, 1.9 mmol) was added 10 mL of a 3M KOH solution in MeOH. The reaction was refluxed for 2 hours, cooled and then quenched with water. Following extraction with ethyl ether (3x), the aqueous layer was acidified with 1 M HCl and then extracted into ethyl ether (3x), washed with brine, dried with $MgSO_4$, and concentrated under reduced pressure to give pure racemic product (450 mg, 96 %). The enantiomers were separated by chiral HPLC using a Chiralpak 1C column (35°C) which was eluted with 98.5% hexanes/0.05% TFA (A) and 1.5% ethanol/0.05% TFA (B). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.59 (d, 3H), 2.57 (s, 3H), 3.88 (q, 1H), 7.36 (dd, *J* = 4, 8 Hz, 1H), 7.43 (dd, *J* = 4, 8 Hz, 1H), 7.57 (s, 1H), 7.69 (s, 2H), 7.71 (d, *J* = 8Hz, 1H); MS *m/z*: 245 $(M - H)^+$.

Suppl. B) Quantification of prostaglandin production by High-Pressure Liquid Chromotography- Tandem Mass Spectrometry.

Reactions were performed as described under "Experimental Procedures" then quenched by the addition of an extraction solution of ethyl acetate containing .5% acetic acid and 1 μM PGE_2-d_4. The organic layer was evaporated to near-dryness under nitrogen and reconstituted in 1:1 MeOH: H_2O. Samples were chromatographed using LC-MS using a Luna C18(2) column (50 × 2 mm, 3 μm) with an isocratic elution method (66:34, 5 mM ammonium acetate pH = 3.3: ACN containing 10% A) at a flow rate of 0.375 mL/min. MS/MS was conducted on a Quantum triple quadrupole mass spectrometer operated in positive ion mode. A mass transition of *m/z* = 370→ 317 was monitored to measure the production of PGE_2/D_2 and *m/z* = 374→ 321 for PGE_2-d_4. Peak areas for PGE_2/D_2 were normalized to PGE_2-d_4. Prostaglandin production for incubations containing inhibitor was normalized to the appropriate DMSO control.

REFERENCES

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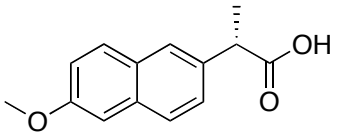
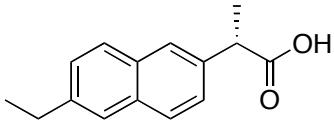
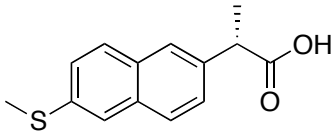
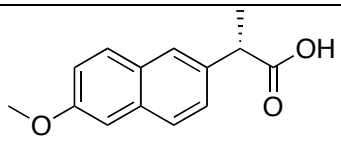
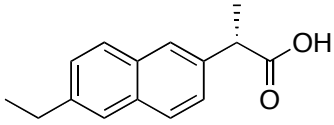
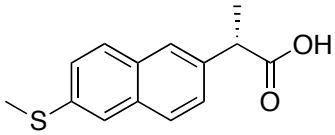
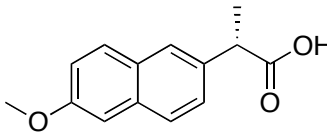
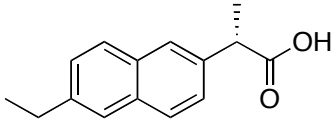
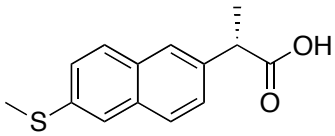
2. Arewang, C. J., Lahmann, M., Oscarson, S., and Tiden, A. (2007) *Carbohydro. Res.* **342**, 970 - 974
3. Kovacs, I., Belanger-Gariepy, F., and Shaver, A. (2003) *Inorgan. Chem.* **42**, 2988-2991

Suppl. C) Additional Tables not shown in manuscript

Supplemental Table I. X-ray data collection and refinement statistics

Data set	**naproxen**	***p*-methylthio naproxen**
Beamline	LS-CAT APS 21 ID-F	SER-CAT APS 22 ID
Wavelength	0.97856	1.0000
Space group	I222	$P2_12_12$
Unit cell dimensions	a=122.3Å, b=133.2Å, c=181.3Å, α=β=γ=90°	a=181.2Å, b=134.2Å, c=122.0Å, α=β=γ=90°
# molecules / ASU	2	4
Resolution (all, highest bin)	35.0-1.73Å, 1.79-1.73Å	20.0-2.27Å, -2.35-2.27Å
R-factor (all, highest bin)	6.7%, 40.9%	12.4%, 50.9%
I/σI (all, highest bin)	23.3, 2.0	12.2, 2.0
Completeness (all, highest bin)	98.6%, 97.8%	97.8%, 91.9%
Redundancy	4.6	4.9
Refinement		
Resolution (all, highest bin)	30.0 – 1.73Å	19.9-2.27Å
# reflections	142,975	131,041
R-factor	16.7%	23.4%
R_{free}	18.6%	26.3%
Rmsd ideal values		
Bond lengths	0.006Å	0.006Å
Bond angles	1.063°	0.895°

Supplemental Table II: Inhibition of WT COX by naproxen and naproxen analogs.

	Analog	COX-1	COX-2
50 mM AA		>25 μM (50%)	0.9 μM (70%)
		>25 μM (45%)	0.77 μM (65%)
		>25 μM (40%)	0.67 μM (70%)
5 mM AA		200 nM (70%)	610 nM (80%)
		2.6 μM (50%)	270 nM (80%)
		1.0 μM (70%)	450 nM (70%)
500 nM AA		227 nM (75%)	53 nM (90%)
		878 nM (75%)	72 nM (90%)
		565 nM (75%)	63 nM (95%)

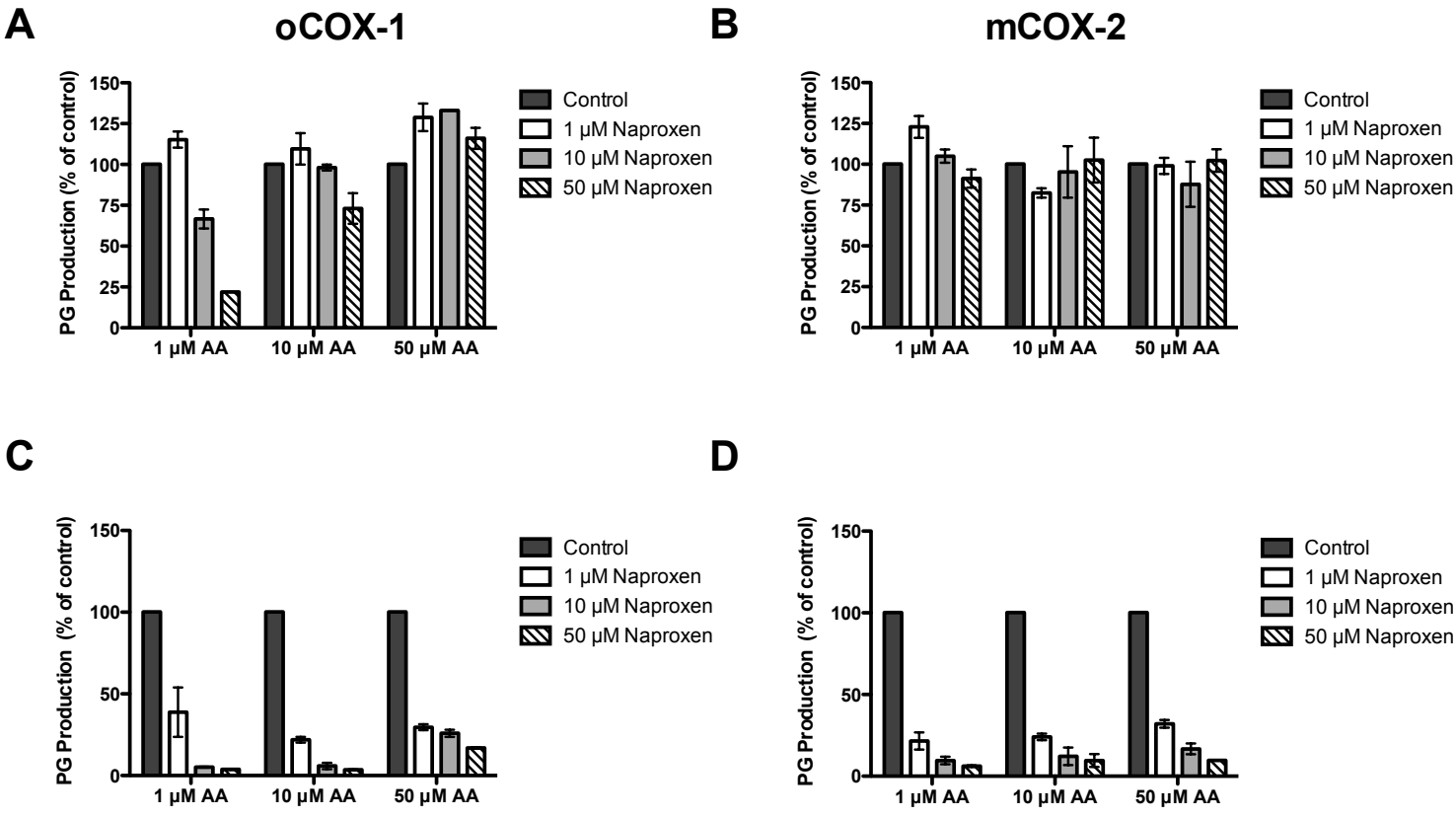
Naproxen and the naproxen analogs were screened against purified oCOX-1 and mCOX-2 as described under experimental procedures for 50 μM and 5 μM substrate concentrations. For 500 nM AA, 6.5 nM oCOX-1 or 21 nM mCOX-2 was pre-incubated with 62.5 to 4000 nM inhibitor for 3 minutes at 37 °C prior to the addition of 500 nM substrate for 8 seconds. Reactions were quenched an extraction solution of ethyl acetate containing .5% acetic acid and 1 μM PGE_2-d_4. PGE_2/D_2 production was analyzed by LC-MS/MS as described under *Suppl. B*. Numbers in paranthesis represent the extent of inhibition.

Suppl. D) Additional Data Not Shown in Manuscript

Suppl. Fig 1. Kinetic basis of COX by naproxen. *Upper panels*: DMSO or 1, 10 or 50 μM naproxen and 500 nM AA were added simultaneously to hematin-reconstitued oCOX-1 (*A*) or mCOX-2 (*B*) and allowed to react for 8 sec. *Lower Panels*: naproxen was incubated with enzyme (*C* - oCOX-1, *D* - mCOX-2) for 3 min at 37 °C prior to the addition of 500 nM AA for 8 sec. PG production was measured by LC/MS/MS as described under *Suppl B*..

Suppl. Fig. 2. Inhibition of Val-349 mCOX-2 mutants by naproxen. Naproxen (0.25 – 25 μM) was preincubated with WT mCOX-2 (○), V349L (▲), and V349I (▼) for 20 min prior to the addition of substrate (50 μM). Each reaction was terminated and analyzed as described under "Experimental Procedures." IC_{50} values are reported under "Results". Each data point is the average of at least two independent determinations.

Supplemental Figure 1.



Supplemental Figure 2.