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Novel non-cyclooxygenase inhibitory derivatives of naproxen for colorectal cancer chemoprevention

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Abstract

A structure-based medicinal chemistry strategy was applied to design new naproxen derivatives that show growth inhibitory activity against human colon tumor cells through a cyclooxygenase

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(COX)-independent mechanism. In vitro testing of the synthesized compounds against the human HT-29 colon tumor cell line revealed enhanced growth inhibitory activity compared to the parent naproxen with **3a** showing IC₅₀ of 11.4 μ M (two orders of magnitude more potent than naproxen). Selectivity of **3a** was investigated against a panel of three tumor and one normal colon cell lines and showed up to six times less toxicity against normal colonocytes. Compound **3a** was shown to induce dose-dependent apoptosis of HT116 colon tumor cells as evidenced by measuring the activity of caspases-3 and 7. None of the synthesized compounds showed activity against COX-1 or COX-2 isozymes, confirming a COX-independent mechanism of action. Compound **3k** was found to have no ulcerogenic effect in rats as indicated by electron microscope scanning of the stomach after oral administration. A pharmacophore model was developed for elucidating structure–activity relationships and subsequent chemical optimization for this series of compounds as colorectal cancer chemopreventive drugs.

Keywords

Naproxen; Colorectal cancer; Chemoprevention; NSAIDs; Cyclooxygenase; Ulcerogenicity; Apoptosis; Pharmacophore

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Introduction

Colorectal cancer is considered as the 4th leading cause of cancer deaths with more than 600,000 documented deaths each year. The worldwide incidence of colorectal cancer is almost one million new malignant cases each year with a survival rate under 50 %, despite chemotherapy (Beck *et al.*, 2009). The high prevalence and mortality from colorectal cancer make the search for effective prevention and/or treatments an urgent medical concern.

The use of chemopreventive approaches to control colon cancer is an attractive strategy, especially for patients with a genetic predisposition as well as for those exposed to environmental carcinogens (Chan, 2002). The relationship between inflammation and cancer is well established that suggests the use of anti-inflammatory drugs for the prevention of colorectal cancer.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a well-known class of anti-inflammatory drugs that inhibit the cyclooxygenase (COX) enzymes which are crucial for the production of eicosanoids from arachidonic acid (Vane and Botting, 1996). Reports have shown the ability of many anti-inflammatory agents, especially the NSAIDs, to inhibit tumor growth. NSAIDs have also been reported to inhibit the migration of tumor cells as well as increase of the rate of apoptosis (Jana, 2008; de Groot *et al.*, 2007; Chan, 2002). Moreover, several studies have revealed a substantial decrease in the mortality from colorectal cancer in association with the use of aspirin and other NSAIDs (Kune *et al.*, 1988; Thun *et al.*, 1991; Giovannucci *et al.*, 1994). A recent 10 year prospective study found that daily use of NSAIDs reduces the risk of colorectal cancer in the general population by as much as 35 % (Sangha *et al.*, 2005). This protective effect was more pronounced (up to 60 %) in high risk candidates, as well as in patients who had a first-degree relative with colorectal cancer (Sangha *et al.*, 2005).

Unfortunately, the depletion of prostaglandins as a result of chronic NSAIDs use is associated with significant side effects, including gastrointestinal ulcers, bleeding, and intestinal perforation (Rainsford, 1999). While COX-2 selective inhibitors display reduced gastrointestinal toxicity, their long-term use is associated with increased risk of myocardial infarction (Cannon and Cannon, 2012; Trelle *et al.*, 2011). These potentially fatal adverse effects outweigh the benefits of NSAIDs or COX-2 inhibitors for preventing colorectal cancer, which tend to require high dosages administration over a long period of time. The mechanism of action that is responsible for the chemopreventive activity of NSAIDs is widely attributed to COX-2 inhibition (Wang and DuBois, 2009; Harris, 2009; Khan *et al.*, 2011). However, in addition to mechanisms that involve the inhibition of COX-2, other reports suggest that the basis for their tumor cell growth inhibitory activity involves a COX-2-independent mechanism (Grösch *et al.*, 2006; Narayanan *et al.*, 2012; Ishikawa and Herschman, 2010). It has been proposed that the anticancer activity of traditional NSAIDs is exerted, at least partially, through other mechanisms that are not based on inhibiting prostaglandin synthesis (Grösch *et al.*, 2006; Tinsley *et al.*, 2013).

Naproxen, (*S*)-6-methoxy- α -methyl-2-naphthaleneacetic acid 1, is a powerful non-selective NSAID that is extensively used as a prescription or an over-the-counter NSAID. The activity of naproxen for the treatment and/or prevention of colorectal cancer has been widely reported (Suh *et al.*, 2011; Elsheikh and Wallace, 2012; Steele *et al.*, 2009; Harris *et al.*, 2008; Lanas, 2009). However, the mechanism by which naproxen exerts its growth inhibitory activity is still a matter of controversy. It has been proposed that inhibition of COX-2 by naproxen prevents the increase in prostaglandin concentrations responsible for cellular transformation into a tumor phenotype (Tuynman *et al.*, 2004). However, increasing number of studies argue against the significance of COX inhibition for the chemopreventive activity of the NSAIDs. Reports have demonstrated that the *in vitro* growth inhibitory activity of the NSAIDs cannot be reversed by the addition of prostaglandins or any of their stable analogs (Hanif *et al.*, 1996). Moreover, sulindac sulfone (a metabolite of sulindac with no COX-1 or COX-2 activity) has shown to have *in vivo* chemoprevention against colorectal cancer (Richter *et al.*, 2001; Piazza *et al.*, 1997). These findings strongly suggest a COX-independent mechanism for the chemopreventive activity of NSAIDs (Hughes *et al.*, 2012).

In the context of our interest in developing new anticancer agents (Radwan *et al.*, 2012) with minimal adverse effects, naproxen derivatives were identified as a potential lead. Our strategy was to develop new naproxen derivatives with enhanced growth inhibitory activity against colorectal tumor cells, while reducing the potential for side effects involving COX inhibition.

Results and discussion

The inspection of the crystal structure for naproxen-bound COX-2 (PDB 3nt1; Fig. 1) indicates the importance of the carboxylic group of naproxen for strong hydrogen bonding at the enzyme active site. Blockade or replacement of this moiety will potentially decrease naproxen binding to the COX active site. Previous studies have demonstrated that carboxylic acid modification can lead to an increase in the anticancer activity of sulindac through a COX-independent mechanism (Whitt *et al.*, 2012; Piazza *et al.*, 2009). Careful selection of

the replacement group or substituent should result in the introduction of new naproxen derivatives that lose their affinity toward the COX enzyme, while maintaining their growth inhibitory activity against cancer cells.

Derivatives with amino acid side chain substituents were chosen for the development of the new derivatives for several important reasons. Firstly, amino acids are normal dietary constituents and generally are non-toxic in moderate doses as compared to other substitutions. Secondly, amino acids can afford a wide range of physicochemical properties for the synthesized molecules. Thirdly, being a nutritional substance, the use of amino acids permit more specific targeting for enzymes involved in the terminal phase of digestion. Accordingly, a novel series of naproxen amides of amino acid esters were designed and evaluated for COX and tumor cell growth inhibitory activity.

Docking of the designed derivatives into COX-2 enzyme

The designed compounds (**3a–m**, Table 1) were docked into COX-2 isozyme in order to insure the lack of potential binding at the enzyme active site. AutoDock 4.2 software (Morris *et al.*, 2009) was used to perform automated docking of the designed compounds inside the active site of COX-2 isozyme (PDB code: 3nt1) after ligand omission (Ibrahim *et al.*, 2012). A validation for the docking method was performed by docking the native co-crystallized naproxen ligand into its binding site of COX-2 and the results deemed satisfactory (RMSD value of 0.17 Å between the docked and co-crystallized structures and an estimated binding free energy (G_{bind}) of –12.6 kcal/mol). All docked compounds showed significantly lower G_{bind} compared to naproxen in the range of –8.3 to –6.7 kcal/mol (Supporting Information, Table S1). Moreover, some designed compounds either failed to be placed inside the pocket or showed steric clashes with amino acid residues at the enzyme active site (Fig. 2). This modeling study provided encouraging evidence of diminished COX inhibitory activity for the designed compounds.

Chemistry

Thirteen derivatives naproxen derivatives (**3a–m**) were synthesized through the reaction of **1** with an appropriate amino acid ester (**2**) under mild conditions using mixed carboxylic–carbonic anhydrides as intermediates (Scheme 1). The carbon dioxide evolution provided a driving force for the major pathway to allow a high yield of the desired product. Analysis of the ^1H NMR data for the obtained compounds revealed retain of the stereochemical properties of the starting materials, while derivatives **3e** and **3f** were obtained as a 1:1 diastereomeric mixture (Supporting Information).

Tumor cell growth inhibitory activity for the synthesized derivatives **3a–m**

Evaluation of the synthesized compounds (**3a–m**) against the human HT-29 colon tumor cell line revealed a general enhancement in growth inhibition activity compared to naproxen (Table 1). Derivatives **3a**, **3g**, **3i**, **3j**, **3l**, and **3m** were among the most active compounds showing up to two orders of magnitude increase in the potency compared to naproxen.

The encouraging activity obtained against the HT-29 cell line prompted further evaluation of the most active derivative (**3a**) against a panel of three human colon tumor cell lines (HT-29,

SW480, and HCT116), in addition to the normal human colonocyte cell line, NCM460 (Fig. 3). Results revealed promising activity for **3a** with IC₅₀ ranging between 11.9 and 17.6 μ M against the tumor cell lines (Fig. 3a). The obtained activity is significantly higher than that of naproxen and the COX-2 selective inhibitor celecoxib (Fig. 3b, c). In terms of selectivity, **3a** has shown a good selectivity against the tumor cells showing up to six times less activity against normal colonocytes (IC₅₀ of 58.5 μ M). Tumor inhibition selectivity was not observed for either naproxen or celecoxib pointing out a different mechanisms of tumor inhibition exerted by **3a**.

Effect of compound **3a** on apoptosis induction

In order to further investigate the cytotoxic mechanism of **3a**, its ability to induce apoptosis was tested through the measurement of two early stage apoptosis biomarkers; caspases-3 and 7. The results shown in Fig. 4 reveal a dose-dependent induction of apoptosis upon treatment of HCT116 cells by **3a**. In addition, a twofold increase of caspase-3 and caspase-7 activity was achieved when treated with 25 μ M of **3a** which correlates well with its IC₅₀ values against tumor cell lines. These results proposed that the observed anti-tumor activity for **3a** is associated with induction of apoptosis, while further investigation is undergoing in order to determine the exact molecular target.

Cyclooxygenase inhibitory activity and ulcerogenicity

In order to confirm the reduced COX inhibitory activity of the synthesized compounds, COX-1 and COX-2 inhibitory activity was measured using an enzymatic assay with purified isozymes. The results revealed the ability of naproxen to non-selectively inhibit COX-1 and COX-2 with IC₅₀ values of 4.2 and 3.6 μ M, respectively. On the other hand, none of the synthesized naproxen derivatives (**3a–m**) showed significant inhibition of either isozyme up to concentrations of 100 μ M (Supplementary Information, Table S2).

GI ulceration is a serious side effect associated with all NSAIDs, which limits their long-term use for cancer chemoprevention. To determine the ulcerogenic activities of synthesized naproxen derivatives, compound **3k** was selected to be tested for ulcerogenicity. Naproxen was used as the positive control in this test. The ulcerogenic effect was measured by the examination of rat stomach using electron microscope scanning technique following a daily oral dose of the tested compound for 4 days. Scanning electromicrographs revealed damage of the protective mucous layer of the stomach in the case of the naproxen-treated group (Fig. 5a). On the other hand, similar toxic effects were not detected in stomach specimens of the **3k**-treated group (Fig. 5b) as shown by their scanning electromicrographs which are almost identical to that of the control group (Fig. 5c). The results are consistent with the lack of COX inhibitory activity and indicate a superior GI safety profile for the synthesized compounds compared to the parent drug naproxen.

Generation of a pharmacophore model for the tested compounds **3a–m**

A common features pharmacophore model was generated in order to elucidate structure activity relationships for the tested compounds. The “HipHop” pharmacophore module implemented in Accelrys Discovery Studio software package (DS) was used for automated ligand-based pharmacophore generation. The tested compounds were divided according to

their relative activities into highly active (**3a**, **3g**, **3i**, **3l**, and **3m**), moderately active (**3b**, **3e**, **3f**, **3h**, **3j**, and **3k**), and inactive (**1**, **3c**, and **3d**) derivatives. For the optimized HipHop run, the highest weight was assigned to the highly active molecules of the training set.

HipHop generated 10 hypotheses (Hypo1-10).each composed of four features (Supporting Information). The top ranked model (Fig. 6) was considered for further analysis.

Results from the mapped compounds (Table 2) show a rough correlation between the derivatives' fit values and their biological activities. The generated model was able to identify inactive compounds (**1**, **3c**, and **3d**) which were assigned low fit value scores.

Mapping of the most active compound **3a** into the pharmacophore model (Fig. 7) shows the fitting of its terminal ethyl group into one of the hydrophobic features. The hydrogen bond acceptor feature was found to map to the side chain carbonyl ester moiety. While these two features are lacked in naproxen, they can be hypothesized to be critical for the improved anticancer activity of the corresponding amides.

The importance of the naphthalene ring could not be tested using the generated model, while a hydrophobic moiety connected to the C_{α} to the naphthalene was assigned to be preferential for activity. The last feature, hydrogen bond acceptor, was found to be occupied by the oxygen atom of the methoxy substituent.

In conclusion, the results revealed the critical importance of the amino acid side chain and its terminal hydrophobic ester for activity. The type of the amino acid side chain plays an important role for activity where the models suggest a hydrophobic side chain for optimal activity. Moreover, modeling results highlight the need for further investigations related to the importance of the naphthalene ring and the attached methoxy group. Current work is focusing on the replacement of the naphthalene ring by a smaller aromatic or heterocyclic ring which is attached through 3–4 carbons side chain to a hydrogen bond accepting moiety.

Experimental

Chemistry

General considerations—All the chemicals used were of commercially available reagent grade and were used without further purification. Melting Points were determined on Stuart melting point apparatus (Stuart Scientific, England) and were uncorrected. Pre-coated silica gel plates, 60G F254, obtained from Merck, Darmstadt (Germany) and were used for thin layer chromatography (TLC). Spots were visualized by using either UV-lamp at 254 nm or iodine. IR Spectra were recorded as KBr disk using Perkin Elmer FT–IR apparatus at the research center, College of Pharmacy, King Saud University, Saudi Arabia. The data are given in ν (cm^{-1}). NMR Spectra: (A) Bruker NMR Spectrophotometer (500 MHz) at the research center, College of Pharmacy, King Saud University, Saudi Arabia, (B) Jeol NMR Spectrophotometer (400 MHz) at the research center, College of Science, King Saud University, Saudi Arabia. The chemical shifts are expressed as δ values (ppm) relative to tetramethylsilane (TMS) as internal standard. Signals are indicated by the following abbreviations: s = singlet, bs = broad singlet, d = doublet, dd = doublet of doublet, t = triplet,

q = quartet, and m = multiplet. The J constant were given in (Hz). Mass spectra carried out using Agilent Triple Quadrupole 6410 QQQ LC/MS with ESI (Electrospray ionization) source. The purity of the synthesized final products (>98 %) was confirmed through the injection into HPLC system (Shimadzu pump LC-10AD, Japan) connected to a variable-wavelength detector (Shimadzu detector SPD-10A, Japan). An analytical reversed phase C18 column (Nucleosil 100/5 μ M C18, 250 $\times$ 4.6 mm, Machery Nagel) was used which is equipped with a cartridge guard C18 column. Chromatographic separations were achieved using a mobile phase of acetonitrile and phosphate buffer (pH 4) at a flow rate of 1.8 ml/min.

General procedure for the synthesis of naproxen derivatives 3a–m—A stirred ice-cold solution of naproxen (0.806 g, 3.5 mmol) in dry dichloromethane, DCM, (30 ml) was treated with triethylamine, TEA, (0.353 g, 3.5 mmol). Ethylchloroformate (0.390 g, 3.5 mmol) was then added dropwise while stirring and the stirring was continued for further 30 min. Solution of appropriate amino acid ester (3.5 mmol) in dry DCM (10 ml) was then added gradually while stirring. The resulting mixture was further stirred at room temperature for 24 h, and the reaction was monitored by TLC using DCM:MeOH 95:5 v/v as a developing system. The solvent was removed under reduced pressure. The obtained mixture was purified by column chromatography using DCM:MeOH 95:5 v/v as a solvent. Pre-coated glass plates of silica gel 60G F254, 20 $\times$ 20 cm, 0.25 mm thick, Merck, were used for preparative chromatography when necessary.

Ethyl 2-(2-(6-methoxynaphthalen-2-yl)propanamido)acetate (3a): Compound **3a** was synthesized from the reaction of naproxen and ethyl glycinate. White powder (yield: 63 %); mp 84–86 °C. IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3290 (NH), 1743 (C=O, ester), 1655 (C=O, amide); ^1H NMR, Jeol 400 MHz, (DMSO- d_6) δ 1.13 (t, 3H, J = 6.6 Hz, $-\text{CH}_3$ of ester), 1.41 (d, 3H, J = 7.32 Hz, $-\text{CH}_3$ of naproxen), 3.79–3.82 (m, 3H, $-\text{CH}$ of naproxen & $-\text{CH}_2$ of glycine), 3.86 (s, 3H, $-\text{OCH}_3$ of naproxen), 4.04 (q, 2H, J = 7.36 Hz, $-\text{CH}_2$ of ester), 7.13–7.15 (m, 1H, ArH), 7.27 (d, 1H, J = 2.2 Hz, ArH), 7.44 (d, 1H, J = 8.04 Hz, ArH), 7.73–7.79 (m, 3H, ArH), 8.42 (t, 1H, NH, J = 5.88 Hz, D_2O -exchangeable); ^{13}C NMR δ 14.56 ($-\text{CH}_3$ of ester), 19.07 ($-\text{CH}_3$ of naproxen), 41.0 ($-\text{CH}_2$ of glycine), 45.0 ($-\text{CH}$ of naproxen), 55.7 ($-\text{OCH}_3$ of naproxen), 61.0 ($-\text{CH}_2$ of ester), 106.22, 119.11, 125.95, 127.09, 128.91, 129.64, 133.71, 137.57, 157.8, 170.40 ($-\text{C}=\text{O}$ amide), 174.51 ($-\text{C}=\text{O}$ ester); MS m/z (%) 315.6 (M^+).

Ethyl 3-(2-(6-methoxynaphthalen-2-yl)propanamido)propanoate (3b): Compound **3b** was synthesized from the reaction of naproxen and β -alanine ethyl ester. White crystals (yield: 59 %); mp 83–84 °C. IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3310 (NH), 1728 (C=O, ester), 1648 (C=O, amide); ^1H NMR, Jeol 400 MHz, (DMSO- d_6) δ 1.09 (t, 3H, J = 6.72 Hz, $-\text{CH}_3$ of ester), 1.41 (d, 3H, J = 7.32 Hz, $-\text{CH}_3$ of naproxen), 2.39–2.42 (m, 2H, $-\text{CH}_2-\text{CO}$ of β -alanine), 3.23–3.28 (m, 2H, $-\text{CH}_2-\text{NH}$ of β -alanine), 3.69 (q, 1H, J = 7.12 Hz, $-\text{CH}$ of naproxen), 3.86 (s, 3H, $-\text{OCH}_3$ of naproxen), 3.97 (q, 2H, J = 7.32 Hz, $-\text{CH}_2$ of ester), 7.13 (dd, J = 9.16, 2.44 Hz, 1H, ArH), 7.26 (d, 1H, J = 1.84 Hz, ArH), 7.41 (d, 1H, J = 7.96 Hz, ArH), 7.69–7.78 (m, 3H, ArH), 8.08 (t, 1H, NH, J = 5.52 Hz, D_2O -exchangeable); ^{13}C NMR δ 14.54 ($-\text{CH}_3$ of ester), 19.03 ($-\text{CH}_3$ of naproxen), 34.30 ($-\text{CH}_2-\text{CO}$ of β -alanine), 35.42 ($-\text{CH}_2-\text{NH}$ of β -alanine), 45.44 ($-\text{CH}$ of naproxen), 55.69 ($-\text{OCH}_3$ of naproxen), 61.41 (–

CH₂ of ester), 106.21, 119.10, 125.78, 126.96, 127.11, 128.92, 129.63, 133.66, 137.89, 157.52, 171.82 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 174.01 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 329.6 (M^+).

(S)-Methyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)propanoate

(3c): Compound **3c** was synthesized from the reaction of naproxen and L-alanine methyl ester. White powder (yield: 64 %); mp 98–99 °C. IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3367 (NH), 1725 ($\text{C}=\text{O}$, ester), 1664 ($\text{C}=\text{O}$, amide); ¹H NMR, Bruker 500 MHz, (DMSO-*d*₆) δ 1.29 (d, 3H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}_3$ of alanine), 1.4 (d, 3H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}_3$ of naproxen), 3.53 (s, 3H, $\text{--}\underline{\text{OCH}}_3$ of ester), 3.81 (q, 1H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}$ of naproxen), 3.87 (s, 3H, $\text{--}\underline{\text{OCH}}_3$ of naproxen), 4.3 (q, 1H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}$ of alanine), 7.14–7.16 (m, 1H, ArH), 7.28 (s, 1H, ArH), 7.45 (d, 1H, J = 7.5 Hz, ArH), 7.71–7.79 (m, 3H, ArH), 8.45 (d, 1H, NH, J = 6.5 Hz, D₂O-exchangeable); ¹³C NMR δ 17.52 ($\text{--}\underline{\text{CH}}_3$ of alanine), 19.15 ($\text{--}\underline{\text{CH}}_3$ of naproxen), 44.59 ($\text{--}\underline{\text{CH}}$ of naproxen), 48.17 ($\text{--}\underline{\text{CH}}$ of alanine) 52.24 ($\text{--}\underline{\text{CH}}_3$ of ester), 55.7 ($\text{--}\underline{\text{OCH}}_3$ of naproxen), 106.24, 119.07, 125.86, 127.01, 127.15, 128.91, 129.64, 133.65, 137.61, 157.53, 173.58 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 173.83 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 315.4 (M^+).

(R)-Methyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)propanoate

(3d): Compound **3d** was synthesized from the reaction of naproxen and D-alanine methyl ester. White powder (yield: 66 %); mp 134–135 °C. IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3332 (NH), 1735 ($\text{C}=\text{O}$, ester), 1663 ($\text{C}=\text{O}$, amide); ¹H NMR, Bruker 500 MHz, (DMSO-*d*₆) δ 1.23 (d, 3H, J = 7.5 Hz, $\text{--}\underline{\text{CH}}_3$ of alanine), 1.4 (d, 3H, J = 6.5 Hz, $\text{--}\underline{\text{C}}\underline{\text{H}}_3$ of naproxen), 3.64 (s, 3H, $\text{--}\underline{\text{OCH}}_3$ of ester), 3.8 (q, 1H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}$ of naproxen), 3.87 (s, 3H, $\text{--}\underline{\text{OCH}}_3$ of naproxen), 4.22 (q, 1H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}$ of alanine), 7.13–7.16 (m, 1H, ArH), 7.27 (s, 1H, ArH), 7.45 (m, 1H, ArH), 7.72–7.79 (m, 3H, ArH), 8.46 (d, 1H, NH, J = 7.0 Hz, D₂O-exchangeable); ¹³C NMR δ 17.42 ($\text{--}\underline{\text{CH}}_3$ of alanine), 19.1 ($\text{--}\underline{\text{CH}}_3$ of naproxen), 45.06 ($\text{--}\underline{\text{CH}}$ of naproxen), 48.22 ($\text{--}\underline{\text{CH}}$ of alanine), 52.38 ($\text{--}\underline{\text{CH}}_3$ of ester), 55.7 ($\text{--}\underline{\text{OCH}}_3$ of naproxen), 106.22, 119.13, 125.85, 127.03, 127.13, 128.9, 129.66, 133.69, 137.62, 157.55, 173.78 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 174.03 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 315.5 (M^+).

Ethyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)propanoate (3e): Compound **3e** was synthesized from the reaction of naproxen and DL-alanine ethyl ester. White powder (yield: 59 %); mp 121–123 °C; the ratio of L/D: 50/50; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3331 (NH), 1745, 1708 ($\text{C}=\text{O}$, ester), 1674 ($\text{C}=\text{O}$, amide); ¹H NMR, Bruker 500 MHz, (DMSO-*d*₆) δ 1.02 (t, 1.5H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}_3$ of D-alanine ester), 1.18 (t, 1.5H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}_3$ of L-alanine ester), 1.23 (d, 1.5H, J = 7.5 Hz, $\text{--}\underline{\text{CH}}_3$ of D-alanine), 1.28 (d, 1.5H, J = 7.5 Hz, $\text{--}\underline{\text{CH}}_3$ of L-alanine), 1.41 (m, 3H, $\text{--}\underline{\text{CH}}_3$ of naproxen), 3.8 (m, 1H, $\text{--}\underline{\text{CH}}$ of naproxen), 3.87 (s, 3H, $\text{--}\underline{\text{OCH}}_3$ of naproxen), 3.97 (q, 1H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}_2$ of D-alanine ester), 4.09 (q, 1H, J = 7.5 Hz, $\text{--}\underline{\text{CH}}_2$ of L-alanine ester), 4.18 (q, 0.5H, J = 7.5 Hz, $\text{--}\underline{\text{CH}}$ of D-alanine), 4.28 (q, 0.5H, J = 7.0 Hz, $\text{--}\underline{\text{CH}}$ of L-alanine), 7.15 (m, 1H, ArH), 7.28 (s, 1H, ArH), 7.45 (m, 1H, ArH), 7.72–7.79 (m, 3H, ArH), 8.45 (m, 1H, NH, Hz, D₂O-exchangeable); ¹³C NMR δ 13.90 ($\text{--}\underline{\text{CH}}_3$ of L-alanine ester), 16.92 ($\text{--}\underline{\text{CH}}_3$ of D-alanine ester), 18.56 ($\text{--}\underline{\text{CH}}_3$ of D-alanine), 18.69 ($\text{--}\underline{\text{CH}}_3$ of L-alanine), 18.95 ($\text{--}\underline{\text{CH}}_3$ of naproxen), 44.11 ($\text{--}\underline{\text{CH}}$ of naproxen), 44.36 ($\text{--}\underline{\text{CH}}$ of naproxen), 47.58 ($\text{--}\underline{\text{CH}}$ of L-alanine), 51.65 ($\text{--}\underline{\text{CH}}$ of D-alanine), 55.1 ($\text{--}\underline{\text{OCH}}_3$ of naproxen), 57.37 ($\text{--}\underline{\text{CH}}_2$ of D-alanine ester), 60.14 ($\text{--}\underline{\text{CH}}_2$ of L-alanine ester), 105.63, 118.48, 125.27, 126.43, 126.56, 128.31, 129.05, 133.6, 137.02, 156.94, 171.38 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 174.03 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 341.5 (M^+).

C=O D-amide), 173.0 (–C=O L-amide), 173.25 (–C=O D-ester), 173.77 (–C=O L-ester); MS m/z (%) 329.4 (M^+).

Methyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)butanoate (3f): Compound **3f** was synthesized from the reaction of naproxen and methyl DL- α -aminobutyrate. White powder (yield: 70 %); mp 97–98 °C; the ratio of L/D: 50/50. IR (KBr) $\nu_{\max}/\text{cm}^{-1}$ 3381 (NH), 1748 (C=O, ester), 1686 (C=O, amide); ^1H NMR, Bruker 500 MHz, (DMSO- d_6) δ 0.75 (t, 1.5H, J = 6.5 Hz, –CH₃ of D-butyrate), 0.89 (t, 1.5H, J = 6.5 Hz, –CH₃ of L-butyrate), 1.40 (d, 3H, J = 7.0 Hz, –CH₃ of naproxen), 1.55–1.57 (m, 2H, –CH₂Me of butyrate), 3.53 (s, 1.5H, –OCH₃ of D-butyrate ester), 3.65 (s, 1.5H, –OCH₃ of L-butyrate ester), 3.86–3.87 (m, 4H, –CH and –OCH₃ of naproxen), 4.1–4.25 (m, 1H, –CH of butyrate), 7.13–7.15 (m, 1H, ArH), 7.28 (s, 1H, ArH), 7.41–7.44 (m, 1H, ArH), 7.72–7.77 (m, 3H, ArH), 8.4 (d, 1H, NH, J = 7.5 Hz, D₂O-exchangeable); ^{13}C NMR δ 13.63 (–CH₃ of butyrate), 18.49 & 20.2 (–CH₃ of naproxen), 35.46 (–CH₂ of butyrate), 44.48 (–CH of naproxen), 48.55 (–CHCO of butyrate), 51.61 & 52.03 (–OCH₃ of ester), 55.11 (–OCH₃ of naproxen), 105.64, 118.52, 125.31, 126.5, 128.31, 129.06, 133.09, 136.84, 156.97, 170.46 (–C=O amide), 171.06 (–C=O amide), 173.4 (2 –C=O ester); MS m/z (%) 329.4 (M^+).

(S)-Ethyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)-3-methylbutanoate (3g): Compound **3g** was synthesized from the reaction of naproxen and L-valine ethyl ester. White powder (yield: 60 %); mp 98 °C. IR (KBr) $\nu_{\max}/\text{cm}^{-1}$ 3348 (NH), 1719 (C=O, ester), 1676 (C=O, amide); ^1H NMR, Jeol 400 MHz, (DMSO- d_6) δ 0.87–0.92 (m, 6H, 2 –CH₃ of valine), 1.04 (t, 3H, J = 7.32 Hz, –CH₃ of ester), 1.41 (d, 3H, J = 7.32 Hz, –CH₃ of naproxen), 2.03–2.06 (m, 1H, –CH(Me)₂ of valine), 3.86 (s, 3H, –OCH₃ of naproxen), 3.9–4.05 (m, 3H, –OCH₂ of ester and –CH of naproxen), 4.16–4.2 (m, 1H, –CHCO of valine), 7.13–7.15 (m, 1H, ArH), 7.27 (s, 1H, ArH), 7.45–7.47 (m, 1H, ArH), 7.72–7.78 (m, 3H, ArH), 8.3 (d, 1H, NH, J = 8.56 Hz, D₂O-exchangeable); ^{13}C NMR δ 14.94 (–CH₃ of ester), 18.85 (2 –CH₃ of valine), 19.29 (–CH₃ of naproxen), 19.54 (2 –CH of naproxen), 30.51 (–CH₃ of valine), 44.71 (–CH(Me)₂ of valine), 51.79 (–CH₃ of ester), 55.69 (–OCH₃ of naproxen), 57.96 (–CHCO of valine), 60.72 (–CH₂ of ester), 106.22, 119.06, 125.83, 126.97, 127.17, 128.89, 129.62, 133.65, 137.64, 157.52, 171.96 (–C=O amide), 174.35 (–C=O ester); MS m/z (%) 357.4 (M^+).

(R)-Methyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)-3-methylbutanoate (3h): Compound **3h** was synthesized from the reaction of naproxen and D-valine methyl ester. White powder (yield: 58 %); mp 73–75 °C. IR (KBr) $\nu_{\max}/\text{cm}^{-1}$ 3381 (NH), 1745 (C=O, ester), 1655 (C=O, amide); ^1H NMR, Bruker 500 MHz, (DMSO- d_6) δ 0.71 (d, 3H, J = 6.5 Hz, –CH₃ of valine), 0.76 (d, 3H, J = 6.5 Hz, –CH₃ of valine), 1.40 (d, 3H, J = 6.5 Hz, –CH₃ of naproxen), 1.95–2.04 (m, 1H, –CH(Me)₂ of valine), 3.67 (s, 3H, –OCH₃ of ester), 3.86 (s, 3H, –OCH₃ of naproxen), 3.96 (q, 1H, J = 6.5 Hz, –CH of naproxen), 4.16 (d, 1H, J = 6.5 Hz, –CHCO of valine), 7.13–7.15 (m, 1H, ArH), 7.27 (s, 1H, ArH), 7.48–7.5 (m, 1H, ArH), 7.74–7.78 (m, 3H, ArH), 8.34 (d, 1H, NH, J = 8.0 Hz, D₂O-exchangeable); ^{13}C NMR δ 16.92 (2 –CH₃ of valine), 19.15 (–CH₃ of naproxen), 44.36 (–CH of naproxen), 47.58 (–CH(Me)₂ of valine), 51.65 (–CHCO of valine), 51.79 (–CH₃ of ester), 55.1 (–OCH₃ of

naproxen), 105.63, 118.49, 125.27, 126.43, 126.56, 128.32, 129.06, 133.06, 137.02, 156.94, 173.0 (–C=O amide), 173.26 (–C=O ester); MS m/z (%) 343.4 (M^+).

(S)-Methyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)-4-methylpentanoate

(3i): Compound **3i** was synthesized from the reaction of naproxen and L-leucine methyl ester. White powder (yield: 60 %); mp 96–98 °C. IR (KBr) $\nu_{\max}/\text{cm}^{-1}$ 3325 (NH), 1734 (C=O, ester), 1670 (C=O, amide); ^1H NMR, Jeol 400 MHz, (DMSO- d_6) δ 0.85 (d, 3H, J = 6.12 Hz, –CH₃ of leucine), 0.89 (d, 3H, J = 6.72 Hz, –CH₃ of leucine), 1.12–1.16 (m, 1H, –CH(Me)₂ of leucine), 1.39 (d, 3H, J = 6.72 Hz, –CH₃ of naproxen), 1.39–1.7 (m, 2H, –CH₂ of leucine), 3.52 (s, 3H, –OCH₃ of ester), 3.81–3.86 (m, 4H, –CH & –OCH₃ of naproxen), 4.3–4.32 (m, 1H, –CH of leucine), 7.13–7.15 (m, 1H, ArH), 7.28 (s, 1H, ArH), 7.44 (d, 1H, J = 8.56 Hz, ArH), 7.70–7.79 (m, 3H, ArH), 8.40 (d, 1H, NH, J = 7.8 Hz, D₂O-exchangeable); ^{13}C NMR δ 19.26 (–CH₃ of naproxen), 21.86 & 23.3 (2 –CH₃ of leucine), 24.87 (–CH(Me)₂), 45.01 (–CH of naproxen), 50.86 (–CHCO of leucine), 52.22 (–CH₃ of ester), 55.69 (–OCH₃ of naproxen), 106.22, 119.07, 125.86, 127.0, 128.89, 129.63, 133.65, 137.55, 157.53, 173.49 (–C=O amide), 174.13 (–C=O ester); MS m/z (%) 357.3 (M^+).

(S)-Ethyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)-4-methylpentanoate

(3j): Compound **3j** was synthesized from the reaction of naproxen and L-leucine ethyl ester. White powder (yield: 54 %); mp 97–99 °C. IR (KBr) $\nu_{\max}/\text{cm}^{-1}$ 3375 (NH), 1717 (C=O, ester), 1669 (C=O, amide); ^1H NMR, Bruker 500 MHz, (DMSO- d_6) δ 0.88 (d, 3H, J = 7.0 Hz, –CH₃ of leucine), 0.91 (d, 3H, J = 6.5 Hz, –CH₃ of leucine), 1.02 (t, 3H, J = 6.5 Hz, –CH₃ of ester), 1.16–1.18 (m, 1H, –CH(Me)₂ of leucine), 1.40 (d, 3H, J = 6.5 Hz, –CH₃ of naproxen), 1.55–1.66 (m, 2H, –CH₂ of leucine), 3.82–3.87 (m, 4H, –CH and –OCH₃ of naproxen), 3.98 (q, 2H, J = 6.5 Hz, –CH₂ of ester), 4.29–4.32 (m, 1H, –CH of leucine), 7.14–7.16 (m, 1H, ArH), 7.28 (s, 1H, ArH), 7.44 (d, 1H, J = 8.0 Hz, ArH), 7.71–7.78 (m, 3H, ArH), 8.39 (d, 1H, NH, J = 7.5 Hz, D₂O-exchangeable); ^{13}C NMR δ 13.79 (–CH₃ of ester), 18.4 (–CH₃ of naproxen), 21.0 (2 –CH₃ of leucine), 24.87 (–CH(Me)₂), 44.45 (–CH of naproxen), 53.53 (–CHCO of leucine), 55.10 (–OCH₃ of naproxen), 60.36 (–CH₂ of ester), 105.62, 118.49, 125.3, 126.44, 126.5, 128.1, 128.28, 129.04, 129.11, 133.09, 156.94, 171.36 (–C=O amide), 173.35 (–C=O ester); MS m/z (%) 371.4 (M^+).

(S)-Dimethyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)succinate

(3k): Compound **3k** was synthesized from the reaction of naproxen and L-aspartic acid dimethyl ester. White powder (yield: 75 %); mp 110–111 °C. IR (KBr) $\nu_{\max}/\text{cm}^{-1}$ 3354 (NH), 1744 (C=O, ester), 1648 (C=O, amide); ^1H NMR, Bruker 500 MHz, (DMSO- d_6) δ 1.39 (d, 3H, J = 7.0 Hz, –CH₃ of naproxen), 2.69–2.78 (m, 1H, H_a of –CH₂ aspartic), 2.79–2.86 (m, 1H, H_b of –CH₂ aspartic), 3.54 (s, 3H, –OCH₃ of ester), 3.57 (s, 3H, –OCH₃ of ester), 3.79–3.80 (m, 1H, –CH of naproxen), 3.87 (s, 3H, –OCH₃ of naproxen), 4.64–4.65 (m, 1H, –CH of aspartic), 7.15 (d, 1H, J = 8.5 Hz, ArH), 7.29 (s, 1H, ArH), 7.42 (d, 1H, J = 8.5 Hz, ArH), 7.70–7.79 (m, 3H, ArH), 8.52 (d, 1H, NH, J = 7.5 Hz, D₂O-exchangeable); ^{13}C NMR δ 18.52 (–CH₃ of naproxen), 35.46 (–CH₂ of aspartic), 44.47 (–CH of naproxen), 48.54 (–CHCO of aspartic), 51.61 (–CH₃ of ester), 52.03 (–CH₃ of ester), 55.10 (–OCH₃ of naproxen), 105.62, 118.53, 125.31, 126.49, 128.31, 129.06, 133.09,

136.85, 156.96, 169.46 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 171.07 ($\text{--}\underline{\text{C}}=\text{O}$ ester), 173.37 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 373.3 (M^+).

(S)-Ethyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)-4-(methylthio)butanoate

(3l): Compound **3l** was synthesized from the reaction of naproxen and L-methionine ethyl ester. White powder (yield: 77 %); mp 85–86 °C. IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3306 (NH), 1737 ($\text{C}=\text{O}$, ester), 1646 ($\text{C}=\text{O}$, amide); ^1H NMR, Jeol 400 MHz, ($\text{DMSO}-d_6$) δ 1.03 (t, 3H, J = 6.72 Hz, $\text{--}\underline{\text{CH}}_3$ of ester), 1.40 (d, 3H, J = 7.32 Hz, $\text{--}\underline{\text{CH}}_3$ of naproxen), 1.88–1.95 (m, 2H, $\text{--}\underline{\text{CH}}_2\text{--}\underline{\text{CH}}_2\text{--}\text{S--}$ of methionine), 2.03 (s, 1H, $\text{--}\text{S--}\underline{\text{CH}}_3$ of methionine), 2.45–2.49 (m, 2H, $\text{--}\underline{\text{CH}}_2\text{--}\underline{\text{CH}}_2\text{--}\text{S--}$ of methionine), 3.81–3.86 (m, 4H, $\text{--}\underline{\text{CH}}$ and $\text{--}\text{O}\underline{\text{CH}}_3$ of naproxen), 3.98 (q, 2H, J = 6.96 Hz, $\text{--}\underline{\text{CH}}_2$ of ester), 4.34–4.36 (m, 1H, $\text{--}\underline{\text{CH}}$ of methionine), 7.13–7.15 (m, 1H, ArH), 7.28 (s, 1H, ArH), 7.44 (d, 1H, J = 8.56 Hz, ArH), 7.71–7.78 (m, 3H, ArH), 8.42 (d, 1H, NH, J = 7.92 Hz, D_2O -exchangeable); ^{13}C NMR δ 14.43 ($\text{--}\underline{\text{CH}}_3$ of ester), 15.14 ($\text{--}\text{SCH}_3$), 19.14 ($\text{--}\underline{\text{CH}}_3$ of naproxen), 30.15 ($\text{--}\text{S--}\underline{\text{CH}}_2$), 31.04 ($\text{--}\text{S--}\underline{\text{CH}}_2\text{--}\underline{\text{CH}}_2$), 45.08 ($\text{--}\underline{\text{CH}}$ of naproxen), 51.66 ($\text{--}\underline{\text{CHCO}}$ of methionine), 55.69 ($\text{--}\text{O}\underline{\text{CH}}_3$ of naproxen), 60.95 ($\text{--}\underline{\text{CH}}_2$ of ester), 106.23, 119.08, 125.87, 127.03, 127.11, 128.9, 129.62, 133.67, 137.56, 157.53, 172.22 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 174.18 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 389.3 (M^+).

(S)-Ethyl 2-((S)-2-(6-methoxynaphthalen-2-yl)propanamido)-3-phenylpropanoate

(3m): Compound **3m** was synthesized from the reaction of naproxen and L-phenylalanine ethyl ester. White powder (yield: 76 %); mp 93–95 °C. IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3346 (NH), 1727 ($\text{C}=\text{O}$, ester), 1643 ($\text{C}=\text{O}$, amide); ^1H NMR, Jeol 400 MHz, ($\text{DMSO}-d_6$) δ 1.0 (t, 3H, J = 7.32 Hz, $\text{--}\underline{\text{CH}}_3$ of ester), 1.3 (d, 3H, J = 6.72 Hz, $\text{--}\underline{\text{CH}}_3$ of naproxen), 2.85–2.93 (m, 1H, H_a of $\text{--}\underline{\text{CH}}_2\text{--}\text{Ph}$ phenylalanine), 2.98–3.02 (m, 1H, H_b of $\text{--}\underline{\text{CH}}_2\text{--}\text{Ph}$ phenylalanine), 3.78 (q, 1H, J = 6.72 Hz, $\text{--}\underline{\text{CH}}$ of naproxen), 3.86 (s, 3H, $\text{--}\text{O}\underline{\text{CH}}_3$ of naproxen), 3.96 (q, 2H, J = 7.32 Hz, $\text{--}\underline{\text{CH}}_2$ of ester), 4.42–4.47 (m, 1H, $\text{--}\underline{\text{CH}}$ of phenylalanine), 7.13–7.41 (m, 8H, ArH), 7.68–7.77 (m, 3H, ArH), 8.47 (d, 1H, NH, J = 7.96 Hz, D_2O -exchangeable); ^{13}C NMR δ 14.4 ($\text{--}\underline{\text{CH}}_3$ of ester), 19.02 ($\text{--}\underline{\text{CH}}_3$ of naproxen), 37.19 ($\text{--}\underline{\text{CH}}_2\text{--}\text{Ph}$), 45.04 ($\text{--}\underline{\text{CH}}$ of naproxen), 54.12 ($\text{--}\underline{\text{CHCO}}$ of phenylalanine), 55.69 ($\text{--}\text{O}\underline{\text{CH}}_3$ of naproxen), 60.93 ($\text{--}\underline{\text{CH}}_2$ of ester), 106.22, 119.07, 127.03, 127.1, 128.69, 128.76, 129.63, 129.71, 137.46, 137.85, 157.53, 171.96 ($\text{--}\underline{\text{C}}=\text{O}$ amide), 173.89 ($\text{--}\underline{\text{C}}=\text{O}$ ester); MS m/z (%) 405.3 (M^+).

Biology

Cell culture—The human HT-29, SW480, and HCT116 colon tumor cell lines were obtained from ATCC and grown under standard cell culture conditions in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5 % CO_2 . The human NCM460 normal colonocyte cell line was obtained from INCELL Corporation and grown in INCELL's enriched M3:10 medium (Cat # M310A500); which is M3 medium plus supplements and 10 % [v/v] fetal bovine serum [FBS], and contains antibiotics. The cells were grown following the manufacture's protocol at 37 °C in a humidified atmosphere with 5 % CO_2 .

Growth assays—Tissue culture treated 96-well microtiter plates were seeded at a density of 5,000 cells/well. For growth inhibition assays, cells were incubated at 37 °C for 18–24 h, treated with the specified compound or vehicle control (0.1 % DMSO final) and incubated at

37 °C for an additional 72 h. Treatment effects on cell growth were determined using the luminescent Cell Titer Glo Assay (Promega) that measures ATP levels as an indicator of viable cell number according to the manufacturer's specifications.

Apoptosis assay—For measuring apoptosis induction, 96-well microtiter plates were seeded at a density of 10,000 cells per well. Cells were treated with the specified compound or vehicle control for 24 h. The Luminescent Caspase-Glo 3/7 Assay (Promega) was performed together with the MultiTox-Fluor assay (Promega), according to the manufacturer's instructions. The live cell signal from the MultiTox-Fluor assay was first done to normalize for cell number and then caspase 3/7 activity was measured.

Cyclooxygenase inhibitory assays—COX-1 and COX-2 activities were measured using purified ovine COX-1 and recombinant human COX-2 enzymes in the COX Inhibitor Screening Assay Kit from Cayman Chemical Company (Ann Arbor, MI). The assay was performed according to the manufacturer's recommendations.

Molecular modeling studies

Pharmacophore generation—All experiments were conducted on an Intel Pentium dual-core processor workstation with 2 MB cache memory and 4 GB RAM. Hypotheses generation were applied against previously described data sets, and their functionality is available as part of Accelrys Discovery Studio 2.5 (DS) software. Molecules were edited using the DS 3D visualizer where conformational models for each compound were generated using the Poling Algorithm. The “best conformational search” option was used, specifying 250 as the maximum number of conformers. The models emphasized a conformational diversity under the constraint of 20 kcal/mol energy threshold above the estimated global minimum based on use of the CHARMM force field. The molecules associated with their conformational models were submitted to HipHop hypothesis generation. Misses, the number of molecules which do not have to map to all features in generated hypotheses; FeatureMisses, the number of maximal molecules which do not have to map to each feature in generated hypotheses and CompleteMisses; the number of molecules which do not have to map to any feature in a given hypothesis, were set as 3, 2, and 2, respectively. A “Principal” value of 2 and MaxOmit features of 0 have been assigned to all training set molecules with an inter-feature value of not less than 3.0 Å.

Molecular docking—Molecular docking of the synthesized compounds into COX-2 enzyme was carried out using the AutoDock 4.2 software package (Radwan *et al.*, 2012). The graphical user interface AutoDockTools (ADT 1.5.4) was employed to setup the enzymes: all hydrogens were added, Gasteiger charges were calculated, and non-polar hydrogens were merged to carbon atoms. The 3D structures of ligand molecules were built, optimized (PM3) level, and saved in pdb format with the aid of Chem3D Ultra 8.0 implemented in ChemOffice Ultra 8.0 package (Chem Draw Ultra 8.0, 2004). In all docking experiments, a grid box size of 50 × 50 × 50 points in *x*, *y*, and *z* directions was built, the box was centered on the ligand naproxen within the binding pocket of the protein. A grid spacing of 0.375 Å and a distances-dependent function of the dielectric constant were used for the calculation of the energetic map. One hundred runs were generated by using

Lamarckian genetic algorithm searches, and the most favorable free energy of binding was selected as the resultant complex structures. Molecular operating environment (MOE 2008.10) was used to optimize and visualize the produced complexes through their relaxation under MMFF94x forcefield.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
2D representation of naproxen at the COX-2 enzyme pocket (PDB code: 3nt1) showing crucial hydrogen bonding between naproxen carboxylic acid and residues at the enzyme pocket. Hydrogen bonds are shown as *green arrows*, hydrophobic amino acids are shown in *green*, and polar amino acids are shown in *purple* (Color figure online)

Fig. 2. Superimposition of **3a** (*green, ball and stick*) and naproxen (*yellow, ball and stick*) both docked at the active site of COX-2 enzyme. Steric clashes were observed between **3a** side chain and residues at the enzyme pocket are shown in *red* contour (Color figure online)

Fig. 3. Dose-dependent growth inhibitory activity of, **a** naproxen derivative **3a**, **b** naproxen, and **c** celecoxib on human colon tumor cell lines, HT-29, SW480, and HCT116 and the normal human colonocyte cell line, NCM460 after 72 h of treatment. Growth inhibitory activity was determined by measuring viable cell number using the Cell Titer Glo ATP assay

Fig. 4.
Apoptosis induction of HCT116 cells by **3a** after 48 h of treatment

Fig. 5. Scanning electromicrographs of rat stomach specimen following a daily oral dose for 4 days: **a** naproxen; **b** compound **3k**; and **c** control

Fig. 6. Top ranked HipHop pharmacophore model showing intra feature distances (Å). Features are *color* coded as follows: hydrogen bond acceptor; *green* and hydrophobic; *cyan* (Color figure online)

Fig. 7.
Compound **3a** (*stick*) mapped onto the generated pharmacophore model

Scheme 1.

Synthesis of the designed naproxen amino acid derivatives (**3a–m**). Reagents and Conditions: (i) Ethyl chloroformate, TEA, DCM, 0 °C, 30 min; (ii) amino acid ester, DCM, RT, 24 h

Table 1

Evaluation of the synthesized compounds **3a–m**^a against the colorectal tumor cell line HT-29

3a-m

Compound	<i>R</i>	<i>R</i> ¹	IC ₅₀ (μM) ^c
Naproxen (1)	–	–	1,150
3a	H	Et	14.6
3b	H	Et	123
3c	Me	Me	>250
3d ^b	Me	Me	>250
3e ^b	Me	Et	175
3f ^b	Et	Me	209
3g	–CH(Me) ₂	Et	29.4
3h	–CH(Me) ₂	Me	102
3i	–CH ₂ –CH(Me) ₂	Me	34.5
3j	–CH ₂ –CH(Me) ₂	Me	74.1
3k	–CH ₂ –COOMe	Me	120
3l	–(CH ₂) ₂ –SMe	Et	49.3
3m	–CH ₂ –Ph	Et	33.6

^a *n* = 1 in case of **3b** and *n* = 0 for the remaining derivatives

^b Diastereomers

^c Tumor cell growth inhibitory activity was measured after 72 h of drug treatment of HT-29 colon tumor cells using the Cell Titer Glo ATP assay

Table 2

Fit values and conformation energies for the mapped compounds into model-1

Compound	IC ₅₀ (μM)	Fit value	<i>E</i> (kcal/mol) ^a
Naproxen	1,150	2.1	9.2
3a	14.6	3.8	2.8
3b	123.4	2.4	7.6
3c	>250	2.3	6.4
3d	>250	2.1	4.1
3e	175.3	3.1	8.1
3f	209	2.9	2.3
3g	29.4	3.4	6.4
3h	102.1	2.5	1.2
3i	34.5	2.6	3.5
3j	74.1	2.7	0.9
3k	120.7	2.1	4.9
3l	49.3	3.9	5.2
3m	33.6	3.6	7.1

^aEnergy of the fitted conformer relative to its global minimum energy