Chemistry & Biology Interface, 2017, 7, 6, 338-350

RESEARCH PAPER ISSN: 2249 –4820

CHEMISTRY & BIOLOGY INTERFACE

An official Journal of ISCB, Journal homepage; www.cbijournal.com

Design, Synthesis and mechanistic Insights into COX-2 selective drugs

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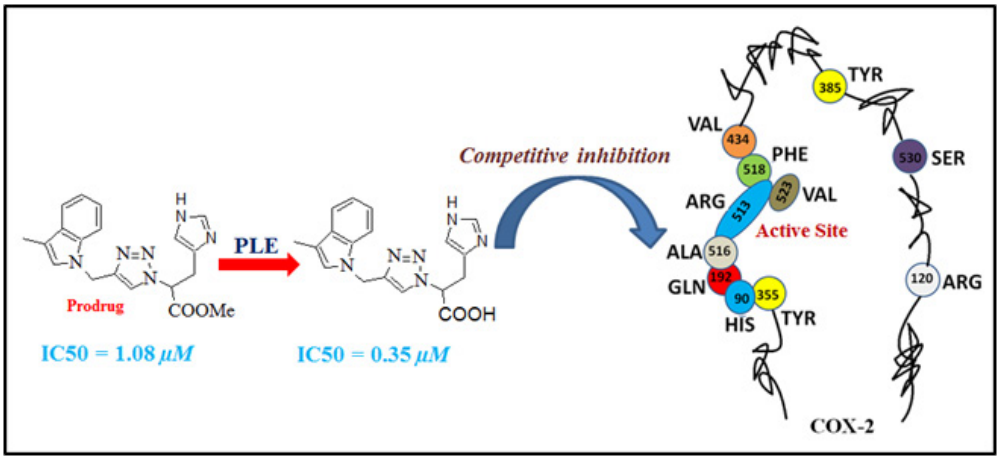
Received 28 June 2017; Accepted 15 January 2018

Abstract: Conjugation of indoles with prudently selected amino acids via a triazole linker led to the development of novel, highly potent inhibitors of COX-2 enzyme witha good lipophilicity. It was well supported by various in-vivo, in-vitro and physical investigations.The compounds displayed a competitive mode of inhibition with the natural substrate of COX-2 enzyme.



Highlights

- Lead compounds which compile the biological activity of indole nucleus appended with amino acids have been developed.
- The test compounds inhibit the COX-2 enzyme by competitive mode of inhibition.
- clogP analysis and in-vitro enzymeimmuoassay analysis indicate the suitability of use of the compounds **5(i-v)** as prodrugs of the compounds **6(i-v)**

Keywords: COX-2, SI, AA, ClogP, IC50, ITC, PLE.

1. INTRODUCTION

An annual report by the World Health Organisation (WHO) revealed that cancer accounted for 8.8 million deaths worldwidein the year 2015 alone. These figures are expected to riseupto 50 percent by the end of the year 2020. The investigators are therefore targeting various factors responsible for the cancer manifestation. One of these factors, which was responsible for about 15% of the total cancer deaths is reported to be associated with the conditions leading to the development of chronic inflammation. Subsequently, an apparent method to minimize the cancer deaths is by capping these chronic inflammation causing conditions. The pioneering work of Sune K. Bergström, Bengt I. Samuelsson and John R. Vane[1] for discovering prostaglandins and related biological substances, which led them to win Nobel Prize in Physiology/Medicine 1982, enabled the scientific community to make extensive exploration of the working of arachidonic acid (AA) pathway[2] and solving several issues related to inflammatory diseases like rheumatoid arthritis and asthma. Dr. J. R. Vane discovered the mechanism of aspirin[3] which was used as a former Non-steroidal anti-inflammatory drug (NSAID) for relieving pain, inflammation and fever by slowing down the production of prostaglandins associated with them. He also discovered prostacyclins[4-6] that relax blood vessels and work for heart and blood vessel diseases and finally led to the development of cyclooxygenase-2 (COX-2) inhibitors. This work further inspired the exploration of arachidonic acid metabolism[7-13] leading to the production of various prostanoids including prostaglandins, prostacyclins, thromboxanes and leukotrienes. Leukotrienes are the regulators of smooth muscle contraction during bronchoconstriction[14]. But a lofty level of LTC4, LTD4 and LTE4 impedes the lung tissues causing asthma. Lipoxygenases are also known to cause atherosclerosis[15],rheumatoidarthritis[16], Inflammatory bowel syndrome[17] and cancer[18]. Albeit, the cyclooxygenases are reported to aggravate mammary cancer due to eminent PGE2 tumour yields isolated from the breast tissues.The prostaglandins such as PGE1, PGF2, PGI2 and TXA2 are formed by human mammary cancers. These studies visiblyindicate that the increased lipoxygenase and cyclooxygenase activity is associated with the development of cancer and this enzyme could be the probable target for cancer treatment as well.

Design of Anti-inflammatory Peptidomimetics: The incorporation of amino acid chains to a heterocycle based mimic is a means to introduce diverse functionalities in the heterocyclic system. The peptide branch has some degree of freedom for the bond rotation which allows the mimic to more flexible and approach the target peptide of the binding protein more effectively. However, this flexibility may raise the entropy of the peptide and conversely, may prohibit the binding at the target protein interface. These dipeptide mimics are known to exhibit some fascinating features including the selective inhibition of COX-2 activity[19]. The cyclooxygenase enzyme has two isoforms, COX-1 and COX-2 with the former functioning as constitutive isoform and the latterworking as an inducible isoform localized primarily to the inflammatory cells like macrophages, fibroblasts and leukocytes. It is expressed in response to inflammatory and other physiologic stimuli and is implicated in the production of those prostaglandins that arbitrate pain and support the inflammatory discourse.Even though both the isoforms of cyclooxygenase have high homology in amino acid sequence (55-60%), but the presence of Val523, Arg513 and Val434 in COX-2 in place of Ile523, His513 and Ile434 in COX-1 provides COX-2 isoform with a much wider substrate entry passage to the active sitethan that of COX-1[20]. The identification of this structural dissimilarity led to the development of COXIBs and NSAIDs which selectively inhibited COX-2 enzyme. Hence a bulky group enhances the COX-2

selective active of a potential anti-inflammatory drug. Peptidomimetics tagged with rigid organic heterocycles have been developed to counter the enzyme mediated inflammation. The rigid heterocyclic tag provides the suitable hydrophilicity to ensure the bioavailability and it provides a base for the attachment of active pharmaceutical to the enzyme. To design and develop an anti-inflammatory pharmaceutical, Indole tag is considered to be the most appropriate standard because its presence has been extensively studied in the development of anti-inflammatory medics. The excretion of indole is also easier from the body compared to other heterocycles which makes it a suitable moiety for drug development. Peptidomimetic acts as the active drug which interacts directly with the target enzyme. To ensure a high drug selectivity towards the problemmatic COX-2 enzyme, the heterocycle is appended with a bulkier group which prevents the entry of the drug in the narrow active site of COX-1 enzyme. The peptidomimetic is appended to the organic heterocycle via a linker which breaks under physiological conditions setting free the active peptidomimetic for unrestricted association with the enzyme. The linker also facilitates the free rotation of the peptidomimetic around the rigid heterocycle. Numerous peptidomimetics[21, 22] which could target both the COX-2 and 5-LOX enzymes have been developed. Preliminarily, the modelling of the peptidomimetics was based on their performance in docking analysis and invitroClogP evaluation. Docking of the designed compounds was done in the active site of the enzyme by using Arguslab.exe software. The crystal structure of the enzyme was downloaded from the RSC protein data bank. While docking it was observed that the compounds with L-amino acids had a better docking score and had a more favourable H-bond interactions with the active site of the enzymes compared to their D-amino acid appended analogues. This significant observation formed one of the comprehensive prerequisites for designing the peptidomimetic. The sole purpose of evaluating the ClogP values was to check whether the test compounds satisfied the Lipinski's criteria for the druglikeness for a compound. ClogP signifies the hydrophilicity index, a criterion validating the bioavailablitiy of a compound to the cells. ClogP was theoretically monitored by molinspiration software and eventually, it was established practically by the traditional shake flask method where the concentration of the compounds in each solvent was found by the quantitative HPLC. For the parent compounds, the most favourable result yielding amino acids were selected to design the daughter peptidomimetic. Based on these initial investigations the screened compounds were synthesised by Scheme 1,2.

2. Materials and Methods

2.1 SYNTHESIS AND CHARACTERISATION:

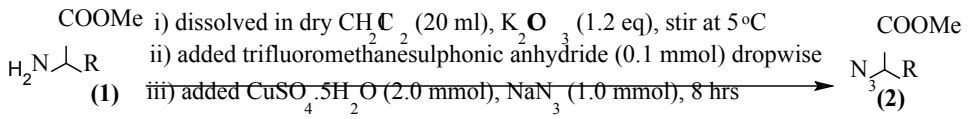
Synthesis: An amount equivalent to 1.2milimolof L-amino esters**(1)** were dissolved in a 25mililitreof dry DCM in a single necked round bottom flaskkept over an ice bath. This solutionwas allowed to stir for 3 minutes till it becomes clear. Afterwards, 1.4 equivalent(w.r.t. amino acid) of perfectly dried K_2CO_3 (activated by preserving at 110 ^{0}C overnight) was added to the clear solution of amino acids in dry DCM. Subsequently, a0.1 milimolequantity of trifluoromethanesulphonic anhydride was very added, dropwise to the reaction mixture inside the vaccum fume hood. On its addition, the colour of the reaction mixture instantaneously changes from colourless to greenish. The contents of reaction mixture were then stirredevenly for 2-3 minutes.Furthur, to enforce the formation of azide from amine functionality, an amount equivalent to 0.8milimol of sodium azide, NaN_3 was added to the reaction mixture with extreme care and left to stir overnight. Theazide formation is completed in about 6-8 hours. After the completion of the reaction, the reaction mixture was washed with 25 ml of distilled

water and the product was extracted with dry etherby using aseparatory funnel. The final product,azide adduct of the amino esters**(2)**, was obtained in a fairly good yield**(Scheme 1)**. 1.5 g of3-methylindole **(3)** was dissolved in 10 ml dry DMSO and allowed to stir till a clear solution is obtained. To this solution was added 1.2 equivalent (w.r.t. methylindole)sodiumhydride, NaH (washed with dry hexane to remove the paraffin coating). The reaction mixture was now stirred for 3-4 minutes till a visible change in the colour of the reaction mixture is observed. The change in colour corresponds to the generation of anion on the indole nucleus after the loss of H^+ from NH position of methylindole. Moreover, H_2 gas is liberated as a result of this reaction. Therefore the addition of NaH to methylindole being a highly exothermic reaction is performed preferably on an ice bath. This serves the purpose of increasing the rate of forward reaction by the Le-Chatelier principle. Furthur, 1.2 equivalents (w.r.t. methylindole) of propargyl bromide was added very , dropwise to the reaction mixture in a vaccum fume hood. The completion of the reaction was monitored with the help of thin layer chromatography. Reaction completes in 20 minutes to give the product **(4)**. The product **(4)** was now dissolved in 10 ml solution of EtOH:H_2O (1:1) and stirredto get a clear solution. To this solution was immediately added 0.02 milimol $CuSO_4.5H_2O$followed by the addition ofazidoaminoesters obtained in Scheme 1, designated as compounds **(2)**. After waiting for another 5 minutes, 0.05 milimole sodium ascorbate was added to the reaction mixture and all the contents were stirredovernight. The monitoring of the reaction was done by thin layer chromatography. The reaction generally completes in 10-12 hours to get the compounds**5 (i-v)**in an average yield. The further purification of the compounds**5 (i-v)** was done by column chromatography using ethylacetate:hexane as eluent. The compounds**5 (i-v)** were finally hydrolysed with LiOH as base in the presence of 10 ml Acetone:water (9:1) as solvent. The reaction completes within minutes to yield the compounds**6 (i-v)(Scheme 2)**. The extraction of the compounds was done with ethylacetate as the solvent using a separatory funnel. All the reactions were performed in vaccum fume hood by using properly cleaned oven-dried borosil glassware. The stirring of the reaction mixtures was done on IKA made magnetic stirrers using teflon coated magnetic beads. Drying of diethyl ether was done over activated anhydrous calcium chloride followed by passing the thin sodium wire through it. Refluxing of acetonitrile was done over anhydrous P_2O_5 followed by distillation over anhydrous K_2CO_3.The dried acetonitrile was finally stored over the activated molecular sieves of size 4 Å to prevent the infilteration of moisture. Reactions were monitored by TLC using silica gel GF254. The chromatograms thus developed were viewed in ultraviolet light and staining was done with iodine. Column chromatography was done to purify the compounds. Silica gel of 100-200 mesh size was used using hexane and ethyl acetate as preferred eluents.All the chemicals and solvents were purchased from Sigma Aldrich chemicals.

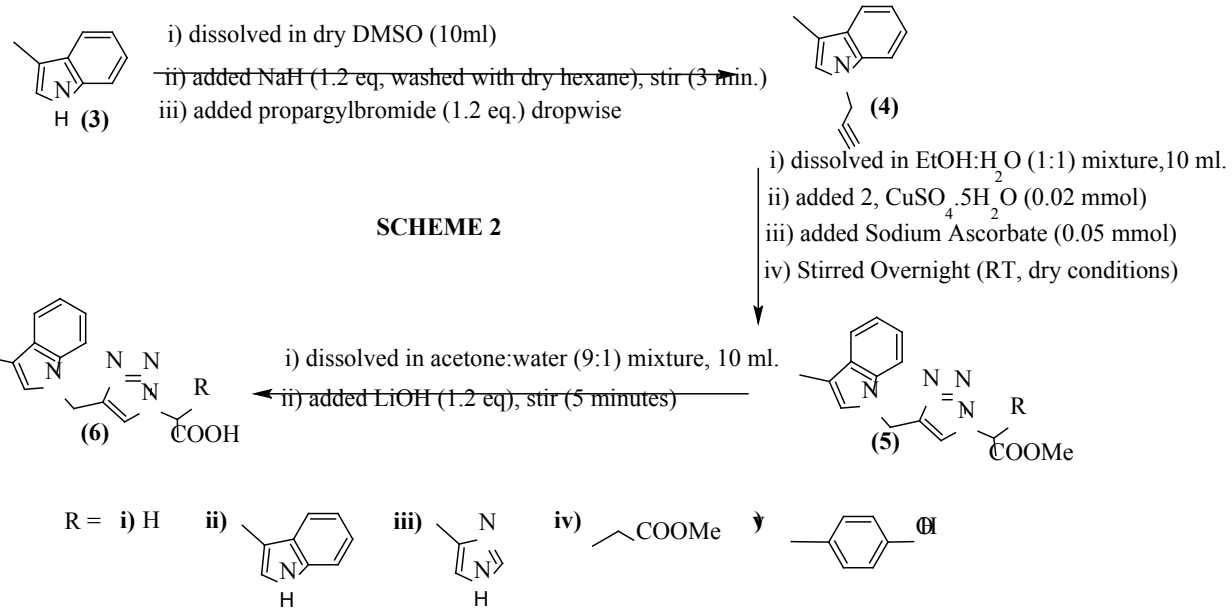
Characterisation

Owing to their very high polarity and a poor solubility in the commonly used solvents, Characterization of compounds **2** was done by using ESI-MS.(supplementary file).

Compound 5i: The compound (4) was dissolved in 9:1solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the azidoacetic acid(2) synthesized in scheme 1, was added to the reaction mixture. The reaction was monitored by thin layerchromatography technique. The reaction generally completes in 8 hours to give a yellow coloured powdery



SCHEME 1



SCHEME 2

Scheme 2

solid, mp 90-95 °C, 80% yield, IR (KBr) νmax 3380 (NH), 1610 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.83-8.401(m, 3H, ArH), 7.39-7.43 (m, 2H, ArH), 4.20 (s, 2H, CH_2), 4.01 (s, 2H, CH_2), 3.21 (s, 3H, OCH_3), 2.25 (s, 3H, CH_3), (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): 181.27 (C=O), 138.66 (C), 134.24 (C), 128.11(CH), 127.93 (CH), 125.74 (C), 122.87(ArCH), 115.79 (ArCH), 113.30 (ArCH), 52.60 (OCH_3), 27.48 (-ve, CH_2), 21.98 (-ve, CH_2), 21.04 (CH_3); ESI-MS (HRMS) calculated for $C_{15}H_{16}N_2O_4$ 285.1087. Found m/z 285.1081($[M+H]^+$).

Compound 5ii: The compound (4) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Lastly, the 2-Azido-3-(1H-indol-3-yl)-propionic acid obtained in scheme 1, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to give a bright yellow coloured solid, mp 137-142 ^{0}C, 69% yield, $[\alpha]_D^{25}$ = +124 (c 0.01, MeOH), IR (KBr) νmax 3390 (NH), 3191 (NH), 1632 (C=O) cm^{-1}; ^1H NMR (500 MHz, DMSO d_6): δ = 6.70-7.50 (m, 9H, ArH), 6.70 (s, 1H, ArH), 4.51(t, J = 10 Hz, 1H, ArH, 1H, CH), 4.20 (s, 2H, CH_2), 3.45-3.63 (m, 2H, CH_2), 3.21 (s, 3H, OCH_3), 2.26 (s, 3H, CH_3) ; (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): d_C = 179.98 (C=O), 136.70 (C), 134.40 (C), 130.92 (ArCH), 127.47 (ArCH), 125.41 (ArCH), 123.83 (ArCH), 121.60 (ArCH), 119.44 (ArCH), 118.00 (ArCH), 114.60 (ArCH), 111.98 (ArCH), 109.85 (C),

107.07 (C), 53.15 (CH), 52.69 (OCH_3), 26.61 (CH_2), 25.45 (CH $_2$), 21.58 (CH_3); ESI-MS (HRMS) calculated for $C_{24}H_{23}N_5O_2$ 414.1788. Found m/z 414.1787 $[M+H]^+$.

Compound 5iii:. Thecompound (4) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-3-(1H-imidazol-4-yl)-propionic acid procured in scheme 1, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours too get a grey coloured solid, mp 121-126 ^{0}C, 65% yield, $[\alpha]_D^{25}$ = +154 (c 0.01, MeOH), IR (KBr) νmax 3299 (NH), 1600 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.45-7.89 (m, 2H, ArH), 6.83-7.39 (m, 5H, ArH), 6.82 (s, 1H, ArH), 4.41 (t, J= 15 Hz, 1H, CH), 4.20 (s, 2H, CH_2), 3.33 (d, J= 10 Hz, 2H, CH_2), 3.21 (s, 3H, OCH_3), 2.27 (s, 3H, CH_3); (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): d_C = 171.97 (COOH), 148.95 (ArCH), 138.64 (ArCH), 136.73 (ArCH), 127.49 (C), 125.96 (C), 121.59 (ArCH), 119.05 (ArCH), 116.64 (ArCH), 111.04 (ArCH), 107.08 (C), 54.27 (CH), 52.70 (OCH_3), 28.61 (CH_2), 25.46 (CH_2), 21.24 (CH_3); ESI-MS (HRMS) calculated for $C_{18}H_{19}N_7O_2$ 366.1431 Found m/z 366.1433 $[M+H]^+$.

Compound 5iv: The compound (4) was dissolvedin 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-4-carboxy-heptanedioic acid obtained in scheme 1, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to give a dark brown solid, mp 111-116 ^{0}C, 85% yield, $[\alpha]_D^{25}$ = +23 (c 0.01, MeOH); IR (KBr) νmax 3412 (NH), 1574 (C=O) cm^{-1}; ^{1}H NMR (DMSO d_6, 500 MHz): δ = 9.81 (s, 1H, COOH), 7.91 (s, 1H, ArH), 7.53-7.82 (m, 2H, ArH), 7.22-7.39 (m, 3H, ArH), 4.39 (t, J = 5 Hz, 1H, CH), 4.20 (s, 2H, CH_2), 3.21 (s, 3H, OCH_3), 2.36-2.72 (m, 2H x CH_2), 2.27 (s, 3H, CH_3); (^{13}C NMR normal/DEPT- 135) (125 MHz, DMSO d_6): d_C = 180.03 (COOH), 179.55 (COOH), 145.55 (ArCH), 134.66 (ArCH), 130.93 (C), 127.14 (C), 123.86 (C), 122.03 (ArCH), 116.85 (C), 114.55 (ArCH), 113.53 (ArCH), 109.82 (C), 53.78 (CH), 52.68 OCH_3), 29.57 (CH_2, -ve), 25.78 (CH_2, -ve), 25.06 (CH_2, - ve), 21.53 (CH_3); ESI-MS (HRMS) calculated for $C_{18}H_{20}N_4O_4$ 356.1401 Found m/z 356.1401 $[M+H]^+$.

Compound 5v: Thecompound (4) was dissolved in 9:1 solution of EtOH:H_2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of $CuSO_4.5H_2O$. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-4-(4-hydroxy-benzyl)-pentanedioic acid obtained by scheme 1 was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to get a brown solid, mp 145-153 ^{0}C, 78% yield, $[\alpha]_D^{25}$ = +35 (c 0.01, MeOH); IR (KBr) νmax 3315 (NH), 1701 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.73-7.94 (m, 2H, ArH), 7.53-7.63 (m, 1H, ArH), 6.87-7.52 (m, 3H, ArH), 4.42 (t, J = 15 Hz, 1H, CH), 4.35 (s, 2H, CH_2), 3.33 (d, J = 3 Hz, 2H, CH_2), 3.21 (s, 3H, OCH_3),2.20 (s, 3H, CH_3); (^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): d_C = 145.30 (C), 144.82 (C), 135.27 (ArCH), 134.94 (ArCH), 130.75 (C), 129.87 (ArCH), 126.81 (ArCH), 126.33 (ArCH), 124.55 (ArCH), 123.30 (ArCH), 121.36 (ArCH), 113.30 (ArCH), 109.04 (C),

107.24 (C), 58.75 (CH), 52.63 OCH_3), 29.29 (CH_2, -ve), 25.19 (CH_2, -ve), 21.53 (CH_3); ESI-MS (HRMS) calculated for $C_{21}H_{20}N_4O_3$ 391.1510 Found m/z 391.1510 $[M+H]^+$.

a) **Compound (6i)**Yellow coloured powdery solid, mp 90 ^oC, 80% yield; ^1H NMR ($CDCl_3$, 500 MHz): δ = 9.42 (s, 1H, COOH) 2.30 7.83-8.401(m, 3H, ArH), 7.39-7.43 (m, 2H, ArH), 4.20 (s, 2H, CH_2), 4.01 (s, 2H, CH_2), 2.25 (s, 3H, CH_3); (^{13}C NMR normal/ DEPT- 135) (125 MHz, $CDCl_3$): 181.27 (C=O), 138.66 (C), 134.24 (C), 128.11(CH), 127.93 (CH), 125.74 (C), 122.87(ArCH), 115.79 (ArCH), 113.30 (ArCH), 27.48 (-ve, CH_2), 21.98 (-ve, CH_2), 21.04 (CH_3); ESI-MS (HRMS) calculated for $C_{14}H_{14}N_2O_4$ 271.1187. Found m/z 271.1181($[M+H]^+$).

b) **Compound (6ii)**Bright yellow coloured solid, mp 137^0C, 69% yield, $[\alpha]_D^{25}$ = +124 (c 0.01, MeOH); ^1H NMR (500 MHz, DMSO d_6): δ = 8.16 (s, 1H, COOH), 6.70-7.50 (m, 9H, ArH), 6.70 (s, 1H, ArH), 4.51(t, J = 10 Hz, 1H, ArH, 2H, CH), 4.20 (s, 2H, CH_2), 3.45-3.63 (m, 2H, CH_2), 2.26 (s, 3H, CH_3).(^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): δ_C = 180.98 (C=O), 136.70 (C), 134.40 (C), 130.92 (ArCH), 127.47 (ArCH), 125.41 (ArCH), 123.83 (ArCH), 121.60 (ArCH), 119.44 (ArCH), 118.00 (ArCH), 114.60 (ArCH), 111.98 (ArCH), 109.85 (C), 107.07 (C), 53.15 (CH), 26.61 (CH_2), 25.45 (CH_2), 21.58 (CH_3); ESI-MS (HRMS) calculated for $C_{23}H_{21}N_5O_2$ 400.1775. Found m/z 400.1700 $[M+H]^+$.

c) **Compound (6iii)**Grey coloured solid, mp 121^0C, 65% yield, $[\alpha]_D^{25}$ = +154 (c 0.01, MeOH); ^1H NMR ($CDCl_3$, 500 MHz): δ = 9.21 (s, 1H, COOH), 7.45-7.89 (m, 2H, ArH), 6.83-7.39 (m, 5H, ArH), 6.82 (s, 1H, ArH), 4.41 (t, J= 15 Hz, 1H, CH), 4.20 (s, 2H, CH_2), 3.33 (d, J= 10 Hz, 2H, CH_2), 2.27 (s, 3H, CH_3).(^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): δ_C = 171.97 (COOH), 148.95 (ArCH), 138.64 (ArCH), 136.73 (ArCH), 127.49 (C), 125.96 (C), 121.59 (ArCH), 119.05 (ArCH), 116.64 (ArCH), 111.04 (ArCH), 107.08 (C), 54.27 (CH), 28.61 (CH_2), 25.46 (CH_2), 21.24 (CH_3); ESI-MS (HRMS) calculated for $C_{17}H_{17}N_7O_2$ 352.1524 Found m/z 352.1523 $[M+H]^+$.

d) **Compound (6iv)**Dark brown solid, mp 111^0C, 85% yield, $[\alpha]_D^{25}$ = +23 (c 0.01, MeOH); ^1H NMR (DMSO d_6, 500 MHz): δ = 10.41 (s, 1H, COOH), 9.81 (s, 1H, COOH), 7.91 (s, 1H, ArH), 7.53-7.82 (m, 2H, ArH), 7.22-7.39 (m, 3H, ArH), 4.39 (t, J = 5 Hz, 1H, CH), 4.20 (s, 2H, CH_2), 2.36-2.72 (m, 2H x CH_2), 2.27 (s, 3H, CH_3).(^{13}C NMR normal/DEPT- 135) (125 MHz, DMSO d_6): δ_C = 180.03 (COOH), 179.55 (COOH), 145.55 (ArCH), 134.66 (ArCH), 130.93 (C), 127.14 (C), 123.86 (C), 122.03 (ArCH), 116.85 (C), 114.55 (ArCH), 113.53 (ArCH), 109.82 (C), 53.78 (CH), 29.57 (CH_2, -ve), 25.78 (CH_2, -ve), 25.06 (CH_2, -ve), 21.53 (CH_3); ESI-MS (HRMS) calculated for $C_{17}H_{18}N_4O_4$ 342.1408 Found m/z 352.1410 $[M+H]^+$.

e) **Compound (6v)**Brown solid, mp 145^0C, 78% yield, $[\alpha]_D^{25}$ = +35 (c 0.01, MeOH); ^1H NMR ($CDCl_3$, 500 MHz): δ = 9.15 (s, 1H, COOH), 7.73-7.94 (m, 2H, ArH), 7.53-7.63 (m, 1H, ArH), 6.87-7.52 (m, 3H, ArH), 4.42 (t, J = 15 Hz, 1H, CH), 4.35 (s, 2H, CH_2), 3.33 (d, J = 3 Hz, 2H, CH_2), 2.20 (s, 3H, CH_3).(^{13}C NMR normal/DEPT- 135) (125 MHz, $CDCl_3$): δ_C = 173.53 (COOH), 145.30 (C), 144.82 (C), 135.27 (ArCH), 134.94 (ArCH), 130.75 (C), 129.87 (ArCH), 126.81 (ArCH), 126.33 (ArCH), 124.55 (ArCH), 123.30 (ArCH), 121.36 (ArCH), 113.30 (ArCH), 109.04 (C), 107.24 (C), 58.75 (CH), 29.29 (CH_2, -ve), 25.19 (CH_2, -ve), 21.53 (CH_3); ESI-MS (HRMS) calculated for $C_{21}H_{20}N_4O_3$ 377.1615 Found m/z 377.1615 $[M+H]^+$.

3. Results and discussions

3.1 Enzyme-immunoassay studies: The compounds were subjected to the in-vitro enzyme immunoassay investigations to check the potency of the compounds against the

COX-1 and COX-2 enzymes.The assay was performedas per the instructionsavailablein the user manual of COX activity assay kit. *To check the suitability of the compounds to act as ester prodrugs, the same assay was performed for the compounds* ***5(i-v)*** *in the presence of pig liver esterase enzyme (PLE). Compound* ***6iii*** *with an imidazole ring and an open, unprotected COOH terminal shows the highest IC50 = 0.32 µM. Its COOH protected analogue, prodrug* ***5iii****has IC50 = 1.08 µM. Another combination with maximum COX-2 inhibition was compound* ***6v****with a phenol ring and open, unprotected COOH grouphaving an IC50 = 0.45 µM. Its COOH protected analogue, prodrug* ***5v****displayed an IC50 = 0.88 µM. Parallel observations were made for the compounds* ***6i****,* ***6ii*** *and* ***6iv****withan IC50 = 1.98 µM, 4.01 µM and 3.07 µM, higher than their ester analogue, prodrugs* ***5i****,* ***5ii*** *and* ***5iv****with IC50 = 3.00 µM, 5.90 µM and 3.77 µM respectively. The expediency of the compounds* ***5(i-v)*** *as ester prodrugs of* ***6(i-v)*** *was also well established. Interestingly, in the presence of PLE, the compounds* ***5(i-v)*** *with ester protected COOH group gave similar results as for the compounds* ***6(i-v)****(similar IC50 as in the absence of PLE) with open, unprotected COOH groups.*

Table 1.COX-1/2 enzyme immunoassay

Cmpd.	IC50(*µM*)			SI*
	COX-1	COX-2	COX-2#	
5i	315.55	3.00	2.00	105.18
5ii	32.12	5.90	3.99	5.44
5iii	>189.90	1.08	0.33	175.83
5iv	>322.22	3.77	3.07	85.46
5v	245.67	0.88	0.44	279.17
6i	355.43	1.98	-	179.51
6ii	135.44	>4.01	-	33.77
6iii	>224.13	0.32	-	700.40
6iv	332.12	3.07	-	108.18
6v	387.89	0.45	-	861.97
Indometacin	0.08	0.96	-	0.08
Celecoxib	15	0.04	-	375

SI* = selectivity index = IC50 (COX-1)/ IC50 (COX-2)

COX-2# = in the presence of pig liver esterase (PLE).

error = ±0.02 of the two experiments

3.2 Calculation of the partitioning coefficient

In order to investigate the lipophilicity data of the compounds, their ClogP values were appraised. ClogPwhich describes the hydrophilic-lipophilic balance of a compound isindispensablefor satisfying the *Lipinski's rule of 5* for drug likeness of a compound.The hydrophilic index was therefore evaluated for the test compounds.100 mg/ml stock solutions of the test compounds were prepared by dissolving the compounds in deionized water with 2% EtOH. The stock solutions of the test compounds were then serially diluted with deionized water and the series of dilutions with known concentrations were thus obtained from which the calibration curves would be prepared. These curves establish a relationship between the aqueous concentration and the absorbance recorded from the UV spectrophotometer. Corresponding volumes with octanol were also prepared. The solutions of the compounds in deionized water were then poured in a separatory funnel followed by the addition of octanol. The funnel was first gently shaken for a few seconds and held with its stem pointing upwards. The stopcock was then gently opened to release the excess pressure and then closed again. This venting procedure was repeated several times to release most of the pressure. Now the separatory funnel was vigorously shaken for 2 minutes and then left to rest on the ring stand in order to allow the compound in the two phases to equilibrate. The equilibrium was assumed to have reached when the two distinct layers started to appear. Both the layers were then separated and their absorbance spectra was recorded.

Data Analysis

Calibration curves were prepared for each compound. The curves relate the measured

absorbance by UV spectrometer to the actual concentration of compounds in aqueous solution. The concentration of the compounds in the aqueous phase was obtained from the calibration curves by the relation:C^{aq} = K.A, where A = absorbance,K = constant which is characteristic for each compound and is obtained from the calibration curves for the aqueous concentration of the compound.The concentration of the compounds in the octanol phase was calculated based on the mass balance of each compound in the two phases. It is evaluated by the relation:$V^{aq}C_e^{aq} + V^{oct}C^{oct} = V^{aq}C^{initial}$,where V is the volume of the respective phase, $C_e^{aq}$is aqueous concentration at equilibrium, $C^{initial}$is the initial aqueous concentrationand $C^{oct}$is octanol phase concentration. Therefore the $C^{oct}$is given by $(V^{aq}C^{initial} - V^{aq}C_e^{aq})/ V^{oct}$. These results were authenticated by another method (method 2) by substituting $C_e^{aq} = K_{ow}C^{oct}$ in the mass balance equation from the relationship:$C^{initial} / C_e^{aq} = (V_{oct}/V_{aq})K_{ow} + 1$. The experimental observations clearly indicate the suitable partitioning coefficient values for the compounds **5(i-v)**, as desired by the *Lipinski's rule of 5*. The compounds **6(i-v)**on the other hand, though highly active, slightly drifted from the rule. This experimental observation claims the suitability of compounds **5(i-v)** as the ester prodrugs of compounds **6(i-v)**.

Table 2. Hydrophilic index for the compounds

Cmpd.	K_{ow}		$\log K_{ow}$	
	Method 1	Method 2	Method 1	Method 2
5i	124.78	124.78	2.09	1.79
5ii	142.58	142.58	2.15	2.11
5iii	162.55	162.55	2.34	2.40
5iv	134.15	134.15	2.12	2.22
5v	168.85	168.85	2.22	2.22
6i	47	50	1.67	1.70
6ii	31	35	1.49	1.48
6iii	67	64	1.82	1.78
6iv	40	35	1.60	1.65
6v	84	80	1.92	1.89

3.3 Competitive/non-competitive interaction of compounds with COX-1 and COX-2

To decipher the mode of inhibition for the test compounds, the lineweaver burke plots were prepared.The rate of change in absorbance on incremental addition of the substrate (arachidonic acid) to a fixed concentration of the enzyme during the progress of the reaction was plotted in the form of a double reciprocal plot (green trail). Likewise, the change in absorbance of the enzyme – inhibitor solution on incremental addition of arachidonic acid was recorded. The red and blue trails in (figure 4) correspond to the solutions containing 10 μM and 20 μM of the inhibitor, respectively. The point where the three trails meet on extrapolation designates the nature of inhibition:competitive or non competitive. Compounds **8, 13, 15** and **17** displayed competitive inhibition of both 5-LOX and COX-2 (Figure 4). The mode of inhibition of the enzyme catalysis by the compounds under present investigation was examined from the Lineweaver-Burk plots. The rate of change in absorbance on incremental addition of the substrate (arachidonic acid) to a fixed concentration of the enzyme during the progress of the reaction was plotted in the form of a double reciprocal plot (green trail). Similarly, the change in absorbance of the enzyme – inhibitor solution on incremental addition of arachidonic acid was recorded. The red and blue trails in figure 4 correspond to the solutions containing 10 μM and 20 μM of the inhibitor, respectively. The point where the three trails meet on extrapolation indicates the nature of inhibition – competitive or non competitive. The test compounds showed a competitive mode of inhibition except the compound **6i** where the mode of inhibition can be stated as non-competitive.

3.4 Isothermal calorimetric data for thermodynamic parameters ΔH, ΔS and ΔG for the enzyme-drug interaction.

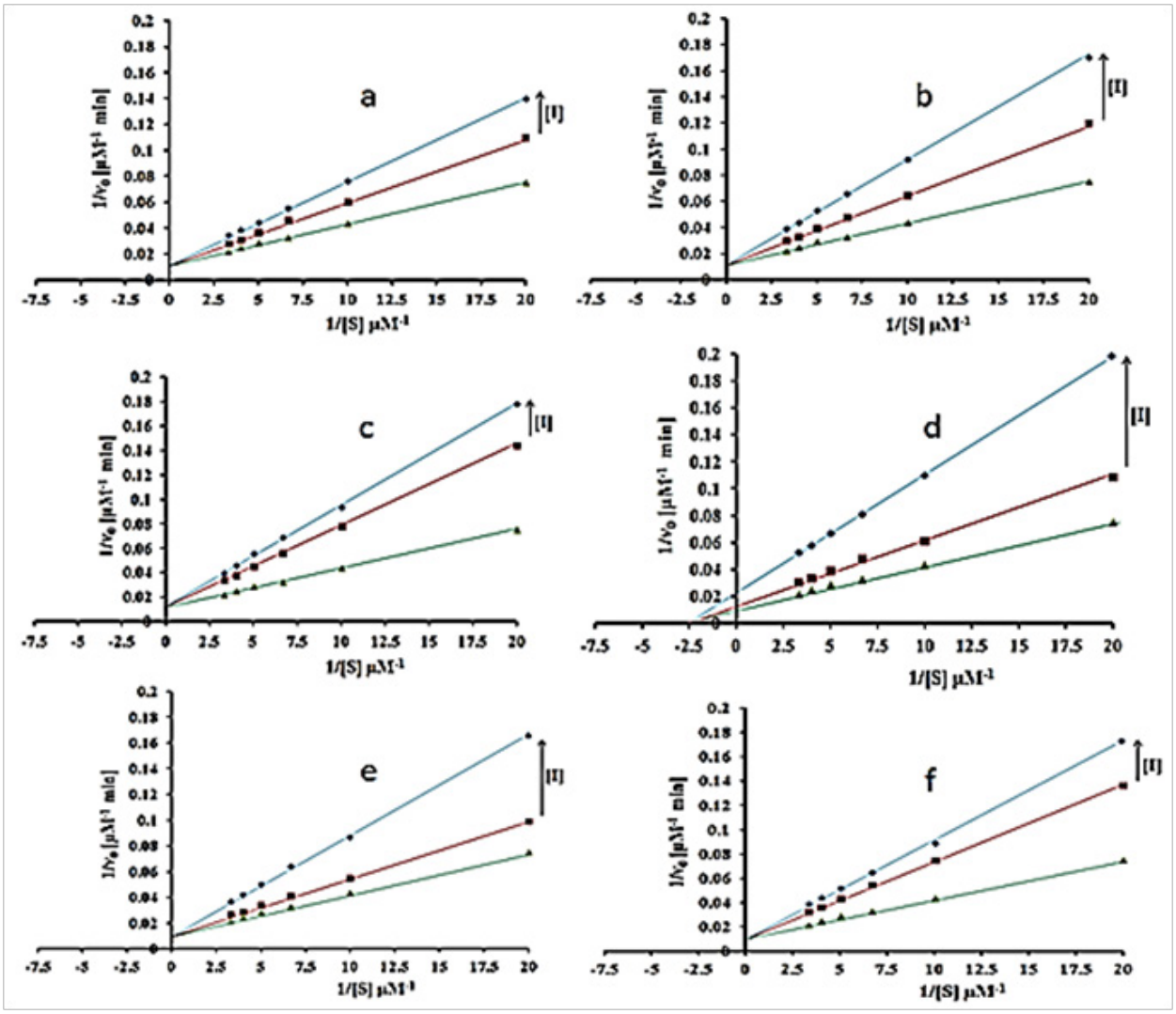


Figure 1. Plots showing lineweaverburk plots for the inhibition of enzymeCOX-2 by: (a) **compound 5i**, (b) **compound 5iii**, (c) **compound 5v**. (d) **compound6i**,(e) **compound 6iii**, (f) **compound 6v**.

The association constant (Ka) for the enzyme compound interaction, standard molar enthalpy (ΔH), standard molar entropy (ΔS), Gibbs free energy of association (ΔH) and stoichiometry (N) of binding of compounds **6(i-v)** for COX-2 enzyme were appraised by using Isothermal Titration Calorimeter technique. The compound solution was titrated into the COX-2 enzyme containing sample cell using a 250 µL rotating stirrer syringe set at 450 rpm. The reference cell contained HEPES buffer. Each of the titration experiment consisted of 19 consecutive injections of 1 µL of 20 µM of the compound to the enzyme contained in the sample cell after regular time intervals of 2 minutes to guarantee the equilibrium at each titration point. The total heat q produced or absorbed in the active cell volume V_a determined at fractional saturation φ after the x^{th} injection is given by equation 1

$$q = n\varphi M_{total}\Delta HV_a \quad (1)$$

Where, M_{total} is the total concentration of the macromolecule

n is the total number of binding sites for the

compound in the macromolecule and

ΔH is the molar heat for ligand binding

The enthalpy change for the x^{th} injection ΔH(x) for an injection volume dVi is then given by equation 2.

$$\Delta H(x) = Q(x) + dV_i/V_a[Q(x) - Q(x-1)/2] - Q(x-1) \quad (2)$$

The various parameters determined from ITC experiments are given in Table 6. The complete ITC profile is given in figure 1. The compounds 6i, 6iii and 6v displayed an appreciable thermodynamics of binding with the enzyme COX-2 with an association constant of $(1.11\pm1.20).10^5$, $(1.77\pm1.20).10^5$and$(2.99\pm0.99).10^5$respectively. Their prodrugs compounds 5i, 5iii and 5v had Kasso. values equal to $(2.43\pm0.90).10^5$, $(0.78\pm0.97).10^5$ and $(1.25\pm0.89).10^5$ respectively. Similarly, the gibbs free energy of association for the test compounds was found to be very favourable for their binding with the COX-2 enzyme. The ITC data therefore confirms the high affinity of the test compounds towards the COX-2 enzyme.

Table 3.Isothermal calorimetric data of compounds **5 (a-e)** for 5-LOX enzyme.

Compd	N	$K_{asso.}$ (M^{-1})	ΔH (Kcal/ mol)	TΔS (Kcal/ mol)	ΔG (Kcal/ mol)
5i	0.99±0.02	$(2.43\pm0.90).10^5$	-6.12±0.3	3.14±0.12	-9.26±0.42
5iii	1.19±0.01	$(0.78\pm0.97).10^5$	-6.10±0.3	1.38±0.34	-7.48±0.64
5v	1.10±0.03	$(1.25\pm0.89).10^5$	-3.45±0.4	1.01±0.88	-4.46±1.28
6i	0.88±0.11	$(1.11\pm1.20).10^5$	-8.10±0.6	0.11±0.56	-8.21±1.16
6iii	0.90±0.21	$(1.77\pm1.20).10^5$	-1.01±0.4	1.32±0.87	-2.33±1.27
6v	0.75±0.02	$(2.99\pm0.99).10^5$	-6.78±0.4	0.76±1.11	-7.54±1.51

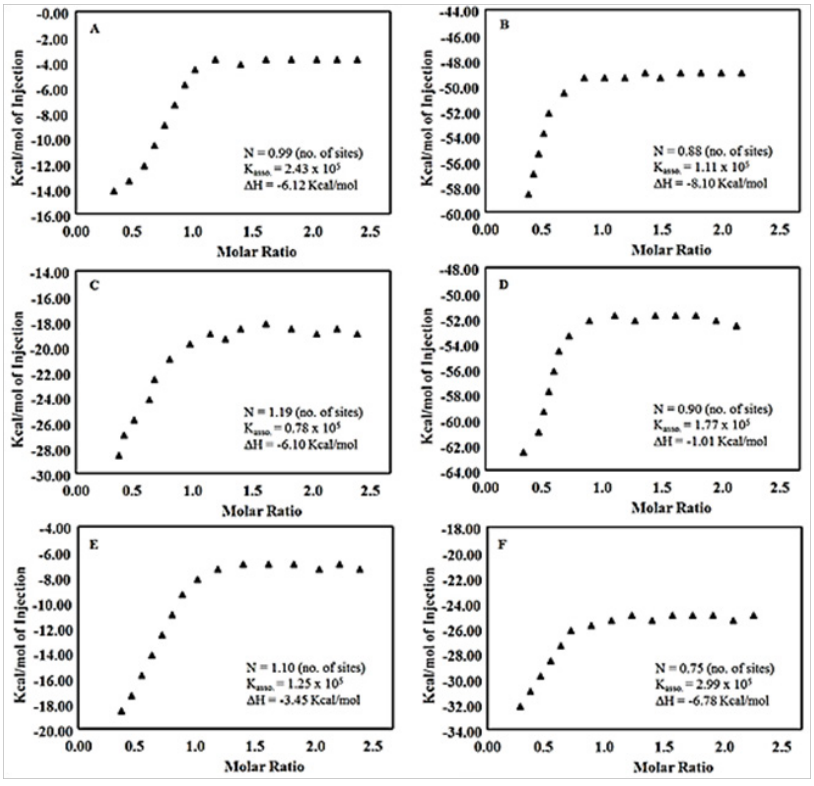


Figure 2. Isothermal calorimetric integrated heat pulse data for **(A)** compound **5i**, **(B)** compound **5iii**, **(C)** compound 5v and **(D)** compound **6i**and **(E)** for compound **6iii** for 5-LOX enzyme, **(F)** for compound **6v** for 5-LOX enzyme.

3.5 Cell Viability Assay

The effect of the compounds **6iii** and **6v**and their prodrugs **5iii** and **5v**on the growth of cells was scrutinized using MTT assay[8]. The experiments were performed as per the protocol available in the kit manual. The experiment was performed in triplicate using a 96-well plate. HeLa cells (sigma Aldrich 93021013),(6000 cells/well) were incubated in a 96-well plate in the presence of 150 g/ml of the test compounds with final volume of 200 µL at 35^{0}C in a humidified chamber. Cells with solvent only (DMSO) were treated as a control. After regular time intervals of 1h, 2h, 3h, 4h, 6h and 24h, a 20 µL of MTT solution (5 mg/ml in PBS) was added to each well and incubatedfor another 6 h. After removing the supernatant at the end of 6 h, the resultant formazan crystals were dissolved in 200 µL DMSO and absorbance (A) was measured at 560 nm by a microplatereader. The percentage viability of the cells was calculated by using the following equation.

Percentage of cell viability =(absorbance of test samples)/(absorbance of the control sample) x100:

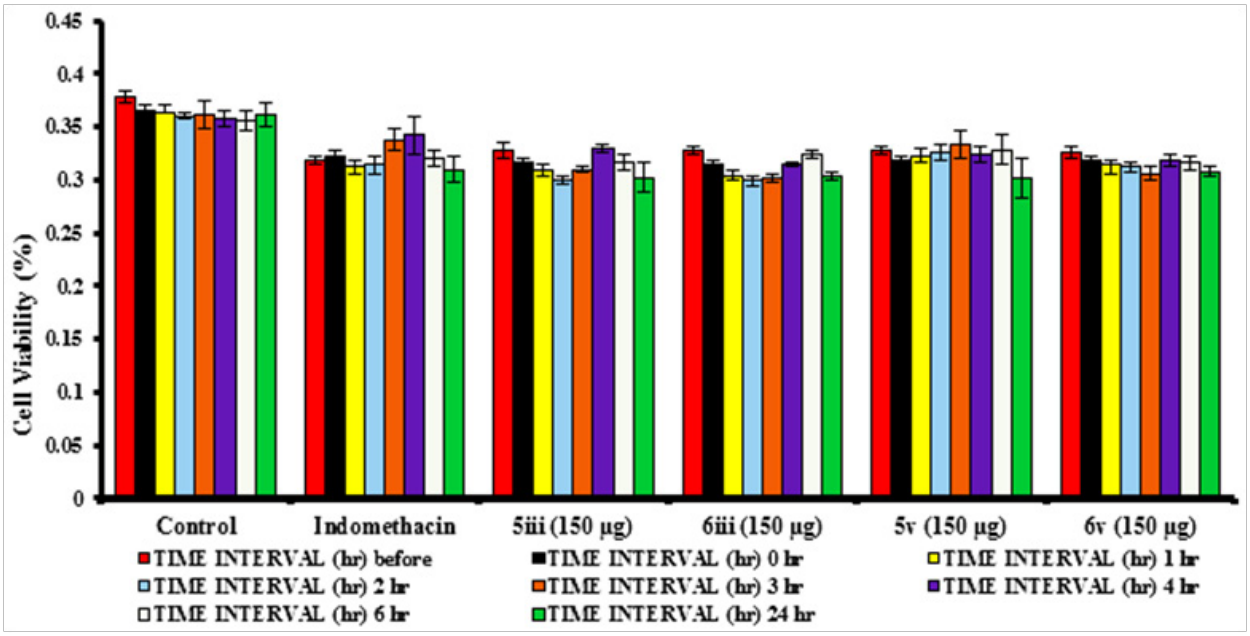


Figure 3. The MTT assay showing the viability of Hela cells at different time intervals in the presence of active compounds and their prodrugs.

4. Conclusion

The bioactive profile of the indole nucleus is retained when appended with amino acids. The bioavailability of the compound is attributed to the triazole linker.The resultant leads exhibited quite excitingCOX-2 inhibitory activities with a competitive mode of inhibiton. The in-vitro results were well supported by kinetic analysis by isothermal titration calorimetry. The bioacceptability of the compounds was ensured by the MTT assay where the compounds did not affected the viability of HeLa cells even at a very high dose. The biological profile of the reported compounds could be extended by introducing a designed peptide appendage in the place of single amino acids.

5. Acknowledgements

PP andMS thanks University of Petroleum and Energy Study, Dehradun for providing the resources. Chemistry Department, Panjab University is also acknowledged for providing the infrastructure for in-vitro analysis. Department of Pharmacy, Lovely Professional University is duly acknowledged for performing the physical investigations. The authors have no conflict of interest.

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