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## Graphical Abstract

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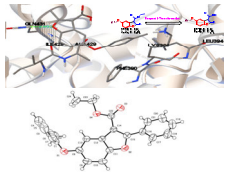

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# Design, synthesis, docking and anti-inflammatory evaluation of novel series of benzofuran based prodrugs

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ABSTRACT

Several new benzofuran derivatives were synthesized, via appropriate synthetic route as anti-inflammatory agents. The anti-inflammatory activity of the prepared compounds was evaluated using carrageenan rat model. Among the synthesized compounds, some compounds showed comparable anti-inflammatory activity to nimesulide, the standard drug taken for anti-inflammatory studies. Docking study of the prepared compounds was performed for the study of interaction of molecules with the active site of COX-2. Preliminary biological studies and docking gave an interesting insight, into the validity of employing benzofuran analogues as good anti-inflammatory agent.

The use of aspirin for the treatment of inflammation, fever and pain, dates back to 1897. Since then many non-steroidal anti-inflammatory drugs (NSAIDs) were developed for the treatment of inflammation, such as ibuprofen, flurbiprofen, indomethacin and diclofenac.[1] NSAIDs have a wide clinical use for the treatment of inflammatory and painful conditions including rheumatoid arthritis, soft tissue lesions, fever and respiratory tract infections.[2] Pharmacological effect of NSAIDs are due to inhibition of cyclooxygenase (COX), which mediates the production of prostaglandins, prostacyclins and thromboxanes from arachidonic acid.[3] There are two isoforms, COX-1 and COX-2. The constitutive COX-1 plays a physiological role in the kidneys and the stomach, whereas, the COX-2 involved in the production of prostaglandins mediating pain and supporting the inflammatory process.[4-6]
Gastrointestinal (GI) erosions and bleeding are two of the most common toxic side effects associated with the administration of NSAIDs, which have been observed even with low prophylactic doses of aspirin (81 mg/day).[7] It is estimated that approximately 50% of patients taking NSAIDs on a long-term basis develop mucosal damage in the small intestine,[8] and 2-4% of these individuals present clinically significant GI ulcers and bleeding, sometimes leading to death.[9] Side effects is due to high COX-1 versus COX-2 selectivity. The development of COX-2 selective NSAIDs (coxibs) (**Figure1**) was meant to circumvent these side effects, by selectively inhibiting the isoenzyme involved in the production of pro-inflammatory mediators. Though clinical trials have shown a reduction of gastrointestinal and renal side effects, an increase in cardiovascular (CV) events was observed suggesting that an exclusive inhibition of COX-2 enzyme could be associated with heart failure and stroke.[10, 11]

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Consequently, the development of new anti-inflammatory drugs is still a strong clinical need, especially after the withdrawal of some selective COX-2 inhibitors such as rofecoxib and valdecoxib.[12, 13]
In an era where new drug pipelines are drying-up and blockbuster agents are facing generic competition, the discovery of novel anti-inflammatory targets continues to propel the development of small molecule therapeutics to treat inflammatory conditions such as prodrugs, that temporarily mask the acidic group of NSAIDs thus reducing or abolishing the GI toxicity due to the local action mechanism.[14]Among the many possible prodrugs, bioreversible esters have received considerable attention because of the presence of enzymes in the living system capable of hydrolyzing them. By use of the prodrug approach, one strategy that could be useful is to temporarily mask the carboxylic acid function of the NSAIDs so that the prodrug hydrolyzes in vivo to release the active parent NSAID.[15-17]

Benzofuran have drawn considerable attention over the last few years due to their profound physiological and chemotherapeutic properties as well as their widespread occurrence in nature.[18]Benzofuran derivatives are versatile biodynamic agents that can be used to design and develop new potentially useful therapeutic agents.[19] Natural and synthetic products possessing the 2-benzylbenzofuran moiety exhibit a broad range of biological and pharmacological activities such as antimicrobial,[20] antioxidant,[21] anti-inflammatory,[22] antifungal,[23] PPARδ agonists,[24] antifeedant, anti-HIV anti-tumor and antiplatelet activities.[25]
As part of our ongoing research program aimed to develop new anti-inflammatory agents with a suitable efficacy/safety profile.[26-30]we now propose the design and biological evaluation of novel benzofuran based ester prodrug.
The synthesis of our target compounds were carried out by adopting a multi-step sequence as outlined in scheme 1. Starting material p-benzoquinone was synthesized according to previously reported procedure.[31] Subsequently benzofuran scaffolds were constructed by Michael addition of ethyl acetoacetate to p-benzoquinone followed by cyclization. Further,

benzofuran scaffolds were subjected to O-arylation in basic condition using CuI catalyst and 8-hydroxyquinoline as co-catalyst.
(**Table 1**).

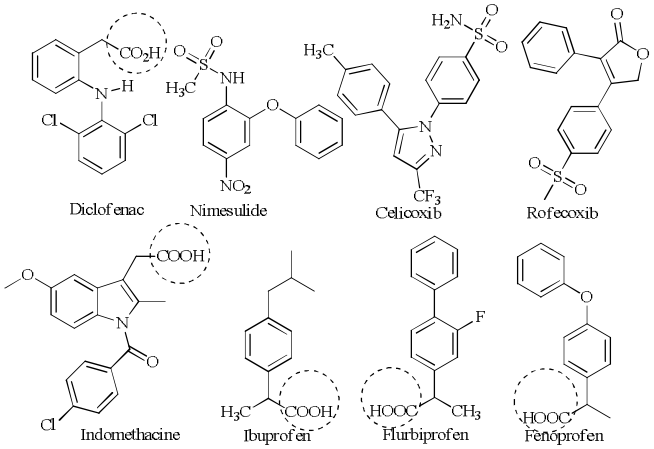
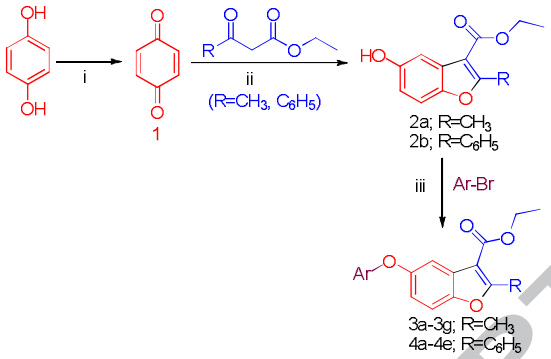



**Figure 1.** Non-steroidal anti-inflammatory drugs (NSAIDs) and coxibs

In preliminary studies with the CuI/NaOH system, the O-arylation of 5-hydroxybenzofuran with iodobenzene was found to give no reaction. Rationalizing that could be resistant to deprotonation; we conducted optimization studies with the same starting materials as the model substrates (**Table 2**). After screening a series of base, 1 eq. of $Cs_2CO_3$ was determined to be the most effective base and gave O-arylated product in 81% yield. While replacing $Cs_2CO_3$ with $K_2CO_3$ afforded **3a** in a slightly lower yield of 75%. Increasing the catalyst loading from 5 to 10 mol % was found to improved the product yield. Finally, on turning our attention to examining solvent effects, we were pleased to find that DMF as a solvent gave the best result, furnishing **3a** in 81% yield. The yield of the products was obtained in the range of 60–80%. Designed series of molecules **3a–3g, 4a-4e** were characterized by IR, $^1$H NMR & $^{13}$C NMR. The molecular structure of a representative compound **4b** was confirmed unambiguously by single crystal X-ray diffraction studies (CCDC 969633) (**Figure 2**).

As prodrug (ester) were rapidly transformed enzymatically to the parent drug (acid) (**Scheme 2**) inside body due to presence of enzymes in the living system capable of hydrolyzing them.[32-33] Therefore, we carried out Molecular docking studies of parent drug (acid) in the active sites of COX-2 in order to get the nature of interactions between the parent drug (acid) and the active site amino acids using the docking program Autodock4.2.[34]The PDB structure 3LN1 (resolution 2.2Å) was used as a receptor for docking the molecules. Firstly, all bound water, ligands, and cofactors were removed from the proteins. The macromolecule was checked for polar hydrogen; torsion bonds of the inhibitors were selected and defined. Gasteiger charges were computed and the AutoDock atom types were defined using AutoDock 4.2, graphical user interface of AutoDock supplied by MGL Tools.[35]




**Scheme1.** Reagents and conditions: (i) Oxidation, KBrO3; (ii) ZnCl2, (1.2 equiv) toluene, reflux 24h, dean stark apparatus; (iii)K2CO3 (1.0 eq.), CuI (0.1 eq.), 8-Hydroxyquinoline (0.01eq.)

**Table 1.** Products and yield of reaction

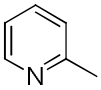
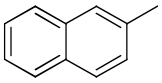
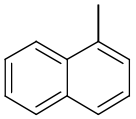
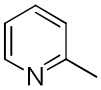
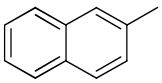
| S. No. | Ar | Product | Yield (%) |
|---|---|---|---|
| 1. | $C_6H_5$ | 3a | 81 |
| 2. | 2-$CH_3$-$C_6H_4$ | 3b | 78 |
| 3. | 4-$CH_3$-$C_6H_4$ | 3c | 73 |
| 4. | 4-$OCH_3$-$C_6H_4$ | 3d | 80 |
| 5. |  | 3e | 74 |
| 6. |  | 3f | 65 |
| 7. |  | 3g | 65 |
| 8. | $C_6H_5$ | 4a | 69 |
| 9. | 2-$CH_3$-$C_6H_4$ | 4b | 73 |
| 10. | 4-$CH_3$-$C_6H_4$ | 4c | 73 |
| 11. |  | 4d | 74 |
| 12. |  | 4e | 60 |

**Table 2.** Optimization of reaction condition

| Entry | [Cu]source | Solvent | Base | Yield % |
|---|---|---|---|---|
| 1. | CuI | DMF | NaOH | 0 |
| 2. | CuI | DMF | $Cs_2CO_3$ | 81 |
| 3. | CuI | DMF | $K_2CO_3$ | 75 |
| 4. | CuI | DMF | $K_3PO_4$ | 70 |
| 5. | CuI | DMSO | $Cs_2CO_3$ | 62 |
| 6. | CuI | NMP | $Cs_2CO_3$ | 60 |
| 7. | CuI | m-xylene | $Cs_2CO_3$ | 59 |
| 8. | CuI | DMA | $Cs_2CO_3$ | 53 |
| 9. | CuI | Toluene | $Cs_2CO_3$ | 22 |
| 10. | CuI | Benzene | $Cs_2CO_3$ | 36 |
| 11. | CuI | Dioxane | $Cs_2CO_3$ | 28 |
| 12. | $Cu_2O$ | DMF | $Cs_2CO_3$ | 55 |
| 13. | $Cu_2O$ | DMF | $K_2CO_3$ | 47 |
| 14. | $Cu_2O$ | DMF | $K_3PO_4$ | 45 |
| 15. | $Cu_2O$ | DMF | $Cs_2CO_3$ | 27 |
| 16. | CuCl | DMF | $Cs_2CO_3$ | 38 |
| 17. | CuBr | DMF | $Cs_2CO_3$ | 41 |
| 18. | $CuBr_2$ | DMF | $Cs_2CO_3$ | 39 |
| 19. | $Cu(OAc)_2$ | DMF | $Cs_2CO_3$ | 41 |
| 20. | $Cu(OTf)_2$ | DMF | $Cs_2CO_3$ | 55 |
| 21. | CuSCN | DMF | $Cs_2CO_3$ | 35 |


**Scheme2.** Enzymatic transformation prodrug (bioreversible ester) to parent drug (acid)

The Lamarckian Genetic Algorithm (LGA), which is considered one of the best docking methods available in AutoDock[36-37], was employed. This algorithm yields superior docking performance compared to simulated annealing or the simple genetic algorithm and the other search algorithms available in AutoDock 4.2. Secondly, the three dimensional grid boxes were created by AutoGrid algorithm to evaluate the binding energies on the macromolecule coordinates. Ligand PDB were prepared using ChemBio3D. The grid maps representing the intact ligand in the actual docking target site were calculated with AutoGrid (part of the AutoDock package). Eventually cubic grids encompassed the binding site where the intact ligand was embedded. Finally, AutoDock was used to calculate the binding free energy of a given inhibitor conformation in the macromolecular structure while the probable structure inaccuracies were ignored in the calculations. The search was extended over the whole receptor protein used as blind docking. Nimesulide (Native Ligand) in the crystal structure was docked as reference. The binding mode of the most active parent drug (acid) **5c** to the COX-2 protein and main interactions are shown in **(Figure 3**). The benzofuran ring of the of compound **5c** is placed close to the side chain of Gln431 to form hydrogen bond at distances of 2.092 Å. **Table 3** shows the docking scores of the active parent drug molecules within the active site of COX-2.

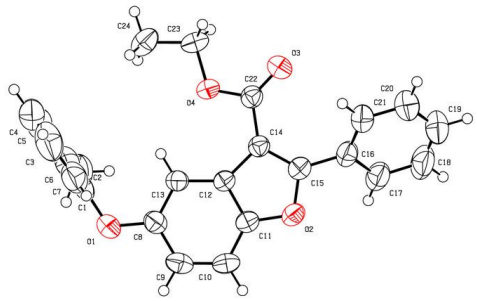


**Figure 2.** ORTEP plot of the X-ray crystal structure of **4b** Displacement ellipsoids are drawn at the 30% probability level

**Table 3**. Dock scores and summary of molecular interactions of compounds after docking into COX-2 active site

| Entry | Dock score | Summary of interactions |
|---|---|---|
| Nimesulide | -12.09 | Arg120, Tyr355, Ser530 |
| 5a | -6.00 | Leu138, Cys21 |
| 5b | -6.87 | Val 335, Ala 513 |
| 5c | -6.68 | Pro139, Pro140,Cys21,Gly121 |
| 5d | -6.52 | Ala 188, His193, Thr 192 |
| 5e | -6.11 | Leu138, Arg455 |
| 5f | -7.07 | Cys32, Leu138, Cys 32 |
| 6a | -7.28 | Lys68, Cys21,Arg 120 |
| 6b | -7.49 | Leu138, Pro139, Arg 455, Glu 451 |
| 6c | -7.62 | Cys32, Gly 30 |
| 6d | -7.29 | Arg 455, Cys32 |
| 6e | -7.30 | Thr61, Phe49, Tyr108 |

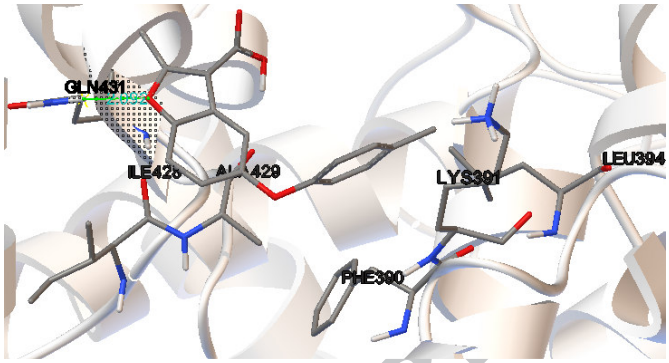



**Figure 3.** Interactions of compound **5c** in the active site of COX-2 Green lines indicate H-bonds formed between the compound and the enzyme active site residues.

Anti-inflammatory study of compounds was performed in order to rationalize the obtained docking results. All the newly synthesized compounds and nimesulide, as a reference drug, were subjected to in vivo anti-inflammatory study using the well-known rat carrageenan induced foot paw edema model.[38]The results of anti-inflammatory activity against carrageenan induced rat paw edema model were shown in **Table 4**, and **Table 5**.The results of present study have shown that compounds **3b**, **3d** had shown to possess maximum inhibitory effect when compared to control group. It was observed that maximum percentage of paw edema growth in control group at 90 min. was 38.7 % which was found to decrease up to 20.4, 12.3 % in the group of rats treated with **3b**, **3d** respectively, compared to nimesulide treated group where it was found 23.4 % also, the values were found statistically very significant. **3c**, **3e** have also been found to possess very good anti-inflammatory property as the percentage paw edema growth was shown to be only 25.8, 18.9 % when compared to that of control group (where it was 38.7 % at 90 min.). Compound **4b** show intermediate effect, while compound **4c**, and **4d** show negligible effect. **(Figure 4)** As shown in **Table 5**. Compound **3b** & **3d** show maximum inhibitory effect, while compound **3c** & **4a-d** to show intermediate effect. Thus a comparison of the SAR data showed that series **3a-d** more potent inhibitor of inflammation relative to **4a-4e** due to electron donating methyl group.

**Table 4.** Percentage Edema Growth Relative to Control at Different Time Intervals (Mean+S.E.M.)

| Group | 0 min | 30min | 90min |
|---|---|---|---|
| Control | 100±0 | 130.1±6.54 (30.1) | 138.7±4.47 (38.7) |
| Nimesulide | 100±0 | 114.1±2.88 (14.1) | 123.4±3.27(23.4)* |
| 3a | 100±0 | 115.7±3.31 (15.7) | 123.8±7.12(23.8) * |
| 3b | 100±0 | 110.1±5.14 (10.1) | 120.4±3.17(20.4)* |
| 3c | 100±0 | 114.1±2.88 (14.1) | 125.8±8.42(25.8)* |
| 3d | 100±0 | 109.5±2.68 (9.5) | 112.3±7.77(12.3)* |
| 4a | 100±0 | 115.8±4.19 (15.8) | 128.4±4.78(28.4)* |
| 4b | 100±0 | 114.8±4.03 (14.8) | 124.4±6.78(24.4)* |
| 4c | 100±0 | 122.1±5.11 (22.1) | 132.6±3.13(32.6)* |
| 4d | 100±0 | 125.1±5.11 (25.1) | 132.6±3.13(32.6)* |
| 4e | 100±0 | 120.1±5.11 (20.1) | 132.6±3.13(32.6)* |

N=number of rats in each group. Results in parentheses indicate percentage change from respective control group. *p-value<0.05

All the synthesized compounds were evaluated for their anti-inflammatory activity by biochemical COX (COX-1 & COX-2) inhibitory assay. The ratio of $IC_{50}$ of COX-2 to $IC_{50}$ of COX-1 (COX- 2/COX-1) would suggest the selectivity of the compound and hence its gastric liability (**Table 6**).[39-41] All prepared

compounds showed weaker COX-2 inhibitory potency and selectivity compared to celecoxib. Among them compounds **3b**, **3c**, **3d**, and **3e** were proved to be potent COX-2 inhibitors with $IC_{50}$ range of 4.2-8.6 µM compare to compound **4a-c**.

**Table 5.** Paw Edema at Different Time Interval (ml/Rat)(Mean+S.E.M.)

| Entry | 0 min | 30min | 90min |
|---|---|---|---|
| Control | 0.99±0.067 | 1.27±0.043 | 1.36±0.070 |
| Nimesulide | 1.02±0.054 | 1.16±0.065 | 1.26±0.038 |
| 3a | 0.78±0.033 | 0.89±0.041 | 1.46±0.068 |
| 3b | 0.78±0.033 | 0.89±0.041 | 0.98±0.044 |
| 3c | 0.98±0.031 | 1.06±0.038 | 1.11±0.045 |
| 3d | 0.76±0.054 | 1.29±0.061 | 1.39±0.068 |
| 4a | 1.08±0.038 | 1.25±0.054 | 1.38±0.058 |
| 4b | 1.06±0.044 | 1.21±0.058 | 1.31±0.061 |
| 4c | 0.98±0.053 | 1.19±0.058 | 1.29±0.061 |
| 4d | 0.96±0.044 | 1.21±0.078 | 1.31±0.066 |
| 4e | 0.75±0.083 | 0.89±0.061 | 0.98±0.044 |

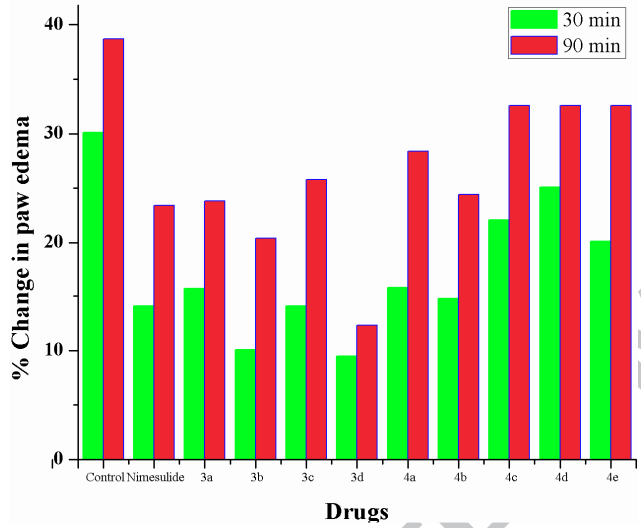



**Figure 4.** Effect of different drugs on carrageenan induced rat pawedema

**Table 6.** Cox-2/Cox-1 enzyme inhibition assay of Benzofuran derivatives.

| Compound | Cox -1 IC50 (µM)[a] | Cox-2 IC50 (µM)[a] | SI[b] |
|---|---|---|---|
| Celecoxib | 15 | 0.04 | 0.0028 |
| 3a | 30.8 | 18.5 | 0.5900 |
| 3b | 35.8 | 6.1 | 0.1700 |
| 3c | 49.7 | 4.2 | 0.0800 |
| 3d | 38.1 | 8.6 | 0.2300 |
| 3e | 27.9 | 8.1 | 0.2900 |
| 3f | 47.5 | 15.8 | 0.3500 |
| 4a | 42.5 | 18.2 | 0.4300 |
| 4b | 29.8 | 20.1 | 0.6700 |
| 4c | 26.2 | 19.2 | 0.7400 |

[a] $IC_{50}$ value is the compound concentration required to produce 50% inhibition of COX-1or COX-2 for means of two determinations.
[b] Selectivity index (COX-2 $IC_{50}$/COX-1 $IC_{50}$)

A series of novel benzofurans analogues were synthesized and their anti-inflammatory activity was determined using carrageenan mouse paw edema bioassay. In synthesized compounds, **3c** exhibited good anti-inflammatory activity, and optimal COX-2 inhibitory potency ($IC_{50}$ = 4.2 µM). Molecular modeling showed that benzofurans analogues interact with COX-2 active site by forming classical hydrogen bonding and this interaction increase the residence time of ligand in the active site consequently augmenting anti-inflammatory activity of compounds.

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