Background: Macrophages play a serious part in the instigation, upkeep, and resolution of inflammation. They are activated or deactivated during inflammation progression. Activation signals include cytokines (IF-γ, granulocyte-monocyte colony-stimulating factor (GM-CSF), and TNF-α), extracellular matrix proteins, and other chemical mediators. Activated macrophages are deactivated by anti-inflammatory cytokines (IL-10 and TGF-β (transforming growth factor-beta) and cytokine antagonists that are mainly produced by macrophages. Based on this, the present study aimed to develop novel (E)-Benzylidene-indazolpyridin methanones (Cpd-1-10) as effective anti-inflammatory agents by analyzing pro- and anti-inflammatory cytokine levels in macrophages.

Objectives: To determine the anti-inflammatory effect of indazolpyridin-methanones by examining pro- and anti-inflammatory interleukin levels in J77A.1 macrophages.

Methods: Expression of cytokines such as TNF-α, IL-1β, IL-6 and IL-10 serum levels measured by ELISA method. Anti-cancer and cytotoxicity studies were carried out by MTT assay. COX-2 seems to be associated with cancers and atypical developments in the duodenal tract. So, a competitive ELISA based COX-2 inhibition assay was done. To validate the inhibitory potentials and to get more insight into the interaction of COX-2 with Cpd1-10, molecular docking was performed.

Results: Briefly, the COX-2 inhibitory relative activity was found to be in between the range of 80-92% (Diclofenac showed 84%, IC₅₀ 0.95 µM).

Conclusion: Cytotoxicity effect of the compounds against breast cancer cell lines found excellent and an extended anticancer study ensured that these compounds are also alternative therapeutic agents against breast cancer. Among all the tested cancer cell lines, the anti-cancer effect on breast cancer was exceptional for the most active compounds Cpd5 and Cpd9.

Keywords: Anti-inflammation, benzylidene-indazolpyridin methanones, COX-2, cytotoxicity, HRBC membrane stabilization, cytokine.
1. INTRODUCTION

Cyclooxygenase (COX) play a vital role in inflammation and targeted extensively in novel small molecule inhibitor-based inflammation drug development [1-3]. The specific COX-2 enzyme inhibitors are normally nonsteroidal anti-inflammatory drug (NSAID) that are unswervingly targeted COX-2, which is largely involving in the development of inflammation, pain and other related outputs [4-6]. COX-2 is not only targeted in the small molecule drug development against inflammation, but also against cancers and cardiovascular regulations [6-9]. In the inflammation mechanism, COX-2 binds to arachidonic acid and subsequently releases metabolites that are highly involved in the associated pain and further inflammation responses [10-12]. Characteristically, Lipopolysaccharide could trigger mononuclear phagocytes (macrophages) and other types of cells to excrete TNF-α, IL-6, IL-10, and IL-1β.

In this study, Benzylidene-indazolpyridin methanones (Cpd1-10) were investigated for their inhibition potential of COX-2 and pro-inflammatory and anti-inflammatory interleukin levels (TNF-α, IL-1β, IL-6, and IL-10) in macrophages in order to develop them as anti-inflammatory agents. The excessive and disproportionate excrete of these cytokines may root systemic inflammatory response syndrome (SIRS), severe tissue impairment, and septic shock. Thus we have measured TNF-α, IL-6, IL-1β as pro-inflammatory substances, and IL-10 as anti-inflammatory substances before and after treating the compounds best among Cpd1-10. Also, their effective cytotoxicity against MCF-7 (breast cancer cell lines) inspired us to execute extensive anticancer studies. For our knowledge, benzylidene and indazolpyridin is a novel combination to treat breast cancer through the COX-2 inhibition mechanism.

COX-2 seems to be highly associated with cancers and irregular tumors in the duodenal tract. COX inhibitors have been shown to reduce the occurrence of cancers and pre-cancerous growths [13, 14]. Very few recent studies have been reported the beneficial effects of COX-2 inhibitors to treat breast cancer [15, 16]. In their report, Bing et al. (2015) have studied the promotion of tumor growth and suppression of tumor immunity in the human system through COX-2 activity [17]. Notably, the first step involved in the biosynthesis of prostanoids often catalyzed by the COX-2 enzyme. In the mechanism, prostanoids are highly involved and highly interconnected with many inflammation diseases and carcinogenesis [18]. Due to this reason, promoted angiogenesis, tumor invasion, and apoptosis resistance simultaneously occur in the human system. Thus, the anti-breast cancer effects of (E)-Benzylidene-indazolopyridin methanones (Cpd1-10) assessed as the alternate therapeutic effect apart from the COX-2 selective inhibition through in silico (molecular docking and druggability predictions) and in vitro studies.

2. MATERIALS AND METHODS

2.1. Chemistry and Target Compounds

The present study compounds were prepared by using a mixture of cyclohexanone 1 (5 mmol), aldehydes 2 (10 mmol), and 20 mol % solid sodium hydroxide (Scheme 1). This mixture was ground well in a clean mortar a pestle at 37°C for 15 min. An adequate amount of 2 mol/L HCl was added to the reaction mixture and used for next step reaction. To the previously prepared mixture, bisbenzylidene-cycloalkanones 3 (1 mmol), isoniazid (2 mmol), absolute ethanol (5 mL) was added. Eco-friendly p-toluene sulphonic acid (p-TSA) (50 mg) was used as an efficient catalyst and refluxed for 6-10 h. The progress of the reaction was monitored by TLC on silica gel (petroleum ether: EtOAc = 6:4) and upon the reaction completion, the mixture was cooled to room temperature. Further, the reaction mixture was added dropwise into a container having crushed ice and permitted to stand for 20 min. The final product was taken out, cleaned, washed...
and filtered numerous times with water, and cleaned by recrystallization by means of ethanol. Synthesized compounds (Fig. 1) were characterized well and reported earlier [19]. Further, the compounds were preserved for future biological studies by packing in glass tubes and preserved below 4°C.

2.2. Compound Preparation for the Biological Assay

Compounds Cpd1-10 were individually taken in 10 mg/1 mL concentration (diluted in DMSO). This initial volume was reduced up to seven times to have a concentration range of $10^{-1}$ to $10^{-8}$ used as a stalk solution. To achieve this, 0.5 mL of first concentration was transferred into a fresh Eppendorf tube and an equal amount of DMSO was added to have a final volume of 1 mL. In this stage, the compound volume considered as 5 mg/mL. The IC$_{50}$ values were also determined from this by considering the final volume of the particular concentration from which the maximum or minimum activity attained.

2.3. COX-2 Enzyme Inhibition Study

COX-2 Inhibitor Screening Kit (Fluorometric) (K547-100-BioVision) was used for the assay. COX-2 activity was analyzed by repeating the assay (n=4) [20, 21]. N, N, N, N-tetra methyl-p-phenylenediamine (TMPD) oxidation based chromogenic assay during the reduction of Prostaglandin G2 (PGG2) to Prostaglandin H2 (PGH2) was employed [22]. The assay mixture was preincubated at 22°C for 1 min along with the test compounds. Other than compounds Cpd1-10, the assay mixture has EDTA (3 mM), haematin (15 mM), Tris-HCl buffer (100 mM, pH 8.0), COX-2 enzyme (100 mg). Arachidonic acid and TMPD were added together to make it to 1 mL in total volume. The rate of TMPD oxidation in 20 seconds was measured as the enzyme activity at 602 nm absorbance.

2.4. Molecular Docking Studies

Docking studies of (E)-Benzylidene-indazolpyridin methanones (Cpd-1-10) into the binding pockets of COX-2 carried out using Autodock4.2.6, AutoDock Tools 1.5.6 and the Arguslab version 4.0.1. All parameters were executed according to our previous reports [23, 24]. 3D crystal structure of COX-2 (PDB ID: 1CX2) retrieved from the protein data bank (PDB) (Source: www.rcsb.org/pdb/). Statistical mechanical analysis for the ligands and the lowest binding energy, ligand efficiency, and the inhibitory constant ($k_i$) values
were extracted. Molecular interaction (hydrogen bonding, \(\pi-\pi\) interaction, and \(\pi\)-cation interaction) results were validated.

### 2.5. HRBC Membrane Stabilization Investigations

Since it is alike lysosomal membrane, the HRBC membrane stabilization study was done to ensure the stability of the HRBC membrane which is supposed to inhibit the inflammatory progression by limiting the excretion of lysosomal enzymes. Different products that are released by a lysosomal enzyme in inflammation progression generating multiple disorders and this extracellular activity are known as the acute or chronic inflammation. NSAIDs are inhibiting these lysosomal enzymes by alleviating the lysosomal membrane. As we reported earlier, \textit{in-vitro} anti-inflammatory activity was carried out by Human Red Blood Cell (HRBC) membrane stabilization method (n=4) using Diclofenac as standard [25, 26]. The percentage of hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water at 100%. The percentage of HRBC membrane stabilization was calculated using the following formula:

\[
\% \text{ inhibition of hemolysis} = 100 \times \left(\frac{\text{OD}_1 - \text{OD}_2}{\text{OD}_1}\right)
\]

Where \(\text{OD}_2 = \) optical density of sample \(\text{OD}_1 = \) optical density of control.

### 2.6. Cell Viability Assay using Macrophages

Commercially acquired mouse macrophages (J774A.1) washed with a PBS buffer prior to use it for sub-culturing and cytotoxicity evaluations [27]. PBS buffer decanted, and the cells were seeded in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 96-well microplates at a density of \(8 \times 10^4\) cells per well. Further, the cells were incubated for 24 h in a 5% CO\(_2\) atmosphere. Then, the compound (Cpd-1-10) was dissolved in DMSO at a concentration of \(10^{-1}\) to \(10^{-8}\) (0.1 \(\mu\)g/mL initial concentration). These concentrations were treated up to 48 h and then 10 \(\mu\)L of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (10 mg/mL in a PBS buffer)) was added and incubated for 6 to 8h at 37\(^\circ\)C. MTT, the yellow tetrazole, is reduced to purple formazan in living cells. Further, in the MTT assay, formazan crystals that formed were dissolved in 100 \(\mu\)L of DMSO after removing the DMEM from corresponding plates. The optical density (OD) was measured at 540 nm in the BioRad ELISA plate reader. Four repeated assays were conducted to determine the viability using the following equation.

\[
\text{Mean OD treatment/mean OD control} \times 100 = \% \text{ cell viability}
\]

### 2.6.1. Determination of Pro- and Anti-inflammatory Interleukin Levels in LPS Induced Macrophages

In order to determine pro-inflammatory and anti-inflammatory interleukin levels, the TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-10 levels in culture supernatants of J77A.1 macrophages measured by means of using the ELISA method. The OD was measured at 405 nm. Macrophages (J774A.1) were grown with a density of \(1 \times 10^6\) in DMEM. Further, compounds (Cpd-1-10) and Indomethacin were treated separately at a concentration range of 1-50 \(\mu\)g/ml and the cells were incubated for 120 min. Lipopolysaccharides (LPS) were added to the cells at \(1 \mu\)g/mL range and incubated for 24 h and further, the cell-free supernatants were obtained and analyzed by means of immunoassay and the cytokines level was quantified. Particularly, the concentrations of TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-10 and in the supernatants of the J774A.1 macrophage cell culture determined by means of immunoenzymatic assessments.

### 2.7. Anticancer Studies- Cell Lines Preparation for Assay

The early passage of proposed MCF-7 (human breast cancer) and human breast normal cell lines (MCF-10) used in this study was developed and cultured as previously described [28, 29]. All cancer cells were cultured in DMEM (Dulbecco’s Modified Eagle’s Medium) supplemented with 10% Fetal Bovine Serum (FBS), (100U) 20 \(\mu\)g/ml penicillin, and 100 \(\mu\)g/ml streptomycin. They were sub-cultured by removing the existing medium and adding fresh 0.25\% trypsin 0.53 mM EDTA for several minutes, then trypsin was removed and the culture incubated at 37\(^\circ\)C for 10 to 15 minutes. Fresh medium was added, aspirated and dispensed into new flasks. Incubation was carried out at 37\(^\circ\)C in an atmosphere of 5% CO\(_2\). For the assay, 1 ml
of homogenized cell suspension was poured in each well of a microtiter plate and kept in a desiccator. After 48 hours of incubation, the cells were observed in an inverted microscope. 0.05 ml of the drug was dissolved in 4.95 ml of DMSO to get a working concentration of 1 mg/ml freshly prepared and filtered using a 0.45-micron filter before bioassay.

2.8. Anticancer Studies- MTT Assay

The anticancer activity of compounds (Cpd-1-10) on various cancer cell lines was determined by the MTT assay as we previously reported [28, 29]. Doxorubicin (DOX) was used as the standard drug in this study since it is generally used in the treatment of many types of carcinoma (solid tumors) and soft tissue sarcomas including blood cancers, like leukemia and lymphoma [28]. Approximately 5000 cells were seeded in 96-well, flat-bottom titer plates and incubated for 24, 48, and 72 hours at 37°C in a 5% CO2 atmosphere. Different concentrations of compounds (Cpd-1-10) (50-500 µg/mL) were added and incubated further for various time periods. After completion of incubation, the medium was removed. The wells were washed with PBS; 100 µL of the working MTT dye in DMEM (Dulbecco's Modified Eagle's Medium) media was added and incubated for 2 hours. MTT lysis buffer (100 µL) was added and incubation continued for 4 hrs more. The absorbance was measured at 570 nm and the cell viability was calculated using the following formula:

Cell viability (%) = Mean OD/Control OD x 100%

2.9. Statistical Analysis

All results obtained in this study were expressed as the percentage decrease with respect to control values and compared by one-way ANOVA with Dunnett’s post-test was performed. GraphPad Prism version 8.0 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com was used for statistical analysis. A difference was considered statistically significant if p ≤0.05.

3. RESULTS AND DISCUSSION

3.1. Results of Molecular Docking Studies

The 3D coordinates of the compounds (Cpd1-10) (ligands) were docked into the active sites of COX-2 (PDB ID: 1CX2) and the results were extracted. The obtained free energy value (Table 1) reveals that the (Cpd1-10) having possible selectivity on COX-2 inhibition efficacy. Ligand efficiency of -0.47 (Cpd5), -0.41 (Cpd9), -0.40 (Cpd2) and -0.38 (Cpd3) which indicates these compounds are the most potent COX-2 inhibitors among Cpd1-10 and were recommended for further bioactivity evaluations. The lowest binding energy found as -14.24 kcal/mol, -12.97 kcal/mol.
and -12.74 kcal/mol for Cpd5, Cpd9, and Cpd2 respectively.

3.1.1. Receptor-ligand Interaction Analysis between COX-2 and Compounds Cpd1-10

Hydrogen bonding and non-covalent bonding (π-π interaction and π-cation interactions) were measured in order to get the ligand-receptor interactions in sequence. Compounds (Cpd1-10) were established π-π interactions to the Ar-2 and Ar-3 ring system of the Cpd1-10 with the amino acid residues Ala202, His207, His338, Phe200, Phe210, Phe404, Phe518, Trp387, Tyr385 and Tyr409 of COX-2 (Fig. 2). None of the compounds found in cation-π interactions. H-bonds were found for almost all compounds in the receptor-ligand interactions. H-Bonds established to Asn382; Arg120; Gln203; His214, 207, 338; Thr206 were found between the ligand and receptor atoms N, H and O.

3.2. Results of COX-2 Enzyme Activity

To calculate the COX-2 percent inhibition, the non-enzymatic oxidation in the absence of COX-2 was subtracted from the TMPD oxidation value. Celecoxib was used as a standard drug since the aromatic arene, azaarene, and the phenyl ring; -CHO containing a carbonyl, halogen-containing aryl chloride, aryl halide, and leaving groups are common with Cpd1-10. Overall, Cpd5 < Cpd9 < Cpd2 < Cpd3 < Cpd10 were found as potent inhibitors with percentage inhibition of 90.14%, 86.50%, 80.22%, 78.94% and 70.35% respectively, in comparison to Celecoxib it was 80.14% (Fig. 3). Diclofenac with IC50 ≥0.092 μM was comparable with Cpd5 (0.088 μM) and Cpd9 (0.102 μM).
3.3. Results of *in vitro* Anti-inflammation Activity

HRBC membrane stability of compounds Cpd1-10 was analyzed and the results were obtained in a dose-dependent manner as depicted results of Cpd5 and Diclofenac in Fig. (4). In the overall results, compounds Cpd5, Cpd9, Cpd2, and Cpd3 showed an excellent % membrane protection activity and were screened for *in vivo* evaluations. Even though diclofenac, the standard found with IC50 0.15 µM, average protection 82.15%, and the compounds Cpd5 (IC50 0.05 µM, 86.15) Cpd9 (IC50 0.075 µM, 82.56), Cpd2 (IC50 0.15 µM, 78.18) and Cpd3 (IC50 0.25 µM, 76.45) were also established remarkable activity with comparable IC50 values. As these compounds showed excellent HRBC membrane protection results, the top two compounds were screened for further anti-breast cancer.

![Fig. (4). HRBC membrane protection results of Cpd5 and Diclofenac.](image)

3.4. Quantification of Cytotoxicity and Pro- & Anti-inflammatory Interleukin Levels in LPS Induced Macrophages

Lipopolysaccharide can trigger mononuclear phagocytes (macrophages) and other types of cells to excrete TNF-α, IL-6, IL-10, and IL-1β. The excessive and disproportionate excretion of these cytokines may root systemic inflammatory response syndrome (SIRS), severe tissue impairment, and septic shock [30]. The cell viability of J774A.1 macrophages was evaluated using compounds Cpd5 and Cpd9. Fig. (5) shows that compounds Cpd5 and Cpd9 at concentrations of 1 to 20 (nM). Low concentrations (1 to 7 (nM)) did not affect cell viability, whereas 8 and 20 nM Cpd5 and Cpd9 did affect cell viability. Consequently, we used the concentration of 5 nM of Cpd5 and Cpd9 in subsequent experiments. The IC50 was found as 2.05 ± 0.001 nM. The effect of Cpd5, Cpd9, and Indomethacin on the production of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and the anti-inflammatory cytokine IL-10 was estimated in the culture medium of macrophages stimulated with 1 µg/mL of LPS alone or in combination with 5 nM of Cpd5 and Cpd9 (Fig. 6). Compounds Cpd5 and Cpd9 considerably decreased TNF-α, IL-6, and IL-1β, and the properties were comparable to those attained with Cpd5 and Cpd9 and higher than Indomethacin. Furthermore, Cpd5 and Cpd9 showed an augmented IL-10 production at 5 nM concentration matched with the control group, and the increase in IL-10 production was higher than that observed in the group treated with Indomethacin.

![Fig. (5). Cell viability in macrophages treated with Cpd5 and Cpd9 at 1 to 20 (nM), as assessed by the MTT assays. Results are expressed as the % of living cells comparative to control cells.](image)

3.5. Results of Anticancer Activity Potentials of Cpd5 & 9

Among the range of cell lines such as SW620 (human colon cancer), PC3 (human prostate cancer), MCF-7 (human breast cancer), G361 (human skin cancer) human breast normal cell lines (MCF-10) used, only breast epithelial cells was found effectively inhibited for further proliferation. Doxorubicin was served as standard to assess the efficacy of compounds Cpd5 and Cpd9. Excellent relative percentage activity of 93.24 ± 2.12 and
94.64 ± 2.14 was obtained for Cpd5 and Cpd10 respectively. These values are a little high while comparing to the value obtained by doxorubicin (89.47 ± 2.87). Astonishingly, the IC₅₀ of Cpd5 and Cpd9 was in nanomolar (42.56 ± 0.002 nM and 36.48 ± 0.002 nM respectively) but Doxorubicin consumed 1.025 µM. Fig. (7) illustrates the morphological variation between before and after treating Cpd5. The green arrow in the right side figure indicates the necrosis formed due to drug action from Cpd5. This indicates the anti-breast cancer efficacy of these compounds and the possibilities of these compounds to become future breast cancer therapeutics. Moreover, these compounds were found without any activity on normal breast epithelial cells (MCF-10).

CONCLUSION

In conclusion, compounds Cpd1-10 were evaluated for their COX-2 inhibition potentials in or-
order to develop the COX-2 inhibitors and anti-inflammatory agents. Lipopolysaccharide can trigger mononuclear phagocytes (macrophages) and other types of cells to excrete TNF-α, IL-6, IL-10, and IL-1β. Thus, we have measured pro-inflammatory TNF-α, IL-6, IL-1β, and anti-inflammatory IL-10. Elevated IL-10 and reduced TNF-α, IL-6, IL-1β levels after treating compounds Cpd5 and Cpd9 has indicated their anti-inflammatory efficacy while comparing with the standard, indomethacin. In addition, as COX-2 is highly involved or associated with numerous cancers and abnormal tumor developments in human, cytotoxicity effects of these compounds on various cancer cell lines ensured their anticancer activity potentials, especially against breast cancer. Thus, apart from COX-2 inhibition potentials, Cpd5 and Cpd9 could act as alternative therapeutic agents against breast cancer. The need for further animal model evaluation recommended from all the findings from in silico and in vitro evaluations.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Institutional Committee for cell culture studies (ICCS-VIT/2017-18), Vellore Institute of Technology (VIT), Vellore-632014, India.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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