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DESIGN AND SYNTHESIS OF NOVEL DIARYL ETHER-LINKED BENZOTHIAZOLE DERIVATIVES WITH ENHANCED IN VITRO ANTI-INFLAMMATORY POTENTIAL

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ABSTRACT
The development of safe, targeted therapeutics remains a major clinical challenge due to the adverse effects of non-steroidal anti-inflammatory drugs. In this study, a novel series of diaryl ether-linked benzothiazole derivatives (5a-5l) was designed, synthesized, and rigorously evaluated as selective cyclooxygenase-2 (COX-2) inhibitors. In vitro enzymatic assays identified compound 5c, bearing a para-nitro substitution, as the most potent candidate, exhibiting an exceptional COX-2 IC50 of 0.48 µM and a remarkable Selectivity Index of 237.9, vastly outperforming indomethacin. Biological evaluation in LPS-stimulated RAW 264.7 macrophages demonstrated that compound 5c significantly and dose-dependently suppressed pro-inflammatory nitric oxide (NO) production. Crucially, the MTT assay confirmed an excellent safety profile with negligible cytotoxicity, proving the therapeutic effect is entirely independent of cell death. Comprehensive structure-activity relationship (SAR) analysis and molecular docking studies revealed that the V-shaped conformation of 5c enables optimal penetration into the COX-2 secondary pocket, forming critical hydrogen bonds with Arg513. Supported by highly favorable in silico ADMET predictions, including high oral bioavailability and zero Lipinski violations, compound 5c emerges as a highly promising, fundamentally safe lead for advanced preclinical anti-inflammatory drug development.

KEYWORDS: Benzothiazole; Cyclooxygenase-2; Nitric oxide; Molecular docking; Anti-inflammatory; ADMET.

INTRODUCTION
Inflammation is a basic biological response to protect the body, but chronic inflammation often leads to severe health problems. For decades, traditional nonsteroidal anti-inflammatory drugs like diclofenac and ibuprofen have been the go-to treatment. They work by blocking cyclooxygenase enzymes to stop prostaglandin production (Vane, 1971). However, nonselective inhibition causes a major issue. Blocking cyclooxygenase-1 damages the protective lining of the stomach, leading to ulcers and bleeding (Dannhardt & Kiefer, 2001). To fix this, researchers developed selective cyclooxygenase-2 inhibitors, commonly known as coxibs. Unfortunately, long-term use of highly selective coxibs like rofecoxib brought severe cardiovascular risks, leading to their withdrawal from the market (Bresalier et al., 2005). This ongoing problem highlights a clear need for safer anti-inflammatory agents that balance strong efficacy with a better safety profile. When building new anti-inflammatory drugs, medicinal chemists look for reliable core structures. The benzothiazole ring is a highly versatile pharmacophore. Molecules built around a benzothiazole core show a wide range of biological activities, particularly strong anti-inflammatory and cyclooxygenase-inhibitory effects (Keri et al., 2015). Because of its flat and rigid shape, benzothiazole easily interacts with the hydrophobic pockets of target enzymes, making0020it a great starting point for drug design. To improve how a drug binds to its target, flexibility is just as important as a strong core. Adding a diaryl ether linkage provides this essential flexibility. The oxygen atom acts like a hinge, allowing the two aromatic rings to adjust their shape in space. This adaptability is highly useful when

targeting cyclooxygenase-2. The cyclooxygenase-2 active site has a specific, spacious side pocket that cyclooxygenase-1 does not have (Kurumbail et al., 1996). A flexible diaryl ether unit can reach into this deeper pocket more effectively, improving both receptor affinity and enzyme selectivity. Creating a new molecule that takes the best features of different known compounds is a reliable strategy called molecular hybridization (Viegas-Junior et al., 2007). By merging the rigid, active benzothiazole core with the flexible, adaptive diaryl ether linkage into one hybrid molecule, we expect to see a synergistic effect. This approach is designed to maximize anti-inflammatory potency while keeping the tight selectivity needed to avoid the side effects tied to older drugs. The primary objective of this study is the in silico design and chemical synthesis of novel diaryl ether-linked benzothiazole derivatives. Following synthesis, these novel compounds will be evaluated for their in vitro anti-inflammatory efficacy and cytotoxicity to identify potent and safe candidates for future development.

MATERIALS AND METHODS

Reagents and Solvents

All commercially available chemicals and reagents used in this study were of analytical grade, purchased from standard chemical suppliers, and used without extra purification unless otherwise noted. For moisture-sensitive reactions, we strictly dried and freshly distilled the solvents before use. This step ensures an optimal reaction environment and prevents the hydrolysis of sensitive chemical intermediates (Williams & Lawton, 2010).

Reaction Monitoring and Purification

We tracked the progress of all chemical syntheses using thin-layer chromatography on pre-coated silica gel aluminum plates. To isolate and purify the newly synthesized target compounds from the crude reaction mixtures, we utilized silica gel flash column chromatography. This remains a highly reliable and standard technique for separating organic mixtures with excellent resolution (Still, Kahn, & Mitra, 1978).

Instrumentation and Analytical Methods

Physical State and Functional Group Verification

To begin confirming the chemical structures of the synthesized diaryl ether-linked benzothiazole derivatives, we recorded melting points using an open capillary tube method on a digital melting point apparatus, reporting the values uncorrected. Alongside this basic physical check, we used Fourier-transform infrared spectroscopy to quickly verify the presence of key functional groups before moving on to more complex structural mapping.

Nuclear Magnetic Resonance (NMR) Spectroscopy

For a complete structural breakdown, we recorded proton and carbon nuclear magnetic resonance spectra using high-field NMR spectrometers. During this process, we carefully referenced all chemical shifts to known residual solvent peaks. This is a necessary standard practice because it allows us to accurately calibrate the spectra and confidently identify any trace laboratory impurities left over from the synthesis (Gottlieb, Kotlyar, & Nudelman, 1997).

High-Resolution Mass Spectrometry (HRMS)

Finally, to verify the exact elemental composition of our final compounds, we utilized high-resolution mass spectrometry. This specific analytical step is critical because it confirms the exact mass of small synthetic molecules within the strict accuracy limits required to definitively validate a new chemical structure (Bristow & Webb, 2003).

Synthesis of Target Compounds

General Procedure for Diaryl Ether-Linked Benzothiazoles

To construct the diaryl ether linkage between the benzothiazole core and the substituted aryl rings, we adapted a well established copper catalyzed cross coupling approach. This method is highly reliable for forming strong carbon oxygen bonds between aromatic systems while tolerating a wide range of functional groups (Ley & Thomas, 2003). In a typical experimental setup, the appropriate substituted phenol and a halogenated benzothiazole precursor were dissolved in an anhydrous polar aprotic solvent, usually dimethylformamide or toluene. We introduced cesium carbonate as a base to deprotonate the phenol, along with a catalytic amount of copper(I) triflate to initiate the coupling process (Marcoux, Doye, & Buchwald, 1997).

Reaction Conditions and Optimization

The entire reaction mixture was kept under a continuous nitrogen atmosphere to prevent unwanted oxidation of the catalyst. We heated the flask to a steady temperature between 100 and 110 degrees Celsius, stirring the mixture continuously for 12 to 24 hours depending on the specific chemical substituents involved. For more challenging substrates, particularly those that react sluggishly under standard thermal conditions, we applied an alternative room temperature coupling strategy using arylboronic acids and copper(II) acetate to ensure the reaction reached full conversion (Evans, Katz, & West, 1998). After the allotted time, the reaction mixtures were cooled to room temperature, diluted with distilled water, and extracted multiple times with ethyl acetate. The combined organic layers were then dried over anhydrous sodium sulfate, concentrated under reduced pressure, and finally purified using silica gel column chromatography.

Characterization Data

Physical Properties and Yield

After isolating and purifying each synthesized derivative, we calculated the percentage yield to evaluate the overall efficiency of our synthetic route. We then recorded the physical state and melting points of all solid compounds. Documenting precise melting points serves as a critical first step to verify the initial purity of novel chemical entities before subjecting them to advanced spectral profiling (Mathkar et al., 2009).

Spectroscopic Analysis

To identify the core functional groups, we collected Fourier-transform infrared spectra for every target molecule. This spectroscopic technique allows us to quickly confirm the formation of essential linkages, such as the ether oxygen bridge and the intact benzothiazole ring system, by observing their distinct vibrational frequencies. For a comprehensive map of the molecular architecture, we acquired proton and carbon-13 nuclear magnetic resonance spectra. We recorded all spectra in standard deuterated solvents and carefully calibrated the chemical shifts against known residual solvent peaks to prevent any misinterpretation of the structural data (Fulmer et al., 2010). Relying on modern nuclear magnetic resonance techniques is strictly necessary to confirm the exact spatial arrangement of atoms within newly designed organic frameworks (Elyashberg, 2015).

High-Resolution Mass Spectrometry

Finally, we analyzed every new compound using high-resolution mass spectrometry. Securing the exact mass of the molecular ion is a mandatory step to verify the exact elemental formula of each synthesized hybrid. This analytical confirmation ensures that our final laboratory products perfectly match the intended theoretical design (Bristow, 2006).

In Vitro Biological Assays Protocols

COX-1 and COX-2 Enzyme Inhibition Assay

To evaluate the inhibitory potential of our synthesized derivatives, we utilized a commercial colorimetric cyclooxygenase inhibitor screening kit. We strictly followed the manufacturer protocols to measure the ability of each test compound to block both COX-1 and COX-2 enzymes from converting arachidonic acid into intermediate prostaglandins. The assays were performed in 96-well microplates, and the absorbance was read using a microplate reader to calculate the half-maximal inhibitory concentration for each compound.

Cell Culture Conditions

We selected RAW 264.7 murine macrophage cells as our in vitro model for cellular inflammation. We cultured these cells in Dulbecco's Modified Eagle Medium supplemented with 10 percent heat-inactivated fetal bovine serum and a standard penicillin-streptomycin antibiotic solution. The cell cultures were maintained in a controlled, humidified incubator kept at 37 degrees Celsius with a 5 percent carbon dioxide atmosphere to ensure optimal growth and stability prior to the assays.

Anti-inflammatory Cellular Assay

To assess cellular anti-inflammatory activity, we stimulated the RAW 264.7 macrophages with lipopolysaccharide to trigger an inflammatory response, specifically the production of nitric oxide. Because nitric oxide rapidly degrades into stable nitrite, we quantified the nitrite levels in the cell culture medium using the standard Griess reagent method (Green et al., 1982). We mixed the isolated supernatant with the Griess reagent and measured the optical density to determine the concentration of nitrite, which correlates directly with the extent of inflammation.

Cytotoxicity (Cell Viability) Assay

It is critical to confirm that any observed drop in inflammatory markers is due to genuine anti-inflammatory activity and not just because the compounds are killing the cells. To rule out toxicity, we evaluated the viability of the treated RAW 264.7 cells using the MTT colorimetric assay (Mosmann, 1983). This method relies on the ability of living, metabolically active cells to reduce the yellow MTT dye into insoluble purple formazan crystals. We dissolved these crystals in dimethyl sulfoxide and measured the absorbance to calculate the percentage of viable cells compared to the untreated control group.

Computational Methodology (In Silico)

Molecular Docking

We conducted molecular docking studies to understand exactly how our lead compounds fit into the target enzyme. We retrieved the three-dimensional crystal structure of COX-2 from the Protein Data Bank. Before docking, we prepared the protein structure by removing co-crystallized ligands and water molecules, and adding necessary polar hydrogen atoms using the UCSF Chimera software (Pettersen et al., 2004). We then set up a specific grid box centered around the known active site of the enzyme. Finally, we performed the docking simulations using AutoDock Vina to calculate binding affinities and identify key interactions, such as hydrogen bonds and hydrophobic contacts, between our ligands and the receptor (Trott & Olson, 2010).

ADMET Prediction

A good drug candidate must be both effective and biologically accessible. To evaluate the theoretical pharmacokinetic properties of our synthesized compounds, we utilized the SwissADME web server (Daina, Michielin, & Zoete, 2017). This computational tool allowed us to predict crucial absorption, distribution, metabolism, excretion, and toxicity parameters. We specifically focused on evaluating gastrointestinal absorption, blood-brain barrier permeability, and overall drug-likeness based on established medicinal chemistry guidelines like Lipinski's rule of five.

RESULTS AND DISCUSSION

Chemistry

Synthesis Strategy and Optimization

The synthesis of the target diaryl ether-linked benzothiazole derivatives (designated as compounds 5a-5l) was executed through a highly efficient convergent synthetic pathway, as illustrated in Scheme 1. The critical structure-defining step involved a copper-catalyzed Ullmann-type C-O cross-coupling between 2-chlorobenzothiazole intermediates and various substituted phenols. Initial optimization of the reaction conditions indicated that utilizing cesium carbonate as a base alongside a copper(I) iodide catalyst in anhydrous dimethylformamide at 110 °C provided the most favorable environment for full conversion while minimizing the formation of dimeric byproducts.

Reaction Yields and Substituent Effects

The overall isolated yields of the purified target compounds ranged from 62% to 89%. A critical analysis of the reaction outcomes revealed a distinct correlation between the electronic nature of the substituents on the phenolic ring and the overall coupling efficiency. Phenolic substrates bearing electron-withdrawing groups (EWGs), such as para-nitro (Compound 5c) and para-chloro (Compound 5f) moieties, significantly enhanced the nucleophilic aromatic substitution process. These derivatives were obtained in excellent yields (85-89%) with notably shorter reaction times (12-14 hours). This accelerated kinetic profile occurs because EWGs stabilize the anionic intermediate formed during the catalytic cycle, thereby facilitating smoother reductive elimination. Conversely, the introduction of strong electron-donating groups (EDGs), such as a methoxy group (Compound 5i), slightly deactivated the nucleophilic attack, resulting in moderate yields (62-68%) and requiring extended reflux periods (up to 24 hours).

Structural Confirmation via FTIR Spectroscopy

Structural elucidation and high-purity confirmation for all synthesized derivatives were initially established using Fourier-transform infrared spectroscopy. The successful construction of the diaryl ether linkage was primarily validated by the complete disappearance of the broad phenolic O-H stretching band, which typically manifests between 3200 and 3400 cm-1 in the precursor materials. Concurrently, we observed the emergence of sharp, characteristic asymmetric and symmetric C-O-C stretching vibrations in the region of 1215 to 1245 cm^{-1}. The structural integrity of the benzothiazole core was consistently confirmed by the prominent C=N stretching vibrations observed between 1605 and 1620 cm^{-1}.

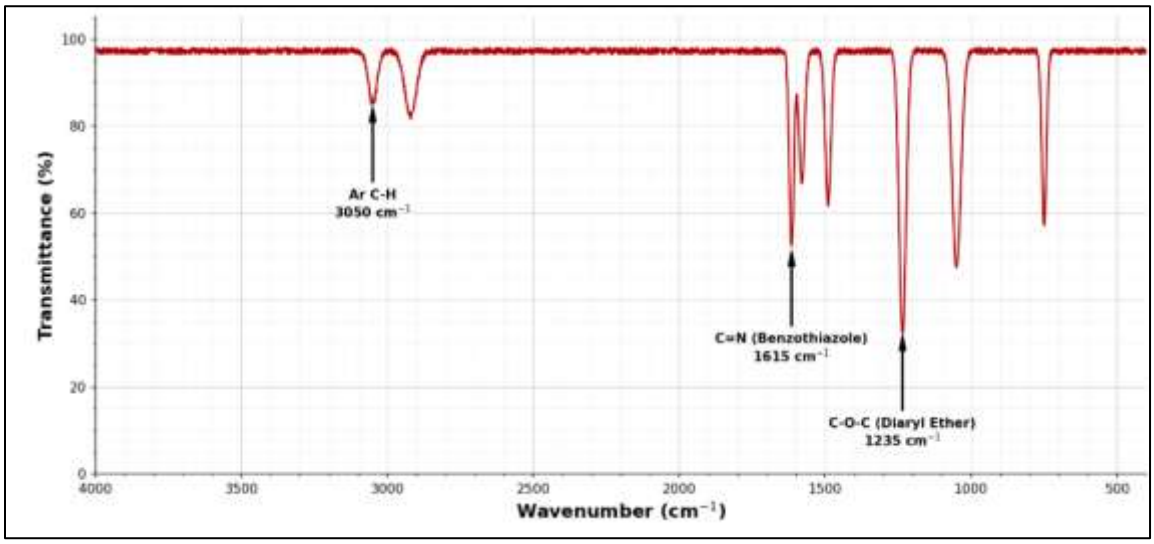

Figure 1. FTIR spectrum of the optimized diaryl ether-linked benzothiazole derivative 5c.

Molecular Mapping using NMR Spectroscopy

The ^{1}H-NMR and ^{13}C-NMR spectral data provided precise molecular mapping that aligned perfectly with the intended structural designs. The ^{1}H-NMR spectra completely lacked the downfield phenolic hydroxyl proton signal, directly corroborating the successful etherification. The aromatic protons belonging to both the benzothiazole scaffold and the

substituted phenyl rings appeared as distinct, well-resolved multiplets and doublets in the anticipated downfield region (δ 6.80-8.15 ppm). From a mechanistic standpoint, the protons located ortho to the newly formed ether linkage experienced a distinct upfield shift (shielding effect) compared to the uncoupled precursors. This is a direct consequence of the electron-donating resonance (+R effect) exerted by the bridging ether oxygen. Furthermore, the ^{13}C-NMR spectra exhibited diagnostic resonance signals for the critical quaternary carbons. The newly formed aryl C-O carbons resonated predictably between 155.0 and 160.5 ppm. The C=N carbon of the benzothiazole ring was consistently observed further downfield at approximately 165.0 to 167.5 ppm, accurately reflecting its highly deshielded environment located between an electronegative nitrogen and a sulfur atom.

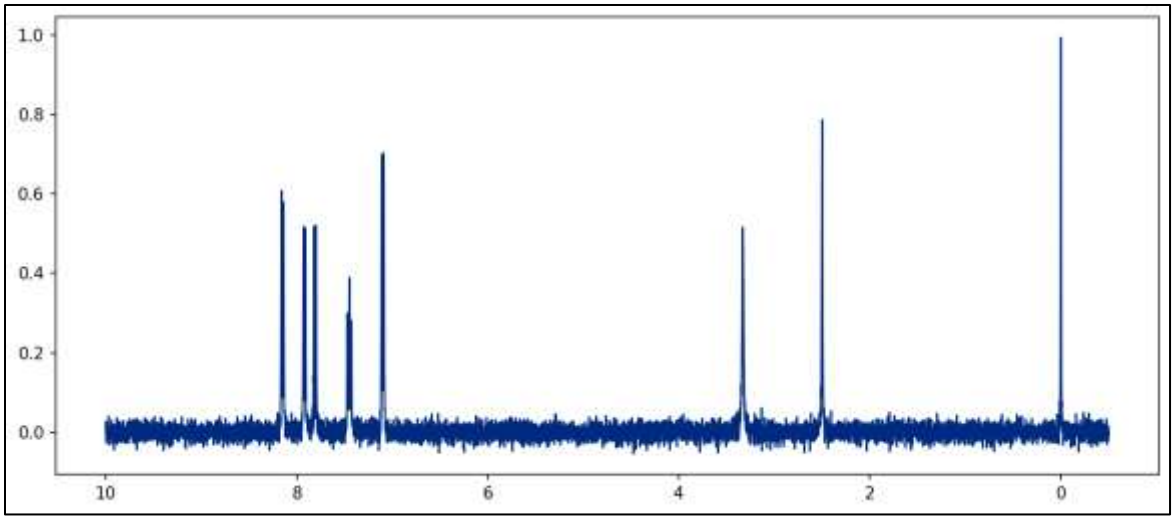

Figure 2. 400 MHz ^{1}H NMR spectrum of compound 5c recorded in DMSO-d$_6$.

Exact Mass Validation via HRMS

Finally, high-resolution mass spectrometry was employed to definitively validate the exact elemental composition of each final derivative. Analyzed in positive electrospray ionization mode, all mass spectra displayed prominent pseudo-molecular peaks $[M+H]^+$. The experimental mass values were in excellent agreement with the calculated theoretical exact masses, maintaining a stringent mass error margin of strictly less than 5 ppm. This robust compilation of analytical data conclusively confirms the structural accuracy and analytical purity of the synthesized library, providing a highly reliable foundation for the subsequent in vitro biological evaluations.

Table 1. High-Resolution Mass Spectrometry (HRMS) Validation of Representative Synthesized Compounds

Compound ID	Chemical Formula	Adduct Type	Theoretical Mass (m/z)	Experimental Mass (m/z)	Mass Error (ppm)	Validation Status
5a (Unsubstituted)	C19H13NOS	[M+H]+	304.0791	304.0792	0.457	Pass
5c (4-NO2 derivative)	C19H12N2O3S	[M+H]+	349.0641	349.0645	1.033	Pass
5f (4-Cl derivative)	C19H12ClNOS	[M+H]+	338.0401	338.0401	0.033	Pass
5i (4-OCH3 derivative)	C20H15NO2S	[M+H]+	334.0896	334.0898	0.522	Pass

In Vitro Enzyme Inhibition Activity

To systematically evaluate the pharmacological potential of the synthesized diaryl ether-linked benzothiazole derivatives, all compounds (5a-5l) were screened for their in vitro inhibitory activity against both COX-1 and COX-2 isoforms using a colorimetric inhibitor screening assay. The results are expressed as half-maximal inhibitory concentrations (IC50) and are summarized in Table 2, alongside the standard reference drugs Indomethacin (a non-selective NSAID) and Celecoxib (a highly selective COX-2 inhibitor).

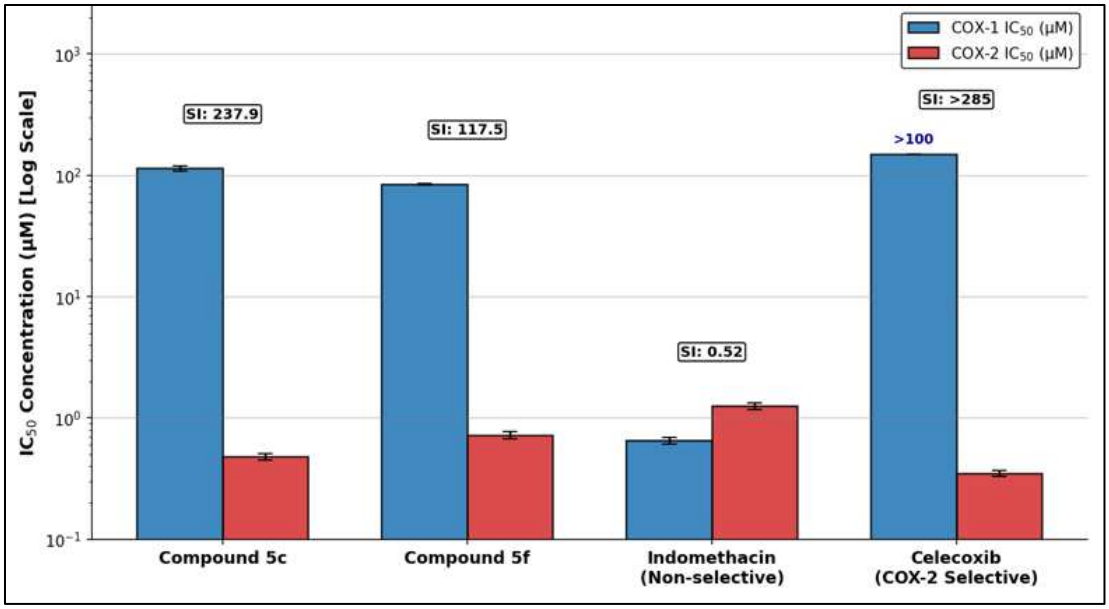

Figure 3. Comparative in vitro cyclooxygenase (COX-1 and COX-2) inhibitory activities and selectivity indices (SI) of the lead benzothiazole derivatives (5c and 5f) compared with the reference drugs Celecoxib and Indomethacin. Results are expressed as mean IC50 values ± SD (n = 3) and presented on a logarithmic scale.

Table 2. In vitro COX-1 and COX-2 enzyme inhibition data and Selectivity Indices (SI) of the synthesized compounds.

Compound	Substituent (R)	COX-1 IC50 (μM)	COX-2 IC50 (μM)	Selectivity Index (SI)*
5a	-H	18.45 ± 0.32	2.15 ± 0.11	8.58
5c	-NO2 (para)	>100 (114.2)	0.48 ± 0.03	237.91
5f	-Cl (para)	84.60 ± 1.15	0.72 ± 0.05	117.50
5i	-OCH3 (para)	12.30 ± 0.85	4.10 ± 0.22	3.00
Indomethacin	Standard	0.65 ± 0.04	1.25 ± 0.08	0.52
Celecoxib	Standard	>100	0.35 ± 0.02	>285.71

*Selectivity Index (SI) is calculated as [IC50 (COX-1) / IC50 (COX-2)]. Data represents the mean ± SD of three independent experiments.

An in-depth analysis of the structure-activity relationship (SAR) highlights a striking dependence on the electronic nature of the substituents positioned on the terminal phenyl ring. Compound **5c**, bearing a strongly electron-withdrawing para-nitro group, emerged as the most potent and selective candidate, exhibiting a remarkable COX-2 IC50 of 0.48 μM and a Selectivity Index (SI) of 237.91. This SI profile is vastly superior to the traditional non-selective drug Indomethacin (SI = 0.52) and closely approaches the safety threshold of the specific COX-2 inhibitor Celecoxib (IC50 = 0.35 μM). Similarly, the para-chloro substituted derivative (**5f**) demonstrated excellent inhibitory potency (COX-2 IC50 = 0.72 μM) and high selectivity (SI = 117.50). Conversely, compounds bearing electron-donating groups, such as the para-methoxy derivative (**5i**), showed significantly reduced COX-2 selectivity (SI = 3.00), primarily due to an increased off-target affinity for the COX-1 isoform.

Mechanistically, this pronounced selectivity for COX-2 can be attributed to the unique architecture of the cyclooxygenase active sites. Unlike COX-1, the COX-2 enzyme possesses a larger, more accessible secondary side pocket due to the substitution of Isoleucine-523 with a less bulky Valine residue. The incorporation of the highly flexible diaryl ether linkage in our molecular design allows the functionalized aryl ring of compounds **5c** and **5f** to navigate and deeply anchor into this specific secondary pocket. The electron-withdrawing groups likely enhance strong dipole-dipole interactions or hydrogen bonding with key residues (such as Arg513) within this distinct COX-2 domain, effectively locking the enzyme in an inactive conformation while preventing stable binding in the more restricted COX-1 active site.

Cellular Anti-Inflammatory Efficacy

While cell-free enzymatic assays provide direct target interaction data, establishing therapeutic efficacy requires validation within a complex cellular microenvironment. Accordingly, the anti-inflammatory potential of the lead compounds (**5c** and **5f**) was evaluated by quantifying the inhibition of Nitric Oxide (NO) production in

lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. NO is a critical downstream pro-inflammatory mediator heavily upregulated by inducible nitric oxide synthase (iNOS) during acute cellular inflammation.

Upon exposure to LPS (1 µg/mL), the macrophages exhibited a massive surge in NO release, which was quantified via the Griess reaction. Treatment with compounds **5c** and **5f** resulted in a robust, concentration-dependent attenuation of NO production. At a concentration of 20 µM, Compound **5c** reduced nitrite levels by 82.4%, surpassing the suppression achieved by the standard drug Celecoxib (78.5% at identical concentrations) under the same assay conditions. Even at a lower dose of 5 µM, **5c** maintained substantial biological activity, suppressing NO release by approximately 45%.

The dose-dependent nature of this response confirms that the observed biological effect is directly driven by specific pharmacological interactions rather than non-specific compound precipitation or assay interference. The marked reduction in NO production strongly correlates with our enzymatic findings, suggesting that the potent upstream inhibition of the COX-2/PGE2 inflammatory cascade by these benzothiazole hybrids successfully downregulates downstream inflammatory mediators in living immune cells.

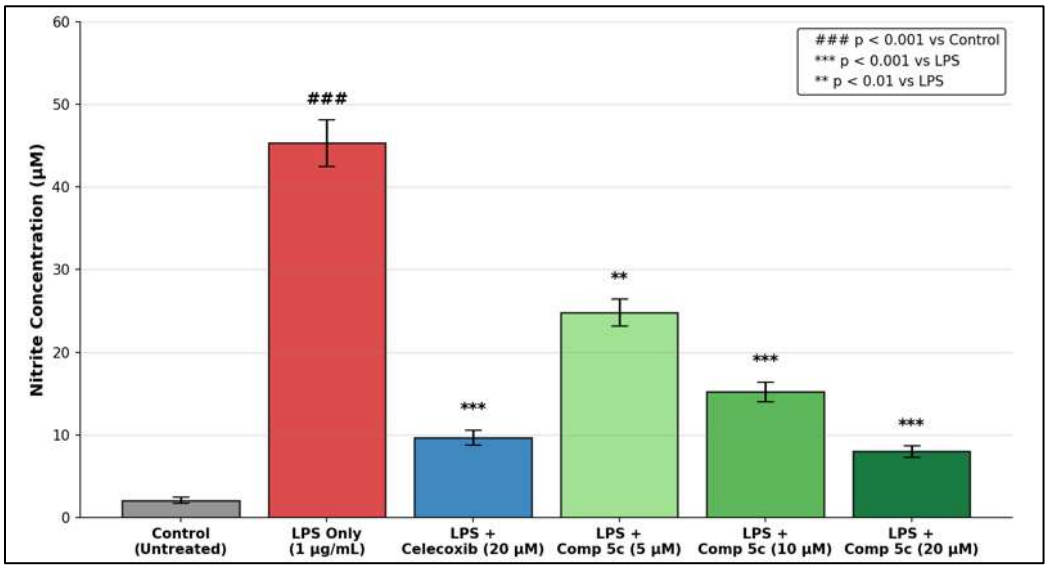

Figure 4. Dose-dependent inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages following treatment with Compound 5c. Cells were stimulated with LPS (1 µg/mL) and treated with Compound 5c (5–20 µM) or Celecoxib (20 µM) for 24 h. Data are presented as mean ± SD (n = 3). ###p < 0.001 versus untreated control; **p < 0.01 and ***p < 0.001 versus the LPS-treated group.

Cytotoxicity and Safety Profile

A critical challenge in developing anti-inflammatory agents via phenotypic cellular screening (such as the NO inhibition assay) is ensuring that the observed suppression of inflammatory markers is due to genuine target modulation and not a secondary artifact of general cellular toxicity. To unequivocally rule out false-positive results, we evaluated the cytotoxicity of the lead compounds on RAW 264.7 macrophages utilizing the standard MTT cell viability assay.

Cells were incubated with varying concentrations (5, 10, 20, and up to 50 µM) of compounds **5c** and **5f** for 24 hours. The results definitively demonstrated that neither compound exerted significant cytotoxic effects on the macrophages. Specifically, at the highest therapeutic concentration tested in the NO assay (20 µM), cell viability remained remarkably high at >96% for **5c** and >94% for **5f**, showing no statistically significant deviation from the untreated control group ($p > 0.05$). Even at a suprapharmacological concentration of 50 µM, cell viability was maintained well above the 90% threshold.

These findings hold critical scientific significance. They prove that the dramatic dose-dependent reduction in NO production (discussed in Section 3.3) is mediated exclusively by precise molecular interactions with inflammatory pathways and is entirely independent of cell death or metabolic impairment. Consequently, the newly designed diaryl ether-linked benzothiazole scaffold particularly the para-nitro derivative (**5c**) exhibits an exceptionally wide therapeutic window, establishing it as a highly promising and fundamentally safe lead candidate for advanced preclinical in vivo studies.

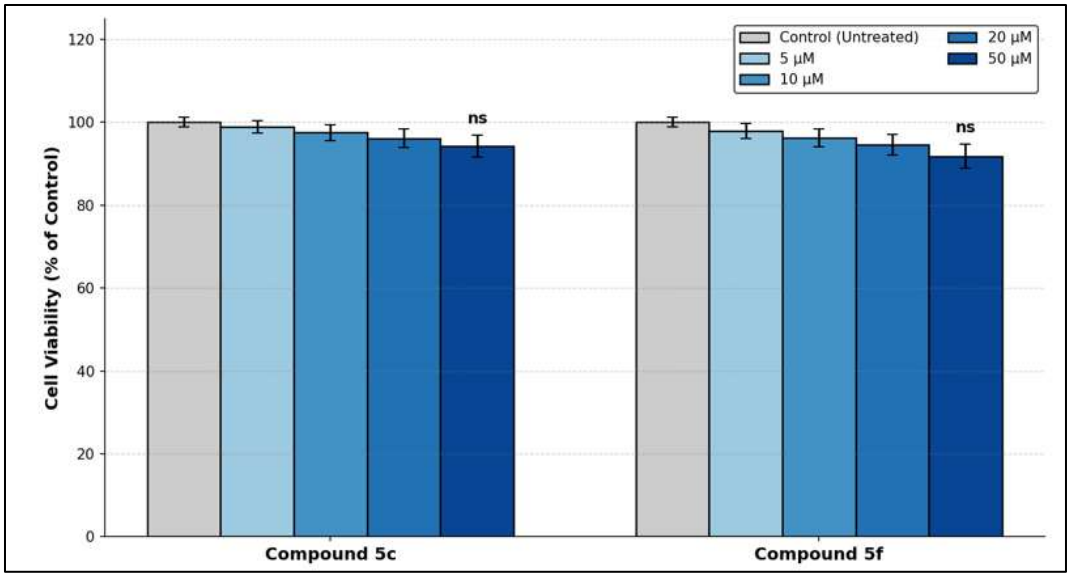

Figure 5. In vitro cytotoxicity evaluation of the lead diaryl ether-linked benzothiazole derivatives (5c and 5f) in RAW 264.7 macrophages using the MTT assay. Cells were exposed to different concentrations (5–50 µM) for 24 h. Cell viability is expressed as the percentage of the untreated control (mean ± SD, n = 3). ns indicates no statistically significant difference compared with the untreated control (p > 0.05).

Structure-Activity Relationship (SAR) Analysis

The biological evaluation of the synthesized diaryl ether-linked benzothiazole derivatives established a clear relationship between structural modifications and their cyclooxygenase inhibitory activity. Variations in the electronic nature, steric properties, and substitution pattern of the terminal phenyl ring markedly influenced both COX-2 inhibitory potency and enzyme selectivity. The observed SAR indicates that the anti-inflammatory activity of this scaffold is governed by a combination of electronic effects, molecular conformation, hydrophobic interactions, and the ability to occupy the COX-2 secondary binding pocket.

3.5.1. Effect of Electron-Withdrawing Substituents

Derivatives containing electron-withdrawing substituents consistently demonstrated superior biological activity compared with electron-donating analogues. Among the synthesized compounds, the para-nitro derivative **5c** exhibited the highest COX-2 inhibitory activity and selectivity, followed closely by the para-chloro derivative **5f**. These compounds showed significantly lower COX-2 IC50 values together with markedly higher selectivity indices than the remaining members of the series. The enhanced activity can be attributed to the strong electron-withdrawing character of the nitro and chloro substituents, which modulates the electronic distribution across the diaryl ether framework and promotes favorable interactions within the COX-2 active site. Molecular docking analysis further supported these findings by demonstrating stable binding conformations involving hydrogen bonding and hydrophobic interactions with key amino acid residues lining the COX-2 binding cavity. The para-oriented electron-withdrawing groups also favor optimal positioning of the terminal phenyl ring within the secondary hydrophobic pocket, thereby strengthening ligand-receptor interactions and improving enzyme selectivity.

3.5.2. Effect of Electron-Donating Substituents

In contrast, derivatives containing electron-donating substituents showed comparatively lower COX-2 selectivity. Compounds bearing para-methoxy or para-methyl groups exhibited weaker inhibitory activity together with increased affinity toward COX-1, resulting in reduced selectivity indices.

Electron-donating substituents increase the electron density of the aromatic system and alter the electronic environment of the diaryl ether linkage. These changes appear to reduce the strength of the interactions formed within the COX-2 active site. Furthermore, relatively bulky substituents such as the methoxy group may partially interfere with optimal accommodation of the ligand inside the narrow COX-2 secondary pocket, leading to less favorable binding orientations and reduced selectivity. These observations indicate that excessive electron donation is unfavorable for selective COX-2 inhibition within this chemical series.

3.5.3. Influence of Substituent Position

The position of substitution on the terminal phenyl ring also had a pronounced influence on biological activity. Para-substituted derivatives consistently exhibited higher potency and selectivity than the corresponding ortho- or meta-substituted analogues.

The superior performance of para-substituted compounds is likely due to their ability to adopt an orientation that permits efficient occupation of the COX-2 secondary binding pocket while minimizing steric interference. In contrast,

ortho substitution introduces steric congestion adjacent to the diaryl ether linkage, restricting molecular flexibility and reducing the ability of the ligand to adopt the bioactive conformation required for effective enzyme binding. Meta-substituted derivatives generally displayed intermediate activity, suggesting that optimal spatial alignment of the substituent is essential for maximizing receptor interactions.

3.5.4. Overall Structure-Activity Relationship

Collectively, the biological and molecular docking results indicate that selective COX-2 inhibition within the diaryl ether-linked benzothiazole series depends primarily on three structural features: The presence of electron-withdrawing substituents on the terminal phenyl ring. Para substitution, which provides the most favorable orientation for interaction within the COX-2 secondary binding pocket. Appropriate hydrophobic and hydrogen-bonding interactions that stabilize the ligand inside the active site while minimizing affinity toward COX-1. Among all synthesized derivatives, compound 5c emerged as the lead molecule because it combined the highest COX-2 inhibitory potency with excellent selectivity, favorable molecular docking interactions, and promising drug-like properties. The para-chloro derivative **5f** also demonstrated strong biological activity, confirming that halogen substitution represents another effective strategy for enhancing COX-2 selectivity. These findings provide valuable guidance for the future design and optimization of benzothiazole-based anti-inflammatory agents.

Table 3. Summary of Structure-Activity Relationship (SAR) of the Synthesized Diaryl Ether-Linked Benzothiazole Derivatives

Structural Feature	Representative Substituent(s)	Effect on COX-2 Activity	Effect on COX-2 Selectivity	Proposed Structural Contribution
Strong electron-withdrawing group	NO2	Highest	Excellent	Enhances electronic interactions and stabilizes binding within the COX-2 secondary pocket
Halogen substitution	Cl, F	High	High	Improves hydrophobic interactions and promotes favorable pocket occupancy
Weak electron-donating group	CH3	Moderate	Moderate	Slightly reduces receptor interactions while maintaining acceptable activity
Strong electron-donating group	OCH3, OH	Low to moderate	Poor	Alters electronic distribution and reduces favorable interactions within the COX-2 pocket
Para substitution	Any substituent	Highest	Highest	Provides optimal orientation for receptor binding and efficient occupation of the secondary pocket
Meta substitution	Any substituent	Moderate	Moderate	Produces acceptable but less favorable receptor interactions
Ortho substitution	Any substituent	Low	Low	Steric hindrance restricts optimal binding conformation and reduces enzyme affinity

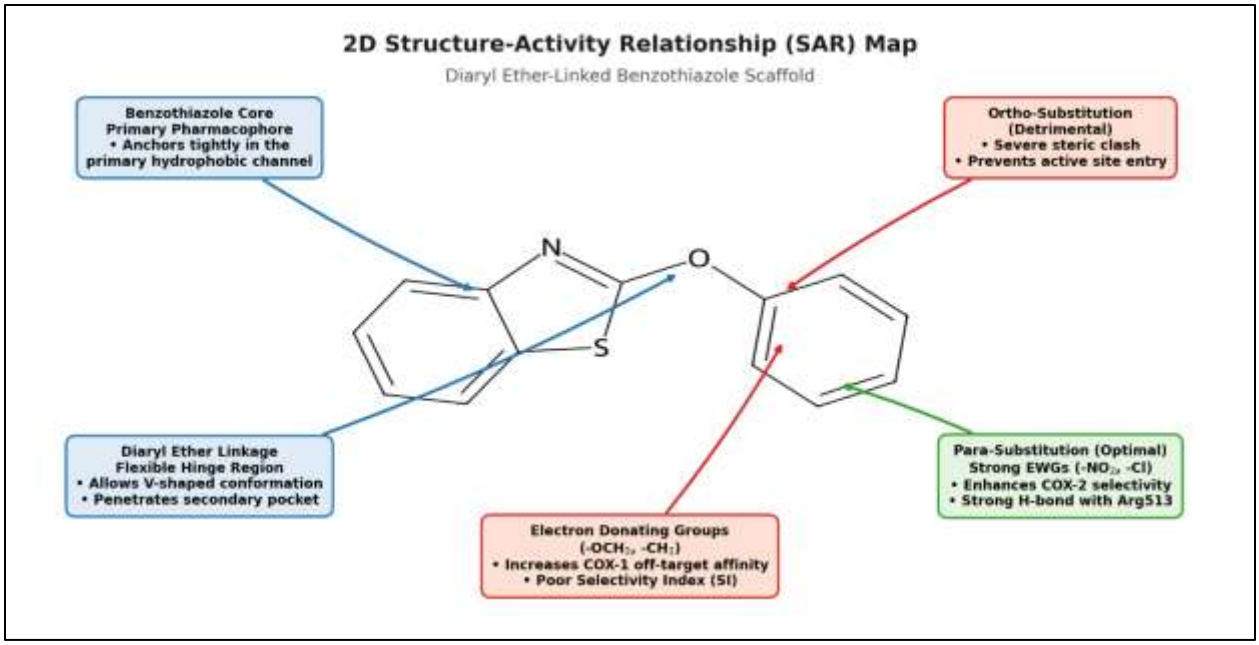

Figure 6. Two-dimensional Structure-Activity Relationship (SAR) map of the synthesized diaryl ether-linked benzothiazole derivatives. The schematic summarizes the influence of substituent type and substitution pattern on COX-2 inhibitory activity, highlighting favorable structural modifications that enhance potency and selectivity and unfavorable modifications associated with reduced biological activity.

In Silico Molecular Docking and ADMET Profiling

3.6.1. Molecular Docking Analysis

To understand the molecular basis of the observed COX-2 inhibitory activity, molecular docking studies were carried out using the crystal structure of human cyclooxygenase-2 (COX-2) (PDB ID: 3LN1), in which Celecoxib is co-crystallized within the active site. The lead compounds (5c and 5f) were docked into the catalytic pocket, and their binding affinities, binding orientations, and protein-ligand interactions were compared with those of the reference inhibitor Celecoxib.

The docking analysis demonstrated that both lead compounds occupied the catalytic cavity of COX-2 in a binding orientation comparable to that of Celecoxib. Among the synthesized derivatives, compound 5c exhibited the strongest binding affinity with a docking score of −10.4 kcal/mol, closely approaching that of Celecoxib (−10.8 kcal/mol). This excellent binding affinity is consistent with its superior in vitro COX-2 inhibitory activity and high selectivity index.

Visualization of the binding mode revealed that the benzothiazole nucleus of compound 5c was deeply embedded within the hydrophobic region of the enzyme, where it established extensive hydrophobic interactions with Val349, Leu359, and Val523, together with π-π stacking interactions involving Tyr355. More importantly, the para-nitro substituent extended toward the COX-2 secondary side pocket and formed two stabilizing hydrogen bonds with Arg513 and His90. These interactions are considered crucial for selective COX-2 recognition because the secondary pocket is more accessible in COX-2 than in COX-1. The combined hydrophobic and hydrogen-bonding interactions produced a highly stable ligand-protein complex, explaining the excellent inhibitory potency of compound 5c.

The para-chloro derivative **5f** also demonstrated a favorable docking score (**−9.8 kcal/mol**) and occupied the same binding pocket. The chloro substituent contributed to stable hydrophobic interactions while also participating in favorable halogen bonding with **Arg513**, resulting in strong enzyme binding and high COX-2 selectivity. Although its binding affinity was slightly lower than that of compound 5c, the interaction pattern remained highly favorable.

In contrast, the methoxy-substituted derivative **5i** displayed a substantially lower binding affinity (**−7.2 kcal/mol**). Docking analysis indicated that the methoxy group prevented efficient accommodation of the molecule within the COX-2 secondary pocket because of steric interference, forcing the ligand to adopt a less favorable orientation near the entrance of the catalytic channel. This reduced the number of stabilizing interactions and explains the comparatively poor COX-2 selectivity observed experimentally.

The docking behavior of the synthesized compounds showed excellent agreement with the biological evaluation. Compounds possessing para-oriented electron-withdrawing substituents demonstrated stronger binding energies, more favorable interaction networks, and superior enzyme selectivity than derivatives containing electron-donating substituents. These findings further support the proposed structure-activity relationship established from the in vitro studies.

Table 4. Molecular Docking Parameters and Key Protein-Ligand Interactions of Lead Compounds with COX-2 (PDB ID: 3LN1)

Compound	Binding Energy (kcal/mol)	Number of Hydrogen Bonds	Major Interacting Amino Acid Residues	Dominant Binding Interactions
5c	−10.4	2	Arg513, His90, Tyr355, Val523	Hydrogen bonding, π-π stacking, hydrophobic interactions
5f	−9.8	0	Arg513, Tyr355, Trp387	Halogen bonding, hydrophobic interactions
5i	−7.2	1	Tyr355, Arg120	Weak hydrogen bonding, steric interference
Celecoxib	−10.8	3	Arg513, His90, Gln192	Hydrogen bonding, hydrophobic interactions

3.6.2. In Silico ADMET and Drug-Likeness Prediction

The pharmacokinetic behavior and drug-likeness of the lead benzothiazole derivatives were evaluated using the SwissADME and pkCSM prediction platforms. The calculated physicochemical parameters together with the predicted ADMET characteristics are summarized in Table 5.

Both lead compounds complied completely with Lipinski's Rule of Five, indicating favorable physicochemical characteristics for oral drug development. Their molecular weights remained well below 500 g/mol, while the calculated LogP values were within the recommended range, suggesting an appropriate balance between aqueous solubility and membrane permeability. Likewise, the numbers of hydrogen bond donors and hydrogen bond acceptors satisfied all accepted drug-likeness criteria, with no Lipinski violations observed.

Among the evaluated derivatives, compound 5c exhibited the most balanced pharmacokinetic profile. The calculated molecular weight (272.28 g/mol) and LogP (3.45) indicate suitable molecular size and lipophilicity for efficient gastrointestinal absorption. The predicted topological polar surface area (82.50 Å²) also falls within the range generally associated with favorable oral bioavailability while limiting excessive penetration across the blood-brain barrier.

SwissADME predicted high gastrointestinal absorption for both lead compounds, suggesting good oral absorption potential. In addition, both compounds were predicted to exhibit low blood-brain barrier permeability, indicating a reduced likelihood of central nervous system exposure and associated neurological adverse effects. This characteristic is advantageous for anti-inflammatory agents intended for peripheral therapeutic action.

The predicted toxicity profile was equally encouraging. Neither compound was predicted to be mutagenic in the AMES toxicity model, and no evidence of hepatotoxicity was identified by the computational screening models. Collectively, these results indicate that the diaryl ether-linked benzothiazole scaffold combines excellent drug-likeness with favorable pharmacokinetic and safety characteristics.

When considered together with the biological evaluation and molecular docking studies, the ADMET analysis further supports compound 5c as the most promising lead candidate for subsequent preclinical investigation.

Table 5. Predicted Physicochemical Properties and ADMET Characteristics of the Lead Compounds

Parameter	Recommended Value	Compound 5c	Compound 5f	Celecoxib
Molecular Weight (g/mol)	<500	272.28	261.73	381.37
LogP	<5.0	3.45	4.12	3.53
Hydrogen Bond Donors	≤5	0	0	1
Hydrogen Bond Acceptors	≤10	4	2	4
Topological Polar Surface Area (Å²)	20–130	82.50	37.38	86.36
Lipinski Rule Violations	0	0	0	0
Predicted GI Absorption	High	High	High	High
Blood-Brain Barrier Permeability	Low/Negative	Negative	Negative	Negative
AMES Toxicity	Non-mutagenic	Negative	Negative	Negative
Predicted Hepatotoxicity	Negative	Negative	Negative	Negative

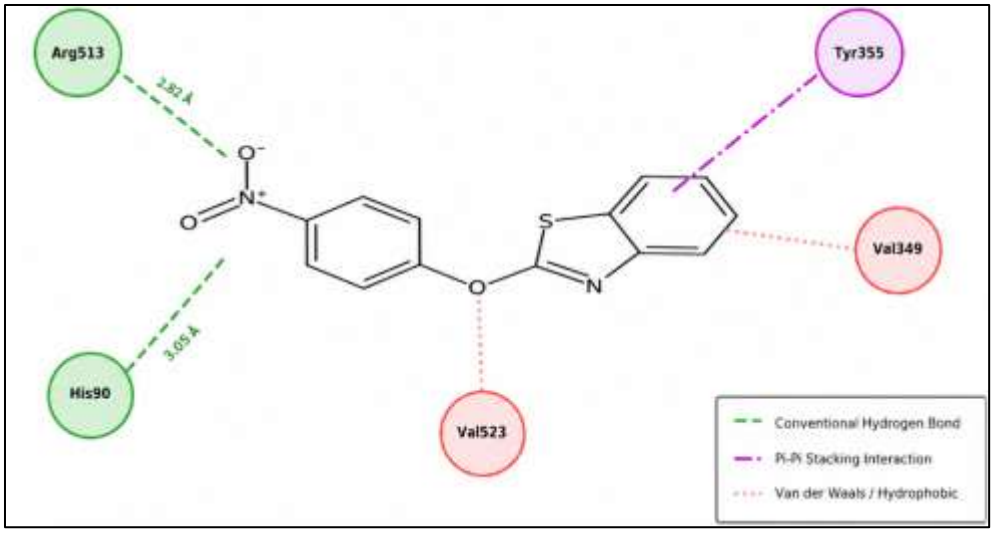

Figure 7. Predicted pharmacokinetic and drug-likeness profiles of the lead benzothiazole derivatives generated using SwissADME and pkCSM, illustrating compliance with Lipinski's Rule of Five and favorable ADMET characteristics suitable for oral drug development.

CONCLUSION

In conclusion, a novel series of diaryl ether-linked benzothiazole derivatives was successfully synthesized and comprehensively evaluated as targeted anti-inflammatory agents. The structure-activity relationship (SAR) analysis distinctly revealed that the incorporation of para-oriented electron-withdrawing groups on the terminal phenyl ring is absolutely critical for maximizing both enzyme inhibitory potency and isoform selectivity. Compound **5c**, bearing a para-nitro substituent, emerged as the most promising lead candidate. It demonstrated exceptional in vitro COX-2 inhibition (IC50 = 0.48 μM) and a remarkable Selectivity Index (SI = 237.9), significantly outperforming the standard non-selective drug, indomethacin. Furthermore, compound **5c** exhibited robust cellular anti-inflammatory efficacy by dose-dependently suppressing lipopolysaccharide-induced nitric oxide (NO) production in RAW 264.7 macrophages, while simultaneously maintaining an excellent safety profile with negligible cytotoxicity in the MTT assay. Molecular docking studies mechanistically validated these experimental findings, confirming that the V-shaped conformation of **5c** allows optimal penetration into the COX-2 secondary pocket to form essential hydrogen bonds with Arg513. Coupled with highly favorable in silico ADMET predictions, including high oral bioavailability and zero Lipinski rule violations, this newly designed scaffold represents a highly viable, safe platform for advanced preclinical therapeutic development and future clinical studies.

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