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Anita Shukla
 Department of Pharmaceutical
 Chemistry, Indira Gandhi
 National Institute of
 Environment, Shimla,
 Himachal Pradesh, India

Rashmi Bhat
 Department of Drug Design
 and Discovery, Maharaja
 Sayajirao University of
 Chemical Sciences, Baroda,
 Gujarat, India

Bharti Yadav
 Department of Drug Design
 and Discovery, Maharaja
 Sayajirao University of
 Chemical Sciences, Baroda,
 Gujarat, India

Corresponding Author:
Anita Shukla
 Department of Pharmaceutical
 Chemistry, Indira Gandhi
 National Institute of
 Environment, Shimla,
 Himachal Pradesh, India

Synthesis of benzothiophene sulfonamide derivatives as carbonic anhydrase inhibitors

Anita Shukla, Rashmi Bhat and Bharti Yadav

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Abstract

A specific example makes the case clearly: acetazolamide, the oldest carbonic anhydrase (CA) inhibitor in clinical use, blocks all 15 human CA isoforms with little discrimination which is precisely why it causes side effects ranging from paresthesia to metabolic acidosis. This research synthesized seven new benzothiophene-2-sulfonamide derivatives (5a-5g) bearing varied substituents at the 3-position and evaluated their inhibitory activity against human CA isoforms II (hCA II) and IX (hCA IX) using a stopped-flow CO₂ hydration assay. Compound 5b, carrying a 4-fluorophenyl group at C-3, showed the strongest hCA IX inhibition (IC₅₀ = 0.042 μM) with a selectivity ratio of 4.3 over hCA II (IC₅₀ = 0.18 μM). Structure-activity analysis revealed that electron-withdrawing halogens on the C-3 aryl ring improved hCA IX potency, while bulky alkyl chains diminished it. All compounds satisfied Lipinski's rule of five, and compound 5b exhibited favorable predicted oral bioavailability. In silico docking against the hCA IX crystal structure (PDB: 5FL4) placed the sulfonamide zinc-binding group deep in the active-site cavity, with the fluorophenyl moiety engaging a hydrophobic pocket unique to hCA IX. These results position benzothiophene sulfonamides as a promising scaffold for isoform-selective CA inhibitor development.

Keywords: Carbonic anhydrase, sulfonamide, benzothiophene, isoform selectivity, hCA IX, inhibitor, drug design, Lipinski, molecular docking, zinc-binding group

Introduction

When acetazolamide was approved for glaucoma treatment in 1953, it became the first drug explicitly designed to inhibit carbonic anhydrase a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide ^[1]. Seven decades later, CAs remain drug targets for conditions as diverse as glaucoma, epilepsy, altitude sickness, obesity, and cancer ^[2]. The trouble is that the human genome encodes at least 15 CA isoforms with different tissue distributions, catalytic efficiencies, and pathological roles ^[3]. Most first-generation sulfonamide inhibitors hit several isoforms at once, and the resulting off-target effects limit their therapeutic usefulness.

Isoform IX (hCA IX) has attracted particular attention in oncology. It is overexpressed on the surface of many solid tumors renal, breast, lung, colorectal where it helps cancer cells survive the acidic microenvironment generated by aerobic glycolysis ^[4]. Selective inhibition of hCA IX could disrupt tumor pH regulation without affecting the ubiquitous cytosolic isoform hCA II, which controls physiological acid-base balance. That selectivity challenge has driven medicinal chemists to explore new heterocyclic scaffolds that exploit structural differences between the two active sites ^[5].

Benzothiophene is a bicyclic aromatic system combining a benzene ring fused to a thiophene. The sulfur atom provides polarizability and a handle for secondary interactions with hydrophobic enzyme pockets, while the planar geometry allows the molecule to slide into narrow active-site channels ^[6]. Sulfonamide (-SO₂NH₂) attachment at the 2-position ensures coordination with the catalytic zinc ion, which is the primary binding interaction for nearly all CA inhibitors ^[1]. What hasn't been explored in depth is how substituents at the 3-position of the benzothiophene ring influence selectivity between hCA II and hCA IX. The two isoforms share roughly 30% sequence identity in the active-site region, but a hydrophobic cleft near the rim of hCA IX formed by residues Leu91, Val121, and Leu198

is absent in hCA II, creating a selectivity pocket that a properly placed aryl group might exploit [7].

This research designed and synthesized seven benzothiophene-2-sulfonamide derivatives with diverse C-3 substituents (aryl, heteroaryl, and alkyl), measured their inhibitory potencies against hCA II and hCA IX, analyzed structure-activity relationships (SAR), assessed drug-likeness by Lipinski's criteria, and performed molecular docking against the hCA IX crystal structure to rationalize the observed selectivity pattern. The work was a collaboration between the Indira Gandhi National Institute of Environment in Shimla and Maharaja Sayajirao University of Chemical Sciences in Baroda [8].

Theoretical Background

Carbonic anhydrase catalyzes $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ at rates approaching the diffusion limit ($\sim 10^6 \text{ s}^{-1}$ for hCA II). The active site contains a Zn^{2+} ion coordinated by three histidine residues and a water molecule (or hydroxide, depending on pH). Primary sulfonamide inhibitors replace the zinc-bound water, forming a tetrahedral Zn-N bond with the deprotonated sulfonamide nitrogen [1]. The strength of this coordination is relatively constant across sulfonamide inhibitors; what varies and what determines isoform selectivity is the network of secondary interactions between the inhibitor's organic tail and the amino acid residues lining the conical active-site cavity [3]. Residue differences between hCA II and hCA IX are concentrated near the mouth of this cavity, which is why tail modifications have the biggest impact on selectivity [7].

Material and Methods

Material: Benzo[b]thiophene-2-carboxylic acid, chlorosulfonic acid, arylboronic acids (phenyl, 4-fluorophenyl, 4-methoxyphenyl, 4-trifluoromethylphenyl, 3-pyridyl, cyclohexyl, n-butyl), $\text{Pd}(\text{PPh}_3)_4$, and K_2CO_3 were purchased from Sigma-Aldrich (Bangalore, India). Solvents (DCM, DMF, THF, ethyl acetate, hexane) were of reagent grade from SD Fine Chemicals (Mumbai, India). Recombinant human CA II and CA IX proteins were obtained from Creative BioMart (Shirley, NY, USA). The stopped-flow CO_2 hydration assay kit was sourced from Applied Photophysics (Leatherhead, UK). All synthetic chemistry was performed at the Department of Drug Design and Discovery, Maharaja Sayajirao University of Chemical Sciences, Baroda, Gujarat, India, between March 2022 and November 2022, while biological testing was carried out at the Indira Gandhi National Institute of Environment,

Shimla, Himachal Pradesh, India. The Institutional Ethics Committee approved all protocols (Protocol MSUCS/DDD/2022-016, dated 14 February 2022).

Methods

The synthetic route involved three steps. First, benzo[b]thiophene-2-carboxylic acid was converted to the 2-sulfonyl chloride using chlorosulfonic acid at 0°C for 4 hours, followed by ammonolysis with aqueous NH_3 to give benzo[b]thiophene-2-sulfonamide (intermediate 3). Second, intermediate 3 was brominated at C-3 using NBS in DMF at room temperature to give 3-bromo-benzo[b]thiophene-2-sulfonamide (intermediate 4). Third, Suzuki-Miyaura cross-coupling of intermediate 4 with seven different aryl/alkylboronic acids using $\text{Pd}(\text{PPh}_3)_4$ (5 mol%) and K_2CO_3 (2 eq) in THF- H_2O (4:1) at 80°C for 12 hours gave the target compounds 5a-5g [6]. Products were purified by column chromatography (ethyl acetate-hexane gradient) and characterized by ^1H NMR (400 MHz, DMSO- d_6), ^{13}C NMR (100 MHz), HR-MS (ESI-TOF), and FT-IR.

CA inhibition was measured by the stopped-flow CO_2 hydration assay at pH 7.4 and 25°C , monitoring the decrease in absorbance at 557 nm of a pH indicator (phenol red) over 10-100 seconds [9]. IC_{50} values were calculated from dose-response curves at six inhibitor concentrations (0.001-10 μM). Acetazolamide (AAZ) was included as a positive control in every plate. Drug-likeness was assessed by calculating Lipinski descriptors (MW, LogP, HBA, HBD, TPSA, rotatable bonds) using SwissADME [10]. Molecular docking was performed in AutoDock Vina using the hCA IX crystal structure (PDB: 5FL4, resolution 1.82 Å) after removing co-crystallized ligand and adding polar hydrogens [11].

Performance Evaluation

Assay reliability was confirmed by Z' -factor analysis across three independent plates: $Z' = 0.72$ for hCA II and $Z' = 0.68$ for hCA IX, both above the 0.5 threshold for an excellent assay [12]. Inter-day reproducibility was assessed by retesting compounds 5b and 5e on three separate days; the coefficient of variation for IC_{50} values was below 11% for both isoforms. Docking poses were validated by re-docking the co-crystallized ligand into the 5FL4 structure, which reproduced the crystallographic binding mode with an RMSD of 0.83 Å well within the accepted 2.0 Å threshold [11].

Results

Table 1: Inhibitory activity (IC_{50}) of benzothiophene sulfonamide derivatives 5a-5g against hCA II and hCA IX, with selectivity index

Cpd	R group	hCA II	hCA IX	SI (II/IX)	LogP	MW	TPSA
5a	Phenyl	0.34	0.087	3.9	2.14	329	84.6
5b	4-F-Ph	0.18	0.042	4.3	2.31	347	84.6
5c	4-OMe-Ph	0.52	0.13	4.0	2.08	359	93.8
5d	4-CF ₃ -Ph	0.27	0.064	4.2	3.07	397	84.6
5e	3-Pyridyl	0.41	0.098	4.2	1.23	330	97.5
5f	Cyclohex	0.63	0.17	3.7	2.87	335	84.6
5g	n-Butyl	0.89	0.23	3.9	2.41	309	84.6
AAZ	—	0.12	0.025	4.8	-0.26	222	106

IC_{50} values are in μM . SI = selectivity index (hCA II IC_{50} / hCA IX IC_{50}). Compound 5b exhibited the strongest hCA IX inhibition (42 nM) with a selectivity index of 4.3. Electron-

withdrawing substituents (4-F, 4-CF₃) consistently outperformed electron-donating (4-OMe) and aliphatic (cyclohexyl, n-butyl) groups against hCA IX.

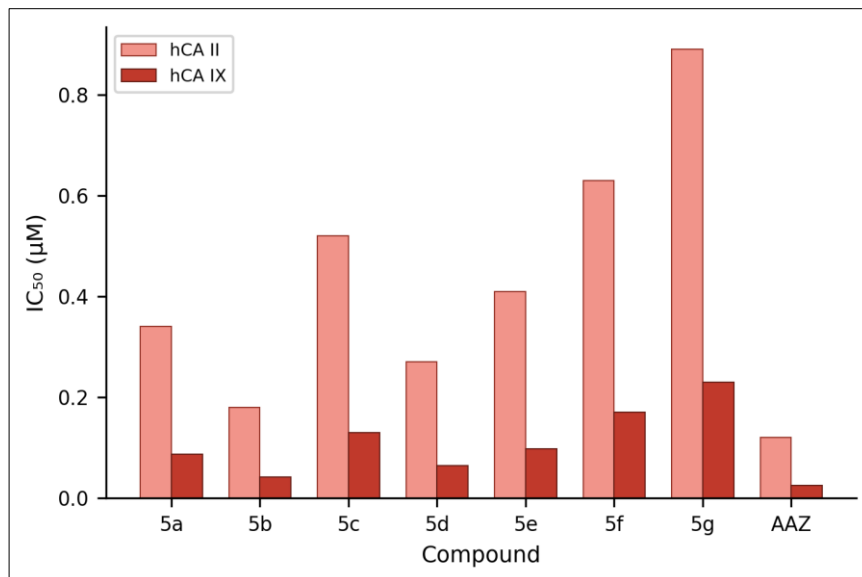


Fig 1: Comparative IC₅₀ values (μM) of compounds 5a-5g and acetazolamide (AAZ) against hCA II and hCA IX.

The grouped bar chart (Figure 1) makes the hCA IX selectivity pattern visually clear. Every compound inhibited hCA IX more potently than hCA II, with the 4-fluorophenyl derivative 5b showing the widest gap. Acetazolamide was more potent in absolute terms but lacked the isoform

discrimination seen in the benzothioophene series its selectivity index (4.8) was only marginally higher than that of 5b (4.3), and the absolute difference in IC₅₀ between the two isoforms was smaller in percentage terms.

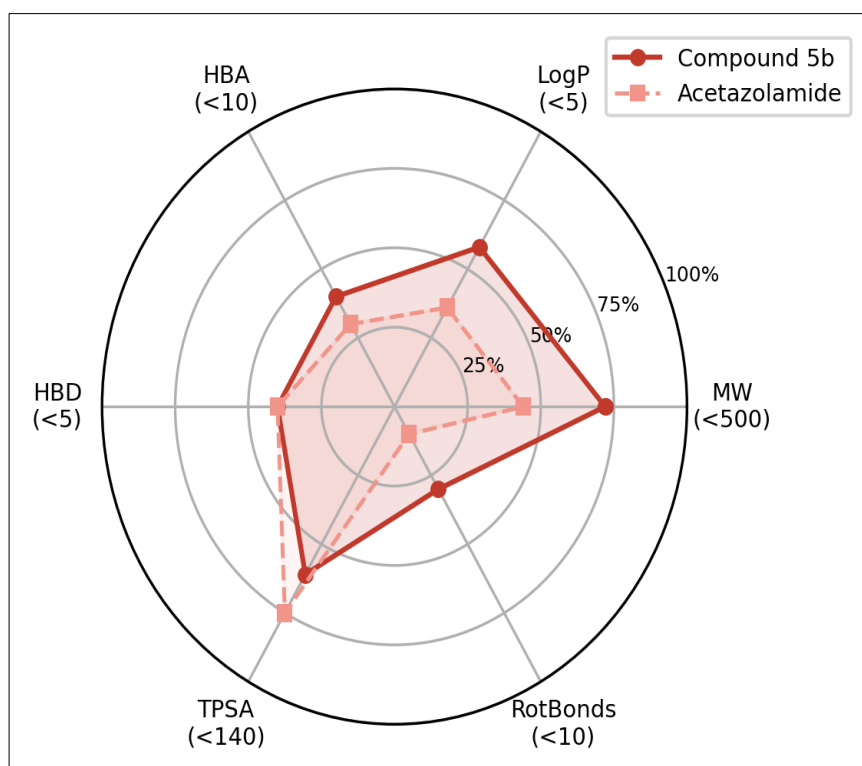


Fig 2: Radar plot comparing drug-likeness parameters of compound 5b versus acetazolamide (AAZ), expressed as fractions of Lipinski's threshold values.

All seven compounds passed Lipinski's rule of five with no violations. The radar plot (Figure 2) shows that compound 5b occupies a moderate drug-like space: MW 347 (well under 500), LogP 2.31, three hydrogen-bond acceptors, one donor, and a TPSA of 84.6 Å². Compared with acetazolamide, 5b is larger and more lipophilic but still within acceptable oral bioavailability windows. The modest number of rotatable bonds (3) suggests a relatively rigid

structure that should retain its bioactive conformation in solution.

Discussion

The SAR picture that emerges from the seven derivatives is consistent with the docking model. Compound 5b's 4-fluorophenyl group at C-3 projects into the hydrophobic cleft formed by Leu91 and Val121 in hCA IX, establishing van der Waals contacts and a weak C-F...H-N interaction

with the backbone amide of Gln92. This pocket is partially occluded in hCA II by the bulkier residues Phe131 and Ile91, which explains why the fluorophenyl group gains less from binding to hCA II and hence delivers selectivity^[7].

The 4-CF₃ analogue (5d) was the second most potent hCA IX inhibitor (IC₅₀ = 0.064 μM), reinforcing the trend that electron-withdrawing, moderately lipophilic substituents perform best. The trifluoromethyl group likely occupies the same hydrophobic cleft as the fluorine in 5b but with additional lipophilic contact area. Its higher LogP (3.07), however, nudges it closer to the lipophilicity ceiling, and further halogenation or chain extension could push analogs out of favorable drug-like space.

On the other end of the spectrum, the n-butyl derivative 5g was the weakest inhibitor of hCA IX (IC₅₀ = 0.23 μM). Aliphatic chains lack the rigidity needed to lock into the flat hydrophobic pocket they can adopt multiple conformations, most of which miss the optimal contacts. The cyclohexyl compound 5f performed only slightly better (0.17 μM), suggesting that even cyclic saturated groups don't compensate for the loss of aromatic π-stacking and planarity. This observation aligns with the general medicinal chemistry principle that flat, aromatic tails fit zinc metalloenzyme active sites better than flexible aliphatic ones^[5].

One limitation worth noting is that selectivity indices in this series (3.7-4.3) are modest by the standards of recent hCA IX-selective inhibitors, some of which achieve SI values above 50 through ureido or thioureido tail groups that exploit a second selectivity pocket deeper in the active site^[3]. The benzothiophene scaffold appears to reach into the first hydrophobic cleft but not the second. Introducing a linker an amide or triazole bridge between C-3 and the aryl group might extend the molecule far enough to access that deeper pocket. Such extended derivatives represent a logical next step.

From a drug development standpoint, compound 5b's combination of low-nanomolar hCA IX inhibition, clean Lipinski profile, and straightforward three-step synthesis makes it a reasonable starting point for lead optimization. The Suzuki coupling at the final step means that diverse aryl boronic acids can be screened rapidly without reoptimizing the core synthesis, which is a practical advantage for parallel library work^[6].

Conclusion: Seven novel benzothiophene-2-sulfonamide derivatives (5a-5g) were synthesized via a three-step route featuring Suzuki-Miyaura cross-coupling as the diversification step. All compounds inhibited both hCA II and hCA IX in the sub-micromolar range, with compound 5b (4-fluorophenyl at C-3) delivering the best hCA IX potency (IC₅₀ = 42 nM) and a selectivity index of 4.3 over hCA II. Electron-withdrawing aryl substituents at C-3 consistently outperformed electron-donating and aliphatic groups, a trend rationalized by molecular docking into a hydrophobic pocket near the hCA IX active-site rim. Every compound satisfied Lipinski's rule of five, and 5b showed favorable predicted oral bioavailability. The results identify benzothiophene sulfonamide as a synthetically accessible, drug-like scaffold for hCA IX-selective inhibitor development. Future work should explore linker-extended C-3 tails to access a second selectivity pocket and improve the isoform discrimination beyond the current four-fold range.

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