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ARTICLE

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Convergent synthesis of carbonic anhydrase inhibiting bi-heterocyclic benzamides: Structure–activity relationship and mechanistic explorations through enzyme inhibition, kinetics, and computational studies

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Abstract

By using a convergent methodology, a novel series of *N*-arylated 4-yl-benzamides containing a bi-heterocyclic thiazole–triazole core was synthesized, and the structures of these hybrid molecules, **9a–k**, were corroborated through spectral analyses. The in vitro studies of these multifunctional molecules demonstrated their potent carbonic anhydrase inhibition relative to the standard used. The kinetics mechanism was exposed by Lineweaver–Burk plots, which revealed that **9j** inhibited carbonic anhydrase non-competitively by forming an enzyme-inhibitor complex. The inhibition constants K_i calculated from Dixon plots for this compound was 1.2 μM. The computational study was also persuasive with the experimental results, and these molecules disclosed good results of all scoring functions and interactions, which suggested a good binding to carbonic anhydrase. So, it was predicted from the inferred results that these molecules might be considered as promising medicinal scaffolds for various diseases related to the uncontrolled production of this enzyme.

1 | INTRODUCTION

Heterocyclic compounds have a wide range of application: they are predominant among the type of compounds used as pharmaceuticals and as veterinary products. They also find applications as antioxidants, as corrosion inhibitors, and as copolymers. Various compounds of heterocyclic amides and thioethers are used in the field of biology, agriculture, optical material, and as intermediates in the synthesis of antibiotics, which would be very promising in the field of medicinal chemistry [1–3].

Thiazole, a heterocyclic compound, is a basic component of all the current penicillins, which modernized the therapy of bacterial diseases [4]. Its derivatives are of

Abbreviations: br.t, broad triplet; d, doublet; dd, doublet of doublets; DMSO-d_6, Deuterated dimethyl sulfoxide; EI-MS, Electron Impact Mass Spectrometry; IR, Infra Red; m, multiplet; q, quartet; quint, quintet s, singlet; sex, sextet; sep, septet; t, triplet; ^{1}H-NMR, Proton Nuclear Magnetic Resonance; ^{13}C-NMR, Carbon Nuclear Magnetic Resonance.

considerable interest for therapeutic point of view because of their utility as antibacterial, antifungal, anti-inflammatory, analgesic, antiviral, antitubercular, central nervous system (CNS) stimulant, anti-HIV agents, and as enzyme inhibitors [5–7]. Moreover, they are also used as anesthetic, sedative, and cardiotonic [8]. Thiazole ring also claims contribution in other fields, such as liquid crystals [9], polymers [10], fluorescent dyes [11], photonucleases [12], and antioxidant [13]. 2-Aminothiazoles and their derivatives are devastating and rapidly emerging in recent heterocyclic chemistry [14,15]. In vitro anticancer studies revealed that various 2-aminothiazole derivatives maintain active inhibitory effects against prevalent human cancerous cells such as colon, prostate, ovarian, leukemia, renal, lung, breast, CNS, and melanoma cell lines [16,17].

Triazoles are five-membered heterocyclic compounds containing two carbons and three nitrogen atoms. These molecules have maintained a unique position in medicinal and organic chemistry due to their numerous biological activities. Various 1,2,4-triazoles and the *N*-bridged heterocycles derived from them are found to be associated with diverse pharmacological activity [18,19]. Triazole derivatives are analgesic [20], anti-inflammatory [21], anticonvulsant [22], antineoplastic [23], antimalarial, antiviral [24], and antiproliferative [25]. Moreover, several derivatives also exhibited antihypertensive, anticholinergic, antibacterial, antifungal, hypoglycemic, antitubercular, and anticancer properties, which made them as important chemotherapeutic agents and a potential targeting agent in multiple types of malignancies [26–28].

Carbonic anhydrases (CAs, EC 4.2.1.1) are Zn(II) metalloenzymes that catalyze the reversible hydration of carbon dioxide to give bicarbonate anion and a proton: $CO_2 + H_2O \rightleftharpoons HCO_3^{-1} + H^{+1}$ [29]. This reaction is involved in vital physiological processes associated with respiration and transport of CO_2/bicarbonate between lungs and metabolizing tissues, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, bone resorption, calcification, tumorigenicity, and many other physiological or pathological processes [30,31]. These enzymes present in prokaryotes and eukaryotes, being determined by four different, evolutionarily distinct gene families: the α-CAs (present in vertebrates, bacteria, algae, and cytoplasm of green plants), the β-CAs (chiefly in bacteria, algae, and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (mostly in archaea and some bacteria), and the δ-CAs, present in some marine diatoms, respectively [32–34].

CA II is a physiologically prime isoform in CA enzymes. It is present in red blood cells and many other tissues [35]. It has also been explored that in several types of cancer cells, the excess amounts of CA II are present either alone or in combination with tumor-associated enzymes CA IX and CA XII [36]. Several CA isozymes are inhibited to treat a series of disorders such as epilepsy, obesity, edema, glaucoma, osteoporosis, and infectious diseases. The inhibitors of CA II have found an extensive range of medical applications as diuretics, antiepileptic agents, and antiglaucoma. Yet, CA inhibitors that have been used until now have not been satisfactorily safe compounds because they are not specific, have very weak efficiency, and have large number of enzyme isoforms. These compounds not only inhibit CA II enzymes but also other isoenzymes and as a result of this, various side effects might be observed such as tingling, drowsiness, fatigue, depression, weight loss, gastrointestinal disturbances, and metabolic acidosis [37–39]. Such situations encourage and lead investigators to study on the discovery of new CA inhibitors.

Although some azole and triazole derivatives have been recently reported as carbonic anhydrase inhibitors as shown in Figure 1 [40–45]. There is a need to discover some unique, hybrid, and safe inhibitors of this enzyme to overcome the problems related to this enzyme. Therefore, in continuation of our previous studies on the bioactivity of related bi-heterocyclic compounds [46], the present investigation was designed to seek some novel hybrid benzamides as carbonic anhydrase inhibitors.

2 | RESULTS AND DISCUSSION

The designed bi-heterocyclic *N*-arylated benzamides were synthesized by a convergent protocol as described in Scheme 1. The synthesis was initiated by refluxing ethyl 2-(2-amino-1,3-thiazol-4-yl)acetate **(1)** in methanol and hydrazine hydrate to transform the starter into 2-(2-amino-1,3-thiazol-4-yl)acetohydrazide **(2).** The hydrazide, **2**, was added in methanol and refluxed with ethyl isothiocyante **(3)** to convert it into a solid intermediary compound 2-[2-(2-amino-1,3-thiazol-4-yl)acetyl]-*N*-ethyl-1-hydrazinecarbothioamide **(4)**, which was cyclized to a nucleophilic molecule 5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4*H*-1,2,4-triazole-3-thiol **(5)**. In a complementary set of reactions, the substituted anilines **(6a–k)** were reacted with 4-chloromethylbenzoyl chloride **(7)** in an aqueous basic medium, to acquire the required electrophiles, 4-chloromethyl-*N*-(aryl)benzamides (**8a–k**). Finally, the nucleophile **5** was dissolved in DMF and a nip of LiH was added to it. To activate its mercapto position, the mixture was stirred for about 30 minutes, and in this last step, the electrophiles, **8a–k**, were coupled in equimolar amounts with the activated **5** to yield the targeted bi-heterocyclic benzamides (**9a–k**).

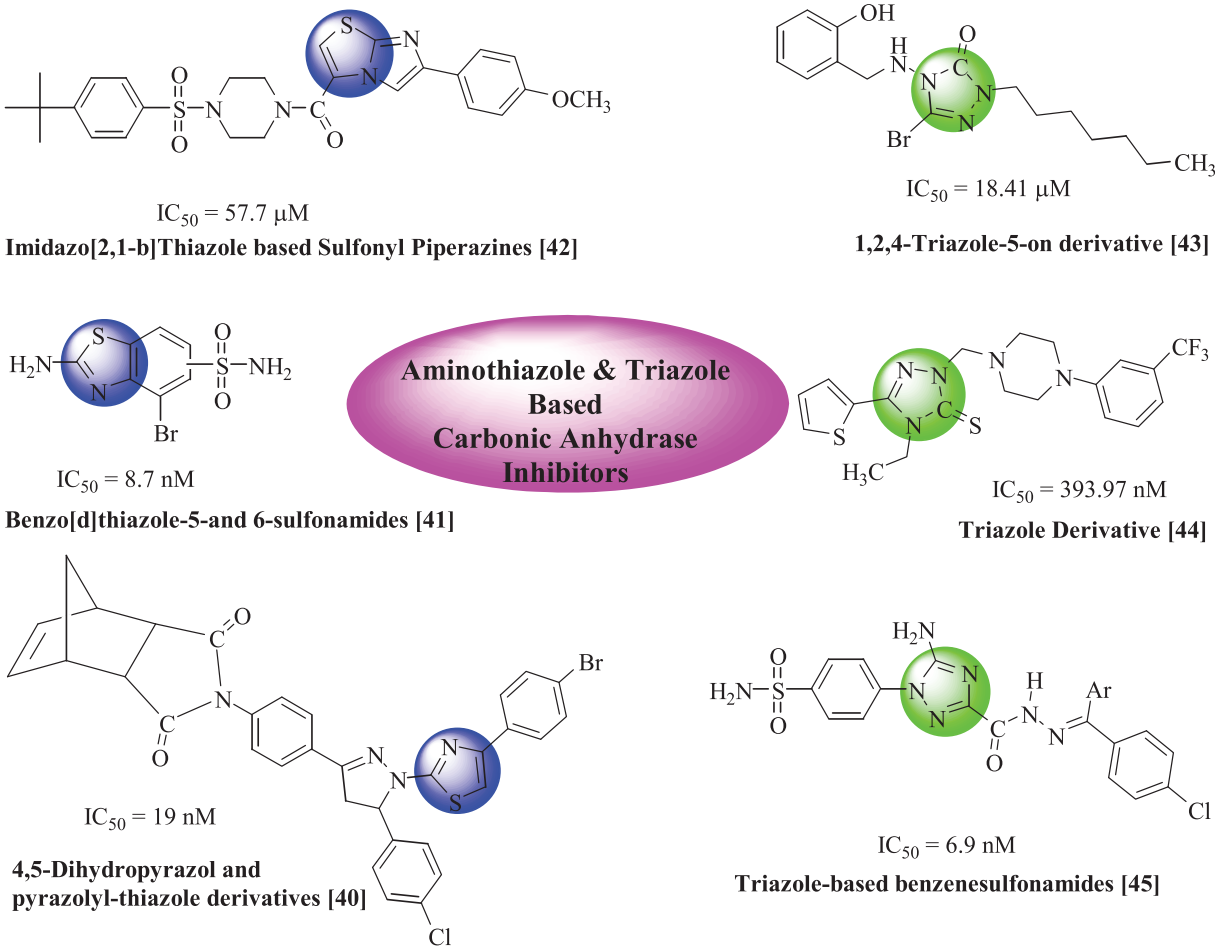
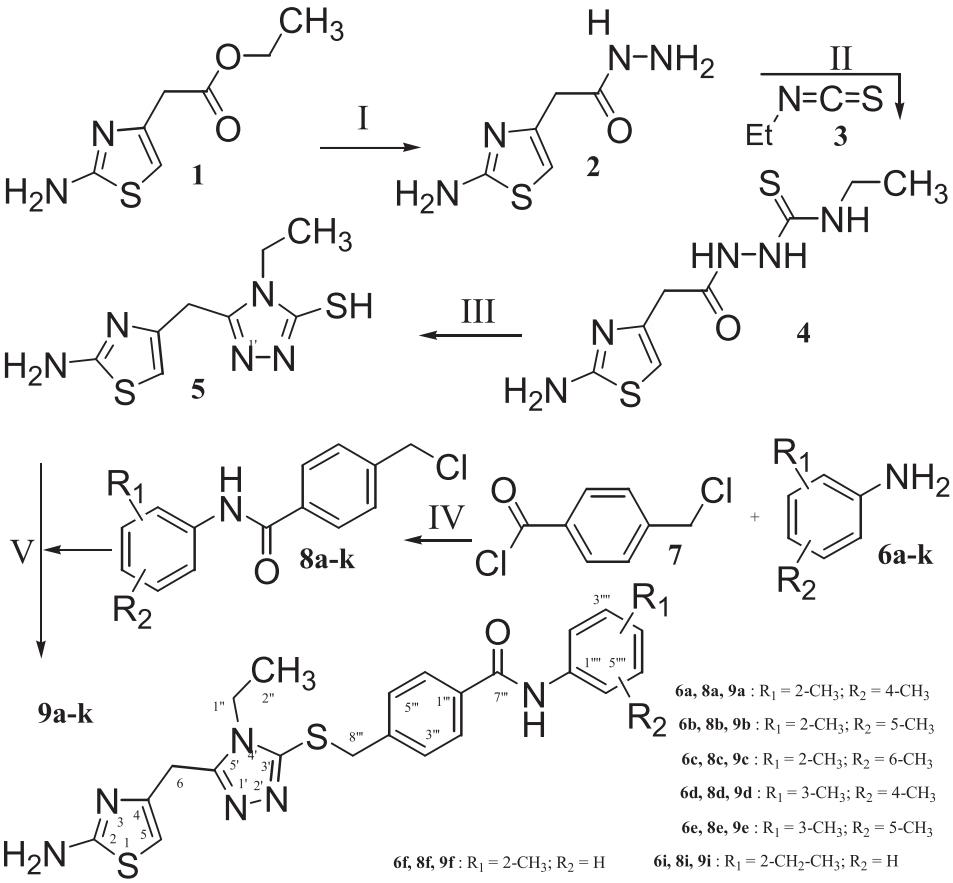


FIGURE 1 Aminothiazole and triazole based CA inhibitors [Colour figure can be viewed at wileyonlinelibrary.com]

Detailed structural analysis of a compound, **9h** is discussed here, which was synthesized as light brown amorphous solid and having melting point of 110–111°C. The molecular formula, $C_{23}H_{24}N_6OS_2$, was confirmed by CHN analysis data, counting the resonance of proton in its ^{1}H-NMR (Figures S1 and S2) and carbon in its ^{13}C-NMR spectrum. Different functionalities in the molecule were approved by IR spectrum. The characteristic peaks appeared at ν 3348 (N—H stretch), 3058 (C—H stretch), 2928 (—CH_2— stretch), 1678 (C═O stretch), 1558 (C═C stretch), 1518 (C═N stretch), 1168 (C—N—C bond stretch), and 628 (C—S stretch) cm^{-1}. In ^{1}H-NMR spectrum, ethyl connected nitrogen (4′) atom was identified by two prominent peaks, a quartet and a triplet. Quartet was positioned at δ 3.80 (q, J = 7.1, 2H, CH_2-1″) while triplet at δ 0.98 (t, J = 7.2, 3H, CH_3-2″). The 4-substituted benzamide unit was predictable by an amidic signal at 10.11 (s, 1H, —CO—NH-1‴′) along with two prominent *ortho*-coupled broad doublets in the aromatic region at δ 7.87 (br. d, J = 8.1, 2H, H-2‴ and H-6‴) and 7.47 (br. d, J = 8.1, 2H, H-3‴, and H-5‴). The 4-methylphenyl moiety connected with nitrogen of benzamide unit was clearly signified by A_2B_2 spin system in the aromatic region as two *ortho*-coupled broad doublets at δ 7.63 (br. d, J = 8.2, 2H, H-2⁗, and C-6⁗) and δ 7.14 (br. d, J = 8.2, 2H, H-3⁗, and C-5⁗), in addition to the typical signal of methyl group represented by a singlet at δ 2.27 (s, 3H, CH_3-4⁗). The 2-amino-1,3-thiazol-4-yl unit was characterized by two singlets at δ 6.91 (br. s, 2H, H_2N-2) and 6.22 (s, 1H, H-5), while a singlet at δ 3.93 (s, 2H, CH_2-6) was assignable to a methylene group joining the two heterocycles in the molecule. Another singlet at δ 4.44 (s, 2H, CH_2-8‴) was rational for the methylene group linking the bezamide unit with mercapto position of triazole moiety.

The 2-amino-1,3-thiazol-4-yl moiety in ^{13}C-NMR spectrum (Figure S3) was clearly indicated by two quaternary signals at δ 168.58 (C-2), and 146.16 (C-4), along with a methine signal at δ 102.37 (C-5). Similarly, the other heterocycle, that is, (1,2,4-triazloe-2-yl)sulfanyl was also identified by two quaternary signals at δ 153.17 (C-5′) and 147.98 (C-3′) while a methylene linking the two heterocycles (4-position of the former heterocycle with 5′-position of the latter heterocycle) was apparent at δ 27.52 (C-6). The 4-methylphenyl moiety was also corroborated



4-[({5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4*H*-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(aryl)benzamides

SCHEME 1 Outline for the synthesis of 4-[({5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4*H*-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(aryl)benzamides. **Reagents and Conditions**: (I) MeOH/$N_2H_4{\cdot}H_2O$/refluxing for 2 h (Yield = 90%). (II) MeOH/**3**/Refluxing for 1 h (Yield = 86%). (III) The ppt. of **4** dissolved in 10% NaOH/filtration/acidification of filtrate in cold state to get ppt. of **5** (Yield = 86%). (IV) Aq. Na_2CO_3 soln./pH 9-10/vigorous manual shaking at RT for 2–3 h (Yield = 86%). (V) DMF/LiH/stirring at 12–24 h (Yield = 90%–96%)

with two quaternary signals at δ 136.54 (C-1′′′′), 132.57 (C-4′′′′) along with two symmetrical methine duplets at δ 128.82 (C-3′′′′ and C-5′′′′) and δ 120.36 (C-2′′′′ and C-6′′′′) for the aromatic ring while the 4-methyl group on it was verified by signal at δ 20.45 (CH_3-4′′′′). The 4-substituted benzamide unit was confirmed by a distinct signal of carbonyl carbon at δ 164.86 (C-7′′′) in addition to other two quaternary signals at δ 141.05 (C-4′′′), and 133.95 (C-1′′′) along with two methine duplet resonances at δ 128.93 (C-3′′′ and C-5′′′) and 127.70 (C-2′′′ and C-6′′′). The signal at δ 36.49 (C-8′′′) was assignable to a methylene joining the benzamide from its 4-position to the sulfur atom joined with 1,2,4-triazole heterocycle. The ethyl group attached to the nitrogen (4′) atom of 1,2,4-triazole ring was distinctive by two signals at δ 38.56 (C-1′′) and 14.65 (C-2′′). The C—H connectivities in the carbon skeleton were comprehensively certified by its HMBC spectrum and the important correlations are demonstrated on this spectrum in Figure 2.

So, the structure of **9h**, 4-[({5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4*H*-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(4-methylphenyl)benzamide and all other synthesized derivatives (Figures S4–S25) were entirely verified through spectral analysis.

2.1 | Carbonic anhydrase inhibition

The novel bi-heterocyclic benzamides (**9a–k**) were synthesized and evaluated against carbonic anhydrase enzyme and their results are arranged in Table 1. All these compounds exhibited potent inhibitory activities against this enzyme, as evident from their lower IC_{50} (μM) values, relative to standard, acetazolamide, having IC_{50} value of 1.091 ± 0.073 μM. Though the rendered activity is accumulative of a whole molecule, yet a limited structure–activity relationship (SAR) was recognized by examining the effect of differently substituted aryl entities on the inhibitory potential, as it was the only varying part and all other parts were intact in all molecules. The general structural parts of the studied benzamides are featured in Figure 3.

2.2 | Structure–activity relationship

Among three di-methylated regio-isomers, compound **9a** in which the methyl groups were present at *ortho* and *para* positions exhibited better inhibitory activity

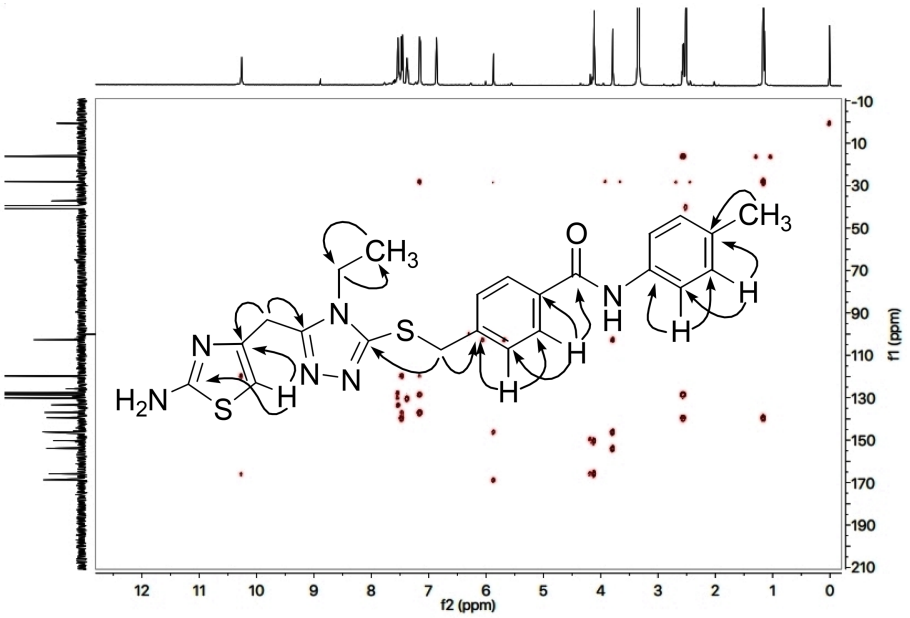


FIGURE 2 HMBC spectrum and significant correlations of **9h** [Colour figure can be viewed at wileyonlinelibrary.com]

(IC_{50} = 7.451 ± 0.074 μM) as compared to other isomers in which methyl groups were not on alternating positions (2,5-dimethylphenyl, 2,6-dimethylphenyl). These analogues **9b** and **9c** possessed comparatively high IC_{50} values; 18.914 ± 0.811 and 10.155 ± 0.099 μM, indicating that the presence of the methyl groups at 2 and 4-positions in former molecule, probably increased its appropriate interactions with the active site of the enzyme (Figure 4).

Similarly, when the inhibitory potential of other two di-methylated isomers was compared in which the small-sized methyl group was fixed at 3-position, it was observed that **9d** with adjacent methyl groups (3,4-dimethylphenyl) had a least activity (IC_{50} = 8.049 ± 0.079 μM), as compared to **9e** (IC_{50} = 3.076 ± 0.006 μM). Indeed, the molecule **9e** was the second most potent among the series bearing the methyl groups at again alternating positions in aryl part, which is presumed to augment the appropriate interactions with the active site of the enzyme and increase its inhibitory activity. However, the steric factor owing to the adjacent methyl groups in **9d** gave an outcome of its decreased interactions with the enzyme (Figure 5).

The three mono-methylated compounds **9f**, **9g,** and **9h** having IC_{50} = 20.236 ± 0.898, 21.111 ± 0.947, and 17.201 ± 0.077 μM showed similar activity with minor differences. It revealed that mono-methylated compounds are less active relative to the di-methylated molecules, probably due to single electron donating factor (+I) in these molecules (Figure 6).

The compound **9i** has a medium-sized non-polar ethyl group at the *ortho* position, while **9j** has the same ethyl group at *para*-position. Among these molecules, a decreasing order of inhibitory activity was observed as **9j** > **9i.** This order was also consistent to some extent with the aforementioned +I observations. As the ethyl group has more +I effect than methyl, so these two compounds are generally impressive inhibitors in the whole series. Compound **9j** (IC_{50} = 0.978 ± 0.004 μM) possessed superb activity relative to **9i**, as in the former molecule the ethyl group was away from the benzamidic entity, thus resulting in lesser steric crowding and hence increased interactions with the active site of the enzyme (Figure 7).

Compound **9k** (IC_{50} = 19.214 ± 0.841 μM) has medium-sized polar group at *meta* position. This group has –I, and + R effect and it is present in a closer proximity of the benzamide moiety. So, it was inferred that –I effect and ample steric crowding resulted in its considerably lower inhibitory potential (Figure 8).

So, it was concluded from the SAR that the molecules bearing groups with substantial +I effect and having less steric crowding in aryl part, such as ethylated bi-heterocyclic benzamides, generally resulted in superb inhibitory potential against this enzyme.

2.3 | Kinetic analysis

To understand the inhibitory mechanism of synthetic compound on carbonic anhydrase inhibition, kinetic study was performed. Based on our IC_{50} results, we selected our most potent compound **9j** to determine their inhibition type. The kinetic results of the enzyme were determined by the Lineweaver–Burk plot of 1/V versus 1/[S] in the presence of different inhibitor concentrations,

TABLE 1 Carbonic anhydrase inhibitory and hemolytic activity of bi-heterocyclic benzamides, **9a–k**

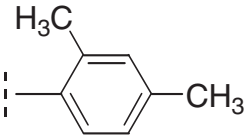
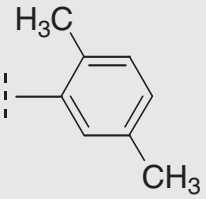
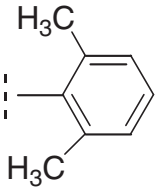
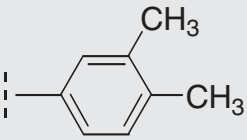
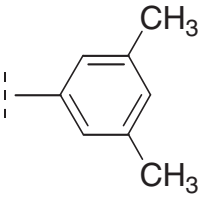
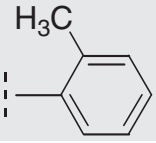
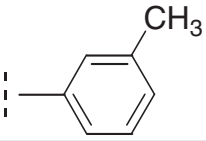
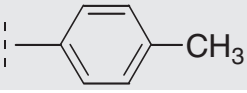
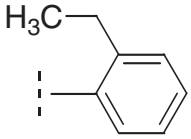
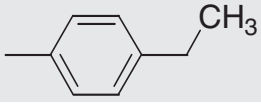
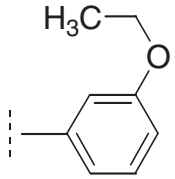
Compounds	Aryl part	Carbonic anhydrase activity IC_{50} ± SEM (μM)	Hemolysis (%) (Mean ± SEM)
9a		7.451 ± 0.074	5.66 ± 0.02
9b		18.914 ± 0.811	15.53 ± 0.03
9c		10.155 ± 0.099	11.13 ± 0.02
9d		8.049 ± 0.079	3.96 ± 0.04
9e		3.076 ± 0.006	13.84 ± 0.02
9f		20.236 ± 0.898	17.61 ± 0.03
9g		21.111 ± 0.947	18.86 ± 0.02
9h		17.201 ± 0.077	19.50 ± 0.04
9i		6.821 ± 0.009	16.98 ± 0.05
9j		0.978 ± 0.004	37.74 ± 0.05

TABLE 1 (Continued)

Compounds	Aryl part	Carbonic anhydrase activity IC_{50} ± SEM (μM)	Hemolysis (%) (Mean ± SEM)
9k		19.214 ± 0.841	3.77 ± 0.03
Acetazolamide		1.091 ± 0.073	-
Triton X		-	87.53 ± 0.01

Note: PBS Hemolysis = 0.934 ± 0.01%.
Abbreviations: SEM, Standard error of the mean; values are expressed in mean ± SEM.

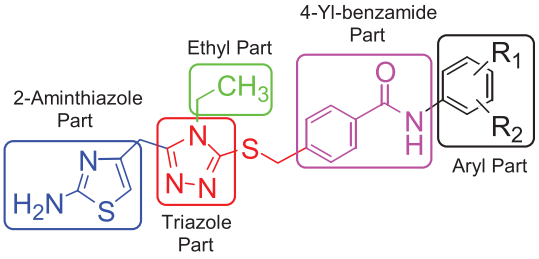


FIGURE 3 General structural features of compounds **9a–k** [Colour figure can be viewed at wileyonlinelibrary.com]

which gave a series of straight lines (Figure 9A). The results of **9j** showed that this compound intersected within the second quadrant. The analysis showed that V_{max} decreased to new increasing doses of inhibitors, on the other hand, K_m remained the same. This behavior indicated that **9j** inhibited the CA II non-competitively to form the enzyme-inhibitor complex. The second plot (Figure 9B) of slope against the concentration of **9j** showed enzyme-inhibitor dissociation constant (K_i). K_i was calculated from the inhibitor concentration of **9j** versus the slope, and it was found to be 1.2 μM. The kinetic results are presented in Table 2.

2.4 | Hemolytic activity

The synthesized compounds, **9a–k**, were also exposed to hemolytic assay to ascertain their safe utility as therapeutic agents. The cytotoxicity was profiled as percentage of hemolysis and the results are shown in Table 1. It was clear from the results that all the derivatives of this series had modest toxicity toward red blood cell membrane as compared to the positive control, Triton X (89.11 ± 0.01%). The minimum toxicity was rendered by **9k** (3.77 ± 0.03) in the series. In general, it was rational that most of the molecules exhibited very moderate cytotoxicity and thus can be considered for further applications in drug designing program.

2.5 | Docking studies of 9a–k into human carbonic anhydrase II

Docking studies were performed in order to better understand the enzyme-inhibitor recognition pattern and to estimate the likelihood of binding of active ligands **9a–k** at the catalytic site of human carbonic anhydrase II (h-CA II). Molecular modeling was conducted starting with a crystal structure of the h-CA II (PDB ID: 3PYK) [47–49] co-crystallized with Ru-complex (SRX). The SRX co-crystallized ligand was removed and re-docked using only Gold Score (GS) to the catalytic pocket with excellent docking overlay, suggesting a plausible binding site is predicted. As GS is the only scoring function able to treat metal complexes [50]. However, reasonable scores were predicted for all the four scoring functions used for ligands **9a–k**, that is, 60–67 for GS, 72–87 for ChemPLP, 26–31 for CS, and lastly 37–43 for ASP (Table 3).

The docked configuration of the most active derivative **9j** into the binding pocket is shown in Figure 10. This molecule exhibited hydrogen bonding interactions through its aminothiazole moiety with Asn67 and Gln92 residues lying in close proximity to the Zn(II). (Table 3) [51,52]. Moreover, it is found to interact through lipophilic contact with His64, His3, Phe20, Pro202, Val121, His119, His94, and His96. It is residing deep in the binding site of the enzyme, showing an excellent fit (Figure 10B), as did all the other ligands (Figures S26–S35), and blocks access of the original substrates to the Zn(II) coordinated to Histidine (His94, His96, and His119) residues. This demonstrated that the said enzyme might be a viable target for its inhibition by such hybrid benzamides.

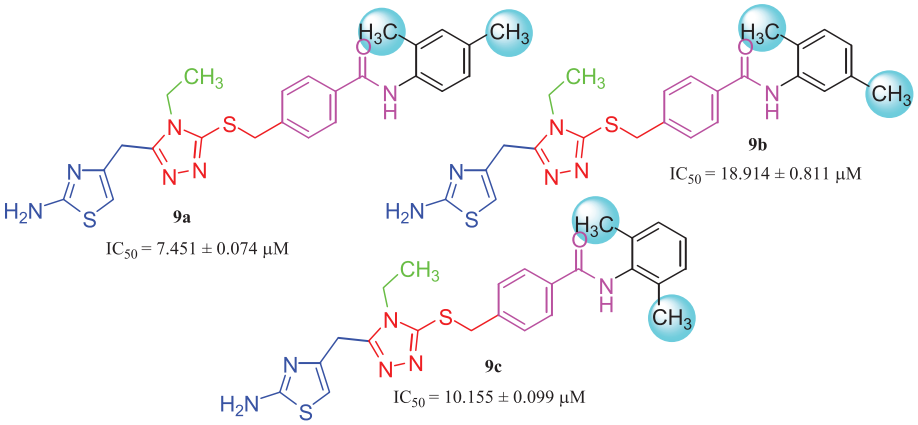


FIGURE 4 SAR of compounds **9a**, **9b,** and **9c** [Colour figure can be viewed at wileyonlinelibrary.com]

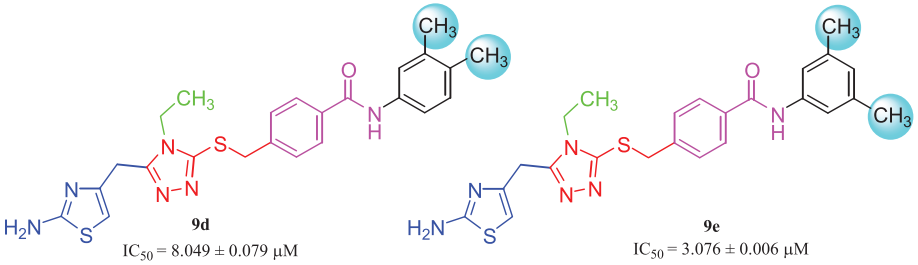


FIGURE 5 SAR of compounds **9d** and **9e** [Colour figure can be viewed at wileyonlinelibrary.com]

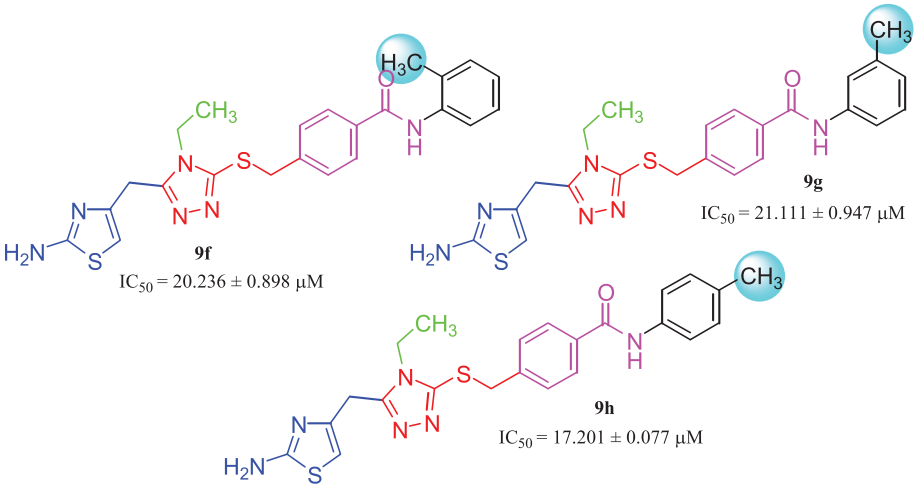


FIGURE 6 SAR of compounds **9f**, **9g,** and **9h** [Colour figure can be viewed at wileyonlinelibrary.com]

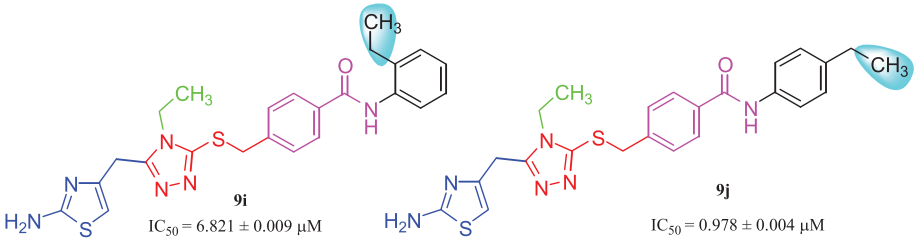


FIGURE 7 SAR of compounds **9i** and **9j** [Colour figure can be viewed at wileyonlinelibrary.com]

3 | EXPERIMENTAL

All the Chemicals were obtained from Sigma Aldrich and Alfa Aesar (Germany). Analytical grade solvents were provided by local suppliers. Melting points were taken on Griffin and George apparatus by using capillary tubes and were found uncorrected. Initial purity of compound was recognized by pre-coated silica gel aluminum plates in thin layer chromatography (TLC). Ethyl acetate and *n*-hexane were used as solvent system (30:70). The spots

were identified by UV_{254}. IR peaks were documented on a Jasco-320-A spectrometer by using KBr pellet method. By using BBO probe in Bruker Advance III 600 Ascend spectrometer, signals were recorded in DMSO-d_6 of ^{1}H-NMR (δ, ppm) at 600 MHz and ^{13}C-NMR at 150 MHz. Foss Heraeus CHN-O-Rapid instrument was used for elemental analyses, and theoretical values were within ±0.4%. Spectra of EI-MS was obtained by JEOL JMS-600H instrument with data processing system. Value of chemical shift (δ) is given in ppm and coupling constant (J) in Hz.

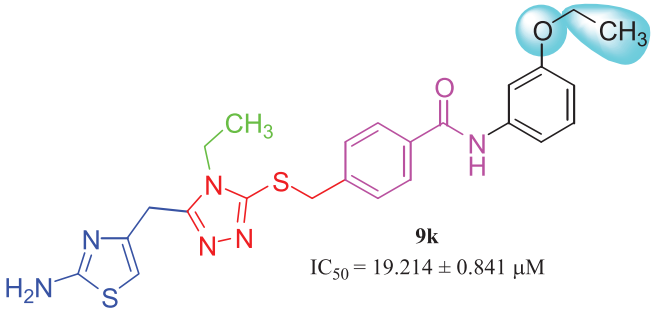


FIGURE 8 SAR of compound **9k** [Colour figure can be viewed at wileyonlinelibrary.com]

Synthesis of 5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazole-3-thiol (5). Starting from ethyl 2-(2-amino-1,3-thiazol-4-yl)acetate (**1**), the compound **5** was synthesized by following the already reported protocols [46].

General Synthesis of 4-[({5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl) methyl]-*N*-(aryl)benzamides (9a–k). *N,N*-Dimethyl formamide (DMF, 3 ml) was taken in 100 ml round bottom flask and 5-[(2-amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4*H*-1,2,4-triazole-3-thiol (0.2 g, **5**) was dissolved in it. Added one pinch of LiH in this solution and mixture was stirred for half an hour at room temperature. After that, equimolar amount of different electrophiles, *N*-aryl-4-chlorobenzamide (**8a–k,** one in each reaction), were added in each respective reaction and stirred for 12–24 h. The completion of reaction was monitored by TLC. *n*-hexane and ethyl acetate were used as solvent system in the ratio of 70:30. Single spot of product was the sign

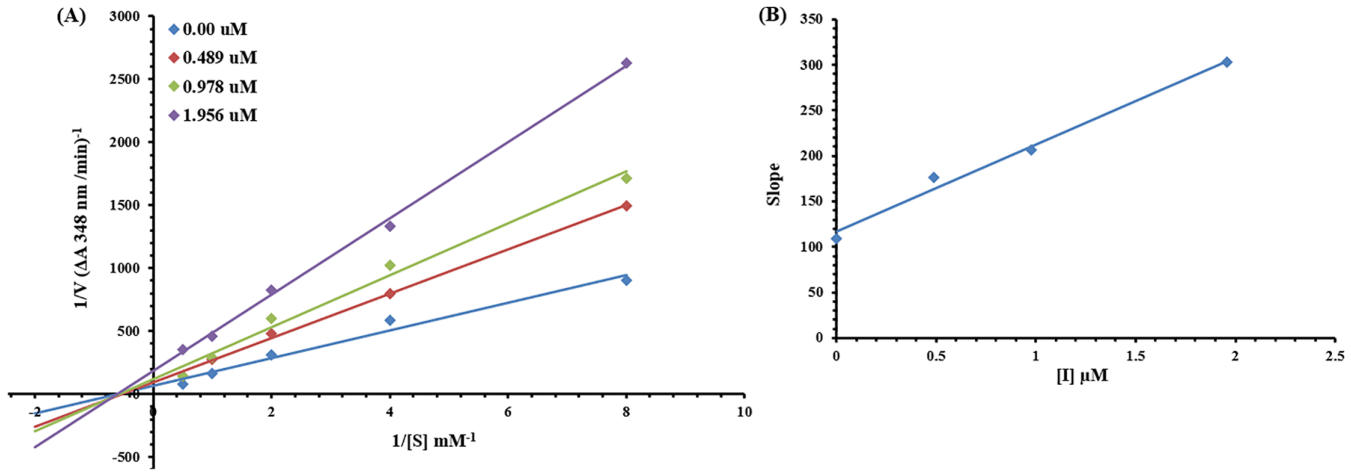


FIGURE 9 Lineweaver–Burk plots for inhibition of carbonic anhydrase in the presence of compound **9j**. (A) Concentrations of **9j were** 0.00, 0.489, 0.978, and 1.956 µM, respectively. Substrate *p*-nitrophenyl acetate concentrations were 0.125, 0.25, 0.5, 1, and 2 mM, respectively. (B) The insets represent the plot of the slope versus inhibitor **9j** concentrations to determine inhibition constant. The lines were drawn using linear least squares fit [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Kinetic parameters of **9j** on carbonic anhydrase

Concentration (µM)	V_{max} (ΔA/min)	K_m (mM)	Inhibition type	K_i (µM)
0.00	0.012233636	0.6	Non-competitive	1.2
0.489	0.007046364	0.6		
0.978	0.006874545	0.6		
1.956	0.002817807	0.6		

Note: V_{max} is the reaction velocity, K_m is the Michaelis–Menten constant, and K_i is the EI dissociation constant.

TABLE 3 The results of the scoring functions and interactions for the compounds **9a–k**

Compound	GS	PLP	CS	ASP	H-bonding/Lipophilic contact
SRX (Co-crystallized ligand)	64.1	-	-	-	Gln92(HB), Thr199(HB), His94(LC), Pro202(LC), Val121 (LC), Val135(LC), Phe131(LC), Leu198LC).
9a	64.9	83.3	30.3	37.8	Val121(LC), Val143(LC), Ile91(LC), Thr200(LC).
9b	63.4	81.8	30.9	39.3	His94(LC), Thr199(LC), Val121(LC), Val135(LC), Phe131(LC), Leu198LC).
9c	66.8	86.8	30.8	40.8	His94(LC), Leu141(LC), Val121(LC), Val135(LC), Phe131(LC), Val143(LC).
9d	65.2	85.5	29.8	40.5	Val121(LC), Ile91(LC), Val143(LC), Phe131(LC), Thr200 (HB).
9e	66.2	73.3	26.2	38.5	Trp5(HB), His3(HB), Leu204(LC), Pro202(LC), His94 (LC), Val135(LC), Phe131(LC).
9f	66.5	74.2	27.2	42.8	Phe20(LC), His94(LC), Trp5(LC), Val121(LC), Val143 (LC), His3(LC), Leu198LC).
9g	63.8	79.2	26.2	42.8	Phe20(LC), His94(LC), Trp209(LC), Trp5(LC), Val121 (LC), Val143(LC),His3(LC), Leu198LC).
9h	60.5	75.1	26.3	40.5	Gln92(HB), Trp5(HB), Phe20(LC), His94(LC), Trp209 (LC), His3(LC), Pro202(LC), Val121(LC), Val143 (LC),His3(LC), Leu198LC).
9i	63.4	72.6	27.8	42.2	Asp72(HB), Thr200(LC), His94(LC), Trp209(LC), Ile91 (LC), Phe131(LC), Val121(LC), Val143(LC)
9j	60.5	77	27.8	40.6	Asn67(HB), Gln92(HB), His64(LC), His3(LC), Phe20 (LC), Pro202(LC),Val121(LC). His119(LC), His94(LC) His96(LC).
9k	62.9	83.5	31.1	39.3	Asp72(HB), Thr199(HB), His96(LC), Leu198(LC), His94 (LC), Val121(LC).

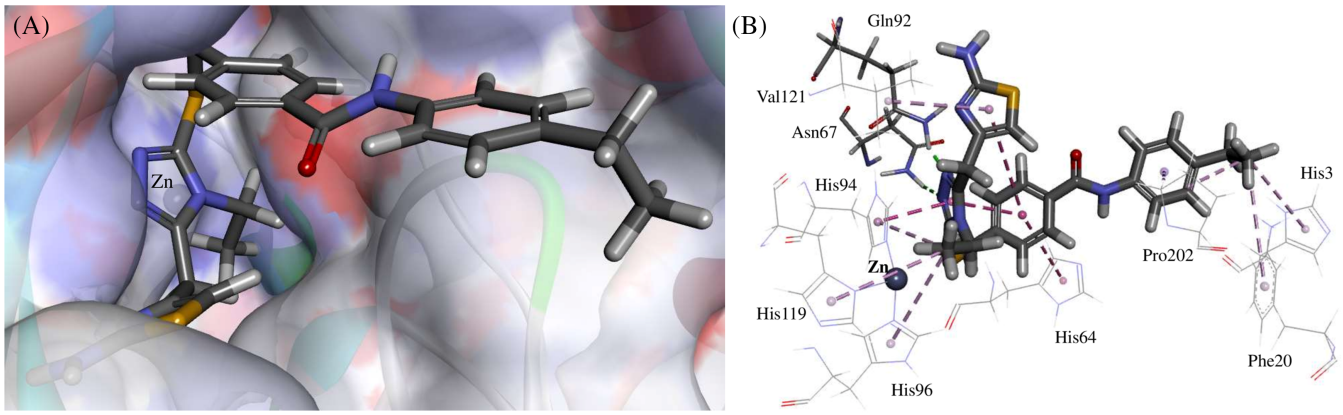


FIGURE 10 The docked configuration of **9j** in the catalytic site of carbonic anhydrase II (PDB ID: 3PYK) using GS. (A) Compound **9j** is shown in the binding pocket with the protein surface rendered. Red depicts a negative partial charge on the surface, blue depicts positive partial charge, and gray shows neutral/lipophilic areas. (B) Hydrogen bonds are depicted as green dotted lines and lipophilic contacts (LC) are represented as purple dotted lines between the ligand and the amino acid [Colour figure can be viewed at wileyonlinelibrary.com]

of completion of reaction. The product was precipitated out by adding excess ice cold distilled water. The targeted products, **9a–k**, were obtained through filtration, excess washing with distilled water, and dried respectively for further use.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(2,4-dimethylphenyl)benzamide (9a). Dark Brown solid; Yield: 92%; m.p. 104–106°C; Mol. Formula: $C_{24}H_{26}N_6OS_2$; Mol. Mass: 478 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3345 (N—H str.), 3054 (C—H

str. of aromatic ring), 2923 (—CH_2— str.), 1673 (C═O str.), 1561 (C═C str. of aromatic ring), 1517 (C═N str.), 1169 (C—N—C bond str.), 623 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 9.80 (s, 1H, —CO—NH-1′′′′), 7.94 (br.d, J = 7.9, 2H, H-2′′′ and H-6′′′), 7.52 (br.d, J = 8.1, 2H, H-3′′′ and H-5′′′), 7.23 (br.d, J = 7.8, 1H, H-6′′′′), 7.12 (br.s, 1H, H-3′′′′), 7.05 (br.d, J = 7.7 1H, H-5′′′′), 6.96 (br.s, 2H, H_2N-2), 6.27 (s, 1H, H-5), 4.49 (s, 2H, CH_2-8′′′), 3.99 (s, 2H, CH_2-6), 3.86 (q, J = 7.0, 2H, CH_2-1′′), 2.32 (s, 3H, CH_3-4′′′′), 2.22 (s, 3H, CH_3-2′′′′), 1.05 (br.t, J = 7.2 3H, CH_3-2′′); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.59 (C-2), 164.87 (C-7′′′), 153.18 (C-5′), 148.03 (C-3′), 146.17 (C-4), 141.02 (C-4′′′), 135.02 (C-1′′′′), 133.72 (C-1′′′), 133.58 (C-2′′′′), 133.47 (C-4′′′′), 130.77 (C-5′′′′), 128.86 (C-3′′′& C-5′′′), 127.69 (C-2′′′ and C-6′′′), 126.49 (C-3′′′′), 126.45 (C-6′′′′), 102.36 (C-5), 38.57 (C-1′′), 36.44 (C-8′′′), 27.53 (C-6), 20.50 (CH_3-4′′′′), 17.77 (CH_3-2′′′′), 14.64 (C-2′′) Anal. Calc. for $C_{24}H_{26}N_6OS_2$ (478.63): C, 60.23; H, 5.48; N, 17.56. Found: C, 60.28; H, 5.44; N, 17.57.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(2,5-dimethyl phenyl)benzamide (9b). Light Brown solid; Yield: 94%; m.p. 82–84°C Mol. Formula: $C_{24}H_{26}N_6OS_2$; Mol. Mass.: 478 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3351 (N—H str.), 3055 (C—H str. of aromatic ring), 2927 (—CH_2— str.), 1671 (C═O str.), 1566 (C═C str. of aromatic ring), 1523 (C═N str.), 1169 (C—N—C bond str.), 638 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 9.82 (s, 1H, —CO—NH-1′′′′), 7.94 (br.d, J = 8.1, 2H, H-2′′′ and H-6′′′), 7.53 (br.d, J = 8.1, 2H, H-3′′′ and H-5′′′), 7.20–7.18 (m, 2H, H-3′′′′ and H-6′′′′), 7.02 (br.d, J = 7.5, 1H, H-4′′′′), 6.97 (br.s, 2H, H_2N-2), 6.28 (s, 1H, H-5), 4.50 (s, 2H, CH_2-8′′′), 4.00 (s, 2H, CH_2-6), 3.87 (q, J = 7.1, 2H, CH_2-1′′) 2.32 (br.s, 3H, CH_3-2′′′′), 2.24 (br.s, 3H, CH_3-5′′′′), 1.04 (t, J = 7.1, CH_3-2′′); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.60 (C-2), 164.84 (C-7′′′), 153.18 (C-5′), 148.04 (C-3′), 146.15 (C-4), 141.06 (C-4′′′), 136.10 (C-1′′′′), 133.58 (C-1′′′), 130.44 (C-2′′′′), 130.05 (C-3′′′′), 128.88 (C-3′′′& C-5′′′), 127.70 (C-2′′′ and 6′′′), 127.03 (C-4′′′′), 126.58 (C-6′′′′), 102.38 (C-5), 38.58 (C-1′′), 36.43 (C-8′′′), 27.53 (C-6), 20.46 (CH_3-5′′′′), 17.41 (CH_3-2′′′′), 14.64 (C-2′′). Anal. Calc. for $C_{24}H_{26}N_6OS_2$ (478.63): C, 60.23; H, 5.48; N, 17.56. Found: C, 60.24; H, 5.46; N, 17.58.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(2,6-dimethyl phenyl)benzamide (9c). Light Brown solid; Yield: 94%; m.p. 132–134°C; Mol. Formula: $C_{24}H_{26}N_6OS_2$; Mol. Mass.: 478 gmol-1; IR (KBr, υ/cm^{-1}): 3353 (N—H str.), 3057 (C—H str. of aromatic ring), 2930 (—CH_2— str.), 1662 (C═O str.), 1575 (C═C str. of aromatic ring), 1529 (C═N str.), 1171 (C—N—C bond str.), 637 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 9.71 (s, 1H, —CO—NH-1′′′′), 7.92 (br.d, J = 8.1, 2H, H-2′′′ and H-6′′′), 7.49 (br.d, J = 8.1, 2H, H-3′′′ and H-5′′′), 7.11 (br.s, 3H, H-3′′′′, H-4′′′′& H-5′′′′), 6.91 (br.s, 2H, H_2N-2), 6.23 (s, 1H, H-5), 4.45 (s, 2H, CH_2-8′′′), 3.94 (s, 2H, CH_2-6), 3.82 (q, J = 7.2 2H, H-1′′), 2.16 (br.s, 6H, CH_3-2′′′′& 5′′′′), 1.00 (t, J = 7.2, 3H, H-2′′); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.58 (C-2), 164.60 (C-7′′′), 153.19 (C-5′), 148.04 (C-3′), 146.18 (C-4), 141.07 (C-4′′′), 135.60 (C-2′′′′ and C-6′′′′), 133.38 (C-1′′′), 128.94 (C-3′′′& C-5′′′), 127.65 (C-2′′′ and 6′′′), 127.59 (C-3′′′′ and C-5′′′′), 126.62 (C-4′′′′), 102.35 (C-5), 38.58 (C-1′′), 36.32 (C-8′′′), 27.53 (C-6), 18.00 (CH_3-2′′′′ and CH_3-2′′′′), 14.64 (C-2′′).. Anal. Calc. for $C_{24}H_{26}N_6OS_2$ (478.63): C, 60.23; H, 5.48; N, 17.56. Found: C, 60.25; H, 5.47; N, 17.59.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(3,4-dimethyl phenyl)benzamide (9d). Orange solid; Yield: 94%; m.p. 192–194°C Mol. Formula: $C_{24}H_{26}N_6OS_2$; Mol. Mass: 478 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3353 (N—H str.), 3039 (C—H str. of aromatic ring), 2910 (—CH_2— str.), 1649 (C═O str.), 1549 (C═C str. of aromatic ring), 1515 (C═N str.), 1145 (C—N—C bond str.), 610 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 10.03 (s, 1H, —CO—NH-1′′′′), 7.87 (br.d, J = 8.1, 2H, H-2′′′ and H-6′′′), 7.53 (s, 1H, H-2′′′′), 7.47 (br.d, J = 8.0, 3H, H-3′′′, H-5′′′ and H-6′′′′), 7.09 (br.d, J = 8.1, 1H, H-5′′′′), 6.91 (br.s, 2H, H_2N-2), 6.22 (s, 1H, H-5), 4.44 (s, 2H, CH_2-8′′′), 3.94 (s, 2H, CH_2-6), 3.80 (q, J = 7.1 2H, H-1′′) 2.21 (br.s, 3H, CH_3-4′′′′), 2.18 (s, 3H, CH_3-3′′′′), 0.98 (t, J = 7.2 3H, H-2′′); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.58 (C-2), 164.77 (C-7′′′), 153.17 (C-5′), 148.00 (C-3′), 146.17 (C-4), 141.00 (C-4′′′), 136.76 (C-3′′′′), 136.07 (C-1′′′′), 133.98 (C-1′′′), 131.37 (C-4′′′′), 129.40 (C-5′′′′), 128.31 (C-3′′′& C-5′′′), 127.68 (C-2′′′ and C-6′′′), 121.61 (C-2′′′′), 117.91 (C-6′′′′), 102.37 (C-5), 38.56 (C-1′′), 36.50 (C-8′′′), 27.53 (C-6), 19.59 (CH_3-3′′′′), 18.77 (CH_3-4′′′′), 14.65 (C-2′′). Anal. Calc. for $C_{24}H_{26}N_6OS_2$ (478.63): C, 60.23; H, 5.48; N, 17.56. Found: C, 60.30; H, 5.42; N, 17.58.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(3,5-dimethyl phenyl)benzamide (9e). Brick Red solid; Yeld: 90%; m.p. 102–104°C; Mol. Formula: $C_{24}H_{26}N_6OS_2$; Mol. Mass.: 478.16 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3340 (N—H str.), 3041 (C—H str. of aromatic ring), 2930 (—CH_2— str.), 1674 (C═O str.), 1561 (C═C str. of aromatic ring), 1510 (C═N str.), 1160 (C—N—C bond str.), 618 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 10.03 (s, 1H, —CO—NH-1′′′′), 7.87 (br.d, J = 8.1, 2H, H-2′′′ and H-6′′′), 7.47 (br.d, J = 8.0, 2H, H-3′′′ and H-5′′′), 7.39 (br.s, 2H, H-2′′′′ and H-6′′′′), 6.91 (br.s, 2H, H_2N-2), 6.74 (br.s, 1H, H-4′′′′), 6.22 (s, 1H, H-5), 4.44 (s, 2H, CH_2-8′′′), 3.94 (s, 2H, CH_2-6), 3.81 (q, J = 7.1 2H, H-1′′) 2.26 (br,s, 6H, CH_3-3′′′′ and CH_3-5′′′′), 0.98 (t, J = 7.1, 3H, H-2′′); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.58 (C-2), 164.92 (C-7′′′), 153.18 (C-5′), 148.00 (C-3′), 146.16 (C-4), 141.07 (C-4′′′), 138.90 (C-1′′′′), 137.46 (C-3′′′′ and C-5′′′′), 133.97 (C-1′′′), 128.83 (C-3′′′& C-5′′′), 127.81 (C-2′′′ and 6′′′), 125.15 (C-4′′′′), 118.12 (C-2′′′′ and C-6′′′′), 102.38 (C-5), 38.56 (C-1′′), 36.45 (C-8′′′), 27.52 (C-6), 21.08 (CH_3-3′′′′ and 5′′′′),

14.64 (C-2″): Anal. Calc. for $C_{24}H_{26}N_6OS_2$ (478.63): C, 60.23; H, 5.48; N, 17.56. Found: C, 60.31; H, 5.41; N, 17.57.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(2-methylphenyl)benzamide (9f). Brick Red solid; Yield: 93%; m.p. 142–144°C; Mol. Formula: $C_{23}H_{24}N_6OS_2$; Mol. Mass.: 464.15 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3364 (N—H str.), 3064 (C—H str. of aromatic ring), 2944 (—CH_2— str.), 1684 (C═O str.), 1544 (C═C str. of aromatic ring), 1534 (C═N str.), 1174 (C—N—C bond str.), 638 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 9.82 (s, 1H, —CO—NH-1‴′), 7.89 (br.d, J = 8.0, 2H, H-2‴ and H-6‴), 7.48 (br.d, J = 8.1, 2H, H-3‴ and H-5‴), 7.33 (br.d, J = 7.5, 1H, H-6⁗), 7.26 (br.d, J = 7.2, 1H, H-3⁗), 7.21 (t, 1H, H-5⁗), 7.16 (t, J = 7.3 1H, H-4⁗), 6.91 (br.s, 2H, H_2N-2), 6.22 (s, 1H, H-5), 4.44 (s, 2H, CH_2-8‴), 3.94 (s, 2H, CH_2-6), 3.82 (q, J = 7.0, 2H, H-1″), 2.22 (br,s, 3H, CH_3-2⁗), 0.99 (t, J = 7.2, 3H, H-2″); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.58 (C-2), 164.86 (C-7‴), 153.18 (C-5′), 148.01 (C-3′), 146.18 (C-4), 141.12 (C-4‴), 136.33 (C-1⁗), 133.65 (C-1‴), 130.25 (C-3⁗) 128.89 (C-3‴& C-5‴), 127.72 (C-2‴ and 6‴), 126.53 (C-6⁗), 125.94 (C-4⁗ and C-5⁗), 102.36 (C-5), 38.57 (C-1″), 36.41 (C-8‴), 27.53 (C-6), 17.84 (CH_3-2⁗). 14.65 (C-2″) Anal. Calc. for $C_{23}H_{24}N_6OS_2$ (464.61): C, 59.46; H, 5.21; N, 18.09. Found: 59.41; H, 5.26; N, 18.12.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(3-methylphenyl)benzamide (9g). Light Brown solid; Yield: 94%; m.p. 140-142°C; Mol. Formula: $C_{23}H_{24}N_6OS_2$; Mol. Mass.: 464.15 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3343 (N—H str.), 3053 (C—H str. of aromatic ring), 2923 (—CH_2— str.), 1673 (C═O str.), 1563 (C═C str. of aromatic ring), 1513 (C═N str.), 1163 (C—N—C bond str.), 621 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm):): 10.11 (s, 1H, —CO—NH-1⁗), 7.88 (br.d, J = 8.1, 2H, H-2‴ and H-6‴), 7.55 (br.d, J = 8.0, 1H, H-6⁗), 7.48 (br.d, J = 8.1, 2H, H-3‴ and H-5‴), 7.22 (t, J = 7.7, 1H, H-4⁗), 6.91 (br.s, 3H, H_2N-2 and H-5⁗), 6.22 (s, 1H, H-5), 4.44 (s, 2H, CH_2-8‴), 3.94 (s, 2H, CH_2-6), 3.81 (q, J = 7.0, 2H, H-1″), 2.30 (br,s, 3H, CH_3-3⁗), 0.98 (t, J = 7.2, 3H, H-2″); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.58 (C-2), 165.00 (C-7‴), 153.18 (C-5′), 147.99 (C-3′), 146.17 (C-4), 141.12 (C-4‴), 138.99 (C-1⁗), 137.68 (C-3⁗), 133.93 (C-1‴), 128.84 (C-3‴& C-5‴) 128.37 (C-5⁗), 127.73 (C-2‴ and 6‴), 124.31 (C-4⁗), 117.53 (C-6⁗), 102.37 (C-5), 38.56 (C-1″), 36.46 (C-8‴), 27.53 (C-6), 21.17 (CH_3-3⁗), 14.65 (C-2″): Anal. Calc. for $C_{23}H_{24}N_6OS_2$ (464.61): C, 59.46; H, 5.21; N, 18.09. Found: 59.42; H, 5.24; N, 18.10.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(4-methylphenyl)benzamide (9h). Light Orange solid; Yield: 96%; m.p. 110-112°C; Mol. Formula: $C_{23}H_{24}N_6OS_2$; Mol. Mass.: 464.15 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3348 (N—H str.), 3058 (C—H str. of aromatic ring), 2928 (—CH_2— str.), 1678 (C═O str.), 1558 (C═C str. of aromatic ring), 1518 (C═N str.), 1168 (C—N—C bond str.), 628 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 10.11 (s, 1H, —CO—NH-1⁗), 7.87 (br.d, J = 8.1, 2H, H-2‴ and H-6‴), 7.63 (br.d, J = 8.2, 2H, H-2⁗ and H-6⁗), 7.47 (br.d, J = 8.1, 2H, H-3‴ and H-5‴) 7.14 (br.d, J = 8.2, 2H, H-3⁗ and H-5⁗), 6.91 (s, 2H, H_2N-2), 6.22 (s, 1H, H-5), 4.44 (s, 2H, CH_2-8‴), 3.93 (s, 2H, CH_2-6), 3.80 (q, J = 7.1, 2H, H-1″), 2.27 (s, 3H, CH_3-4⁗), 0.98 (t, J = 7.2, 3H, H-2″); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm):): 168.58 (C-2), 164.86 (C-7‴), 153.17 (C-5′), 147.98 (C-3′), 146.16 (C-4), 141.05 (C-4‴), 136.54 (C-1⁗), 133.95 (C-1‴), 128.93 (C-3‴& C-5‴), 128.82 (C-3⁗ and C-5⁗), 127.70 (C-2‴ and C-6‴), 120.36 (C-2⁗ and C-6⁗), 102.37 (C-5), 38.56 (C-1″), 36.49 (C-8‴), 27.52 (C-6), 20.45 (CH_3-4⁗), 14.65 (C-2″): Anal. Calc. for $C_{23}H_{24}N_6OS_2$ (464.61): C, 59.46; H, 5.21; N, 18.09. Found: 59.41; H, 5.29; N, 18.14.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(2-ethylphenyl)benzamide (9i). Orange solid; Yield: 92%; m.p. 88-90°C; Mol. Formula: $C_{28}H_{26}N_6OS_2$; Mol. Mass.: 478.16 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3356 (N—H str.), 3036 (C—H str. of aromatic ring), 2916 (—CH_2— str.), 1686 (C═O str.), 1576 (C═C str. of aromatic ring), 1536 (C═N str.), 1166 (C—N—C bond str.), 626 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 9.84 (s, 1H, —CO—NH-1⁗), 7.90 (br.d, J = 8.0, 2H, H-2‴ and H-6‴), 7.48 (br.d, J = 8.1, 2H, H-3‴ and H-5‴), 7.30–7.27 (m, 2H,, H-3⁗ and H-6⁗), 7.23–7.21 (m, 2H, H-4⁗& H-5⁗), 6.92 (br.s, 2H, H_2N-2), 6.23 (s, 1H, H-5), 4.45 (s, 2H, CH_2-8‴), 3.94 (s, 2H, CH_2-6), 3.81 (q, J = 7.1, 2H, H-1″) 2.60 (q, 2H, J = 7.5, CH_3—CH_2—2⁗), 1.11 (t, 3H, J = 7.5, $\underline{CH_3}$—CH_2—2⁗), 0.99 (t, J = 7.2, 3H, H-2″); ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.59 (C-2), 165.24 (C-7‴), 153.19 (C-5′), 148.03 (C-3′), 146.17 (C-4), 141.11 (C-4‴), 139.78 (C-1⁗), 135.69 (C-2⁗), 133.49 (C-1‴), 128.91 (C-3‴ and C-5‴), 128.44 (C-3⁗), 127.68 (C-2‴ and C-6‴), 127.51 (C-5⁗), 126.45 (C-4⁗), 125.96 (C-6⁗), 102.36 (C-5), 38.56 (C-1″), 36.38 (C-8‴), 27.53 (C-6), 23.94 ($\underline{CH_2}$-CH_3-2⁗), 14.64 (C-2″), 14.07 (CH_2-$\underline{CH_3}$-2⁗). Anal. Calc. for $C_{24}H_{26}N_6OS_2$ (478.63): C, 60.23; H, 5.48; N, 17.56. Found: C, 60.29; H, 5.45; N, 17.59.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(4-ethylphenyl)benzamide (9j). Dark Orange solid; Yield: 93%; m.p. 86-88°C; Mol. Formula: $C_{28}H_{26}N_6OS_2$; Mol. Mass.: 526.16 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3349 (N—H str.), 3049 (C—H str. of aromatic ring), 2929 (—CH_2— str.), 1679 (C═O str.), 1569 (C═C str. of aromatic ring), 1529 (C═N str.), 1181 (C—N—C bond str.), 633 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 9.84 (s, 1H, —CO—NH-1⁗), 7.90 (d, J = 7.9, 2H, H-2‴ and H-6‴),

7.30–7.28 (m, 2H, H-2′′′′, H-6′′′′), 7.23–7.21 (m, 2H, H-3′′′′& H-5′′′′), 6.92 (br.s, 2H, H_2N-2), 6.23 (s, 1H, H-5), 4.45 (s, 2H, CH_2-8′′′), 3.94 (s, 2H, CH_2-6), 3.82 (q, J = 7.1, 2H, H-1′′), 2.61 (q, 2H, J = 7.5, CH_3—CH_2—4′′′′), 1.11 (t, 3H, J = 7.5, $\underline{CH_3}$—CH_2—4′′′′**),** 0.99 (t, J = 7.2 3H, H-2′′); ^{13}C-N'MR (150 MHz, DMSO-d_6, δ/ppm): 168.60 (C-2), 165.25 (C-7′′′), 153.19 (C-5′), 148.04 (C-3′), 146.17 (C-4), 141.11 (C-4′′′), 139.78 (C-4′′′′), 135.69 (C-1′′′′), 133.50 (C-1′′′), 128.96 (C-3′′′ and C-5′′′), 128.44 (C-3′′′′ and C-5′′′′), 127.69 (C-2′′′ and C-6′′′), 125.96 (C-2′′′′ and C-6′′′′), 102.37 (C-5), 38.58 (C-1′′), 36.69 (C-8′′′), 27.53 (C-6), 23.94 (CH_3—CH_2—4′′′′), 14.64 (C-2′′), 14.07 ($\underline{CH_3}$—CH_2—4′′′′). Anal. Calc. for $C_{28}H_{26}N_6OS_2$ (526.68): C, 63.85; H, 4.98; N, 15.96. Found: C, 63.90; H, 4.96; N, 15.91.

4-[({5-[(2-Amino-1,3-thiazol-4-yl)methyl]-4-ethyl-4H-1,2,4-triazol-3-yl}sulfanyl)methyl]-*N*-(3-ethoxyphenyl)benzamide (9k). Light Orange solid; Yield: 95%; m.p. 174-175°C; Mol. Formula: $C_{24}H_{26}N_6O_2S_2$; Mol. Mass.: 494.16 gmol^{-1}; IR (KBr, υ/cm^{-1}): 3365 (N—H str.), 3065 (C—H str. of aromatic ring), 2945 (—CH_2— str.), 1685 (C═O str.), 1545 (C═C str. of aromatic ring), 1505 (C═N str.), 1145 (C—N—C bond str.), 605 (C—S str.); ^{1}H-NMR (600 MHz, DMSO-d_6, δ/ppm): 10.15 (s, 1H, —CO—NH-1′′′′), 7.87 (br.d, J = 8.0, 2H, H-2′′′ and H-6′′′), 7.48 (br.d, J = 8.1, 2H, H-3′′′ and H-5′′′), 7.45 (brs, 1H, H-2′′′′), 7.33 (br,d, J = 7.9, 1H, H-6′′′′), 7.22 (br,t, J = 8.1, 1H, H-5′′′′), 6.91 (s, 2H, H_2N-2), 6.65 (dd, J = 1.6 and 8.0, H-4′′′′), 6.22 (s, 1H, H-5), 4.44 (s, 2H, CH_2-8′′′), 4.01 (q, 2H, J = 6.9, O—CH_2—CH_3-2′′′′), 3.94 (s, 2H, CH_2-6), 3.80 (q, J = 7.0, 2H, H-1′′), 1.33 (t, 3H, J = 6.9, O—CH_2—$\underline{CH_3}$-3′′′′), 0.99 (t, J = 7.1, 3H, H-2′′),; ^{13}C-NMR (150 MHz, DMSO-d_6, δ/ppm): 168.58 (C-2), 165.10 (C-7′′′), 158.63 (C-3′′′′), 153.17 (C-5′), 147.98 (C-3′), 146.16 (C-4), 141.18 (C-4′′′), 140.26 (C-1′′′′), 133.90 (C-1′′′), 129.29 (C-5′′′′), 128.85 (C-3′′′ and C-5′′′), 127.74 (C-2′′′ and C-6′′′), 112.41 (C-6′′′′), 109.71 (C-4′′′′), 106.48 (C-2′′′′), 102.37 (C-5), 62.90 (O—CH_2—CH_3-3′′′′), 38.56 (C-1′′), 36.64 (C-8′′′), 27.52 (C-6), 14.65 (C-2′′), 14.63 (O—CH_2—$\underline{CH_3}$-3′′′′). Anal. Calc. for $C_{24}H_{26}N_6O_2S_2$ (494.63): C, 58.28; H, 5.30; N, 16.99. Found: C, 58.30; H, 5.38; N, 16.94.

Carbonic anhydrase inhibition assay. Carbonic anhydrase inhibition was measured as described previously with some modifications [53–55]. The method is based on the principle that *p*-nitrophenyl acetate is hydrolyzed by carbonic anhydrase to form yellow colored *p*-nitrophenol, which was measured spectrophotometrically. Briefly, reaction mixture contained 120 µl of 50 mM Tris-Sulfate buffer (pH 7.6 containing 0.1 mM $ZnCl_2$), 20 µl of inhibitor, and 20 µl of (50 U) bovine enzyme per well. Contents were well mixed and pre-incubated at 25°C for 10 min. Substrate *p*-nitrophenyl acetate was prepared (6 mM stock using <5% acetonitrile in buffer and used fresh every time) and 40 µl was added per well to achieve 0.6 mM concentration per well. Total reaction volume was made to 200 µl. After 30 min incubation at 25°C, contents were mixed and absorbance of reaction mixture was recorded at 348 nm using a microplate reader (SpectraMax ABS, USA). Acetazolamide was used as a standard. Each concentration was analyzed in three independent experiments. IC_{50} values were calculated by nonlinear regression using GraphPad Prism 5.0 (GraphPad, San Diego, CA USA).

$$\text{Inhibition}\,(\%) = [(\text{B}-\text{S})/\text{B}] \times 100$$

Here, the B and S are the absorbance for the blank and samples.

Kinetic analysis of the inhibition of carbonic anhydrase. To determine the kinetic parameters of compound **9j**, a series of experiments were performed. Compound **9j** was selected on the basis of IC_{50} value, which was used at different concentrations (0.00, 0.489, 0.978, and 1.956 µM, respectively), the substrate (*p*-nitrophenyl acetate) concentrations were varied (2, 1, 0.5, 0.25, and 0.125 mM). Other experiment conditions were the same as discussed in the Carbonic anhydrase assay section. Maximal initial velocity was determined from initial linear portion of absorbance up to 10 min at a 1 min interval. The inhibition type on the enzyme was assayed by Lineweaver–Burk plots of inverse of velocities (1/V) versus inverse of substrate concentration 1/[S] mM^{-1}. The EI dissociation constant K_i was determined by the secondary plot of 1/V versus inhibitors concentrations.

Hemolytic activity. Bovine blood sample was collected in EDTA that was diluted with saline (0.9% NaCl), and centrifuge at 1000xg for 10 min. The erythrocytes separated diluted in phosphate buffer saline of pH 7.4 and a suspension was made. We added 20 µl of synthetic compounds solution (10 mg/ml) in 180 µl of RBCs suspension and incubated for 30 min at room temperature. PBS was used as negative control and Triton 100-X was taken as positive control [56,57]. The %age of hemolysis was obtained from the following formula:

$$(\%)\ of\ Hemolysis = \frac{Absorbance\ of\ Sample - Absorbance\ of\ Negative\ Control}{Absorbance\ of\ Positive\ Control} \times 100.$$

Molecular docking process. The compounds were docked to the crystal structure of the human carbonic anhydrase II (PDB ID: 3PYK) [47], which was obtained from the Protein Data Bank (PDB) [58,59]. The Scigress v2.6 [60] was used to prepare the crystal structure for docking, that is, the hydrogen atoms were added, and the co-crystallized ligands as well as crystallographic water molecules were removed. The Scigress software suite was

also used to build the chemical structures, which were optimized using the MM2 force field [61]. The center of the binding was defined in the protein crystal structure as C4 of the co-crystallized SRX molecule (x = 16.785, y = 5.439, z = 15.072) with a 10 Å radius. Fifty docking runs were allowed for each ligand with default search efficiency (100%). The basic amino acids lysine and arginine were defined as protonated. Furthermore, aspartic and glutamic acids were assumed to be deprotonated. The GoldScore (GS) [62], ChemScore (CS) [59,60], Chem Piecewise Linear Potential (ChemPLP) [63] and Astex Statistical Potential (ASP) [64] scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the GOLD v5.7.3 software suite.

4 | CONCLUSION

A structurally distinctive series of novel molecules, amalgamated with a thiazole, an ethyl triazole, and a benzamide moiety, were synthesized and recognized with super carbonic anhydrase inhibition. It was postulated from their SAR studies that molecules, particularly bearing medium-sized non-polar group with +I effect at *para*-position in aryl part of the compound, generally inhibited the carbonic anhydrase in an excellent manner. All these molecules also showed mild cytotoxicity toward red blood cell membranes. Therefore, it was pertinent to conclude that these bi-heterocyclic benzamides can find their utility as leading medicinal scaffolds for the treatment of ailments related to overexpression of carbonic anhydrase enzyme, such as cancer, glaucoma, and epilepsy.

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DATA AVAILABILITY STATEMENT

The data is shared as the supplementary material.

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