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1. Introduction

There is a rising rate of research in medicinal chemistry to discover new effective and specific chemotherapeutic agents for cancer treatment.¹ Although many available anticancer drugs exist, drug resistance is still a major problem for their effective use. The hybridization approach is considered one of the most effective methods in drug design to attain novel anticancer agents capable of limiting their proliferation.² The synergistic effects of the hybrid structures can display pharmacological properties that are higher than those of individual structures, as well as decreased toxicity and fewer adverse effects. This trend in drug design is most obvious in thiazole chemistry,³ where

Tail-approach based design, synthesis, and cytotoxic evaluation of novel disubstituted and trisubstituted 1,3-thiazole benzenesulfonamide derivatives with suggested carbonic anhydrase IX inhibition mechanism[†]

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2,4,5-А novel series of and 2,3,4-trisubstituted thiazole hybrids with 1,3,4thiadiazolylbenzenesulfonamide was designed following the tail approach as possible hCAIX inhibitors. The key intermediate 1 was condensed with thiosemicarbazide 2a to give 1,3,4thiadiazolylthiosemicarbazone 3, which upon hetero-cyclization with substituted α -haloketones and esters afforded 2,4,5-trisubstituted thiazole-1,3,4-thiadiazole conjugates 4-8. Furthermore, the trisubstituted thiazole-1,3,4-thiadiazole hybrids 12a-d were synthesized via the regioselective cyclization of 4-substituted-1,3,4-thiadiazolylthiosemicarbazones with phenacyl bromide. The cyclized 2,4-disubstituted thiazole 4 enhanced cytotoxicity by nine, four and two times against HepG-2, Caco2, and MCF-7, respectively. Moreover, the simple methyl substitution on the thiosemicarbazone terminus 9a improved the parent derivative 3 cytotoxicity by nine, fourteen, and six times against HepG-2, Caco2, and MCF-7, respectively. This astonishing cytotoxicity was elaborated with hCAIX molecular docking simulation of 4, 9a, and 12d demonstrating binding to zinc and its catalytic His94. Furthermore, molecular dynamic simulation 9a revealed stable hydrogen bonding with hCAIX with interaction energy of -61.07 kcal mol⁻¹ and $\Delta G_{\text{binding}}$ MM-PBSA of -9.6 kcal mol⁻¹.

> a growing number of novel thiazole-derived hybrids have been described with a strong emphasis on their various enzymeinhibitory properties.⁴ Among the several thiazole derivative platforms, promising and expanding applications with the sulfonamide system have been well exploited for producing carbonic anhydrase inhibitors.⁵ Furthermore, sulfonamide-substituted 1,3,4-thiadiazoles have been demonstrated to have potential carbonic anhydrase inhibitory characteristics with anticancer activity.⁶

> Carbonic anhydrases (hCAs, EC 4.2.1.1) are metalloenzymes that control the balance between carbon dioxide and bicarbonate ions through the adding-removing water mechanism. Their catalytic activity involved zinc(n) ions as a cofactor to regulate the cellular pH.^{7,8} To date, fifteen human isoforms have been isolated; 12 of them are catalytic isoforms with a zincbinding site.⁹⁻¹¹ Out of the 12 catalytic isoforms, the transmembrane hCAIX and hCAXII were strongly correlated with cancer progression, which assisted the cancerous cell in accommodating the hypoxia conditions promoting metastases.¹² They were considered an interesting target for new anticancer agents' development, especially with their established protein structures. Despite their variable tissue distribution, the hCA isoforms show high similarity in their protein

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structure. The main chain structure of the tumor-associated hCAIX demonstrated RMSD 2.02, 2.05, and 1.87 Å with hCAI, hCAII, and hCAXII, respectively.¹³ Moreover, hCAIX shared 83 conserved amino acids with the other tumor-associated isoform hCAXII while sharing 79 and 75 conserved residues with hCAI and hCAII, respectively.¹³

Among the **hCAIX** inhibitors. thiadiazolebenzenesulfonamide derivatives had established appreciated activity, with the sulfonamide moiety acting as the zinc-binding group (ZBG). For instance, acetazolamide AAZ and methazolamide MAZ are clinically proven non-selective hCA inhibitors with thiadiazole sulfonamide core that possess the common structural features of classical inhibitors (Fig. 1).14,15 Merging the thiadiazole scaffold with the ZBG benzenesulfonamide was followed into Ia showed hCAXII Ki of 34.2 nM and Ib had hCAIX Ki of 86.4 nM.¹⁶ Several thiazole benzenesulfonamides had established valued hCA inhibition, such as II with Ki 0.4 and 0.11 µM of isoforms hCAI and hCAII, respectively.17 Similarly, the thiazole-benzenesulfonamide hybrids IIIa-c were reported with a potential inhibition of hCAIX, especially when increasing the lipophilicity of IIIa. This lipophilicity optimization improved their inhibitory potentials by three and 1.5 times against hCAIX and hCAXII, respectively, upon adding a phenyl IIIb or 4-chlorophenyl IIIc moieties to the C₄-thiazole ring (Fig. 1).18

On the other hand, several thiosemicarbazone derivatives were reported as hCA isoform inhibitors, such as **IV–V**, which showed hCAII IC₅₀ of 26.9 and 0.13 μ M, respectively.^{19,20} Moreover, the aromatic linked thiosemicarbazone proved appreciated inhibition against both hCA isoforms hCAI and hCAII with sub-micromolar IC₅₀ values, including **VIa** and **VIb** with IC₅₀ range 0.19–0.28 μ M (Fig. 1).²¹ Similarly, the naphthalene–

thiosemicarbazone **VIIa** and **VIIb** hybrids were reported to exhibit hCAII inhibition at the micromolar level (Fig. 1), with their molecular docking simulation highlighting the importance of the thiosemicarbazone sulfur and nitrogen groups to anchor zinc ion and the binding site residues Gln91, Thr198, and Thr199.²⁰

Based on the information above, we designed novel hCAIX inhibitors by optimizing the structure of the non-selective hCA inhibitor AAZ (Fig. 2). In the target compounds, we kept the sulfonamide moiety as a zinc-binding group. Besides its ability to bind to the zinc in the active site, the sulfonamide moiety could bind to His94, Leu198, and/or Thr199 through H-bond formation. We then replaced the 1,3,4-thiadiazole spacer of AAZ with 5-imino-4,5-dihydro-4-(phenyl)-1,3,4-thiadiazole moiety. The main reason is the ability of the imino group at C_5 -thiadiazole to form a hydrogen bond with His64. It was proved that the role of His64 is shuttling the proton during hCAs catalytic activity.¹³

Moreover, the phenyl thiadiazole could form hydrophobic interactions with the amino acids within the active site of hCA, such as Leu198 and Leu135. Also, we replaced the small acetyl tail of **AAZ** with longer and flexible substituted thiosemicarbazide moieties. This moiety allows hydrogen bond formation with the amino acids Gln67, Asn62, Arg60, and/or Gln92 of the active site of hCA. On the other hand, the ring closure strategy was applied to introduce the thiazole moiety in the tail part by cyclization of the thiosemicarbazide moiety. Aromatic substitution at C_4 of the thiazole ring might form hydrophobic interaction with the Val131 or Leu91. Besides, the aromatic group at C_3 -thiazole might form extra hydrophobic interaction with Gln92 and/or Val131. In a trial for finding the thiazole ring's best substitution pattern, we tried a set of



Fig. 1 Reported thiadiazole, thiazole, and thiosemicarbazone derivatives with carbonic anhydrase inhibition and anticancer activity.



Fig. 2 Design the targeted 2,4,5- and 2,3,4-trisubstituted thiazole hybrids with 1,3,4-thiadiazolylbenzenesulfonaminde derivatives as hCAIX inhibitors.

substitutions to get 2,3,4- and 2,4,5-trisubstituted thiazole derivatives to adopt a conformation that might fill the unoccupied region of hCAIX active site.

2. Results and discussion

2.1. Chemistry

The synthetic strategy for constructing the target two series of disubstituted and trisubstituted 1,3-thiazole sulfonamide derivatives (Schemes 1 and 2).

2.1.1. Synthesis of 1,3,4-thiadiazolylbenzenesulfonaminde tagged with 2,4-disubstituted and 2,4,5-trisubstituted thiazole derivatives (4–8). Thiazole is easily obtainable *via* the Hantzsch reaction of α -haloketones with thiourea fragment.²² In this context, the primary step for preparing novel functionalized 1,3-thiazole-based 1,3,4-thiadiazole is the reaction of 5-acetyl-3-*N*-(4-sulfamoylphenyl)-2-imino-1,3,4-thiadiazoline **1** with

thiosemicarbazide 2a followed by cyclization of product with sets of α -halocarbonyl reagents. Thus, treatment of 1 with thiosemicarbazide in boiling ethanol with a few drops of conc. HCl gave the corresponding condensation product 3 (Scheme 1). Spectral data and chemical transformation proved the structure of the later product. The IR spectrum of 3 revealed absorption bands at 3434-3151, 3141, 3102,1640, 1335, and 1248 cm⁻¹ characteristic for 2NH₂, 2NH, C=N, SO₂ groups, and C=S, respectively. Its ¹H-NMR spectrum exhibits the expected signals from aromatic protons and six singlet signals at δ 2.32, 7.39, 7.64, 8.60, 9.41, and 10.83 ppm assigned to CH₃, SO₂NH₂, ⁴N-H^b, ⁴N-H^a, NH and ²N-H protons, respectively. The ¹³C-NMR spectrum of 3 showed the existence of nine carbon signals which compatible with the suggested structure. The most significant carbon signals resonate at δ 12.50, 158.56, and 179.07 ppm, assignable to CH₃, thiadiazole-C₅, and C=S carbon, respectively. The mass spectrum (MS) of 3 revealed



Scheme 1 Synthesis of 1,3,4-thiadiazolylbenzenesulfonaminde tagged with 2,4-disubstituted and 2,4,5-trisubstituted thiazole derivatives 4-8.

a molecular ion peak (M^+) at m/z = 371 confirming its molecular weight.

It is worth mentioning that the ⁴*N*-unsubstituted thiosemicarbazone 3 can adopt *syn* conformation (NHC=S) and 1*E* (C=N) configuration (Scheme 1) based on previous studies.²³ The suggested molecular configuration with the ⁴N-H^b *cis* to the C=S group, leaving the second ⁴N-H^a disposed of intramolecular H-bond formation with ¹N=C. The proposed configuration of 3 supports the appearance of the germinal protons of the NH₂ group in the ¹H-NMR spectrum as magnetically nonequivalent protons.

Next, the reactivity of **3** with various α -chlorocarbonyl reagents was explored to prepare bioactive 1,3-thiazole-1,3,4-thiadiazole conjugates. Thus, the reaction of **3** with each of phenacyl bromide and chloroacetone in ethanol in the presence of sodium acetate under reflux afforded benzenesulfonamides **4** and **5**, respectively (Scheme 1). The structures of **4** and **5** were confirmed based on microanalyses and spectral data. For example, the IR spectrum of **5** displayed the lack of NH₂ and C=S functions and the presence of two bands at 3142, 3101 cm⁻¹ for two NH groups besides the azomethine (C=N) band at 1602 cm⁻¹. The ¹H-NMR spectrum of **5** indicated six

singlet signals at δ 2.13, 2.26, 6.30, 7.37, 9.29, and 11.79 ppm related to the two CH₃ groups, thiazole-H₅, NH₂, imine NH, and NH, as well as to the expected two doublets for the four aromatic protons. Its ¹³C-NMR spectrum revealed two sp³ hybridized carbon atoms at δ 13.34 and 17.46 ppm due to two methyl carbons, besides ten carbon signals assigned to sp² hybridized carbon atoms. The M⁺ peaks at m/z = 471 and 409 in the mass spectra of 4 and 5 are compatible with their molecular formula (C₁₉H₁₇N₇O₂S₃) and (C₁₄H₁₅N₇O₂S₃), respectively.

Furthermore, the mass fragmentation pattern of 5 is depicted in Scheme S1.† Compound 5 underwent cleavage to give two ion peaks at m/z = 128 and 281 due to the formation of 2-methylamino-4-methylthiazole and 5-cyano-2-imino-3-(4-sulfamoylphenyl)-1,3,4-thiadiazoline, respectively. The formed thiazole cleavages further to produce the base peak at m/z = 59 (100%), which is assigned to 1,2-thiazine cation radical. On the other hand, the thiadiazole fragment cleavages further to give rise to a sulfanilamide ion peak at m/z = 172.

Similarly, 2,4,5-trisubstituted thiazoles 6 and 7 were obtained in good yields by heating 3 with each of 3-chloro-2,4pentanedione and ethyl 2-chloroacetoacetate in ethanol in the presence of sodium acetate. Heterocyclization of 3 with ethyl

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Scheme 2 Synthesis of 1,3,4-thiadiazolylbenzenesulfonaminde tagged with 2,3,4-trisubstituted thiazole derivatives 12a-d.

chloroacetate in ethanol under reflux in sodium acetate furnished the thiazolidine-4-one 8 (Scheme 1). Their correct elemental analyses and spectral data clarified the structures of 6, 7, and 8. Their IR spectra exhibited, in each case, a strong absorption band in the region 1713–1668 cm⁻¹, characteristic of the carbonyl function. The ¹H-NMR spectrum of 6, as a representative example, showed, along with aromatic proton signals, six singlet signals, three signals exchangeable with D₂O, resonate downfield at δ 7.35, 9.25, 11.79 ppm corresponding to NH₂ and two NH protons, respectively, and three signals appeared upfield at δ 2.19, 2.26, 2.39 ppm related to three methyl protons. Its ¹³C-NMR spectrum revealed the appearance of 14 carbon signals. The sp³-hybridized carbon atoms resonated at δ 12.98, 27.48, 30.87 ppm is assignable to the three methyl carbons. Furthermore, the characteristic signal of the C=O resonates at δ 189.00 ppm. The mass spectrum of 6 showed a M^+ peak at m/z 451, which is adaptable to a molecular formula (C₁₆H₁₇N₇O₃S₃).

2.1.2. Synthesis of 1,3,4-thiadiazolylbenzenesulfonaminde tagged with 2,3,4-trisubstituted thiazole derivatives (12a–d). The cyclization reaction of 4-substituted thiosemicarbazones with α -haloketones represents the simple and convenient route

to obtain 2,3,4-trisubstituted thiazoles.²⁴ we studied the site selectivity for the cyclization reaction of 4-substituted thiosemicarbazones with phenacyl bromide. Thus, condensation of 4-substituted thiosemicarbazide **2a–d**, obtained from treating substituted isothiocyanates with hydrazine hydrate in ethanol,^{25,26} with 5-acetyl-1,3,4-thiadiazoline **1** in ethanol and the presence of HCl under reflux, afforded the corresponding compounds **9a–d** (Scheme 2).

The structures **9a–d** were proved based on their elemental analyses, spectral data, and chemical transformations. Their IR spectra exhibited characteristic absorption bands in the region $3339-3030 \text{ cm}^{-1}$ attributed to NH₂ and three NH groups and at $1635-1625 \text{ cm}^{-1}$ characteristics for conjugated azomethine (C=N) groups and 1247–1187 cm⁻¹ due to thiocarbonyl groups, respectively. The mass spectra of **9a–d** exhibited a M⁺ peak in each case, which was compatible with its molecular weight. The ¹H-NMR spectrum of **9a**, as an example of these series, revealed a doublet signal at δ 3.04 ppm due to methylamino protons, five singlet signals at δ 2.28, 7.66, 8.70, 11.04, and 11.43 ppm due to CH₃, NH₂, and three NH protons, respectively, and two doublet signals at δ 7.92 and 8.05 ppm due to four aromatic protons. Its ¹³C-NMR spectrum exhibited 10 carbon signals. The sp³ hybridized carbon signals resonate at δ 12.78 and 31.53 ppm, attributed to two methyl carbons. The two downfield carbon signals appear at δ 167.79 and 178.67 ppm characteristics for C=NH and C=S, respectively. Hence, NMR spectra for these analogues, 9a-d, showed only one set of peaks, demonstrating the *E* configuration's preferential formation. The $Z_{C=N}$ configuration is not observed because of steric hindrance, and the non-planar conformation of C=N-NH does not exist because it would violate the n- π conjugation.²⁷⁻²⁹ To get more evidence on the geometry of our molecular structure, we performed Quantum DFT-B3LYP/6-31G(d) calculations for each isomer of 9a as an example. Accordingly, the calculated energy for optimized geometry of the (E) isomer of -2194.475905 Hartree appears to be slightly lower than that of the (Z) isomer (-2194.465113 Hartree). Theoretically, the (E) isomer is more stable than the (Z) isomer, with an energy difference of 6.77 kcal mol⁻¹ (ESI, Fig. S57[†]).

2,3,4-Trisubstituted thiazoles were prepared to investigate the effect of substituents inserted on the N-3 position for anticancer activity. Thus, cyclocondensation of 9a-d with phenacyl bromide furnished a sole product, in each case, identified as 2,3,4-trisubstituted thiazoles 12a-d (Scheme 2). Spectral data and microanalyses secured the structures of the later products. Their IR spectra were free of absorption bands in regions 3130-3100 cm⁻¹ and 1247-1187 cm⁻¹ related to two secondary NH groups and a thiocarbonyl group. For example, the ¹H-NMR spectrum of 12b revealed four singlet signals at δ 2.27, 6.62, 7.46, 9.98 ppm due to CH₃, thiazole-H₅, NH₂, and NH protons, respectively, and a doublet signal resonates at δ 4.47 ppm due NCH₂ protons. It also exhibited two doublets of doublets, and one multiplet signal resonated around δ 5.12, 5.19, 5.82 ppm due to the vinyl protons (CH₂=CH), and two doublets resonate at δ 7.96 and 8.08 ppm, indicating the existence of 4-disubstituted benzene, besides the other expected signals for five aromatic ring residues. The ¹³C-NMR spectrum of **12b** revealed eighteen carbon signals. The aliphatic carbons resonate at δ 12.49, 47.95 ppm, assignable for the carbons of CH₃, and NCH₂ groups, respectively. The olefinic carbons appear at δ 117.09, 132.07 ppm due to the carbons of the vinyl group (CH₂=CH). Further, the signals resonate at δ 102.35, 146.27, 155.51, 159.51, 171.48 ppm assignable to the carbons of thiazole- C_5 , C=N, thiadiazole- C_5 , thiazole- C_2 , and thiadiazole- C_2 , respectively. The mass spectrum showed a M^+ peak at m/z =511, corresponding to a molecular formula C₂₂H₂₁N₇O₂S₃. The mass spectrometry fragmentations are characteristic of the appearance of the fragment at m/z = 496 and 470 due to the loss of methyl and allyl groups from the parent ion peak, respectively. The peak showed at m/z = 102 (59%), assigned to the loss of phenylacetylene molecule [M⁺-C₈H₆] and the base peak appeared at m/z = 134 (100%) due to the formation of 2-phenylthirine cation radical [C₈H₆S]⁺⁻ that confirms the formation of thiazole ring.

The formation of **12a–d**, as depicted in Scheme 2, is expected to start with the S_N^2 -displacement reaction of bromine atom by the thiolate anion of **9a–d**, generated from deprotonation of more acidic N–H² proton, to afford the non-isolable thioether intermediates **10a–d** that subsequently undergoes tandem 5-

exo-trig ring closure *via* nucleophilic addition of a secondary NH group to C=O group to give intermediates **11a–d** that loss water molecule to afford the target compounds **12a–d**. Another structural feature of the synthesized thiazoles **12a–d** is they can theoretically exist in *EZ* or/and *EE* diastereomers. The preference formation of the thermodynamically stable diastereomers was demonstrated for **12a**-as example using B3LYP/6-31G(d) calculation. The results revealed that the optimized geometry for the diastereomer *EZ* has an energy of -2501.718252 Hartree, which is lower than that calculated for the *EE*-diastereomer (-2501.705865 Hartree) by about 7.77 kcal mol⁻¹ (ESI, Fig. S58†).

2.2. Bio-evaluation

2.2.1. Cytotoxicity evaluation. The cytotoxicity potential of the synthesized derivatives 1, 3-8, 9a-d, 12b, and 12d was evaluated in vitro against three human cancer cell lines: hepatic carcinoma HepG-2, colon carcinoma Caco2, and breast MCF-7 in addition to the normal lung fibroblast WI-38 (Table 1). The obtained results demonstrated that 3, 4, 8, 9a, and 12d showed the most potent growth inhibition among all derivatives against the three tested cancer cell lines (Fig. 3). Moreover, derivatives 3 and 4 exhibited more cytotoxic effect than the reference staurosporine against HepG-2 with IC50s 4.57, 0.50 and 13.60 µM, respectively. Furthermore, derivatives 7 and 8 showed substantial cytotoxic activity against HepG-2 with IC_{50s} 19.60 and 11.30 µM, respectively. Similarly, both 4 and 8 revealed better inhibition against Caco2 than staurosporine showing IC_{50s} of 3.19, 5.31, and 8.18 µM, respectively. Likewise, the ethyl ester-containing 2,4,5-trisubstituted thiazole 7 showed slightly better Caco2 cytotoxicity than staurosporine, revealing IC₅₀ of 7.39 μ M. On the other hand, derivative 4 (IC₅₀ = 3.52 \pm 0.2 μ M) was more potent against MCF-7 than staurosporine ($IC_{50} = 6.19$ \pm 0.3 μ M) while 3 demonstrated equipotent activity to staurosporine. Furthermore, remarkable MCF-7 growth inhibition was shown by compounds 1, 6, and 7 with IC₅₀ 14.90, 21.20, and 12.60 µM, respectively.

In the same context, derivatives 9a and 12d were more potent than staurosporine against the three tested cancer cell lines. Yet, the open chain thiosemicarbazone 9a showed the best growth inhibition potential among all the synthesized derivatives demonstrating IC₅₀ 0.53, 1.01, 1.12 µM against HepG-2, Caco2, and MCF-7, respectively. Nevertheless, it showed appreciable inhibition of the normal WI-38 giving IC_{50} 18.4 μ M. The most intriguing finding was that derivatives 1, 6, and 7 were less hazardous to the normal fibroblast cell line than staurosporine, as indicated by their IC₅₀ values (Fig. 3). These findings indicated that these compounds could be considered safer than staurosporine. Similarly, IC₅₀ of compounds 9c, 12b, and 12d on normal fibroblasts were higher than staurosporine, confirming their safety preferences. The remaining derivatives exhibited weak to moderate inhibitory activity against the three cancer cell lines with IC50 range of 34-153 µM.

2.2.2. Structure-activity relationship analysis. Converting the acetyl moiety of the thiadiazole nucleus **1** to thiosemicarbazone **3** greatly improved the cytotoxic effect towards

Table 1	The cytotoxicity evaluation of derivatives 1, 3–8, 9a–d, 12b, and 12d as IC ₅₀ in μ M \pm SD and their corresponding hCAIX binding energy
score	

	h CATV his dia a	$\mathrm{IC}_{50} \ (\mu\mathrm{M}\pm\mathrm{SD})$						
Compound	energy (kcal mol^{-1})	HepG-2	Caco2	MCF-7	WI-38			
1	-10.13	59.60 ± 3.40	11.1 ± 0.60	14.90 ± 0.70	91.50 ± 4.70			
3	-10.64	4.57 ± 0.30	14.9 ± 0.70	6.38 ± 0.30	44.40 ± 2.30			
4	-11.61	0.50 ± 0.04	3.19 ± 0.20	3.52 ± 0.20	21.40 ± 1.10			
6	-11.65	32.90 ± 1.90	33.8 ± 1.70	21.20 ± 1.20	110.00 ± 5.70			
7	-11.96	19.60 ± 1.10	7.39 ± 0.40	12.60 ± 0.60	42.90 ± 2.20			
8	-11.39	11.30 ± 0.60	5.31 ± 0.30	27.20 ± 1.20	22.90 ± 1.20			
9a	-11.47	0.53 ± 0.01	1.01 ± 0.10	1.12 ± 0.10	18.40 ± 1.70			
9b	-11.21	149.00 ± 8.40	74.80 ± 3.70	73.00 ± 3.30	92.90 ± 4.80			
9c	-11.51	37.10 ± 2.10	34.70 ± 1.70	21.10 ± 0.90	28.50 ± 1.50			
9d	-11.52	153.00 ± 8.60	98.70 ± 4.90	42.60 ± 1.80	60.70 ± 3.10			
12b	-11.13	17.50 ± 1.50	25.50 ± 1.30	15.10 ± 0.70	63.50 ± 3.90			
12d	-12.04	4.26 ± 0.20	1.06 ± 0.10	3.79 ± 0.20	46.60 ± 2.40			
Staurosporine	NA^a	13.60 ± 0.80	8.18 ± 0.70	6.19 ± 0.30	25.20 ± 1.30			

HepG-2 by twelve times and 2.5 times against MCF-7 while preserving 1 cytotoxicity against Caco2 (Table 1). Further increment in the molecule hydrophobicity by cyclizing the thiosemicarbazone terminus 3 into a thiazole ring 4–8 would improve the cell permeability and, ultimately, the cytotoxic effect. This was observed upon mono-substituting the cyclized thiazole with a large hydrophobic moiety like phenyl at C_4 to get 4, which revealed better cytotoxicity against the three tested cell lines. The added phenyl moiety could increase the overall derivative's lipophilicity, enhancing its cellular uptake. This assumption was supported by the decrement of the cytotoxicity when cyclizing the thiosemicarbazone terminus **3** with the relatively polar thiazolidine-4-one **8** against HepG-2 and MCF-7 by approximately three times.

On the other hand, preserving a small hydrophobic group like a methyl substitution at thiazole C_4 with another H-bond



Fig. 3 Graphical presentation of the cytotoxic activity of derivatives 3, 4, 8, 9a and 12d against staurosporine as the positive control expressed as IC_{50} (μ M).

forming moieties like ketone **6** or ester 7 at C_5 did not add much to their cytotoxicity. As concluded, the di-substitution of the thiazole at C_4 and C_5 was not preferable but rather monosubstitution with a phenyl moiety at C_4 .

Nevertheless, N3 and C₄ di-substitution of the thiazole ring was evaluated in series **12a–d**. It was observed that incorporating the *para*-chlorophenyl group to N3 with another phenyl ring at C₄ significantly improved the lipophilicity of **12d** compared to the aliphatic allyl substitution at N3 of **12b**. This modification led to 4–25 times improvement of cytotoxicity of **12d** over **12b** against the three tested cell lines, which might be due to the lipophilicity improvement to increase the drug's cellular permeability, as discussed earlier.

In the same context, increasing the lipophilicity of 3 was implemented by another strategy through adding lipophilic moieties such as methyl, allyl, and phenyl groups to the open chain thiosemicarbazone giving derivatives 9a-d. In this case, the best cytotoxic activity was achieved with the small methyl group substitution at 9a, showing three to nine times improvement against the three cell lines. Nonetheless, increasing the lipophilic moiety size by adding a phenyl group to the thiosemicarbazone terminus 9c showed drop-in cytotoxicity by 10 times. Further reduction in the cytotoxicity was observed upon substituting the thiosemicarbazone terminus with an allyl or para-chlorophenyl group of 9b and 9d, respectively. The size of the added substitution to the thiosemicarbazone terminus of 3 reduced the cytotoxicity probably by interfering with the possible interactions of the terminal sulfur of 3 with the active site residues of hCAIX, as discussed later in the molecular docking simulation (Fig. 5a). A summary

of the effect of the structural modification of **3** on the activity is illustrated in Fig. 4.

2.2.3. *In silico* molecular docking simulation. Molecular docking simulations of derivatives **1**, **3–8**, **9a–d**, and **12a–d** were conducted using hCAIX (PDB 3IAI, 2.20 Å) as a possible target to rationalize the obtained cytotoxic result.³⁰ The protein crystal was prepared as mentioned in the methodology section then the docking protocol was validated before starting the docking simulation. The validation included re-docking the co-crystallized AAZ to the binding site using different algorithms until getting the best binding orientation retaining the interaction pattern with the lowest RMSD. The validated molecular docking algorithm of hCAIX showed RMSD 0.374 Å (ESI, Fig. S59†).

The achieved molecular docking results (Tables 1 and 2, Fig. 5-7 and S60 and S61[†]) revealed better binding energy scores of all the tested benzenesulfonamide derivatives than AAZ ranging from -10.13 to -12.04 kcal mol⁻¹ compared to -8.83 kcal mol⁻¹ of AAZ. Moreover, all derivatives conserved the usual zinc coordination between the deprotonated nitrogen and one of the oxygen groups of the sulfonamide moiety (Table 2). It is well established that the sulfonamide nitrogen gets deprotonated in the physiological pH unveiling a negative charge that anchors the zinc ion.31,32 The zinc ion is an essential cofactor in maintaining the catalytic activity of the hCA isoforms.7,10,33 Therefore interfering with it would ultimately hinder hCA activity. The bi-dentate coordination of the derivatives sulfonamide moiety to zinc ion would replace the bound water/ hydroxide ion from the zinc at the enzyme's active site as an establishing mechanism to initiate the catalytic inhibition.11,34



Fig. 4 Schematic representation of the structure-activity relationship of the synthesized derivatives.



Fig. 5 The 2D and 3D binding pattern of molecular docking simulation of 3 (a and b) and 4 (c and d) using hCAIX (PDB 3IAI, 2.20 Å). The tested derivatives were shown in the stick model in green relative to AAZ in magenta color, with the H-bonds and hydrophobic interaction appearing in blue and red dotted lines, respectively.

Moreover, all tested derivatives maintained the H-bond formation with the active site Thr199, similar to **AAZ**. Additionally, the most potent derivatives **3**, **4**, **8**, **9a**, **9c**, and **12d** managed to form H-bond with one of the hCAIX catalytic histidine triad His94 with an average distance of 3.30 Å that augmented with their biological activity (Fig. 5–7).¹⁰

It was observed that replacing the terminal acetyl moiety of **1** with thiourea in **3** allowed the derivative to interact with Gln67, Asn62, and Arg60 through H-bonds. In addition, it adopted a conformation that allowed the deprotonated nitrogen of **3** to approach His94 and Thr200, thus enhancing its cytotoxicity against HepG-2 and MCF-7 (Fig. 5a and b and S60a†). Conversely, cyclizing the terminal thiourea into a thiazole ring with phenyl group **4** preserved the H-bond with Thr199 and the catalytic His94 with extra hydrophobic interaction with Val131 (Fig. 5c and d). However, replacing the terminal phenyl moiety of **4** with an acetyl group, **6**, greatly decreased its cytotoxic effect by losing the interaction with His94 and Val131 (Fig. S60c†).

Changing the acetyl group with an ethyl ester 7 achieved a proper conformation to interact with His64, Leu135, Thr199, Leu198, and Pro201 (Fig. 6a) that was translated into double the cytotoxic effect on HepG-2 and MCF-7. Unlike the small molecule **AAZ**, this extended thiazole ring with ethyl ester of 7 adopted a conformation that filled the unoccupied active site region with the interaction with residues His64 and Pro201 (Fig. 6b). Similarly, altering the ester group with a carbonyl moiety **8** maintained the interaction with His64, and Gln67 at the unoccupied pocket of the active site (Fig. 6c and d). Moreover, it demonstrated further improvement in the cytotoxicity by regaining the H-bond with His94 and additional H-bond with the active site His64 (Fig. 6c). The role of His64 in shuttling the proton during hCAs catalytic activity was valued, especially with its proven inward and outward conformation.¹³

Among the aliphatic substituted thiourea derivatives **9a–d**, derivatives **9a** and **9c** exhibited the best binding conformation inside hCAIX active site while maintaining the H-bond with Table 2 The molecular docking simulation results of derivatives 1, 3-8, 9a-d, and 12a-d using hCAIX (PDB 3IAI, 2.20 Å)

	Ligand		hCAIX (PDB: 3	IAI)		Interaction		
Compound	Interactin moiety	g	Interacting moiety	Interact residue	ing	Interaction type	Distance (Å)	Energy (kcal mol ⁻¹)
AAZ	N3	11	OG1	Thr	200	H-acceptor	3.04	-0.90
	01	14	Ν	Thr	199	H-acceptor	2.98	-4.40
	O3	16	NE2	Gln	92	H-acceptor	3.07	-0.70
	N1	8	ZN	Zn	262	Ionic	2.03	-15.60
	O2	15	ZN	Zn	262	Ionic	3.15	-3.50
	5-ring		CD2	Leu	198	Pi-H	3.76	-0.70
1	0	17	Ν	Thr	199	H-acceptor	2.97	-4.70
	Ν	16	ZN	Zn	262	Ionic	2.68	-7.00
	0	18	ZN	Zn	262	Ionic	2.00	-16.20
	6-ring		CD2	Leu	198	Pi-H	3.87	-0.20
3	Ν	4	ND1	His	64	H-acceptor	3.18	-4.80
	S	13	CB	Arg	60	H-acceptor	3.82	-0.70
	S	13	CD	Arg	60	H-acceptor	3.60	-1.00
	S	13	NH1	Arg	60	H-acceptor	3.54	-2.50
	S	13	CB	Asn	62	H-acceptor	4.03	-0.80
	S	13	ND2	Asn	62	H-acceptor	3.64	-3.90
	S	13	CB	Gln	67	H-acceptor	3.74	-0.70
	0	21	N	Thr	199	H-acceptor	2.84	-3.00
	0	21	OG1	Thr	200	H-acceptor	3.49	-0.50
	N	23	CE1	His	94	H-acceptor	3.23	-0.30
	0	22	ZN	Zn	262	Ionic	1.97	-16.80
	N	23	ZN	Zn	262	Ionic	3.12	-3.70
4	N	23	CE1	His	94	H-acceptor	3.23	-0.30
	0	24	N	Thr	199	H-acceptor	3.01	-4.50
	N	23	ZN	Zn	262	Ionic	2.95	-4.80
	0	25	ZN	Zn	262	Ionic	1.98	-16.60
	6-ring		CG2	Val	131	Pi-H	4.13	-0.60
	6-ring		CB	Leu	198	Pi-H	4.31	-0.50
	5-ring		CD1	Leu	198	Pi-H	4.01	-0.30
	6-ring		CD1	Leu	198	Pi-H	4.25	-0.40
_	6-ring		CD2	Leu	198	P1-H	3.59	-0.40
5	0	23	N	Thr	199	H-acceptor	3.02	-4.40
	N	22	ZN	Zn	262	Ionic	2.02	-15.90
	0	24	ZN	Zn	262	lonic	3.03	-4.20
<i>c</i>	6-ring	4-	CD2	Leu	198	PI-H	4.26	-0.40
6	0	1/	ND1	H1S	64	H-acceptor	2.85	-3.90
	U N	26	IN ZNI	1 nr	199	H-acceptor	2.92	-4.90
	N	24	ZIN	Zn	262	Ionic	2.08	-14.70
	0 Crina	25	ZN	Zn	262	IONIC D: LL	2.78	-6.20
-	6-ring	10	CD2	Leu	198	PI-H II dan an	3.54	-0.40
1	N	10	0	Pro	201	H-donor	2.98	-1.50
	3	12	U ND1	PIO	201	H-uonor	2.02	-2.90
	0	17	ND1 N	HIS	04 100	H-acceptor	2.98	-2.60
	N	23	IN ZNI	7n	199	H-acceptor Ionia	2.90	-3.00
	N O	24	ZN	Zn	202	Ionic	2.09	-6.90
	5 ring	20	ZIN CD2	Lou	125		1.97	-10.70
	5-mg		CD2	Leu	100	гі-11 р; ц	4.87	-0.70
o	6-ing	10	CD2 OF1	Cln	198	H-donor	2.39	-0.00
0	N	12	ND1	Hic	64	H-acceptor	2 20	-1.80
	1	0 16	CB	Hic	04 64	H-acceptor	3.30	-2.00
	N	22	CA	Len	100	H-acceptor	3.30	-0.20
	IN N	∠3 23	N	LCU Thr	190	H-acceptor	3.43	-0.90
	N11	23 25	CE1	111 Lie	133	H-acceptor	3.33 2.20	-5.90
	0	20	UE1 7N	П15 7n	94	Ionic	3.30 1.02	-0.20
	0	24	ZIN	211 7n	202	Ionic	2.95	-17.70
	6-ring	25		Lu	202	DiLU	3.43 4 55	-2.20
09	N	11	NE2	Clr	199	ri-n H-accentor	4.00	-0.20
<i>7</i> a	N11	14	CE1	UII UIa	92	H-acceptor	3.30 2.47	2.0U
	0	23		Lou	94 100	H acceptor	0.4/ 2.10	-0.30
	U	24	UA	Leu	198	n-acceptor	3.18	-0.40

	Ligand		hCAIX (PDB: 3	IAI)		Interaction		
Compound	Interactin moiety	g	Interacting moiety	Interact residue	ing	Interaction type	Distance (Å)	Energy (kcal mol ⁻¹)
	0	24	Ν	Thr	199	H-acceptor	3.03	-4.00
	Ν	22	ZN	Zn	262	Ionic	2.00	-16.30
9b	Ν	7	NE2	Gln	92	H-acceptor	3.50	-0.70
	0	23	Ν	Thr	199	H-acceptor	2.96	-4.80
	Ν	22	ZN	Zn	262	Ionic	2.67	-7.10
	0	24	ZN	Zn	262	Ionic	1.98	-16.60
9c	Ν	22	CE1	His	94	H-acceptor	3.28	-0.80
	0	23	Ν	Thr	199	H-acceptor	3.05	-4.00
	Ν	22	ZN	Zn	262	Ionic	3.35	-2.50
	0	24	ZN	Zn	262	Ionic	1.94	-17.40
	5-ring		CZ2	Trp	5	Pi-H	4.34	-0.50
	6-ring		CG1	Val	131	Pi-H	3.60	-0.40
9d	0	24	Ν	Thr	199	H-acceptor	2.92	-4.90
	Ν	22	ZN	Zn	262	Ionic	2.08	-14.80
	0	23	ZN	Zn	262	Ionic	2.59	-7.90
	6-ring		CB	Asp	132	Pi-H	3.74	-0.80
12a	0	25	Ν	Thr	199	H-acceptor	2.88	-5.10
	Ν	24	ZN	Zn	262	Ionic	2.48	-9.10
	0	26	ZN	Zn	262	Ionic	1.93	-17.60
12b	0	25	ZN	Zn	262	H-acceptor	2.92	-0.40
	0	26	Ν	Thr	199	H-acceptor	2.96	-4.70
	Ν	24	ZN	Zn	262	Ionic	2.06	-15.10
	0	25	ZN	Zn	262	Ionic	2.92	-5.00
	6-ring		CG2	Val	131	Pi-H	4.22	-0.50
	5-ring		CD1	Leu	198	Pi-H	4.12	-0.30
	6-ring		CD2	Leu	198	Pi-H	3.54	-0.30
12c	0	25	Ν	Thr	199	H-acceptor	2.95	-4.80
	Ν	24	ZN	Zn	262	Ionic	2.68	-7.00
	0	26	ZN	Zn	262	Ionic	1.97	-16.70
	6-ring		CD2	Leu	198	Pi-H	4.24	-0.30
12d	N	53	CE1	His	94	H-acceptor	3.33	-0.60
	0	56	CA	Leu	198	H-acceptor	3.27	-0.30
	0	56	N	Thr	199	H-acceptor	3.07	-3.90
	N	53	ZN	Zn	262	Ionic	3 34	-2.60
	0	55	ZN	Zn	262	Ionic	1.93	-17.60
	6-ring	50	CD2	Leu	91	Pi-H	4.81	-0.20
	6-ring		NE2	Gln	92	Pi-H	4.55	-0.80
	6-ring		CG1	Val	131	Pi-H	4 61	-0.30
	6-ring		CG2	Val	131	Pi-H	4 27	-0.30

His94 by their sulfonamide moiety (Fig. 7a and b and S60e†). Moreover, **9a** showed other H-bonds with Gln92, Leu198, and Thr199 with an average distance of 3.20 Å (Fig. 7a). In a similar way; among the cyclized thiazole derivatives **12a–d**, derivative **12d** was the best oriented inside hCAIX with the lowest binding energy of -12.04 kcal mol⁻¹. The deprotonated nitrogen of **12d** conserved the H-bond with His94, while the terminal *para* chloro-substituted phenyl displayed additional hydrophobic interactions with Gln92 and Val131 (Fig. 7c and d). Furthermore, **12d** showed multiple interactions with hCAIX active site residues Leu198, Thr199, and Leu91 that rationalized its good cytotoxicity.

Acknowledging the similarity between the CA isoenzymes, molecular docking simulation was performed using hCAI, hCAII, and hCAXII for the most potent derivatives **4**, **9a**, and **12d** to assess their off-target potentials. Their molecular docking simulation used the reported PDB: 3W6H,³⁵ 3HS4,³⁶ and 1JD0 (ref. 37) for hCAI, hCAII, and hCAXII, respectively. The attained data revealed the unfavorable binding pattern of the tested derivatives to the evaluated isoenzymes in contrast to hCAIX (Table 3 and ESI, Fig. S62–S64†). The deprotonated sulfonamide moiety of the three derivatives exhibited the classical coordination with zinc among the evaluated isoenzymes similar to hCAIX. Although **4** and **9a** demonstrated H-bond formation with Thr199, this solely formed bond was not expected to be sufficient for appreciable enzyme inhibition. Moreover, **12d** is only bound to Thr199 in the case of hCAII, not the other isoenzymes and showed single coordination with zinc of 3.27 Å length. In the same context, the predicted binding conformation and interaction of these promising derivatives with the



Fig. 6 The 2D binding pattern of molecular docking simulation of 7 (a and b) and 8 (c and d) using hCAIX (PDB 3IAI, 2.20 Å) showing distance in Å. The tested derivatives were shown in the stick model in green relative to AAZ in magenta color, with the H-bonds and hydrophobic interaction appearing in blue and red dotted lines, respectively.

tumor-associated hCAXII supported the assumption of exerting their anticancer activity through hCAIX inhibition, not hCAXII.

2.2.4. Molecular dynamics simulation of 9a and 9d. To further verify the suggested hCAIX mechanism for the obtained anticancer activity, the most potent derivative, 9a, was submitted for molecular dynamic simulation against its least potent congener, 9d, to test their hCAIX binding complex's stability. Their docked poses were validated using 100 ns-long molecular dynamics (MD) simulation where 9a demonstrated good stability inside the hCAIX binding site (PDB code: 3IAI) across the simulation (Fig. 8a). Moreover, its simulation revealed RMSD of 1.95 Å demonstrating stable H-bonds (~3 H-bonds on average) throughout MD simulation (Fig. 8b). In contrast, 9d was less stable exhibiting higher RMSD fluctuation with average 4.1 Å (Fig. 8a) which established from 1 to 2 H-

bonds on average showing highly fluctuating H-bond pattern (Fig. 8c).

Accordingly, the overall interaction energies of both derivatives averaged around -61.07 and -22.58 kcal mol⁻¹, respectively (Fig. 9). Furthermore, their calculated binding free energies ($\Delta G_{\text{binding}}$) in terms of MM-PBSA were found to be -9.6and -4.3 kcal mol⁻¹, respectively showing a significant difference in affinities between the two structures towards the active site of hCAIX (Table 4). Accordingly, **9a** was likely more active as an inhibitor of hCAIX in comparison to **9d**.

2.2.5. Physicochemical, pharmacokinetics properties and drug-likeness prediction. The promising derivatives 3, 4, 7, 8, 9a, 12b, and 12d were *in silico* evaluated in terms of their physicochemical, pharmacokinetics, and drug-likeness using the SwissADME® interface (Table 5).³⁸ Despite their structural



Fig. 7 The 2D and 3D binding pattern of molecular docking simulation of 9a (a and b) and 12d (c and d) using hCAIX (PDB 3IAI, 2.20 Å). The tested derivatives were shown in the stick model in green relative to AAZ in magenta color, with the H-bonds and hydrophobic interaction appearing in blue and red dotted lines, respectively.

differences, derivatives **9a**, **12b** and **12d** shared the same lipophilicity values. As expected from their chemical structures, all evaluated derivatives showed high polarity and were moderately soluble in an aqueous medium. This polarity was demonstrated in their topological polar surface area TPSA that exceeded 140 Å,^{38,39} which was beneficial for their low CNS permeation, and negligible binding to the efflux P-glycoprotein.⁴⁰ The low lipophilic characters of the tested derivatives did not affect the liver cytochrome P-450 isoenzymes. Nonetheless, it negatively affected GI absorption and skin permeation, requiring better lipophilic characteristics.

On the other hand, all tested derivatives had acceptable drug-likeness properties according to Lipinski's rule, with one violation of molecular weight of more than 500 g mol⁻¹ for **12b** and **12d**.^{41,42} However, their bioavailability scores revealed good

physiological activity according to Abbott's bioavailability score. $^{\rm 43}$

3. Experimental section

3.1. Synthesis and spectroscopic characterization

3.1.1. Synthesis of 2-(1-(5-imino-4-(4-sulfamoylphenyl)-4,5-dihydro-1,3,4-thiadiazol-2-yl)ethylidene)hydrazine-1-

carbothioamide (3). A mixture of 1 (2.22 g, 0.007 mol) and thiosemicarbazide 2a (0.67 g, 0.007 mol) in EtOH (30 ml), concentrated HCl (0.15 ml), was refluxed for 4 h. Compound 3 was obtained by filtering the precipitate, washing it with EtOH, drying it, and recrystallizing it from EtOH. Orange powder; yield 76%; mp > 300 °C; IR $\nu_{max}/cm^{-1} = 3434$, 3340, 3244, 3151 (2NH₂), 3141, 3102 (2NH), 1640 (C=N), 1596 (C=C), 1335, 1282

	hCAI (PDB: 3V	(H9V				hCAII (PDB: 3F	IS4)				hCAXII (PDB: 1	lJD0)			
Compound	Binding energy $(kcal mol^{-1})$	Intera residu	acting ue	Interaction type	Distance (Å)	Binding energy (kcal mol ⁻¹)	Interac residu	cting e	Interaction type	Distance (Å)	Binding energy (kcal mol ⁻¹)	Intera residu	cting e	Interaction type	Distance (Å)
4	-10.24	Thr Zn	199 302	H-acceptor Ionic	3.21 2.03	-10.59	Thr Zn	199 301	H-acceptor Ionic	2.96 2.76	-11.53	Thr Zn	199 901	H-acceptor Ionic	3.01 2.82
50	11 13	Zn Asn	302 69	Ionic H-accentor	2.68 3.53	-10.35	Zn Thr	301 199	Ionic H-accentor	2.05 3.10	10.02	Zn	901 67	Ionic H-accentor	$\begin{array}{c} 1.95 \\ 3.35 \end{array}$
24	CT'11_	Thr	199	H-acceptor	2.93	CC.U1-	Zn	301	Ionic	2.03	C6.01-	Thr	199	H-acceptor	3.01
		zn Zn	302 302	Ionic Ionic	2.01 3.10		Zn	301	Ionic	3.02		zn Zn	901 901	Ionic Ionic	2.85 1.95
12d	-7.43	Gln Zn	92 262	H-acceptor Ionic	3.56 3.23	-7.35	Thr Zn	199 262	H-acceptor Ionic	2.95 2.67	-8.42	Zn	262	Ionic	3.27
		Val	131	Pi-H	4.14		Zn Val	262 131	Ionic Pi-H	$1.99 \\ 4.21$					

(SO₂), 1248 (C=S); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.32$ (s, 3H, CH₃), 7.39 (s, 2H, NH₂), 7.64 (s, 1H, ⁴N-H_b), 7.87 (d, J = 8.5 Hz, 2H, Ar-H_{2,6}), 8.23 (d, J = 8.5 Hz, 2H, Ar-H_{3,5}), 8.60 (s, 1H, ⁴N-H_a), 9.41 (s, 1H, C=NH), 10.83 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.50$ (CH₃), 121.52 (2CH Ar-C_{2,6}), 126.45 (2CH Ar-C_{3,5}), 140.26 (Ar-C₄), 141.38 (Ar-C₁), 141.94 (C=N), 148.62 (thiadiazole-C₂), 158.56 (thiadiazole-C₅), 179.07 (C=S); MS *m/z* (%): 371 (M⁺, 1.3), 370 (1.9), 367 (1.8), 366 (2.0), 356 (1.7), 339 (2.1), 326 (1.3), 312 (1.6), 297 (5.1), 285 (2.3), 281 (1.7), 273 (1.5), 258 (1.6), 172 (1.5), 93 (12.7), 83 (58.1), 57 (100). Anal. calc. For C₁₁H₁₃N₇O₂S₃ (371.45): C, 35.57; H, 3.53; N, 26.40%; found: C, 35.56; H, 3.54; N, 26.41%.

3.1.2. General procedure for the preparation of thiazole derivatives (4–8). To a solution of 3 (0.8 g, 0.002 mol) in EtOH (30 ml) containing AcONa (1 g, 0.01 mol) and DMF (5 mL), α -chlorocarbonyl compounds (0.002 mol) was added. The reaction mixture was heated under reflux for 3–6 h, then cooled before being put into ice-cold water (50 ml). The precipitate was filtered and recrystallized from EtOH to yield the desired compounds 4–8.

3.1.2.1. 4-(2-Imino-5-(1-(2-(4-phenylthiazol-2-yl)hydrazineylidene)ethyl)-1,3,4-thiadiazol-3(2H)yl)benzenesulfonamide (4). The compound was obtained by heating 2-bromo-1-phenylethan-1one (0.42 g, 0.002 mol) for 3 h under reflux. Brown powder; yield 84%; mp 279–281 °C; IR $\nu_{\text{max}}/\text{cm}^{-1} = 3305, 3237 (\text{NH}_2),$ 3197, 3109 (2NH), 1657 (C=N), 1548 (C=C), 1328, 1267 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.33$ (s, 3H, CH₃), 7.49 (s, 2H, NH₂), 7.55 (s, 1H, thiazole-C₅), 7.32-8.22 (m, 9H, Ar-H), 9.45 (s, 1H, NH), 12.05 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.86$ (CH₃), 106.82 (thiazole-C₅), 121.80 (2CHAr-C_{2.6}), 126.01 (2CHAr-C_{2',6}'), 126.45 (1CHAr-C_{4'}), 126.99 (2CHAr-C_{3',5}'), 127.32 (2CHAr-C_{3,5}), 128.28 (Ar-C₄), 129.19 (Ar-C₁), 140.30 (Ar-C₁), 142.54 (C=N), 152.82 (thiadiazole- C_5), 158.83 (thiazole- C_4), 162.82 (thiadiazole-C₂), 168.83 (thiazole-C₂); MS m/z (%): 471 $(M^+, 15.6), 470(0.7), 464(0.2), 456(0.4), 392(2.4), 382(0.3), 356$ (0.6), 259 (1.0), 217 (0.7), 191 (0.9), 176 (9.6), 134 (100), 92 (1.1), 66 (7.7), 54 (6.1). Anal. calc. For C₁₉H₁₇N₇O₂S₃ (471.57): C, 48.39; H, 3.63; N, 20.79%; found: C, 48.38; H, 3.64; N, 20.78%.

3.1.2.2. 4-(2-Imino-5-(1-(2-(4-methylthiazol-2-yl)hydrazineylidene)ethyl)-1,3,4-thiadiazol-3(2H)-yl)benzenesulfonamide (5). The compound was obtained by heating 1-chloropropan-2-one (0.19 g, 0.002 mol) for 7 h under reflux. Brown powder; yield 94%; mp 259–260 °C; IR $\nu_{\text{max}}/\text{cm}^{-1}$ = 3291, 3186 (NH₂), 3142, 3101 (NH), 1602 (C=N), 1558 (C=C), 1320, 1304 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.13$ (s, 3H, CH₃), 2.26 (s, 3H, CH₃), 6.30 (s, 1H, thiazole- C_2), 7.37 (s, 2H, NH₂), 7.87 (d, J = 8.5 Hz, 2H, Ar- $H_{2,6}$), 8.24 (d, J = 8.5 Hz, 2H, Ar- $H_{3,5}$), 9.29 (s, 1H, NH), 11.79 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{\text{ppm}} = 13.34$ (CH₃), 17.46 (CH₃), 106.82 (thiazole-C₅), 121.21 (2CHAr-C_{2,6}), 127.42 (2CHAr-C_{3,5}), 129.29 (Ar-C₄), 140.01.42 (Ar-C₁), 142.09 (C=N), 149.77 (thiazole-C₄), 158.89 (thiadiazole-C₅), 162.82 (thiadiazole-C₂), 168.83 (thiazole-C₂); MS m/z (%): 409 (M⁺, 4.4), 367 (3.0), 313 (4.3), 299 (1.9), 269 (2.1), 262 (1.6), 207 (4.5), 199 (1,6), 177 (2.4), 153 (8.8),117 (9.0), 99 (23.9), 76 (71.7), 65 (62.7), 59 (100), 51 (20.5); anal. calc. For C₁₄H₁₅N₇O₂S₃ (409.50): C, 41.06; H, 3.69; N, 23.94%; found: C, 41.05; H, 3.68; N, 23.93%.

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Fig. 8 (a) RMSDs of derivatives 9a and 9d inside the active site of hCAIX (PDB ID: 3IAI) throughout 100 ns-long MD simulation. (b) and (c) The number of H-bonds detected for 9a and 9d, respectively inside the active site of hCAIX over the course of 100 ns-long MD simulation. The cut-off distance for H-bonds was set to be 2.5 Å.

3.1.2.3. 4-(5-(1-(2-(5-Acetyl-4-methylthiazol-2-yl)

hydrazineylidene)ethyl)-2-imino-1,3,4-thiadi azol-3(2H)-yl)benzenesulfonamide (6). The compound was obtained by heating 3chloro-2,4-pentanedione (0.29 ml, 0.002 mol) for 5 h under reflux. Brown powder; yield 82%; mp 260–262 °C; IR $\nu_{\rm max}/{\rm cm}^{-1}$ = 3300, 3237 (NH₂), 3197, 3105 (2NH), 1668 (C=O), 1585 (C= N), 1488 (C=C), 1305, 1277 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{\rm ppm}$ = 2.19 (s, 3H, CH₃), 2.26 (s, 3H, CH₃), 2.39 (s, 3H, CH₃), 7.35 (s, 2H, NH₂), 7.85 (d, *J* = 8.5 Hz, 2CH, Ar-H_{2,6}), 8.22 (d, *J* = 8.5 Hz, 2CH, Ar-H_{3,5}), 9.25 (s, 1H, NH), 11.79 (s, 1H, NH); ¹³C-NMR (DMSO- d₆): $\delta_{ppm} = 12.98$ (CH₃), 27.48 (CH₃), 30.87 (CH₃), 113.48 (2CHAr-C_{2,6}), 126.43 (2CHAr-C_{3,5}), 126.54 (Ar-C₄), 140.05 (thiazole-C₅), 141.05 (Ar-C₁), 143.25 (C=N), 149.39 (thiadiazole-C₅), 158.73 (thiazole-C₄), 162.45 (thiadiazole-C₂), 169.63 (thiazole-C₂), 189.00 (C=O); MS *m*/*z* (%): 451 (M⁺, 32.9), 450 (1.9), 393 (2.9), 326 (1.4), 305 (1.8), 414 (2.1), 296 (1.8), 253 (1.5), 209 (2.1), 171 (5.6), 118 (9.5), 92 (65.1), 77 (27.0), 67 (100), 56 (19.5); anal. calc. For C₁₆H₁₇N₇O₃S₃ (451.54): C, 42.56; H, 3.80; N, 21.71%; found: C, 42.55; H, 3.81; N, 21.72%.



Fig. 9 Interaction energies (*i.e.*, electrostatic and van der Waals interaction energies) of 9a and 9d (A and B, respectively) inside the active site of hCAIX (PDB ID: 3IAI) for 100 ns-long MD simulation.

Table 4 Binding free energies ($\Delta G_{\text{binding}}$) of compounds **9a** and **9d** in complex with the hCAIX (PDB ID: 31AI) calculated in kcal mol⁻¹

Energy component (kcal mol ⁻¹)	9a	9d
$\Delta G_{\rm gas}$	-21.7654	-14.1167
$\Delta G_{ m solv}$	12.1875	9.8145
$\Delta G_{ m total}$	-9.5779	-4.3022

3.1.2.4. Ethyl-2-(2-(1-(5-imino-4-(4-sulfamoylphenyl)-4,5dihydro-1,3,4-thiadiazol-2-yl)ethylidene)hydrazineyl)-4-

methylthiazole-5-carboxylate (7). The compound was obtained by heating ethyl 2-chloro-3-oxobutanoate (0.35 ml, 0.002 mol) for 6 h under reflux. Brown powder; yield 80%; mp 100–102 °C; IR $\nu_{\rm max}/{\rm cm^{-1}} = 3310, 3232$ (NH₂), 3190, 3115 (2NH), 1683 (C=O), 1654 (C=N), 1540 (C=C), 1320, 1268 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{\rm ppm} = 1.23$ (t, J = 6.8 Hz, 3H, CH₃), 2.26 (s, 3H, CH₃), 2.87 (s, 3H, CH₃), 4.19 (q, J = 6.8 Hz, 2H, CH₂), 7.40 (s, 2H, NH₂), 7.89 (d, J = 8.5 Hz, 2H, Ar–H_{2,6}), 8.15 (d, J = 8.5 Hz, 2H, Ar–H_{3,5}), 9.53

(s, 1H, NH), 12.33 (s, 1H, NH–N); ¹³C-NMR (DMSO-d₆): $\delta_{\text{ppm}} =$ 12.52 (CH₃), 16.31 (CH₃), 30.86 (CH₃), 60.58 (CH₂), 113.48 (2CHAr–C_{2,6}), 121.35 (thiazole-C₅), 126.90 (2CHAr–C_{3,5}), 127.35 (Ar–C₄), 141.22 (Ar–C₁), 142.15 (C=N), 146.56 (thiadiazole-C₅), 161.5 (thiazole-C₄), 162.45 (thiadiazole-C₂), 168.38 (thiazole-C₂), 173.72 (C=O); MS *m*/*z* (%): 481 (M⁺, 11.5); MS *m*/*z* (%): 481 (M⁺, 11.5), 437 (3.3), 401 (0.9), 382 (1.2), 373 (1.1), 302 (0.8), 294 (0.7), 255 (0.9), 222 (1.7), 199 (3.0), 134 (9.9), 127 (5.7), 96 (14.4), 67 (100), 56 (14.2); anal. calc. For C₁₇H₁₉N₇O₄S₃ (481.56): C, 42.40; H, 3.98; N, 20.36%; found: C, 4.41; H, 3.97; N, 20.35%.

3.1.2.5. 4-(2-Imino-5-(1-(2-(4-oxo-4,5-dihydrothiazol-2-yl) hydrazineylidene)ethyl)-1,3,4-thiadi azol-3(2H)-yl)benzenesulfonamide (8). The compound was obtained by heating ethyl 2chloroacetate (0.26 ml, 0.002 mol) for 4 h under reflux. Orange powder; yield 86%; mp 193–195 °C; IR ν_{max} /cm⁻¹ = 3303, 3278 (NH₂), 3189, 3102 (2NH), 1713 (C=O), 1599 (C=N), 1563 (C= C), 1329, 1305 (SO₂); ¹H-NMR (DMSO-d₆): δ_{ppm} = 2.32 (s, 3H, CH₃), 3.91 (s, 2H, CH₂), 7.38 (s, 2H, NH₂), 7.88 (d, *J* = 8.5 Hz, 2H, Ar-H_{2,6}), 8.22 (d, *J* = 8.5 Hz, 2H, Ar-H_{3,5}), 9.35 (s, 1H, NH), 12.24

Table 5 The calculated physicochemical descriptors, predicted pharmacokinetics properties and drug-likeness of 3, 4, 7, 8, 9a, 12b and 12d by SwissADME

Compounds	3	4	7	8	9a	12b	12d
Physicochemical properties							
Mol. weight in g mol^{-1}	371.5	471.6	481.6	411.5	385.5	511.6	582.1
#Heavy atoms	23	31	31	26	24	34	38
#Aromatic heavy atoms	11	22	16	11	11	22	28
Fraction Csp ³	0.09	0.05	0.24	0.15	0.17	0.09	0.04
#Rotatable bonds	5	6	8	5	6	6	6
#H-bond acceptors	6	7	9	8	6	7	7
#H-bond donors	4	3	3	3	4	2	2
MR	90.5	122.4	118.0	102.3	95.4	135.9	151.0
TPSA $(Å^2)$	221.0	204.0	230.3	217.6	207.0	196.3	196.3
Lipophilicity							
ilogp	1.42	2.48	2.59	1.34	1.53	1.53	1.53
XLOGP3	0.40	3.77	3.06	0.98	0.80	0.80	0.80
Water solubility							
ESOL log S	-2.42	-5.27	-4.61	-2.99	-2.68	-2.68	-2.68
ESOL solubility (mg ml $^{-1}$)	$1.42 imes 10^{0}$	$2.54 imes10^{-3}$	$1.19 imes10^{-2}$	$4.19 imes 10^{-1}$	$8.11 imes 10^{-1}$	$8.11 imes 10^{-1}$	$8.11 imes 10^{-1}$
ESOL class	Soluble	Moderately soluble	Moderately soluble	Soluble	Soluble	Soluble	Soluble
Pharmacokinetics							
GI absorption	Low	Low	Low	Low	Low	Low	Low
BBB permeant	No	No	No	No	No	No	No
P-gp substrate	No	No	No	No	No	No	No
CYP1A2 inhibitor	No	No	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	Yes	No	No	No	No
Skin permeation $\log K_{\rm p} ({\rm cm \ s^{-1}})$	-8.28	-6.5	-7.06	-8.11	-8.08	-8.08	-8.08
Drug-likeness							
Lipinski #violations	0	0	1	0	0	0	0
Ghose #violations	0	0	1	0	0	0	0
Veber #violations	1	1	1	1	1	1	1
Egan #violations	1	1	1	1	1	1	1
Muegge #violations	1	1	1	1	1	1	1
Bioavailability score	0.55	0.55	0.55	0.55	0.55	0.55	0.55

(s, 1H, N–H); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.80$ (CH₃), 33.15 (CH₂), 120.79 (2CHAr–C_{2,6}), 126.89 (2CHAr–C_{3,5}), 141.92 (Ar–C₄), 149.17 (Ar–C₁), 153.85 (C=N), 155.85 (thiadiazole-C₅), 158.49 (thiadiazole-C₂), 165.37 (thiazole-C₂), 171.77 (C=O); MS *m*/*z* (%): 411 (M⁺, 2.9), 410 (2.2), 367 (1.9), 340 (1.8), 313 (7.7), 297 (1.9), 242 (5.6), 208 (2.1), 197 (1.6), 177 (1.7), 153 (3.7), 111 (17.0), 90 (8.3), 77 (11.3), 61 (8.6), 55 (100); anal. calc. For C₁₃H₁₃N₇O₃S₃ (411.47): C, 37.95; H, 3.18; N, 23.83%; found: C, 37.94; H, 3.19; N, 23.82%.

3.1.3. General procedure for the preparation of 2-(1-(5imino-4-(4-sulfamoylphenyl)-4,5-dihydro-1,3,4-thiadiazol-2-yl) ethylidene)hydrazine-1-carbothioamide derivatives (9a-d). A mixture of 1 (1 g, 0.003 mol) and 4-substituted thiosemicarbazides 2a-d (0.003 mol) in EtOH (30 ml) containing concentrated HCl (0.25 ml) was refluxed for 2–4 h. The formed precipitate was filtered off, washed with ethanol, dried, and recrystallization from EtOH to afford compounds 9a-d, respectively.

3.1.3.1. 2-(1-(5-Imino-4-(4-sulfamoylphenyl)-4,5-dihydro-1,3,4-thiadiazol-2-yl)ethylidene)-N-methylhydrazine-1-

carbothioamide (9a). The compound was obtained by heating 2a (0.35 g, 0.003 mol) for 2 h under reflux. Orange powder; yield 78%; mp 251–253 °C; IR $\nu_{\text{max}}/\text{cm}^{-1}$ = 3339, 3301 (NH₂), 3199, 3115, 3109 (3NH), 1628 (C=N), 1559 (C=C), 1327, 1292 (SO₂), 1217 (CS); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.28$ (s, 3H, CH₃), 3.04 (d, J = 5.1 Hz, 3H, NH-CH₃), 7.66 (s, 2H, NH₂), 7.92 (d, J = 8.5 Hz, 2H, Ar-H_{2,6}), 8.05 (d, *J* = 8.5 Hz, 2H, Ar-H_{3,5}), 8.70 (s, 1H, NH), 11.04 (s, 1H, NH), 11.43 (s, 1H, NH); 13 C-NMR (DMSO-d₆): δ_{ppm} = 12.78 (CH₃), 31.53 (CH₃), 127.20 (2CHAr-C_{2,6}), 127.55 (2CHAr-C_{3,5}), 137.68 (Ar-C₄), 139.07 (Ar-C₁), 145.91 (C=N), 155.60 (thiadiazole- C_2), 167.79 (thiadiazole- C_5), 178.67 (C=S); MS m/z (%): 385 (M⁺, 0.1), 384 (0.1), 379 (0.1), 375 (0.1), 372 (0.1), 369 (0.1), 364 (0.1), 352 (0.2), 346 (0.1), 265 (0.7), 259 (0.2), 245 (0.3), 194 (1.2), 165 (1.7), 95 (22.2), 57 (100). Anal. calc. For C₁₂H₁₅N₇O₂S₃ (385.48): C, 37.39; H, 3.92; N, 25.44%; found: C, 37.38; H, 3.93; N, 25.45%.

3.1.3.2. N-Allyl-2-(1-(5-imino-4-(4-sulfamoylphenyl)-4,5-di-

hydro-1,3,4-thiadiazol-2-yl)ethylid ene)hydrazine-1carbothioamide (9b). The compound was obtained by heating 2b (0.44 g, 0.003 mol) for 4 h under reflux. Orange powder; yield 70%; mp 263–265 °C; IR $\nu_{\text{max}}/\text{cm}^{-1}$ = 3303, 3268 (NH₂), 3197, 3120, 3100 (3NH), 1630 (C=N), 1555 (C=C), 1313, 1293 (SO₂), 1187 (CS); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.30$ (s, 3H, CH₃), 4.24 (t, J = 5.9 Hz, 2H, N-CH₂), 5.11 (dd, J = 8.5, 1.5 Hz, 1H, CH=), 5.16 (dd, J = 15.3, 1.5 Hz, 1H, CH=), 5.88 (m, 1H, CH=), 7.64 (s, 2H, NH_2 , 7.93 (d, J = 8.5 Hz, 2H, Ar- $H_{2.6}$), 8.05 (d, J = 8.5 Hz, 2H, Ar-H_{3,5}), 8.83 (s, 1H, NH), 9.48 (s, 1H, NH), 11.13 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.81$ (CH₃), 46.19 (CH₂), 115.83 (2CHAr-C_{2,6}), 127.03 (CH₂), 127.52 (2CHAr-C_{3,5}), 134.36 (Ar-C₄), 137.74 (Ar-C₁), 139.34 (CH), 145.80 (C=N), 155.51 (thiadiazole-C₂), 167.64 (thiadiazole-C₅), 178.33 (C=S); MS m/z (%): 411 (M⁺, 0.7), 410 (1.1), 396 (1.0), 384 (1.1), 369 (1.9), 354 (2.2), 290 (0.7), 264 (4.5), 126 (3.5), 112 (10.4), 100 (3.4), 57 (100); anal. calc. For C₁₄H₁₇N₇O₂S₃ (411.52): C, 40.86; H, 4.16; N, 23.83%; found: C, 40.87; H, 4.17; N, 23.82%.

3.1.3.3. Synthesis of 2-(1-(5-imino-4-(4-sulfamoylphenyl)-4,5di-hydro-1,3,4-thiadiazol-2-yl)ethylidene)-N-phenylhydrazine-1carbothioamide (9c). This compound was obtained by heating 2c (0.56 g, 0.003 mol) for 4 h under reflux. Orange powder; yield 86%; mp 260–261 °C; IR $\nu_{\text{max}}/\text{cm}^{-1} = 3295$, 3257 (NH₂), 3187, 3114, 3102 (3NH), 1625 (C=N), 1554 (C=C), 1318, 1294 (SO₂), 1237 (CS); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.35$ (s, 3H, CH₃), 7.24– 756 (m, 5H, Ar-H), 7.65 (s, 2H, NH₂), 7.94 (d, J = 8.5 Hz, 2H, Ar- $H_{2,6}$), 8.06 (d, J = 8.5 Hz, 2H, Ar- $H_{3,5}$), 10.49 (s, 1H, NH), 11.11 (s, 1H, NH), 11.48 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} =$ 13.06 (CH₃), 121.54 (2CHAr-C_{2,6}), 125.34 (2CHAr-C_{2',6}'), 125.84 (1CHAr-C₄), 127.15 (2CHAr-C_{3',5}'), 127.59 (2CHAr-C_{3,5}), 128.47 (Ar-C₄), 137.73 (Ar-C₁), 138.94 (Ar-C₁), 140.04 (C=N), 145.91 (thiadiazole- C_2), 167.85 (thiadiazole- C_5), 177.61 (C=S); MS m/z(%): 447 (M⁺, 0.2), 423 (0.3), 412 (0.2), 400 (0.2), 382 (0.2), 368 (0.2), 208 (0.2), 179 (0.5), 167 (1.1), 152 (1.3), 137 (0.7), 93 (100), 78 (3.9), 66 (40). Anal. calc. For C₁₇H₁₇N₇O₂S₃ (447.55): C, 45.62; H, 3.83; N, 21.91%; found: C, 45.61; H, 3.82; N, 21.92%.

3.1.3.4. N-(4-Chlorophenyl)-2-(1-(5-imino-4-(4-

sulfamoylphenyl)-4,5-dihydro-1,3,4-thiadiazol-2-yl)-ethylidene) hydrazine-1-carbothioamide (9d). The compound was obtained by heating 2d (0.67 g, 0.003 mol) for 3 h under reflux. Orange powder, yield 87%; mp 262–264 °C; IR $\nu_{max}/cm^{-1} = 3339, 3258$ (NH₂), 3165, 3130, 3104 (3NH), 1635 (C=N), 1553 (C=C), 1363, 1292 (SO₂), 1247 (CS); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.37$ (s, 3H, CH₃), 7.47 (d, J = 8.5 Hz, 2H, Ar-H_{3',5}), 7.61 (d, J = 8.5 Hz, 2H, $Ar-H_{2',6'}$), 7.67 (s, 2H, NH₂), 7.96 (d, J = 8.5 Hz, 2H, $Ar-H_{2.6}$), 8.07 $(d, J = 8.5 Hz, 2H, Ar-H_{3.5}), 10.51 (s, 1H, NH), 10.99 (s, 1H, HN),$ 11.56 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 13.50$ (CH₃), 127.59 (2CHAr-C_{2,6}), 127.96 (2CHAr-C_{3',5}'), 128.78 (2CHAr-C_{3,5}), 130.21 (Ar- C_4), 138.18 (2CHAr- $C_{2',6}$), 138.36 (Ar- C_4), 140.80 (Ar-C₁), 140.99 (Ar-C₁), 143.32 (C=N), 146.23 (thiadiazole-C₂), 155.76 (thiadiazole-C₅), 178.08 (C=S); MS m/z (%): 481 (M⁺, 0.2), 480 (0.3),467 (0.3), 397 (0.3), 381 (0.2), 367 (0.2), 333 (0.2), 325 (0.2), 295 (0.2), 268 (0.2), 264 (0.4), 254 (0.2), 250 (0.5), 239 (0.5),227 (0.2), 201 (0.2), 199 (0.2), 191 (0.3), 182 (0.2), 171 (1.4), 127 (100), 93 (4.5), 78 (3.03), 66 (8.9). Anal. calc. For C₁₇H₁₆ClN₇O₂S₃ (481.99): C, 42.36; H, 3.35; N, 20.34%; found: C, 42.37; H, 3.35; N, 20.33%.

3.1.4. General procedure for the preparation of 4-(2-imino-5-(1-((4-phenylthiazol-2(3*H*)-ylidene)hydrazono)ethyl)-1,3,4thiadiazol-3(2*H*)-yl)benzenesulfonamide derivatives (12a–d). To a mixture of compound 9a–d (0.5 g, 0.001 mol) and 2-bromo-1phenylethan-1-one (0.001 mol) in EtOH (20 ml) containing TEA (0.15 ml) and DMF (5 ml) was refluxed for 3–6 h. The reaction mixture was allowed to cool to RT before being poured into ice water (40 ml). The formed precipitate was filtered off, dried, and recrystallized from EtOH to yield compounds 12a–d.

3.1.4.1. 4-(2-Imino-5-(1-((3-methyl-4-phenylthiazol-2(3H)-ylidene)hydrazono)ethyl)-1,3,4-thiadiazol-3(2H)-yl)benzenesulfonamide (**12a**). Orange powder; yield 77%; mp 231–233 °C; IR ν_{max} / cm⁻¹ = 3334, 3245 (NH₂), 3172 (NH), 1652 (C=N), 1582 (C=C), 1324, 1297 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.33$ (s, 3H, CH₃), 2.5 (s, 3H, NCH₃), 6.56 (s, H, thiazole-H₅), 7.39 (s, 2H, NH₂),7.51 (s, 5H, Ar–H), 7.88 (d, *J* = 8.5 Hz, 2H, Ar–H_{2,6}), 8.25 (d, *J* = 8.5 Hz, 2H, Ar–H_{3,5}), 9.33 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} =$ 12.50 (CH₃), 33.78 (CH₃), 101.48 (thiazole-C₅), 120.58 (2CHAr– C_{2.6}), 126.46 (1CHAr–C₄), 128.85 (2CHAr–C_{2.6}'), 128.87 (2CHAr– $\begin{array}{l} C_{3',5}'), \ 129.09 \ (2CHAr-C_{3,5}), \ 129.41 \ (Ar-C_{1'}), \ 130.11 \ (Ar-C_4), \\ 140.14 \ (Ar-C_1), \ 142.01 \ (thiazole-C_4), \ 146.83 \ (C==N), \ 153.00 \\ (thiadiazole-C_5), \ 159.19 \ (thiazole-C_2), \ 171.56 \ (thiadiazole-C_2); \\ MS \ m/z \ (\%): \ 485 \ (M^+, \ 25.4), \ 484 \ (1.1), \ 443 \ (0.1), \ 412 \ (0.1), \ 394 \\ (0.1), \ 325 \ (0.1), \ 315 \ (0.1), \ 298 \ (0.1), \ 256 \ (12.0), \ 190 \ (2.8), \ 160 \ (1.2), \\ 102 \ \ (100), \ 72 \ \ (4.1); \ anal. \ calc. \ For \ C_{20}H_{19}N_7O_2S_3 \ (485.60): \ C, \\ 49.47; \ H, \ 3.94; \ N, \ 20.19\%; \ found: \ C, \ 49.48; \ H, \ 3.93; \ N, \ 20.18\%. \end{array}$

3.1.4.2. 4-(5-(1-((3-Allyl-4-phenylthiazol-2(3H)-ylidene)hydrazono)ethyl)-2-imino-1,3,4-thiadiazol-3(2H)-yl)benzenesulfonamide (12b). Orange powder; yield 80%; mp 202–204 °C; IR $\nu_{\rm max}/{\rm cm}^{-1}$ = 3303, 3237 (NH₂), 3165 (NH), 1637 (C=N), 1584 (C=C), 1326, 1287 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{\text{ppm}} = 2.27$ (s, 3H, CH₃), 4.47 $(d, J = 4.2 \text{ Hz}, 2H, CH_2), 5.12 (dd, J = 91, 1.7 \text{ Hz}, 1H, CH=), 5.19$ (dd, *J* = 16, 1.7 Hz, 1H, CH=), 5.82 (m, 1H, CH=), 6.62 (s, 1H, thiazole-H₅), 7.46 (s, 2H, NH₂), 7.47-7.49 (m, 5H, Ar-H), 7.96 (d, J = 8.5 Hz, 2H, Ar-H_{2.6}), 8.08 (d, J = 8.5 Hz, 2H, Ar-H_{3.5}), 9.98 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.49$ (CH₃), 47.95 (N-CH₂), 102.35 (thiazole-C₅), 117.09 (CH₂), 121.19 (2CHAr-C_{2,6}), 126.00 (1CHAr-C_{4'}), 126.87 (2CHAr-C_{2',6}'), 127.00 (2CHAr-C_{3',5}'), 128.82 (2CHAr-C_{3.5}), 129.00 (Ar-C_{1'}), 129.61 (Ar-C₄), 130.04 (Ar-C₁), 132.07 (CH), 140.75 (thiazole-C₄), 146.27 (C=N), 155.51 (thiadiazole- C_5), 159.51 (thiazole- C_2), 171.48 (thiadiazole- C_2); MS m/z (%): 511 (M⁺, 15.8), 509 (1.6), 497 (1.3), 454 (3.6), 437 (6.7), 419 (2.4), 391 (1.4), 368 (2.1), 277 (2.6), 256 (9.8), 177 (2.4), 134 (100); anal. calc. For C₂₂H₂₁N₇O₂S₃ (511.64): C, 51.65; H, 4.14; N, 19.16%; found: C, 51.66; H, 4.15; N, 19.17%.

3.1.4.3. 4-(5-(1-((3,4-Diphenylthiazol-2(3H)-ylidene)hydrazono)ethyl)-2-imino-1,3,4-thiadiazol-3(2H)-yl)benzenesulfonamide (12c). Green powder; yield 74%; mp 210–214 °C; IR $\nu_{\rm max}/{\rm cm}^{-1}$ = 3330, 3274 (2NH), 3177 (NH), 1653 (C=N), 1586 (C=C), 1325, 1280 (SO₂); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.07$ (s, 3H, CH₃), 6.78 (s, 1H, thiazole-H₅), 7.15–7.31 (m, 10H, Ar-H), 7.44 (s, 2H, NH₂), 7.90 (d, J = 9.3 Hz, 2H, Ar-H_{2,6}), 7.99 (d, J = 9.3 Hz, 2H, Ar-H_{3,5}), 9.68 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.61$ (CH₃), 103.16 (thiazole-C₅), 121.28 (2CHAr-C_{2.6}), 126.78 (1CHAr-C_{4"}), 126.89 (2CHAr-C_{2",6}"), 127.31 (1CHAr-C_{4'}), 128.32 (2CHAr- $C_{2',6}$), 128.38 (2CHAr- $C_{3',5}$), 128.48 (2CHAr- $C_{3',5}$), 128.50 (2CHAr-C_{3,5}), 128.68 (Ar-C_{1'}), 128.76 (Ar-C₄), 140.25 (Ar-C_{1"}), 140.40 (Ar-C1), 147.79 (C-N), 148.31 (C=N), 152.73 (thiadiazole- C_5), 162.45 (thiazole- C_2), 172.46 (thiadiazole- C_2); MS m/z (%): 547 (M⁺, 45.4), 546 (1.9), 523 (1.3), 520 (1.6), 488 (2.6), 487 (4.5), 479 (1.5), 468 (1.9), 443 (1.3), 435 (1.4), 407 (1.7), 393 (1.4), 368 (1.9), 252 (4.9), 239 (4.2), 163 (2.4), 107 (10.9), 92 (21.1), 77 (100), 65 (25.0). Anal. calc. For C₂₅H₂₁N₇O₂S₃ (547.67): C, 54.83; H, 3.87; N, 17.90%; found: C, 54.82; H, 3.86; N, 17.91%.

3.1.4.4. 4-(5-(1-((3-(4-Chlorophenyl)-4-phenylthiazol-2(3H)-ylidene)hydrazono)ethyl)-2-imino-1,3,4-thiadiazol-3(2H)-yl)benzenesulfonamide (12d). Dark green powder; yield 84%; mp 227-230 ° C; IR ν_{max} /cm⁻¹ = 3303, 3276 (NH₂), 3155 (NH), 1654 (C=N), 1584 (C=C), 1306, 1279 (SO₂), 738 (C-Cl); ¹H-NMR (DMSO-d₆): $\delta_{ppm} = 2.12$ (s, 3H, CH₃), 6.81 (s, 1H, thiazole-H₅), 7.18 (d, J = 2.5 Hz, 2H, Ar-H_{2',6}'), 7.29 (d, J = 4.2 Hz, 2H, Ar-H_{3',5}'), 7.34-7.45 (m, 5H, Ar-H), 7.59 (d, J = 8.5 Hz, 2H, Ar-H_{2,6}), 7.90 (d, J = 8.5 Hz, 2H, Ar-H_{3,5}), 8.19 (s, 2H, NH₂), 9.49 (s, 1H, NH); ¹³C-NMR (DMSO-d₆): $\delta_{ppm} = 12.42$ (CH₃), 103.67 (thiazole-C₅), 114.33 (2CHAr-C_{2,6}), 121.69 (2CHAr-C_{2',6}''), 127.03 (1CHAr-C_{4'}), 128.69 (2CHAr-C_{2',6}'), 128.91 (2CHAr-C_{3',5}'), 129.37 (2CHAr- $C_{3'',5}''$), 130.03 (2CHAr- $C_{3,5}$), 130.54 (Ar- C_4), 130.83 (Ar- C_1), 131.12 (Ar- $C_{1''}$), 133.01 (Ar- C_1), 136.43 (thiazole- C_4), 140.25 (C=N), 148.87 (thiadiazole- C_5), 158.73 (thiazole- C_2), 162.45 (thiadiazole- C_2); MS *m*/*z* (%): 582 (M⁺, 22.1) 584 (M²⁺, 8.1), 581 (28.5), 524 (1.9), 503 (1.6), 488 (1.9), 425 (1.6), 381 (2.6), 355 (2.6), 351 (1.9), 339 (2.1), 315 (1.7), 294 (1.7), 279 (1.8), 2.51 (2.7), 210 (2.4), 155 (4.1), 127 (7.6), 93 (15.2), 71 (52.1), 57 (100). Anal. calc. For $C_{25}H_{20}ClN_7O_2S_3$ (582.11): C, 51.58; H, 3.46; N, 16.84%; found: C, 51.59; H, 3.47; N, 16.85%.

3.2. Biological evaluation

The evaluated human cancer cell lines were cultivated to ensure their presence in the log phase then 3 mL of the cultivation medium was introduced to 96-well microplate. The total cells count in the medium should not exceed 10^6 cells per cm² and the medium did not contain phenol or serum. Afterward, an equivalent volume of MTT (M-5655) to 10% of the medium was added and incubated at room temperature for 2–4 hours. The deposited formazan crystals were dissolved in an equivalent MTT solubilizing solution (M-8910) in the culture medium. The intensity of the obtained solution was measured as absorbance at wavelength 570 nm against the blank solution. The blank solution was prepared following the same conditions without cells at every test.^{44,45}

3.3. Molecular docking simulation

The protein structure of hCAIX was recovered from the Protein Data Bank using PDB: 3IAI.⁴⁶ Its crystal structure was subjected to the preparation step by deleting the unnecessary chains, co-factors, and water molecules while keeping the zinc ion. The Molecular Operating Environment (MOE) softwareembedded autocorrection tool was used to check the validity of the chain conformation then hydrogens were added at a cutoff of 15 Å using AMBER10:EHT forcefield. The molecular docking site was adjusted to a radius of 4.5 Å around the cocrystallized ligand. Afterward, the actual docking simulation was implemented using Triangle Matcher, London dG, and GBVI/WSA as the placement, and rescoring functions 1 and 2, respectively. On the other hand, the chemical structures of the derivatives were constructed using ChemAxon Marvin JS, then pasted as smiles to MOE builder, protonated, and energy minimized at the same forcefield before commencing the simulation protocol.

3.4. Molecular dynamic simulation

The simulation was performed using NAMD 3.0.0, applying Charmm-36 as the forcefield. According to the literature,^{47,48} Protein systems were built, corrected and prepared using the QwikMD.^{48,49} The binding free energy was calculated according to Molecular Mechanics Poisson–Boltzmann Surface Area (MM-PBSA) embedded in the MMPBSA.py module of AMBER18 (ref. 50) using the following equation:

$$\Delta G_{\text{binding}} = \Delta G_{\text{complex}} - \Delta G_{\text{receptor}} - \Delta G_{\text{inhibitor}}$$

Each equation required calculating multiple energy components, including van der Waals energy, electrostatic energy, internal energy from molecular mechanics, and polar contribution to solvation energy.

4. Conclusion

Paper

Herein, the tail approach synthesized a bundle of different hybrids of disubstituted/trisubstituted 1,3-thiazoles with 1,3,4thiadiazolylbenzenesulfonaminde started with 1. The best cytotoxicity was observed with 4 and 9a against HepG-2, Caco2, and MCF-7 compared to staurosporine, with 4 showing a better safety margin as declared from its WI38 IC₅₀ 21.4 µM and 18.4 µM, respectively. Similarly, the phenylthiazole trisubstituted analogue 12d showed good cytotoxicity against the three tested cancer cell lines with higher safety than both 4 and 9a, as concluded from its WI38 IC₅₀ 46.6 µM. Moreover, the molecular modeling simulation of 4, 9a, and 12d against hCAIX revealed the bidentate coordination with zinc and forming H-bond with the catalytic His94, which explained their astonishing cytotoxicity. The molecular dynamic simulation of the more potent 9a and the least potent 9d with hCAIX demonstrated appreciable support of the proposed mechanism of their anticancer activity.

Conflicts of interest

There are no conflicts to declare.

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