New Sulfamethoxazole Derivatives as Selective Carbonic Anhydrase IX and XII Inhibitors: Design, Synthesis, Cytotoxic Activity and Molecular Modeling

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Abstract: In this study new sulphamethoxazole derivatives (S1–S4, S6–S12, and S14–S22) were designed and synthesized and their structures were fully characterized and validated using NMR, mass, and IR spectroscopy, as well as elemental analyses. All new derivatives (S1–S22) were assayed against human carbonic anhydrase (hCAs IX and XII) for their inhibitory activities. hCAs IX and XII were chosen due to the fact that CAIX expression is recognized as a hypoxia marker with a poor prognosis in breast cancer. When compared to Dorzolamide HCl as a standard reference, derivatives S2, S3, S8, S9, and S15 had the most effective inhibition with low IC₅₀ values. The active compounds were further evaluated against hCAs I and II inhibitory activity and compounds S8, S9 and S15 showed the least inhibitory effect compared to the reference standard, acetazolamide, indicating that their effect in normal cells is the lowest. Cell viability tests for the selected compounds were carried out on MCF7 (normoxia and hypoxia) and on the normal breast cell line (MCF10a) with Staurosporine as a standard. The results showed that compound S15 had a highly potent cytotoxic effect. Furthermore, cell cycle analysis results showed that compound S15 triggered cell cycle arrest and apoptosis in G1/S of MCF7 cancer cells. Finally, molecular docking was performed to point out the possible explanation for the vital structural features and key-interactions exerted by our ligands with hCAs IX and XII that might share additional designs and highlight possible leads for a hopeful anticancer agent. Consequently, sulphamethoxazole Derivative S15 could be the potential lead for emerging selective cytotoxic compounds directing h CAs IX and XII.

Keywords: carbonic anhydrase; anticancer; sulfamethoxazole; apoptosis; molecular docking
1. Introduction

Cancer is a disease that has a wide range of effects on humanity, starting with its negative influence on a patient’s physical and emotional health, and capacity to work and live a full life, and ending with its financial impact due to the high costs and prolonged course of cancer treatment [1]. In view of the seriousness of the disease and its harmful impact on human life, scientists have accelerated their endeavors to discover new and effective drugs [2].

In order to combat cancer, researchers need to find medications with the ability to target cancer cells without affecting healthy cells at a high pace of growth. As a result, identifying novel anticancer medicines relies heavily on the ability to identify specific and powerful anticancer targets. Human carbonic anhydrase enzymes (hCAs) are a recent target for anticancer drugs. These metalloenzymes, known as carbonic anhydrases (hCAs), are abundantly found in the human body and are involved in the synthesis of bicarbonate and proton from water and carbon dioxide (CO2) [3,4]. The hCAs catalyze various crucial reactions involved in pathological and physiological functions: for example, several metabolic processes (such as gluconeogenesis, ureagenesis and lipogenesis), respiration, bone resorption, pH and CO2 homeostasis, electrolyte excretion, and tumorigenicity [5].

A total of fifteen distinct hCA isoforms have been identified, and all but three of them are catalytically active, with the exclusion of CA-VIII, X, and XI, which lack the crucial histidine residues that are required for the coordination of the zinc ion [6]. Inhibition of hCAs has been shown to have substantial druggable therapeutic actions in many diseases, including cancer (hCAs-IX and XII), CNS-related disorders (hCAs-VII and XIV), glaucoma (hCAs-II, IV and XII), and edema (hCAs-II, IV and XIV) [7].

Overexpression of hCAs-IX and XII in malignancies has motivated medicinal chemists to design a novel target scaffold for finding new anticancer drugs to treat hypoxic cancers. However, the design of high selective inhibitors for hCAs IX and XII, other than physiologically hCAs I and II isoforms, is a challenge for drug designers.

It was shown that the “tail style” of hCAs inhibitor development was the most developed, as well as the most effective [8].

Two arms with a broad range of chemical character, such as primary carboxylic and sulfamoyl function groups, are connected to a heterocyclic or aromatic ring comprising a zinc binding group (ZBG) by an adaptive and flexible linker. Sulphonamide or carboxylic groups of carbonic anhydrase inhibitors react with zinc, which is essential for hCAs activation.

Currently in Phase I/II clinical trials, a newly found hCAs inhibitor, SLC-0111, is highly selective for hCA IX over hCAs I and II isoforms in the treatment of solid hypoxic cancers [9,10].

Additional research has shown that, in combination with 3-O-acetylbetulin, SLC-0111 can increase radiation sensitivity, cytotoxicity and DNA damage while inhibiting cell motility. On the basis of this investigation, it has been indicated that a combination of SLC-0111 and other hypoxic tumour-targeting agents will have a promising therapeutic strategy [11].

Bioisosteric substitution was also used by medicinal chemists to prepare new SLC-0111 derivatives. The SLC-0111 ureido bond has been substituted with selenoureido and thioureido (drug A), piperazinyl-ureido (drug B), enaminone (drug C), cyanoguanidine (drug D), and 1,3-triazene (drug E) linkers (Figure 1) [12–16].

These SLC-0111 bioisoteres, on the other hand, improved hCAs-IX and XI1 inhibitory activity, but they did not show that they were more effective in blocking hCAs-IX than hCAs-I and hCAs-II. First, there is ZBG, which can be either sulpomyl or carboxylic or N-substituted sulfonamide. Then, there are flexible and variable linkers, and then the tail, which can be substituted with electron-withdrawing or electron-giving groups.
In this study, based on isoxazol-3-ylbenzenesulfonamide scaffold, we aim to develop and prepare new SLC-0111 derivatives with diverse functional moieties Figure 2.

**Figure 1.** The main structural feature and similarity of reported h CA IX and XII inhibitors (A–E) with SLC-0111 (patent compound).

**Figure 2.** The main structural features and similarity of novel design h CA IX and XII inhibitors (S1 to S22) with SLC-0111.
The applied design approach depends on the substitution of the SLC-0111 ureido linker with substituted amino (S1–S4), chloroacylamido (S5–S7), substituted arylamido (S8–S11), benzoyl ethylamido (S12), substituted arylamido-acetamido (S14–S17), thiazolidinone-amino (S18) and substituted thiazolidinone-amino (S19–S22).

Moreover, a bioisosteric sulfonamide SLC-0111 with N-sunstitued-sulfamoyl as ZBG, but the tail part of novel derivatives S1 to S22.

Additionally, the 4-florophenyl group in the tail of SLC-0111 was replaced with substituted phenyl groups. All new compounds (S1–S22) were structurally verified, characterized, and pharmacologically assessed against hCAs I, II, IX, and XII, as well as tested for cytotoxicity against MCF7 and MCF10a cell lines. Additionally, the cell cycle and apoptosis of compound S15 were analyzed.

The appropriate format for writing articles was followed: Abstract, Introduction, Methods, Results, Discussion, and Conclusions. In the current format, the writ-up is chaotic and boring to read. Hence, readers can lose interest.

The main objective of this study is to design and synthesize novel sulfamethoxazole derivatives that have the essential features of the reported compounds that have proven effective against carbonic anhydrase IX and XII. The novel sulfamethoxazole should be a potent and selective inhibitor for hCA IX and XII, which increase its secretion in the case of cancer and, at the same time, do not affect hCA 1 and II, which have vital and important physiological effects.

As result, the new scaffold has proven to be effective against hCA IX and XII without harmful side effects resulting from action on physiologically important h CA 1 and II.

2. Results and Discussion

2.1. Chemistry

The preparation of sulphamethoxazole derivatives was performed according to the reaction orders demonstrated in (Schemes 1–3).

![Scheme 1. Synthesis of S1 to S7.](image-url)
Candidate derivatives S1–S12 and S14–S17 were prepared in an excellent yield via the nucleophilic substitution of sulphasalazine with different alkyl halide (for compounds S1–S4), acid chlorides (for compounds S5 [17–19]–S11), 3-chloropropiophenone (for compound S12) or 2-chloroacetamide derivatives S13a–d [20–23] (for compounds S14–S17) in
dry dimethylformamide in the presence of anhydrous potassium carbonate or triethylamine (Schemes 1 and 2).

The structure of novel prepared derivatives was well-characterized using NMR, IR and Mass spectroscopy, as well as elemental analyses.

The $^1$H NMR and $^{13}$C NMR of alkyl compounds S1–S4 revealed the presence of aliphatic protons of different alkyl moieties at $\delta$ 1.16–5.85 ppm. Allyl group in compound S2 appeared as a doublet, doublet of doublet, and multiple signals at $\delta$ 4.30, 5.20, and 5.75–5.85 ppm, respectively, in the $^1$HNMR spectrum.

The IR spectra of sulphamethoxazole amide derivatives S5–S11 showed absorption peaks at 1666–1687 cm$^{-1}$ attributed to the carbonyl group. $^1$H NMR spectrum of S6 displayed two triplets at $\delta$ 2.87 ppm and $\delta$ 3.88 ppm, each of them integrating two protons, which were accounted for CH$_2$-CH$_2$ of propionyl moiety. $^1$H-NMR of benzamide derivatives S8–S11 displayed added signals at the aromatic region.

The structure of benzenesulfonamide derivative S12 was confirmed by its spectral data. IR spectrum revealed a stretching band at 3331 cm$^{-1}$ due to NH$_2$ groups and a stretching band at 1765 cm$^{-1}$ was ascribed to the carbonyl group. $^1$H NMR spectrum reported two triplets at $\delta$ 3.33 and 3.46 ppm, each equivalent to two protons with the same $J$ value corresponding to CH$_2$-CH$_2$ protons. The presence of 3-oxo-3-phenylpropyl moiety was also confirmed by carbon NMR spectrum, which appeared at $\delta$ 37.72 (CH$_2$CO), 39.71 (CH$_2$-N) and 198.86 (CO).

In $^1$H NMR spectra of compounds S14–S17, additional protons for CH$_2$ group and aryl moieties were detected at $\delta$ 4.47–4.59 and 6.63–8.23 ppm, respectively; in $^{13}$C NMR, the CH$_2$ group appeared at a range of $\delta$ 50.66–50.90 ppm.

Cyclization of acetamide derivative S5 with ammonium thiocyanate in ethanol under reflux conditions afforded thiazol-2-ylamino benzenesulfonamide derivative S18, followed by condensation with different aromatic aldehydes in glacial acetic acid to yield benzenesulfonamide derivatives S19–S22 see Scheme 3. The structure of the new derivatives was assigned and established depending on their spectral data. The $^1$H NMR of compounds S19–S22 confirmed the presence of benzylidene CH at $\delta$ 7.65–8.36 ppm.

### 2.2. Biological Evaluation

#### 2.2.1. hCA IX and XII Inhibiting Effect

In the current experiment, hCA inhibiting effect was performed on 22 compounds and Dorzolamide HCL (DZM) was used as a reference standard. Out of these compounds, S2, S3, S8, S9 and S15 showed potent hCA IX inhibiting effect with IC$_{50}$ of 0.083 ± 0.004, 0.042 ± 0.002, 0.042 ± 0.002, 0.074 ± 0.004 and 0.037 ± 0.002, respectively, compared to DZM (IC$_{50}$ of 0.036 ± 0.002). Similarly, S2, S3, S8, S9 and S15 showed strong hCA XII inhibiting action with IC$_{50}$ of 0.056 ± 0.003, 0.07 ± 0.003, 0.04 ± 0.002, 0.047 ± 0.002 and 0.061 ± 0.003, respectively, compared to DZM with IC$_{50}$ of 0.024 ± 0.001 (Table 1 and Figure 3A,B).

#### Table 1. Inhibitory activity of compounds S1–S22 against hCAIX, and hCAXII using Dorzolamide HCL as reference standard.

| Compound | Carbonic Anhydrase (IC$_{50}$, nM) |
|----------|----------------------------------|
|          | CA-IX   | CA-XII   |
| S1       | 0.119 ± 0.006 | 0.162 ± 0.008 |
| S2       | 0.083 ± 0.004 | 0.056 ± 0.003 |
| S3       | 0.042 ± 0.002 | 0.07 ± 0.003 |
| S4       | 0.172 ± 0.008 | 0.208 ± 0.01 |
| S5       | 0.398 ± 0.019 | 0.651 ± 0.032 |
| S6       | 1.664 ± 0.081 | 0.877 ± 0.043 |
| S7       | 0.413 ± 0.02  | 0.18 ± 0.009 |
| S8       | 0.042 ± 0.002 | 0.04 ± 0.002 |
| S9       | 0.074 ± 0.004 | 0.047 ± 0.002 |
Table 1. Cont.

| Compound | CA-IX (IC_{50}, nM) | CA-XII (IC_{50}, nM) |
|----------|---------------------|-----------------------|
| S10      | 0.789 ± 0.039       | 0.392 ± 0.019         |
| S11      | 1.977 ± 0.097       | 0.991 ± 0.048         |
| S12      | 0.902 ± 0.044       | 0.437 ± 0.021         |
| S14      | 0.152 ± 0.007       | 0.081 ± 0.004         |
| S15      | 0.037 ± 0.002       | 0.061 ± 0.003         |
| S16      | 0.165 ± 0.008       | 0.164 ± 0.008         |
| S17      | 0.235 ± 0.011       | 0.247 ± 0.012         |
| S19      | 1.587 ± 0.078       | 0.917 ± 0.045         |
| S20      | 0.51 ± 0.025        | 0.069 ± 0.003         |
| S21      | 0.698 ± 0.034       | 0.284 ± 0.014         |
| S22      | 0.186 ± 0.009       | 0.249 ± 0.012         |
| Dorzolamide HCL | 0.036 ± 0.002 | 0.024 ± 0.001 |

The data in the table represent the average ± SEM after three independent experiments. Consider statistically significant at \( p < 0.05 \) if compared to DZM for CA-IX and CA-XII, respectively.

Figure 3. (A) Human CAIX, XII inhibitory effect of S1–S22 compounds using Dorzolamide HCL as reference standard. \(^a\) and \(^b\) consider statistically significant at \( p < 0.05 \) if compared to DZM for CA-IX and CA-XII, respectively. (B) Human CAI, II inhibitory effect of S2, S8, S9, and S15 compounds. Data represent mean ± SEM after three independent experiments. \(^a\) and \(^b\) consider statistically significant at \( p < 0.05 \) if compared to AAZ for hCAI and hCAII, respectively.
2.2.2. hCA I and II Inhibiting Effect

In the current experiment, compounds S2, S3, S8, S9 and S15 were selected for hCA I and II inhibiting activity based on the results of screening analysis on CA IX and IX inhibiting effect. Remarkably, compounds S2, S3, S8, S9 and S15 revealed a potent inhibitory effect on hCA IX with IC$_{50}$ of 0.177 ± 0.008, 0.08 ± 0.003, 1.017 ± 0.049, 0.121 ± 0.006 and 0.411 ± 0.02, respectively, compared to the reference standard, Acetazolamide (AAZ), with IC$_{50}$ of 0.281 ± 0.014 (Table 2 and Figure 3). Likewise, compounds S2, S3, S8, S9 and S15 revealed potent inhibitory action on CA XII with IC$_{50}$ of 0.102 ± 0.006, 0.199 ± 0.011, 1.848 ± 0.12, 0.543 ± 0.03 and 0.158 ± 0.009, respectively, compared to AAZ with IC$_{50}$ of 0.117 ± 0.007 (Table 2 and Figure 3). Notably, compounds S8, S9 and S15 showed the least inhibitory effect compared to compound S2 and S3 and the reference standard, AAZ, indicating that their effect in normal cells is the lowest.

Table 2. Human CAI, II inhibitory effect of S2, S3, S8, S9 and S15 compounds.

| Compound | MW (g/mol) | hCA I IC$_{50}$ (µM) | hCA II IC$_{50}$ (µM) |
|----------|------------|-----------------------|------------------------|
| S2       | 293        | 0.177 ± 0.008         | 0.102 ± 0.006          |
| S3       | 295        | 0.08 ± 0.003          | 0.199 ± 0.011          |
| S8       | 471        | 1.017 ± 0.049         | 1.848 ± 0.12           |
| S9       | 391        | 0.121 ± 0.006         | 0.543 ± 0.03           |
| S15      | 420        | 0.411 ± 0.02          | 0.158 ± 0.009          |
| AAZ      | 222,245    | 0.281 ± 0.014         | 0.117 ± 0.007          |

The data in the table represent the average ± SEM after three independent experiments. * and ** consider statistically significant at p < 0.05 if compared to AAZ for hCAI and hCAII, respectively.

2.2.3. Cytotoxic Activity against MCF7 and MCF10a Cell Lines

The in vitro cytotoxic activity for S8, S9 and S15 compounds was estimated on MCF7 and MCF10a cell lines. Staurosporine was used as a reference standard in the current experiment. The concentration of the derivative that causes the 50% inhibition of cell survivability (IC$_{50}$) was calculated. Table 3 and Figure 4 show the IC$_{50}$ of the synthesized compounds compared to Staurosporine. Based on the results of the CA assay, S8, S9 and S15 compounds were selected for the cytotoxicity study. In the current study, the results displayed that compounds S15 and S8 had strong cytotoxic activity on MCF7 in the hypoxic conditions with IC$_{50}$ of 0.73 ± 0.04 and 3.64 ± 0.2 µg/mL, respectively, compared to Staurosporine (IC$_{50}$ of 1.63 ± 0.09 µg/mL). Similarly, compounds S15 and S8 had strong cytotoxic activity on MCF7 in the normoxic condition with IC$_{50}$ of 4.15 ± 0.2 and 7.68 ± 0.41 µg/mL, respectively, compared to Staurosporine (IC$_{50}$ of 4.78 ± 0.26 µg/mL). Instead, compounds S8 and S15 showed potent cytotoxic activity on MCF701a recording IC$_{50}$ of 31.65 ± 1.71 and 34.71 ± 1.87 µg/mL, respectively, compared to Staurosporine (IC$_{50}$ of 23.65 ± 1.27 µg/mL). Notably, compound S9 showed moderate IC$_{50}$ activity in hypoxic and normoxic conditions on MCF7 and IC$_{50}$ on MCF10a, counting 0.73 ± 0.04, 4.15 ± 0.22 and 34.71 ± 1.87, respectively (Table 3 and Figure 4).

Table 3. Effect of S8, S9 and S15 compounds on cell viability of MCF7 (normoxia and hypoxia) and MCF10a cells using Staurosporine as a reference standard.

| Compound | MW (g/mol) | Cytotoxicity (IC$_{50}$ µg/mL) |
|----------|------------|-------------------------------|
|          |            | MCF7 Normoxia | MCF7 Hypoxia | MCF10a Normoxia | MCF10a Hypoxia |
| S8       | 471        | 3.64 ± 0.2     | 7.68 ± 0.41  | 31.65 ± 1.71    | 34.71 ± 1.87   |
| S9       | 391        | 9.81 ± 0.53    | 24.69 ± 1.33 | 46.72 ± 2.52    | 34.71 ± 1.87   |
| S15      | 420        | 0.73 ± 0.04    | 4.15 ± 0.22  | 34.71 ± 1.87    | 34.71 ± 1.87   |
| Staurosporine | 466.5 | 1.63 ± 0.09    | 4.78 ± 0.26  | 23.65 ± 1.27    | 23.65 ± 1.27   |

The data in the table represent the average ± SEM after three independent experiments.
2.2.4. Cell Cycle Analysis and Apoptosis of Compound S15

The most potent compound, S15, was selected for cell cycle analysis and the initiation of apoptosis in the MCF7 cell line. The MCF7 cell line was incubated with an IC_{50} concentration for compound S15 and its effects on the cell cycle profile and induction of apoptosis were inspected. Incubation of MCF7 cells with compounds S15 resulted in an interference with the normal cell cycle distribution on this cell line. In MCF7, compound S15 increased the percentage of cells in pre-G1 by more than 40-fold. The current results showed that compound S15 resulted in arrest cell growth at G1/S (Table 4 and Figure 5). The initial G1/S checkpoint suggests that the cell is organized in order to start DNA replication and the damaged DNA is repaired. The increase in the number of the cells in the pre-G1 phase demonstrated a potential role of apoptosis (Table 4 and Figure 5).

Table 4. Effect of compound S15 treatment of MCF7 cancer cell line on necrosis and apoptosis.

|                  | Apoptosis          | Necrosis |
|------------------|--------------------|----------|
|                  | Total | Early | Late | Total | Early | Late |
| S15/MCF7         | 41.03 | 2.92  | 22.8 | 15.31 |
| Control/MCF7     | 1.35  | 0.29  | 0.07 | 0.99  |

The data in the table represent the average ± SEM after three independent experiments.

Treatment of the MCF7 cancer cell line with compound S15 increased the percentage of necrosis by 15-fold compared to control untreated MCF7. Interestingly, compound S15 increased apoptosis by about 30-fold (Table 4 and Figure 6).
**Figure 5.** Percentage apoptosis and necrosis of MCF without treatment (A) or with S15 treatment (B) on Effect of Compound S15 on cell cycle distribution. Results were collected analyzed and blotted (C). The cells were treated with IC\textsubscript{50} (µM) of compound S15 and the cells were harvested and exposed to the analysis of cell-cycle. Data expressed as mean ± SEM.
Inhibition of CA IX has been newly confirmed as a recent method for targeting hypoxic tumors [25]. CA IX play a critical role in regulating the pH of the breast cancer microenvironment, while CAXII is accompanied by a better prognosis, although both have the same catalytic function [26]. Furthermore, CA XII may endorse the invasion and migration of breast cancer cells [27]. In the current experiment, CA IX and CAXII inhibiting effects were implemented on 22 compounds using Dorzolamide HCL (DZM) as a reference standard. Out of 22 compounds, compounds S2, S3, S8, S9 and S15 showed superior inhibitory activity on CA IX and CA XII. Subsequently, effective compounds were then applied for CAI and CAII inhibitory activity. Earlier work has revealed that CA I has an important role in the migration of breast cancer [28]. Likewise, amplification of the CA1 gene was identified in nearly 25% studies on breast cancer [29]. On the other hand, in breast cancer CA II increased expression has been found to be allied with tumor progression, poor prognosis, and metastasis [30]. The current results showed that compounds S2, S3, S8, S9 and S15 have potent inhibitory effect on CA I and CAII, using acetazolamide as a reference standard. The current results confirm the superior inhibitory activity of compounds S2, S3, S8, S9 and S15 on CA I, CA II, CA IX and CA XII.

Breast cancer is the most commonly diagnosed cancer type in women. Hypoxic distributed regions are frequently identified in invasive breast cancer and is considered the worse type of cancer as it is metastatic and largely invasive [31]. Using hypoxia-effective anticancer is one of the prominent tactics to avoid hypoxic challenges [32]. The use of this strategy offers a discriminating tumor inhibition, providing lower toxicity to normoxic tissues. Although many trials have aimed to provide hypoxia-effective drugs for different tumor types, their activities against breast cancer are still low [33]. In order to confirm the possible anticancer activity, we selected compounds S8, S9 and S15 based on CAs assay. We explored the action of the derivatives on cell viability using breast cancer cell line, MCF7, below hypoxic and normoxic conditions and non-tumorigenic epithelial breast cell line, MCF10a, using Staurosporine as a reference standard. MTT assay displayed that compounds S15 and S8 reduced MCF7 cell viability more significantly under hypoxia than normoxia, with little effect on the cell viability of MCF10a, suggesting that our compounds could be promising hypoxia effective agents for breast cancer. We found in the current study that compounds S15 triggered cell cycle arrest and apoptosis in G1/S of MCF7 cancer cells.
2.3. Molecular Docking Study

Carbonic anhydrases (Cas) are a group of Zn-metalloenzymes, in which Zn ions play a crucial role in its function [34,35]. Thus, drugs targeting such enzymes should engage in a coordination interaction with the Zn ion. Consequently, computational docking studies are utilized to accurately predict these kinds of interactions between ligands and Zn containing proteins.

In this regard, the most recently released version of AutoDock Vina 1.2.0 is used to model and describe the Zn-coordinating ligands utilizing a specialized AutoDock4Zn force-field [36–38]. This method does not describe only the energetic components of the interaction but also the geometric ones. This novel approach acts through creating a pseudo-atom (TZ) to describe the preferred position and the optimal tetrahedral coordination geometry of Zn ion complexed in proteins. Moreover, it defines the possibility for the interaction of Zn with coordinating elements such as nitrogen, oxygen and sulfur incorporated in the ligand. Additionally, the coordination geometry is encoded in the grid maps for the standard AutoDock4 (AD4) atom types. The new method includes an expanded force-field, an exceedingly advanced scoring function, and can replicate the improved docking presentation related to an AD4 engine.

Utilizing this highly improved capability in AutoDock Vina 1.2.0, a docking study was conducted to delineate a plausible explanation for the binding patterns and key interactions adopted by the most active (S3 and S15) inside the active site of tumor-associated isoenzymes CA-IX and compounds (S15 and S19) inside the active site CA-XII. In doing so, the available 3D crystal structures of hCA-IX enzyme (Protein Data Bank (PDB): 5FL4, resolution = 1.82 Å, complexed with 2-thiophene-sulfonamide ligand; 9FK) and hCA-XII (PDB code: 1JD0, resolution = 1.50 Å, complexed with acetazolamide) on the PDB (https://www.rcsb.org, accessed on 07/18/2022) were downloaded and used for this simulation. Initially, the docking procedure was validated by re-docking the co-crystallized ligand 9FK into the active site of CA-IX and then inspected visually, and the RMSD was calculated. It revealed similar conformations and good alignment with low RMSD value of 0.63 Å, Figure 1A. The superimposition of the highest docking poses of the greatest potent compounds; S3 and S15, along with the co-crystallized ligands; 9FK and acetazolamide into the active place of hCA-IX and hCA-XII, respectively, displays an amazing shape complementarity and similar orientations, Figure 7B,C.

Upon inspection of the docking results, it was revealed that the most potent compounds S3 and S15 (IC50 = 0.042 and 0.037 nM, respectively) bind within the CA-IX active site in a similar manner orientation compared to that of the co-crystallized ligand, 9FK, where the anilino moiety was directed to the bottom of the active site near Zn cation, while their tails were allocated at the entrance of the catalytic binding pocket cleft engaging in some vital hydrophobic interactions with Leu91, Val130 and Leu140 residues. However, the coordinating points with Zn ion were different. In compounds S3 and S15, the pseudoatom (TZ) was created on the N atom of amino group of the anilino moiety rather than NH of the sulfonamide moiety as in 9FK. This shift could be explained by the increased length of the compounds’ tail and size compared to 9FK. This amino group was also involved in an important H-bond interaction with Thr200 amino acid residue. Furthermore, the isoxazole ring in S15 was involved in a set of hydrogen bonds with the well-known key residue Gln92, where sulfonamide group formed two H-bonds, while NH was involved in an additional H-bond. Moreover, the p-chlorophenyl ring was engaged in π-π stacking with the imidazole ring of His94 and π-cation interaction with His68 residue. Nevertheless, S3 engaged in the same interactions, except one of the H-bond interactions of isoxazole with Gln92 residue, and this might be the reason behind the very slight decrease in activity. The 2D and 3D binding modes and key interactions of S3 and S15 against CA-IX isoform were depicted in Figure 8A–D and interaction results are illustrated in Table 5.
Figure 7. (A) ligand (9FK, green) co-crystallized and its redocked pose (yellow) inside the active place of hCA IX isoenzyme (PDB code: 5FL4); (B) Alignment of the top docked poses of compounds S3 (cyan), S15 (yellow) and 9FK (green) as a co-crystallized ligand into the binding pocket of hCA IX isoenzyme (PDB code: 5FL4); (C) Alignment of the top docked poses of compounds S3 (cyan), S15 (yellow), S19 (violet) and acetazolamide (green) as a co-crystallized ligand into the binding site of hCA-XII isoenzyme (PDB code: 1JD0). The protein structures were displayed as solvent surfaces colored by atom charge.

Table 5. The collective docking results and the interactions of compounds S3 and S15 into h-CA-IX isoenzyme and S15 and S19 into h-CA-XII isoenzyme.

| Ligand (Isoenzyme) | ΔG (kcal/mol) | H-Bonding Interactions | Hydrophobic Interactions | π-π Stacking |
|--------------------|---------------|------------------------|--------------------------|--------------|
| S3 (h-CA-IX)       | −29.86        | Amino and sulfonamide  | Thr200, Gln92            | Leu91, Val130 and Leu140, p-chlorophenyl ring with His94 |
| S15 (h-CA-IX)      | −28.98        | Amino, isoxazole and sulfonamide | Thr200, Gln92 | Leu91, Val130 and Leu140, p-chlorophenyl ring with His94 |
| S15 (h-CA-XII)     | −27.83        | Amino and sulfonamide  | Thr199, Gln92 and Thr200 | Val121, Ala131, Leu141 and Leu198 |
| S19 (h-CA-XII)     | −25.21        | sulfonamide            | Thr199                   | Val121, Ala131, Leu141 and Val143 |
Figure 8. The docking results and interactions of compounds S15 (yellow) and S3 (cyan) into the active site of hCA-IX isoenzyme (PDB code: 5FL4); (A,B) 3D and 2D binding modalities of S15, respectively; (C,D) 3D and 2D binding modalities of S3, respectively. The docked poses were displayed in ball & stick style. Zn atom was shown as a blue ball while pseudoatom TZ was represented as a pink ball. Favorable co-ordinate bond with Zn cation, hydrogen bonds and π-Stacking were showed in dashed blue, green and orange dashed lines, respectively. Hydrogen bond surfaces around docked poses were created. Hydrogen atoms were omitted for clarity purpose. The alignment of the most dominant conformations of S3, S15 and co-crystallized ligand, 9FK into the active place of CA-IX was shown in Figure 9A,B. All compounds maintained almost the same conformation in the active site. Interestingly, the visual inspection was in agreement with the obtained docking scores and were much higher than that of the redocked pose 9FK where compounds S3 and S15 recorded the ΔG values of −29.86, −28.98 kcal/mol, respectively compared with docking score of 9FK with ΔG values of −17.87 kcal/mol.
On the other hand, compound **S15** with an IC$_{50}$ value of 0.06 nM and **S19** with lower activity (IC$_{50}$ = 0.9 nM) were docked into the catalytic binding site of hCA-XII isoenzymes. The results showed that **S15** coordinates with the Zn cation with N atom of anilino moiety in the same manner that has been observed with hCA-IX. In addition, it was involved in a crucial H-bonding interaction with Thr199 residue. However, the two oxygens of the sulfonamide group were involved into two vital hydrogen bonds with Gln92 and Thr200 amino acids residues. Some hydrophobic interactions were noticed with Ala121, Val131, Leu141 and Leu198 residues, which greatly contributed to its stability inside the active pocket, Figure 10A,B. However, compound **S19** adopted a different orientation, where the pseudoatom (TZ) preferred to be on the NH of the sulfonamide, which resulted in some steric clashes, unfavorable coordination with Zn ion and unfavorable acceptor-acceptor interaction with Thr199 amino acid. This could explain the significant difference in activity compared to **S15**. Nevertheless, **S19** was engaged in other important H-bonding, hydrophobic, Van der Waal and π-sulfur interactions, which significantly have a great impact on keeping its activity within the nanomolar range, Figure 10C,D. Compounds **S15** and **S19** recorded the Δ$_G$ values of $-27.83$ and $-25.21$ kcal/mol, respectively. Taken together, our in silico docking analysis strongly confirmed the potential inhibitory activity of our new hits against both hCA-IX and hCA-XII isoenzymes, which in turn might participate in the design and development of promising leads that could be utilized in the future as anticancer agents.

![Figure 9.](image-url)

**Figure 9.** (A) 3D binding pattern of 9fk (green) into active place of hCA-IX isoenzyme; (B) Overlay of S3, S15 and 9FK to show the shape complementarities with hCA-IX active place and the difference in torsions and conformations. The docked and redocked poses were displayed in ball & stick style. Zn atom was shown as a blue ball while pseudoatom TZ was represented as a pink ball. Favorable co-ordinate bond with Zn cation, hydrogen bonds and π-Stacking were showed in dashed blue, green and orange dashed lines, respectively. Hydrogen bond surfaces around docked poses were created. Hydrogen atoms were omitted for clarity purpose.
Figure 10. The docking results and interactions of compounds S15 (yellow) and S19 (violet) into the active place of hCA-XII isoenzyme (PDB code: 1JD0); (A,B) 3D and 2D binding modalities of S15, respectively; (C,D) 3D and 2D binding modalities of S19, respectively. The docked poses were displayed in ball & stick style. Zn atom was shown as a blue ball while pseudoatom TZ was represented as a pink ball. Unfavorable co-ordinate bond with Zn cation or clashes were displayed in dashed red lines. π-Sulfur and hydrophobic interactions were represented as orange and light violet dashed lines, respectively. Hydrogen bond surfaces around docked poses were created. Hydrogen atoms were omitted for clarity purpose.

3. Materials and Methods

3.1. Chemistry

The instruments specifically used for NMR, IR, mass spectroscopy and elemental analysis of synthesis compounds are presented in the Supplementary Data (Section S2). Additionally, TLC plates and chemical supplies are illustrated in Section S2.

Derivatives S5, S13a–d, and S18 were synthesized following known methods.

3.1.1. General Method for Preparation of Sulfamethoxazole S1–S4

To a mixture of sulfamethoxazole (2.53 g, 10 mmol) and anhydrous potassium carbonate (5.52 g, 40 mmol) in dry DMF (30 mL), the corresponding alkyl chloride (10 mmol) was added dropwise. The reaction mixture was heated under reflux for 24 h then poured onto
ice-cold water. The formed product was filtered, washed with water, dried and crystallized from absolute ethanol to give compounds S1–S4.

4-Amino-N-ethyl-N-(5-methylisoxazol-3-yl)benzenesulfonamide (S1):

Yield (84%); yellow crystals; m.p. 90–92 °C; IR (KBr): 3261 (NH) cm⁻¹. ¹HNMR at δ 1.16 (t, J = 6.8 Hz, 3H, CH₂-CH₃); 2.35 (s, 3H, CH₃ isoxazole ring); 2.70 (q, J = 6.8 Hz, 2H, CH₂-CH₃); 6.19 (s, 2H, NH₂); 6.41 (s, 1H, isoxazole H-4); 6.61 (d, J = 8.8 Hz, 2H, benzenesulfonamide H-3,5); 7.44 (d, J = 8.8 Hz, 2H, benzenesulfonamide H-2,6). ¹³CNMR at δ: 12.57 (CH₃ isoxazole), 14.18 (CH₂), 97.86 (isoxazole C-4), 113.29, 122.85, 129.44, 154.24, 159.72, 170.71. EIMS (m/z) 281.41 M⁻² (11.27%), 268.41 (100%). Anal. Calcd. for C₁₂H₁₅N₂O₃S: C, 52.37; H, 5.37; N, 14.94; Found; C, 51.61; H, 5.02; N, 14.86.

N-Allyl-4-amino-N-(5-methylisoxazol-3-yl)benzenesulfonamide (S2):

Yield (83%); yellowish white powder; m.p. 105–107 °C; IR (KBr): 3345 (NH) cm⁻¹. ¹HNMR at δ: 2.34 (s, 3H, CH₃ isoxazole ring); 4.30 (d, J = 5.2 Hz, 2H, NH-CH₂); 5.20 (dd, J = 1.2, 17.2 Hz, 2H, CH=CH₂); 5.75–5.85 (m, 1H, CH allyl); 6.22 (s, 2H, NH₂); 6.40 (s, 1H, isoxazole H-4); 6.62 (d, J = 8.4 Hz, 2H, benzenesulfonamide H-3,5); 7.48 (d, J = 8.4 Hz, 2H, benzenesulfonamide H-2,6). ¹³CNMR at δ: 12.63 (CH₃ isoxazole), 50.57 (NH-CH₂), 97.82 (isoxazole C-4), 113.25, 116.38, 118.14, 122.55, 129.60, 133.14, 154.25, 159.79, 170.85. EIMS (m/z) 293.84 M⁻² (15.73%), 166.27 (100%). Anal. Calcd. for C₁₃H₁₅N₂O₃S: C, 53.23; H, 5.15; N, 14.32; Found; C, 53.11; H, 5.22; N, 14.06.

4-Amino-N-(5-methylisoxazol-3-yl)-N-propylbenzenesulfonamide (S3):

Yield (85%); white crystals; m.p. 75–77 °C; IR (KBr): 3330 (NH) cm⁻¹. ¹HNMR at δ: 0.85 (t, J = 8.0 Hz, 3H, CH₂-CH₃); 1.54–1.63 (m, 2H, CH₂-CH₃); 2.35 (s, 3H, CH₃ isoxazole ring); 3.59 (t, J = 8.0 Hz, 2H, NH-CH₂); 6.20 (s, 2H, NH₂); 6.41 (s, 1H, isoxazole H-4); 6.60 (d, J = 8.0 Hz, 2H, benzenesulfonamide H-3,5); 7.42 (d, J = 8.0 Hz, 2H, benzenesulfonamide H-2,6). ¹³CNMR at δ: 12.60 (CH₃ isoxazole), 21.48 (CH₂CH₃), 50.02 (NH-CH₂), 98.09 (isoxazole C-4), 113.26, 122.66, 129.45, 154.22, 159.95, 170.74. EIMS (m/z) 295.00 M⁻² (21.00%), 63.17 (100%). Anal. Calcd. for C₁₃H₁₇N₂O₃S: C, 52.87; H, 5.80; N, 14.23; Found; C, 52.91; H, 6.02; N, 14.15.

4-Amino-N-butyl-N-(5-methylisoxazol-3-yl)benzenesulfonamide (S4):

Yield (79%); white crystals; m.p. 85–87 °C; IR (KBr): 3230 (NH) cm⁻¹. ¹HNMR at δ: 0.86 (t, J = 7.6 Hz, 3H, CH₂-CH₃); 1.25–1.30 (m, 2H, CH₂-CH₃); 1.52–1.56 (m, 2H, -CH₂-CH₂-CH₂); 2.35 (s, 3H, CH₃ isoxazole ring); 3.62 (t, J = 7.6 Hz, 2H, NH-CH₂); 6.19 (s, 2H, NH₂); 6.40 (s, 1H, isoxazole H-4); 6.60 (d, J = 8.4 Hz, 2H, benzenesulfonamide H-3,5); 7.41 (d, J = 8.4 Hz, 2H, benzenesulfonamide H-2,6). ¹³CNMR at δ: 12.58 (CH₃ isoxazole), 13.95 (CH₃), 19.73 (CH₂CH₃), 30.23(CH₂-CH₂-CH₂), 48.17, (NH-CH₂), 98.15 (isoxazole C-4), 113.28, 122.64, 129.44, 154.19, 159.94, 170.78. EIMS (m/z) 309.79 M⁻² (12.93%), 189.82 (100%). Anal. Calcd. for C₁₄H₁₉N₂O₃S: C, 54.35; H, 6.19; N, 13.58; Found; C, 54.23; H, 6.07; N, 13.66.

3.1.2. General Method for Preparation of Compounds S6–S11

A solution of sulfamethoxazole (2.53 g, 10 mmol) in dry DMF (30 mL) was cooled to 0–5 °C. The appropriate acid chloride (10 mmol) was added slowly with vigorous stirring, followed by the addition of triethylamine (0.5 mL). The reaction mixture was then stirred at rt for 24 h (for compounds S6 and S7) or heated under reflux (for compounds S8–S11). At the end of the reaction, the mixture was poured into ice-cooled water and the precipitate was filtered off, dried and crystallized from methanol to afford the target compounds S6–S11.
4-Chloro-N-(4-(N-(5-methylisoxazol-3-yl)sulfonyl)phenyl)butanamide (S7)

Yield (77%); white powder; m.p. 212–214 °C; IR (KBr): 3303 (NH), 1682 (C=O) cm⁻¹.

1H NMR at δ: 2.00–2.07 (m, 2H, CH₂-CH₂-CH₂); 2.29 (s, 3H, CH₃ isoxazole ring); 2.53 (t, J = 7.6 Hz, 2H, CO-CH₂); 3.70 (t, J = 6.4 Hz, 2H, CH₂-CH₂); 6.13 (s, 1H, isoxazole H-4); 7.79–7.82 (m, 4H, phenyl H-2, 3, 5, 6); 10.40 (s, 1H, NH); 11.32 (s, 1H, NH). 13C NMR at δ: 12.51 (CH₃ isoxazole), 28.12 (CH₂-CH₂-CH₂), 33.94 (CH₂CO), 45.38 (CH₂-Cl), 95.83 (isoxazole C-4), 119.27, 129.29, 133.90, 158.03, 170.75 (CO), 171.49. EIMS (m/z) 357.07 M⁺ (26.08%), 87.68 (100%). Anal. Calcd. for C₁₄H₁₆ClN₃O₄S: C, 45.42; H, 4.10%; N, 12.22; Found: C, 45.71; H, 4.18; N, 12.37.

4-Methyl-N-(4-(N-(5-methylisoxazol-3-yl)sulfonyl)phenyl)benzamide (S8)

Yield (73%); yellowish white powder; m.p. 225–227 °C; IR (KBr): 3283 (NH), 1666 (C=O) cm⁻¹. 1H NMR at δ: 2.28 (s, 3H, CH₃ isoxazole ring); 2.39 (s, 3H, CH₂); 6.10 (s, 1H, isoxazole H-4); 7.35 (d, J = 7.6 Hz, 2H, benzamide H-3,5); 7.80–7.96 (m, 6H, phenyl H-2, 3, 5, and benzamide H-2, 6); 10.51 (s, 1H, NH); 11.72 (s, 1H, NH). 13C NMR at δ: 12.57 (CH₃ isoxazole), 21.50 (CH₃), 96.13 (isoxazole C-4), 120.38, 128.16, 128.31, 129.48, 131.95, 142.63, 143.59, 166.37. EIMS (m/z) 371.62 M⁺ (11.27%), 285.69 (100%). Anal. Calcd. for C₁₄H₁₂N₂O₄S: C, 58.21; H, 4.61%; N, 11.31; Found: C, 58.40; H, 4.88; N, 11.16.

4-Chloro-N-(4-(N-(5-methylisoxazol-3-yl)sulfonyl)phenyl)benzamide (S9)

Yield (79%); yellow crystals; m.p. 233–235 °C; IR (KBr): 3242 (NH), 1673 (C=O) cm⁻¹. 1H NMR at δ: 2.30 (s, 3H, CH₃ isoxazole ring); 6.16 (s, 1H, isoxazole H-4); 7.63 (d, J = 8.4 Hz, 2H, benzamide H-3,5); 7.87 (d, J = 8.8 Hz, 2H, phenyl H-3,5); 7.98–8.00 (m, 4H, phenyl H-2, 6 and benzamide H-2, 6); 10.69 (s, 1H, NH); 11.38 (s, 1H, NH). 13C NMR at δ: 12.53 (CH₃ isoxazole), 95.88 (isoxazole C-4), 113.06, 120.57, 128.38, 128.68, 129.04, 130.27, 133.51, 133.54, 134.15, 137.36, 143.82, 150.05, 156.50 (CO), 170.77. EIMS (m/z) 391.73 M⁺ (61.57%), 60.02 (100%). Anal. Calcd. for C₁₇H₁₃N₄O₆S: C, 50.74; H, 3.51%; N, 13.92; Found: C, 51.01; H, 3.32; N, 13.64.

N-(4-(N-(5-Methylisoxazol-3-yl)sulfonyl)phenyl)-4-nitrobenzamide (S10)

Yield (73%); yellow crystals; m.p. 188–190 °C; IR (KBr): 3235 (NH), 1681 (C=O) cm⁻¹. 1H NMR at δ: 2.30 (s, 3H, CH₃ isoxazole ring); 6.16 (s, 1H, isoxazole H-4); 7.89 (d, J = 8.8 Hz, 2H, phenyl H-3,5); 8.00 (d, J = 8.8 Hz, 2H, phenyl H-2,6); 8.19 (d, J = 8.4 Hz, 2H, nitrobenzamide H-2,6); 8.39 (d, J = 8.4 Hz, 2H, nitrobenzamide H-3,5); 10.94 (s, 1H, NH); 11.41 (s, 1H, NH). 13C NMR at δ: 12.52 (CH₃ isoxazole), 95.88 (isoxazole C-4), 113.06, 120.72, 124.08, 128.44, 129.30, 129.86, 134.51, 140.48, 143.51, 149.84, 154.03, 165.00 (CO), 170.80. EIMS (m/z) 402.81 M⁺ (66.30%), 243.77 (100%). Anal. Calcd. for C₁₇H₁₄N₄O₆S: C, 52.11; H, 3.60%; N, 10.72; Found: C, 52.03; H, 3.92; N, 10.56.

3,4,5-Trimethoxy-N-(4-(N-(5-methylisoxazol-3-yl)sulfonyl)phenyl)benzamide (S11)

Yield (69%); white powder; m.p. 242–244 °C; IR (KBr): 3295 (NH), 1687 (C=O) cm⁻¹. 1H NMR at δ: 2.31 (s, 3H, CH₃ isoxazole ring); 3.74 (s, 3H, OCH₃); 3.87 (s, 6H, 2 OCH₃); 6.15 (s, 1H, isoxazole H-4); 7.27 (s, 2H, benzamide H-2,6); 7.86 (d, J = 8.4 Hz, 2H, phenyl H-3,5); 7.96 (d, J = 8.4 Hz, 2H, phenyl H-2,6); 10.49 (s, 1H, NH); 11.37 (s, 1H, NH). 13C NMR at δ: 12.52 (CH₃ isoxazole), 55.69 (2 OCH₃), 60.64 (OCH₃), 95.88 (isoxazole C-4), 105.93, 120.67, 128.35, 129.92, 134.05, 141.11, 143.87, 153.13, 158.12, 165.99 (CO), 170.77. EIMS (m/z) 447.01 M⁺ (39.39%), 402.65 (100%). Anal. Calcd. for C₂₀H₂₁N₃O₈S: C, 53.69; H, 4.73%; N, 9.39; Found: C, 53.41; H, 4.85; N, 9.57.

3.1.3. Preparation Method of Compounds S12 and S14–S17

A mixture of sulphamethoxazole (2.53 g, 10 mmol), 3-chloropropiophenone or compounds S13a–d (10 mmol) and triethylamine (0.5 mL) in dry dimethylformamide (30 mL) was heated under reflux for 24 h. The reaction mixture was poured into ice-cooled water, the precipitate was filtered, dried and crystallized from methanol to obtain the target compounds S12 and S14–S17.
4-Amino-N-(5-methylisoxazol-3-yl)-N-(3-oxo-3-phenylpropyl)benzenesulfonamide (S12)

Yield (64%); pale yellow powder; m.p. 200–202 °C; IR (KBr): 3331 (NH2), 1765 (C=O) cm⁻¹. ¹H NMR at δ: 2.29 (s, 3H, CH3 isoxazole ring); 3.33 (t, J = 6.0 Hz, 2H, CO-CH2); 3.46 (t, J = 6.0 Hz, 2H, CH2-N); 6.11 (s, 2H, NH2); 6.64–6.70 (m, 3H, benzenesulfonamide H-3,5 and oxazole CH); 7.51–7.56 (m, 4H, benzenesulfonamide H-2,6 phenyl H-3,5); 7.64 (t, J = 7.6, 1H, phenyl H-4); 7.98 (d, J = 7.2, 2H, phenyl H-2,6); ¹³C NMR at δ: 12.51 (CH3 isoxazole), 37.72 (CH3-CO), 39.71 (CH2-N), 95.75 (isoxazole C-4), 111.36, 124.68, 128.39, 129.20, 129.24, 133.75, 137.01, 152.84, 158.44, 170.38, 198.86 (CO). EIMS (m/z) 383.54 M⁺ ² (33.65%), 204.82 (100%). Anal. Calcd. for C19H19N3O3S: C, 59.21; H, 4.97; N, 10.90; Found; C, 59.33; H, 4.87; N, 10.76.

4-Amino-N-(5-methylisoxazol-3-yl)-N-(2-oxo-2-(p-toly)ethyl)benzenesulfonamide (S14)

Yield (66%); yellow powder; m.p. 180–182 °C; IR (KBr): 3355, 3262 (NH2), 1673 (C=O) cm⁻¹. ¹H NMR at δ: 2.25 (s, 3H, CH3); 2.34 (s, 3H, CH3 isoxazole ring); 4.49 (s, 2H, CH2); 6.25 (s, 2H, NH2); 6.41 (s, 1H, isoxazole H-4); 6.63 (d, J = 8.8 Hz, 2H, tolyl H-3,5); 7.11 (d, J = 8.4 Hz, 2H, phenyl H-2,6); 7.45 (d, J = 8.4 Hz, 2H, phenyl H-3,5); 7.55 (d, J = 8.8 Hz, 2H, tolyl H-2,6); 10.06 (s, 1H, NH). ¹³C NMR at δ: 12.58 (CH3 isoxazole), 21.15 (CH3), 50.66 (CH2), 97.18 (isoxazole C-4), 113.23, 119.48, 122.43, 129.64, 129.89, 132.73, 136.78, 154.45, 160.02, 165.27 (CO), 170.82. EIMS (m/z) 400.26 M⁺ ² (26.30%), 211.22 (100%). Anal. Calcd. for C19H19N3O3S: C, 56.99; H, 5.03; N, 13.99; Found; C, 56.41; H, 4.89; N, 14.16.

4-Amino-N-(2-(4-chlorophenyl)-2-oxoethyl)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (S15)

Yield (75%); brown powder; m.p. 182–184 °C; IR (KBr): 3333, 3281 (NH), 1661 (C=O) cm⁻¹. ¹H NMR at δ: 2.34 (s, 3H, CH3 isoxazole ring); 4.50 (s, 2H, CH2); 6.25 (s, 2H, NH2); 6.41 (s, 1H, isoxazole H-4); 6.62 (d, J = 8.8 Hz, 2H, phenyl H-2,6); 7.37 (d, J = 8.8 Hz, 2H, chlorophenyl H-3,5); 7.53 (d, J = 8.8 Hz, 2H, phenyl H-3,5); 7.58 (d, J = 8.4 Hz, 2H, chlorophenyl H-2,6); 10.29 (s, 1H, NH). ¹³C NMR at δ: 12.58 (CH3 isoxazole), 50.69 (CH2), 97.08 (isoxazole C-4), 113.23, 121.05, 122.31, 127.41, 129.20, 129.89, 138.16, 154.45, 159.95, 165.74 (CO), 170.95. EIMS (m/z) 420.99 M⁺ ² (97.40%), 402.69 (100%). Anal. Calcd. for C18H17ClN3O3S: C, 51.37; H, 4.07; N, 13.31; Found; C, 51.50; H, 4.19; N, 13.38.

4-Amino-N-(5-methylisoxazol-3-yl)-N-(2-(4-nitrophenyl)-2-oxoethyl)benzenesulfonamide (S16)

Yield (72%); yellowish brown powder; m.p. 186–188 °C; IR (KBr): 3354, 3221 (NH2), 1654 (C=O) cm⁻¹. ¹H NMR at δ: 2.34 (s, 3H, CH3 isoxazole ring); 4.59 (s, 2H, CH2); 6.27 (s, 2H, NH2); 6.43 (s, 1H, isoxazole H-4); 6.64 (d, J = 8.4 Hz, 2H, phenyl H-2,6); 7.55 (d, J = 8.4 Hz, 2H, phenyl H-3,5); 7.81 (d, J = 8.8 Hz, 2H, nitrophenyl H-2,6); 8.23 (d, J = 8.8 Hz, 2H, nitrophenyl H-3,5); 10.83 (s, 1H, NH). ¹³C NMR at δ: 12.59 (CH3 isoxazole), 50.90 (CH2), 97.03 (isoxazole C-4), 113.25, 119.26, 122.20, 125.32, 130.54, 142.78, 145.36, 154.53, 159.92, 166.65 (CO), 171.05. EIMS (m/z) 431.54 M⁺ ² (25.98%), 231.96 (100%). Anal. Calcd. for C18H17N3O4S: C, 50.11; H, 3.97; N, 16.23; Found; C, 50.23; H, 4.09; N, 16.48.

4-Amino-N-(5-methylisoxazol-3-yl)-N-(2-oxo-2-(3,4,5-trimethoxyphenyl)ethyl)benzenesulfonamide (S17)

Yield (64%); pale yellow powder; m.p. 201–203 °C; IR (KBr): 3291, 3236 (NH2), 1667 (C=O) cm⁻¹. ¹H NMR at δ: 2.34 (s, 3H, CH3 isoxazole ring); 3.68 (s, 3H, OCH3); 3.74 (s, 6H, 2 OCH3); 4.47 (s, 2H, CH2); 6.24 (s, 2H, NH2); 6.41 (s, 1H, isoxazole H-4); 6.63 (d, J = 8.4 Hz, 2H, phenyl H-2,6); 6.95 (s, 2H, trimethoxyphenyl H-2,6); 7.52 (d, J = 8.4 Hz, 2H, phenyl H-3,5); 10.09 (s, 1H, NH). ¹³C NMR at δ: 12.59 (CH3 isoxazole), 50.67 (CH2), 56.16 (2 OCH3), 60.59 (OCH3), 97.18 (isoxazole C-4), 113.24, 122.44, 129.82, 133.91, 135.43, 153.23, 154.46, 160.00, 165.36 (CO), 170.87. EIMS (m/z) 476.18 M⁺ ² (15.15%), 384.64 (100%). Anal. Calcd. for C21H24N4O7S: C, 52.93; H, 5.08; N, 11.76; Found; C, 52.64; H, 4.99; N, 11.56.

3.1.4. Method for Preparation of Benzenesulfonamides S19–S22

A mixture of thiazol-2-ylamino benzenesulfonamide derivative S18 (3.52 g, 10 mmol), appropriate aromatic aldehyde (10 mmol) and CH3COONa (1.23 g, 15 mmol) in glacial
Acetic acid (20 mL) was heated under reflux for 24 h. The mixture was poured onto crushed ice, and the precipitate was filtered, dried, and crystallized from aqueous ethanol 95% to give the target products S19–S22.

\((Z)-4-(5-(4-Methylbenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (S19)\)

Yield (86%); white powder; m.p. 228–230 °C; IR (KBr): 3362 (NH), 1753 (C=O) cm⁻¹. ¹HNMR at δ: 2.32 (s, 6H, 2 CH₃); 6.18 (s, 1H, isoxazole H-4); 7.25–7.52 (m, 7H, benzenesulfonamide H-2,3,5,6, methylbenzylidene H-3,5 and NH); 7.65–7.89 (m, 3H, methylbenzylidene H-2,6 and CH); 11.97 (s, 1H, NH). ¹³CNMR at δ: 12.55 (CH₃isoxazole), 21.54 (CH₃), 95.96 (isoxazole C-4), 122.47, 128.91, 130.28, 130.38, 135.75, 140.74, 158.08, 170.83. EIMS (m/z) 456.94 M + 1 (7.45%), 250.90 (100%). Anal. Calcd. for C₂₁H₁₈N₄O₄S₂: C, 55.49; H, 3.99; N, 12.33; Found; C, 55.36; H, 3.61; N, 12.66.

\((Z)-4-(5-(4-Chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (S20)\)

Yield (75%); yellow crystals; m.p. 275–277 °C; IR (KBr): 3295 (NH), 1711 (C=O) cm⁻¹. ¹HNMR at δ: 2.32 (s, 6H, CH₃isoxazole); 6.18 (s, 1H, isoxazole H-4); 7.25 (d, J = 7.2 Hz, 2H, benzenesulfonamide H-3,5); 7.55–7.68 (m, 5H, benzenesulfonamide H-2,6, chlorobenzylidene H-3,5 and NH); 7.87–7.97 (m, 3H, chlorobenzylidene H-2,6 and CH); 11.56 (s, 1H, NH). ¹³CNMR at δ: 12.55 (CH₃isoxazole), 95.93 (isoxazole C-4), 122.47, 128.96, 129.84, 130.38, 131.86, 135.07, 140.74, 158.01, 170.89. EIMS (m/z) 474.21 M + 1 (20.45%), 344.74 (100%). Anal. Calcd. for C₂₀H₁₅ClN₄O₄S₂: C, 50.58; H, 3.18; N, 11.80; Found; C, 50.66; H, 3.44; N, 12.06.

\((Z)-N-(5-Methylisoxazol-3-yl)-4-(5-(4-nitrobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (S21)\)

Yield (69%); yellow powder; m.p. 266–268 °C; IR (KBr): 3235 (NH), 1706 (C=O) cm⁻¹. ¹HNMR at δ: 2.32 (s, 3H, CH₃isoxazole); 6.18 (s, 1H, isoxazole H-4); 7.26 (d, J = 8 Hz, 2H, benzenesulfonamide H-3,5); 7.77–8.05 (m, 5H, benzenesulfonamide H-2,6, nitrobenzylidene H-2,6 and NH); 8.27–8.36 (m, 3H, nitrobenzylidene H-3,5 and CH); 11.95 (s, 1H, NH). ¹³CNMR at δ: 12.54 (CH₃isoxazole), 95.93 (isoxazole C-4), 122.47, 128.00, 128.80, 129.01, 129.84, 131.14, 135.96, 147.66, 148.77, 153.64, 157.98, 170.91. EIMS (m/z) 485.49 M + 1 (24.56%), 253.28 (100%). Anal. Calcd. for C₂₀H₁₅N₅O₆S₂: C, 49.48; H, 3.11; N, 14.43; Found; C, 49.83; H, 3.53; N, 14.76.

\((Z)-N-(5-Methylisoxazol-3-yl)-4-(4-oxo-5-(3,4,5-trimethoxybenzylidene)-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (S22)\)

Yield (71%); yellowish brown crystals; m.p. 240–242 °C; IR (KBr): 3382 (NH), 1723 (C=O) cm⁻¹. ¹HNMR at δ: 2.31 (s, 3H, CH₃isoxazole), 3.72 (s, 6H, 2 OCH₃), 3.77 (s, 3H, OCH₃), 6.18 (s, 1H, isoxazole H-4); 6.81–7.26 (m, 3H, benzenesulfonamide H-3,5 and NH); 7.64–7.97 (m, 5H, trimethoxybenzylidene H-2,6, benzenesulfonamide H-2,6 and CH); 11.47 (s, 1H, NH). ¹³CNMR at δ: 12.52 (CH₃isoxazole), 56.42 (2 OCH₃), 60.67 (OCH₃), 95.95 (isoxazole C-4), 107.21, 107.79, 120.94, 122.49, 122.47, 128.82, 130.98, 132.44, 135.40, 139.68, 153.64, 157.98, 170.86. EIMS (m/z) 530.65 M + 1 (29.89%), 49.79 (100%). Anal. Calcd. for C₂₃H₂₂N₄O₇S₂: C, 52.07; H, 4.18; N, 10.56; Found; C, 51.86; H, 4.41; N, 10.79.

3.2. Biological Activities
3.2.1. Materials and Methods

Chemicals and Kits
The specifications and suppliers of the chemicals and kits are presented in Supplementary Section S2 (attached file).

3.2.2. CA IX Inhibitory Assay
The hCA IX was assayed following the manufacturer’s instruction and reported method. (Table 1, Figure 3A,B) [39] (Supplementary Material Section S2).
3.2.3. hCA XII Inhibitory Assay

The hCA XII was assayed following the manufacturer’s instruction and reported method [40] (Supplementary Material Section S2) (Table 1, Figure 3A,B).

3.2.4. CA1 Inhibitory Assay

The hCA I was assayed following the manufacturer’s instruction and reported method [41] (Supplementary Material Section S2) (Table 2).

3.2.5. CAII Inhibitory Assay

CAII was assayed following the manufacturer’s instruction and reported method [42] (Supplementary Material Section S2) (Table 2).

3.2.6. Cell Culture Protocol

Suppling, specification and incubation of MCF7 and MCF10a cell lines were carried out according to the method mentioned in detail in Supplementary Material Section S2.

3.2.7. MTT–Cytotoxicity Assay Protocol

MTT assay is a method used for in vitro cytotoxicity of different compounds. The method details are illustrated in Supplementary Material Section S2. (Table 3, Figure 4).

3.2.8. Annexin V-FITC Assay for Apoptosis

Treated MCF7 and MCF10a cells of 1–5 \times 10^5 were collected and suspended with binding buffer and cultured and incubated with 5 \mu L of both propidium iodide (PI 50mg/mL) and Annexin V-FITC. The details regarding the method of detection are listed in Supplementary Material Section S2. (Table 4, Figures 5 and 6).

3.3. Docking Study

This study was achieved using Autodock Vina program version 1.2.0 0 [42,43]. The details of the docking method, visualization and crystal structure were downloaded from the protein data bank and carried out according to reported methods; the details are presented in Supplementary Material Section S2.3 [43,44] (Table 5, Figures 7–10).

4. Conclusions

The new synthesized compounds possess carbonic anhydrase I, II, IX and XII inhibitory activities. The activity of compounds S9 and S15 towards hCAs may be attributed to 4-chlorophenyl groups; however, the presence of methyl groups in compounds S8 and S18 also showed activity and selectivity, but less than chloride. Additionally, amido groups in the synthesized compounds (S8–S11, S14–S17) showed a vital role in binding with the zinc atom in receptor (ZBG) and this reflected on its activity.

The sulphomyl moieties possess effects similar to the urido linker. The thiazolidinone rings (S18–S22) did not enhance activity as expected, as it was weakly bonded with ZBG.

The strong electron withdrawn group (nitro, S10) or donating group (trimethoxy groups; S11) had no impact on activity. The S15 has selective inhibitory activity towards hCAs IX and X11 compared to hCAs I and II, paving the way for the discovery of new selective anticancer agents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ph15091134/s1.

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