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Targeting the Carbonic Anhydrase Enzyme with Synthesized Benzenesulfonamide Derivatives: Inhibiting Tumor Growth

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Abstract

Objectives: To assess the anticancer effects of recently developed compounds, Sa, Sb, Sc, and Sd. These compounds were designed to specifically target the carbonic anhydrase enzyme in solid tumors.

Methods: The chemical synthesis involved the use of sulfanilamide, chloroacetyl chloride, GABA, thionyl chloride, methanol, hydrazine hydrate, potassium hydroxide, carbon disulfide, and benzyl chloride derivatives. Docking studies were conducted using the MOE software program version 2015.10, and cytotoxic activity was predicted using the MTT assay.

Results: The newly synthesized compounds exhibited notable antineoplastic activity in both in silico and cell line investigations. Although they showed a significant difference in potency compared to cisplatin against cancer cells, they also demonstrated significant differences in toxicity towards normal cells. When compared to acetazolamide, compounds Sb displayed an IC50 = 28.41 µM, which was significantly different, and compound Sd showed a non-significant difference with an IC50 = 61.20 µM against MCF7 cells. Additionally, Sb and Sd demonstrated significant difference in toxicity, with IC50 = 279.02 µM and 194.00 µM, respectively, against MCF10a cells. These findings indicate a significant difference compared to acetazolamide for the Sb compound and suggest that the synthesized compounds hold potential for further development as antineoplastic agents. Furthermore, the results from the cell line study align with the in silico study, where both compounds Sb and Sd exhibited higher S scores compared to acetazolamide, implying a stronger binding affinity with the receptor's catalytic site. The presence of a substituted 1, 2, 4-triazole ring in these compounds contributed to enhanced flexibility and improved interaction with the receptor.

Conclusion: A new synthesized compounds exhibited cytotoxicity and demonstrated inhibitory potencies against carbonic anhydrase.
Keywords: In Silico, Triazoles, Moieties, Carbonic Anhydrase Inhibitors.

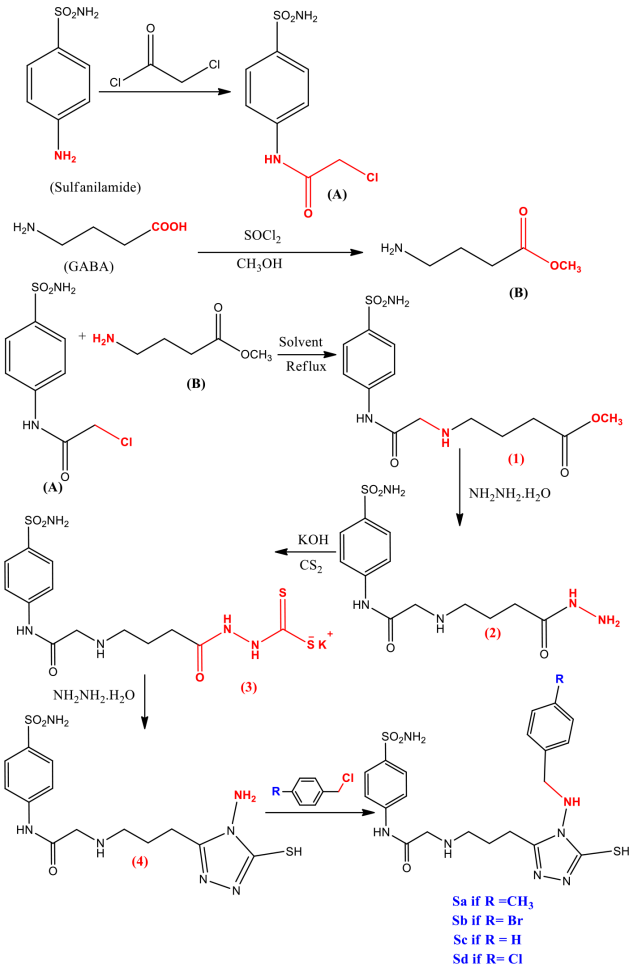
Introduction

Diseases in which abnormal cells develop and spread uncontrollably are called cancers. If the metastatic spread of cancer cells is not stopped, the patient is going to die.[1] In 2018, the World Health Organization (WHO) estimated that 18.1 million people worldwide had been diagnosed with cancer, and 9.6 million died from the disease. The estimate for 2040 puts the number at 29.4 million.[2] The effective management of cancer seems to be a significant hurdle at the beginning of the current century. This obstacle arises from the challenge of finding new targeted substances that can hinder the growth of cancerous cells without being toxic to normal cells.[3] Tumor cells and normal cells exhibit several differences, and one notable difference is the lower oxygen levels (known as hypoxia) observed in tumor cells compared to normal cells.[4] Under hypoxic conditions, tumor cells shift their metabolism from oxidative phosphorylation to aerobic glycolysis. Consequently, this metabolic change leads to the accumulation of lactic acid and extracellular H^+ in the tumor's nearby areas.[5] The function of carbonic anhydrase (CA) aids in balancing the environment by enabling the reversible hydration of carbon dioxide (CO_2) into hydrogen ions (H^+) and bicarbonate ions ($HCO^3–$). These metalloenzymes, known as carbonic anhydrases, are present in a wide range of living organisms and are classified into eight distinct families: α-CAs, β-CAs, γ- CAs, δ-CAs, ζ- CAs, η-CAs, θ-CAs, and ι-CAs. In humans, all carbonic anhydrases belong to the α-class, and thus far, 16 different variations of α-CAs have been identified.[6-8] CA IX and XII have been extensively associated with cancer among the identified isoforms.[9] Developing an effective and low-side-effect anticancer treatment shows promise through the targeted inhibition of two transmembrane isoforms, CA IX and XII, while sparing the ubiquitous and cytosolic CA I and II.[10,11] Over the past few decades, extensive research has been carried out to discover effective inhibitors that can target CA IX, with the aim of creating innovative and effective anti-cancer drugs. As a result, a number of hopeful candidates have been found.[12] Sulfonamide/sulfamate carbonic anhydrase inhibitors (CAIs) have been under investigation for many years and continue to be employed in medical practice as diuretics, anti-glaucoma, anti-epileptic, and anti-obesity medications. However, over the last two decades, CAIs have received considerable attention and investigation as potential anticancer agents.[13, 14] Sulfonamide derivatives have a shared molecular structure consisting of an aromatic or heterocyclic motif. Depending on the substitution pattern on the aromatic ring, which can be a tail or linker moiety, they can establish specific interactions within the typical bipolar structure of the enzyme. These interactions can occur either at the center of the active site or at its edge.[15-17] Different heterocyclic compounds have different chemical properties and biological activity, making them of interest to medicinal chemists.[18] Extensive research has been conducted to explore its potential as a therapeutic option for a wide range of illnesses, including cancer. The importance of heterocyclic compounds in medicine has grown significantly due to their presence in essential biological

components like DNA, RNA, and vitamins, which have naturally occurring heterocyclic core rings.[19] Triazole ring is a significant nitrogen-containing heterocyclic compound composed of three nitrogen atoms and two carbon atoms.[20] It has been noted that molecules incorporating the triazole structure possess significant practical applications across various industries.[21] Several chemicals utilizing this nucleus are already available in the market.[22] The 1, 2, 4-triazole nucleus serves as both a hydrogen bond acceptor and donor in the active site of a receptor. Moreover, it exhibits resistance to metabolic degradation, which can be advantageous for improving solubility and facilitating bimolecular binding.[23] Based on this context, new sulfonamide derivatives containing a 1, 2, 4-triazole ring connected to benzyl chloride were specifically designed and synthesized to function as carbonic anhydrase inhibitors (CAIs).

Materials and Methods

From (Sigma-Aldrich Germany, Riedel deHaën Germany, Hangzhou Hyper Chemicals, and Merck Germany), all reagents and anhydrous solvents were provided. The Thomas Hover device, using the capillary tube method, was employed for determining the melting points (England). Ascending thin-layer chromatography was utilized to confirm the reaction steps, purification process of the formed compounds, and to determine the retention factor (Rf) values, with a mobile phase consisting of acetone and methanol (1:1).[24] The College of Pharmacy at the University of Kufa employed a Shimadzu spectrophotometer (Japan) for scanning FT-IR and estimating spectra using KBr discs. The Bruker 300 MHz at the University of Mashhad was used to record H1NMR, with DMSO used as the solvent.



General procedure

Sulfanilamide was dissolved in a mixture of benzene and DMF, and TEA was added to the mixture. The entire mixture was stirred in an ice bath. Then, chloroacetyl chloride, dissolved in benzene, was gradually added drop by drop to the sulfanilamide mixture, resulting in the formation of Acetyl sulfanilamide. For the esterification of GABA carboxyl (-COOH), thionyl chloride was used in the presence of cold methanol. The methyl ester of GABA reacted with Acetyl sulfanilamide, forming a secondary amine linker. Additionally, the methyl ester of GABA was reacted with 99% hydrazine hyadrate, leading to the production of hydrazide. The hydrazide was then treated with carbon disulfide in the presence of potassium hydroxide to generate the potassium dithiocarbazate derivative. Subsequently, the derivative was cyclized with 99% hydrazine hydrate to produce the heterocyclic ring (1, 2, 4-triazole-5-thiol) derivative of sulfanilamide. This process was repeated multiple times, targeting the primary amine group of the synthesized ring.

Synthesis of 2-chloro-N-(4-sulfamoylphenyl) acetamide (compound A); this compound synthesized by reaction of sulfanilamide with chloroacetylchlorid according to Mina et. Al.[25]

To a solution of DMF:Benzen (1:3) (40 ml), 2g of sulfanilamide (11.6 mmol) and 1.6ml of TEA (11.6 mmol) were added. Chloroacetylchloride (0.92 ml, 11.6 mmol in 10 ml benzene) was added drop wise over the period of an hour while the reaction mixture was being stirred on an ice bath, and then the mixture was refluxed for three hours. Then, excess cold water was added, the precipitated compound was filtered, and recrystallization from ethanol to give compound (A). Table1 showed Rf values and percent yield are included in the physical data.

Synthesis of methyl-4-aminobutyrate hydrochloride Compound (B) synthesized according to Li, J. and Y. Sha.[26]

GABA (1 g, 9.7 mmol) was dissolved in 10 mL of methanol, chilled to 0°C. Thionyl chloride (0.7 mL, 9.7 mmol) was gradually added to the solution, which was then allowed to stand at room temperature for 45 minutes before refluxing for 12 hours. After the completion of the reaction, the solvent was evaporated to dryness. The resulting residue was collected and washed multiple times with diethyl ether. Further refinement was achieved through recrystallization using methanol: diethyl ether, resulting in the formation of compound A. Table 1 provides the Rf values and percent yield, which are included in the physical data. Table1 showed Rf values and percent yield are included in the physical data.

Synthesis of methyl 4-((2-oxo-2-((4-sulfamoylphenyl) amino)ethyl)amino)butanoate compound (1) according to the method described by Park, H.-S., et al.[27]

Compound B (0.6 g, 4 mmol) was placed in a round flask before being dissolved in a mixture of ethanol 99%: DMF (1:1). (30 ml). Then TEA (0.75 mL) was adding gradually to the mixture of the reaction, then compound A (1 gm, 4 mmol)

Table 1. **Presents the percent yield, physical data, and Rf values**

Benzyl chloride	Aromatic benzyl chloride names	P. number	R- group
1	4-methyl benzyl chloride	Sa	CH_3
2	4-bromo benzyl chloride	Sb	Br
3	benzyl chloride	Sc	H
4	4-chloro benzyl chloride	Sd	Cl

was added. Followed by 10 hours reflux. The solvent was evaporated, and the resulting residue was collected and washed with diethyl ether, yielding a sticky oil compound (1). Table1 showed Rf values and percent yield are included in the physical data.

Synthesis of 2-((4-hydrazinyl-4-oxobutyl)amino)-N-(4-sulfamoylphenyl)acetamide compound (2) according to Jubie et. Al.[28]

Compound (1) (1.5 g 4.5mmol) was dissolved in absolute ethanol and transferred into a round-bottom flask (RBF). Hydrazine hydrate solution 99%, (0.63 ml) was added to the flask, and the reaction mixture was refluxed for 10 hours. After the completion of the reaction, the solvent was reduced to half, cooled, and a white solid product was obtained. The product was filtered, dried, and subjected to recrystallization from ethanol to yield compound (2). Table1 showed Rf values and percent yield are included in the physical data.

Synthesis potassium 2-(4-((2-oxo-2-((4-sulfamoylphenyl) amino)ethyl)amino)butanoyl)hydrazinecarbodithioate compound (3) was synthesized according to Husain et al.[29]

Compound (2) (1.35 g, 4.1 mmol) was added with stirring to a cold solution of potassium hydroxide (0.35 g, 6.1 mmol) in absolute ethanol (100 ml).then the addition of carbon disulfide (0.47 ml, 7.8 mmol) was added. The reaction mixture was continuously stirred at room temperature for 36 hours. The resulting precipitate, which was the potassium dithiocarbazinate derivative, was obtained by filtration, washed with anhydrous ether, and then dried without further purification, the resulting potassium salt compound (3) was utilized in the next step. Physical data are shown in Table 1 for Rf values and percent yield.

Synthesis 2-((3-(4-amino-5-mercapto-4H-1, 2, 4-triazol-3-yl) propyl) amino)-N-(4-sulfamoylphenyl)acetamide Compound (4) was synthesized according to Jubie et al.[28]

A mixture containing 1g (2.2mmol) of potassium dithiocarbazinate compound (3), 0.22ml (4.4mmol) of 99% hydrazine hydrate, and 10ml of distilled water was subjected to reflux for a duration of 7 hours. During the reaction, the color of the mixture turned green, and the release of hydrogen sulfide gas evolved, resulting in a homogeneous solution. Upon dilution with 25ml of cold water and acidification with a few drops of 35% HCl, a white solid precipitated. The precipitate was filtered, washed twice with 5ml portions of cold water, and subsequently crystallized from ethanol, resulting in the formation of a white powder identified as compound (4). The physical data, Rf values, and percent yield can be found in Table 1.

Synthesized into different final products, according to Park, H.-S., et al. (Sa-Sd).[27]

Compound (4) (1g, 2.6 mmol) was dissolved in a mixture of 99% ethanol and DMF (1:1). To this solution, 0.33 ml of triethylamine was added. In a separate solution, the same mixture was used to dissolve 0.34 ml of Sa, 0.53 g of Sb, 0.29 ml of Sc, and 0.42 g of Sd. This second solution was added to the first one, and the mixture was refluxed for different durations: 12 hours for Sa, 24 hours for Sb, 36 hours for Sc, and 24 hours for Sd.

After refluxing, the solvent was evaporated, leaving behind a residue. This residue was dissolved in ethyl acetate and washed with distilled water. The solution was then filtered over magnesium sulfate to remove impurities. Finally, the filtrate was evaporated to obtain the desired compound.

The target molecules' characterization and physical properties, as well as their intermediates

1. Sulfanilamide ($C_6H_8N_2O_2S$) M.WT = 172.20, off white crystals, Melting point in °C = 165. Rf value= 0.94.
2. GABA ($C_4H_9NO_2$) = M.WT = 103.12, white crystals, melting point in °C = 203 and Rf value = 0.35.
3. Compound A ($C_8H_9ClN2O_3S$) M.WT = is 248.69, %yield 62.979, grey powder, melting point in °C = 197-200 and Rf value is 0.75.
4. Compound B ($C_5H_{12}ClNO_2$) M.WT = is 153.06, %yield 98, white crystal, melting point in °C = 117-120 and Rf value is 0.48.
5. Compound 1 ($C_{13}H_{19}N_3O_5S2$) M.WT = 329.37, Brown, yield = %97 and RF value = 0.66.
6. Compound 2 ($C_{12}H_{19}N_5O_4S$) M.WT = 329.38, yellowish white powder, yield = %82, melting point in °C = 50-52 and Rf value = 0.57.
7. Compound 3 ($C_{13}H_{18}KN_5O_4S_3$) M.WT = 443.61, white powder, yield = %79, melting point in °C = 270-272 and Rf value = 0.90.
8. Compound 4 ($C_{13}H_{19}N_7O_3S_2$) M.WT = 385.47, white powder, yield = %58, melting point in °C = 154-157 and Rf value = 0.60.
9. Compound Sa ($C_{21}H_{27}N_7O_3S_2$) M.WT = 489.61, white powder, yield = %50, melting point in °C = 129-131 and Rf value = 0.69.
10. Compound Sb ($C_{21}H_{27}N_7O_3S_2$) M.WT = 554.48, white powder, yield = %75, melting point in °C = 111-113 and Rf value is 0.87.
11. Compound Sc ($C_{21}H_{27}N_7O_3S_2$) M.WT = 475.59, brown, yield = %60, and Rf value = 0.82.
12. Compound Sd ($C_{21}H_{27}N_7O_3S_2$) M.WT = 510.03, whitish brown powder, yield = %72, melting point in °C = 95-97 and Rf value = 0.88.

Spectroscopic analysis[30]

Compound A ($C_8H_9ClN2O_3S$) **FT-IR (cm^{-1})** 3327-3215 stretching of N-H_2 sulfonamide, 3134 N-H of amide, 1689 C = O amide, 1602-1546 C=C of aromatic ring 684 C-Cl. **Compound B** ($C_5H_{12}ClNO_2$) **FT-IR(cm^{-1})** 3018 N-H stretching of primary amine salt , 1735 C=O stretching of ester 1132 C-N stretching. **Compound 1** ($C_{16}H_{14}N_4O_3S_2$) FT-IR (cm^{-1}) 3317-3232 stretching of N-H_2 sulfonamide, 1699 C = O stretching of ester,1660 C = O stretching vibration of amide,1597 1468 aromatic C = C stretching. **Compound 2** ($C_{12}H_{19}N_5O_4S$) **FT-IR (cm^{-1})** 3323-3261 stretching of NH_2 sulfonamide overlap with

NH_2 primary amine, 1668 C = O stretching vibration of amide. **Compound 3** ($C_{13}H_{18}KN_5O_4S_3$) **FT-IR(cm^{-1})** 3441-3342 stretching vibration NH_2 of sulfonamide,1668 C = O amide stretching vibration, 1045 C = S stretching vibration. **Compound 4** ($C_{13}H_{19}N_7O_3S_2$) **FT-IR (cm^{-1})** 3475-3415 stretching vibration of NH_2 sulfonamide overlap with NH_2 of heterocyclic ring ,2578 S-H stretching vibration, 1691 C = O stretching vibration of amide, 1620 C = N stretching vibration, 1047 C-S stretching vibration.

Compound Sa ($C_{21}H_{27}N_7O_3S_2$) **FT-IR (cm^{-1})** 3475-3415 stretching vibration of NH_2 sulfonamide,3361 NH stretching vibration of amine, 3242 NH stretching vibration of amide,1693 C = O stretching vibration, 1595 C = N stretching vibration, 1031 C-S. **^{1}H NMR (ppm):**1.3 Multiplet 2H of CH_2, 2.3 Singlet 3H of CH_3-Ar, 2.4-2.6 Multiplet 4H of CH_2-CH_2, 3.4 Multiplet 1H of secondary amine, 4.1 Doublet 2H of CH_2-C = O, 4.5 Doublet 2H of CH_2-NH, 6.7 singlet 2H of sulfonamide, 7.01 Multiplet 1H of secondary amine, 7.2-8.1 Multiplet, 8H of H-AR, 10.1 Singlet, 1H of amide and 10.5 Singlet 1H of S-H.

Compound Sb ($C_{21}H_{27}N_7O_3S_2$) **FT-IR (cm^{-1})** 3471-3415 stretching vibration of NH_2 sulfonamide, 3232 NH stretching of amine overlap with NH stretching vibration of amide, 1689 C = O stretching vibration,1593 C = N stretching vibration of triazole ring, 829 C-Br stretching vibration. **^{1}H NMR (ppm):**1.2 Multiplet 2H of CH_2 , 2.3-2.5 Multiplet 4H of CH_2-CH_2, 3.4 Multiplet 1H of secondary amine, 4.43 Doublet 2H of CH_2-C = O 4.49 Doublet 2H of CH_2-NH, 6.6 singlet 2H of sulfonamide, 7.01 Multiplet 1H of secondary amine, 7.3-8.1 Multiplet, 8H of H-AR, 10.3 Singlet, 1H of amide and 14.5 Singlet 1H of S-H.

Compound Sc ($C_{21}H_{27}N_7O_3S_2$) **FT-IR (cm^{-1})** 3485-3414 stretching vibration of NH_2 sulfonamide, 3309 NH stretching vibration of amine, 3217 NH stretching vibration of amide, 1680 C = O amide stretching vibration, 1595 C = N stretching vibration, 1049 C-S. **^{1}H NMR (ppm):**1.3 Multiplet 2H of CH_2, 2.3-2.5 Multiplet 4H of CH_2-CH_2, 3.4 Multiplet 1H of secondary amine, 4.35 Doublet 2H of CH_2-C = O, 4.52 Doublet 2H of CH_2-NH, 6.8 singlet 2H of sulfonamide, 7.1 Multiplet 1H of secondary amine, 7.2-7.9 Multiplet, 9H of H-AR, 10.5 Singlet, 1H of amide and 12.44 Singlet 1H of S-H.

Compound Sd ($C_{21}H_{27}N_7O_3S_2$) **FT-IR (cm^{-1})** 3452-3415 stretching vibration of NH_2 sulfonamide, 3236 NH stretching of amine overlap with NH stretching vibration of amide, 1687 C=O amide stretching vibration, 1593 C = N stretching vibration, 748 C-Cl. **^{1}H NMR (ppm):**1.9 Multiplet 2H of CH_2, 2.2-2.5 Multiplet 4H of CH_2-CH_2, 3.4 Multiplet 1H of secondary amine, 4.3 Doublet 2H of CH_2-C = O, 4.52 Doublet 2H of CH_2-NH, 6.7 singlet 2H of sulfonamide, 7.1 Multiplet 1H of secondary amine, 7.3-8.7 Multiplet, 8H of H-AR, 10.2 Singlet, 1H of amide and 10.3 Singlet 1H of S-H.

Docking Study

The research involved the use of the MOE (Molecular Operating Environment) software version 2015.10 to perform a molecular docking analysis, which included preparing both the protein and ligand structures. The ligand structures were accurately drawn using ChemDraw Professional 12.0. Subsequently, in the Molecular Operating Environment (MOE), the ligands were protonated in their three-dimensional form, had partial charges added, and underwent energy minimization before saving the results. For the receptor, the crystal structure of genetically engineered carbonic anhydrase IX (29.25 kDa) (PDB code: 4YWP) was downloaded from the PDB website and imported into MOE. The target protein was prepared by selecting only the chain sequences involved in the protein's action, while deleting the remaining chains and small molecules. Water molecules were also removed. Hydrogen bonds were added and the protein's atom potentials were adjusted, followed by identification of the active site. Finally, the previously prepared ligands were loaded into MOE from the saved data, and the docking process was conducted.

Cytotoxic Study

The MCF7 cell line, which represents a specific type of human breast cancer cells, and the MCF10A cells, known as non-malignant breast epithelial cells, were acquired from the National Cell Bank of Iran at the Pasteur Institute. These cells were cultivated using two different types of media: RPMI-1640 and DMEM, both supplemented with 10% FBS (fetal bovine serum) and antibiotics (penicillin at 100 U/mL and streptomycin at 100 µg/mL). The cells were maintained in a controlled environment with a temperature of 37 °C, 5% CO_2, and appropriate humidity. For cell passage, trypsin/EDTA and phosphate-buffered saline (PBS) solution were used. The same culture conditions and media were utilized for growing 3D colonies as in standard monolayer cell culture.

To assess cell growth and viability, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) assay was employed. For monolayer cell culture, cells were detached using trypsin, counted, and seeded into 96-well plates at a density of 1.4 × 104 cells/well in 200 µl of fresh medium. Once a monolayer was formed, the cells were exposed to various concentrations of compounds (ranging from 100 µg/mL to 6.25 µg/mL) for 24 hours at 37 °C with 5% CO_2. After treatment, the supernatant was removed, and the cells were incubated with an MTT solution (0.5 mg/mL in PBS) for an additional 4 hours at 37 °C. Subsequently, the supernatant was replaced with dimethyl sulfoxide (100 µL per well), and the cells were incubated on a shaker at 37 °C until the crystals were completely dissolved. The absorbance at 570 nm was measured using an ELISA reader to determine cell viability. The IC50, representing the concentration of compounds resulting in 50% cell death, was calculated based on the corresponding dose-response curves

Result and Discussion

The nucleophilic attack of electron pairs on the nitrogen atom in the primary amine of sulfanilamide towards chloroacetyl chloride and the synthesis of the 2-chloroacetamide derivative are evident from the presence of a distinct sharp band at 1689 cm^{-1}. Esterification of GABA was achieved by utilizing methanol, with the addition of thionyl chloride to provide the methyl group ester of GABA. A 1735 cm^{-1} C = O band confirms the conversion of GABA to Methyl 4-aminobutanoate. The formed ester was then treated with hydrated hydrazine 99% to yield the hydrazide. The carbonyl of the ester disappeared, while the amide band stretched at 1668 cm^{-1} remained, with the primary amine group overlapping with NH_2 sulfonamide in the range of 3323-3261 cm^{-1}. The potassium salt of compound (3) was synthesized through a nucleophilic

addition reaction, where the amine from compound (2) reacted with carbon disulfide through a nucleophilic mechanism. This reaction was confirmed by the disappearance of the primary amine in the IR reading.

Afterward, cyclization of the potassium salt intermediate was achieved by utilizing hydrated hydrazine to produce the triazole ring intermediate (4), which was confirmed by the reappearance of the primary amine band on the heterocyclic ring at 3475-3415 cm^{-1}, indicating the stretching vibration of NH2, overlapping with sulfonamide NH2, and characteristic bands at 2578 cm^{-1} due to S-H stretching vibration. The unrestricted amine in the formed five-membered ring reacted with four benzyl chloride derivatives to produce several final derivatives of sulfanilamide. All of the final products were supported by FT-IR and HNMR readings, with values for C-Br at 829 cm^{-1}, C-Cl at 748 cm^{-1}, and C = N at 1595 cm^{-1}. The sulfonamide group was observed as a singlet at 6.6 ppm, the peak of secondary amine nucleophilic attack appeared as a multiplet at 7.01 ppm, and the hydrogens of the two aromatic rings showed as a multiplet from 7.3 ppm to 8.1 ppm. The 1H of the amide appeared as a singlet at 10.3 ppm, and the singlet H at 14.5 ppm corresponded to S-H. This reaction involves nucleophilic substitution. Finally, the changes observed in the FT-IR spectrum, the alteration in melting point, and the results obtained from the FT-IR and ^{1}H NMR spectra strongly support the successful synthesis of these compounds. The physical properties of the formed agents (A-Sd), such as melting points and Rf data, are arranged in Table 1.

Cytotoxic Evaluation

As previously mentioned in the introduction, there is an urgent need for the development of novel anticancer drugs that exhibit reduced side effects and increased selectivity towards cancer cells. In the human body, tumor cells are surrounded by normal cells.[2] As a result, compounds intended for future clinical use should exhibit a greater degree of cytotoxicity against tumor cells compared to normal cells. Hence, the development of these compounds relies on a strategy that ensures they have a lower impact on normal cells compared to cancerous cells. The cytotoxicity effect of compounds (Sa, Sb, Se, and Sd) was assessed using cell line MTT assays in a cell culture setting. Two types of cells, MCF7 (representing cancer cells) and MCF10 (representing normal cells), were utilized to examine the impact of the compounds. The IC50 values, which indicate the concentration at which a 50% decrease in cell viability is observed, were calculated for each one.

All the compounds mentioned in this study were assessed for their inhibitory activity compared to acetazolamide and their cytotoxic activity compared to cisplatin, as shown in Table 2. The results clearly demonstrate that all synthesized compounds exhibited inhibition activity, albeit with varying degrees of effectiveness. The IC50 values of the compounds against MCF7 and MCF10a cell lines ranged from 28.41 µM to 128.07 µM and 142 µM to 279 µM, respec4 tively. Notably, Compounds Sb exhibited a significant difference in cytotoxic activity against tumor cells (IC50 = 28.41 µM) compared to acetazolamide, while also demonstrating a significant difference in the inhibition of normal cell viability (IC50 = 279.02 µM).

Compound Sd showed a non-significant difference from Acetazolamide but a significant difference from cisplatin, against MCF7 (IC50 = 61.20 µM), and significant difference against MCF10a (IC50 = 194.00 µM) cells when compared to acetazolamide and cisplatin.

On the other hand, the lowest inhibition activity against MCF7 cells was observed for compounds Sa (IC50 = 128.07 µM) and Sc (IC50 = 114.18 µM).

In terms of cisplatin, all the synthesized compounds showed significant difference cytotoxicity, indicating that they are less potent than cisplatin against cancer cells. However, they have significant difference toxicity compered to cisplatin for normal cell, suggest that these compounds are less harmful to these cells.

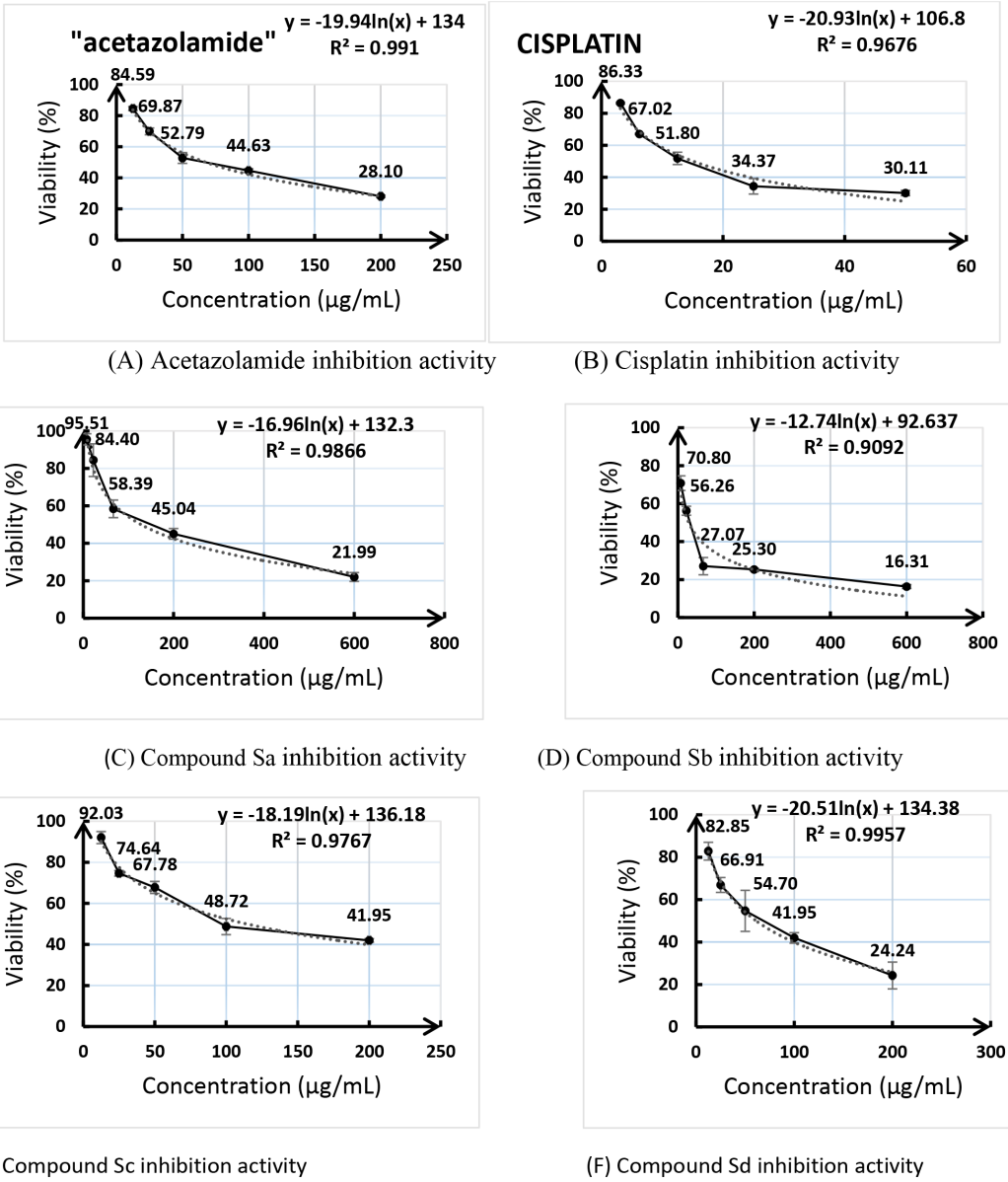
Overall, these findings highlight the potential of the synthesized compounds as selective inhibitors of tumor cells with reduced toxicity to normal cells.

Structure-activity relationship studies revealed that the presence of 2-((3-(4-(benzylamino)-5-mercapto-4H-1, 2,4-triazol-3-yl)propyl)amino)acetamide (with different substitution of a halogen atom Br or Cl) as a substituent at the para-position of benzenesulfonamide is highly beneficial for inhibitory activity for carbonic anhydrase against MCF7 cells compered to acetazolamide while exhibiting less activity

Table 2. **IC50 values for the tested compounds. The values are represented by the mean ± SD of triplicate measurements**

Compound	MCF7		MCF10a	
	IC50 (µM) ± SD	*P*-value	IC50 (µM) ± SD	*P*-value
Acetazolamide	67.53 ± 1.80	Standard	53.91 ± 5.636	Standard
Cisplatin	15.09 ± 2.25	Standard	13.55 ± 3.45	Standard
Sa	128.07 ± 4.31	0.0001 a	214.97 ± 5.49	0.0001 a
		0.0001 b		0.0001 b
Sb	28.41 ± 2.47	0.0001 a	279.02 ± 1.62	0.0001 a
		0.038 b		0.0001 b
Sc	114.18 ± 2.41	0.0278 a	142.96 ± 2.36	0.0001 a
		0.0001 b		0.0001 b
Sd	61.20 ± 5.23	0.072 ns	194.00 ± 4.69	0.0001 a
		0.0001 b		0.0001 b

a , b latters: Significant difference that the comparison with Acetazolamide and Cisplatin respectively . ns: non-significant.



(A) Acetazolamide inhibition activity (B) Cisplatin inhibition activity

(C) Compound Sa inhibition activity (D) Compound Sb inhibition activity

(E) Compound Sc inhibition activity (F) Compound Sd inhibition activity

Fig. 1 **illustrates a cytotoxicity analysis that investigates the effects of standards and the final compound on MCF7 cells.**

against MCF10a cells compered to cisplatin. Moreover, replacing the para substituent halogen atom of benzyl chloride with H or CH3 led to compounds with decreased activity.

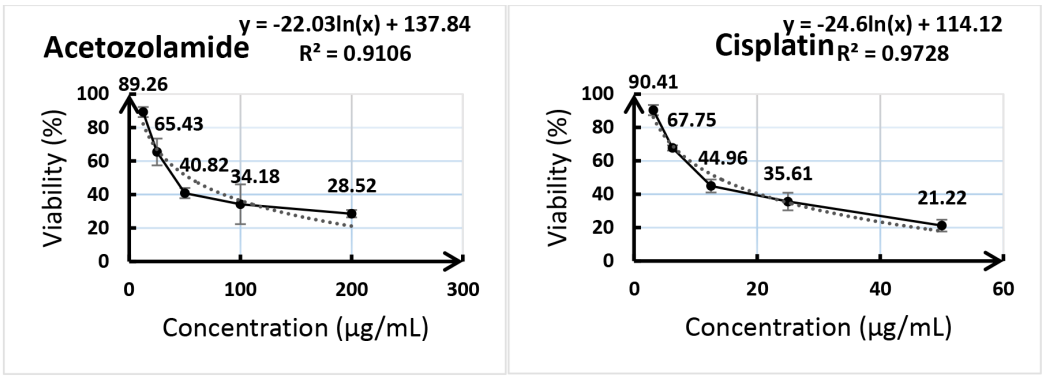
Figure 1 (A) demonstrates the inhibition activity of acetazolamide, while Figure 1 (B) represents the inhibition activity of cisplatin. Notably, Figures 1(C), 1(D), 1(E), and 1(F) correspond to the inhibition activities of compounds Sa, Sb, Sc, and Sd respectively.

Figure 2 illustrates the correlation between concentration and effect by graphing the viability data of MCF10a cells against the corresponding concentrations of various compounds. The curves depicted in the figure represent the suppressive effects of different compounds, namely acetazolamide (Figure 2A), cisplatin (Figure 2B), compound Sa (Figure 2C), compound Sb (Figure 2D), compound Sc (Figure 2E), and compound Sd (Figure 2F). Each curve represents the response of MCF10a cells to increasing concentrations of the respective compound, highlighting the inhibitory effects on cell viability.

Figure 2 illustrates a cytotoxicity analysis that investigates the effects of standards and the final compound on MCF10a cells.

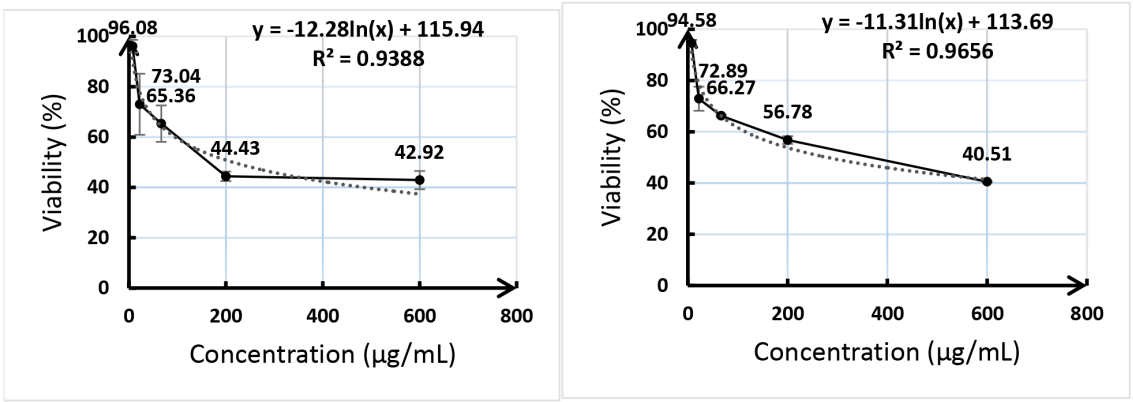
Docking Study

The molecular operating environment analysis revealed that the designed compounds exhibited binding selectivity towards the carbonic anhydrase IX enzyme, targeting the same main active site as acetazolamide. To evaluate the inhibitory effects of the synthesized compounds and assess the similarity between the interacting amino acids within the active site, S. Score and rmsd (root mean square deviation) values were employed. The efficacy of the compounds was evaluated based on this data. Acetazolamide interacts with its interaction site, which consists of Zn301, Thr199, and Thr200. Table 3 presents the S. score, rmsd, and the primary amino acids involved in the interaction for the final products of the reaction. Comparatively, all the final



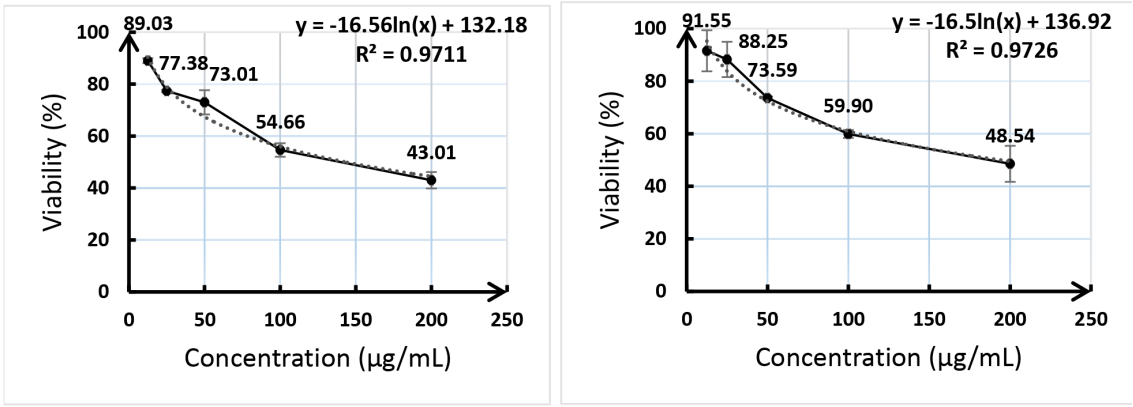
(A) Acetazolamide inhibition activity

(B) Cisplatin inhibition activity



(C) Compound Sa inhibition activity

(D) Compound Sb inhibition activity



(E) Compound Sc inhibition activity

(F) Compound Sd inhibition activity

Fig. 2 **illustrates a cytotoxicity analysis that investigates the effects of standards and the final compound on MCF10a cells.**

Table 3. **S score and rmsd values for the tested compounds**

Compound	R group	S. score	rmsd	No. of binding site	Binding amino acids
acetazolamide	-------	-5.06	1.7	1	Thr199, Thr200, Zn301.
Topiramate	-------	-6.69	1.9	3	Gln92,Thr200,Zn301.
Sa	CH_3	-7.64	1.6	3	Gln A92, Thr199, Zn301.
Sb	Br	-8.37	1.8	3	Thr199,Thr69, Zn301.
Sc	H	-8.09	2.2	3	Thr199, Gln., Zn301.
Sd	Cl	-8.32	1.6	7	His, Gln67, Gln92, Gly, Thr199, Th200, Zn301.

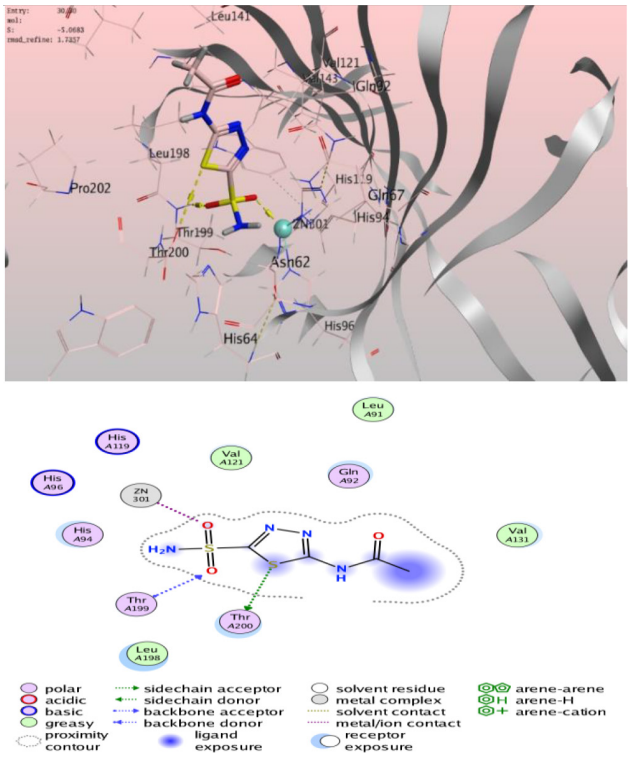


Fig. 3 **Acetazolamide compound with carbonic anhydrase IX (PDB code: 4ywp).**

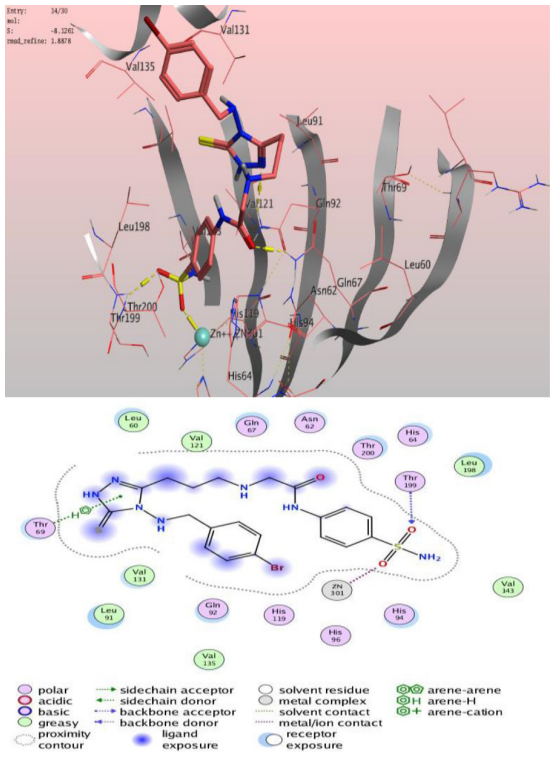


Fig. 5 **Sb final compound with carbonic anhydrase IX (PDB code: 4ywp).**

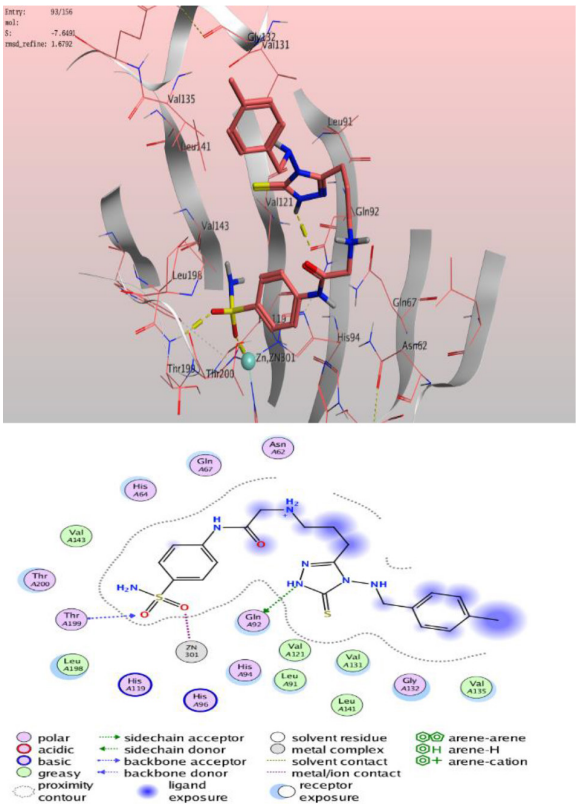


Fig. 4 **Sa final compound with carbonic anhydrase IX (PDB code: 4ywp).**

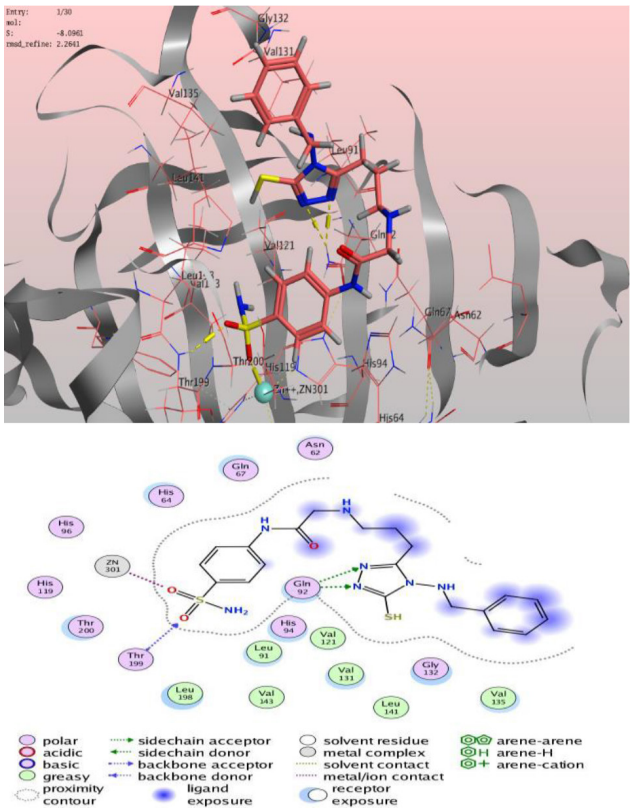


Fig. 6 **Sc final compound with carbonic anhydrase IX (PDB code: 4ywp).**

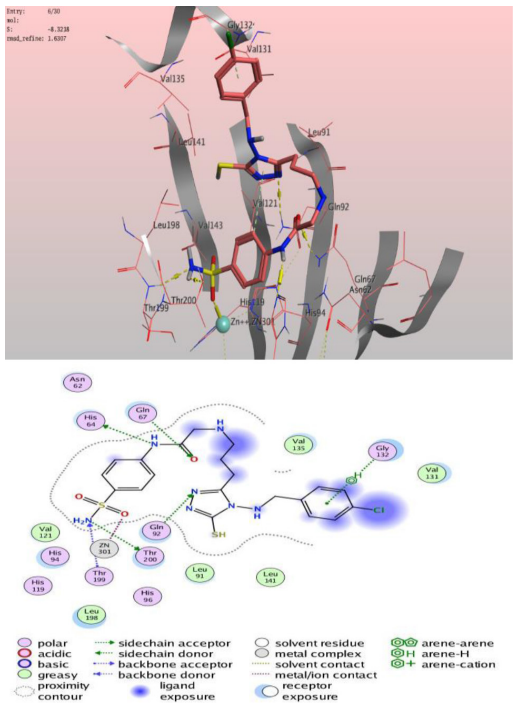


Fig. 7. **Sd final compound with carbonic anhydrase IX (PDB code: 4ywp)**

products (Sa, Sb, and Sd) exhibited stronger binding affinity with the target proteins compared to acetazolamide. They had S. score values of -7.6491, -8.3789, and -8.3218, respectively, and rmsd values of 1.6792, 1.8982, and 1.6307, respectively.

Conclusion

A new series of benzenesulfonamide compounds were synthesized and evaluated for their cytotoxicity and carbonic anhydrase inhibitory potencies. Through docking studies and cytotoxicity results, compounds Sb exhibited significant difference inhibitory effects compared to the standard compound (acetazolamide). The cytotoxicity of the compounds against tumor cell lines ranged from 28.41 μM to 128.07 μM. Sb and Sd demonstrated the lowest IC50 values in the cytotoxicity assays, making them the most promising candidates as antineoplastic agents. The introduction of bromine (Br) or chlorine (Cl) substituents in the para position of benzyl chloride affected the bioactivity. The docking study suggested that the cytotoxic activity may be attributed to selectivity towards carbonic anhydrase IX (CA IX). The compounds exhibiting notable bioactivities against the target have the potential to serve as lead compounds for future investigations.

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