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Design, in silico study, synthesis and cytotoxic evaluation of new isatin-thiazole-sulfanilamide derivatives as potential carbonic anhydrase inhibitors

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ABSTRACT: One of the promising strategies in treatment of cancer is inhibiting the overexpression of carbonic anhydrase enzyme. This target represents a viable target for cancer therapy. In order to do this, four new compounds containing sulfonamide were prepared and manufactured utilizing different isatin derivatives. To choose the most appropriate compounds with the greatest S score prior to their production, docking research was carried out using MOE software (Molecular Operating Environment). Furthermore, the synthesis process involved the preparation of Schiff base derivatives by condensation of ketone (isatin and its derivatives) with 4-amino ethyl benzoate using Glacial acetic acid, then these derivatives combined with 4-aminobenzene sulfonamide to synthesize the final product (sulfonamide-containing isatin derivatives, compound A1-A3).

In order to evaluate how well the synthesized compounds could fight cancer, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used. The results of the assay revealed that the compound A2 demonstrated the greatest cytotoxic action against cancer cells within the group IC50=9.95 µM in MCF-7 and 135.18 µM in MCF10A. Whereas, compound A1 displayed the least amount of cytotoxicity IC50=16.30 µM in MCF-7 and 291.98 µM in MCF10A. In comparison with acetazolamide IC50=19.71 µM in MCF-7 and 67.53 µM in MCF10A, these results demonstrate that these chemicals' potential as subjects for additional research in the realm of cancer therapies.

As mentioned before, this study aimed to design and synthesis of new compounds A1-A3, which have isatin-thiazole-sulfanilamide derivatives and then evaluate the cytotoxic activity of these compunds. The synthesized compounds demonstrated significant inhibition of carbonic anhydrase IX activity through molecular docking and significant inhibition of cancer cell viability. Compounds A1-A3 have (IC50 16.30µM, 9.95 µM, 15.44 µM) respectively for MCF7 which is better than IC50 for acetazolamide which is 19.71 µM. Additionally they show higher selectivity toward cancer cells than normal cells. Additionally, compounds A1-A3 have S scores in docking studies higher than acetazolamide so they possess a higher affinity for binding to the receptor's active pocket. Flexibility and receptor interaction are enhanced by the substituted thiazole ring and isatin. In conclusion, the synthesized derivatives have a valuable cytotoxic activity against cancer cell, accordingly this enhance the ability to inhibit cancer growth.

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INTRODUCTION

Cancer is a major global health concern that causes a large number of fatalities and impedes attempts to increase life hope globally. Cancer is one of the top two causes of mortality for those under 70 in 112 out of 183 nations, according to data from the World Health Organization (WHO). Moreover, in twenty-three additional countries, it ranks as the third or fourth most prevalent cause of death [1]. In terms of breast cancer, certain cases of the disease show long-term resistance to treatment, despite a range of available therapeutic alternatives. Such resistance may result in treatment failure and the progression of the illness [2]. In order to overcome this barrier, further research and creative solutions will likely be needed. Anhydrases are a family of enzymes that are one of the new targets for tumor cells. (CAs). Chemotherapy resistance is intended to be decreased by treating the tumor microenvironment. This protein is encoded by eight gene families, ranging from α-to ι. CAs are widely distributed enzymes that are part of the metalloenzymes group and found in different species [3,4]. Carbon dioxide's reversible conversion to a bicarbonate ion and a proton, which is an essential reaction for many bodily processes is aided by these enzymes [5]. Interestingly, there are 15 different isoform of α-carbonic anhydrase (hCAs) with differing catalytic effectiveness, tissue and subcellular distribution, and physiological roles in humans. Catalytic activity is present in twelve isoforms (hCA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV) [6]. Carbonic anhydrases (CAs), specifically, hypoxia-inducible factor-1 (HIF-1) enhances isoforms IX and XII, which are nearly nonexistent in normal cells, in hypoxic tumor conditions. These isoforms play a crucial role in the acidity of the tumor microenvironment, which affects tumor survival and proliferation. They work in tandem with anaerobic glycolysis [7]. As a result, Research on inhibiting this enzyme has proven to be beneficial for chemotherapy [8]. These enzymes offer particular therapeutic targets that may decrease adverse effects due to their cancer cell selectivity [9]. For this enzyme, the zinc-binding group is a classic type of inhibitor. In the enzyme's active site, CAIs collaborate with the required Zn^{+2}[10-11]. Zn^{+2} can take on geometries of trigonal or tetrahedral bipyramids throughout the inhibitory process. Known examples of CAIs that use this form of binding include sulfonamides [12-13]. Understanding the complexities of the carbonic anhydrase active site provides a viable avenue for the development of targeted inhibitors. A major class of inhibitors of carbonic anhydrase that is known for its strong binding affinity for CAs is aryl-sulfonamides. Although producing these compounds is not too difficult, obtaining a high level of selectivity remains a challenging task. But the "tail approach," which involves changing a substituent on the benzene ring containing the sulfonamide group, has shown to be effective in achieving significant selectivity toward the desired target CAs [14]. Analogously, thiazole is a unique ring system made up of five hetero aryl ring members that contain atoms of sulfur and nitrogen. This property gives thiazole a remarkable degree of flexibility in a wide range of chemical reactions and biological effects. The thiazole ring is highly reactive due to the presence of an H-proton at C-2, which makes it an essential synthon for the creation of a large variety of chemical substances [15-16]. Many medications are used, recently utilized experiments on cancer treatments both in vivo and in vitro. Tiazofurin, dabrafenib, and bleomycin are a few medications with thiazole rings that are employed as anticancer agents. In order to treat specific types of breast cancer, the FDA approved alpelisib (Pigray®) in 2019 [16]. Additionally, one fascinating property of metal complexes produced from inhibitors of carbonic anhydrase sulfonamide is their capacity to demonstrate inhibitory efficacy that is ten to one hundred times higher than that of the original sulfonamide substance. It is thought that sulfonamide anions and metal ions work together in a dual action mechanism to provide this increased inhibition. In low concentrations, dissociating the coordination molecules leads to the desired state. Using this method, In the enzyme's active site, sulfonamide anions are created and bound to the Zn (II) ion. Parallel to this, adding metal ions prevents the carbonic anhydrase's proton shuttle residues from functioning [17]. We have used the knowledge from the aforementioned discoveries to develop a composition for our novel chemicals. Furthermore, the efficacy of these compounds has been confirmed by the computational analysis, providing us with confidence and motivation to continue producing their chemicals.

MATERIALS AND METHODS

The experiment's chemical materials and anhydrous solvents came from a number of sources, including Merck, Germany, Reidal Dehean, Germany, Hangzhou Hyper Chemicals, and Sigma-Aldrich, Germany. The melting point was determined using the capillary tube method with the Thomas hover apparatus (England). Ascending thin-layer chromatography was employed to confirm the phases of the reaction, purify the products, and determine the retention factor (Rf) values using methanol and acetone (1:1) as the mobile phase [18]. The University of Kufa's College of Pharmacy employed a spectrophotometer made by Shimadzu Japan to estimate spectra using KBr discs and scan for FT-IR. The Bruker was utilized by the Mashhad University of Medical Sciences to record ^{1}HNMR and ^{13}CNMR data, with DMSO-d6 serving as the solvent.

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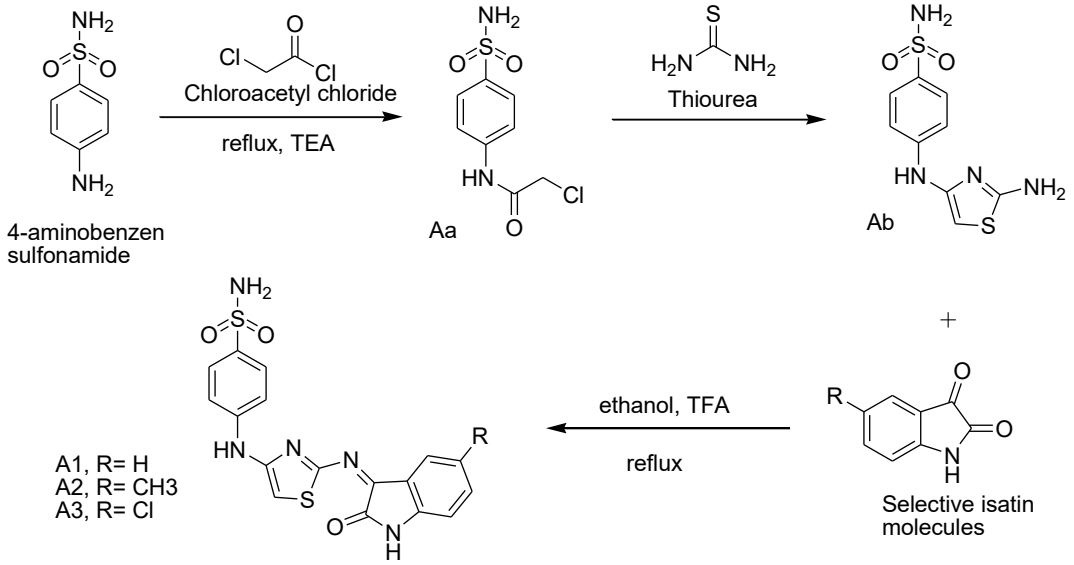
General procedure

As shown in scheme 1, the 4-aminobenzen sulfonamide was dissolve in a benzene: DMF combination, then TEA was added and the mixture was placed in an ice bath. Subsequently, benzene was used to dissolve chloro acetyl chloride, which was then gradually added to the sulfanilamide mixture. Then, the mixture was reacted with thiourea in ethanol as solvent and the result was a thiazole ring with a primary amine in position 2. Schiff base was produced by the final primary amine and isatin derivatives after refluxing in the presence of a few drops of glacial acetic acid.



Scheme 1: Synthetic route of isatin-thiazole-sulfanilamide derivatives A1-A3 and intermediates Aa and Ab.

Synthesis of 2-Chloro-N-(4-sulfamoyl phenyl) acetamide (Aa): Made by reacting 4-aminobenzen sulfonamide with chloroacetylchlorid [19]. In 40 milliliters of DMF: benzene (1:3), 4-aminobenzen sulfonamide (2 grams, 11.6 mmol) was dissolved, and then TEA (1.6 milliliters, 11.6 mmol) was added. Drop by drop, chloroacetyl chloride (0.92 ml, 11.6 mmol in 10 ml benzene) was added while this mixture was continuously stirred over an ice bath. The mixture was refluxed for three hours following the addition operation, which took around an hour. TLC was used to track the reaction's development. After adding cold water at the conclusion of the reaction, the liquid was filtered to separate the precipitate. Diethyl ether was then used to wash the precipitate in the filter paper. The final compound was gray powder, yield 85%, M.p 201-203 ^{0}C.

Synthesis of 4-((2-aminothiazol-4-yl) amino) benzenesulfonamide (Ab) [20]: After dissolving 2 mmol, 0.53 gm of the compound Aa in 50 ml of 99% ethanol, 2 mmol, 0.17 gm of thiourea were added. After eight hours of reflux, the reaction mixture is exposed to solvent evaporation and then recrystallization using ether. The final compound was beige powder, yield 82%, M.p 105-106 ^{0}C.

Synthesis of Schiff base (Z)-4-((2-((2-oxoindolin-3-ylidene)amino)thiazol-4-yl)amino)benzene sulfonamide (A1)[21]: Involves dissolving the intermediate (Ab) (2.05 mmol, 0.554 gm) and (2.05 mmol, 0.300 gm) of isatin in 50 ml of 99% ethanol along with a few drops of glacial acetic acid; the mixture is then refluxed for 24 hours. Following that, the precipitate from the ethanol was recrystallized and the extra solvent was evaporated. ^{1}H NMR (ppm) show the following data: δ 10.41 (s, 1H), 9.93 (s, 1H), 7.85 – 7.77 (m, 1H), 7.72 – 7.60 (m, 7H), 7.38 – 7.27 (m, 2H), 6.99 (s, 2H), 6.54 (s, 1H). ^{13}C NMR (ppm) show the following data: δ 167.39 of C=N of thiazole ring, 167.24 of C=O of isatin, 138.55 singlets of carbon of Schiff base, 136.31, 129.87, 128.69, 125.67of aromatic ring of sulfonamide 118.05 of aromatic ring of isatin, 90.59 of C=C of thiazole ring. The final compound was yellowish brown powder, yield 84%, M.p 245-247 ^{0}C.

Synthesis of (Z)-4-((2-((5-methyl-2-oxoindolin-3-ylidene)amino)thiazol-4-yl)amino)benzene sulfonamide (A2): The intermediate (Ab) and final product (2.05 mmol, 0.554 gm) of 5-methyl isatin were mixed in 50 ml of 99% ethanol together with a few drops of glacial acetic acid. The mixture was then refluxed for a whole day. Following that, the precipitate from the ethanol was recrystallized and the extra solvent was evaporated. ^{1}H NMR (ppm) δ 10.48 (s, 1H of NH of isatin), 9.93 (s, 1H of

NH of sulfonamide), 7.81 (d, 1H of H4 of isatin), 7.73 – 7.61 (m, 5H, H6 of isatin and 4H of Ar sulfonamide), 7.02 (s, 1H of NH_2 of sulfonamide), 2.33 (m, 3H of CH_3). ^{13}C NMR (ppm) δ 167.58 of C=N of thiazole ring, 166.75 of C=O of isatin, 138.71 singlets of carbon of Schiff base, 136.31, 134.26, 130.82, 128.69 of aromatic ring of sulfonamide 118.05 of aromatic ring of isatin, 90.60 of C=C of thiazole ring, 21.18 of CH_3. The final compound was brown powder, yield 85%, M.p 262-264 ^{0}C.

Synthesis of (Z)-4-((2-((5-chloro-2-oxoindolin-3-ylidene)amino)thiazol-4-yl)amino) benzene sulfonamide (A3)[22]: The intermediate(Ab) (2.05mmol, 0.554gm) and (2.05mmol,1.189gm) of 5-Chloro isatin were dissolved in 50ml 99% ethanol, with a couple of drops of glacial acetic acid, subsequently, the mixture is refluxed for 24h. Subsequently, the surplus solvent was eliminated, and the ethanol precipitate was once again crystallized [21]. ^1H NMR (ppm) δ 10.51 (s, 1H of NH of isatin), 9.90 (s, 1H of NH of sulfonamide), 7.89 (d, 1H of H4 of isatin), 7.73 – 7.65 (m, 5H, H6 of isatin and 4H of Ar sulfonamide), 7.08 (s, 1H of NH_2 of sulfonamide), ^{13}C NMR (ppm) δ 167.58 C=N of thiazole ring, 167.06 of C=O of isatin, 138.71 singlets of carbon of Schiff base, 136.26, 131.69, 128.69, 128.54 of aromatic ring of sulfonamide, 118.07 of aromatic ring of isatin, 90.62 of C=C of thiazole ring. The final compound was brown powder, yield 82%, M.p 268-270 ^{0}C.

Spectroscopic analysis [23, 24]

4-aminobenzen sulfonamide show the following characteristic IR spectrum value: The primary aniline amine's 3475 N-H asymmetric stretching vibration, 3373 N-H symmetric stretching vibration that overlaps with the primary sulfonamide's asymmetric vibration, 3267 N-H symmetric stretching vibration of the primary amine, 1629 N-H bending vibration of the aniline group, and 1591 C=C extending the aromatic ring's vibration, 1436 C-N Sulfonamide group stretching vibration, 1309, 1134 SO_2 symmetric and asymmetric stretching vibration, respectively, 896 C-S Extending the vibration.

Compound (Aa) show the following characteristic IR spectrum value: 3327, 3215 Primary Amine N-H Asymmetric Stretching Vibration, Primary Amine N-H Symmetric Stretching Vibration, 1689 C=O Middle band stretching, 1602,1546 C=C stretching band of the aromatic ring, C-N 1406 Stretching vibration of the band, 1319,1157 SO_2 asymmetric and symmetric, respectively, 839 C-S Elastic band, 684 C-Cl elastic band.

Compound (Ab) show the following characteristic IR spectrum value: 3469, 3373 N-H Asymmetric and symmetric primary amine stretching bands, 3331, 3242 N-H, respectively Primary sulfonamide's asymmetric and symmetric stretching bands, measured at 3180 N-H Secondary amine stretching band, 3074 C-H Aromatic stretching band1600 C=N, 1629 C-H bending band Thiazole ring stretching band, 1510 C=C aromatic ring stretching band, 1404 C-N Stretching band, symmetric stretching vibration, 1309, 1151 SO_2, and so forth.

Compound (**A1**) show the following characteristic IR (cm^{-1}) spectrum value: 3469, 3414 N-H symmetric and asymmetric stretching bands of primary amine of sulfonamide, 3244 N-H Secondary amine stretching band, 3421 N–H stretching band of isatin, 1734 C=O stretching of amide of isatin, 1602 C=N Thiazole stretching band overlaying imine stretching band, 1514–1448 C=C aromatic ring stretching band, 1400 C-N Bands for stretching, 1313, 1155 SO_2 Stretching vibrations that are symmetric and asymmetric, respectively.

Compound (**A2**) show the following characteristic IR (cm^{-1}) spectrum value: 3478, 3465 N-H symmetric and asymmetric stretching bands of primary amine of sulfonamide, 3423 N–H stretching band of isatin, 3111 C-H stretching of aromatic ring, 1734 C=O stretching of amide of isatin, 1691 C=N of imine, 1623 C=N Thiazole stretching band overlaying imine stretching band, 1525-1498 C=C of aromatic ring, 1417 C-N Bands for stretching, 1289, 1134 SO_2 Stretching vibrations that are symmetric and asymmetric, respectively.

Compound (**A3**) show the following characteristic IR (cm^{-1}) spectrum value: 3459, 3443 N-H symmetric and asymmetric stretching bands of primary amine of sulfonamide, 3416 N–H stretching band of isatin, 3114 C-H stretching of aromatic ring, 1723 C=O stretching of amide of isatin, 1687 C=N of imine, 1614 C=N Thiazole stretching band overlaying imine stretching band, 1519-1494 C=C of aromatic ring, 1412 C-N Bands for stretching, 1251, 1127 SO_2 Stretching vibrations that are symmetric and asymmetric, respectively.

Docking study

Our molecular docking investigation has involved the creation of protein and ligand structures, and we have been utilizing the Molecular Operating Environment (MOE) software program version 2015.10. In order to produce the MOE ligand, a three-

dimensional structure was protonated, partial charge was added, and energy was minimized. In order to facilitate interaction with ligands, Water molecules and other non-essential sites were eliminated in order to process the 4z0q protein from the carbonic anhydrase IX website (www.rcsb.org). To make it easier to retrieve and deposit the protein from the PDB, the protein molecule's potential was ascertained, damaged bonds were repaired, and the removed protons were added. The carbonic anhydrase active site was located using the MOE program, which made it easier to identify the specific amino acids that were present in the site [25].

Cytotoxic cell line study [26]

The MCF10A (non-malignant breast epithelial cells) and MCF7 (a type of human breast cancer cell line) were purchased from the NCB (National Cell Bank) of Iran, located in the Institute Pasteur. The two types of media used to cultivate these cells were RPMI-1640 and DMEM. 10% fetal bovine serum (FBS) and antibiotics were added to each medium. 100 µg/mL streptomycin and 100 U/mL penicillin. PBS (phosphate-buffered saline) solution and trypsin/EDTA were used to pass the cells, which were kept in a humidified atmosphere with 5% CO_2 at 37 °C. 3D colonies were grown using the same medium and culturing conditions as the monolayer cell culture. Cell growth and viability were evaluated using the MTT "3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide" test. For monolayer cell culture, 200 µl of fresh media was used to seed cells at a density of 1.4 × 104 cells/well onto 96-well plates after they had been trypsin digested and counted. The chemicals were added to the cells at several doses (from 100 µg/mL to 6.25 µg/mL) and left them for 24 hours at 37 °C with 5% CO_2. This was done after the cells had formed a monolayer. The MTT solution (0.5 mg/mL in PBS) was added to the supernatant after the treatment was completed. After the supernatant was removed and dimethyl sulfoxide (DMSO) was added, the mixture was incubated for 4 hours at 37 °C. (one hundred microliters per well), Additionally, until the crystals entirely dissolved, the cells were maintained at 37°C on a shaker. Using an ELISA reader, the absorbance at 570 nm was measured in order to determine the cell viability. According to the related dose-response curves, the compounds' IC_{50}, or concentration, was determined to cause 50% cell death.

RESULT AND DISCUSSION

Cytotoxicity evaluation

Cell culture-based antitumor activity of compounds (A1, A2 and A3) were evaluated by performing cell line MTT assays. We have employed MCF7 cells to evaluate the cytotoxic effect of compound on cancer cell. While MCF10A cells to study the effects of the same compounds on normal cells. Using IC_{50} estimates, which show the concentration needed to cause a 50% drop in cell viability. Acetazolamide (AZ) and a newly synthesized sulfonamide-isatin carbonic anhydrase inhibitor derivatives were all tested for cytotoxic potential. The selection of these medications was based on the fact that AZ is the model for inhibitors of CA and has a long history of research on its possible application in antineoplastic therapy. Several studies have been carried out to investigate its efficacy and activity in preventing malignant development. Moreover, clinical trials are now being conducted on carbonic anhydrase inhibitors that particularly target antineoplastic efficacy. The ability of this inhibitor to specifically block carbonic anhydrase enzymes implicated in the development and spread of cancer has been demonstrated. Compounds A1 and A3 exhibit notable deviations from AZ in relation to MCF7, in addition to noteworthy variations in the MCF10A cell line. IC_{50} of prepared compound and standers tabulated in table 1

Table 1: Demonstrate the synthesized compounds' and standers' inhibitory activity.

Compound	MCF7 $IC_{50}(\mu M)$	MCF10A $IC_{50}(\mu M)$
Acetazolamide	19.71	67.53
A1	16.30	63.91
A2	9.95	135.18
A3	15.44	291.98

Docking study

Multiple binding mechanisms for the ligands within the 4z0q protein's receptor region were discovered by the docking simulations. By forming a coordination connection with the Zn (II) ion, the negatively charged nitrogen atom in the sulfonamide

group improves binding inside the catalytic site, as demonstrated by the compounds' deprotonated structural structures. An elaborate network of hydrogen bonds interacting with the Thr199 residue strengthens this binding contact even further.

To assess the drug's efficacy, we utilize the S. score and RMSD (root mean square deviation) values. An elevated binding affinity with the receptor is shown by a lower S score, which measures how well the ligand and target protein attach to each other. The posing ligand's average distance from the tested ligand's atoms is displayed by the RMSD values for the anti-cancer site. The produced molecule exhibited a variety of binding affinities; some of them revealed stronger interactions than acetazolamide did with the target protein.

Out of all the compounds under study, compound A3 has the lowest S score, suggesting a potential improved ligand-protein complex and a stronger binding connection. Conversely, compound A1 shows a reduced RMSD, showing a closer match between the actual and anticipated ligand positions. Acetazolamide, in contrast, has the greatest RMSD values and S score. Furthermore, it is impossible to dispute the role that the thiazole ring plays in improving the orientation of the imine group and the compound's interaction with the receptor's active site. The docking study results are shown in the table below.

In conclusion, the docking study shows that the synthesized compounds A1 - A3 in particular pose positive binding contacts and perhaps bind with affinity to the active region of the target protein. It is thought that the sulfonamide group plays a crucial role in encouraging these interactions.

Table 2: Result of docking study of prepared compounds and standers

Compound	Docking S- scores in ΔG (Kcal/mol)	RMSD	Number of binding sites	Molecules that involve in binding
acetazolamide	-5.572	2.0942	5	Zn1001, Thr A199, His A 96, His A 94, Asn A 67
A1	-6.751	1.598	6	Zn1001, Thr A199, His A 94, His A 96, Asn A67, Glu A 69
A2	-6.557	1.901	6	Zn1001, Thr A199, Leu A 198, His A 96, His A 94
A3	-6.894	1.385	8	Zn1001, Thr A199, Leu A 198, His A 96, His A 94, His A 64, Asn A 62

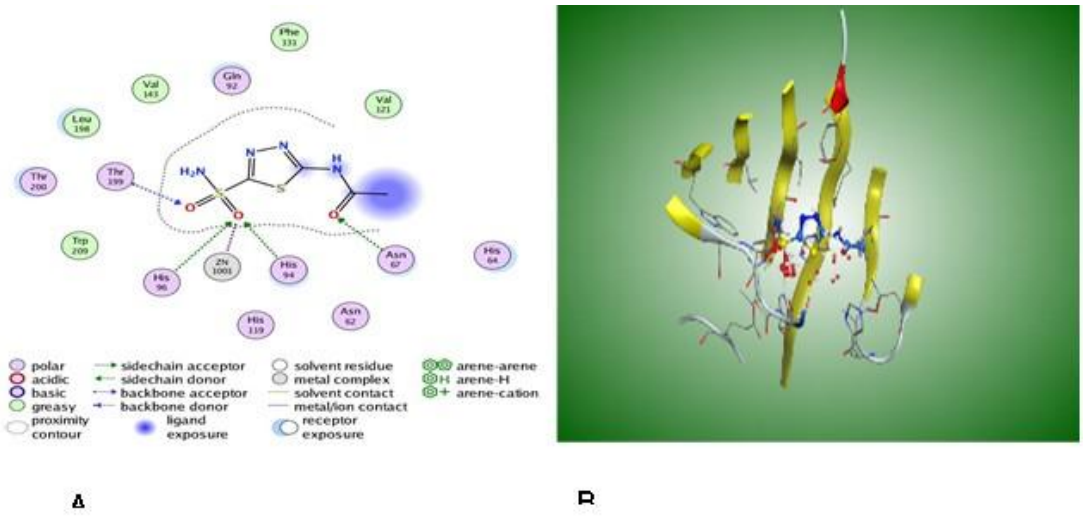


Figure (1) Acetazolamide with Carbonic anhydrase IX (PDB code: 4z0q). Where (A) elucidates the two-dimensional image of the active site binding of acetazolamide, (B) elucidates the three-dimensional image of the active site binding of acetazolamide.

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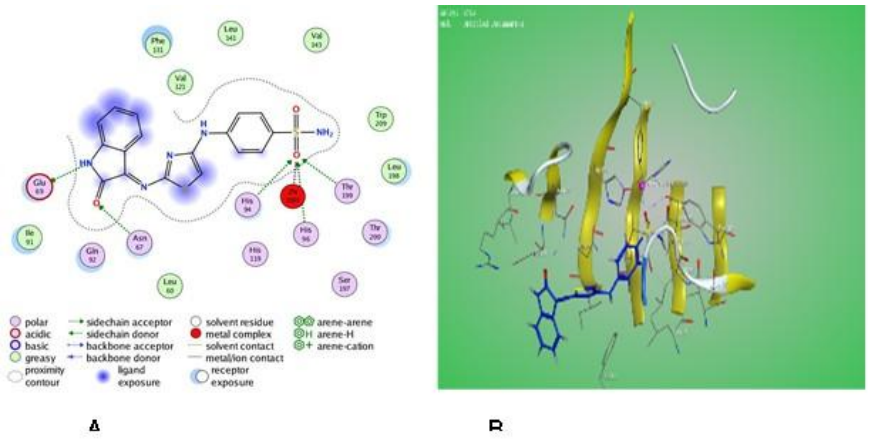

Figure (2) Compound **A1** with Carbonic anhydrase IX (PDB code: 4z0q). Where (A) elucidates the two-dimensional image of the active site binding of compound **A1**, (B) elucidates the three-dimensional image of the active site binding of compound **A1**.

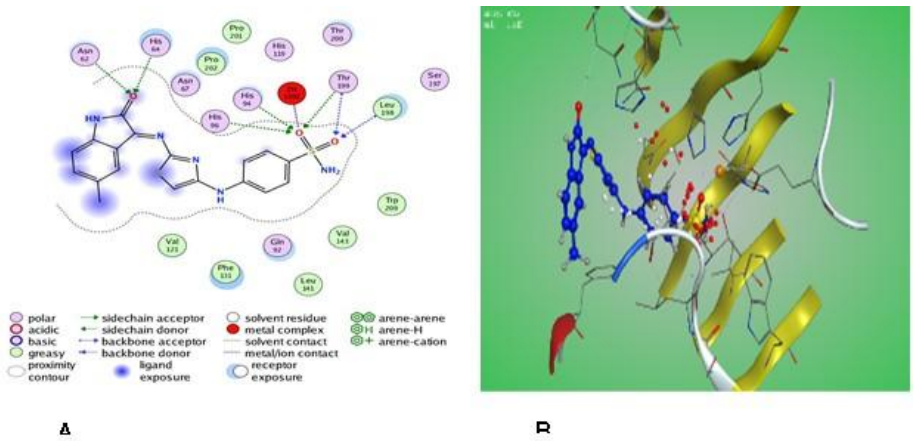

Figure (3) Compound **A2** with Carbonic anhydrase IX (PDB code: 4z0q). Where (A) elucidates the two-dimensional image of the active site binding of compound **A2**, (B) elucidates the three-dimensional image of the active site binding of compound **A2.**

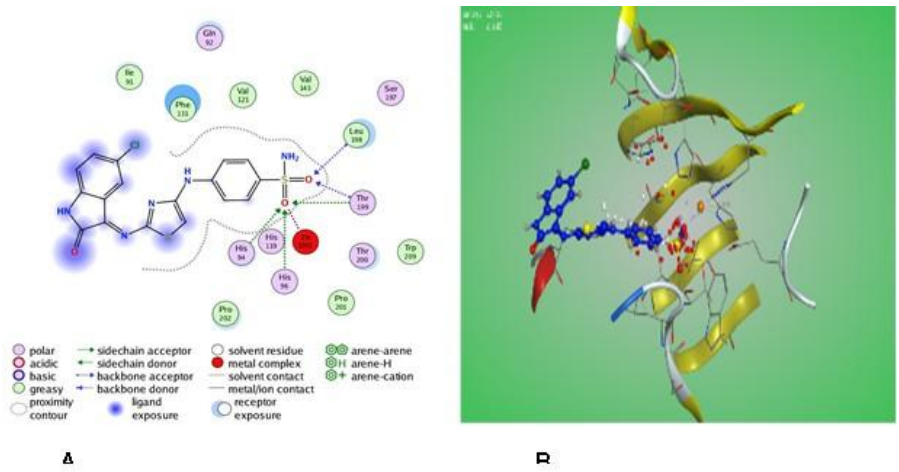

Figure (4) Compound **A3** with Carbonic anhydrase IX (PDB code: 4z0q). Where (A) elucidates the two-dimensional image of the active site binding of compound **A3**, (B) elucidates the three-dimensional image of the active site binding of compound **A3**.

Figure (5) Morphology of the cell lines control and after treatment with Acetazolamide at the IC_{50}

Figure (6) Morphology of the cell lines control and after treatment with compounds (A1-A3) at the IC_{50}.

CONCLUSION

Using 4-aminobenzen sulfonamide as the starting precursor, the compounds (A1, A2, and A3) were successfully synthesized. The molecules were characterized by spectroscopic examination using techniques such as FT-IR, ^{1}H NMR, and ^{13}C NMR. The human cancer cell line MCF-7 and the normal epithelial breast cell MCF10A were then employed as targets for the MTT test to evaluate their cytotoxic characteristics. The collected data from docking study and cytotoxic activity study show that these compounds can be good candidate as carbonic anhydrase inhibitors for treatment of cancer diseases.

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