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Insilco Design, Synthesis and Assessment of the Beta-Lactamases Inhibitory Activity for New 4,4′-Methylenedianiline Monocyclic Azetidinone Compounds

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Abstract: Infectious diseases are a leading cause of death globally. While beta-lactam antibiotics remain highly effective treatments, their efficacy is increasingly threatened by microbial beta-lactamase, an enzyme that deactivates these vital drugs. This escalating antimicrobial resistance has been widely reported internationally. Beta-lactamase inhibitors (BLIs), like clavulanic acid, are co-administered with beta-lactams to mitigate this resistance. However, these inhibitors themselves are becoming susceptible to similar resistance mechanisms. This necessitates the urgent discovery of innovative compounds with potent anti-beta-lactamase activities. This study leveraged in silico docking to design and synthesize novel N,N'-diaroyl-4,4'-methylenedianiline monocyclic azetidinone compounds (which resist the Beta-lactamase hydrolysis). Our docking efforts guided the synthesis of ten new compounds, created by reacting selected acid chlorides with synthesized Schiff bases to form the azetidinone ring. We characterized the structures of these synthesized compounds using physical and spectral data. We then biologically tested the monobactam compounds, determining their Minimum Inhibitory Concentration (MIC) against three strains of beta-lactamase-producing Gram-negative bacteria. Their anti-beta-lactamase activities were subsequently compared to clavulanic acid, used as a co-inhibitor with amoxicillin against the same bacterial strains using disk diffusion method. Our results indicate that all synthesized monobactam compounds exhibited beta-lactamase inhibitory actions. Notably, their activity against *Pseudomonas aeruginosa* and *Escherichia coli* was stronger than against *Klebsiella pneumoniae*. The compounds Az2, Az3, Az6, and Az8 having the stronger activities, they representing 14- and 17-mm inhibition zones (moderate activities) against *Pseudomonas aeruginosa* and *Escherichia coli respectively*. While all compounds demonstrated varying degrees of anti-beta-lactamase activity, their potency remained lower than that of the standard, clavulanic acid. Notably, chloride substituents showed promising results. these electronegative atoms facilitate stronger interactions with the enzyme's active sites. The nitro groups and halogen atoms appear to enhance the binding of hydrophobic residues within the beta-lactamase active site, thereby strengthening the overall interaction. This study underscores the critical importance of developing new beta-lactamase inhibitors to overcome the pervasive challenge of bacterial resistance.

Keywords: Insilco design, Beta-lactamases, 4,4′-methylenedianiline, monocyclic, azetidinone compounds.

Introduction

Beta-lactam antibiotics remain a cornerstone in the fight against bacterial infections. However, the escalating global threat of antibacterial resistance has severely diminished their effectiveness, posing a significant public health challenge [1]. Antimicrobial resistance (AMR) is a critical global health crisis, rendering infections untreatable and threatening modern medicine. It imposes immense economic and health burdens, leading to prolonged illness, disability, and death worldwide. Recent data reveal AMR directly caused 1.14 million deaths in 2021 and was associated with nearly 5 million. Projections suggest a catastrophic rise to 1.91 million direct deaths annually by 2050, totaling over 39 million by mid-century. Addressing this escalating threat urgently requires global collaboration, robust surveillance, and accelerated development of new antimicrobials and strategies. [1,2] Infections caused by drug-resistant bacteria are a persistent concern, with the rising prevalence of beta-lactamases—enzymes that inactivate beta-lactam antibiotics—being particularly alarming across bacterial strains [2]. This concerning trend demands immediate attention and action. Among the numerous mechanisms of bacterial resistance to beta-lactams currently recognized, the production of beta-lactamases is the most prominent[3]. Serine beta-lactamases, encompassing classes A, C, and D, are crucial contributors to bacterial resistance [4]. These enzymes hydrolyze the beta-lactam ring by forming a covalent acyl-intermediate, a vital step in breaking down the antibiotic molecule [5,6]. In contrast, Class B metallo-β-lactamases require one or two zinc ions for their catalytic activity [7].

Beta-lactamase inhibitors (BLIs) are crucial in combating bacterial resistance to beta-lactam antibiotics. These inhibitors are specifically designed to bind to the active site of beta-lactamase enzymes, effectively neutralizing their activity [3]. When co-administered with beta-lactam antibiotics, BLIs

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enhance the antibiotics' efficacy by preventing the breakdown of the beta-lactam ring, a structure essential for their antibacterial action [8]. However, a significant limitation of currently available BLIs is their inherent vulnerability. Many of these inhibitors also contain a beta-lactam ring, rendering them susceptible to degradation by beta-lactamases. This susceptibility means that BLIs often face the same time-limited efficacy as the antibiotics they are meant to protect [9]. Consequently, their effectiveness in combating bacterial resistance can diminish over time, posing a persistent challenge in antimicrobial therapy [10,11].

The escalating challenge of bacterial resistance necessitates the urgent development of more selective beta-lactamase inhibitors (BLIs) for co-administration with beta-lactam antibiotics [12]. Despite numerous studies focused on advancing new antibiotics and anti-beta-lactamases, sustained efforts remain crucial to overcome this formidable challenge.[13].

Monocyclic beta-lactams, unlike other beta-lactams, exhibit inherent resistance to degradation by beta-lactamases [12]. These monobactams are a class of monocyclic azetidinone compounds [14]. Aas an example aztreonam is typically known for their inherent resistance to many beta-lactamases, rather than directly inhibiting them [14,15]. Consequently, research focusing on such compounds presents a unique opportunity to discover novel and original BLIs.

4,4′-Methylenedianiline (MDA) is primarily an industrial chemical and its biological importance is predominantly observed through beneficial biological roles. It's not a naturally occurring compound. Its derivatives have many biological applications like antibacterial and antioxidant activities [10].

This study aimed to leverage in silico docking to design and synthesize novel monocyclic azetidinone beta-lactam inhibitors derived from a 4,4′-methylenedianiline core. Subsequently, the study evaluated their antibacterial and anti-beta-lactamase inhibitory activities.

Materials and Methods

General approach

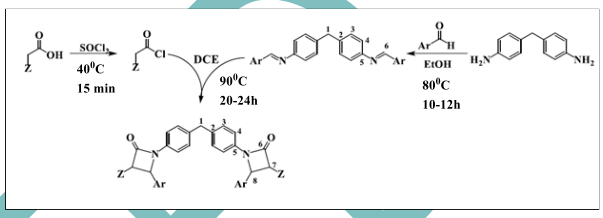
In this study, all the materials utilized were obtained from reputable commercial suppliers, ensuring their quality and consistency. The implied chemical are1- 4-Methyl benzaldehyde (Fluka, Switzerland), 3-Chloro benzaldehyde (Fluka, Switzerland), 4-Chloro phenoxy acetic acid (Fluka, Switzerland), 4-methylbenzoic acid (Thomas Baker,India), Absolute Ethanol (Scharlau,Spain), Dichloroethane (Scharlau, Spain), Dimethylsulfoxide (DMSO) (Fluka, Switzerland), Naphthaldehyde (Scharlau, Spain), P-nitro benzaldehyde (Fluka,Switzerland), Pyridine (Fluka, Switzerland), Thionyl chloride (Alpha, India). The melting points of the synthesized compounds were determined using open capillaries. Moreover, the FTIR spectra were recorded using a PerkinElmer infrared spectrophotometer. Additionally, ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 using a Bruker Avance DPX 400 MHz spectrometer. Tetramethylsilane was used as an internal reference in these NMR experiments to standardize the chemical shift values

Docking study

Molecular docking simulations were performed using the Mcule 1-click-docking online platform to identify potential inhibitors for a broad spectrum of beta-lactamases [15]. Docking was specifically conducted against two key enzymes: the Class A TEM-1 beta-lactamase (PDB ID: 1PZP) and the metallo IMP-1 beta-lactamase (PDB ID: 1JJE). This dual-enzyme approach aimed to identify compounds with universal inhibitory potential. Optimal compounds were selected based on their docking scores, prioritizing those exhibiting the most favorable (lowest) binding energies across both enzymes [16]. This assessment leveraged the principle of geometric shape complementarity between the candidate compounds and the enzyme's active site. Molecular structures of all synthesized and depicted chemical compounds were generated using ChemDraw software (version 16.0.0.82(68) from PerkinElmer). Docking simulation outcomes were quantified as energy scores in kcal/mol. Compounds with the lowest energy scores and best shape complementarity, indicative of the strongest predicted interactions with the enzyme's binding site, were selected for subsequent synthesis. The spatial arrangement of the docked compounds was then visualized and analyzed using BIOVIA Discovery Studio Visualizer v20.1.0.19295.

Chemical Synthesis:

The synthetic rout is denoted in the subsequent scheme:



Method utilized for Schiff base synthesis [17]:

The synthesis commenced by dissolving 0.008 moles of the selected substituted benzaldehyde in 20 ml of absolute ethanol under heating. Concurrently, 0.004 moles of 4,4'-methylenedianiline were dissolved in 20 ml of hot absolute ethanol. These two solutions were then combined and refluxed for 10–15 hours. Upon cooling, the resulting precipitate was isolated by filtration, dried, and subsequently washed with cold ethanol followed by two washes with distilled water before final drying.

Method utilized for acid chloride synthesis [17]:

To prepare the acid chloride, 0.2 moles of the selected carboxylic acid were combined with 15 ml of thionyl chloride. This mixture was refluxed under heating for 30 minutes. Following reflux, the excess thionyl chloride was removed under reduced pressure, and the crude acid chloride obtained was used immediately in the subsequent reaction step.

Method utilized for monocyclic derivatives synthesis [18,19]:

The final synthesis involved the dropwise addition of the corresponding acid chloride (0.0032 moles), dissolved in 10 ml of dichloroethane, to a pre-cooled mixture (0°C) containing the synthesized Schiff base (0.0016 moles) and pyridine (0.0032 moles) in 30 ml of dichloroethane. The resulting reaction mixture was then refluxed for 22–26 hours. After cooling, the mixture underwent a series of washes: twice with 30 ml of water, once with 30 ml of a saturated aqueous sodium bicarbonate solution, and finally with 30 ml of a sodium chloride solution. The organic layer was subsequently separated, dried over anhydrous sodium sulfate, and the solvent was evaporated. The crude product was then purified by washing with ethanol.

Biological section

Beta-lactamases detection in bacterial isolates[20]:

The presence of beta-lactamase was detected using the acidimetric method, which guided the selection of three pathogenic Gram-negative isolates: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. A color change from pink to yellow within five minutes indicated beta-lactamase activity. Positive control samples were run in parallel to validate the results. The selected isolates demonstrated sensitivity to Augmentin and resistance to amoxicillin at a specific

concentration. This finding confirms that clavulanic acid in Augmentin effectively inhibits beta-lactamase activity in these isolates, whereas amoxicillin alone lacks antibacterial activity in the absence of clavulanic acid.

Minimum inhibitory concentration (MIC) determination:

The broth microdilution method was employed to determine the Minimum Inhibitory Concentration (MIC) of the synthesized compounds, amoxicillin, and Augmentin (serving as control antibacterial agents). [21] Ten consecutive two-fold dilutions were prepared for each agent, commencing from an initial concentration of 2000 µg/ml.

These diluted solutions were then aliquoted into test tubes, and bacterial strains were introduced to achieve a final concentration of 5×105 CFU/ml, followed by the addition of 1 ml of Mueller-Hinton broth. The test tubes were incubated for 18 hours at 37◦C. Mueller-Hinton broth with bacterial isolates served as the positive control, while Mueller-Hinton broth alone constituted the negative control. [22]

The primary objective of MIC determination was to evaluate the antibacterial activity of both standard and synthesized compounds. For the standard, the MIC specified the concentration at which Augmentin remained effective (sensitive), whereas amoxicillin consistently displayed resistance, as observed in preliminary assessments. For the synthesized compounds, MIC evaluation established their intrinsic antibacterial activity, if any, and, critically, identified a concentration below the MIC for use in subsequent experiments. This sub-MIC concentration ensures that any observed antibacterial activity of amoxicillin in later stages is attributable solely to beta-lactamase inhibition by either clavulanic acid or the synthesized compound, following co-inoculation with amoxicillin.

Anti beta-lactamase activities evaluation[23,24]**:**

In this study, the disk diffusion method was employed to measure the anti-beta-lactamase activity of the new compounds. For the experiment, each compound was tested as a co-inhibitor alongside 1000 µg of amoxicillin (equivalent to the quantity in Augmentin), applied to the disks at a volume of 5 µl per disk. These disks, comprising amoxicillin and the synthesized compound (at sub-MIC concentrations), were placed on Mueller-Hinton agar in Petri dishes, which had been inoculated with bacterial strains using sterile cotton swabs. The setup was then incubated for 24 hours at 37°C. The zones of microbial growth inhibition around the disks were measured to determine the diameters of inhibition. As a control, 1000 µg disks of amoxicillin alone (5 µl per disk) were also prepared and included in the experiment. Dimethyl sulfoxide (DMSO) was used for dissolving the new compounds, ensuring a final concentration of less than 2% to avoid any adverse effects on bacterial growth.

Results and discussion

The Docking section:

The standard beta-lactamase inhibitors clavulanic acid, avibactam, tazobactam, and sulbactam (Table 1), along with the newly designed amide compounds, were subjected to molecular docking simulations. Docking was performed against two distinct beta-lactamases: TEM-1 (PDB ID: 1PZP), a Class A enzyme, and IMP-1 (PDB ID: 1JJE), a metallo-beta-lactamase. The chemical structures of the substituents employed in designing the novel compounds are presented in Table 2, while the comprehensive docking results are detailed in Table 3.

Table (1): The docking scores for the standard inhibitors.

Name	Docking Score	
	1PZP	1JJE
Sulbactam	-5.5	-5.3
Clavulanic acid	-6.1	-6.1
Avibactam	-5.4	-7.6

Table (2): Substitution used to design the new azetidinone derivatives.

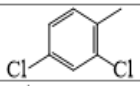
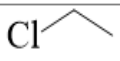
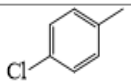
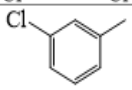
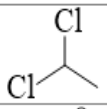
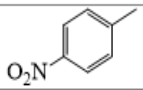
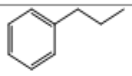
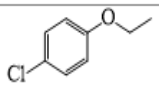
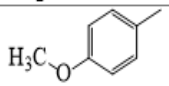
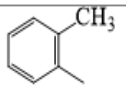
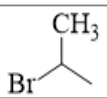
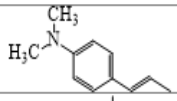
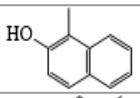
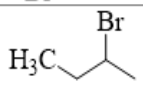
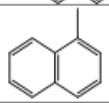
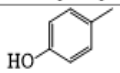
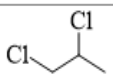
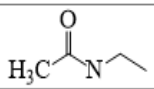
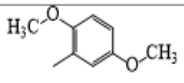
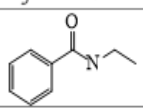
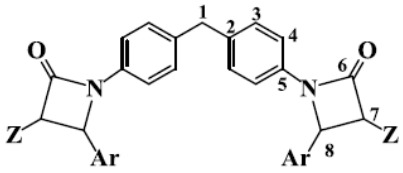
Number	Structure	Number	structure	Number	Structure
Ar1		Ar9		Z1	
Ar2		Ar10		Z2	
Ar3		Ar11		Z3	
Ar4		Ar12		Z4	
Ar5		Ar13		Z5	
Ar6		Ar14		Z6	
Ar7				Z7	
Ar8				Z8	

Table (3). Docking study results for the new azetidinone derivatives with TEM-1 beta-lactamase (1PZP) and the IMP-1 beta-lactamase (1JJE) enzymes (kcal/mol)



Groups	Docking Score		Groups	Docking Score		Groups	Docking Score	
	1PZP	1JJE		1PZP	1JJE		1PZP	1JJE
Ar1+Z1	-8.2	-8.0	Ar5+Z7	-8.4	-8.6	Ar10+Z5	-8.4	-8.6
Ar1+Z2	-8.0	-8.7	Ar5+Z8	-7.8	-8.6	Ar10+Z6	-8.8	-8.6
Ar1+Z3	-10.8	-10.1	Ar6+Z1	-10.3	-10.5	Ar10+Z7	-8.2	-8.0
Ar1+Z4	-8.6	-9.4	Ar6+Z2	-8.4	-8.0	Ar10+Z8	-8.0	-8.7
Ar1+Z5	-8.5	-7.0	Ar6+Z3	-10.0	-10.1	Ar11+Z1	-8.4	-7.6
Ar1+Z6	-8.5	-9.1	Ar6+Z4	-8.8	-7.0	Ar11+Z2	-8.8	-8.6
Ar1+Z7	-8.5	-8.0	Ar6+Z5	-8.4	-8.0	Ar11+Z3	-8.2	-8.0
Ar1+Z8	-8.0	-8.7	Ar6+Z6	-8.4	-8.4	Ar11+Z4	-8.0	-8.7
Ar2+Z1	-8.4	-8.0	Ar6+Z7	-8.3	-7.9	Ar11+Z5	-8.4	-7.6
Ar2+Z2	-8.4	-8.4	Ar6+Z8	-8.4	-8.0	Ar11+Z6	-7.2	-8.0
Ar2+Z3	-8.0	-10.0	Ar7+Z1	-9.6	-8.1	Ar11+Z7	-9.3	-10.7
Ar2+Z4	-8.5	-8.0	Ar7+Z2	-8.4	-9.4	Ar11+Z8	-8.4	-9.4
Ar2+Z5	-8.2	-8.0	Ar7+Z3	-8.4	-7.6	Ar12+Z1	-10.9	-10.7
Ar2+Z6	-8.0	-7.7	Ar7+Z4	-7.8	-8.6	Ar12+Z2	-8.8	-8.6
Ar2+Z8	-9.0	-8.1	Ar8+Z5	-9.1	-8.3	Ar12+Z3	-10.8	-10.0
Ar2+Z8	-7.6	-9.4	Ar7+Z6	-8.4	-8.0	Ar12+Z4	-8.0	-8.7
Ar3+Z1	-10.5	-10.2	Ar7+Z7	-7.4	-8.4	Ar12+Z5	-8.4	-7.6
Ar3+Z2	-8.5	-9.1	Ar7+Z8	-7.8	-8.0	Ar12+Z6	-8.8	-8.6
Ar3+Z3	-10.2	-10.3	Ar8+Z1	-8.4	-8.0	Ar12+Z7	-8.2	-8.0
Ar3+Z4	-8.0	-8.7	Ar8+Z2	-8.4	-8.4	Ar12+Z8	-8.0	-7.7
Ar3+Z5	-7.4	-8.0	Ar8+Z3	-8.3	-8.9	Ar13+Z1	-8.4	-8.6
Ar3+Z6	-8.4	-9.4	Ar8+Z4	-8.4	-8.0	Ar13+Z2	-8.2	-8.0
Ar3+Z7	-8.0	-10.0	Ar8+Z5	-9.6	-8.1	Ar13+Z3	-9.3	-10.7
Ar3+Z8	-8.4	-9.4	Ar8+Z6	-8.4	-9.4	Ar13+Z4	-8.4	-9.4
Ar4+Z1	-8.2	-8.0	Ar8+Z7	-8.4	-8.6	Ar13+Z5	-8.4	-8.6
Ar4+Z2	-8.0	-8.7	Ar8+Z8	-8.8	-8.6	Ar13+Z6	-9.8	-8.6
Ar4+Z3	-9.0	-8.1	Ar9+Z1	-10.8	-10.5	Ar13+Z9	-9.2	-8.0
Ar4+Z4	-9.6	-9.4	Ar9+Z2	-9.4	-8.0	Ar13+Z8	-9.0	-9.7
Ar4+Z5	-8.5	-9.0	Ar9+Z3	-9.4	-8.4	Ar14+Z1	-9.4	-9.6
Ar4+Z6	-8.5	-9.1	Ar9+Z4	-9.8	-9.0	Ar14+Z2	-9.8	-8.6
Ar4+Z7	-8.5	-9.0	Ar9+Z5	-9.4	-8.0	Ar14+Z3	-9.2	-8.0
Ar4+Z8	-9.0	-9.9	Ar9+Z6	-9.4	-8.4	Ar14+Z4	-9.0	-9.7
Ar5+Z1	-9.4	-8.0	Ar9+Z7	-9.3	-9.9	Ar14+Z5	-9.4	-9.6
Ar5+Z2	-8.4	-9.4	Ar9+Z8	-9.4	-8.0	Ar14+Z6	-9.2	-8.0
Ar5+Z3	-9.0	-10.0	Ar10+Z1	-9.6	-8.1	Ar14+Z7	-9.3	-10.7
Ar5+Z4	-9.2	-8.0	Ar10+Z2	-10.8	-10.3	Ar14+Z8	-9.4	-9.6
Ar5+Z5	-9.0	-9.7	Ar10+Z3	-10.5	-10.0			
Ar5+Z6	-9.0	-8.1	Ar10+Z4	-9.1	-8.3			

The molecular docking procedure for standard inhibitors served a dual purpose: to establish control binding scores and to delineate the key amino acid residues involved in active site interactions. For TEM-1 beta-lactamase (PDB ID: 1PZP) [15,25]. the amino acids LEU196, ALA199, ILE200, GLY211, ALA253, and GLY256 were consistently associated with the binding of most standard inhibitors. Conversely, VAL25, TRP28, PHE51, HIS79, SER80, ASP81, and ASN167 were implicated in binding with the majority of standard inhibitors in IMP-1 beta-lactamase (PDB ID: 1JJE) [26] These specific sets of amino acids (six for TEM-1 and seven for IMP-1) define the respective active binding sites of these enzymes.

Analysis of the interactions between the synthesized azetidinone compounds and both enzymes (Table 3) revealed promising results. Ten compounds, identified by their substituents (Ar1+Z3, Ar3+Z1, Ar3+Z3, Ar6+Z1, Ar6+Z3, Ar9+Z1, Ar10+Z2, Ar10+Z3, Ar12+Z1, and Ar12+Z3), demonstrated the ability to bind to 4-5 of the 6 and 7 critical amino acids defining the active sites of TEM-1 (PDB ID: 1PZP) and IMP-1 (PDB ID: 1JJE) beta-lactamases, respectively (Figure 1). Furthermore, these azetidinones engaged with an additional 3 to 5 amino acids, potentially enhancing their interactions within the enzyme binding pockets.

The selected compounds exhibited encouraging docking scores, in many cases equaling or surpassing those of the standard inhibitors against both enzymes. Their promising activity can be attributed to the incorporation of one or two aromatic hydrophobic benzene rings (some with an additional methyl hydrophobic moiety) in their substitution patterns. Moreover, the presence of electronegative aromatic halogenated chloride groups (at ortho, meta, or para positions) or nitro substituents appears to play a significant role. It is noteworthy that among the various halogenated substituents explored during the docking process, only the chloride substituents consistently yielded favorable docking results. This suggests that these electronegative atoms or groups lead to stronger interactions with the enzyme active sites, exhibiting high binding scores and affinities for the binding pocket, and potentially synergizing with the hydrophobic moieties favored by beta-lactamases [27].

Chemical study results:

The chemical structures of the synthesized Schiff bases were confirmed through their physical properties and IR spectral

data, as detailed in Table 4. Key spectroscopic evidence included the presence of characteristic stretching bands at 1600−1612 cm−1, corresponding to the C=N imine bonds. Crucially, the disappearance of the 3343 cm−1 band, typically attributed to the N-H bond of 4,4'-methylenedianiline, further validated the successful formation of the imine linkages.

The physical properties of the newly synthesized monocyclic azetidinone compounds are presented in Table 5. The formation of the azetidinone monocycles was unequivocally confirmed by the presence of stretching bands at 1637−1654 cm−1 for the carbonyl (C=O) group. Further structural elucidation of these new azetidinones was achieved using 1H-NMR and 13C-NMR spectroscopic analysis. Specifically, the appearance of the C6 signal in the range of 132.66−139.90 ppm and the C7 signal in the range of 132.06−139.90 ppm in the 13C-NMR spectra provided strong confirmation of the formation of the monobactam rings in the synthesized compounds. These signals correspond to the carbon atoms derived from the acid chlorides incorporated into the Schiff base structures.

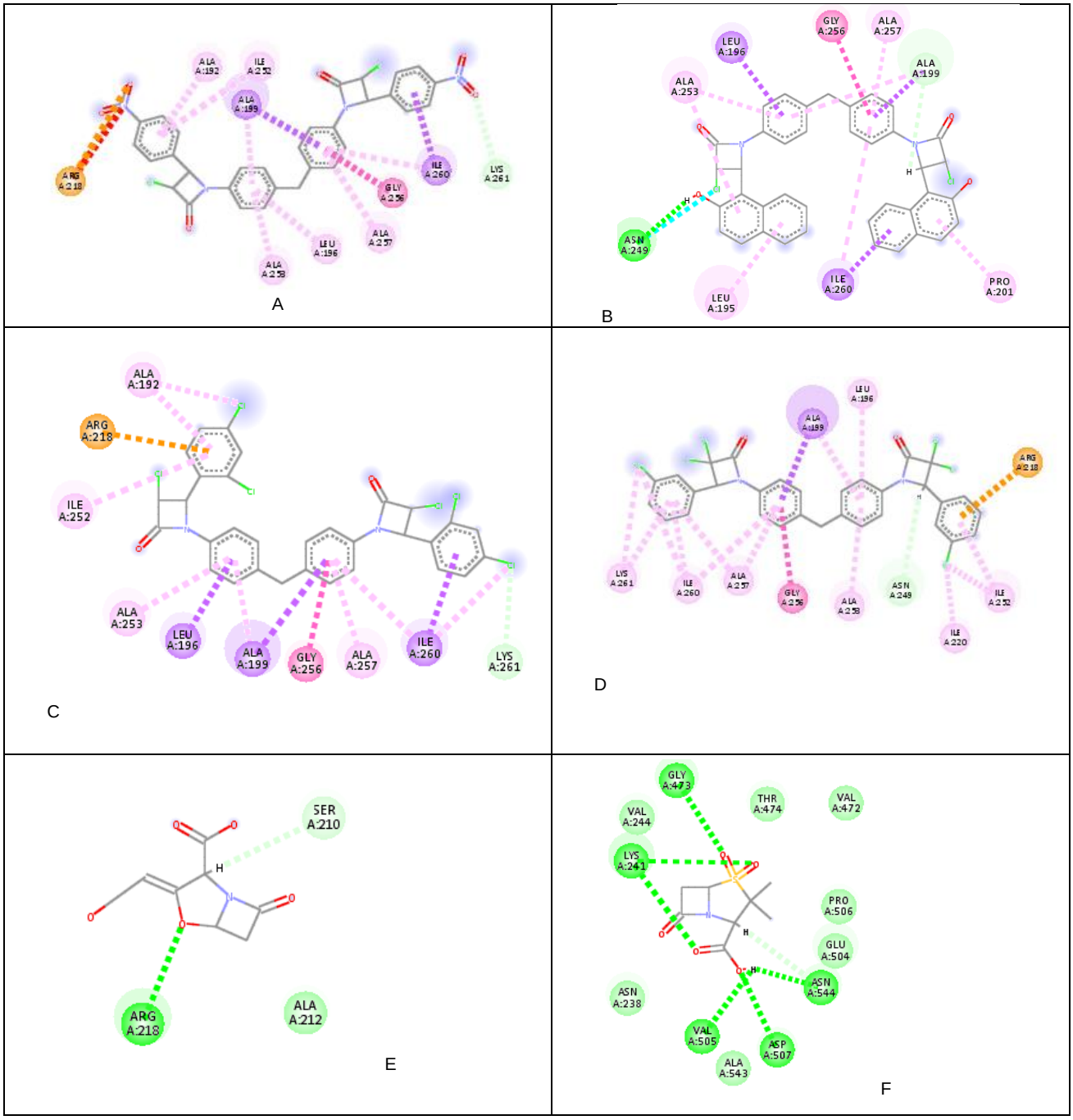
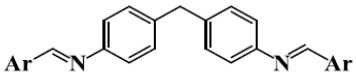


Figure (1): 2D chemical structure for selected Az2 (A) Az4 (B) Az6 (C) Az7 (D) clavulanic acid (E) sulbactam (F).

Table (4): physical properties and the FT-IR bands for the Schiff bases.



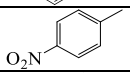
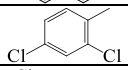
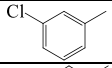
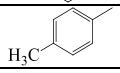
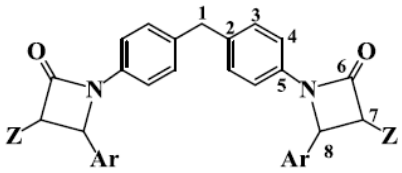
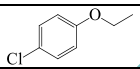
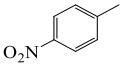
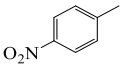
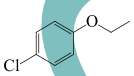
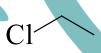
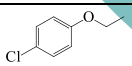
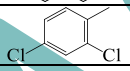
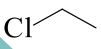
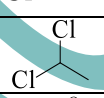
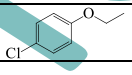
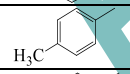
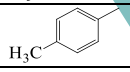
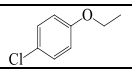
No.	Ar	m.p(ºC)	Color	Yield%	C-H	C=N	Other
1		145-148	Yellow	90	w 3024	S 1608	-----
2		153-158	Pale yellow	84	w 3068	S 1610	N-O m 1433
3		160-163	Yellow	80	w 3052	S 1612	-------
4		128-131	Yellow	91	w 3090	S 1600	C-Cl m 767
5		155-158	Dark yellow	84	w 3071	S 1609	C-Cl m 760
6		137-140	Yellow	89	w 3058	S 1611	--------

Table (5): The physical properties and the FT-IR bands for the new compounds (Az1-Az10).



No.	Ar	Z	M wt.	m.p(ºC)	Color	Yield%	C-H	C=O	Other
Az 1			711.64	275-277	Gray	86	w 3046	S 1654	C-Cl m 768
Az 2			617.4 4	293-296	Brown	83	w 3070	S 1637	C-Cl m 774 N-O m 1420
Az 3			801.63	237-240	Brown	82	w 3076	S 1649	C-Cl m 752 N-O m 1420
Az 4			627.57	246-250	Gray	80	w 3052	S 1647	C-Cl m 778
Az 5			811.76	286-289	Gray	87	w 3032	S 1650	C-Cl m 759
Az 6			665.21	268-271	Dark brown	80	w 3054	S 1648	C-Cl m 736
Az 7			665.21	246-249	Gray	77	w 3052	S 1647	C-Cl m 758
Az 8			780.52	273-276	Dark brown	90	w 3070	S 1650	C-Cl m 728
Az 9			555.50	238-240	Brown	81	w 3076	S 1649	C-Cl m 794
Az 10			739.69	273-277	Dark gray	78	w 3052	S 1647	C-Cl m 785

The presence of stretching bands at 1637-1654 cm^{-1} for the carbonyl (C=O) group confirms the formation of the azetidinone monocycles. Additionally, these new azetidinones were characterized by ^{1}H-NMR and ^{13}C-NMR spectroscopic analysis. The appearance of the C6 signal in the range of 132.66-139.90 ppm and the C7 signal in the range of 132.06-139.90 ppm in the ^{13}C-NMR spectra (both corresponding to the acid chlorides added to the Schiff base structures) further confirms the formation of the monobactam rings in the synthesized compounds.

The scientific names and the NMR characterizations bands for the azetidinone compounds.

Az1[1,1'-(methylenebis(4,1-phenylene))bis(3-(4-chlorophenoxy)-4-phenylazetidin-2-one)**]The ^{1}HNMR of Az1** (δ, ppm) (DMSO-d6): 7.15 (dt, 2H, C 8,12), 7.38 (m, 2H, C 13,17), 7.28-7.33 (m, 5H, C 14,15,16,22,24), 7.08 (dt, 2H, C 9,11), 6.95 (dt, 2H, C 21,25), 5.55 (d, 1H, C 1), 5.45 (dt, 1H, C 4), 3.87 (t, 1H, C 19). **The ^{13}C-NMR of Az1** (δ, ppm) (DMSO-d6): 165.75 (C 2), 156.03 (C 20), 137.62 (C 7), 134.55 (C 6), 133.73 (C 10), 129.87 (C 22,24), 129.75 (C9,11), 129.27 (C23), 128.42

(C14,16), 127.86 (C15), 127.12 (C13,17), 121.85 (C8,12), 118.58 (C21,25), 81.55 (C1), 61.26 (C4), 42.12 (C19).

Az2[1,1'-(methylenebis(4,1-phenylene))bis(3-chloro-4-(4-nitrophenyl)azetidin-2-one)**] The ^{1}HNMR of Az2** (δ, ppm) (DMSO-d6): 8.30 (dt, 2H, C 14,16), 7.63 (dq, 2H, C 13,17), 7.54 (dt, 2H, C 8,12), 7.08 (dt, 2H, C 9,11), 5.42 (dt, 1H, C 4), 5.21 (d, 1H, C 1), 3.87 (q, 1H, C 19). **The ^{13}C-NMR of Az2** (δ, ppm) (DMSO-d6): 163.75 (C 2), 147.28 (C 15), 139.90 (C6), 138.73 (C10), 129.72 (C 9,11), 128.45 (C 13,7), 123.59 (C14,16), 121.79 (C8,12), 65.04 (C4), 62.10 (C1), 42.15 (C19).

Az3[1,1'-(methylenebis(4,1-phenylene))bis(3-(4-chlorophenoxy)-4-(4-nitrophenyl)azetidin-2-one)**] The ^{1}HNMR of Az3** (δ, ppm) (DMSO-d6): 8.40 (dt, 2H, C 14,16), 7.62 (dq, 2H, C 13,17), 7.51 (dt, 2H, C 8,12), 7.33 (dt, 2H, C 22,24), 7.08 (dt, 2H, C 9,11), 6.95 (dq, 2H, C 21,25), 5.52 (d, 1H, C 1), 5.44 (dd, 1H, C 4), 3.87 (t, 1H, C 19). **The ^{13}C-NMR of Az3** (δ, ppm) (DMSO-d6): 165.72 (C 2), 156.03 (C 20), 147.3 (C 15), 137.80 (C 6), 137.62 (C 7), 133.73 (C 10), 129.87 (C 22,24), 129.75 (C9,11,23), 127.89 (C13,17), 123.61 (C14,16), 121.85 (C8,12), 118.58 (C21,25), 81.63 (C1), 61.17 (C4), 42.12 (C19).

Az4[1,1'-(methylenebis(4,1-phenylene))bis(3-chloro-4-(naphthalen-1-yl)azetidin-2-one)**] The ^{1}HNMR of Az4** (δ, ppm) (DMSO-d6): 8.04 (dd, 1H, C 20), 7.83-7.88 (m, 2H, C 15,23), 7.53-7.58 (m, 4H, C 8,12,21,22), 7.44 (m, 2H, C 13,14), 7.18 (dt, 2H, C 9,11), 5.87 (d, 1H, C 4), 5.23 (d, 1H, C 1), 3.87 (q, 1H, C 19). **The ^{13}C-NMR of Az4** (δ, ppm) (DMSO-d6): 163.77 (C 2), 138.45 (C 7), 135.03 (C 16), 133.73 (C 10), 132.27 (C 17), 131.03 (C6), 129.72 (C 9,11), 128.20 (C15,23), 126.75 (C21,22), 125.96 (C14), 125.20 (C13), 124.91 (C20), 121.74 (C8,12), 65.54 (C4), 62.36 (C1), 42.12 (C19).

Az5[1,1'-(methylenebis(4,1-phenylene))bis(3-(4-chlorophenoxy)-4-(naphthalen-1-yl)azetidin-2-one)**] The ^{1}HNMR of Az5** (δ, ppm) (DMSO-d6): 8.07 (dd, 1H, C 27), 7.85 (m, 2H, C 15,30), 7.46-7.53 (m, 6H, C 8,12,13,14,28,29), 7.33 (dt, 2H, C 22,24), 7.08 (dq, 2H, C 9,11), 6.95 (dt, 2H, C 21.25), 5.69 (d, 1H, C 4), 5.64 (d, 1H, C 1), 3.87 (q, 1H, C 19). **The ^{13}C-NMR of Az5** (δ, ppm) (DMSO-d6): 165.83 (C 2), 156.08 (C 20), 137.19 (C 7), 135.13 (C 16), 133.73 (C 10), 132.12 (C17), 129.87 (C 22,24), 129.75 (C9,11), 139.27 (C6), 129.14 (C23), 128.14 (C15,30), 126.77 (C28,29), 125.96 (C14), 125.63 (C13), 124.90 (C27), 121.85 (C8,12), 118.58 (C21,25), 81.87 (C1), 61.52 (C4), 42.12 (C19).

Az6[1,1'-(methylenebis(4,1-phenylene))bis(3-chloro-4-(2,4-dichlorophenyl)azetidin-2-one)**] The ^{1}HNMR of Az6** (δ, ppm) (DMSO-d6): 7.58 (d, 1H, C 16), 7.54 (dt, 2H, C 8,12), 7.35 (dd, 1H, C14), 7.27 (dd, 1H, C 13), 7.07 (dt, 2H, C 9,11), 5.62 (d, 1H, C 4), 5.22 (d, 1H, C 1), 3.87 (q, 1H, C 19). **The ^{13}C-NMR of Az6** (δ, ppm) (DMSO-d6): 163.74 (C 2), 138.32 (C 7), 134.70 (C 17), 133.73 (C 10), 133.62 (C 15), 132.06 (C6), 129.72 (C9,11,13), 129.45 (C16), 127.20 (C14), 121.79 (C8,12), 61.93 (C1), 61.79 (C4), 42.12 (C19).

Az7[1,1'-(methylenebis(4,1-phenylene))bis(3,3-dichloro-4-(3-chlorophenyl)azetidin-2-one)**] The ^{1}HNMR of Az7** (δ, ppm) (DMSO-d6): 7.53 (t, 1H, C 17), 7.45 (dt, 2H, C 8,12), 7.35-7.41 (m, 3H, C13,14,15), 7.32 (dt, 1H, C 15), 7.07 (dt, 2H, C 9,11), 5.70 (s, 1H, C 4), 3.87 (t, 1H, C 19). **The ^{13}C-NMR of Az7** (δ, ppm) (DMSO-d6): 164.55 (C 2), 137.80 (C 7), 135.10 (C 6), 134.09 (C 16), 133.54 (C 10), 130.54 (C17), 129.76 (C9,11), 129.60 (C14), 128.81 (C15), 127.84 (C13), 120.67 (C8,12), 86.53 (C1), 76.54 (C4), 42.12 (C19).

Az8[1,1'-(methylenebis(4,1-phenylene))bis(3-(4-chlorophenoxy)-4-(3-chlorophenyl)azetidin-2-one)**] The ^{1}H NMR of Az8** (δ, ppm) (DMSO-d6): 7.51 (m, 3H, C 8,12,17), 7.32-7.38 (m, 5H, C 13,14,15,22.24), 7.07 (dq, 2H, C9,11), 6.95 (dt, 2H, C 21,25), 5.55 (d, 1H, C 1), 5.46 (dt, 1H, C 4), 3.87 (t, 1H, C 19). **The ^{13}C-NMR of Az8** (δ, ppm) (DMSO-d6): 165.76 (C 2), 156.03 (C 20), 137.62 (C7), 135.45 (C6), 134.05 (C16), 133.73 (C10), 129.87 (C22,24), 129.75 (C9,11), 129.27 (C23), 129.04 (C14), 128.67 (C15), 127.52 (C17), 126.21 (C13), 121.85 (C8,12), 118.58 (C21,25), 81.69 (C1), 61.28 (C4), 42.14 (C19).

Az9[1,1'-(methylenebis(4,1-phenylene))bis(3-chloro-4-(o-tolyl)azetidin-2-one)**] The ^{1}H NMR of Az9** (δ, ppm) (DMSO-d6): 7.55 (dt, 2H, C 8,12), 7.24 (m, 1H, C 13), 7.06-7.17 (m, 5H, C 9,11,14,15,16), 5.55 (dd, 1H, C 4), 5.16 (d, 1H, C 1), 3.87 (t, 1H, C 19), 2.48 (s, 3H, C 20). **The ^{13}C-NMR of Az9** (δ, ppm) (DMSO-d6): 163.75 (C 2), 138.90 (C 17), 138.41 (C7), 134.35 (C6), 133.73 (C10), 129.72 (C9,11), 129.37 (C16), 127.15 (C15), 126.45 (C13,14), 121.79 (C8,12), 63.42 (C4), 61.72 (C1), 42.12 (C19), 19.42 (C26).

Az10[1,1'-(methylenebis(4,1-phenylene))bis(3-(4-chlorophenoxy)-4-(o-tolyl)azetidin-2-one)**] The ^{1}H NMR of Az10** (δ, ppm) (DMSO-d6): 7.52 (dt, 2H, C 8,12), 7.33 (dt, 2H, C 22,24), 7.24 (m, 1H, C13), 7.06-7.17 (m, 5H, C 9,11,14,15,16), 6.96 (dt, 2H, C 21,25), 5.56 (d, 1H, C 1), 5.51 (d, 1H, C 4), 3.87 (t, 1H, C 19), 2.45 (s, 3H, C 27). **The ^{13}C-NMR of Az10** (δ, ppm) (DMSO-d6): 165.81 (C 2), 156.03 (C 20), 137.26 (C7,17), 133.73 (C6,10), 129.87 (C22,24), 129.75 (C9,11), 129.27 (C16,23), 127.11 (C15), 126.56 (C3,14), 126.18 (C13), 121.85 (C8,12), 118.58 (C21,25) 81.76 (C1), 60.63 (C4), 42.154 (C19), 19.43 (C27).

Biological results

Following the selection of bacterial strains by the established method, the Minimum Inhibitory Concentrations (MICs) were determined for both the newly synthesized azetidinone compounds and reference antibacterial agents. Results showed that Augmentin exhibited effective antibacterial activity against all tested strains at a concentration of 1500 µg/ml. In contrast, amoxicillin demonstrated antibacterial action solely against *Pseudomonas aeruginosa* at this same concentration. Notably, the new azetidinone compounds displayed no inherent antibacterial activity at any tested concentration against the three bacterial species.

The MIC study for the new azetidinones was crucial to confirm that, when co-administered with amoxicillin as beta-lactamase inhibitors, they would not exert direct antibacterial effects. Therefore, sub-MIC concentrations of the azetidinones will be employed in subsequent experiments. This ensures that any observed antibacterial activity will be attributed to amoxicillin, with the azetidinone compounds contributing solely through their beta-lactamase inhibitory action.

For evaluating their beta-lactamase inhibitory effects, two concentrations, 450 µg and 900 µg, were selected from the MIC study of the new azetidinones. These will be compared against 200 µg of clavulanic acid, the concentration present in Augmentin.

In the subsequent phase, the beta-lactamase inhibitory effect of the azetidinone compounds against bacterial strains was evaluated. Each of the new compounds was applied as a co-inhibitor with amoxicillin (1000 µg), employed as disks (5 µl per disk) at concentrations of 450 µg and 900 µg. Moreover, amoxicillin (1000 µg) alone and Augmentin (1000/200 mg) were also incubated, and their results were utilized as references for assessment with the newly synthesized azetidinone compounds, as shown in Table 6.

Table (6): The results for the new monobactame compounds when used as co-inhibitors with Amoxicillin represented as inhibition zones.

Com. No.	Inhibition zone diameter (mm)					
	Gram (-)ve					
	p. aeruginosa		*E. coli*		*K. pneumonia*	
	1:1	1:2	1:1	1:2	1:1	1:2
Augmentin	19	20	20	20	21	23
Amoxicillin	8	9	0	0	0	0
Az1	7	12	6	14	5	5
Az2	0	14	0	17	5	7
Az3	0	14	5	17	0	8
Az4	7	12	6	14	5	5
Az5	7	12	6	14	5	5
Az6	0	14	0	17	0	0
Az7	9	15	5	14	15	9
Az8	0	14	0	17	0	0
Az9	7	12	6	14	5	5
Az10	7	12	6	14	5	5

1:1 = 1000 µg/ml Amoxicillin: 450 µg/ml synthesized compound
1:2 = 1000 µg/ml Amoxicillin: 900 µg/ml synthesized compound

≤10 very weak activities; 10-10 moderate activities; ≥20 strong activities

Our findings indicate that the newly synthesized azetidinones exhibited weaker beta-lactamase inhibitory action against *K. pneumoniae* compared to *P. aeruginosa* and *E. coli*. This variation in performance could be attributed to the unique enzyme structures present in the *K. pneumoniae* strain, which may differ from those employed in our docking study or belong to distinct beta-lactamase families or types.

All synthesized azetidinones demonstrated variable levels of anti-beta-lactamase activity. It's crucial to reiterate that none of these compounds showed intrinsic antibacterial activity when tested individually (as confirmed by MIC results). Compounds Az2, Az3, Az6, and Az8 displayed the most potent activity against both *P. aeruginosa* and *E. coli*. While these activities are notable, they remain weaker than that of the reference standard, clavulanic acid.

The compounds exhibiting favorable inhibitory activity consistently incorporate one or more hydrophobic residues in their structure, alongside either a nitro group or halogen atoms. Specifically, they feature at least one aromatic hydrophobic benzene ring or an additional methyl hydrophobic moiety in their substitutions, and include electronegative aromatic chlorinated groups (located at ortho, meta, or para positions) or nitro substituents. It's important to highlight again that among the halogenated substituents investigated, only the chloride groups yielded promising results.

This suggests that these electronegative atoms or groups lead to stronger interactions with the enzyme active sites. The presence of a nitro group and halogen atoms appears to enhance the binding of hydrophobic residues within the beta-lactamase active site, thereby strengthening their overall interaction. These observations are further supported by our docking outcomes and align with conclusions drawn from previous and other research studies [28,29].

This paper contributes to an ongoing project focused on developing novel and effective universal anti-beta-lactamases. We explored and tested a new approach utilizing compounds with two identical arms, each incorporating an azetidinone monobactam moiety. While the outcomes of this study, as presented, may not be highly encouraging in terms of superior potency, they nonetheless represent a notable enhancement over previous research. To definitively confirm the observed activities, more comprehensive studies are essential, particularly those involving bacterial strains harboring beta-lactamase enzymes analogous to those used in our initial docking investigations.

Conclusion

The combination of beta-lactamase inhibitors (BLIs), such as clavulanic acid, with beta-lactam antibiotics has proven crucial in overcoming bacterial resistance. Incorporating novel compounds with anti-beta-lactamase properties holds significant potential for improving infectious disease treatment. By understanding the characteristics of the beta-lactamase active site and designing compounds with appropriate hydrophobic substituents, researchers can enhance the binding affinity and efficacy of these inhibitors. Our findings indicate that while all new monobactam compounds exhibit beta-lactamase inhibitory action, their efficacy against *P. aeruginosa* is weaker compared to *S. aureus* and *E. coli*. The new compounds displayed varying levels of anti-beta-lactamase activity, though none reached the potency of the reference compound, clavulanic acid. Notably, chloride substituents showed promising results. The data suggest that these electronegative atoms facilitate stronger interactions with the enzyme's active sites. Furthermore, the presence of nitro groups and halogen atoms appears to enhance the binding of hydrophobic residues within the beta-lactamase active site, thereby strengthening the overall interaction.

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- **Ethics approval and consent to participate:** Not applicable
- **Consent for publication:** Not applicable
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- **Author's contribution:** The authors confirm contribution to the paper as follows: study conception and design: Ahmed A. J. Mahmood, theoretical calculations and modeling: Ahmed A. J. Mahmood and Islam T. Qasim; data analysis and validation, Ahmed A. J. Mahmood and Islam T. Qasim. draft manuscript preparation: Ali Alazzo, Ahmed A. J. Mahmood All authors reviewed the results and approved the final version of the manuscript.
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