Docking and Biological Screening of Bezo[A]phenothiazinones as Novel Inhibitors of Bacterial Peptidogloycan Transpeptidase

Ibezim, Akachukwu E.*[+•]

Department of Pharmaceutical and Medicinal Chemistry, University of Nigeria, Nsukka, NIGERIA

Onoabedje, Efeturi A.*[+]***; Akpomie, Kovo G.***

Department of Pure and Industrial Chemistry, University of University, Nsukka, NIGERIA

ABSTRACT: *Rising cases of antibiotic-resistant bacteria is a public health concern. Many approved antibiotics target penicillin-binding proteins example peptidoglycan transpeptidase (PTPase). Due to wide pharmacological activity of phenothiazines, new styryl, aryl, alkynyl, and thiophenyl benzo[a]phenothiazines were synthesized and their inhibitory potency against PTPasein silico and Gram-positive/Gram-negative bacteria evaluated. The compounds inhibited the activity of PTPase at 18.93 - 75.48 µM and their best-docked poses identified interaction with PTPase Tyr318, His336, and His352. Experimental results agreed with computational predictions and further confirmed the benzo[a]phenothiazines as potential antibiotics. Also, the identified essential residues could be targeted during the rational optimization of the analogs.*

KEYWORDS: *Phenothiazines; Antimicrobial; Peptidoglycan transpeptidase; Docking, binding mode.*

INTRODUCTION

Multi-resistant organisms have become a serious public health challenge. Most infection-causing bacteria have persisted in the face of known antibiotic medications [1]. Because these bacteria have developed resistance against known antibiotics, researchers in both academia and industry are in constant search for new antibiotic agents and modification of known ones [2]. Phenothiazines are an important class of nitrogen-sulfur heterocycles with a broad spectrum of pharmacological activities [3]. These compounds and their derivatives were known to exhibit to antibacterial, antifungal, anti-inflammatory, antimalarial, analgesic, anticancer, antiviral, multidrug-resistant, neuroleptic, and tranquilizer properties [4-7]. As a result of their promising therapeutic properties, efforts had been directed to the synthesis and biological screening of various derivatives of the parent compound. *Motohashi* and *Co-workers* over the last two decades had made a significant contribution to the synthesis

***** *To whom correspondence should be addressed.*

+ E-mail: akachukwu.ibezim@unn.edu.ng ; efeturi.onoabedje@unn.edu.ng

• Other Address: Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, California, USA

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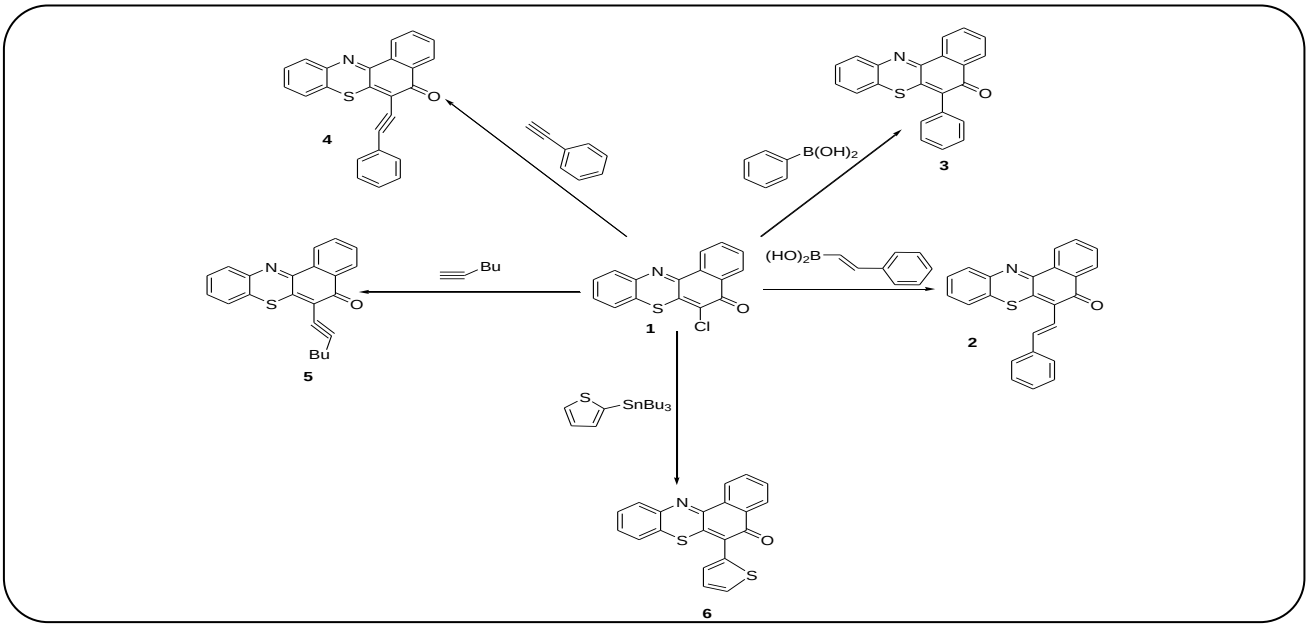

Fig 1: Functionalization of 6-chloro-5H-benzo[a]phenothiazine via palladium/Xphos catalyzed cross couplings.

and biological study of phenothiazines [9]. The synthesis of new derivative of phenothiazine has been a subject of interest in our research work [10-12]; therefore, in this study, computational methods which are generally cost and time effective and routinely employed in rational drug discovery processes were used. These methods have been used to mine inhibitors from databases of both synthetic and natural compounds [13-14] in order to study binding modes of lead compounds and hence to suggest derivatives with possible improved activities [15]. It also enables one to propose hypothetical inhibitor against a novel disease target [16]. Moreover, the growing coherence between computational and experimental results, have raised the acceptance of computational methods within the scientific community [17]. In this article, the synthesis antimicrobial and computational study of new 5-substituted benzo[a]phenothiazinone is described.

RESULTS AND DISCUSSION

Synthesis of 5H-benzo[a]phenothiazin-5-one derivatives

The derivatives were synthesized from 6-chloro-5*H*-benzo[a]phenothiazin-5-one **1**. Compounds 6-styryl-5*H*-benzo[a]phenothiazin-5-one **2**, 6-phenyl-5*H*-benzo[a]phenothiazin-5-one **3** were obtained in good yields via palladium(0)/Xphos mediated Suzuki-Miyaura cross-coupling of styryl- and phenyl boronic acids with 6-chloro-5*H*-benzo[a]phenothiazine **1**. Similarly, the cross-coupling of 6-chloro-5*H*-benzo[a]phenothiazine with ethynylbenzene and hex-1-yne supplied compounds 6-(phenylethynyl)-5*H*-benzo[a]phenothiazin-5-one **4**, and 6-(hex-1-yn-1-yl)-5*H*-benzo[a]phenothiazin-5-one **5** in moderate to high yields respectively. The cross-coupling of 6-chloro-5*H*-benzo[a]phenothiazine with tributylthiophenylstannane afforded 6-(thiophen-2-yl)-5*H*-benzo[a]phenothiazin-5-one **6** at a yield of 57%. The synthesized compounds were established by spectral and elemental analytical data (Fig 1) [10-12].

Docking and binding prediction

Validation of dock protocol is required before performing docking simulation. This was carried out by positioning the grid box size of 24 18 32 A^3 points which centered on the mass center (-0.124, 6.828, 53.916) of the crystallographic macromolecule and covers all the essential active site residues. This method was used to carry out docking calculation in this study because it gave co-crystallized ligand dock pose which differed from that of X-ray crystallographic pose by 1.163 Å. The protocol was, therefore, adopted having satisfied the criteria for root mean square deviation (rmsd) method of validating docking protocol (rmsd $\leq$ 2.0 Å).

The newly synthesized phenothiazines (**1** – **6**) were screened for antibiotic potency, in-silico, by docking them toward a validated antibacterial drug target - peptidoglycan transpeptidase (PTPase). This enzyme, which belongs

Table 1: Dock result for compound 1 – 6.

Compounds	ΔG (Kcal/mol)	K_i (μM)	Ligand efficiency
nl	-6.44	18.93	0.25
1	-6.30	24.08	0.32
2	-6.88	8.99	0.25
3	-6.32	23.19	0.25
4	-5.81	54.81	0.22
5	-5.62	75.48	0.22
6	-6.59	14.74	0.27

nl = native ligand, ΔG = free binding energy, K_i= inhibition constant

to a group of proteins known as Penicillin Binding Proteins (PBPs), is involved in synthesis of peptidoglycan, the major constituent of bacterial cell wall. By inhibiting the action of PTPase, the integrity of the exoskeleton that encapsulates the bacterial cytoplasmic membrane will be compromised, the bacteria suffer lysis and finally dies [18-19]. Record shows that most common mode of action for antibiotics is the inhibition of cell wall synthesis. Also, the fact that many successful antibiotics (penicillin, ampicillin, bacitracin, carbapenems, cephalosporin, methicillin, oxacillin and vancomycin) target PBPs, validates PTPase as antibacterial drug target and its use in the docking process [20].

Docking studies showed that compounds **1 - 6** interacted with PTPase at varying degrees (Table 1). The relatively small size of compound **1** allowed it to penetrate deep into the protein binding cavity and made more favourable interactions with the protein residues (K_i = 24.08 μM). From Fig 2a (compound **1** in red), it appears that the entire molecule (**1**) was engulfed by the protein, which made it bind more tightly. It was observed that the most active compound, **2** (K_i = 8.99 μM), showed a similar docked conformation as **4** (Fig 2c). Both docking poses used the phenothiazinone aromatic rings to make π-π contact with Trp340 while the substituent moieties (styryl and phenylethynyl respectively) were accommodated within inner side of the protein's lipophilic pocket. The fact that double bond is longer than triple bond, might explain why compound **2** exhibited greater affinity/interaction for PTPase over compound **4**.This is because its aromatic ring in compound **2** had closer and therefore stronger π-π contact with Tyr318, His336 and His352 aromatic rings than in compound **4**.The key role of aromatic ring substituent at the C6 position is obvious by the dock result of compounds **3** and **5**. Both compounds showed similar binding modes (Fig 2d), made the same usual hydrogen bond contact with His352. However, compound **3** (K_i = 23.19 μM) exhibited greater inhibitory potency against PTPase over compound **5** (K_i = 75.48 μM). Compound **6** emerged as the second most active compound to inhibit the activity of PTPase (K_i = 14.74 μM). The unique binding conformation of the compound within the binding cavity indicated π-π bond interaction between the phenothiazinone aromatic rings and Trp340, and between the benzo ring and Tyr318, His336 and His352 (Fig 2b). In addition, the thiophenylsulphur and carbonyl oxygen atoms were found to make hydrogen bond contacts with Thr350 and His352 of PTPase, respectively. The importance of having substituent at C6 position is the propensity to form hydrogen bonds, as was the case with thiophenyl moiety, as supported by the fact that inhibitory potency reduced with other substituents except in compound **2**.

Drug resistance occurs when the antibiotic fails to cross Gram negative outer membrane layer of bacterial [2]. The size (molecular weight - MW), lipophilicity (log P) and flexibility (NRB) are the basic physicochemical properties of a molecule which affect its permeability across cell membrane. As shown in Table 2, it was found that **1** – **6** have the requisite MW, log P and NRB, according to Lipinski's "rule of five" [21], which will enable them to pass across bacterial cell lipopolysaccharide layer and membrane. Based on the promising features of **1**-**6**, they were screened

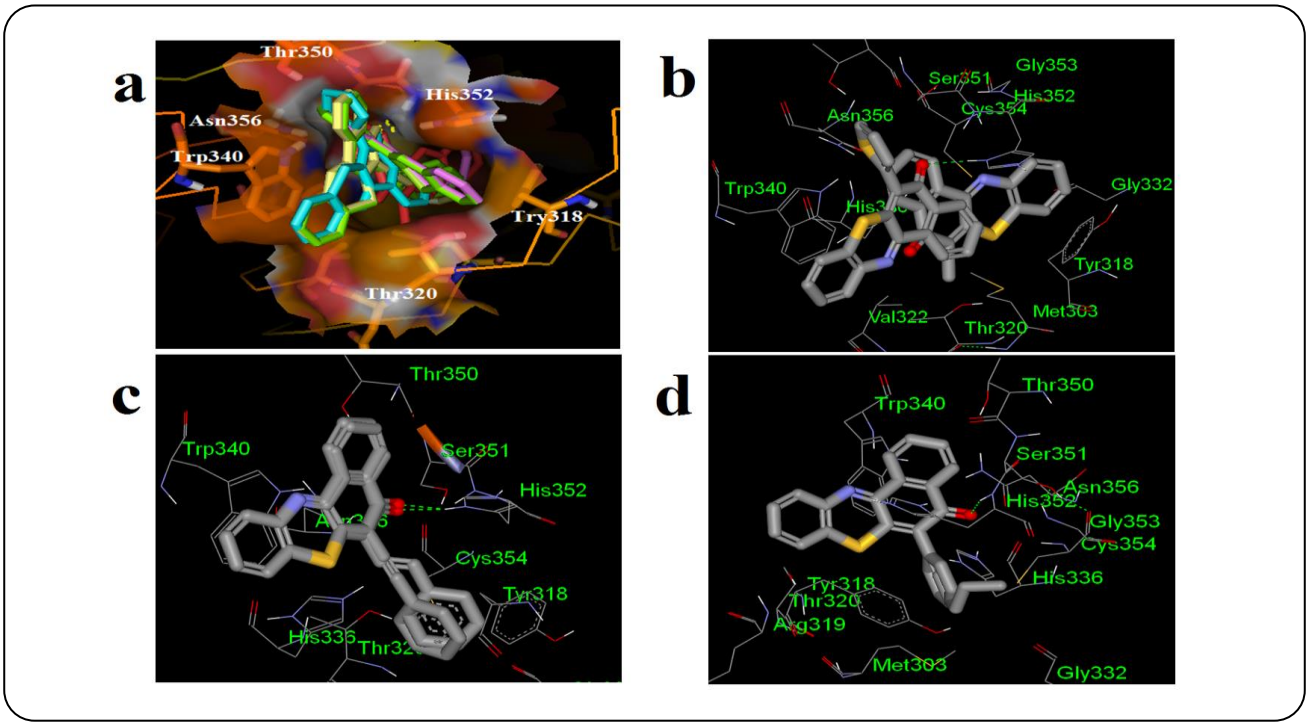

Fig 2: (a) Overlap of docked poses of compounds 1-6 within the binding cavity of PTPase (the compounds are coloured as follows: 1 - red, 2 – green, 3 – blue, 4 – yellow, 5 – magenta, 6 – cyan. (b-d) binding poses of compounds (1 and 6, 2 and 4 and 3 and 5 respectively) with similar binding conformation toward PTPase. Throughout, polar contacts are shown in green broken lines while atoms are colour coded as follows: carbon in gray, hydrogen in white, oxygen in red, sulphur in yellow, nitrogen in blue.

biologically against two Gram positive and Gram negative bacterial.

Biological Screening

All the compounds, **1-6**, were evaluated for *in vitro* antibacterial activity and their inhibition zone diameter (mm) are shown in Table 3. Compound **1** – **6** showed varying inhibitory activity against the test bacteria. The activity of compound is dependent on the position 6 substituent. *Bacillus cereus* is susceptible to all compounds except compound **1** which retain chloro group at position 6 of the tetracyclic molecule. Compounds **3**, **4** and **5** having phenyl and alkynyl groups at positions 6 of the compounds exhibited mild antibiotic effect towards *Staphylocuccusaureus, Escherichia coli, Pseudomonas aeruginosa* but show moderate activity towards Gram positive bacteria. As was expected from in-silico prediction, **2 (**with styryl group**)** and **6** (with heterocyclic thiophenyl group) demonstrated interesting activities against all the test organisms, with **6** possessing broad and potent comparable activity to tetracycline. Interestingly, the MIC of **6** (3.10 µg/mL) was also comparable to that of tetracycline (4.00 µg/mL) for *B. cereus* micro-organism. Therefore, it appears the presence of heterocyclic group on position 6 has an enhancing effect on the activity of the derivatives.

The activity of phenothiazine derivatives against micro-organisms is as result of interaction of pharmacophoric substituents and multicyclic ring (π-π interaction, intercalation in DNA) as well as the penetration of biological membrane as result of the lipophilic character of the molecule [22].

EXPERIMENTAL SECTION

General information

All chemicals were purchased from Aldrich Chemical Company UK and were used without further purification. Otherwise stated, all compounds were synthesized and characterized in the School of Chemistry of Cardiff University UK. Melting points was determined with a Fischer Johns apparatus and were uncorrected. ^{1}H and ^{13}C NMR data were recorded with Brucker DPX 400 MHz spectrometers relative to TMS as internal standard. All and chemical shifts reported in ppm (δ) and coupling

Table 2: Main physical properties of the 1 - 6.

Compounds	MW	log P	NRB
1	339.42	5.48	1
2	363.44	6.23	2
3	343.45	5.92	4
4	345.45	5.01	1
5	297.77	4.38	0
6	365.46	6.13	2

Table 3: Antimicrobial evaluation of 6-chlorobenzo[a]phenothiazine and its derivatives.

Compound	Antibacterial activity			
	Gram positive		Gram negative	
	B. c	S. a	E. c	P. a
1	-	-	++	++
2	++	+	+	++
3	+	+	-	-
4	++	-	-	-
5	+	-	-	-
6	++	++	+++	+++
Tetracycline	++++	++++	+++	+++

B. c (Bacillus cereus); S. a (Staphylocuccusaureus); E. c (Escherichia coli); P. s (Pseudomonas aeruginosa); (-) = ≤ 5; (+) = 6 – 10; (++) = 11 – 15; (+++) = 16 – 25; (++++) = 26 – 40. Conc. = 0.1 mg/mL of DMSO.

constants (J), reported in Hz. Multiplicity is indicated using the following abbreviations: br, for broad; s, for singlet; d, for doublet; t, for triplet; dd, for doublet of doublets and; m, for multiplet. The mass spectra data were obtained on a Varian 1200 Quadruple Mass and MicromassQuadro II Spectrometers. Elemental Analysis was carried out with Thermo Quest Flash series (CHNS) Elemental Analyzer. UV-Visible spectra were recorded on Cecil 7500 Aquarius 7000 Series Spectrometer at Chemistry Advance Laboratory (CAL), Sheda Science & Technology Complex (Shestco) Abuja, Nigeria, using matched 1cm quartz cells and methanol as solvent. The absorption maxima are recorded in nanometers (nm) and figures in parenthesis are log ε. Antimicrobial evaluations were carried out in Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka , Nigeria. The detail procedures of syntheses and characterizations of compounds **1** – **6** were first published in our previous papers [10-12].

Molecular simulation

The 3 dimensional structure of peptidoglycan transpeptidase in complex with an inhibitor (PDB code 5E1G) was retrieved from the protein data bank [23]. Molecular operating environment (MOE) was used to carry out the following: treat the complex dimers as described in our earlier work [14], generate the 3D chemical structure of compounds **1** - **6** and calculate the following molecular descriptors: Molecular Weight (MW), lipophilic (log P) and number of rotatable bonds (NRB). AutoDock 4.2.0 program [24] was used to compute the energies of binding and to score the binding conformations of **1**– **6** towards the target site. In order to dock using Autodock 4.2.0, preparation of the Grid (.gpf) and Docking (.dpf) parameter files were carried out using the AutoDockTools 4.0 graphical user interface. The grid box was centered on the macromolecule and restricted to the binding pocket of the target, and then saved in the gpf file format. The docking parameter file was prepared

using the Docking option menu using the Lamarckian Genetic Algorithms (LGA). This was carried out using the default docking parameters. In order to adopt a protocol for docking the synthesized molecules towards the peptidoglycan transpeptidase binding site, the native ligands present within the binding pocket of each of the protein-ligand complexes were docked towards their respective receptor sites using different grid parameters. This was an attempt to identify the best docking parameters which reproduces the ligand conformation (docking poses) within the binding pocket, with the lowest root-mean-square deviation (RMSD) values, with respect to the experimental binding mode (X-ray crystal structure), initially retrieved from the PDB. Docking experiments were performed using the AutoDockTools 4.0 docking interface in order to find the preferred binding conformations of the ligands in the receptor. The analysis of the binding conformation was carried out using the scoring function, based on the free energy of binding [25]. The grid parameter file of each receptor was generated using AutoDockTools4.0. A grid box was generated. Precautions were taken to make this grid box originally large enough to cover the entire receptor binding site, as well as taking into the ligand sizes into consideration. This was then progressively adjusted to get the ‘‘best’’ box size for the reproduction of the native ligand pose. The number of grid points in x-, y-, and z-axes were recorded. The distance between two connecting grid points was 0.375 A. Receptor-fixed ligand-flexible docking calculations were then carried our using AutoDock4.2 and a Lamarckian Genetic Algorithm [24]. All docking and search parameters were set to default. A total of 10 search attempts (GA run parameters) were performed for each ligand. This resulted in 10 conformations of ligand in complex with the receptor. These were finally ranked on the basis of binding energy. The resulting conformations were visualized in the Discovery Studio Visualizer [26] and PyMol [27]. The docking protocol used in this study was validated by checking the ability of the docking protocol to reproduce the experimental binding modes rmsd value of $\leq$ 2.0 Å. The spacing between the grid points was constant at 0.375 Å, while the AutoDockTools were used to add atomic Gasteiger partial charges.

General antimicrobial sensitivity testing of compounds

A pure culture of human pathogenic microbes were obtained from culture collection center, Bishop Shahanan Hospital, Nsukka, Enugu State. The agar cup diffusion method was applied to determine the sensitivity of compounds against bacteria using Muller Hinton Agar [28]. The MHA plates were inoculated with 1 x 104 CFU culture of test organism. After which cups were made in each sector after previously dividing the plate into six segments and labeled. Using the sterile pipette, each cup was filled with four drops of compound (0.1 mg/mL). Pre-diffused time of 30 min was allowed before all the plates were incubated at 37°C for 24 h for bacteria. After incubation the Inhibition Zone Diameter (IZD) resulting were measured and result recorded after subtracting the diameter of the cork borer. The cork borer used to make the cup is 8 mm in diameter. The procedure was repeated for tetracycline (standard bacteria) and DMSO (solvent). 2.10.

Minimum inhibitory concentration (MIC) testing

The MIC was evaluated by the broth dilution technique approved by the National Committee for Clinical Laboratory Standards (NCCLS) [28, 29]. Serial dilution of 0.1 mg/mL DMSO solution of each sample was carried out to have 0.05, 0.025, 0.0125, 0.00625 mg/mL solutions. Fours drops of each dilution were added to the corresponding cup previously cut in the Mueller Hinton Agar (MHA) plate. The plates were incubated at 37 °C for 24 h for bacteria. The diameter of zone of inhibition was measured and the value subtracted from the diameter of the borer to give the Inhibition Zone Diameter (IZD). The graph of IZD against the log of concentrations was plotted for each plate containing a specific compound and a microorganism. The anti-log of the intercept on x-axis gives the MIC. The procedure was repeated for tetracycline and ketoconazole.

CONCLUSIONS

Newly synthesized styryl, aryl, alkynyl and thiophenyl benzo[a]phenothiazines exhibited inhibitory potency against PTPase *in silico* and Gram positive/Gram negative bacteria. The compounds inhibited the activity of PTPase at 18.93 - 75.48 µM and their best docked poses identified interaction with PTPase Tyr318, His336 and His352. The *in vitro* assay of derivatives agreed

with computational predictions and further validate the antibiotic potentials of the benzo[a]phenothiazines especially 6-(thiophen-2-yl)-5H-benzo[a]phenothiazin-5-one which possesses the broadest and most potent antimicrobial activity compared to the standard.

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