Original Article:

Inhibitory Effects of *Salinispora*-derived Metabolites Against Multidrug Resistance: An In-silico Study

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ABSTRACT

Background: Multi Drug Resistance (MDR) is known to defeat most chemotherapies as one of the main anticancer strategies. The role of overexpression or overactivation of ATP-Binding Cassette (ABC) transporters, especially P-glycoprotein (P-gp), in the development of chemotherapy has long been demonstrated. *Salinispora* is a marine actinomycete genus known for the production of novel bioactive metabolites.

Objectives: In this study, the potential of Salinispora derived metabolites as inhibitor of ATP-binding cassette (ABC) transports have been investigated using in-silico approaches.

Methods: Physicochemical, pharmacokinetic and drug likeness of the Salinispora derived metabolites have been analyzed using SwissADME server. This was accompanied by the employment of docking strategy to evaluate anti-MDR potential of the metabolites using P-gp, Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance Protein 1 (MRP-1) as target proteins.

Results: Nineteen metabolites were found to have demonstrated appropriate physicochemical, pharmacokinetic, and drug-likeness properties and were involved in the docking studies. Based on docking studies, saliniquinones, cyclomarazine, and cyanosporoside A demonstrated ABC transporters inhibitory potential.

Conclusion: Our results suggest that further in vivo and in vitro studies on anti-MDR effects of *Salinispora*-derived metabolites are warranted.

Introduction

Therapy of a disease can be hindered through drug resistance, in other words, attenuating a drug's pharmacologic effects [1]. Intrinsic or acquired drug resistance impedes the efficiency of chemotherapy as one of the main anticancer strategies. Notably, intrinsic drug resistance arises when resistance to chemotherapeutic agents is revealed from the first cycles of chemotherapy, regardless of the history of exposure to the chemotherapeutic agents. Despite the initial response to an

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anticancer agent, tumors develop resistance and become unresponsive and acquire drug resistance. Multi Drug Resistance (MDR) is a condition in which tumors develop intrinsic or acquired resistance to several chemically and pharmacologically unrelated anticancer agents [2]. Various cellular and molecular mechanisms are involved in MDR development [3]. Enhancement of drug efflux by ATP-binding cassette transporters (ABC transporters) has long been mentioned as one of the primary mechanisms promoting MDR. Transfer of the substrate across the membrane by these transmembrane proteins is coupled with ATP hydrolysis to supply the energy of transportation. ABC-transporters contain two transmembrane substrate-binding domains recognizing the substrate and two cytoplasmic nucleotide-binding domains hydrolyzing ATP to provide the energy of transporting substrate regardless of the concentration gradient. Despite nucleotide-binding domain, which demonstrates similar structure and function in various ABC transporters, substrate-binding domains are highly heterogenous, enabling recognition and transport of heterogeneous substrates. Substrate specificity is different in the various members of ABC transporters. Members with a broad range of substrate specificity can be involved in the MDR transporting of various anticancer medicines [4]. Among 48 ABC transporters, overexpression and or overactivation of Multi Drug Resistance protein 1 (MDR1), also called P-glycoprotein 1 (P-gp), Multidrug Resistance-associated Proteins (MRPs), and Breast Cancer Resistance Protein (BCRP) have been reported in various experimental models of MDR and clinical studies [5].

The marine actinomycete genus *Salinispora* was discovered in 1989 for the first time [6]. *Salinispora* genus is the first obligate marine actinomycete genus and can only be grown in the growth medium containing seawater [7]. This taxon is well known for the production of unique and biologically active secondary metabolites. It has been estimated that about 10% of the *Salinispora*'s genome is devoted to the secondary metabolites synthesis gene clusters [8]. Table 1 presents that secondary metabolites have been isolated from the various genus of the taxon. As mentioned in Table 1, *Salinispora*-derived secondary metabolites are predominantly supporting the idea of investigating new taxa from poorly studies environment for new secondary metabolite discovery [9]. Salinosporamide A is a potent proteasome inhibitor isolated from *Salinispora tropica*. It has entered phase III clinical trials as an anticancer agent (www.clinicaltrials.gov). Limited biomedical assays have demonstrated various biological activities for *Salinispora*-derived compounds necessitating further studies to complete elucidation of their biological effects.

Computer-assisted drug design and structural molecular biology are highly benefited from molecular docking tools [10-12]. The ligand-protein docking binding mode and free binding energy of ligand in the 3D structure of a protein have been predicted. The docking approach is routinely used to help elucidation of drug-receptor interactions as well as the screening of a library of ligands against a protein to find ligands with appropriate binding mode and free binding energy [13-16]. Here we studied the anti-MDR potential of *Salinispora*-derived compounds using in silico approaches, including molecular docking, drug-likeness, and pharmacokinetic factors (Adsorption, Distribution, Metabolism, and Excretion; ADME) prediction.

Materials and Methods

Ligand preparation

Two-dimensional (2D) structures of compounds were drawn using ChemDraw ultra 8 software. Energy minimization and optimization of the three-dimensional (3D) geometries of each compound have been done in HyperChem software version 7.5 using molecular mechanics force field (MM+) and Austin Model 1 (AM1) semi-empirical molecular orbital calculations [17-19]. MM^+ is a general-purpose force-field in HyperChem software useful for simple molecules. It has been developed based on Allinger's MM2 force-field and has been set as the default force-field in the HyperChem software. It has been reported that MM^+ force-filed will provide reasonable accurate conformational energies for the vast majority of organic compounds [18]. Finally, the format of each compound has been changed to the pdb, and all compounds were saved to a ligand database using Molecular Operating Environment (MOE) 2008.10 software.

Drug-likeness and Pharmacokinetic Properties Analysis

The Simplified Molecular-Input Line-entry System (SMILES) format of all compounds are made in ChemDraw ultra 8 software, and drug-likeness was investigated using various rules, including Lipinski [20], Veber [21], Egan [22], Muegge [23], and Ghose [24]. Furthermore, the pharmacokinetics of the ligands was analyzed using the SwissADME online server [25].

Target proteins

To investigate the anti-MDR effects of *Salinispora*-derived compounds, three proteins have been selected as targets: P-gp, MRP1, and BCRP. As mentioned before,

Table 1. List of *Salinispora*-derived compounds

Species	Metabolites	Biosynthetic Pathway	Novelty	Bioactivity (Target)	Ref.
S. tropica	Salinosporamide A	PKS-NRPS	New	Proteasome	[52]
	Sporolide A	ePKS	New	Reverse transcriptase	[53]
	Salinilactam	Type I PKS	New	ND	[8]
	Sioxanthin	Terpene	New	ND	[54]
	Antiprotealide	PKS-NRPS	New	Proteasome	[55]
S. pacifica	Pacificanone A	Type I PKS	New	ND	[56]
	Salinipyrone A	Type I PKS	New	ND	[56]
	Cyanosporoside A	PKSe	New	ND	[41]
	Lomaiviticin A	Type II PKS	New	Cytotoxic	[48]
	Enterocin	Type II PKS	Known	Antibiotic	[57]
S. arenicola	Saliniketal A	Type I PKS	New	Ornithine decarboxylase	[58]
	Arenicolide A	Type I PKS	New	ND	[59]
	Saliniquinones A-F	Type II PKS	New	Cytotoxic	[38]
	Cyclomarin A	NRPS	Known	Anti-inflammatory	[60]
	Cyclomarazine	NRPS	New	ND	[39]
	Arenimycin	NRPS	New	Antibiotic	[61]
	Arenamide A	Type II PKS	New	Anti-inflammatory (NF-κB)	[62]
	Staurosporine	Alkaloid	Known	Protein kinase	[63]
	Isopimara-8,15-dien-19-ol	Terpene	New	ND	[64]
	Rifamycin B	Type I PKS	Known	RNA polymerase	[65]
	Mevinolin	PKS	Known	HMG-CoA reductase	[66]
St, *Sa*, and *Sp*	Desferrioxamine B	NRPS	Known	Iron chelator	[67]
	Lymphostin	NRPS-PKS	Known	Immunosuppressant	[68]

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Polyketide Synthase (PKS); Non-Ribosomal Peptide Synthase (NRPS); 3Enediyne Polyketide Synthase (ePKS); 4Not Determined (ND); 5Nuclear Factor Kappa B (NF-κB); 63-hydroxy-3-methylglutaryl-CoA (HMG-coA)

ABC transporters have nucleotide and substrate binding sites. An ABC transporter can be inhibited by suppressing ATP hydrolysis in the nucleotide-binding site or inhibiting drug binding in the transmembrane substrate-binding domain. Therefore, to evaluate anti-MDR effects on the compounds, docking simulations have been performed in both nucleotide and substrate binding sites of every protein. Crystal coordinate of P-gp (6c0v for ATP binding site and 6qex for substrate binding site), BCRP (6hzm for ATP binding site and 6eti for substrate binding site), and MRP1 (2cbz for ATP binding site and 5uja for substrate binding site) were retrieved from Protein Data Bank at the Research Collaboratory for Structural Bioinformatics (RCSB) [26].

Molecular docking study

Molecular docking studies have performed using MOE 2008.10 software based on the previous studies [27]. Briefly, the binding sites have set as all atoms within 15

Table 2. Physicochemical, pharmacokinetics, and drug-likeness of the compounds

Compounds	Physicochemical Properties		Lipo.	Water Sol.	Pharmacokinetics				Drug Likeness (Violation)
	MW	TPSA	CLog P		GI Abs.	BBB Per.	P-gp Sub.	CYP Inh.	
Antiprotealide	275.73	75.63	1.2	Soluble	High	No	No	None	None
Cyclomarazine	397.51	74.57	2.47	Soluble	High	No	Yes	None	None
Enterocin	444.39	163.73	-0.05	Soluble	Low	No	No	CYP3A4	Veber, Egan, Muegge, and Ghose
Isopimara-8,15-dien-19-ol	302.49	20.23	5.11	Moderate	High	Yes	No	CYP2C19, CYP2C9	Lipinski, Ghose, and Muegge
Lymphostin	295.27	107.53	0.13	Soluble	High	No	Yes	CYP1A2	None
Pacificanone A	322.48	57.53	3.79	Moderate	High	Yes	No	CYP2D6, CYP3A4	None
Saliniketal A	395.53	102.01	2.51	Soluble	High	No	Yes	CYP3A4	None
Salinilactam	469.61	110.02	2.59	Moderate	High	No	Yes	CYP3A4	Ghose
Salinipyrone A	292.37	70.67	3.22	Soluble	High	Yes	No	None	None
Saliniquinone A	404.37	117.34	2.38	Soluble	High	No	No	CYP3A4	None
Saliniquinone B	406.38	117.34	2.53	Moderate	High	No	Yes	CYP2C9, CYP3A4	None
Saliniquinone C	440.38	125.04	2.5	Moderate	High	No	No	CYP2C9, CYP3A4	None
Saliniquinone D	390.39	104.81	3.18	Moderate	High	No	No	CYP1A2, CYP2C9, CYP3A4	None
Saliniquinone E	390.39	104.81	3.12	Moderate	High	No	No	CYP2C9, CYP3A4	None
Saliniquinone F	390.39	104.81	3.11	Moderate	High	No	No	CYP2C9, CYP3A4	None
Salinosporamide A	313.78	75.63	1.72	Soluble	High	No	Yes	None	None
Cyanosporoside A	417.84	120.01	1.28	Soluble	High	No	Yes	None	None
Staurosporine	466.53	69.45	3.03	Moderate	High	Yes	Yes	CYP2C19, CYP3A4, CYP2D6	Ghose, Muegge
Lovastatin	404.54	72.78	3.88	Moderate	High	Yes	No	CYP2C9, CYP3A4	None

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Lipophilicity (Lipo); Molecular Weight (MW); Topological Polar Surface Area (TPSA); Gastrointestinal Absorption (GI Abs); Blood-brain Barrier Permeability (BBB Per); P-glycoprotein Substrates (P-gp Sub); Cytochrome P Inhibition (CYP Inh.).

Å of co-crystallized ligands. In protein preparation, following the elimination of all water molecules, hydrogen atoms and partial charges were added using Protonate 3D application of MOE 2008.10 with all default options. The docking procedure was performed based on the standard protocol implemented in the MOE 2008.10 software using flexible ligand and rigid receptor docking. Triangle matcher as placement algorithm combined with London dG scoring function was used to set the docking simulation. We also used the force field in the refinement to energy minimize the docked poses in the binding pockets and the rotating bonds option to flexible ligand-rigid receptor docking. Validation of docking procedure has been evaluated by re-docking of co-crystalized ligand and calculating Root-Mean-Square Deviation (RMSD). The top-score docking pose of each compound was selected for further ligand-receptor interaction analysis using the Lig-X module of MOE 2008.10 software.

Results

Ligands, drug-Likeness, and ADME investigations

Table 2 presents the results of drug-likeness and ADME investigations for *Salinispora*-derived compounds, which were involved in this study. In silico calculation of physicochemical and pharmacokinetic properties and drug-likeness analysis, ligands were per-

Table 3. Percentage of residues in the Ramachandran plot analysis

Target Proteins PDB IDs	Percentage of Residues			
	Most Favored Regions	Additional Allowed Regions	Generously Allowed Regions	Disallowed Regions
6c0v	92	7.9	0.1	0
6qex	88.4	11.4	0.2	0
2cbz	91	9	0	0
5uja	92.6	6.8	0.3	0.3
6eti	87.3	12.6	0.1	0
6hzm	90.1	9.9	0	0

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formed using the SwissADME server [25]. SwissADME server investigates drug-likeness by various rules, including Lipinski [20], Veber [21], Egan [22], Muegge [23], and Ghose [24]. Table 2 presents physicochemical, pharmacokinetic, and drug-likeness properties of 19 compounds that have passed SwissADME drug-likeness filters and showed no violation based on at least one of the above-mentioned rules. Among 19 compounds presented in Table 2, enterocin violated Veber, Egan, Muegge, and Ghose rules because of WLOGP<0.4 and TPSA>140, isopimara-8,15-dien-19-ol violated Lipinski (MLOGP>4.1), Ghose (WLOGP>5.6), and Muegge rules (XLOGP3>5, Heteroatoms<2), staurosporine violated Ghose (MR>130) and Muegge rules (Ring>7) and salinilactam violated Ghose rule (MR>130, atoms>70). Nine compounds of sioxanthin, sporolide A, arenicolide A, arenamide A, arenimycin, cyclomarin A, desferrioxamine B, lomaiviticin A, and rifamycin B have violated all of the rules and did not represent appropriate drug-likeness, so they were excluded from the docking studies.

About pharmacokinetic properties, almost all compounds represented promising oral bioavailability. Enterocin was the only compound with low GI absorption. Other pharmacokinetic properties, including blood-brain-barrier permeability and cytochromes P450 (CYPs) inhibition, have also been summarized in Table 2.

Protein validation

Validation of protein structures has been investigated using Ramachandran plot. In this plot, psi and phi angles of amino acid residues are calculated, and the distribution of amino acids in the 3D structure of the protein is divided into the energetically allowed and disallowed regions [28]. Amino acids in disallowed regions lead to steric hindrance or clashes between atoms and are not favored. Analysis of protein structures has been done using

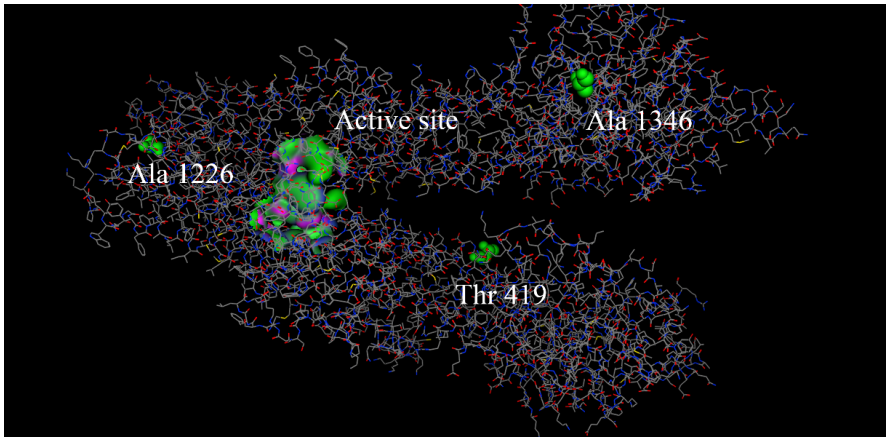

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Figure 1. 3D structure of MRP1 (PDB ID: 5uja). Active site and position of three outlier residues (Thr419, Ala 1226, and Ala1346) based on the Ramachandran plot analysis have been drawn in green space-filling mode

As illustrated, outlier residues are not involved in the active site of the protein and do not interfere with the docking procedure.

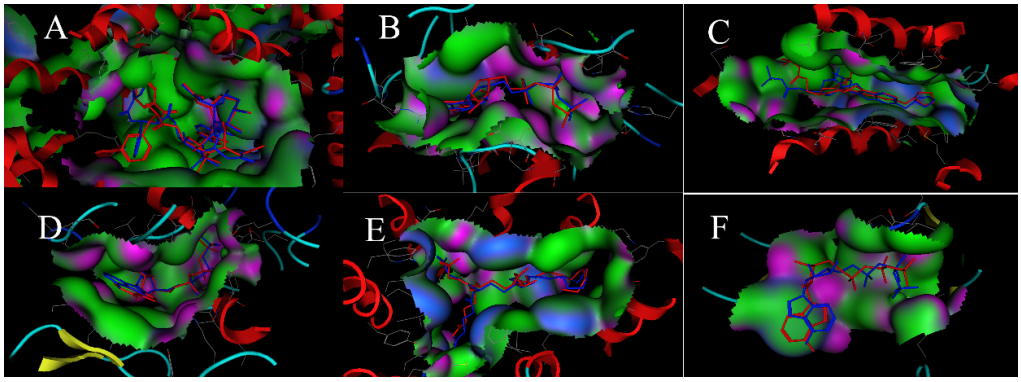

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Figure 2. Docked co-crystallized ligands (Blue) superimposed on the co-crystallized one (Red)
In the active site of P-gp (A: substrate binding site, B: ATP binding site), BCRP (C: substrate binding site, D: ATP binding site), and MRP1 (E: substrate binding site, F: ATP binding site). As shown docked ligands could appropriately simulate the co-crystalized one (RMSD<2 Å in all target proteins) demonstrating validity of docking analysis.

PDBsum web-based tool [29]. As mentioned in Table 3, all residues of most protein structures are distributed in the allowed region. Actually, PDBsum divides amino acids into 4 categories in the Ramachandran plot: most favored areas, additionally allowed regions, generously allowed regions, and disallowed regions. 6c0v, 6qex, 2cbz, 6eti, and 6hzm have demonstrated 92%, 88.4%, 91%, 87.3%, and 90.1% of amino acids in the most favored region, respectively, and no amino acid in the disallowed area have reported for these structures. Three amino acids (0.3%) of the MRP1 protein structure (PDB code: 5uja) are in the disallowed region. These are Thr419, Ala 1226, and Ala1346. Since the active site of this protein was defined based on the co-crystalized ligand in the substrate-binding site of the protein, these amino acids are not involved in the active site and do not interfere with the docking procedure (Figure 1).

Docking studies

Docking studies were utilized to evaluate the anti-MDR potential of *Salinispora*-derived compounds. Since ABC-transporters have two binding sites of ATP-binding site and substrate/drug binding site. All compounds were docked to the ATP-binding site and substrate/drug binding site of P-gp, BCRP, and MRP1 proteins. Validation of the docking process has been done by re-docking of co-crystalized ligand found in the crystal structures of the proteins. As illustrated in Figure 2, the top score pose of the docked ligands has shown a similar orientation with the co-crystallized one in the binding site of the proteins demonstrating the validity of the docking process. The RMSD between docked ligands and co-crystallized ones were lower than 2 Å for all target proteins (Figure 2). Table 4 illustrates three compounds with the lowest free binding energy following docking into each target protein. As demonstrated in Table 4, saliniquinones seem the most potent ligands inhibiting various MDR pumps in the ATP and substrate binding sites. For example, saliniquinone C developed free binding energy of 21.85 kcal/mol in the P-gp substrate-binding site. In comparison, verapamil, as a known P-gp inhibitor, developed free binding energy of 23.02 kcal/mol when docked using a similar protocol (data not shown), demonstrating appropriate potency of saliniquinone C. Salinilactam were also effective in the MRP1 and P-gp substrate-binding site, but it should be mentioned that based on the ADME studies in the SwissADME server, salinilactam is a P-gp substrate and it may not be a right candidate as P-gp inhibitor. Furthermore, cyclomarazine and cyanosporoside A are also demonstrated low free binding energy in the MRP1 substrate and ATP binding site, respectively.

The mode of interaction between selected ligands and target proteins have been investigated using the LigX module of MOE software. Figure 3 shows a 2D diagram of interactions between the most favorable ligand in the target proteins. As illustrated, the most prevalent interaction is hydrogen bonds. In addition to hydrogen bonds, the phenol ring of saliniquinone A demonstrated a π-π interaction with Tyr 401 in the P-gp ATP binding site (Figure 3A). Saliniquinone D also developed π-π interactions between benzyl alcohol ring and Phe 439 residue of substrate binding site of BCRP (Figure 3D). Cyclomarazine is another compound creating π-π interactions between pyrrole ring and Phe 594 in MRP1 substrate binding site

Table 4. List of compounds with the best binding energy in the target proteins

Target Protein/Site	Three Compounds with the Best Binding Energy	Binding Energy
P-gp substrate binding site	Salinilactam	-21.9308
	Saliniquinone C	-21.8489
	Cyclomarazine	-21.4831
P-gp ATP binding site	Saliniquinone A	-47.5554
	Saliniquinone D	-44.7063
	Saliniquinone C	-41.3541
BCRP substrate binding site	Saliniquinone D	-35.1877
	Saliniquinone F	-33.9537
	Saliniquinone A	-33.9383
BCRP ATP binding site	Saliniquinone C	-18.4901
	Saliniquinone E	-17.835
	Saliniquinone D	-17.0762
MRP1 substrate binding site	Salinilactam	-27.1068
	Cyclomarazine	-24.0158
	Saliniquinone F	-23.9899
MRP1ATP binding site	Cyanosporoside A	-65.8714
	Saliniquinone C	-35.509
	Saliniquinone D	-32.3809

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(Figure 3F). Overall, it seems that developing hydrogen bonds and π-π interactions may be crucial for ABC transporters inhibitors.

Discussion

The role of ABC transporters in the MDR and subsequently the failure of chemotherapy has been reported in various studies [30]. These ABC transporters are involved in protecting cells by exporting xenobiotics from a cell, but their overexpression/overactivation leads to the development of MDR in cancer. P-gp is the most prevalent ABC transporter, which its role in MDR has been demonstrated in human studies [31, 32]. Furthermore, BCRP and MRP1 have been frequently reported as a cause of MDR in animal and in vitro studies [33-35]. Overexpression of BCRP has also been associated with poor prognosis in acute myeloid leukemia patients [36]. Unfortunately, clinical trials investigating P-gp inhibitors have failed due to the adverse effects and or low efficacy; thus, the recommendation of a new class of ABC transporters inhibitors, especially P-gp inhibitors, is essential to circumvent MDR and improve the efficacy of chemotherapy [4, 37].

We have screened drug-likeness and anti-MDR potential of secondary metabolites derived from marine actinomycete genus *Salinispora* using in silico approaches. Saliniquinones have demonstrated promising effects as P-gp, BCRP, and MRP1 inhibitors. Furthermore, cyclomarazine A and cyanosporoside A may also be an appropriate candidate as MRP-1 inhibitors. Saliniquinones are anthraquinone-γ-pyrones compounds isolated from *Salinispora arenicola*. Saliniquinone A has exerted cytotoxic effects in the human colon adenocarcinoma cell line (HCT-116) with an IC50 of 9.9×10^{-9} M [38]. Cyclomarazine A, which is demonstrated anti-MRP1 potential in this study, is a diketopiperazine dipeptide

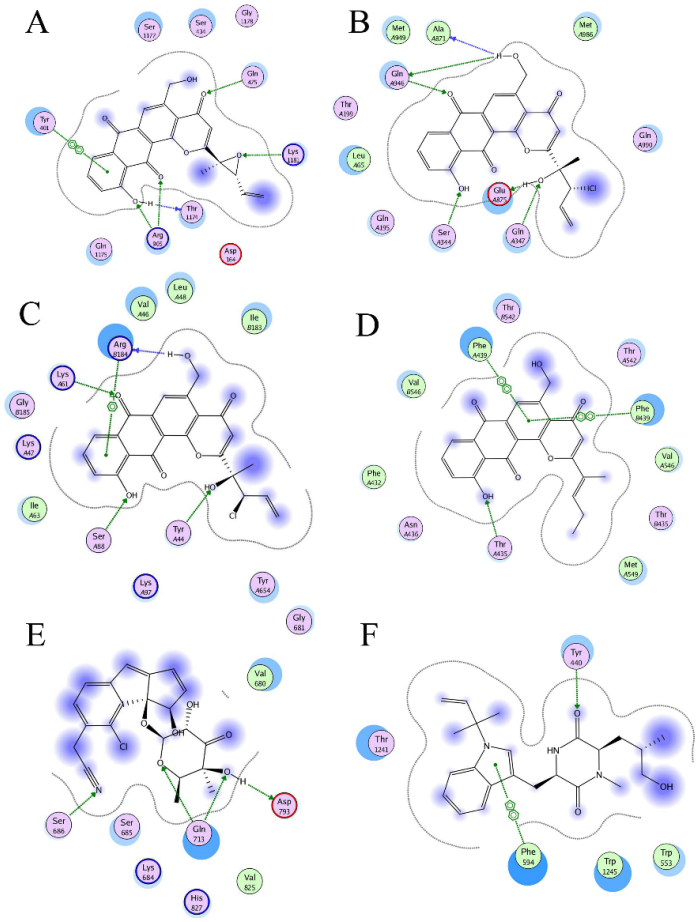

Figure 3. 2D diagram of interactions between the ligands and target proteins

These ligands have been chosen based on the lowest free binding energy and are not P-gp substrates. A represents saliniquinone A in 6c0v, B represents saliniquinone C in 6qex, C represents saliniquinone C in 6hzm, D represents saliniquinone D in 6eti, E represents cyanosporoside A in 2cbz and F represents cyclomarazine in 5uja. Doted green and royal blue arrows illustrate H-bonds, and the dotted green line with aromatic rings in the middle represents π-π interactions.

isolated and extracted from S. arenicola. It is an intermediate compound in the biosynthesis of cyclic heptapeptide compounds called cyclomarins representing anti-mycobacterial effects [39, 40]. Cyanosporoside A is another compound demonstrating anti-MRP1 potential based on our docking studies. It is a glycoside compound derived from *Salinispora pacifica* [41]. To the best of our knowledge, the biological effects of cyclomarazine A and cyanosporoside A have not been studied. Since cyclomarazine A and cyanosporoside A have passed all of the drug-likeness filters and demonstrated appropriate pharmacokinetic properties in the SwissADME server, it is worth investigating their biological effects.

Despite a few decades of extensive investigations, actinomycetes are still a rich source of novel bioactive metabolites. Investigating deserted and faraway places, especially marine environments, have led to the discovery of new actinomycetes producing new bioactive metabolites [42]. Although common genera of terrestrial actinomycetes, including Streptomyces, have been isolated from marine samples but at least 5 marine-specific actinomycete genera have been reported demonstrating the potential of marine environments in isolation of taxonomically new actinomycete genera [43-47]. *Salinispora* is a marine actinomycete genera demonstrating a model microorganism producing novel metabolites [9]. *S. tropica*, *S. arenicola*, and *S. pacifica* are three species of the *Salinispora* genus. These actinomycetes are mostly isolated from sediment, while some studies have also isolated *Salinispora spp*. from an ascidian [48], seaweeds [49], and marine sponges [50, 51]. The genus is known as a rich source of new and bioactive metabolites. Genome sequencing and bioinformatics analysis revealed about 10% of the genome of *Salinispora spp*. is dedicated to metabolite production [8]. Table 1 presents metabolites, which have been derived from *Salin-*

ispora spp. Among 23 compounds, 16 compounds are new. Salinosporamide A, also known as marizomib, is a potent proteasome inhibitor isolated from S. tropica. Its anticancer effects have been extensively studied, and it is in phase III clinical trial for the treatment of newly-diagnosed glioblastoma (www.clinicaltrials.gov) [52].

Based on the novelty and appropriate bioactivity, we investigated the anti-MDR potential of the *Salinispora*-derived metabolites using a docking approach. Physicochemical, pharmacokinetic, and drug-likeness of the compounds were investigated, and compounds with proper properties were involved in the docking studies. Docking of compounds in a substrate and ATP binding site of ABC transporters, including P-gp, BCRP, and MRP1 as a cause of cancer MDR revealed saliniquinones, cyclomarazine, and cyanosporoside A that can inhibit ABC transporters and reverse MDR. Further in vitro and in vivo studies are warranted to evaluate the results of this in silico study.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed of the purpose of the research and its implementation stages.

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Conflict of interest

The author declared no conflict of interest.

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