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IN SILICO MOLECULAR DOCKING STUDIES OF OVARIAN EXTRACT SINGKARAK LAKE PUFFERFISH (*TETRAODON LEIURUS*) AGAINST BREAST CANCER

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ABSTRACT

Targets the development of breast cancer chemoprevention by blocking the expression of Na_V 1.5 channels and NHE, decreasing the expression of Bcl-2 and Bcl-X_L, and increasing the expression of Bax. The ovarian extract Singkarak Lake Pufferfish (*Tetraodon leiurus*) has the potential as chemoprevention in breast cancer cell lines (MCF-7). This study aimed to analyze the interaction of STX, neoSTX, and dcSTX ligands against Na_V1.5, NHE, Bcl-2, Bcl-X_L, and Bax receptors. This study used the molecular docking method using STX, neoSTX, and dcSTX ligands against Na_V1.5, NHE, Bcl-2, Bcl-X_L, and Bax receptors. The result showed that the STX ligand has a more stable interaction with Na_V1.5, Bcl-2, Bcl-X_L, and Bax receptors with ΔG values of -8.72, -7.32 -6.86, and -6.31 kcal/mol compared to neoSTX and dcSTX ligands. Furthermore, the neoSTX ligand has a more stable interaction with the NHE receptor with an ΔG value of -7.5 kcal/mol compared to the STX and dcSTX ligands. This study shows that the ovarian extract of Singkarak Lake Pufferfish (*Tetraodon leiurus*) has the potential to be developed as cancer chemoprevention.

Keywords: Autodock, Breast cancer, *In silico*, Saxitoxin, Pufferfish

INTRODUCTION

Cancer cells have the characteristics of invasion and metastasis throughout the body and grow uncontrollably (**Shan *et al.*, 2019**). Invasion and metastasis are caused by the expression of Na_V channels which increase the intracellular Na^+ concentration, thereby activating NHE (sodium hydrogen exchanger) and NCX (sodium-calcium exchanger) in cancer cells (**Angus and Ruben 2019**). Breast cancer cells specifically express Na_V1.5 channels (**Gradek *et al.*, 2019**), so inhibition of Na_V1.5 channel expression is a target in the treatment of breast cancer (**Luo *et al.*, 2020**).

In addition to the characteristics of invasion and metastasis, cancer cells also obtain immortality by escaping programmed cell death (apoptosis) (**Chen *et al.*, 2018**) caused by overexpressing anti-apoptotic protein such as Bcl-2 (B-cell lymphoma 2) (**Li *et al.*, 2017**) and Bcl-X_L (B-cell lymphoma-extralarge) (**Trisciuoglio *et al.*, 2017**), and also underexpression proapoptotic protein such as Bax in cancer cells (Bcl-2 associated X-protein) (**Wang *et al.*, 2019**). Therefore, decreased expression of anti-apoptotic proteins and increased expression of proapoptotic proteins (**Pistritto *et al.*, 2016**) are targeted in the development of cancer chemoprevention (**Jagadeeshan *et al.*, 2018**).

Cancer chemoprevention is the process of using chemicals, either natural or synthetic to prevent cancer, which is a complex interplay of a multitude of biological processes (**Nahar and Sarker, 2020**). Cancer chemoprevention using natural compounds has minimal side effects and toxicity compared to synthetic (**Ko and Moon, 2015**). A natural compound such as toxin Pufferfish (Tetrodotoxin (TTX)/saxitoxin (STX) can be used as cancer chemoprevention. Pufferfish in Lake Singkarak is a toxic fish, with the scientific name is *Tetraodon leiurus.* **Kungsuwan *et al.* (1997)** reported that the ovaries of *Tetraodon leiurus* from Thailand contained the toxins STX, neoSTX (neosaxitoxin), and dcSTX (decarbamoyl saxitoxin). **Hanif *et al.* (2021)**, ovarian extract Singkarak Lake Pufferfish (*Tetraodon leiurus*) has potential as cancer chemoprevention in MCF-7 cells.

The development of cancer chemoprevention compounds can be carried out by several tests, namely *in vitro, in vivo* (**Singh *et al.*, 2014**), and *in silico* (**Chen *et al.*, 2012**). *In vitro* tests aim to evaluate various biological phenomena in certain cells in a controlled environment and free from systemic variations (**Arango *et al.*, 2013**). *In vivo* tests are used to evaluate the biological response of living organisms to given chemoprevention (**Haas *et al.*, 2012**). *In silico* test with molecular docking to predict the interaction between compounds (ligands) and protein receptors with computational procedures (**Meng *et al.*, 2011**). Molecular docking has been shown to contribute to cancer progression (**Edelman *et al.*, 2009**).

Research on the Na_V1.5, NHE, Bcl-2, Bcl-X_L, and Bax proteins induced by the ovarian extract Singkarak Lake Pufferfish (*Tetraodon leiurus*) in breast cancer cells has not been carried out. In this study, *in silico* test with molecular docking using the STX, neoSTX, and dcSTX ligands against Na_V1.5, NHE, Bcl-2, Bcl-X_L, and Bax receptors. Therefore, it is necessary to conduct this research to utilize toxins from the ovarian extract Singkarak Lake Pufferfish (*Tetraodon leiurus*) as cancer chemoprevention.

MATERIAL AND METHODS

Ligand preparation

The ligands used in this study were Pufferfish toxin compounds obtained from the PubChem database, namely STX (CID: 56947150), neoSTX (CID: 135562690), and dcSTX (CID: 21117969) (Figure 1). The SDF ligand file format was converted into a PDB file using Discovery Studio Visualizer 2021.

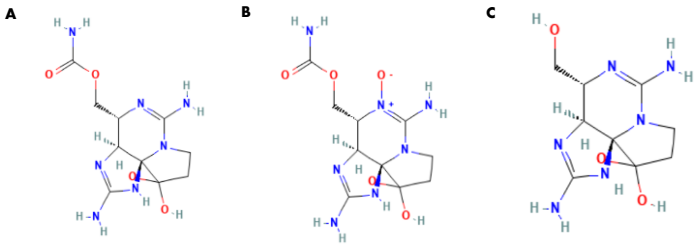

Figure 1 Ligands 2D Interaction. A. STX; B. neoSTX; C. dcSTX

Receptor preparation

The receptors used in this study were obtained from the Protein Data Bank database, namely Na_V1.5 channel (PDB ID: 4DJC), NHE (PDB ID: 2E30), Bcl-2 (PDB ID: 4IEH), Bcl-X_L (PDB ID: 4QVF), and Bax (PDB ID: 1F16). The macromolecular crystal structure obtained was prepared using Discovery Studio Visualizer 2021 and MGLTools 1.5.6 equipped with AutoDock 4.2.6 by removing water molecules, native ligands, and adding polar hydrogen atoms and Kollman partial charge.

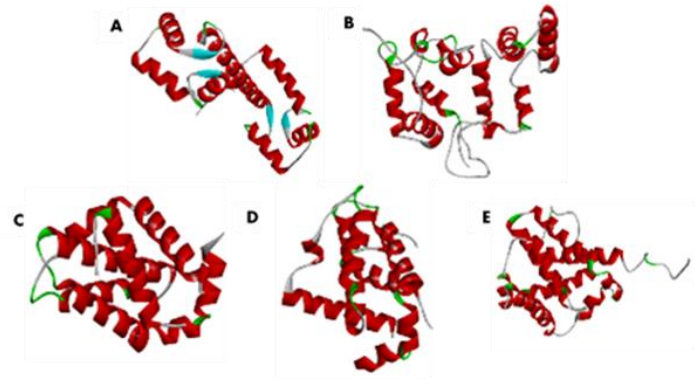

Figure 2 Crystal structure of the receptor macromolecule. A. $Na_V1.5$; B. NHE; C. Bcl-2; D. Bcl-X_L; E. Bax

Molecular docking method validation

Docking studies using the docking tool, MGL Tools 1.5.6 equipped AutoDock 4.2.6 with the re-docking method and RMSD (Root Mean Square Deviation) value < 2Å. The distance between the surface of the receptor and the ligand is limited by a maximum radius of 0.375Å, a grid box size of 126Å × 126Å × 126Å in the x, y, and z dimensions, and the Lamarckian Genetic Algorithm method with 100 conformations.

Molecular docking results visualization

The molecular docking results were visualized using PyMOL for molecular surface and BIOVIA Discovery Studio Visualizer for docked complex, 3D interaction, and 2D interaction.

RESULTS AND DISCUSSION

In silico test with molecular docking, results are displayed based on the interaction of STX, neoSTX, and dcSTX ligands against $Na_V1.5$, NHE, Bcl-2, Bcl-X_L, and Bax receptors.

$Na_V1.5$ receptor

The results of the molecular docking of the test ligands to the $Na_V1.5$ receptor with several parameters (Table 1) and the interaction between the test ligand to the $Na_V1.5$ receptor (Fig. 3).

Table 1 Molecular docking of STX, neoSTX and dcSTX ligands with $Na_V1.5$ receptor

Ligands	ΔG (kcal/mol)	KI (μM)	Interacting Residues
STX	-8.72	406.57	Glu121; 124; 128
neoSTX	-8.42	673.3	Glu124; 128, Met145
dcSTX	-7.71	2.22	Glu121; 124; 128

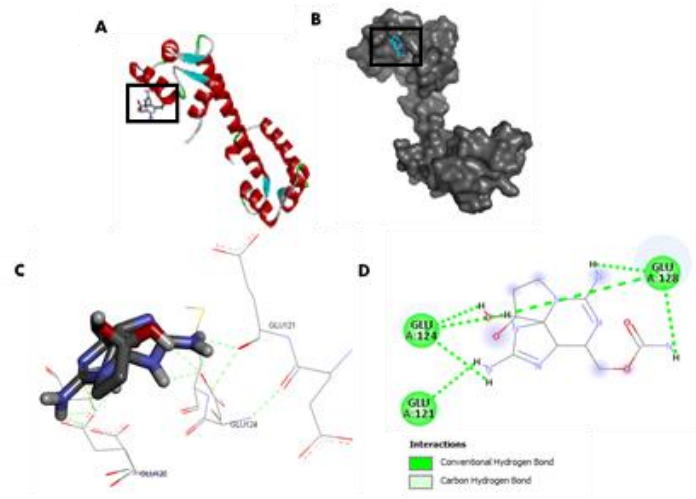

Figure 3 STX ligand interactions with $Na_V1.5$ receptor. A. Docked complex; B. Molecular Surface; C. 3D Interaction; D. 2D Interaction

Values of ΔG, KI, and interacting residues of STX, neoSTX, and dcSTX ligands with $Na_V1.5$ receptor (Table 1). The lowest ΔG value for STX ligand was -8.72 kcal/mol. The smaller value of ΔG, the more stable the interaction of the ligand with the receptor (**Pantsar and Poso, 2018**). Based on the ΔG value, the interaction of the STX ligand with the $Na_V1.5$ receptor was more stable than the interaction of the neoSTX and dcSTX ligands. The lowest KI value for the dcSTX ligand was 2.22 μM. The smaller value of KI indicates the smaller concentration of the ligand required to inhibit the target receptor (**Kim *et al.*, 2021**). Based on the KI value, the interaction of the dcSTX ligand was more effective in inhibiting the $Na_V1.5$ receptor in a small concentration than the STX and neoSTX ligands.

The amino acid residues involved in these interactions have hydrogen bonds, hydrophobic interactions, and electrostatic interactions. The STX ligand has seven conventional hydrogen bonds (Glu121; 124; 128) (Fig. 3). The neoSTX ligand has three conventional hydrogen bonds (Glu124; 128), one alkyl hydrophobic interaction (Met145), and one attractive charge electrostatic interaction (Glu128). The dcSTX ligand has five hydrogen bonds consisting of four conventional hydrogen bonds (Glu121; 124; 128) and one carbon-hydrogen bond (Glu124). Based on the interaction of the ligand with the receptor, the STX ligand has a more stable binding to the $Na_V1.5$ receptor than the neoSTX and dcSTX ligands. Hydrogen bonding is the main bond that provides stability to the protein structure, the more hydrogen bonds, the more stable the ligand bond with the receptor (**Hubbard and Kamran, 2010**). Based on the value of ΔG, KI and molecular interactions on STX, neoSTX, and dcSTX ligands have the potential as inhibitors of $Na_V1.5$ activity to suppress invasion in cancer cells.

NHE receptor

The results of the molecular docking of the test ligands to the NHE receptor with several parameters (Table 2) and the interaction between the test ligand to the NHE receptor (Fig. 4).

Table 2 Molecular docking of STX, neoSTX and dcSTX ligands with NHE receptor

Ligands	ΔG (kcal/mol)	KI (μM)	Interacting Residues
STX	-7.16	5.6	Arg132, Asp127; 168, Glu128, Ser131
neoSTX	-7.5	3.19	Asp39;50;76, Gln77, Glu49, Lys40, Thr45
dcSTX	-6.87	9.21	Asp39;49;50;76, Lys40, Thr45

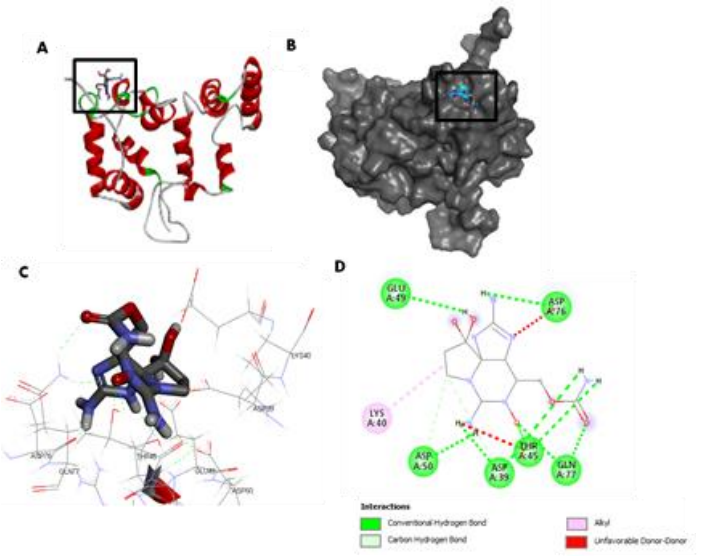

Figure 4 neoSTX ligand interactions with NHE receptor. A. Docked complex; B. Molecular Surface; C. 3D Interaction; D. 2D Interaction

Molecular interactions at NHE receptor, the lowest ΔG and KI values for the neoSTX ligand were -7.5 kcal/mol and 3.19 μM (Table 2). Based on ΔG and KI values, the neoSTX ligand interaction was more stable and more effective in inhibiting NHE activity compared to STX and dcSTX ligands. The amino acid residues involved in these interactions have hydrogen bonds and hydrophobic interactions. The STX ligand has ten hydrogen bond interactions consisting of eight conventional hydrogen bonds (Arg132, Asp127; 168, Glu128, and Ser131) and two carbon-hydrogen bonds (Asp168 and Ser131). The neoSTX ligand has eleven hydrogen bonds interactions is nine conventional hydrogen bonds (Asp39;50;76, Gln77, Glu49, Thr45) and two carbon-hydrogen bonds (Asp39;50), one alkyl hydrophobic interaction (Lys40) and there is an unfavorable donor-donor bond (Asp76 and Thr45) (Fig. 4). Unfavorable donor-donor bonds affect the ligand-receptor complex and reduce the stability of the complex because this type of bond exhibits repulsive forces that occur between two molecules and atoms (**Dhorajiwala *et al.*, 2019**). The dcSTX ligand has seven hydrogen bonds is six conventional hydrogen bonds (Asp39;49;50;76 and Thr45) and one carbon-hydrogen bond (Asp50), and one alkyl hydrophobic interaction (Lys40). Based on the interaction of the ligand with the receptor, the neoSTX ligand has a more stable

binding to the NHE receptor than the STX and dcSTX ligands. Based on the value of ΔG, KI, and molecular interactions on STX ligands, neoSTX, and dcSTX have the potential as inhibitors of NHE activity.

Bcl-2 receptor

The results of the molecular docking of the test ligands to the Bcl-2 receptor with several parameters (Table 3) and the interaction between the test ligand to the Bcl-2 receptor (Fig. 5).

Table 3 Molecular docking of STX, neoSTX and dcSTX ligands with Bcl-2 receptor

Ligands	ΔG (kcal/mol)	KI (μM)	Interacting Residues
STX	-7.32	4.32	Ala59, Asp62, Gln58, Gly162, Leu160, Tyr161
neoSTX	-6.76	11.12	Asp62, Gln58, Gly162, Thr55, Tyr161
dcSTX	-6.22	27.8	Asp62, Gln58, Gly162, Thr55, Tyr161

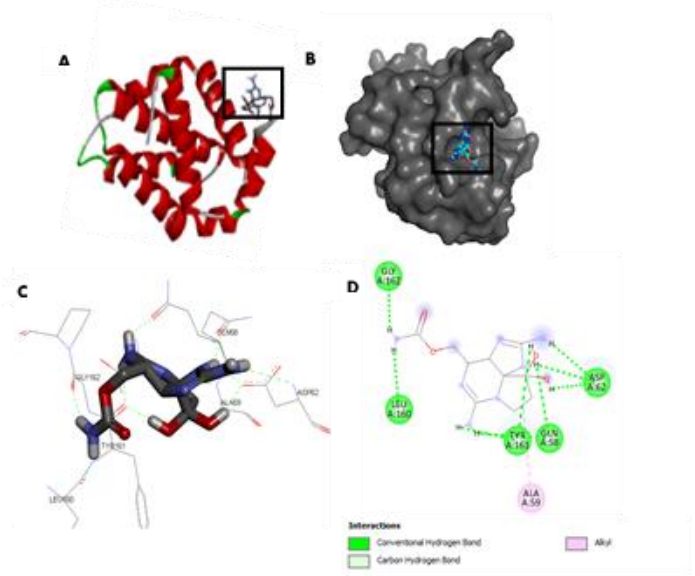

Figure 5 STX ligand interactions with Bcl-2 receptor. A. Docked complex; B. Molecular Surface; C. 3D Interaction; D. 2D Interaction

At the Bcl-2 receptor, the lowest ΔG and KI values for the STX ligand were -7.32 kcal/mol and 4.32 μM (Table 3). Based on the values of ΔG and KI, STX ligand interaction was more stable and effective in inhibiting Bcl-2 activity than neoSTX and dcSTX ligands. The amino acid residues involved in these interactions have hydrogen bonds and hydrophobic interactions. STX ligands have nine conventional hydrogen bonds (Asp62, Gln58, Gly162, Leu160, and Tyr161) and one alkyl hydrophobic interaction (Ala59) (Fig. 5). The neoSTX and dcSTX ligands have seven conventional hydrogen bonds (Asp62, Gln58, Gly162, and Thr55) and one alkyl hydrophobic interaction (Tyr161). Based on the interaction of the ligand with the receptor, the STX ligand has a more stable binding to the Bcl-2 receptor than the neoSTX and dcSTX ligands. Based on the value of ΔG, KI, and molecular interactions on STX ligands, neoSTX, and dcSTX have potential as inhibitors of Bcl-2 activity.

Bcl-X_L receptor

The results of the molecular docking of the test ligands to the Bcl-X_L receptor with several parameters (Table 4) and the interaction between the test ligand to the Bcl-X_L receptor (Fig. 6).

Table 4 Molecular docking of STX, neoSTX and dcSTX ligands with Bcl-X_L receptor

Ligands	ΔG (kcal/mol)	KI (μM)	Interacting Residues
STX	-6.86	9.33	Asn128, Asp176, Glu124, Trp169, Tyr120
neoSTX	-6.16	30.33	Glu124, His177, Trp169
dcSTX	-6.23	27.09	Asp176, Glu124, Trp169, Tyr120

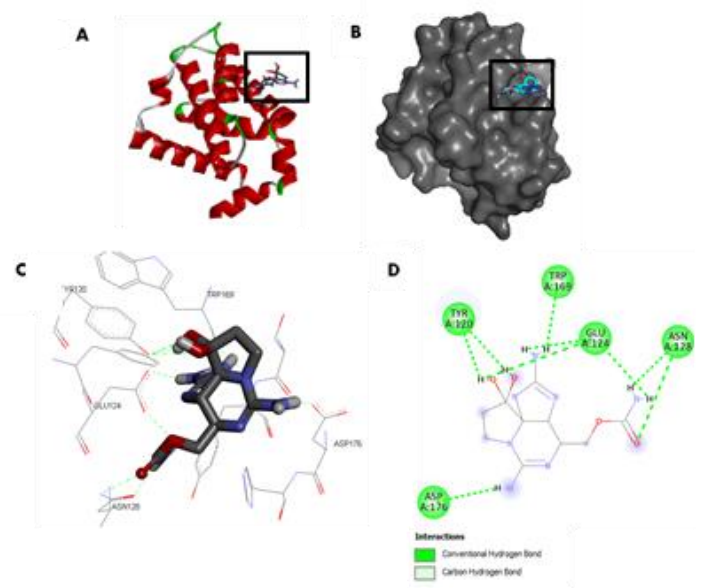

Figure 6 STX ligand interactions with Bcl-X_L receptor. A. Docked complex; B. Molecular Surface; C. 3D Interaction; D. 2D Interaction

Interactions with Bcl-X_L receptor, the lowest ΔG and KI values for STX ligands were -6.86 kcal/mol and 9.33 μM. Based on the values of ΔG and KI, the interaction of the STX ligand was more stable and more effective in inhibiting Bcl-X_L activity than neoSTX and dcSTX ligands. The amino acid residues involved in these interactions have hydrogen bonding, hydrophobic interactions, and electrostatic interactions. The STX ligand has nine conventional hydrogen bonds (Asn128, Asp176, Glu124, Trp169, and Tyr120) (Fig. 6). The neoSTX ligand has four conventional hydrogen bonds (Glu124, His177, and Trp169) and one attractive charge electrostatically interacting (Glu124). The dcSTX ligand has five conventional hydrogen bonds (Asp176, Glu124, Trp169, and Tyr120). Based on the interaction of the ligand with the receptor, the STX ligand has a more stable binding to the Bcl-X_L receptor than the neoSTX and dcSTX ligands. Based on the value of ΔG, KI, and molecular interactions on STX ligands, neoSTX and dcSTX have potential as inhibitors of Bcl-X_L activity.

Bax receptor

The results of the molecular docking of the test ligands to the Bax receptor with several parameters (Table 5) and the interaction between the test ligand to the Bax receptor (Fig. 7).

Table 5 Molecular docking of STX, neoSTX and dcSTX ligands with Bax receptor

Ligands	ΔG (kcal/mol)	KI (μM)	Interacting Residues
STX	-6.31	23.64	Ala46, Asp48, Gly39, Leu45
neoSTX	-5.6	78.98	Ala183, Asp 98; 102, Ser 184, Val180
dcSTX	-5.71	65.66	Ala183, Asp 98; 102, Val180

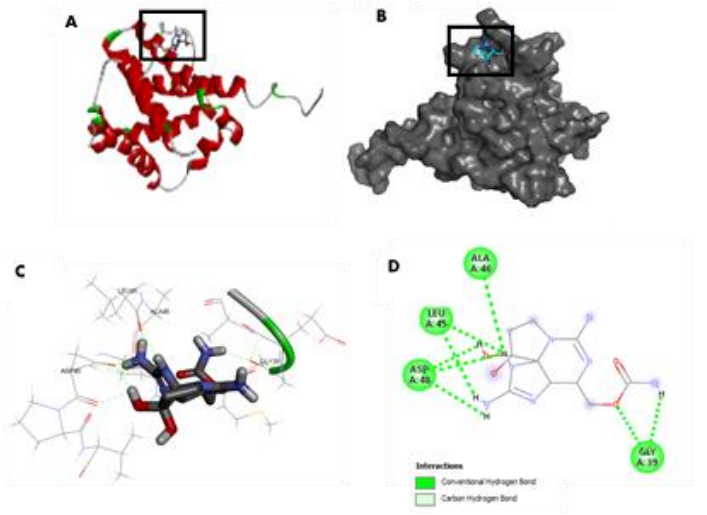

Figure 7 STX ligand interactions with Bax receptor. A. Docked complex; B. Molecular Surface; C. 3D Interaction; D. 2D Interaction

Molecular interactions at Bax receptor, the lowest ΔG and KI values for the STX ligand were -6.31 kcal/mol and 23.64 μM (Table 5). Based on the values of ΔG and KI, the STX ligand interaction was more stable and effective in activating Bax than neoSTX and dcSTX ligands. The amino acid residues involved in these interactions have hydrogen bonding, hydrophobic interactions, and electrostatic interactions. The STX ligand has eight conventional hydrogen bonds (Ala46, Asp48, Gly39, and Leu45) (Fig. 7). The neoSTX ligand has six hydrogen bonds is five conventional hydrogen bonds (Asp102, Ser184, and Val180) and one carbon-hydrogen bond (Ser184), one alkyl hydrophobic interaction (Ala183), and a salt bridge bond (Asp98). The salt bridge bond is a combination of two non-covalent interactions, namely hydrogen bonds and ionic bonds, these bonds contribute to protein stability (**Kumar and Nussinov, 2002**). The dcSTX ligand has six conventional hydrogen bonds (Asp98;102, Val180) and one alkyl hydrophobic interaction (Ala183). Based on the interaction of the ligand with the receptor, the STX ligand has a more stable binding to the Bax receptor than the neoSTX and dcSTX ligands. Based on the value of ΔG, KI and molecular interactions on STX ligands, neoSTX and dcSTX have potential as compounds that can activate Bax activity. Cytotoxic compounds can activate Bax which triggers the mechanism of apoptosis and is developed in the treatment of cancer (**Jensen *et al.*, 2019**).

Comparison of ΔG and KI values between ligands and receptors

Molecular docking results based on ΔG and KI values are shown in Table 6. Comparing the ΔG and KI values between STX, neoSTX, and dcSTX ligands with the receptors (Table 6), it was found that the STX ligand with the $Na_V1.5$ receptor had the lowest ΔG value of -8.72 kcal/mol. Based on the ΔG value, the interaction of STX ligand with the $Na_V1.5$ receptor has a more stable interaction than the interaction of the neoSTX and dcSTX ligands with the $Na_V1.5$ receptor and also the interaction between the STX, neoSTX, and dcSTX ligands with the NHE, Bcl-2, Bcl-XL, and Bax receptors. On the KI value, the interaction of dcSTX ligand with the $Na_V1.5$ receptor had the lowest KI value of 2.22 μM. Based on the KI value, the interaction of the dcSTX ligand was more effective in inhibiting the $Na_V1.5$ receptor in a small concentration than the STX and neoSTX ligands and also the interaction between the STX, neoSTX, and dcSTX ligands with the NHE, Bcl-2, Bcl-XL, and Bax receptors. The mechanism of action of STX and STX derivative compounds that inhibit and block the action of Na_V channels (**Walker *et al.*, 2012**).

Table 6 Comparison of ΔG and KI values between ligands and receptors

Receptors	STX		neoSTX		dcSTX	
	ΔG (kcal/mol)	KI (μM)	ΔG (kcal/mol)	KI (μM)	ΔG (kcal/mol)	KI (μM)
$Na_V1.5$	-8.72	406.57	-8.42	673.3	-7.71	2.22
NHE	-7.16	5.6	-7.5	3.19	-6.87	9.21
Bcl-2	-7.32	4.32	-6.76	11.12	-6.22	27.8
Bcl- X_L	-6.86	9.33	-6.16	30.33	-6.23	27.09
Bax	-6.31	23.64	-5.6	78.98	-5.71	65.66

The main component of Pufferfish toxin (TTX/STX) is the guanidine structure that interacts with the carboxylate group on the Na_V channel so that it can block the Na_V channel (**Mahdavi and Kucuyak, 2015**). The STX binding site is located in the α subunit, site 1 is formed by four P-loop (**Ruiz and Kraus, 2015**), the subunit has four homologous domains (DI-DIV) arranged to form a symmetrical channel (**Sheets *et al.*, 2015**). STX binds to selectivity filter residues in the DEKA ring (Asp-Glu-Lys-Ala) which plays an important role in the selectivity of Na_V channels (**Yen *et al.*, 2019**).

CONCLUSION

Molecular docking using STX, neoSTX, and dcSTX ligands against $Na_V1.5$, NHE, Bcl-2, Bcl-X_L, and Bax receptors has low ΔG and KI values so that the ligand and receptor interactions have a stable and effective interaction in inhibiting and activating the protein receptor. Based on the results of this molecular docking, the ovarian extract of Singkarak Lake Pufferfish (*Tetraodon leiurus*) has the potential as alternative cancer chemoprevention.

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