## CHAPTER 5

# IN-SILICO SCREENING OF ESTETROL AS A POSSIBLE NEUROTHERAPEUTIC

### Chapter Highlights

- ***Estrogen group of hormones and Necrostatins are docked against RIPK1 using AutoDock Tools***
- ***Binding affinity and binding patterns of the Estrogens are compared with Necrostatins***
- ***Based on binding affinity, analysis of hydrogen bonding and hydrophobic interaction patterns, Estetrol is identified as RIPK1 inhibitor***


### ABSTRACT

Receptor Interacting Protein Kinase-1 (RIPK1) is the molecular mediator of programmed necroptosis, a pathway intrinsically involved in ischemic cell death. It has been previously reported that inhibiting RIPK1 can ameliorate cerebral ischemic conditions. Necrostatins reportedly inhibit RIPK1, but their low pharmacokinetics property and lower metabolic stability necessitates the need of designing new RIPK1 inhibitors. Neuroprotective ability of Estrogen molecules is long studied and Estetrol has been recently reported to combat neonatal ischemic hypoxia. This study is designed to explore the capability of Estrogen molecules in inhibiting RIPK1. *In-silico* interactions between RIPK-1 and the Estrogen molecules were studied using Auto Dock Tools 1.5.6 and interaction patterns were analyzed using Ligand Interaction script in Maestro (Schrödinger Inc.). Results show that Estradiol, Estetrol and Estriol binds in the hydrophobic back pocket of RIPK1 with greater binding affinity than the reported RIPK1 inhibitors Necrostatin-1 (Nec-1) and Necrostatin-4 (Nec-4). Analysis of the interaction patterns revealed that all the three hormones form hydrogen bonds and interact hydrophobically with the

key RIPK1 residues. This study establishes estrogen hormones inhibit RIPK1 with higher affinity than Nec-1 and Nec-4, simultaneously identifying Estetrol as a potent RIPK1 inhibitor, based on its binding affinity, hydrogen bonding and hydrophobic interaction patterns.

## 5.1. INTRODUCTION

Caspase mediated apoptosis via stimulation of various death-domain receptors (DRs) is a common phenomenon leading to cell death [1]. However, a number of recent scientific studies have established that merely inhibiting caspase signaling pathway does not prevent DR-induced cell death [2], and cell death occurring under such scenario exhibits striking resemblance to necrosis rather than normal apoptotic events [3-6]. Since necrosis is conventionally considered to be a non-regulated pathway, not much investigation was carried out to identify potential targets for development of therapeutics. In 2005, Degterev et al reported a non-apoptotic cell death pathway manifesting necrotic morphology which is stimulated by DR activation and not cellular damage [2]. This non-apoptotic DR-mediated cell death pathway is controlled by cellular and molecular machineries and have been termed as programmed necrosis or necroptosis. Being a relatively new discovery as compared to apoptosis [7], underlying mechanisms of this pathway are yet to be elaborated. Binding of DRs like TNFR1, TNFR2 and Fas with their agonists induces the necroptotic pathway, which is mediated by Receptor-Interacting Protein kinases (RIPKs) [7]. Receptor-Interacting Protein kinase1 (RIPK1) is recruited to the complex I, formed as a response to binding of TNFα and TNFR1, by TNFR1 and acts as a key controlling protein of necroptotic pathway [7, 8]. Polyubiquitination of RIPK1 by TRAF2/5, cIAP1, and cIAP2 promotes cell survival [8, 9] whereas its de-ubiquitination by cylindromatosis (CYLD) releases it into the cytoplasm, initiating complex II formation with caspase-8, TRADD, Receptor-Interacting Protein kinase3 (RIPK3) and FADD [10].When caspase-8 is blocked and apoptosis is halted,

phosphorylated RIPK1 and RIPK3 form a "necrosome" which mediates necroptosis and thereby leads to cell death [11; 12]. Therefore, RIPK1 has been established as the key protein contributing to bifurcation of necroptosis from other DR-induced cell death pathways (Fig. 5.1.) [11].

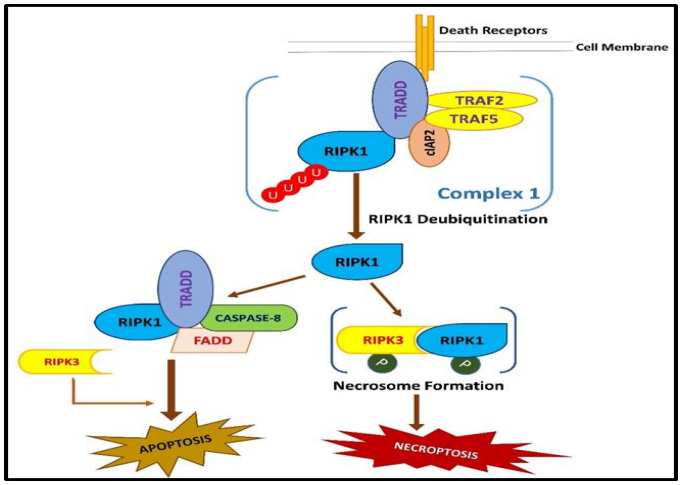


**Fig. 5.1: Role of Rip-1 Kinase in necroptosis pathway**

The fact that necroptosis has been established as a delayed response in neuronal ischemia, makes the pathway and RIPK1 a potential target for designing of therapeutics for combating cerebral ischemic pathophysiology [2]. Inhibition of RIPK1 by Necrostatins (mainly Nec-1, Nec-4, Nec-3 and Nec-5), the only reported group of RIPK1 inhibitors, successfully combats necroptotic cell death. Therefore, identifying inhibitors of RIPK1 seems a promising therapeutic approach against cerebral ischemia. Cerebral ischemia is a leading cause of death and disability worldwide with very limited options of therapeutic treatment. The only existing Food & Drug Administration (FDA) approved drug for treatment of cerebral ischemia is human tissue type plasminogen activator (tPA), but very often, tPA converts ischemic damage to cerebral hemorrhage and contributes to the morbidity [13]. Such scenario calls for designing of new drugs that can effectively combat the pathogenesis of cerebral ischemic insult.

Estrogen, classically known as a "reproductive" hormone extends its non-reproductive effects on brain, by exhibiting neuroprotection against neuro-inflammmation [14], oxidative stress induced neurodegeneration [15], Parkinson's disease [16] and cerebral ischemia [17]. Among the four estrogen hormones, namely, Estrone (E1), Estradiol (E2), Estriol (E3), and Estetrol (E4), present in human body, Estetrol was pronounced as a weak estrogen and researchers virtually abandoned the compound in mid 1980s [18]. But recent scientific studies regarding effect of estetrol in menopausal hot flushes has established that the hormone can directly affect the central nervous system (CNS) [19]. Also, estetrol has been rendered as a neuroprotectant in animal model of neonatal hypoxic-ischemic brain injury [20]. The neuroprotective behavior of estetrol opens up a new horizon in therapeutics against cerebral ischemia since estetrol might be a potential therapy against ischemic pathophysiology. The ability of estetrol to provide neuroprotection motivated this study to explore the ability of estrogen hormones to inhibit RIPK1, the molecule which mediates necroptosis and is thereby responsible for cell death in ischemic assault of brain. The study also compares the ability of the four estrogen hormones with that of Necrostatin-1 (Nec-1) (Fig. 5.2.a) and Necrostatin-4 (Nec-4) (Fig. 5.2.b), the reported potent inhibitor of RIPK1, to investigate the plausibility of establishing the natural reproductive hormones as potent neuroprotective therapeutics for ameliorating cerebral ischemic conditions.

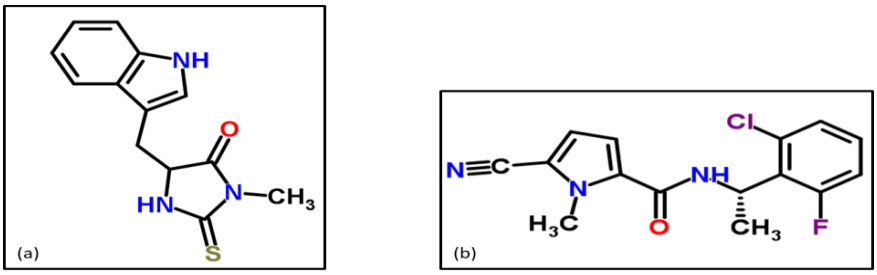


**Fig. 5.2.: Chemical structures of (a) Necrostatin-1; (b) Necrostatin-4**

Estradiol has been long reported for its neuroprotective ability and recent studies suggest that estetrol, till now considered as a weak estrogen, has potential to ameliorate neonatal hypoxic ischemia in rat pups [20]. On the other hand, several researchers have associated necroptosis with cerebral ischemia and RIPK1 has been established as a key molecule in the necroptosis pathway [21]. Hence, this study was designed to evaluate the effect of estrogen molecules in inhibiting RIPK1 and to compare their ability with existing RIPK1 inhibitors Nec-1 and Nec-4.

## 5.2. METHODS

### 5.2.1. Selection and preparation of inhibitors

The structures for estrogen group of hormones, namely, estradiol, estetrol, estrone and estriol were downloaded from PubChem database in .sdf format. Structures of Necrostatin-1 (Nec-1) and Necrostatin-4 (Nec-4), inhibitors of RIPK1, were downloaded in .sdf format from RCSB-Protein Database (PDB) [22]. The structures of all the hormones and the inhibitors were converted from .sdf to .pdb format using PyRx-Python prescription 0.8 [23]. The same software was also used for energy minimization of all the ligands by application of mmff94 force field and conjugate gradients optimization algorithm for 200 steps [24].

### 5.2.2. Study of drug like properties of inhibitors

All four hormones were studied for their drug like properties. The molecules were assessed for drug-likeness (complying with Lipinski's Rule of Five) using Molsoft Drug-likeness and Molecular Property Prediction server (https://www.molsoft.com/mprop/). Mutagenicity (Ames test) and Human Intestinal Absorption (HIA) of the hormones were analyzed using PreADMET server [25]. Brain penetration ability of the compounds were predicted using Online BBB

Prediction software with AdaBoost algorithm and MACCS fingerprint (www.cbligand.org/BBB/predictor.php).

### 5.2.3. Retrieval and preparation of RIPK1 Enzyme Structure

The 3-dimensional (3D) structure of human RIPK1 was downloaded from PDB (PDB ID: 4ITJ) [22]. The structure was deposited by Xie *et al* which reveals RIPK1 in complex with its known inhibitor necrostatin-4 and contains both chain A and B of the enzyme. The enzyme was cloned and expressed in *Spodoptera frugiperda* expression system. The protein was crystallized and resolved by X-ray diffraction at a resolution of 1.8 Å. USCF chimera was used for energy minimization of the downloaded 3-D structure of RIPK1 [26]. The method involved removal of the ligand 1-HX and the heteroatom iodide from the enzyme structure, followed by energy minimization using steepest descent method for 100 steps (0.02 Å step size) and then by conjugate gradient method which has ten steps with step size of 0.02 Å.

### 5.2.4. Simulating Molecular Docking Studies

Auto Dock Tools 1.5.6 (ADT) [27] was used to perform the docking studies of RIPK1 with their respective inhibitors and estrogen hormones. The non-polar hydrogen present in the enzyme were merged and torsions were applied to the ligand by rotation of all the rotatable bonds. The molecule was then assigned Gestgeiger partial charges. Using ADT, polar hydrogen atoms, solvation parameters and Kollman charges were also added to the enzyme. Among the three search algorithm options offered by ADT, Lamarckian genetic algorithm (LGA) was selected to analyze active binding of RIPK1 with various inhibitors. Docking for RIPK1 was performed into a grid box near the enzyme's catalytic site with number of points 88,112 and 100 in the X, Y and Z direction respectively with center grid box value of 25.147, 2.253 and 54.305 for X, Y and Z-

center. Spacing for grid box for docking studies of the enzyme was kept at 0.375 Å while ensuring that all the active site residues are entirely covered by the grid box and the ligands are provided space for translational and rotational walk.

For docking study of every ligand, 30 independent runs were performed with maximum number of 27,000 GA operations generated on a single population of 150 individuals. Parameters like rate of crossover, rate of gene mutation, and elitism were set to their default values of 0.80, 0.02, and 1, respectively. For further analysis and visualization of protein-ligand interaction patterns, 2D protein-ligand interaction diagram was generated using the Ligand Interaction script in Maestro (Schrödinger Inc., www.schrodinger.com).

## 5.3. RESULTS

### 5.3.1. Analyses of drug-like properties of estrogen molecules

In order to analyze the drug like properties of a compound, study of its structure and parameters like drug distribution and absorption, metabolism and excretion (ADME) become crucial. These parameters are considered as Lipinski's Rule of Five (Ro5) [28], which a candidate compound should satisfy to establish itself as a drug. According to a study conducted by Lipinski and his colleagues, when a compound complies with three of the following four criteria, it will be quickly absorbed by the body. Those four criteria are: a) the number of hydrogen bond donors ≤5; b) the number of hydrogen bond acceptors ≤10; c) the molecular weight ≤500 daltons and d) the calculated 1octanol–water partition coefficient (cLogP) ≤5 [29, 30]. If a candidate drug obeys LRo5, it possesses a better chance of commercialization [31]. A potential candidate drug should also be checked for its mutagenic properties, and the mutagenicity of such compounds is checked using Ames test [32].

Oral administration is the most preferred delivery mode as compared to other routes of systemic administration. To be successful orally administered drug, it has to be absorbed by intestinal membranes so that it can enter the systemic circulation. Lower intestinal absorption of a drug will limit its probability of clinical development [33]. In this study, human intestinal absorption (% HIA) is predicted using PreADMET server. The % HIA is defined as the drug percentage reaching the hepatic portal vein.

All the four estrogen molecules comply with LRo5, are predicted as non-mutagen and BBB permeable. Also, all of them exhibit high probability of intestinal absorption due to the high HIA score (80-100 %). Detailed values of these parameters are provided in Table-5.1.

**Table-5.1.: Detailed values Drug-Likeness and Molecular Properties of Estrogen Hormones of Lipinski's Rule of 5, Mutagenicity, BBB permeability and percentage of Human Intestinal Absorption of Estrogen molecules.**

| SN | PubChem CID | Ligands | Lipinski's (Ro5) Criteria | | | | HIA % |
|---|---|---|---|---|---|---|---|
| | | | Mol. Wt. (≤500) | HBA (≤10) | HBD (≤5) | LogP (≤5) | |
| 1 | 5757 | Estradiol | 272.388 | 3 | 1 | 1.93 | 92.39% |
| 2 | 27125 | Estetrol | 304.386 | 3 | 1 | 1.93 | 81.31% |
| 3 | 5756 | Estriol | 288.387 | 3 | 1 | 1.93 | 100% |
| 4 | 5870 | Estrone | 270.372 | 3 | 1 | 1.58 | 95.58% |

### 5.3.2. Analyzing the inhibition efficacy of the estrogen hormones

Binding interaction between the ligands and the substrate (RIPK1) was studied by docking the inhibitors near the catalytic site of RIPK1. Table 2 summarizes the obtained results including inhibition constant and lowest binding energy and hydrogen bonding patterns for docking studies

between all the ligands and the enzyme (30 docking runs for each ligand). The ligand showing highest binding affinity is deduced by plotting the lowest binding energies obtained against the respective ligands. The plot reveals Estradiol as the compound with highest affinity towards RIPK1 since it has lowest binding energy (-9.57 kcal/mol) as compared to the other ligands, even the Necrostatins. Nec-1 and Nec-4 exhibits binding energies -8.97 kcal/mol and -8.99 kcal/mol, respectively, which are quite higher as compared to that of Estradiol. Estriol (binding energy -9.38 kcal/mol) and Estetrol (binding energy -9.33 kcal/mol) also show higher affinity towards RIPK1 as compared to the Necrostatins. Estrone shows low affinity towards RIPK1 since it has a high binding energy of -7.01 kcal/mol. The lowest binding energy, estimated inhibition constants and amino acids participating in hydrogen bonds are listed in Table-5.2.

**Table-5.2.: Analysis of AutoDock results for lowest binding energies, estimated inhibition constant and hydrogen bond forming residues of RIPK1 with Estrogen hormones.**

| Serial No | Ligand | PubChem ID | Lowest Binding Energy (kcal/mol) | Estimated Inhibition Constant ($K_i$) | Amino Acid residues Participating in Hydrogen Bonding with RIPK1 (Residues essential for Hydrogen bond formation with RIPK1) |
|---|---|---|---|---|---|
| | Nec-4 | | -8.99 | 256.58 nM | Asn 99, Asp 156, Lys 45, Tyr 94 |
| | Nec-1 | 2828334 | -8.97 | 266.11 nM | Asn 99, Asp 156, Leu 78, Lys 45 |
| **Estrogen Hormones:** | | | | | |
| **1** | Estradiol | 5757 | -9.57 | 96.11 nM | Leu 70, Met 67 |
| **2** | Estrone | 27125 | -7.01 | 7.34 µM | Asn 148, Glu 96, Asn 99, Phe 28, Lys 45 |
| **3** | Estetrol | 5756 | -9.33 | 144.17 nM | Leu 70, Ile 154, Met 67, Val 91, Phe 28, Tyr 94, Glu 96, Met 95, Lys 45 |
| **4** | Estriol | 5870 | -9.38 | 133.22 nM | Leu 70, Ile 154, Met 67 |

### 5.3.3. Analysis of enzyme-ligand interaction

Since Estradiol, Estetrol and Estriol display binding energy lower than the known inhibitors Nec-1 and Nec-4, their binding patterns were visualized and analyzed using 2-D Protein-Ligand Interaction script and compared to the later mentioned compounds. Estradiol shows a single hydrogen bond with Leucine (Leu) 70 amino acid backbone (Fig. 5.3.a.), whereas Estriol exhibits two hydrogen bonding with backbones of Leu 70 and Isoleucine (Ile) 154 (Fig. 5.3.b.). Estradiol hydrophobically interacts with hydrophobic amino acid residues Valine (Val) 76, Alanine (Ala) 155, Leu 90, Val 91, Methionine (Met) 92, Leu 78, Met 67 and Phenylalanine (Phe) 162 (Fig. 5.3.a.). It also interacts with two positively charged Lysine (Lys) residues in position 77 and 45 and one negatively charged Aspartate (Asp) 156 residue (Fig. 5.3.a.). Estriol shows hydrophobic interaction pattern almost similar to Estradiol, but further interacts with another hydrophobic residue Val 75 (Fig. 5.3.b.). Estetrol manifests highest number of hydrogen bonds (Fig. 5.3.c.). It shows three hydrogen bonds, one with Leu 70 backbone and two with backbone of Ile 154 (Fig. 5.3.c.). Hydrophobic interaction of Estetrol with the amino acid residues is similar to that of Estriol's. The known inhibitor Nec-1 shows hydrogen bonding with side chain of amino acid residues Lys 45 and Asp 156 (Fig. 5.4.a), whereas Nec-4 exerts a single hydrogen bond with side chain of Asp 156 only (Fig. 5.4.b). None of the inhibitors display hydrogen bond formation with backbone of any amino acid residue (Fig.5.3.a-5.3.b.). Nec-1 shows hydrophobic interaction with Val 76, Leu 78, Leu 90, Val 91, Met 92, Ile 43, Leu 157, Phe 162 and Ala 155 (Fig. 5.4.a), compared to which Nec-4 has an elaborate hydrophobic interaction network comprising the polar amnio acid Serine (Ser) 161, the positively charged Lys 45, the hydrophobic residues Phe 162, Met 67, Val 76, Leu 78, Met 92, Leu 90. Leu 70, Val 75, Ala 155, Ile 154 and Leu 129 (Fig. 5.4.b). All three of these estrogen hormones binds with RIPK1 with binding energies lower than that of the known inhibitors, Nec-1 and Nec-4.

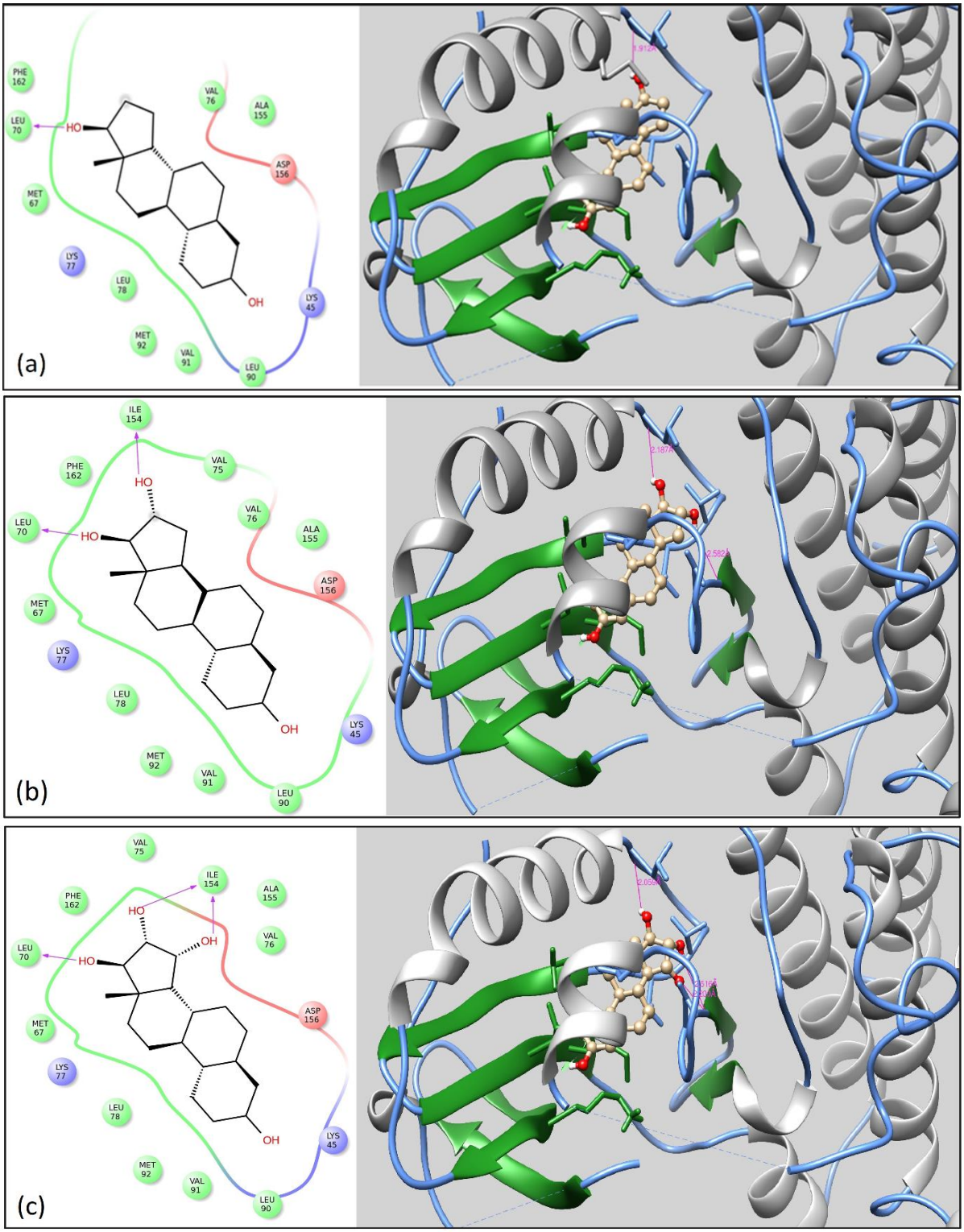


**Fig. 5.3. Interation pattern of RIPK1 and Estrogen hormones. (a) Binding pattern of Estradiol (ball and stick) with RIPK1 (ribbon); (b) Interaction of Estriol (ball and stick with RIPK1 (ribbon); (c) Hydrogen bond formation and hydrophobic interaction of Estetrol (ball and stick) with RIPK1 (ribbon) amino acid residues.**

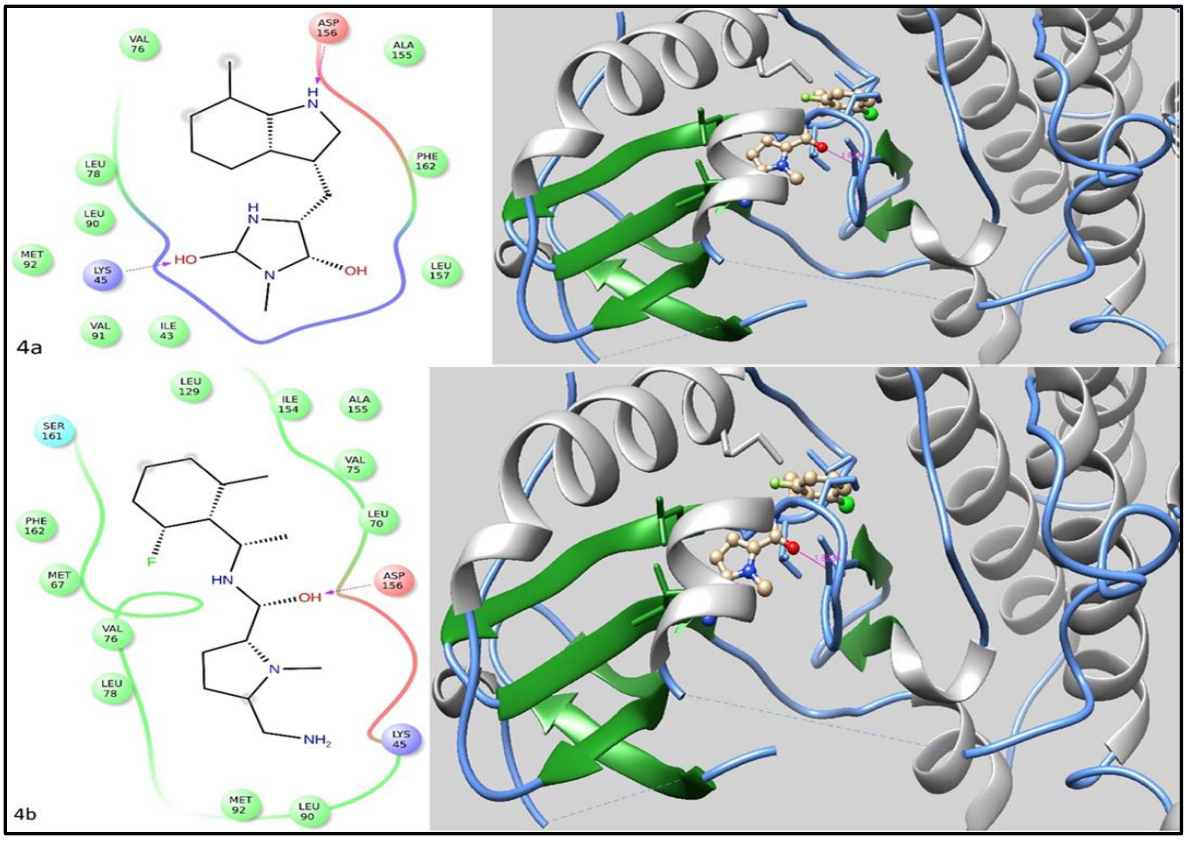


**Fig. 5.4. (a) Interaction pattern of Necrostatin-1 (ball and stick) with RIPK1 (ribbon); (b) Interaction pattern of Necrostatin-4 (ball and stick) with RIPK1 (ribbon).**

## 5.4. DISCUSSION

Necroptosis has been established as an important paradigm involved in ischemic pathophysiology of brain [2,34] and the key step involves RIPK1 activity [21]. Following the activation of TNFR1 due to the binding of the inflammatory cytokine TNFα, a receptor associated complex I is formed which constitutes of the enzyme RIPK1, the death domain TRADD, TRAF2 and cellular inhibitor of apoptosis proteins (cIAPs) 1 and 2 [35]. Ubiquitination of the constitute proteins of the complex I is mediated by both the cIAP1 and 2, which further causes recruitment of IκB-kinase (IKK) complex [35]. IKK is composed of the NF-κB essential modulator protein or NEMO which binds to the non-degradative ubiquitin chains of the complex I [35]. The IKK complex also modulates transcritption of genes necessary for cell survival [35].

Internalization of the complex I contatining TNFR1 subsequently causes activation of caspase-8, which in absence of NEMO enhances apoptosis and inactivates the key necroptosis components like RIPK1 [35]. When the Caspase-8 activity is compromised, RIPK1 and RIPK3 complex is formed, which further activates necroptotic cell death [35]. Several studies have reported that RIPK1 mediated cell death is observed in absence of NEMO [35,36]. In absence of NEMO, the RIPK3 protein binds the FADD complex, which is necessary for promoting necroptosis [35]. Besides its ability to mediate cell death, RIPK1 is also involved in the inflammatory pathways involved with innate immunity, but it has been reported that these two activities of RIPK1 are independent of each other [37].

The fact that RIPK1 is involved in an alternative programmed cell death pathway [7] and its inhibition ameliorates ischemia-reperfusion mediated brain injury [21], makes RIPK1 an attractive target for therapeutics designing. Necrostatins have been designed to act as strong inhibitors of RIPK1 and Nec-1 mediated RIPK1 inhibition was reported to be a promising approach. But recent studies reveal limitations of Nec-1 to be lower metabolic stability, poor pharmacokinetics and modest inhibition capability [38], thus necessitating designing of novel inhibitors of RIPK1. In this context, we have performed *in-silico* evaluation of the potency of estrogen hormones in inhibiting RIPK1 and compared with Nec-1 and Nec-4 inhibition pattern. Structure of RIPK1 consists of an N-lobe and a C-lobe [39] and a hydrophobic back pocket located in between them [39, 40]. The hydrophobic back pocket constitutes of amino acids Leu70, Val76, Leu78, Leu90, Asp156, Ser161 and Phe162 [41] and the amino acids Lys 45, Glu 63 and Asp 156 are responsible for the catalytic activity of the enzyme [38]. These residues seem to be highly conserved [39] and binding of molecules to the hydrophobic back pocket of RIPK1 reportedly contributes to RIPK1 inhibition [41]. The molecular docking results suggest that Estradiol, Estetrol and Estriol bind to RIPK1 with higher affinity as compared to that of Nec-1

and Nec-4, the established RIPK1 inhibitors, since they bind to the enzyme with lower binding energy than that of Nec-1 (-8.97 kcal/mol) and Nec-4 (-8.99 kcal/mol) (Table. 5.2). Also, similar to Nec-1 and Nec-4, all the Estrogen molecules bind into the back pocket of the enzyme RIPK1 (Fig. 5.5.a, 5.5.b).

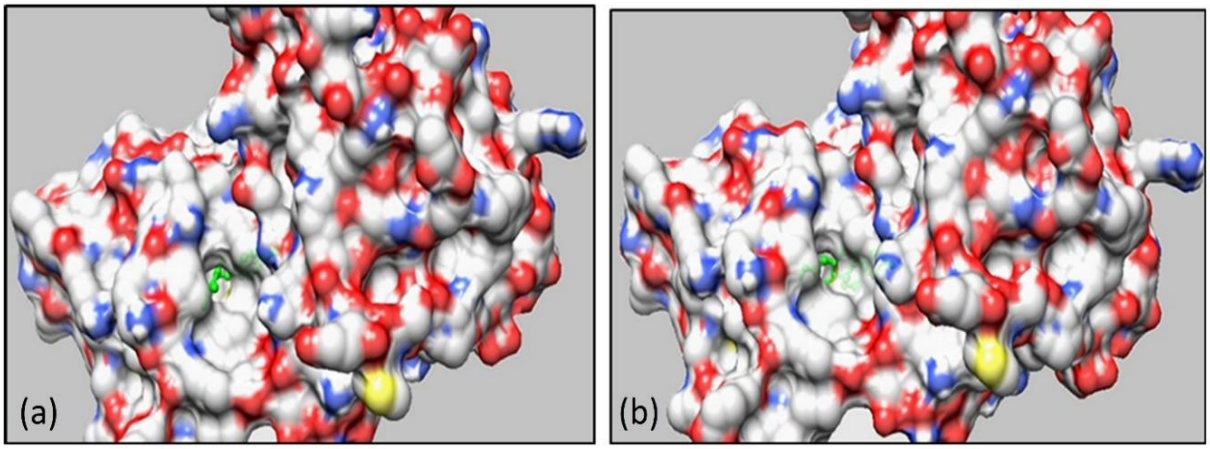


**Fig 5.5. Surface structure of RIPK1 (heteroatom) showing the hydrophobic back-pocket and bonded inhibitors (green ball and stick) (a) Necrostatin-1 and (b) Estetrol.**

The molecular docking results reveal that Nec-1 forms hydrogen bonds with side chains of two residues of the catalytic triad, Asp 156 and Lys 45 and shows further hydrophobic interaction with four amino acids from hydrophobic back pocket of RIPK1 (Val76, Leu78, Leu90 and Phe162) (Fig. 5.4a) and also with the gatekeeper residue of RIPK1, Met 92 [41, 2] (Fig. 5.4a).On the other hand, Nec-4 shows a single hydrogen bond formation with side chain of a single amino acid residue from the catalytic triad, Asp 156 and manifests hydrophobic interaction with Lys 45, another residue from the conserved catalytic domain (Fig. 5.4b). Nec-4 also demonstrates hydrophobic interaction with all of the other residues of the hydrophobic back pocket as well as with the gate keeper residue Met 92 and another significant residue Met 67 (Fig. 5.4b). Analyses of binding patterns of estrogen molecules reveal that Estradiol forms hydrogen bond with the backbone of the amino acid residue Leu 70, which is a constituent

residue of the hydrophobic back pocket of RIPK1 [41] (Fig. 5.3a). The molecule also interacts hydrophobically with most of the other residues of the hydrophobic back pocket, i.e., Val 76, Leu 78, Leu 90, Asp 156 and Phe 162, except for Ser 161 (Fig. 5.3a). Among these residues, Asp 156 is an important member of the catalytic triad [39, 41]. Estradiol shows hydrophobic interaction with an important catalytic residue, Lys 45 and also with Met 92, the gatekeeper residue [41, 2]. Estetrol reveals the highest number of hydrogen bond formation, two with backbone of the residue Ile 154, previously reported as a key residue in RIPK1 inhibition [39, 41] and another one with Leu 70 backbone (Fig. 5.3c), one of the crucial residues of the hydrophobic back pocket of RIPK1. The molecule also exhibits extensive interactions with most of the other critical amino acids of the hydrophobic back-pocket of the enzyme, viz., Val76, Leu78, Leu90, Asp156 and Phe162 and with gate keeper residue Met 92 (Fig. 5.3.c). Estriol forms two hydrogen bonds with backbones of amino acid residues Ile 154 and Leu 70 as well as exhibits hydrophobic interactions with most of significant residues of the hydrophobic back pocket (Fig. 5.3b). The molecule also manifests hydrophobic interactions with the catalytic residues Asp 156, Lys 45and the gate keeper residue Met 92. All the three estrogen molecules also interact with the hydrophobic residues Met 67 and Lys 77, reported as significant for RIPK1 inhibition [41] (Fig. 5.3c).

From the above study, it can be inferred that Estradiol, Estriol and Estetrol can act as potent RIPK1 inhibitors, since all of them manifest lower binding energies as compared to the known inhibitors Nec-1 and Nec-4, which represent that Estrogen molecules possess higher binding affinity towards RIPK1. Though in terms of binding energy, Estradiol can be denoted as most potent inhibitor among the three compounds, but an overall evaluation of hydrogen bond formation, hydrophobic interaction pattern, suggests Estetrol as a potent candidate for RIPK1

inhibition. Three hydroxyl groups of Estetrol show hydrogen bonding with two important amino acid residues of RIPK1 back pocket, which is responsible for inhibition of the enzyme [41] and the compound also interacts hydrophobically with an extensive number of amino acid residues present in the RIPK1 back pocket. The fact that it has higher binding affinity towards RIPK1, forms hydrogen bonds with critical amino acid residues and manifests elaborate hydrophobic interaction pattern with the RIPK1 back pocket residues including Asp 156 and Lys 45 (members of the catalytic triad of RIPK1) ascertain its ability as a potent RIPK1 inhibitor.

## 5.5. CONCLUSION

This study evaluates the potential of the estrogen hormones in inhibiting RIPK1, an enzyme involved with necroptotic cell death during cerebral ischemia. Out of four estrogen hormones, three, namely Estradiol, Estriol and Estetrol, displays lower binding energy towards RIPK1 compared to Nec-1 and Nec-4, known inhibitors of RIPK1. The hormones also interact with the reported catalytic residues of the enzyme via both hydrogen bonding and hydrophobic interaction. Among the three estrogen molecules, Estetrol exhibits three hydrogen bonds with amino acid residues of RIPK1, which is more than hydrogen bonds formed by rest of the ligands including the reported RIPK1 inhibitors Nec-1 (2 hydrogen bonds) and Nec4 (1 hydrogen bond). The residues that Estetrol binds are critical for RIPK1 inhibition and it also interacts hydrophobically with back pocket residues of RIPK1 in a fashion similar to Nec-1 and Nec-4. Hence, we can conclude that Estetrol has the potential to inhibit RIPK1 and thereby necroptosis, which is explicitly associated with cerebral ischemia. Thereby, Estetrol can be exploited for its neuroprotective abilities and can be promising candidate for further drug

development in the field of neurotherapeutics. Presently, *in-vitro* and *in-vivo* studies are being carried out to delve deeper into the mechanism of Estetrol's neuroprotective ability.

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