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1 # *In silico* modeling and simulation of neuroendocrine-immune modulation through
2 adrenergic and 17β-estradiol receptors in lymphocytes show differential activation of cyclic
3 adenosine monophosphate (cAMP)

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24 **Abstract**

25 Sympathetic innervation of lymphoid organs and presence of 17β-estradiol and adrenergic
26 receptors (ARs) on lymphocytes suggests that sympathetic stimulation and hormonal activation
27 may influence immune functions. Simulation of these pathways may help to understand the
28 dynamics of neuroendocrine-immune modulation at the cellular and molecular level.

29 Dose- and receptor-dependent effects of 17β-estradiol and AR sub-type-specific agonists
30 were established in vitro on lymphocytes from young male Sprague-Dawley rats and modeled in
31 silico using MATLAB Simbiology toolbox. Kinetic principles were assigned to define receptor-
32 ligand dynamics and state/time plots were obtained using Ode15s solvers at different time intervals
33 for key regulatory molecules. Comparisons were drawn between in silico and in vitro data for
34 validating the constructed model with sensitivity analysis of key regulatory molecules to assess
35 their individual impacts on the dynamics of the system. Finally docking studies were conducted
36 with key ligands 17β-estradiol and norepinephrine to understand the mechanistic principles
37 underlying their interactions.

38 Adrenergic activation triggered pro-apoptotic signals while 17β-estradiol enhanced
39 survival signals showing contradictory effects as observed in vitro. Treatment of lymphocytes with
40 17β-estradiol shows ten-fold increase in survival signals concentration-dependently manner.
41 cAMP activation is crucial for the activation of survival signals through p-ERK (Extracellular
42 Signal-Regulated Kinase) and p-CREB (cAMP Responsive Element Binding (protein). Inhibition
43 of p-ERK by norepinephrine and β2-AR by 17β-estradiol indicate direct influence on the down-
44 stream signals altering apoptotic v/s survival signals.

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45 Thus, the cross-talk between 17β-estradiol and adrenergic signaling pathways determines
46 lymphocyte functions in a receptor subtype- and co-activation-dependent manner in health and
47 disease.

48 **1.0 Introduction**

49 The neuroendocrine-immune network is a complex inter-regulatory system with wide
50 plasticity in order to maintain systemic homeostasis [1-3]. In females, the cyclic fluctuations in
51 the levels of gonadal hormones especially, 17β-estradiol affect the functioning of immune effector
52 cells by binding to specific receptors [4-7].

53 In the periphery, *in vitro* and *in vivo* 17β-estradiol-stimulation has been shown to enhance
54 splenocyte proliferation and cytokine production through the alteration of specific signaling
55 molecules [2, 4, 8]. In the resting state, the close apposition of T-lymphocytes in direct synaptic
56 association with sympathetic noradrenergic (NA) nerve fibers that innervate the lymphoid organs
57 renders them highly responsive to norepinephrine [NE; 7, 9-13]. Since both 17β-estradiol and NE
58 mediate their effects on lymphocytes through their specific receptors, their down-stream effects
59 are dependent upon the kinetic parameters that govern receptor-ligand interactions [10, 14-16].
60 Previous studies from our laboratory and others have shown that both 17β-estradiol and
61 norepinephrine-induced signaling cascades involve similar signaling molecules (p-ERK, p-CREB,
62 cAMP and p-Akt) in modulating the expression of similar cytokines (IFN-γ and IL-2) thereby,
63 altering cell-mediated immune functions [2, 4, 17-18]. Thus, the scope of cross-talk between the
64 two pathways during multiple ligand-receptor interactions is infinite. Considering that NE can bind
65 to α1-/α2- or β1-/β2-/β3- adrenoceptors (AR) on lymphocytes and 17β-estradiol can bind to
66 cytosolic (cERs) or nuclear (nERs) 17β-estradiol receptors and initiate signals that target similar
67 down-stream signaling molecules, the underlying kinetic parameters will influence the outcome of

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68 the cross-talk through synergistic, additive or diminutive effects. Hence in this study, we have
69 created a dynamic model of resting lymphocyte functions in the presence of 17β-estradiol and/or
70 NE using kinetic principles that govern receptor-ligand activation from secondary data with
71 concentration and down-stream signaling data from *in vitro* studies published from our laboratory.
72 Also, in an attempt to understand the multiple levels of interactions between these two pathways,
73 we have also conducted docking studies of 17β-estradiol with the β2-AR and NE with the key
74 signaling molecule in 17β-estradiol signaling cascade p-ERK.

75 2.0 Methods

76 *2.1 Model building*

77 The model was built using both primary and secondary data sources. The dose- and
78 receptor-dependent effects of 17β-estradiol and AR sub-type-specific agonists were established *in*
79 *vitro* on lymphocytes from young male Sprague-Dawley rats in previous studies published by our
80 laboratory [17,18]. In these studies, the concentration of the signaling molecules, cytokines, and
81 other secondary molecules was determined using ELISA and this data was used to construct the
82 model (Tables 1, 2). The possible cross-talk pathway was mapped using receptor-specific
83 inhibitors and inhibitors of signal transduction molecules and was modeled *in silico* using
84 MATLAB Simbiology toolbox. Receptor-ligand dynamics were defined based on kinetic
85 principles from available literature and previous studies from our laboratory. State versus time
86 plots were obtained using Ode15s solvers at 1ns-1s time intervals in accordance with
87 neurotransmitter kinetics and at 24-72 hrs for long-term effects on lymphocyte functions.
88 Comparisons were drawn between *in silico* and *in vitro* data for validating the constructed model.
89 Sensitivity analysis was performed on key regulatory molecules to assess their individual impacts
90 on the dynamics of the system.

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91 *2.1.1 Model*

92 The model was built to show an interconnected network of four signaling pathways on the
93 basis of in vitro studies conducted in our laboratory including:

94 1. α1-adrenoceptor signaling [17]; (Table 1A)
95 2. α2-adrenoceptor signaling [17]; (Table 1B)
96 3. β2-adrenoceptor signaling [18]; (Table 1C)
97 4. 17β-estradiol signaling through ERs and GPCRs [4]; (Table 1D) and their respective
98 kinetic parameters outlined in Table 2.

99 **Table 1:** Reaction table and kinetics for α1-adrenoceptor signaling (1A), α2-adrenoceptor
100 signaling (1B), β2-adrenoceptor signaling (1C) and 17β-estradiol signaling through ERs and
101 GPCRs (1D).

102 **1A**

S.No	Reaction	Kinetics
1	[AR-α1]+[Phenylephrine]=>[RL1]	vm*[Phenylephrine]/(km+[Phenylephrine])
2	[CREB]+[RL1]=>[p-CREB]	nkn.[CREB].[RL1]
3	[p-CREB]+[CBP]=>[CBP-CREB]	ct.[p-CREB].[CBP]
4	[CBP]-[p-CREB]+[DNA]=>[mRNA1]+[mRNA2]	tr.[CBP-p-CREB].[DNA]
5	[mRNA1]=>[survival signal]	as.[mRNA`1]

103 **1B**

S.No	Reaction	Kinetics
1	[AR-α2]+[Clonidine]=>[RL2]	va*[Clonidine]/(kn+[Clonidine])
2	[pi3k*]+[Akt]+[RL2]=>[p-Akt]	at.[pi3k*].[Akt].[RL2]
3	[RL2]+[PKA*]=>[PKA]	ml.[RL2].[PKA*]
4	[p-Akt]+[BCL2]=>[BCL2*]	bc.[p-Akt].[BCL2]
5	[BCL2*]=>[survival signal]	aps.[BCL2*]

104 **1C**

S.No	Reaction	Kinetics
1	[β2-AR]+[Terbutaline]=>[RL3]	bt.[Terbutaline]/(kbt+[Terbutaline])
2	[RL3]+[PKA]=>[PKA*]	gh.[RL3].[PKA]
3	[RL3]+[pi3k]=>[pi3k*]	jk.[RL3].[pi3k]
4	[PKA*]+[ERK]=>[p-ERK]	fd.[PKA*].[ERK]
5	[p-ERK]+[CREB]=>[p-CREB]+[ERK]	pe.[p-ERK].[CREB]
6	[p-CREB]+[CBP]=>[CBP-CREB]	ct.[p-CREB].[CBP]
7	[CBP]-[p-CREB]+[DNA]=>[mRNA1]+[mRNA2]	tr.[CBP-p-CREB].[DNA]
8	[p-Akt]+[DNA]=>[mRNA2-1-1]	kj.[p-Akt].[DNA]

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9	[mRNA2-1-1]=>[iNOS]	hg.[mRNA2-1-1]
10	L-Arg+iNOS=>NO	hg.L-Arg/(fd+[L-Arg])
11	[NO]+[CAAT]=>[C/EBP]	vm.[NO].[CAAT]
12	[C/EBP]=>[CHOP]	cvc.[CAAT]/(kc+[CAAT])
13	[DNA]+[CHOP]=>[mRNA2-1]	vch.[CHOP]/(kch+[CHOP])
14	[mRNA2-1]=>[BAX]	fbax.[mRNA2-1]
15	[BAX]+[BCL2]=>[BCL2-BAX]2`	vbax.[BAX]/(kbax+[BAX])
16	[BCL2-BAX]2=> [Apoptotic signal]	vx.[BCL2-BAX]2
17	[DNA]+[p-Akt]=>[mRNA3]	kj.[DNA].[p-Akt]
18	[mRNA3]=>[IL-2]	kl.[mRNA3]
19	[IL-2]=>[Survival Signal]	kj.[IL-2]

105 **1D**

S.No	Reaction	Kinetics
1	[17β-estradiol]+[GPCR]=>[cAMP]`	Vm2.[17β-estradiol]/(Km1+[17β-estradiol])
2	[cAMP]+[PKA]=>[PKA*]	pk.[cAMP].[PKA]
3	[ER]+[17β-estradiol]=>[E-ER]	erb.[ER].[17β-estradiol]
4	[E-ER]+[shc-grb2sos]=>[E-ER-shc-grb2sos]	eg.[E-ER].[shc-grb2sos]
5	[E-ER-shcgrb2sos]+[pi3k]=>[pi3k*]	ep.[E-ER-shc-grb2sos].[pi3k]
6	[E-ER]+[shc-grb2sos]+[RAS]=>[RAS*]	era.[E-ER].[shc-grb2sos].[RAS]
7	[RAF]+[RAS*]=>[RAF*]	kc.RAF*.RAS*
8	[MEK]+[RAF*]=>[MEK-RAF*]	mek.[MEK].[RAF*]
9	[MEK-RAF*]=>[MEK-p]+[RAF]	mekp.[MEK-RAF*]
10	[MEK-p]+[RAF*]=>[MEK-p-RAF*]2	rm.[MEK-p].[RAF*]
11	[MEK-p-RAF*]2=>[MEK-pp]+[RAF]	mpp.[MEK-p-RAF*]2
12	[ERK]+[MEK-pp]=>[p-ERK]	ka2.[ERK].[MEK-pp]
13	[p-ERK]+[CREB]=>[p-CREB]+[ERK]	pe.[p-ERK].[CREB]
14	[p-CREB]+[CBP]=>[CBP-CREB]	ct.[p-CREB].[CBP]
15	[CBP]-[p-CREB]+[DNA]=>[mRNA1]+[mRNA2]	tr.[CBP-p-CREB].[DNA]
16	[mRNA2]=>[IFN-γ]	ifp.[mRNA2]
17	[mRNA2]=>[Survival signal]	bnb.[mRNA2]
18	[pi3k*]+[Akt]=>[p-Akt]	at.[pi3k*].[Akt]
19	[p-Akt]+[DNA]=>[mRNA2-1-1]	kj.[p-Akt].[DNA]
20	[mRNA2-1-1]=>[iNOS]	hg.[mRNA2-1-1]
21	L-Arg+iNOS=>NO	hg.L-Arg/(fd+[L-Arg])
22	[NO]+[CAAT]=>[C/EBP]	vm.[NO].[CAAT]
23	[C/EBP]=>[CHOP]	cvc.[CAAT]/(kc+[CAAT])
24	[DNA]+[CHOP]=>[mRNA2-1]	vch.[CHOP]/(kch+[CHOP])
25	[mRNA2-1]=>[BAX]	fbax.[mRNA2-1]
26	[BAX]+[BCL2]=>[BCL2-BAX]2`	vbax.[BAX]/(kbax+[BAX])
27	[BCL2-BAX]2=> [Apoptotic signal]	vx.[BCL2-BAX]2
28	[17β-estradiol]+[cER]=>[E-cER]	ecr.[17β-estradiol].[cER]
29	E-cER=>[E-cER]2	ed.[E-cER]

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30	[E-cER]2+[DNA]=>[mRNA2]	nt.[E-cER]2.[DNA]
31	[mRNA2]=>[IFN-γ]	ifp.[mRNA2]
32	[IFNGR]+[IFN-γ]=>[IFNγ-IFNGR]	vm.[CREB]/(kd+[CREB])
33	[IFNγ-IFNGR]=>[IFNγ-IFNGR]2	rd.[IFNγ-IFNGR]
34	[IFNγ-IFNGR]2=>[IFNγ-IFNGR*]2	rc.[IFNγ-IFNGR]2
35	[IFNγ-IFNGR*]2+[RAS]+[shc-grb2sos]=>[RAS*`]	kx.[IFNγ-IFNGR*]2.[RAS*].[shc-grb2sos]

106

107 **Table 2:** Receptor Binding maxima and Kd values (A), Signaling Molecule concentrations (B)

108 and Ligand concentrations (C) as incorporated in the model.

109 **2A**

S.No.	Receptor	Bmax	Kd (nM)	Reference
1.	α1-AR	175.3(fM/10^6 cells)	0.65	[27]
2.	α2-AR	19.9(fM/10^6 cells)	3.7	[28]
3.	β2-AR	1222 sites/cell	19.9	[29, 30]
4.	IFN-gR	708±14 receptors/cell	0.9±0.2	[30]
5.	cER	0.6204 (fM/mg protein)	5.5	[31]
6.	nER	(0.136 fm/mcg dna)	-	[31]

110

111 **2B**

S.No.	Signaling Moelcules	Concentration	Reference
1.	ERK	251	[17]
2.	CREB	245.6	
3.	Akt	219	
4.	IFN-g	560	

112

113 **2C**

S.No.	Signaling Moelcules	Concentration	Reference
1.	Phenylephrine	10^{-6} M	

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2.	Clonidine	10^{-6} M	[17, 18]
3.	Terbutaline	10^{-6} M	
4.	Estrogen	10^{-8} M	

114

115

116 **2.2 Simulations**

117 Simulations were performed using Ode15s solvers at different time points depending
118 upon the signals studied. For signaling molecules including p-ERK, p-Akt, p-CREB, and cAMP,
119 simulations were performed for 0.05 seconds in order to capture the rapid
120 neurotransmitter/steroid hormone-mediated transduction. For the cytokines including IL-2 and
121 IFN-γ, the simulations were performed for 24 hours. For antioxidant enzymes and ROS/RNS the
122 simulations were performed up to 3000 seconds. Finally, for survival v/s apoptotic signals, the
123 simulations were carried out for 72 hours.

124 **2.3 Sensitivity Analysis**

125 Key regulatory molecules were scanned and their sensitivities were analyzed at 24 hours
126 for each of the ligands used. In this case, phenylephrine-, clonidine-, terbutaline-, and 17β-
127 estradiol-mediated signals were scanned for their sensitivities in two groups: (a) molecular
128 markers (p-ERK, p-CREB, p-Akt and cAMP) and (b) cytokines (IL-2 and IFN-γ).

129 **2.4 Docking**

130 Molecular docking was performed by keeping the ERK molecule or the β2-AR molecule
131 rigid and ligand (NE or 17β-estradiol) flexible (Rigid Docking). This resulted in different
132 conformations in each run and the best conformer which fits with lowest binding energy
133 (kcal/mol) was chosen and written as a PDB file using UCSF Chimera. Auto Dock4.2.6 was used

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134 to automatically dock the ligands to the enzyme. In this version the windows platform, cygwin
135 was used for our analyses. The enzyme/receptor molecule was loaded and stored as a .pdb file
136 after assigning hydrogen bonds and kollman charges. The investigation ligands were loaded and
137 their torsions along with rotatable bonds were assigned and the file saved as ligand .pdbqt. Grid
138 menu is toggled, after loading enzyme .pdbqt the map files were selected directly with setting up
139 grid points with 80 X 106 X 90 dimensions for the searching of ligand within the active site of
140 the enzyme molecule. This way the grid parameter files were created. The docking parameter
141 files were then set up with search parameter as genetic algorithm and docking parameter utilizing
142 Lamarckian genetic algorithm. Following up of grid parameter files (gpf) and docking log files
143 (dlg), the command prompt was toggled and commands were typed in stepwise for autogrid and
144 autodock execution. The docked structures were generated after maximum number of
145 evaluations using UCSF Chimera.

146 **3.0 Results**

147 ***3.1 17β-estradiol treatment alters signaling molecule expression in lymphocytes stimulated***
148 ***with adrenergic agonists***

149 Simulation of lymphocytes with adrenergic agonists, terbutaline, clonidine, and
150 phenylephrine (Fig. 2A), or 17β-estradiol (Fig. 2B) or both (Fig. 2C) significantly alters the
151 expression of p-ERK, p-CREB, p-Akt, and cAMP. State v/s time diagrams at 0.05 seconds show
152 significantly enhanced p-CREB and p-ERK expression alone upon stimulation with adrenergic
153 agonists (Fig. 2A). Stimulation with 17β-estradiol in the absence of adrenergic agonists (Fig. 2B)
154 or with adrenergic agonists (Fig. 2C) significantly enhanced cAMP, p-ERK, and p-CREB
155 expression. p-Akt expression was unaltered upon stimulation of lymphocytes with 17β-estradiol
156 and/or adrenergic agonists.

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157 ### *3.2 17β-estradiol and adrenergic agonists decrease superoxides but enhance peroxynitrites*
158 ### *in lymphocytes*

159 Simulation of lymphocytes with adrenergic agonists terbutaline, clonidine, and
160 phenylephrine (Fig. 3A), or 17β-estradiol (Fig. 3B) or both (Fig. 3C) similarly decrease production
161 of superoxides and enhance production of peroxynitrite in lymphocytes within the 0.1 second time
162 interval. State v/s time diagrams up to 0.3 seconds show decreased catalase (CAT) and superoxide
163 dismutase (SOD) activities in all treatment groups.

164 ### *3.3 17β-estradiol with or without adrenergic agonists increases CAT and SOD activities in*
165 ### *lymphocytes but not adrenergic agonists alone*

166 Simulation of lymphocytes with adrenergic agonists terbutaline, clonidine, and
167 phenylephrine (Fig. 4A), or 17β-estradiol (Fig. 4B) or both (Fig. 4C) significantly alters CAT and
168 SOD activities and production of peroxynitrites and superoxides within 3000 seconds of
169 stimulation. State v/s time diagrams at 3000 seconds show significantly enhanced CAT and SOD
170 activities upon stimulation with 17β-estradiol alone (Fig. 4B) or 17β-estradiol with adrenergic
171 agonists (Fig. 4C). Stimulation with adrenergic agonists (Fig. 4A), or with 17β-estradiol alone
172 (Fig. 4B) or 17β-estradiol with adrenergic agonists (Fig. 4C) significantly decreased peroxynitrite
173 production similarly.

174 ### *3.4 17β-estradiol treatment alters cytokine production in lymphocytes stimulated with*
175 ### *adrenergic agonists*

176 Treatment with adrenergic agonists (Fig. 5A) does not alter IL-2 or IFN-γ production in
177 lymphocytes after 24 hours (86420 seconds) of treatment. However, treatment with 17β-estradiol
178 alone (Fig. 5B) or 17β-estradiol with adrenergic agonists (Fig. 5C) significantly enhance IFN-γ
179 and IL-2 production after 24 hours of treatment.

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180 ***3.5 17β-estradiol co-stimulation of lymphocytes stimulated with adrenergic agonists***
181 ***enhances proliferation dose-dependently***

182 Adrenergic stimulation of lymphocytes significantly enhanced pro-apoptotic signals (Fig.
183 6A) above proliferation and favoured lymphocyte apoptosis. Co-stimulation with 17β-estradiol
184 significantly enhanced proliferation and shifts the balance between pro-apoptotic and apoptotic
185 signals (Fig. 6B). Ten-fold increase in 17β-estradiol concentration (Fig. 6C) further increases
186 proliferation signals and reverses the balance in favor of proliferation over apoptosis after 24 hours.

187 ***3.6 Phenylephrine stimulation shows significantly increased sensitivity to IFN-γ and p-***
188 ***CREB expression***

189 Stimulation of lymphocytes with α1-AR agonist, phenylephrine, showed increased
190 sensitivity to IFN-γ ($2X10^{-25}$) but not IL-2 expression (Fig. 7A). Similarly, signaling molecules
191 also showed altered sensitivities upon stimulation with phenylephrine, whereas p-CREB
192 expression alone showed increased sensitivity of $2X10^{-22}$ (Fig. 7B).

193 ***3.7 Clonidine stimulation shows no alteration in sensitivity to cytokines or signaling***
194 ***molecules***

195 There was no significant alterations in the sensitivities of IFN-γ or IL-2 upon stimulation
196 of lymphocytes with α2-AR agonist, clonidine (Fig. 8A). Similarly, sensitivities of signaling
197 molecules p-ERK, p-CREB, p-Akt, and cAMP were not altered upon treatment of lymphocytes
198 with clonidine (Fig. 8B).

199 ***3.8 Terbutaline stimulation of lymphocytes shows significantly enhanced sensitivity to IFN-***
200 ***γ, p-ERK, and p-CREB expression***

201 Stimulation of lymphocytes with β2-AR agonist, terbutaline, showed increased sensitivity
202 to IFN-γ ($3.5X10^{-24}$) but not IL-2 production (Fig. 9A). Similarly, signaling molecules also showed

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203 altered sensitivities upon stimulation with terbutaline, whereas p-ERK and p-CREB expression
204 alone showed increased sensitivity of $7X10^{-21}$ (Fig. 9B).

205 ***3.9 17β-estradiol stimulation of lymphocytes shows significantly enhanced sensitivity to***
206 ***IFN-γ, p-ERK, p-CREB and cAMP expression***

207 There was a significant increase in the sensitivity of IFN-γ ($3.5X10^{-16}$) upon stimulation of
208 lymphocytes with 17β-estradiol (Fig. 10A). Also, there was a significantly high sensitivity to
209 signaling molecules including p-ERK, p-CREB, and cAMP upon treatment of lymphocytes with
210 17β-estradiol ($6X10^{-15}$) (Fig. 10B).

211 ***3.10 Direct inhibition of ERK by NE***

212 On the basis of the data obtained, the enzyme ERK (PDB ID: 2ERK; *Rattus rattus;* was
213 inhibited by NE showing significant inhibitory activity. Analysis of the results obtained
214 demonstrate that NE binds to the Catalytic residue Asp 147 and Glu 69; the residue that forms an
215 ion pair with Lys 52 in the phosphate binding site of ERK with a Ki=207.69 µM, bond distance
216 of 2Åand an energy of -5.02Kcal/mol (Fig. 11A) and to Asp 177 which is close to the catalytic
217 pocket with a Ki=365.7µM and an energy of -4.69Kcal/mol (Fig. 11B and 11C).

218 ***3.11 Direct inhibition of β2-AR by 17β-estradiol:***

219 17β-estradiol also bound to β2-AR receptor (PDB ID: 3SN6; *Rattus norvegicus)* at the
220 Asp 173 residue with a Ki of 22.09 µM, bond distance of 1.99 Åand an energy of -6.35Kcal/mol
221 (Fig. 12A), the Met 61 residue with a Ki=11.0 µMand an energy of -6.76Kcal/mol(Fig. 12B) and
222 the Arg 314 residue with a Ki= 8.77 µM, and an energy of -6.9Kcal/mol(Fig. 12C) indicating
223 potent inhibitory effect.

224 **4.0 Discussion**

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225 Immune functions of innate, humoral or cell-mediated origin are modulated by sympathetic
226 signals and circulating hormone levels during health and disease in a dose and receptor-type-
227 dependent manner [1, 10, 12, 19]. *In vitro* studies from our laboratory have shown that adrenergic
228 stimulation through α1-, α2- or β2- ARs using specific agonists non-specifically inhibit
229 lymphoproliferation through distinct signaling pathways: α1-ARs mediated immunosuppressive
230 effects by inhibiting IFN-γ production, α2-AR activated the NF-κB, p-Akt and NO pathways and
231 β2-AR activation involved IL-6, NO and NF-κB signaling cascades mediating immunosuppression
232 [17, 18]. On the other hand, treatment of lymphocytes with 17β-estradiol enhanced proliferation
233 in a dose and receptor subtype specific manner through p-ERK, p-CREB and p-Akt involving IFN-
234 γ and compensatory mechanisms including anitioxidant enzymes [4, 20]. Our studies have shown
235 that co-treatment of lymphocytes with 17β-estradiol and adrenergic agonists lead to 17β-estradiol-
236 mediated over-ride of adrenergic immunosuppression in a dose-dependent, AR-subtype
237 independent manner [17, 18].

238 In the present study, we have modeled these signaling pathways *in silico* using MATLAB
239 Simbiology toolbox. Due to difficulties in conducting the experiments using NE, the *in vitro*
240 studies were conducted using receptor-specific agonists and antagonists. While these experiments
241 are highly beneficial in elucidating the dose-dependent effects on specific adrenoceptor sub-types,
242 they cannot be considered as absolute indicators of NE-mediated effects. This is because when NE
243 is added to the cells, depending on its relative affinity for the individual receptor subtypes, it may
244 bind proportionally to all the ARs triggering a wide variety of down-stream signals. The
245 cumulative effects of these signals are likely to be different from the outcomes of receptor-subtype-
246 specific signaling fates. In order to overcome this problem, the simulation model was constructed
247 on the basis of the data obtained from *in vitro* studies and all three ARs studied were

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248 simultaneously activated to study the outcome. Based on previous findings, an attempt was made
249 to incorporate cross-talks between these pathways by using common signaling molecule pools.

250 As expected, the simulation of lymphocytes with adrenergic agonists, phenylephrine,
251 clonidine, and terbutaline enhanced p-CREB and p-ERK expression in accordance with classical
252 adrenergic signaling cascades within a few milliseconds. 17β-estradiol behaved similarly in the
253 presence and absence of adrenergic agonists enhancing cAMP, p-ERK, and p-CREB expression
254 suggesting that activation of cAMP may be crucial to 17β-estradiol-mediated over-ride of
255 adrenergic immunosuppression.

256 A significant role is played by compensatory mechanisms such as antioxidant enzyme apart
257 from signaling molecules, to influence the proliferative/apoptotic milieu of a cell by balancing free
258 radical load including superoxides and peroxynitrites to name a few. These free radicals released
259 as by-products following immune responses or during neurotransmitter release in secondary
260 lymphoid organs may accumulate over the years and contribute to denervation of sympathetic
261 noradrenergic fibers leading to impaired neuroendocrine-immune homeostasis setting the stage for
262 age-associated diseases [7]. Studies from our lab and others have documented ER-subtype
263 dependent increase in SOD and GPx activities in lymphocytes stimulated with estrogen [4, 21-24].
264 Adrenergic stimulation also enhanced SOD and catalase activities *in vitro* which were suppressed
265 upon coincubation with 17β-estradiol indicating cross-talk between the two pathways leading to
266 diminutive effects [18]. Along these lines, previous studies have implicated the involvement of
267 PKA and ERK pathways, which when inhibited, reversed terbutaline-mediated increase in SOD
268 and CAT activities. It is possible that these signaling molecules play a regulatory role in the
269 expression and activity of these enzymes, thereby, exerting immunomodulatory effects [25, 26].
270 In our model, adrenergic activation, 17β-estradiol treatment, and their co-treatment decrease

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271 superoxide formation but enhance peroxynitrites, although 17β-estradiol treatment or co-treatment
272 alone was accompanied by an induction of SOD and CAT activities within the 3000 second time
273 frame.

274 Cytokines play a crucial role in influencing immune functions thereby affecting
275 survival/apoptosis. Previously we have shown that while activation of α1-ARs decrease IFN-γ and
276 increase IL-2 production, α2- and β2-AR activation did not alter both IFN-γ and IL-2 production.
277 Co-activation of all three signals show that adrenergic stimulation does not alter IL-2 or IFN-γ
278 production in lymphocytes after 24 hours (86420 seconds) of treatment. However, treatment with
279 17β-estradiol alone or 17β-estradiol with adrenergic agonists significantly enhance IFN-γ
280 production alone after 24 hours of treatment. We have reported dose-dependent increase in IFN-γ
281 production with 17β-estradiol alone or co-treated with α1- and β2-AR agonists. In agreement with
282 these findings, sensitivity analysis show that stimulation of lymphocytes with α1-AR agonist,
283 phenylephrine, showed increased sensitivity to IFN-γ ($2X10^{-25}$) and p-CREB expression $2X10^{-22}$
284 while β2-AR agonist terbutaline showed increased sensitivity to IFN-γ ($3.5X10^{-24}$), p-ERK and p-
285 CREB expression $7X10^{-21}$. Interestingly 17β-estradiol signaling showed the highest sensitivity to
286 IFN-γ ($3.5X10^{-16}$), p-ERK, p-CREB, and cAMP ($6X10^{-15}$) suggesting a probable cause in the
287 dynamics of 17β-estradiol-mediated signals that predispose it to over-ride adrenergic signals.

288 In order to assess the likely effects of the signals and their cross-talks on proliferation, end-
289 point signals were classified as either pro-apoptotic or survival signals. Plots obtained at the end
290 of 72 hours indicate that while adrenergic stimulation was predominantly pro-apoptotic signals,
291 17β-estradiol signals were pro-survival. Dose-dependent role of 17β-estradiol could be seen by
292 increasing the 17β-estradiol concentration by 10-fold leading to further increase in proliferation
293 signals reversing the balance in favor of proliferation over apoptosis after 72 hours. This is in

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294 accordance with the *in vitro* evidence generated in previous studies where all the adrenergic
295 agonists decreased proliferation while 17β-estradiol played a dose-dependent role in increasing
296 proliferation of lymphocytes.

297 Finally, docking studies show that apart from modulating signaling mechanisms,
298 NE can directly inhibit ERK and estrogen can directly bind to key residues on β_2-AR inhibiting its
299 subsequent signal transduction processes. Thus, *in silico* modeling of estrogen and adrenergic
300 signaling pathways in lymphocytes implicates the involvement of cAMP and ERK as key
301 regulatory molecules that influence the cross-talk between the signaling cascades mediating
302 immunomodulatory effects. Modeling estrogen and adrenergic stimulation-mediated signals will
303 help us to understand variations in the expression of downstream molecular markers with age- and
304 disease-associated variations in kinetic parameters and concentrations and thereby providing an
305 effective tool for understanding the alterations in the cross talk between the pathways in health,
306 aging, and diseases.

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399 **Figure Legends**

400 **Figure 1** *In silico model of adrenergic and estrogen signaling cascades in lymphocytes:* The
401 cross-talk between the adrenergic and estrogen-mediated signals result in specific
402 immunomodulatory effects depending upon the estrogen concentration. The layout of the signaling
403 pathway is elucidated from receptors to transduction into the cytosol and nuclear translocation of
404 signaling molecules leading to specific outcomes.

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405 **Figure 2** *Alteration of signaling molecules with adrenergic agonists and 17β-estradiol*
406 *treatment:* While adrenergic agonists enhanced p-CREB and p-ERK expression (A), 17β-estradiol
407 treatment alone (B) or with adrenergic agonists (C) enhanced cAMP, p-ERK and p-CREB
408 expression.

409 **Figure 3** *Adrenergic and estrogen signaling alter superoxides and peroxynitrites:* Both
410 adrenergic (A) and estrogen (B) signaling, alone or together (C) decrease superoxides and enhance
411 production of peroxynitrite in lymphocytes.

412 **Figure 4** *Adrenergic and estrogen signaling alter superoxides and peroxynitrites*
413 *differentially:* Estrogen treatment alone (B) or on adrenergic agonist-treated lymphocytes (C)
414 enhanced CAT and SOD activities and decreased peroxynitrite production. These effects were not
415 observed on adrenergic stimulation alone (A).

416 **Figure 5** *17β-estradiol but not adrenergic stimulation alters cytokine production:* Treatment
417 with 17β-estradiol alone (B) or 17β-estradiol with adrenergic agonists (C) but not adrenergic
418 agonists alone (A) significantly enhances IFN-γ production after 24 hours of treatment.

419 **Figure 6** *17β-estradiol enhances survival signals while adrenergic signals are pro-*
420 *apoptotic:* Adrenergic stimulation of lymphocytes favors lymphocyte apoptosis over survival (A)
421 while treatment with estrogen alone (B) or co-stimulation with 17β-estradiol shifts the balance in
422 favor survival signals (C).

423 **Figure 7** *α1-AR stimulation in sensitive to IFN-γ and p-CREB:* Sensitivity analysis showed
424 that α1-AR stimulation was sensitive to IFN-γ ($2X10^{-25}$; A) and p-CREB ($2X10^{-22}$; B).

425 **Figure 8** *α2-AR stimulation is not sensitive to cytokine production or signaling molecules:*
426 Clonidine mediated signaling is not sensitive to cytokines (A) or signaling molecules (B).

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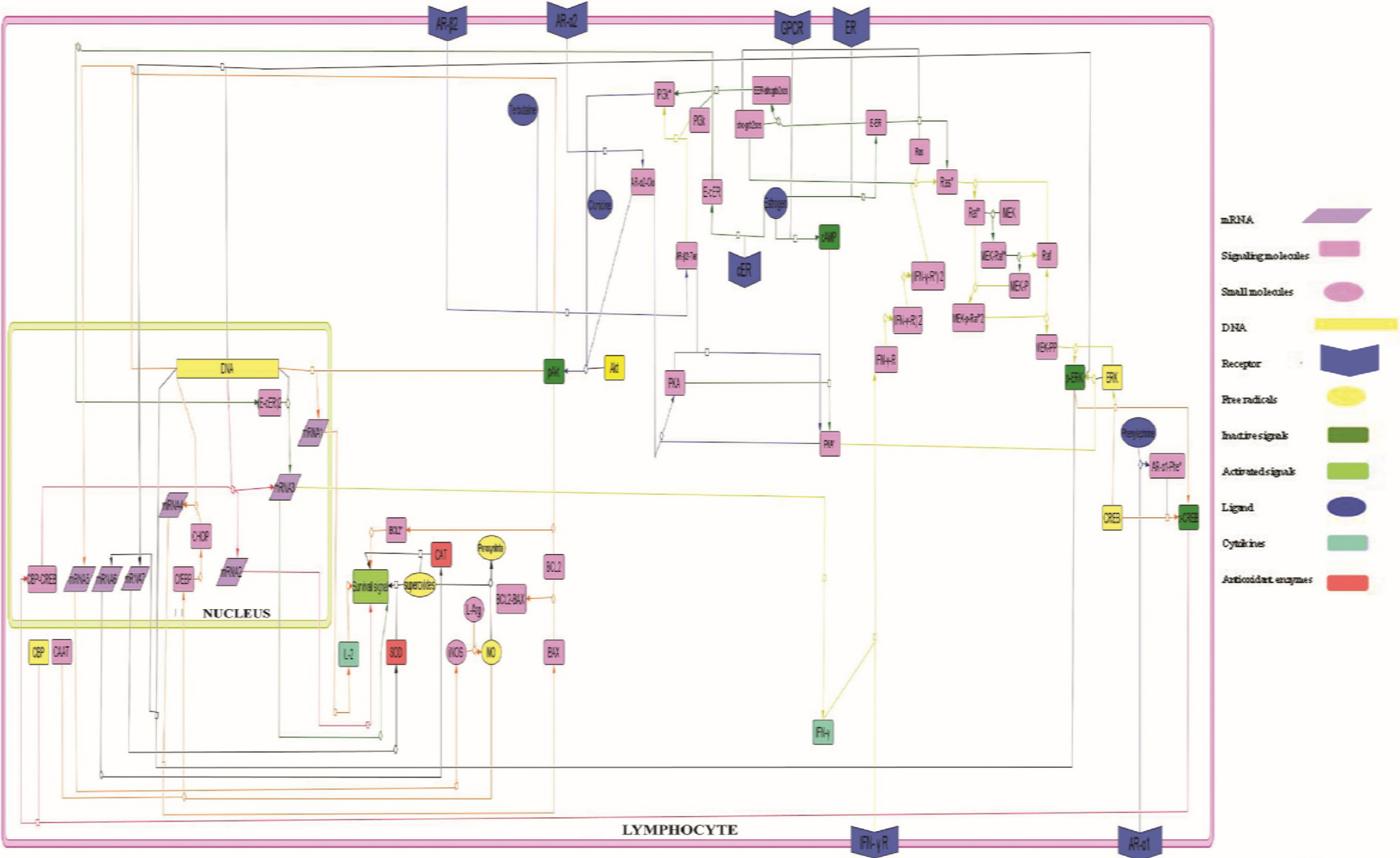
427 **Figure 9** *β2-AR stimulation of lymphocytes is sensitive to IFN-g, p-ERK and p-CREB*
428 *expression:* Terbutaline treatment –mediated signals are sensitive to IFN-γ ($3.5X10^{-24}$; A) and p-
429 ERK and p-CREB expression ($7X10^{-21}$; B).

430 **Figure 10** *Estrogen receptor stimulation is sensitive to IFN-g, p-ERK, p-CREB and cAMP*
431 *expression:* 17β-estradiol –mediated signals are sensitive to IFN-γ ($3.5X10^{-16}$; A), p-ERK, p-
432 CREB, and cAMP ($6X10^{-15}$; B).

433 **Figure 11** *Norepinephrine inhibits catalytic and phosphate binding residues of ERK:*
434 Norepinephrine inhibits ERK (PDB ID: 2ERK; *Rattus rattus*) by binding to the catalytic and
435 phosphate binding residues (A), and residues close to the catalytic pocket (B and C).

436 **Figure 12** *17β-estradiol inhibits β2-AR binding to key residues:*17β-estradiol inhibitsβ2-AR
437 receptor (PDB ID: 3SN6; *Rattus norvegicus)* by binding to the G-protein binding sites (A, B and
438 C).

Figure 1



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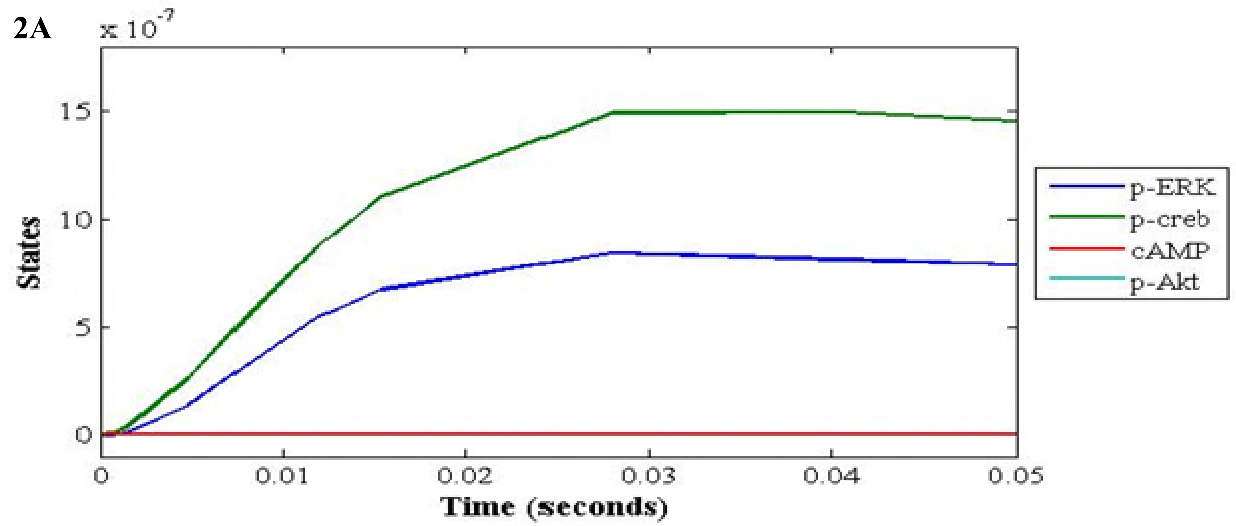

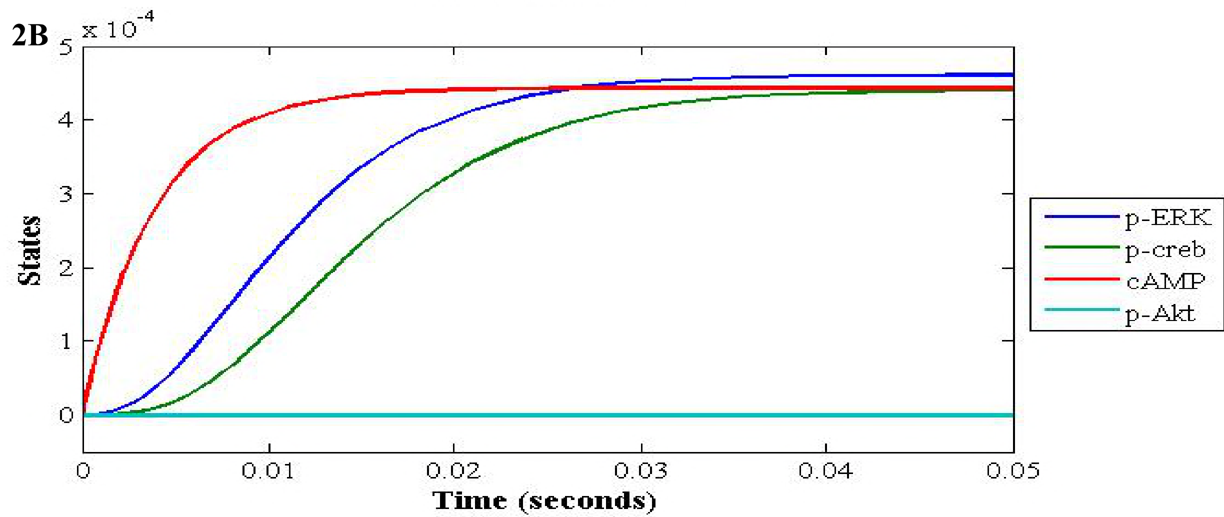

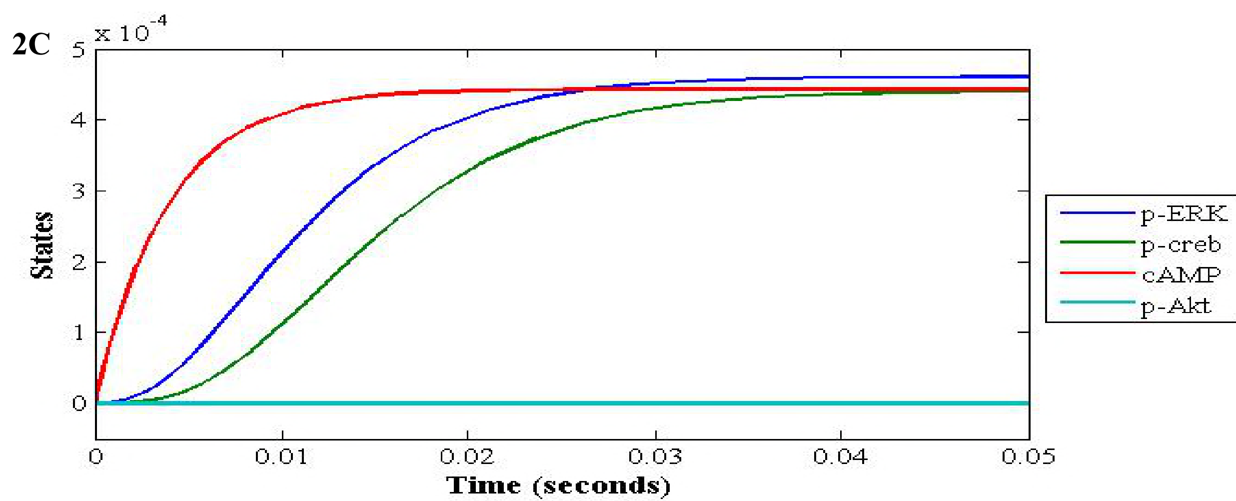

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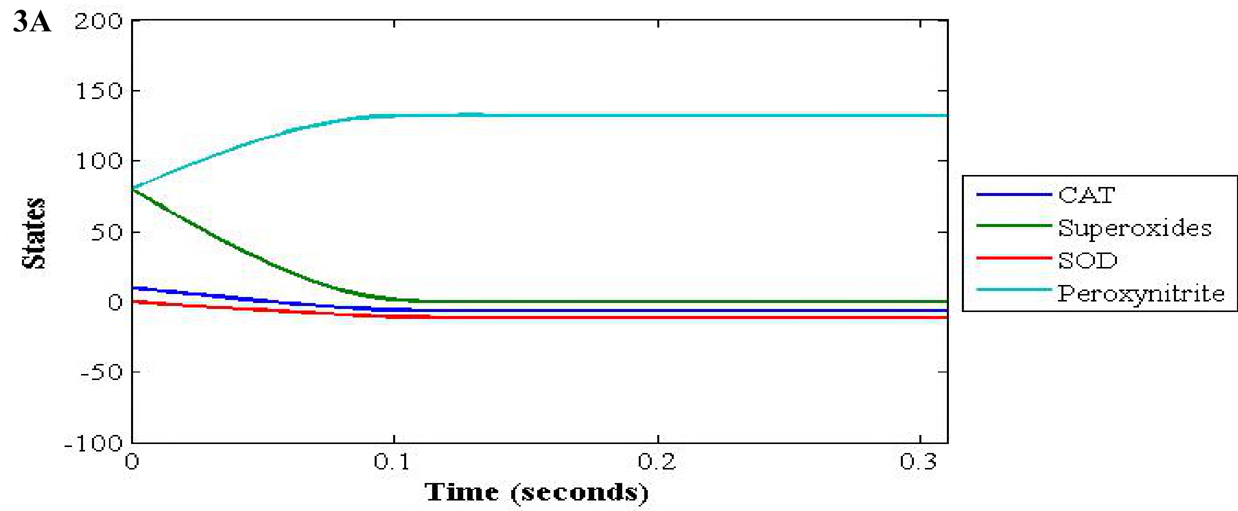

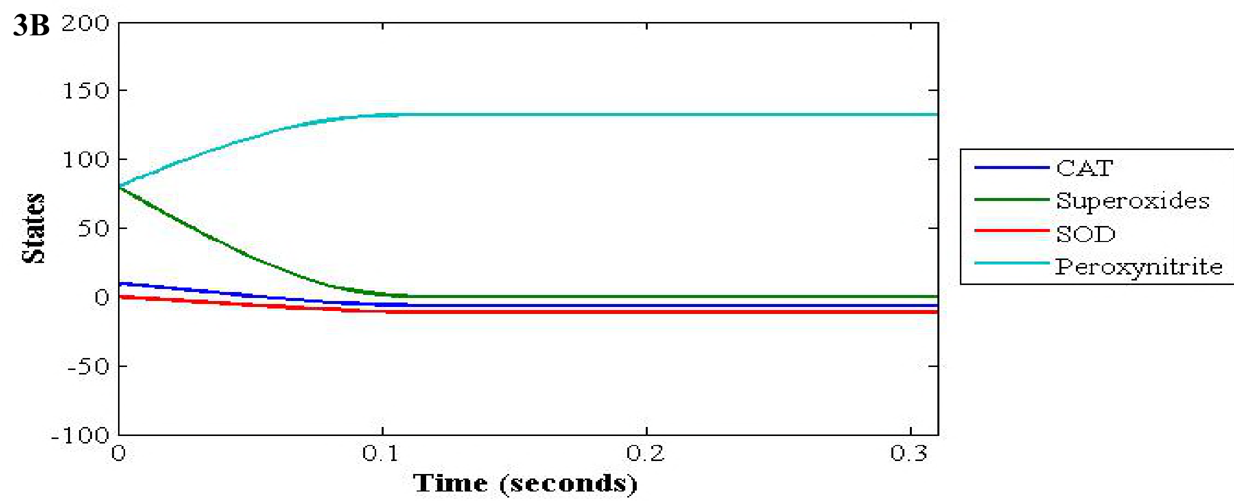

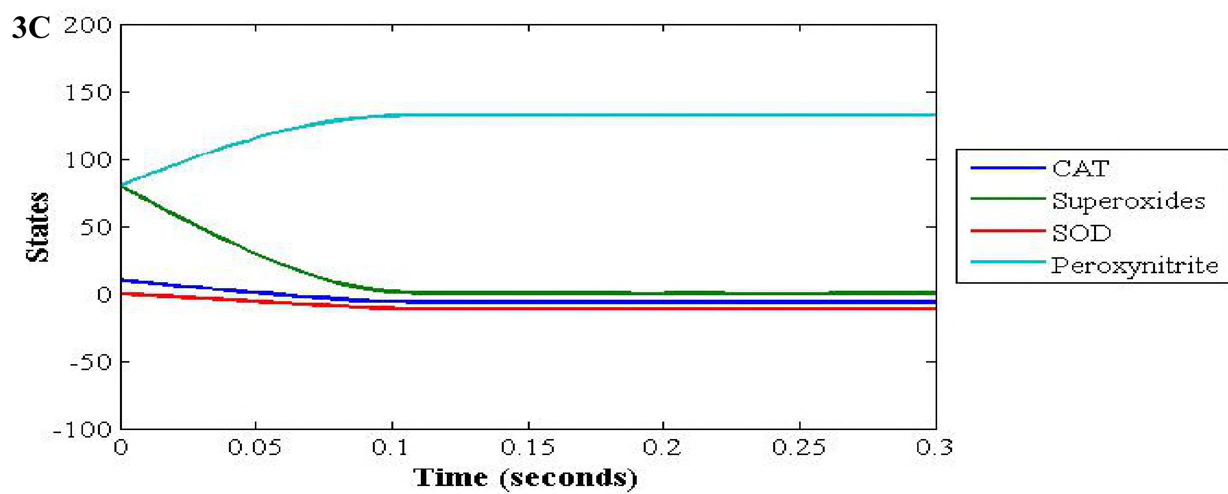

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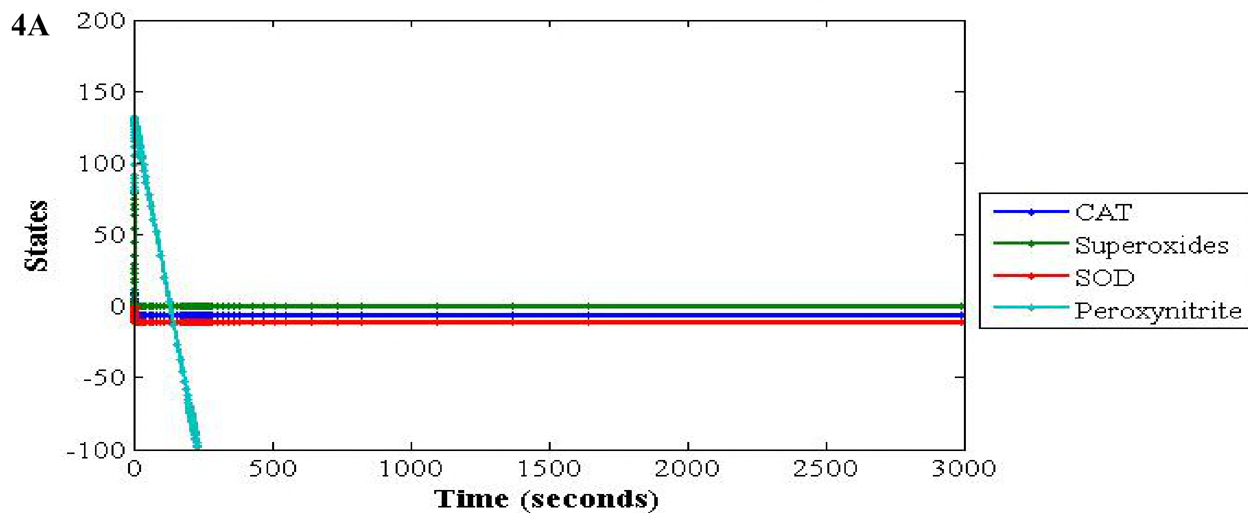

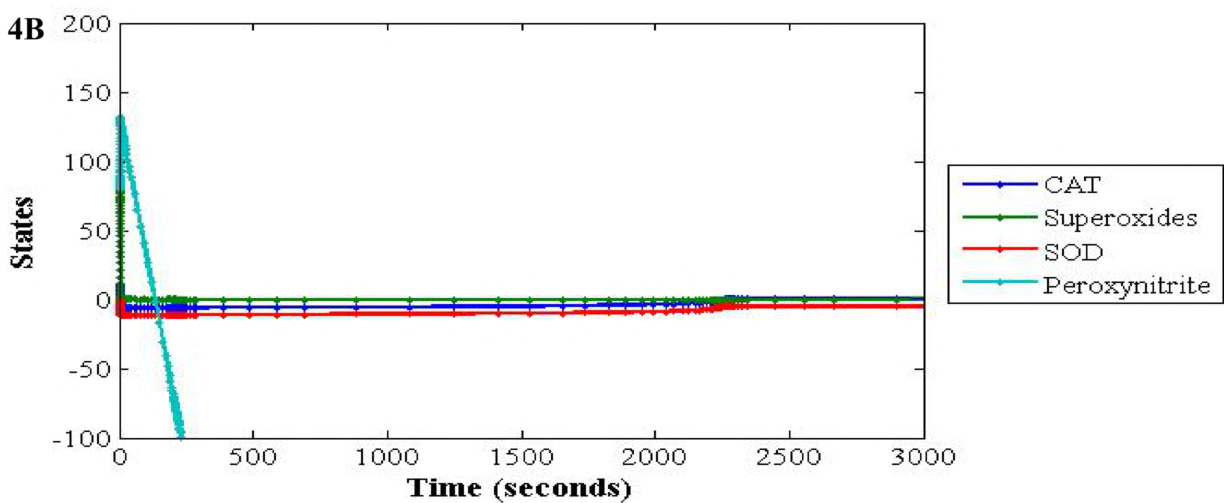

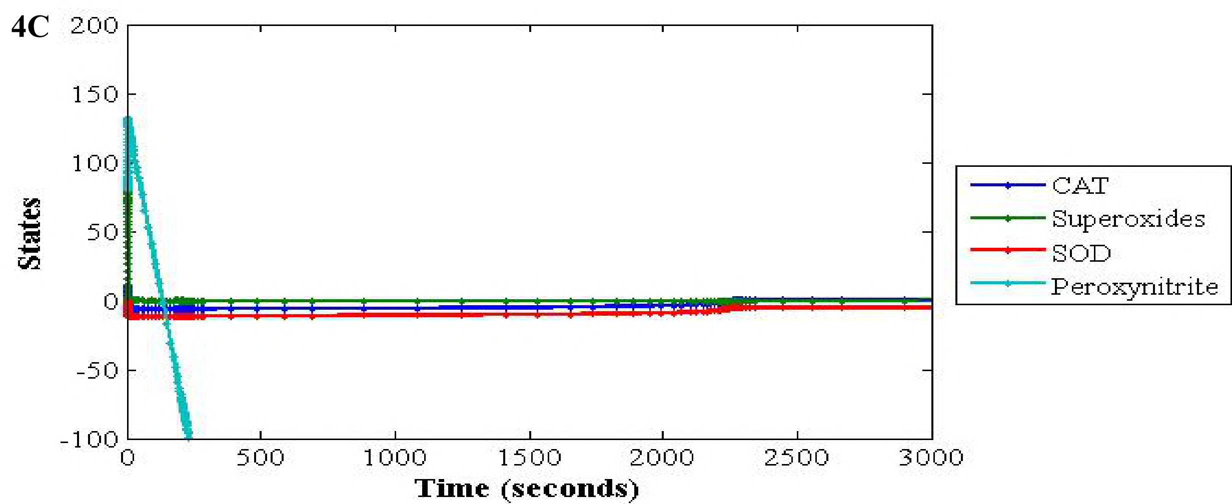

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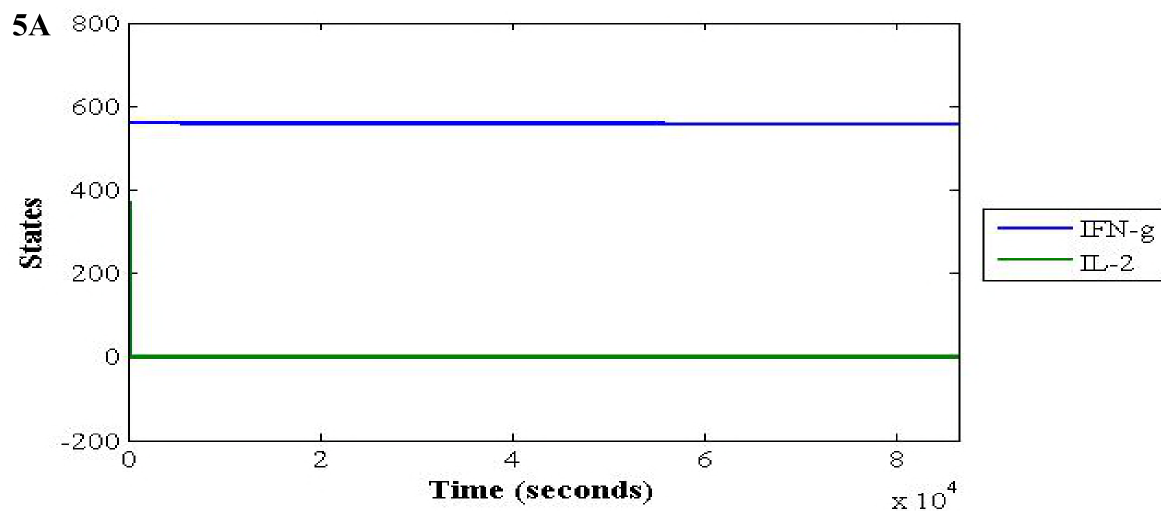

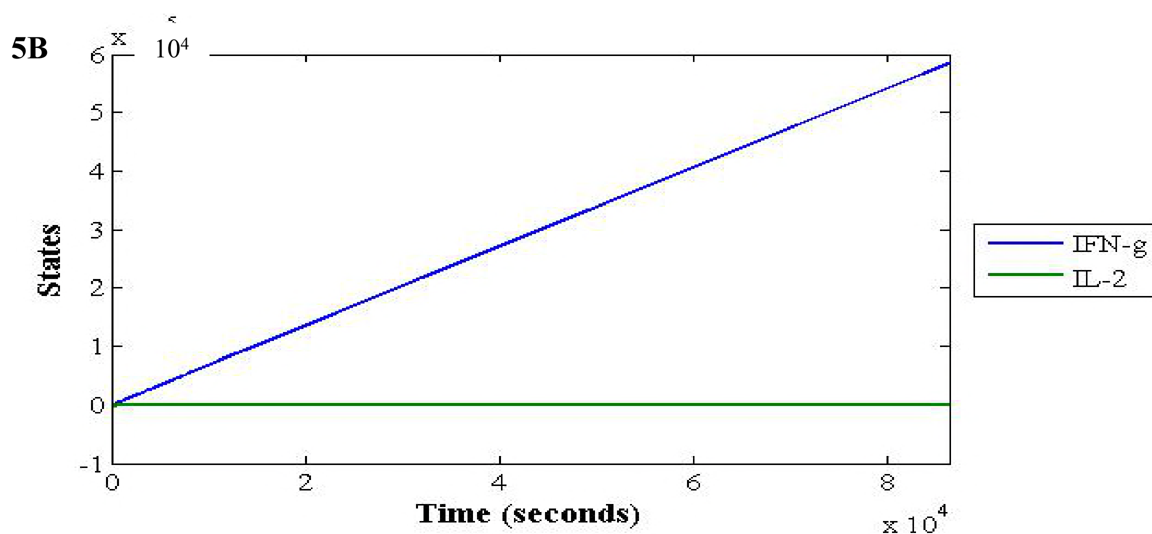

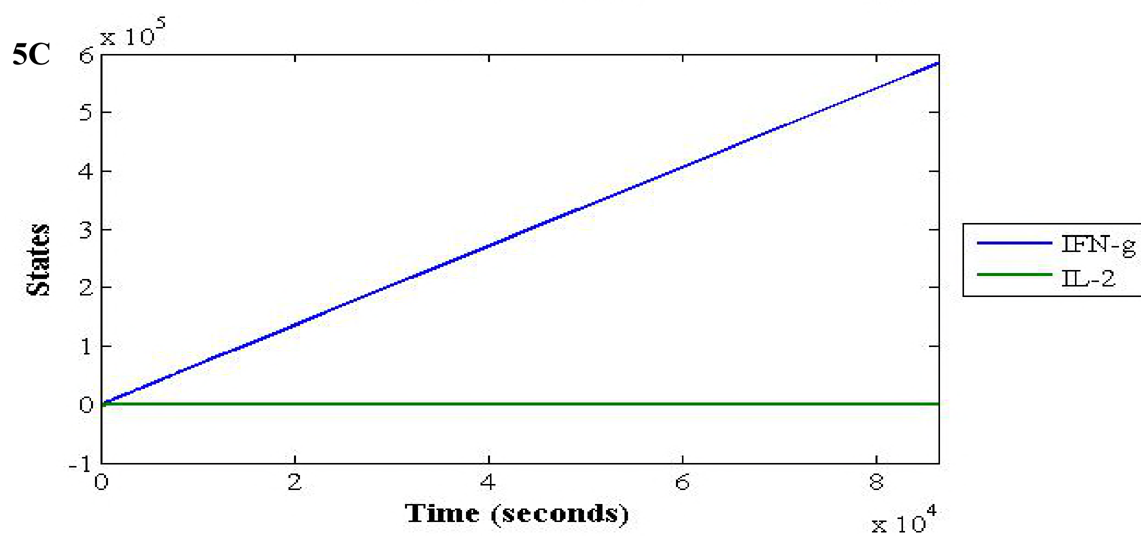

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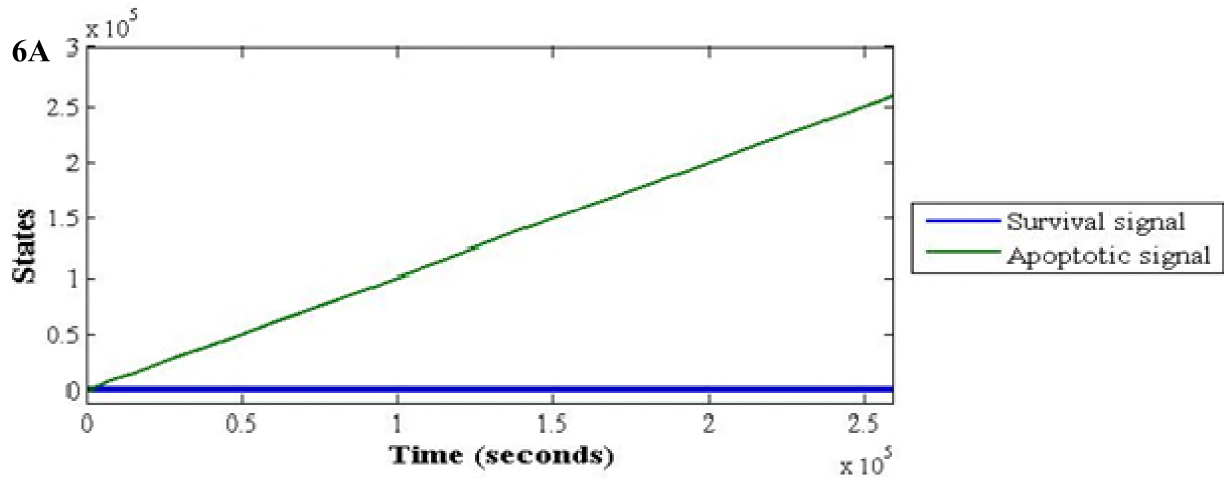

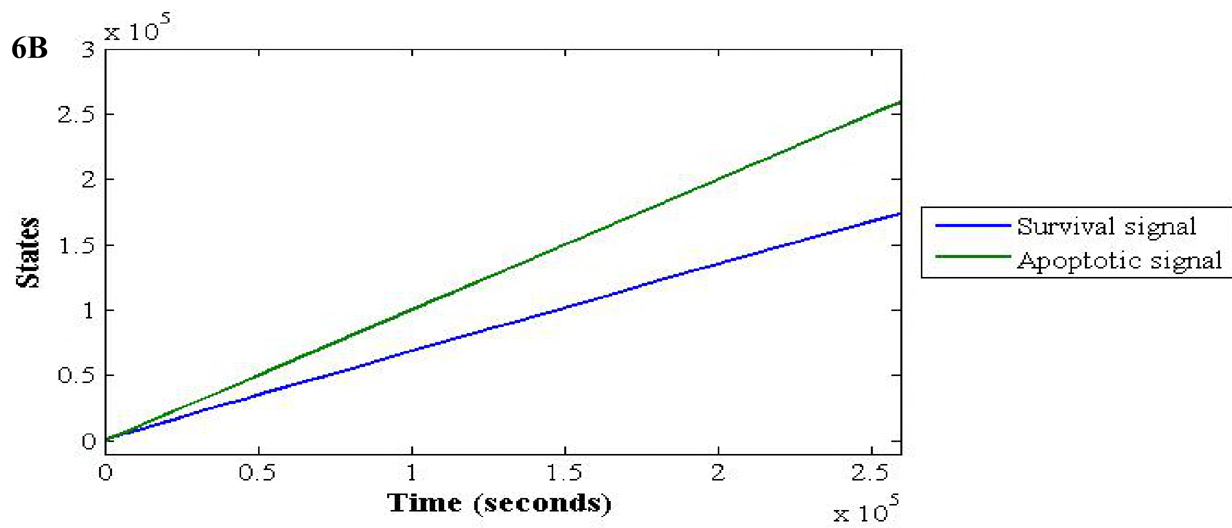

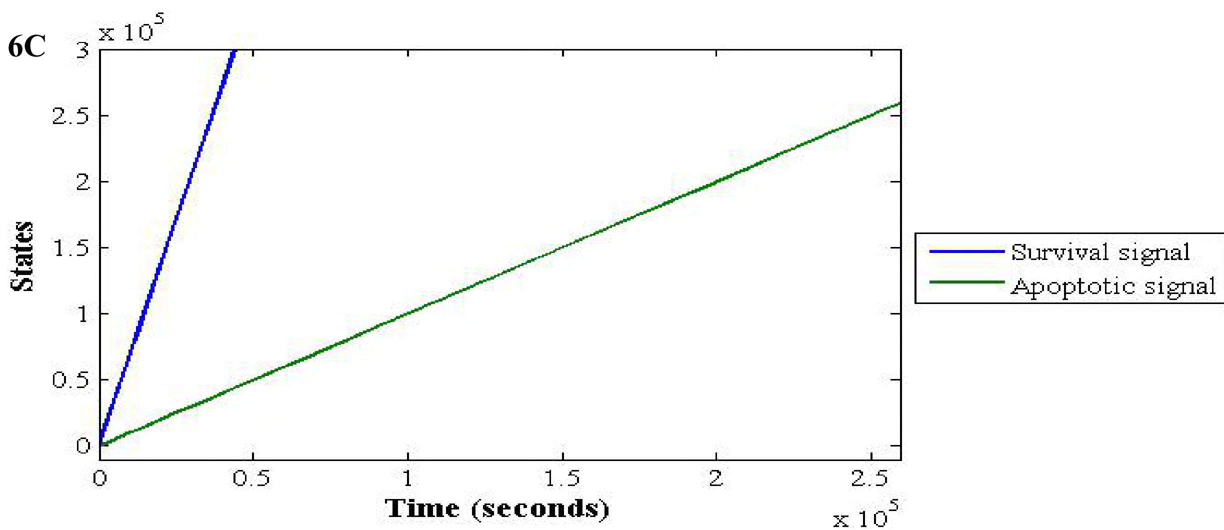

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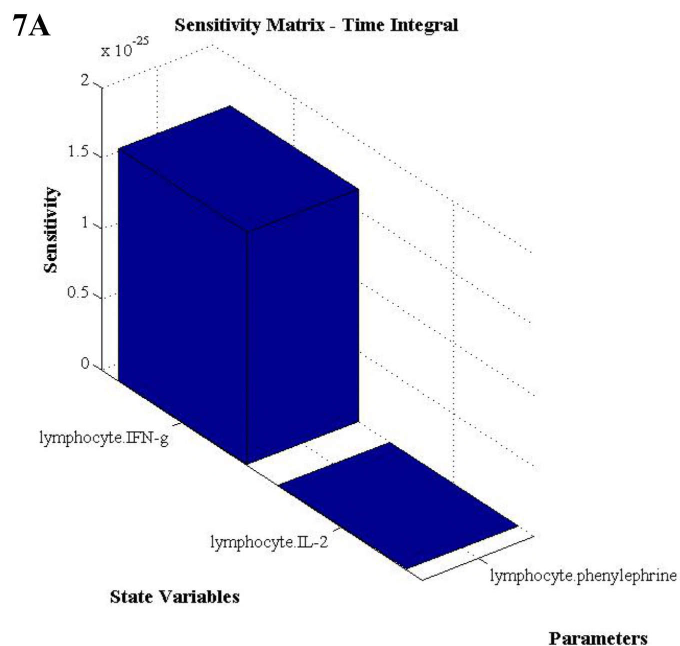

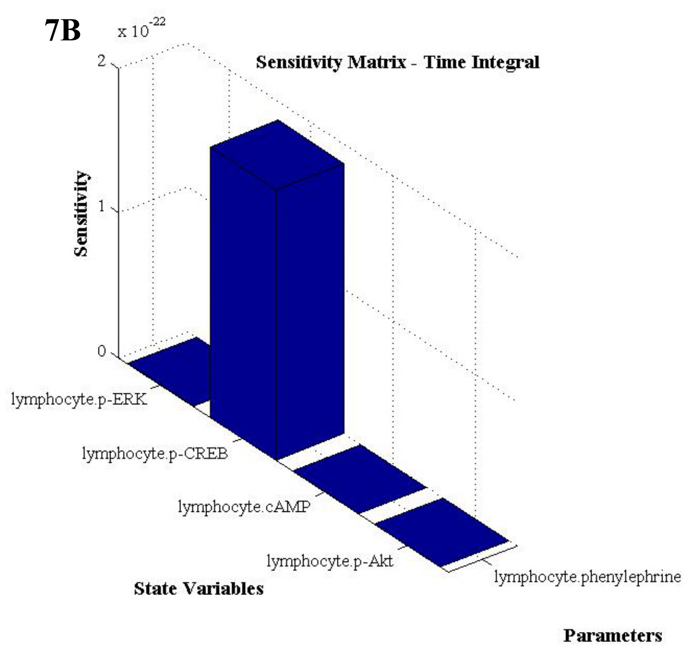

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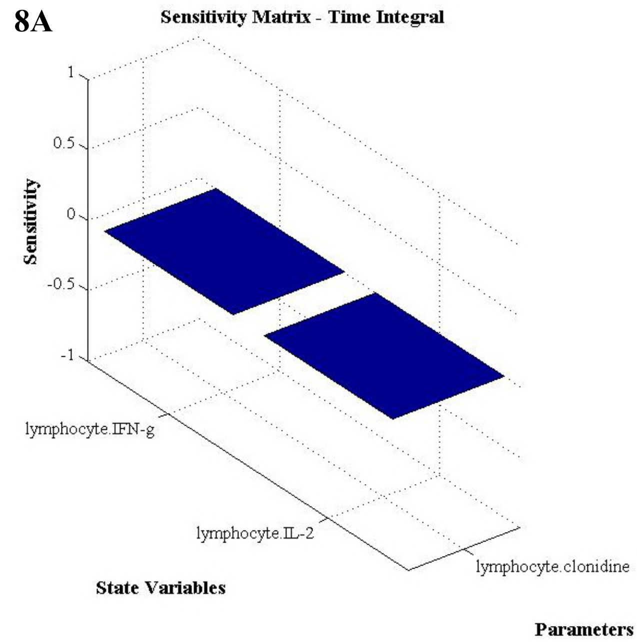

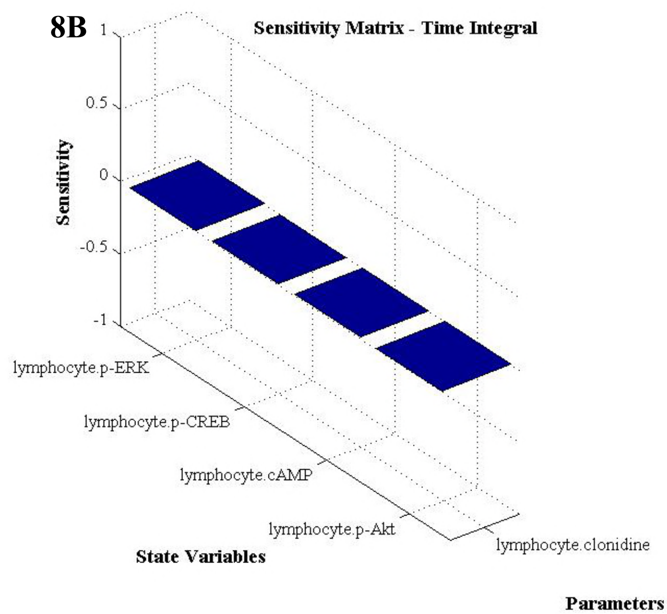

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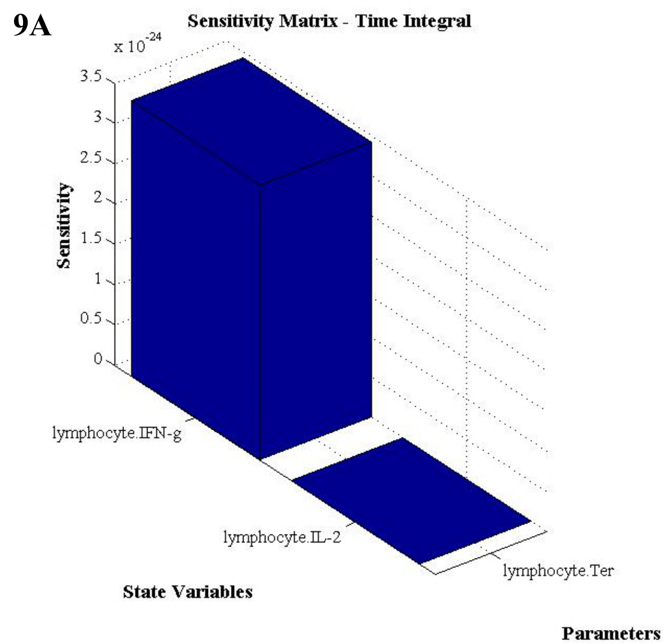

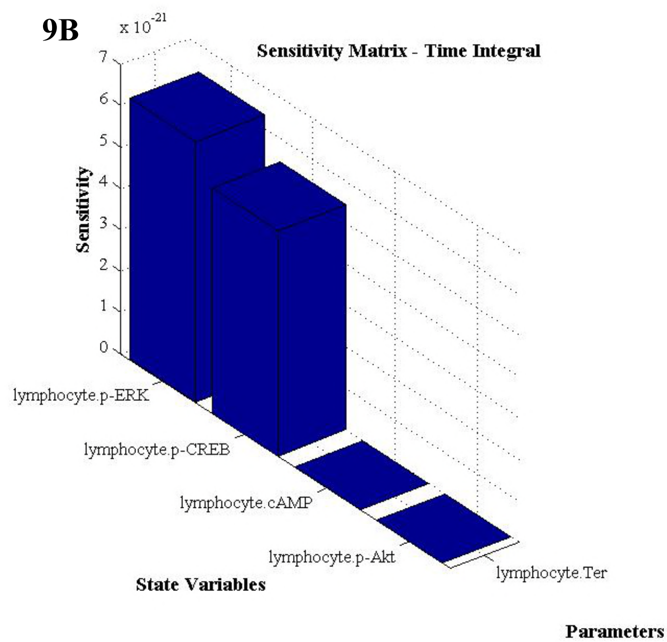

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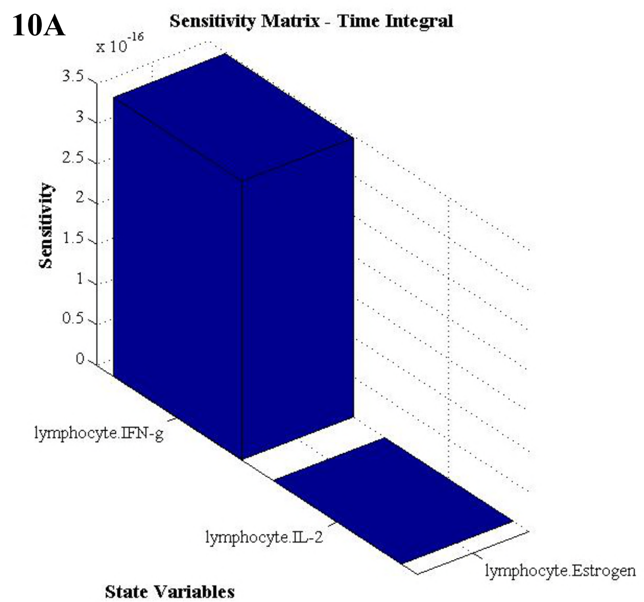

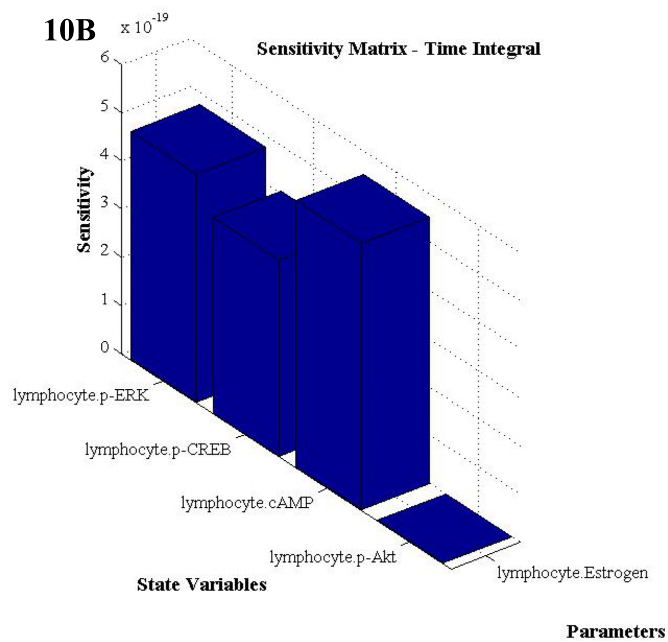

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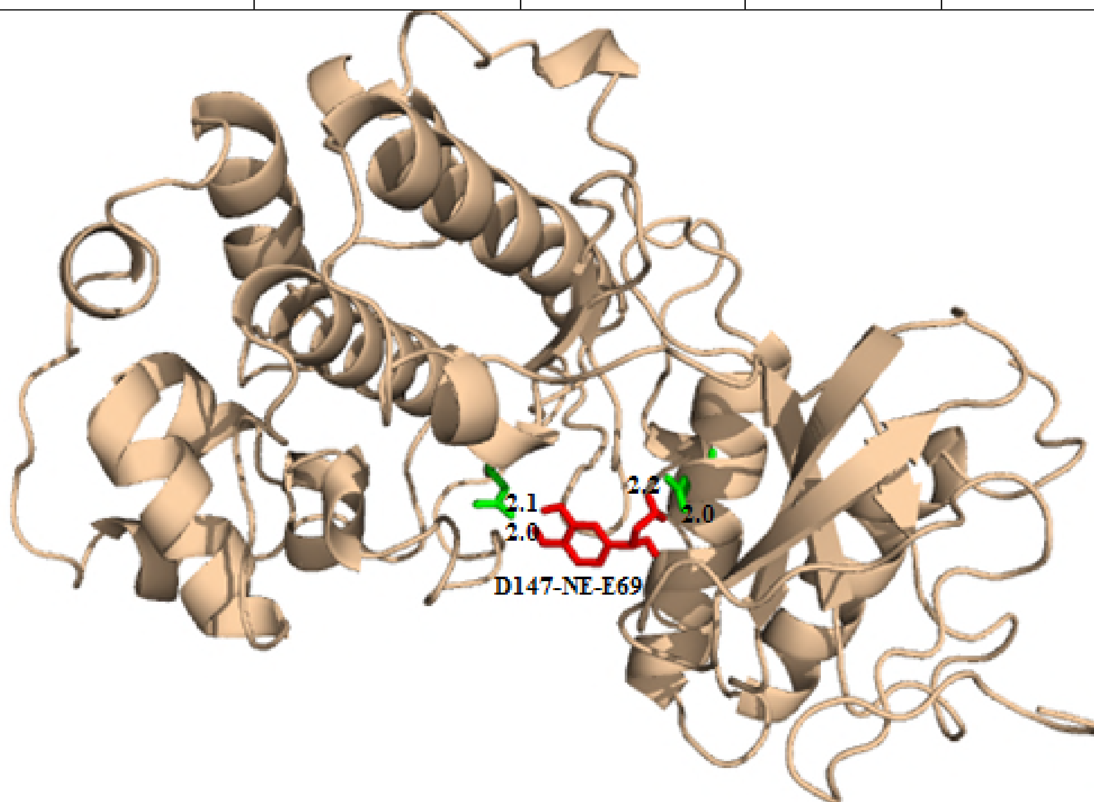
11A Ligand	**Residues of ERK that interact**	**Binding energy (Kcal/mol)**	**Ligand efficiency**	**Inhibitor constant**
Norepinephrine	Asp 147 Glu 69	-5.02	-0.42	207.69 µM



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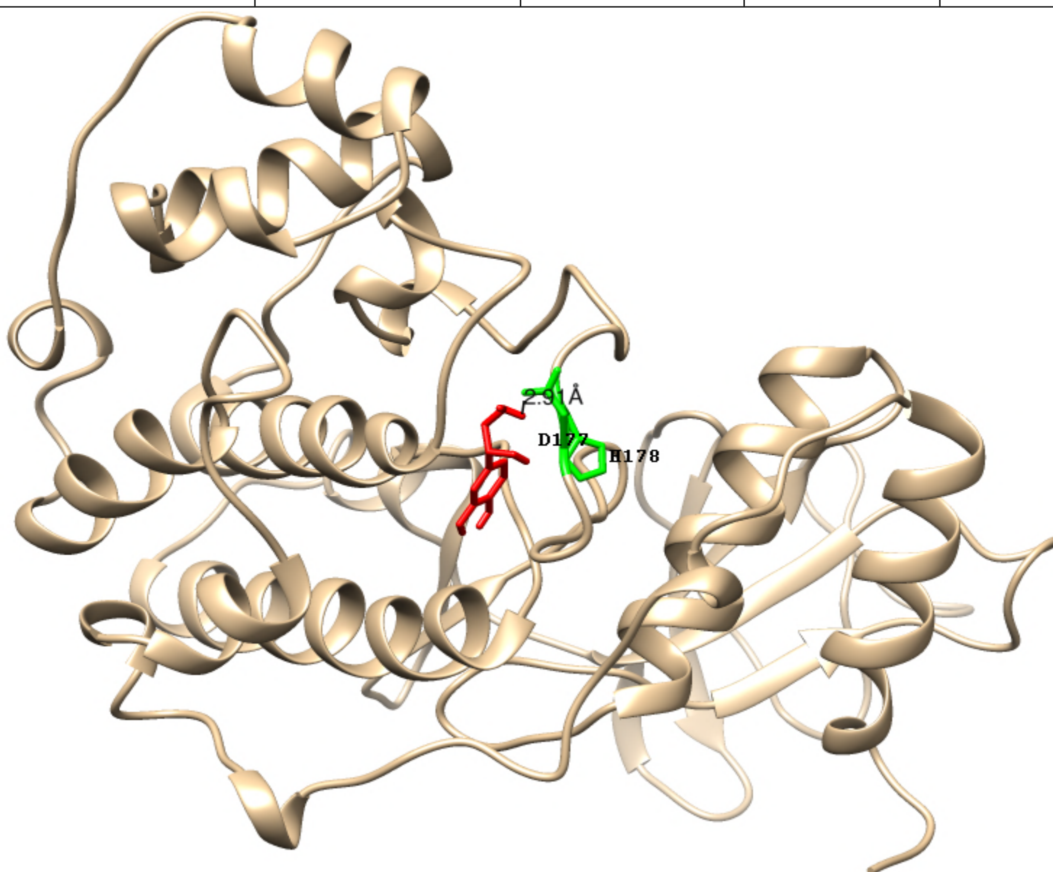
11B Ligand	**Residues of ERK that interact**	**Binding energy (Kcal/mol)**	**Ligand efficiency**	**Inhibitor constant**
Norepinephrine	His 178 Asp 177	-4.69	-0.39	365.7 µM



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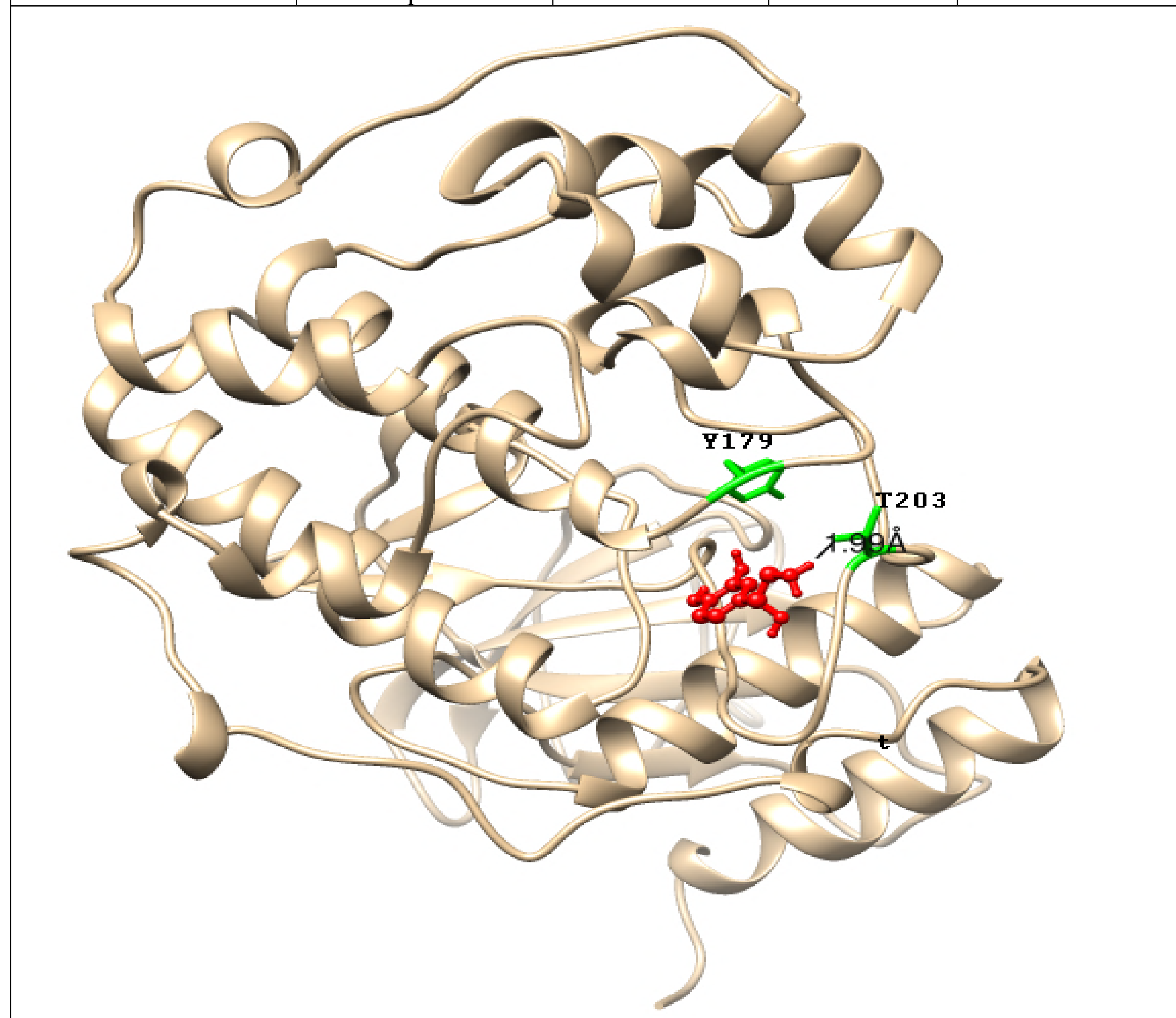
11C Ligand	Residues of ERK that interact	Binding energy (Kcal/mol)	Ligand efficiency	Inhibitor constant
Norepinephrine	Thr 179 Tyr 203	-4.48	-0.37	523.7 µM
	Tyr 203 Asp 177	-4.47	-0.37	528.44 µM



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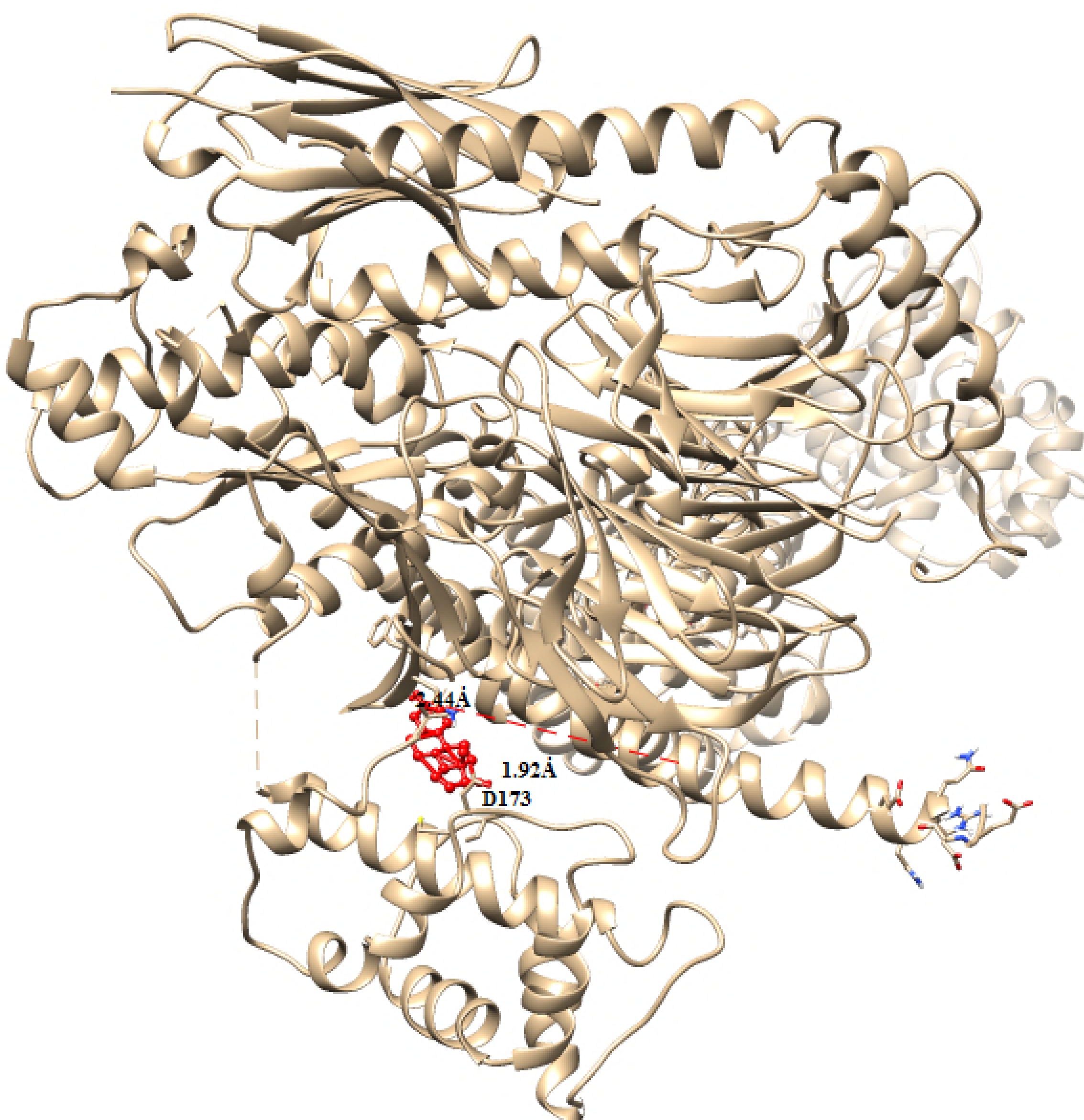
12A Ligand	Residues of β2-AR that interact	Binding energy (Kcal/mol)	Ligand efficiency	Inhibitor constant
17β-estradiol	Asp 173	-6.35	-0.32	22.09 µM



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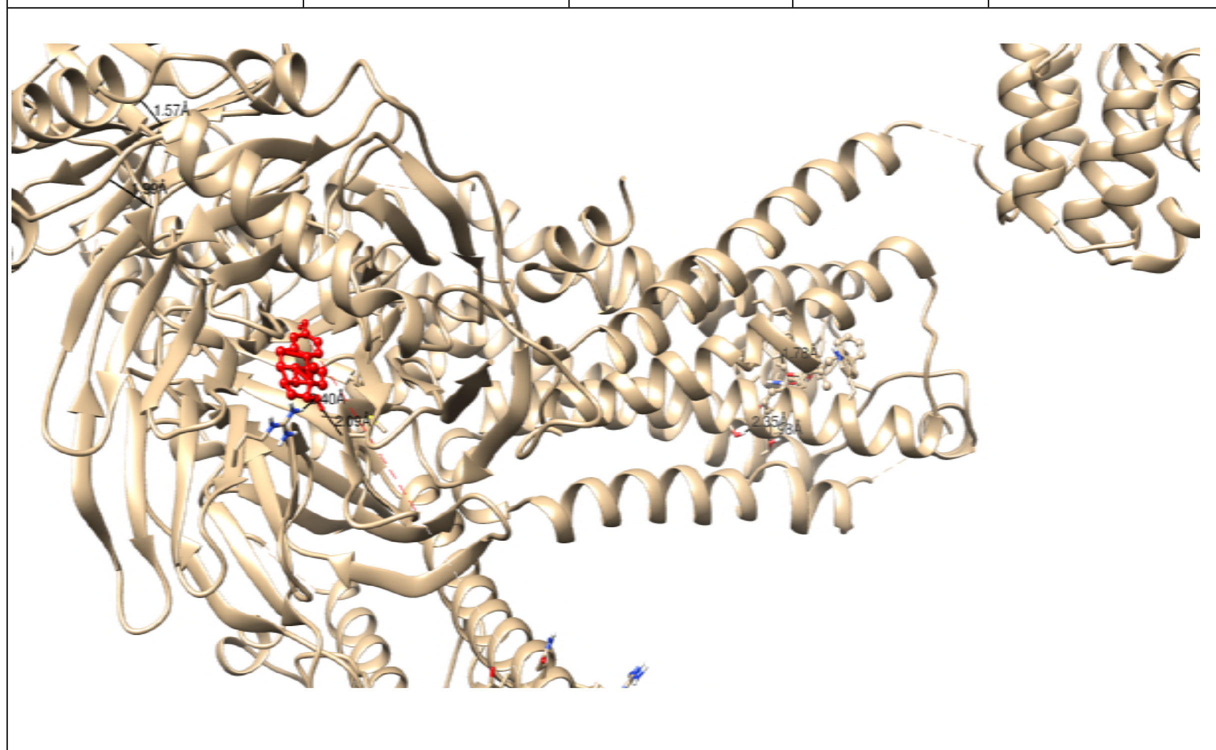
12B **Ligand**	**Residues of β2-AR that interact**	**Binding energy (Kcal/mol)**	**Ligand efficiency**	**Inhibitor constant**
17β-estradiol	Met 61	-6.76	-0.34	11.0 µM



12C **Ligand**	**Residues of β2-AR that interact**	**Binding energy (Kcal/mol)**	**Ligand efficiency**	**Inhibitor constant**

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17β-estradiol	Arg 314	-6.9	-0.35	8.77 µM
	Arg 314	-6.79	-0.34	10.49 µM