Chapter 3

MATERIAL AND METHODS

3.1 IN-SILICO STUDIES

3.1.1 Molecular target 1: Bcl-2

3.1.1.1 Approach 1

Autodock 4.0 suite was used as molecular-docking tool in order to carry out the docking simulations. PDB id: 2XA0, obtained from RCSB protein data bank, was used as initial structure for Bcl-2. The structure of ligands (quercetin and taxifolin) was generated from smile strings followed by energy minimization. Hydrogen atoms were added to protein crystal structures using Autodock program while all non polar hydrogen atoms were merged. Lamarckian genetic algorithm was used as a search parameter which is based on adaptive local search. Short range van der Waals and electrostatic interactions, hydrogen bonding, entropy losses were included for energy based Autodock scoring function (Morris et al., 1998, 2009). The Lamarckian GA parameters used in the study were numbers of run, 30; population size, 150; maximum number of eval; 25000000, number of generation; 27000, rate of gene mutation; 0.02 and rate of cross over; 0.8. Docking was carried out using grid size 126, 126 and 126 along the X, Y and Z axes with 0.375 Å spacing. RMS cluster tolerance was set to 2.0 Å. Semi-flexible docking was performed which includes a flexible ligand and a rigid receptor. All the protein and ligand structural images were generated using PYMOL (DeLano, http://www.pymol.org).

MD simulation of the complex was carried out with the GROMACS 4.5.4 package using the GROMOS96 43a1 force field (Berendsen et al., 1995; Lindah et al., 2001). The lowest binding energy (most negative) docking conformation generated by Autodock was taken as initial conformation for MD simulation. The topology parameters of proteins were created by using the Gromacs program. The topology parameters of taxifolin and quercetin were built by the Dundee PRODRG server (Schuttelkopf and van Aalten, 2004). The complex was immersed in an octahedron box of simple point charge (SPC) water molecules (Van Gunsteren et al., 1996, 1998). The solvated system (Bcl2, ligand and water) was neutralized by adding 4 Na ions in all simulation. To release conflicting contacts, energy minimization was performed using the steepest descent method of 10000 steps followed by the conjugate gradient method for 10000 steps. MD simulation studies consist of equilibration and production phases. To equilibrate the system, the solute (protein, counterions, and ligand) were subjected to the position-restrained dynamics simulation (NVT and NPT) at 300 K for 300 ps. Finally, the full system was subjected to MD production run at 300 K temperature and 1 bar pressure for 10000 ps. For analysis, the atom coordinates were recorded at every 0.5 ps during the MD simulation.

3.1.1.2 Approach 2

In approach 2 Bcl2-Bax complex was used as initial structure (PDB id:2XA0) for performing molecular docking and molecular dynamics simulation. Methodology was same as used in approach 1. Obatoclax was also used along with quercetin and taxifolin

as control. The lowest binding energy (most negative) docking conformation generated by Autodock was taken as initial conformation for MD simulation. The solvated system (Bcl2-Bax, ligand and water) was neutralized by adding 5 Na ions in all simulation. The full system was subjected to MD production run at 300 K temperature and 1 bar pressure for 12000 ps.

3.1.2 Molecular target 2: Heat shock protein 90

Pdb id 3K97 and 3K5B, obtained from RCSB protein data bank, were used as initial structure for Hsp90 NTD (N-terminal domain) apo form and Hsp90–cdc37 complex respectively. The structure of ligand taxifolin was generated from smile strings followed by energy minimization. All the heteroatom was removed except Mg ion. Hydrogen atoms were added to protein crystal structures using Autodock program while all non polar hydrogen atoms were merged. Lamarckian genetic algorithm was used as a search parameter, in Autodock 4.0, which is based on adaptive local search. Short range van der Waals and electrostatic interactions, hydrogen bonding, entropy losses were included for energy based Autodock scoring function (Morris et al., 1998, 2009). The Lamarckian GA parameters used in the study were numbers of run, 30; population size, 150; maximum number of eval; 25,000,000, number of generation; 27,000, rate of gene mutation; 0.02 and rate of cross over; 0.8. Blind docking was carried out using grid size 126, 126 and 126 along the X, Y and Z-axes with 0.375Å spacing. RMS cluster tolerance was set to 2.0 Å. Semi flexible docking was performed which includes a flexible ligand and a rigid

receptor. All the protein and ligand structural images were generated using PyMol (DeLano, http://www.pymol.org). MD simulation of the complex was carried out with the GROMACS 4.5.4 package using the GROMOS96 43a1 force field (Berendsen et al., 1995; Lindah et al., 2001). The lowest binding energy (most negative) docking conformation generated by Autodock was taken as initial conformation for MD simulation. The topology parameters of proteins were created by using the Gromacs program. The topology parameters of taxifolin were built by the Dundee PRODRG server (Schuttelkopf and van Aalten, 2004). The complex was immersed in an octahedron box of simple point charge (SPC) water molecules (Van Gunsteren et al., 1996, 1998). The solvated system (Hsp90, taxifolin and water) was neutralized by adding 8 Na ions, and 7 Na ions in case of Hsp90 NTD-taxifolin and Hsp90–cdc37–taxifolin complex respectively. To release conflicting contacts, energy minimization was performed using the steepest descent method of 10,000 steps followed by the conjugate gradient method for 10,000 steps. MD simulation studies consist of equilibration and production phases. To equilibrate the system, the solute (protein, counterions, and taxifolin) were subjected to the position-restrained dynamics simulation (NVT and NPT) at 300 K for 300 ps. Finally, the full system was subjected to MD production run at 300 K temperature and 1 bar pressure for 10,000 ps. For analysis, the atom coordinates were recorded at every 0.5 ps during the MD simulation.

3.1.3 Molecular target 3: Mouse double minute 2 (MDM2)

3.1.3.1 Approach 1

PDB id: 1T4E, obtained from RCSB protein data bank, was used as initial structure for MDM2. The structure of ligands (quercetin and taxifolin) was generated from smile strings followed by energy minimization. Hydrogen atoms were added to protein crystal structures using Autodock program while all non polar hydrogen atoms were merged. Lamarckian genetic algorithm was used as a search parameter which is based on adaptive local search. Short range van der Waals and electrostatic interactions, hydrogen bonding, entropy losses were included for energy based Autodock scoring function (Morris et al., 1998, 2009). The Lamarckian GA parameters used in the study were numbers of run, 30; population size, 150; maximum number of eval; 25000000, number of generation; 27000, rate of gene mutation; 0.02 and rate of cross over; 0.8. Blind docking was carried out using grid size 126, 126 and 126 along the X, Y and Z axes with 0.375 Å spacing. RMS cluster tolerance was set to 2.0 Å. Semi-flexible docking was performed which includes a flexible ligand and a rigid receptor. All the protein and ligand structural images were generated using PYMOL (DeLano, http://www.pymol.org). MD simulation of the complex was carried out with the GROMACS 4.5.4 package using the GROMOS96 43a1 force field (Berendsen et al., 1995; Lindah et al., 2001). The lowest binding energy (most negative) docking conformation generated by Autodock was taken as initial conformation for MD simulation. The topology parameters of proteins were created by using the Gromacs program. The topology parameters of taxifolin and quercetin were built by the

Dundee PRODRG server (Schuttelkopf and van Aalten, 2004). The complex was immersed in an octahedron box of simple point charge (SPC) water molecules (Van Gunsteren et al., 1996, 1998). The solvated system (MDM2, ligand and water) was neutralized by adding 4 Cl ions in all simulation. To release conflicting contacts, energy minimization was performed using the steepest descent method of 10000 steps followed by the conjugate gradient method for 10000 steps. MD simulation studies consist of equilibration and production phases. To equilibrate the system, the solute (protein, counterions, and ligand) were subjected to the position-restrained dynamics simulation (NVT and NPT) at 300 K for 300 ps. Finally, the full system was subjected to MD production run at 300 K temperature and 1 bar pressure for 65000 ps. For analysis, the atom coordinates were recorded at every 0.5 ps during the MD simulation.

3.1.3.1.1 Calculation of Binding Free Energy

The binding free energies were calculated using molecular mechanics/Poisson-Boltzman surface area (MMPBSA) approach (Swanson et al., 2004; Jogalekar et al., 2010; Thanyada et al., 2010) supplied with Amber 10 package. A total number of 200 snapshots evenly from the last 10 ns on the MD trajectory was chosen for the study. The MM-PBSA method can be conceptually summarized as:

$$\Delta G_{\text{bind}} = \Delta G_{\text{complex}} - [\Delta G_{\text{protein}} + \Delta G_{\text{lig}}] \quad (3.1)$$

$$\Delta G_{\text{bind}} = \Delta H - T\Delta S, \quad (3.2)$$

Where ΔH of the system is composed of the enthalpy changes in the gas phase upon complex formation (ΔE_{MM}) and the solvated free energy contribution (ΔG_{sol}), while $-T\Delta S$ refers to the entropy contribution to the binding. Equation (3.2) can then be approximated as shown in Eq. (3.3):

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G_{sol} - T\Delta S, \quad (3.3)$$

where ΔE_{MM} is the summation of the van der Waals (ΔE_{vdw}) and the electrostatic (ΔE_{ele}) interaction energies.

$$\Delta E_{MM} = \Delta E_{vdw} + \Delta E_{ele} \quad (3.4)$$

In addition, $\Delta Gsol$, which denotes the solvation free energy, can be computed as the summation of an electrostatic component ($\Delta G_{ele,sol}$) and a nonpolar component ($\Delta G_{nonpolar,sol}$), as shown in Eq. (3.5):

$$\Delta G_{sol} = \Delta G_{ele,sol} + \Delta G_{nonpolar,sol} \quad (3.5)$$

3.1.3.2 Approach 2

In approach 2 MDM2-p53 complex was used as initial structure (PDB id: 1YCQ) for erforming molecular docking and molecular dynamics simulation. Methodology used was same as Approach 1. The lowest binding energy (most negative) docking conformation generated by Autodock was taken as initial conformation for MD simulation. The solvated system (MDM2-p53, ligand and water) was neutralized by adding 5 Cl ions in all simulation. The full system was subjected to MD production run at 300 K temperature

and 1 bar pressure for 15000 ps. 20000 ps MD simulation of MDM2-p53 complex was performed without ligand in same condition as control.

3.1.4 Molecular target 4: Vascular epithelial growth factor receptor 2 (VEGFR-2) kinase

The crystal structure of VEGFR-2 kinase (pdb id 1YWN) obtained from RCSB protein data bank and the structures of ligands taxifolin generated from smile strings. Hydrogen atoms were added to VEGFR-2 kinase crystal structure using Autodock program while all non polar hydrogen atoms were merged. Lamarkian genetic algorithm was used as a search parameter which is based on adaptive local search. Short range vanderwaal and electrostatic interactions, hydrogen bonding, entropy losses were included for energy based Autodock scoring function (Berendsen et al., 2005; Sudhamalla et al., 2010). The lamarkian GA parameters used in the study were: numbers of run, 30; population size, 150; maximum number of evals; 25000000, number of generation; 27000, rate of gene mutation; 0.02 and rate of cross over; 0.8. Blind docking is carried out using grid size 126, 126 and 126 along the X, Y and Z axes with 0.480 Å spacing. The grid center was set to 5.033 38.562 and 23.562 Å. RMS cluster tolerance was set to 2 Å. Semi-flexible docking was performed which includes a flexible ligand and a rigid receptor.

A 3800 ps MD simulation of the complex was carried out with the GROMACS4.5.4 package using the GROMOS96 43a1 force field (Van Gunsteren et al., 1996; Lindah et al., 2001). The lowest binding energy docking conformation generated

by Autodock was taken as initial conformation for MD simulation. The topology parameters of VEGFR-2 were created by using the Gromacs program. The topology parameters of taxifolin were built by the Dundee PRODRG server (Schuttelkopf and van Aalten, 2004). The complex was immersed in an octahedron box of extended simple point charge (SPC) water molecules (Van Gunsteren et al., 1998). The solvated system was neutralized by adding 3 chloride ions. To release conflicting contacts, energy minimization was performed using the steepest descent method of 1000 steps followed by the conjugate gradient method for 1000 steps. MD simulation studies consist of equilibration and production phases. In the first stage of equilibration, the solute (protein, counterions, and taxifolin) was fixed and the position-restrained dynamics simulation of the system, in which the atom positions of VEGFR-2 were restrained at 300 K for 300 ps. Finally, the full system was subjected to 3800 ps MD at 300 K temperature and 1 bar pressure. For analysis, the atom coordinates were recorded every 0.5 ps during the MD simulation

3.2 IN-VITRO STUDIES

3.2.1 Cell culture and maintenance

HeLa cells were procured from the National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco's modified Eagles medium (DMEM) (Sigma, india) containing 10% FBS (Sigma, india) and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). Cells were maintained as monolayer cultures in a humidified atmosphere of 5% CO2 at 37 °C (Vidya Priyadarsini et al., 2010).

3.2.2 Determination of cell viability

Cell viability was assessed by the MTT assay based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazon product (Mosmann, 1983). Briefly, cells were plated at a density of 1×10^5 cells/ml into 96-well plates. After overnight growth, cells were treated with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 110 and, 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 and 550 µM concentrations of quercetin and taxifolin respectively for 24 h. Subsequently, the cells were washed with 200 µl of PBS, and incubated with 100 µl of 500 µg/ml MTT in PBS at 37°C for 3 h. The MTT-formazon product dissolved in 200 µl of DMSO was estimated by measuring the absorbance at 570 nm in an ELISA plate reader. The percent of cell survival was determined by comparing the average absorbance of the treated cells with the corresponding absorbance of untreated cells. All doses were tested in triplicates and the experiment was repeated at least three times.

3.2.3 DNA fragmentation assay

DNA extraction and electrophoresis were performed as described previously by An et al., 2004. In brief, HeLa cells containing adherent and non-adherent were collected by centrifugation at 1, 000 × g for 5 min. The cell pellet was suspended in cell lysis buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 10 mmol/L pH 8.0, Triton-100 0.5 %) and kept at 4 °C for 30 min. The lysate was centrifuged at 25, 000 × g for 20 min. The supernatant was incubated with RNase A 40 µg/L at 37 °C for 1 h, then incubated with proteinase K 40 µg/L at 37 °C for 1 h. The supernatant was mixed with NaCl 0.5 mol/L and 50 % 2-

propanol overnight at -20 °C, followed by centrifugation at 25, 000 × g for 15 min. After drying, DNA was dissolved in TE buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 1 mmol/L pH 8.0) and separated by 2 % agarose gel electrophoresis at 100 V for 50 min (Yanga et al., 2006).

3.2.4 RNA isolation and cDNA synthesis

Following treatment, cells grown in 60 mm Petri dishes were washed with ice-cold PBS and 1 ml of trizol was added and flushed gently to disrupt the cells. The lysates were collected and mixed with 300 µl of chloroform by inversion. The tubes were then centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phases from the tubes were collected and the RNA was precipitated using 700 µl of isopropanol and centrifuged at 10,000 rpm for 10 min at 4 °C. The pellets were washed twice with 70% ethanol and air-dried for about 20–40 min. The pellets were resuspended in 100 µl of DEPC treated water. Isolated total RNA (1 µg) was reverse-transcribed to cDNA in a reaction mixture containing 4 µl of 5× reaction buffer, 2 µl of dNTPs mixture (10 mM), 20 units of RNase inhibitor, 200 units of avianmyeloblastosis virus (AMV) reverse transcriptase and 0.5 µg of oligo (dT) primer (Sigma, India) in a total volume of 20 µl. The reaction mixture was incubated at 42°C for 60 min and the reaction was terminated by heating at 70°C for 10 min. The resultant cDNA was stored at −80°C until further use (Vidya Priyadarsini et al., 2010).

3.2.5 PCR amplification

All oligonucleotide primers were purchased from Sigma Genosys, India. Details about the primers are given in Table 1.

Gene product	Primer sequences	Product size (bp)
Bax	Sense 5′-ACCAAG CTGAGCGA GTGTC-3′ Antisense 5′-ACAAAGATGGTCACGGTCTGCC-3′	293
Bcl-2	Sense 5′-ACCAAG CTGAGCGA GTGTC-3 Antisense 5′-ACAAAGATGGTCACGGTCTGCC-3′	415
Caspase-9	Sense 5′-TGTGGTGGTCATCCTCTCTCA-3′ Antisense 5′-GTCACTGGGGGTAGGCAAACT-3′	282
Cytochrome C	Sense 5′-GGAGGCAAGCATAAGACTGG-3′ Antisense 5′-GTCTGCCCTTTCTCCCTTCT-3′	267
β-Actin	Sense 5′-AACCGCGAGAAGATGACCCAGATCATGTTT-3′ Antisense 5′-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3′	350

The PCR amplification reaction mixture (in a final volume of 25 µl) contained1 µl of cDNA, 0.5 µl of forward primer, 0.5 µl of reverse primer and 10 µl of Hot Master Mix (2.5×) (Sigma, India). The PCR was carried out in a thermal cycler (Eppendorf). Negative controls without cDNA were also performed. Amplification products were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide with 400 bp DNA ladder (Vidya Priyadarsini et al., 2010). The PCR products were visualized as bands with a UV-transilluminator and photographs were taken using gel documentation system.