Results & Discussion
Chapter 5 Results and discussion

5.0 Results and Discussion

The primary focus of our research is to determine the biological activities of new potent phytochemicals and to elucidate their molecular mechanisms (Park and Schoene, 2002; Park, 1999). The phytochemical and their analogues can then hopefully be utilized as preventive and/or therapeutic compounds for specific diseases such as cancers, heart diseases, and diabetes (Park, 2001; Mans et al., 2000). This objective can be more easily accomplished by investigating a potent phytochemical and its analogues together to determine which structural moieties influence biological activities (Mitra et al., 2002). For this purpose we have chosen resveratrol, a naturally occurring phytoalexin produced by plants to protect them against fungal pathogens, oxidative stress, and/or UV radiation and has been shown to act as a potential cancer chemopreventive agent based on its striking inhibitory effects on cellular events associated with cancer initiation, promotion and progression. The simplicity of resveratrol, associated with its interesting anticancer activity, offers promises for the rational design of new chemotherapeutic agents, and efforts have recently been devoted regarding a detail study on the structure-activity relationship of this type of substituted stilbene derivatives.

Although resveratrol shows tremendous promise as a preventive lead, it is not without some complicating problems. For instance, because of the many targets known to interact with resveratrol, it is very difficult to pinpoint which target is most important for the treatment of a given disease state. The anti-cancer activity could result from inhibition of COX-1, COX-2, NF-kB or activation of p53 or any combination thereof. Such an ambiguity makes lead optimization studies difficult and the broad-spectrum of activities exhibited by resveratrol can lead to side effects. One possible solution to these problems is to develop resveratrol analogues that exhibit selectivity for only one target. Yet another documented problem with resveratrol is its limited bioavailability owing to its metabolism in the liver. Studies have shown that circulating resveratrol has a serum half-life of 8-14 min because it is rapidly metabolized by sulfation (Marier et al., 2002) and glucuronation (Yu et al., 2002) at its OH groups. To be useful as a chemotherapeutic, resveratrol must have
greater bioavailability. A straightforward approach to increasing bioavailability involves finding analogues with comparable activity that lack the hydroxyl groups of resveratrol and consequently cannot be sulfated and glucuronated.

To begin to address these problems with resveratrol, the design, synthesis and screening of a library of analogues were envisaged to find lead compounds displaying increased selectivity and/or potency and to begin to elucidate the structure-activity relationships of resveratrol with respect to the inhibition of NF-kB, p53, AP1 etc. Another goal of these experiments was the discovery of resveratrol analogues with activity comparable and more to that of the natural product but which lacked the hydroxyl groups known to lead to decreased bioavailability.

5.1 Designing the library of Resveratrol analogues

The library of resveratrol analogues were designed to probe one of the three different structural features of resveratrol into consideration: substitution on each of the three hydroxyl groups together or substitution on only one group or substitution of two of the three available hydroxyl groups. To probe the electronic and steric demands on each of the hydroxyl groups, electron-donating (OEt, Ally, OMe and NMe2) and withdrawing (F, Cl, CF3, and NO2) substituents were chosen as well as naphthyl substituents were chosen to probe the required disposition of the two rings and the steric requirement around the parental rings. The dataset designed for this study contains 1000 resveratrol analogs, characterized by adequate biological and structural diversity. All the compounds in the data set were designed by side chain manipulations without disturbing the seed structure by taking into consideration of the linkers that are available in molinspiration cheminformatics database (Lipinski et al, 2001). These collections of substituents and spacers are extracted by substructure analysis of a collection of current drugs, developing drugs and other molecules with biological activity containing about 17000 entries (Lipinski et al, 2001).

The analogues designed for this study are classified into 7 types. Type 1 compounds having substituents in R1 position by replacing the hydroxyl group and type 2 are having substituents in R2 position by substituting the hydroxyl group where as type 3 compounds are designed by substituting the hydroxyl in R3
position. Group 4, 5 and 6 compounds are prepared by substituting the hydroxyl groups in R1 and R2, R2 and R3 as well as in R1 and R3 positions. The group 7 compounds are designed by replacing hydroxyl groups in R1, R2 and R3 positions. All the compounds were analyzed for their bioavailability using Lipinski’s rule of five (Lipinski et al, 2001) by Molinspiration (http://www.molinspiration.com). Briefly, this rule is based on the observation that most orally administered drugs have a molecular weight of 500 or less, a log P no higher than five, 5 or fewer hydrogen bond donor sites and 10 or fewer hydrogen bond acceptor sites (N and O atoms). Among all the designed analogues 50 were found to follow Lipinski’s rule and were represented in Table.5.1.1 along with their properties. The structural modifications induced on side chains for these molecules were represented in Table.5.1.2. From these compounds top 10 compounds (Table.5.1.1) were selected based on their polar surface areas (PSA) (since it is key property linked to drug adsorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood-brain barrier penetration, thus, passively absorbed molecules with a PSA>140 Å² are thought to have low oral bioavailability) and suggested for synthesis. The physiochemical, ADME and toxicological properties for these 10 compounds were predicted through l-labs (www.ilab.acdlabs.com) and their properties are represented in Table 5.1.3. All these properties for the 10 compounds are within the limit and can be suggested for synthesis. The inhibitor design concept of the present study triggered the design and selection of resveratrol analogues as potential new lead structures by extending modifications onto the established molecular scaffold of resveratrol via introduction of modifications in the hydroxyl positions.
Table 5.1.1 Lipinski’s Values for 50 designed resveratrol analogues (Bold ones are suggested for synthesis)

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## Results and discussion

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## Chapter 5

### Results and discussion

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Table 5.1.3 ADME and toxicity prediction data of the top 10 designed resveratrol analogues. (High lightened are the molecules synthesized and studied in this work)

<table>
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<tr>
<th>Resveratrol analogs</th>
<th>Solubility (LogS)</th>
<th>Buffer (pH 7.4)</th>
<th>LogD</th>
<th>LogP</th>
<th>Pka (Base)</th>
<th>Pka (Acid)</th>
<th>Physico-Chemical Determinants</th>
<th>Acute toxicity LD50 Rat (mg/Kg)</th>
<th>Oral LD50 Mouse (mg/Kg)</th>
<th>Ames test (positive)</th>
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<td>&lt;85%</td>
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<td>676</td>
<td>623</td>
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<td>893</td>
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<td>&lt;85%</td>
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</table>

<70%<77%<85%<94%<83%<58%<90%<87%
5.2. Synthesis of resveratrol analogues

Among the 10 suggested resveratrol analogues we were able to synthesize only 4 compounds. These four compounds are named as RA1, RA2, RA3 and RA4 respectively. RA1 is formed by fusion of two resveratrol molecules. RA2 is formed by the addition of cyclopropyl group to the 4'-OH group of resveratrol. RA3 and RA4 are synthesized addition of allyl groups.

5.2.1 Synthesis of RA1

A mixture of the styrylphenol (Acros Organics, 98% purity) (58.9 mg, 0.30 mmol) and AgOAc (50.1 mg, 0.30 mmol) in dry MeOH (5.0 ml) was heated at 50°C for 1 hr. The formation of a silver mirror during the reaction was observed. After removal of the solvent under reduced pressure, the resulting residue was subjected to column chromatography by eluting with chloroform to isolate the optically inactive 4-(3-phenyl-5-styryl-2,3-dihydrobenzofuran-2-yl)phenol, a dehydrodimer of the strating styrylphenol (57.2 mg, 97%) as a colorless amorphous powder: mp 179-181°C (from Et2O) (lit. 175-178°C); Mass (m/z, intensity) 390 (M⁺, 100), 373 (2), 299 (5), 296 (7), 265 (5), 252 (5); UV (MeOH) 320, 310, 228 nm; IR (KBr) 3441, 1609, 1516, 1490, 1451 cm⁻¹; H NMR δ 4.60 (1H, br d, J= 8.7 Hz), 5.48 (1H, d, J= 8.7 Hz), 6.81 (2H, d, J= 8.3 Hz), 6.87 (1H, d, J= 8.3 Hz), 6.97 (1H, d, J= 16.1 Hz), 7.13 (1H, d, J= 16.1 Hz), 7.16-7.33 (11H, m), 7.42 (1H, d, J= 8.3 Hz), 7.46 (2H, d, J= 8.3 Hz), 8.38 (1H, br s); Anal. Calcd for C₂₈H₂₂O₂ m/z 390.1620. Found m/z 390.1614.

![Figure 5.2.1 Schematic representation of chemical synthesis of RA1](image-url)
Figure 5.2.2 $^1$H NMR spectra obtained for RA1

Figure 5.2.3 Chemical ionization mass spectrum of RA1
5.2.2 Synthesis of RA2

A mixture of resveratrol (1) (SIGMA, 99% purity; 100 mg, 1 eq) and K₂CO₃ (2 eq) in dry acetone (4.0 ml) was added cyclopropyl methyl bromide (1.2 eq) and heated to reflux. After completion of starting material (TLC monitoring) the reaction mixture was evaporated and the crude product was purified using column chromatography by eluting with chloroform-MeOH (10/1) to isolate (E)-5-(4-(cyclopropyl methoxy)styryl)benzene-1,3-diol (20 mg) as a colorless amorphous powder.¹H NMR (400 MHz, acetone) ppm 0.38-0.30 (m, 2H), 0.58 (m, 2H), 1.29-1.21 (m, 1H), 3.85 (d, J = 6.87 Hz, 2H), 6.27 (t, J = 1.85 Hz, 1H), 6.54 (d, J = 2.07 Hz, 2H), 6.95-6.88 (m, 3H), 7.03 (d, J = 16.34 Hz, 1H), 7.48 (d, J = 8.71 Hz, 2H)

![Figure 5.2.4 Schematic representation of chemical synthesis of RA2](image)

![Figure 5.2.5 Proton NMR spectra for RA2](image)
5.2.3 Synthesis of RA3

A mixture of resveratro! (SIGMA, 99% purity, 100 mg, 1eq) and K₂CO₃ (2 eq) in dry Acetone (4.0 ml) was added allyl bromide (3 eq) and heated to reflux. After completion of starting material (TLC monitoring) the reaction mixture was evaporated and the crude product was purified using column chromatography by eluting with chloroform-MeOH (10/1) to isolate (E)-1,3-bis(allyloxy)-5-(4-(allyloxy)styryl) benzene as a colorless amorphous powder. ¹H NMR (400 MHz, CDCl₃) δ ppm 4.61-4.50 (m, 6H), 5.30 (d, J = 10.49 Hz, 3H), 5.43 (d, J = 17.25 Hz, 3H), 6.18-5.98 (m, 3H), 6.41 (s, 1H), 6.66 (d, J = 2.05 Hz, 2H), 6.89 (dd, J = 12.18, 10.95 Hz, 3H), 7.01 (d, J = 16.24 Hz, 1H), 7.43 (d, J = 8.70 Hz, 2H)
Chapter 5 Results and discussion

Figure 5.2.7 Schematic representation of RA3 synthesis

Resveratrol

 Allyl bromide, K2CO3
Acetone, reflux, 6h

RA3

Figure 5.2.8 1H NMR spectra obtained for RA3

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5.2.4 Synthesis of RA4

A mixture of resveratrol (SIGMA, 99% purity; 100 mg, 1eq) and K₂CO₃ (2 eq) in dry Acetone (4.0 ml) was added allyl bromide (2 eq) and heated to reflux. After completion of starting material (TLC monitoring) the reaction mixture was evaporated and the crude product was purified using column chromatography by eluting with chloroform-MeOH (10/1) to isolate (E)-3-(allyloxy)-5-(4-(allyloxy)styryl)phenol benzene as a colorless amorphous powder. 1H NMR (400 MHz, CDCl₃) δ ppm 4.55 (dd, J = 6.51, 5.47 Hz, 4H), 5.30 (d, J = 10.51 Hz, 2H), 5.42 (dd, J = 17.26, 1.36 Hz, 2H), 6.06 (dtd, J = 6.27, 5.21, 2.52 Hz, 2H), 6.36-6.31 (m, 1H), 6.57 (s, 1H), 6.64 (s, 1H), 6.94-6.81 (m, 3H), 7.00 (dd, J = 16.21, 4.55 Hz, 1H), 7.40 (t, J = 8.41 Hz, 2H).
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Figure 5.2.10 Schematic representation of RA4 synthesis.

Figure 5.2.11 Proton NMR spectra obtained for RA4
The tumor growth inhibition activities of resveratrol were variable based on different modifications to its structure (Roberti et al., 2003). All the four synthesized resveratrol analogues RA1, RA2, RA3, RA4 and resveratrol were evaluated for their activity and molecular mechanism through in-vitro analysis using U937 human histocytic lymphoma cell lines. In-vitro evaluation methods were started with cytotoxicity assays. Resveratrol was found to induce apoptosis in cancerous cells (Manna et al., 2000). This made us to screen the ability of resveratrol analogues to induce apoptosis. For this purpose we have done apoptotic screening and conformation assays. Resveratrol has been shown to interfere in the binding of NF-kB to DNA (Manna et al., 2000). Based on these results we conducted EMSA for NF-kB in resveratrol analogues treated cells. Finally we performed semi-quantitative RT-PCR to estimate the expression of genes depending on NF-kB.
5.3.1 Cytotoxicity assays

Cytotoxic activity of resveratrol and its synthesized analogues was estimated by MTT assay. Cytotoxic activity found to be increased as a positive function with the increase in the concentration of the tested resveratrol analogues. Among the different doses (1μM, 3μM, 5μM and 10μM) tested for all the compounds (Res, RA1, RA2, RA3 and RA4) at 10 μM concentration all of them have found to produce marked inhibition on U937 cell proliferation. Based on the gained data evaluated from cytotoxicity assays, RA4 among the four resveratrol analogues demonstrated lowest inhibition between 35-38 % at 10 μM (Fig.5.3.1) concentration where as the other three (RA1, RA2 and RA3) compounds demonstrated inhibition between 45-70 % at 10 μM concentration (Fig.5.3.1). The inhibition rates for RA1 and RA3 was found to be 45-50 % at 10 μM concentration. Resveratrol has shown 15-18 % of inhibition at 10 μM concentration (Fig.5.3.1). Among all the compounds, the highest inhibition rates were shown by RA2 (60-70%) (Fig.5.3.1) at 10 μM concentration. IC\textsubscript{50} values of all the tested compounds were calculated based on their percentage of cell death. Experimental IC\textsubscript{50} value for resveratrol is 32.01 μM, for that of RA1 11.22 μM, for RA2 8.0945 μM, for RA3 10.372 μM and for RA4 13.372 μM. Among all synthesized compounds, RA2 has shown IC\textsubscript{50} values at low concentrations. This may be due to higher rates of cell death by RA2. RA2 is showing cytotoxic activities four times to that of resveratrol (Fig.5.3.2). Whereas for other 3 compounds RA1, RA3 and RA4 are three times highly potent to that of resveratrol. The cytotoxic activity values obtained for resveratrol is same to that of previous studies obtained by Manna\textit{et al.}, (Manna et al., 2000). The findings from Minutolo\textit{et al.} (Minutolo\textit{et al.}, 2005) and Simoni\textit{et al.} (Simoni\textit{et al.}, 2006) also demonstrated that treatment with a methoxy resveratrol derivatives, yielded higher cytotoxicity than resveratrol in MDA-MB-231 and HL-60 cells. As mentioned in Minutolo\textit{et al.}, and Simoni\textit{et al} work, due to capping of hydroxyl groups for methoxy derivatives the bioavailability got increased. Resveratrol analogues also might have displayed higher pharmaceutical activity and bioavailability than the parental molecule resveratrol due to capping of hydroxyl groups.
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Figure 5.3.1  Resveratrol and its analogues RA1, RA2, RA3 and RA4 inhibited the cell viability. U937 cells treated with various concentrations of resveratrol and its analogues for 72 h. Cell viability was determined by adding MTT reagent and measured the OD at 570 nm.

Figure 5.3.2  Estimated IC₅₀ values for resveratrol and its analogues RA1, RA2, RA3 and RA4 on U937 cells.
Table 5.3.1. The dose response analysis of viability in human cancer cells U937 treated with resveratrol and its four analogues RA1, RA2, RA3 and RA4.

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<th>Concentration</th>
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<tr>
<td>Resveratrol</td>
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</tr>
<tr>
<td>RA1</td>
<td>84.82</td>
</tr>
<tr>
<td>RA2</td>
<td>88.47</td>
</tr>
<tr>
<td>RA3</td>
<td>89.93</td>
</tr>
<tr>
<td>RA4</td>
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5.3.2 Nuclear fragmentation analysis using Propidium iodide staining

The possible role of apoptosis in the present study was examined with propidium iodide staining of treated and untreated cells. U937 cells were treated with resveratrol and its analogues (RA1, RA2, RA3 and RA4) in a 6 well plate at 10 µM concentration for 72 h and the extent of apoptosis was assessed by propidium iodide staining through nuclear fragmentation. The treated U937 cells contained more apoptotic cells when compared to untreated cells (Fig. 5.3.3). There was characteristic nuclear fragmentation of nuclei in treated cells; whereas untreated cells did not show any nuclear fragmentation. The treated cells displayed the characteristic features of reduced size, intense florescence of condensed nuclear chromatin and formation of membrane blebs. The results revealed that the mode of cell death induced by treatment of resveratrol and its analogues is apoptosis. RA1, RA2 and RA3 among all treated cells induced higher apoptotic cell death in U937 cells (Fig. 5.3.3). However treatment of RA4 did not significantly increased the percentage of U937 cells undergoing apoptosis compared to those with RA1, RA2 and RA3 (Fig. 5.3.3). PI staining results revealed that the amount of cell death induced by resveratrol is lesser than its analogues RA1, RA2 and RA3. PI staining results shows that resveratrol and its analogues are inducing the death of cancerous cells through apoptosis. Again this can be due to increase in
bioavailability and half-life period of resveratrol analogues. The increase in bioavailability is due to capping of its hydroxyl groups. This capping might have decreased the easily excretion of these compounds thereby half life periods might have increased (Wen and Walle, 2006; Walle, 2004).

Figure 5.3.3 Propidium iodide stained U937 cells showing nuclear fragmentation after treatment with resveratrol and its analogues for 72h.

5.3.3 Characterization of apoptosis via examination of DNA fragmentation

As apoptosis plays an important role in anticancer effect. It is a highly regulated death process by which cells undergo inducible non-necrotic cellular suicide (Kaufmann and Hengartner, 2001) and in order to ascertain the induction of apoptosis by resveratrol and its analogues (RA1, RA2, RA3 and RA4) is DNA fragmentation, which is reflecting the endonuclease activity characteristic of apoptosis, we performed DNA fragmentation analysis. U937 cells treated with resveratrol and its analogues RA1, RA2, RA3 and RA4 at 10 μM concentration for
48h resulted in the formation of definite fragments, which could be seen via electrophoresis as a characteristic ladder pattern (Fig. 5.3.4). Whereas untreated cells did not provide such ladders. The four tested analogues of resveratrol RA1, RA2, RA3, and RA4 as well as resveratrol have given characteristic ladder formation (Fig. 5.3.4). Among all the analogues RA2 and RA3 had shown to induce prominent ladder formation (Fig. 5.3.4). The pattern of ladder formation by resveratrol and its analogues supports the mode of cell death in treated U937 cells is apoptosis. These hallmark features of morphological changes suggested that the treated resveratrol analogues caused apoptosis of U937 cancer cells. This further supports the high potent action of resveratrol analogues than that of resveratrol. This may be due to the increase in bioavailability of the resveratrol analogues by the decrease of the free hydroxyl groups (Wen and Walle, 2006).

Figure 5.3.4 Agarose gel electrophoresis demonstrating DNA fragmentation in U937 cells treated with resveratrol and its analogues RA1, RA2, RA3, and RA4 with 10 μM concentration for 48 h
5.3.4. Western blot analysis for PARP cleavage

Data obtained from flow cytometric PI staining and DNA fragmentation analysis showed that resveratrol and its analogues treated U937 cells shows apoptosis. We know that during apoptosis cytochrome C binds with Apaf-1 and procaspase-9 in a dATP-dependent manner to form the apoptosome (Mignotte and Vayssiere, 1998). The apoptosome can induce activation of caspase-9 which activates the effector pro-caspases, including pro-caspase-3, an effector caspase of apoptosis (Cohen, 1997). Caspase-3 is a well-known downstream adaptor caspase which can be proteolytically activated by caspase-9 via mitochondrial or cell death receptor signaling pathways. PARP represents an intrinsic substrate for caspase-3 (Lazebnik et al., 1994). and is cleaved upon caspase-3 activation. PARP is a highly conserved nuclear enzyme tightly binding to DNA with importance for DNA repair, (Dey et al., 2007) recombination, proliferation and genomic stability. Cleavage of PARP is an early and critical event required for tumor cell apoptosis (Thati et al., 2009). Cleavage of PARP is an early and critical event required in tumor cell apoptosis. PARP cleavage in resveratrol and its analogues RA1, RA2, RA3 and RA4 treated cells was detected by western blot analysis. It was found that PARP cleavage was induced and detected with resveratrol and its analogue treatment at 10 μM concentration after 72 h. Western blot analysis of PARP clearly showed that resveratrol and its analogue treated cells induced degradation of 116 KDa PARP protein into two fragments of which larger fragment is having MW of 89 KDa (Fig.5.3.6b). Among all tested resveratrol analogues, RA2 and RA3 had induced large amounts of PARP cleavage. This has been shown in Fig.5.3.6b by the formation of a thick band. This cleavage was higher than resveratrol and almost equal to oleandrin (Positive) induced cleavage. Even-though RA1 and RA4 induced PARP cleavage, it is very less when compared to RA2 and RA3. This confirms the mode cell death induced by of resveratrol and its analogues is apoptosis. The increase in rates of apoptosis by resveratrol and its analogues is attributed to the
increase in bioavailability and pharmacological activities by the capping of hydroxyl groups (Minutolo et al., 2005).

5.3.5 Effects of resveratrol and its analogues on NF-kB/DNA interactions

In this work we have tested ability of resveratrol and its analogues on NF-kB activation in U937 cells induced with TNF. TNF is a potent inducer of inflammation and inflammatory response is always mediated by activation of NF-kB. NF-kB plays very important role in the regulation of genes involved in inflammation and tumorigenesis. Activation and nuclear translocation of transcription factor NF-kB is important for expression of genes involved in the development and progression of cancer. To investigate the effects of resveratrol and its analogues, on TNF induced NF-kB DNA interactions we performed electro phoretic mobility shift assay (EMSA) as described in materials and methods (Borgatti et al., 2007; Bezzerri et al., 2008).

U937 cells were incubated with resveratrol and its analogues at 10 μM concentration for 72 h and from the treated cells nuclear extract (NE) was prepared. EMSA was performed by incubating 4μg of protein of the nuclear extract with 16 fmol of 32P end-labelled, 45 bp, double-stranded NF-kB oligonucleotide from the HIV-1 long terminal repeat,5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3' (Nabel and Baltimore, 1987), in the presence of binding buffer for 15 min at 37 °C (Collart et al., 1990) and the DNA proteins formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. EMSA was performed in two ways. First, we performed EMSA with 10 μM concentration of resveratrol and its analogues RA1, RA2, RA3 and RA4. Oleandrin is used as reference molecule in the assay. The results of gel retardation analysis are shown in Fig.5.3.5a and it clearly demonstrates that all the four resveratrol analogues along with resveratrol inhibited the DNA binding interactions of NF-kB even in presence of TNF and among all RA2 followed by RA3 suppressed the NF-kB interactions with DNA at higher rates and it is similar to that exhibited by reference compound oleandrin. Later on, we performed EMSA for NF-kB in the presence of increasing concentration (1 μM, 3 μM, 5 μM and 10 μM) of RA2 (Fig.5.3.5b). It was found that RA2 at 5 μM and 10 μM concentrations inhibited DNA binding interactions of NF-kB at higher rates where as at 3 μM concentration at very low
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rates. These results support that resveratrol and its analogues, by suppressing NF-kB DNA interactions, inhibited the growth of cancerous cells. It has been already shown that resveratrol suppress the DNA binding activity of NF-kB (Manna et al., 2000; McNary and Baldwin Jr, 2000). In this work EMSA analysis clearly showed that resveratrol analogues also suppressed the DNA binding ability of NF-kB (Fig. 5.3.5a). Two possible reasons can be given for these results; one is resveratrol and its analogues might have interfered in the activation of NF-kB (Manna et al., 2000; McNary and Baldwin Jr, 2000) and the second is resveratrol and its analogues by binding to NF-kB may have interfered in the binding of NF-kB to DNA.

Figure 5.3.5 a) Nuclear NF-kB DNA binding activities of TNF-induced resveratrol and its analogues RA1, RA2, RA3 and RA4 treated cells U937 cells b) Dose dependent EMSA performed with nuclear extracts of RA2 treated U937 cells.
5.3.6 Expression of IL-8, Actin, FASL, FASR, TNF1 and TNFR

Since NF-kB is one of the most important transcription factor regulating the expression of IL-8 gene, and the data reported in Fig. 5.3.5 demonstrates that the compounds RA2 and RA3 at 10 μM concentration inhibited the NF-kB/DNA interactions at higher rates, we analyzed the activity of RA2, RA3 and resveratrol on controlling the rates expression of IL-8 genes in U937 cells. Studies have shown that several members of TNF receptor family regulate the transcription factor NF-kB through a common adaptor protein and regulate NF-kB dependent IL-8 production (Malinin et al., 1997; Ponton et al., 1996). Based on these studies we have analyzed the expression of TNF1/ TNFR genes in treated and untreated cells. FasL/FasR system is one of the most important mechanisms in the activation of apoptosis. It has been shown that FasL/FasR ligation also affects IL-8 productivity in addition to apoptosis. Based on this, in this work we analyzed the effect of resveratrol and its analogues RA2 and RA3 on FasL/FasR gene expression. As the concentration of NF-kB was found to influence the actin cytoskeleton, here we have tested the expression of actin genes in resveratrol and its analogues RA2 and RA3 treated cells.

U937 cells were exposed to 10 μM concentration of RA2 and RA3 for 72 h. After the completion of incubation, the cells were harvested, RNA was extracted and semi-quantitative RT-PCR followed by PCR for amplification of genes using suitable primers was performed. The results obtained (Fig. 5.3.6a) demonstrated that RA2 is a strong inhibitor of IL-8, TNF1, TNFR and actin whereas FasR, and FasL, were activated in presence of RA2. RA3 and resveratrol had also given the same results but lesser than that of RA2 (Fig. 5.3.6a). These results indicated that the down regulation of NF-kB binding to DNA in resveratrol and its analogues treated cells might have activated the expression of FasR/FasL complex, and suppression of TNF1/TNFR association, IL-8 and actin gene expression, and is responsible for potentiating the mechanism of apoptosis. Through these results, we hypothesized that the parental molecule resveratrol and its synthesized analogues were known to elicit anticarcinogenic activities by regulating the NF-kB interactions with DNA. Increase in the expression of FasL/FasR genes further supports the activation of
apoptosis in resveratrol and its analogues treated cells, Whereas TNF1/TNFR, IL-8, actin gene expressions are down regulated in resveratrol and its analogues treated cells. This supports the decrease in the activation NF-kB in the treated U937 cells.

![Figure 5.3.6](image-url)  
**Figure 5.3.6** a) Effect of resveratrol and its analogues RA2 and RA3 treated U937 cells expression on IL-8, Actin, TNF1, TNFR, FasL and FasR expression. b) Dose dependent EMSA performed with nuclear extracts of RA2 treated U937 cells.

5.4. **In-silico evaluation**  

*In-vitro* evaluation of resveratrol and its analogues RA1, RA2, RA3 and RA4 on U937 cells showed that all these molecules, by targeting NF-kB, decreased the growth of U937 cells. To investigate the molecular mechanism of the inhibition, we performed docking and dynamic simulations. For this purpose, we retrieved the 3D structure of NF-kB (p65/p50 dimer) from PDB.

5.4.1 **Preparation of protein**  
The crystal structure of NF-kB heterodimer of the rel homology region (RHR) of human p65 (39-350 amino acid residues) and p50 (amino acid residues 19-291) bound to a 26 bp double stranded DNA with PDB id:3GUT was retrieved from PDB for *in-silico* analyses. 3UGT (Stroud et al., 2009) was found to contain tetrameric
RelA (chain A, C, E and G) sequences and tetrameric p50 sequences (chain B, D, F and H) sequences. Among these we have taken chain A from RelA and chain B from p50 (heterodimer of p65/p50) (Fig. 5.4.1) for our studies along with 26 bp double stranded DNA (Fig. 5.4.1). The residues involved in the binding of DNA are Met$^{32}$, Arg$^{33}$, Arg$^{35}$, Tyr$^{36}$, Cys$^{38}$, Glu$^{39}$, Arg$^{41}$, Ser$^{42}$, Ala$^{43}$, Gly$^{44}$, Ser$^{45}$, Lys$^{122}$, Lys$^{123}$, Asn$^{156}$, Arg$^{187}$, Gln$^{220}$, Arg$^{246}$ from p65 subunit and Arg$^{354}$, Arg$^{356}$, Tyr$^{357}$, Cys$^{359}$, Glu$^{360}$, His$^{364}$, Gly$^{365}$, Val$^{442}$, Thr$^{443}$, Lys$^{444}$, Lys$^{541}$, Pro$^{543}$, Lys$^{572}$, Gln$^{574}$, Lys$^{575}$, Arg$^{605}$, Gln$^{606}$ from p50 (Fig. 5.4.2). The DNA was separated from the protein and was saved for further use of docking with the complex. The protein alone was subjected to energy minimization using Gromacs to get relaxed conformation for to use in the docking experiments.

Figure 5.4.1 3D structure of NF-kB heterodimer (p65/p50) along with its DNA binding domain represented in cartoon conformation. P65 subunit rendered forest green, p50 subunit rendered slate blue and DNA binding domain rendered in magenta pink.
Figure 5.4.2 Amino acid residues of NF-κB heterodimer (p50/p65) interacting with kB site of DNA. P65 subunit rendered forest green represented in ball and stick conformation, p50 subunit rendered slate blue and represented in ball and stick conformation and DNA binding domain is rendered in magenta pink represented in sticks.
5.4.2 Molecular docking of resveratrol analogues onto NF-kB

Experimental results of resveratrol analogues with U937 cell revealed that all these analogues by targeting NF-kB induce tumor cell death. To determine mechanism of action of resveratrol analogues at molecular level, we performed docking of resveratrol analogues onto NF-kB. The energy minimized structure of NF-kB (3GUT) was used as receptor for docking with resveratrol and its analogues. The obtained docking orientations (Fig. 5.4.3 to 5.4.7) and corresponding scores (Table 5.4.1) of resveratrol and its analogues showed that the binding site of these compounds on NF-kB is located in the central core of the protein that is involved in DNA binding for both p65 and p50 subunits. Fig 5.4.3 shows the resveratrol binding site onto NF-kB and the residues within 10 Å from the resveratrol are displayed.

The binding site of resveratrol consists of Met32, Arg33, Arg35, Tyr36, Cys38, Glu39, Arg41, Ala43, Lys122 and Lys123 from p65 subunit and Arg354, Arg356, Tyr357, Cys359, Glu360, His364, Gly365, Val442, Thr443, Lys444, Pro543, Lys572, Gln574, Lys575, Arg605 and Gln606 from p50. Residues at the NF-kB site that form hydrogen bonding with resveratrol are Arg33 (p65), Arg41(p65), Arg354(p50) and Gln606(p50) whereas residues Tyr36(p65), Lys122(p65), Lys123(p65), Lys572(p50) and Arg606(p50) are forming hydrophobic and aromatic interactions with the resveratrol. The molecular docking results of RA1 are shows (Fig.5.4.4) that the binding mode is same as that of resveratrol. The binding site is formed of Met32, Arg33, Arg35, Tyr36, Cys38, Glu39, Ala43, Gly44, Lys122, Lys123 and Arg354 from p65 subunit and Arg356, Arg358, Tyr357, Cys359, Glu360, His364, Gly365, Val442, Thr443, Lys444, Pro543, Gln574, Lys575, Arg605 and Gln606 from p50. Among these residues Arg33(p65), Arg187(p65), Arg356(p50), His364(p50) and Gln606(p50) are forming hydrogen bonding interactions with the RA1 and the residues Tyr36(p65), Lys122(p65), Arg187(p65), Tyr357(p50), Cys359(p50) and Arg605(p50) are forming hydrophobic and aromatic interactions.
Figure 5.4.3 Binding mode of resveratrol (ball and sticks) onto NF-κB obtained from AutoDock dockings. P65 subunit rendered forest green represented in sticks p50 subunit rendered slate blue and represented in stick conformation.

Figure 5.4.4 Binding mode of resveratrol analogue RA1 (ball and sticks) onto NF-κB obtained from AutoDock docking. P65 subunit rendered forest green and represented in sticks p50 subunit rendered slate blue and represented in stick conformation.
The molecular docking performed for compound RA2 suggests (Fig 5.4.5) the interacting site on NF-kB for RA2 is formed of Met32, Arg33, Arg35, Tyr36, Cys38, Glu39, Ala41, Gly44, Lys122, Lys123, and Arg187 from p65 subunit and Arg354, Arg356, Tyr357, Cys359, Glu360, His364, Gly365, Val442, Thr443, Lys444, Pro543, Lys572, Gln574, Lys575, Arg605 and Gln606 from p50. The residues that are involved in hydrogen bonding interactions with RA2 are Arg35(p65), Arg187(p65), Arg354(p50), Arg356(p50), Tyr357(p50) and Cys359(p50). The residues present in the binding pocket are involved in hydrophobic and electrostatic interactions with RA2 are Glu360(p50), Val442(p50), Thr443(p50) and Gln606(p50).

The binding mode for RA3 with NF-kB is shown in Fig5.4.2.6. It shows that the allyl group added to this compound made this compound to bind to region on NF-kB formed of residues Arg33(p65), Arg354(p50), Tyr357(p50) and Lys444(p50) by involving in hydrogen bonding interactions and the residues Arg187(p65), Val442(p50), and Thr443(p50) are showing hydrophobic and electrostatic interactions.
The data obtained for RA4 shows that pocket is formed with Met^{32}, Arg^{33}, Arg^{35}, Tyr^{36}, Cys^{38}, Glu^{39}, Ala^{43}, Gly^{44}, Lys^{122}, Lys^{123}, and Arg^{187} from p65 subunit and Arg^{354}, Arg^{356}, Tyr^{357}, Cys^{359}, Glu^{360}, His^{364}, Gly^{365}, Val^{442}, Thr^{443}, Lys^{444}, Pro^{543}, Lys^{572}, Gln^{574}, Lys^{575}, Arg^{605} and Gln^{606} from p50 on NF-kB protein. Among these three residues Arg^{33} (p65), Arg^{35} (p65) and Lys^{444} (p50) are showing hydrogen bonding interactions with RA4 and Tyr^{36} (p65), Arg^{187} (p65) and Tyr^{357} (p50) are involved in hydrophobic and aromatic interactions. The obtained results of molecular docking of resveratrol and its analogues allowed us to propose a general binding mode of these compounds onto NF-kB and to determine residues involved in the ligand recognition. The obtained models demonstrate that Arg^{33} (p65), Arg^{35} (p65), Tyr^{36} (p65), Arg^{41} (p65), Lys^{122} (p65), Lys^{123} (p65), Arg^{187} (p65), Arg^{354} (p50), Arg^{356} (p50), Tyr^{357} (p50), Cys^{359} (p50), Glu^{360} (p50), His^{364} (p50), Val^{442} (p50), Thr^{443} (p50), Lys^{572} (p50), Arg^{605} (p50) and Gln^{606} (p50) are forming binding pocket for anchoring the docked ligands onto NF-kB. Most of these residues (Arg^{33} (p65), Arg^{35} (p65), Arg^{354} (p50) Arg^{356} (p50) and His^{364} (p50)) are functionally important in binding of NF-kB dimer to DNA. Obtained docking results of NF-kB with resveratrol and its analogues are supporting the in-vitro results. Where all the resveratrol analogues were able establish strong interaction with NF-kB by forming hydrogen bonding, hydrophobic interactions and aromatic interactions. The interaction energies generated and the number interactions established by the resveratrol analogues are higher than resveratrol (Table.5.4.1). Among all the resveratrol analogues RA2 has been shown to establish more number of interactions with NF-kB (Fig.5.4.5).
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Figure 5.4.6 Binding conformation of RA3 (ball and sticks) onto to NF-κB. P65 subunit rendered forest green and represented in sticks p50 subunit rendered slate blue and represented in stick conformation.

Figure 5.4.7 Binding mode of RA3 (ball and sticks) onto to NF-κB obtained through AutoDock. P65 subunit rendered forest green and represented in sticks p50 subunit rendered slate blue and represented in stick conformation.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding energy (kcal/mol)</th>
<th>Docked energy (Kcal/mol)</th>
<th>RMSD (Å)</th>
<th>Cluster Number</th>
<th>Estimated Kᵢ (µM)</th>
<th>H-bonding residues &amp; distances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>-6.12</td>
<td>-6.01</td>
<td>0.8943</td>
<td>23</td>
<td>32.64</td>
<td>Arg&lt;sup&gt;33&lt;/sup&gt; (p65) (1.3025 Å) Arg&lt;sup&gt;41&lt;/sup&gt; (p65) (0.6825 Å) Arg&lt;sup&gt;52&lt;/sup&gt; (p50) (0.6527 Å) Gln&lt;sup&gt;66&lt;/sup&gt; (p50) (0.4432 Å)</td>
</tr>
<tr>
<td>RA1</td>
<td>-7.08</td>
<td>-6.92</td>
<td>0.7948</td>
<td>35</td>
<td>6.4</td>
<td>Arg&lt;sup&gt;33&lt;/sup&gt; (p65) (2.0825 Å) Arg&lt;sup&gt;167&lt;/sup&gt; (p65) (0.3983 Å) Arg&lt;sup&gt;59&lt;/sup&gt; (p50) (0.8983 Å) His&lt;sup&gt;364&lt;/sup&gt; (p50) (0.3628 Å) Gln&lt;sup&gt;660&lt;/sup&gt; (p50) (0.7432 Å)</td>
</tr>
<tr>
<td>RA2</td>
<td>-10.89</td>
<td>-10.46</td>
<td>0.2468</td>
<td>48</td>
<td>0.1041</td>
<td>Lys&lt;sup&gt;122&lt;/sup&gt; (p65) (2.3214 Å) Arg&lt;sup&gt;187&lt;/sup&gt; (p65) (0.9983 Å) Arg&lt;sup&gt;364&lt;/sup&gt; (p50) (1.0652 Å) Arg&lt;sup&gt;59&lt;/sup&gt; (p50) (0.8983 Å) Tyr&lt;sup&gt;357&lt;/sup&gt; (p50) (1.6214 Å) Cys&lt;sup&gt;359&lt;/sup&gt; (p50) (0.2350 Å)</td>
</tr>
<tr>
<td>RA3</td>
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<td>-7.93</td>
<td>1.3698</td>
<td>40</td>
<td>1.322</td>
<td>Arg&lt;sup&gt;33&lt;/sup&gt; (p65) (0.6825 Å) Arg&lt;sup&gt;364&lt;/sup&gt; (p50) (1.2652 Å) Tyr&lt;sup&gt;357&lt;/sup&gt; (p50) (0.7314 Å) Lys&lt;sup&gt;444&lt;/sup&gt; (p50) (0.2450 Å)</td>
</tr>
<tr>
<td>RA4</td>
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<td>-5.30</td>
<td>1.4219</td>
<td>26</td>
<td>22.150</td>
<td>Arg&lt;sup&gt;33&lt;/sup&gt; (p65) (0.1925 Å) Arg&lt;sup&gt;364&lt;/sup&gt; (p50) (0.9652 Å) Lys&lt;sup&gt;444&lt;/sup&gt; (p50) (0.7350 Å)</td>
</tr>
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5.4.3 Dynamic simulations of complexes

MD simulations were performed and analyzed to understand the protein internal motions and conformational changes within nanosecond time scale for NF-kB-Res, NF-kB-RA1, NF-kB-RA2, NF-kB-RA3 and NF-kB-Ra4 complexes. To check the stability of the simulations, the root mean square deviations (RMSDs), radii of gyration (Rg) and root mean square fluctuations (RMSFs) of the complexes of NF-kB-resveratrol, NF-kB-RA1, NF-kB-RA2, NF-kB-RA3 and NF-kB-RA4 were calculated and monitored over the course of simulations. The RMSDs of the six complexes (NF-kB, NF-kB-resveratrol, NF-kB-RA1, NF-kB-RA2, NF-kB-RA3 and NF-kB-RA4) with respect to the starting structure, for the six simulations as a function of time, are plotted in Fig.5.4.8. The equilibration time for NF-kB is ~6000 ps (Fig.5.4.8) whereas for NF-kB-resveratrol it is at ~7000 ps (Fig.5.4.8). NF-kB-RA1 reached equilibration at ~8000 ps and NF-kB-RA2 complex reached equilibration at ~2500 ps (Fig.5.4.8). The other two NF-kB-RA3 and NF-kB-RA4 reached equilibration at ~4000 and ~6000 ps (Fig.5.4.8). After 2000 ps of simulations, the NF-kB protein alone and after 7000 ps the complex of NF-kB-resveratrol had undergone larger fluctuations with respect to RMSD values. The same variations in fluctuations with respect to RMSD values were seen for the complexes of NF-kB-RA1 after 3000 ps and for NF-kB-RA4 after 6000 ps. Where as the complexes of NF-kB-RA2 and NF-kB-RA3 maintained relatively low fluctuations throughout the simulation time with respect to RMSD values. The relatively high values and irregular profile of the plots reflect the structural changes of the flexible protein regions. The average values of RMSD for NF-kB, NF-kB-resveratrol are ~0.6725 and ~0.7895 nm (Fig.5.4.8). Whereas the other four complexes (NF-kB-RA1, NF-kB-RA2, NF-kB-RA3 and NF-kB-RA4) has shown the average RMSD values ~0.4925, ~0.2428, ~0.3249 and ~0.5765 nm respectively. Among all, the complex of NF-kB-RA2 reached equilibrium in earlier periods (~2000 ps) of simulations and was maintained stably throughout the simulation time (Fig.5.4.8).
The plot of radius of gyration $R_g$ of the protein and complexes versus time is represented in Fig. 5.4.9. It is evident that the $R_g$ value of NF-kB is varied between 2.4268 nm and 2.5280 nm (Fig. 5.4.9), for NF-kB-Resveratrol complex this value is varied between 2.4569 nm and 2.5536 nm (Fig. 5.4.9), for NF-kB-RA1 and NF-kB-RA4 complexes this variation is in between 2.458-2.5521 nm (Fig. 5.4.9) and 2.3764-2.5614 nm (Fig. 5.4.9). Whereas for the complexes of NF-kB-RA2 (Fig. 5.4.9) and NF-kB-RA3 (Fig. 5.4.9) the variation is high in the initial periods of simulation and reached up to 2.7534 nm and after 6000 ps simulations for both the complexes the values of $R_g$ decreased and maintained at 2.23264-23598 nm. These data revealed that the complex of NF-kB-RA2 and NF-kB-RA3 increased in the initial periods of simulations but later their $R_g$ value was decreased and maintained stably. For other four complexes, these kinds of larger fluctuations are not seen (Fig. 5.4.9). Interesting information comes from the RMSFs of each amino acid (Fig. 5.4.10 and 5.4.11) for the NF-kB, NF-kB-resveratrol, NF-kB-RA1, NF-kB-RA2, NF-kB-RA3 and NF-kB-RA4. In each graph, the RMS fluctuations of NF-kB alone and NF-kB-resveratrol are compared with the other. The RMSF difference indicates
that the dynamics of the core were different for NF-kB and complexes of NF-kB-resveratrol, NF-kB-RA1, NF-kB-RA2, NF-kB-RA3 and NF-kBRA4.

RMS fluctuations of the NF-kB alone had shown larger fluctuations when compared to the complexes (Fig.5.4.10 and 5.4.11). Among all complexes for NF-kB-RA2 complex, the flexibility decreased throughout simulation time. The increasing deviations observed along the complex of NF-kB-resveratrol are related to the deformations of the protein and are not decreased due to the binding of resveratrol. Whereas for others, these fluctuations are lowered this might be due to the binding of resveratrol analogues there will be decrease in the deformations formed on the protein. Among all the complexes of resveratrol analogues RA4 complex has shown larger fluctuations. Distance variations in DNA binding site of NF-kB protein environment are represented in (Fig.5.4.11). From these results it has been shown that the fluctuations of the DNA binding residues Lys$^{122}$(p65), Tyr$^{207}$(p50), Cys$^{359}$(p50), Lys$^{444}$(p50), Lys$^{541}$(p50), Pro$^{543}$(p50), Lys$^{572}$(p50), Gln$^{574}$(p50), Lys$^{575}$(p50), Arg$^{605}$(p50) got increased due to the binding of Resveratrol to NF-kB
and by the binding of RA4 to NF-kB the fluctuations of Tyr^{36}\(_{\text{p65}}\) at DNA binding site got increased. Whereas by the binding of RA1, RA2 and

![Image of RMSF as a function amino acids of complexes of a) NF-kB (cyan), NF-kB-resveratrol (black) and NF-kB-RA1 (red), b) NF-kB (cyan), NF-kB-resveratrol (black) and NF-kB-RA2 (green) obtained during 10 ns of MD simulations.](image-url)

Figure 5.4.10 The RMSF as a function amino acids of complexes of a) NF-kB (cyan), NF-kB-resveratrol (black) and NF-kB-RA1 (red), b) NF-kB (cyan), NF-kB-resveratrol (black) and NF-kB-RA2 (green) obtained during 10 ns of MD simulations.
Figure 5.4.11 The RMSF of MD simulated complexes of A) NF-kB (cyan), NF-kB-resveratrol (black) and NF-kB-RA3 (blue). B) NF-kB (cyan), NF-kB-resveratrol (black) and NF-kB-RA4 (yellow) obtained during 10 ns of MD simulations.
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RA3 to NF-kB the fluctuations for all the residues involved in DNA binding got decreased (Fig.5.4.12). These results revealed that the binding of resveratrol analogues stabilized the DNA binding residues by decreasing their fluctuations at DNA binding site of NF-kB.

![Graph showing RMS fluctuations of residues involved in DNA binding across six complexes during 10 ns of MD simulations.](image)

Figure 5.4.12 The RMS fluctuations of residues, involved in DNA binding of six studied complexes during 10 ns of MD simulations

The total number of hydrogen bonds between the compounds resveratrol, RA1, RA2, RA3 RA4 and NF-kB are measured for all snap shots of the simulations (Fig.5.4.13). The average number of hydrogen bonds formed for resveratrol is 2, for RA1 is 3, for RA2 is 3.75, for RA3 is 2.5 and RA4 is 2.5 (Fig.5.4.14). This indicates that resveratrol and its analogues maintained stably in forming hydrogen bonding with NF-kB. Among all RA2 is more stably maintained than that of other compounds by forming more number of hydrogen bonds with that of NF-kB. The hydrogen bonds seemed to be stronger in the compound RA2-NF-kB complex than in other. The energy profile (total, kinetic, potential) of the simulated complexes was
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represented in Table. 5.4.2. The energies generated for NF-KB and its complexes shows that when compared with the NF-kB alone by the binding of the ligand to the protein the energies of the protein got decreased (i.e more stable) and maintained in equilibrium throughout the simulation time (Table.5.4.3.1).

Figure 5.4.13 The number of hydrogen bonds formed by five studied complexes of NF-kB during 10 ns of MD simulations.

Figure 5.4.14 The average number of hydrogen bonds formed by five studied complexes of NF-kB during 10 ns of MD simulations.
Table 5.4.2 Time averaged energies obtained for NF-κB and its complexes with resveratrol and its analogues

<table>
<thead>
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<th>complex</th>
<th>Average total energy (Kcal/mol)</th>
<th>Average kinetic energy (Kcal/mol)</th>
<th>Average potential energy (Kcal/mol)</th>
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<td>NF-κB</td>
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<td>-211438</td>
<td>-1201820</td>
</tr>
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<td>NF-κB-resveratrol</td>
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<td>-212556</td>
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</tr>
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</tr>
<tr>
<td>NF-κB-RA4</td>
<td>-968788</td>
<td>-212536</td>
<td>-1181324</td>
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</table>

Linear interaction energies were used to estimate the Δ_G_bind values from MD simulation averages of the non-bonded intermolecular ligand potential energies (Hansson et al., 1998). Most precisely, this approach is based on thermal conformational sampling of the ligand, both in the free state (i.e., solvated in water) and bound to the solvated protein. The estimated energy of binding is calculated as a linear combination of the differences in the averages ligand-environment interactions. In the bound state, the environment is represented by both the protein and the solvating water molecules, while in the unbound state, the environment is represented by the only water molecules. We used β value of 0.5 for the electrostatic interactions and α value of 0.181 for the non-polar interactions. The constant parameter γ≠0 can be adjusted by least-squares optimization in order to improve the calculated absolute binding energies quantitatively with respect to the experimental ones. In the present inspection, we did not optimize this parameter, since we were mainly interested in calculating a relative binding free energy. With the above mentioned approach, we achieved the Δ_G_bind for all five simulated compounds resveratrol, RA1, RA2, RA3, RA4 and the results are represented in Table 5.4.3.
LIE calculations were helpful in gaining major insights into the main reasons responsible for the observed interactions of NF-κB towards the inspected ligands. Infact, analysis of the non bonded interactions (electrostatic and van der Waals) between the protein and compounds resveratrol, RA1, RA2, RA3 and RA4 clearly shows that polar as well as hydrophobic interactions mainly contribute to the binding of the inspected ligands. Moreover, by comparing the electrostatic contributions calculated for all the ligands one can see that high differences between the compounds were found. Most precisely, the electrostatic energy of interaction in the RA2 analogue is higher than other compounds. Our dynamics study shows that all the four resveratrol analogues can steadily anchor to NF-κB to suppress its activity. Compared to resveratrol, its analogues can provide enough hydrogen bond strength and proper orientation to bind with NF-κB.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_{ij}$</th>
<th>$\Delta E_{ij}$</th>
<th>$E_{\text{coul}}$</th>
<th>$\Delta E_{\text{coul}}$</th>
<th>$\Delta G_{\text{bind}}$</th>
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<tr>
<td>RA3</td>
<td>-239.114</td>
<td>-100.904</td>
<td>-138.210</td>
<td>-35.219</td>
<td>-10.119</td>
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5.4.4 Docking of NF-κB resveratrol and NF-κB-resveratrol analogues complexes onto DNA

The docked results of DNA protein and ligand complexes shows that during docking the amino acids involved in the DNA binding by interacting with ligands changed overall topology of the protein. Table 5.4.4 lists the estimated binding energies and RMSDs of docked DNA, protein and ligand complexes. Fig.5.4.15 shows NF-κB (minimized) docked onto DNA. Fig.5.4.15 shows that 17 residues from p65 subunit Met32, Arg33, Arg35, Tyr36, Cys38, Glu39, Arg41, Ser42, Ala43, Gly44, Ser45, Lys122, Lys123, Asn155, Arg187, Gln220, Arg246 and 17 residues from p50 subunit Arg354, Arg356, Tyr357, Cys359, Glu360, His364, Gly365, Val364, Thr443, Lys444, Lys445, Pro541, Pro543, Lys572, Gln574, Lys575, Arg505, Gln606 are involved in binding of NF-κB to DNA by occupying the major grooves on DNA. Among these Arg33, Arg35, Lys122, Gln220, Arg354, Arg356, Glu360, His364 and Lys444 are important in establishing tight binding interactions of NF-κB onto DNA. (Fig.5.4.15)

Figure 5.4.15 Docking pose of NF-κB onto DNA. a) NF-κB represented in cartoon (p65-forest green, p50-slate blue), DNA binding residues are represented in sticks DNA in lines (magenta pink) b) enlarged NF-κB DNA binding site.
Fig. 5.4.16 shows docked results of NF-kB-resveratrol complex onto DNA. Through this figure it was found that resveratrol decreases the binding affinity of NF-kB to DNA by establishing hydrogen bonding interactions with DNA binding residues Arg^{33}(p65), Arg^{41}(p65), Arg^{354}(p50), Gln^{606}(p50). Due to these interactions the amino acids might have changed their orientation and this became the reason for decreasing in the binding affinity of the complex to DNA. The RMSD superposition of NF-kB-resveratrol-DNA complex onto NF-kB-DNA complex is 0.9652 Å. Through Fig. 5.4.17 it was found that RA1 by interacting with DNA binding residues (Arg^{33}(p65), Arg^{187}(p65), Arg^{356}(p50), His^{364}(p50) and Gln^{606}(p50)) of NF-kB interferes with the binding of NF-kB to the DNA. When the complex of NF-kB-RA1-DNA was supposed onto the complex of NF-kB-DNA the RMSD of superposition was found to be 1.2563 Å.

Figure 5.4.16 Docking pose of NF-kB-resveratrol complex onto DNA a) NF-kB represented in cartoon (p65-forest green, p50-slate blue), DNA binding residues are represented in sticks DNA in lines (magenta pink) and resveratrol in ball and stick conformations b) enlarged NF-kB-resveratrol complex DNA binding site.
Docked results of NF-kB-RA2-DNA complex are shown in Fig.5.4.18. From this figure it is shown that RA2 by interacting with residues Arg$^{33}(p65)$, Lys$^{122}(p65)$, Arg$^{187}(p65)$, Arg$^{354}(p50)$, Arg$^{356}(p50)$, Tyr$^{357}(p50)$ and Cys$^{359}(p50)$ involved in DNA binding through hydrogen bonding interactions, interferes in the binding of NF-kB to the major groove of the DNA. This may be due to the change in the degree of orientation of these residues. In presence of RA2, NF-kB is unable to bind to DNA due change in the orientation of DNA binding residues. The RMSD value of superposition of NF-kB-RA2-DNA complex onto NF-kB-DNA is 1.6523 Å. Fig.5.4.19 and 5.4.20 represent the binding of NF-KB-RA3 and NF-kB-RA4 complexes to the DNA. From these results it is known that RA3 and RA4 by establishing interactions with DNA binding residues of NF-kb interfering with DNA binding of NF-kB. The RMSD value of superposition of NF-kB-RA3-DNA complex onto NF-kB-DNA was 1.3269 Å and that of NF-kB-RA4-DNA onto NF-kB-DNA was found to be 1.0288 Å.
Figure 5.4.18 Docking pose of NF-kB-RA2 complex onto DNA a) NF-kB represented in cartoon (p65-forest green, p50-slate blue), DNA binding residues are represented in sticks, DNA in lines (magenta pink) and RA2 in ball and stick conformations b) enlarged NF-kB-RA2 complex DNA binding site.

Figure 5.4.19 Docking pose of NF-kB-RA3 complex onto DNA a) NF-kB represented in cartoon (p65-forest green, p50-slate blue), residues involved in DNA binding are represented in sticks, DNA in lines (magenta pink) and RA3 in ball and stick conformations b) enlarged NF-kB-RA3 complex DNA binding site.
Figure 5.4.20 Docking pose of NF-kB-RA4 complex onto DNA a) NF-kB represented in cartoon (p65-forest green, p50-slate blue), residues involved in DNA binding are represented in sticks, DNA in lines (magenta pink) and RA4 in ball and stick conformations b) enlarged NF-kB-RA4 complex DNA binding site.

From these docking results, it was found that when NF-kB alone was docked onto DNA high DNA protein interaction energies were established. These interactions energies got decreased in case of resveratrol and its analogues complex with NF-kB was docked onto DNA. This might be due to the binding of resveratrol and its analogues (RA1, RA2, RA3 and RA4) in the DNA binding pocket of NF-kB by interacting with residues involved in DNA binding. Most of these molecules are bound to the area occupied by the major groove of DNA by NF-kB. This predicted binding mode of complexes would significantly interfere with the binding of NF-kB to DNA by changing the topology of the molecules at the site.

Based on experimental data, molecular docking and dynamics study were performed to explore the mechanism of action of resveratrol and its analogues towards NF-kB. Our results suggested that resveratrol and its analogues bound to NF-kB exactly at the DNA binding site to display their action. Due to this binding
they are altering the topology of the amino acid residues playing key role in the binding of NF-κB to the DNA. These molecules are oriented in the NF-κB binding site in such a way that, the ligand-receptor complex stability was greatly enhanced and this further suppressed the binding of these complexes to DNA. RA2 and RA3 are the two compounds, among the four synthesized resveratrol analogues, that displayed more potent action both in in-vitro and in in-silico studies and even proved to be more potent than resveratrol. With these studies we were able to find out the binding mechanism of resveratrol and its analogues to represent the critical features responsible for NF-κB activity.

Table 5.4.4: Hex docking results of NF-κB Resveratrol and its analogues RA1, RA2, RA3 and RA4 with DNA

<table>
<thead>
<tr>
<th>S.No</th>
<th>Complex</th>
<th>E-Total (Kcal/mol)</th>
<th>RMSD (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NF-κB-DNA</td>
<td>-3005.72</td>
<td>--------</td>
</tr>
<tr>
<td>2</td>
<td>NF-κB-resveratrol-DNA</td>
<td>-628.25</td>
<td>0.9652</td>
</tr>
<tr>
<td>3</td>
<td>NF-κB-RA1-DNA</td>
<td>-756.85</td>
<td>1.2563</td>
</tr>
<tr>
<td>4</td>
<td>NF-κB-RA2-DNA</td>
<td>-894.28</td>
<td>1.6523</td>
</tr>
<tr>
<td>5</td>
<td>NF-κB-RA3-DNA</td>
<td>-787.86</td>
<td>1.3269</td>
</tr>
<tr>
<td>6</td>
<td>NF-κB-RA4-DNA</td>
<td>-714.95</td>
<td>1.0285</td>
</tr>
</tbody>
</table>

In this study, resveratrol, a natural polyphenol, and its analogues RA1, RA2, RA3 and RA4 were chemically synthesized in order to investigate their anti-proliferation effects. Examination of the relation of the structures to anti-proliferation activity through in-vitro and in-silico analyses suggests that the cyclopropyl group added by replacing 4' - hydroxyl in RA2 has shown highly potent anticancer activity. This may be due to the capping of free 4'-hydroxyl group the pharmacological activity, bioavailability and half-life of RA2 has increased. The allyl groups added to RA3 by
replacing the three hydroxyl groups has shown more potent activity may be due to increase in half-life and availability of the compound. Whereas when allyl groups are added to two of the three hydroxyl groups (3, 4'-hydroxyl) deduction in antiproliferation activity was seen. RA1 formed by the fusion of two resveratrol moieties even though does not display hydroxyl groups, unable to show potent antiproliferative activity than other analogues. This may be due its high molecular weight it might not have crossed the cell membrane. In all synthesized resveratrol analogues the hydroxyl groups are capped, this might be reason for the enhancement of their bioavailability and half-life (Wen and Walle, 2006; Walle, 2004). It is generally accepted that capping of hydroxyl groups prevents glucuronidation in liver and intestinal cells and may enhances bioavailability and half-life in \textit{in-vivo} (Wen and Walle, 2006; Walle, 2004). The decrease in antiproliferative activities of resveratrol than its synthesized analogues is due to the presence of free hydroxyl groups. It has been known that compounds possessing free hydroxyl groups may present reduced pharmacological and antiproliferative activities, due to rapid excretion. A recent study underlined the importance of methoxylation in trans-stilbenes in increasing the toxicity towards \textit{Caenorhabditis elegans} (Wilson \textit{et al.}, 2008). The order of potency of all the synthesized resveratrol analogues is RA2>RA3>RA1> RA4>Resveratrol.