Summary & Conclusions
Chapter 6

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6.0 Summary and Conclusions

6.1 Summary

Chemoprevention is a concept that has become increasingly appreciated as a new strategy in the fight against cancer (Hong and Sporn, 1997; Sporn and Suh 2000). Increasing evidence has shown that resveratrol (3, 5, 4'-trihydroxy-trans-stilbene), a phytoalexin found in a multitude of dietary plants, including grapes and peanuts, is one of the most promising chemopreventive agents against cancer (Mgbonyebi et al., 1998; Carbo et al., 1999; Clement et al., 1998; Huang et al., 1999). Resveratrol has been shown to inhibit tumor initiation, promotion, and progression in different systems. This is partly attributable to its antioxidant activities and to its inhibition of cyclooxygenase 1 and 2 (Mgbonyebi et al., 1998, Subbaramaih et al., 1998). In addition, many studies have shown that the anticancer properties of resveratrol are related to its ability to cause cell-cycle arrest in the G1 phase (Hsieh and Wu, 1999) or the S-G2 phase transition or to trigger apoptosis in a variety of cancer cell lines (Surh et al., 1999; Subbaramaih et al., 1998).

Although resveratrol shows tremendous promise as a preventative lead, it is not without some complicating problems. One among them is broad-spectrum activities of resveratrol which can lead to development of side effects. Another problem associated with resveratrol usage directly as an anti-cancer compound is lack of potency. The possible solution to these problems is to develop resveratrol analogues that exhibit selectivity for a specific target. Another advantage of this study is to suggest resveratrol analogues with significantly greater potency, thereby reducing the need for larger dosages. One more advantage associated with this study is to suggest resveratrol analogues that possess increasing bioavailability than that of resveratrol with comparable or more activity. With an aim to suggest a selective and potent inhibitor for cancer using resveratrol, we divided our work into four parts.
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1. Designing and screening of resveratrol analogues: by inducing side chain modifications onto the seed structure of resveratrol, we designed 500 structural analogues of resveratrol. All these were screened for their ability to follow Lipinski rule-of-five. Among these, based on TPSA, we selected 50 compounds to follow the rule. The 50 selected compounds were further screened for their ADME properties, toxicities, as well their biological features. Among these top 10 compounds which are satisfying all above said properties were separated and they were suggested for chemical synthesis.

2. Synthesis of resveratrol analogues: Among the 10 suggested compounds, we were able to synthesize 4 compounds. All these compounds were synthesized by taking the raw molecule as resveratrol and they given the names as RA1, RA2, RA3 and RA4.

3. In-vitro evaluation of resveratrol analogues: The four synthesized resveratrol analogues and resveratrol were evaluated for their ability to inhibit the growth of tumor cells by in vitro evaluation methods using U937 cell lines. They were initially screened for their cytotoxic activity using MTT assay. The obtained results had shown that all the four analogues are potent inducers of tumor cell death at lower concentrations than resveratrol. In the next step, the apoptosis screening assay was performed using PI staining. The result shows that all the resveratrol analogues were able to induce apoptosis. This was further confirmed by DNA fragmentation assay. All the resveratrol analogues were shown to induce clear fragmentation of DNA and it was proved to be more than resveratrol. Another confirmation for apoptosis came from PARP assay. Western blotting analysis for PARP shows that resveratrol analogues were able to induce fragmentation of PARP and this might be the reason for the inhibition of tumor cell growth. Further to determine the clear target for tumor cell death, we performed EMSA for transcriptional factor NF-kB. EMSA result has shown that resveratrol analogue by suppressing the DNA binding ability of NF-kB is inhibited the growth of cancerous cells. We isolated RNA from resveratrol treated cells and performed semi-quantitative RT-PCR for NF-kB dependent genes. PCR result shows that TNF, TNFR, IL-8 and actin genes expression got suppressed.
due to the treatment of resveratrol analogues onto U937 cell, whereas Fas and FasL genes got expressed. All these results showed that resveratrol analogues by suppressing the NF-κB activity induced the cancerous cells to undergo apoptosis.

4. *In-silico evaluation:* To investigate the molecular mechanism of action of the synthesized resveratrol analogues, we performed *in-silico* analyses. During these experiments, docking and dynamic studies were performed with all the four resveratrol analogues using NF-κB as receptor. The 3D structure of heterodimer of NF-κB (3GUT) was obtained from PDB. This protein after manual modifications was subjected to energy minimization using Gromacs. The minimized structure of NF-KB was used as receptor for docking with resveratrol analogues. Docking results showed that resveratrol and its analogues were able bind to NF-κB with high affinity at its DNA binding domain. The stability of these complexes was determined by performing dynamic simulation. Dynamic simulations showed that all resveratrol analogues in the bound state maintained high stability of the protein and its higher to that of resveratrol binding. The stability of the complex of resveratrol analogues with NF-κB is higher than the protein was present in unbound state. To find out the effect of complexes in interacting with DNA we performed docking studies of resveratrol and its analogues complex with NF-κB onto DNA. These results clearly showed that interaction of resveratrol analogues, with DNA binding residues of NF-κB interferes in the binding of NF-κB 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-silico* studies and even proved to be more potent than resveratrol.

6.2 Conclusion

Resveratrol, a trihydroxystilbene found in grapes and other plants, has been shown to be active in inhibiting multistage carcinogenesis, was found to associate with some problems for its direct usage. To begin to address the problems associated with resveratrol, the synthesis and screening of a small library of
analogues were envisaged to find lead compounds that are displaying increased selectivity and potency to elucidate the structure activity relationships of resveratrol with respect to inhibition of NF-kB activation and induction of apoptosis. Using resveratrol as a prototype, we have synthesized four resveratrol analogues and tested their anti-proliferative effect through both in-vitro and in-silico analyses in U937 cells. Here, we have shown that two of the resveratrol analogues RA2 and RA3 inhibited the growth of U937 cells at 10 μM concentration more potently than resveratrol and other analogues. EMSA analyses showed that resveratrol analogues significantly suppressed the binding of NF-kB to DNA and induced the suppression of TNF1/TNFR and expression of FasL/FasR genes for inhibiting the growth of tumor cells. Molecular mechanism of action of resveratrol analogues revealed that these molecules by establishing association with the residues involved in the DNA binding pocket of NF-kB interferes in the binding of NF-kB to DNA. Among the tested analogues of resveratrol, RA2 and RA3 exhibited clear differential growth inhibitory activities. Cancerous cells underwent apoptosis after treatment with resveratrol and its analogues suggesting that apoptosis is the major cause for growth inhibitory effect. After treatment the transformed cells significantly inhibited the binding of NF-kB to DNA may the reason the activation of apoptosis. Our results indicate that the some synthesized resveratrol analogues possess more potent apoptosis-inducing activity than resveratrol and these compounds are of great interest for further investigation.

In present work, we offered a possible molecular mechanism by which the antitumor activity of resveratrol and its analogues occurs through extracellular pro-inflammatory cytokines, mediated through suppression of NF-kB binding to DNA and induction of apoptosis. As the parental molecule resveratrol and its synthesized analogues were known to elicit anticarcinogenic activities by regulating the NF-kB, we hypothesized that these effects are mediated through the suppression of NF-kB DNA binding activity. In this work we have chosen U937, a histolytic lymphoma cell line for our study. Our results clearly demonstrated that resveratrol and its analogues RA1, RA2, RA3 and RA4 suppresses NF-kB activation is mediated by proinflammatory cytokines such TNF1 and other inflammatory agents. We also
found that NF-kB regulated genes involved in angiogenesis (IL-8, TNF1/TNFR) are down regulated and the genes involved in apoptosis (FasL/FasR) are up regulated by treatment of resveratrol and its analogues. There are various ways by which resveratrol and its analogues might have inhibited TNF1 induced NF-kB activation. This involves the suppression TNF1 followed by sequential decrease of TNFR association with TRADD, TRAF2 etc, which maintains inactivated IKK2 that in turn is unable to phosphorylates IKBα, so that it binds to NF-kB and prevents its activation thereby its function. This indicates that resveratrol and its analogues might have suppressed the NF-kB activation and also binding of NF-kB to DNA through a wide variety of agents, including TNF1, FasL etc in U937 cells.
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a) Predicted mode of action of resveratrol analogues by activating FasL/FasR system.

b) Schematic representation of NF-kB inactivation by resveratrol analogues.

Figure 6.2.1 Predicted schematic representation of mode of action the synthesized resveratrol analogues. a) Predicted mode of action of resveratrol analogues by activating FasL/FasR system. b) Schematic representation of NF-kB inactivation by resveratrol analogues.