Chapter - V

DISCUSSION
Natural products have proven to be the most reliable source of new and effective anticancer agents. Several in vitro and in vivo studies have shown that combinational treatments with synthetic drugs and/or natural product based drugs is more effective in inhibiting cancer growth than treatment with a single drug (Aggarwal, 2007). Chemotherapy, one type of cancer treatment plays a major role in treating various cancers especially to control advanced stages of malignancies in clinical situations (Kinghorn et al., 2003). Most of these chemotherapeutic agents show considerable adverse side effects. For example the clinical use of anthracyclines like doxorubicin induces life threatening cardiomyopathy and congestive heart failure, which is a major difficulty for optimum use of doxorubicin (Minotti et al., 2004). This aspect can be minimized by reducing its dose and adding some natural polyphenols (Zang et al., 2012). For example, Curcumin inhibits MAPK, p38, c-JNK activation and scavenges reactive oxygen species (ROS) which minimizes the cardiotoxicity of doxorubicin (Misra et al., 2011) and is a potent suppressor of NFkB pathway which promotes cell proliferation and inhibits apoptosis (You et al., 2011). In the same manner, GA has shown to sensitize a variety of human cancer cell lines for apoptosis induced by different anti-cancer drugs (Hseu et al., 2008).

Shi et al., (2000) evaluated the combination of an orally available FTI, SCH66336, and taxanes: paclitaxel or docetaxel. The combination was synergistic in a number of lung, pancreatic, ovarian, and breast cancer cell lines in vitro. The combination study was carried out in two breast cancer cell lines: MDA MB-231 and MDA MB-468, when combined to paclitaxel, SCH66336 showed synergism with MDA MB-468 and antagonism with MDA MB-231, while the SCH 66336/docetaxel combination showed synergism with MDA MB-468 and an additive effect with MDA MB-231 cells. The effect of drug pharmacokinetics on the combination should also be considered as drugs with very different half lives have different interactions at different time points (Peters et al., 2000).

Hence, combining polyphenols with conventional therapies may help to overcome drug resistance and reduce the side effects of standard anticancer treatments. Therefore, the present study focused on combinational therapy of curcumin, gallic acid and doxorubicin on cervical cancer cells. Tumor resistance to apoptotic cell death is an
important hallmark of cancer and contributes to increased survival of cells that have acquired oncogenic mutations, eventually leading to uncontrolled cell proliferation, invasion, metastasis, angiogenesis and chemoresistance. Combination of polyphenols, that of EGCG and Cur, strongly suppressed the growth of NSCLC cells and breast cancer cells both in vitro and in vivo. In the present study, results of MTT assay showed that Cu is more effective than GA in inhibiting cell viability and cell proliferation. Our study indicated that curcumin in combination with gallic acid is more effective, and curcumin + gallic acid in combination with doxorubicin showed still more significant anti proliferative activity both in dose and time dependent manner in human cervical cancer (HeLa) cell lines. A Checkerboard assay analysis to systematically analyze the drug combination effects revealed that the exposure of both compounds and dox at the same time. Similar results were observed in other drug combination studies. Abuzeid et al, (2013), studied the effect of a novel Cur analog on cisplatin-sensitive (UM-SCC-74B) and cisplatin-resistant (UM-SCC-29) HNSCC cell lines in vitro. FLLL32, a novel small inhibitor derived from CUR, down-regulated the phosphorylated form of STAT3 protein and increased the number of apoptotic cells in both cell lines when used either alone or in combination with cisplatin. In particular, FLLL32 sensitized UM-SCC-29 cells to cisplatin treatment, allowing for a 4-fold reduction in the dose of cisplatin compared to the dose required for cisplatin as monotherapy.

Cell migration and invasion are pivotal events in progression of tumors. In the present study HeLa cells migration was inhibited 60% with Cur + GA treatment and 70% with Cur + GA + Dox combinational treatments which showed far better inhibition than individual treatments (Fig. 4.22 A and 4.22 B). Our data supports the current cancer treatment strategies in favour of combination therapies which would offer low toxicities to the cancer patients.

Pramanik et al., (2012) developed a DOX-curcumin composite polymer nanoparticle formulation called Nano DOXCurc (NDC) for overcoming MDR and showed that NDC ameliorates DOX associated cardiomyopathy. Duan et al. showed that reversion of MDR can be accomplished by co-encapsulation of DOX and curcumin in chitosan/poly (butylcyanoacrylate) nanoparticles (Duan et al., 2012). Since integrin receptors over expressed on tumor endothelial cells are emerging as potential
therapeutic target in anti-angiogenic cancer therapy (Weis and Cheresh 2011; Desgrosellier and Cheresh, 2010).

In the present study, combinational treatment with Cur, GA and Dox exhibited significant suppressive effect on HeLa cells colony formation than individual treatments (fig 4.17 A - 4.17 C ). Curcumin, gallic acid and doxorubicin individually shown 66.67%, 61.11%, 75% colony formation, while Cur + GA shown 47.22% and Cur +GA+Dox shown 30.56% colony formation. Our results suggest that concurrent treatment of Cur, GA and Dox shown synergistic effect in human cervical cancer (HeLa) cells and these drugs may have future clinical utility for treating cervical cancer. These observations may be of value while carefully considering the combination therapies in a clinical setting to enhance efficacies of potent chemotherapeutics by simultaneously delivering them with curcumin to tumor vasculature via integrin receptors over expressed on tumors and tumor endothelial cells and ultimately beneficial in cervical cancer therapy. With the availability of the presently described combinations of curcumin, gallic acid and doxorubicin, it will now be possible to analyse the mechanistic origin of anti-cancer properties of Dox. It has been reported previously that Dox inhibits transforming growth factor b (TGF β) signaling pathway in cancer cells (Vogelstein and Kinzler, 2004, Filyak et al., 2008). Similar kind of reports in combination studies with s-allylcysteine and lycopene were reported to induce apoptosis by modulating Bcl-2, Bax, Bim and caspases during experimental gastric carcinogenesis (Velmurugan et al, 2005). With regard to antioxidant activities, Dox did not contribute while Cur and GA have shown potent antioxidant effects as shown in figures 4.18-4.21. Similar kind of antioxidant activity was reported from several fruits earlier (Wolfe, 2003). Lacking antioxidant activity may be one of the reasons for possible side effects of Dox (Kaur et al., 2009; Sethi et al., 2012; Tacar et al., 2013).

In this study, we investigated the morphological changes through microscope to notice the synergistic anticancer activity of curcumin, gallic acid and doxorubicin in cervical cancer cell line HeLa. Cellular morphological changes such as cell bulging, shrinkage, rounding, and flouting in the HeLa cells were seen in cells incubated with curcumin, gallic acid and doxorubicin. The morphological changes were highest (Fig.4.
13-4.16) for the cells incubated in combination of curcumin, gallic acid and doxorubicin. Similar observations were made by previous studies who have confirmed the apoptotic effect of curcumin or doxorubicin or gallic acid in various cancer cell lines (Aggarwal, 2009).

The apoptosis is the outcome of a complex interplay of pro apoptotic and anti-apoptotic molecules. In present study curcumin, gallic acid and doxorubicin induces apoptosis which is proved by western blotting analysis of pAkt, pro-caspase-3 and PARP protein expression, the key indicators of apoptotic death. We observed decreased levels of pAkt, Pro-caspase-3 and PARP indicating the involvement of apoptosis in curcumin, gallic acid and doxorubicin induced cell death. Probably, these compounds induce the release of cytochrome c from mitochondria, causing activation of caspase 3 from pro-caspase-3 and concomitant PARP cleavage, which is the hallmark of caspase-dependent apoptosis. Furthermore, it was shown that curcumin-induced ROS generation leads to induction of pro apoptotic protein p53 as well as its effector protein p21 and down-regulation of cell cycle regulatory proteins such as Rb, cyclin D1, and D3. Both glutathione and N-acetyl cysteine pretreatment resulted in the complete inhibition of curcumin- induced ROS generation, apoptosis inducing factor released from mitochondria, and caspase activation (Yang et al., 2006, Thayyullathil et al., 2008). In another study, EGCG-induced down regulation of IAP family member X chromosome linked inhibitor of apoptosis protein, which might be helpful to facilitate cytochrome c mediated downstream caspase activation and DNA fragmentation was shown (Qanungo et al., 2005).

The results of the real time PCR showed differential expression of p53, p21, STAT 1, VEGF and TGF β at mRNA level in the presence and absence of Cur, GA and Dox, in combinations as well as individually. The p53 mRNA expression was 2-3 fold higher compared to individual treatments (Fig.4.27), The p21 mRNA expression was 1.5-3 fold higher compared to individual treatments (Fig.4.28), The STAT 1 mRNA expression was 2 fold higher compared to individual treatments (Fig.4.29), The VEGF mRNA expression was no change compared to individual treatments (Fig. 4.29), The VEGF mRNA expression was no change compared to individual treatments (Fig. 4.30) and the TGF β mRNA expression was 3-4 fold higher compared to individual treatments (Fig.4. 31). The mechanism of TGF β signaling in cancer therapy was earlier explained
by Shi and Massague, (2003). Similar studies with p21 were reported in regulation of cell cycle by Singh et al (2004).

Additionally, in present study curcumin, gallic acid and doxorubicin induces apoptosis proved by real time analysis p53, p21, STAT 1, VEGF and TGF β. m RNA expression, the key indicators of apoptotic death. We observed increased levels of pAkt, Pro-caspase-3 and PARP and decreased levels VEGF and TGF β of indicating the involvement of apoptosis with curcumin, gallic acid and doxorubicin induced cell death. EGFR binds with its ligands and subsequently activates downstream MAPK/ERK and PI3K/Akt pathways, which contribute to invasiveness and other malignant phenotypes (Gaffney et al., 2003; Triratanachat et al., 2006; Zheng et al., 2009; Lin et al., 2012; Xiao et al., 2012). Gallic acid significantly decreases the phosphorylation of members of the PI3K/AKT and MAPK/ERK signaling pathways, which play key roles in cell proliferation and invasion. The present results indicated that the inhibition of pAkt by combinations may be responsible for decreased invasiveness through the suppression of the EGFR/PI3K/AKT and EGFR/MAPK/ERK pathways. Regulation of tumor cell apoptotic sensitivity involving the above genes were previously reported by Smith et al, (2000).
SUMMARY AND CONCLUSION
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In view of the challenges faced in treatment of different cancers, combinational therapy has gained importance in recent decades. In the context of considerable side effects encountered with synthetic drugs, there is a growing focus towards natural product based therapeutics like polyphenols. Hence, the present work was aimed to evaluate the combinational effect of curcumin (Cur), gallic acid (GA) in the presence and absence of doxorubicin (Dox) in HeLa cells. After designing the experiments HeLa cells were maintained and grown in uncontaminated conditions and treatments were given with the selected drugs/compounds both individually and in combinations. Initially both time dependant and dose dependent experiments were conducted to optimize the concentrations and treatment time for Cur, GA and Dox to conduct further experiments.

Based on cell culture experiments like MTT assay, Chekkar board assay, cell migration assay, clonogenic assay, western blot analysis and Rt-PCR, Cur and GA in combination were found to be more effective in inhibiting HeLa cells growth and proliferation and causing apoptosis in them, than when they were administered individually. More interestingly, addition of Dox to Cur+ GA was found to be still more efficacious in treating cancer cells causing synergistic effect.

Although Dox has found its place as an efficient drug in cancer therapy, its potent cardiotoxic effects has dwindled its use. Therefore, involving Cur and GA as part of combinational studies has the potential to overcome the adverse effects posed by Dox. Infact, the results of our antioxidant studies has highlighted the role of Cur+ GA and discarded the role of Dox as potent antioxidant.

The western blot analysis and RT-PCR analysis of apoptotic related genes and proteins have further supported the more effective therapeutic nature of combinational studies. In conclusion, our studies pave the way for more combinational studies that may lead to development of effective drugs/formulations, ultimately benefitting the mankind against different cancers and to lead a healthy life.