Chapter 4

Discussion
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CRC is the third most common cancer with significant mortality and morbidity in both men and women throughout the world. Although a decline in CRC mortality has been observed in recent past from economically developed countries possibly due to availability of better screening tests, changes in risk factors and improved treatments, they continue to increase in low-resource countries.

Chemotherapeutic agents in current clinical practice have contributed significantly in reducing mortality/morbidity but often limited due to lack of specificity leading to undesirable side effects. In addition, development of drug resistance is another major hindrance to successful chemotherapy and thus narrowing anticancer arsenal. Therefore, the availability of anticancer agents that combine efficacy, safety and patient compliance remains a great challenge.

As most of the cancers develop due to multiple genetic alterations or abnormalities, current chemotherapy practices employ combination of anticancer agents to obtain clinical efficacy; however, identifying rational combination that could lead to cancer cure is also an active area of research now.

Natural products have played a major role in developing novel anticancer drugs. Today many of the existing chemotherapeutic drugs are developed either from natural source or their semi synthetic derivatives. Combination of these phytochemicals with known anticancer drugs has been shown to be highly efficacious, as in combination, they not only lower the adverse effects and thus, more patient compliance but overcome drug resistance.

Therefore, the objective of the present study was to evaluate the combination effects of a natural anticancer agent: limonene (122) and a synthetic drug: NVP BEZ-235, in human CRC cell lines: COLO-320, HCT-116, HT-29 and SW-620 in a bid to explore better efficacy compared to monotherapy. Testing the drugs/combination in CRC cells has more clinical relevance in the context of drug resistance, because CRC cells may be of useful model for representing clinical cancer drug resistance as mentioned before.
The presence or absence of a variety of cellular targets/biomarkers in various CRCs guide not only to design therapeutic regimen, but provides information on clinical outcome. K-ras and PI3K signaling pathways are one of such biomarkers and key regulators of cell proliferation and apoptosis. These markers also exert profound influence on drug sensitivity of cancer cells in vitro as well as in vivo. Therefore, in the present study the drug combination was tested in CRC cells with distinct expression of these pathways, in order to find, if there is any significant difference in chemosensitivity.

The cell viability results indicated that both drugs in combination exhibited significant antiproliferative effects in concentration and time dependent manner in CRC cells. In the combination study for antiproliferative activity, the response of the cells varied widely. COLO-320 and SW-620 cells were found to be more sensitive with 83% antiproliferative activity for combination of limonene and BEZ (1000 μM+100 nM) than HCT-116 and HT-29 cells with 77% and 74% respectively at 2000 μM+100 nM concentrations. The disparity in the response could be largely attributed to the distinct genetic makeup of the cell lines with regards to PI3K and K-ras pathways. The presence of K-ras and PI3K mutations in HCT-116 and HT-29 cells could possibly be one of the reasons for the observed outcome as compared to the wild type expressions of these genes in COLO-320 cells. However, surprisingly, SW-620 cell line, which is a mutant for K-ras also showed a nearly the same degree of sensitivity as COLO-320. Such a scenario could be partially explained by the varied sensitivity of mutant phenotypes as different types of mutations might exhibit varied response.

CompuSyn analysis for systematic evaluation of drug combination effects revealed that the exposure of both drugs at the same time was more efficacious than pre-treatment with either limonene or BEZ followed by exposure to the other. However, for combination effects, pre-treatment with BEZ followed by exposure to limonene was marginally more effective than pre-treatment with limonene followed by BEZ exposure. This observation is of critical importance in clinical applications of combination therapy, where not only the drug proportions but spacing between the drug administrations is also considered vital as varying outcomes were reported for combination studies based on the dosing schedule. In breast and ovarian cancer cells, the combination was antagonistic, when the cells were exposed to simultaneous or sequential treatment of carboplatin followed by paclitaxel (123). Similarly, the combination of docetaxel
and doxorubicin, in the same tumor cells were proved antagonistic by simultaneous or sequential treatment of doxorubicin followed by docetaxel (124). Therefore, the optimal schedule for both of these combinations should be sequential exposure to paclitaxel or docetaxel followed by carboplatin or doxorubicin respectively.

Drug induced cytotoxicity on tumor cell clonogenicity can be more precisely determined by in vitro colony-forming assay than conventional cell viability methods (117). This assay essentially tests every cell in the population for its ability to undergo unlimited division to produce a large colony or clone, which is the proof that it has retained capacity to reproduce and potentially become tumorigenic (125). Moreover, the in vitro colony formation assay results most often positively correlate with in vivo antitumor activity, therefore, prediction of chemotherapeutic sensitivity could be possible.

In the present study, the inhibition of colony formation was more effective in COLO-320 cells (53.6%) and least effective in HT-29 cells (38.4%) at the maximum concentration of limonene and BEZ (500 μM+50 nM) tested. In general, in vitro drug resistance in clonogenicity tests is strongly correlated with lack of tumor response in patients (126). So, in the present scenario, where high in vitro response was observed for limonene and BEZ combination, the in vivo antitumor response may be highly predictable for this combination in human CRCs and their xenografts and probably in other types of cancers also.

Cell migration and invasion are essential physiologic process but, also implicated in the cancer related mortality by promoting metastasis. Therefore, chemotherapeutic drugs, in general, are expected to decrease cancer cell migration by manipulating the molecular mechanisms that regulate the distinct steps of metastasis such as detachment, invasion and dissemination of cancer cells.

The scratch assay for cell migration in vitro most often correlates/mimics cell migration in vivo. Therefore, the present study was designed to investigate the effects of limonene and BEZ combination on cell migration in cancer cells. Consequently, the inhibition of cell migration was highest in HCT-116 cells (67.7%) and least response was found in SW-620 cells (20%) at the highest concentration of (500 μM+50 nM) drug combination.
The anti-metastatic activity of limonene and BEZ in CRC cells was almost comparable to those obtained in highly metastatic triple negative (estrogen receptor negative, progesterone receptor negative and HER2-negative) breast cancer cells (MDA-MB-231), where a combination of dasatinib (inhibit Src kinase that regulate cell migration and invasion) in combination with doxorubicin showed synergistic anticancer activity by inhibiting cell migration and invasion (127) indicating that such an activity may be relevant for anticancer effect of present combination also. Although the effects of limonene and BEZ on molecular mechanisms that regulate cell migration were not investigated in the present study, such an effect might be the underlying mechanism for the observed outcome in CRC cells. In addition, the differential expression of these (Src) kinases in CRC cells might be responsible for the varied outcomes observed. In agreement with the notion that cellular signals for proliferation or survival response also regulate the migratory behaviors of the tumor cells, the potent antiproliferative activity of the drug combination positively correlated with its effects on inhibition of cell migration (128).

The CompuSyn analysis of combination effects on inhibition of colony formation and cell migration showed either synergistic or additive effects in all the cell lines tested (except for SW-620 cells, which showed an antagonistic effect for cell migration at 500 μM+50 nM concentration of limonene and BEZ) for the highest concentration of limonene (500 μM) with all BEZ combinations (25 and 50 nM). Overall, the results indicate that the profound anticancer activity of this drug combination is positively correlating with its anti-tumor and anti-metastatic effects in CRC cells and such activities for this drug combination has not been tested in any other cancer cells before.

As uncontrolled cell proliferation is the hallmark of cancer, precise regulation of cell proliferation by cyclins and cyclin dependent kinases (Cdk) in different phases of cell cycle is well delineated process. In general, the cells in G₁ phase integrate growth inhibitory or mitotic signals and either proceeds towards S phase for DNA synthesis or exit cell cycle into quiescent stage (G₀). Therefore, arrest in G₁ phase can prevent a cell from entering into proliferation and even stimulates it to undergo apoptosis, if something is awry, whereas, altered regulation of G₁/S transition can make the cells divide independently of mitogenic stimuli and thus leads to cancer.
The results of the present study also indicate that G1 phase of the cell cycle has been the target for drug combination. The combination of limonene and BEZ significantly inhibited proliferation of CRC cells resulting in the increased accumulation of cells in G1 phase with concomitant decrease in S phase and G2 phase in COLO-320, HCT-116, HT-29 and SW-620 cells. While the combination effect on different cell cycle phases (G1, S and G2) was nearly similar in all the cells, COLO-320 cells were found to be more responsive to the drug combination with regards to G1 (1.6x increase) and G2 (4.3x decrease) phases. So, the altered pattern of distribution of cells in different cell cycle phases following combination treatment in COLO-320 could be very likely due to the wild type expression of K-ras and PI3K pathways in COLO-320 cells compared to their mutant phenotype in HCT-116, HT-29 and SW-620 CRC cells. As a consequence, with more proportion of cells being arrested at G1 phase and eliminated further by apoptosis might be the reason for very less proportion of cells enter into G2 phase for proliferation/division and thus, limiting the chances of cancer development.

As evident, the p53 is a critical regulator of G1 check point (halt the cells at G1 phase) following DNA damage (129) and loss of p53 activity due to mutations may allow the cells to progress through the cycle for proliferation. However, limonene and BEZ combination effects on cell cycle analysis were not significantly differ in p53 wild (HCT-116) and p53 mutant (COLO-320, HT-29 and SW-620) cells. HCT-116 cells did not appear to have an additional benefit because of wild p53 (1.3 fold increase in G1 and 2.3 fold decrease in G2 phase) compared to its mutant counterpart COLO-320 (1.6 fold increase in G1 and 4.3 fold decrease in G2 phase) showing that, additive signaling by mutant K-ras and PI3K pathway might have strongest input for cell proliferation than p53 induced apoptosis in those cells.

Overall, the effects of limonene and BEZ on cell cycle phases in CRC cells were also closely comparable to their effects in other cancer cells. Such as, limonene and BEZ when tested individually resulted in accumulation of cells in G1 phase and concomitantly decreased cells accumulating in S phase in breast (130, 54) and gastric (131, 55) cancer cells and such events are preceded by decreased cyclin D1 expression in breast cancer cells due to limonene. Similarly, BEZ induced cell cycle arrest in G1 phase was mediated by down regulation of cyclin A and cyclin D1 in glioma stem cells (56), indicating that G1 phase or its regulators could be the main target for the drugs/combination effects on cell cycle in CRC cells also.
Based on the results of cell cycle analysis the following conclusions can be derived.

- $G_1$ phase might be the target for drug effects on cell cycle
- Cells with K-ras and PI3K wild type expression were more sensitive for cell cycle alterations to combination treatment than their mutant counterparts
- Tumor suppressor p53 did not appear to play significant role in the drug induced alterations in the cell cycle phases

As the combination results on cell cycle analysis evidently varied based on molecular mutations of key signaling pathways, the further experiments were planned to rationally connect the antiproliferative activities of drug combination with alterations in apoptosis pathway.

Apoptosis or programmed cell death is an essential physiological and an underlying pathological process in many diseases including cancer and numerous methods for confirmation of apoptosis in cancer cells are available (132). Therefore, to validate the effects of combination treatment on apoptosis two different methods were performed, such as fluorescent microscopic examination of apoptotic cells and estimation of caspases activities by colorimetric method.

Apoptotic cells were differentiated from necrotic cell death based on features such as cell shrinkage, nuclear condensation and fragmentation and were also quantified. The % apoptotic cells for combination of limonene and BEZ were 32.7%, 27%, 24.3% and 28.4% respectively in COLO-320, HCT-116, HT-29 and SW-620. Based on the results, it was evident that COLO-320 cells were more sensitive for limonene and BEZ induced apoptosis and HT-29 was found to be least responsive. When tested individually, the effect of limonene in apoptosis was marginally more than the BEZ in CRC cells used in the study (20%, 18%, 16.7%, 20.3% vs. 16.3%, 16.7%, 18.3%, 17.3%). In leukemia, prostate cancer cells and human gastric cancer implanted in nude mice (in vivo) (133) also, limonene exerted anticancer activity that was mediated through induction of apoptosis and as the data indicate, it might be the basic underlying principle for in vitro and in vivo anticancer activity of limonene, which is strongly corroborating with our findings. Similarly, BEZ also induced apoptosis in breast, colon and glioma stem cells, suggesting that apoptosis pathway could be the main target for both the drugs in CRC cells.
In order to authenticate the combination effects on apoptosis and also to identify the underlying mechanism for increased apoptosis, further studies were performed to determine the caspases activities, the critical components of apoptotic pathway.

Caspases activity is like a double-edged sword, defective caspase activation leads to cancer and extreme activity may cause neurodegenerative diseases. Although caspases are key players in the apoptotic mechanism, mutations in caspase genes are not as frequent as p53 mutations and cancer cells may gain a survival advantage by inactivating signaling cascade upstream of caspase activation, emphasizing the critical role they play in apoptosis (134).

The combination treatment on caspases activities showed an increase of 1.7x, 1.6x, 1.6x and 1.6 fold for caspase-3 and 1.5x, 1.3x, 1.4x and 1.4 fold for caspase-9 respectively in COLO-320, HCT-116, HT-29 and SW-620 cells. It appeared that COLO-320 was more sensitive than other cells for caspase induced apoptosis. The combination was more efficacious for induction of caspase-3 activity than caspase-9 in all the cells, indicating the profound influence of caspase-3 in overall cell killing efficacy of tested drugs. The combination treatment was more potent on increasing the caspases activities compared to either of the drugs alone. The antiproliferative effects of drug combination were further authenticated here, by establishing an intense correlation between caspases activity and alterations in apoptosis.

Because of pivotal role they play in apoptosis, compounds have been developed capable of directly activating caspase-3 for use in cancer therapy (135, 136) or indirectly activate caspases by blocking endogenous caspase inhibitors such as Bcl-2 and inhibitor of apoptosis proteins (137). Although limonene and BEZ combination induced caspases activity, it was not clear, whether these drugs act by direct or indirect manner to promote such activity.

Although the caspases mediated proteolytic cascade represents a central point in the apoptotic pathway, its initiation is closely regulated upstream by Bcl-2 family proteins. Therefore, further experiments were intended to determine the effects of drugs on regulation of pro-apoptotic (BAD and BAX) and anti-apoptotic (Bcl-2) proteins of Bcl-2 family in order to promote release of cytochrome c from mitochondria, eventually, activating downstream caspase cascade (138).
While BAX functions were antagonistic to Bcl-2, abrogating the ability of Bcl-2 to prolong cell survival, it is uncertain which of these proteins is an effector and which is a regulator. However, it is suggested that homodimerization of BAX generates cell death signals whereas, heterodimerization of Bcl-2 and BAX abrogates BAX function leading to cell survival (139). In the present study, both the drugs were shown to increase the activity of BAX with concomitant decrease of Bcl-2. It is assumed that translocation and dimerization of BAX, which are essential events for its activity might have augmented following treatment. As the ratio of BAX to Bcl-2 determines whether a cell should undergo apoptosis or not, the drug combination clearly altered the balance by not only increasing the BAX ratio, but causing a significant decline in Bcl-2 also. With regard to cells, both COLO-320 and SW-620 cells were more sensitive for apoptotic changes probably, because of elevation of BAX was more potent in these two cell lines (4.7x and 2.0x increase respectively) compared to other cells (1.3x increase in HCT-116 only) in this study. Similarly, reduction in the Bcl-2 activity was also highly significant in COLO-320 and SW-620 cell lines (0.9x decrease in both cells, which corresponds to approximately, 90% reduction).

Phosphorylation of BAD by survival promoters AKT and protein kinase A, inactivates BAD and promote cell survival (140), whereas, in response to death signals, it gets dephosphorylated and translocated to the mitochondrial membrane to promote cytochrome c release. Hence, the results clearly indicate that limonene and BEZ combination increased the activity of BAD, which in part, might be due to their ability to down regulate AKT activity, therefore, alleviating the phosphorylation and inactivation of BAD by AKT.

As the effects of limonene on Bcl-2 family members were widely investigated in various types of cancer cells, our results are in agreement with those studies highlighting that mitochondrial death pathway could be the potential target for drug effects in CRC. In LS174T CRC cells (50) and hormone refractory prostate cancer cells (48), limonene induced apoptosis was mediated by inducing caspases (3 and 9) activities and increasing the ratio of BAX to Bcl-2 with concomitant release of cytochrome C, therefore, favoring apoptosis. However, when compared to limonene, BEZ might have modest effect on apoptotic pathway, which might be achieved through inhibition of AKT activity therefore, alleviating the inactivation signal for BAD and caspase-9.
Subsequent experiments for combination effects on p53 showed only marginal increase of p53 activity in HCT-116 cells (wild p53), indicating that drug combination induced apoptosis in CRC cells was to a large extent is mediated by induction of caspases and Bcl-2 family proteins and p53 was suggested to have very minimal role to play. Although these results were not encouraging, the role of p53 in drug induced apoptosis can be fully investigated further in order to get inclusive information on p53 role in mediating apoptosis.

The following conclusions can be drawn to highlight the summative effects of combination treatment on overall apoptosis pathway.

- Limonene and BEZ induced apoptosis in CRC cells was regulated by mitochondria mediated intrinsic death pathway as shown by induction of caspases activity and alterations of pro and anti-apoptotic proteins of Bcl-2 family in promoting apoptosis
- The induction of caspases activity and resultant apoptosis was significant following combination treatment and the ability to induce caspase-3 was strongly correlated with antiproliferative activity of the combination, as caspase-3 is an effector caspase
- Combination treatment produced significant alterations in BAX (high increase) and Bcl-2 (high decrease) predominantly in COLO-320 and SW-620 cells, which might be the strong rationale for high sensitivity observed in these two cell lines for induction of apoptosis

The effect of limonene and BEZ on PI3K, mTORC1 and mTORC2 activity was monitored by measuring the phosphorylation of their downstream targets AKT-Thr308, p70S6K-Thr389 and AKT-Ser473 respectively.

The anticancer activity of BEZ is equally effective against PI3KCA mutant (HCT-116 and HT-29) as well as PI3KCA wild type (COLO-320 and SW-620) CRC cells. It may be due to the fact that these mutations do not interfere with ATP binding site, where the drug binds reversibly (141).

The inhibition of p70S6K-Thr389 and AKT-Ser473 phosphorylation was more effective than AKT-308 following BEZ treatment. It shows more profound influence of BEZ on inhibition of mTORC1 and mTORC2 activity than PI3K. When critically observed, the inhibition of p70S6K-
Thr389 phosphorylation was more sensitive to BEZ than AKT-Ser473, indicating that the effect of BEZ was more potent on mTORC1 activity than mTORC2. The relatively less sensitivity of p-AKT-Thr308 (PI3K) to BEZ, could be due to disruption of the negative feedback loop, when exposed to low concentrations of BEZ (100 nM) for 48 h (142). While majority of the in vitro and in vivo studies also demonstrated that sustained inhibition of mTORC1 and mTORC2 (as indicated by decreased phosphorylation of p70S6K-Thr389 and AKT-Ser473 respectively) and transient blockade of PI3K activation (p-AKT-Thr308) following BEZ treatment for 48 h (143). However, such an effect on p-AKT was completely abolished with higher concentrations (500 nM) of BEZ. As Violeta et al demonstrated in DU145 prostate cancer cells, treated with 500 nM BEZ for 48 h, completely blocked AKT phosphorylation regardless of time duration (54). In the present study we did not investigate BEZ at higher concentrations (500 nM) and time course experiments. However, the combination of BEZ (100 nM) with limonene treatment for 48 h demonstrated a significant decrease in p-AKT level, indicating that limonene might have sustained the transient effect of BEZ on AKT.

Although we used less concentrations of BEZ (100 nM), the effect on inhibition of phosphorylation was almost comparable to the results obtained with high concentrations of BEZ (500 nM), which is the most important observation with considerable clinical implications. Indeed, the superior efficacy at lower concentrations of BEZ with limonene combination might be beneficial clinically by avoiding additional toxicities due to high BEZ concentrations. It was achieved because of combination with limonene might have continued or sustained the transient effects of BEZ on the phosphorylation.

On the other hand, limonene showed only meager effects on the phosphorylation of AKT-Thr308 and AKT-Ser473, which indicates the least influence imposed on these targets at the tested concentrations. However, the phosphorylation of p70S6K-Thr389 was more responsive to limonene also, indicating its general sensitivity to drug effects. Overall, the effects of limonene on the PI3K/AKT pathway were quantitatively inferior to BEZ, because of lack of specificity on these signaling pathways. In general, the anticancer properties of most of the natural compounds are derived from nonspecific mechanisms on diverse cellular targets as opposed to specific targeted molecules like BEZ.
Collectively, these findings suggest that inhibition of PI3K/AKT pathway by limonene and BEZ combination may contribute, at least in part, for inducing apoptotic cell death in CRC cells possibly by sensitizing the tumor cells to chemotherapy.

The chemotherapeutic potential of limonene had been demonstrated in several preclinical and clinical studies and is briefly highlighted below. In a number of animal models of chemically induced tumors, oral administration of limonene, at different doses for several weeks, significantly reduced the frequencies of tumors such as lung tumor in mouse (144), aberrant crypt foci in rat colon (145) and pancreatic tumors in golden hamsters (146). Similarly, evidence from phase I clinical trial showed a partial response in patient with breast cancer and stable disease for more than six months in three patients with CRC. In another study, limonene at a dose of 0.5 g/M²/day was able to halt the progression of cancer for nine months in a patient with mucinous cystadenocarcinoma of appendix. Another patient with local retrovesical recurrence of colorectal adenocarcinoma remained stabilized on 2 g/day for 7.5 months (40).

In addition to the anticancer activities that are well described, limonene also has well established chemopreventive activity against many types of cancers. It acts on many key cellular targets in cancer cells. Limonene possesses strong antimutagenic activity both \textit{in vitro} and \textit{in vivo} as demonstrated by us using different test systems (147). It also prevents lipid peroxidation and an excellent free radical scavenger by virtue of its antioxidant activity and ability to enhance endogenous antioxidant systems (148). Another possible mechanism that strongly corroborate with limonene’s chemopreventive activity is that it induces phase I and phase II detoxifying/carcinogen-metabolizing enzymes (cytochrome p450), which metabolize carcinogens to less toxic forms and prevent their interaction with DNA (149).

Another widely supported mechanism to explain the anticancer activity of limonene is the inhibition of ras prenylation (122), by inhibiting farnesyl transferase (FTI) enzyme, which is involved in ras prenylation. Therefore, a series of compounds such as R115777, SCH66336 and BMS-214662 have been introduced into the cancer drug research as potent FTIs and soon discontinued because of toxicities (150). Although initially, limonene was highlighted as FTI with relatively low toxicity but soon it was suggested to have modest anticancer effects as FTI when used alone. Therefore, because of their limited efficacy, FTIs were not projected as potent
anticancer agents and thus, recommended to be used in combination with other compounds for better clinical efficacy. Accordingly, the present study results also imply that the anticancer activities shown by limonene may not be predominantly carried out by inhibition of ras via FTI, but might be due to more potent alternative mechanisms such as induction of apoptosis, caspases activity and AKT inhibition.

Based on the results of the study, the concept of combining chemotherapeutic drugs with natural compounds could be more promising in clinical perspective, if critical aspects such as sequence of administration, proportion of each drug in the combination and drug interactions were carefully considered so as to obtain synergistic or additive effects.