Chapter 5

Summary
Summary

Availability of anticancer agents that combine safety and clinical efficacy are highly challenging. Therefore, the rationale is combination of chemotherapeutic drugs with natural compounds could be more beneficial. Limonene and BEZ were both reported to have anticancer activity; however, the combination of both was not tested before in CRC cells. Human CRC cells: COLO-320 (wt-K-ras and PI3K), HCT-116 (mt-K-ras and PI3K), HT-29 (wt-K-ras and mt-PI3K) and SW-620 (mt-K-ras and wt-PI3K) were selected to test the combination effects. High incidence of K-ras and PI3K mutations in CRCs and their ability to confer chemoresistance, poor prognosis in CRC patients could be directly correlated with results obtained in these cells for probable clinical efficacy of drug combination in CRCs. Moreover, BEZ induced transient effect on AKT phosphorylation could be sustained by limonene leading to sensitize the cancer cells for apoptosis.

5.0 Combination effects on antiproliferative activity in colon cancer cells

- The drug combination produced concentration and time dependent cytotoxicity/antiproliferative activity in all the cancer cells used in the study
- COLO-320 and SW-620 cells were more sensitive to drug effects than other cell lines used in the study. However, the concentration of limonene required to achieve the same effect in HCT-116 and HT-29 cells was much high (twice) compared to what was used for COLO-320 and SW-620 cells
- In combination studies, exposure of the drugs at the same time produced maximum antiproliferative response compared to sequential exposures, where the cells were pre-treated with a drug followed by exposure to the other
- Pre-treatment with BEZ followed by limonene was marginally more effective than the pre-treatment with limonene followed by BEZ
- CompuSyn analysis of combination effects also suggested that simultaneous treatment of limonene and BEZ at the same time exhibited additive effect in human CRC cells
- From the CompuSyn analysis, it was evident that the pre-treatment with either of the drugs may have diversified impact, based on which drug was used for pre-treatment and followed by the other, indicating a caution that the drug combination needs to be
implemented quite carefully. This kind of treatment regimens may be of clinical relevance for any combination anticancer treatment strategies.

5.1 Combination effects on inhibition of colony formation and cell migration

- The anticancer efficacy of combination of limonene and BEZ was further investigated by their effects on inhibition of colony formation and cell migration in CRC cells.
- Compared to single agents, combination was exhibiting additive, inhibitory effects on both colony formation and cell migration in all the cells used in the study, the results of which strongly corroborate with the profound anti-tumor and anti-metastatic activity of the drug combination respectively.

5.2 Combination of limonene and BEZ induced G₁ phase arrest in human colon cancer cell lines

- The drug combination produced significant alterations in the distribution of cells in different phases. Though all the cells responded with an increase in G₁ phase cells with concomitant decrease in S phase and G₂ phase cells. However, the response in COLO-320 cells was higher probably, due to wild type PI3K and K-ras pathway in contrast to their mutant phenotypes in other CRC cells.

5.3 Limonene and BEZ induced apoptosis in colon cancer cells is regulated by mitochondria mediated intrinsic death pathway

- The cytotoxicity/antiproliferative activity of drug combination was further rationally connected to their ability to induce apoptosis with substantial authentication.
- The drug combination tested in different assays for apoptosis increased the activities of caspases-3 and -9 and expression of pro-apoptotic BAD and BAX with concomitant decrease in anti-apoptotic Bcl-2. The results indicate the pivotal role of mitochondria mediated intrinsic death pathway in the anticancer activity of limonene and BEZ.
- Although, p53 activity was not significantly altered by combination treatment, it appears that it may have minimal role in apoptosis at least in cells having wild type p53 (HCT-116).
5.4 Combination of Limonene and BEZ down regulates PI3K/AKT pathway in colon cancer cells

- Inhibition of phosphorylation of PI3K, mTORC1 and mTORC2 was more effective with drug combination than individual treatments, but, BEZ was found more effective than limonene
- Overall, the inhibition of mTORC1 and mTORC2 was higher than PI3K when exposed to these drugs
- However, limonene appeared to enhance the transient effects of BEZ by imposing a sustained blockade on PI3K signaling. Also, inhibition of AKT might have further contributed, or at least in part, to the induction of apoptosis as increased activity of AKT make the cells less sensitive to apoptosis

While evaluating the anticancer agents, the combination studies of limonene and BEZ in CRC cells with distinct mutations have reemphasized the significance of K-ras and PI3K mutations, not only in the lab, but in the clinic also. However, the quantitative differences in anticancer activities could be anticipated for COLO-320 by virtue of its wild type expressions of K-ras and PI3K pathways compared to their mutant versions in other cells used in the study. In addition, the expression of various other markers controlling cellular behavior may also responsible for differential sensitivities of CRC cells tested. Though the effective concentration of BEZ used did not vary among the cells tested, the concentration of limonene required to yield significant activities among the cells varied widely, probably, due to distinct genetic makeup of the cells. Such a phenomenon may have clinical implications while designing the chemotherapeutic regimens based on the molecular types of CRC.