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Limonene and BEZ 235 inhibits growth of COLO-320 and HCT-116 colon cancer cells

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Abstract

D-Limonene is a dietary monoterpene with significant anticancer activity against many cancer types in preclinical and clinical studies. The study is designed to investigate synergistic anticancer effects of limonene and BEZ235 combination in COLO-320 and HCT-116 colon cancer cells. Cells were treated with both the drugs alone and in combination and the effects on cell viability; cell migration and clonogenic potential were examined. Results show that both drugs exhibited dose and time dependant cytotoxicity on the cell lines tested. Composing analysis of the drug combination effects revealed the strong synergistic interaction of the combination. Our results also indicate that COLO-320 cells were more sensitive for anticancer effects of the drugs than HCT-116 cells. The presence of Ras and PI3K mutations in HCT-116 cells could possibly be one of the main reasons for the observed outcome as compared to the wild type expressions of them in COLO-320 cells.

Kewords: Colorectal cancer, limonene, BEZ235, Ras, PI3K.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in both men and women and the second leading cause of cancer death when men and women are combined [1]. It shows significant geographical, racial and ethnic variation in its incidence rate and pattern. Most of CRCs are sporadic, whereas 10% have a clear genetic background. These include familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) are dominantly inherited conditions with 100% and 80% life-time risk of developing colorectal cancer, respectively. The mutations in adenomatous polyposis coli (APC) and mismatch repair (MMR) genes are responsible for these two conditions [2]. These same genes also play a key role in the formation of sporadic colorectal cancers.

Most of CRCs are thought to develop slowly through an orderly series of events known as adenoma carcinoma sequence. Here, normal colonic mucosa is transformed into adenoma, which then transforms into adenocarcinoma [3]. Therefore early detection and removal of adenomatous polyps can prevent colorectal cancer. Majority of the colorectal cancers (approximately 96%) are adenocarcinoma type which is cancer that develops in glandular cells on the inner lining of the colon and rectum [4].

Advances in molecular pathogenesis of CRC have aided in formulating both preventive and therapeutic strategies. Ras and PI3K are complex signaling pathways that are critical for cell proliferation and apoptosis. Aberrant activation of these signaling pathways has been observed in the development of CRC with enhanced risk of tumor growth, angiogenesis and metastasis [5]. K-ras and PI3K mutations are one of the commonest genetic alterations seen in CRC with a frequency of about 30% and 17 % respectively [6]. Therefore, inhibition of these signaling pathways requires a multitargeted approach. Natural compounds with anticancer activity are better chemo preventive and/or therapeutic alternatives because of their pleiotropic properties.

D-limonene is a monocyclic monoterpene with a lemon-like odor and is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). It is widely used as a flavor and fragrance additive in food industry. Limonene has been clinically used to dissolve cholesterol containing gallstones [7] and because of its potential for gastric acid neutralization, it has also been used to relieve heartburn and gastro esophageal reflux disorder (GERD) [8]. Limonene acts on many cellular targets in cancer cells such as; immune modulation, modulating chemical carcinogenesis, anti-oxidant activity and apoptosis. In preclinical cancer models, it has been shown to prevent or delay the growth of a number of cancer types including lymphomas [9], mammary [10], gastric [11], liver [12], lung [13], and prostate cancer [14]. It is also shown to decrease the number of aberrant crypt foci (ACF) in azoxymethane induced putative preneoplastic carcinogenesis in rodents [15]. For many years azoxymethane induced ACF has been used as surrogate biomarkers in the screening of anticancer agents.

NVP-BEZ 235 (Novartis) is a dual pan-class I PI3K and mTOR kinase inhibitor that has been demonstrated to reduce tumor growth in a number of different xenograft and several genetically engineered mouse models and is currently in clinical trials [16]. It shows anti-proliferative effects and cytotoxicity in various tumor

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types, including breast cancer, multiple myeloma, and sarcoma [17, 18]. The compound has also been tested in colorectal cancer with coexistent mutations of KRAS and PIK3CA [19].

The objective of this study is to determine synergistic anticancer activity of limonene and BEZ in colon cancer cells and also to find if there is any difference in response between K-ras and PI3K mutation vs. wild type cells.

Materials and methods

Cell lines and drugs

COLO-320 (wild type KRAS and PI3KCA) and HCT-116 (mutant KRAS-G13D; and PI3KCA-H1047R) colon cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and was maintained in DMEM culture medium (Gibco BRL-Invitrogen) supplemented with 10% FBS (Hi-Media), 100 U/ml penicillin and 100 μg/ml streptomycin (Hi-Media) at 37° C in an incubator with 5% CO_2. D-Limonene was procured from Sigma and NVP-BEZ235 (Novartis) was procured from Cayman chemicals (Prolab Marketing Pvt Ltd, New Delhi, India).

Cell viability assay

The effect of limonene and/or BEZ235 on the cell viability of colon cancer cells was determined by MTT assay. Briefly, cells were seeded at a density of 1 X 10^4 cells per well in 96-well plates and treated either with limonene or BEZ235 and also with their combinations, at specified concentrations for 24 and 48h. For combination effects the cells were treated with both the drugs simultaneously as well as sequentially. In simultaneous treatment, the cells were treated with limonene and BEZ for 24 and 48h continuously. While in sequential treatment, cells were pre-treated with either limonene or BEZ for 12h, followed by the exposure to the other agent for a total of 24 and 48h.

Cells were incubated with the MTT reagent (5 mg/ml in DMEM) for 3h at 37° C, followed by solubilization of the formazan crystals with DMSO for 10min. Absorbance was measured at 570 nm using a microplate analyzer (BioTEK, synergy 4 multimode microplate reader). The percent cell viability was calculated using the following formula; % cell viability= OD of test/OD of control x 100.

In vitro scratch assay

The *in vitro* scratch assay is an easy, low-cost and well-developed method to measure cell migration *in vitro*. Compared to other methods, the *in vitro* scratch assay is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration, mimic cell migration *in vivo*, which in general, occurs during embryogenesis or tissue regeneration or cancer metastasis.

Cells were seeded at a density of 5 ×10^4 in 6 well plates and the monolayer of 80% confluent cells were treated for 48h either with limonene or BEZ235 and also with their combinations (in the format of both drugs at once) at the doses indicated. The serum concentration in the growth media was decreased to minimize cell proliferation, but just sufficient to prevent apoptosis and/or cell detachment. After 48h of treatment, the drug containing medium was removed and a scratch was created with a sterile p200 pipette tip and washed twice with PBS to remove floating cells and plates were incubated after adding complete DMEM with 5% FBS. Cell migration was monitored by capturing images at 0, 24 and 48h using an inverted phase microscope (Olympus, CKX41). Gap area was measured relative to the total cell-covered area with Wimasis image analysis software. The effect of drug treatment was measured by a reduction in the % cell migration at each time interval compared to untreated control using the following formula; % Cell migration= (Scratch area at 0h-Scratch area at specific time point)/Scratch area at 0h x100.

Colony formation assay

Clonogenic cell survival is the method of choice to determine cell reproductive death after treatment with ionizing radiation, but can also be suitably adopted to determine the effects of cytotoxic agents. More over colony-forming assays are good indicators of drug- induced cytotoxicity and effects on tumor cell clonogenicity than the conventional cell viability methods [20]. The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone which is visible to the naked eye. Cells were seeded (5 10^4) in 6-well culture plates and treated with various doses of drugs either single or in combination, as indicated for 48h. Cells were trypsinised and approximately 100 cells for each drug concentration was seeded into fresh 6-well culture plates and incubated for another 2 -3 weeks with change of fresh media once in 3days. Colonies were fixed in methanol and stained with 0.5% crystal violet dye for 30min and colonies containing >50 cells were counted under inverted phase contrast microscope.

Analysis of combination effects using CompuSyn software

Further the anticancer effects of limonene and BEZ235 combination in COLO-320 and HCT-116 cells were analyzed using CompuSyn software [21] for synergism, additive or antagonistic effects based on Combination Index (CI). If the CI of less than, equal to, and more than 1 indicate synergistic, additive and antagonistic effects, respectively.

Statistical analysis

Statistical analysis was carried out using 'GraphPad Prism Software' (San Diego, USA). All experiments were performed in triplicate and repeated three times. Data were presented as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The level of significance was set at p 0.05.

Results

Limonene and BEZ235 inhibits cell proliferation

Cells were treated with increasing doses of limonene (100-3000µm) and BEZ235 (10-1000nm) for 24 & 48h and viability was determined by MTT assay. Drug treatment exhibited significant difference in viability compared to control (P<0.05). When tested individually both the drugs inhibited cell proliferation in dose and time dependent manner (Figure 1). Under the experimental conditions, the calculated IC50 values in COLO-320 were at 950µm and 104nm for limonene and BEZ respectively; whereas in HCT-116 the IC 50 values for limonene and BEZ were at 1995µm and 98nm respectively.

Cells were treated with different combinations of the drugs added simultaneously as well as sequentially and cell viability was determined as described previously by MTT assay. The combination of varying doses of limonene and BEZ produced maximum antiproliferative activity at 48h when compared with the treatment of either agents alone in both the cell lines. The combination of LIM+BEZ at a concentration of 1000+100 produced highest antiproliferative activity in COLO-320; whereas in HCT-116 the combination at a concentration of 1500+100 showed highest activity (Figure 2.A.D).

While testing combination effects the cells were exposed to drugs in different formats as mentioned above. Antiproliferative effect was more when the cells were exposed to both drugs simultaneously than sequential treatment (83% vs. 60% or 68% for COLO-320: Figure 2.A.B.C) and (86% vs. 68% or 70% for HCT-116: Figure 2.D.E.F).

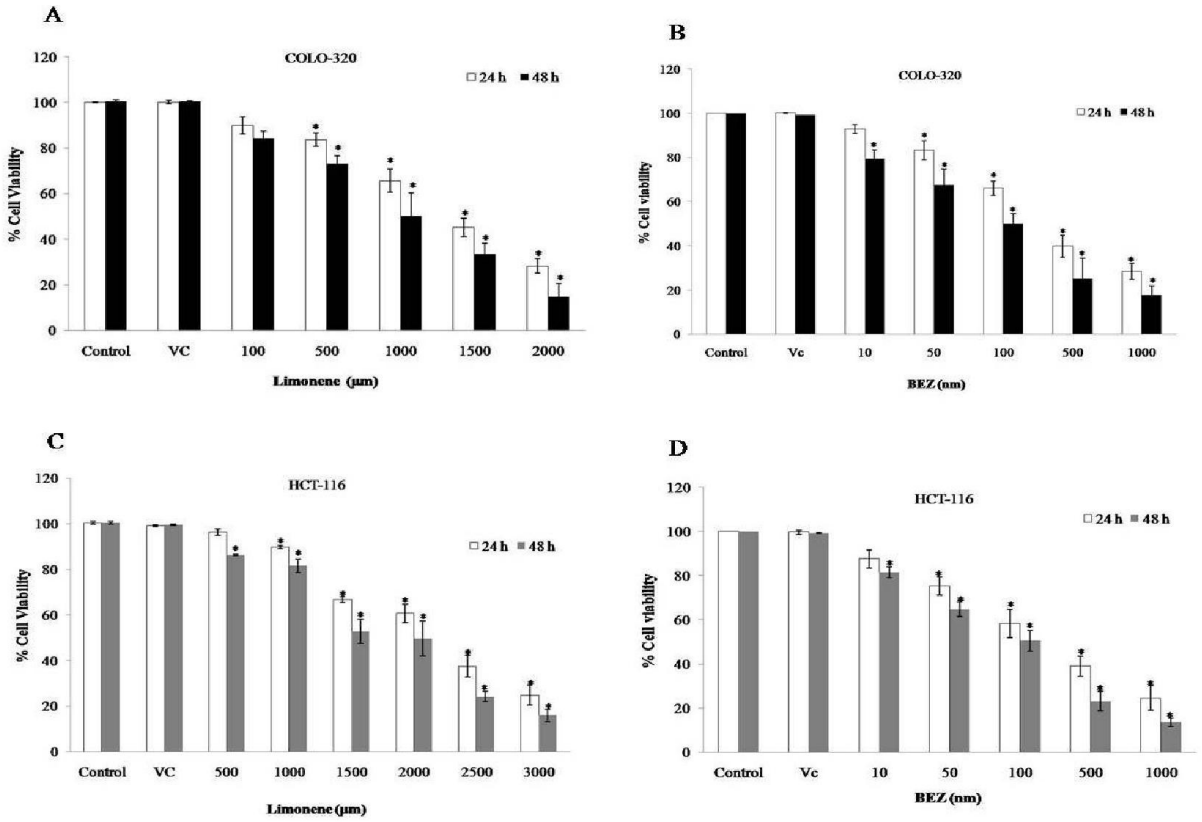

Figure 1: Effect of Limonene and BEZ on cell viability Showing the effect of limonene and BEZ on cell viability in COLO-320 (A.B); HCT-116 (C.D) CRC cell lines. Cells were treated for 24 & 48h and viability was determined by MTT. Data were expressed as mean ± SD (n=3). *p < 0.05

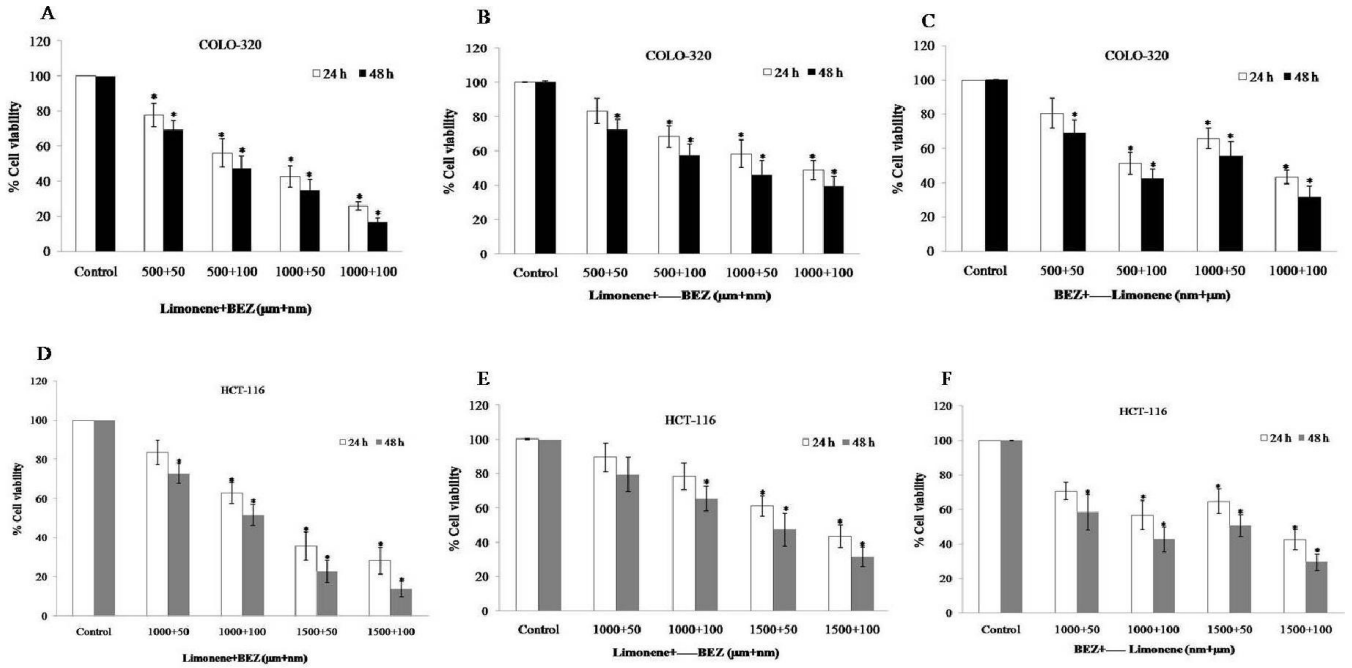

Figure 2: Effect of limonene and BEZ combination of on cell viability**.** Combination effect of limonene (LIM) and BEZ on anti-proliferative activity in COLO-320 & HCT-116 cells. A.D: Cells were treated with limonene in combination with BEZ at the same time. B.E: Cells were pre-treated with limonene for 12h followed by exposure to BEZ. C.F: Cells were pre-treated with BEZ for 12h followed by exposure to limonene. Cells were treated for a total of 24 & 48h and viability was determined by MTT. Data were expressed as mean ± SD (n=3). *$p < 0.05$

Effects of limonene and BEZ235 on cell migration

In vitro scratch assay was performed to study the effects of limonene and BEZ235 on cell migration in COLO-320 & HCT-116 cell lines (Figure 3.A.B). Lower doses of limonene (250, 500µm) and BEZ (25, 50nm) and their possible combinations were selected for the study only to minimize the cytotoxicity. In control group cell migration into the scratch was 100% in 48h resulting complete closure of the gap, whereas drug treatment alone or in combination caused a significant inhibition of cell migration in both the cell lines ($P<0.05$). The maximum inhibition of % cell migration observed at 48h was 54.75, 67.7 in COLO-320 and HCT-116 cells respectively at the highest concentration of LIM+BEZ (500+50).

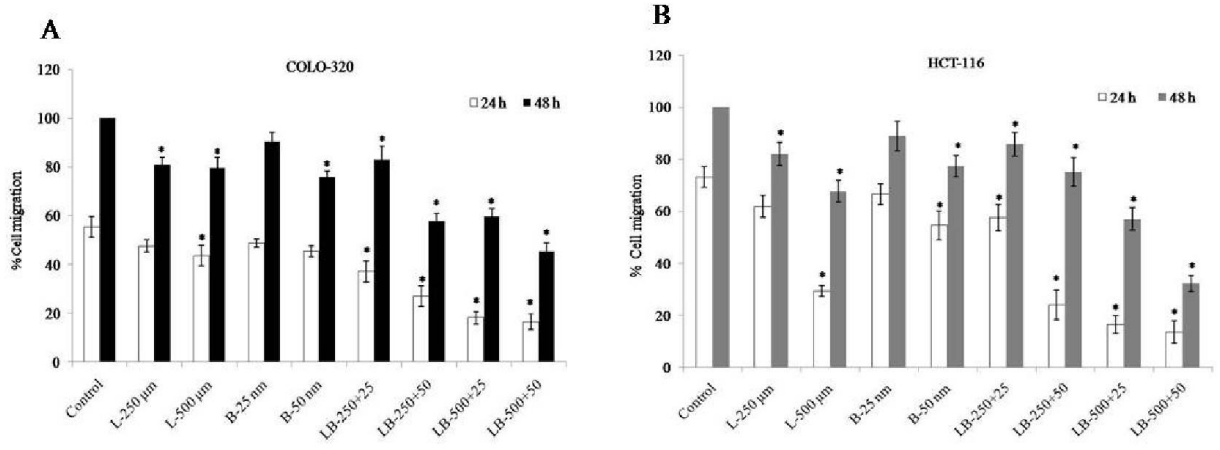

Figure 3: Limonene and BEZ inhibits cell migration. Effect of limonene and BEZ combinations on cell migration in COLO-320 (A); HCT-116 (B) cell lines. Cells were treated for 48h and scratch images were captured at 0, 24 and 48h and analyzed as described in materials and methods. The percent cell migration was expressed as mean ± SD (n=3). *$p < 0.05$

Combination of limonene and BEZ235 inhibits colony formation

Clonogenic assay was performed to study the effects of limonene (250, 500µm), BEZ (25, 50nm) and their combinations (Figure 4.A). Represent images of the assay were shown in Figure 4.B. Both the drugs at high concentrations resulted a significant inhibition of colony formation (%) compared to control ($P<0.05$). Drug combinations were more effective than either of the drugs alone. LIM+BEZ at high concentration (500+50) showed maximum inhibition of 53.66; 51.76 in COLO-320 & HCT-116 cells respectively.

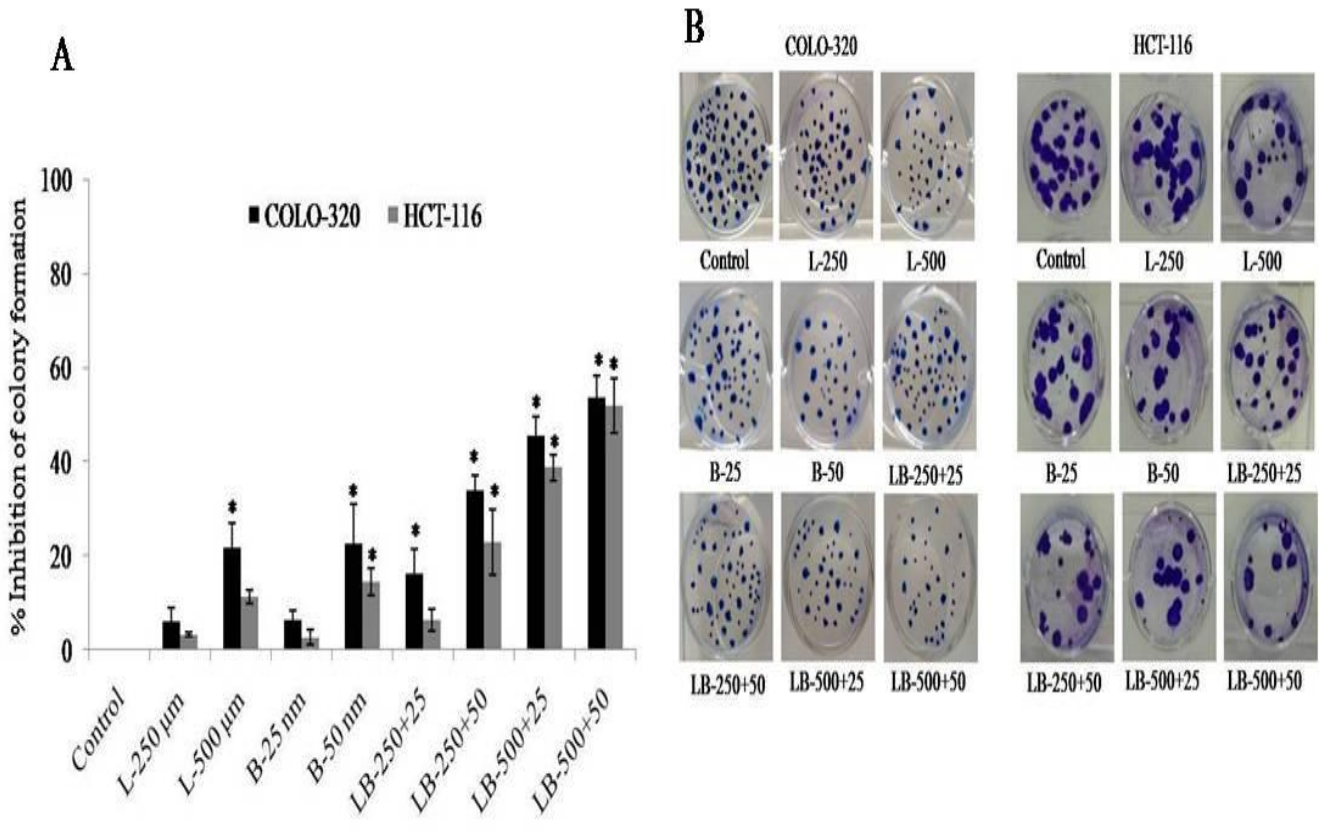

Figure 4: Limonene and BEZ inhibits colony formation. Effect of limonene and BEZ combinations on colony formation of COLO-320 and HCT-116 cell lines. A: The percent inhibition of colony formation at different drug combinations expressed relative to untreated cell control considering as zero; B: Images of colonies that were stained with 0.5% crystal violet reagent. The data were expressed as mean ± SD (n=3). *$p < 0.05$

Synergistic anticancer activity of limonene and BEZ

The interaction of drug combinations was analyzed by CompuSyn software and CI values were generated to determine synergy, additive or antagonistic effects. The combination of LIM+BEZ on cell viability was strongly synergistic (CI<1) at 1000+50; 1000+100 in COLO-320 when the drugs were exposed at the same time. In HCT-116 also the combination at 1500+50; 1500+100 produced strong synergism in the same format. While in pretreatment formats all the combinations were shown to be antagonistic (CI >1) in both the cell lines (Table 1.A).

In COLO-320 the combination shows strong synergism on inhibition of cell migration at 250+50; 500+25 and 500+50; whereas it shows a weak synergistic interaction on colony formation at the dose of 500+25. However in HCT-116 the combination at 500+25; 500+50 produced strong synergism on inhibition of cell migration and clonogenic ability (Table 2.B).

Table 1: Combination Index (CI) values of limonene and BEZ combination effect on COLO-320 and HCT-116 cell lines

A

	LIM+BEZ (µm+nm)	Cell viability		
		Both at once	Pre-treat with LIM	Pre-treat with BEZ
COLO-320	500+50	2	2.2	2
	500+100	1.3	2	1.1
	1000+50	0.9	1.3	1.8
	1000+100	0.5	1.4	1.1
HCT-116	1000+50	2.4	3.4	1.4
	1000+100	1.7	2.8	1.2
	1500+50	0.7	1.3	1.4
	1500+100	0.6	1.1	1.1

B

LIM+BEZ (µm+nm)	COLO-320		HCT-116	
	Cell migration	Colony formation assay	Cell migration	Colony formation assay
250+25	5	1.1	2	1.3
250+50	0.6	1.1	1.6	1.1
500+25	0.3	0.9	0.9	0.7
500+50	0.4	1	0.4	0.8

A: Effect of combination treatment on anti-proliferative activity. Cells were treated with the drug combination simultaneously as well as sequentially as described in material & methods. B: Effect on cell migration and colony formation. Synergistic interaction is determined if CI<1, an additive interaction if CI=1 and antagonistic if CI >1.

Discussion

The presence or absence of a variety of cellular targets in various colon cancers guide not only to design therapeutic regimen but provides information on clinical outcome. A range of molecular markers are known and currently being investigated for prognostic and predictive utility in colorectal cancer. Ras and PI3K signaling pathways are one of such biomarkers and key regulators of cell proliferation and apoptosis. Mutations in these signaling pathways are frequently observed in the genesis of CRC. Generally point mutations in K-ras gene are believed to be among the earliest events in colorectal tumor genesis [21]. Therefore we designed the study to investigate how K-ras and PI3K mutations influence the anticancer effects of the drugs by testing on COLO-320 (wild) and HCT-116 (mutant) colon cancer cell lines.

Although limonene has been tested as an anticancer agent against various cancers but its potential application in combination with other anticancer drugs has not been thoroughly explored. Therefore in this study we tested limonene and BEZ235 combination for synergistic anticancer activity. Limonene is a natural dietary monoterpene with lemon like flavor. Evidence from a phase I clinical trial showed that limonene is well tolerated and may have clinical activity in cancer patients [22]. Our study demonstrated that limonene shows anticancer effects relatively at high concentrations, but lack of toxicity even at high doses as shown in various clinical studies makes it a potential anticancer agent when used in combination with other cytotoxic drugs.

We found that combination treatments were more effective than either of the drugs alone. In CompuSyn analysis also the drug combination effects revealed the strong synergistic interaction. In particular, we noted that all the combinations with high concentrations of limonene were strongly synergistic in both the cells. It is also evident from our results that exposure of both drugs at the same time was more effective than pre-treatment format. Our results also indicate that COLO-320 cells were more sensitive for anticancer effects of the drugs than HCT-116 cells. The presence of Ras and PI3K mutations in HCT-116 cells could possibly be one of the main reasons for the observed outcome as compared to the wild type expressions of them in COLO-320 cells.

Conclusion

In conclusion the results demonstrate that combination treatment of limonene and BEZ235 elicits synergistic anticancer effects in COLO-320 and HCT-116 colon cancer cell lines. Although further studies are required to determine whether or not this drug combination yield similar anticancer effects in other cancer cell lines, we suggest that this study may be useful to identify potential anticancer compounds. Also studies are under progress to determine the anticancer mechanism of the drug combination.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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