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RESEARCH ARTICLE

Limonene and BEZ 235 induce apoptosis in COLO-320 and HCT-116 colon cancer cells

Y. Raja RatnaReddy[1,2,3], Manisha Singh[4], Vasanti Kabra[4], P. Chandra Sekhar[2,3], K Sreeja Vamsi[4], B. Ramachandra Reddy[4], S Ramamoorthy[3] and C. Damodar Reddy[1*]

Abstract

Deregulated apoptosis is the hall mark of many cancers, therefore every defect in apoptosis pathway could be a potential target for cancer treatment.The anticancer mechanism of limonene could be multifactorial. However, induction of apoptosis in cancer cells is proposed as the predominant mechanism in several of preclinical studies. Therefore, we determined to investigate the role of apoptosis in the anticancer activity of limonene and BEZ235 combination in COLO-320 and HCT-116 colon cancer cells. Cells after treatments were assessed for apoptosis by DAPI staining for fluorescent microscopic examination of apoptotic cells, estimation of caspases activities, Bcl-2 family proteins in addition to cell cycle analysis by flowcytometry. Results show that both drugs induced apoptosis as demonstrated by increased caspases activity, significant alterations in pro and anti-apoptotic proteins of Bcl-2 family in promoting apoptosis and cell cycle arrest at G1 phase. Over all, it is indicated that limonene and BEZ exerted anticancer activity is mediated through induction of apoptosis involving mitochondria mediated intrinsic death pathway in the selected CRC cells.

Keywords: Colorectal cancer; limonene; BEZ; apoptosis; caspases

Introduction

Apoptosis or programmed cell death is an essential physiological process and an underlying cause of many diseases including cancer. There are two pathways by which it is activated such as extrinsic and intrinsic pathways of apoptosis. Extrinsic pathway is initiated by binding of death ligands to death receptors, whereas, intrinsic pathway is initiated due to stimuli such as genetic damage, hypoxia and severe oxidative stress and is regulated by Bcl-2 family proteins [1]. Apoptosisis characterized by series of morphological changes and activation of specific group of enzymes namely caspases leading to cleavage of nuclear and cytoskeleton scaffold [2]. As deregulated apoptosis is the hall mark of many cancers, thus every defect or deregulation in apoptosis pathway can be a potential target for cancer treatment and many of such apoptosis based therapeutic strategies are under development [3]. D-limonene is a dietary monoterpene abundantly present in several citrus oils [4]. It is shown to have wide clinical applications in cancer as well as in other disease conditions. The anticancer mechanism of limonene could be multifactorial. Several of preclinical studies indicate that induction of apoptosis in cancer cells could be one of the most important mechanisms for its anticancer activity [5]. NVP-BEZ 235 is a dual pan-class I PI3K and mTOR kinase inhibitor and is currently in clinical trials against solid tumors [6]. Additionally, the diverse mechanisms proposed for its clinical efficacy including induction of apoptosis, autophagy, impair DNA repair mechanisms, increase of radio sensitivity and cell cycle arrest. The anticancer activity of limonene is modest when used alone,

*Correspondence: cdr@sugenlife.com

[1]Sugen Life Sciences Pvt Ltd.,, Tirupati, A P, India

Full list of author information is available at the end of the article.

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hence it is proposed to use in combination with BEZ in a bid to explore better efficacy compared to monotherapy.

Human colorectal cancer (CRC) cells COLO-320 (*wt*-K-ras and PI3K) and HCT-116 (*mt*-K-ras and PI3K) have been chosen to study the role of apoptosis in the anticancer activity of limonene and BEZ 235 combination using *in vitro* anticancer tests. In general, K-ras and PI3K mutations are used as valid biomarkers in the clinical management of CRC, and also a significant proportion of human tumors display coexisting mutations of these pathways leading to poor prognosis. In addition, CRCs are considered as the most resistant type because of their constant exposure to ingested food and toxins and thus CRC cells are considered as an ideal model for representing clinical cancer drug resistance. Therefore, testing the drug combination in selected CRC cells may explore the clinical utility of the drug combination even against resistant cancer phenotypes.

Material and Methods

Cell lines and drugs

COLO-320 and HCT-116 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).

DAPI staining for fluorescent microscopic examination of apoptotic cells

Cells (1×10^5/well) in 6 well culture plates were treated with limonene and BEZ alone and with their combinations for 48h. Cells were harvested, washed 3x in PBS and fixed in 3.7% formaldehyde in PBS for 10min at room temperature. The fixative was removed and the cells were washed 3x, 5min each in PBS and incubated with 0.1% Triton X-100 (Sigma) for 5min, to make the cells permeable. The cells were washed 3x in PBS and incubated with DAPI (Sigma) staining solution (stock=1mg/ml in dH_2O -diluted 1:1000 in PBS before use) for 10min at room temperature in the dark; washed 3x in PBS and mounted with cover slip and examined under fluorescent microscope with blue filter. The slides were screened for the quantification of cells showing features of apoptosis, and were expressed as % apoptotic cells [7].

Estimation of caspases-3 and -9 activities

The activities of caspases-3 and 9 were measured using colorimetric assay kit [8]. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA (caspase 3) and LEHD-*p*NA (caspase 9). The *p*NA light emission from the respective substrates can be quantified using a spectrophotometer. Cells (1×10^6) in 25cm^2 flask were treated with limonene, BEZ and also with their combinations for 48h. Cells were harvested and suspended in 50μl of chilled lysis buffer for 10min and centrifuged at 10000x g for 1min and the supernatant was transferred to a fresh tube for protein quantification. Approximately, 100μg protein was diluted in 50μl lysis buffer for each assay. Then to each sample, 50μl of 2X reaction buffer containing 10mM DTT and 5μl of 4mM DEVD-*p*NA (for caspase 3) and 4mM LEHD- *p*NA (for caspase 9) substrates were added and incubated at 37°C for 2h. The OD was measured at 400nm in a plate reader (BioTEK, synergy 4 multimode microplate reader). Increase in number of fold of caspase activity of test samples was determined by comparing the results with the untreated control.

Effect of limonene and BEZ combination on regulation of Bcl-2 family proteins

Cells (1×10^6) following treatments were washed with ice cold PBS, lysed with ice cold RIPA buffer containing protease inhibitor cocktail (RIPA + PI - 1ml+40μl) (Roche, Indianapolis, IN, USA) and centrifuged at 12000x g for 15min at 4ºC. Supernatants were collected and total protein quantification was performed with Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany). Equal amounts (40μg) of protein were fractionated by performing 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked for 1h at room temperature with fresh 5% non-fat dried milk in TBST (10mMTris, 150mMNaCl, 0.5% Tween-20; pH7.4) and incubated with primary antibodies (1:1000) from Cell Signaling Technology (Beverly, MA) overnight at 4ºC. The antibodies used were against; BAD, BAX, Bcl-2 and β-actin. The membranes were washed three times with TBST and incubated with goat anti-rabbit or anti mouse IgG conjugated secondary antibody (Cell Signaling Technology, 1:5000 dilutions in 5% non fat milk in TBST) for 1h at room temperature. Following washing with TBST buffer, the protein bands were developed using enhanced chemiluminescence kit (ECL, Bio-Rad) according to the manufacturer's instructions [9].

Cell cycle analysis by Flow cytometry

The cell cycle analysis was performed to evaluate the distribution of cells in different phases. Asynchronized cells were

seeded (1×10^6) in 25cm^2 flask. Following after overnight incubation, the cells were treated with limonene, BEZ alone and also with combinations for 48h. The cells were harvested, washed with cold PBS and fixed in ice-cold 70% ethanol and kept at -20ºC for 24 –48h. Before the analysis, the cells were washed with cold PBS and then suspended in PBS containing DNAse free-RNAse A (420μg/ml, Bio-medicals, LLC, France) at 37ºC for 30min and stained with propidium iodide (1μg/ml, BD Pharmingen^{TM}). The samples were analyzed by flow cytometry using BD LSRFortessa^{TM} cell analyzer instrument (BD Bioscience, San Jose, CA, USA) and the results were analyzed using BD FACSDivasoftware (BD Biosciences, San Jose, CA, USA) to determine the percentage distribution of cells in different phases for untreated and drug treated groups of each cell line [10].

Statistical analysis

Statistical analysis was carried out using 'GraphPad Prism Software' version 5.0 (San Diego, USA). All the experiments were performed in triplicate. 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 & Discussion

Results

Limonene and BEZ induce apoptosis in colorectal cancer cells

Both the drugs exhibited significant increase in apoptosis compared to corresponding untreated control ($p< 0.05$). In COLO-320 cells the % apoptotic cells for limonene (1000 μM), BEZ (100 nM) and their combination treatment were 20%, 16.3% and 32.7% respectively. Similarly, the % apoptotic cells in HCT-116 cells treated with limonene (2000 μM), BEZ (100 nM) and their combination were 18%, 16.7% and 27% respectively. Drug combination was more effective than either agent alone (Fig 1). COLO-320 cells appear to be more sensitive to apoptosis than HCT-116 possibly because of wild type expressions of the PI3K and K-ras signaling pathways in the former cell line.

Effects on caspases-3 and -9 activities

The cells were treated and fold increase in caspase activities were estimated by comparing to those of untreated control (Fig 2). The activities of both the caspases were significantly increased ($p< 0.05$) by combination treatment, whereas, individually only limonene showed significant increase in the caspase-3 in both the cells.

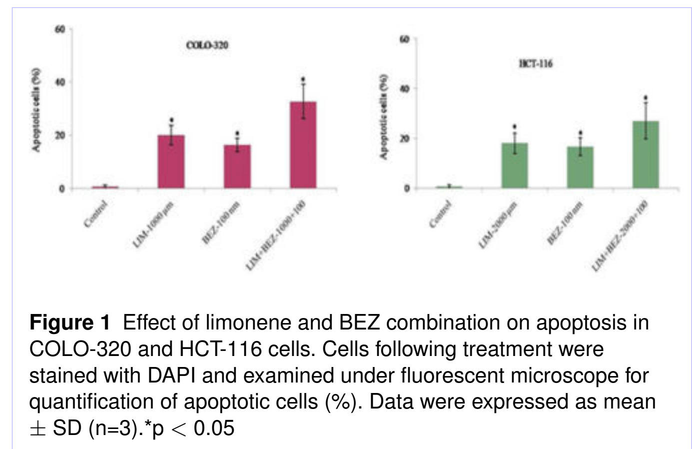

Figure 1 Effect of limonene and BEZ combination on apoptosis in COLO-320 and HCT-116 cells. Cells following treatment were stained with DAPI and examined under fluorescent microscope for quantification of apoptotic cells (%). Data were expressed as mean ± SD (n=3).*p < 0.05

In COLO-320 cells, treatment with limonene (1000 μM), BEZ (100nM) and their combination showed 1.5x, 1.4x and 1.7 fold increase in caspase-3 activity and 1.4x, 1.1x and 1.5 fold increase in caspase-9 activity respectively. Whereas, in HCT-116 cells, treatment with limonene (2000 μM), BEZ (100 nM) and their combination showed 1.5x, 1.3x and 1.6 fold increase in caspase-3 activity and 1.1x, 1.1x and 1.3 fold increase in caspase-9 activity respectively.

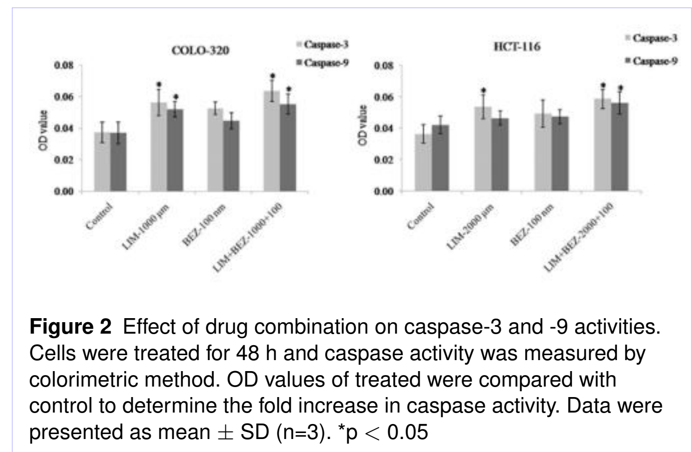

Figure 2 Effect of drug combination on caspase-3 and -9 activities. Cells were treated for 48 h and caspase activity was measured by colorimetric method. OD values of treated were compared with control to determine the fold increase in caspase activity. Data were presented as mean ± SD (n=3). *p < 0.05

Limonene and BEZinduce apoptosis by regulation of Bcl-2 family proteins

Western blot analysis was performed and the proteins were quantified to determine the combination effect on the expression of pro and anti-apoptotic proteins. Drug treatments exhibited significant alterations in the expression of proteins compared to untreated control (Fig 3). In COLO-320 cells, treatment with limonene (1000 μM), BEZ (100 nM) and their combination respectively resulted 1.4x, 1.5x and 1.7 fold increase in the expression of BAD and 3.5x, 4.2x and 4.7 fold increase in BAX with a concomitant decrease of 0.4x, 0.9x and 0.9 fold in the

expression of Bcl-2 ($p < 0.05$). Whereas, HCT-116 cells treated with limonene (2000 μM) did not significantly alter the pro- and anti-apoptotic proteins, but treatment with BEZ (100 nM) and combination showed an increase of 1.3x and 1.5 fold respectively in BAD with a concomitant decrease of Bcl-2 by 0.6 fold ($p < 0.05$). However, the expression of BAX was significantly increased (1.3x) following combination treatment without much responsive to the drugs individually.

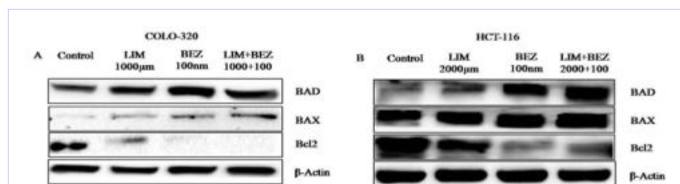

Figure 3 Western blot analysis showing the effect of drug treatment on expression of pro and anti-apoptotic proteins of Bcl-2 family in COLO-320 (A) and HCT-116 (B) cells. β-Actin was used as loading control.

Combination effect on distribution of cell cycle phases

In general, there is a high potential for cells in G_2 phase to enter into the mitosis for proliferation and eventually become tumorigenic. However, when the combination effects on cell cycle phases were analyzed, it clearly showed a significant increase in G1 phase cells compared to respective control, indicating that G1 phase might be the target for both the drugs (Fig 4). Consequently, there were very few cells in G_2 phase that can enter in mitosis phase, indicating the profound antiproliferative effects of the combination.

Discussion

CRC is the third most common cancer with significant mortality and morbidity. As they develop due to multiple genetic alterations or abnormalities, it is seldom possible to treat with monotherapy.Therefore, current chemotherapy practices employ combination of anticancer agents to obtain clinical efficacy. Induction of apoptosis in cancer cells is the most effective approach for clinical regression of tumors. Therefore, we designed the study to determine the role of apoptosis in the anticancer activity of limonene and BEZ combination in the CRC cells selected. In earlier studies we demonstrated the synergistic interaction of limonene and BEZ combination effects on inhibition of cell viability, migration and clonogenicity in the same cancer cells [11].

DAPI is a cell permeable fluorescent DNA binding dye that enables microscopic detection of apoptotic cells based on features such as, cell shrinkage, nuclear condensation and fragmentation. COLO-320 cells (32.7%) were found to be highly sensitive to the combination induced apoptosis than HCT-116 (27%) possibly because of marked genetic variations. In leukemia, prostate cancer cells and human gastric cancer implanted in nude mice also, limonene exerted anticancer activity that was mediated through induction of apoptosis [12]. 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 the tested CRC cells.

Caspases are cysteine-aspartic acid protease family enzymes that are key players in the apoptotic mechanism. However, mutations in caspase genes are not as frequent as p53 mutations, emphasizing the critical role they play in apoptosis [13]. Therefore, we estimated the caspases activities in order to identify the underlying mechanism for increased apoptosis. Results show that, the combination was more efficacious for induction of caspase-3 activity than caspase-9 in both the cells, which is strongly corroborating with antiproliferative activity of the combination, as caspase-3 is an effector caspase. Because of its critical role in apoptosis, compounds have been developed capable of directly activating caspase-3 for use in cancer therapy [14, 15] or indirectly activate caspases by blocking endogenous caspase inhibitors [16].

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 [17]. 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 also causing a significant decline in Bcl-2. Further, 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 survival promoter AKT activity, therefore, alleviating the phosphorylation and inactivation of BAD by AKT [18]. 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 cells tested. As stated before COLO-320 cells were more sensitive to the induction of apoptosis possibly because of significant alterations in BAX (high increase) and Bcl-2 (high decrease) compared to HCT-116 cells.

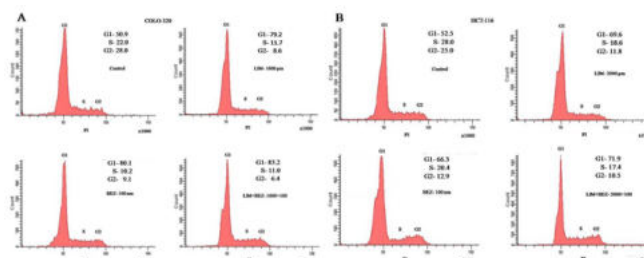

Figure 4 Flow cytometric analysis for combination effects of limonene and BEZ on distribution of cells in different phases of cell cycle in COLO-320 (A) and HCT-116(B) cells. The percentage of cells in each phase of cell cycle (G1, S and G2) is indicated.

Conclusions

The anticancer activity of limonene and BEZ combination in CRC cells tested was shown to be mediated through induction of apoptosis involving mitochondria mediated intrinsic death pathway as demonstrated by increased caspases activity and alterations of pro and anti-apoptotic proteins of Bcl-2 family in promoting apoptosis.

Conflict of Interest

The authors declare no conflict of interest.

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Author details

[1]Sugen Life Sciences Pvt Ltd.,, Tirupati, A P, India. [2]Manipal University, Manipal, Karnataka, India. [3]Palamur Biosciences Pvt Ltd.,, Mahabubnagar, Telangana, India. [4]S.V.S Medical College, Mahabubnagar, Telangana, India.

References

[1] Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. J Cell Mol Med . 2003;7(3):249–257.

[2] Kumar V, Abbas A, Fausto N, Aster J, Cotran R. Pathologic basis of disease. 8th ed. Philadelphia: Saunders Elsevier; 2010.

[3] Wong RS. Apoptosis in cancer: from pathogenesis to treatment. Wong Journal of Experimental & Clinical Cancer Research. 2011;30(87):1–14.

[4] The United States Code of the Federal Regulations, Title 21, Part 182.60;.

[5] Ji J, Zhang L, Wu YY, Zhu XY, SQ L, XZ S. Induction of apoptosis by d-limonene is mediated by a caspase-dependent mitochondrial death pathway in human leukemia cells. Leuk Lymphoma. 2006;47:2617–2624.

[6] Serra V, Markman B, Scaltriti M, JA P, Eichhorn, Valero V, et al. NVP-BEZ235, a Dual PI3K/mTOR Inhibitor, Prevents PI3K Signaling and Inhibits the Growth of Cancer Cells with Activating PI3K Mutations. Cancer Res. Cancer Res. 2008;68(19):8022–8030.

[7] Cold Spring Harb Protoc; 2011. Available from: 10.1101/pdb.prot5556.2011.

[8] Enzo Life Sciences Inc; 2012.

[9] Clarity™ Western ECL Substrate Instruction Manual. Bio-Rad Laboratories, Inc;.

[10] Protocol for Staining DNA with Propidium Iodide for Cell-Cycle Analysis Using the BD FACSArray™ Bioanalyzer;.

[11] RatnaReddy R, sekhar C, Reddy B, Ramamoorthy, Suresh R, Lakshmi Y, et al. Limonene and BEZ 235 inhibits growth of COLO-320 and HCT-11 colon cancer cells. International Journal of Drug Delivery. 2016;8:89–95.

[12] Lu XG, Zhan LB, Feng BA, Qu MY, Yu LH, Xie JH. Inhibition of growth and metastasis of human gastric cancer Implanted in nude mice by d-limonene. World J Gastroenterol. 2004;10(14):2140–2144.

[13] RMcIlwain D, Berger T, Mak TW. Caspase Functions in Cell Death and Disease. Cold Spring HarbPerspect Biol . 2013;5:a008656.

[14] Zhang HZ, Kasibhatla S, Wang Y, Herich J, Guastella J, Tseng B, et al. Characterization and SAR of gambogic acid as a potent apoptosis Inducer by a HTS assay. Bioorg Med Chem. 2004;12:309–331.

[15] Putt KS, Chen GW, Pearson JM, Sandhorst JS, Hoagland M, Kwon J, et al. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. . Nat Chem Biol. 2006;2:543–550.

[16] Vogler M, Dinsdale D, Dyer MJ, Cohen GM. Bcl-2 inhibitors: Small Molecules with a big impact on cancer therapy. Cell Death Differ. 2009;16:360–367.

[17] Hockenbery D, Nunez G, Milliman C, Schreiber RD, SKorsmeyerJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 1990;348:334.

[18] Datta D, Tao H, S M, Fu H, Gotoh Y, Greenberg M. AKT phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91:231.