Molecular Biology Reports
https://doi.org/10.1007/s11033-020-05327-4

ORIGINAL ARTICLE

The synergistic anti-proliferative effect of the combination of diosmin and BEZ-235 (dactolisib) on the HCT-116 colorectal cancer cell line occurs through inhibition of the PI3K/Akt/mTOR/NF-κB axis

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Received: 4 October 2019 / Accepted: 13 February 2020

Abstract
One of the most lethal malignancies worldwide is colorectal cancer (CRC). Alterations in various signalling pathways, including PI3K-mTOR and NF-κB, have been reported in CRC with subsequent dysregulation of proliferation, apoptosis, angiogenesis and, questionably, autophagy processes. BEZ-235 (dactolisib) is a dual PI3K-mTOR inhibitor with potent anti-tumour activity. However, the observed toxicity of BEZ-235 necessitated the termination of its clinical trials. Hence, we aimed to evaluate the potential long-lasting anti-carcinogenic effects of adding diosmin (DIO, a natural NF-κB inhibitor) to BEZ-235 in HCT-116 CRC cells. The median inhibitory concentrations (IC50s) of BEZ-235 and/or DIO were evaluated in the HCT-116 CRC cell line. Caspase-3 activity was assessed colorimetrically, and p-Akt, NF-κB, CD1, VEGF and LC3B levels were assessed by ELISA. Additionally, *LC3-II* and *P62* gene expression were assessed using qRT-PCR. The observed CIs (combination indices) and DRIs (dose reduction indices) confirmed the synergistic effect of DIO and BEZ-235. Co-administration of both drugs either in combination-1 (1 μM for BEZ-235, 250 μM for DIO) or in combination-2 (0.51 μM for BEZ-235 + 101.99 μM for DIO) inhibited the PI3K/Akt/mTOR/NF-κB axis, leading to the induction of apoptosis (via active caspase-3), and the inhibition of proliferation marker (CD1), angiogenesis marker (VEGF), autophagy protein (LC3B) and altered effects on *LC3-II and P62* gene expression. Our results reveal the synergistic chemotherapeutic effects of DIO combined with BEZ-235 in the HCT-116 CRC cell line and encourage future preclinical and clinical studies of this combination with reduced BEZ-235 concentrations to avoid its reported toxicity.

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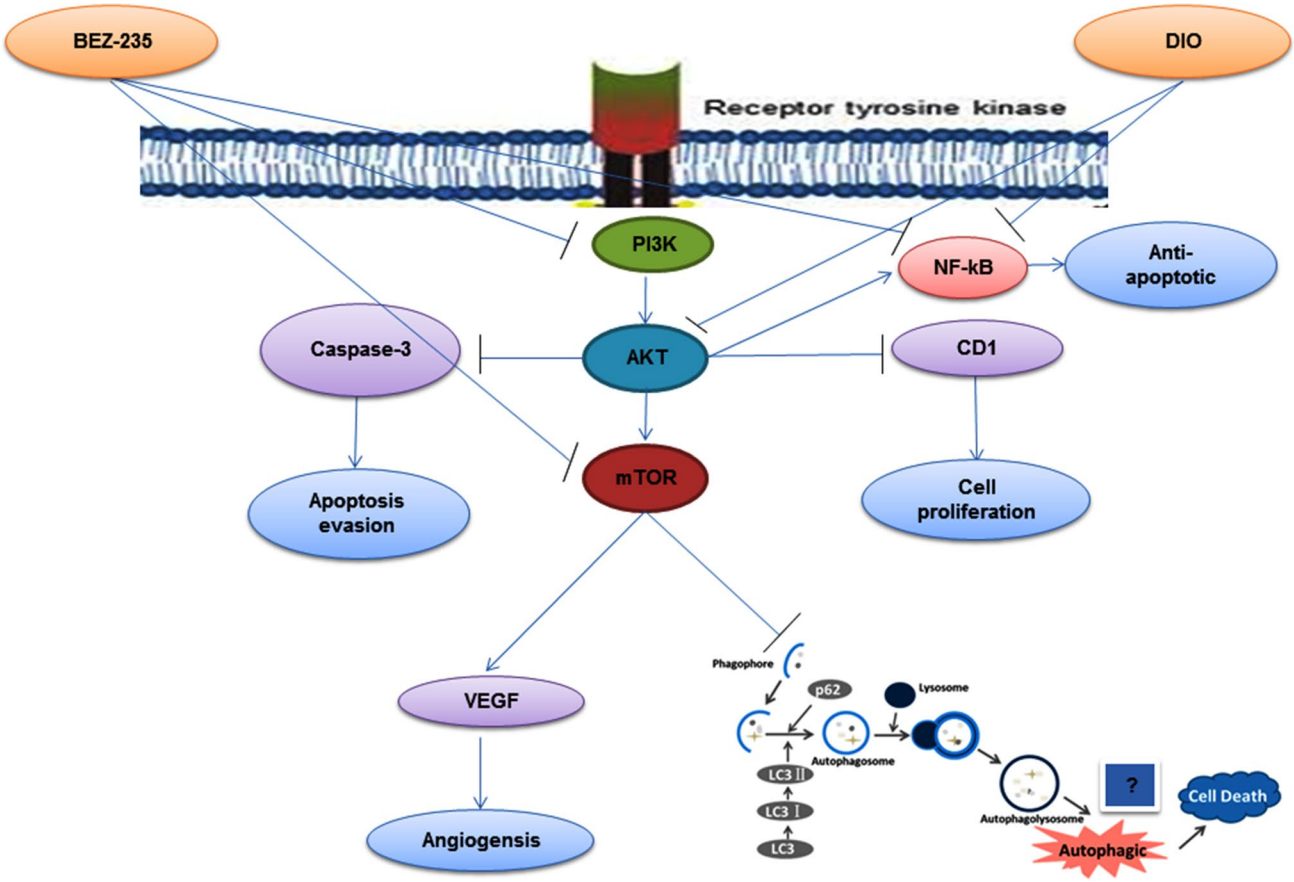
Published online: 22 February 2020

Graphic abstract



Keywords Colorectal cancer · BEZ-235 · Diosmin · PI3K · NF-κb

Abbreviations

CD1	Cyclin D1
CRC	Colorectal cancer
HIF1α	Hypoxia-inducible factor-1 alpha
LC3B	Human microtubule-associated proteins 1A/1B light chain 3 beta
LC3-II	Microtubule-associated protein 1 light chain 3
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-kappa B
p-Akt	Phospho-Akt
PI3K	Phosphatidylinositol-3-kinase
STAT-3	Signal transducer and activator of transcription-3
VEGF	Vascular endothelial growth factor

Introduction

Colorectal cancer (CRC) is an enormous global health problem. The main curative treatment for CRC is surgical resection followed by radiation and chemotherapy. However, metastasis and rapid progression remain the main obstacles that necessitate the development of new therapeutic strategies [1].

CRC is characterized by extensive dysregulation of various oncogenes, tumour suppressor genes [2] and signalling pathways that play a central role in the development and progression of CRC [3]. Sonic hedgehog (SHH) [4], Notch, wingless-type mouse mammary tumour virus (Wnt)/β-catenin, transforming growth factor-β/SMAD [2–4], p53 [2, 5], epidermal growth factor receptor (EGFR) [4, 6, 7], and its downstream targets phosphatidylinositol-3-kinase (PI3K) [4, 7, 8], nuclear factor-kappa B (NF-κB) [5, 9, 10], and vascular endothelial growth factor (VEGF) [5, 10–12] are the main signalling pathways involved in

the progression of CRC. Recently, alterations in autophagy have been shown to play a mysterious role in CRC [13].

PI3K/Akt/mTOR is the second most frequently dysregulated signalling network in human cancers [14]. PI3K is dysregulatedin almost 30% of human cancers [14]. The PI3K/Akt/mTOR signalling axis plays a key role in the proliferation, resistance to apoptosis, angiogenesis and metastasis that are critical for the development and progression of CRCs, and inhibition of the PI3K/Akt pathway decreases the growth, increases the apoptosis, increases the sensitivity to chemotherapy and decreases the metastasis of CRCs [15–17].

NF-κB plays an important role in many physiological processes, such as innate and adaptive immunity, cell proliferation, cell death, and inflammation [18]. It has become obvious that the aberrant regulation of NF-κB and the signalling pathways controlling its activity are implicated in the development and maintenance of cancer [19]. NF-kB is constitutively activated in 66% of CRC cell lines and 40% of human CRCs [20, 21], leading to resistance to chemotherapy and radiotherapy [20, 21].

Angiogenesis is the growth of new capillary blood vessels in the body. Tumours release angiogenic growth factors that stimulate blood vessel growth, providing oxygen and nutrients needed for tumour growth. VEGF is the predominant angiogenic growth factor [22]. VEGF expression is upregulated in the majority of human tumours and inversely associated with survival [23]. VEGF is expressed in approximately 50% of CRCs [24] and is associated with the metastasis and poor prognosis [25]. Furthermore, it has been indicated that VEGF is expressed in hypoxicHCT-116 CRC cells [26]. Thus, targeting VEGF is a rational approach to inhibit tumour growth and prolong survival [27].

Autophagy is a cellular degradation mechanism involving protein turnover as well as the recycling of surplus and dysfunctional organelles [28–30]. In cancer, autophagy has both anti- and pro-tumorigenic effects [31, 32]. Under basal conditions, autophagy exhibits cytoprotective activity by recycling impaired cellular organelles, eliminating ROS and preventing carcinogenic transformation [33, 34]. Once cancer progresses, autophagy is activated in tumour cells to meet the increased metabolic requirements essential for survival and rapid proliferation [34]. Thus, the inhibition of autophagy has been extensively shown to sensitize tumour cells to anticancer therapy [35]. Therefore, it is worth including autophagy as an additional molecular target in developing a targeted strategy for the enhanced management of cancer progression.

BEZ-235 (dactolisib) is an oral imidazoquinoline derivative, targeting both PI3K and mammalian target of rapamycin (mTOR) [36, 37]. BEZ-235 exerts inhibitory activity through binding to the ATP binding cleft of PI3K and mTOR kinase enzymes [36]. The efficacy of BEZ-235 has been proven in different preclinical investigations with multiple tumour types, including CRC [38–43]. The anti-carcinogenic activity of BEZ-235 is attributable to the inhibition of various signalling pathways, including the PI3K/Akt/mTOR [36], NF-κB [44, 45] and VEGF [46, 47] pathways. However, the clinical trials in various types of cancer were terminated due to toxicity and intolerability [48, 49]

Diosmin (DIO) is a naturally occurring flavonoid glycoside isolated from *Scrophularianodosa* [50]. The effects of DIO have been described in various venous diseases, including chronic venous insufficiency and haemorrhoids [51, 52]. The typical dosage of diosmin is 1000 mg daily in two divided doses. In the case of an acute haemorrhoidal attack, a loading dose of 3000 mg per day for the first 4 days is needed, followed by 1000 mg twice a day (bid) for 3 days [53]. In addition, DIO displays anti-carcinogenic activity in many types of cancer, including CRC [50, 54–59]. Furthermore, DIO is the most potentgenotoxic agent of three flavonoid glycosides tested in DU145 prostate cancer cells and the most effective phytochemical of 30 nutraceuticals tested in MCF-7 breast cancer cells, showing pro-apoptotic activity and chemo-preventive potential in a cancer cell-specific manner [35, 60]. DIO has been shown to exert its anti-tumour effect through the inhibition of multiple signalling pathways, including the PI3K-Akt [61], NF-κB [62] and VEGF [63] pathways.

The HCT-116 cell line was selected as a CRC model for several reasons. First, HCT-116 is a human colon cancer cell line that is commonly used to study cancer biology, and it is a growth factor-independent cell line that has been shown to be invasive and highly motile in in vitro studies [64, 65]. Second, Kirsten rat sarcoma viral oncogene homologue (KRAS) [66, 67] and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutant cells have increased Akt expression [66, 68] and mTOR activity [66], which are needed to investigate the PI3K/Akt/mTOR signalling pathway inhibitors used in our study. Additionally, HCT-116 cells show dysregulated NF-κB [69] and VEGF [26] activity, which enables the evaluation of the potential effects of the proposed drugs on these carcinogenic signalling pathways.

The co-targeting of more than one molecular carcinogenic pathway could overcome the complexity and interplay of these signalling pathways and offers increased efficacy and decreased toxicity [70]. Based on this hypothesis and the inhibitory activities of both BEZ-235 and DIO on the PI3K/Akt/mTOR, NF-κB and VEGF pathways in CRC, our aim was to investigate the possible long-lasting anti-carcinogenic effects and pathways of DIO and/or BEZ-235 in a CRC cell line (HCT-116) using 72 h as our time point and to determine whether the combination of DIO and BEZ-235 displays beneficial anti-tumour efficacy over the individual

compounds alone through potential signalling (PI3K/Akt/mTOR, NF-κB and VEGF) pathways interactions.

Materials and methods

Chemicals

BEZ-235 (cat. No. # S1009) and diosmin (cat. No. # S2292) were purchased from Selleckchem (Houston, TX, USA). Foetal bovine serum (FBS), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (St. Louis, USA). Trypsin, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and penicillin/streptomycin antibiotic mixtures were purchased from Lonza® (Basel, Switzerland). Ethanol was purchased from El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt).

Experimental cell lines

Two types of cell lines were used in the study: the HCT-116 CRC cell line and normal fibroblast CCD-18Co cells. Both cell lines were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and then maintained in an incubator with 5% carbon dioxide and humidified air at 37 °C.

Stock solutions of either BEZ-235 or diosmin were dissolved in DMSO (1%) and diluted in DMEM to reach the specified concentration used in the study. DMEM containing the equivalent amount of DMSO used in the other treatment groups (1%) was used as a control.

Cell viability assessment

The effect of BEZ-235 and/or DIO on cell viability was evaluated by MTT assay as described by Van Meerloo et al. [71]. Cells were seeded in 96-well plates (4000 cells/well) [72, 73] and maintained overnight at 37 °C. Old media was aspirated, and 100 µl of treatment media (drugs dissolved in DMEM to give final culture concentration) containing BEZ-235 concentrations ranging from 0.12 to 4 µM [42] and/or DIO concentrations ranging from 25 to 800 µM [50, 56] were added to all wells and incubated for an additional 72 h. The cell viability was assessed for the monotherapies and combination therapy after 24 h, 48 h and 72 h. The 72 h time point [43, 50, 74] showed the maximum cell viability inhibition compared to the viability of the control untreated group; thus, we chose this time point for further investigation of the proposed signalling pathways. Finally, cells were incubated with 20 µl MTT reagent (5 mg/ml) for 4 h, and the resulting formazan crystals were dissolved in 150 µl of DMSO. The absorbance was recorded using a Bio-Rad microplate reader at 590 nm. All treatment experiments were performed in triplicate. Cell viability was expressed as a percentage relative to that in the control wells. CompuSyn software (CompuSyn, Inc., version 1) was utilized to determine the median inhibitory concentrations (IC50) values [75].

Calculation of the selectivity index (SI)

The cell viability inhibition of the individual drugs alone and their combination is shown as the SI, which was calculated as the ratio of the IC50 values of BEZ-235, DIO and their combination (using both drugs at their IC50 levels) on normal fibroblasts (CCD-18Co) to the IC50 values of the same treatment regimens on HCT-116 cancer cells. The higher the SI value was, the greater the cancer cell specificity and selectivity of the probed drugs and their combination [76].

Analysis of the anti-proliferative effect of the drug combination

The hypothesized anti-tumour interaction between BEZ-235 and DIO on HCT-116 cells was evaluated using an MTT assay. Cells were incubated with BEZ-235 and/or DIO using the same concentration ranges used in the former study for 72 h, and the cytotoxicity was assessed. To quantify the interaction synergism or antagonism, the combination index (CI) was determined as described by *Chou 2010*, where $CI < 1$ indicates synergism, $= 1$ indicates additive action and > 1 indicates antagonistic effects. Moreover, the dose reduction index (DRI), expressed as the synergy of the combination of two drugs, was calculated as the fold-decrease in the dose of each drug independently related to their dose in combination using CompuSyn software [75].

Treatment of HCT-116 cells with drugs

Cells were seeded in 12 T-75 flasks (2×10^5 cells/flask) [72] Cells were seeded in 12 T-75 and allowed to adhere overnight. The next day, cells were segregated into groups and treated as follows: (i) vehicle-treated group (1% DMSO); (ii) BEZ-235-treated group; (iii) DIO-treated group; (iv) combination-1-treated group (using both drugs at their IC50 levels); (v) combination-2-treated group (at the synergistic level obtained from DRI calculation, 0.51 µM for BEZ-235 + 101.99 µM for DIO) and incubated for 72 h. Then, the cells were harvested and divided into aliquots. For each aliquot, the total protein concentration was determined using the method described by Bradford MM, 1976 [77]. Finally, the aliquots were stored at − 80 °C until use in the subsequent investigations.

Preparation of cell lysates

Cell lysates were obtained using RIPA lysis and extraction buffer, purchased from Thermo Scientific, USA (#89900), containing 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. According to the manufacturer's instructions, 1 ml of RIPA buffer (containing a protease inhibitor cocktail) was added to HCT-116 cell pellets, which were subsequently shaken gently for 15 min on ice and then centrifuged at 14,000×*g* for 15 min to pellet the cell debris. The supernatants were then obtained and stored at − 20 °C for further experiments.

Biomarker analysis using the sandwich ELISA technique

The following tumour biomarkers were evaluated in the HCT-116 cell lysates from different treatment groups using the sandwich ELISA technique, which offers a trustworthy quantitative method with high sensitivity and specificity [78]. The biomarkers included phospho-Akt (p-Akt [pS473] kit, DRG International, Inc., Massachusetts, USA), phospho-NF-κB (p-NF-κB p65 (S536) kit, Ray Biotech, Georgia, USA), CD1 (MBS724349 kit, MyBioSource, CA, USA), VEGF (measured in the supernatant rather than in the cell lysates; CSB-E11718h kit, CUSABIO, Maryland, USA) and MAP1LC3B (#MBS917498). All of the aforementioned markers were measured according to the manufacturer's protocol. The values are presented as the mean ± SEM of three separate experiments, each performed in triplicate. The total protein content was measured in each sample, and each measured parameter was expressed relevant to the total protein content in the same sample.

Caspase-3 activity assay

A colorimetric kit (# ab39401, Abcam) was utilized to detect the level of active caspase-3**.** Briefly, active caspase-3 in cell lysates hydrolyses the peptide substrate (Ac-DEVD-pNA), releasing a p-nitroaniline moiety. The cleaved p-nitroaniline concentration was calculated from a calibration curve constructed from absorbance at 405 nm using a microtitre plate as described by Nicholson et al. [79]. Data were expressed as the mean ± SEM of three separate experiments, each performed in triplicate.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using an easy-RED™ Total RNA Extraction Kit (#170630, iNtRON Biotechnology, Inc.). Using equivalent concentrations of isolated RNA, the RNA was reverse-transcribed by a TOPscript™ cDNA Synthesis kit (#EZ005S, Enzynomics, Korea); both were performed according to the manufacturer's protocols. The expression of the *LC3-II* and *p62* gene was measured by qRT-PCR (Dtlite Real-Time system) using a RealMOD™ Green Real-time PCR master mix kit, iNtRON Biotechnology, Inc., using primer pairs with the following sequences (Sigma-Aldrich, St. Louis, MD, USA): *LC3-II*, 5′-GAT GTC CGA CTT ATT CGA GAG C-3′/5′-TTG AGC TGT AAG CGC CTT CTA-3′, *P62,*5′-GGG GAC TTG GTT GCC TTT T-3′/5′-CAG CCA TCG CAG ATC ACA TT-3′and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACC ACA GTC CAT GCC ATC AC-3′/5′-TCC ACC ACC CTG TTC CTG TA-3′.The assessment of each specimen was carried out in triplicate. GAPDH was used as a reference gene, and the fold changes in *LC3-II* and *P62* gene expression were calculated as described by Livak et al. [80].

Statistical analysis

Data were expressed as the mean ± SEM. A one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test was used to analyse multiple comparisons, and the differences were considered significant at $p < 0.05$. All statistical analyses and graphical data presentations were performed using Graph Pad Prism® software package version 6 (GraphPad Software Inc., CA, USA)**.**

Results

IC50 of BEZ-235 and DIO in a CRC cell line (HCT-116)

The effect of BEZ-235 and/or DIO on the growth of HCT-116 cells is presented in Fig. 1. BEZ-235 exhibited a concentration-dependent cytotoxic effect, where exposure to the BEZ-235 concentration range (0.125–4 µM) inhibited cell viability with an IC50 of 1 ± 0.06 µM (Fig. 1a). Similarly, DIO at different concentrations (from 25 to 800 µM) produced potent growth inhibition with an IC50 of 250 ± 2.08 µM (Fig. 1b). Additionally, the combination of BEZ-235 and DIO in the same concentration ranges resulted in cell viability inhibition with a combined IC50 value of 102.43 ± 1.46 (0.51 µM for BEZ-235 + 101.99 µM for DIO; Fig. 1c).

Combination index (CI) and dose reduction index (DRI) of BEZ-235 and DIO

To examine the combined effects of BEZ-235 and DIO on HCT-116 CRC cells, synergy experiments were performed. These cells were treated experimentally with BEZ-235, diosmin, or combination of both drugs, and CompuSyn software was used to determine the type of drug interaction

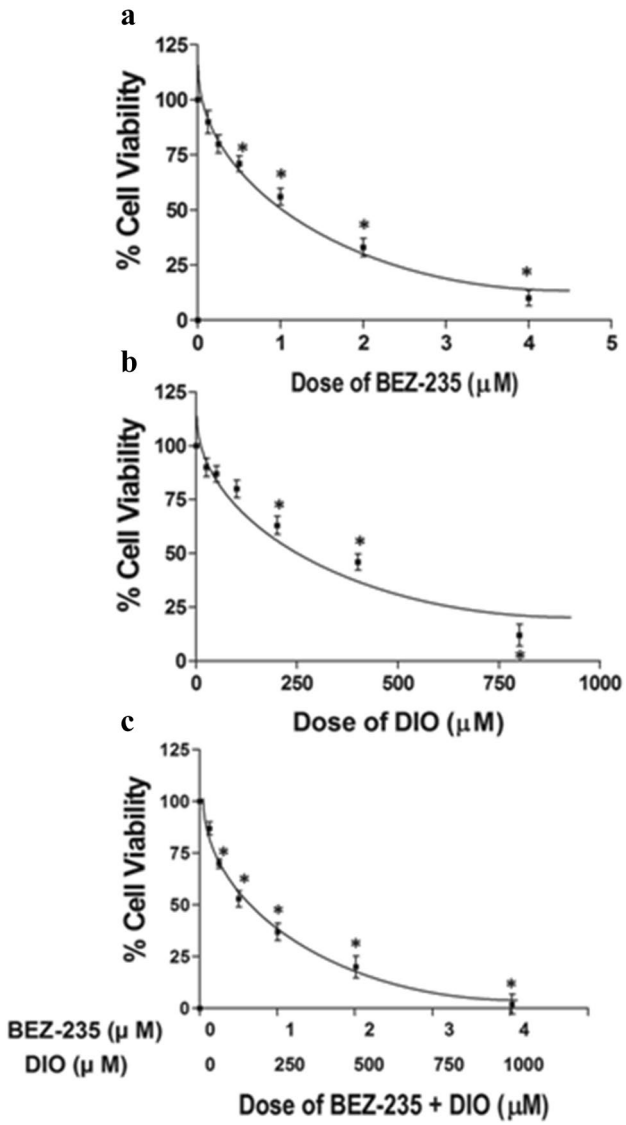


Fig. 1 The viability of HCT-116 cells treated dactolisib (BEZ-235, 0.125–4 μM) (**a**), diosmin (DIO, 25–800 μM) (**b**), and a combination of the two drugs (**c**). Cells were cultured and treated with increasing concentrations of BEZ-235 and/or DIO for 72 h in DMEM containing 10% FBS. An MTT assay was performed to assess the cell viability. Data points represent the mean ± SEM, each performed in triplicate. *p < 0.05 indicates a significant difference for BEZ-235 and/or DIO vs. the corresponding control group

between the agents. Table 1 presents the CIs detected using CompuSyn software after treatment of HCT-116 CRC cells with different combinations of the two agents. The DIO and BEZ-235 CI values at ED50, ED75, ED90, and ED95 were 0.97 ± 0.01, 0.79 ± 0.01, 0.65 ± 0.01 and 0.57 ± 0.01, respectively; these results demonstrate a CI value < 1, revealing a synergistic relationship between the two drugs at all levels in the HCT-116 CRC cell line (Table 1). Additionally, Table 1 shows that at ED50 (50% inhibition achieved by the combination), the concentration of BEZ-235 alone was 0.91 μM, and the concentration of DIO alone was 247.15 μM; however, the concentration of each drug in the combination at ED50 was 0.51 and 101.92, respectively. The relevant DRI values were > 1 for BEZ-235 and DIO individually, at 1.79 and 2.42, respectively, when used in combination. These observations support the hypothesis that adding DIO to BEZ-235 reduces its toxicity.

Selectivity index (SI) results

The relative selectivity indices of BEZ-235 and DIO individually and in combination in normal fibroblast cells and HCT-116 CRC cells are shown in Table 2. The SI analysis revealed that the IC50 of BEZ-235 in normal fibroblast cells was 7.14-fold greater than that in HCT-116 cells, and the IC50 of DIO in normal fibroblasts was 4.52-fold greater than that in HCT-116 cells. The combination group showed a 7.04-fold greater IC50 in normal fibroblasts than HCT-116 cells. These results indicate the cancer cell specificity and enhanced safety profile of BEZ-235, DIO and the combination of BEZ-235 and DIO.

BEZ-235 and/or DIO treatment reduced the phosphorylated Akt (S473)

Figure 2a shows the effect of BEZ-235 and/or DIO on the PI3K/Akt/mTOR proliferation axis. The highest concentration of p-Akt (S473) was observed in the control group, whereas the administration of BEZ-235 and DIO reduced the concentration of p-Akt (S473) significantly, by 37.025% and 33.617%, respectively. Remarkably, the maximum inhibition of p-Akt was observed in the combination-1 and

Table 1 CIs (combination indices) and DRIs (dose reduction indices) obtained using CompuSyn software to analyse HCT-116 colorectal cell viability inhibition resulting from treatment with the combination of BEZ-235 (0.125–4 μM) and diosmin (25–800 μM) for 72 h

Effective dose (ED) of cellular viability inhibition	CI value	Concentration of each drug alone (μM)		Concentration of each drug in combination(μM)		DRI DIO	DRI BEZ-235
		Concentration DIO (μM)	Concentration BEZ-235 (μM)	Concentration DIO (μM)	Concentration BEZ-235 (μM)		
10	1.45 ± 0.01	0.14	36.79	0.12	23.49	1.23	1.57
50	0.97 ± 0.01	0.91	247.15	0.51	101.92	1.79	2.42
90	0.65 ± 0.01	5.76	1660.17	2.21	442.29	2.60	3.75

Table 2 SI (selectivity index)analysis calculated by comparing the IC50 of BEZ-235, DIO and the combination of BEZ-235 + DIO in normal fibroblast cells against their IC50 values in HCT-116 cells using CompuSyn software

	IC50 in HCT-116 (μM)	IC50 in normal fibroblast cells (μM)	Selectivity index (SI)
BEZ-235	1 ± 0.06	7.14 ± 0.14	7.14
DIO	250 ± 2.08	1130.67 ± 8.95	4.52
Combination	102.43 ± 1.46	720.82 ± 5.87	7.04

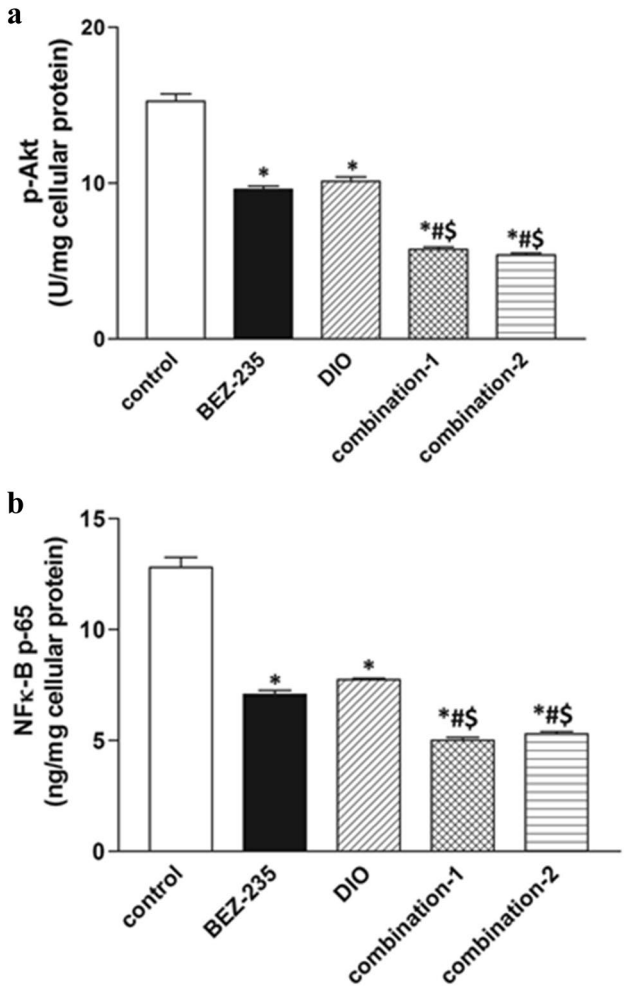


Fig. 2 Effects of dactolisib (BEZ-235, 1 μM), diosmin (DIO, 250 μM), combination-1 (1 μM for BEZ-235 + 250 μM for DIO 250 μM) and combination-2 (0.51 μM for BEZ-235 + 101.99 μM for DIO) on the PI3K/Akt/mTOR/NF-κB axis in HCT-116 cells. Cells were treated with BEZ-235 and/or DIO for 72 h. The levels of p-Akt (**a**) and NF-κB p65 (**b**) were measured using different ELISAs, as described in the "Materials and methods" section. Data are expressed as the mean ± SEM (standard error of mean) of three samples each performed in triplicate. Statistically significant differences between groups are designated as *$p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. the BEZ-235 group and $^{\$}p < 0.05$ vs. the DIO group

combination 2—treated group with 62.255% and 64.681% inhibition respectively.

BEZ-235 and/or DIO treatment inhibited NF-κB p65 pathway phosphorylation

Figure 2b shows that compared to that in the control group, the NF-κB p65 content was reduced by 44.687%, 39.532%, 58.672% and 60.375% in the BEZ-235-treated group, DIO-treated group, combination-2 group and combination-1 group, respectively.

BEZ-235 and/or DIO treatment downregulated the expression of the proliferation marker cyclin D1 (CD1)

The different experimental treatments affected the expression of the proliferation marker CD1 to different extents. The highest concentration of CD1 was found in the untreated group at 14.29 ± 0.60 ng/ml; the CD1 concentration in the BEZ-235, DIO, combination-2 and combination-1 groups was 7.99 ± 01.60, 9.44 ± 0.170, 6.53 ± 0.19 and 6.010 ± 0.220 ng/ml, respectively (Fig. 3a).

BEZ-235 and/or DIO treatment induced apoptosis and caspase-3 activity

Caspase-3 activity was measured to investigate apoptosis. The lowest level of apoptosis was found in the control group, as evidenced by the lowest level of the apoptotic marker active caspase-3, whereas the inhibition of apoptosis was reduced in all other treatment groups, with 1.889-, 1.579-, 2.482- and 2.571-fold increases in active caspase-3 in the BEZ-235, DIO, combination-1 and combination-2 groups, respectively (Fig. 3b).

BEZ-235 and/or DIO treatment downregulated the expression of the angiogenic factor VEGF

As shown in Fig. 3c, the expression of the angiogenesis factor VEGF was the highest in the untreated group compared to the other treatment groups. However, the treatment with BEZ-235, DIO, or both drugs either incombination-1 or combination-2, reduced VEGF expression to different extents. Relative to that in the control group, the protein expression of VEGF was inhibited by 56.908% in the BEZ-235-treated group and 47.401% in the DIO-treated group, and the most significant inhibition, 63.133% and 68.81%, was found in the BEZ-235 and DIO co-administered combination-2 and combination-1 group respectively.

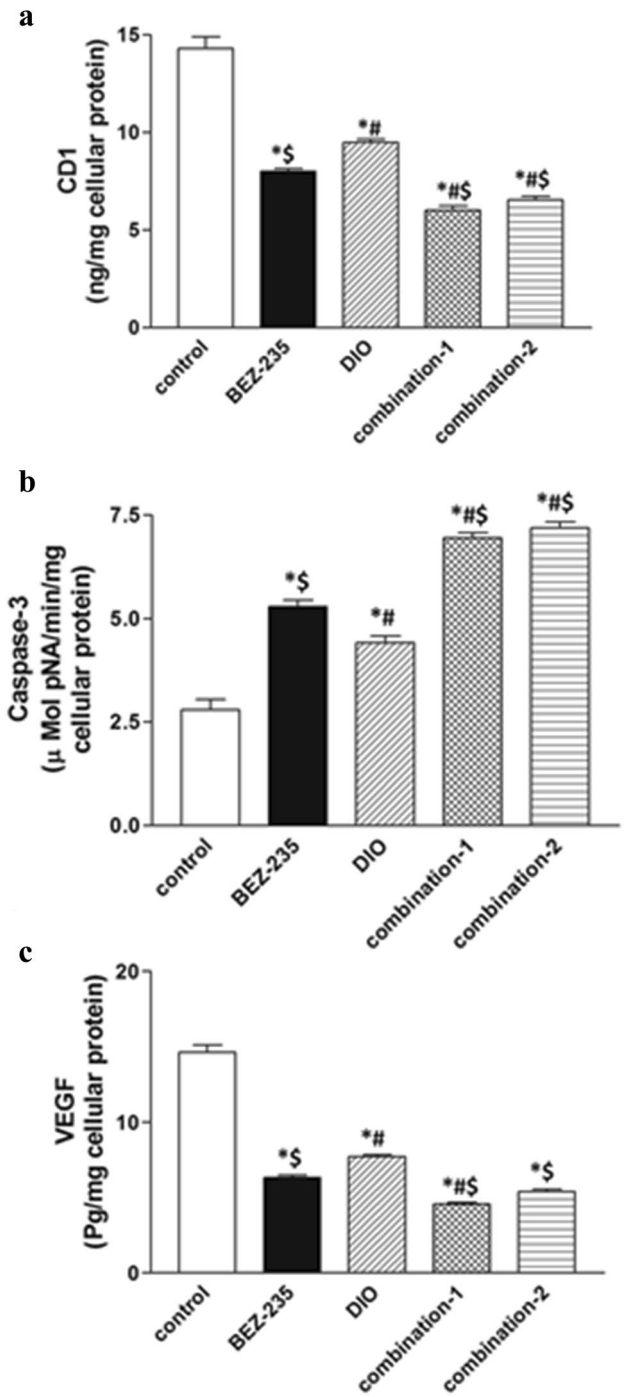


Fig. 3 Effects of BEZ-235 (1 μM), DIO (250 μM), combination-1 (1 μM for BEZ-235 + 250 μM for DIO 250 μM) and combination-2 (0.51 μM for BEZ-235 + 101.99 μM for DIO) on markers of proliferation, apoptosis and angiogenesis in HCT-116 cells. The levels of tumour markers of proliferation (Cyclin D1; CD1) (**a**), apoptosis (active caspase-3) (**b**) or angiogenesis (Vascular endothelial growth factor; VEGF) (**c**) were measured using an ELISA or colorimetrically, as described in the "Materials and methods" section. Data are expressed as the mean ± SEM (standard error of mean) of three samples each performed in triplicate. Statistically significant differences between groups are designated as $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. the BEZ-235 group and $^{\$}p < 0.05$ vs. the DIO group

Effect of BEZ-235 and/or DIO treatment on the level of the autophagy protein LC3B (Human microtubule-associated proteins 1A/1B light chain 3 beta), *LC3-II* (Microtubule-associated protein 1 light chain 3) gene expression and P62 (Sequestosome 1, SQSTM1) gene expression

First, the effect of BEZ-235, DIO and their combinations (combination-1 and combination-2) on autophagy was investigated through the expression of the autophagosome markers, LC3B protein and LC3-II gene expression, in HCT-116 cells (Fig. 4a, b). The expression of LC3B was markedly reduced in the BEZ-235- and DIO-treated groups by 49.033% and 43.292%, respectively. Interestingly, the maximum reduction in the LC3B protein level was found in the co-treatment, combination-2 and combination-1, groups where it was 62.277% and 65.495% respectively lower than that in the control group (Fig. 4a). *LC3-II* gene expression was lowest in both the BEZ-235 and combination-1 treatment groups compared to the untreated group, reaching 92.51232% and 97.8627%inhibition, respectively. However, DIO treatment and combination-2 induced a 2.5- and 9.5-fold increase in *LC3-II* gene expression respectively, greater than that in the control group (Fig. 4b).

Second, to confirm the effect of the different treatment regimens on autophagy, *P62* (a marker for autophagic flux) gene expression levels were assessed (Fig. 4c). The results revealed that the expression of *P62* gene was reduced in the BEZ-235- and combination-1-treated groups by 50% and 84.088% respectively lower than that in the control group. Nevertheless, DIO-treated group induced 2.3-fold increase in P62 gene expression greater than that in the control group, while combination-2 treatment showed non-significant results compared to control group (Fig. 4c).

Discussion

The multiplicity of overlapping signalling pathways involved in cancer has emerged as a potential benefit of combinatorial strategies in cancer management. The results of the present study clearly highlight the beneficial effects of the co-targeted inhibition of the PI3K/mTOR cascade and NF-κB in the human CRC HCT-116 cell line using BEZ-235 and DIO. The synergistic effect of DIO on the anti-proliferative capacity of BEZ-235 was confirmed at two levels: first, at the cellular level, as indicated by the cell viability assay and calculated CIs and DRIs. Second, at the molecular level, as indicated by the marked inhibition of the PI3K/Akt/mTOR/NF-κB axis and downregulated expression of the CD1, VEGF, and LC3B proteins and altered expression of *LC3-II and P62* genes together with restored caspase-3 activity in

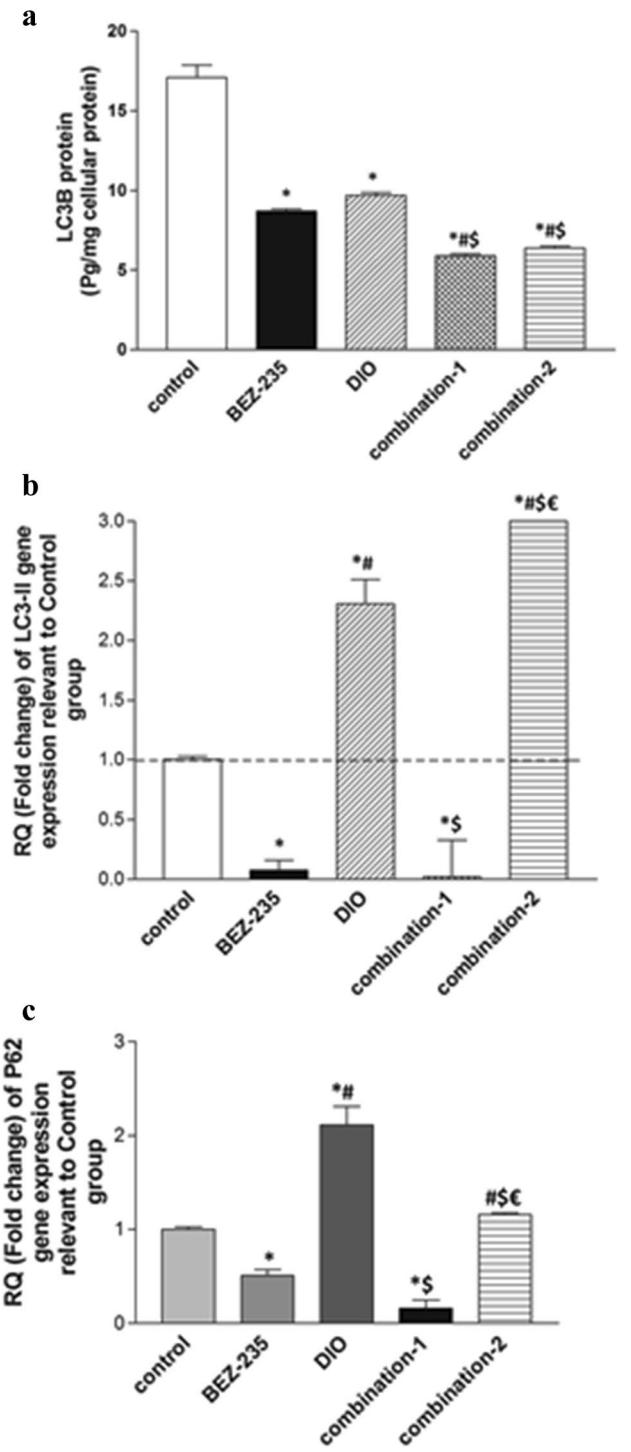


Fig. 4 Effects of dactolisib (BEZ-235, 1 μM), diosmin (DIO, 250 μM), combination-1 (1 μM for BEZ-235 + 250 μM for DIO 250 μM) and combination-2 (0.51 μM for BEZ-235 + 101.99 μM for DIO) on autophagy in HCT-116 cells. The levels of the autophagy protein LC3B (Human microtubule-associated proteins 1A/1B light chain 3 beta) (**a**), measured using an ELISA, the fold change (RQ) in LC3-II (Microtubule-associated protein 1 light chain 3) gene expression in each treated group relative to the control untreated group (**b**) and the fold change (RQ) in P62 (Sequestosome 1, SQSTM1) gene expression in each treated group relative to the control untreated group (**c**), measured using qRT-PCR. Data are expressed as the mean ± SEM (standard error of mean) of three samples each performed in triplicate. Statistically significant differences between groups are designated as $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. the BEZ-235 group, $^{\$}p < 0.05$ vs. the DIO group and $^{€}p < 0.05$ vs. the combination-1 group

cells treated with the drug combination compared to cells treated with either drug alone.

The inhibition of the PI3K/Akt/mTOR/NF-κB axis, the keystone of the current study, has emerged as an enhanced approach for cancer management. The PI3K/Akt/mTOR/NF-κB axis is involved in different important aspects of tumorigenesis, including cell proliferation, angiogenesis and invasiveness [81]. Additionally, the hyper-regulation of this axis has been identified in different types of cancer [82, 83].

In the current study, the control group demonstrated increased levels of p-Akt (S473), NF-κB, CD1, VEGF, LC3Band a decreased level of active caspase-3, which confirms that the PI3K/Akt/mTOR/NF-κB machinery is hyper-regulated in CRC, as reported previously in [82] and [21], and reflects an increase in proliferation, angiogenesis and resistance to apoptosis, as evidenced here and in Roy et al. [84]. Surprisingly, the elevated PI3K/Akt/mTOR/NF-κB activity increased the level of the autophagy protein LC3B, which is in contrast to a substantial amount of evidence showing that mTOR is a negative regulator of autophagy and that the downregulation of PI3K/Akt/mTOR signalling activates autophagy in CRC [40] and prostate cancer [85].

In this study, compared to the no treatment control, the dual inhibition of PI3K/mTOR by BEZ-235 treatment reduced the level of p-Akt (S473), leading to PI3K/Akt/mTOR/NF-κB cascade inhibition. This result is consistent with findings in glioblastoma, where BEZ-235 treatment, through the dual blockade of PI3K and mTOR complex 2, inhibits Akt phosphorylation (S473) [36]. Similarly, in human lung cancer [86] and in colon cancer stem cells [87], BEZ-235 treatment downregulates p-Akt expression. The observed inhibition of NF-κB in the current study could be attributed to the suppression of inhibitor of kappa B kinase (IKK), as shown by Dan et al. and Roman et al. [44, 45].

Our results showed that CD1 expression was reduced and caspase-3 activity restored in the BEZ-235-treated group compared to the control group, causing proliferation abrogation and apoptosis activation. This result is attributed to the inhibition of PI3K/Akt/mTOR signalling as depicted herein and in HCT15 cells, where it causes cell cycle arrest with a marginal effect on apoptosis [40], and in various types of cancer, including chronic myelogenous leukaemia [88] and breast cancer [46].

Herein, the demonstrated ability of BEZ-235 treatment to reduce the VEGF expression could be explained by the inhibition of the PI3K/Akt axis and its downstream target

hypoxia-inducible factor-1 alpha (HIF1α), leading to a blockage of the positive feedback loop of the VEGF pathway [89, 90]. This blockage results in decreased angiogenesis as evidenced here and in HER2-defined breast cancer via the downregulation of PI3K/mTOR/HIF1α signalling [46]. Similar findings have also been described in colonic tumours in vivo [47].

The role of autophagy in cancer is still a mystery and has many controversies [34]. In the current study, compared to the control group, the BEZ-235-treated group had the lowest LC3B protein, *LC3-II* and *p62* gene expression levels. The inhibition of *p62* gene expression reflects induction of autophagy, apoptosis and cell death. However, the inhibition of LC3B protein and *LC3-II* gene expression may be associated with the inability of turnover to keep pace with increased autophagosome formation [91]. The induction of autophagy shown in BEZ-235 treatment could be attributed to inhibition of mTOR, the negative regulator of autophagy [92, 93], which is parallel to many studies that consider the dual PI3K-mTOR inhibitor, BEZ-235, an autophagy inducer in diverse cancer cell lines, such as CRC [40], renal cell carcinoma [94] and hepatocellular carcinoma (HCC) cell lines [95], which limits the anti-tumour effects of BEZ235 and necessitates the further use of autophagy inhibitors, such as chloroquine (CQ), with BEZ-235. The current work is the first to evaluate the effect of BEZ-235 on autophagy after incubation for 72 h. However, we recommend further investigation to evaluate time-dependent effect of BEZ-235 on the expression of LC3B protein, *LC3-II* gene expression and autophagy process.

Flavonoids, including DIO, are considered promising candidates for cancer prevention and treatment because they possess anti-proliferation, growth inhibition and apoptosis induction activity in different types of cancer [50, 96].

In our study, DIO showed IC50 values of 247.15 μM and 101.92 μM in DIO-treated group and in combination-2-treated group respectively. Although, the IC50 values of DIO either alone or in combination-2 seems to behigher than the physiological C max of DIO and unachievable clinically, the obtained promising combinational results encourage further preclinical studies using DIO nano-vehicular delivery to overcome this problem. In the nano-vehicular delivery study, the IC50 of DIO reached 56.96 μM [97] which can be achieved clinically and in context with physiological Cmax of tested micronized DIO that reached 50.3 ± 22.6 μM [98] which nominates DIO nano-vehicular delivery as a future candidate for further preclinical studies in cancer research that overcome high IC50 values of free DIO.

In the present work, the p-Akt (S473) and NF-κB levels were decreased in the DIO-treated group compared to the control group. The latter reduction has been documented by Dung et al. who showed that protein phosphatase 2A (PP2A) mediates DIO-induced PI3K-Akt-mouse double minute-2 homologue (MDM2) signalling suppression in HA22T HCC cells and experimental tumour growth in a nude mouse xenograft model [61]. Additionally, Tahir et al. stated that the inhibition of oxidative stress, cell proliferation and inflammatory markers involving NF-κB may be another underlying mechanism by which DIO inhibits liver tumour formation [62].

Our data demonstrate that DIO treatment inhibits CD1 expression and maintains caspase-3 activity, causing proliferation inhibition, cell death and apoptosis. This result is explained by the downregulation of the PI3K/Akt/mTOR pathway, as proven here, and by Dung et al. [61]. Additional mechanisms could be involved in the anti-proliferative and pro-apoptotic activity of DIO. These mechanisms include ROS-mediated apoptotic induction, as shown in the androgen-independent DU145 prostate cancer cell line and A431 skin cancer cells [56, 99], signal transducer and activator of transcription-3 (STAT-3) signalling inhibition [63] and cytotoxic autophagy in breast cancer [60].

Similar to that of BEZ-235, the inhibitory action of DIO on the PI3K/Akt/mTOR pathway is also reflected in the reduction in VEGF expression demonstrated in our study. The latter effect could be mediated by DIO-induced inhibitory effects on HIF1α and STAT-3 expression, as demonstrated by Rajasekar et al. [63].

Surprisingly, our results show that the level of the autophagy protein LC3B was reduced, and the expression of the *LC3-II and P62* gene was increased in the DIO-treated group compared to the untreated control group. The reduced LC3B protein and increased *p62* gene expression levels are associated with autophagy inhibition, increased apoptosis and cell death, while, increased levels of *LC3-II* gene may be a reflex mechanism. In contrast to the previous results, DIO treatment at low micro-molar concentrations induces oxidative stress and DNA damage that promotes autophagy in breast cancer cells [60]. These results recommend further investigations to evaluate the effect of the DIO on *LC3-II* gene expression and whole autophagy process as well. Currently, most efforts to modulate autophagy in cancer cells are focused on autophagy inhibition because this may overcome any insensitivity to other chemotherapeutic drugs [100] and the blockage of autophagy rescues BEZ-235-induced growth inhibition in CRC [40] and other types of cancer [94, 95]; in the same context, our results are consistent with the latter approach, where the autophagy inhibition demonstrated by DIO could play at least in part a role in the synergistic interaction between DIO and BEZ-235.

To our knowledge, the current study is the first to demonstrate the effect of the BEZ-235/DIO combinations (combination-1 and combination-2) on CRC and other cancer types. Additionally, we are the first to investigate the synergetic inhibitory effect of the BEZ-235/DIO combinations on HCT-116 cell viability, which could be

attributed to the increased inhibition of p-Akt (S473) and NF-κB expression by the BEZ-235/DIO different combination treatments compared to each drug treatment alone. The latter effect is reflected in the carcinogenesis hallmarks demonstrated in the current study: inhibition of proliferation marker CD1, angiogenesis marker VEGF, autophagy protein LC3B, altered effects on *LC3-II* and *p62* gene expression and activation of the pro-apoptotic marker caspase-3.Combination-1 reduced LC3B, *LC3-II* and *p62* gene expression compared to control group. The previous reduction in *P62* gene expression levels reflects induction of autophagic flux, and the reduction in LC3B and *LC3-II* levels may be associated with the inability of turnover to keep pace with increased autophagosome formation [91]. Whereascombination-2 decreased LC3B protein that reflects inhibition in the autophagosome synthesis, increased *LC3-II* levels that may be a reflex mechanism as previously shown in DIO-treated group in addition to non-significant effect on *p62* gene expression, compared to control group. Further investigations are recommended to evaluate both dose- and time- dependent effects of BEZ-235 and DIO combinations on *LC3-II*, *p62* gene expression and autophagy process.

In conclusion, the pivotally increased inhibition of the PI3K/Akt/mTOR/NF-κB pathway induced by the co-administration of BEZ-235 with DIO indicates that this combination is a promising candidate for further preclinical and clinical investigation to enhance CRC management. Notably, adding DIO over BEZ-235 allows achieving therapeutic outcomes of BEZ-235 at lower levels with subsequent reduction of its reported toxicity.

Author contributions MWH: Main research idea, experimental design, work supervision, data analysis and presentation, manuscript preparation and revision. AIG: Experimental design, sharing research ideas and work supervision, data analysis and presentation, and manuscript preparation and revision. MAK: Sharing ideas and work supervision, and manuscript preparation. RKE: Experimental design, sharing ideas, performance of the experiments, data analysis and presentation, manuscript preparation.

Funding The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval The current research followed accepted principles of ethical and professional conduct according to approval reference number 1216PO3 issued by the Research Ethics Committee of the Faculty of Pharmacy, Damanhour University, regarding originality, risk control, and community service.

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