

BETULIN INDUCES CYTOTOXIC EFFECTS IN AN OSTEOSARCOMA CELL LINE

IOAN SMULTEA^{1,2}, ȘTEFANIA-IRINA DUMITREL^{1,2,3#}, JENEL MARIAN PĂTRAȘCU^{4*},
DANIELA SAȘCO^{1,2}, CASIANA BORU^{5,6}

¹Doctoral School, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041, Timișoara, Romania

²Research Centre for Pharmaco-Toxicological Evaluations, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041, Timișoara, Romania

³Department of Toxicology, Drug Industry, Management and Legislation, Faculty of Pharmacy, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041, Timișoara, Romania

⁴Department of Orthopedics-Traumatology, Faculty of Medicine, “Victor Babeș” University of Medicine and Pharmacy, 300041 Timișoara, Romania

⁵Department of Medicine, University of Medicine and Pharmacy “Vasile Goldis”, 310045 Arad, Romania

⁶Arad County Emergency Clinical Hospital, 310037 Arad, Romania

*corresponding author: jenel.patrascu@umft.ro

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Abstract

Although advances in multimodal chemotherapy and oncological surgery have improved outcomes in osteosarcoma (OS), prognosis remains poor for patients with metastatic or treatment-resistant disease. Natural compounds, including betulin (BET), a pentacyclic triterpenoid, have attracted interest for their anticancer properties. Based on these considerations, the main objective of this study was to evaluate the *in vitro* cytotoxicity of BET on the human OS cell line U-2 OS. Following 24 h of BET treatment (10 - 50 μ M), cell viability decreased in a dose-dependent manner relative to untreated controls ($p < 0.0001$). JC-1 staining showed reduced red/green fluorescence ratios, consistent with mitochondrial membrane depolarization ($p < 0.001$ and $p < 0.0001$). Microscopy revealed nuclear and mitochondrial morphological alterations, while AO/PI staining showed increased PI-positive cells at all BET concentrations. These findings indicate that BET reduces U-2 OS cell viability and is associated with mitochondrial and cell death-related morphological changes.

Rezumat

Deși introducerea chimioterapiei multimodale și a tehnicilor chirurgicale oncologice moderne a îmbunătățit semnificativ prognosticul pacienților cu osteosarcom, ratele de supraviețuire ale celor care dezvoltă metastaze sau prezintă rezistență la tratament rămân limitate. În acest context, compușii naturali au devenit un domeniu important de cercetare în oncologie. Betulina (BET), un triterpenoid pentaciclic, a atras atenția considerabilă datorită efectului său asupra celulelor canceroase. Pe baza acestor considerente, obiectivul principal al acestui studiu a fost evaluarea activității antitumorale a BET asupra liniei celulare U-2 OS de osteosarcom uman (OS) în ceea ce privește activitatea sa citotoxică, impactul asupra morfologiei mitocondriale și nucleare, modificările potențialului membranei mitocondriale și tipul de moarte celulară apoptoză/necroză.

Keywords: betulin, osteosarcoma, U-2 OS, cytotoxicity, apoptosis, mitochondrial membrane potential

Introduction

Osteosarcoma (OS) is the most frequent primary malignant bone tumor and occurs mainly in children, adolescents and young adults. The advent of multimodal chemotherapy and advanced oncological surgical techniques has dramatically improved the prognosis for patients with localized disease. However, survival rates for those who develop metastases or are resistant to therapy are still limited. Additionally, the toxicities of classical chemotherapy regimens and the absence of efficient targeted treatments underline the importance of finding new molecules with antitumor activity and a good safety profile (1).

Natural compounds have become an important research area within the discipline of oncology as they can be used to modulate multiple molecular pathways simultaneously involved in the development and progression of tumors. Among them, BET, a pentacyclic triterpenoid isolated mainly from the bark of *Betula* species, has been shown to have anti-tumor, anti-inflammatory, antioxidant and antimicrobial properties in many studies. This compound is of interest due to its ability to decrease cell proliferation, impair mitochondrial function and induce death of tumor cells, but with relatively low toxicity toward normal cells (2).

In recent years, international research on BET has increased, and its effects have been increasingly

explored in multiple cancer types (melanoma, breast, colorectal, lung, pancreatic, and ovarian cancers), particularly its ability to inhibit tumor proliferation through the PI3K/AKT/mTOR, AMPK, and MAPK signaling pathways. BET has also been shown to induce mitochondrial apoptosis by disturbing the Bcl-2/Bax balance, cytochrome c release and caspase activation. Depending on the tumor type, it can activate NOXA, inhibit NF- κ B and induce G2/M phase arrest. However, the number of studies dedicated to OS is limited, and the mechanisms of action of this compound on the viability and morphological changes of OS cells have not been fully elucidated yet. The literature on the use of this triterpenoid in OS at the national and regional levels is scarce, which justifies further exploration of its antitumor potential and contributes to the advancement of studies in the field of natural compounds with oncological applications (2, 3).

Based on these considerations, the main objective of this study was to evaluate the *in vitro* cytotoxicity of BET on the human OS cell line U-2 OS. To this end, we sought to determine the effect of the compound on cell viability and to investigate the morphological changes induced at the cellular, nuclear, and mitochondrial levels. In addition, changes in mitochondrial membrane potential and the characteristics of cell death were evaluated to obtain as complete a picture as possible of the biological effects exerted by BET on OS cells. To achieve these objectives, an *in vitro* experimental study was conducted using the U-2 OS cell line. Cytotoxic activity was assessed using a cell viability assay, and morphological changes were investigated by fluorescence microscopy using Hoechst 33342, MitoTracker™ Red CMXRos, JC-1, and acridine orange/propidium iodide (AO/PI) stains. The combination of these methods allowed for a complementary assessment of nuclear and mitochondrial changes, mitochondrial membrane potential, and the characteristics of cell death induced by the compound of interest.

Materials and Methods

Reagents and instruments

BET (purity $\geq$ 98%; Lot No. MKBX3894V; Cat. No. B9757-5G), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay kit, trypsin-EDTA solution, phosphate-buffered saline (PBS), 0.4% trypan blue solution, acridine orange hydrochloride solution (10 μ g/mL), and propidium iodide solution (10 μ g/mL) were obtained from Sigma-Aldrich, MerckKGaA (Darmstadt, Germany). JC-1 (Invitrogen, Thermo Fisher Scientific, USA; Cat. No. T3168) was prepared as a 1.5 mM stock solution. The cell culture medium, McCoy's 5A, as well as fetal bovine serum (FBS), were purchased from ATCC (American Type Culture

Collection, Łomianki, Poland). Paraformaldehyde 4% was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Dimethyl sulfoxide (DMSO) and the antibiotic solution (penicillin 100 U/mL - streptomycin 100 μ g/mL) were purchased from PanBiotech (Aidenbach, Germany). Hoechst 33342 and MitoTracker Red CMXRos were supplied by Thermo Fisher Scientific (Waltham, MA, USA). The Cytation 5, the Lionheart FX automated microscope, and the Gen5™ Microplate Data Collection and Analysis software (v3.14) were purchased from BioTek Instruments Inc. (Winooski, VT, USA).

Preparation of working solutions for in vitro experiment

BET was initially dissolved in DMSO to obtain a 10 mM stock solution. Subsequently, the solution was diluted in McCoy's 5A cell culture medium to achieve final concentrations of 10, 20, 30, 40, and 50 μ M. The maximum DMSO concentration in the final working solutions did not exceed 0.5%, the threshold considered safe for cells (4).

MTT viability assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to analyze the effect of BET on the viability of U-2 OS cells. Cells were counted using trypan blue solution and were then seeded in transparent 96-well plates at a density of 1×10^4 cells/well and incubated until they reached about 70% confluency. They were then treated with 10, 20, 30, 40, and 50 μ M for 24 h. After that, 10 μ L/well MTT reagent was added to each well. The plates were then incubated for 3 hours at 37 °C, 5% CO₂. After 3 h, 100 μ L MTT solubilizing solution was pipetted into each well and plates were incubated for another 30 min at room temperature. All steps with the MTT reagent and the solubilization solution were performed under light-protected conditions. Finally, the absorbance of each well was read at 570 nm and 630 nm in a Cytation 5 plate reader. Cell viability was calculated as a relative percentage of untreated control cells grown in the indicated culture medium. The MTT assay was performed using three independent biological replicates, with three technical replicates per experimental condition on each plate. Blank wells that contained only culture medium. The mean absorbance of the blank wells was subtracted from the absorbance values of the experimental wells.

Morphological analysis

The effects of BET on U-2 OS cell morphology were assessed 24 hours after treatment at concentrations of 10, 20, 30, 40, and 50 μ M. The cells were cultured in the same manner as for the viability assay. After the treatment period, cell morphology was examined and photographed at 20 $\times$ magnification using the Lionheart FX automated microscope and Gen5™ Microplate Data Collection and Analysis software (v. 3.14) (5).

Mitochondrial and nuclear analysis

To observe changes at the nuclear and mitochondrial levels, Hoechst 33342 and MitoTracker™ Red CMXRos were used, respectively. The cells were cultured as described previously and allowed to adhere under standard culture conditions. Once the desired confluence was reached, BET was applied at concentrations of 10, 30, and 50 μ M for 24 hours. Initially, MitoTracker™ Red CMXRos was dissolved in DMSO at a concentration of 1 mM. Afterwards, the stock solution was diluted in the cell culture medium to 300 nM. Following treatment, the culture medium was aspirated, and the cells were washed once with PBS. Subsequently, the staining solution was added to each well, and the cells were incubated for 30 min at room temperature in the dark. The cells were then washed twice with PBS to remove excess dye and fixed with 4% paraformaldehyde for 10 min at room temperature. Before fluorescence imaging, the cells were washed again with PBS before fluorescence imaging. After rinsing, Hoechst 33342 (stock concentration: 10 mg/mL in water) was diluted 1:2000 in PBS by adding 3 μ L of the stock solution to 6 mL of PBS, yielding a final working concentration of approximately 5 μ g/mL. After rinsing, the dye was left for 10 minutes at room temperature in the dark. The final step consisted of rinsing the plate three times with PBS. Finally, to take photographs to observe any mitochondrial and/or nuclear changes, the following step was performed: the cells were fixed by adding 100 μ L of 4% paraformaldehyde per well, and the plate was incubated for 10 minutes at room temperature (5).

Analysis of mitochondrial membrane potential using JC-1 staining

The cells were cultured according to the protocol described above, except that 96-well black plates were used for this experiment. Twenty-four hours after treating the cells with 10, 30, and 50 μ M of BET, they were washed with PBS to remove residual culture medium. Next, 100 μ L of JC-1 staining solution (5 μ M) was added to each well, and the cells were incubated for 45 minutes at 37 °C in 5% CO₂. After incubation, 100 μ L of PBS was pipetted into each well, and fluorescence was measured at 485/530 nm for JC-1 monomers and 540/590 nm for JC-1 aggregates on the Cytation 5. After measuring the fluorescence, the PBS was discarded from the plate, and the plate was then photographed using the Lionheart FX automated microscope and Gen5™ Microplate Data Collection and Analysis software (v. 3.14) (5).

Assessment of Cell Death by Double Staining with Acridine Orange and Propidium Iodide

Double staining with acridine orange and propidium iodide was used to identify apoptotic and necrotic morphological changes in U-2 OS cells. The cells were seeded as previously described. After treatment with 10, 30, and 50 μ M of BET was completed, 100

μ L of AO/PI solution was added to each well; this solution was prepared by mixing Acridine Orange and Propidium Iodide in a 1:1 ratio and diluted in the culture medium to a final concentration of 10%. Images were then acquired within a maximum of 30 minutes to minimize potential changes in fluorescence intensity and staining patterns associated with prolonged dye exposure and photobleaching, using a Lionheart FX automated microscope at 20 $\times$ magnification, with Gen5 Microplate Data Collection and Analysis software (5).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9.3.1; GraphPad Software, San Diego, CA, USA; www.graphpad.com). The normality of the data distribution was assessed using the Shapiro-Wilk test. Differences between the experimental groups and the control group were evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test for multiple comparisons. Values of $p < 0.05$ were considered statistically significant, with significance levels denoted as follows: *** $p < 0.001$; **** $p < 0.0001$. All experiments were performed in three independent biological experiments, with three technical replicates per experimental condition in each independent experiment. The values obtained from the three technical replicates were averaged within each independent experiment, and the results were presented as mean $\pm$ standard deviation (SD), calculated across the three independent biological experiments ($n = 3$). The results were expressed relative to the control group, set at 100%, consisting of untreated cells cultured in complete growth medium.

Results and Discussion

To date, numerous studies have been conducted demonstrating the anticancer effects of BET in various types of cancer (colorectal, breast, pancreatic, lung, etc.) (2). BET is known to have protective effects on bones due to its ability to reduce osteoclast activity and increase osteoblast activity; however, in the case of OS, data on the effects of this compound are still limited (6, 7). This lack of evidence regarding the efficacy of this compound in OS served as the basis for this study. The objective of the study was to investigate the *in vitro* effects of BET on the U-2 OS osteosarcoma cell line at concentrations of 10–50 μ M for 24 hours. To investigate the antitumor potential, its effects were analyzed on cell viability and on nuclear and mitochondrial morphology; to specifically determine how they affect mitochondria, mitochondrial membrane potential was assessed using JC-1 staining. The final stage of the analysis involved differentiating the type of cell death induced by the compound using acridine orange and propidium iodide staining. The U-2 OS cell line is an OS

model frequently used in toxicology to test the activity of active substances *in vitro*. The concentrations and duration of treatment were selected based on previous studies in the literature in which antitumor effects were observed (2, 8, 9).

MTT viability assay

The viability of U-2 OS cells was assessed 24 hours after treatment with BET at concentrations ranging from 10 to 50 μM (Figure 1) to determine its potential cytotoxic effect. BET produced a dose-dependent cytotoxic effect, reducing cell viability from 56% at 10 μM to 28% at 50 μM .

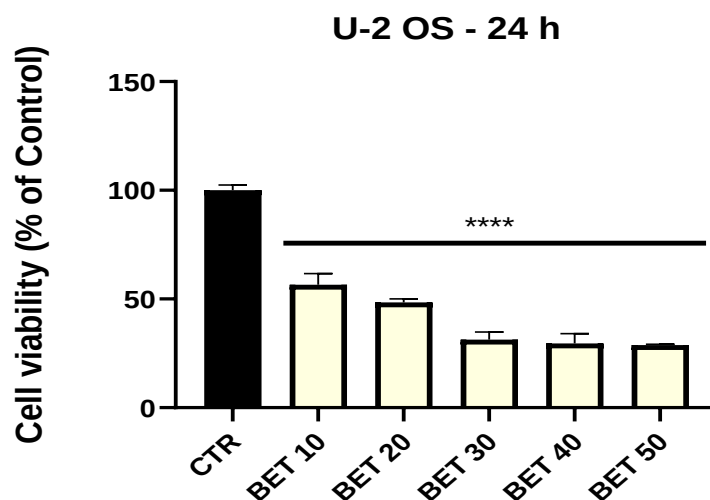


Figure 1.

Graphical representation of cell viability following 24-hour treatment with BET at concentrations of 10, 20, 30, 40, and 50 μM in the U-2 OS osteosarcoma cell line. The results are presented as percentages relative to the untreated control group. Values are expressed as mean $\pm$ standard deviation (SD) and were obtained from three independent experiments. Statistical differences between the treated groups and the control group were evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Statistical significance is indicated by **** $p < 0.0001$

The MTT method is commonly applied as a colorimetric assay to determine cell viability, proliferation, and cytotoxicity. The mechanistic principle of the MTT assay is founded on the ability of living cells to convert the yellow tetrazolium salt MTT into purple formazan crystals. This conversion from yellow to purple provides a visual indicator of cell viability (10, 11). Cell viability was reduced to less than 50% starting from 20 μM and further decreased with increasing concentrations, indicating a significant dose-dependent cytotoxic effect. BET has shown antitumor and bone-protective effects in other studies involving bone-associated malignancies (7, 8). BET has been shown to up-regulate the expression of osteogenic markers, including RUNX2, ALP, COL1A1 and osteopontin, and to increase osteoblast differentiation and mineralization of the extracellular matrix. BET has been shown to exert cytotoxic effects on the Saos-2 osteosarcoma cell line, significantly reducing cell viability at concentrations ranging from 1 to 25 μM (7). In other OS cell lines, BET showed significant toxic effects at 1 - 20 μM in MG-63 cells and 5 - 20 μM in HOS cells (8). Research on the antitumor potential of betulin and its synthetic derivatives has

revealed a significant reduction in cell viability, dependent on dose and duration of exposure, in a wide variety of cancer cell lines. In the case of lung and breast cancer, evaluation using the MTT assay after 48 hours showed IC_{50} values of 7.8 μM and 9.5 μM . Cytotoxic effects and dynamic changes in cell adhesion were confirmed using the ECIS method at concentrations ranging from 5 to 20 μM (12). In metastatic colorectal cancer treatment, betulin at concentrations of 0 - 8 μM for 24 - 72 hours inhibited cell viability (13). In addition, a recent study on pharyngeal carcinoma showed a reduction in cell viability to 55 - 60% for betulin at a dose of 50 μM after 24 hours of exposure (5). Moreover, chemical modification through the synthesis of 38 ester derivatives at the C-28 position improved cytotoxic potential in MTT and SRB assays at 72 hours on leukemia, prostate cancer, breast cancer, and lung cancer cell lines, with the most active derivatives achieving IC_{50} values of 3.94 - 7.04 μM by inducing caspase-3/7-mediated apoptosis (14).

The cytotoxic effect on U-2 OS cells was maximal at 30 μM , and higher concentrations resulted in only a limited further decrease in cell viability. This result

is in agreement with previous studies on OS cell lines which have demonstrated significant cytotoxic effects of the compound in the low micromolar range, usually up to 25 μM (7,8).

Morphological analysis

To evaluate the cytotoxicity of BET, U-2 OS cells were photographed 24 hours after treatment (Figure 2) to observe whether any morphological changes had occurred. At the lowest concentration, following BET application, a decrease in confluency was

observed, accompanied by cell rounding and shrinkage; at a concentration of 20 μM , these signs became somewhat more obvious. At concentrations ranging from 30 to 50 μM , it was already evident that most cells had lost their characteristic elongated shape; cell confluence was greatly reduced, and the cells were smaller, rounded, and had lost their adhesion to one another, detaching from the culture dish, with cellular debris also appearing.

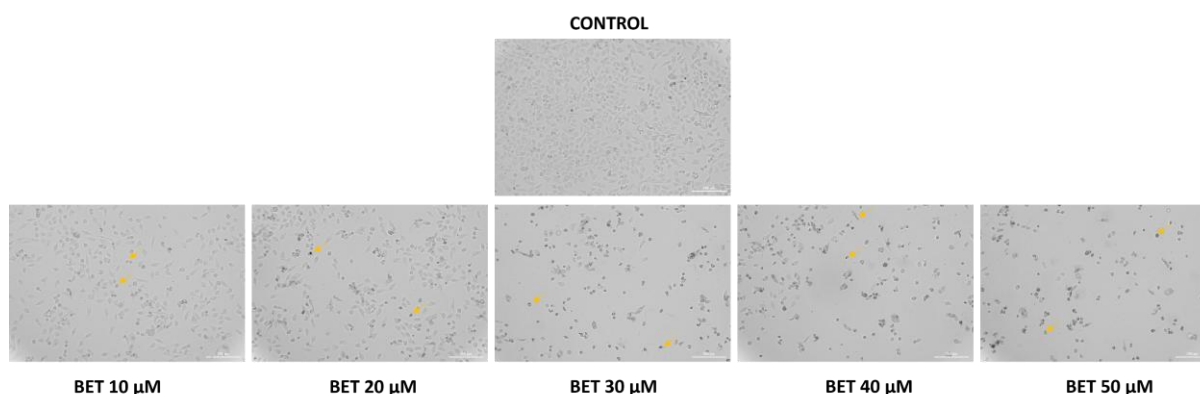


Figure 2.

Morphology of U-2 OS osteosarcoma cells after 24-hour treatment with BET at concentrations of 10, 20, 30, 40, and 50 μM . Scale bar = 200 μm

Cell morphology assessment by bright-field microscopy is a critical step in the evaluation of the effects of treatment on cells, as morphological changes can provide information concerning treatment-induced cellular stress and cytotoxicity. Following morphological analysis, dose-dependent effects were observed, characterized by a decrease in cell confluence, rounding and shrinkage of the cells, changes which may be related to cellular stress. Moreover, an increase in cellular debris and detached cells at 30 - 50 μM was observed, which might indicate the initiation of cell death or high cellular stress. Similar effects, observed via bright-field microscopy and caused by treatment with BET, have been reported in the literature, but in other cancer models. In non-small-cell lung cancer cells, morphological investigations showed that BET, at a concentration of 30 μM , caused blebbing on the cell membrane surface and the formation of apoptotic bodies after 24 hours (15). In a thyroid carcinoma cell line, BET induced dysmorphologies like cell shrinkage and elongation at 5 and 10 μM after 24 h (16). Exposure to a compound related to BET in colorectal cancer cell lines, at concentrations of 1.25 - 10 μM for 24 hours, led to visible changes in cell morphology, characterized

by cell flattening and loss of adhesion (17). Another study showed that the inhibitory effect of BET on HeLa cell proliferation was associated with morphological changes characteristic of apoptosis. After 24 hours of exposure to BET, a dose-dependent effect was observed: the cells were smaller, and their morphology exhibited karyopinosis (18). Furthermore, testing of a betulin-loaded liposomal nanoformulation (1 - 10 μM for 24 h) on pharyngeal carcinoma cells Detroit-562 has shown severe morphological changes at a dose of 10 μM , with cell rounding, massive detachment of cells from the culture plate, and a collapse of monolayer confluency (19).

Analysis of Nuclear and Mitochondrial Morphology
Nuclear and mitochondrial alterations were analyzed (Figure 3), as they serve as evidence of a compound's cytotoxicity. At the lowest concentration, BET induces nuclear and mitochondrial condensation, which are characteristic features of the early stages of apoptosis. At intermediate and maximum concentrations, these signs become somewhat more evident, and cell confluence is visibly reduced; in addition, nuclear fragmentation and the presence of apoptotic bodies also appear.

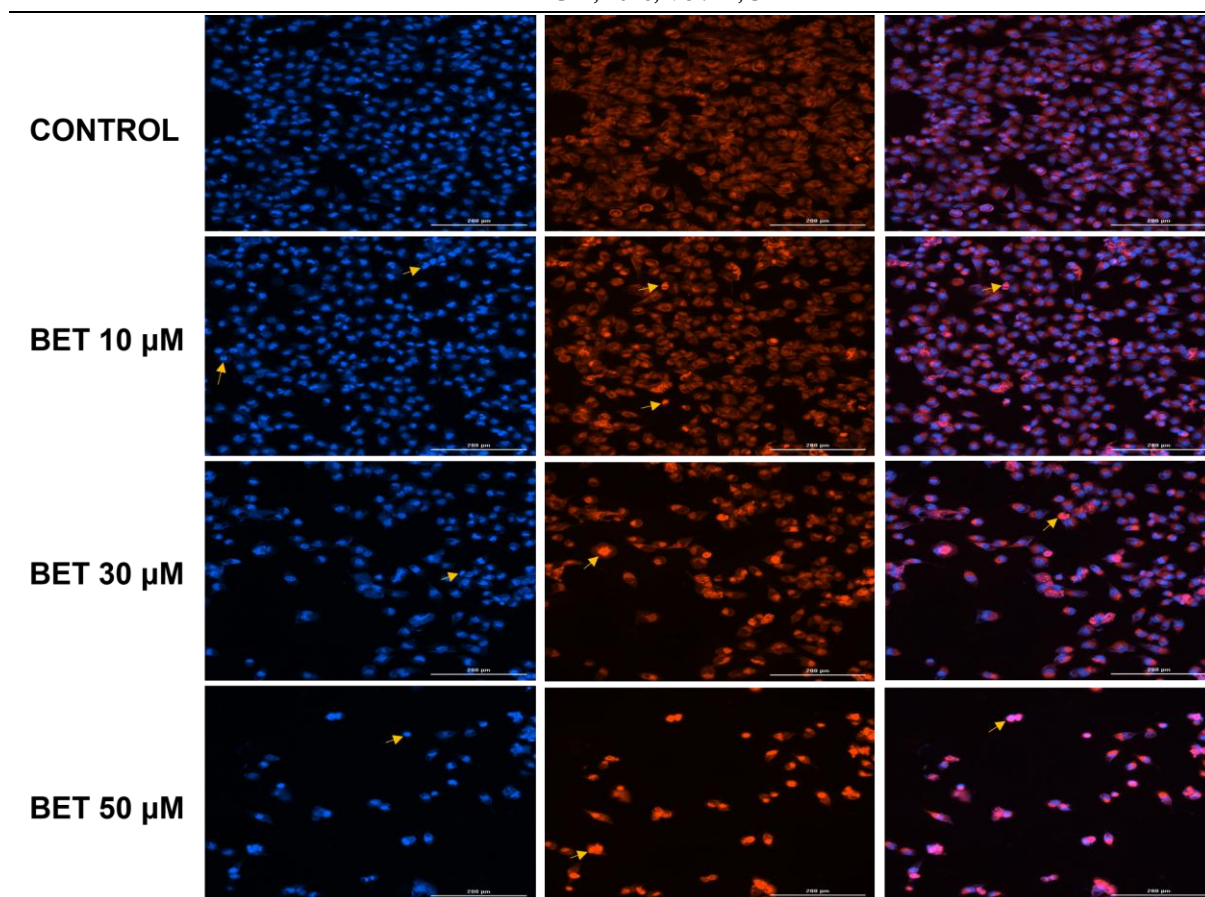


Figure 3.

Representative images obtained 24 hours after treatment with BET at concentrations of 10, 30, and 50 μM , following staining with Hoechst 33342 and MitoTracker™ Red CMXRos. Scale = 200 μm

Nuclear morphology was visualized by staining with Hoechst 33342. This method allows the evaluation of chromatin condensation and nuclear fragmentation, which are morphological features typical of apoptosis (20). In agreement with the results obtained in the present study, BET at a concentration of approximately 22.6 μM induced chromatin condensation and micronucleation in colon cancer cells and nuclear fragmentation of up to $40 \pm 4\%$ after 48 h of treatment (21). Cells treated with a BET-related compound produced dose-dependent cytotoxic effects at 10 - 50 μM , including nuclear fragmentation, apoptotic bodies, and changes in mitochondrial morphology (19).

Mitochondrial morphology was assessed by MitoTracker staining after treatment with the compound. Changes in mitochondrial organization and integrity are important markers of mitochondrial dysfunction and can shed light on the mechanisms of compound-induced cell death. The effects observed in our study were similar to those reported by Biță *et al.*, who demonstrated that BET induced mitochondrial condensation in a concentration-dependent manner in Detroit 562 pharyngeal cancer cells. Similar mitochondrial morphological changes were observed in both Detroit 562 and U-2 OS cells, suggesting that

BET may have similar effects on mitochondrial morphology in different cancer cell models (5). An integrity assessment of organelles in melanoma cells at concentrations ranging from 10 to 50 μM revealed a significant decrease in the fluorescent signal, reflecting mitochondrial membrane depolarization and mitochondrial structural damage (22). Furthermore, the biosafety assessment of betulinic acid-loaded oleogel (BA-O) at concentrations up to 500 $\mu\text{g/mL}$ on non-tumor skin cells (HaCaT and JB6 Cl 41-5a) showed, via the Hoechst 33342 assay, that nuclear confluence was maintained, and there were no significant differences in the apoptotic index compared to the control. In addition, staining with MitoTracker Red CMXRos revealed an intact and well-structured mitochondrial network, confirming the high biocompatibility of the formulation and the absence of cytotoxicity in healthy cells (23). Another study showed that a high-purity triterpene extract from *Betulae* cortex, which contains BET as its main component, produces dose-dependent cytotoxic and apoptotic effects on HaCaT and A431 cells (24).

JC-1 staining for mitochondrial membrane potential analysis

The influence of the sample on mitochondrial membrane potential was demonstrated by JC-1 dye. The

control group exhibited predominantly red fluorescence, indicating a preserved mitochondrial membrane potential, whereas BET-treated cells showed increased green fluorescence, suggesting mitochondrial membrane depolarization at all tested

concentrations (Figure 4A). The ratio of red aggregates to green monomers was greater than 2 in the cells of the untreated group, indicating a healthy, polarized membrane, whereas after treatment, the ratio fell below 2 (Figure 4B).

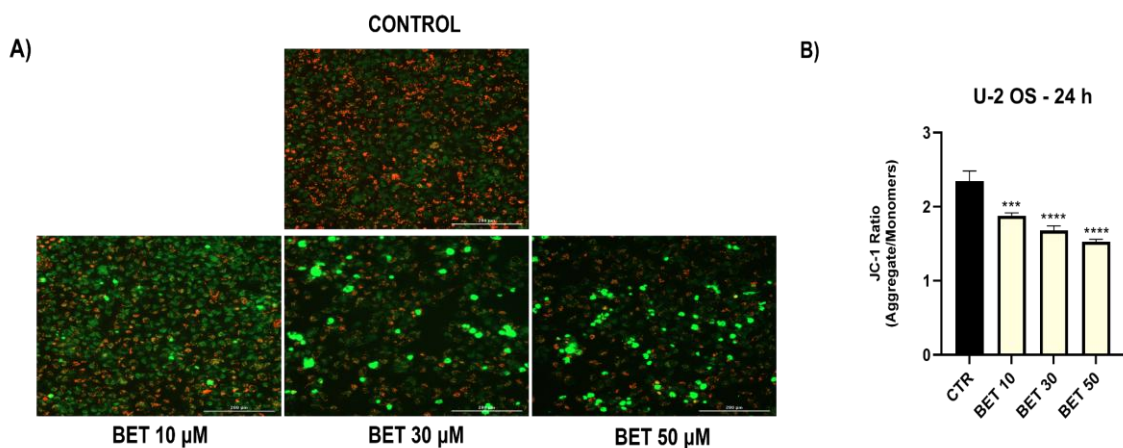


Figure 4.

A) Representative images obtained after JC-1 staining following treatment with the indicated concentrations of BET for 24 hours. B) Quantitative analysis of the ratio of red to green fluorescence. Data are presented as mean $\pm$ standard deviation (SD) from three independent biological experiments ($n = 3$), with three technical replicates per experimental condition. Statistical differences between treated groups and the untreated control were evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc multiple comparisons test.

*** $p < 0.001$; **** $p < 0.0001$

JC-1 staining is a widely used method that allows for the examination of changes in mitochondrial membrane potential, which is an important indicator of mitochondrial health. A healthy, polarized membrane accumulates JC-1 aggregates that fluoresce red, whereas a depolarized membrane retains JC-1 as monomers that fluoresce green. Thus, the red/green fluorescence ratio decreases after stimulation with the tested compound, which signifies loss of mitochondrial membrane potential and mitochondrial dysfunction (25). These results are in line with other studies showing that BET acts via the mitochondrial pathway, inducing a loss of mitochondrial membrane potential in other cancers (10, 26, 27). BET induced apoptosis through activation of the intrinsic mitochondrial pathway, as demonstrated by Li *et al.* BET treatment increased reactive oxygen species (ROS) levels, which favored the accumulation of the pro-apoptotic proteins Bax and Bak in mitochondria and the decrease of the anti-apoptotic proteins Bcl-2 and XIAP. These changes are associated with mitochondrial membrane depolarization and the release of cytochrome c, which activates caspase-9 and then caspases-3/7, leading to apoptosis (25, 26). Treatment with a compound related to BET in human laryngeal carcinoma cells induced apoptosis via a mitochondrial pathway mediated by ROS, with the JC-

1 assay showing a marked loss of mitochondrial membrane potential due to a collapse in the ratio of JC-1 aggregates to JC-1 monomers (28). Also, exposure of cervical cancer cells to a dose of 30 $\mu\text{mol/l}$ of the same compound showed that the mitochondrial permeability transition pore opened after the first hour of incubation, with the JC-1 assay indicating a dramatic change in the monomer-to-aggregate ratio as a trigger for the early phase of apoptosis (29). *Analysis of cell death types by double staining with acridine orange and propidium iodide*

Double staining with acridine orange and propidium iodide was used to identify and differentiate between viable, apoptotic, and necrotic cells. Treatment with BET at a concentration of 10 μM resulted in a marked increase in the number of cells with apoptotic morphology, accompanied by the appearance of a small number of PI-positive cells, compared to the control group, in which viable cells predominated (Figure 5). At 30 μM , a change in the cell death profile was observed, characterized by an increase in the number of cells stained with PI and a decrease in those with apoptotic morphology. At a concentration of 50 μM , most cells were PI-positive, indicating extensive loss of plasma membrane integrity and suggesting progression toward late-stage cell death.

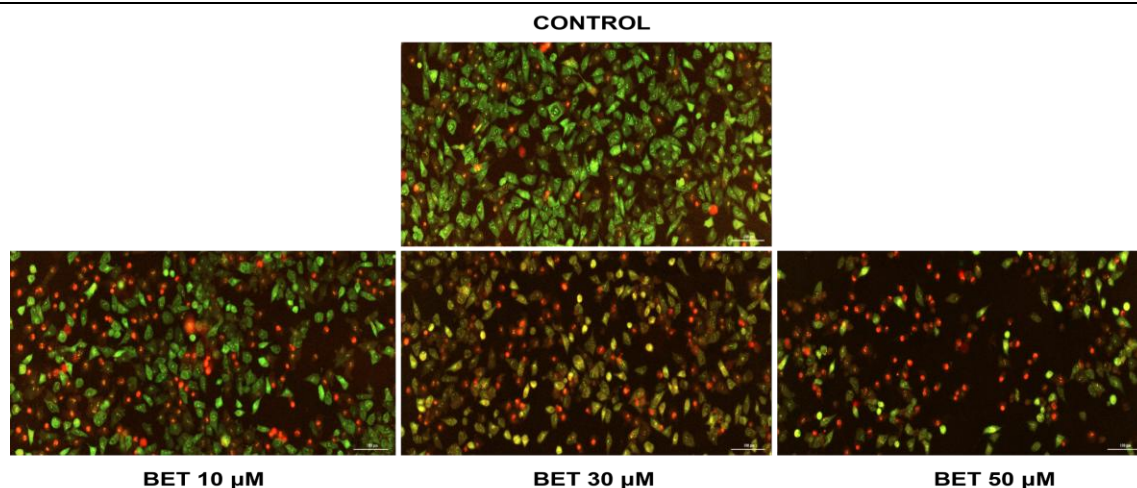


Figure 5.

Images obtained after staining with acridine orange and propidium iodide, used to determine apoptosis in U-2 OS cells following stimulation with BET for 24 hours at 10, 30, and 50 μM . Scale bar = 100 μm

AO and PI staining is a fluorescence-based method to differentiate viable, apoptotic, and necrotic cells based on plasma membrane integrity and nuclear morphology. Acridine orange enters living cells and stains them green. Propidium iodide enters cells with damaged membranes and stains them fluorescent red. Our results demonstrated that this experiment induced BET-mediated cell death in a concentration-dependent manner. At the lowest concentration tested (10 μM), it mainly induced apoptotic morphological features, whereas at increasing concentrations it caused a progressive appearance of cells with necrotic characteristics. A similar pattern was observed following BET treatment in a pharyngeal carcinoma cell line, where the proportion of PI-positive cells increased in a concentration-dependent manner (5). In addition, BET induced cell death via apoptosis in RCC4 renal carcinoma cells, which was associated with the activation of caspases and proteins involved in the intrinsic and extrinsic apoptotic pathways (30).

Although the cytotoxic and pro-apoptotic effects of BET have been previously demonstrated in other osteosarcoma cell lines (8), its effects on mitochondrial membrane potential and mitochondrial and nuclear morphology in U-2 OS cells remain insufficiently characterized. Therefore, this study extends previous findings by providing additional insights into betulin-induced mitochondrial alterations and cell death-associated morphological changes in U-2 OS osteosarcoma cells.

The present study has several limitations. First, the lack of a non-tumor cell line limits the evaluation of the cytotoxic selectivity of the compound. Secondly, the experiments were performed at a single time point (24 h) and studies at longer exposure times could give more information about the progression of the effects induced by BET. Third, further

analysis is needed to clarify the mechanism of action of BET. For example, Annexin V/PI staining would allow for a more precise differentiation between the stages of cell death and would clarify whether the PI-positive cells observed by acridine orange/propidium iodide staining correspond to primary necrosis or secondary necrosis. Finally, in vivo studies are needed to evaluate the translational potential of BET and their applicability in clinical practice.

Conclusions

In this study, it was shown that BET induces significant cytotoxic effects on the U-2 OS osteosarcoma cell line even at the lowest concentrations, as initially confirmed by the viability assay. Subsequently, morphological analysis revealed a dose-dependent response in the OS cell line after 24 hours, characterized by cell rounding and shrinkage, detachment from the culture dish, and a dramatic decrease in confluence. At the nuclear and mitochondrial levels, BET induced morphological alterations, including nuclear and mitochondrial condensation and nuclear fragmentation. The impact of the sample on mitochondria was also confirmed by JC-1 staining, which demonstrated dose-dependent depolarization of the membrane through a decrease in the ratio of red aggregates to green monomers. Finally, through AO/PI double staining, it was possible to distinguish between necrotic and apoptotic cell populations, which predominated in the treated cells compared to the untreated ones, where viable cells were in the majority. Thus, the present study may represent a first step toward a more detailed exploration of the potential of BET in osteosarcoma. Future studies could also benefit from the inclusion of non-tumor cell lines, the evaluation of effects at multiple time points, the use of complementary methods to characterize the type of cell death and the

mechanisms by which they induce toxicity, as well as the validation of results in in vivo models.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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