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ORIGINAL PAPER

Mechanism of anticancer effect of ETP-45658, a PI3K/AKT/mTOR pathway inhibitor on HT-29 Cells

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Abstract

The PI3K pathway plays a crucial role in tumor cell proliferation across various cancers, including colon cancer, making it a promising treatment target. This study aims to investigate the antiproliferative activity of ETP-45658, a PI3K/AKT/mTOR pathway inhibitor, on colon cancer and elucidate the underlying mechanisms. HT-29 colon cancer cells were treated with varying doses of ETP 45658 and its cytotoxic effect assessed using the XTT cell viability assay.ELISA was also used to measure TAS, TOS, Bax, BCL-2, cleaved caspase 3, cleaved PARP, and 8-oxo-dG levels. Flow cytometry was performed to investigate apoptosis, cell cycle, caspase 3/7 activity, and mitochondrial membrane potential. Additionally, following the administration of DAPI (4,6-diamidino-2-phenylindole) dye, the cells were visualized using an immunofluorescence microscope. It was observed that ETP-45658 exerted a dose-dependent and statistically significant antiproliferative effect on HT-29 colon cancer cells. Further investigations using the IC_{50} dose showed that ETP-45658 decreased TAS levels and increased TOS levels and revealed that it upregulated apoptotic proteins while downregulating anti-apoptotic proteins. Our findings also showed that it increased Annexin V binding, arrested the cell cycle at G0/G1 phase, induced caspase 3/7 activity, impaired mitochondrial membrane potential, and ultimately triggered apoptosis in HT-29 cells. ETP-45658 shows promise against colon cancer by inducing cell death, and oxidative stress, and arresting the cell cycle. Targeting the PI3K/AKT/mTOR pathway with ETP-45658 offers exciting potential for colon cancer treatment.

Keywords Anticancer · PI3K/AKT/mTOR inhibition · ETP-45658 · Cell cycle · Apoptosis

Introduction

Cancer represents a critical public health issue, presenting substantial economic and social burdens. It has a profound impact on both mortality and morbidity rates, with its prevalence steadily on the rise. Alongside surgical interventions, advancements have been made in non-surgical treatment options like chemotherapy, immunotherapy, and radiotherapy for cancer patients [1]. Among the various types of cancer, colon cancer stands out as the third most frequently detected form, persistently ranking as the second leading cause of cancer-related fatalities [2]. Despite the use of numerous chemotherapeutic agents in the management of colon cancer, the search for an ideal chemotherapeutic agent continues. Several pathways and mechanisms have emerged in this context, including the phosphatidylinositol-3-kinase (PI3K) pathway. Various hormones and growth factors can activate the PI3K pathway, which is an intracellular signaling pathway. AKT (Protein Kinase B) and mTOR (mammalian Target Of Rapamycin) are two serine-threonine protein kinases that serve as downstream effectors of the pathway. The interconnections between PI3K, AKT, and mTOR are highly complex, forming an intricate network. Consequently, it is widely acknowledged that the PI3K/AKT/mTOR pathway functions as a unified signaling pathway [3]. The activation of this pathway regulates essential cellular processes, including cell growth, proliferation, survival, enhanced cell migration, and can also influence apoptosis and the development of cancer [4]. PI3-kinase enzymes possess a common structural motif comprising a C2 domain, a

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helical domain, and a catalytic (kinase) domain. In mammals, these enzymes are categorized into three classes based on their specific coding genes, unique structures, and preferences for different substrates [5]. Typically, the isoforms of Class IA are activated through receptor tyrosine kinases, while Class IB isoforms are often activated via G-protein-coupled receptors. The ligands of growth factors activate tyrosine kinase receptors, leading to the autophosphorylation of tyrosine residues and subsequent recruitment of PI3Ks to the cellular membrane [3]. Once activated, PI3K catalyzes the conversion of PIP2 (phosphatidylinositol 4,5-bisphosphate) into PIP3 (inositol 1,4,5-trisphosphate). PIP3 acts as a docking site for AKT, triggering mTOR signaling [6]. The activity of PI3K is physiologically balanced by the phosphatase and tensin homolog (PTEN). PTEN primarily acts on the lipid substrate PI(3,4,5)P3, converting it into PI(4,5)P2, thereby terminating or inactivating the PI3K/AKT signaling pathway. Thus, PTEN serves as a negative regulator of the PI3K/AKT signaling pathway [7]. Deregulation of the PI3K/AKT/mTOR pathway plays a significant role in tumor cell proliferation, growth, and apoptosis in many human cancers. Currently, this pathway is being investigated and implemented as a targeted therapy in various cancers, including colon cancer. However, there is still a lack of feasible and effective drugs targeting this pathway in the clinical treatment of colon cancer. The intricate nature of the PI3K/mTOR signaling pathway and the potential for targeted anticancer therapies focusing on mTOR have gained significant attention. Despite the limited efficacy of mTOR inhibitors in certain cancer types such as metastatic breast cancer, they have shown significant benefits in tumors associated with the tumor suppressor complex, where limited treatment options are available [8]. In a preclinical study using colon cancer cell lines, increased activation of the PI3K/AKT/mTOR pathway was observed. Targeting PI3K in colon cancer has been deemed biologically feasible, and in vitro targeting of this pathway in colon cancer models has shown promising preclinical results, highlighting the potential of new therapeutic agents related to these pathways [6]. It was shown that BEZ235, a PI3K/mTOR inhibitor, can suppress the proliferation of colorectal cancer cells [9]. Similarly, it was demonstrated that NVP-BEZ235, a PI3K/mTOR inhibitor, can induce apoptosis by arresting BL (Burkitt lymphoma) cells in the G0/G1 phase of the cell cycle [10]. In vitro studies have demonstrated that ETP-45658, an inhibitor of the PI3K/AKT/mTOR pathway, effectively inhibits the proliferation of various cancer cell lines. Furthermore, in vivo experiments have shown that ETP-45658 reduces AKT phosphorylation activity [11]. While numerous studies have demonstrated the anticancer actions of ETP-45658 in various cancer cell lines the specific molecular mechanisms underlying its activity remain to be fully understood. Our study aims to determine the antiproliferative activity of ETP-45658 on the colon cancer HT-29 cells and elucidate the underlying mechanisms involved in this action.

Materials and methods

Cell line and cell culture

The HT-29 colon cancer cell line (HTB-38) was obtained from the American Type Culture Collection (ATCC) located in the USA. To culture these cells, they were routinely grown in Roswell Park Memorial Institute Medium (RPMI) at a temperature of 37 °C, in a humidified atmosphere containing 5% CO_2. The culture medium utilized in this study consisted of 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. ETP-45658, a compound of interest, was liquefied in DMSO and then diluted in the culture medium. Care was taken to adjust the final concentration of DMSO in the culture medium to be less than 0.1% before treating the cells. All the materials used in the experiment, excluding the cell line, were acquired from Sigma–Aldrich (United Kingdom).

Cell viability assay

The impact of ETP-45658 on the viability of the HT-29 cell line was evaluated by conducting the XTT assay (Roche Diagnostic, Germany). The cells were initially seeded at a density of 1×10^4 cells per well in a 96- well plate and allowed to incubate for 24 h. Following that, the HT-29 cells were subjected to treatment using different concentrations of ETP-45658, (1, 10, 25, 50, and 100 µM). Untreated cells served as the control group. After the incubation period, each well received 50 µL of an XTT mixture. Following a 4-hour incubation, the cells were agitated, and the absorbance at 450 nm was quantified using a microplate reader provided by Thermo Fisher Scientific in Altrincham, United Kingdom. The experiment was conducted in triplicate, and the cell viability was determined by calculating the percentage of live cells relative to the untreated control cells.

Determination of total antioxidant status (TAS) and total oxidant status (TOS)

The present study aimed to examine the TAS and TOS values in HT-29 cells treated with ETP-45658, as well as in untreated cells. The Total Antioxidant Status Assay Kit (Rel Assay Diagnostics, Turkey) was employed to assess the TAS values, while the Total Oxidant Status Assay Kit (Rel Assay Diagnostics, Turkey) was used to assess the TOS values. A

total of approximately 10^6 HT-29 cells per well were plated in 6-well plates and allowed to adhere overnight. HT-29 cells were exposed to ETP-45658 at a dose of 19.3 µM (IC_{50}) for a duration of 24 h, following the manufacturer's guidelines. The findings were reported as mmol Trolox Equiv./L for TAS and µmol H_2O_2 equivalent/L for TOS [12] [13].

Determination of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-hydroxy-deoxyguanosine levels

In order to assess the levels of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-hydroxy-deoxyguanosine (8-oxo-dG) in both ETP-45658-treated and untreated HT-29 cells, specific ELISA kits were employed. The human ELISA kits of Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-Hydroxy-Desoxyguanosine (BT Lab Shanghai, China) were utilized for their respective measurements. To perform the experiments, HT-29 cells were seeded in a 6-well plate with 10^6 cells per welland exposed to ETP-45658 at the dose of 19.3 µM (IC_{50}) for 24 h. Following the treatment, both the ETP-45658-treated and untreated HT-29 cells were collected, diluted with PBS, and subjected to multiple freeze-thaw cycles to induce cellular damage. The levels these markers in the resulting cell lysates were quantified according to the instructions provided by the manufacturer. Furthermore, the total protein concentrations of both the ETP-45658 treated and untreated HT-29 cells were assessed using the BCA assay from Pierce Biotechnology in the United States.

Annexin V binding assay

A total of approximately 5×10^5 HT-29 cells per well were plated in 6-well plates and allowed to adhere overnight. The next day, the HT-29 cells were exposed to ETP-45658 at dose of 19.3 µM (IC_{50}) and incubated for an additional 24 h. Following the incubation period, the cells were detached using trypsin, collected, and resuspended in PBS containing at least 1% FBS. Subsequently, the cell suspension was mixed with an equivalent volume of Annexin V & Dead Cell reagent, following the manufacturer's instructions. The Muse Cell Analyzer from Merck Millipore (USA) was employed to quantify the various cell populations, which included live cells, dead cells, early apoptotic cells, and late apoptotic cells.

Cell cycle analysis

The development of the cell cycle phases was evaluated using the Muse Cell Cycle Assay Kit in accordance with the manufacturer's instructions. A total of approximately 5×10^5 HT-29 cells per well were plated in 6-well plates and allowed to adhere overnight. After the addition of ETP-45658 at a dose of 19.3 µM (IC_{50}), the cells were subjected to incubation for 24 h. The cells that had been exposed to ETP-45658 as well as the control cells were then separated by centrifugation, rinsed in ice-cold PBS, and fixed in 70% ethyl alcohol. Before detection, the cells that were fixed were then rinsed with PBS, resuspended in 200 µL of Muse™ Cell Cycle Reagent, and cultured for 30 min at room temperature in a dark place. Finally, the Muse Cell Analyzer (Merck, Millipore, USA) was utilized to categorize the cell cycle phases (G0/G1, S, and G2/M phases).

Evaluation of caspase 3/7 by flow cytometry

The detection of caspase 3/7 was conducted using the Muse Caspase-3/7 Kit. Initially, HT-29 cells (5×10^5 per well) were cultivated in a 6-well plate and allowed to attach overnight. The following day, the cells were treated with ETP-45658 at a dose of 19.3 µM (IC_{50}) and incubated for 24 h. On the next day, the cells were collected in each tube with a volume of 50 µL of cell suspension. Subsequently, the cells were then given 5 µL of caspase 3/7 working solution and and they underwent incubation at 37 °C for 30 min. After that, the cells were treated for 5 min at room temperature in a dark environment with 150 µL of 7-AAD (7-Aminoactinomycin D) dye, and the cells were underwent incubation in the dark at room temperature for 5 min. The Muse Cell Analyzer (Merck, Millipore, USA) was then used to examine the cells.

Evaluation of mitochondrial membrane potential by flow cytometry

The Muse MitoPotential Kit was utilized to assess the mitochondrial transmembrane potential. Initially, HT-29 cells (5×10^5 per well) were cultivated in a 6-well plate and allowed to attach overnight. The following day, the cells were treated with ETP-45658 at a dose of 19.3 µM (IC_{50}) and incubated for 24 h. On the next day, the cells were collected in each tube with a volume of 100 µL of cell suspension. The cells were then stained with Muse™ MitoPotential Dye and incubated at 37 °C for 20 min. Finally, the cells were treated for 5 min at room temperature in a dark environment with 5 µL of Muse MitoPotential 7-AAD reagent. Using the Muse Cell Analyzer (Merck, Millipore, USA), the percentages of live, depolarized, depolarized/dead, and dead cells were calculated. Each measurement was also performed as 5000 events.

Fluorescence microscopy

HT-29 cells (5×10^5 per well) were cultivated in a 6-well plate and allowed to attach overnight. The following day, the cells were treated with ETP-45658 at a dose of 19.3

μM (IC_{50}) for 24 h. On the next day, the DAPI (Thermo Fisher, United Kingdom) staining kit was used according to the kit protocol, and the cells were visualized using an immunofluorescence microscope (ZEISS AXIO Vert. A1, Germany). The immunofluorescence microscope was used with an excitation value of 359 nm and an emission value of 457 nm. ZEN 2.3 (blue edition) software was used to analyze the data.

Statistical analysis of the data

The laboratory findings were presented as mean ± standard error and analyzed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey to assess multiple comparison tests. A p-value of less than 0.05 was chosen for accepting statistically significant differences. The statistical evaluations were performed using the SPSS Statistics Program v.22.

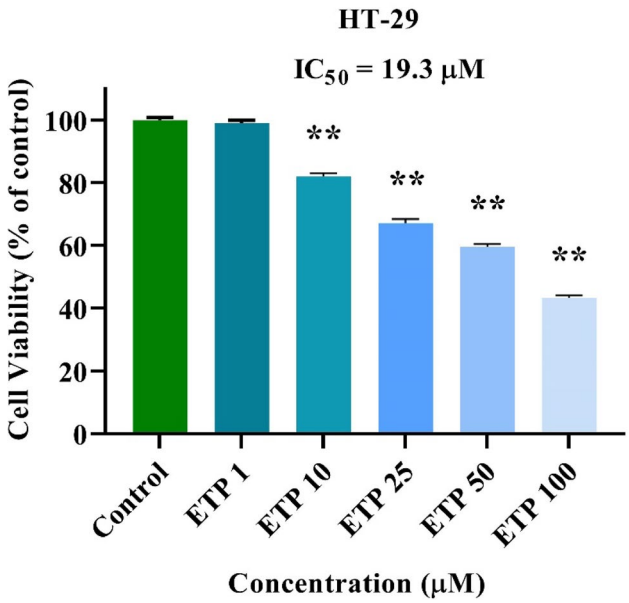

Fig. 1 Evaluation of the antiproliferative effect of ETP-45658 on HT-29 colon cancer cells. ETP-45658 exhibited a statistically significant antiproliferative effect compared to the control at doses of 10 and above (**p < 0.01)

Results

The antiproliferative effect of ETP-45658 in HT-29 cells

The cells were exposed to ETP-45658 in a concentration range of 1 to 100 μM for 24 h, and the viable cell count was determined using the XTT cell viability assay. ETP-45658 did not show any effect at a dose of 1 μM, but exhibited a dose-dependent antiproliferative effect on HT-29 colon cancer cells at doses of 10 μM and above ($p < 0.01$; Fig. 1). The IC_{50} value of ETP-45658 was calculated as 19.3 μM using GraphPad Prism. Light microscopic differences in the ETP-45658 group at a dose of 19.3 μM compared to the control are shown in Fig. 2.

The effect of ETP-45658 on TAS and TOS in HT-29 cells

The effects of ETP-45658 on TAS and TOS levels in HT-29 colon cancer cells were evaluated. ETP-45658 significantly decreased the TAS levels while significantly increasing the TOS levels in HT-29 colon cancer cells. This effect indicates that ETP-45658 significantly affects the oxidant-antioxidant system in HT-29 colon cancer cells ($p < 0.01$; Fig. 3A, B). Disruption of the oxidant-antioxidant system in the cells suggests its significant role in the antiproliferative effect of ETP-45658.

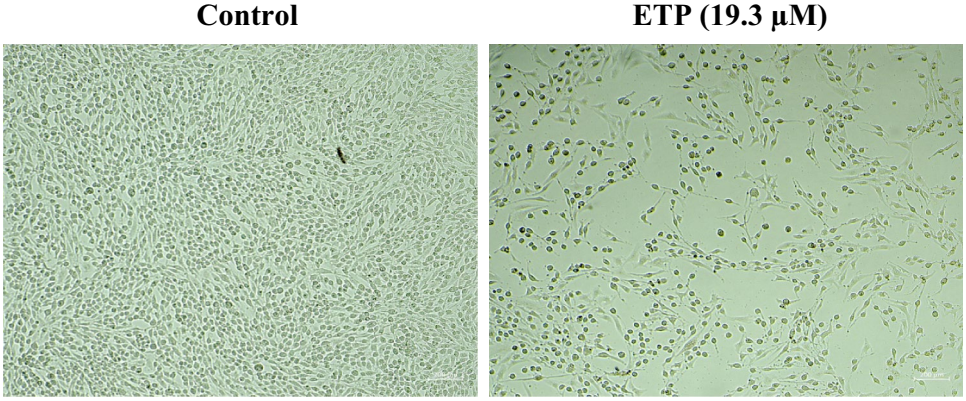

Fig. 2 Light microscopic images of cells in the control and ETP-45658 (19.3 μM) groups

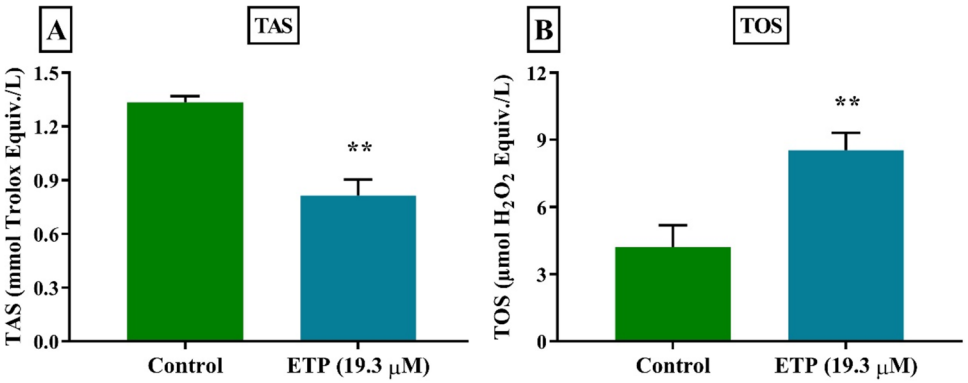

Fig. 3 Evaluation of the effects of ETP-45658 (19.3 μM) on TAS (**A**) and TOS (**B**) compared to the control. ETP-45658 at a dose of 19.3 μM significantly decreased TAS levels compared to the control, while significantly increasing TOS levels (**p < 0.01)

The effect of ETP-45658 on Bax, cleaved caspase-3, BCL-2, cleaved PARP, and 8-oxo-dG proteins in HT-29 cells

ETP-45658's effects on the levels of Bax, cleaved caspase-3, BCL-2, cleaved PARP, and 8-oxo-dG proteins in HT-29 colon cancer cells were evaluated using ELISA kits specific to these proteins. The decrease in the anti-apoptotic protein BCL-2 and the increase in the cleaved caspase-3, resulting from the degradation of the apoptotic protein Bax, suggest that ETP-45658 induces apoptosis and exhibits an anti-proliferative effect in HT-29 cells. Additionally, PARP, an enzyme involved in DNA damage and repair mechanisms, is cleaved by caspase-3 during apoptosis, leading to the formation of cleaved PARP. The increased levels of cleaved PARP in HT-29 cells upon treatment with ETP-45658 support its apoptotic effect. Furthermore, 8-oxo-dG is a protein associated with DNA damage in cells, and the observed increase in its expression level after ETP-45658 treatment indicates that ETP-45658 induces DNA damage in HT-29 cells ($p < 0.05$ to $p < 0.01$; Fig. 4A–E).

The apoptotic effect of ETP-45658 on HT-29 cells

Flow cytometry device and appropriate kits were used to evaluate the apoptosis profile of ETP-45658 in HT-29 colon

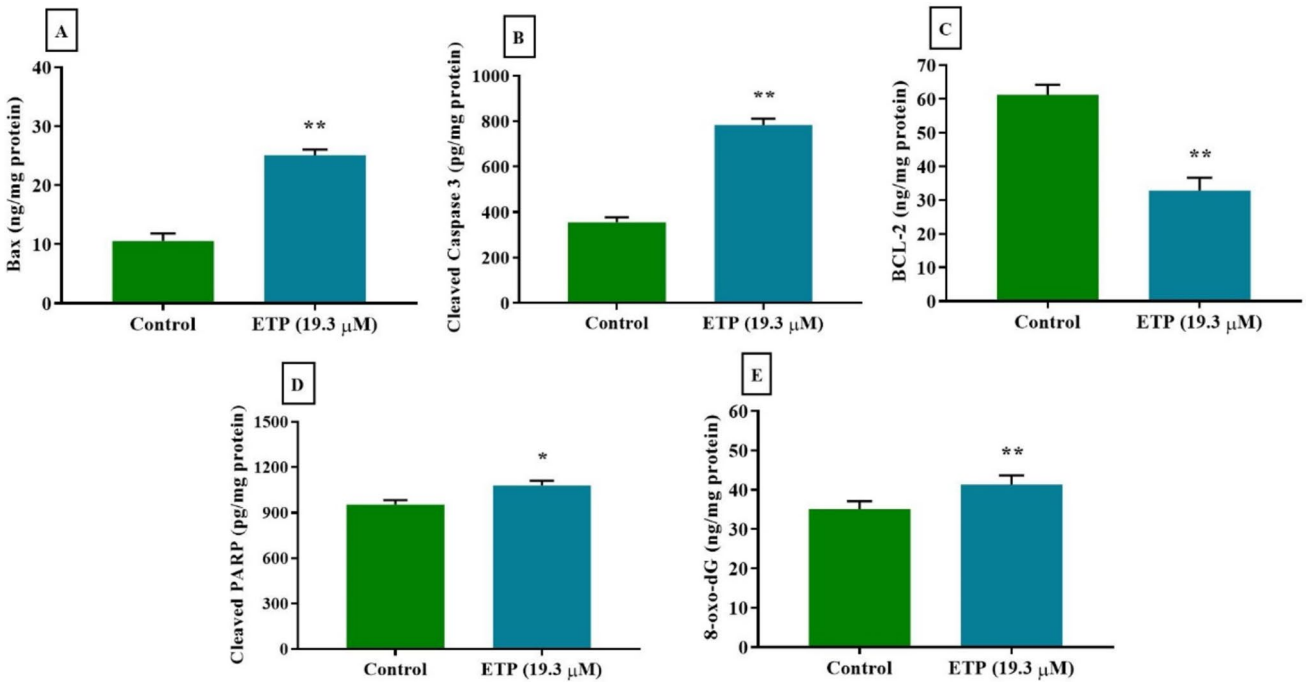

Fig. 4 Evaluation of the effects of ETP-45658 (19.3 μM) on Bax (**A**), cleaved caspase-3 (**B**), BCL-2 (**C**), cleaved PARP (**D**), and 8-oxo-dG (**E**) proteins compared to the control. ETP-45658 at a dose of 19.3 μM significantly increased the levels of Bax, cleaved caspase-3, and 8-oxo-dG proteins compared to the control (**p < 0.01). It also significantly decreased the level of BCL-2 protein (p < 0.01). ETP-45658 at a dose of 19.3 μM significantly increased the level of cleaved PARP compared to the control (*p < 0.05)

cancer cells. In the control group, the percentage of early apoptotic cells was measured as 1.62 ± 1.21% and the percentage of late apoptotic cells was 5.58 ± 1.43%. However, these percentages showed a significant increase in the group treated with ETP-45658, with the early apoptotic cell percentage measuring 6.44 ± 2.12% and the late apoptotic cell percentage measuring 28.51 ± 1.22%. The percentage of dead cells exhibited a slight increase from 2.21 ± 1.89% in the control group to 5.08 ± 0.87% in the ETP-45658 group. Looking at the percentages of live cells, there was a significant decrease from 90.59 ± 2.01% to 59.96 ± 3.26%. Based on our findings, it can be observed that ETP-45658 significantly induces apoptosis in HT-29 colon cancer cells and plays a significant role in its antiproliferative effect ($p < 0.05$ to $p < 0.01$; Fig. 5).

The effect of ETP-45658 on cell cycle in HT-29 cells

Flow cytometry device and appropriate kits were used to evaluate the cell cycle profile of ETP-45658 in HT-29 colon cancer cells. It was observed that in the group treated with ETP-45658 (60.6 ± 2.4%), there was a significant accumulation of cells in the G0/G1 phase compared to the control group (46.3 ± 2.1%). One of the underlying mechanisms of the antiproliferative effect of ETP-45658 is its ability to arrest HT-29 cells in the G0/G1 phase of the cell cycle ($p < 0.01$; Fig. 6).

The effect of ETP-45658 on caspase 3/7 in HT-29 cells

Another method called caspase-3/7 was used to evaluate the apoptotic profile of ETP-45658 in HT-29 colon cancer cells. The test was performed using a flow cytometry device. In the control group, the percentage of apoptotic cells was measured as 2.51 ± 1.34%, and the apoptotic/dead cell ratio was 4.27 ± 1.32%. However, in the group treated with ETP-45658, these ratios showed a significant increase, with the percentage of apoptotic cells measuring 7.53 ± 1.97% and the apoptotic/dead cell ratio measuring 14.17 ± 2.12%. The dead cell ratio was evaluated as 2.47 ± 1.79% in the control group, while it was 3.99 ± 1.23% in the ETP-45658 group. When looking at the percentages of live cells, they decreased significantly from 90.75 ± 1.80% to 74.30 ± 2.95% ($p < 0.01$; Fig. 7). Based on our findings, ETP-45658

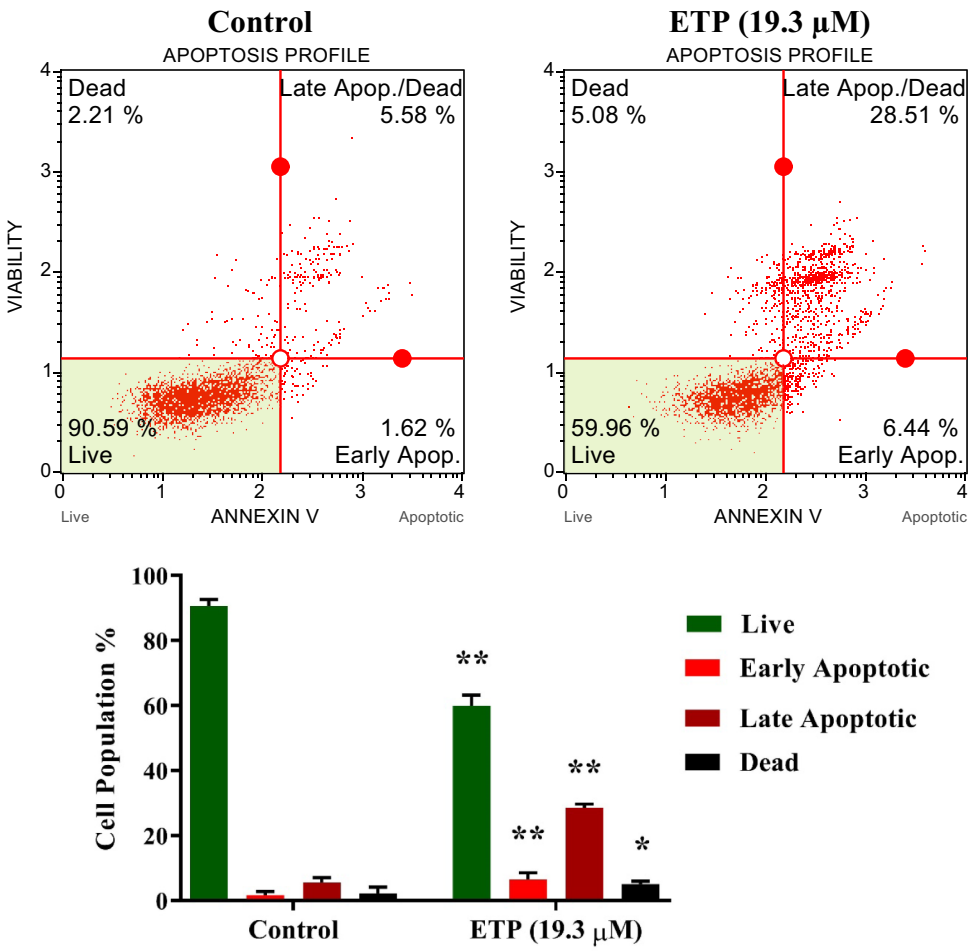

Fig. 5 Evaluation of the effects of cells in the ETP-45658 (19.3 µM) group compared to the control on the apoptosis profile using flow cytometry. ETP-45658 at a dose of 19.3 µM significantly increased the percentage of early and late apoptotic cells compared to the control group, while significantly decreased the percentage of live cells (**$p < 0.01$). ETP-45658 at a dose of 19.3 µM significantly increased the percentage of dead cells compared to the control group (*$p < 0.05$)

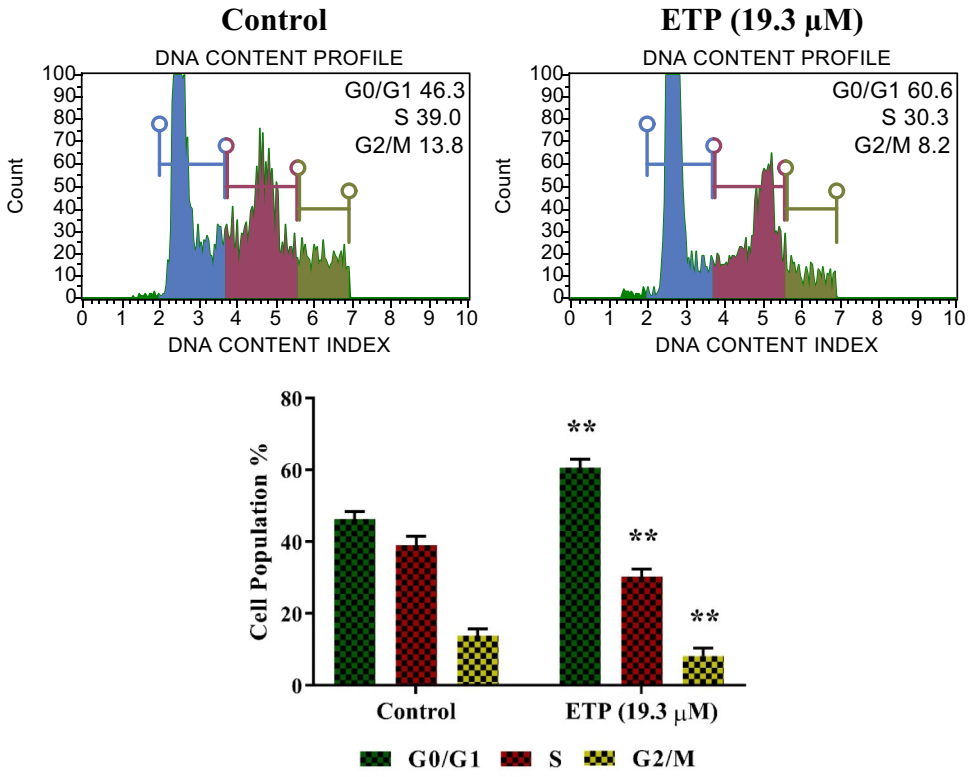

Fig. 6 Evaluation of the effects of cells in the ETP-45658 (19.3 µM) group compared to the control on the cell cycle profile using flow cytometry. ETP-45658 at a dose of 19.3 µM significantly increased the percentage of cells in the G0/G1 phase compared to the control group, while significantly decreased the percentage of cells in the S and G2/M phases (**$p < 0.01$)

significantly increased the amount of caspase-3/7 in HT-29 colon cancer cells, thereby promoting apoptosis to a significant extent. This supports the notion that apoptosis induction is one of the key mechanisms underlying the antiproliferative effects of ETP-45658, which was further supported by another method.

The effect of ETP-45658 on mitochondrial membrane potential in HT-29 cells

To assess the mitochondrial membrane potential in HT-29 colon cancer cells, flow cytometry was used. In the control group, the depolarized/live cell ratio was measured as 9.63 ± 1.11%. However, in the ETP-45658 group, there was a significant increase, reaching 40.60 ± 2.47% ($p < 0.01$; Fig. 8). Disruption of the mitochondrial membrane potential triggers apoptotic pathways in cells. Increased depolarization in HT-29 cell mitochondria leads to mitochondrial dysfunction and initiation of apoptotic pathways in the cell. Once again, through another method of measuring mitochondrial membrane potential, ETP-45658 stimulates apoptosis by disrupting the mitochondrial membrane potential in HT-29 cells.

Evaluation of the effect of ETP-45658 on HT-29 cell nucleus using DAPI staining

The effect of ETP-45658 on the nuclear status in HT-29 cells was determined using DAPI staining. Examining the results, it is observed that apoptotic cells exhibit concentrated nuclear chromatin condensation and deep staining on one side of the nuclear membrane (indicated by the arrow marked with 'a' in fig. 9). Additionally, nuclear shrinkage and chromatin condensation are observed in apoptotic cells (indicated by the arrow marked with 'b' in fig. 9).

Discussion

The PI3K/AKT/mTOR pathway is frequently implicated in the development of various types of cancer, including colon cancer [14–17]. A key regulator of cell growth, proliferation, differentiation, and survival, is this pathway. As a result, aberrant PI3K/AKT/mTOR signaling pathway activation frequently contributes to the initiation and development of different cancers [18–22]. A number of different proteins, including p70S6K, cyclin D1, BCL-2/Bax, and others, are regulated by PI3K activation in the growth of tumors [23], [24]. The BCL-2 family of proapoptotic proteins are inhibited by AKT activation, which encourages cell growth and survival [25]. Activated AKT can induce mTOR, which is a serine/threonine kinase [26]. Evidence suggests that both AKT and mTOR exhibit high expression levels in colon cancer tissue and HT-29 cells. Moreover, activated mTOR participates in gene transcription and protein translation processes [27]. Inhibition of the PI3K/AKT/mTOR pathway holds promise

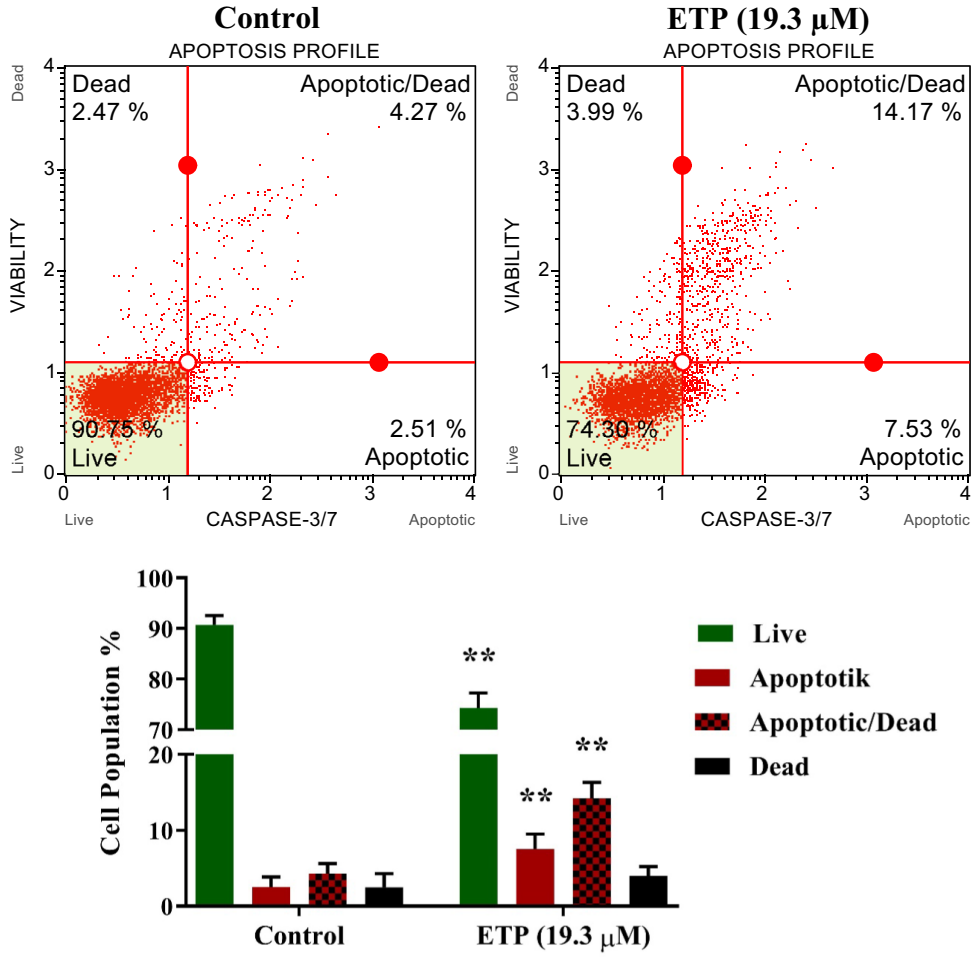

Fig. 7 Evaluation of the effects of ETP-45658 (19.3 µM) on caspase 3/7 profile in cells compared to the control using flow cytometry. ETP-45658 at a dose of 19.3 µM significantly increased the apoptotic and apoptotic/dead cell ratios compared to the control ($p < 0.01$), while significantly decreased the live and dead cell ratios (**$p < 0.01$)

as a potential cancer treatment strategy that can augment the effectiveness of chemotherapy and radiotherapy by increasing their sensitivity. The anticancer effects of ETP45658, a member of the inhibitors of the PI3K/AKT/mTOR signaling pathway, have been demonstrated in several reports [28], [29]. ETP45658, a pyrazolopyrimidine derivative, has been demonstrated to have potent antiproliferative properties with specificity towards PI3K and mTOR [29], [30]. Although many investigations have demonstrated the anticancer activity of ETP45658 in various cancer cell types, including colon cancer, the underlying molecular mechanisms of this activity have not yet been elucidated.

In this study, we initially exposed HT-29 colon cancer cells to varying doses of ETP-45658 for 24 h. The results showed a substantial suppression of cell proliferation in colon cancer cells, with IC_{50} values of 19.3 µM. Consistent with our results, other experimenters have also documented various activities of ETP-45658 on various tumor cell lines, including prostate, lung, colon, lymphoma, and breast [29], [30]. However, evaluation of the toxic effect of ETP-45658 on normal cells is also important for prevent possible systemic toxicity.

Apoptosis, known as programmed cell death, plays a pivotal role in regulating cell growth and proliferation. When cells receive specific signals to undergo apoptosis, they initiate internal processes that lead to distinct physiological alterations. The externalization of phosphatidylserine (PS) on the cell surface, the fragmentation and cleavage of particular cellular proteins, the condensation and fragmentation of nuclear chromatin, and, in later stages, the degradation of membrane integrity are all examples of these alterations [31–35]. Annexin V is a protein that has an affinity for phosphatidylserine (PS), a phospholipid predominantly found on the inner side of the cell membrane. In early apoptosis, PS molecules are relocated to the outer surface of the cell membrane, where Annexin V can readily bind to them. By virtue of its ability to bind to externalized PS molecules on the cell membrane surface, Annexin V serves as a commonly employed marker for detecting apoptotic cells [36–38]. Caspases, which are cysteine proteases, assume a pivotal role in the execution of programmed cell death following

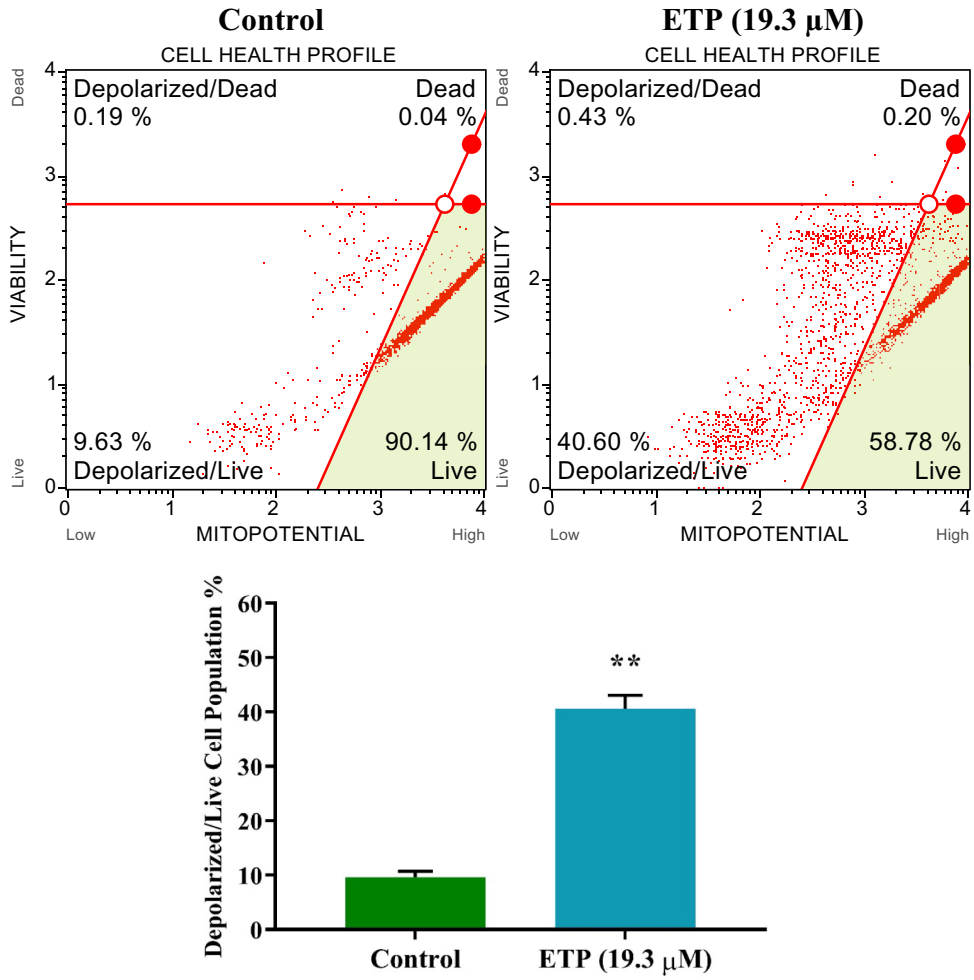

Fig. 8 Evaluation of the effects of ETP-45658 (19.3 µM) on mitochondrial membrane potential in cells compared to the control using flow cytometry. ETP-45658 at a dose of 19.3 µM significantly increased the depolarized/live cell ratio compared to the control (**$p < 0.01$)

proapoptotic signals [39]. Certain caspases primarily serve as initiators of the intracellular signaling cascade, while effector caspases directly facilitate cellular disintegration by cleaving structural proteins (Caspase-3 and Caspase-7). Activation of Caspase-3/7 is therefore a hallmark of apoptosis [40]. The cell cycle is a crucial and fundamental process in eukaryotic cells, playing a vital role in cell growth and the formation of two daughter cells [41]. Because it regulates the repair of genetic damage and inhibits unregulated reproduction, the cell cycle must be precisely regulated for cells to survive. Tumor cells exhibit abnormalities in cell cycle control, and cancer is characterized by a high frequency of mutations in genes that control the cell cycle. Unrestrained cell proliferation is a defining feature of cancer. Inhibiting the cell cycle presents an effective approach to impede the proliferation of cancer cells. The cell cycle is comprised of sequential phases, namely G0/G1, S, G2, and M, which occur in a specific order between cell divisions [42]. The significance of analyzing the cell cycle has grown in importance for understanding the effects of compounds that prevent cancer and exploring the mechanisms underlying cell division [43], [44]. Due to their similar morphological features, mitosis and apoptosis can be assumed to have a direct connection with the cell cycle [45]. Research has demonstrated that the FOXO transcription factor can induce both cell cycle arrest and apoptosis, highlighting its capability to trigger programmed cell death [29]. On the other hand, the G0/G1 checkpoint serves as a protective mechanism that prevents cells with damaged DNA from progressing into mitosis. This checkpoint creates an opportunity for DNA repair and effectively halts the proliferation of cells with genetic damage [42]. Flow cytometry analyses were employed in this study to examine the effects of ETP45658 treatment on HT-29 cells, specifically focusing on cell cycle arrest and apoptosis. The results of our investigation demonstrated that ETP45658 treatment caused cell cycle arrest at the G0/G1 phase and facilitated apoptosis through enhanced Annexin V binding and increased caspase 3/7 activity. Similarly, Richard et al. [29] demonstrated that ETP-45658-induced inhibition of the FOXO-mediated PI3K/AKT/mTOR signaling pathway resulted in G0/G1 cell cycle arrest in various cancers such as breast, osteosarcoma, and

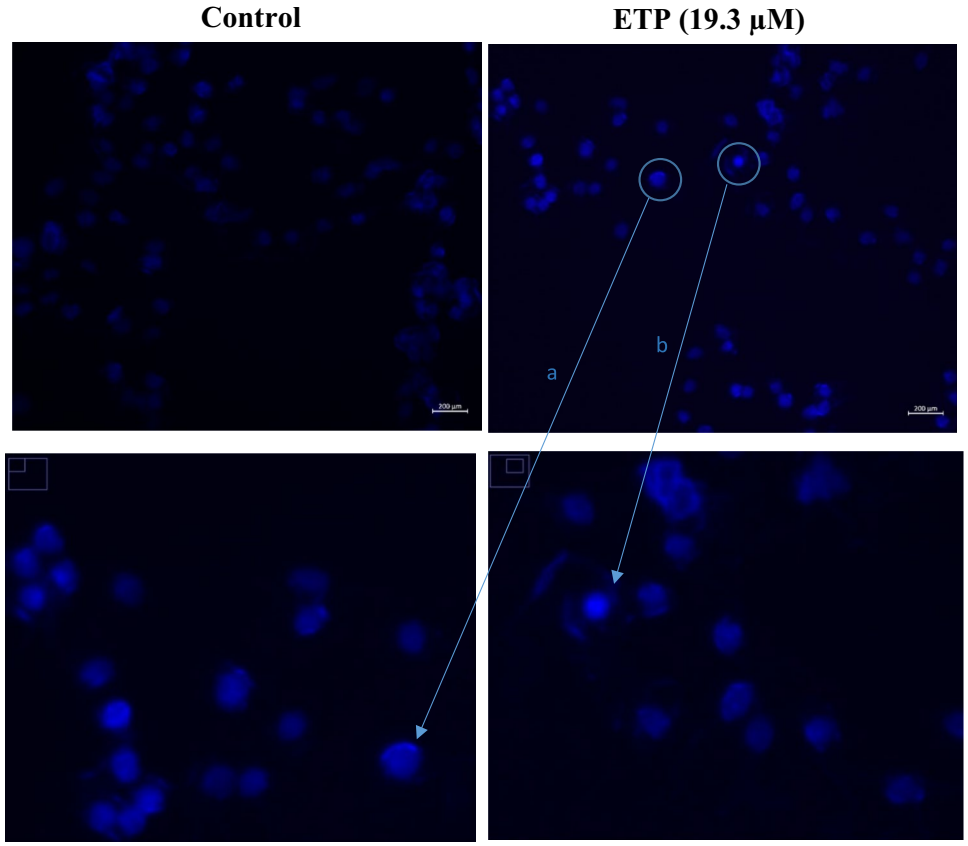

Fig. 9 Evaluation of cells in the ETP-45658 (19.3 µM) group compared to the control using DAPI staining. **a** Read as concentrated nuclear chromatin condensation in a crescent shape on one side of the nuclear membrane. **b** Read as nuclear shrinkage and chromatin condensation indicating an apoptotic state in cell nuclei

colon cancer. In another study, Yang et al. [46] showed that derivatives of ursodeoxycholic acid (UDCA) halted the cell cycle in the G0/G1 phase through PI3K/AKT/mTOR signaling pathway inhibition, leading to apoptosis and exhibiting an antiproliferative effect in hepatocellular carcinoma (HCC) cells. Consistent with our results, Qu et al. [47] demonstrated that Lappaconitine sulfate (LS), a compound inducing PI3K/AKT/mTOR inhibition, arrested the cell cycle in the G0/G1 phase, induced apoptosis, and inhibited cell proliferation in small cell lung cancer. Another study utilizing Imperatorin (IMP), a bioactive coumarin compound, showed similar results to our findings, as it halted the cell cycle in the G0/G1 phase through PI3K/AKT/mTOR inhibition, leading to apoptosis in osteosarcoma cells both in vitro and in vivo [48]. Our data, demonstrating the cell cycle arrest in the G0/G1 phase and induction of apoptosis by ETP-45658, are consistent with the findings mentioned above. Several other studies also support our findings, showing induction of G0/G1 cell cycle arrest and apoptosis by promising anticancer agents [49–52]. ELISA studies were performed to validate the apoptotic effect of ETP-45658 on HT-29 cells by measuring the levels of Bax, cleaved caspase 3, BCL-2, and cleaved PARP. The activation of apoptotic cell death, which is a crucial defense mechanism against cancer development and progression, is a primary objective in cancer treatment [53]. In the apoptotic process, the pro-apoptotic protein Bax plays a role in disrupting the mitochondrial membrane and releasing cytochrome c, which then forms a complex known as the apoptosome. This complex includes cytochrome c, caspase-9, and Apaf-1 (Apoptotic protease-activating factor-1), which activate effector caspases and induce apoptosis [54]. In contrast, BCL-2 maintains membrane stability, inhibits the release of cytochrome c, and suppresses apoptosis [55]. Cell death mechanism is closely related to the upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic BCL-2 proteins. The relative balance between these pro-apoptotic and anti-apoptotic proteins, namely Bax and BCL-2, is frequently employed to evaluate the fate of cells [56], [57]. It has been shown that imbalance in the apoptotic proteins causes mitochondria dysfunction and induces apoptosis [58]. The involvement of caspases in the apoptotic process is vital, particularly through their ability to proteolytically cleave proteins. Caspases are initially synthesized as inactive zymogens within cells. However, upon receiving apoptotic signals, they undergo cleavage, activating their substrates and ultimately promoting apoptosis [59]. Caspase 3 is considered the predominant caspase in the apoptotic process. It plays a crucial role in activating caspase-activated DNase (CAD), an endonuclease responsible for inducing chromosomal DNA fragmentation and chromatin condensation [60]. PARP (Poly ADP-ribose polymerase) is a significant protein involved in

DNA repair pathways, specifically in the repair of base excisions. Its cleavage or inhibition can exploit DNA repair defects, leading to cell death [61]. This protein is also one of the most well-studied substrates of active caspases [53]. Additionally, to investigate whether the cytotoxic effect of ETP-45658 is associated with DNA damage, we analyzed DNA fragmentation in HT-29 cells after 24 h of ETP-45658 treatment using the 8-oxo-dG ELISA method. 8-oxo-dG is a well-known biomarker of oxidative damage in DNA [62]. Our findings demonstrated that ETP-45658 treatment significantly induced levels of 8-oxo-dG in PC3 cells, supporting its cytotoxic and apoptotic effects. In the present study, the apoptotic-inducing effect of ETP-45658 was further confirmed in apoptotic cells through fluorescent imaging of chromatin condensation using DAPI staining, in addition to the ELISA studies. ETP-45658 treatment significantly reduces the expression levels of the anti-apoptotic protein BCL-2, while simultaneously increasing the expression of the pro-apoptotic proteins Bax and cleaved caspase 3. These findings provide strong evidence for the apoptotic impact of ETP-45658 on HT-29 cells. Similar observations have been reported with other inhibitors of the PI3K/AKT/mTOR pathway, where their inhibition resulted in a substantial increase in the expression of Bax and cleaved caspase 3, along with a decrease in BCL-2 expression, across various cancer cell lines [63–65]. Oxidative stress is clearly implicated in cancer, similar to its involvement in numerous other diseases. The level of reactive oxygen species (ROS) in the body is a crucial factor in the development and metastasis of cancer. Moderate levels of ROS can influence the tumor microenvironment by inducing various signaling cascades that initiate angiogenesis, metastasis, and cell survival. Although reactive oxygen species (ROS) can contribute to cancer development, it is noteworthy that elevated levels of ROS can induce apoptosis in cancer cells. This highlights the critical roleof ROS concentrations in determining whether tumor formation or apoptosis occurs [66]. Most anticancer drugs work by producing oxidative stress, which is thought to be the main factor in most of the cell's macro-molecular alterations. ROS can target proteins, membrane lipids, and DNA among these macromolecules [67]. TOS, which is a parameter used to assess oxidative stress, is commonly utilized to estimate the overall oxidation status of the body [13]. Likewise, TAS is employed to measure the overall antioxidant capacity of the body [68]. In this study, we investigated whether ETP-45658 induces a cytotoxic effect by modulating TAS and TOS levels. Our findings revealed that a 24-hour exposure to ETP-45658 significantly elevated TOS levels and concurrently decreased TAS levels compared to the control group cells. This observation suggests that treatment with ETP-45658 induces oxidative stress in HT-29 cells, as indicated by the increased TOS and decreased TAS levels. Mitochondria, known as the powerhouses of the cell, play a vital role in maintaining cellular energy balance and are primarily responsible for generating reactive oxygen species (ROS). They also contain important regulators of cell death processes, including apoptosis. Alterations in mitochondrial structure and function serve as sensitive indicators of cellular well-being and stress. Through mitochondrial respiration, cellular energy is produced and stored as an electrochemical gradient across the mitochondrial membrane. In healthy cells, this energy accumulation establishes a mitochondrial transmembrane potential that drives ATP synthesis. However, in the early stages of apoptosis, there is a loss of inner mitochondrial transmembrane potential, often associated with the opening of mitochondrial permeability transition pores. This event leads to the release of cytochrome c into the cytosol, triggering the apoptotic cascade. Changes in mitochondrial membrane potential are implicated in various cell death processes, including apoptosis, necrosis, and caspase-independent cell death. The depolarization of the inner mitochondrial membrane potential serves as a reliable indicator of mitochondrial dysfunction and cellular health. It plays an increasingly important role in studies related to apoptosis, drug toxicity, and the understanding of various disease conditions. Excessive ROS production in conjunction with mitochondrial membrane damage leads to the activation of the intrinsic pathway of apoptosis [69]. Considering our findings on mitochondrial membrane potential, it can be said that ETP-45658 induces apoptosis through the mitochondrial pathway. Consistent with our findings, Chen et al. [70] demonstrated the induction of ROS-mediated apoptosis in gallbladder cancer through PI3K/AKT/mTOR inhibition. Similarly, itraconazole, an antifungal agent, induced apoptosis in hepatocellular carcinoma cells by increasing ROS levels and reducing mitochondrial membrane potential through PI3K/AKT/mTOR inhibition [71]. Zuo et al. [72] showed that voacamine induced apoptosis in breast cancer cells through increased ROS levels and decreased mitochondrial membrane potential via PI3K/AKT/mTOR inhibition. Furthermore, Hambright et al. [73] found that inhibition of the PI3K/AKT/mTOR pathway induced ROS formation in melanoma cells.Consistent with the relevant literature, our current findings suggest that oxidative stress and loss of mitochondrial membrane potential can contribute to ETP-45658-induced apoptosis. The findings of the current study and relevant literature indicate that the PI3K/AKT/mTOR pathway plays a significant role in the development and progression of human cancers, and its inhibition is particularly associated with the reduction of cancer cell growth. The results presented in our study demonstrate the potential of ETP-45658 as an innovative treatment for colon cancer, warranting further evaluation in this direction. However, there is a lack of sufficient research on the possible mechanisms of action of ETP-45658 in colon cancer, and further

studies are needed both in vitro and in vivo to investigate its effects on colon cancer.

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Declarations

Competing interests The authors disclose that they do not possess any conflicts of interest.

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