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RESEARCH ARTICLE

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Zosterabisphenone B, a new diarylheptanoid heterodimer from the seagrass *Zostera marina*, induces apoptosis cell death in colon cancer cells and reduces tumour growth in mice

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Abstract

Colorectal cancer (CRC) is one of the most common malignant tumours worldwide. Diarylheptanoids, secondary metabolites isolated from *Zostera marina*, are of interest in natural products research due to their biological activities. Zosterabisphenone B (ZBP B) has recently been shown to inhibit the viability of CRC cells. The aim of this study was to investigate the therapeutic potential of ZBP B for targeting human CRC cells. Cell viability was determined using the MTT assay. Flow cytometry and Western blot analyses were used to assess apoptosis and autophagy. A CRC xenograft model was used to evaluate the in vivo effect of ZBP B. No cytotoxic effect on HCEC cells was observed in the *in vitro* experiments. ZBP B caused morphological changes in HCT116 colon cancer cells due to an increase in early and late apoptotic cell populations. Mechanistically, ZBP B led to an increase in cleaved caspase-3, caspase-8, caspase-9, PARP and BID proteins and a decrease in Bcl-2 and c-Myc proteins. In the xenograft model of CRC, ZBP B led to a reduction in tumour growth. These results indicate that ZBP B exerts a selective cytotoxic effect on CRC cells by affecting apoptotic signalling pathways and reducing tumour growth in mice. Taken together, our results suggest that ZBP B could be a lead compound for the synthesis and development of CRC drugs.

KEYWORDS
apoptosis, autophagy, colorectal cancer, marine compounds, phytochemicals, xenografts

1 | BACKGROUND

Several marine-derived drugs have been approved by the Food and Drug Administration (FDA) and other global regulatory agencies for the treatment of various cancers. There are about 240,000 known marine species, including various types of organisms: *Animalia*, *Bacteria*, *Plantae*, *Protozoa*, *Archaea*, *Fungi* and so forth, and they could offer numerous and interesting new bioactive compounds against cancer (Santaniello et al., 2022).

Zostera marina L (Zosteraceae) is the most widespread temperate seagrass and is widely distributed in the Northern Hemisphere. It is one of the best-studied seagrass species in the world, both in terms of the diversity of natural products and their bioactivity. Most of the natural compounds reported are phenolics, mainly flavonoids and sulphated flavonoids (Zidorn, 2016). Other phenols described from *Z. marina* are simple phenylpropanoids, caffeic acid, coumaric acid,

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ferulic acid, zosteric acid and rosmarinic acid (Sævdal Dybsland et al., 2021). For these compounds, several studies have investigated the anticoagulant, anti-inflammatory, antiviral and anti-tumour activities (Sevimli-Gur & Yesil-Celiktas, 2019; Teles et al., 2018).

Recently, a new class of secondary metabolites from *Z. marina* has also been described. This includes seven diarylheptanoids: one tricyclic diarylheptanoid (isotedarene A), three tetracyclic diarylheptanoids, tedarene B, zosteraphenol A and B, and three unique diarylheptanoid dimers, ZBPs A, B, and C (Grauso et al., 2020; Grauso et al., 2022; Li et al., 2021). Diarylheptanoids consist of two phenolic aromatic rings linked by a linear seven-carbon chain, and they are classified into linear, cyclic and dimeric diarylheptanoids or diarylheptanoids with special moieties.

Diarylheptanoids are mainly found in the roots, rhizomes and bark of various genera of plants such as *Alpinia*, *Zingiber*, *Curcuma* and *Alnus* species. In the last 20 years, they have become of interest in natural products research due to their remarkable anticancer, antiemetic, estrogenic, antimicrobial and antioxidant activities (Ganapathy et al., 2019). Ginger is one of the major producers of diarylheptanoids in nature. Diarylheptanoids isolated from *Zingiber officinale* rhizomes showed cytotoxic effects against five tumour cell lines (A549, HepG2, HeLa, MDA-MB-231 and HCT116) by affecting DNA damage signalling pathway.

In a previous paper, it was reported that zosterabisphenones isolated from *Z. marina* exerted a cytotoxic effect on a human colon carcinoma cell line (HCT116), with zosterabisphenone B (ZBP B) being more effective and potent (Li et al., 2021). In this study, we thoroughly investigated the anti-tumour activity of ZBP B against the human colon cancer cell line HCT116 and in vivo using a human colon cancer xenograft model.

2 | MATERIALS AND METHODS

2.1 | Drugs

Zosterabisphenone B (ZBP B) (Figure 1a) is a dimeric diarylheptanoid found in the seagrass *Z. marina*. The biological source, isolation, purification and characterization of ZBP B have been reported in detail in a previous paper (Li et al., 2021). The purity of the sample of ZBP B used for the experiments was judged >98% by HPLC analysis as well as by ^{1}H NMR spectroscopy (see Supplementary Material S1). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was obtained from Sigma-Aldrich (Milan, Italy). All cell culture reagents were purchased from Sigma-Aldrich (Milan, Italy), Bio-Rad Laboratories (Milan, Italy) and Aurogene Srl (Rome, Italy). The vehicles used for the in vitro (0.1% v/v ethanol) and in vivo (ethanol/Tween 20/saline in a 5:1:4 ratio) experiments had no effect on the responses under study.

2.2 | Cell lines

The human colon adenocarcinoma cell line (HCT116) was purchased from the American Type Culture Collection (ATCC from LGC Standards, Milan, Italy). The immortalised healthy human colonic epithelial

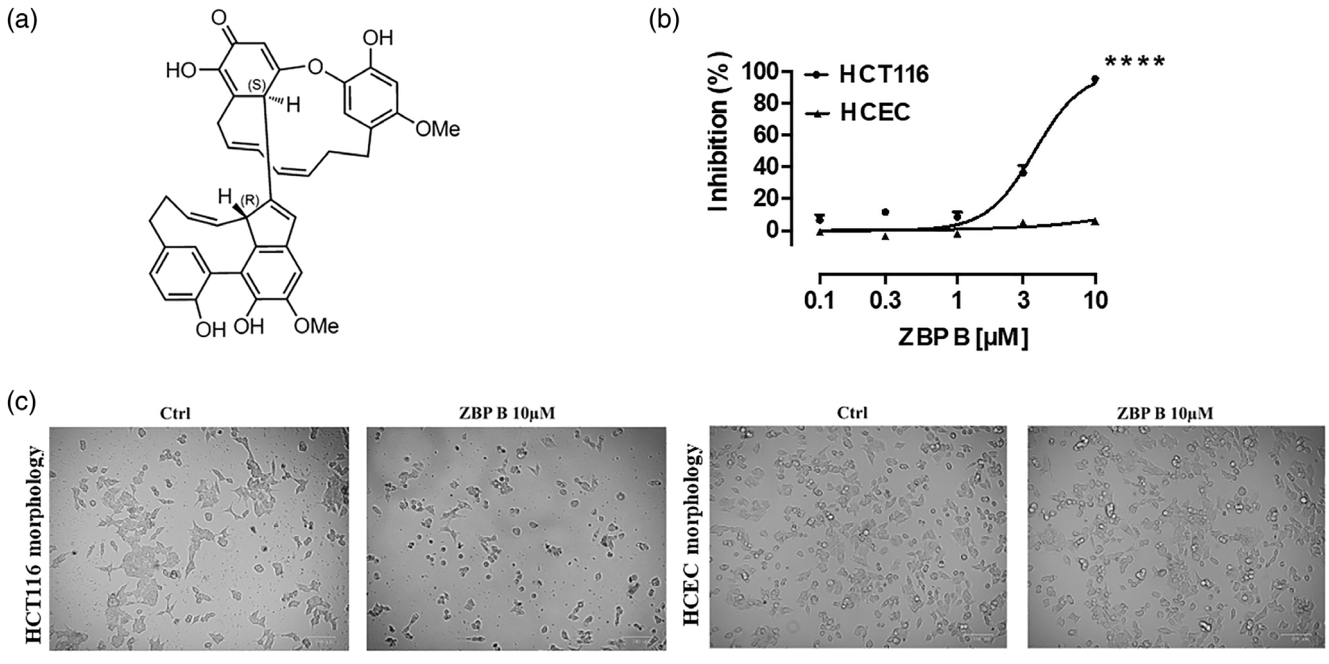

FIGURE 1 (a) Chemical structure of zosterabisphenone B (ZBP B). (b) Effect of different concentrations of ZBP B (0.1–10 μM) in HCT116 and HCEC cells, expressed as a percentage of cell viability inhibition. ****$P < 0.0001$ as assessed by two-way ANOVA. Each value represents the mean ± SEM of 3 experiments including 5–6 replicates for each treatment. (c) Representative images of morphological changes in HCT116 and HCEC cells. The cells were incubated in the absence (vehicle) or presence of 10 μM ZBP for 48 h (×100).

cells (HCEC), derived from human colon biopsies, were kindly gifted by Fondazione Callerio Onlus (Trieste, Italy). HCT116 cells (passage 15–25) were cultured in McCoy's 5A medium (catalog number 10-050-CV, Corning) while HCEC (passage 14–18) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Milan, Italy) both supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, Milan, Italy) 100 U/mL penicillin, and 100 μg/mL streptomycin (Euroclone). Cell lines were cultured at 37°C in a humidified incubator with 5% CO_2.

2.3 | Cell viability assay

The MTT assay was used to assess cell viability. Briefly, HCT116 cells (5×10^3 cells/well) and HCEC cells (1×10^4 cells/well) were seeded in 96-well culture plates for 24 h in medium containing 10% FBS. Cells were then treated with or without ZBP B at concentrations of 0.1–10 μM for 48 h in medium containing 1% FBS. At the end of the treatment period, a stock MTT solution (250 μg/mL) was added to each well. After an incubation period of 1 h at 37°C, the intracellular formazan crystals were dissolved with DMSO and the absorbance of the solution was measured at 570 nm using a microplate reader (Cytation 3, BioTek Instruments, Inc.). The morphological changes of the cells were recorded with the ZOE™ Fluorescent Cell Imager (Bio-Rad).

2.4 | Apoptosis assay

Apoptosis was assessed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, CA, USA) according to the manufacturer's instructions. In brief, HCT116 seeded in 6-well plates at a density of 4×10^5 cells/well, were treated with ZBP B (3–10 μM) in medium containing 1% FBS for 24 h. After the treatment period, cells were detached by trypsinisation, washed with PBS and incubated in 200 μL binding buffer 1× containing 5 μL Annexin V-FITC and 5 μL PI for 15 min. Viable cells, early apoptotic cells, late apoptotic cells and necrotic cells were detected by flow cytometry analysis (BD Accuri C6).

2.5 | Protein extraction and Western blot analysis

Whole-cell extracts were prepared from HCT116 cells (1×10^6 cells) treated or not with ZBP B (10 μM, 24 h). Briefly, cells were lysed in RIPA buffer (20 mM Tris-Cl [pH 7.5], 1 mM Na_2EDTA, 150 mM NaCl, 1% NP-40, 1% [v/v] sodium deoxycholate) supplemented with protease (Roche, Monza, Italy) and phosphatase inhibitors (Sigma-Aldrich, Milan, Italy). Thirty-sixty micrograms of protein extract were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated for 1 h at room temperature (RT) with blocking buffer solution Tris-buffered saline (TBS) solution containing 25 mM Tris (pH 7.4), 150 mM NaCl, and 5% non-fat dry milk (Bio-Rad) under a gentle shaker. Blot membranes were cut using standard band positions and then incubated with the appropriate antibodies. Membranes were then incubated overnight at 4°C with specific primary antibodies: cleaved caspase-8 (#9496 Cell Signaling Technology Inc., USA, 1:1000), cleaved caspase-9 (#20750 Cell Signaling Technology, 1:1000), cleaved caspase-3 (#9664 Cell Signaling Technology, 1:1000), cleaved PARP (#5625, Cell Signaling Technology, 1:1000), c-Myc (#18583, Cell Signaling Technology, 1:1000), Bcl-2 (E-AB-22004, 1:1000), BID (#2002, Cell Signaling Technology, 1:1000), anti-tubulin (1:20,000, E-AB-20036, Elabscience Biotechnology Inc.); anti-actin (1:20,000, sc-47778, Santa Cruz Biotechnology Inc., USA). After 1 h incubation with HRP-conjugated secondary antibodies (anti-rabbit, #7074 or anti-mouse, #7076.) the immunoreactive bands were detected on x-ray films using an enhanced chemiluminescent kit and quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.6 | Animals

Female BALB/c nude mice (age 6–8 weeks, 24–26 g) were purchased from Charles River (Sant'Angelo Lodigiano, Italy). Mice were housed in the conventional animal house of the Department of Molecular Medicine and Biotechnologies of the University of Naples Federico II (Naples, Italy) and kept in cages equipped with additional environmental conditions. The mice were fed ad libitum with sterile mouse chow and kept under pathogen-free conditions in IVC cages. All experimental procedures and protocols were performed in compliance with European (EEC Directive 2010/63) and Italian (D.L. 4 March 2014 n. 26) legislation. All studies involving animals are reported in accordance with the guidelines of ARRIVE. Every effort has been made to minimise the number of animals used and their suffering.

2.7 | Xenograft model

The xenograft model of CRC was performed by subcutaneous injection of HCT116 cells (2.5×10^6, 200-μL PBS) into the back of BALB/c nude mice (Charles River, Sant'Angelo Lodigiano, Italy). Once the tumours reached a volume of about 200 mm^3, the mice were randomly divided into a control and a treatment group. The control group received a peritumoural injection of a vehicle solution (ethanol/Tween20/saline in a ratio of 5:1:4), while the treatment groups received a daily peritumoural injection of 20 mg/kg ZBP B for the entire duration of the experiment. The peritumoural route of administration was chosen because the therapeutic treatment of CRC consists of the combination of immunotherapeutic and chemotherapeutic drugs and peritumoural injection has proven to be the best route of administration for combined immuno-chemotherapy to avoid systemic side effects and to achieve a direct effect on the tumour cells (Ma et al., 2021; Wu et al., 2017). Tumour size was measured daily with a

digital calliper and tumour volume was calculated using the modified ellipsoid volume formula (volume = $\pi/6 \times$ length $\times$ width^2). Six days after the start of treatment, the mice were humanely killed, the tumours removed, measured (volume), weighted and stored at −80°C for later analysis. All outcome assessments were performed in blind.

2.8 | Statistical analysis

Statistical analysis, conducted using GraphPad Prism 9.2.0, was performed using Student's *t*-test for comparing a single treatment mean with a control mean and one-way ANOVA or two-way ANOVA followed by Dunnett's multiple comparisons test for the comparison of multiple groups. Two-way ANOVA was used to compare different concentration/day-effect curves. A *p*-value <0.05 was considered statistically significant. Data were expressed as mean ± mean standard error (SEM) of n experiments. The study was designed to form groups of equal size, with randomisation and blinded analysis.

3 | RESULTS

3.1 | ZBP B cytotoxic effect is selective for colon cancer cells

The cytotoxic effect of ZBP B against the colon human cancer cell line HCT116, as well as its selectivity against another cancer cell line, was previously investigated using the MTT assay (Li et al., 2021). Here, we investigated the anti-tumour effect of ZBP B in detail and evaluated its selectivity compared to healthy cells. Exposure of the human healthy colonic epithelial cell line (HCEC) to various concentrations of ZBP B (0.1–10 μM) for 48 h resulted in a significant decrease in cell viability only at the highest concentrations tested [% cell viability (mean ± SEM): control, 100 ± 0.68; ZBP B 0.1 μM, 101 ± 1.33; ZBP B 0.3 μM, 103 ± 1.17; ZBP B 1 μM, 102 ± 1.21; ZBP B 3 μM, 94.5 ± 0.90**; ZBP B 10 μM, 94 ± 1.25***; **$P < 0.01$, ***$P < 0.001$; three experiments including 5–6 replicates for each treatment]. Compared to HCT116 cells, the cytotoxic effect of ZBP B was less effective and potent (HCEC % of viability: 95% and 94% at 3 and 10 μM, respectively) (Figure 1b). The selective cytotoxic effect of ZBP B on HCT116 compared to HCEC was also demonstrated by morphological analysis. Indeed, treatment of HCEC and HCT116 cells with 10 μM ZBP B for 48 h resulted in morphological cellular changes indicative of cell death and growth inhibition only in HCT116 cells; no morphological changes were observed in HCEC (Figure 1c).

3.2 | ZBP B induces apoptosis in CRC cells

To gain insight into the function of ZBP B in triggering cell death, we performed apoptosis studies. HCT116 cells treated with or without ZBP B were stained with Annexin V-FITC/PI and analysed by flow cytometry. Analysis of the results showed that apoptotic cells accounted for 7.6% and 15.1% of cells in early apoptosis (lower right quadrant) and for 18.9% and 43.5% of cells in late apoptosis (upper right quadrant) following a 24 h treatment with 3 and 10 μM ZBP B, respectively (Figure 2a, b). The pro-apoptotic effect of ZBP B was further confirmed by the increased expression of cleaved caspase-3 and its downstream effector PARP1 (Figure 2c).

3.3 | ZBP B activates both the intrinsic and the extrinsic pathways of apoptosis in colon cancer cells

To date, it is known that there are two main pathways of apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. To explore the molecular mechanism(s) by which ZBP B induces apoptosis of HCT116 cells, the levels of key proteins involved in both pathways were measured by western blotting. Our results showed that caspase-8, the key initiator caspase in the death receptor-dependent apoptosis pathway (Huang et al., 2016), and its downstream substrate BID were cleaved in HCT116 cells treated with ZBP B (Figure 3a). As shown in Figure 3b, ZBP B also promoted the induction of the mitochondrial apoptotic pathway, as indicated by the increased levels of cleaved caspase-9 and the decreased protein expression levels of the anti-apoptotic protein Bcl-2 in HCT116 cells (Figure 3b). The c-Myc protein has been shown to be an important regulator of the apoptotic pathway (Huang et al., 2022). We sought to investigate the effect of ZBP B on c-Myc expression. As shown in Figure 3b, protein expression of c-Myc was also decreased by treatment with ZBP-B in HCT116 cells. These results suggest that ZBP B regulates both the intrinsic and extrinsic pathways of apoptosis in HCT116 cells.

3.4 | ZBP B induced autophagic cell death in colon cancer cells

There is evidence that autophagy and apoptosis occur simultaneously in the cell and may have synergistic effects in inducing cancer cell death (Xie et al., 2020). To investigate whether ZBP B was able to trigger autophagic cell death in HCT116 cells, we analysed the conversion of LC3 from LC3A (cytosolic form) to a lipidated LC3B form (autophagosome membrane-bound form). As shown in Figure 4a, treatment with ZBP B resulted in accumulation of LC3B protein in HCT116 cells. We also examined the level of the autophagic marker p62. As shown in Figure 4b, treatment with ZBP B promoted the upregulation of p62. Our results suggest that ZBP B induces autophagic cell death in HCT116 cells.

3.5 | ZBP B significantly reduces tumour growth in xenograft mice

To investigate the anticancer effect of ZBP B in vivo, we used a human colon cancer xenograft model in which HCT116 cells were

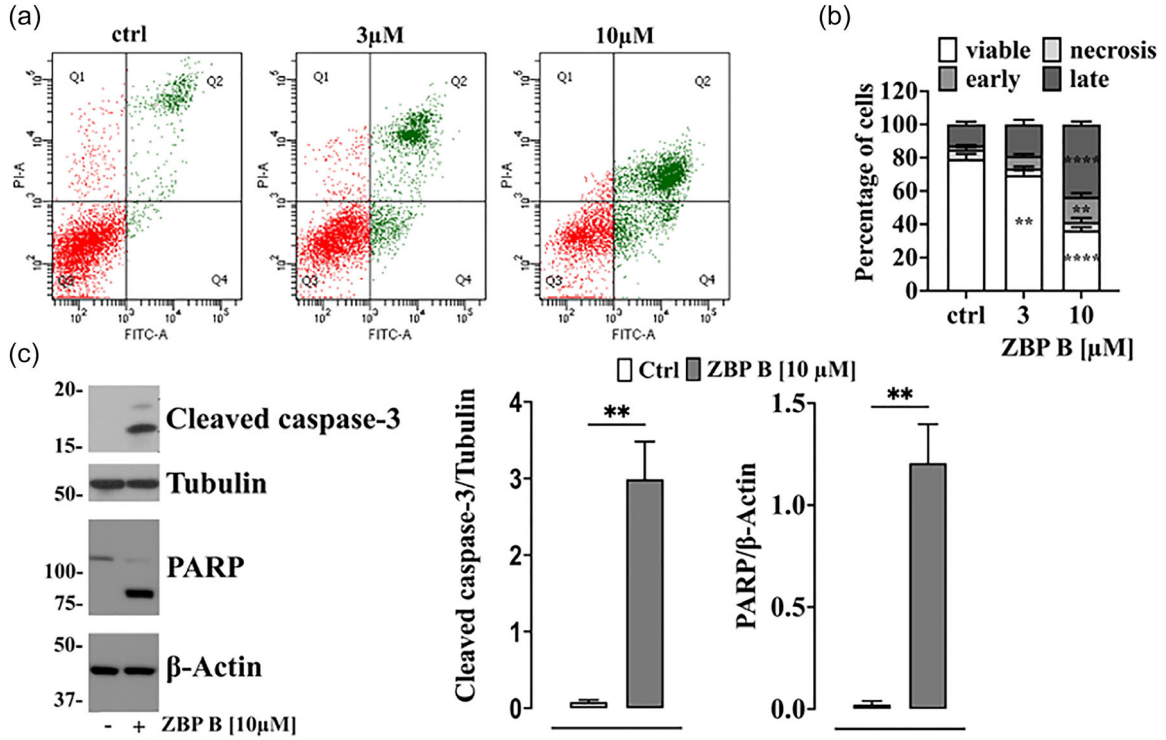

FIGURE 2 (a) Flow cytometry of Annexin V and PI double staining of HCT116 cells treated or not with zosterabisphenone (ZBP) B at different concentrations (3 and 10 M) for 24 h. (b) Columns show the percentage of viable, early apoptotic, late apoptotic and necrotic HCT116 cells incubated or not with 3 and 10 μM of ZBP B for 24 h. Values are expressed as mean ± SEM of 3 experiments including 3 replicates; $^{**}p < 0.01$ and $^{****}p < 0.0001$ versus control (Ctrl, vehicle) as assessed by two-way ANOVA followed by Dunnett's multiple comparisons test. (c) Representative immunoblots and densitometric analysis of cleaved caspase-3 and PARP protein expression in HCT116 cells treated or not with 10 μM ZBP B for 24 h. Values are expressed as mean ± SEM of 3 experiments including 3 replicates; $^{**}p < 0.01$ versus control (Ctrl, vehicle) as assessed by Student's *t*-test.

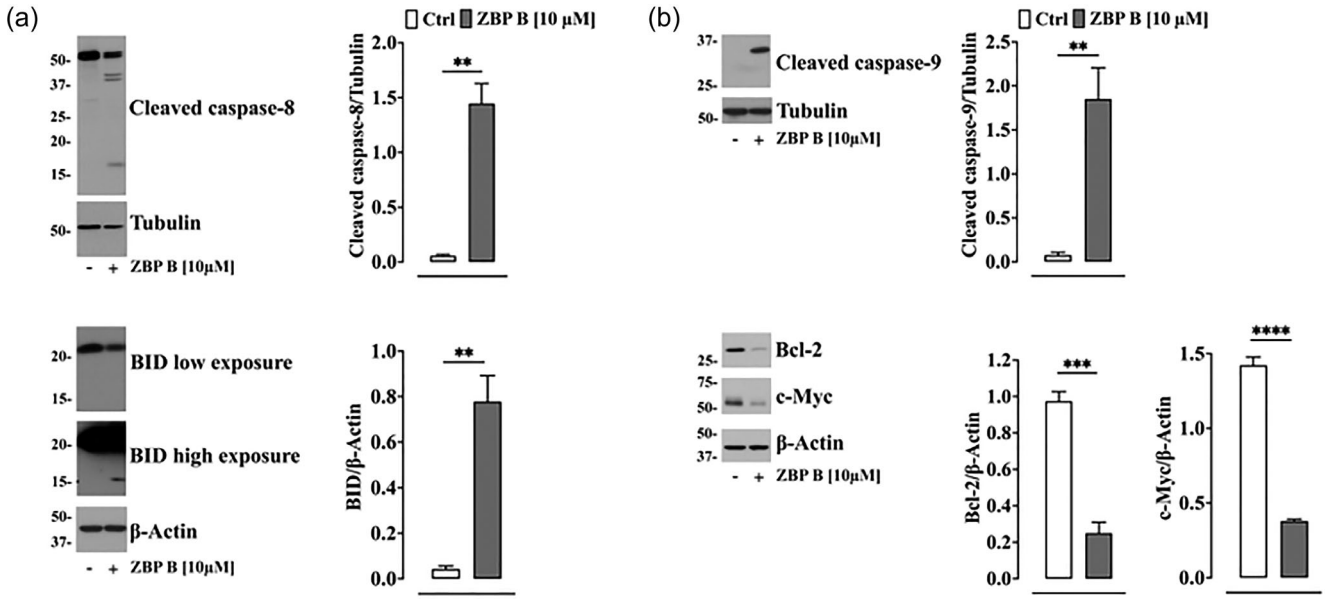

FIGURE 3 Effect of zosterabisphenone (ZBP) B on the protein expression of apoptotic related proteins. Representative immunoblots and densitometric analysis of (a) cleaved caspase-8 and BID, (b) cleaved caspase-9, Bcl-2 and c-Myc protein expression in HCT116 cells treated or not with ZBP B (10 μM). Protein expression was evaluated by Western blot analysis and normalized to a housekeeping protein. Values are expressed as mean ± SEM of 3 experiments including 3 replicates; $^{**}p < 0.01$ and $^{****}p < 0.0001$ versus control (Ctrl, vehicle) as assessed by Student's *t*-test.

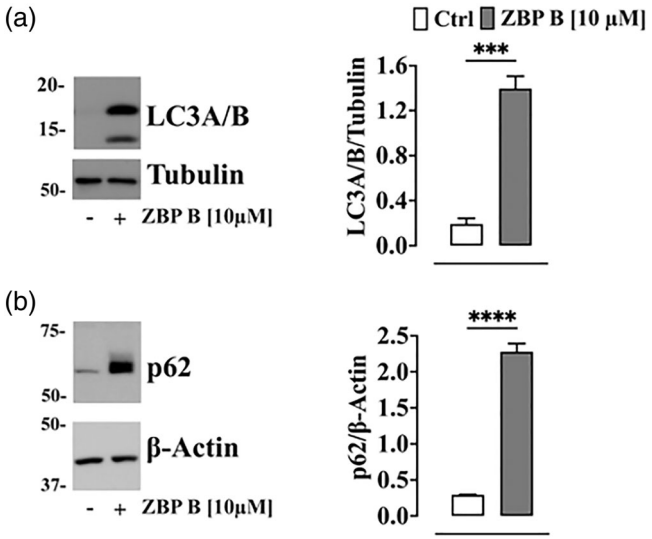

FIGURE 4 Effect of zosterabisphenone (ZBP) B on the protein expression of autophagic related proteins. Representative immunoblots and densitometric analysis of (a) LC3A/B and (b) p62 protein expression in HCT116 cells treated or not with ZBP B (10 µM). Protein expression was evaluated by Western blot analysis and normalized to the housekeeping protein. Values are expressed as mean ± SEM of 3 experiments including 3 replicates; ***$p < 0.001$ and ****$p < 0.0001$ versus control (Ctrl, vehicle) as assessed by Student's *t*-test.

transplanted into the back of immunodeficient mice to induce tumour development. ZBP B was injected peritumourally every day (20 mg/kg). As shown in the Figure 5, 6 days after the first administration, ZBP B significantly reduced tumour volume compared to the vehicle group. Similarly, the volume and weight of explanted tumours from ZBP B-treated mice were significantly reduced compared to the control group.

4 | DISCUSSION

Numerous studies have reported that seaweeds are rich in secondary metabolites that are not only economically important in the field of food additives and nutraceuticals, but also possess a variety of biological activities in the pharmaceutical industry (e.g., antibacterial, antifungal, anticancer and anti-inflammatory) (Li et al., 2021; Ioanna et al., 2005; Ioanna et al., 2008; Kuo-Feng et al., 2006; Cliff et al., 2008; Khotimchenko et al., 2012). We have previously shown that ZBP B is a bioactive compound with cytotoxic effects on human colon cancer cells (HCT116). To date, the mechanism by which ZBP B exerts its anti-tumour effect on colon cancer cells is unknown. We have shown here for the first time that the diarylheptanoid heterodimer ZBP B isolated from the seaweed *Zostera marina* is able to induce morphological changes in the colon cancer cell line HCT116, and that

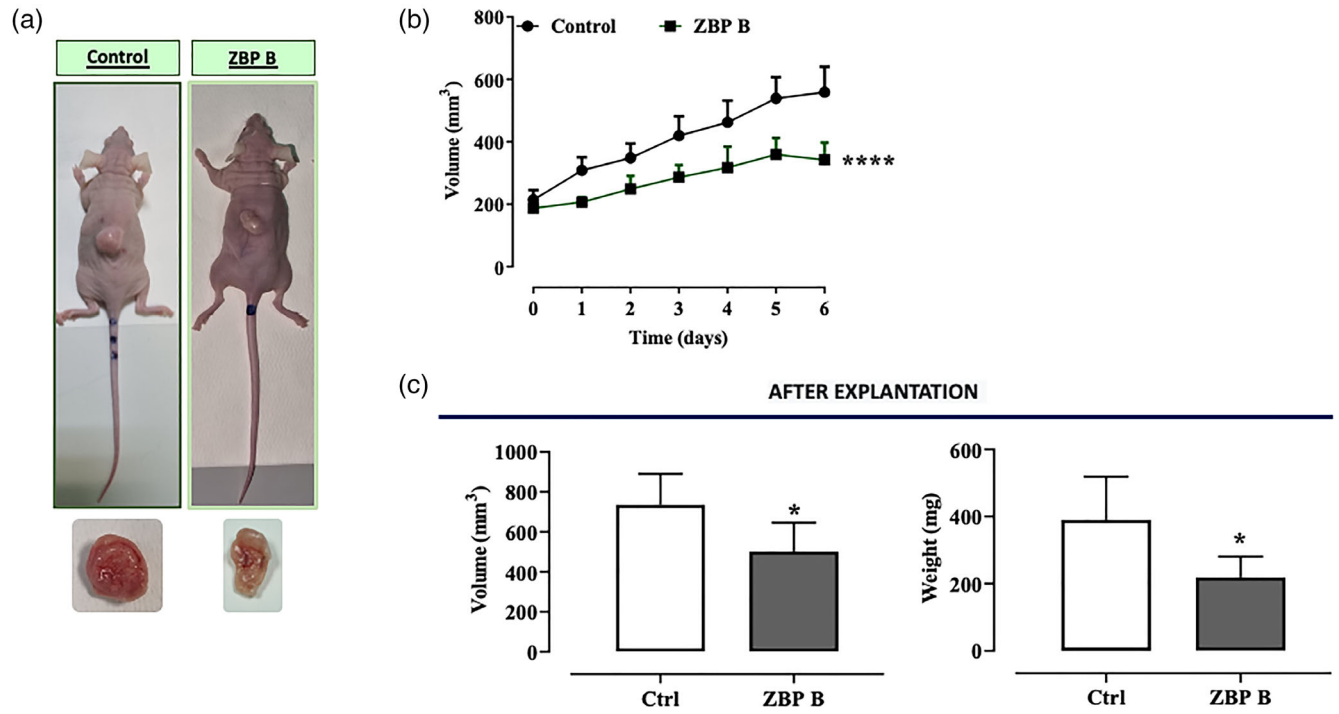

FIGURE 5 Effect of zosterabisphenone (ZBP) B on tumour growth in the xenograft model of CRC. Xenograft was performed by subcutaneous injection of HCT 116 cells (2.5×10^6, 200-µL PBS) into the back of BALB/c nude mice. Treatment started 8 days after cell inoculation (i.e., once tumours had reached a size of about 200 mm^3). Tumour size was measured each day with a digital caliper and tumour volume was calculated. ZBP B (20 mg/kg, peritumoural) was administered every day for the entire duration of the experiment. (a) Representative images of animals with tumours and of explanted tumours treated or not with ZBP B. (b) Analysis of tumour volume of mice receiving vehicle (control) or ZBP B in a 6-day time course. Each dot represents the means ± SEM of 5/6 mice; ****$p < 0.0001$ versus control as assessed by Two-way ANOVA. (c) Analysis of tumour volume and weight of the explanted tumours from mice treated with or not with ZBP B. *$p < 0.05$ versus control (Ctrl, vehicle) as assessed by Student's *t*-test.

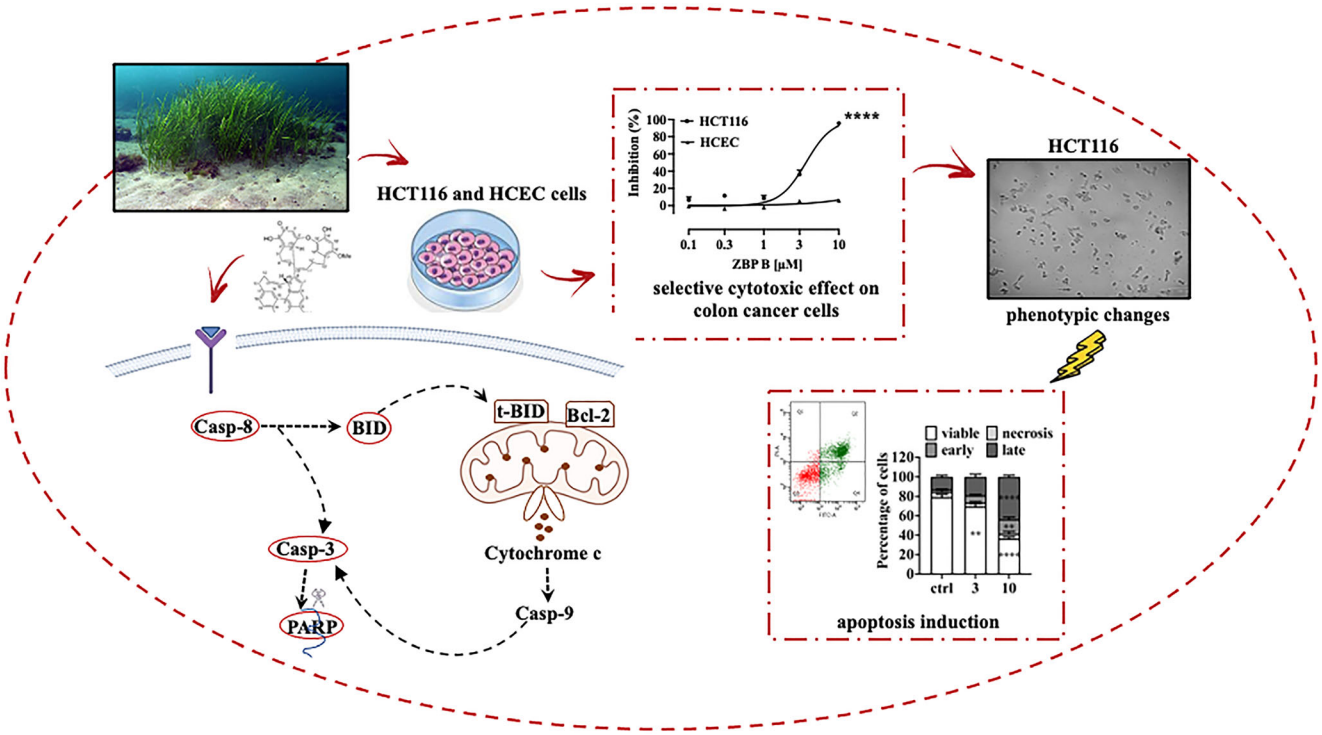

FIGURE 6 Proposed schematic representation of zosterabisphenone (ZBP) B-mediated effects in HCT116 cells. ZBP B-induced apoptosis suggests a central role of apoptosis in its cytotoxic effects.

these morphological changes are associated with the induction of the apoptotic pathway, as demonstrated by flow cytometry and Western blot analyses. We also showed that ZBP B induces autophagic cell death in HCT116 cells. When we investigated the cytotoxic activity of ZBP B on normal immortalised human colon epithelial cells (HCEC), we found that this compound has an excellent safety profile, as the cytotoxic effect is selective against cancer cells. Furthermore, in vivo studies showed that ZBP B was able to reduce the growth of human colon cancer xenografts in mice.

It is well known that chemotherapeutic agents can induce cell death through various mechanisms (Okada & Mak, 2004; Qi et al., 2022). Among them, apoptosis or programmed cell death is a multistep process characterised by specific morphological and biochemical changes such as cell shrinkage, cell condensation, increased cytoplasmic density, protein-DNA degradation and phagocytosis (Kari et al., 2022). Consistent with these observations, we detected morphological changes in HCT116 ZBP B-treated cells using light microscopy, indicating activation of the apoptotic pathway. Indeed, after exposure to 10 μM ZBP B, HCT116 cells had a round shape and floated in the cell culture medium. Using flow cytometry and western blot assay, we confirmed that ZBP B induced apoptosis by leading to an increase in both early and apoptotic cell populations and cleaving caspase 3 and its substrate poly (ADP-ribose) polymerase 1 (PARP1). Apoptosis can occur via two major signalling pathways, namely the cell death receptor (extrinsic pathway) involving caspase 8 and the mitochondrial or the ER stress-mediated (intrinsic pathway) involving caspase 8 and 9 (Ashkenazi, 2002; Fan et al., 2005; Gupta, 2003; Thornberry & Lazebnik, 1998). Interestingly, exposure of HCT116 cells to ZBP B resulted in a marked increase in cleaved caspase-8, cleaved caspase-9 and their substrate BID and a concomitant reduction in Bcl-2 protein expression levels, suggesting simultaneous induction of both the extrinsic and intrinsic pathways.

C-Myc is a proto-oncogene that is increased in more than 70% of CRC (Arango et al., 2003) and is involved in cell growth, proliferation, cell cycle, metabolism and apoptosis in cancer cells (Dang, 2013). Our results showed that ZBP B decreased the expression of c-Myc, which is another indication of a possible mechanism for its anti-tumour effect.

Autophagy and apoptosis are both typical types of programmed cell death and have a reciprocal relationship in anti-tumour therapy (Xie et al., 2020). Consistent with these findings, we have shown that treatment with ZBP B promotes an increase in LC3B and p62 expression, both of which are required for the induction of autophagy (Puissant et al., 2010). To translate the in vitro results in vivo, we used a colon cancer xenograft model obtained by subcutaneous injection of human HCT116 cells into BALB/c nude mice. We showed that ZBP B inhibited tumour growth by about 40% compared to the control.

We speculated that ZBP B acts by cleaving caspase-8, which in turn activates Bid at Asp60, leading to translocation of the carboxyl-terminal p15 fragment (tBid) to the outer mitochondrial membrane. Translocation of Bid is accompanied by the release of cytochrome c from the mitochondria and leads to complex formation with Apaf-1 and caspase-9, resulting in caspase-9 activation. ZBP B thus transmits an apoptotic signal from the cell surface to the mitochondria and

triggers caspase activation. However, this is only a hypothesis that still needs to be confirmed (Figure 6).

In summary, this study shows that ZBP B both triggers caspase-dependent apoptosis in HCT116 cells by activating intrinsic and extrinsic signalling pathways and downregulates c-Myc expression. Finally, ZBP B reduces the growth of CRC in vivo. Taken together, our results suggest that ZBP B may represent a novel compound that could be used in its current form or as a lead compound for the synthesis and development of CRC drugs.

AUTHOR CONTRIBUTIONS

Nunzio Antonio Cacciola: Conceptualization; methodology; writing – original draft. **Paola De Cicco:** Conceptualization; methodology; writing – original draft. **Rebecca Amico:** Methodology. **Fabrizia Sepe:** Methodology. **Yan Li:** Methodology. **Laura Grauso:** Methodology. **Maria Francesca Nanì:** Methodology. **Silvia Scarpato:** Methodology. **Christian Zidorn:** Methodology; writing – review and editing. **Alfonso Mangoni:** Conceptualization; writing – review and editing. **Francesca Borrelli:** Conceptualization; data curation; funding acquisition; supervision; validation; visualization; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this research has been included in this published study.

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REFERENCES

Arango, D., Mariadason, J., Wilson, A., Yang, W., Corner, G. A., Nicholas, C., Aranes, M. J., & Augenlicht, L. H. (2003). c-Myc overexpression sensitises colon cancer cells to camptothecin-induced apoptosis. *British Journal of Cancer*, *89*, 1757–1765.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nature Reviews. Cancer*, *2*(6), 420–430.

Cliff, R., Melany, P. P., & Valerie, J. P. (2008). Antifungal defenses of seagrass from the Indian River lagoon, Florida. *Aqua Bot*, *88*, 134–141.

Dang, C. V. (2013). MYC, metabolism, cell growth, and tumourigenesis. *Cold Spring Harbor Perspectives in Medicine*, *3*(8), a014217.

Fan, T. J., Han, L. H., Cong, R. S., & Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica et Biophysica Sinica (Shanghai)*, *37*(11), 719–727.

Ganapathy, G., Preethi, R., Moses, J. A., & Anandharamakrishnan, C. (2019). Diarylheptanoids as nutraceutical: A review. *Biocatalysis and Agricultural Biotechnology*, *19*, 101109.

Grauso, L., Li, Y., Scarpato, S., Cacciola, N. A., De Cicco, P., Zidorn, C., & Mangoni, A. (2022). A cytotoxic heterodimeric cyclic diarylheptanoid with a rearranged benzene ring from the seagrass *Zostera marina*. *Journal of Natural Products*, *85*(10), 2468–2473.

Grauso, L., Li, Y., Scarpato, S., Shulha, O., Árová, L., Strnad, M., Teta, R., Mangoni, A., & Zidorn, C. (2020). Structure and conformation of Zosteraphenols, tetracyclic diarylheptanoids from the seagrass *Zostera marina*: An NMR and DFT study. *Organic Letters*, *22*(1), 78–82.

Gupta, S. (2003). Molecular signaling in death receptor and mitochondrial pathways of apoptosis. *International Journal of Oncology*, *22*, 15–20.

Huang, K., Zhang, J., O'Neill, K. L., Gurumurthy, C. B., Quadros, R. M., Tu, Y., & Luo, X. (2016). Cleavage by caspase 8 and mitochondrial membrane association activate the BH3-only protein bid during TRAIL-induced apoptosis. *The Journal of Biological Chemistry*, *291*(22), 11843–11851.

Huang, Q., Pu, C., Tan, L., Wang, S., Zhang, H., Yu, S., Deng, R., Luo, D., Ma, X., & Li, R. (2022). Synthesis and biological evaluation of a novel c-Myc inhibitor against colorectal cancer via blocking c-Myc/max heterodimerization and disturbing its DNA binding. *European Journal of Medicinal Chemistry*, *243*, 114779.

Ioanna, K., Constantinos, V., Jasmin, J., Dimitri, M., Christos, R., & Vassilios, R. (2005). Cymodienol and cymodiene: New cytotoxic diarylheptanoids from the seagrass *Cymodocea nodosa*. *Tetrahedron Letters*, *46*, 2845–2847.

Ioanna, K., Michael, S., Mire, Z., Constantinos, V., Simon, G., & Vassilios, R. (2008). New metabolite with antibacterial activity from the marine angiosperm *Cymodocea nodosa*. *Tetrahedron*, *64*, 1696–1702.

Kari, S., Subramanian, K., Altomonte, I. A., Murugesan, A., Yli-Harja, O., & Kandhavelu, M. (2022). Programmed cell death detection methods: A systematic review and a categorical comparison. *Apoptosis*, *27*, 482–508.

Khotimchenko, Y., Khozhaenko, E., Kovalev, V., & Khotimchenko, M. (2012). Cerium binding activity of pectins isolated from the seagrasses *Zostera marina* and *Phyllospadix iwatensis*. *Marine Drugs*, *10*(4), 834–848.

Kuo-Feng, H., Hsien-Yeh, H., Yu-Chang, S., I-Fan, L., Su-Sing, Y., Ya-Mai, C., & Louis, K. C. (2006). Study on the anti-inflammatory activity of methanol extract from seagrass *Zostera japonica*. *Journal of Agricultural and Food Chemistry*, *54*, 306–311.

Li, Y., Grauso, L., Scarpato, S., Cacciola, N. A., Borrelli, F., Zidorn, C., & Mangoni, A. (2021). Stable catechol keto tautomers in cytotoxic heterodimeric cyclic diarylheptanoids from the seagrass *Zostera marina*. *Organic Letters*, *23*(18), 7134–7138.

Ma, H., He, C., & Chen, X. (2021). Injectable hydrogels as local depots at tumor sites for antitumor immunotherapy and immune-based combination therapy. *Macromolecular Bioscience*, *21*(6), e2100039.

Okada, H., & Mak, T. W. (2004). Pathways of apoptotic and non-apoptotic death in tumour cells. *Nature Reviews. Cancer*, *4*(8), 592–603.

Puissant, A., Robert, G., Fenouille, N., Luciano, F., Cassuto, J. P., Raynaud, S., & Auberger, P. (2010). Resveratrol promotes autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation. *Cancer Research*, *70*(3), 1042–1052.

Qi, X., Li, Q., Che, X., Wang, Q., & Wu, G. (2022). Application of regulatory cell death in cancer: Based on targeted therapy and immunotherapy. *Frontiers in Immunology*, *13*, 837293.

Sævdal Dybsland, C., Bekkby, T., Hasle Enerstvedt, K., Kvalheim, O. M., Rinde, E., & Jordheim, M. (2021). Variation in phenolic chemistry in

Zostera marina seagrass along environmental gradients. *Plants (Basel)*, *10*(2), 334.

Santaniello, G., Nebbioso, A., Altucci, L., & Conte, M. (2022). Recent advancement in anticancer compounds from marine organisms: Approval, use and bioinformatic approaches to predict new targets. *Marine Drugs*, *21*(1), 24.

Sevimli-Gur, C., & Yesil-Celiktas, O. (2019). Cytotoxicity screening of supercritical fluid extracted seaweeds and phenylpropanoids. *Molecular Biology Reports*, *46*(4), 3691–3699.

Teles, Y. C. F., Souza, M. S. R., & Souza, M. F. V. (2018). Sulphated flavonoids: Biosynthesis, structures, and biological activities. *Molecules*, *23*(2), 480.

Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: Enemies within. *Science*, *281*(5381), 1312–1316.

Wu, X., Wu, Y., Ye, H., Yu, S., He, C., & Chen, X. (2017). Interleukin-15 and cisplatin co-encapsulated thermosensitive polypeptide hydrogels for combined immuno-chemotherapy. *Journal of Controlled Release*, *255*, 81–93.

Xie, Q., Liu, Y., & Li, X. (2020). The interaction mechanism between autophagy and apoptosis in colon cancer. *Translational Oncology*, *13*(12), 100871.

Zidorn, C. (2016). Secondary metabolites of seagrasses (Alismatales and Potamogetonales; Alismatidae): Chemical diversity, bioactivity, and ecological function. *Phytochemistry*, *124*, 5–28.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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