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Original article

The efficacy of bortezomib in human multiple myeloma cells is enhanced by combination with omega-3 fatty acids DHA and EPA: Timing is essential

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SUMMARY

Background & aims: Although bortezomib as one of the first line medicines that has greatly improved the overall survival of patients with multiple myeloma (MM), undesired drug resistance is frequently observed. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been shown to be able to enhance the efficacy of chemotherapeutic drugs in many cancer types. The aim of the present study was to further evaluate the anticancer activity of DHA and EPA in relation to bortezomib chemosensitivity in human MM cells. The potential involvement of NF-κB signaling pathway was studied.
Methods: MM cells were treated with DHA/EPA with or without bortezomib. Cell viability was estimated by WST-1 assay. Apoptotic cells were determined through flow cytometry using annexin V and propidium iodide (PI) staining. Protein expression and phosphorylation was investigated by western blotting.
Results: Cell type dependent anticancer potential of DHA and EPA was observed in the cell viability assay. DHA and EPA induced apoptosis in L363, OPM2, MM.1S and U266 cell lines through both mitochondrial and death receptor pathways. Treating MM cells with DHA and EPA significantly downregulated IκBα and upregulated phosphorylation of p65, indicating that they triggered NF-κB activation in MM cells. Treating cells with DHA or EPA prior to bortezomib enhanced the induced cell death. However, concomitant use of bortezomib in combination with either of DHA or EPA decreased the cell death induced by bortezomib, indicating that timing of coincubation is important for the effects on chemosensitivity.
Conclusions: The present study provides novel evidence for the anticancer effects of DHA and EPA, and highlights their rational utilization in combination with bortezomib to achieve improved therapeutic outcome for MM.

1. Introduction

Multiple myeloma (MM), a hematologic neoplasm of terminally differentiated B cells, is the second most frequent hematological malignancy characterized by the clonal expansion of malignant plasma cells within bone marrow [1]. Although introduction of several effective therapeutic agents into the clinic, such as proteasome inhibitors (PI) (e.g., bortezomib, carfilzomib, and ixazomib), immunomodulatory drugs (e.g., thalidomide, lenalidomide, and pomalidomide), and monoclonal antibodies (mAbs) (e.g., elotuzumab and daratumumab), has achieved tremendous improvement in the treatment of MM, it still remains incurable in most patients due to its highly heterogenous genomic, phenotypic nature, high-risk and relapse [2]. Moreover, almost always, the long-term use of these agents incurs numerous unfavorable side-effects and subsequently develops resistance to the therapeutic agents after an initial period of responsiveness, eventually becoming refractory [3]. Thus, there remains an urgent need for developing of novel anti-myeloma drugs or new therapeutic strategies to increase or retain the therapeutic efficacy of existing drugs and reduce their toxicity to normal cells.

Bortezomib (Velcade), a reversible 26S PI, is the first FDA-approved PI drug for the treatment of relapsed and/or refractory, or newly diagnosed MM patients [4]. In spite of the great success of bortezomib in the treatment of MM, most patients develop drug resistance during bortezomib-based therapy. The underlying mechanisms for this acquired resistance have been revealed gradually, including clonal evolution, glucose metabolic alterations,

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upregulation or mutations of proteasomal system and abnormal activation of cellular pro-survival pathways [3,5,6]. Therefore, the development of rational combinations of bortezomib with other anticancer agents to overcome drug resistance will continue to be a promising therapeutic strategy for future bortezomib-based therapies. Recently, omega-3 polyunsaturated fatty acids (PUFAs) DHA and EPA, which are largely contained in fish or fish oils, have been found to enhance the efficacy of chemotherapeutic drugs in clinical studies in multiple cancer types. Preclinical studies suggested that DHA and EPA have direct cytotoxic effect in a variety of cancer cells, including multiple human solid tumors, leukemia and lymphomas [7], but showed no cytotoxicity in normal cells [8]. Moreover, these fatty acids showed selective toxicity for PBMCs from multiple myeloma patients, but not for mononuclear cells from healthy donors [9]. These findings further support that the anticancer use of these fatty acids in combination with chemotherapy might be a rational strategy for MM treatment.

The nuclear transcription factor-kappa B (NF-κB) signaling pathway is frequently found to be constitutively activated in a number of human cancers including MM [10,11]. Because of its critical role in cancer cell survival, proliferation, invasion, metastasis, angiogenesis and chemoresistance, we assumed that DHA and EPA might mediate cell death of MM cells by modulating the NF-kB signaling cascade. The canonical mammalian NF-κB is a heterodimer composed of a p65 and a p50 subunit. In normal cells, the NF-κB binds to Inhibitor of κB (IκBα) and is constitutively present in the cytoplasm as an inactive complex. Upon activation -for example by tumor necrosis factor α (TNFα)- IκBα is phosphorylated by IκB kinases (IKKs) at Ser32, which results in ubiquitination and following degradation of IκBα by the 26S proteasome. The IκBα degradation leads to the release of NF-κB that allows NF-κB to translocate into the nucleus where it initiates the transcription of multiple pro-inflammatory and pro-survival genes [12].

In this study, we evaluated the cytotoxic effects of DHA and EPA as well as their synergistic anticancer efficacy with bortezomib in a set of MM cell lines. Our results demonstrated the selective anticancer potency of DHA and EPA against different MM cell lines and also revealed a novel role of the DHA and EPA in bortezomib-induced MM cell death. We also explored the potential mechanism involving NF-κB signaling activity. Our results showed that DHA and EPA downregulated IκBα and upregulated phosphorylation of p65, indicating that they activated the canonical NF-κB pathway in MM cells. The present work highlights that proper utilization of DHA and EPA in combination with chemotherapy reagents might be able to improve the clinical efficacy in the treatment of MM.

2. Materials and methods

2.1. Reagents and antibodies

DHA (D2534, ≥98% purity), EPA (E2011, ≥99% purity) and cell proliferation reagent WST-1 (5015944001) were purchased from Sigma. DHA and EPA were dissolved in ethanol to produce a 100 mM stock solution and stored at −20 °C for 3 months (without loss of activity). Annexin V apoptosis detection kit (88-8005-74) was obtained from Thermo Fisher. Bortezomib was obtained from LC laboratories and dissolved in DMSO. Antibodies for phospho-Ser536-p65 (#3033), p65 (#8242), IκBα (#4814), cleaved-caspase3 (#9661), cleaved-caspase8 (#9496) and cleaved-caspase9 (#9505) were purchased from Cell Signaling Technology; anti-GAPDH (sc-47724) was obtained from Santa Cruz Biotechnology. All primary antibodies were used with 1:1000 dilution. Horse radish peroxidase-conjugated secondary antibodies were purchased from Dako.

2.2. Cell culture

Human MM cell lines L363, OPM2, U266 and MM.1S were maintained in RPMI-1640 medium, which was supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU penicillin, and 100 mg/ml streptomycin, at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Cell viability assay

MM cells in fresh culture medium were seeded in triplicate into a 96-well plate at density of 10×10^3 cells/well and incubated with the indicated concentrations of compounds for 24 h. After 24 h, 10 μl of WST-1 reagent was added to the medium and incubated for 3 h at 37 °C and 5% CO_2. The absorbance at 490 nm was determined using a microplate reader.

2.4. Flow cytometric analysis of apoptosis

FITC-conjugated annexin V and propidium iodide (PI) staining to detect apoptotic cells was performed according to manufacturer's protocol. Briefly, MM cells were seeded in triplicate into a 12-well plate at density of 200×10^3 cells/well and incubated with indicated concentrations of compounds. After treatment, cells were washed once in PBS and once in 1 × binding buffer, adjusted to a concentration of 1×10^6 cells in 100 μl 1 × binding buffer, then stained with 5 μl Annexin V-FITC antibody for 15 min at room temperature in dark. Cells were washed once with 1 × binding buffer and resuspended in 100 μl 1 × binding buffer, then 2.5 μl of PI was then added and incubated in dark at 4 °C for 5 min. The percentage of apoptotic cells (Annexin $V^+PI^{-/+}$) was determined by a BD FACS Canto II flow cytometer. Flowlogic software (Miltenyi Biotec) was used for data analysis.

2.5. Western blotting

Treated cells were lysed with RIPA buffer [#89900 Thermo Fisher, 1 × protease inhibitor cocktail (Roche), 1 × Phosphatase Inhibitor Cocktail (Roche)] on ice for 30 min. Then, cell lysate was harvested after centrifugation for 10 min at 12 000 rpm at 4 °C. Equal amounts of protein (25–50 μg) per sample (determined with Pierce BCA Protein Assay Kit; Thermo Fisher) were resolved by reducing SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% skimmed milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h and probed with specific primary antibodies overnight at 4 °C. Membranes were then washed three times with TBST (10 min each time) followed by incubation with relevant HRP-conjugated secondary antibody for 1 h at room temperature. After washing with TBST (10 min, 3 times), membranes were developed using ECL (#1705061; Biorad) and detected by a ChemiDoc Imaging System (Bio-Rad).

2.6. Statistics and data analysis

All experiments were performed independently at least three times. Relative cell viability was presented as a percentage (%) relative to the untreated control cells. Data were analyzed using GraphPad Prism software. Statistical significance between the tested groups was determined using one-way ANOVA. $P < 0.05$ was considered significant. Western blot image signal intensity was quantified using ImageJ software. P-p65 fold changes were normalized to total p65, while IκBα fold changes were normalized to GAPDH. Quantification numbers are presented below the blot images.

3. Results

1. DHA and EPA showed selective anticancer potential against different MM cell lines.

The anticancer effect of DHA and EPA was studied in L363, OPM2, U266 and MM.1S cell lines. All the MM cell lines were treated with a range of concentrations of DHA or EPA for 24 h and cell viability was determined by a WST-1 assay (Fig. 1). The viability of L363 and OPM2 cells treated with either of DHA or EPA was significantly decreased in a concentration-dependent manner. Meanwhile, DHA and EPA showed only a moderate effect on the viability of U266 cells when the concentrations reached to 100 μM. Interestingly, the MM.1S cell line was sensitive to DHA treatment at a high concentration (100 μM), but it was completely resistant to EPA in the range of concentrations used in this experiment. Additionally, by comparing the effects of DHA/EPA on cell viability among these cell lines, DHA exhibited a higher cytotoxic potential than EPA. These results suggest that DHA and EPA have selective anticancer activity in these cell lines.

2. DHA and EPA induced apoptosis in MM cells via both mitochondrial and death receptor pathways.

To further confirm whether DHA and EPA affected cell viability in these cell lines by inducing apoptosis, MM cells were incubated with vehicle as negative control, 50 μM or 100 μM of DHA/EPA for 24 h, and then apoptosis and cell death were determined through flow cytometry using annexin V-FITC and propidium iodide (PI) staining. As shown in Fig. 2A and B, consistent with our cell viability results, DHA and EPA induced pronounced increases of apoptotic (Annexin V^+) cells in L363 and OPM2 cells in concentration-dependent manners but had only a minor effect on the U266 cell line. Meanwhile, in MM.1S cells only 100 μM of DHA treatment significantly increased the percentage of apoptotic cells. In contrast, DHA exhibited stronger induction of apoptosis than EPA in OPM2 and L363 cells at both 50 μM and 100 μM concentrations. Western blot analysis of apoptotic markers further validated that DHA and EPA induced cell apoptosis (Fig. 2C). Caspases are the central enzymes in apoptotic pathways, as they are both the initiators and executioners for apoptosis. Cleavage of caspase-8 or caspase-9 is the marker of mitochondrial (intrinsic) and death receptor (extrinsic) apoptotic pathways, respectively [13]. After treating OPM2 cells with DHA or EPA for 24 h, a concentration-dependent increase of cleaved caspase-3 was observed. Furthermore, the levels of cleaved caspase-8 and cleaved caspase-9 were evidently increased after DHA/EPA treatment. These results indicate that DHA and EPA induced apoptosis through both intrinsic (mitochondrial) and extrinsic (death receptor) pathways.

3. Simultaneous administration of DHA/EPA and bortezomib reduced bortezomib chemosensitivity.

The proteasome inhibitor bortezomib has been widely used for the treatment of MM, but undesired chemoresistance is frequently observed. We next examined whether DHA and EPA could improve the chemosensitivity of bortezomib in MM cells. Treatment with 10 nM bortezomib increased the percentage of Annexin V^+ cells to 42.7% in OPM2 cells, 73.7% in L363 cells, 84.8% in U266 cells and 96.5% in MM.1S cells, indicating that OPM2 cell line is most resistant to bortezomib among these cell lines (Fig. S1).

To examine the effects of DHA/EPA on bortezomib chemosensitivity, OPM2 and L363 cells were incubated with 25 μM or 50 μM of DHA/EPA in the presence of 10 nM or 25 nM bortezomib for 24 h, after which Annexin V-FITC and PI staining was performed to measure apoptosis. Surprisingly, simultaneous treatment of cells with bortezomib plus either DHA or EPA decreased bortezomib chemosensitivity in MM cells (Fig. 3 A and B). Only 50 μM DHA and 25 nM bortezomib treated OPM2 cells showed a slight increase of apoptotic cells from 54.1% to 65.84%. Additionally, we used lower concentrations (5 nM and 10 nM) of bortezomib in MM.1S and U266 cells due to their hypersensitivity to bortezomib. Similarly, prominent inhibitory effects on bortezomib chemosensitivity were also observed in these two cell lines (Fig. 3C and D). Noteworthily, EPA exhibited stronger inhibitory effects on bortezomib efficacy compared to DHA in all MM cell lines at both 25 μM and 50 μM. These results suggest that possible interactions between DHA/EPA and bortezomib may exist leading to a decreased chemosensitivity of bortezomib when they are used concomitantly.

4. Pretreating OPM2 and L363 cells with DHA or EPA prior to bortezomib enhanced bortezomib chemosensitivity.

To avoid possible “drug–drug” interactions, we next tried to treat cells with DHA or EPA prior to bortezomib and then determined MM cell apoptosis. MM cells were pretreated with 50 μM DHA or EPA for 6 h and then the incubation continued with 10 nM (in L363 and OPM2 cells) or 5 nM (in U266 and MM.1S cells) bortezomib for an additional 24 h. Interestingly, it was found that pretreating cells with DHA/EPA before bortezomib in OPM2 and L363 cells significantly increased cytotoxicity of bortezomib

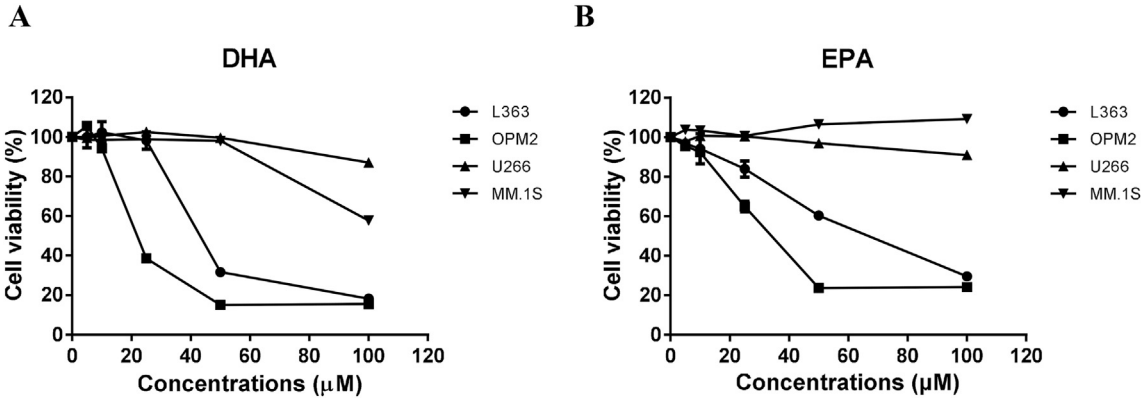


Fig. 1. The effect of DHA and EPA on cell viability in MM cells. MM cells were incubated with 5, 10, 25, 50 100 μM DHA **A)** or EPA **B)** for 24 h, and then cell viabilities were estimated using WST-1 assay. Ethanol was used as negative control. Relative cell viability was presented as a percentage (%) relative to control cells. Data are presented as mean ± SD of three independent repeats.

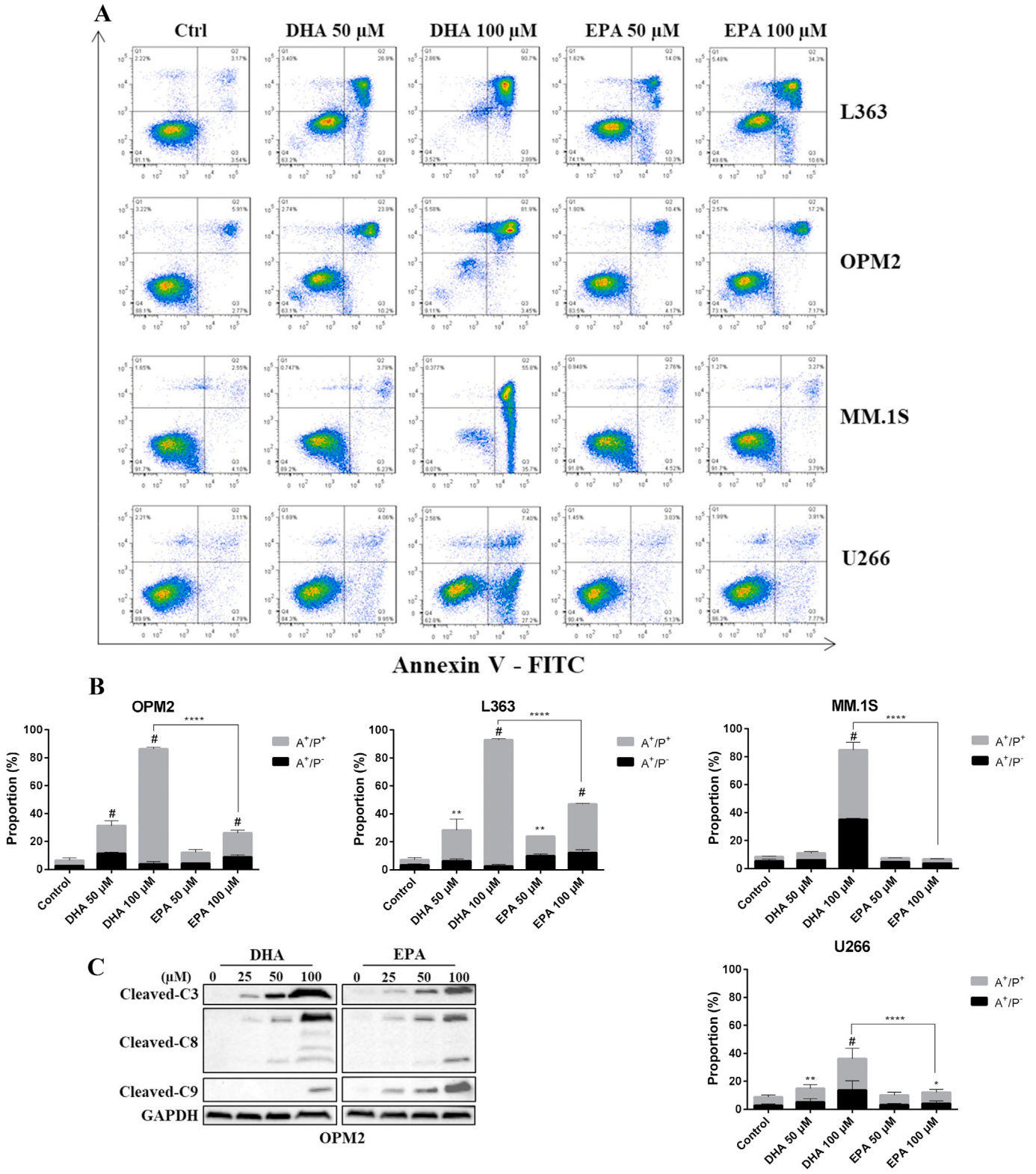


Fig. 2. DHA and EPA induce apoptosis through mitochondrial and death receptor pathways in MM cells. MM cells were treated with 50 or 100 μM DHA/EPA for 24 h **A)** apoptotic cells were determined by Annexin-V and PI staining. **B)** Quantification of **A**. Data are presented as mean ± SD of three independent repeats. $^{**}p < 0.01$, $^{*}p < 0.05$, $\#p < 0.0001$when compared with control. $^{****}p < 0.0001$. **C)** whole cell lysates were analyzed by western blotting with antibodies for cleaved-3/8/9 and GAPDH. Ethanol was used as control.

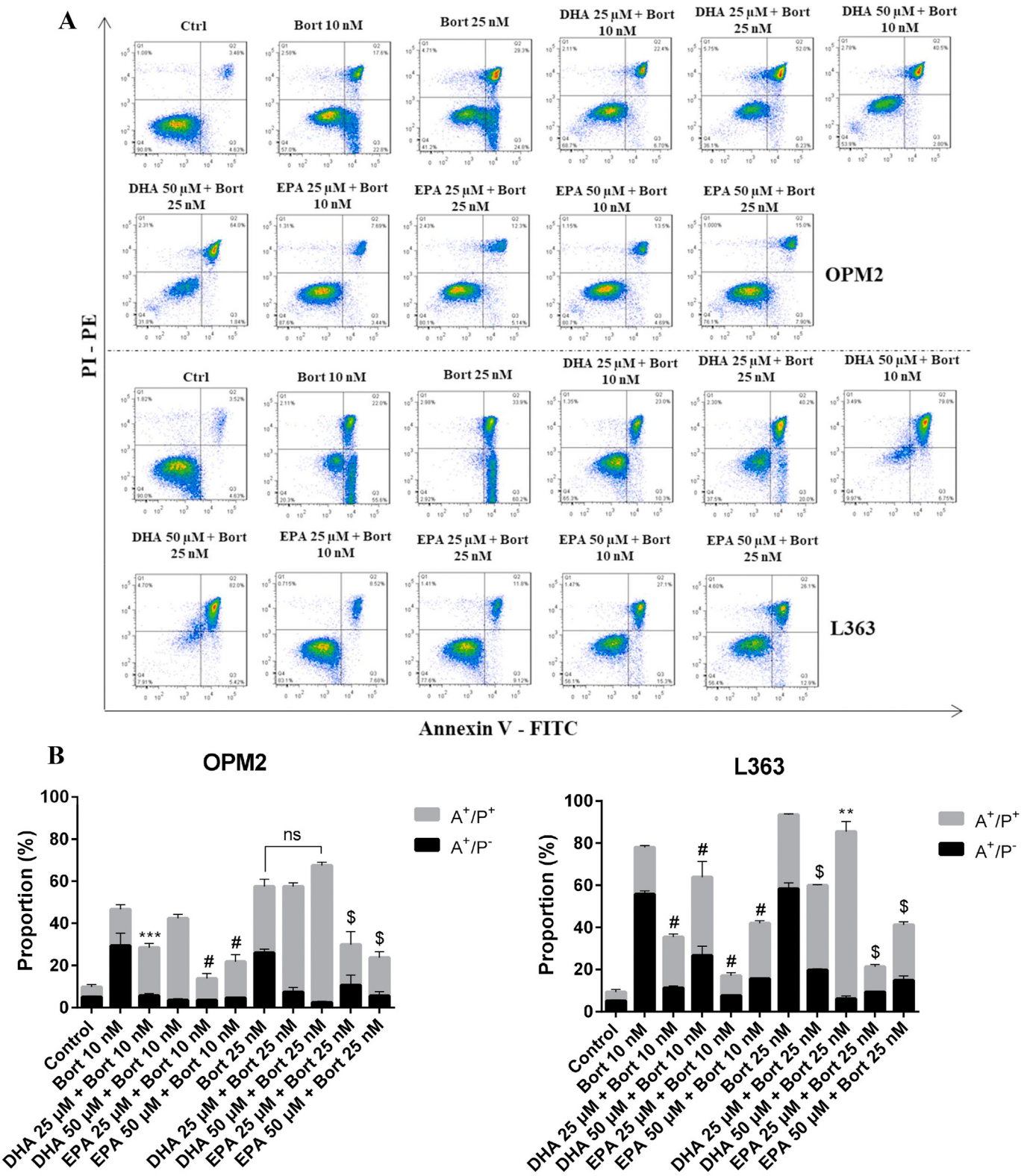
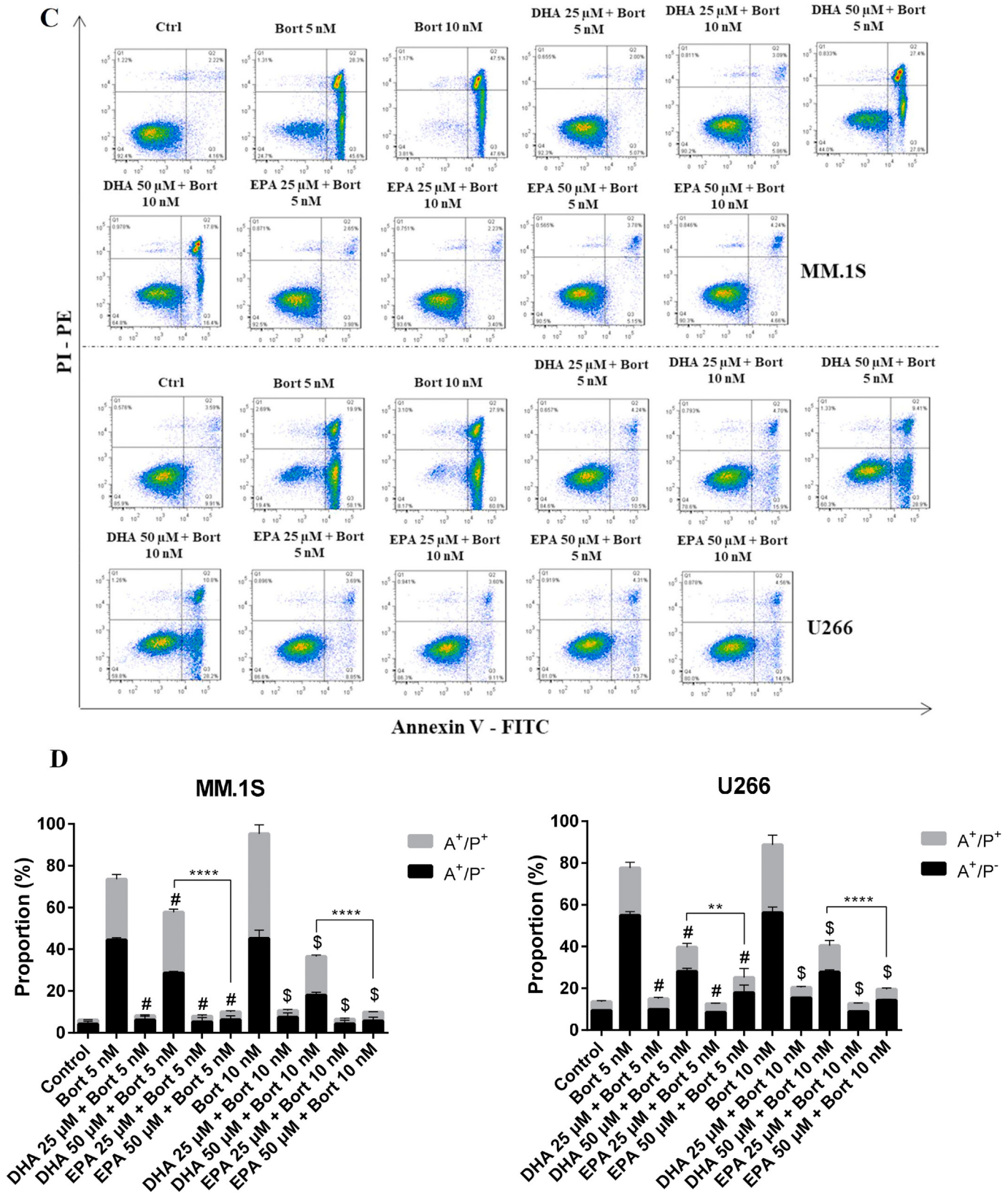


Fig. 3. DHA and EPA decrease bortezomib chemosensitivity in MM cells when DHA or EPA are added simultaneously with bortezomib. A) OPM2 and L363 **C)** MM.1S and U266 cells were treated with 25 μM or 50 μM DHA or EPA in the presence or absent of bortezomib for 24 h, then apoptotic cells were determined by Annexin-V and PI staining. **B)** Quantification of **A**. **D**) Quantification of **C**. Data are presented as mean ± SD of three independent repeats. ***$p < 0.001$, #$p < 0.0001$when compared with bortezomib 5 nM group in MM.1S and U266 cells or bortezomib 10 nM group in OPM2 and L363 cells. **$p < 0.01$, $ $p < 0.0001$when compared with bortezomib 10 nM group in MM.1S and U266 cells or bortezomib 25 nM group in OPM2 and L363 cells. ****$p < 0.0001$. Ns, not significant.



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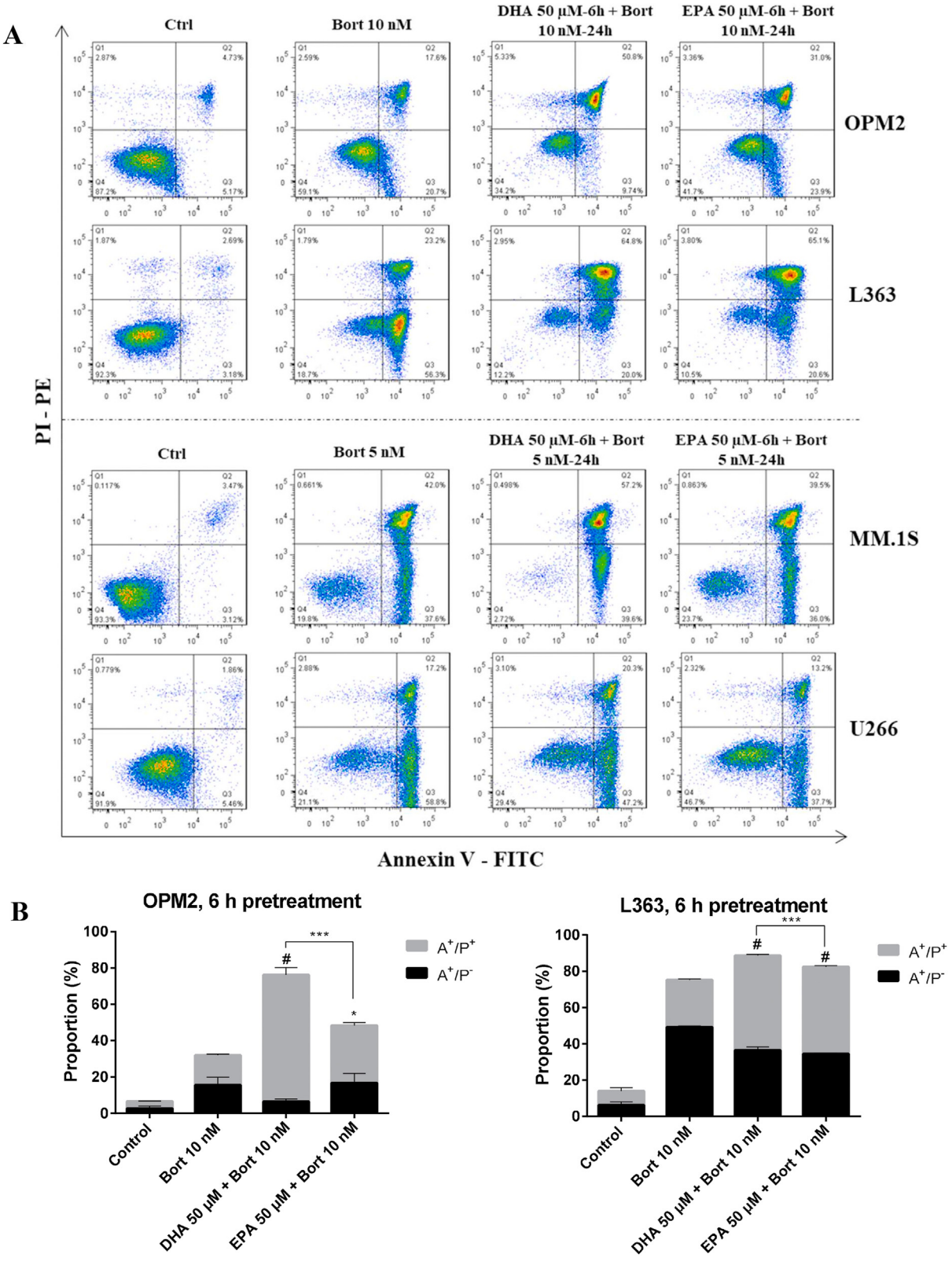


Fig. 4. DHA and EPA enhance bortezomib chemosensitivity in MM cells when DHA or EPA are added before bortezomib. A) MM cells were pretreated with 50 μM DHA or EPA for 6 h and then treated with bortezomib for 24 h. **B)** and **D)** Quantification of **A**. **C)** OPM2 and L363 cells were pretreated with 50 μM DHA or EPA for 0, 0.5, 1, 2, 4 and 6 h, then bortezomib (10 nM) was added for 24 h treatment. Then apoptotic cells were determined by Annexin-V and PI staining. **E)** Quantification of **C**. Data are presented as mean ± SD of three independent repeats. *$p < 0.05$, **$p < 0.01$, #$p < 0.0001$when compared with the bortezomib-treated group in each experiment. ***$p < 0.001$, ****$p < 0.0001$. Ns, not significant.

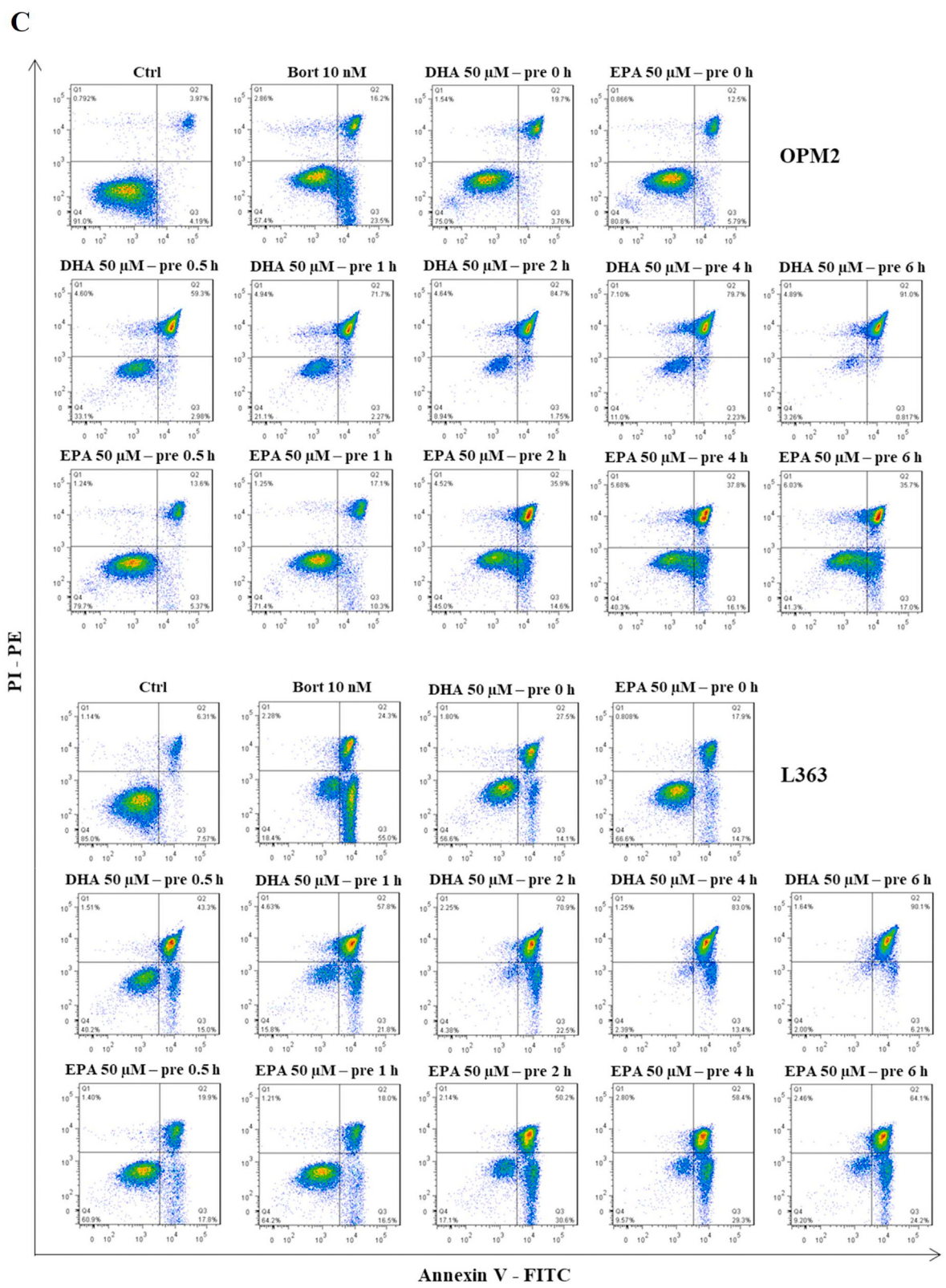


Fig. 4. (*continued*).

(Fig. 4A and B). However, pretreating U266 cells with either of DHA or EPA up to 6 h, surprisingly, still showed inhibitory effects on bortezomib cytotoxicity and pretreatment with EPA in MM.1S also induced inhibition (Fig. 4A and D).

Next, we examined the effects of different starting time points of DHA/EPA pretreatment on bortezomib induced MM cell apoptosis. L363 and OPM2 cells were pretreated with 50 μM DHA or EPA from 0.5 h to 6 h before 10 nM bortezomib treatment. Interestingly, as shown in Fig. 4C and E, 0.5 h and 1 h pretreatment with EPA in OPM2 cells still showed pronounced inhibition. Meanwhile, short time pretreatment of L363 cells (0.5 h with DHA and 0.5 or 1 h with EPA) decreased the anticancer efficacy of bortezomib (Fig. 4C and E). After 2 h, 4 h, and 6 h pretreatment with DHA/EPA an equal enhancing effect on bortezomib chemosensitivity in L363 and OPM2 cells was found. These experiments indicate that the timing of DHA/EPA treatment is important for its effect on bortezomib chemosensitivity in MM cells.

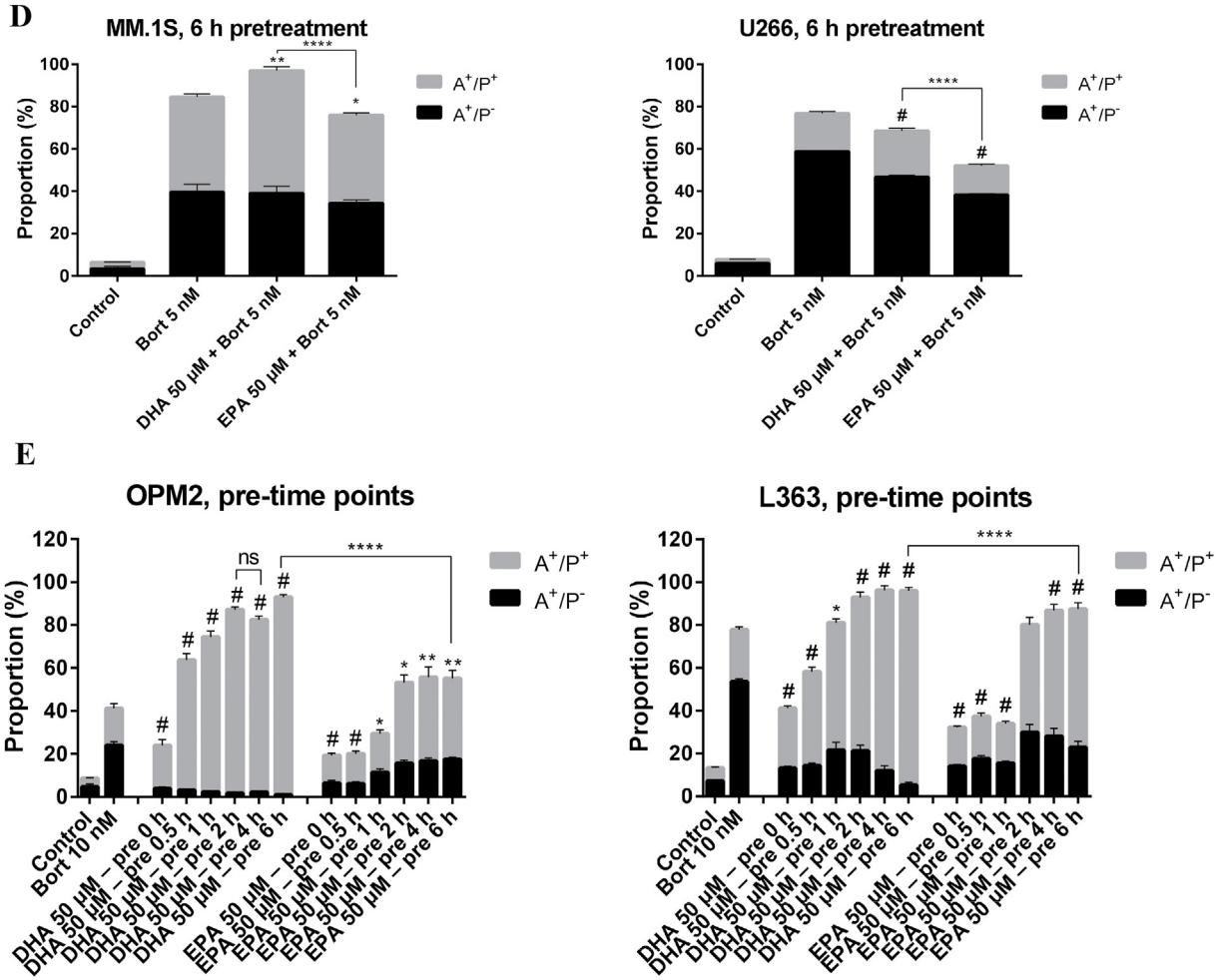
5. DHA and EPA induced canonical NF-κB activation in MM cells.

The underlying mechanism of EPA- and DHA-induced cytotoxicity in MM cells was further investigated. Because of the critical role of NF-κB regulated gene products in tumorigenesis, we assumed that DHA and EPA might mediate MM cell apoptosis by blocking the NF-κB signaling cascade. It has been well demonstrated that the phosphorylation of p65 at Ser536 is a key event to activate the NF-κB signaling pathway. For this reason, we first examined the effect of DHA and EPA on phosphorylation of p65 in OPM2 and U266 cells. Western blot analysis showed that DHA and EPA increased the phosphorylation of p65 in OPM2 cells without alteration of p65 expression (Fig. 5A). However, in U266 cells the phosphorylation of p65 was not changed in the presence of DHA or EPA (Fig. 5B). Noteworthily, the DHA/EPA resistant cell line U266 showed a higher level of phosphorylated p65, indicating that NF-κB signaling is constitutively activated in this cell line.

Because the proteasome-mediated degradation of IκBα is crucial in the activation of the NF-κB signaling pathway, we also tested the effect of DHA and EPA on IκBα protein level. DHA and EPA decreased IκBα in OPM2 cells but had only a slight impact in U266 cells (Fig. 5). Taken together, these data indicate that DHA and EPA selectively triggered NF-κB activation in DHA/EPA sensitive cells, but not in resistant cells.

4. Discussion

Despite the promising therapeutic benefits of the proteasome inhibitor bortezomib in MM, this disease is still considered incurable due to therapeutic refractoriness, disease relapse and acquisition of drug resistance. Furthermore, numerous severe side-effects have been observed in MM patients treated with bortezomib, such as peripheral neuropathy, gastrointestinal symptoms, anemia, fatigue, venous thrombocytopenia, cardiac dysfunctions,



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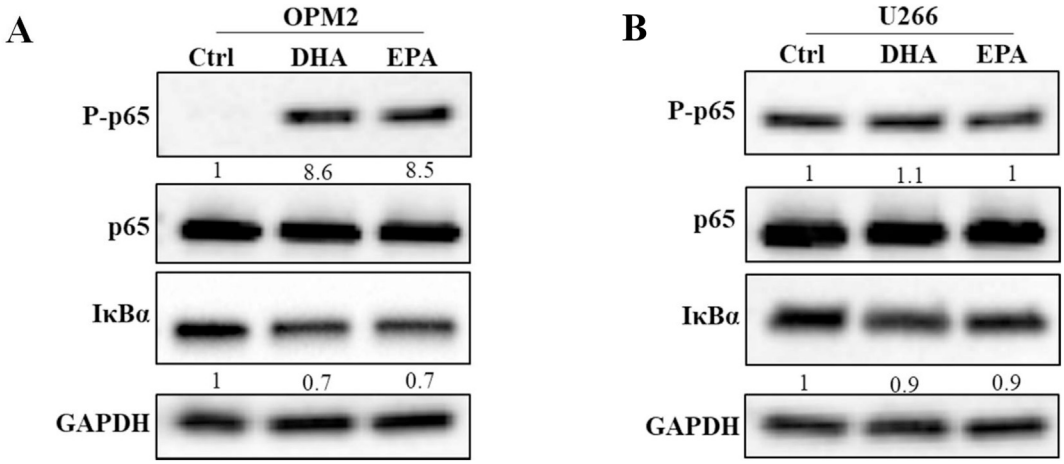

Fig. 5. DHA and EPA activate the canonical NF-κB pathway in MM cells. A) OPM2 and **B)** U266 cells were incubated with 50 μM DHA or EPA for 24 h, then whole cell lysates were analyzed by western blotting with antibodies for p-p65, p65, IκBα and GAPDH. The fold changes of p-p65 and IκBα were normalized to total p65 and GAPDH, respectively. Quantification numbers are presented in the bottom of p-p65 and IκBα.

severe pulmonary complication, hepatic toxicity and reversible posterior encephalopathy [14]. It remains an unmet and urgent need to develop novel therapeutic strategies to circumvent acquired drug resistance and reduce related side-effects. In the present study, we evaluated the anticancer effect of DHA and EPA as well as their synergistic anticancer efficacy with bortezomib in a set of human MM cell lines and explored a rationale for an effective combinational therapy for MM.

In the present work, we found that DHA and EPA showed selective anticancer activity in different MM cell lines by inducing apoptosis. Both L363 and OPM2 MM cells were sensitive to DHA and EPA. It is interesting to note that MM.1S cells were only sensitive to DHA but resistant to EPA in the present study, while U266 cells showed resistance to both DHA and EPA. These results are consistent with our previous study which showed that L363 and OPM2 cell lines were more sensitive to DHA/EPA than U266 [8]. Furthermore, it has been documented that the U266 cell line is resistant to a variety of apoptotic stimuli, as well as to some chemotherapeutic drugs. One of the mechanisms underlying drug resistance in U266 cell line is associated with the constitutive activation of STAT3 signaling pathway which regulates expression of various pro-survival proteins, such as antiapoptotic proteins Bcl-xL and Bcl-2 [15]. As shown in our results, another pro-survival transcriptional factor NF-κB is also constitutively activated in U266 cell line. Several studies have shown that these fatty acids could induce apoptosis through a caspase-dependent pathway in multiple cancer cells, including oral squamous cell carcinomas, breast cancer, colon cancer and pancreatic cancer [16–19]. Furthermore, our data show that the levels of cleaved caspase-3/8/9 were markedly increased in response to DHA and EPA in the sensitive cell line OPM2, suggesting that both mitochondrial and death receptor apoptotic pathways might be activated.

Currently, the clinical treatment of MM with existing chemotherapeutic drugs is facing two major obstacles, i.e. acquisition of drug resistance [20,21] and the induction of adverse side-effects [14]. Thus, the future direction of bortezomib-based therapeutic strategies for MM should further aim for overcoming drug resistance and preserving non-tumor cells. Many pre-clinical studies have reported that Ω-3 fatty acids DHA/EPA as single agent exerted their anticancer activity in multiple human cancer cells by inducing apoptotic cell death [22,23]. Notably, in 2018, FDA approved the use of fish oil triglycerides, marketed under the name Omegaven, for the treatment of parenteral nutrition-associated cholestasis in pediatric patients, suggesting the potential clinical use of Ω-3 fatty acids as a novel pharmaceutical drug. DHA and EPA have shown synergistic anticancer activities with conventional cytotoxic therapies against multiple types of cancers, especially those resistant to chemotherapies [24–27], thereby suggesting their potential to be used for overcoming chemoresistance. Inspired by these findings, we investigated their effects on the chemotherapy with bortezomib in MM cells. Our initial experiments showed that simultaneously treating MM cells with DHA/EPA and bortezomib inhibited bortezomib-induced cell death in four MM cell lines. On the other hand, our previous work showed that pretreating L363 and OPM2 cells with 30 μM of DHA/EPA for 3 days before bortezomib markedly increased bortezomib cytotoxicity [8]. It can be speculated that DHA and EPA may interfere with the uptake of bortezomib in MM cells. The critical micelle concentration for DHA is approximately 60 μM [28,29] and effects on solubilization and uptake of bortezomib may be expected at higher concentrations of the Ω-3 fatty acids. On the other hand, inhibitory effects of simultaneous incubation of EPA/DHA on bortezomib efficacy were already observed at concentrations of 25 μM of the Ω-3 fatty acids (Fig. 3). This suggests that this induced inhibition may not solely due to effects on drug solubilization or uptake. To circumvent direct interaction with bortezomib, we further examined the effects of a pretreatment approach of DHA/EPA on bortezomib chemosensitivity. Intriguingly, significant synergistic anticancer effects were observed in L363 and OPM2 cells when bortezomib was introduced after 2 h of DHA/EPA pretreatment. These results indicate that administering DHA or EPA prior to chemotherapy may be a promising strategy to overcome chemotherapy resistance in MM. However, surprisingly pretreating U266 cells with DHA/EPA or MM.1S cells with EPA up to 6 h still showed inhibitory effects on bortezomib cytotoxicity, indicating that DHA/EPA might be able to be only used as adjuvants to potentiate chemosensitivity in MM cells which are sensitive to DHA/EPA. Interestingly, DHA and EPA have been found to specifically target cancer cells without affecting normal cells [30–32]. Our previous work also showed they had no detectable toxicity in normal human PBMCs [8].

After oral administration of EPA or DHA, these fatty acids are shown to be incorporated into circulating phospholipids, triacylglycerol and cholesteryl esters, and into the phospholipid components of RBC membrane [33], while EPA and DHA fatty acids are also measurable in human serum. There are several studies published on the bioavailability and pharmacokinetics of oral

administered EPA and EPA/DHA formulations [33–35]. The studies of Rusca et al. [33] and Marangoni et al. [34] show that multiple oral dosing of capsules containing EPA and DHA resulted in steady state plasma concentrations of approximately 100 mg/L at day 14–18. The study of Braeckman et al. [35] analyzed only EPA formulations and showed similar results. Notably, the dosage regimen of 2 × 4 g EPA even reached steady state concentrations of 200 mg/L. The formulations used in these studies contained ethyl-esters of EPA/DHA, but also free acid preparations of EPA/DHA were shown to reach plasma concentrations in the same range [36]. However, while most studies measured the total -free unesterified and esterified-concentrations of EPA or DHA, Braeckman et al. [35] showed that circulating concentrations of free unesterified EPA are only a fraction (0.4–0.6%) of these total concentrations. Given the equilibrium between esterified and unesterified fatty acids in vivo, it needs to be established if the effects of EPA and DHA on chemosensitivity may be independent on the free acid fraction of the fatty acids and may rather depend on the fraction incorporated into the cell membrane of myeloma cells. Therefore, further studies need to determine if oral intake of EPA/DHA will lead to sufficient exposure of multiple myeloma cells to induce cell death as shown in the presented in vitro experiments. Taken together, our study suggests that proper using of DHA/EPA in combination with chemotherapy reagents may improve the clinical efficacy in the treatment of MM through reducing side-effects and increasing chemosensitivity, but further research e.g. in preclinical models of MM is needed to further substantiate our findings and its clinical translation.

DHA and EPA-induced cell death has been associated with multiple signaling pathways which are well known to contribute to cancer cell proliferation, anti-apoptosis, inflammation, and chemoresistance [37]. Constitutively elevated activity of NF-κB is frequently observed in primary MM cells and MM cell lines [38]. Hence, blocking the NF-κB pathway is always the primary or secondary therapeutic strategy in MM. The degradation of IκBα by the ubiquitin-proteasome system is required for NF-κB activation. Thus, proteasome inhibitors are widely used as first-line and second-line therapy in MM due to their capability in blocking IκBα degradation via disrupting proteasome function. Another very important mechanism involved in proteasome inhibitor-induced cell death is mediated by retarding the widespread degradation of unfolded or misfolded proteins, specifically immunoglobulins in MM cells, which ultimately triggers programmed cell death [39,40]. Initially, as a proteasome inhibitor, bortezomib was supposed to inhibit NF-κB by blocking IκBα degradation similar to other proteasome inhibitors. However, in 2009, a paradoxical study showed bortezomib could decrease IκBα and induce activation of canonical NF-κB pathway in MM cell lines and patient cells [41]. Furthermore, later it was confirmed that bortezomib can induce IκBα downregulation in multiple cancer types, including lung cancer, prostate cancer, cervical cancer and breast cancer [21]. In our study, surprisingly, we also found that these fatty acids triggered NF-κB activation in OPM2 cells. Our time course study showed that DHA induced NF-κB activation after long time treatment (more than 16 h) (Fig. S2), suggesting that these fatty acids may not activate NF-κB through direct targeting the key regulators of this pathway like TNFα. A recent paper showed necroptotic cell death can induce autocrine TNFα and enhance NF-κB activity [42]. Apoptosis is mediated by the activation of caspases. Under apoptotic-deficient conditions, for example, in the absence of caspase-8, RIPK1/RIPK3/MLKL-mediated necroptosis can be activated to lead necroptotic cell death. Until now, caspase-8 inhibition has been found to be essential for TNFα-induced necroptosis. Our results showed caspase-8 was activated by DHA/EPA in MM cells, which did not meet the requirement for necroptosis induction. However, interestingly, it was shown that the proteasome inhibitors MG132 and bortezomib can activate the necroptotic pathway in mouse fibroblasts as well as human leukemia cells without caspase-8 inhibition [43]. This insight suggested the further possibility that DHA/EPA and bortezomib might induce necroptosis in MM cells in addition to apoptosis. Moreover, large populations of Annexin V^+/PI^+ cells (late stage of apoptotic cells or necroptotic cells) were observed in treated-MM cells. Further investigations are needed to unravel the mechanism of NF-κB activation and induction of necroptosis in DHA/EPA-promoted MM cell death (work in progress).

Our study showed that pretreatment of EPA- and DHA-sensitive MM cells enhanced bortezomib chemotoxicity, whereas simultaneous treatment with bortezomib and either of DHA or EPA decreases bortezomib chemosensitivity in all MM cell lines. This indicates that timing of administration is essential to direct the effect of EPA/DHA on efficacy of chemotherapy with bortezomib. Since our study also showed that pretreatment of DHA/EPA-refractive U266 MM cells significantly decreased chemosensitivity to bortezomib, it appears of importance to determine if MM cells are sensitive to EPA/DHA before combining omega-3 fatty acids with chemotherapeutics therapies. Therefore, because of the individual variability in clonal heterogeneity in MM patients, personalized intervention would be imminent for introduction of DHA/EPA in their cancer therapy.

Collectively, our results demonstrated that the order for the combinational use of DHA or EPA with chemotherapy bortezomib determines different synergetic anticancer effect against MM cells and administration of DHA or EPA prior to chemotherapy might serve as a new strategy to enhance chemosensitivity and may prevent chemotherapy resistance in MM.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2020.09.009.

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