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Esterified derivatives of DHA and EPA increase bortezomib cytotoxicity in human multiple myeloma cells

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A R T I C L E I N F O

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A B S T R A C T

Background & aims: Although the proteasome inhibitor bortezomib has greatly improved the clinical outcome of patients with multiple myeloma (MM), acquired drug resistance remains the greatest obstacle on the road of treating MM. We previously showed that omega-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) with the chemotherapeutic agent bortezomib can overcome its chemoresistance in MM cells. However, most DHA/EPA are esterified shortly after oral administration, which may affect their bioactivity. This study was to evaluate the cytotoxicity of ethyl ester-DHA/EPA in human MM cells. The mechanisms relevant for the cytotoxicity of these esterified-fatty acids were further investigated.
Methods: Human MM cell lines L363, OPM2, U266 were treated with ethyl ester-DHA/EPA with or without bortezomib. The percentage of dead cells and intracellular reactive oxygen species (ROS) levels were analyzed by flow cytometry.
Results: Ethyl ester-DHA and -EPA were much more potent than DHA/EPA to induce cytotoxicity in MM cells, even in DHA/EPA-resistant MM cells. Pretreating MM cells with esterified-DHA/EPA before bortezomib potently increased its cytotoxicity. Additionally, intracellular ROS levels were upregulated in MM cells after treatment with ethyl ester-DHA/EPA, which reflected the enhanced oxidative stress in treated cells.
Conclusions: This study provides evidence that ethyl ester-DHA/EPA in combination with bortezomib may improve the overall efficacy in MM cells, similar to DHA/EPA, relieving the concern that esterification of DHA/EPA may affect its bioactivity and further supporting the potential clinical use of fatty acids DHA/EPA for combating drug resistance during MM therapy.

1. Introduction

MM is the second most prevalent hematological malignancy characterized by the clonal expansion of malignant plasma cells within the bone marrow. This incurable disease predominantly affects elderly people, with a median age at diagnosis of about 70 years (Rollig et al., 2015). Patients with MM often have poor prognosis, and the 5-year overall survival rate is only 54% (Cancer.Net Editorial Board, 01/2021). The first-in-class proteasome inhibitor bortezomib, marketed under the name Velcade, has been approved by the FDA for the treatment of patients with relapsed/refractory or newly diagnosed MM (McBride and Ryan, 2013). However, long-term treatment with bortezomib often incurs serious side effects, such as severe headache, fast or irregular heartbeat, fainting, convulsions, and trouble breathing (Schlafer et al., 2017), many of which lead to a rapid and fatal outcome. Moreover, most MM patients who received bortezomib-based therapy suffer from repeated relapses or become refractory after initial therapeutic responses due to the development of drug resistance (Bazarbachi et al., 2019; Robak et al., 2018). To date, combination therapy approaches using bortezomib with daratumumab, a CD38-targeting human monoclonal antibody, have been found to be able to overcome bortezomib resistance (Lamb, 2020; Lokhorst et al., 2015). However, developing novel combination therapeutic strategies is still urgently needed to overcome bortezomib-associated resistance for MM treatment.

Omega-3 fatty acids DHA and EPA, largely contained in fish or fish oils, are traditionally used as nutrient additives in regular food. In our recent studies, using a combinational treatment of DHA/EPA and

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bortezomib, it was found that pretreating MM cells with DHA/EPA potently increased bortezomib cytotoxicity by triggering glutathione (GSH) degradation (Chen et al., 2020, 2021). GSH is a tripeptide consisting of glutamic acid, cysteine, and glycine. It is a major intracellular antioxidant to maintain cellular oxidative balance. Normally, cancer cells produce high levels of ROS (reactive oxygen species) to maintain cell survival and proliferation. However, when its concentrations reach to toxic levels, cells go to apoptosis (Cui et al., 2018). Thus, as antioxidant GSH is important in cancer cells to preserve the oxidative balance. The degradation of GSH in DHA/EPA pretreated MM cells may induce ROS accumulation within cells, which eventually induce MM cell death. Moreover, DHA/EPA selectively killed patient-derived primary MM cells but showed almost no effect on normal PBMCs (Abdi et al., 2014; Mortaz et al., 2020). These findings highlight the potential use of these fatty acids as adjuvants for combating drug resistance in MM. However, after oral administration, these fatty acids are mainly incorporated into circulating phospholipids, triacylglycerol and cholesteryl esters, and the phospholipid of cell membranes as esterified forms (Arterburn et al., 2006; Subbaiah et al., 1993) and only a small fraction (0.4%–0.6%) of total is free (unesterified) (Braeckman et al., 2014), which provokes a question of whether esterified-DHA and -EPA have the same potential to affect cell viability and bortezomib efficacy in MM cells.

The present study evaluated the cytotoxicity of ethyl ester-DHA and -EPA and their synergistic effects with bortezomib in MM cells. Furthermore, ROS, a key factor that contributes to intracellular oxidative stress, was measured in esterified-DHA/EPA treated MM cells to assess the potential involvement of oxidative stress in their cytotoxicity.

2. Materials and methods

2.1. Reagents

DHA (D2534, ≥98% purity), EPA (E2011, ≥99% purity) were purchased from Sigma. Ethyl ester-DHA (FD59690, ≥90% purity) and ethyl ester-EPA (86227-47-6, ≥98% purity) were purchased from Biosynth Carbosynth and Sanbio, respectively. All fatty acids were dissolved in ethanol to produce a 100 mM stock solution and stored at −20 °C. Bortezomib (179324-69-7, >99%) was purchased from LC laboratories and dissolved in DMSO. AnnexinV apoptosis detection kit (88-8005-74) and CellROX® deep red flow cytometry assay kit (C10491) were obtained from Thermo Fisher.

2.2. Cell culture

MM cell lines L363, OPM2, U266 were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU penicillin, and 100 mg/ml streptomycin. Human mast cells were cultured from buffy coat as previously described (Folkerts et al., 2020).

2.3. Cell death analysis

The percentage of dead cells was determined by flow cytometry using Annexin V-FITC and propidium iodide (PI)-PE staining, as described previously (Chen et al., 2020). For pretreatment, MM cells were seeded into a 12-well plate at density of 200,000 cells/well and treated with the indicated concentrations of ethyl ester-DHA/EPA for 2 h. After 2 h of pretreatment, bortezomib was added directly into the medium for an additional 24 h incubation. Subsequently, annexin V and PI staining-based flow cytometry was performed for cell death analysis (Chen et al., 2020).

2.4. Intracellular ROS measurement

MM cells were seeded into a 12-well plate at density of 200,000 cells/well and treated with the indicated concentrations of compounds for 2 h. After treatment, cells were incubated with CellROX® Deep Red reagent at a final concentration of 500 nM for 1 h at 37 °C in the dark. Then, cells were washed and resuspended with cold PBS. Intracellular ROS levels were determined by flow cytometry immediately.

2.5. Statistics

Statistical analysis was performed using GraphPad Prism software. Statistical significance between the tested groups was determined using one-way ANOVA. $P < 0.05$ was considered significant. In each bar of all graphs, the upper portion and the lower portion, differentiated by two colors, represent percentage of Annexin V+/PI+ and Annexin V+/PI-, respectively.

3. Results

3.1. Ethyl ester-DHA/EPA were more potent than DHA/EPA for inducing apoptosis in MM cells

In our previous studies, it was demonstrated that various MM cell lines responded differently to DHA/EPA treatment. L363 and OPM2 cell lines were sensitive to DHA and EPA, while U266 cell line was resistant to both (Chen et al., 2020). The cytotoxicity of ethyl ester-DHA/EPA in DHA/EPA-sensitive MM cells were first evaluated. L363 and OPM2 cells were incubated with a range of concentrations of these esterified-fatty acids for 24 h, and cell death was determined by flow cytometry using Annexin V and PI staining. As shown in Fig. 1, esterified-DHA/EPA potently increased the percentage of apoptotic cells (Annexin V+) in a concentration-dependent manner and ethyl ester-DHA showed a stronger induction of apoptosis than ethyl ester-EPA in these cells, in line with the earlier observations with DHA and EPA (Chen et al., 2020).

Moreover, the esterified-DHA/EPA exhibited remarkably higher toxicity than DHA/EPA in DHA/EPA-sensitive MM cell lines L363 and OPM2 (Fig. 2A and B). Interestingly, these esterified-fatty acids induced pronounced apoptotic cells in the DHA/EPA-resistant cell line U266 as well (Fig. 2C). It is notable that U266 is also the least sensitive cell line to the esterified-DHA/EPA among these MM cell lines (Figs. 1 and 2C).

3.2. Pretreatment with ethyl ester-DHA/EPA before chemotherapy bortezomib synergistically enhanced bortezomib efficacy

The first experiments revealed the potent cytotoxic bioactivity of ethyl ester-DHA/EPA in MM cell lines, including DHA/EPA-resistant cell line U266. Interestingly, consistent with the previous results of DHA/EPA (Chen et al., 2020), pretreating L363 cells with nontoxic concentrations of esterified-DHA/EPA before bortezomib synergistically enhanced the efficacy of bortezomib, whereas simultaneous treatment of cells with same concentrations of these fatty acids had no such effect (Fig. 3A and B). Therefore, different treatment schedules with esterified-DHA/EPA and bortezomib lead to different anticancer activity in DHA/EPA-sensitive MM cells, depending on the timing of the treatment.

The result of the remarkable cytotoxicity of the esterified-DHA/EPA in U266 cell line encouraged us to further evaluate their effect on bortezomib efficacy. Interestingly, simultaneous treatment with these esterified-fatty acids inhibited bortezomib efficacy in U266 cells, while pretreatment had almost no effect on bortezomib cytotoxicity (Fig. 3A and C). Together, these results provide evidence that the timing of the ethyl ester-DHA/EPA treatment may be important for bortezomib cytotoxicity in MM cells.

3.3. Ethyl ester-DHA/EPA increase intracellular ROS levels in MM cells

It has been demonstrated that pretreatment with DHA/EPA increase bortezomib cytotoxicity by promoting oxidative stress-induced GSH degradation in MM cells (Chen et al., 2021). Mechanistically, decrease of cellular GSH can induce the accumulation of ROS in cancer cells, which

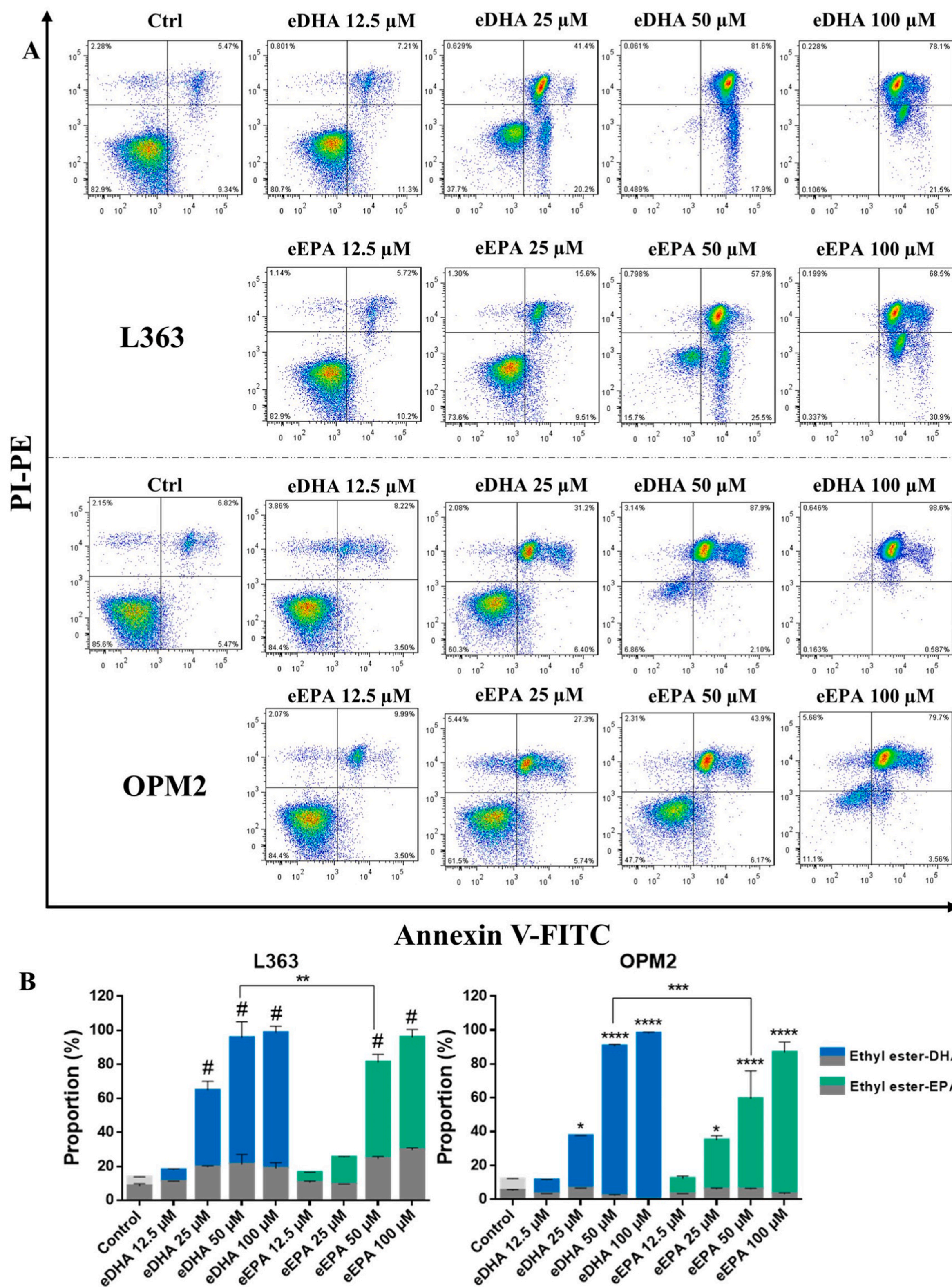
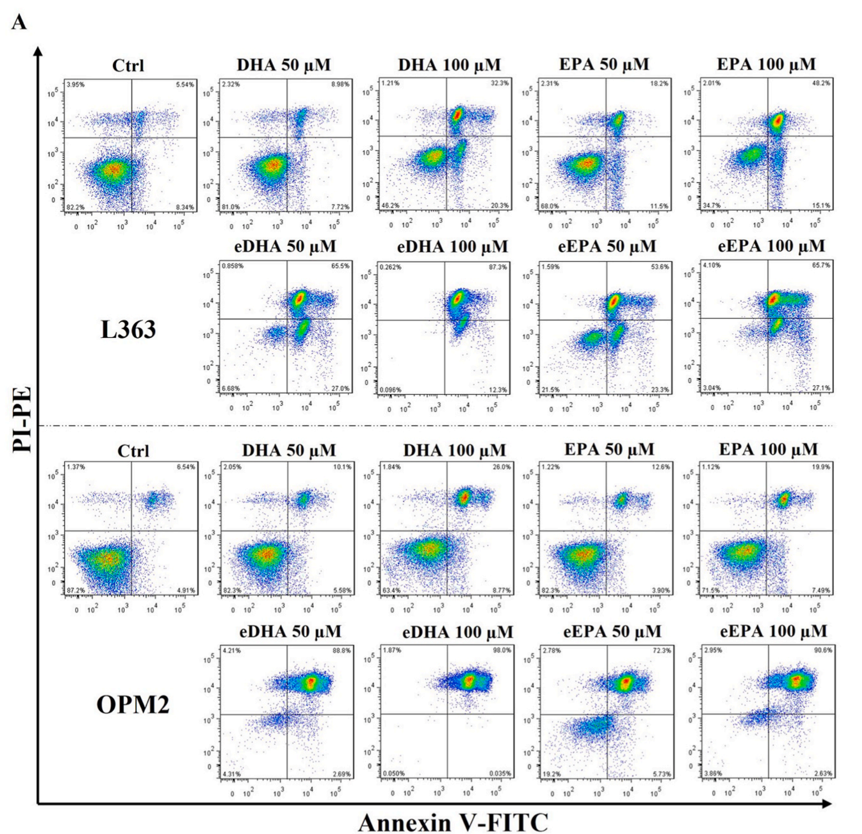
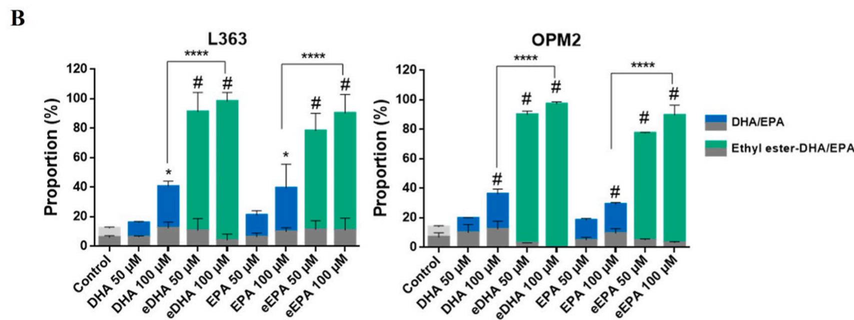


Fig. 1. Cytotoxicity of ethyl ester-DHA/EPA in DHA/EPA sensitive MM cells. A) L363 and OPM2 cells were treated with indicated concentrations of ethyl ester-DHA/EPA for 24 h. Apoptotic cells were determined by Annexin V and PI staining. **B)** Quantification of A. The upper portion of the bar (blue for EPA or DHA; green for esterified-EPA or -DHA) and the lower portion (grey)represent percentage of Annexin V+/PI+ and Annexin V+/PI-, respectively. Data are presented as mean ± SD of three independent repeats. $^{*}p < 0.05$, $^{\#}p < 0.0001$ compared with control.





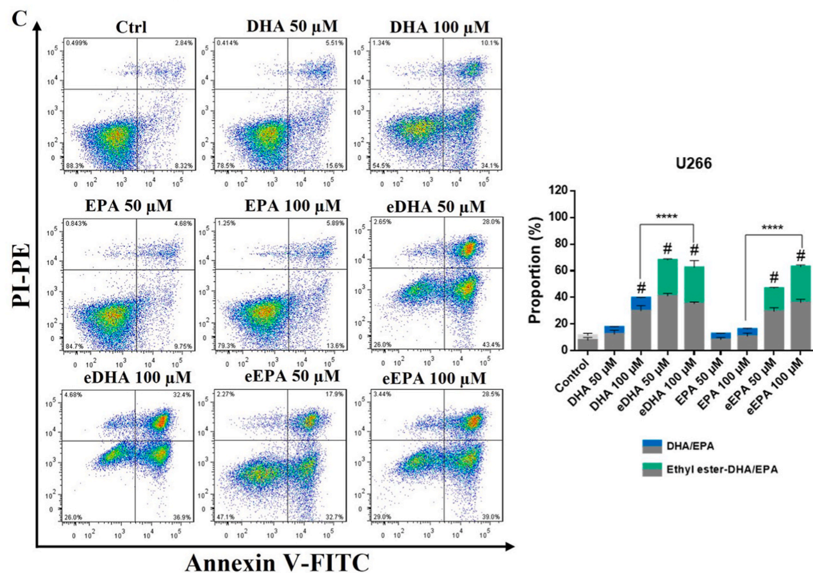
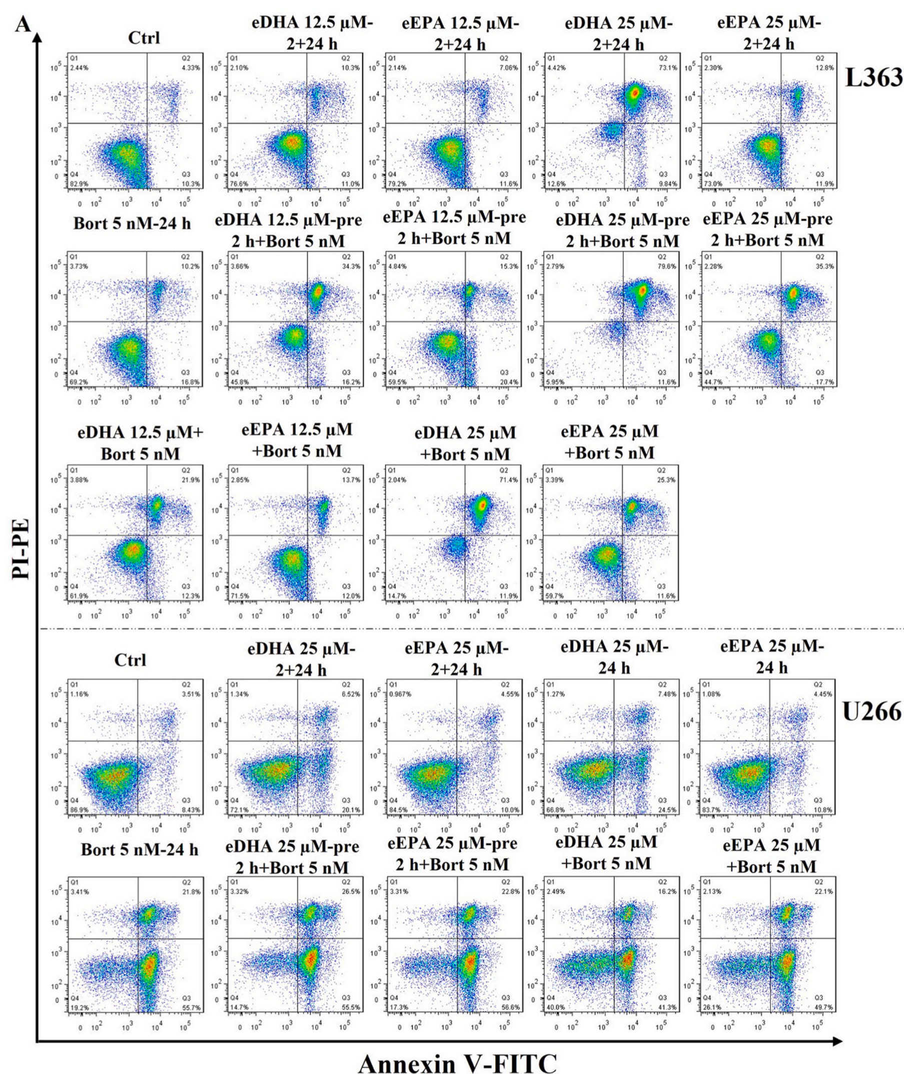


Fig. 2. The effect of ethyl ester-DHA/EPA on cell death (proportion cell death (%) of MM cells. A) L363, OPM2 and **C)** U266 cells were treated with indicated concentration of compounds for 24 h. Apoptotic cells were determined by Annexin V and PI staining. **B)** Quantification of A. The upper portion of the bar (blue for EPA or DHA; green for esterified-EPA or -DHA) and the lower portion (grey) represent percentage of Annexin V+/PI+ and Annexin V+/PI-, respectively. The upper portion of the bar (blue for EPA or DHA; green for esterified-EPA or -DHA) and the lower portion (grey) represent percentage of Annexin V+/PI+ and Annexin V+/PI-, respectively. Data are presented as mean ± SD of two independent repeats. $^{**}P < 0.01$, $^{****}P < 0.0001$. $^{\#}P < 0.0001$ compared with control.



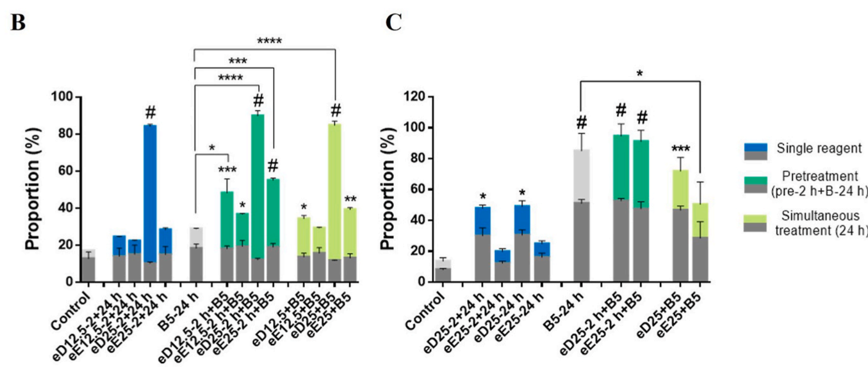


Fig. 3. The effect of ethyl ester-DHA/EPA on the bortezomib cytotoxicity in MM cells. A) L363 and U266 cells was pretreated with esterified-DHA/EPA for 0 or 2 h and then incubated with bortezomib (5 nM) for 24 h. Apoptotic cells were determined by Annexin V and PI staining. **B)** and **C)** Quantification of A. In each bar graph, the upper portion and the lower portion (grey) represent percentage of Annexin V+/PI+ and Annexin V+/PI-, respectively. Data are presented as mean ± SD of two independent repeats. *$p < 0.05$, **$p < 0.01$, #$p < 0.0001$ compared with control. ***$p < 0.001$, ****$P < 0.0001$.

therefore can lead to DNA damage and more cell death when its concentration reaches toxic levels (Caillot et al., 2021; Starheim et al., 2016). Intracellular ROS was determined in MM cells using flow cytometry. Interestingly, as the concentrations of the esterified-DHA/EPA increased, a right shift of the ROS peaks was observed in treated MM cells (Fig. 4), indicating the increase of ROS levels after treatment. This result provides support that the ethyl ester-DHA/EPA may induce MM cell apoptosis through enhancing oxidative stress. In addition, these esterified-fatty acids showed no effect on human primary mast cells even at high concentrations (Fig. 5), suggesting their selective cytotoxicity against human primary cells and MM cells.

4. Discussion

One of the major frustrations for MM treatment comes from the fact that many patients, sooner or later, suffer from repeated relapses after initial therapeutic successes. Recently, we found that rational combination of omega-3 fatty acids DHA/EPA with chemotherapy bortezomib may have the potential to overcome chemoresistance in MM cells through activating GSH degradation-induced apoptosis (Chen et al., 2020, 2021). The present study provides further evidence that

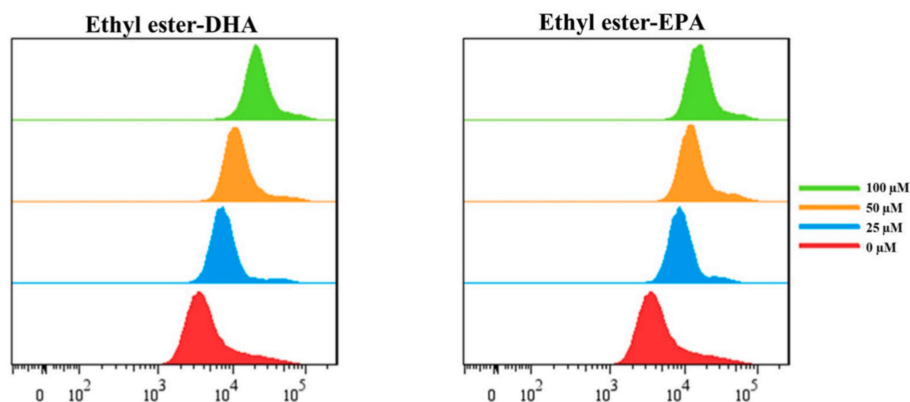


Fig. 4. Ethyl ester-DHA/EPA increase ROS level in MM cells. L363 cells were treated with 0, 25, 50 or 100 μM of Ethyl ester-DHA/EPA for 2 h. The intracellular ROS level was analyzed using CellROX® Deep Red Flow Cytometry Assay Kit.

Fig. 5. The effect of ethyl ester-DHA/EPA on cell death of human primary mast cells. Human primary mast cells were isolated form surplus autologous stem cell concentrates and treated with indicated concentrations of compounds for 24 h. Apoptotic cells were determined by Annexin V and PI staining. Data are presented as mean ± SD of two independent repeats. The upper portion (blue and green) and the lower portion (grey) represent percentage of Annexin V+/PI+ and Annexin V+/PI-, respectively.

pretreatment with esterified-DHA/EPA, the main forms of DHA/EPA present after oral intake, potently enhance bortezomib efficacy, similar to DHA/EPA. Therefore, this shows that esterification of DHA/EPA does not inhibit their tumor-cytotoxic activity.

Studies have demonstrated that the majority of DHA/EPA are present as esterified molecules in blood shortly after oral administration (Braeckman et al., 2014; Rusca et al., 2009). Therefore, our previous studies on the synergistic anticancer efficacy with bortezomib caused by DHA/EPA pretreatment in MM cells raised a question of how esterified-DHA/EPA affect bortezomib efficacy in these same cells. Interestingly, current results suggest that administration of the ethyl ester-DHA/EPA before bortezomib can overcome bortezomib-associated resistance in DHA/EPA-sensitive MM cells, which is consistent with the findings of DHA/EPA (Chen et al., 2020). Our results provide a

promising therapeutic strategy using ethyl ester-DHA/EPA in combination with bortezomib for overcoming drug resistance in MM. Interestingly, a preparation of omega-3 acid ethyl esters, Omacor, contains approximately 46.5% ethyl ester-EPA and 37.5% ethyl ester-DHA, has been used as a pharmaceutical drug for lowering triglycerides to reduce the risk of coronary artery disease, heart disease and stroke (Bays, 2006). Omacor has been advanced into clinical trials in prevention of breast cancer (NCT01282580) and colorectal cancer (NCT03831698). Due to the prominent anti-myeloma activities and high stability after oral intake, ethyl ester-DHA/EPA may be more desirable for combating bortezomib-associated resistance in MM.

Our observation of the decreased toxicity of bortezomib in ethyl ester-DHA/EPA simultaneously treated MM cells is consistent with our previous study of DHA/EPA (Chen et al., 2020). Using a cell-permeable fluorescent proteasome activity probe (Berkers et al., 2005), we evaluated the proteasome activity by different treatment in MM cells. As shown in the supplementary data, simultaneous treatment, especially by DHA, almost fully blocked the inhibitory effect of bortezomib on proteasome activity, which reflected the fact that less bortezomib entered simultaneously treated cells compared to only bortezomib-treated cells, indicating a conclusion that these fatty acids somewhat impede drug influx in MM cells when they are used concomitantly with chemotherapeutic drugs. Because ethyl ester-DHA/EPA can also affect cell membranes, it is reasonable to assume that these esterified fatty acids may interfere with the uptake of bortezomib in MM cells when they are used simultaneously with bortezomib.

In addition to apoptosis, RNA-seq analysis has shown that necroptosis and ferroptosis, two recently recognized forms of programmed cell death, have been strongly implicated in DHA/EPA-induced MM cell death (Chen et al., 2021). Necroptosis has been associated with a variety of human pathological processes, including neurodegenerative disorders, inflammatory diseases, kidney injury and multiple cancers, mechanistically characterized by MLKL phosphorylation, oligomerization of the phosphorylated MLKL and eventual plasma membrane rupture (Chen et al., 2019). We have found that these fatty acids induced MLKL oligomerization in MM cells (manuscript submitted), thereby confirming their capacity to induce necroptosis. This is mainly due to the quite high expression level of the effector protein of necroptosis (MLKL) in MM cells. Due to the high toxicity of ethyl ester-DHA/EPA in MM cells and their functional similarity to DHA/EPA, it is reasonable to assume that these esterified fatty acids can also induce necroptosis in MM cells. Ferroptosis can be initiated by the failure of the GSH-dependent antioxidant defense, which results in lipid peroxidation and eventual cell death (Tang et al., 2021). The increased ROS level in ethyl ester-DHA/EPA treated MM cells may suggest the failure of the GSH-dependent antioxidant defense, which tips the oxidative balance in these cells in favor of cell death. Therefore, ferroptosis may also be involved in the cell death induced by ethyl ester-DHA/EPA in MM cells. Further investigation is ongoing to confirm the involvement of necroptosis and ferroptosis in the esterified DHA/EPA-induced MM cell death.

It has been reported that CHAC1-mediated GSH degradation is strongly implicated in the potentiation of bortezomib efficacy by DHA/EPA (Chen et al., 2021). GSH is an intracellular "master antioxidant" that plays key roles in maintaining cellular redox homeostasis (Kennedy et al., 2020). GSH degradation has been reported to increase intracellular ROS levels, leading to oxidative stress-induced cell death when it accumulates to toxic levels (Chen et al., 2017). Moreover, we have revealed that NRF2-ATF3/4 signaling pathway may be associated with CHAC1 expression level. Interestingly, in addition to upregulation of *CHAC1* for GSH degradation, NRF2-ATF3/4 signaling pathway also can upregulate the expression of a series of genes for GSH synthesis, including *GCLM*, *GSR*, *PSAT1*, *PSPH*, *MTFHD2*, *SHMT2*, *TAK* and *TALDO*. Therefore, it is reasonable to assume that these esterified DHA/EPA may affect GSH metabolism by upregulation of genes for GSH synthesis and degradation. CHAC1-mediated GSH degradation may be critical for inducing MM cell death, while GSH synthesis may function to recover the cellular redox homeostasis during cell death. The increase of ROS level in esterified-DHA/EPA-treated MM cells reflected the enhanced oxidative stress in these cells, indicating that oxidative stress may be correlated with the cytotoxicity of esterified-DHA/EPA in MM cells.

Taken together, the current study provides evidence that ethyl ester-DHA/EPA exhibit considerable cytotoxic bioactivity against MM cells and administration of these esterified-DHA/EPA prior to chemotherapy bortezomib could improve the cytotoxicity of bortezomib, supporting the rationale of using DHA/EPA or ethyl ester-DHA/EPA as adjuvants for overcoming drug resistance during MM therapy.

CRediT authorship contribution statement

Jing Chen: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original. **Rob Ruijtenbeek:** Writing – review & editing. **Johan Garssen:** Writing – review & editing, All authors read and approved the final manuscript. **Frank A. Redegeld:** Conceptualization, Data curation, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that no conflicts 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.ejphar.2022.174883.

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