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Original article

Molecular mechanisms underlying antitumor activity of camel whey protein against multiple myeloma cells

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ABSTRACT

Treating drug-resistant cancer cells is a clinical challenge and it is also vital to screen for new cancer drugs. Multiple myeloma (MM) is a plasma cell clonal cancer that, despite many experimental therapeutics, remains incurable. In this study, two MM cell line lines U266 and RPMI 8226 were used to determine the impact of camel whey protein (CWP). The CWP IC_{50} was calculated by MTT examination, while the flow cytometry analysis was used to investigate the chemotaxis responses of MM cells in relation to CXCL12 and the pro-apoptotic effect of CHP. MM cells were treated with CWP and Western blot analysis was used to determine the underlying molecular mechanisms. Dose and time based on the impact of CWP on the cell viability of MM cells with IC_{50} of 50 μg/ml, without affecting the viability of normal healthy PBMCs. CWP reduced chemotaxis of MM cells significantly from the CXC chemokine ligand 12 (CXCL12). Using Western blot analysis, we found that CWP decreased the activation of AKT, mTOR, PLCβ3, NFαB and ERK, which was mechanistically mediated by CXCL12/CXCR4. In both U266 and RPMI 8226, CWP induced apoptosis by upregulating cytochrome *C* expression. In addition, CWP mediated the growth arrest of MM cells by robustly decreasing the expression of the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-XL and Mcl-1. Conversely, the expression of pro-apoptotic Bcl-2 family members Bak, Bax and Bim was increased after treatment with CWP. Our data indicates CWP's therapeutic potential for MM cells.

1. Introduction

Multiple myeloma is the second most prevalent hematological disease in the US and is a clonal long-lived plasma-cylinder malignancy which burdens about 20,000 people and kills around 10,000 annually (Picot et al., 2011; Tuchman et al., 2012). Several studies have clarified the function of chemokines and their receptors in MM pathogenesis (Aggarwal et al., 2006). Chemokine receptors are found in cells of cancer and function in all tumor stages (Arya et al., 2003). In many other malignancies CXCR4 is the most deeply expressed chemokine receptor in MM cells and cells (Möller et al., 2003). The chemokine receptor expressed by MM cells is the most abundant and functional and hence could play an important role in the disease pathogenesis (Fulton 2009). In lung, liver, bone marrow and lymph nodes that are a typical metastatic site for many forms of cancer, the CXCR4 Ligand, CXCL12, is commonly represented. Continuing studies indicate that the association between CXCL12 and CXCR4 (Al Ghamdi et al., 2015; Alsayed et al., 2007; Badr et al., 2011) is significant in the maintenance and survival of MM cells in in vivo and in vitro models.

Apoptosis dysregulation tends to be a major contributor to both chemoresistance and pathogenesis in cancer (Tiberio et al., 2012). Agents that induce apoptosis in MM cells can lead to improved disease or rebound, and can play an important role in the treatment of myeloma. Mainly myeloma patients with plasma cell lines are

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overexpressed with anti-apoptotic molecules such as Bcl-2 and Bcl-XL (Pettersson et al., 1992) and with Bcl-2 overexpressions (van de Donk et al., 2003). The blockage of cytochrome *C* blockades prevents the activation of caspo-3 and the initiation of apoptosis (Nie et al. 2014), by inhibiting pro-apoptotic proteins of the Bcl-2 community (for example, Bax and Puma) and by blocking cytohrome C releases from the mitochondria. Upregulated expression of Bcl-2 is also associated with resilience of either etoposide or doxorubicin-treated tumor cells (Feinman et al., 1999). The abrogation or overcoming of Bcl-2′s anti-apoptotic potential may therefore increase chemosensitivity and decrease chemoresistance in myeloma tumor cells.

Numerous natural products for use in the treatment of malignancies have been tested in recent years. Some were used to minimize side-effects and improve efficacy as adjuvants in combination with chemotherapy (Bose et al., 2015). Camel whey protein (CWP) is immunomodulated and is an important system for the supply of glutathione to cysteine (GSH) in diseases involving immunodeficiency (Beaulieu et al., 2006). In our earlier trials CWP has been shown (Badr et al., 2019), protected from apoptosis and protected the lymphocytes (Badr et al., 2018a; Badr et al., 2018b). In addition, the explained by El-Shinnawy et al. (2018).

The CWP effects on MM cells are nevertheless unaware. This research has thus investigated the impacts of cellular and molecular mechanism on the growth arrest of human MM cell line (U266 and RPMI 8226) in the U266 and RPMI 8226.

2. Materials and methods

2.1. CWP preparation

The milk of healthy female camels from Marsa Matrouh, Egypt was isolate and distilled from the CWP, as mentioned before (El-Shinnawy et al., 2018).

2.2. Cell culture

The American Type of Culture Collection (Rockville, MD, USA) developed RPMI8226 and U266 MM cell lines. In $5X10^5$ viable cells/mL the cells were grown at 37 °C below 5% CO_2 by means of R-10 media (RPMI 1640 containing 10% fetal calf serum and 1% L–glutamate and 1% penicillin – streptomycin). In accordance with the Ficoll-Paque Standard Manufacturer Direction (Amersham Pharmacia, Uppsala, Sweden), peripheral blood mononuclear cells (PBMCs) of healthy donors were purified.

2.3. Cytotoxicity assay

A MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used for evaluation of cytotoxic effects on both cell lines and PBMCs. The CWP (0,1 μg/ml, 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/mL, 100 μg/mL and 200 ug/ml) or 100 μg/mL of bovine serum albumin (BSA) have been put and treated in 2 ml of culture medium as negative controls for 0, 1, 3, 6, 11, 12, 24, 48 h, at a variety of CWP concentrations. Absorption was estimated at 570 nm and a relative portion of optical density values were determined as a percentage of cell viability. The reverse phase microscope (Olympus, Tokyo, Japan) used morphologic changes following exposure to medium (0), BSA, and CWP.

2.4. Chemotaxis and transwell cell migration assay

In the transwell top chamber (10 μg/mL; Costar, Cambridge, MA, USA) were added cells RPMI8226 and U266 (4.0 to 105 cells). The lowness of the chamber was supplemented by CXCL12 (250 ng/mL; R&D systems) or a 600 μL migration buffer alone. After three hours of incubation with a temperature of 37°, input cells and cells were analyzed using a FACSCalibur (BD-PharMingen) flow cytometer (Badr et al., 2011) and counted by flow cytometry. The data is shown as the percentage of CXCL12-induced migration input cells.

2.5. Apoptosis detection assay

A double-staining (ABCam, Canada), was used for the determination of the percent of the apoptotic cells mentioned earlier (Sayed et al., 2012). ABCam is also a targeted product for the determination of apoptotic cells as defined above. After 24 h of CWP therapy, cells (1–106 cells/mL) were harvested. After staining Annexin V-FITC and PI in the dark for 15 min at 25 °C followed by cytometrical analysis of flow, the percentage of cells subject to apoptosis was identified.

2.6. Western blot analysis

CWP was treated or untreated with RPMI8226 and U266 cells (0) for 12 h, as defined previously (Badr et al., 2016). Some untreated cells first had to be pretreated with the following inhibitors for a period of 12 h at a temperatures of 37 C (1 μM wortmannin (WM), a phosphatidylinositol-3 kinase (PI3K) inhibitor, 10 μM PD98059, inhibitor of mitogen-active protein kinase (MEQ1/2) and 10 nM of arctigenine inhibitors (PHC); For 2 min, the medium (0) or 250 ng/mL CXCL12 stimulated both treated and untreated samples.

The same quantities (50 μg) of the total cellular protein were separated by a phospho-AKT (s473), a phospho-ERK1/2 (t202/y204), and phospho-IκBα (S32/36), a phospho-PLCβ3 (s537), a phospho-mTOR, a pan-AKT, pan-ERK, a pan-PLCβb3, and a pan-IκBα (all of the N-AKT) antibodies. Fifty micrograms of lysates were transferred onto polyvinyl Fluoride membranes for immunoblotting with: phospho-AKT (S474). The ECL signal has been reported using LI-COR C-DiGit Chemiluminescence Western Blot Scanner, analyzed by software NIH Image J (National Institutes of Health, Bethesda, MD, United States), which can be used for the analysis and analysis of the ESD scanner.

2.7. Statistical analysis

The software Prism 6.0 (GraphPad Software, San Diego, CA, USA) used to perform statistical analyzes. Data is expressed as a ± default error. In order to equate the cell handled with untreated cells, the *t*-test of the student's two tailed. In order to measure variations between three classes, one or both forms ANOVA, accompanied by Bonferroni's multiple comparison tests was utilized. Data were tested with a single- or bidirectional ANOVA followed by a post-hoc test by Tukey. $P < 0.05$ was regarded as meaningful (Khan et al., 2019).

3. Results

3.1. CWP prevents MM cell growth

In the cell line U266, MMI 8226 and PBMCs, isolated from ordinary healthy humans, the cytotoxically effects of CWP have been studied. Different CWP concentrations (0, 1, 5, 10, 25, 50, 100 and 200 μg/mL) were used to treat cells with the aid of an MTT test shown in Fig. 1 for different incubation periods (0, 1, 3, 6, 12, 24 and 48 h). CWP treatment in a concentration and time-dependent manner substantially inhibited the growth of U266 and RPMI 8226 cells, but not normal PBMCs. The CWP IC_{50} was

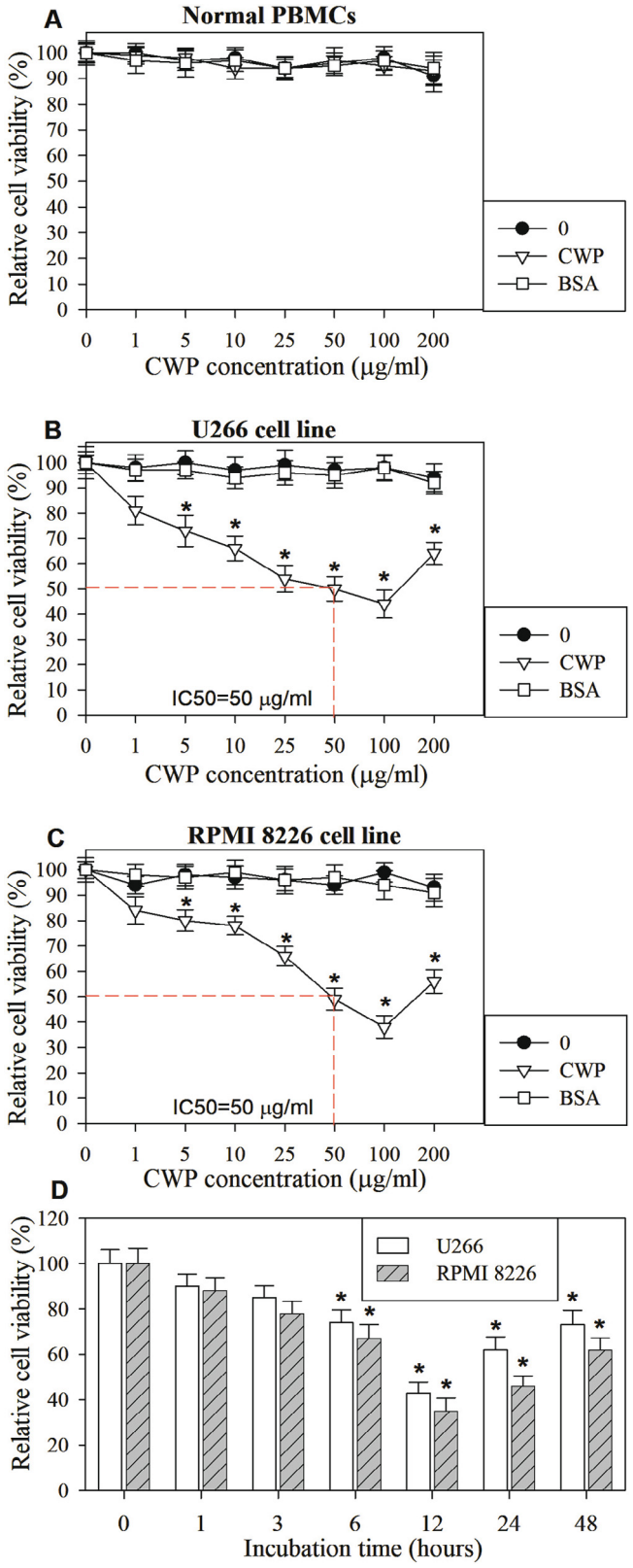

Fig. 1. CWP therapy has a time and dose-based effect on the viability of cells. The MTT test measured cell viability. Cell viability. The median (0) and varying concentrations (1, 5, 10, 25, 5, 100, 200 µg/mL) for CWP (open triangles) and 100 µg/mL for BSA (open squares) were handled for standard PBMCs (A), U266 cells (B) and RPMI 8226 cells (C). (d) The cells were then incubated with CWP for various cycles (0, 1, 3, 6, 12, 24, 48 h) with a calculation of their percentage of relative cell viability. The collected data from five independent experiments are displayed as medium percentages of viable cells ± standard error and the findings are expressed.

50 µg/mL and after 12 h of treatment, cells were highly sensitive. A negative control was used for the BSA (100 µg/mL) that does not affect the viability of the myeloma cell line.

3.2. CWP blunts MM cell migration encouraged by CXCL12

In U266, RPMI 8226 cell lines for 12 h after CWP (50 µg/mL) treatment with the use of Transwell Plates and flow cytometry analysis were checked on the effects of CWP on CXCL112-promoted migration, an important parameter to metastasize the myeloma cell. The dot plots display the RPMI 8226 cells to median or CXCL12 directional migration (Fig. 2A). As can be seen from the collected data from 10 independent experiments (Fig. 2B), the CWP reduced significantly the CXCL12-fostered migration of both cell lines.

3.3. Therapy with CWP abrogates ACT, mTOR, PLCβ3, NFκB and ERK by CXCL12/CXCR4

Since the treatment of both U266 and RPMI 8226 cell CXCL12-mediated chemotaxis with CWP has decreased significantly, AKT,

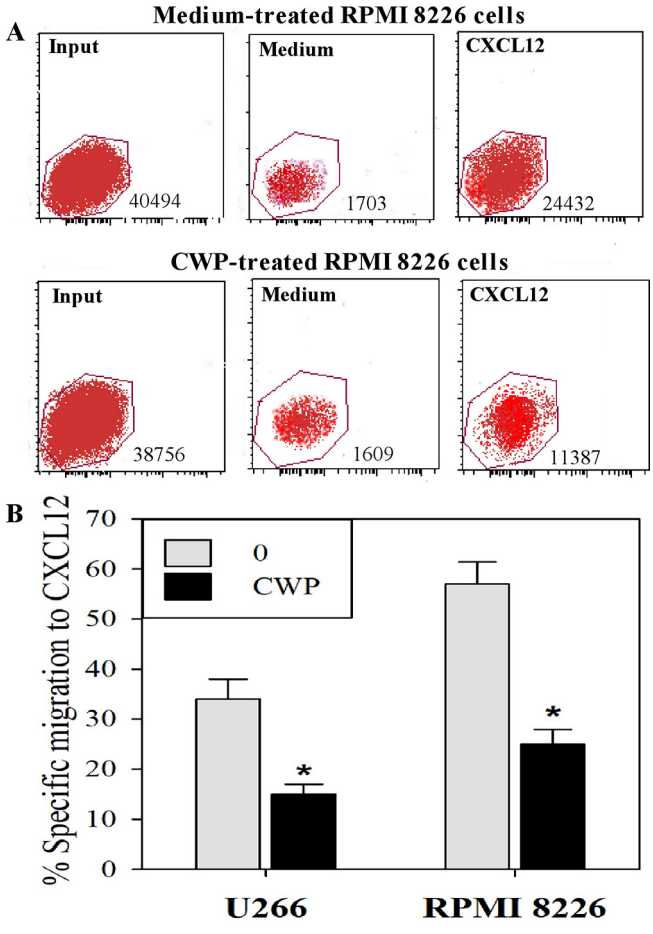

Fig. 2. CWP treatment effect on chemotaxis mediated in MM cells by CXCL12. Following treatment for 12 h with CWP, Transwell's plates and the cytometry flow study have been used to calculate the migratory response from cells U266 and RPMI 8226 to CXCL12 (250 ng/mL). (a) The total number of untreated, CWP-treated RPMI 8226 cells and cell number that migrated to medium, or CXCL12 was demonstrated by one representative experiment. (b) Cumulative data of ten independent experiments is represented in the medium-treated (gray) and CWP (closed black bar) cell, as the mean percentage of real migration ± standard errors in response to CXCL12.

mTOR, PLCß3, IκBα and ERK1/2 phosphorylation status and Rho-A active status after stimulation with CXCL12 were analyzed. Medium (0), 1 μM WM, 10 nM RAPM, 10 μM PD98059, 100 nM U73122 and 10 nM of arctigenin have been used to treat MM cells for 12 h. Moreover, untreated, inhibitor-treated and CWP-treated cells were then treated 2 min with 250 ng/mL CXCL12 before lysation, and were subjected to pAKT, pmTOR, pPLCβ3, pIκBα, pERK, pan-AKT, pan-mTOR, pan-IκBα, and pan-ERK antibodies to Western blotting. Like in the Fig. 3A, CWP therapy has lowered the CXCL12-mediated phosphorylated levels of AKT, mTOR, PLCβ3, IκBα and ERK.

Phospho AKT (pAKT) levels were calibrated for the overall specific protein levels, with PLCβ3 (pPLCβ3), pIκBα and pERK1/2. Collected data is shown in the Fig. 3B & C from 10 independent tests carried out on cell lines of RPMI 8226 and U266. CXCL12/CXCR4-enhanced phosphorylation of AKT, mTOR, PLCβ3, IκBα and ERK was significantly reduced by treatment with CWP ($P < 0.05$). While Rho-A activation was increased by CXCL12, and CWP therapy had no effect (data not shown).

3.4. CWP induces MM cell apoptosis

Using the annexin V FITC/PI kit, the effect of CWP on induction of apoptosis in cell lines U266 and RPMI 8226 were examined. Like in the Fig. 4A, in the untreated cells (0) U266 and RPMI 8226 respectively, the percentage of apoptotic cells was 4 and 5. The percentage of apoptotic cells was 51% in the U266 cells and 75% in the RPMI-8226 cells has increased significantly with CWP therapy. The percentage of viable cells in the U266 cells (96% vs 49%) and in the RPMI 8226 cells (95% vs 25%), also decreased after CWP treatment. Pooled results from 10 studies have shown that CWP induces apoptosis significantly in myeloma cells (Fig. 4B).

We examined the expression of cytochrome *C* by Western blotting in U266 and RPMI 8226 cells in order to better understand the role of CWP in apoptosis induction. The treatment of MM cells with CWP clearly increased the cytochrome *C* expression, as shown in Fig. 5A. Complete normalization of the cytochrome *C* with total protein β-actin levels was performed. The data obtained from ten U266 and RPMI cell line experiments (Fig. 5B) show clearly that

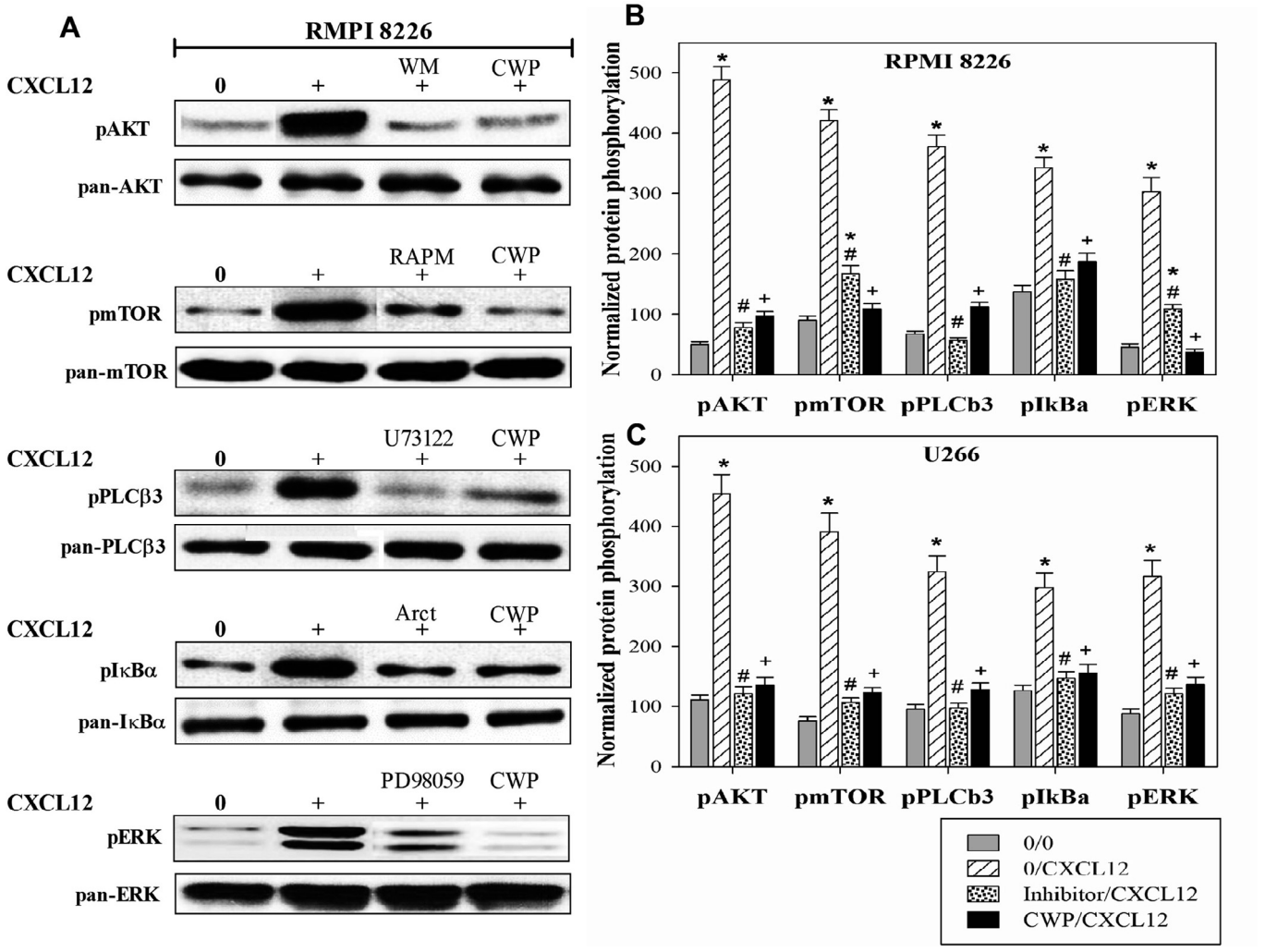

Fig. 3. CWP impact on signaling via CXCL12. The medium (0), 1 μM WM, 10 nM RAPM, 10 nM PD98059, 100 nM U73122, and 10 nM arctigenin were treated to untreated RPMI 8226 cells for 12 h. (A) Through the use of pAKT, pmTOR, pPLCβ3, pIκBα, pan-AKT, pan-PLCβ3 and pan-ERK antibodies, the untreated, inhibitor-treated and CWP-treated cells were then treated for 2 min with medium, or 250 ng/mL, CXCL12 until they were lysed and subjected to western blotting. A representative experiment reveals the protein bands. Phosphorylated AKT (pAKT), PLCβ3 (pPCβ3), IκBα (pIκBα) and ERK1/2 (p-ERK1/2) levels were calibrated for the overall relevant protein levels. A representative blot from 10 separate experiments is shown for each downstream effector. Average values for standardized, unique phosphorylated proteins ± standard defect from 10 different (B) RPMI 8226 and (C) U266 (experiment) experiments are accreted from ten experiments. *P < 0.05, treatment with CXCL12 vs. untreated + P < 0.05, untreated inhibitor vs. + P < 0.05, treated with CWP, vs. unrecited.

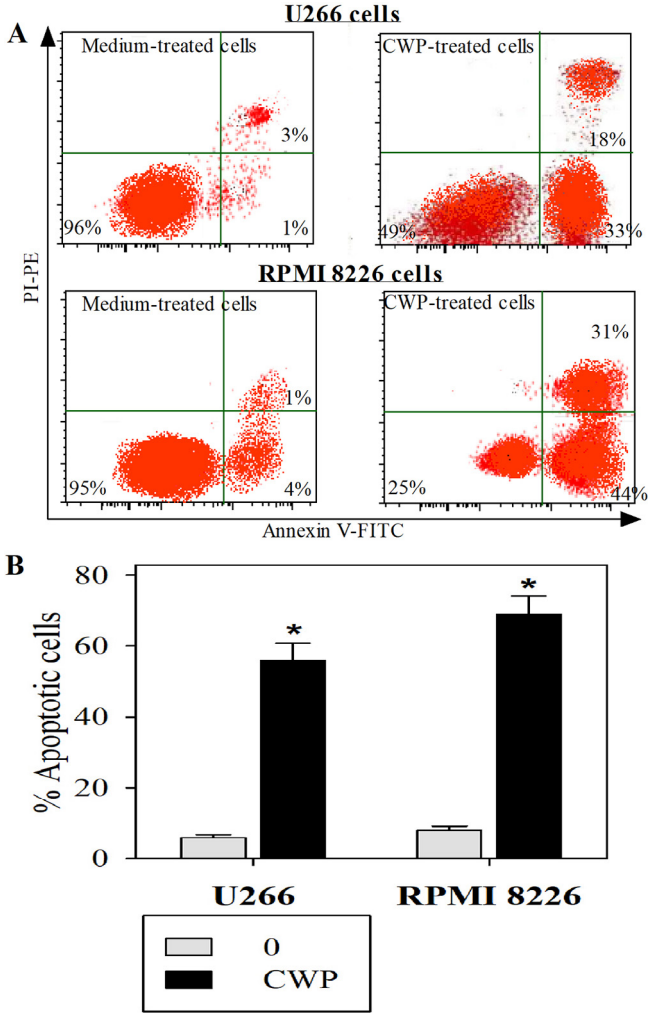

Fig. 4. Apoptosis caused by CWP in MM cells. A flow cytometry based on annexin V FITC/PI stain pattern was used to assess the ability of CWP to lead to apoptosis or necrosis of MM cancer cells. (a) A representative data set consisting of ten independent tests shall be shown. (b) Cumulative results from 10 experiments are indicated as the average apoptotic percentage ± standard cancer cell error for untreated (0, gray bar) and CSB (closed black bar) cells. *CWP < 0.05, untreated cells. * P < 0.05.

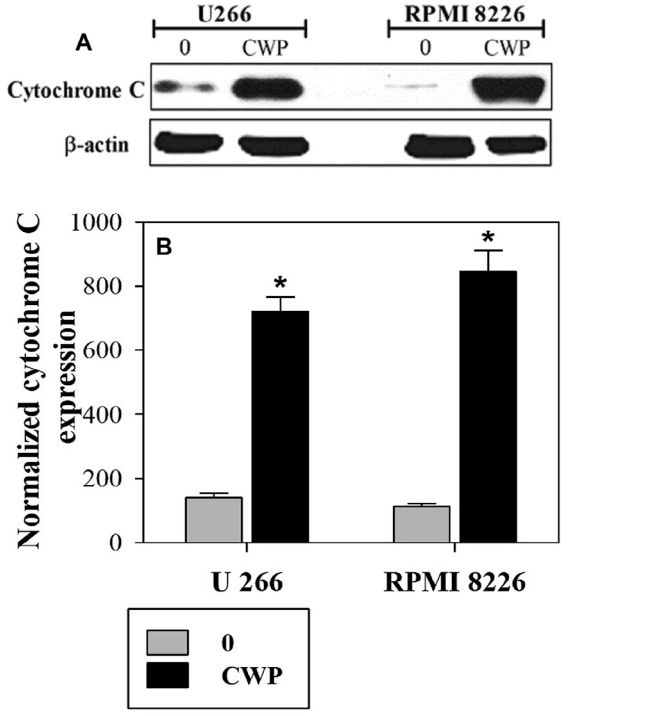

Fig. 5. The cytochrome *C* expression in MM cells has been upgraded by CWP. The U266 and RPMI 8226 cells were lysed and subjected, untreated (0) and CWP-treated, to western blot analysis, with cytochrome *C*-reconnaissance antibodies and complete β-actin. (a) A representative experiment reveals the protein bands. Cytochrome *C* expression levels were standardized to a complete β-actin level. A representative blot of ten independent experiments for each C cytochrome and a total β-actin are seen. Average values of normalized protein ± SEM are suggested by the cumulative results from 10 independent U266 and RMPI 8226 cells (b) experiments. *P < 0.05, CWP-processed versus unprocessed cells.

the expression of cytochrome *C* is significantly higher than that of untreated cells (0) in the treatment with CWP.

3.5. CWP induces cell mortality in MM cells by changes in the Bcl-2 family members

The effect of the CWP treatment was studied using western blotting on the expression of anti-apoptotic and pro-apoptotic Bcl-2 family members in U266 and RPMI-8226 cells. Like in the Fig. 6A, CWP therapy reduced Bcl-2, Bcl-XL and Mc-1 expression and increased Bak, Bax and Bim expression in myeloma cells. The acquired data (n = 10) showed that CWP treatment substantially abolished anti-apoptotic protein expression (see Fig. 6B) and significantly increased pro-apoptotic protein expression in both U266 and RPMI 8226 cell lines (see Fig. 6C).

4. Discussion

This study provides the first data on the effects of CWP on growth arresting in in vitro human MM cells in U266 and RPMI 8226. We find that CWP inhibits concentration and time-dependent growth of myeloma cells. Its IC_{50} was 50 μg/mL and after 12 h of therapy, cells were highly sensitive. Chemokines and their receptors play an important role in their immune function and interactions in B cell malignancies may have tumor-promoting effects (Jöhrer et al. 2012). Meads et al. reported that CXCR4 over-expression directs tumor cell migration to CXCL12 (Meads et al. 2008). Our findings demonstrated CWP's effects on U266, RPMI8226 and PBMC's isolated from ordinary healthy people. Here, we assessed the effects of CWP on signalization of CXCL12 to facilitate migration of myeloma cells.

Antagonists of CXCL12 and CXCR4 trigger new and default MM cells to become susceptible by blocking downstream CXCL12/CXCR4 (Azab et al., 2009). In line with our results, MM cell chemotaxis mediated with CXCL12 includes PI3K, ERK, and AMD3100, an antagonist with CXCL12, which substantially inhibits the homing of MM cells in the bone marrow (Alsayed et al., 2007). CXCL12 attracts and induces the propagation by the endothelial boundary of human mm cells and MM derived cell lines, including 5T33MM cells (Menu et al., 2006). In turn the PI3K allows the serine-threonine kinase AKT to be activated, which is likely a key factor in tumor cell proliferation and survival (Barbero et al., 2003). Other proteins, including p38 and ERK1/2 (Vlahakis et al., 2002), may also be involved in signaling the survivor of tumor cells. The activation of PLCß3 results in rapid hydrolyses with inositol-1,4,5-trisphosphate, diacylglycerin, intracellular mediators that increase Ca^{2+} and activate protein C activity (Venkatakrishnan and Exton 1996). The result is a high-speed process for phosphatidylinositol-4,5-bisphosphate development. Without cell cycle progression, CXCL12 will improve cell survival through PI3-kinase and MAP-kinase cascades. CXCL12-mediated activation of

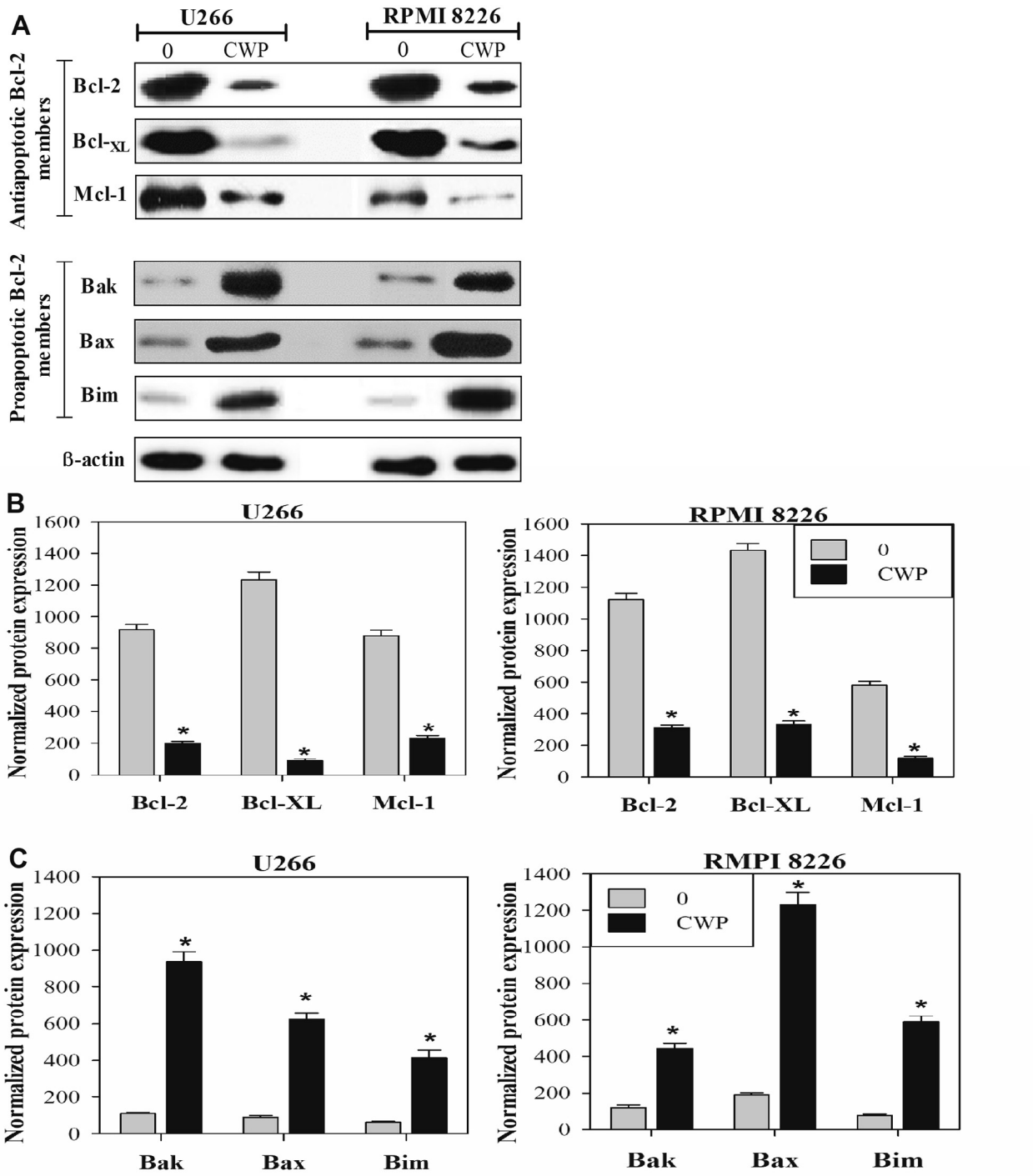

Fig. 6. CWP modified the family members' expression. Bcl-2 family members were analyzed using western blot analysis on U266 and RPMI 8226 cell lines. (a) Protein bands from one representative U266 and RPMI 8226 cell experiment will be shown. (b) Anti-apoptotic protein expression has normalized to the complete amount of β-actin. Tens of independent test results from U266 and RPMI 8226 cells are indicated in untreated (0 Gy bar) and CWP (closed black bar) cells as usual ± defect in normalized values for the anti-apoptotic protein. (c) Proapoptotic and antiapoptotic protein expression to total β-actin level has been standardized. The findings obtained from the ten independent cell U266 and RPMI 8226 experiments are expressed as meaning ± standard defect of normalized protein values pro-apoptotic/anti-apoptotic. *CWP < 0.05, untreated cells. * P < 0.05.

MAPK extracellular signal-regulated kinases, extracellular signal-regulated kinasae-riptosomal S6 and PI3K pathways may be inactivated by the pro-apoptotic Bcl-2 cell death protein (BAD) antagonist. Cell survival genes can be upregulated with CXCL12 exposure. CXCL12 can thus facilitate cell survival by using two mechanisms: post-translation of cell death machines and improved cell gene survival transcripts (Suzuki et al., 2001).

Tumor growth, angiogenesis, metastasis, and survival is aimed at the CXCL12/CXCR4 axis, with therapeutic agents preventing or inhibiting the CXCL12/CXCR4′s interaction (Teicher and Fricker, 2010). Different chemotherapy methods for treatment of MM target activation of apoptosis and cessation of cell cycle progression in cancer cells. Our research showed that CWP treatment results in dose-dependent cell proliferation inhibition in the cell lines of

U266 and RPMI8226MM inducing a pre-emptive and advanced strategy of clearing tumor cells instead of necrosis.

Family members of Bcl-2 have a crucial role in controlling the death and progression of mitochondrial-mediated cells in cancer cells (Chao and Korsmeyer 1998). CWP's data have shown that CWP decreases the expression of anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-XL and Mcl-1, and increases the expression of the pro-apoptotic Bcl-2 family proteins Bak, Bax and Bim in the U266 and RPMI 8226 cell lines. CWP therefore changes the Bax/Bcl-2 ratio, resulting in the loss of cytochrome *C* withholding potential. In addition, a previous study stated that concurrent targeting of multiple familial Bcl-2 proteins may be a resistance solution, consistent with our finding, when redundant between Mcl-1 Bcl-2 and MM BCcl-XL (Slomp and Peperzak, 2018).

5. Conclusion

In conclusion, the CWP research showed that CXCL12 chemotaxis, inhibited propagation in the U266 and RPMI 8226 MM cell lines, was decreased substantially by a CWP induced apoptosis. CWP could be an effective alternative MM treatment technique.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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