Original article

Simvastatin enhances irinotecan-induced apoptosis in prostate cancer via inhibition of MCL-1

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A R T I C L E   I N F O

A B S T R A C T

Prostate cancer is one of the most common malignant tumors around the world. Hyperlipidemia is considered as one of the most important risk factors for the development of prostate cancer. Simvastatin is widely used for the treatment of hyperlipidemia and was previously shown to induce apoptosis in several cancer types including lung, colon, pancreas, breast, and prostate cancer. In this study we aimed to explore the potential role of simvastatin in enhancing irinotecan-induced apoptosis in prostate cancer cells. In addition, the underlying molecular mechanisms driving this potential effect of simvastatin were also explored. PC3 cells were treated with simvastatin, irinotecan or combination. Cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay. Flow cytometry technique was used to analyze apoptosis and cell cycle progression. Western blot was used for detection of protein expression. Results showed that simvastatin has a significant antiproliferative activity on PC3 cells. Combined treatment of simvastatin with irinotecan exhibited a significant inhibition of PC3 cell growth compared to each treatment alone. Flow cytometry analysis showed that PC3 cell treatment with simvastatin and irinotecan combination demonstrated a remarkable increase in the percentage of apoptotic cells and those accumulated at G0/G1 phase when compared to each treatment alone. Moreover, induction of apoptosis was caspase-independent. Western blot showed that apoptosis was accompanied by upregulation of GRP-78 level and downregulation of Mcl-1 levels in a time-dependent manner. The results of this study demonstrated that combined treatment of simvastatin with chemotherapeutic agents such as irinotecan resulted in enhancement of growth inhibition and induction of prostate cancer cell apoptosis.

1. Introduction

Prostate cancer is commonly diagnosed in men over the age of 50 and its incidence rates increase with age; representing the most common cancer diagnosed in US men accounting for 28% of all cancers diagnosed (Daniyal et al., 2014; Hsing and Chokkalingam, 2006; Siegel et al., 2012). The exact etiology of prostate cancer is still elusive with several suggested factors. However, the only well-known risk factors are advancing age, race, and a family history of prostate cancer (Hsing and Chokkalingam, 2006). Several putative risk factors have been implicated in the pathogenesis of prostate cancer. These include androgens, diet, physical activity, sexual factors, inflammation, and obesity (Hsing and Chokkalingam, 2006). However, their exact significance in prostate cancer etiology is still unclear.

Despite the development of new agents, chemotherapeutic resistance has not improved in the last few decades (Singh et al., 2012). In addition, combination of two or more chemotherapies usually adds more toxicity and increase the economic burden on health care system thus underscoring the need for a better understanding of disease pathophysiology and a fresh approach to treatment. Obesity and hyperlipidemia are among the risk factors for prostate cancer (Jespersen et al., 2014; Mittal et al., 2011). In a retrospective study by Mittal et al., prostate cancer patients with elevated prostate specific antigen (PSA) levels have been shown to have relatively higher low density lipoprotein (LDL) levels compared to healthy controls (Mittal et al., 2011). In another study

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by Shannon et al., statin use (lipid lowering agents) has been shown to be associated with a significant reduction in prostate cancer risk (Chan et al., 2012; Shannon et al., 2005). Therefore, proposing cholesterol as a vital player in signal transduction events in prostate cancer will open new deterministic insights in its pathogenesis and new routes for prostate cancer therapeutic management.

Statins (HMG-CoA reductase inhibitors) are class of drugs used to lower cholesterol levels by inhibiting the conversion of HMG-CoA to L-mevalonic acid and subsequently inhibit cholesterol synthesis in the liver. In addition, they lower LDL cholesterol levels up to approximately 60% since the liver produces about 70% of total cholesterol in the body (Papadopoulos et al., 2011). Simvastatin, a member of statin family, has been recently shown to possess immunomodulatory, anti-inflammatory, antioxidant, anti-proliferative and anti-cancer properties (Kochuparambil et al., 2011). The exact molecular mechanisms responsible for statin-mediated anti-cancer effects posed a real focus of research over the last decade. In fact, statins interfere with cholesterol-mediated regulation of prostate cancer cell functions. On the other hand, statins suppress the synthesis of lipid-anchoring units for a number of oncogenic signaling molecules such as Ras and Rho which consequently represents the non–cholesterol mediated regulation of prostate cancer by statins (Karreth and Tuveson, 2009). Irinotecan acts as a topoisomerase inhibitor that disrupts rejoining of DNA single strand breaks during DNA synthesis and has been shown to have no significant activity against in hormone refractory prostate cancer (Reese et al., 1998). In this study we aimed to explore the potential role of simvastatin in enhancing irinotecan-induced apoptosis in prostate cancer cells. In addition, the underlying molecular mechanisms driving this potential effect of simvastatin were also explored.

2. Materials and methods

2.1. Cell culture

PC3 human prostate cancer cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 media supplemented with 10% fetal calf serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. All cells were maintained at 37 °C in an environment of 95% air and 5% CO_2 in a humidified incubator. At 90% confluence, the cells were passed and harvested for future experiments.

2.2. MTT assay

The MTT (3-[4,5-Dimethylthiazol-2-yl]-diphenyltetrazolium Bromide) colorimetric assay was used to assess cell viability (Riss et al., 2004). Briefly, 1×10^4 cells/well were plated in 96-well plate, in quadruplicates for each treatment concentration. After 24 h, cells were treated with DMSO, simvastatin (Sigma-Aldrich, St Louis, MO, USA), irinotecan or combination. Simvastatin concentrations used were 10, 25, or 50 μM, while irinotecan concentrations used were 0.5, 1.0, 2.5, 5.0, or 10.0 μM. Treated cells were incubated for 72 h and then cell viability was examined. MTT (Sigma-Aldrich, Missouri, USA) solution was added to each well and incubated for 2 h at 37 °C. After incubation, the MTT solution was removed and the formazan crystals were solubilized using DMSO. Then the absorbance was measured at 540 nm. The following equation was used to calculate cell viability.

∗ Cell viability in each well = (absorbance of treatment well/Average absorbance of control in 4 replicates) ∗ 100

∗ Cell viability for each treatment = Average cell viability of 4 replicates

For each treatment, there were 4 replicates. The results were shown as a percentage of viable cells in comparison to control cells.

2.3. Flow cytometry analysis of the cell cycle and apoptosis

The cell sorting assay using flow cytometry (FCM) analysis with propidium iodide (PI, Sigma–Aldrich, St Louis, MO, USA) was used to evaluate apoptosis, and to analyze cell cycle. Flow Cytometry was also used to determine whether apoptosis depends on activation of caspases or not. Cell-permeable pan-caspase inhibitor Z-ValAla-Asp (OMe)-CH2F (z-VAD-fmk) was purchased form Calbiochem (La Jolla, CA). For both cell cycle analysis and apoptosis detection, $15 * 10^4$ cells/well were seeded in a 24-well plate, in triplicate for each treatment concentration and allowed for overnight incubation. With or without z-VAD, cells were pretreated with 25 μM of freshly prepared simvastatin, and after 48 h of cell seeding, cells were treated with 10 μM of irinotecan. For cells that supposed to be treated with the combination treatment; irinotecan was prepared in 2X (20 μM) concentration and mixed with 1X (25 μM) of simvastatin. Treated cells were incubated for 72 h, and then were transferred for centrifugation at 2000 rpm for 5 min. PI stain was added to each well after solubilizing with PBS (1:20 w/v) followed by incubation for 30 min. Then stained cells were added to the pellet in the previous centrifuge tube. After that cells in the centrifuge tube were incubated for 24 h in the fridge and protected from light using aluminum foil. Finally; cells were analyzed by flow cytometry. Percentage of apoptotic cells and cells in each phase of the cell cycle were determined.

2.4. Western blot for protein expression levels analysis

PC3 cells were seeded in $45 * 10^4$ cells/well in 6-well plate in RPMI growth medium, and then incubated overnight to allow cell attachment. Cells were seeded in triplicate for the indicated time points (16, 24, and 36 h). After 24 h, cells in combination wells were pretreated with 25 μM of freshly prepared simvastatin. For cells that were treated with simvastatin only; 25 μM of simvastatin was added before 36, 24, and 16 h of cell harvesting and protein extraction step. For cells that were treated with either irinotecan only or combination; 10 μM of irinotecan was added before 36, 24, and 16 h of cell harvesting and protein extraction step. After the treatment periods (16, 24, and 36 h), the cells were washed with PBS and harvested using radio-immunoprecipitation assay lysis buffer. The cell lysate was centrifuged at 3000 rpm for 1 h 4 °C, and the supernatants that contain proteins were used. The protein content was measured using Bio-Rad reagent kit. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method was carried out to separate proteins. Then proteins were transferred to nitrocellulose membranes, and the membranes were blocked for 1 h at room temperature using the blocking buffer solution that contains 5% Bovine serum albumin (BSA) in Tris-buffered saline/Tween-20 (TBST). The membranes were incubated with primary antibodies against Mcl-1 (Santa Cruz Biotechnology, USA) and GRP-78 (Santa Cruz Biotechnology, USA) proteins overnight at 4 °C. GAPDH monoclonal antibody (Abcam, UK) was used as loading control antibody. After that, the membrane was washed using TBST buffer, and then the horseradish peroxidase-conjugated secondary antibodies were added to the membrane and incubated for 1 h at room temperature on a platform shaker. The membrane was washed using TBST buffer, and finally the immune complexes or bands were visualized using enhanced chemiluminescence (ECL) according to the manufacturer's protocol.

2.5. Statistical analysis

Graphpad Prism statistical software version 5.0 was used to conduct all statistical analysis. ANOVA and post-hoc Tukey's tests were used. Results were expressed as mean ± standard deviation. $P < .05$ was considered statistically significant.

3. Results

3.1. Simvastatin and irinotecan mono-treatments inhibit PC3 cell proliferation

The biological effect of simvastatin or irinotecan treatments on PC3 cell proliferation was determined by MTT viability assay. Interestingly, simvastatin reduced the viability of PC3 cells in a dose-dependent manner compared with untreated control cells. PC3 cell viability was significantly lowered by 35%, 50%, and 60% using concentrations of 10, 25, and 50 μM, respectively (Fig. 1A). In addition, irinotecan anti-proliferative effect was studied using various concentrations. Results indicate that irinotecan induced cytotoxic effect against PC3 cells, and significantly reduces cell viability by 15%, 20%, and 25% using concentrations of 2.5 μM, 5.0 μM, and 10.0 μM, respectively. In contrast, the viability of PC3 cells was minimally affected using 0.5 μM and 1 μM irinotecan compared to control cells (Fig. 1B).

3.2. Simvastatin sensitized PC3 cells to irinotecan- induced cytotoxicity

To study whether simvastatin treatment could sensitize prostate cancer cells to irinotecan treatment, PC3 cells were pretreated with simvastatin at 50 μM for 24 h before the addition of irinotecan at various concentrations. As shown in Fig. 2A, results obtained from MTT assay revealed that combinations of 50 μM simvastatin with the various concentrations of irinotecan augmented irinote-
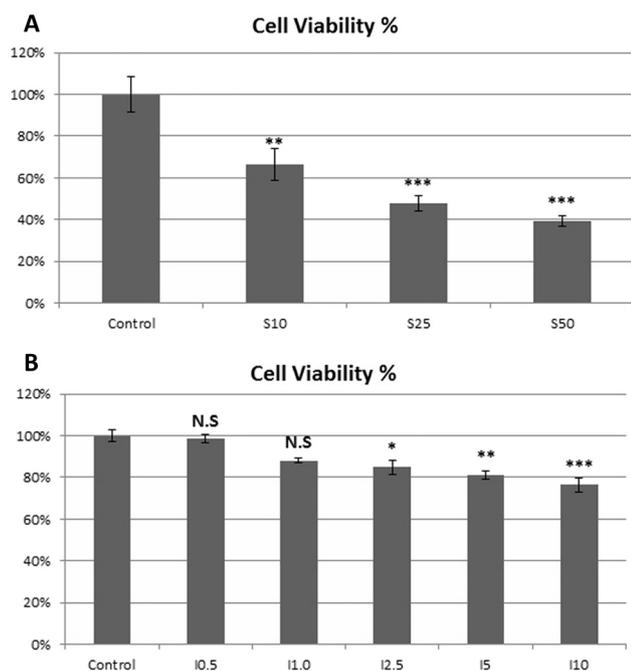

Fig. 1. Antiproliferative effects of simvastatin and irinotecan single treatment. Cell viabilities of PC3 cells following simvastatin (A) or irinotecan (B) treatments were determined using MTT assay. The data represent mean ± SE of three independent experiments performed in triplicate compared with control (DMSO). *indicates p < .05, **indicates p < .01 and ***indicates p < .001 (ANOVA and Tukey's multiple comparison test).
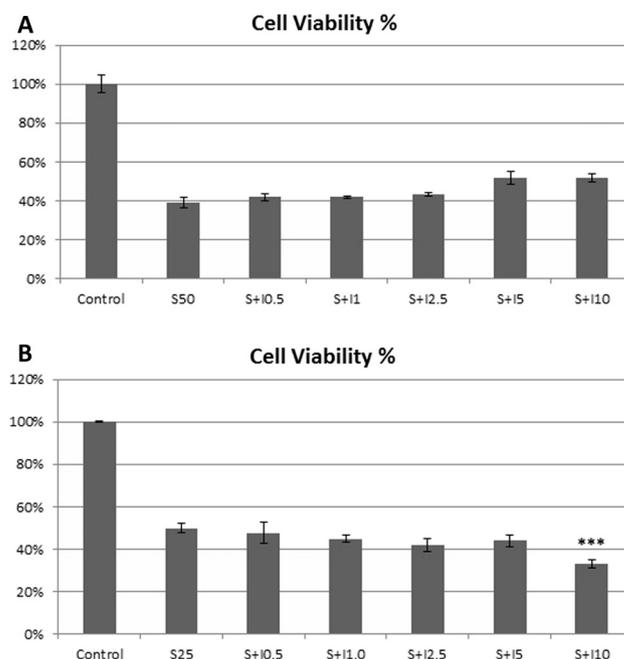

Fig. 2. Antiproliferative effects of simvastatin and irinotecan combined treatment Cell viabilities of PC3 cells following simvastatin 50 μM (A) or 25 μM (B) treatments combined with serial concentrations of irinotecan were determined using MTT assay. The data represent mean ± SE of three independent experiments performed in triplicate compared with simvastatin alone. ***indicates p < .001 (ANOVA and Tukey's multiple comparison test).

can cytotoxic potential with a significant reduction in cells proliferation compared to control. However, there was no significant difference in cell viability among combination treatments in comparison to 50 μM simvastatin treated cells. Possibly, high simvastatin concentration masked the effect of combined treatments. Therefore, we decided to use a lower concentration of simvastatin (25 μM) with the various concentrations of irinotecan. Similarly, all combination treatments significantly reduced cell viability compared to control. However, compared with cells that treated with 25 μM simvastatin alone, only the combination of 25 μM simvastatin with 10 μM irinotecan showed significant effect (Fig. 2B).

3.3. Simvastatin enhanced irinotecan-induced apoptosis and G0/G1 arrest in caspase-independent manner

Since simvastatin treatment resulted in sensitizing PC3 cells to irinotecan treatment, we further investigated the underlying molecular mechanism related to this growth inhibition by analyzing cell cycle and apoptosis using flow cytometry following simvastatin and irinotecan treatment. Cell cycle kinetics in Fig. 3A and C showed that combination of 10 μM irinotecan and 25 μM simvastatin significantly increased the percentage of PC3 cells at G0/G1 phase compared to each treatment alone. In addition, there was a significant increase in percentage of apoptotic cells after using combination treatment compared to each treatment alone (Fig. 3A and B).

To determine the apoptotic pathway that prostate cancer cells follow when treated with simvastatin and irinotecan, PC3 cells were pretreated with simvastatin for 24 h before the addition of irinotecan with or without Z-VAD treatment, a caspase inhibitor. Apoptotic cells were measured using flow cytometry with propidium iodide. Results showed that there was no significant difference in the percentage of apoptotic cells treated with or without Z-VAD (Fig. 4). Therefore, simvastatin and irinotecan single or combination treatments induced PC3 cell apoptosis in a caspase-
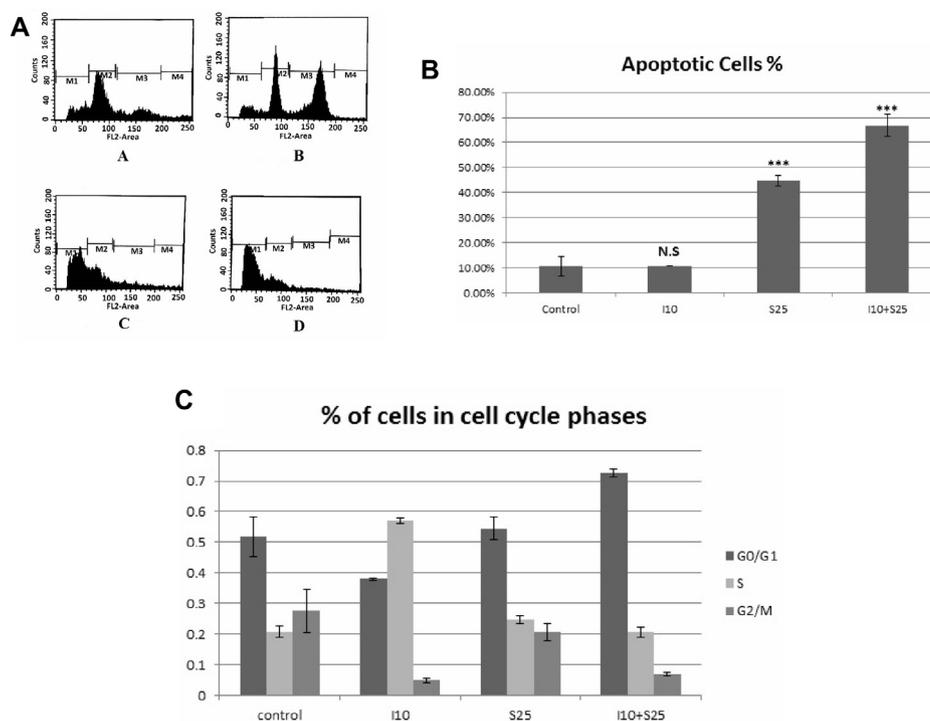
Fig. 3. Simvastatin irinotecan combined treatment alters cell cycle progression and induces apoptosis in PC3 cells. (A) Cell cycle profile of PC3 cells post-treatment. Cell cycle was assessed by propidium iodide staining followed by FACS analysis. (B) Histogram showing apoptotic cell percentages. (C) Histogram distribution of the 3 major cell cycle steps. ***indicates p < .001 (ANOVA and Tukey's multiple comparison test).

independent manner due to the absence of Z-VAD effect on treated cells.

3.4. Simvastatin/irinotecan combination treatment downregulates Mcl-1, and upregulates GRP-78

To further elucidate the mechanism by which simvastatin sensitize prostate cells to irinotecan by induction of apoptosis, protein levels of potential targets were assessed using immunoblot. As shown in Fig. 5A, the anti-apoptotic protein, Mcl-1 was downregulated in time-dependent manner after treatment with 25 μM simvastatin, 10 μM irinotecan and their combination at different time points (16, 24, and 36 h). In contrast, as shown in Fig. 5B, the level of the endoplasmic reticulum (ER) chaperon, GRP-78, was upregulated in time-dependent manner after treatment with 25 μM simvastatin, 10 μM irinotecan and their combination at different time points (16, 24, and 36 h).

4. Discussion

Prostate cancer is considered among the top five cancers in the world. The five-year survival rate for patients with established metastatic prostate cancer remains low despite the advancement in the diagnosis and treatment of prostate cancer. Chemotherapy is usually used as a last treatment option in the advanced stage of PC (Flam, 1996). However, several studies have shown that prostate cancer cells are resistant to many chemotherapeutic agents including irinotecan most chemotherapeutics show nonselective toxicity and adverse effects (Alfarouk et al., 2015; Xu and Villalona-Calero, 2002).

Simvastatin is a cholesterol lowering agent that is commonly used in reducing the risk of cardiovascular events. Beside its potency to reduce serum lipid, simvastatin has been recently associated with pleotropic effects including antioxidant, anti-

inflammatory, and antitumor properties. Simvastatin has shown significant anticancer effect against different types of malignancies including lung, colon, pancreas, breast, skin, liver, bladder, renal cell, multiple myeloma, and prostate cancer (Jakobisiak and Golab, 2010). Data obtained from human based studies have revealed that simvastain could reduce the risk of prostate cancer development and or death (Babcook et al., 2016; Yu et al., 2014). In addition, simvastatin has demonstrated several cellular and molecular effects against tumor cells including anti-proliferative, anti-metastatic, anti-angiogenic, and pro-apoptotic effects (Ghosh-Choudhury et al., 2010; Schointuch et al., 2014).

In the present study, consistent with previous studies, we confirmed the antiproliferative effect of simvastatin in prostate cancer in vitro. Moreover, we showed that simvastatin sensitized PC3 cells to irinotecan-induced cytotoxicity via induction of apoptosis and arresting cell cycle. Further, we demonstrated that these effects might be attributed to decreased expression of the antiapoptotic protein, Mcl-1 and elevated stress on ER represented by increase expression of GRP-78. These data are in agreement with previous studies that demonstrated the sensitizing effect of simvastatin on lung and colorectal cancer cells that are resistant to irinotecan (Jang et al., 2016; Park et al., 2011).

We examined the sensitizing effects of simvastatin on irinotecan cytotoxicity on PC3 cell viability by exploring the effect of this combination on cell cycle and apoptosis. Our findings showed that this combination significantly arrested PC3 cells at G0/G1 phase of cell cycle and induced apoptosis to higher extent compared to either drug alone. These results are consistent with the previous studies in lung, hepatic, melanoma and prostate cancer (Park et al., 2011; Relja et al., 2010; Saito et al., 2008).

There are three common pathways for cellular apoptosis: the extrinsic or death-receptor pathway, the intrinsic pathway or mitochondrial pathway, and ER stress-mediated pathway (Hengartner, 2000). Mitochondrial–mediated apoptosis is regu-
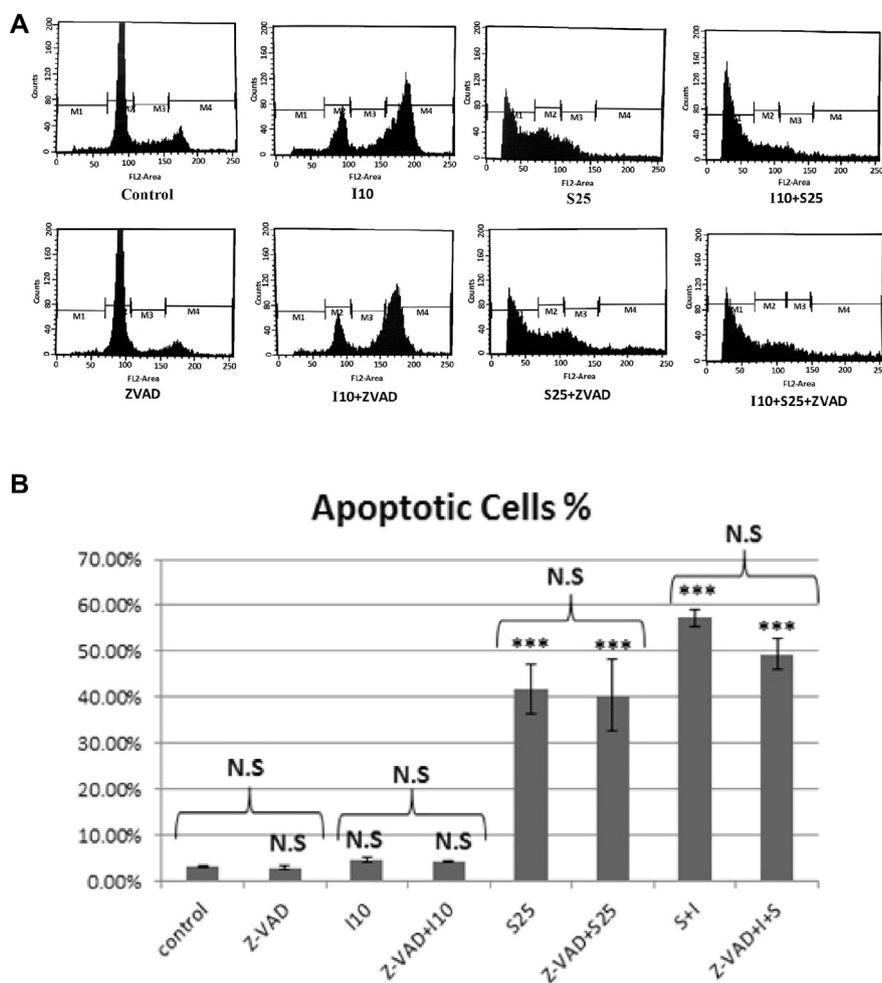
Fig. 4. Percentage of apoptotic cells with or without Z-VAD treatment. (A) Cell cycle profile of PC3 cells post-treatment. Cell cycle was assessed by propidium iodide staining followed by FACS analysis. (B) Histogram showing apoptotic cell percentages with or without Z-VAD treatment. NS indicate no significant difference, ***indicates p < .001 (ANOVA and Tukey's multiple comparison test).
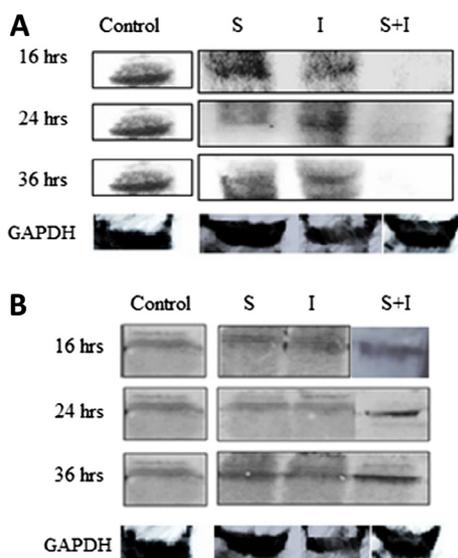

Fig. 5. Western blot analysis of (A) Mcl-1 and (B) GRP-78 expression levels in PC3 cells. Simvastatin and irinotecan combined treatment resulted in inhibition of Mcl-1 expression and accumulation of GRP-78 protein content in a time dependent manner.

lated by BCL-2 family proteins represented by the balance between pro-apoptotic and anti-apoptotic proteins (Youle and Strasser, 2008). In addition, apoptosis can occur in caspase-dependent or caspase-independent manners. Among the pro-apoptotic proteins, cytochrome-c has been found to be important in the stimulation of caspase-induced cell death (Garrido et al., 2006). Alternatively, the second group of pro-apoptotic proteins, such as endonuclease G and apoptosis-inducing factor (AIF) activate the caspase-independent apoptotic pathway (van Loo et al., 2001). Mcl-1, an anti-apoptotic protein of BCL-2 family proteins, plays important roles in cell survival and tumorigenesis, and its downregulation promotes cellular apoptosis (Youle and Strasser, 2008). Finally, ER stress- is a crucial event in cellular apoptosis. In particular, ER activates the unfolded protein response (UPR) pathway which protects cells against accumulation of misfolded proteins within ER and therefore restoring normal cellular function (Breckenridge et al., 2003). However; UPR initiate apoptosis when adaptation to ER stress fails. GRP78 is one of important proteins in UPR signaling pathway, and its overexpression is a hallmark of severe ER stress (Xiong et al., 2015).

Consistent with previous literature, we demonstrated that apoptosis process in our conditions was caspase independent. However, some studies have shown that simvastatin induce caspase-dependent apoptosis in several cancers (Cafforio et al.,

2005; Hwang et al., 2014). In addition, a recent study has demonstrated that simvastatin/irinotecan combination significantly enhanced caspases-mediated death of colorectal cancer cells (Jang et al., 2016). Moreover, one study has found that simvastatin could activate AIF-mediated apoptosis (an alternative pathway for caspase-dependent apoptosis) in macrophage cells (Kim et al., 2006).

Mcl-1 is highly expressed in advanced stage prostate cancer, which has been associated with resistance to chemotherapy-induced apoptosis (Karnak and Xu, 2010). Mcl-1 knockdown in an *in vitro* or *in vivo* PC model has been shown to enhance chemotherapeutic sensitivity in prostate cancer (Reiner et al., 2015). Moreover, depletion of Mcl-1 has been found to augment irinotecan efficacy and induce colorectal cancer cell death (Jonchere et al., 2015). Further, downregulation of Mcl-1 expression has been shown by other statins such as lovastatin in myeloma plasma cells (van de Donk et al., 2003a) and malignant lymphocytes (van de Donk et al., 2003b). In addition, the combination of simvastatin and tipifarnib (a small molecule) in leukemia has been shown to induce apoptosis via downregulation of Mcl-1 (Ahmed et al., 2014). Finally, our findings showed that simvastatin-irinotecan combination upregulated GRP78 protein levels which was previously shown in leukemic cells (Xu and Villalona-Calero, 2002) and macrophages (Chen et al., 2008). Consistent with previous studies, our results showed that simvastatin-irinotecan combination downregulates Mcl-1 protein expression and upregulates GRP-78 as one of the molecular mechanisms of cellular apoptosis.

5. Conclusions

Our findings elucidate, the ability of simvastatin to alter aggressive behavior of prostate cells which render these cells more sensitive to irinotecan induced apoptosis. This modulatory effect of simvastatin can be predominantly attributed to simvastatin targets such as Mcl-1 and GRP78.

Acknowledgements

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