Saudi Pharmaceutical Journal xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

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

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ARTICLE INFO

Article history:
Received 22 November 2017
Accepted 12 December 2017
Available online xxxx

Keywords:
Prostate cancer
Simvastatin
Irinotecan
Apoptosis

ABSTRACT

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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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2017.12.012
1319-0164/

Please cite this article in press as: Alqudah, M.A.Y., et al. Simvastatin enhances irinotecan-induced apoptosis in prostate cancer via inhibition of MCL-1. Saudi Pharmaceutical Journal (2017), https://doi.org/10.1016/j.jsps.2017.12.012

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.

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