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Assessment of the Anticancer Effects of Simvastatin on Human Osteosarcoma and Colorectal Cancer Cell Lines

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ABSTRACT

Background: *Colorectal cancer (CRC) and osteosarcoma (OS) and all other cancers represents one of the most challenger issues to the humanity for long time, due to their associated sever health lesions, emotional and psychological hardships, and significant economic encumbrances. Adding to that, the poor curing fate achieved with current therapeutic strategies of cancer. The invention of new drugs for cancer treatment required long time reaching to years as well as consuming the huge money budget, so that the focusing on the aspect of the repositioning of conventional drugs (like simvastatin) has gradually take a large consideration as they can participate in the alleviation of cancer treatment burden in view of this work promising results and preceding hopeful findings and conclusions have been obtained from the studies conducted in this field.* ***The Aim:*** *assessment of the cytotoxic effects of a lipid lowering drug, simvastatin, in human CRC and OS cells.* ***Methods:*** *SW480 (CRC) and MG-63 (OS) cell lines were utilized in this work and have been treated with various doses of simvastatin, then its cytotoxic effects have been evaluated by MTT assay. Thereafter, the VEGF and GDF-15 suppression effects of simvastatin have been measured in SW480 and MG-63 cells.* ***Results:*** *exposure of CRC and OS cells to simvastatin resulted in significant cellular growth inhibition, and VEGF and GDF-15 suppression effects.* ***Conclusions:*** *Simvastatin has a promising antineoplastic potential and its role in cancer therapy cannot be underestimated, and encourage the utilization and surveying of other preexisting drugs in cancer treatment path.*

Key Words: Simvastatin, Colorectal Cancer, Osteosarcoma, VEGF, GDF-15.

Article Information

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INTRODUCTION

Cancer is a very dangerous multi-step disease due to its aggressive behavior in the body, as its main features are: the dividing of the cells in continuous and out of control manner, the ability of the pathological cells to invade the neighboring tissues , and migration potential to the distant organs of the body (1). Among the global leading causes of deaths, cancer is the second one (2).

Colorectal cancer is a type of malignant tumors that originates from the colonic and rectal epithelium, it can invade and spread to various distant parts of the body (3). CRC is awfully the third most prevalent cause of cancer mortality (4). It is the second repeatedly and exceedingly diagnosed type of malignant tumors in women and third in men(5,6). Globally, there is around 1.9 million new CRC cases have been annually reported, according to the database of the World Health Organization GLOBOCAN (7–9). The situation of CRC emergence is commonly coincided with a not possible to be modified risk factors such as age and hereditary factors, as well as with adjustable environmental and

lifestyle factors (10). More research are needed to improve treatment options for CRC subtypes without effective therapies (11).

Osteosarcoma is the most common complex type of primary bone cancers, characterized by a tremendous degree of malignancy, robust invasiveness, mounting disease progression, and dramatically high mortality rate; it is regarded as a serious threat to the human health globally (12–14). Mainly, OS has been originated and developed in long bones of the arms and legs usually around the knee and shoulder. Principally, two-thirds of the primary OS are positioned around the knee joint, with the most widespread sites in the distal femur, the proximal tibia, and the proximal humerus. It is characterized by its rapid growth and high ability of metastasis, commonly to the lungs. The currently recommended chemotherapeutic procedures showed incommensurate response rates in patients with recurrent and metastatic phases (15–18). The age and gender relevant incidence of OS is biaxial, since it reaches its topmost reported cases at 18 and 60 years of age, and it is more common in males than females. For the patients with metastatic OS, the cancer-free survival outcomes are still poor (19,20).

Despite of the scored advances in cancer therapeutic outcomes, till now great efforts are needed to be done in making the life-threatening disease (cancer) progressively curable. One of the most frequent problematic situations in cancer treatment is the repetitive cycle of remission and recurrence making distinctive chronic peculiarities in this timeframe (21).

The currently recommended anticancer drugs are the mainstay cancer therapeutic modalities so far. But unfortunately, they are coincided with severe multisystemic unwanted adverse effects such as: myelosuppression, cardio-cytotoxicity, nephrotoxicity, hepatotoxicity, neurotoxicity, mucositis, gastrointestinal toxicity, and alopecia, which tragically influence the quality of life of cancer patients (22). Adding to that, the chemotherapeutic resistance is one of the paramount causes of treatment failure. The competent fight against cancer mandates a multidimensional approaches for example combinations of multitargets drugs (23).

New, practical, and innovative anticancer drug discovery has been acknowledged as an arduous, complicated, expensive, time-consuming, and challenging project during which most if the candidate agents fail (24). The alternative serendipitous, faster and cheaper approach than new anticancer drugs discovery is the repurposing or repositioning of the already available drugs that employed for the management of other diseases. This concept has been occupied a great therapeutic stature in cancer fighting field (25,26).

Simvastatin is a currently available antihyperlipidemic medication belongs to a group or family of drugs under the class called statin, it specifically a 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) enzyme inhibitor. It is primarily intended to be utilized lowering cholesterol levels in the blood, particularly low-density lipoprotein (LDL) cholesterol, which is often referred to as "bad" cholesterol (27–29).

Among statins family, simvastatin has been frequently examined (alone or in combination with other drugs) for its anticancer behavior and revealed a promising impact on the proliferation, migration, and survival of cancer cells. Since 1990s the concept of recommendation of simvastatin in cancer therapy has been espoused. in vitro and in vivo experiments and cohort studies utilizing other statins' family members have been conducted to evaluate their anticancer impacts, for example the diminishing of the proliferation, invasion, and migration of malignant cells via manipulating inflammatory and oxidative stress-related tumorigenesis. Despite of these efforts, the biological targets and pathways for these actions expressed by simvastatin and other

statins are not fully illuminated and required further investigations (30–32). In this work the cytotoxic and anticancer effects of simvastatin have been assessed and evaluated in human CRC and OS cell lines.

MATERIALS AND METHODS

Materials: Cell culture medium RPMI-1640 and trypsin–EDTA were from Usbiological,/USA. Fetal bovine serum (FBS) was provided from Gibco/Germany. Penicillin and streptomycin mixture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, and simvastatin were obtained from Sigma–Aldrich/USA. Simvastatin has been dissolved with dimethylsulfoxide (DMSO) from Santacruze biotech/Europe and then diluted with PBS at a final concentration not exceed 0.5%. The Vascular Endothelial Growth Factor (VEGF) and Growth Differentiation Factor-15 (GDF-15) ELIZA kits were purchased from BT-LAB bioassay technology laboratory/Shanghahai/China.

Cell culture: The human osteosarcoma cells MG-63 and colorectal cancer cells SW480 were used to evaluate the cytotoxic effects of simvastatin. Both cell lines were cultivated as monolayer and maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin mixture in a humidified cell culture incubator (5% CO2,37 Cº). All concentrations of the experiments were conducted triplicate and from 3 different experiments (33,34).

Cytotoxicity Assessment with MTT Assay: The cells of each line MG-63 and SW480 were cultivated in 96-well plate with 200µl of a warm RPMI-1640 culture medium supplemented with 10% FBS and incubated until they reached a confluent monolayer on the well's bottom. After incubation, the culture media in each well was aspirated and replaced with 200 µl of serial two-fold dilutions of simvastatin along with RPMI-1640 media and incubated for 24 hours. The concentrations of simvastatin were (500, 250, 125, 62.5, 31.25, 15.625, and 7.8125) µg/ml in each cell line MG-63 and SW480. The control wells of DMSO with culture media also involved in all plates. After incubation, the culture media has been removed from each well, then MTT dye solution has been administered at final concentration of 50mg/ml. four hours incubation has been done followed by MTT solution removal. Next, 150µl of DMSO has been added to each well with shaking for ten minutes to solubilize the formed formazan crystals by viable cells' enzymes. The optical density (OD) of each worked plate was read at 570 nm, with a plate reader and the average OD_{570} of the 3 wells without cells (blank wells) was subtracted from the OD_{570} of each well in the plate. The percent of cytotoxicity was calculated according to the below formula: (35,36)

Percent of cellular viability = (At-Ab/Ac-Ab) ×100

Percent of growth inhibition = 100- Percent of cellular viability

Where: At: Absorbance value of simvastatin. Ab: Absorbance value of blank. Ac: Absorbance value of control

Dose-response Curve and Determination of IC50: The dose-response curve relationship was utilized for describing the changes in cellular growth inhibition (GI) occurred in OS and CRC cells in response to various dose levels of simvastatin after a certain time exposure. The IC50 values were extrapolated and calculated from the dose-response curves of tested agents, the IC50 of the drug is the dose that corresponding to 50% reduction in the viability(37–39) .

Assessment of Cancer Biomarkers Levels: the ELIZA technique has been applied for in vitro quantitative determination of the levels VEGF and GDF-15 in SW480 an MG-63 cells treated and incubated with (125, 62.5, 31.25, 15.625, and 7.8125) µg/ml of simvastatin. The control wells of DMSO with culture media also involved (40–43).

According to the manufacturer's constructions (BT-LAB bioassay technology laboratory) (44): the ELIZA 96-wells plates of VEGF and GDF-15 biomarkers was loaded with 50µl of the previously prepared Standard Solution dilutions to the standard's wells (each concentration was loaded to one well of its corresponding plate). After incubation of cancer cell with simvastatin, A forty microliters of SW480 and MG-63 cell lines' samples treated with simvastatin and control samples were added to the samples' wells, then 10µl of human biotinylated VEGF and GDF-15 antibodies were added to each well of their specific plates. After that, A fifty microliters of streptavidin-HRP were administered to both CRC and OS cells' samples wells and standards' wells with well mixing then the plates of VEGF and GDF-15 were covered with sealer, after that, they were incubated for one hour at 37Cº. Next, the sealers were taken away and the wash buffer was utilized to wash its respective plate 5 times. As at each washing process the plates were soaked with 300µl of wash buffer for 50 seconds. Then they blotted onto paper towels. After that, a fifty microliters of substrate solution A and another 50µl of the substrate solution B were administered to each well of their specific VEGF and GDF-15 ELIZA plates, next to that, they covered and incubated in dark for ten minutes at 37Cº. The final step was the addition of the Stop Solution in the amount of 50µl to each well and the color has been changed. The optical density values of each well of the worked biomarkers (VEGF and GDF-15) were measured immediately at 450nm by microplate reader.

Statistical Analysis: Data collection and statistical analysis was performed using the "Microsoft Office Excel 2010" and "IBM SPSS (statistical package for social science) version 26 software". The data were expressed as mean ± Standard error (S.E.). One way ANOVA technique was used for comparisons between groups followed by post-hoc tests using LSD (45).

RESULTS

MTT Assay: The Serial two-fold concentrations of simvastatin that have been administered to SW480 cell, displayed a statistically significant reduction in cellular viability as compared with control SW480 cells. Simvastatin IC50 value was 40.21 µg/ml. The cellular growth inhibition mode was in a dose-dependent manner, by which the SW480 cells' growth inhibition increased by increasing simvastatin concentration. Fig.1

The MG-63 cells treatment with serial doses of simvastatin resulting in statistically significant reduction in the cell viability which was happened in a dose-dependent manner. Simvastatin IC50 value in MG-63 line was equal to 32.76 µg/ml. Fig.2

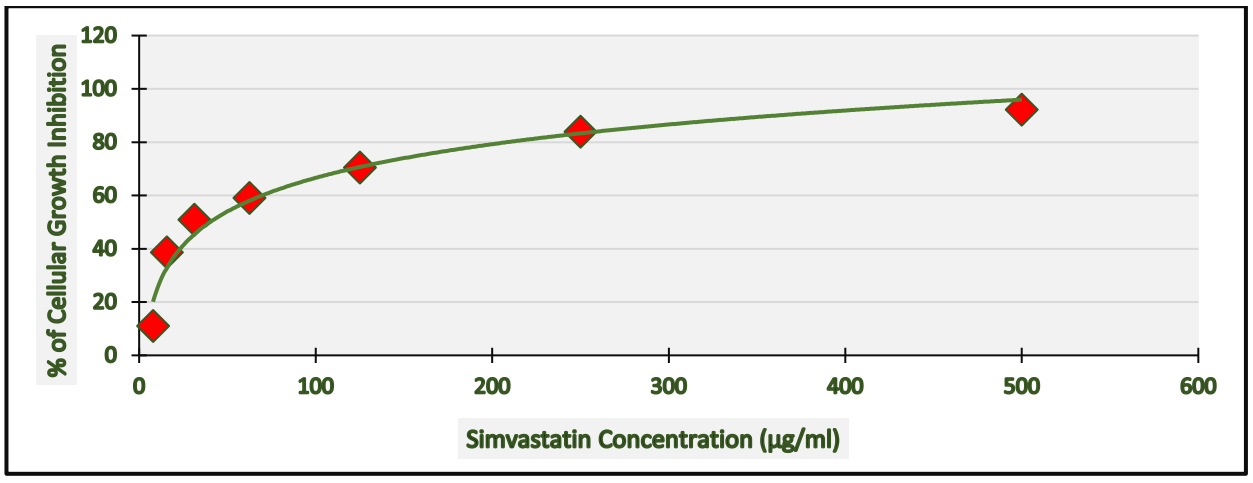

Figure 1: Dose-Response Curve of Simvastatin in SW480 Cells.

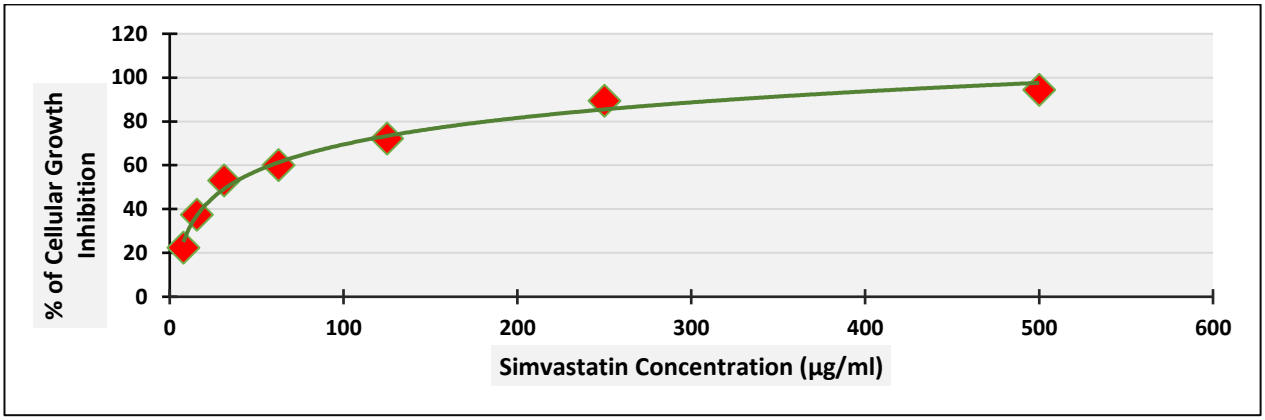

Figure 2: Dose-Response Curve of Simvastatin in MG-63 Cells

Biomarkers Levels: The results of the effects simvastatin on the VEGF levels in CRC and OS cells indicate that the incubation of SW480 and MG-63 cells with various dose levels of simvastatin, resulting in statistically significant decreasing in VEGF levels between the simvastatin exposed cells and the control untreated cells. Tab.2**,** Concerning the GDF-15 biomarker, the obtained results revealed that incubation of SW480 and MG-63 cells with multiple doses of simvastatin resulting in statistically significant reduction in GDF-15 level as compared with control SW480 and MG-63 cells. Tab.3.

Table 1: VEGF Levels in SW480 and MG-63 Cell Lines.

Concentration (µg/ml)	VEGF Level (ng/L) SW480 Cells Mean± SE	VEGF Level (ng/L) MG-63 Cells Mean± SE	P-value (with control)
0.00 (Control)	282.69±1.01	372.83±0.73	< 0.001(HS)
7.8125	232.47±1.01	365.24± 0.91	
15.625	209.77±1.78	329.35± 0.81	
31.250	166.06±1.31	302.66± 1.13	
62.5	99.26±1.44	246.25± 0.53	
125	79.23±0.53	158.52± 0.81	

HS: high significance.

Table 2: GDF-15 Levels in SW480 and MG-63 Cell Lines.

Concentration (µg/ml)	GDF-15 Level (ng/L) SW480 Cells Mean± SE	GDF-15 Level (ng/L) MG-63 Cells Mean± SE	P-value (with control)
0.00 (Control)	1559.02± 1.41	1028.46± 4.12	< 0.001(HS)
7.8125	1291.57± 3.63	945.81± 3.37	
15.625	1063.99± 2.06	925.48± 2.21	
31.250	632.56± 2.33	662.55± 2.01	
62.5	327.63± 3.52	517.36± 1.02	
125	238.90± 2.19	376.08± 1.57	

HS: high significance.

DISCUSSION

The obtained results by this work showed that simvastatin exerts a significant cytotoxic effect in CRC (SW480) and OS (MG-63) cells as compared to control untreated cells. The results revealed by the following previous studies that have been conducted in various types of cancers are in agreement with this research results concerning the anticancer activity of simvastatin: Recent studies have mentioned that simvastatin may has astonishing contribution power in the treatment of CRC, as achieved from clinical studies that have used simvastatin as an adjuvant drug to assist in the curing of metastatic colorectal cancer (46). Also, statins was identified to produce a remarkable higher cellular viability reduction in the mutated APC gene, in in vivo patient derived xenograft models and cell lines, than the wild-type APC samples of CRC cells (47). It was evidenced, by a recent study, that simvastatin played an anti-osteosarcoma role due to the study's findings that showed the local delivery of simvastatin could stimulate and potentiate the ferroptosis and cell death along with its osteogenic activity in osteosarcoma animal model (48).

On the other hand, the stromal cells in Giant cell tumor of bone (GCTB) represent the primary neoplastic cells and are known as inadequately differentiated pre-osteoblasts. It was observed that simvastatin efficiently knocks down tumor cell viability by terminating proliferation and by promoting apoptosis in GCTB stromal cells. Furthermore, the upregulation of the osteogenic maturation genes was associated with simvastatin treatment. As concluded from these findings, the antitumor and differentiation-promoting potentials of simvastatin could be exploited to be recommended as an adjuvant therapy for GCTB in order to diminishing the chances of recurrence and distant metastasis after surgical treatment (49). In 2023, a study utilized four prostate cancer cell lines to assess the anticancer potential of simvastatin. It was reported that all prostate line suffered from a significant autophagy elevation, as well as paramount concentration-dependent growth inhibition effects on all four cell lines (50).

The secretory proteins have been utilized and recommended in this research (VEGF and GDF-15) as another model for further detection and confirmation of CRC and OS response to the simvastatin exposure. As the biomarkers are used to evaluate the anticancer drugs and their response to therapeutic agents in various cancer models, the biomarker discovery is a fundamental part of the development of cancer biology and disease management (51). The ELIZA technique results of this work appeared a highly significant reduction in VEGF and GDF-15 levels exhibited by various concentrations of simvastatin in OS and CRC cells, which were consistent with MTT assay outcomes. This may be of great value in treatment path of CRC and OS patients.

The correlation between VEGF and colorectal cancer was assessed and systematically reviewed via a meta-analysis conducted by Zhu and his colleagues in 2019. They concluded that VEGF serum levels could be recommended as an eligible biomarker for colorectal cancer patients' management, due to the remarkable higher tremendous VEGF levels associated with CRC group than with control group (52).

C. Zhang and his work team, in 2021, have been done a systematic literature retrieval of available databases about the correlation between VEGF levels and osteosarcoma. They found in their meta-analysis that the VEGF high expression levels may be a candidate biomarker that assist in the prediction of poor prognosis and unwanted clinicopathological features in osteosarcoma patients (53). The conclusions mentioned in the study performed in 2022, reported that VEGF expression and chemotherapy response were exceedingly interconnected and it may serve as an index

regarding clinical decision-making about therapy and outcomes (54).

As mentioned by Mirzaei and his collagenous in 2022, the prostate cancer cell lines, treated with simvastatin alone and in combination with Arsenic trioxide (which has anti- neoplastic properties), have been suffered from significant raise in the percentage of apoptotic cells as well as VEFG and Osteopontin expressions downregulation in both alone and combined forms of simvastatin exposure (55).

According to the meta-analysis findings, it was reported that GDF-15 can be used as a beneficial biomarker diagnostic and prognostic means in colorectal cancer (56). In 2019, ELISA technique was utilized to assess concentrations of GDF-15 in serum and cell culture medium samples of osteosarcoma patients and cell lines models respectively. The results presented in the study revealed that the overall survival and pulmonary metastasis-free survival time were reduced in patients who reported with elevated serum GDF-15 as compared with patients expressed low serum GDF-15 values. Adding to that, as revealed in ELIZA assay, the osteosarcoma cells a showed higher GDF-15 values than the noncancerous cells (57).

Xie and his work team, in 2016, found that simvastatin was able to forbid the epithelial-mesenchymal transition (critical initial step and a lineament for cancer metastasis) in prostate cancer cells through the attenuation of GDF signaling (58). The aforementioned results and observations by previous studies support this work findings and emphasize the role of simvastatin in cancer treatment path. The recommended doses of simvastatin can be employed alone or in combination with other anticancer medications according to the type, stage, and grade of cancer.

CONCLUSION

Simvastatin has a promising antineoplastic potential and its role in cancer therapy cannot be underestimated, and encourage the utilization and surveying of other preexisting drugs in cancer treatment path. These findings support further preclinical and clinical investigations about the prospective outcomes of using simvastatin as a neoadjuvant and/or adjuvant therapy for cancer to achieve better curing results and reduce recurrence and distant metastasis.

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