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RESEARCH ARTICLE

Antitumor activity of doxycycline in HepG-2 cells

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Abstract

Hepatocellular carcinoma (HCC) is a major health problem worldwide. While considerable advances have been made in diagnosis and treatment of HCC, it is still associated with high rate of mortality and poor prognosis, even with therapies that are considered potentially curative. The current study sought to evaluate the anti-tumor/cytotoxic activity of doxycycline in HepG2 cells. Following 48-hr treatments with increasing concentrations of doxycycline, HepG2 cell survival was measured using MTT and lactate dehydrogenase (LDH) assays. Matrix metalloproteinase-9 (MMP-9), heparan sulfate proteoglycans (HSPGs), and Fascin levels were assessed via ELISA. In addition, indicators of potential induction of apoptosis and anti-oxidant activity of the drug were assessed via measures of cell caspase-3 activity and both superoxide anion production and superoxide dismutase activity, respectively. The results indicate that treatment of the HepG2 cells with doxycycline caused reductions in cell survival in a dose-related manner. In addition, it was seen that doxycycline was able to stimulate cellular apoptosis (measured by caspase-3 activity) in these cells. In conclusion, doxycycline proved promising cytotoxic/antitumor activity and opens, thereby, a new horizon against vascular migration ability of the tumor cells.

1. Introduction

Hepatocellular carcinoma (HCC) is a major health problem worldwide; it is the fifth most common cancer and the third most common cause of cancer-related death (Hashiguchi et al., 2013). Tumor invasion is a complex biological process that involves the loss of cell–cell contact, followed by detachment from the primary tumor, active cell migration, invasion, and infiltration in the surrounding tissue. The mechanisms underlying local invasion and distant metastasis are still unclear (Bao et al., 2013). Thus, understanding the mechanisms involved in the development of HCC invasion and metastasis is crucial to improve future treatment strategies and prognosis.

Heparan sulfate proteoglycans (HSPGs) are present on the cell surface of most cells and are major elements of the extracellular matrix (ECM) (Bishop et al., 2007). HSPGs comprise core proteins covalently attached to one or more sugar chains called heparan sulfate chains. The ability of HSPGs to bind to diverse protein ligands, including growth factors, proteases, matrix proteins, and cell adhesion molecules, facilitates a multitude of structural and signaling functions (Iozzo, 2001). HSPGs also play an important defensive role against tumor cell invasion. It has been shown that the activities of HSPGs-degrading enzymes were noticeably higher in invasive cancer cells than in cells with less invasive potentials (Toyoshima and Nakajima, 1999).

Numerous clinical and experimental studies have demonstrated that elevated levels of matrix metalloproteinases (MMPs) are associated with increased tumor growth, cancer progression, and metastasis, and shortened survival in patients. Among the various MMPs, MMP-9 (gelatinase B) has been postulated to play a

critical role in HCC cell invasion and metastasis by degradation of Type IV collagen, a major component of the ECM and basement membranes (Watanobe and Takebe, 1987; Roomi et al., 2014). Interestingly, MMP-9 activities are often elevated in tumors and in malignant cells (and low\undetectable in normal tissues) (Lou et al., 2013; Roomi et al., 2013; Zhang et al., 2013; Ordonez et al., 2014; Zhu et al., 2014).

Fascin, an actin-binding protein, is a cytoskeleton regulatory protein that plays a pivotal role in cell movement under physiologic and pathologic conditions. Fascin is mainly expressed in mesenchymal tissues and the nervous system (Hayashi et al., 2011). Recent studies have demonstrated that fascin is highly expressed in many human tumors, including hepatocellular carcinoma and that its high expression significantly correlates with tumor cell invasiveness/metastatic potential and reductions in patient prognosis (Huang et al., 2012; Oh et al., 2012).

Tetracycline derivatives are novel agents that block HCC invasion and metastasis. These do so, in part, by inhibiting both the activity and production of MMP (Hidalgo and Eckhardt, 2001). Doxycycline is an antibiotic that is considered a non-selective and broad-spectrum MMP inhibitor (Tae et al., 2012). Recent studies have shown that it is also a pluripotent drug that affects many cellular functions, and imparts cytotoxicity against cell lines of various tumor origins (Al-Gayyar et al., 2011), induces apoptosis in cultured tumor cells (Iwasaki et al., 2002), and inhibits several MMPs (Fife and Sledge, 1995). Accordingly, the current study sought to investigate if the anti-tumor activity of doxycycline in HepG2 cells (as well as human HCC cells) was, in part, through targeting of MMP-9 and/or fascin associated with these cells.

2. Materials and Methods

2.1 Cell lines and cell cultures

Human hepatocellular carcinoma (HepG2) cell lines were obtained from VacSERA (Cairo, Egypt). HepG2 cell lines were ensured to be mycoplasma-free through microbiological culture. HepG2 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin and 1% penicillin. Cells were incubated for 24 hr at 37°C in a 5% CO_2 incubator to allow the cells to grow. Aliquots containing 10^4cells were plated in each well of 96-well plates, and incubated in a humidified 5% CO_2 incubator at 37°C to for 24hr. The medium was then removed and replaced with serum-free medium to allow for the exposures to different concentrations of doxycycline (20, 40, or 60 μM). The treated cells were then incubated at 37°C for 48 hr. Cells incubated in culture medium alone served as a control for cell viability. Each experiment was repeated three times. After doxycycline treatment, cells were recovered, re-suspended in phosphate-buffered saline (PBS, pH 7.4), and then centrifuged (5 min, 4000 rpm) to remove cell debris and obtain a clear supernatant.

2.2 MTT assay

Cell viability was determined using MTT assay (Al-Gayyar et al, 2011). In brief, viability of HepG2 cells was determined by incubating cells for 4 hrs at 37°C with 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;(Sigma-Aldrich, St. Louis, MO, USA) in PBS. The purple formazan precipitate that was formed in the viable cells was then dissolved by addition of 20 μl acid isopropanol (1:9 of 1 N HCl/isopropanol) to each well. Optical density in each well was then measured at 540 nm and 690 nm using a microplate reader (BioTek Instruments, Wisnooski, VT).

2.3 Evaluation of cytotoxicity with LDH

Equal volumes of the supernatant were placed in a 96-well plate. The LDH solution was added to each well including controls and cell-free wells. The plate was allowed to develop for 20 min in the dark at room temperature. The cytotoxicity with LDH was determined by subtracting the normalized absorbance at 680 nm of the cell-free wells from the normalized absorbance of wells with cells. Relative cytotoxicity was determined by normalizing against the positive cytotoxicity control, 1% Triton-X.

2.4 Assessments of oxidative stress, Fascin and HSPG levels, and Caspase-3 activity

The level of oxidative stress associated with the HepG2 cells was quantified by measures of superoxide anion formation (Baehner et al., 1976) and superoxide dismutase (SOD) activity (DeChatelet et al, 1974). Fascin and HSPGs levels were measured in cells homogenates using commercially available ELISA kit (New East Biosciences, Malvern, Pennsylvania) , and Enzo Life Science Inc., Farmingdale, NY, respectively) following manufacturer protocols. Lastly, caspase-3 activity within the cells was measured using a commercial kit (GenScript, Piscataway, NJ) and following manufacturer protocols.

2.5 Statistical analysis

Values are reported as mean ± SE. For comparison between two groups, a Student's t-test was used. Statistical computations were done using SPSS version 13 software (Chicago, IL). Statistical significance was accepted at $p < 0.05$.

3. Results:

3.1. Effect of doxycycline on HepG2 cells

Matrix metalloproteinase-9 (MMP-9) was measured in HepG-2 cell line cultured with doxycycline with three different doses (20, 40and 60 µm) in relation to the zero concentration serving as the control group. Relative measures of concentration among all four groups are shown in figure 1.

Doxycycline was found to block MMP-9 in a dose-dependent manner. Maximum reduction in MMP-9 concentration was significant by dose 60 µm.

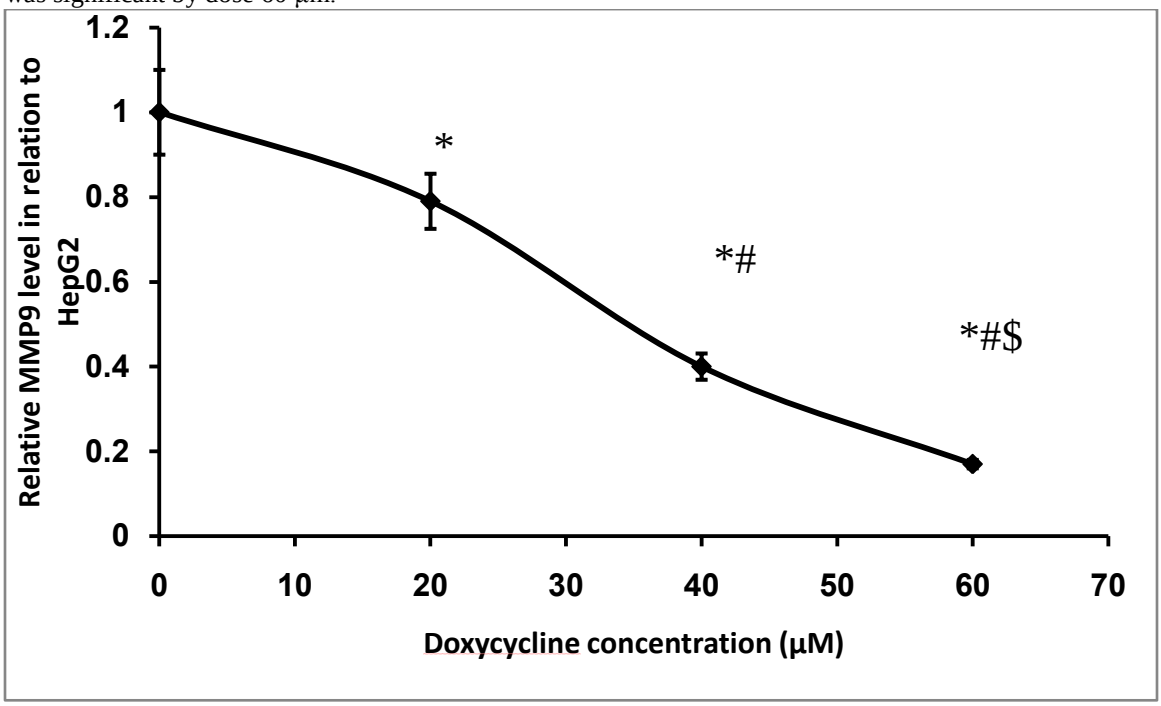

Figure 1. Relative MMP-9 level (relative mean ±SE) in HepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group. (*) Significant difference as compared to HepG2 group at P<0.05. (#) Significant difference as compared to doxycycline (20µm) at P<0.05, ($) Significant difference as compared to doxycycline (40µm) at P<0.05.

3.2. Cytotoxic activity of doxycycline on HepG2 cells

The antitumor activity of doxycycline of different doses (20, 40and 60 µm) was assayed by MTT assay and LDH assay in relation to the zero concentration serving as the control group. Relative cell survival was carried among all four groups and is shown in figure 2a.

Relative cell survival measure showed significant decrease in cell survival by both doses 40 and 60 µm against control. Moreover, HepG2 cell line cultured with 20µm showed insignificance against HepG2 control. Doxycycline reduced cell survival in a dose-dependent manner. Moreover, statistical analysis of relative cell cytotoxicity, in relation to triton-X, revealed that the cytotoxicity of doxycycline is in a dose-dependent manner. Cytotoxicity was significantly increased in HepG2 by all doses (20, 40and 60 µm) when compared to HepG2 without treatment, as shown in figure 2b.

A.

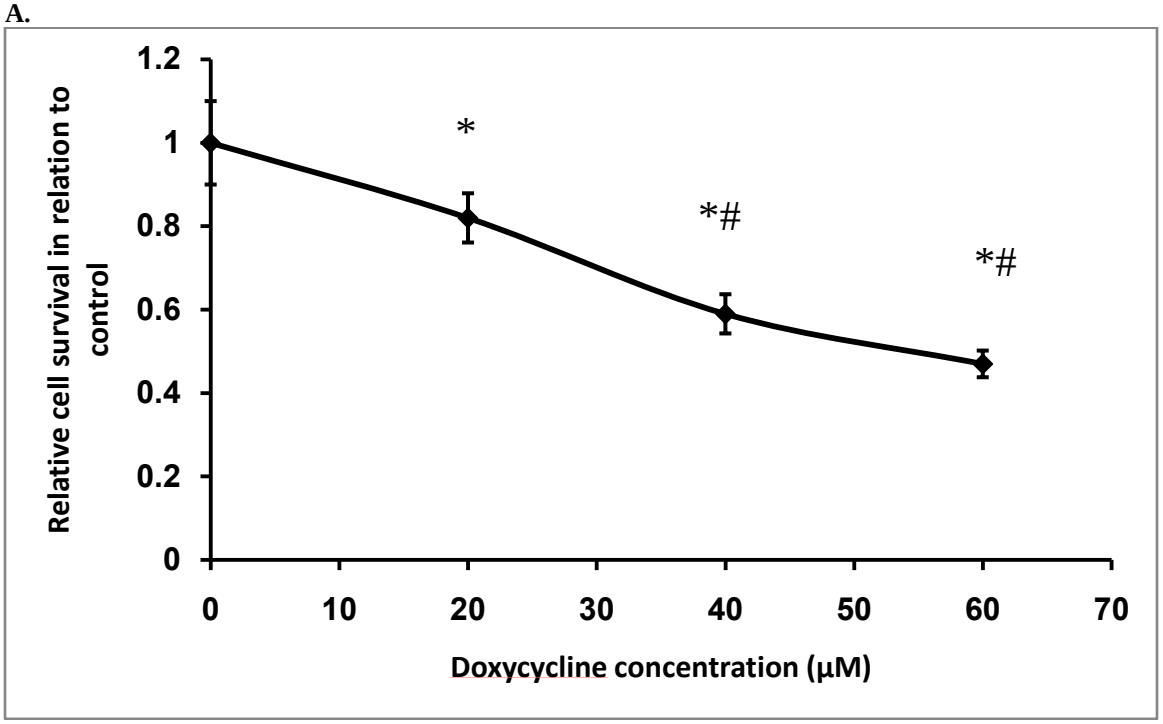

Figure 2A. Relative cell survival level (relative mean ±SE) in HepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group (Figure 2a). (*) Significantly different vs. HepG2 group at p < 0.05; (#) vs. doxycycline (20 µm) at p < 0.05.

B.

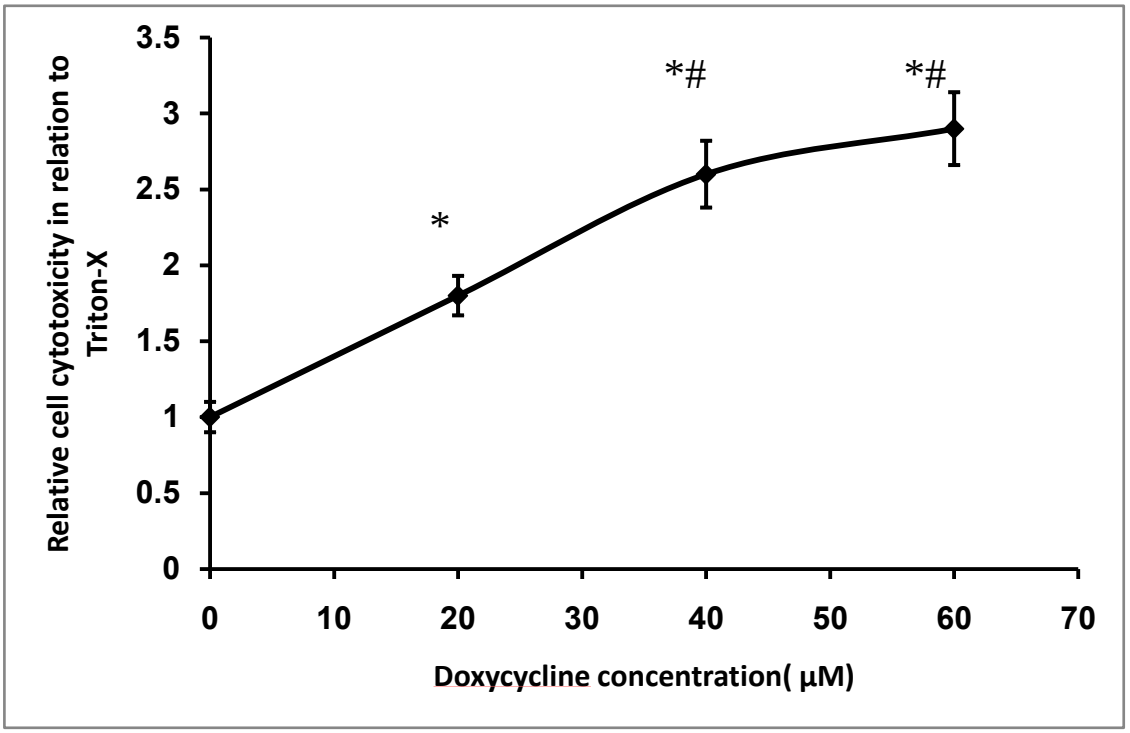

Figure 2B. Relative cell cytotoxicity (relative mean ±SE) in HepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group (Figure 2b). (*) Significantly different vs. HepG2 group at p < 0.05; (#) vs. doxycycline (20 µm) at p < 0.05.

3.3. Effects of doxycycline against HCC-induced oxidative stress in HepG2 cells

The oxidative stress superoxide anion was measured colorimetrically in HepG2 cultured with doxycycline of different doses (20, 40and 60 µm) in relation to the zero concentration serving as the control group. The treatment of HepG2 cells with doxcycline showed dose-dependent reduction in hydrogen peroxidase (figure3a). Moreover, the antioxidant enzyme, superoxide dismutase (SOD) activity, was measured kinetically in HepG2 cells cultured with doxycycline of different doses (20, 40and 60 µm) in relation to the zero concentration serving as the control group. Doxycycline showed an elevation in superoxide dismutase in a dose-dependent manner (figure3b).

A.

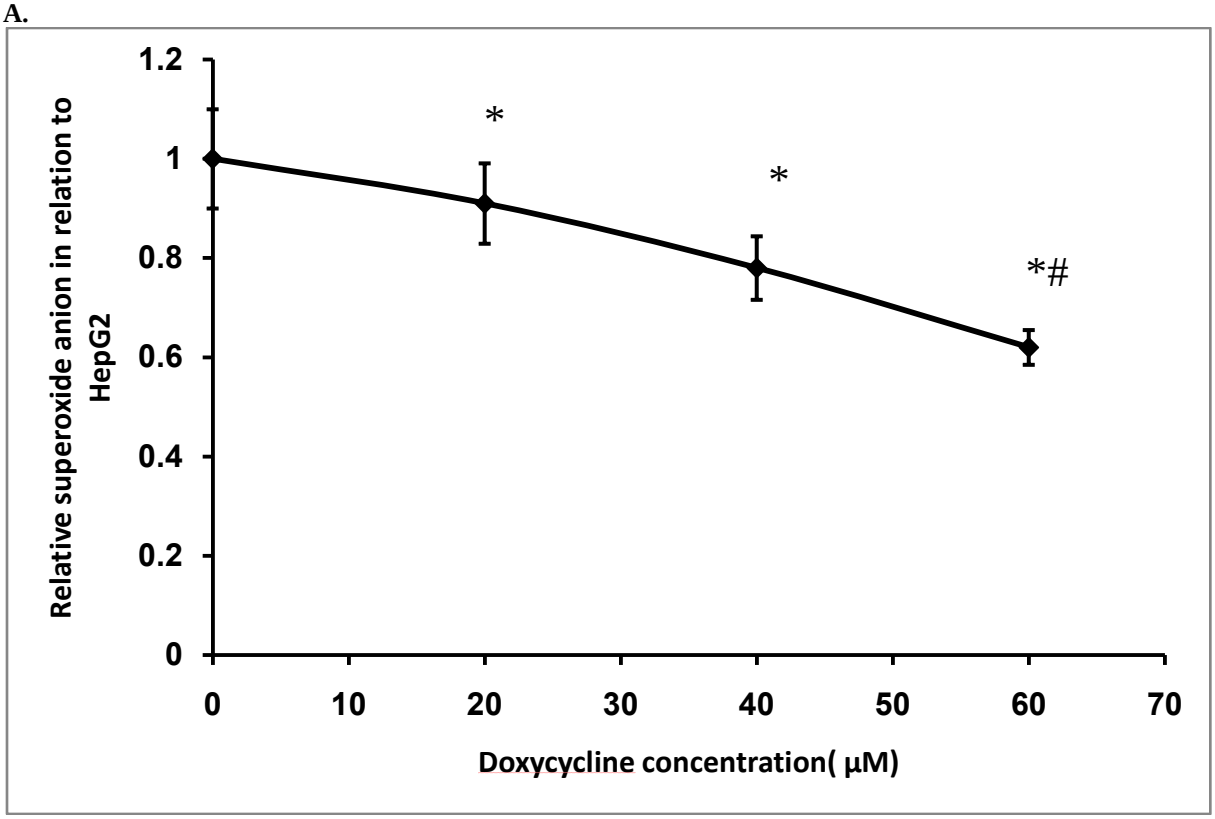

Figure 3A. Relative superoxide anion (relative mean ±SE) in HepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group (Figure 3a). (*) Significant difference as compared to HepG2 group at P<0.05. (#) Significant difference as compared to doxycycline (20 µm) at P<0.05

B.

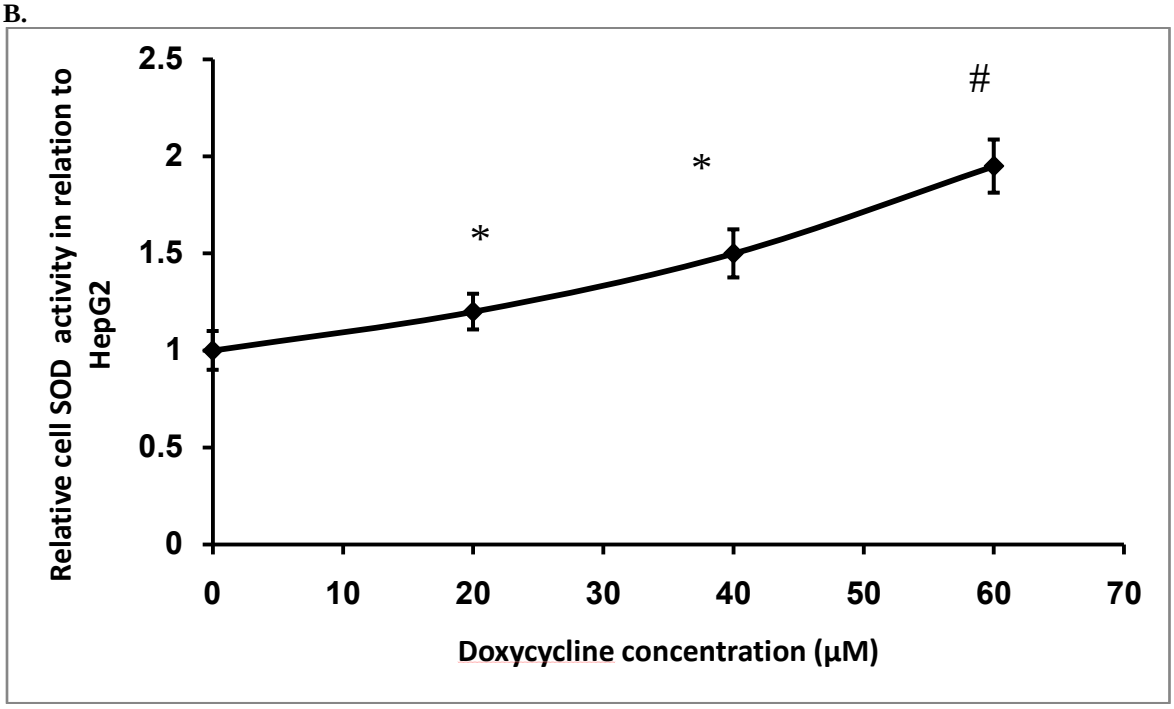

Figure 3B. Relative superoxide dismutase (SOD) activity (relative mean ±SE) in HepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group (Figure 3b). (*) Significantly different vs. HepG2 group at p < 0.05; (#) vs. doxycycline (20 µm) at p < 0.05.

3.4. Effects of doxycycline against HCC-induced reduction of HSPGs in HepG2 cells

Heparan sulfate proteoglycan (HSPG) level was measured in HepG2 cell line cultured with doxycycline of different doses (20, 40and 60 µm) in relation to the zero concentration serving as the control group. Levels of HSPGs were found to be significantly reduced in HepG2 untreated control. Figure 4 depicts the dose-dependent increase in heparan sulfate proteoglycans (HSPGs) in response to doxycycline treatment of HepG2 cells.

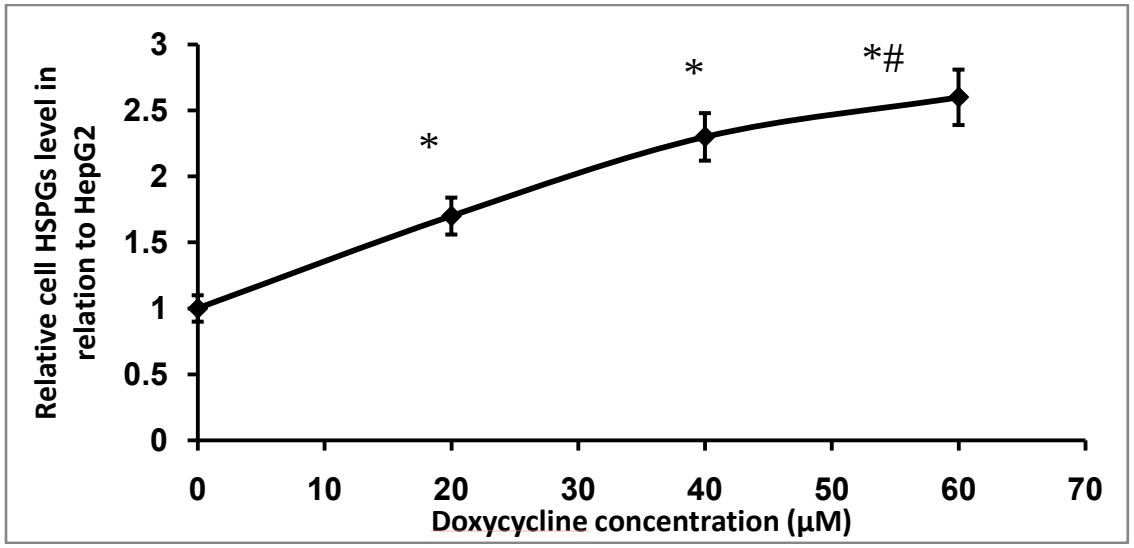

Figure 4. Relative HSPG level (relative mean ±SE) in HepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group. (*) Significantly different vs. HepG2 group at p < 0.05; (#) vs. doxycycline (20 µm) at p < 0.05.

3.5. Effects of doxycycline against HCC-induced increase in invasion marker in HepG2 cells

The ability of the investigated doxycycline in three different doses (20, 40and 60 µm) to show dose dependent reduction in fascin levels in HepG2 cells in relation to the zero concentration serving as the control group is illustrated in figure 5. Maximum reduction in fascin concentration was significant by dose 60 µm.

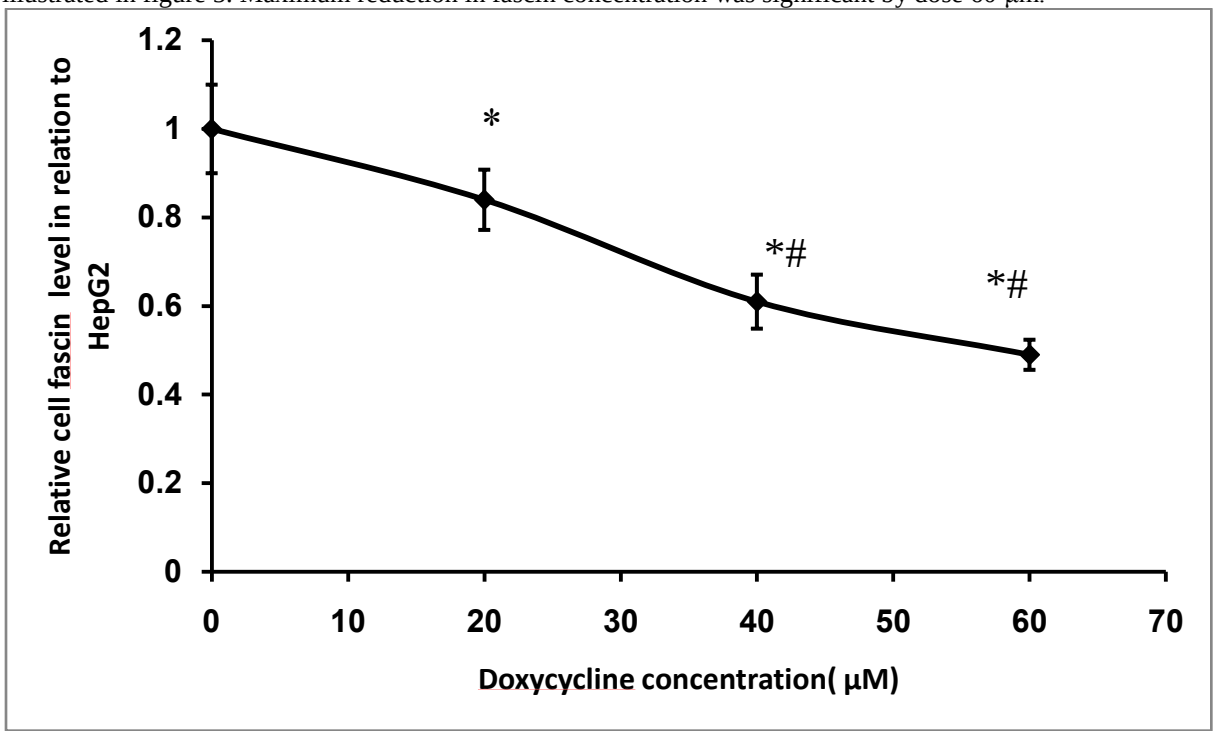

Figure 5. Relative Fascin level (relative mean ±SE) in hepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group. (*) Significantly different vs. HepG2 group at $p < 0.05$; (#) vs. doxycycline (20 µm) at $p < 0.05$.

3.6. Effects of doxycycline against HCC-induced increase in apoptosis marker in HepG2 cells

Caspase-3 enzyme activity was measured in HepG2 cell line cultured with doxycycline of different doses (20, 40and 60 µm) in relation to the zero concentration serving as the control group.

Caspase-3 activity was found to be significantly low in HCC untreated control HepG2 cells**.** Treatment of HepG2 with doxycycline resulted in a dose-dependent increase in the activity of caspase-3 as shown in Figure 6.

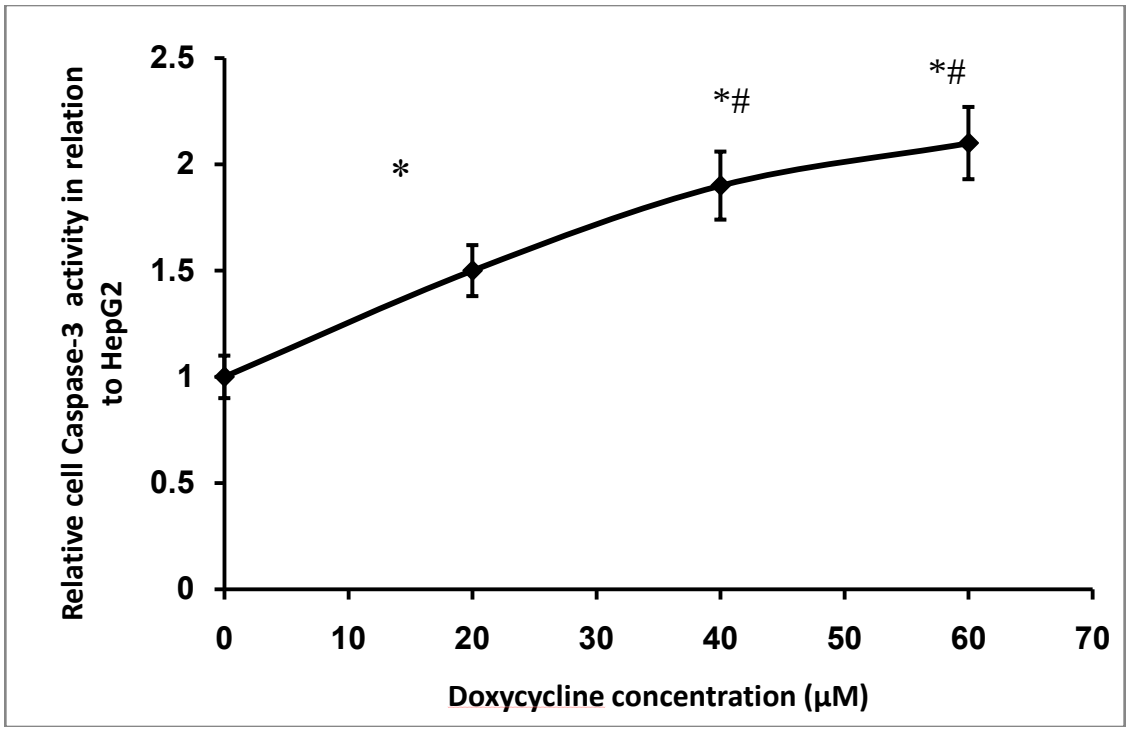

Figure 6. Relative caspase-3 enzyme activity (relative mean ±SE) in hepG2 cell line of (0, 20, 40 and 60µm of doxycycline) in relation to the zero concentration serving as the control group (*) Significantly different vs. HepG2 group at p < 0.05; (#) vs. doxycycline (20µm) at p <0.05.

4. Discussion

Primary liver cancer represents a major health burden and mortality in the world. HCC is a very aggressive cancer with poor clinical outcomes, as patients usually survive< 1 year after diagnosis (Liang et al., 2010). Albeit a wide range of therapeutic options is available, the efficacy of these methods and the survival of patients with HCC remain poor. Currently, the most effective treatment for early-stage HCC patients with preserved liver function and without distant metastasis is surgical resection (Yao et al., 2007). Though encouraging progress has been made in the treatment of primary tumors, metastases still affect these patients' prognoses, with an even poorer treatment effect seen during advanced metastatic disease. Thus, developments of novel small-molecular agents to block HCC invasion are primary objectives of hepatic cancer research (Lou et al., 2013).

Tumor aggression and metastasis have been correlated with increased MMP-9 and fascin expression (Al-Alwan et al., 2011; Roomi et al., 2014). MMP are thought to contribute to tumor metastasis by their matrix-degrading activity. Heparan sulfate proteoglycans (HSPGs), when degraded by MMP-9, permit the release of their cell-membrane–bound precursors of some growth factors such as fibroblast growth factors which contribute in tumor invasion (Egeblad and Werb, 2002). MMPs degrade the basement membrane and extracellular matrix, thus facilitating the invasion of malignant cells through connective tissues and blood vessel walls and resulting in the establishment of metastasis (Chambers and Matrisian, 1997). Furthermore, cytoskeleton proteins - such as Fascin - regulate multiple cellular processes, including morphological changes and motility, both are critical events for metastasis. As with MMP, fascin expression is associated with a bad prognosis, metastasis, and reduced disease-free survival in cancer patients (Al-Alwan et al., 2011).

In the present study, the anti-tumor activity of doxycycline was investigated through assessments of its inhibitory effects on key players of metastasis (e.g., MMP-9, HSPGs, and Fascin); ability to induce apoptosis; and anti-oxidant effects. It has been known for years that members of the tetracycline family, such as doxycycline inhibits the growth of various tumor cells in vitro (Fife et al., 1997). Doxycycline was found to be the most effective tetracycline analogue at inhibiting survival of a human adenocarcinoma cell line (Duivenvoorden et al., 1997). The mechanisms for doxycycline anti-proliferative effects have been previously reported, and include impairment of

mitochondrial protein synthesis (Kroon et al., 1984; van den Bogert et al., 1986), proliferation arrest in the G1 cell cycle phase (van den Bogert et al, 1986), and induction of apoptosis by caspase-3 activation (Iwasaki et al., 2002).

The results of the present study showed that doxycycline was able to reduce MMP-9 levels (Figure1) in a dose–related fashion in HepG2 cell lines, an outcome that is in accordance with previous studies (Sun et al., 2007; Shen et al., 2010; Roomi et al., 2014). Moreover, Nowak et al., (2013) clarified that this inhibitory effect may be through one of several mechanisms, i.e., chelating of Zn^{2+} ions, which is crucial for MMP-9 activity or influencing the mRNA stability of MMP-9. Recently, Roomi et al., (2013) showed that doxycycline completely blocked MMP-9, that functions as hydrolyzer of extracellular matrix components and elements of the endothelial cell basement membrane. This results in removing physical/structural barriers and promotes cell migration and invasion (Krylova et al., 1991; Nowak et al., 2013). In the current study, doxycycline helped to maintain the integrity of HSPG by preventing protease enzyme (mainly MMP-9) from destroying the HSPG - an outcome that could result in release of a multitude of growth factors (like fibroblast growth factor [FGF]) that could contribute to tumor cell proliferation, HCC progression, and metastasis. The doxycycline also led to reduced fascin levels in the HepG2 cells. Whether this is a direct effect or secondary to changes in MMP levels/activity remains to be determined. Hashimoto et al., (2007) is the only other study that supports our results of the effect of doxycycline on fascin but in human colon carcinoma cells.

Doxcycline has recently been shown to possess cytotoxic activity against several tumor types (Sekeroglu et al., 2012). The use of doxycycline, in the current study, resulted in a dose-dependent decrease in the cell survival as indicated by MTT assay as well as significant dose-dependent elevation of cytotoxicity as indicated by LDH level in relation to Triton-X. These findings are in agreement with a previous study of Sekeroglu et al., (2012) who investigated the cytotoxic effects of doxycycline in cultured human peripheral blood lymphocytes. They showed that if doxycycline has cytotoxic potential to different types of cancer cells, it may also have been used as antitumor drug. Furthermore, some studies demonstrate that doxycycline can induce its antitumor activity through induction of apoptosis via caspase-dependent way (Onoda et al., 2006; Mouratidis et al., 2007). However the mechanism by which doxycycline exerts its apoptotic effect remains unclear. It is proposed that doxycycline induces apoptosis through a Fas/Fas-ligand dependent pathway in jurkat T-lymphocytes (Liu et al., 1999). In the present study, the treatment of HepG-2 cells with doxycycline resulted in a significant elevation of caspase-3 activity in all three dose levels (20, 40, 60 μm) compared to their control. It was previously shown that the cytotoxic activity of doxycycline exceeding 20μg/ml in pancreatic cancer cells is mediated by the induction of apoptosis which was evidenced by DNA fragmentation (Son et al., 2009).

Oxidative stress is known to have a key role in HCC development and progression, as it can affect cell proliferation, apoptosis, cell cycle arrest and cell senescence (Marra et al., 2011). Furthermore, it was shown that the function of MMPs can be influenced by reactive oxygen species (ROS) via the inflammatory response which occurs at the tumor site that creates large amounts of ROS. These oxidants initially activate MMPs via oxidation of the pro-domain cysteine (Weiss et al., 1985). These pieces of evidences are of a great significance to be highlighted in this study, hence the effect of doxycycline on two of the oxidative stress markers; superoxide anion and superoxide dismutase was assessed .In the current study, treatment of HepG2 cells with doxycycline resulted in a significant decrease in superoxide anion levels and significant increase in superoxide dismutase. Doxycyline improved the compromised antioxidant defense and ameliorated high levels of protein oxidation in a tissues-dependent manner (Kinnunen et al., 2005).

5. Conclusions

The main findings of the current study revealed that doxycycline exhibited significant anti-tumor activity that can be partially explained by inhibition of key players of metastasis (MMP-9 and Fascin levels), restoration of HSPG integrity, and anti-oxidant activity as indicated by the inhibition of superoxide anion persistence. However, other mechanisms involved include cytotoxity via activation of caspase-3 apoptotic pathways. To our knowledge, the current study demonstrates for the first time the inhibitory effect of doxycycline on Fascin in the HepG-2 cell line.

Statement of Conflict of interest

The authors declare no conflict of interest. The authors alone are responsible for the content of this manuscript.

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