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# The Autophagy Inhibitor Hydroxychloroquine Enhances Sensitivity of Osteosarcoma Cell Line MG-63 to Doxorubicin Treatment

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## ABSTRACT

Autophagy is a self-energy supplier process response to stressful or fasting conditions where cell degrades and recycles intracellular constituents as an alternative way of energy source. Unfortunately Autophagy process play important role in tumor cell resistance to anticancer drugs. Osteosarcoma is the most commonly diagnosed primary malignant tumor of the bone. The present study evaluates the role of Autophagy inhibitor hydroxychloroquine (HCQ) on doxorubicin-induced cell death in Osteosarcoma cell line (MG-63). The results of current study found that doxorubicin induced Autophagy in Osteosarcoma (MG-63) cells, exhibiting an increased microtubule associated protein 1 light chain 3 (LC3)level, a decrease in SQSTM1 (p62) level, moreover using of Autophagy modulator increased in generation of reactive oxygen species (ROS) and decreased Autophagy activity and increased Osteosarcoma-cell death.

Although doxorubicin-induced cell death was enhanced by combined with HCQ but at high concentration of doxorubicin the cytotoxicity was decreased with increased Autophagy activity. These finding suggest that Autophagy attenuate cytotoxicity effect of doxorubicin by decreasing level of ROS, while HCQ improved doxorubicin-cytotoxicity on Osteosarcoma by inhibiting of Autophagy process.

**Keywords**: Osteosarcoma, Autophagy, doxorubicin, hydroxychloroquine, MG-63.

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## INTRODUCTION

Autophagy is a natural, regulated, intracellular recycling system by which cells decay and recycle those cytoplasmic components through the lysosome-dependent path Cao, 2017. Autophagy has a definitive role in regulating cellular homeostasis and plays a pivotal function in disease conditions Islam, 2017. Although cancerous cells often show deregulation of Autophagy, the two roles of Autophagy; as tumor suppressor and tumor promoter, have been supported by numerous scientific researches. The role of Autophagy depends on type of tumor, stage of disease and conditions of tissue Cheong, 2012 [1]. Osteosarcoma is the most commonly diagnosed primary malignant tumor of the bone. In addition to over 56% of all bone tumors, Osteosarcoma is the third most frequent cause of cancer in adolescents Yang, 2017. The distal femur and proximal tibia are most frequently involved bones and is thought to be more frequent in males than females Smeland, 2019. Approximately 8.7 per million in persons fewer than 20 years suffer from Osteosarcoma Zamborsky, 2019. The 5-year survival rate of patient with a non-metastatic disease is 65-70 %, but its only 20% for patient with metastatic disease Camuzard, 2019. Chemotherapy: Depending on the stage of Osteosarcoma at time of diagnosis, chemotherapy is routinely used to treat patients with advanced Osteosarcoma Jain, 2016. The most commonly used chemotherapeutic agents are high-dose Methotrexate, doxorubicin, cisplatin, and ifosfamide Mustafa, 2018. Doxorubicin: Doxorubicin is anthracyclines member has broad spectrum of anticancer activity, currently is the most effective chemotherapeutic drug used to treat many forms of cancers Christowitz, 2019. Although doxorubicin is one of two only cytotoxic single agents with objective response rates over 10% Chugh, 2015, but there is Osteosarcoma resistance to doxorubicin. This resistance may related to the ATP-binding cassette (ABC) transporters mediated drug efflux Yang, 2014; Lovitt, 2018. However the mechanisms of resistance to doxorubicin in human may be caused by:

- Increased efflux of doxorubicin out of tumor cell
- More efficient intracellular detoxification of tumor cell

Alterations of topoisomerase II and increased repair of damaged DNA, He, 2014; Yang, 2014). Additionally He review observed diverse studies indicate that type II programmed cell death (Autophagy) mediates drug resistance of cancers cells, the cytotoxic drugs like doxorubicin, Methotrexate and cisplatin induce protein expression of high-mobility group box 1 (HMGB1) which induce Autophagy (He, 2014; Kim, 2015). Autophagy Inhibition in Cancer Therapy: In the last decades Autophagy pathway becomes a targeting as line of treatment of cancer. However this line confronts challenges due to its variant and opposite roles of Autophagy in tumor formation and progression Cheong, 2012 [2], Autophagy inhibitor plus cytotoxic drug combination is attractive line of cancer treatment, which depend on ability of Autophagy to increase ability of cancer cell to survive in stressful conditions. So inhibition of Autophagy will increase the cytotoxic effect of anticancer drugs in eradication of cancer cell Amaravadi, 2011. Hydroxychloroquine: HCQ is a well-described ant malarial drug, its derivative of chloroquine and has the same effect but less toxic and more tolerable

than parent drug chloroquine Xu, 2016. HCQ has long been used in prophylaxis and treatment of parasites falciparum malaria Adeel, 2012. Most patients of rheumatoid arthritis and SLE use HCQ Jorge, 2018. Recently HCQ used as Autophagy inhibitor and its only drug established to use as Autophagy inhibitor in clinical study Xu, 2018. HCQ inhibits Autophagy by stabilized lysosome enzymes via increase the pH in the lumen of lysosome vesicles Morgan, 2014. The role of Autophagy inhibitors in cancer treatment renders HCQ as one of common item of anticancer combination in clinical trials Jones, 2019.

## MATERIAL AND METHODS

Cell culture: MG-63 human Osteosarcoma cells were kindly provided by cell culture unit of Babylon medical college, Babylon University. This line was seeded in Roswell park memorial institute (RPMI-1640) medium (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco, UK) with different concentrations of doxorubicin (Ebewe, Austria), and/or hydroxychloroquine (HCQ) (Bristol lab, UK), at 37ºC in a humidified chamber with 5% carbon dioxide.

### Cell viability assay

Cell viability was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Fluka, Switzerland). Cells of Osteosarcoma were seeded in 96-well plates and grown to 70% confluence. Doxorubicin plus 100µm HCQ was added to culture medium at the indicated concentration (Osteosarcoma MG-63 cell line was already pretreated with 100µm HCQ for 3 hours). After 24 hours twenty micro liters of MTT (5mg/ml) was added to each well, then the 96 well plates was incubated at 37ºC, then after 4hours150 µl of DMSO was added to adherent cells of each well for dissolving of crystal. Then the luminescence of each well was measured using a micro plate reader Human, Germany at a wavelength (570 nm).

### Measurement of intracellular reactive oxygen species (ROS)

The intracellular ROS were measured by human reactive oxygen species ELISA kit. This kit was purchased from bioassay technology laboratory (Shanghai, China). Human Osteosarcoma cells were cultured in 96 wells plate, then when growth were reaching 70% confluence, the cells were treated by experimental drugs and incubated for 24 hours at 37ºC. Then cell lysate of wells transported into kit wells which has been pre-coated with human ROS antibody, so ROS present in sample bound to pre-coated antibody, then the biotinylated human ROS antibody was added and followed by Streptavidin-HRP which bound to biotinylated ROS antibody and incubated for 60 minutes at 37ºC. then unbound Streptavidin-HRP was removed by washing step, then substrates of coloring reaction were added for a couple of minutes and followed by termination of reaction and optical density (which proportion to the amount of human ROS) was measured by micro plate reader (Bio Tek, USA) at 450 nm. Measurement of levels of microtubule associated protein 1 light chain 3 alpha (MAP1LC3a) and sequestosome 1 (P62) by ELISA kits:

Human Osteosarcoma cells were cultured in 96 wells plate, then when growth were reaching 70% confluence, the cells were treated by experimental drugs and incubated for 24 hours at 37ºC. Then cell lysate of wells were used for measuring of LC3 and P62 by ELISA kits, and these kits were purchased from cloud- clone corp. (USA).The measurement of levels of two Autophagy markers LC3 and P62 were performed according to manufacture protocol of kits: cat no SEL701Hu for LC3 and cat no SED198Hu for P62.

### Statistical Analysis

Statistical package for social sciences (SPSS) version 23 was used for statistical analysis. Results were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons and LSD post-hoc test. The results variability was expressed as mean± standard error of mean (SEM). P value of 0.05 was considered statistically significant.

## RESULTS

Autophagy attenuates the anticancer effect of doxorubicin against Osteosarcoma cell line (MG-63): An Osteosarcoma cell line (MG-63) was chosen to investigate the effect of Autophagy on Osteosarcoma treatment. MTT assay was used to reveal the inhibitory effect of doxorubicin with and without Autophagy inhibitor HCQ against MG-63 cell line. The data of figure (1) reveals that the cytotoxic effects of serial concentrations of doxorubicin (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) (after 24hours incubation) on the Osteosarcoma cell line (MG-63) were significantly increased versus the control group($p < 0.001$). Further it showed the best effect at high concentration or in other word the percentage of cells viability of Osteosarcoma cell line MG-63 decreases with increasing of doxorubicin concentration. To modulate the role of Autophagy in reducing the inhibitory effect of doxorubicin we treated MG-63 cell line with 100µm HCQ as an Autophagy inhibitor for three hours before exposure to combination of serial concentrations of doxorubicin and 100µm HCQ (incubation for 24 hours) then assess the inhibitory effect of combination by MTT test and results was represented by figure (2) which shows a significant decrease in percentage of cell viability of MG-63 at all concentrations of doxorubicin (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) plus 100µm of HCQ (after 24hours incubation) with comparison to control group ($p < 0.001$). The figure also showed decreasing of inhibitory effect with increasing of concentration and the best effect was observed at low concentrations (3.125, 6.25 and 12.5 µg/ml). While the figure (3) shows that HCQ was significantly enhances the cytotoxic effect of doxorubicin at lower concentrations (3.125 and 50 µg/ml ($p =0.03$)) and (6.25, 12.5 and 25 µg/ml ($p < 0.001$)) and not significantly difference at concentration (100 µg/ml) in comparison with non-pre-treated MG-63 cell line. Although HCQ is Autophagy inhibitor but we have to detect the activity of Autophagy, so the Autophagy markers MAP1LC3 (LC3) and SQSTM1 (P62) have been used as Autophagy indicators. LC3 is one important protein required by Autophagy process and its recruited to the autophagosomal membrane. The increment of LC3 expression indicates induction of Autophagy activity

(Weinberger al., 2011). LC3 plays vital role in mediation the specificity of targeting cargo for clearance of waste product. Also many Autophagy proteins like Sequestosome 1 (P62/SQSTM1) are recognized by Autophagy substrates as cargo receptors for same process (Goldsmith et at., 2014). A decrease in P62 expression indicates early Autophagy degradation (Lin et al., 2017). The present study explained the effect of tested drugs on these two markers and figures (4 to 5) reveal these finding. The figure (4) shows there was a significant decrease in level of LC3 of HCQ pre-treated mg-63 cell line at all concentrations of doxorubicin (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) with except low concentration in comparison with non HCQ pre-pretreated MG-63 cell line ($p < 0.001$). The figure (5) shows there level P62 of HCQ pre-treated MG-63 cell line was a significantly higher at all concentrations (100, 50, 25, 12.5, and 6.25 µg/ml) with except low concentration in comparison with non HCQ pre-pretreated MG-63 cell line ($p < 0.001$) and non-significant difference at concentration 3.125 µg/ml i.e. doxorubicin alone induce Autophagy activity of Osteosarcoma MG-63 cell line.

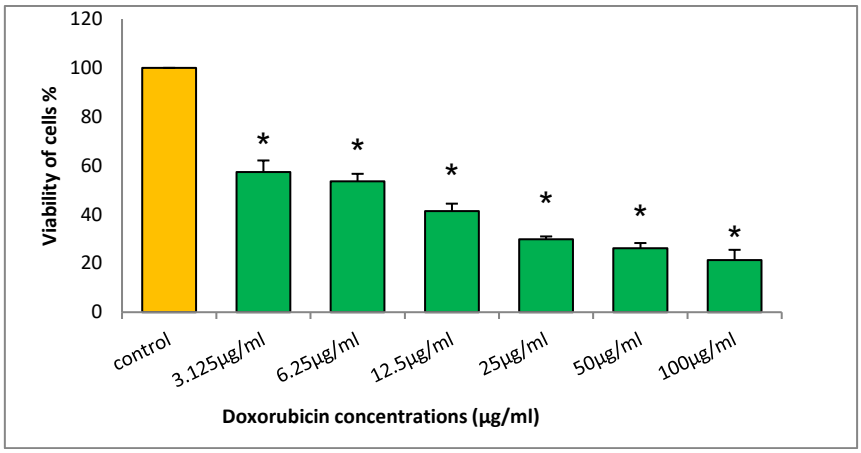


Figure 1: The effect of different concentrations of Doxorubicin on MG-63 cell line (after incubation for 24 hours) versus control group represented by mean± SEM ($p < 0.001$).

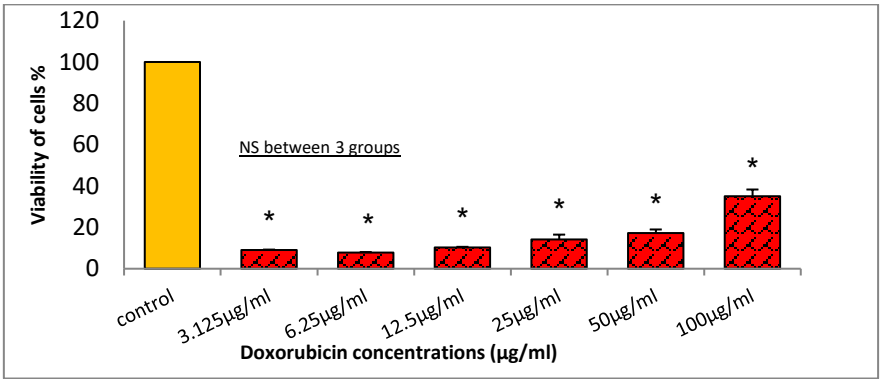


Figure 2: The effect of combination of different concentrations of doxorubicin plus 100 µM HCQ on pre-treated MG-63 cell line (after incubation for 24 hours) versus control group represented by mean± SEM (*= $P < 0.001$), NS= non-significant difference.

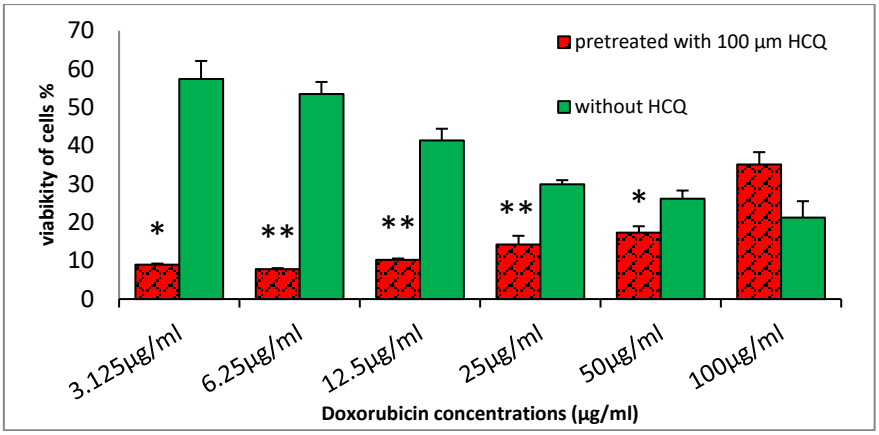


Figure 3: Comparison the effect of serial concentration of doxorubicin on MG-63 with HCQ versus without HCQ represented by mean± SEM (P*=0.03) and (p**< 0.001).

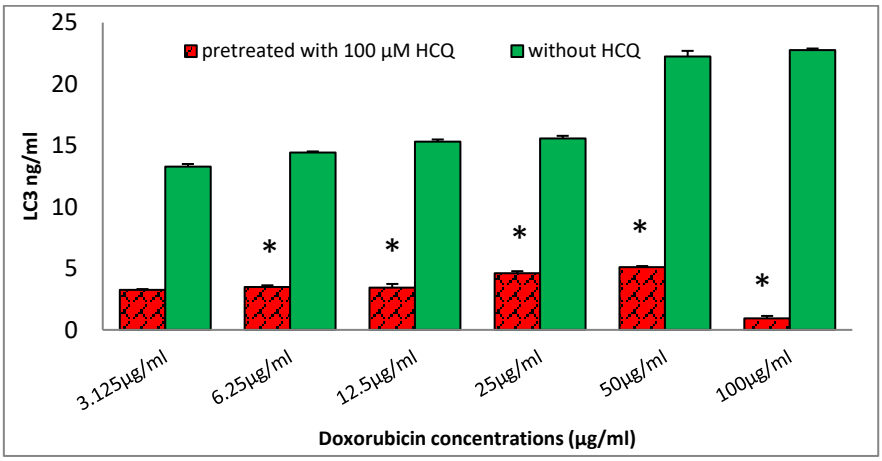


Figure 4: The effect of serial concentrations of doxorubicin on level of LC3 of MG-63 with and without HCQ represented by mean± SEM (*p<0.001).

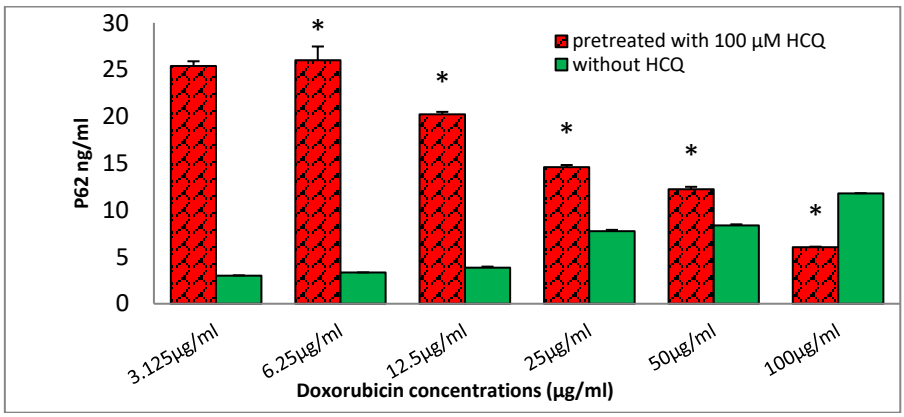


Figure 5: The effect of serial concentrations of doxorubicin on level of P62 of MG-63 with and without HCQ represented by mean± SEM (*p<0.001).

HCQ increases ROS level of doxorubicin-treated-MG-63 cell line: To confirm the role of Autophagy in decreasing the cytotoxicity of doxorubicin by decreasing ROS generation. Figure (6) illustrates the levels of ROS of HCQ-pre-treated MG-63 cell line was a significantly higher than the levels of non HCQ pre-treated MG-63 cell line at concentrations (3.125, 6.25, 12.5 and 50 µg/ml) ($p < 0.001$) and at concentration 100 µg/ml ($p = 0.002$) and non-significant difference at concentration 25 µg/ml.

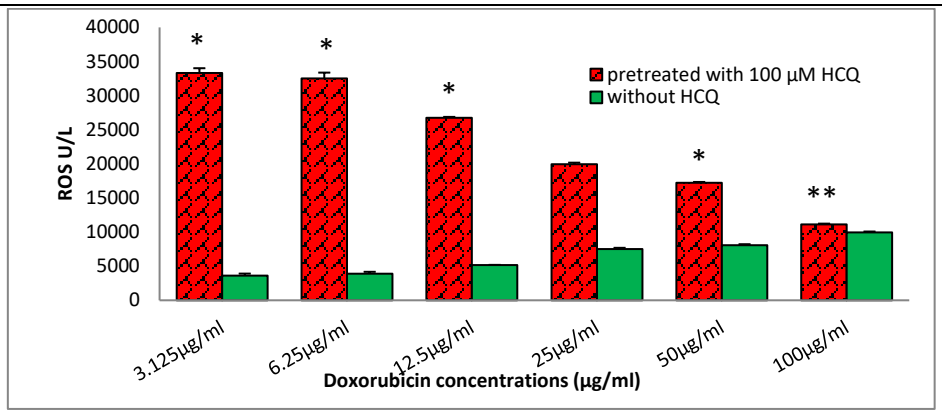


Figure 6: The effect of serial concentrations of Doxorubicin on levels of ROS of MG-63 with and without HCQ represented by mean± SEM (*p<0.001), (**p=0.002).

## DISCUSSION

The two main obstacles face management of cancerous diseases is drug adverse effect and drug resistance. Scientists attempt to solve these problems through combination treatment Kim, 2015. Combination treatment may enhance therapeutic effect of drug Jin, 2019 and reducing of drug adverse effect of anticancer drugs via reducing of drug dose Daughton and Ruhoy, 2013. The adverse effects are responsible for discontinuation a lot of medication Lavan and Gallagher, 2016 and even doxorubicin has dose-dependent adverse effect like cardio toxicity which causes cardiomyopathy and leads to congestive heart failure and death Carvalho, 2009. One of great challenges faced improvement of the prognosis of Osteosarcoma patients is the deleterious long-term consequence of doxorubicin treatment for Osteosarcoma survival, and doxorubicin causes severe cardio toxicity in 28% Osteosarcoma-pediatric patients Mandell, 2019. Also tumor resistance has limited the effectiveness of the agent in single drug treatment regimen Lovitt, 2018. In addition to adverse effect, doxorubicin can induce drug resistance and even tumor growth resulting in poor patient prognosis and survival Christowitz, 2019. The present study reported new combination doxorubicin with HCQ for treatment of Osteosarcoma in-vitro study. The Autophagy inhibitor (HCQ) enhances sensitivity of Osteosarcoma cell line (MG-63) to low concentration of doxorubicin. The finding of present study was compatible with previous recommendation and it observed that cytotoxic effect of doxorubicin on Osteosarcoma cell line MG-63 was increased directly to concentration of doxorubicin and showed the best effect at high concentration. Doxorubicin is already member of first line treatment of guideline of Osteosarcoma chemotherapy regimens by national comprehensive cancer network Zhang Y., 2018. Additionally these finding agreed with work of Yang et al. who indicated that doxorubicin inhibits the percentage of cell viability of Osteosarcoma (MG-63) in concentration-dependent manner Yang, 2017. The article of Pilco-Ferreto is additional assay that consistence with the observation of current study, Pilco-Ferreto illustrated that increasing concentration of doxorubicin lead to decrease the viability of all cell line included in study in a time- and dose-dependent manner Pilco-Ferreto and Calaf, 2016. The mechanism of doxorubicin in decreasing cell viability is:

1- inhibiting protein synthesis,
2- Inhibiting topoisomerase-II
3- Increasing of intracellular free radical generation of cancerous cells Wenningmann, 2019.

Measuring of percentage of cell viability for the HCQ-pre-treated Osteosarcoma MG-63 cell lines, that treated with combination of serial concentration of doxorubicin plus 100µm of HCQ (after 24hours incubation) revealed that the best inhibitory effect was observed at concentrations (3.125, 6.25, 12.5 and 25 µg/ml) and these four concentrations had same cytotoxic effect and better than highest concentration 100 µg/ml. The present study observed the ability of combination of HCQ and doxorubicin in reducing of percentage of cell viability of MG-63 cell line specifically at low concentrations (3.125, 6.25 12.5 and 25 µg/ml) of doxorubicin is better than what produce by doxorubicin alone, that means higher cytotoxicity in comparison with non-pre-treated MG-63 cell line. These reductions in percentage of cell viability of MG-63 indicates enhancement of cytotoxicity effect of Doxorubicin by combination with HCQ. These suggestion are compatible with work of Gupta's team, they mentioned that Autophagy inhibitor HCQ increases sensitization of cancerous cells to antitumor agent Imatinib Gupta, 2010. In 2018 an article related the ability of HCQ in enhancing cytotoxicity effect of antitumor drugs to role of HCQ in blocking Autophagy processing at end stage through increasing lysosome pH and stabilizing lysosome enzymes Xu, 2018. The present study used two Autophagy markers (LC3 and P62) to evaluate the effect of study reagents including HCQ against Autophagy activity and it found that using of combination of HCQ with doxorubicin inhibited Autophagy activity by decreasing the level of LC3 and increase level of P62 Lin, 2017. While doxorubicin that did not combined with HCQ showed opposite results of P62 and LC3 with high Autophagy activity that indicated the induction of Autophagy by doxorubicin in concentration-dependent manner. These finding of current study was agreement with a large body of evidences those reported many anticancer drugs induce Autophagy, like Bevacizumab

and everolimus, Bevacizumab is an ant angiogenesis treatment increases expression of Autophagy-related genes (Beclin1 and LC3) and increases formation of autophagosomal in hepatocarcinoma; Bevacizumab causes hypoxia and nutrient stress which induce Autophagy Guo, 2013. While everolimus induces Autophagy activity in mantle cell lymphoma which reported by Rosich and his coworkers who said that Autophagy processes are used by tumor cells to overcome stress conditions like antitumor activity and survival Rosich, 2012, also Autophagy processes induced in resistant H358 cell line (non-small cell lung cancer) by Li, 2013. The present study found ROS concentration is high due to low Autophagy activity with using of HCQ and consequently resulted low percentage of cell viability. While cell treated with only doxorubicin, revealed low ROS concentration due to high Autophagy activity that resulted in low inhibitory effect. These result indicated the role of Autophagy inhibitor HCQ in improving of anticancer drugs which proved previously with other type of cancer Barnard, 2014. Although ROS generation is a corner stone of antitumor activity of doxorubicin Wang, 2004, ROS induces Autophagy activity leading to attenuate antitumor activity and increase drug resistance Liu, 2015. The present study revealed inversing of pattern of concentration-dependent response of doxorubicin when combined with HCQ and showed the best effect at low concentration and this may be due to induction of Notch signaling pathway by doxorubicin in concentration-dependent manner and Notch pathway has role in cell proliferation as demonstrated by Mei et al, where Mei's paper revealed that doxorubicin increases Notch target gene expression in Osteosarcoma cell especially at high concentration Mei, 2015. On the other hand in 2018 Schott paper showed the ability of Autophagy inhibitor to improve cytotoxicity of doxorubicin against Osteosarcoma cell line was decreased with increase concentration of doxorubicin Schott, 2018.

## CONCLUSION

Doxorubicin monotherapy induced Autophagy activity of Osteosarcoma cell line (MG-63) with low ROS concentration. While the Autophagy inhibitor HCQ enhances sensitivity of Osteosarcoma cell line MG-63 to doxorubicin especially at low concentration of doxorubicin. Additionally HCQ reverses the pattern of concentration-dependent inhibitory response of doxorubicin-treated cell line (MG-63).

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