

RESEARCH ARTICLE

Oral and IV Dosages of Doxorubicin-Methotrexate loaded-Nanoparticles Inhibit Progression of Oral Cancer by Down-Regulation of Matrix Metalloproteinase 2 Expression *in Vivo*

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Abstract

Oral cancer is one of the most common and lethal cancers in the world. Combination chemotherapy coupled with nanoparticle drug delivery holds substantial promise in cancer therapy. This study aimed to evaluate the efficacy and safety of two dosages of our novel pH and temperature sensitive doxorubicin-methotrexate-loaded nanoparticles (DOX-MTX NPs) with attention to the MMP-2 mRNA profile in a 4-nitroquinoline-1-oxide induced oral squamous cell carcinoma (OSCC) model in the rat. Our results showed that both IV and oral dosages of DOX-MTX NP caused significant decrease in mRNA levels of MMP-2 compared to the untreated group ($p < 0.003$). Surprisingly, MMP-2 mRNA was not affected in DOX treated compared to cancer group ($p > 0.05$). Our results indicated that IV dosage of MTX-DOX is more effective than free DOX (12 fold) in inhibiting the activity of MMP-2 in OSCCs ($P < 0.001$). Furthermore, MMP-2 mRNA expression in the DOX-MTX treated group showed a significant relation with histopathological changes ($P = 0.011$). Compared to the untreated cancer group, we observed no pathological changes and neither a significant alteration in MMP-2 amount in either of healthy controls that were treated with oral and IV dosages of DOX-MTX NPs whilst cancer group showed a high level of MMP-2 expression compared to healthy controls ($p < 0.001$). Taking together our results indicate that DOX-MTX NPs is a safe chemotherapeutic nanodrug that its oral and IV forms possess potent anti-cancer properties on aggressive tumors like OSCC, possibly by affecting the expression of genes that drive tumor invasion and metastasis.

Keywords: MMP-2 - DOX-MTX-nanoparticles - oral squamous cell carcinoma - oral and IV dosage

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Introduction

Squamous cell carcinomas encompass approximately 95% of all oral malignancies. Oral cancer holds the sixth position in cancer incidence ranking worldwide (Albano et al., 2013; Baykara et al., 2013). Squamous cell carcinoma (SSC) of the tongue has historically been shown to be associated with a poor prognosis, much more an aggressive biological and clinical behavior and a high metastatic potential (Jones et al., 1992; Brandwein-Gensler et al., 2005; Kademani et al., 2005; Bell et al., 2007; Montoro et al., 2008). Patients with premalignant lesions and early stage cancers have a high rate of survival, but the vast majority of Stages III and IV cases are fatal (Zwetyenga et al., 2003; Massano et al., 2006; Montoro et al., 2008).

The prognosis of SCC depends on a series of factors such as proliferative activity of the tumor,

degree of differentiation, and invasion and metastatic potential (Nasiri et al., 2013; Mesgari Abbasi et al., 2014b). The last two processes involve multiple steps, such as degradation of the basement membrane and extracellular matrix (ECM), alterations in cell adhesiveness, tumor cell motility, and angiogenesis. Without doubt, ECM degradation plays a crucial role in tumor invasion and metastasis (Schliephake, 2003; Kademani et al., 2005; Pardis et al., 2012).

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases that are able to degrade all ECM proteins. There are multiple members of the family, however there are several studies that indicate to the metastatic potential of MMP2 and 9 in HNSCCs. (Thomas et al., 1999; Sharma et al., 2013). These enzymes are abundantly expressed in various malignant neoplasms and are implicated in all stages of tumor proliferation, progression, angiogenesis, and metastasis (Hong et al.,

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2000; Lippman and Hong, 2001; Zwetyenga et al., 2003). As disease outcome depends on the invasion potential of tumor, hence drugs that reduce MMPs activity may improve the clinical outcome in OSCC (Vilen et al., 2013).

Combination chemotherapy and nanoparticle drug delivery have shown great promise in cancer treatment. Cooperative medication of two or more drugs results in synergism or additive cytotoxic that can conquer drug resistance through distinct mechanisms of action (Valiyari S et al., 2013). On the other side, nanoparticle drug delivery enhances therapeutic effectiveness whilst reduces toxicity on normal cells and vital organs by improving their pharmacokinetics. (Kalaria et al., 2009; Rossi et al., 2010; Wang et al., 2010; Benival and PV, 2012; Duong and Yung, 2013; Tacar et al., 2013; Liboiron and Mayer 2014).

In its unaltered form, Doxorubicin still being regarded as a one of the most powerful anti-neoplastic agents, the only limitation is its unspecific effects on healthy cells. However, combined to nanodelivery systems, DOX-nanoparticles not only increase intracellular uptake of DOX, at the same time reduce its side effects significantly compared to conventional DOX formulations (Tacar et al., 2013).

In other side, Methotrexate (MTX) is another central chemotherapeutic drug that is widely used either in monotherapy or in combination with other biologic and synthetic disease modifying anti cancer drugs (Huang et al., 2011; Cipriani et al., 2014).

DOX- MTX NPs is new combination chemotherapy and nanoparticle drug delivery systems that have shown initial promising results in affecting the OSCC in rat model. However more studies require evaluating its efficacy, safety and also the mechanism of action.

In this respect, this study conducted to evaluate the efficacy of IV and oral administration of DOX-MTX-loaded nanoparticles in term of their ability in affecting the expression level of MMP-2 gene that promote invasion and metastasis of OSCC - as a new combination chemotherapy and nanoparticle drug delivery system for treatment of aggressive cancers, per se oral cancer .

Materials and Methods

Dual anticancer drug loaded nanoparticles

The synthesis procedure of nanoparticles was fully explained by Salehi et al. (2014). Briefly, appropriate amount of novel synthesized nanoparticles were ultrasonically dispersed in the MTX solution for 5 min. After stirring for 24h under dark conditions, DOX-HCl was added to MTX-loaded nanoparticles mixture and

dispersed with the aid of ultrasonication (Sonics Vibra cell, Model: VCX 130 PB, Newton, CT) for 3 min. The final carrier/drug ratio was 5 to 1 for both of drugs. The mixture was kept under magnetic stirring at room temperature for another 24h under dark conditions. Then, dispersion of MTX-DOX-loaded nanoparticles was left for 2h to allow the sedimentation of the fine precipitates. DOX-MTX-loaded nanocomposites were collected by centrifugation at 14000 rpm for 15 min and vacuum dried for 24h at room temperature and stored in a desiccators until used. The dual anticancer drug loaded nanoparticles were diluted with physiologic saline solution in appropriate concentration before administration to rats.

Animals

120 male Sprague-Dawley rats weighing 180±20 grams were randomly divided into 8 groups. The animals were housed in the polycarbonate standard cages in a temperature-controlled animal room (22±2°C) with a 12/12 hours light/dark cycle during the experiments. The animals were provided by a standard rat pellet diet ad libitum. Drinking water containing 4-NQO was prepared three times a week by dissolving the carcinogen in distilled water and was given in light-opaque bottles.

Establishment of Oral Squamous Cell Carcinoma (OSCC) model in rat

OSCC carcinogenesis usually develops through a multistep process that begins from hyperplasia and passes to mild, moderate and severe dysplasia before OSCC. 4-NQO induced OSCC have been used to study the various stages of oral carcinogenesis, because of its capability of inducing sequentially the phases of carcinogenesis (hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ and OSSC). We have previously confirmed that 4-NQO successfully induces different stages of tongue carcinogenesis process in all cancer groups. High mortality rate, low weight gain, and frequency of OSCC and high proliferation severity of cancer control group compared to other groups demonstrate the efficacy of 4-NQO induced OSCC model in our study (Mehdipour et al., 2013).

Experimental design

120 rats were divided into 8 groups, with 15 animals included in each one as following (Table1 is provided for a quick review of our experimental design): *i*) Group I served as a carcinoma control and received 4-NQO (Sigma) at the concentration of 30ppm in their drinking water for 14 weeks without any treatment; *ii*) Group II - III served as the treatment groups and received 4-NQO at

Table 1. Characteristic of Studied Animals in Each Group

Route of drug administration	Groups classification	Groups names	Treatment	Number of cases(N) (Beginning of the study)	Number of cases (N) (End of study)
Oral (5mg/kg body weight)	I	Cancerous control	-	15	11
	II-III	Cancerous groups	DOX	15	13
			DOX-MTX NPs	15	14
	IV (1.5mg/kg body weight)	IV	Healthy control	DOX-MTX NPs	15
V-VI		Cancerous groups	DOX	15	12
			DOX-MTX NPs	15	14
			DOX-MTX NPs	15	11
-	VIII	Healthy control	-	15	15

the concentration of 30ppm in their drinking water for 14 weeks and oral doses (Gavage) of Doxorubicin and the DOX-MTX-loaded nanoparticles respectively at the dose 5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study; *iii*) Group IV - V served as the treatment groups and received 4-NQO at the concentration of 30ppm in their drinking water for 14 weeks and intravascular (IV) dosages of doxorubicin and the DOX-MTX-loaded nanoparticles at the dose 1.5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study. *iv*) Group VI and VII served as the treated control group that received oral and IV the dose DOX-MTX-NPs (5mg/kg and 1.5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study, respectively). *v*) Group VIII served as normal control group and the rats of this group didn't get any carcinogen or treatment material. *vi*) Death rate of the animals was also recorded during the study.

Ethics

All the ethical and the humanity considerations were performed according to the Helsinki humanity research declaration during the experiments and the euthanasia of the animals. All the animals' experiments were approved by the Ethics Committee of the Tabriz University of Medical Sciences.

Histological evaluations

At the end of the interventional period, the animals were euthanized under anesthetic condition (Pentobarbital, 150mg/kg IP). The tongue tissue samples were taken from each animal and were immediately fixed in 10% phosphate-buffered formalin. The 5µm thick microscopic sections were prepared after embedding of tissue samples in paraffin. Afterward, the sections were stained by hematoxylin-eosin staining method and histological evaluations were performed with light microscopy (Mehdipour et al., 2013).

Quantification of MMP2 mRNA expression by real time PCR

Briefly, total RNA (2µg) extracted from homogenized fine powder of removed tongue tissues as described in detail by jahanban et al. (2011a; 2011b; 2012). RNA was reverse transcribed to cDNA using Revert Aid first strand cDNA synthesis kit (fermentase). The resulting cDNA was diluted 1:30 fold and the PCR reaction was performed with 2µl cDNA, 10pM each forward and reverse primers, 12.5µl SYBR Green PCR Master Mix (Fermentase) in a final volume of 25µl. The thermal profile for the real-time Q-PCR was 95°C for 10 min and followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 min. The gene expression was expressed as fold change from the GAPDH level which is calculated as $2^{-\Delta\Delta Ct}$. In addition, melting curve analysis was performed to assure the specificity of PCR product in this experiment. The following rat primers were used: MMP-2 (NM_031054.2): TCTGGTGTTCACCACGTACAAC-3' (forward), 5'-ACCCATGGTAAACAAGGCTTCG -3' (reverse); GAPDH (AF 106860): 5'-ATGACTCTACCCACG GCAAG-3' (forward), 5'-CTGGAAGATGGTGTATGGT TT-3' (reverse).

Data analysis

The data were analyzed by SPSS 13. One-Way Analysis Of Variance (ANOVA) was used to compare fold change differences of MMP-2 in studied groups followed by the multiple comparisons with the Tukey post-hoc test. A p value <0.05 was considered significant. For analyzing the possible relation between mRNA expression of MMP-2 gene with pathological changes in tissue samples, a Fischer's exact test were used and a p value <0.05 was considered significant.

Results

Effect of DOX-MTX NP on mRNA expression of MMP-2 in tongue tissues of OSCC rats models

Compared to (untreated) healthy group, MMP-2 mRNA approximately 25.28 fold over-expressed in cancerous group (p=0.02) (Figure 1). In groups that received oral doses of DOX and DOX-MTX NP (5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study), results showed that compared to untreated cancerous group, mRNA expression of MMP-2 decreased 1.4 and 2.5 folds in DOX (p=0.30) and DOX-MTX treated cancerous group (p=0.026) (Figure 2) (Fold changes represented as mean ±SE).

After IV treatment with DOX and DOX-MTX NP

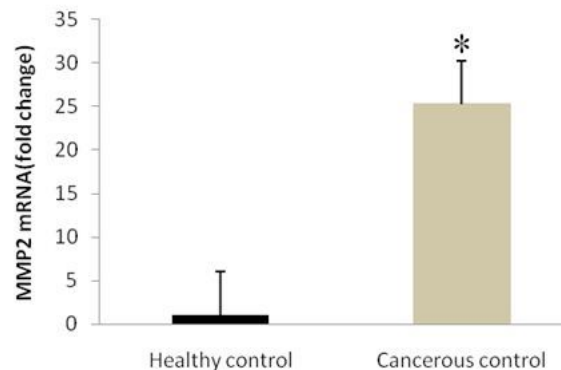


Figure 1. MMP-2 mRNA Expression in OSCC Cancerous Group and Healthy Control. Compared to healthy group, MMP-2 over-expressed ~ 25.5 fold in untreated cancerous group. *Indicates to a significant P value (p<0.001)

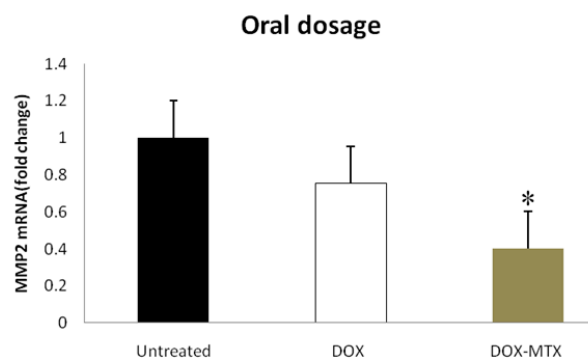


Figure 2. The Effect Of Oral Dosage of DOX and DOX-MTX NP on mRNA level of MMP-2 in OSCC cancer Model in Rat. MMP-2 mRNA level decreased significantly in DOX-MTX NPs compared to untreated cancerous group whilst oral dosages of DOX could not alter amount of MMP-2 in treated group. *Indicates to a significant P value (p<0.05)

(1.5mg/kg of body weight once a day on the days of 2, 5 and 8 of the study), mRNA expression of MMP-2 decreased 10 folds compared to untreated cancerous that was statistically significant ($p=0.003$), in contrast in DOX treated group, we observed no significant decrease in MMP-2 mRNA level ($p=0.36$) (Figure 3).

Moreover, there was no significant difference between IV and oral administration of DOX-MTX NP ($p=0.223$) and neither DOX ($p=0.070$) (Figure 4). All three healthy controls showed significant difference in MMP-2 mRNA expression compared to untreated cancerous group

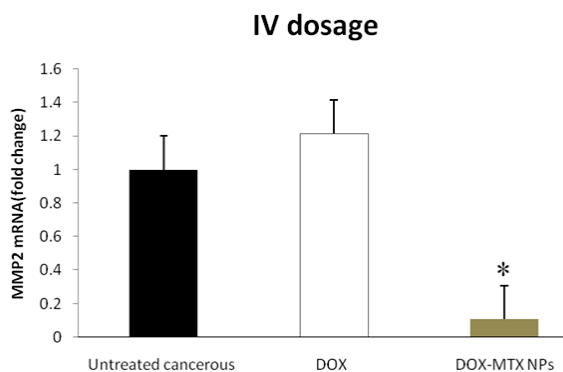


Figure 3. The Effect of IV Dosage of DOX and DOX-MTX NP on mRNA level of MMP-2 in OSCC Cancer Model In Rat. MMP-2 mRNA level decreased significantly in DOX-MTX NPs compared to untreated cancerous group whilst IV dosages of DOX could not alter amount of MMP-2 in treated group. *Indicate to a significant P value($p<0.05$)

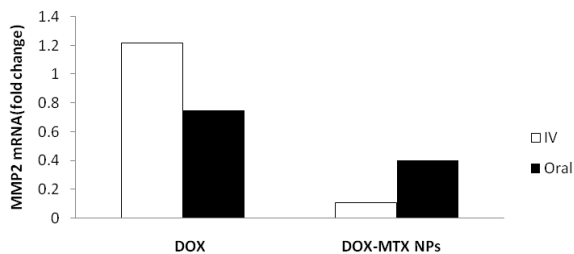


Figure 4. Comparison between the Efficacy of IV and oral Administration of DOX-MTX NP in Affecting the MMP-2 mRNA Expression in OSCC Model in Rat. In both DOX and DOX-MTX NPs treated groups, there was no difference between IV and oral route in decreasing the amount of MMP-2 mRNA level in OSCC model in rat. *Indicates to a significant P value ($p<0.05$)

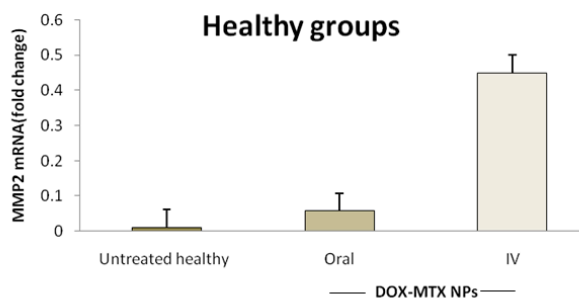


Figure 5. The Effect of oral and IV Dosage of DOX-MTX NP on mRNA Level of MMP-2 in Healthy Rats. There was no significant difference in MMP-2 amount of treated healthy in both modalities of nanodrug with that of untreated healthy control. *Indicates to a significant P value ($p<0.05$)

($p<0.01$). In case of evaluation of the safety of our nanodrug, both healthy group that were treated with oral and IV doses of DOX-MTX NP showed no significant difference in mRNA level of MMP-2 compared to untreated healthy group ($p>0.05$), although in oral administration, normal cells 8 folds were less affected than the systemic administration of nanodrug (IV), however this difference was not statistically significant ($p=0.087$) (Figure 5). In addition, compared to DOX, our result indicate that IV administration of MTX-DOX is more effective (12 folds) in inhibiting the activity of MMP-2 in OSCC ($P=0.001$) (Figure 6). However, there was no difference between the efficacy of oral administration of two forms of drugs in affecting the MMP-2 mRNA level ($p=0.151$).

Histopathological changes in DOX and DOX-MTX NPs groups

AS IV mode of nanodrug showed superior performance over oral form, hence this group was subjected for evaluation of histopathological changes.

Our data showed that in DOX treated group 6/13 of lesion showed a low stage (No/Mild/moderate dysplasia) while 7/13 were advanced (Severe dysplasia, Carcinoma in situ and OSCC). At the other hand, we observed markedly increase in the frequency of low staged tumor (12/14 vs. 2/14) in group treated with IV doses of nonodrug (Figure 7). Pathological changes significantly were different between the groups ($p<0.05$). Furthermore, no pathological changes detected in either of healthy controls, whilst all rats of cancerous group developed aggressive lesions.

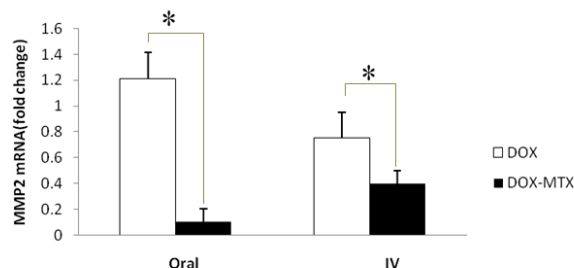


Figure 6. Comparison between the efficacy DOX and DOX-MTX NPs in affecting the MMP-2 mRNA expression. In both forms of oral and IV administration, nanodrug group showed superior activity over free DOX in down-regulation the MMP-2 mRNA level. *Indicate to a significant P value ($p<0.05$)

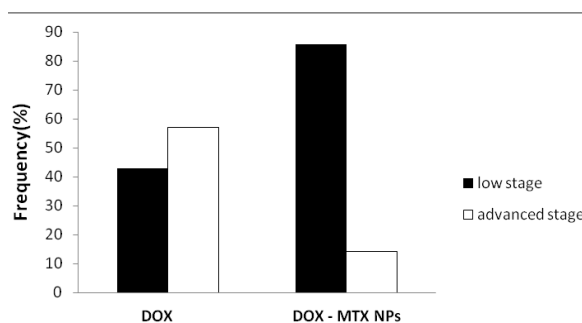


Figure 7. Frequency of Observed Histopatological Changes in Groups of Rats that were Treated with IV Dosages of DOX and DOX-MTX NP

Table 2. Relation between MMP-2 mRNA Expression and Tumor Stage in Cancerous Group that Treated with IV doses of DOX-MTX NP

DOX-MTX NP	MMP-2 mRNA			P-value
	Low	Moderate/High	Total	
Tumor stage				
Low stage	12(85.7%)	0 (0%)	12(85.7%)	p=0.011
High stage	0 (0%)	2(14.3%)	2(14.3%)	
Total	12(85.7%)	2(14.3%)	14(100%)	

Relation between MMP-2 mRNA with tumor stage in OSCC samples

As we observed a significant reduction in the number of pathological changes in group treated with nanodrug, we thought that this could be related to the MMP-2 content. To examine this hypothesis, in cancerous group that was treated with IV doses of DOX-MTX NP and showed better response to treatment, lesions according to their pathologic profile categorized in two main groups: group 1 (Low stage) that consist of those that represent No, Mild or Moderate dysplasia and group 2 (High stage) that included the severe dysplasia, Carcinoma insitu and OSCC. Following, Fisher exact test showed that as we predicted high stages of tongue cancer related with high expression of MMP-2 while low level of MMP-2 in samples exhibit better prognosis and less aggressive lesions (p=0.011) (Table 2).

Discussion

Squamous cell carcinoma (SCC) is the most common head and neck cancer with poor clinical outcome. One of the factors governing the poor prognosis of oral SCC is frequent metastasis of cancer cells to regional lymph nodes or distant organs (Massano et al., 2006; Rusthoven et al., 2010). Combinatorial chemotherapy coupled with nanomedicine have opened appealing window to the current therapeutic approaches that always failed due to tumor cell resistance and unwanted side effects of drug on normal cells, However aggressive advances made when nano-based drug delivery systems paired with combination chemotherapeutic agents (Rossi et al., 2010; Liboiron and Mayer 2014).

Invasion and metastasis potential of tumor is a critical factor in predicting the survival in OSCC. Among the ECM-degrading enzymes recruited by cancer cells, MMPs have been reported to play an important role in determining the invasiveness of neoplasm. Both MMP-2 and -9 proteolysis type IV collagen and destroy the basement membrane as a prelude to invasion. The action of MMP-2 and -9 has been shown to correlate well, in particular, with the metastatic potential of tumor cells in particular the SCC of tongue cancer. In this view, the evaluation of MMP-2 and -9 expression in oral SCCs could be a valuable tool for predicting their prognosis and designing drugs with potential to inhibit their activity (Hong et al., 2000; Montoro et al., 2008; Lin et al., 2013; Vilen et al., 2013).

Doxorubicin is a potent anti-cancer drug, however because of its high cardiotoxicity, new formulation of doxorubicin based on nanodelivery systems developed

(Mesgari Abbasi et al., 2014c). In this view, doxorubicin encapsulated or conjugated with divergent nanocarriers, subsequently in order to enhance its specificity on targeting the cancerous cells DOX-nanocarrier complex attached to specific antibodies, i.e. folat receptors or EGFR which is abundantly express on surface of cancerous cells. Both oral and IV formulation of Doxorubicin is available, however oral route is better accepted by patients, although it may be sometimes less effective and also better tolerable than the systemic doses. For instance, targeted delivery of DOX to solid tumors with reduced side effects of drug could be achieved by nanoparticulated doxorubicin which is chemically conjugated with dextran and encapsulated in chitosan nanoparticles. Regulated particle size and enhanced circulation time in blood stream allow these hydrogel nanoparticles to exit where the blood vessel are leaking at the site of cancer tumor and let them to concentrate specifically within tumor cells. In this way, the cardiotoxicity of DOX can be reduced by coupling the drug with dextran and encapsulating it in chitosan nanoparticles (Yoo and Park, 2004; Kalaria et al., 2009; Bae, 2010; Guhagarkar et al., 2010; Chen et al., 2011; Benival and PV, 2012; Jain et al., 2012; Deng and Zhang, 2013; Duong and Yung, 2013; Liboiron and Mayer 2014). In unaltered form, divergent nanodelivery systems exploit to enhance efficacy of DOX based therapeutic.

For example, Guhagarkar, S.A et al used polyethylene sebacate (PES)-doxorubicin (DOX) nanoparticles (PES-DOX NP) using pullulan as asialoglycoprotein receptor (ASGPR) ligand for hepatic targeting (Guhagarkar et al., 2010).

In another study, in order to reduce the toxic effects of free DOX and increase the bioavailability of oral delivery, Benival D.M, et al designed doxorubicin hydrochloride (DOX) loaded lipid based nanocarrier (LIPOMER) for oral delivery. A 384 % enhancement in oral bioavailability compared to Dox solution was observed following Dox-Lipomer administration at 10mg/kg body weight in rats (Benival and PV, 2012).

Kalaria et al. (2009) designed a biodegradable nanoparticles for oral delivery of DOX. In this way, their DOX loaded PLGA demonstrated superior performance in vivo as evident by enhanced bioavailability and lower toxicity.

Duong and Yung (2013) used the synergistic co-delivery of doxorubicin and paclitaxel using multi-functional micelles for cancer treatment.

In this view, our novel stimuli-responsive cationic mesoporous silica nanoparticles (MSNs) loaded with dual anticancers DOX- MTX designed with the aim to enhance their cytotoxic effects on cancerous cells. We have tested the efficacy of both oral and systemic delivery of our dual action nanoparticle and compared the results with free doxorubicin.

The main mechanism of action described for DOX and MTX is induction of apoptosis via activation / increase in p53 level (Huang et al., 2011; Tacar et al., 2013). In our earlier study (data not published), we observed remarkable increase in p53 mRNA level after treatment of cancerous group with oral and IV forms of DOX and DOX-MTX NPs, However both oral and IV forms of

DOX-MTX NP showed superior performance over DOX in increasing p53 amount (Mesgari Abbasi et al., 2014a). To our knowledge, this is the first record that a new formulation of Doxorubicin possesses anti-MMP activity besides its enhanced apoptotic potential in the new format, whilst such a potential has never described for free DOX. However, it worth to note that Matrix metalloproteinase (MMP)-activated prodrugs could be generated by coupling MMP-cleavable peptides to doxorubicin in order to enhance the specific accumulation of DOX within tumor and also to reduce its toxic effects on healthy cells. MMPs are attractive enzymes that tend to activate prodrugs in the tumor environment because MMPs are intimately connected with tumorigenesis (Albright et al., 2005; Hu et al., 2010).

Our result shows that both oral and IV forms of DOX-MTX NP are more effective than the free DOX against aggressive OSCC with no debilitating side effects on healthy cells. However, we find IV administration of DOX-MTX NP more effective than oral route in affecting the mRNA level of MMP-2. We have demonstrated that decrease in MMP-2 level was correlated with less aggressive tumor stage. In case of the safety of dual action nanodrug, neither of treated healthy controls showed any pathological lesion and nor alteration in MMP-2 mRNA level compared to untreated healthy, this implies that DOX-MTX NPs exert preferential and tumor specific effects without any harmful effects on the normal cells. As MMP-2 is one of the key factors that govern the invasion and metastasis of cancer cells, dual action

In conclusion, MMPs are the main prognosticators for metastasis and invasion potential of aggressive malignancies including OSCC. Down-regulation of MMP-2 is a new feature acquired in group treated with dual action DOX-MTX-NPs whilst such a potential never reported for free DOX. By affecting the MMP-2 expression in OSCC, DOX-MTX-NP inhibit effectively and specifically progression and invasion of tumoral cells without affecting the normal ones. However, further investigations acquire to clarify the underlying mechanism of action and its further therapeutic potentials in different types of cancer.

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