RESEARCH ARTICLE

DOX-MTX-NPs Augment p53 mRNA Expression in OSCC Model in Rat: Effects of IV and Oral Routes

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy worldwide. Cancer development and progression require inactivation of tumor suppressor genes and activation of proto-oncogenes. The well recognized mechanism of action demonstrated for chemotherapeutic agents is induction of apoptosis via reactivation of p53. In this context, we evaluate the efficacy of IV and oral routes of our novel PH and temperature sensitive doxorubicin-methotrexate-loaded nanoparticles (DOX-MTX NP) in affecting p53 profile in an OSCC rat model. Methods: In this study, 120 male rats were divided into 8 groups of 15 animals each. The new formulated DOX-MTX NP and free doxorubicin were IV and orally given to rats with 4-nitroquinoline-1-oxide induced OSCC. Results: Results showed that both DOX and DOX-MTX-NP caused significant increase in mRNA levels of P53 compared to the untreated group ($p<0.000$). With both DOX and DOX-MTX NP, the IV mode was more effective than the oral (gavage) route ($p<0.000$). Surprisingly, in oral mode, p53 mRNA was not affected in DOX treated groups ($p>0.05$), Nonetheless, both IV and oral administration of MTX-DOX NP showed superior activity (~3 fold) over free DOX in reactivation of p53 in OSCC ($p<0.000$). The effectiveness of oral route in group treated with nanodrug accounts for the enhanced bioavailability of nanoparticulated DOX-MTX compared to free DOX. Moreover, in treated groups, tumor stage was markedly related to the amount of p53 mRNA ($p<0.05$). Conclusion: Both oral and IV application of our novel nanodrug possesses superior activity over free DOX-in up-regulation of p53 in a OSCC model and this increase in p53 level associated with less aggressive tumors in our study. Although, impressive results obtained with IV form of nanodrug (-21 fold increase in p53 mRNA level) but both forms of nanodrug are effective in OSCC, with less toxicity normal cells.

Keywords: p53 - DOX-MTX-NPs - oral squamous cell carcinoma - oral and IV route - rat model

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Introduction

Oral squamous cell carcinoma (OSCC) ranks the sixth most common cancer worldwide as it accounts near to 95% of all oral neaoplasms and 38% of all head and neck cancers in particular tongue and lip. In contrast to lip, tongue cancers tend to show aggressive biological and clinical behavior (Jones et al., 1992). Unfortunately, the increase in incidence has not been paralleled by the development of new therapeutic agents(Lasrado et al., 2014). In contrast to the measurable progress made in surgery, chemotherapy, and radiotherapy the survival rate has only elevated slightly, with the 5-year survival rate remaining at 50% over the past 30 years (Kademani et al., 2005). Patients with premalignant lesions and early tage cancers, if they get a chance to be diagnosed earlier, have a high rate of survival, however unfortunately oral cancer most often show off in advanced forms and a fatal fate predicted for the vast majority of Stages III and IV cases (Kim et al., 2001).

The p53 tumor suppressor gene is one of the frequently studied biomarkers in OSCC. Functional inactivation of p53 causes defects in DNA repair and apoptosis, with a subsequent increase in genetic instability that can lead to the accumulation of mutations (Abusail et al., 2013; Liu L et al., 2014). The high expression of p53 due to high rate of mutations has been linked with a unfavorable prognosis in OSCC patients (Lippman and Hong, 2001; Schliephake, 2003; Massano et al., 2006; Montoro et al., 2008).

Combination chemotherapy and nanoparticle drug delivery have shown substantial promise in cancer treatment (Baykara et al., 2013). Cooperative medication of two or more drugs results in synergism among the different drugs against cancer cells and can conquer

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drug resistance through distinct mechanisms of action (Nasiri et al., 2013; 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 ameliorating their pharmacokinetics and bioavalibilities. Current aggressive multidisciplinary advances in improving the efficacy of cancer therapeutics are due to combination of these two dynamic research fields (Mollazade et al., 2013). There are also some challenges and design specifications that need to be addressed in optimizing nanoparticle-based combination chemotherapy (Kalaria et al., 2009; Guhagarkar et al., 2010; Hu et al., 2010; Benival and PV, 2012; Jain et al., 2012; Deng and Zhang, 2013; Duong and Yung, 2013; Liboiron and Mayer 2014; Salehi et al., 2014).

In its unchanged form, doxorubicin has shown measurable treatment potential, being regarded as one of the most potent of the FDA approved chemotherapeutic Drug. The ability to target rapidly dividing cells and slow disease progression has been widely appreciated for several decades, limited only by its bodily toxicity (Tacar et al., 2013). However, combined to nanodelivery systems, DOX-nanoparticles not only increase intracellular uptake of DOX, at the same time reduce its side effects significantly compared with conventional DOX formulations (Wang et al., 2010; Chen et al., 2011). Methotrexate (MTX) is another central chemotherapeutic drug that is widely used either in monotherapy or in combination with other anti cancer drugs (Rossi et al., 2010; Cipriani et al., 2014).

DOX-MTX NP is a new combination chemotherapy and nanoparticle drug delivery system that showed initial promising results in vitro. However more studies require evaluating its efficacy, safety and also the mechanism of action in animals.

In this respect, this study conducted to evaluate the efficacy of IV and oral administration of DOX-MTX-loaded nanoparticles in term of their therapeutic potential to affect the expression level of p53 compared to free DOX, as a new combination chemotherapy and nanoparticle drug delivery system for treatment of aggressive tumors like 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 minutes. After stirring for 24 hours 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 minutes. 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 24 hours under dark conditions. Then MTX-DOX-loaded nanoparticles dispersion was left for 2 hours to allow the sedimentation of the fine precipitates. DOX-MTX-loaded nanocomposites were collected by centrifugation at 14000 rpm for 15 minutes, and vacuum dried for 24 hours 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 of 15 animals each. 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 fold a week by dissolving the carcinogen in distilled water and was given in light-opaque bottles.

Experimental design

120 animals were divided into 15 groups (see Table 1):

Group I served as a carcinoma control and received 4-NQO (Sigma) at the concentration of 30 ppm in their drinking water for 14 weeks without any treatment.

Groups II-III served as the treatment groups and received 4-NQO at the concentration of 30 ppm in their drinking water for 14 weeks and oral doses (Gavage) of Doxorubicin and the DOX-MTX-loaded nanoparticles respectively at the dose 5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study.

Groups V-VI served as the treatment groups and received 4-NQO at the concentration of 30 ppm in their drinking water for 14 weeks and intravascular (IV) dosages of doxorubicin and the DOX-MTX-loaded nanoparticles at the dose 1.5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study.

Group IV and VII served as the treated control group that received oral and IV dose DOX-MTX-NPs (5 mg\kg and 1.5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study, respectively).

Table 1. Characteristic of Studied Animals in Each Group

Route of drug administration	Groups classification	Groups names	Treatment	No. of cases (Beginning of the study)	No. of cases (End of study)
-	I	Cancer control	-	15	11
Oral	II-III	Cancer groups	DOX	15	12
(5 mg/kg body weight)			DOX-MTX NPs	15	14
	IV	Healthy control	DOX-MTX NPs	15	15
IV	V-VI	Cancer groups	DOX	15	13
(1.5 mg/kg body weight)			DOX-MTX NPs	15	14
	VII	Healthy control	DOX-MTX NPs	15	15
-	VIII	Healthy control	-	15	15

Group VIII served as normal control group and the rats of this group didn't get any carcinogen or treatment material.

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 5 µm 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. Histopathological changes in tumors evaluated blindly by two pathologists.

Detection of p53 mRNA expression by quantitative real time PCR

Briefly, total RNA (2 μg) extracted from homogenized fine powder of removed tongue tissues as described in detail elsewhere (Jahanban Esfahlan et al., 2011a). RNA were 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 2.5 μl cDNA, 10 pM each forward and reverse primers, 12.5 μl SYBR Green PCR Master Mix (Fermentase) in a final volume of 25 μl. The thermal profile for the real-time Q-PCR was 95°C for 10 minutes and followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. 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: p53 (NM_030989.3): 5'- TCGAGATGTTCCGAGAGCTGAATG-3' (forward), 5'-CTTCTTGGTCTTCGGGTAGCTG -3' (reverse). GAPDH (AF 106860): 5'-ATGACTCTA CCCACGGCAAG-3' (forward), 5'-CTGGAGATGGTGATGGGTT-3' (reverse).

Data analyses

The data were analyzed by SPSS 13. One-Way Analysis Of Variance (ANOVA) was used to compare fold change differences of p-53 between and within studied groups followed by the multiple comparisons with the Tukey post-hoc test. Fischer's exact test used for analyzing pathological changes in groups. Chi square test used to verify the possible relation between expression of p53 gene and pathological changes in tissue samples. A p value <0.05 was considered significant.

Results

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 OSSC. 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 verified 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 (Mehdipour et al., 2013).

In this study, 120 Sprague Dawley rats divided into 8 equal groups as following: healthy control group (n=15), healthy control group that received IV doses of DOX-MTX (n=15), healthy control group that received oral doses of DOX-MTX (n=15), cancerous group that received IV doses of DOX (n=13), cancerous group that received oral doses of DOX (n=12), Cancerous group that received IV doses DOX-MTX NP (n=14) and Cancerous group that received oral doses DOX-MTX NP (n=14) ,untreated cancerous group (n=11). During experiment, 2/15 rat from DOX (IV) group, 1/15 in DOX-MTX (IV), 3/15 rat from DOX (oral) group, 1/15 in DOX-MTX (oral) and 4/15 of untreated cancerous group and 0\15 in healthy group died.

Effect of IV administration of DOX-MTX NP and free DOX on mRNA expression of P53 in tongue tissues of OSCC rat model

Our results indicated that after IV treatment with DOX and DOX-MTX NP (1.5 mg/kg of body weight once a day on the days of 2, 5 and 8 of the study), mRNA expression of p53 increased 12.3 fold and 20.54 fold respectively compared to untreated cancerous that was statistically significant (p<0.000) (Figure 1) (fold changes represented as mean ±SE).

At the other hand, DOX-MTX NP treated healthy control showed significant difference in p53 mRNA expression compared to untreated cancerous group (p<0.05). DOX-MTX NP had no effect on p53 content

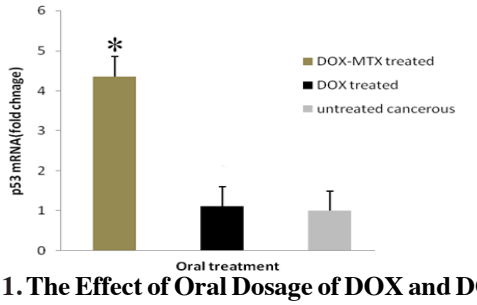

Figure 1. The Effect of Oral Dosage of DOX and DOX-MTX NP on mRNA Level of p53 in OSCC Cancer Model in Rat. *Indicate to a significant p value (p<0.05) when compared to cancerous group

of treated healthy group compared to untreated healthy control (p>0.05) (Figure 2).

Effect of oral administration of DOX-MTX NP and free DOX on mRNA level of P53 in tongue tissues of OSCC rat model

In groups that were treated with oral doses of DOX and DOX-MTX NP (5 mg/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, mRNA expression of p53 increased 1.6 and 5 fold in DOX (p=0.776) and DOX-MTX treated groups, respectively (p=0.026) (Figure 3). Oral form of free DOX was not effective in up-regulating of p53 compared to DOX-MTX-NP, (p>0.05). Oral treatment of healthy control with the same dose of DOX-MTX NP had no significant effect on p53 mRNA level compared to untreated healthy control (p>0.05) (Figure 2).

Comparison between the efficacies of DOX-MTX NP and free DOX in affecting mRNA level of P53 in tongue tissues of OSCC rat model

Overall, our result indicate that both IV and oral modalities of DOX-MTX-NPs possessed superior activity (~3 fold) over free DOX in reactivation of p53 in OSCC model *in vivo* (p<0.000) (Figure 4).

Comparison between efficacy of oral and IV treatment of rats with DOX-MTX NP and free DOX in affecting mRNA level of P53 in tongue tissues of OSCC rat model

Our result indicated that IV administration was 12 fold and 5 fold effective than oral route in up-regulating\ reactivation of p53 expression in both DOX and DOX-MTX treated group, respectively (Figure 5). In both groups that treated with free DOX and new formulated version of it (DOX-MTX NP), results showed that IV mode was effective than oral route and this difference was statistically significant (p<0.000).

Histopatological changes in DOX and DOX-MTX NP treated groups

AS IV mode of nanodrug showed significant results compared to oral form, hence these group subjected for evaluation of histopathological changes as following: cancerous groups treated with DOX (n=13) and DOX-MTX NP (n=14), untreated healthy controls (n=15), DOX treated healthy control (15), DOX-MTX NP treated healthy (15), untreated cancerous group (n=11).

Our results 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) (Figure 6). At the other hand, we observed markedly increase in frequency of low stage tumor (12/14 vs 2/14) in group treated with IV doses of nonodrug. 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.

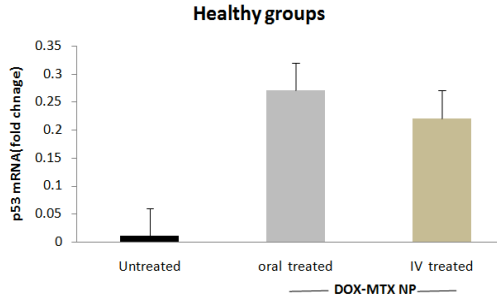

Figure 2. The Effect of Oral and IV Dosage of DOX-MTX NP on mRNA Level of p53 in Healthy Rats. *Indicate to a significant p value (p<0.05) compared to untreated healthy group

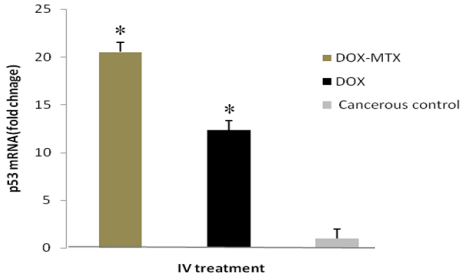

Figure 3. The Effect of IV Dosage of DOX and DOX-MTX NP on mRNA Level of p53 in OSCC Cancer Model in Rat. *Indicate to a significant P value (P<0.05) when compared to cancerous group

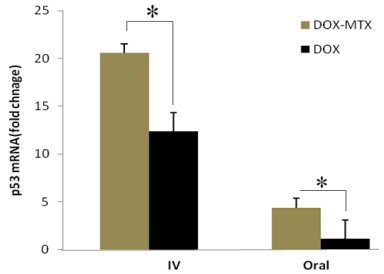

Figure 4. Comparison between The Efficacy of Oral and IV Dosage of DOX and DOX-MTX NP in Affecting p53 mRNA Expression in OSCC Model in Rat. *Indicate to a significant p value (p<0.05)

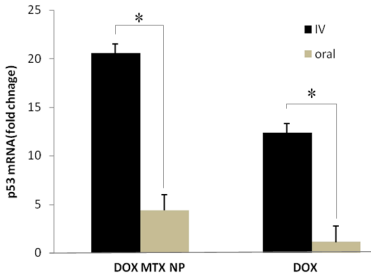

Figure 5. Comparison between The Efficacy of IV and Oral Administration of DOX-MTX NP in Affecting p53 mRNA Expression in OSCC Model in Rat. *Indicate to a significant p value (p<0.05)

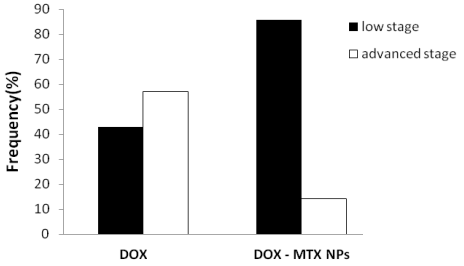

Figure 6. Frequency of Observed Histopatological Changes in DOX and DOX-MTX NP Treated Groups (IV)

Table 2. Relation between p53 mRNA Level and Tumor Stage in DOX Treated Group (IV)

DOX	p53 mRNA			p value
	Low/moderate	High	Total	
Pathologic changes				p=0.004
Low stage	0 (0%)	6 (100%)	6 (100%)	
High stage	6 (85.7%)	1 (14.3%)	7 (100%)	
Total	6 (46.2.3%)	7 (53.8.7%)	13 (100%)	

Table 3. Relation between p53 mRNA Level and Tumor Stage in DOX-MTX NPs Treated Group(IV)

DOX-MTX NPs	p53 mRNA			p value
	Low/moderate	High	Total	
Pathologic changes				p=0.033
Low stage	1 (8.3%)	11 (91.7%)	12 (100%)	
High stage	2 (100%)	0 (0%)	2 (100%)	
Total	3 (21.4%)	11 (78.6%)	14 (100%)	

Relation between p53 mRNA level and tumor progression in OSCC samples

Subsequently, we tested the relation between p53 mRNA profile and the tumor stage in DOX and DOX-MTX NP treated group. In this respect according to the observed mRNA fold changes, samples categorized in two main groups: group with high mRNA and group with low/moderate mRNA level. p53 mRNA was detectable in all studied samples.

As shown in Table 2 and Table 3, our results indicated that in DOX treated group, all 6/6 low staged lesions expressed high mRNA level of p53 (100%) whilst in high staged lesions, 1/7 showed a high level of p53 level (14.3%) while 6/7 of them represent low amount of p53 mRNA (62.5%) (p=0.004). In group treated with DOX-MTX NP, results were as following: 11/12 low staged tumors showed high level of p53 (91.7%) and 1/12 showed low level of p53 (8.3%). All advanced lesions (2/2) displayed low amount of p53 (100%) (p=0.033).

Discussion

It takes two to tango and it takes two or even more than single agent therapy in order to conquer the limited success in cancer therapy due to the toxicity at high drug dosage, the heterogenic tumors and more importantly the acquired drug resistance (Jahanban Esfahlan et al., 2011; Jahanban Esfahlan et al., 2012). Hence, combination therapy exploit combined regimes that multiply the synergistic effects to the targeted cancer cells. In the second step, when synergistic combination with higher therapeutic effects get combined with an appropriate nanodelivery system such as the nanoparticles , a much powerful weapon create that could efficiently combat tumor cells with less toxic effects on normal cells (Bae, 2010; Rossi et al., 2010; Wang et al., 2010; Chen et al., 2011; Deng and Zhang, 2013; Duong and Yung, 2013; Tacar et al., 2013; Liboiron and Mayer 2014; Salehi et al., 2014).

With distinct activities to cancer cells, the pairing of chemotherapeutic agents doxorubicin (DOX) and Methotroxate (MTX) for combination treatment may have higher and synergistic therapeutic effect. DOX intercalate to DNA that suppress nucleic acid synthesis while MTX is an antimetabolite, with different activities, both induce apoptosis via reactivation of p53 (Duong and Yung, 2013).

According to the study by Huang WY et al, MTX promote p53 phosphorylation at Ser15 and acetylaion at Lys373/382, which increase its stability and expression. In their study, apoptosis and inhibition of cell viability induced by MTX were dependent on p53 and partially, on p21 (Huang et al., 2011).

At the other side, doxorubicin binds to DNA, causes the activation of various molecular signals from AMPK (AMP-activated protein kinase inducing apoptosis) to influence the Bcl-2/Bax apoptosis pathway. By altering the Bcl-2/Bax ratio, downstream activation of different caspases can occur resulting in apoptosis. When a chemotherapeutic drug such as doxorubicin is administered, p53 levels are often increased, therefore activating the p53 pathway. This suggests that the doxorubicin effect on Bcl-2 expression is mediated by p53 pathways (Tacar et al., 2013).

DOX and MTX ,two potent chemotherapeutic agents with distinct mechanism of action but one single aim: "induction of apoptosis by affecting the p53 gene expression within tumoral cells", although this is not the whole of story, they also influence other genes and pathways to combat cancerous cells (Mesgari Abbasi et al., 2014).

Modified mesoporous silica nanoparticles (MSNs) provide specific release of entrapped drugs at tumor tissue environment (lower pH and higher temperature than physiological condition). An efficient anticancer performance of Multi anticancer drug-loaded MSNs previously verified by DAPI staining and MTT assay tests. This formulation provides cooperative thermo and pH-responsive targeted delivery of DOX and MTX to the cancerous tissues (Salehi et al., 2014).

In this study we evaluate the efficiency and safety potential of oral and systemic dosages of novel stimuli-responsive cationic MSNs loaded with DOX-MTX in improving OSCC clinical outcome possibly by increasing/reactivating the p53 mRNA level .Our result indicated that both oral and IV forms of DOX-MTX NP showed superior performance in increasing p53 mRNA level compared to free DOX and interestingly the increase in p53 accompanied by substantial clinical improvement. However although p53 is one the most frequent affected genes in DOX and MTX induced apoptosis, in our study the observed clinical improvement achieved by DOX-MTX NP treatment is not fully attributed to the improvements in p53 expression, nonetheless other studies by our group shows the implication of none-apoptosis related genes as well, surprisingly their expression only affected by IV/oral form of DOX-MTX NPs and none of oral and IV forms of free DOX exhibit such a potential (Mesgari et al., 2014).

Although much impressive results obtained by IV forms of nanodrug (12 fold effective than its oral form) but both oral and systemic forms of nanodrug showed superior potential to up-regulate p53 gene (compared to the free DOX) which provide the implementation of both formulation. At the other hand, oral form of DOX was not effective however we find significant results with oral mode of DOX-MTX nanoparticles. This observation

indicate to the enhanced oral bioavailability of DOX-MTX compared to free DOX. Overall, both oral and IV forms of DOX-MTX NP are effective against OSCC with less side effects/alterations on normal cells.

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