**In vitro** anti-cancer activity of Doxycycline on some human cancer cell lines

Nadia Matter ALMhana¹, Israa Ghassan Zainal², Nahi Y. Yaseen³

1 AL-Mustansirya University, College Of Engineering, Environmental Engineering Department, Baghdad-Iraq. 
2 Kirkuk University, College Of Science, Chemistry Department, Kirkuk-Iraq. 
3 Iraqi Center for Cancer and Medical Genetics Research, Baghdad-Iraq.

Abstract:

Doxycycline (DC) is an antibiotic that is used in the treatment of number types of infections diseases caused by bacteria and protozoa. Doxycycline is a kind of second-generation tetracyclines which is commonly used to treat a variety of infections. Three cancer cell lines include: human cerebral glioblastoma-multiforme (AMGM) at passages 75-84, human cervical cancer (HeLa) at 70 passages, and human pelvic rhabdomyosarcoma (RD) at 75 passages cell lines were used in this study. The results showed that DC exerted significant cytotoxic effects with all concentrations used (50, 100, 150, 200, 250, 300, 350 and 400) µg/ml on all types of cell lines. Because of cytotoxic activity, good pharmacokinetic characteristics and the safety of drug which used for many years in the treatment of infectious disease, we can conclude that these characteristics make DC a valuable treatment for many types of cancer.

**Key Words:** Doxycycline, Anticancer, Cell Line, Glioblastoma, cervical cancer, rhabdomyosarcoma.

Introduction:

Tetracyclines are a family of antibiotics with effectiveness against several diseases caused by microbial infection. They were discovered in 1953 and feature a 4-ring structure to which a variety of side chains is attached. Doxycycline (DC) is a member of the tetracycline family. It has good water and lipid solubility and in bacteria, it is known to inhibit protein synthesis of pathogens [1]. As such it has found applications in the treatment of a large number of diseases, including Lyme disease [2], Legionnaire’s disease [3] and malaria [4]. Treatments are well tolerated by patients [5] with little side effects. In addition to its anti-microbial action, DC has also shown therapeutic potential against rheumatoid arthritis [6], abdominal aortic aneurysms [7,8], malignant pleural effusions[9] and mesenteric ischemia [10]. Doxycycline are also becoming known as anti-tumor properties in the case of prostate, breast and colon cancer [11,12,13,14]. Its role as a nonspecific matrix metalloproteinase and angiogenesis inhibitor is well established [15,16,17,18,19]. Its pro-apoptotic effects are also known as in the case of T-lymphocytes [20]. Apoptosis is a gene-directed form of cell death. It is important in a number of physiological processes like in normal development and maintenance of tissue homeostasis [21,22]. It also plays a role in tumor regulation because dysregulation of apoptosis may cause cancer cells to increase in numbers. Also chemotherapeutic agents may exert their anti-tumor action via activation of apoptosis in target cells [23,24,25]. Doxycycline is an antibiotic that is used in the treatment of a number of types of infections caused by bacteria and protozoa [26]. Current studies have demonstrated that DC is a pluripotent drug that affects many anticarcinogenic functions, including anti-tumor growth effect on human. It is useful for bacterial pneumonia, acne, infections, early Lyme disease, cholera and syphilis. It is also useful for the treatment of malaria when used with quinine and for the prevention of malaria. Doxycycline can be used either by mouth or intravenously [26]. It is a broad spectrum antibiotic of the tetracycline class [27]. Like other agents of this class it kills bacteria and protozoa by inhibiting protein production[26,28]. Although chemotherapy plays significant role in the treatment of cancer, morbidity and mortality due to drug resistance and cancer metastasis are yet to be eliminated. Doxycycline has been reported to have cytotoxic and anti-proliferating properties in various cancer cells [29]. In a study whether DC was apoptosis threshold lowering agent in colorectal can-
cancer cells by targeting mitochondria. The tetracycline family have been used clinically since the mid twentieth century. Since then, they have found application beyond their antimicrobial activity in both the clinic and biomedical research [30,31]. Tetracycline controlled transcriptional activation the method of inducible gene where transcription is reversibly turned on or off in the presence of the antibiotic tetracycline or one of its derivatives (e.g. Doxycycline)[32]. To investigate the potential of DC as a therapeutic agent, this study aimed to determine the effect of DC on three human cancer cell lines growth including (AMGM, HeLa and RD) cell lines and study its cytotoxic effect in vitro.

Methods:

Cells and Cell Culture
Three cancer cell lines, human cerebral glioblastoma-multiforme (AMGM, at passages 75-84), human pelvic rhabdomyosarcoma (RD, at 75 passages), and human cervical cancer (HeLa, at 70 passages) cell lines were kindly provided by ICCMGR (Baghdad, Iraq) and used throughout this study. AMGM was propagated and maintained on Roswell Park Memorial Institute medium (RPMI-1640, US biological, USA), while HeLa and RD cultured on minimal essential medium (MEM, Sigma) [33]. To these media, 10% fetal bovine serum (Cellgro, USA) and 1% penicillin/streptomycin (Cellgro, USA) were added and incubated in a humidified 5% CO2 incubator (HeraCell 150, Thermo Electron Corp.) at 37°C. The cells were subculture after they had achieved 80-90% confluency which can be observed under inverted microscope (Nicon Eclipse TS100). Cell viability was assessed by using trypan blue (Pharma, Sweden) exclusion test and found to be greater than 99% [34].

Cytotoxicity Measurement of Doxycycline
The cytotoxicity of DC on cancer cell lines was examined according to the inhibition of proliferation rate (IR %) [35]. In a 96 well tissue culture plate, seed 2 x 104 cells per well in 200 μl antibiotic-free normal growth medium supplemented with FBS. Incubate the cells at 37°C in a CO2 incubator until the cells are 60-80% confluent. This will usually take 18-24 hours. Then it will exposure with different concentrations of DC (50,100,150,200,250,300,350 and 400) μg/ml in 100 μl and incubated at 37°C for 24, 48 and 72 hrs. After incubation, 20 μl of MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide)] was added and incubated for further 3 hrs at 37°C. The untreated cells were also done as control. The absorbance of treated and untreated cells was measured at 492 nm. The inhibitory rate of cell proliferation was calculated according the equation: IR%= A−B/A×100

Where A represents the absorbance of untreated cells, while B represents the absorbance of treated cells. The results of IR% was plotted on Y-axis while the concentration of DC where plotted on X-axis. The concentrations that caused a 50% reduction in cell growth (IC 50) was determined by non-linear regression analysis using Graph pad Prism software [36].

Statistical Analysis
The Statistical Analysis System- SAS (2012) program was used to effect of difference factors (concentration, cell lines and time) in study parameters (inhibition rate and Knockdown percentage). Least significant difference –LSD test was used to significant compare between means in this study [37].

Results:
According to our knowledge this is the first research which study the cytotoxic effect of DC on (AMGM, HeLa and RD) cell lines. The results showed that DC decreased the growth of AMGM cells significantly as compared to untreated control cells; it appeared that the growth inhibition was concentration and exposure time dependent. The inhibition rates for the concentrations 50, 100, 150, 200, 250, 300, 350 and 400 μg/ml were 32.87, 33.79, 44.55, 41.08, 51.22, 57.14, 60.40 and 62.64% respectively after 24 hrs of exposure. When the exposure time increased to 48 hrs, the inhibition rates for these concentrations reached 34.82, 42.82, 43.36, 42.87, 56.06, 65.62, 78.71 and 71.48% respectively. However after 72 hrs exposure the inhibition rates increased to 31.08, 42.49, 46.00, 50.84, 53.36, 67.54, 72.33 and 69.42% respectively (Figure 1).

Using low concentration of DC was used, cells continued to proliferate for 48 hrs, as shown by the comparison of 48 hrs to 24 hrs (P<0.05). There was a trend for MTT values to increase from 48 hrs to 72 hrs under low concentration conditions, the P values were a significant, including comparison with untreated as control. This suggests that cell proliferation slowed considerably by 72 hrs in this assay condition. Thus, there was a growth-inhibitory but not a cytotoxic effect from DC on AMGM cell line treated in serum-free culture conditions. The cell viability rate of DC compared to untreated as control cells; it appeared that the growth inhibition was concentration and exposure time dependent. The inhibition rates for the concentrations 50, 100, 150, 200, 250, 300, 350 and 400 μg/ml were 32.87, 33.79, 44.55, 41.08, 51.22, 57.14, 60.40 and 62.64% respectively after 24 hrs of exposure. When the exposure time increased to 48 hrs, the inhibition rates for these concentrations reached 34.82, 42.82, 43.36, 42.87, 56.06, 65.62, 78.71 and 71.48% respectively. However after 72 hrs exposure the inhibition rates increased to 31.08, 42.49, 46.00, 50.84, 53.36, 67.54, 72.33 and 69.42% respectively (Figure 1).
Doxycycline also significantly decreased the growth of HeLa cells in comparison to untreated control cells with a concentration and exposure time dependent manner. When HeLa cell line exposed to 50, 100, 150, 200, 250, 300, 350 and 400 μg/ml concentrations of Doxycycline, the growth rates inhibited by 40.52, 49.31, 55.60, 57.97, 61.31, 70.91 and 76.97% respectively after 24 hrs exposure. The same concentrations of the drugs exerted inhibition of growth rates 32.05, 43.63, 46.51, 50.42, 52.37, 61.07, 75.53 and 75.53%, when the exposure time increased to 48 hrs. While after 72 hrs of exposure, the growth rates inhibition reached 39.10, 39.10, 47.43, 59.73, 61.76, 71.17, 79.02 and 77.56% for the same concentrations respectively (Figure 3).

Based on the above observations, the highest inhibition effect by using 350 μg/ml concentration of the DC after 72hrs of the treatment duration on HeLa cell line where it was inhibited by 79.02%. Figure 4 represents the cell viability rate of DC effect on HeLa cell line for 24, 48 and 72 hrs for treated and untreated cells.

The results also showed that cimetidine decreased the growth of RD cells significantly as compared to untreated control cells with a concentration and exposure time dependent manner. The inhibition rates for the concentrations 50, 100, 150, 200, 250, 300, 350 and 400 μg/ml were 33.92, 40.85, 46.29, 51.38, 54.54, 55.20, 55.02 and 63.41 respectively after 24 hrs of exposure. When the exposure time increased to 48 hrs, the inhibition rates for these concentrations reached 34.81, 38.72, 43.15, 46.81, 54.68, 62.38, 73.79 and 74.51% respectively. After 72 hrs exposure the inhibition rates increased to 33.20, 37.04, 50.95, 55.92, 57.41, 61.09, 73.59 and 79.84% respectively (Figures 5).

Figure 6 shows the gradually decreasing in the viability rate with increasing the DC concentration, which it is inverse the inhibition rate in RD cell line.
Finally, the comparison between AMGM, HeLa and RD cell lines inhibition rates for treated and untreated cells for the same day presented in figure 7, it was clear that there were a significant effect for each concentration to all cell lines under study after 24 hrs. The highest inhibition rate were noticed for HeLa cell line using 350 µg/ml and the least one for RD cells.

After 48 hour there were no significant effect (P>0.05) of each concentration for the three cell lines except for the concentration 200 µg/ml, there were a significant effect (P<0.05). As shown in figure 8 below. The higher DC concentration was noticed in AMGM cell line using 350 µg/ml by 78.71% (P>0.05), then HeLa cells with 75.58% using 400 µg/ml (P>0.05).

From the above results the most DC concentrations effectives on the cells viability is 350 and 400 µg/ml after 48 and 72 hours. The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. The values are typically expressed as molar concentration. It is commonly used as a measure of antagonist.
drug potency in pharmacological research. According to the FDA, IC50 represents the concentration of a drug that is required for 50% inhibition in vitro. It is comparable to an IC50 for agonist drugs. IC50 also represents the plasma concentration required for obtaining 50% of a maximum effect in vivo [38].

Table 1 represents the mean IC50 values for AMGM, HeLa and RD cell lines after 24, 48 and 72 hours treatment with DC.

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>AMGM Mean IC50 µg/ml</th>
<th>HeLa Mean IC50 µg/ml</th>
<th>RD Mean IC50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>250</td>
<td>115</td>
<td>200</td>
</tr>
<tr>
<td>48</td>
<td>200</td>
<td>110</td>
<td>150</td>
</tr>
<tr>
<td>72</td>
<td>150</td>
<td>100</td>
<td>130</td>
</tr>
</tbody>
</table>

From the data above, it was clear that for AMGM cells, the higher concentration noticed in 24 hour by 250 µg/ml but the lower one after 72 hour was 150 µg/ml, that is mean the concentration decreased with increasing the days. Which noticed the same thing for HeLa cell line, where after 24 hour, the IC50 was 115 µg/ml and in 72 hour was 100 µg/ml. But for the RD cell line, it’s clear that after 24 hour the IC50 was 200 µg/ml, but in 72 hour, the IC50 was 130 µg/ml. The highest IC50 values were for AMGM cell line, but the lower one was for HeLa cell line, as shown in figure 10.

Figure 10: The IC50 of cell viability for AMGM, HeLa and RD cell lines for 24, 48 and 72 hours.

Discussion:

Doxycycline belongs to the tetracycline family of drugs, members of which function as anti-microbial agents by preventing the binding of amino acyl tRNA to the ribosome, thereby inhibiting protein synthesis [39]. It has been known for years that members of the tetracycline family, such as DC and minocycline, inhibit the growth of various tumor cells in vitro [40]. Doxycycline was found to be the most effective analogue of tetracycline at inhibiting cell survival in a human adenocarcinoma cell line [41]. The mechanisms for DC anti-proliferative effects have been reported, and include impairment of mitochondrial protein synthesis [42,43], proliferation arrest in the G1 phase of cell cycle [44], and induction of apoptosis by caspase-3 activation in human T-lymphoblastic leukemia cells [45]. However, the serum concentrations of DC required to achieve these cytotoxic effects are generally higher (20 to 50) µg/ml than the typical therapeutic serum concentration used to treat infections, generally from 0 to 5 µg/ml and occasionally as high as 10 µg/ml [46], making DC less desirable as a cytotoxic agent. There are reports showing that different cancer cell lines have different sensitivities to DC. Doxycycline at 10 µg/ml was sufficient to induce G0/G1 arrest, and a concentration of 20 µg/ml was able to provoke mitochondrion mediated apoptosis in HT 29 cells [47,48]. In prostate cancer cell culture, necrosis and apoptosis were not apparent until DC concentrations exceeded 20 µg/ml [49]. In clinical settings, GBM is highly resistant to cytotoxic intervention as compared to other solid tumors, and this may be a clinical reflection of our observation that glioma cells require higher concentrations of DC in vitro to achieve its effect on growth inhibition [50].

Wang et al. [51] found that the glioma cells under serum-free culture conditions were exposed to low concentrations of DC displayed a similar behavior to those cultured in 5% serum. However, viability of U87 and LN229 cells tended to be reduced in response to high concentrations of DC treatments. A similar, but less obvious trend was observed with lower concentrations as well. For example, the cell viability of U87 cells after 72 hrs was reduced to 79% compared to that after 48 hrs in the higher concentration group (P < 0.05), indicating a cytotoxic effect of DC on U87 cells in serum-free conditions. In U251HF cells, the curve for cell viability was essentially the same between 48 hrs and 72 hrs for all treated groups including the untreated control cells. These data are consistent with the study by Sagar et al [52], they demonstrated cytotoxic activity of DC in colorectal
cancer cell (HT29 cell line). Zhao et al [53] found that in HeLa and CaLo cell lines, DC inhibited proliferation in a dose-dependent manner with IC50 (10–20 µmol/L). While Chen et al [54] explained that DC significantly inhibited the proliferation of many cancer cells in a dose-dependent manner. IC50 of most cells was less than 5 µM, which was quite low. This result demonstrated that many cancer cell lines were very sensitive to DC. Wei et al [55] found after 48 hrs treatment with DC in different concentrations, cell viability was decreased in a dose-dependent manner in HO8910, SKOV3 and SKOV3/DDP. The IC50 of DC in SKOV3 was compared with that in SKOV3/DDP. The results showed that the IC50 was 16.33 mg/ml in SKOV3 and 8.53 mg/ml in SKOV3/DDP respectively, which indicated that SKOV3/DDP cells were more sensitive to DC treatment than SKOV3 cells. But Hector et al [56], when they study on HuTu-80 human duodenal adenocarcinoma cells 1x104, after being exposed to DC for 72 hr, a cellular viability of 55±2% and 10±1.6% at 5 and 10 µM, respectively, was determined. These concentrations corresponded to 2.22 and 4.44 µg/ml. The maximum plasma concentration attainable in humans varies from 1.5 to 3.6 µg/ml after therapeutic doses of DC. Thus, the concentrations employed in this study were similar to those that can be obtained in human plasma at therapeutic doses.

References:

فعالية عقار الدوكسيسيكلين على بعض الخطوط السرطانية البشرية (في الزجاج)

نادية مطر المحتة، إسرا عثمان، ناهي يوسف بايين

1 الجامعة المستنصرية، كلية الهندسة، قسم هندسة البيئة، بغداد-العراق.
2 جامعة كركوك، كلية العلوم، قسم الكيمياء، كركوك-العراق.
3 الجامعة المستنصرية، المركز العراقي لبحوث السرطان والوراثة الطبية، بغداد-العراق.

الخلاصة:
الدوخسيكلين مضاد حيوي من الجيل الثاني للتتراسيكلينات يستخدم في علاج العديد من الأمراض التي تعنيها البكتيريا والبروتوزوا. تم استخدامه في هذه الدراسة ثلاثة خطوط سرطانية هي سرطان عنق الرحم البشري (AMGM) عند تمريرة الـ75، سرطان عق عق الدماغ البشري (glioblastoma) عند تمريرة الـ75، و سرطان الخلايا العضلية المخططة البشرية (RD) عند مقطع الـ70 و سرطان الخلايا العضلية المخططة البشري (HeLa) عند تمريرة الـ75. أشارت النتائج إلى أن الدوكسيكلين يظهر تأثير معنوي على جميع أنواع الخلايا. المراقبة أظهرت أن الدوكسيكلين علاج جيد لعديد من السرطان. }

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