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Research paper

Inhibitory Effect of 5-Aza-2'-Deoxytidine on Cell Viability of the Oral Cancer Cell Line, ORL-48T

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Abstract

Oral cancer is recorded as the sixth highest malignancy globally. 5-Aza-2'-deoxycytidine (5-Aza-dC) has been applied as an inhibitory agent in various tumourous cells. Previous studies showed that 5-Aza-dC can inverse genes silencing resulting from hypermethylation process. Inhibitory effects of 5-Aza-dC on oral cancer cell lines, ORL-48T proliferation was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT)-based assay in this study. The results demonstrated the treatment with 20 µM 5-Aza-dC for 144 h and 40 µM with 72 h respectively, revealed a significantly decreased percentage of viable tumour cells (p= 0.03). This current study provides an evidence of the inhibitive effect of 5-Aza-dC on the ORL-48T cells proliferation.

Keywords: *Oral Cancer; 5-aza-2'-deoxycytidine; MTT Assay; Cell Viability.*

1. Introduction

Oral cancer is a heterogeneous disease in nature with a dismal survival rate of approximately 50% which has not changed for last decade [1]. Based on the annual death rate caused by oral cancer, Malaysia ranked number 14 in the world with a total number of 1,587 deaths [2], [3]. In addition, the reported rate of oral cancer patients by the World Health Organization (WHO) in 2011 has reached more than 1% of the total deaths [4].

The rate of oral cancer cases in Malaysia differs between ethnic communities due to the different sociocultural risk factors such as smoking and the betel chewing habit [3] [5]. These risk factors increase the number of oral cancer cases especially of Indians in Malaysia [6]. Moreover, this cancer is having a huge impact on family and society since it normally occurs among middle age males [3].

5-aza-2'-deoxycytidine (5-Aza-dC), a cytosine analogue demonstrates a vital role in inhibiting the function of DNA Methyltransferases (DNMT), therefore consequently causing demethylation. Because of its structural similarity to cytosine, it gets incorporated into the DNA molecules [7]. This cytosine analogue might cause cell death by irreversibly binding the methyltransferase [8]. This depletion of DNMT in the cell results in demethylation and can stop the proliferation of cancer cells [9]. A previous study by Balch et al., investigating the effectiveness of 5-Aza-dC on hematologic malignancies, demonstrated the most positive effect in comparison to other treatments [10]. Besides that, the sensitization or reactivation of silenced genes has been observed in the cancer cells [8].

Although the general principle of 5-Aza-dC is known, it is not clear if and how it will affect the oral cancer cells proliferation. Thus, in this study, the inhibitory effect of 5-Aza-dC on the proliferation of the oral cancer cells, ORL-48T, was determined using the MTT-based *in vitro* assay. The MTT assay is one of the most widely exploited approaches in cancer research for measuring cell viability, proliferation, and drug cytotoxicity over the years [11].

2. Results and discussion

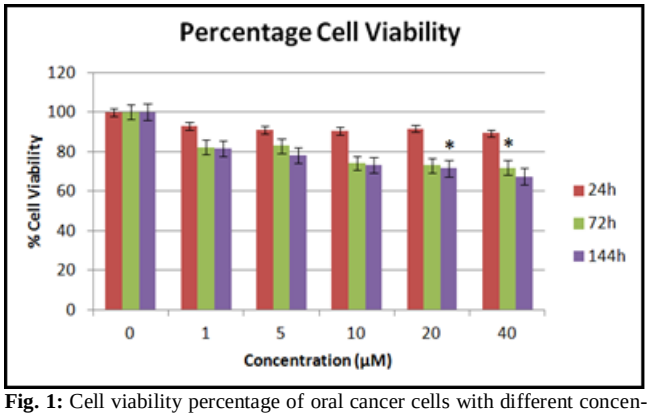


Fig. 1: Cell viability percentage of oral cancer cells with different concentrations of 5-Aza-dC for 24, 72, and 144 h.

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The percentage of viable oral cancer cells with and without 5-Aza-dC treatment for 24, 72 and 144h is shown in **Fig. 1**. The cell viability with 40 µM for 72 h and 20 µM 5-aza-dC for 144 h treatments dropped approximately to 71% (p=0.03) and therefore was significantly decreased in comparison to untreated cells. The 5-Aza-dC treatment with doses of 10 µM or less, for 72 h and 144 h did not show any significant reduction of the cell proliferation as more than 90% of cells were still viable.

Previous studies, determining the inhibitory effects of 5-aza-dC on other cancer cell lines such as salivary adenoid cystic carcinoma [12], colorectal cancer [8], [13], and breast cancer [14], reported a lower effective concentration of 5-Aza-dC with values below than 10 µM. In contrast, the result of our present study may differ from previous reports due to the different type of cancer cell lines used and also technical skills in handling cell cultures.

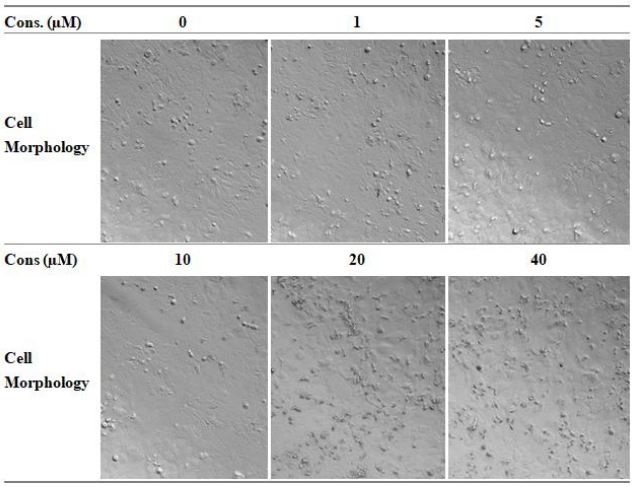


Fig. 2: The cell morphologies of the oral cancer cells, ORL-48T after 72h incubated with different concentrations of 5-aza-dC.

The cell morphology between treated and untreated ORL-48T cells was observed using an inverted microscope with 20 times of magnification. From **Fig. 2**, it can be observed that at a higher concentration of 5-Aza-dC, the cell morphology of ORL-48T cells has changed when compared to untreated ORL-48T cells. The oral cancer cells changed from a rounded epithelial-like shape to a more mesenchymal-like shape and these cell morphological changes (**Fig. 2**) were seen around 20 µM to 40 µM of 5-aza-dC. The changes might also be due to apoptotic activities induced by the drug, although no blebbing was observed. A study done by Hemberger et al. demonstrated that the 5-Aza-dC treatment induced a transition from mesenchymal into epithelial morphology of cancer cells [15]

On the other hand, when a concentration of 5-Aza-dC lower than 20 µM was used, no morphological changes were observed (**Fig. 2**). This might be due to the lack of inhibition by 5-Aza-dC towards oral cancer cell lines.

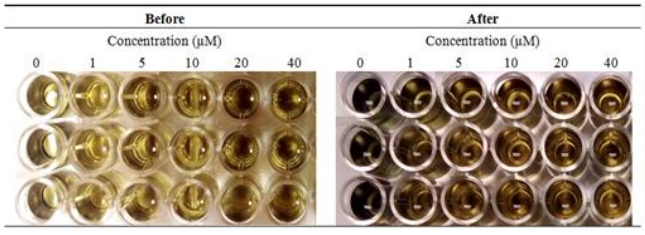


Fig. 3: The colour intensity of the cell supernatant before and after MTT reagent was added to different 5-Aza-dC concentrations (0, 1, 5, 10, 20, and 40 µM) at 144h treatment.

The colour changes of the cell supernatant before and after the addition of MTT reagent were noticeable in the cell viability assay as shown in **Fig. 3**. The difference in media colour changes is due to the resulted formation of the purple coloured formazan derivatives after each treatment of 5-aza-dC with MTT reagent [16]. The higher the number of viable cells with an active mitochondrial metabolism is, the more of the purple coloured formazan product (absorbance maximum of 570nm) is formed [17]. The colour formation is only applicable for viable cells as dead cells lose the ability to convert MTT into formazan [18]. Besides that, it was observed that the intensity of purple coloured formazan decreases from 0 µM to 50 µM for 72 h and 144 h treatment.

This result also was supported by the optical density (OD) measurement of the cultured cell line, ORL-48T (**Fig. 4**). It has been observed that the colour intensity of the purple coloured formazan was the darkest and the OD measurement obtained was the highest at a concentration of 0 µM. The obtained results suggest that lowest intensity of purple coloured formazan signifies the presence of non-actively proliferating viable cells that transform from the purple coloured formazan derivatives into yellow coloured tetrazolium salts [19], therefore give lower OD measurement. While the higher intensity of purple colour formazan indicates the presence of actively proliferating viable cells which expressed higher OD measurement [20].

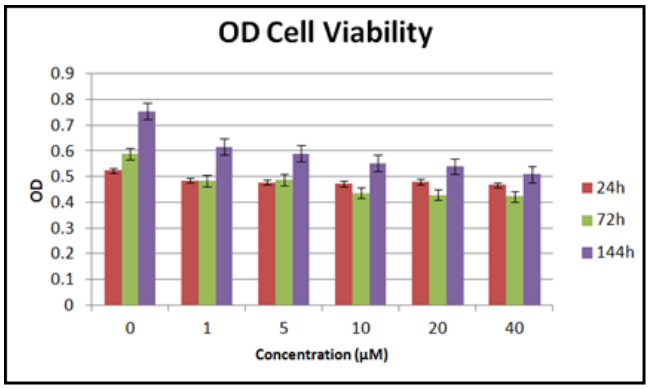


Fig. 4: The optical density (OD) measurement of cell viability for 24 h, 72 h and 144 h of various concentration of 5-aza-dC treatment.

However, the amount of generated formazan signals also depends on the optimization of different parameters such as the number of cells per well, cell type and culture medium [17]. Therefore, from the obtained OD measurement, this suggests that the cancer cells with a lower concentration of 5-Aza-dC treatment contained a higher number of viable cells, whereas cells with a higher concentration of 5-aza-dC contained a lower concentration of viable cells. From the results, it was observed that 5-Aza-dC decreased the proliferation of cancer cells significantly in a concentration dependent manner. This suggests that 5-Aza-dC has effectively functioned in inhibiting the proliferation of oral cancer cells. Hence, this may imply that 5-Aza-dC may have the potential to be used in the treatment therapy for oral cancer in the future. In addition, we will investigate the apoptotic genes' expressions involved for a better understanding of the effect of 5-aza-dC on oral cancer cell lines in the future.

3. Experimental

3.1. Preparation of 5-aza-2'-Deoxycytidine

5-aza-2'-deoxycytidine (5-Aza-dC) powder was purchased (Sigma-Aldrich, USA) and dissolved in dimethyl sulfoxide (DMSO) to prepare a 50 mg/mL stock solution.

3.2. Cell Line and Culture Condition

Oral squamous cell carcinoma cell line, ORL-48T acquired from Cancer Research Malaysia was used in this study. The cells were cultured in a mixture of DMEM (Dulbecco's Modified Eagle Me-

dium) F-12 medium (Gibco, USA) added with 10 % of foetal bovine serum (Gibco, USA) and 2% of penicillin-streptomycin (Gibco, USA). The cells were kept in a 5 % CO_2 at 37 ˚C humidified atmosphere.

3.3. Cell Viability Assay

Cell viability was obtained using the MTT-based assay of the Thermofisher Scientific, USA. A density of 1.4×10^4 cells/mL/ well of ORL-48T cells was seeded and left to adhere for 24h in a 96-well plate. They were then treated with different concentrations of 5-Aza-dC for 24, 72 and 144 h. Six different concentrations (0, 1, 5, 10, 20, 40 µM) were applied and the experiment was conducted in triplicates. The negative or test control did not receive any 5-Aza-dC (0 µM) treatment. After 24, 72 and 144 h respectively, 20 µL of MTT reagent was subjected into each well, then incubated for 4 h at 37 °C in 5 % CO_2 in a humidified atmosphere. Later, 50 µL SDS-0.1 M hydrochloric acid was added and the mixture was incubated overnight. The absorbance of each cell viability was measured by a microplate reader (TECAN) at a wavelength of 540 nm.

3.4. Data Analysis

Percentage of cell viability at every concentration at different hours was evaluated using the formula below.

Cell viability perrcentage = (OD sample / OD control) x 100%

Percentage of cell viability was plotted against concentration and the significant difference between the concentrations of 5-aza-dC at different time points was then determined by one-way ANOVA using SPSS software (version 23). The experiment was repeated three times (n=9) in this study.

3. Conclusion

In conclusion, this present study demonstrated that 5-Aza-dC may inhibit the cell proliferation of oral cancer cells. The oral cancer cells treated with 40 µM and 20 µM of 5-Aza-dC for 72 h and 144 h respectively had significantly reduced cell viability. Moreover, the treatment of 5-Aza-dC towards oral cancer cells may be of importance in clinical treatment and may contribute to the future development of treatment for oral cancer.

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