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Thioridazine enhances the effect of Doxorubicin on HEp-2 Cell Line

Bassant Mahmoud[1], Iman Mohamed Helmy[2], Hala El Kammar[3], Nermeen Sami Afifi[4]

Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers occupying the sixth position worldwide. PI3k/Akt signaling pathway plays a significant role in regulating diverse cellular functions. This includes cell growth, proliferation and survival via inhibition of apoptosis, transcription and protein synthesis. One of the most acknowledged and approved chemotherapeutic agents is Doxorubicin (DOX) which is a non- selective class I anthracycline. In spite of DOX being one of the most acknowledged chemotherapeutic agents, its use has been limited by its toxic side effects and the development of chemoresistance. Nowadays, Thioridazine (TZ) is a newly repurposed drug that was originally used in the treatment of psychosis, schizophrenia and anxiety. Recently, TZ has been tried in the treatment of breast, ovarian, gastric cancers and leukemias.

Methods: The viability was assessed on the effect of TZ and DOX separately and in combination, with different doses and durations in HEp2 cell line.

Results: Our results revealed that the highest viability was shown with the control group followed by the TZ group I (low dose) compared to the other groups. While, it showed the lowest mean viability with the combination group VI (high dose) compared to other groups.

Conclusion: Our data suggested that the combination group enhanced DOX's cytotoxic effects in addition to the anticancer effects of TZ (acting synergistically). Leading to the best outcome compared to other groups in eradicating cancer cells.

1. Assistant Lecturer, Oral Pathology Department, Faculty of Dentistry, Future University in Egypt
2. Professor of Oral Pathology, Faculty of Dentistry, Ain Shams University
3. Assistant Professor of Oral Pathology, Faculty of Dentistry, Future University in Egypt
4. Lecturer of Oral Pathology, Faculty of Dentistry, Ain Shams University

Introduction:

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers occupying the sixth position worldwide. More than 600,000 cases are diagnosed annually and are often lethal with a minority of HNSCC patients being able to survive 5 years after their diagnosis [1]. In spite of the emergence of new treatment modalities, the mortality rates are still high owing to the development of chemoresistance [2,3].

Apoptosis is one of the main mechanisms by which cytotoxic drugs mediate their effect. Failure of activation of the apoptotic pathway represents an important mode of chemoresistance. Survival signals induced by several receptors are mediated mainly by the phosphatidylinositol 3-kinase/Akt (PI3k/Akt) signaling pathway. Hence this pathway may decisively contribute to the resistant phenotype[4].

PI3k/Akt signaling pathway plays a significant role in regulating diverse cellular functions. This includes cell growth, proliferation and survival via inhibition of apoptosis, transcription and protein synthesis. The activation of Akt inhibits apoptosis, increases migration, metabolism and enhances cell cycle induction. PI3k/Akt signaling pathway is dys-regulated in many human cancers making it an appealing target for cancer therapy[5].

One of the most acknowledged and approved chemotherapeutic agents is Doxorubicin (DOX) which is a non- selective class I anthracycline. This drug is used in the treatment of various cancers among of which are lung, gastric, breast, thyroid, ovarian and Hodgkin's and non-Hodgkin's lymphoma[6,7].

In spite of DOX being one of the most acknowledged chemotherapeutic agents, its use has been limited by its toxic side effects and the development of chemoresistance. One of the mechanisms involved in developing drug resistance is the up regulation of PI3k/Akt pathway which transmits anti-apoptotic and survival signals[8,9].

Nowadays, Thioridazine (TZ) is a newly repurposed drug that was originally used in the treatment of psychosis, schizophrenia and anxiety. TZ belongs to the phenothiazine drug group as well as an antagonist of the dopamine receptor D2 family proteins. Lately, it was shown that patients suffering from schizophrenia had a lower risk of getting cancer than in patients without [10].

Recently, TZ has been tried in the treatment of breast, ovarian, gastric cancers and leukemias. Investigations showed that THIO reduced cell proliferation mediated through cell cycle arrest. Furthermore, it was found that TZ has anti-proliferative effect that could be attributed to the inhibition of PI3k/Akt signaling pathway[11].

The emergence of TZ as an anticancer therapeutic agent is exciting with the benefit of being moderately safe for use for over 40 years of psychosis therapy as well as having the potential to serve as an adjuvant with anticancer agents, yet further studies should be done to transition from bench to bedside for cancer treatment[11].

Our study was conducted to evaluate the effect of TZ and DOX separately and in combination, with different doses and durations in HEp2 cell line.

Materials and Methods

Materials

Doxorubicin hydrochloride (1mg), 98.0-102.0% (HPLC), Thioridazine hydrochloride (5g), 99% were purchased from sigma Aldrich. HEp2 cell line was supplied from Cell Culture Department of holding company for biological products and vaccines-VACSERA-Egypt.

Cell Culture

Human laryngeal squamous cell carcinoma, HEp2, cell line was purchased from Cell Culture Unit - VACSERA, Egypt. HEp2 cells were imported from the "American Type Culture Collection (ATCC)" in the form of frozen vials. Hep2 cells were cultured in Dulbeco"s Modified Eagle's Medium (DMEM) supplemented in 10% fetal bovine serum (FBS) (Cambrex BioScience, Copenhagen, Denmark) and 1% streptomycin-penicillin. The cells were grown at 37°c in humidified atmosphere of 5% CO2 in air.

Cell Viability Assay

Using the MTT assay, the IC50 for each substance was calculated .All the experiments there-after were performed using the calculated

IC50 for 24 and 48 hours duration for each group.

The HEp2 cell line was divided into 20 groups. Groups I and II were the control groups after 24 and 48 hrs, respectively. Groups from 3 to 8 were treated with different doses (low dose, intermediate dose and high dose) of Thioridazine hydrochloride for 24 and 48 hours. Groups 9 to 14 were treated by different doses (low dose, intermediate dose and high dose) of Doxorubicin hydrochloride for 24 and 48 hours. Groups from 15 to 20 were treated with different doses (low dose, intermediate dose and high dose) of combined Thioridazine hydrochloride and Doxorubicin hydrochloride, for 24 and 48 hours.

Cells were incubated with or without Thioridazine, Doxorubicin and combinations of Thioridazine and Doxorubicin into 96-well plates ($5x10^3$ cells / well) for 24 h, cell viability was evaluated using MTT assay. The relative cell viability and the IC50 for each substance was calculated .All the experiments there-after were performed using the calculated IC50 for 24 and 48 hours duration for each group.

Results

On comparing cell viability between groups for 48 hours, statistically significant differences were detected. Control group II showed statistically significant differences with all groups using ANOVA.

There was no statistically significant difference upon comparing TZ group VI with TZ group V and DOX group IV, V (they showed statistically significant highest mean viability). There was no statistically significant difference between TZ group V, TZ group VI, DOX group VI, V, and combination groups IV. TZ group VI showed statistically significant difference between all groups except DOX group IV, V, VI and combination groups IV, V and VI (they showed statistically significantly lowest mean viability).

There was a statistically significant difference between DOX group VI with all groups except DOX group V and combination group IV. On comparing DOX group V with all groups, there was a statistically significant difference with all groups except DOX group VI and combination groups VI, V and VI. There was a statistically significant difference between combination group VI with all groups except combination group V and VI.

Regarding the comparison of viability between all studied groups at 24 and 48 hours, all drugs at different concentrations showed no statistically significant change in mean viability after 48 hours using Wilcoxon signed- rank test.

Discussion

TZ a phenothiazine is an antipsychotic agent used in treating psychosis and schizophrenia [12]. Yet, there are an increasing number of studies demonstrating the anticancer effects of TZ. These anticancer effects include TZ induced apoptosis, inhibition of angiogenesis and metastasis in cancer cells. Moreover, TZ was shown to be a selective inducer of CSC differentiation. Researchers have attributed its effects to inhibition of PI3K/Akt and mTOR signaling and the antagonism of D2 family.

Twenty groups of HEp2 cell line were included in this study; two control groups. Six groups were treated with different doses of TZ. Another six groups were treated with different doses of DOX. Additional, six groups were treated with different doses of combined TZ and DOX. All groups were evaluated after 24 and 48 hours. The doses were prepared according to the calculated IC50 value as referenced in the study group.

The value of the doses used in this study were calculated based on the IC50 that was comparative to the mean effective doses used by other researchers in previous studies [12-18] and the doses used were estimated as follows: a dose of (0.5, 5 and 10 µg) concentration of TZ, a dose of (0.5, 5 and 10 µg) concentration of DOX and a dose of (0.5, 5 and 10 µg) of a combination of TZ and DOX.

MTT results showed the highest viability with the control group followed by the TZ group I (low dose) compared to the other groups. While, it showed the lowest mean viability with the combination group VI (high dose) compared to other groups. Even though the best effect on

the cell viability were obtained by combination group VI (high dose), there was no significant difference between combination group I (low dose) and combination group VI (high dose). Further studies are required to see the feasibility of using low doses of the drugs in order to avoid high dose induced toxicity of DOX.

The highest viability in the control group (un treated HEp2 cell line) could be attributed to the number of acquired accumulated mutations such as P53 mutations, in activating mutations in NOTCH1 and dys-regulation of PI3k/Akt pathway which has been associated with multiple biological functions, such as regulation of self-renewal capacity, cell cycle exit and survival. All these factors probably resulted in uncontrolled cell proliferation and decreased cell apoptosis that was reflected on the MTT results in this study [19-21].

On the other hand, the least viability that was noted in the combination group VI, could be attributed to the growth inhibitory effects of TZ which include anti-proliferative and anti-apoptotic properties such as the down regulation of cyclin D1 and cyclin dependent kinase 4 (CDK4), which are associated with the transition from G1 to S phase, the up -regulation of the CDK inhibitors p16 and p27, which interrupts cell cycle procession at the G1 or G2/M phase. Also, it up- regulates anti-apoptotic markers such as Bax and p53 [22-26].

In conclusion, in spite of the fact that DOX markedly reduced the viability of cancer cells, it didn't inhibit metastasis. Where the difference in the migration ability of the cells between DOX treated and control groups was insignificant. However, the combination group was able to overcome DOX's drug limitations by inhibiting PI3K/Akt pathway, inhibiting metastasis via TZ's anticancer effects. Consequently, the combination group enhanced DOX's cytotoxic effects in addition to the anticancer effects of TZ (acting synergistically). Leading to the best outcome compared to other groups in eradicating cancer cells.

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