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Baghdad Science Journal **Vol.16(3)2019**

DOI: http://dx.doi.org/10.21123/bsj.2019.16.3.0555

Trypan Blue Exclusion Assay Verifies *in Vitro* Cytotoxicity of New *Cis*-Platinum (II) Complex in Human Cells

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Received 5/5/2018, Accepted 4/2/2019, Published 1/9/2019

Abstract:

Various assays are used to determine the toxic effects of drugs at cellular levels in *vitro.* One of these methods is the dye exclusion assay, which measures membrane integrity in the presence of Trypan blue. Trypan blue the dye which was used in this study to investigate cytotoxic effect of a new *Cis* – dichloroplatinum (II) complex [$(Qu)_2PtCl_2$] on the viability of polymorphonuclear cells (PMNs). Three concentrations of platinum complex were prepared (70, 35and 17.5 µg/ ml) and the results revealed that the percentage of cell viability decreased as the platinum complex concentration increased in comparison with control. The platinum complex exhibited low cytotoxic effects towards healthy cells at the concentrations of 17.5 µg/ ml and 35 µg/ ml, in which the percentage of cell viability was (77.01 ± 6.3) and (72.3± 0.50)respectively, with no significant differences as compared with the control(90.66 ±0.577). The viability was significantly decreased (67.59 ± 3.16) when the cells were treated with the concentration of 70 µg/ ml in comparison with control. These results indicated that the percentage of living cells decreased when treated with high concentrations of [$(Qu)_2PtCl_2$], which causes cells death, while low concentrations of the compound show low toxicity. This data indicates that this compound, at these concentrations may be suitable for use as a cancer treatment because it has low toxic effects on the healthy cells.

Key words: *Cytotoxicity, Pt(II) complex, Trypan blue exclusion assay*

Introduction:

Cancer is defined as a disease which characterized by abnormal growth of the body's own cells. Four features distinguished cancer cells from normal cells: de-differentiation, loss of function, invasiveness and uncontrolled proliferation leading to metastasis (1). Globally, cancer is the second greatest cause of mortality, it affecting people of all races, ages, genders and nationalities. In 2012, about (8.2) million people died from cancer, this number is expected to increase to (13.1) million by 2030 according to the World Health Organization (2).

Chemotherapy is still one of the major courses of treatments for various cancers. Cisplatin, *cis*-DDP (*Cis*-diamminedichloroplatinum II) is one of the most successful compounds for treating wide varieties of cancers (3). Even though cisplatin is a valuable antitumor drug, it has numerous drawbacks side effects, such as nephrotoxicity, ototoxicity, neurotoxicity, myelotoxicity, peripheral neuropathy and hematological toxicity (4).

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In fact, several studies are focused to develop new platinum complexes with decreased side effects, an extensive range of antitumor action, and which have enhanced pharmacokinetic properties (5).

In cancer treatment, chemotherapeutic agents are designed to be toxic to the cancer cells but not to the normal cells. However, this selective toxicity is difficult to achieve, and therefore, vast amount of research is conducted worldwide with the aim to develop novel and improved anticancer drugs. Such studies frequently begin at the chemical level to design and synthesize compounds, which is followed by screening the cytotoxic properties *in vitro* using various biological studies (6).

Cytotoxicity refers to the quality of being toxic to cells. A chemical substance and immune cell or some kinds of venom are examples of toxic agents (7). The effect of a drug on cells propagated *in vitro* is studied using various assays. These assays can be simple tests, which estimate cell viability directly after drug exposure or can measure viability indirectly, such as assessing the capability of the cell to reduce compounds or to produce ATP (8, 9).

The trypan blue dye exclusion test is one of the first techniques established for evaluating cell viability, which remains broadly used today. It is a simple and inexpensive method in which the non-viable cells retain the trypan blue dye, and seem blue in color at the end, while viable cells can eliminate the trypan blue and will not retain the dye (10).

In the trypan blue dye exclusion assay, the cells must be in a single cell suspension and then must be visualized using a hemocytometer under a microscope. Automated counting can also be used to count the cells. A ratio of cells retaining the dye compared to those with no dye can be used to determine the percent of viable cells inside a population after counting the total number of cells, and thus determine the toxicity of the compound (11).

During recent years and due to resistance of some cancers to anticancer drugs, the research community has increased its efforts to overcome this obstacle. For this reason, many platinum(II) complexes have been synthesized and screened. One of them was a platinum (II) complex, which used the quinoline amine as a ligand, with an objective of finding a compound that displays extensive activity, less toxicity to non-cancer cells, and therefore the present project came to investigate the cytotoxicity of this compound by using trypan blue exclusion assay.

Materials and Methods:

Preparation of Tissue Culture Medium RPMI-1640

Media was prepared by dissolving one liter of medium in one liter of sterilized distilled water, and then 10% of fetal calf serum that was suppressed in 56^{o} C for 30 minutes was added. The pH was adjusted to 7.2 and the entire solution was filtered by using a Millipore filter (0.20 Micrometer). This was partitioned and preserved in tightly closed plain tubes at 4^{o}C for future usage. This medium was used to culture, a wash of lymphocytes, as well as, a preparation of different concentrations of the reagent.

Preparation of Trypan Blue Dye Solution

Trypan blue was prepared as follows: 0.2 g of trypan blue powder was dissolved in 100 ml of phosphate buffer saline solution (PBS). This solution was then filtrated using a Millipore filter (0.20µm) and preserved in a dark container at 4^{o}C until usage. This dye is commonly used for cell counting and assessment of tissue viability.

Preparation of Phosphate Buffer- Saline Solution (PBS)

Phosphate buffer saline solution was prepared by adding 0.795 g of Na_2HPO_4 to 9 g of NaCl in one liter of distilled water and the pH was adjusted to 7.2, then autoclaved and preserved at 4^{o}C for future usage.

Preparation of Physiological Saline Solution (PSS)

To prepare the PSS, 8.5 g of Sodium Chloride was dissolved in 1 liter of distilled water, then autoclaved and preserved at 4^{o}C until use. This solution was used to prepare serial dilutions of the platinum complex as well as for washing of cells.

Preparation of Dextran (70 T) Solution

This solution was prepared by dissolving 6 g of Dextran (70 T) in 1 liter of distilled water, then was preserved at 4^{o}C until use. It was used to the isolation of PMNs.

Preparation of Platinum (II) Compound Concentrations:

Cis-dichloro-platinum (II) complex $[Pt(Qu.)_2Cl_2]$was prepared and chemically identified in previous work (12).A stock solution of the complex was prepared by using dimethyl sulfoxide (DMSO) as a solvent (the final concentration of the solvent should be 1%). After that, different concentrations (70, 35, and 17.5) µg /ml were prepared by using RPMI-1640 medium.

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs):

In the present study, about 10 ml of blood from five normal volunteers was collected. Volunteers were screened to ensure that no infection or indications of infection for one week were present previous to taking the sample, no exercise or alcohol was used for a day previous the blood sample withdrawal, no systemic antihistamines or corticosteroid used for 1 week prior to gaining the sample, nor were topical corticosteroid or aspirin drugs were used for the previous 48 hours in order to prevent results interference. Before the experiment, the blood and all the chemicals used for PBMC separation were maintained at room temperature. An equal amount of PBS was added to precipitate red blood cells; 3 ml of dextran was used for every 10 ml of blood. Subsequently, all substances were mixed gently together and incubated for 45 minutes at 37^{o}C. There were two layers formed the lower one that contained RBC was neglected, and the upper layer contained plasma rich in WBCs that were transferred to another test tube. Hank's Balanced Salt Solution (HBSS) was added, the mixture was centrifuged twice to wash WBCs. Finally, the cell suspensions were adjusted to the concentration of 1.0 $x10^7$ cells/ml (10).

Cytotoxicity Assay by Trypan Blue Dye Exclusion Technique:

To evaluate the count of viable cells existent in a sample, Trypan Blue was used. First, a cell suspension was made in a fixed volume of cells (e.g. 1ml). Human peripheral blood lymphocytes ($1x10^7$ cell/well) were incubated in culture RPMI-1640 medium (100 µl) in 96- well microplate for 1hour at 37°C, with an aliquot of Pt(II) compound solutions (50 µl) for each concentration (70, 35, and 17.5) µg/ml. Cells were washed and re-suspended in RPMI-1640 medium.

Fifty µl of cell suspension was taken and mixed with an equivalent volume of trypan blue dye, and then incubated for 3 minutes at 37°C. This mixture was transferred to a hemocytometer to count the viable cell and non-viable cells. Counting should begin in fewer than 5minutes after staining with trypan blue solution. After that time the cells will begin to take up the dye. Some of the cell suspension was placed with a pipette and the trypan blue mixture was transferred into a hemocytometer and covered using a coverslip. Finally, the hemocytometer was placed on the stage of an inverted microscope and the focus and power were adjusted till a single calculating square appeared filling the field, then the number of cells was counted per ml, percent viability (%) was evaluated using the formula (11): % viability = (viable cell count/total cell count)*100

The assay was performed in quadruplicate for each test group. The control cells only RPMI-1640 medium and solvent was added without test compound.

Statistical Analysis:

All investigations were accomplished in triplicate. Data were accessed as (mean ± SD). Results were investigated by the SPSS Base 16(SPSS Inc. Chicago, IL). Statistical significance of variances between data was estimated by ANOVA test (analysis of variance). A value of $p<0.05$ was considered statistically significant.

Results and Discussion:

Results of a new *Cis* –dichloroplatinum (II) complex $[(Qu)_2PtCl_2]$ on the viability of polymorphonuclear cells (PMNs) was tabulated in Table (1) and graphically represented in Fig. (1). The percentage viability was found to be increasing with decreasing concentration of test compound. In general, the concentrations of $[PtCl_2(Qu.)_2]$ complex (70, 35, and 17.5 µg/ml) exhibited relatively low cytotoxic effects on the viability of cells as compared with control. At the concentration of 70 µg/ml, the percentage of cell viability was 67.59%, which was statistically significant as compared with control (90.66 %), whereas the percentage of viability was 72.3% at the concentration 35 µg/ml and 77.01 % at the concentration 17.5 µg/ml contained no significant differences in comparison with control($p< 0.05$).

From the results, the percentage of viability was found to be cumulative with reducing concentrations of the test compound. These results indicated that the percentage of viable cells was decreased when treated with high concentrations of $[(Qu)_2PtCl_2]$ compound, which caused cells death that lead to loss the membrane integrity making trypan blue to penetrate them. In contrast, low concentrations exhibit low toxicity. Therefore, use of this compound at these concentrations may be suitable for use as a treatment because they have low toxic effects on the healthy cells. Relatively high toxicity is due to side reactions with other biomolecules separately from DNA such as proteins, was one of the most common disadvantages of the currently used chemotherapeutic agents. For this reason, the cytotoxicity study was carried out in healthy cells in order to evaluate this factor (13).

In fact, several platinum complexes have been shown to inhibit the growth of cancer cells. In general, one of the most important means for this activity was the presence of at least one amino group, which is essential for hydrogen-bonding to DNA. Therefore, a valuable strategy for modeling the physicochemical characteristics was by the variation of the ligands on the metal center which enhances the uptake of platinum compounds (14, 15). DNA binding properties, and/or the relative amounts of inter-strand crosslinks in double-stranded DNA, may be changed by simple chemical alterations in the structure of a certain compound. This governs its cytotoxic and anti-proliferative activities (10). Finally, other in vivo assays could be used beside trypan blue to confirm the cytotoxicity of *Cis* –dichloroplatinum (II) complex $[(Qu)_2PtCl_2]$ against different cancer cell lines.

Table 1: Viability Percent (%) after Exposure to Different Concentrations of Platinum (II) Complex.

Concentrations of [(Qu.)2PtCl2] µg/ ml	Percentage of cell viability (Mean ± SD)
70 µg/ ml	67.59± 3.16*
35 µg/ ml	72.3± 0.50 **
17.5 µg/ ml	77.01± 6.3 **
0 µg/ ml(control)	90.66± 0.577

* Significant difference ($p< 0.05$)
** No Significant difference ($p>0.05$)

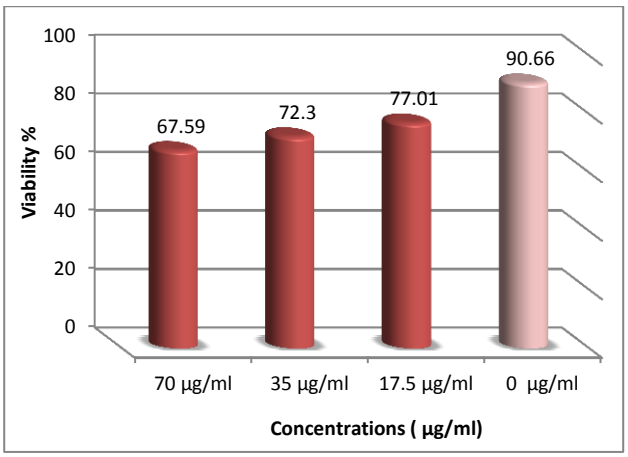

Figure 1. Cell viability percent (%) after exposure to different concentrations of Platinum (II) complex.

Conflicts of Interest: None.

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التحقق من السمية الخلوية لمعقد بلاتيني ثنائي جديد خارج الجسم الحي باستخدام اختباراقصاء صبغة التريبان الزرقاء

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الخلاصة:

استخدمت عدة طرق لتقييم التأثيرات السمية للأدوية على المستويات الخلوية ،واحدة من اهم هذه الطرق طريقة اقصاءالصبغة التي تقيس سلامة الغشاء الخلوي باستخدام صبغة التريبان الزرقاء وهي صبغة حيوية استخدمت للتحري عن التاثير السمي الخلوي لمعقد البلاتين الثنائي مع الكوينولين $[(Qu)_2PtCl_2]$ على حيوية الخلايا البلعمية متعددة اشكال النوى ،حيث حضرت ثلاثة تراكيزمن هذا المركب وهي (*70 µg/ ml , 35 µg/ ml, 17.5 µg/ ml*).

اظهرت النتائج ان نسبة حيوية الخلايا تنخفض بزيادة التركيز مقارنة بمعامل السيطرة، اذ كانت النسبة المئوية لحيوية الخلايا هي (*77.01 ± 6.3*) بالمئة عند التركيز *17.5 µg/ ml* و *(72.3± 0.50)*بالمئة عند التركيز *35 µg/ ml* مع عدم وجود فارق معنوي عند مقارنتها مع عينة السيطرة*(90.66 ±0.577)* ، بينما كانت النسبة المئوية لحيوية الخلايا في التركيز*70 µg/ ml* هي *(67.59 ± 3.16)* بالمئة بفارق معنوي مقارنة مع السيطرة . تشيرهذه النتائج الى ان نسبة الخلايا الحية تقل بزيادة التركيز لهذا المعقد*$[(Qu)_2PtCl_2]$* اذ تكون هذه التراكيز قاتلة للخلايا الطبيعية بينما تكون التراكيز المنخفضة ذات سمية قليلة للخلايا السليمة في الجسم وبذلك يمكن التوصية باستخدام هذه التراكيزكعلاج.

الكلمات المفتاحية: اختبار اقصاء التريبان الزرقاء، السمية الخلوية، معقد بلاتين ثنائي.