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Potential Chemoprotectants Platinum- Based Therapy with S-ligand Using ATP Bioluminescence Assay

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ABSTRACT- *The critical dose limiting side effect of cisplatin is nephrotoxicity; 20% of patients receiving a high dose of cisplatin have suffered from severe renal dysfunction; the ability to manage this induced toxicity is crucial for the success of chemotherapy. The concomitant administration of chemoprotective agents become an important approach in a platinum chemotherapy, the potential reversibility of Pt-S bonds in presence of other sulfur ligands suggests that certain Pt-bound sulfur ligand can be substituted by other sulfur nucleophiles, and Pt can be transferred between various S-containing molecules which form the chemical basis to alleviate the acute platinum toxicity using chemoprotectants. This research concerned with the cytotoxicity studies of already prepared new Pt(II) complexes with S-donor ligands, mesna and dimesna; using ATP bioluminescence assay against HeLa, BEAS-2B and A549. The best results were obtained with A549 cell line and were not differing significalnty from the contol (cisplatin), Our fininding sugggest that there are potenitainls in delivering the chemoprotection with acceptable cytotoxicity.*

Keywords- *chemoprotectants, S-based ligand, Mesna, ATP bioluminescence assay*

I INTRODUCTION

High toxicity is a serious and annoying disadvantage of cisplatin, The major toxicities arise from cisplatin therapy are nephrotoxicity, ototoxicity, hepatotoxicity, gastrointestinal, neurotoxicity (S. Dasari, P.B. Tchounwou 2014, Rabbab et al 2018 , S. Manohar, N. Leung 2018)

These side effects are limiting the dose which can be administered and is thought to arise through a combination of the drug's non-specificity resulting in the damage of tissues rather than tumor such as the rapidly dividing cells of

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certain normal tissues including the ones in hair follicles, bone marrow, lining of the gastrointestinal tract, and platination of the sulfur residues on proteins by the soft platinum (II) center (Hannon, 2007; Rafique et al., 2010; Richa Prakash1et al., 2018. The ability to manage this induced toxicity is crucial for the success of chemotherapy (Richa et al., 2018).

The chief dose limiting side effect of cisplatin is nephrotoxicity; 20% of patients receiving high dose of cisplatin would suffer from severe renal dysfunction (Duffy et al., 2018). The kidney appears to take up and retain platinum to greater extent than other organs (Hausheer et al., 2011). Nephrotoxicity is seen because of increase in blood urea nitrogen (BUN) and creatinine, serum uric acid and/or a decrease in creatinine clearance and imbalanced electrolytes (http://www.accessdata.fda.gov. 2015 and Marija Petrovic, Danijela Todorovic 2016)

On the other hand, the long-term effects of cisplatin on renal function are not fully understood, but it's believed that cisplatin treatment may lead to a substantial and permanent reduction in glomerular filtration rate (Pabla and Dong, 2008).

Cisplatin is cleared by the kidney through both glomerular filtration and tubular secretion. Cisplatin concentration within the kidney exceeds that in blood suggesting an active accumulation of drug by renal parenchymal cells (Miller et al., 2010). Studies in rats and mice indicate that cisplatin undergoes metabolic activation in kidney turning it into a more potent toxin. This process begins with the formation of glutathione conjugates in the circulation, perhaps mediated by glutathione-S-transferase (Miller et al., 2010). The Pt-GSH conjugate is cleared to toxic metabolite mostly by gamma-glutamyl transpeptidase (GGT), an enzyme located in the brush boarder of the renal proximal tubule, forming a cysteine-glycine conjugate and are then cleaved by cell surface amino peptides, to form a cysteine conjugate, which is then reabsorbed by the proximal tubules and ultimately metabolized by C-S lyase into toxic thiol compounds resulting in nephrotoxicity (Peres and da Cunha Junior, 2013) as illustrated in figure 1.

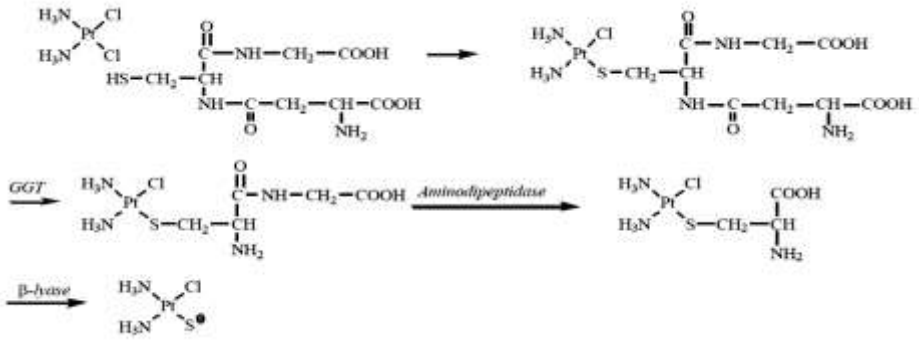

Figure 1. The pathway for the metabolism of cisplatin to a nephrotoxin, GGT: gammaglutamyl transpeptidase adopted from (Wang and Guo, 2007).

The concomitant administration of chemoprotective agents become an important approach in a platinum chemotherapy (Abhishek et al., 2016; Rohilla et al., 2019). Cytoprotectant or chemoprotectant agents are

administered to protect normal tissues and organs from the damaging effect of certain chemotherapy drug, radiation treatment and disease process (Kintzel, 2006). The affinity of thiols for platinum (II) complexes has led to the investigation of numerous sulfur-containing compounds as inhibitors of platinum nephrotoxicity (YukaYajima et al., 2017). As a chemoprotectant, it should modulate the side effects in a beneficial way, but not affect the antitumor activity of the drug, and with no or mild toxicity. Because of the preference of platinum for S donor ligands, the majority of the potential chemoprotectant explored for platinum-based therapy thus far are sulfur-containing compounds. The protective nature of these compounds is involved in prevention or reversal of Pt- S adduct in proteins. The potential reversibility of Pt-S bonds in presence of other sulfur ligands suggests that certain Pt-bound sulfur ligand can be substituted by other sulfur nucleophiles, and Pt can be transferred between various S-containing molecules (see Figure 2), which form the chemical basis to alleviate the acute platinum toxicity using chemoprotectants (Wang and Guo, 2007).

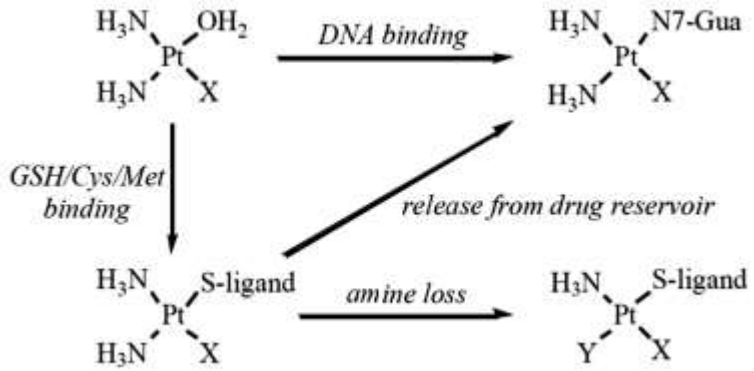

Figure 2. Intracellular competitive binding and inactivation of cisplatin derivatives in presence of sulfur-ligand and DNA (charge of complexes not shown; X= spectator ligand, Y= nucleophile) adapted from (Wang and Guo, 2007).

The Pt-S (thiol) bond can be broken in the presence of compounds known as "rescue agents" that are exclusively S-containing compounds (Bugarcic et al., 2012), like WR-2721, mesna, diethyl dithiocarbamate and thiosulfate (Lippert, 1999). The substitution of platinum- thioether to –thiolate is an important mechanism in the circumvention of cisplatin induced toxicity by thiol containing protective agent (Wang and Guo, 2007) as seen in figure 2.

The illustration in figure 3 shows that Mesna (Sodium 2-mercaptoethane sulfonate), (coenzyme M) (Cutler, 2010) are clinically used to prevent oxazaphosphorine-induced nephrotoxicity (Verschraagen et al., 2004). Mesna contains reactive thiol group with high nucleophilic reactivity that can bind Pt(II) complexes. Kangarloo et al., found that mesna has only a minor influence on the pharmacokinetics of cisplatin in plasma, which results in a decrease in the distribution of total Pt-species, providing that mesna is not mixed in solution with Pt-drug or infused through the same infusion line as the Pt-Drug (Kangarloo et al., 2004). Sadwitz et al, found that endogenous thiol intercept

cellular cisplatin. Their data showed that this phenomenon is less significant at a higher cisplatin concentration while therapeutic concentration of mesna does not decrease DNA platination by cisplatin (Sadwitz et al., 2002).

Yeh et al., found that the addition of mesna to cisplatin administration in female rats decreased the rate of fatal loss and provided protection against the loss of the reproduction function (Yeh et al., 2011).

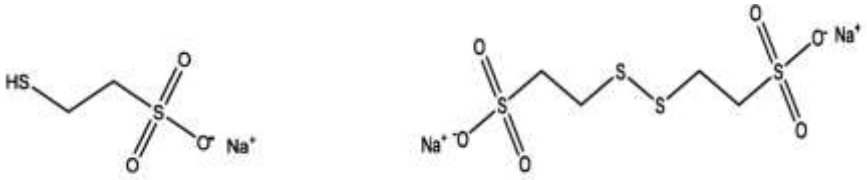

Figure 3. Mesna and dimesna adopted from (Cutler, 2010).

Martin et al., have synthesized rhenium and technetium complexes with mesna to be used as kidney radiopharmaceutical marker (Martin et al., 2003). Disulfied of Mesna (BNP7787, disodium 2,2`-dithio-bis-ethane sulfonate) (Verschraagen et al., 2004) is showen in figure 3. It is a water soluble investigational agent that is undergoing clinical development (Hausheer et al., 2011). As a disulfide, dimesna cannot conjugate cisplatin or its hydrated reactive metabolite. Upon entrying to the organ parenchyma, dimesna may be chemically reduced to active mesna which may form inactive adduct with intracellular cisplatin or its metabolites (YukaYajima et al., 2017) as explained in figure 4.

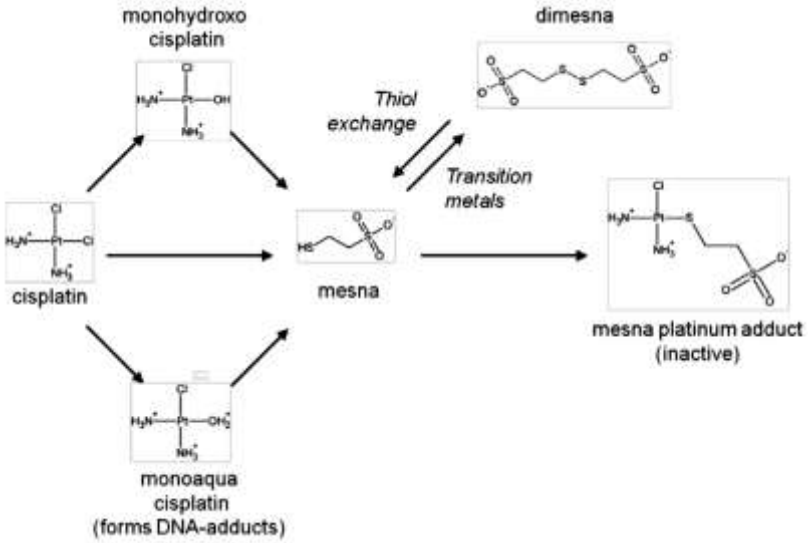

Figure 4. Conjugation of cisplatin and metabolites by mesna adopted from (Cutler, 2010).

Pendyala et al., found that BNP7787 has the potential to enhance antitumor activity by depleting the reactive thiols in plasma (Pendyala et al., 2003). Many efforts have been made to reduce the toxicity of Pt anticancer complexes. One of them is using a variety of sulfur-containing ligands as detoxificant agents against metal-containing drugs (Patel et al., 2014). The use of dimethylsulfoxide (DMSO) as a leaving group for anticancer

platinum (II) complexes was first proposed by Farell et al and this led to the preparation of a series of [PtCl(diamine)RR`SO]+ complexes. These complexes are more water soluble and less toxic than their chloride counterparts, and they maintain a high degree of biological activity, the antitumor activity of these complexes was found to be dependent on the nature of both the amine and the sulfoxide ligands and on the chirality of the sulfoxide leaving group (Farrell, 1982; Datt, 2001) as expressed in figure 5.

Pasini et al., prepared complexes of [pt(diamine) (L-S,O)]+ type, where (L-S,O) represent the leaving group; their mode of action is thought to be similar to the [PtCl(diamine)RR`SO]+ complexes which is mentioned previously (Pasini et al., 1995; Pasini and Moroni, 1997) as illustrated in figure 6.

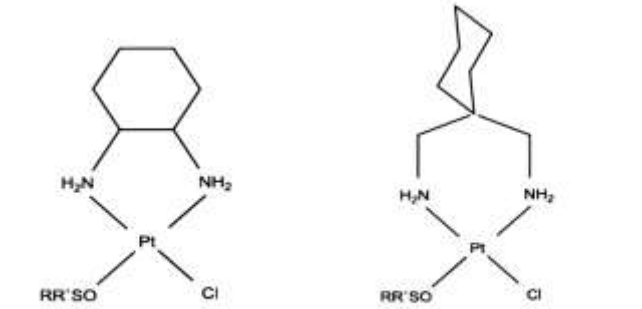

Figure 5. First prepared Pt(II) complexes with sulfur-donor ligands adopted from (Farrell, 1982).

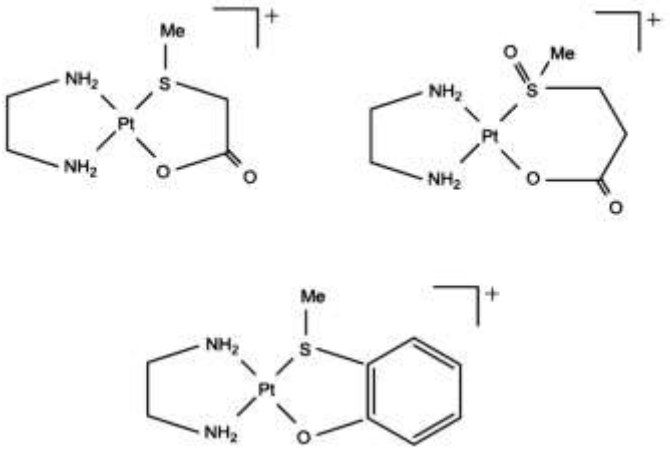

Figure 6. Examples of [Pt(diamine)(L-S,O)](Pasini and Moroni, 1997)

Torshizi et al., synthesized two new platinum (II) and palladium (II) complexes, both of which bear planar 2,2`-bipyridine ligand and attached a bidentate dithiocarbamate to Pt(II) and Pd(II) centers which can protect a variety of animal species from renal, gastrointestinal and bone marrow toxicity induced by cisplatin (Torshizi et al., 2014) as shown in figure 7.

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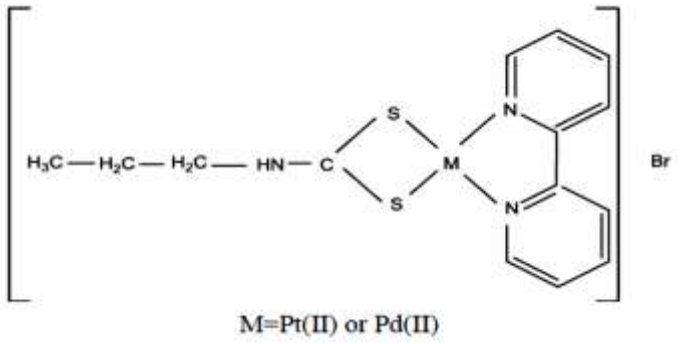


Figure 7. Platinum (II) complex with dithiocarbamate adopted from (Torshizi et al., 2014).

Some data showed that polysulfide also exhibited strong renal protective effects in cisplatin-induced nephrotoxicity by preventing renal dysfunction and apoptosis (Cao, et al 2018)

Based on the previous literatures the use of metal complexes as anticancer agent and the development of new Pt-containing drugs always continue with the aim of decreasing the severe side effects of Pt-drugs and enhance stability to metabolic deactivation by the coordination of S-donor nucleophiles.

A. SYNTHESIS OF DISODIUM2-MERCAPTOETHANESULFONATE (L2)

The previously designed compounds briefly prepared as follows (eq.1). A reaction of equivalent amount of sodium hydroxide (0.001mol, 0.04g) and mesna (0.001mol, 0.164 g) was prepared in (3 ml) of ethanol. Ethanol and it was boiled under reflux for 3 hours. Then the product was obtained through evaporation of the solvent, and the precipitate was washed with diethyl ether, then dried under vacuum for 4 hours. It is important to mention that mesna was dissolved in few drops of water before addition to the refluxing mixture because it will not dissolved in ethanol. The obtained product is an off-white powder, yield 77.7% (Nohad and Seema, 2017).

$$NaSO3CH2CH2SH + NaOH \rightarrow NaSO3CH2CH2SNa + H2O \quad \text{eq. 1}$$

B. SYNTHESIS OF THE COMPLEXES)

1:1 Complex (C1): Potassium tetrachloroplatinate (0.001 mol, 0.415g) was dissolved in 5 ml water and mixed with a solution of 0.001 mol mesna, which contained in 5 ml water and the resulting mixture was stirred continuously for about 2 hr. The reaction completion was checked by TLC and the solvent was later evaporated at room temperature near completion and a precipitate was formed by the addition of ethanol and then washed with (5 ml) diethylether and left to dry at room temperature over CaCl2 pellets in a desiccator. A ratio of 1:2 Complexes (C2 and C3): The same method was used but by taking (0.002 mol) of the ligand (L1 for C2 and L3 for C3). Solvent system used for TLC is Acetone: Hexane (5:1) (Nohad and Seema, 2017).

Our research is concerned with the cytotoxicity studies of a previously prepared novel Pt(II) complexes with S-donor ligands, mesna and dimesna (Seema, 2017); which are already in the clinical use as chemoprotectors. The cytotoxicity of these complexes was studied using ATP bioluminescence assay against HeLa, BEAS-2B and A549 cell lines.

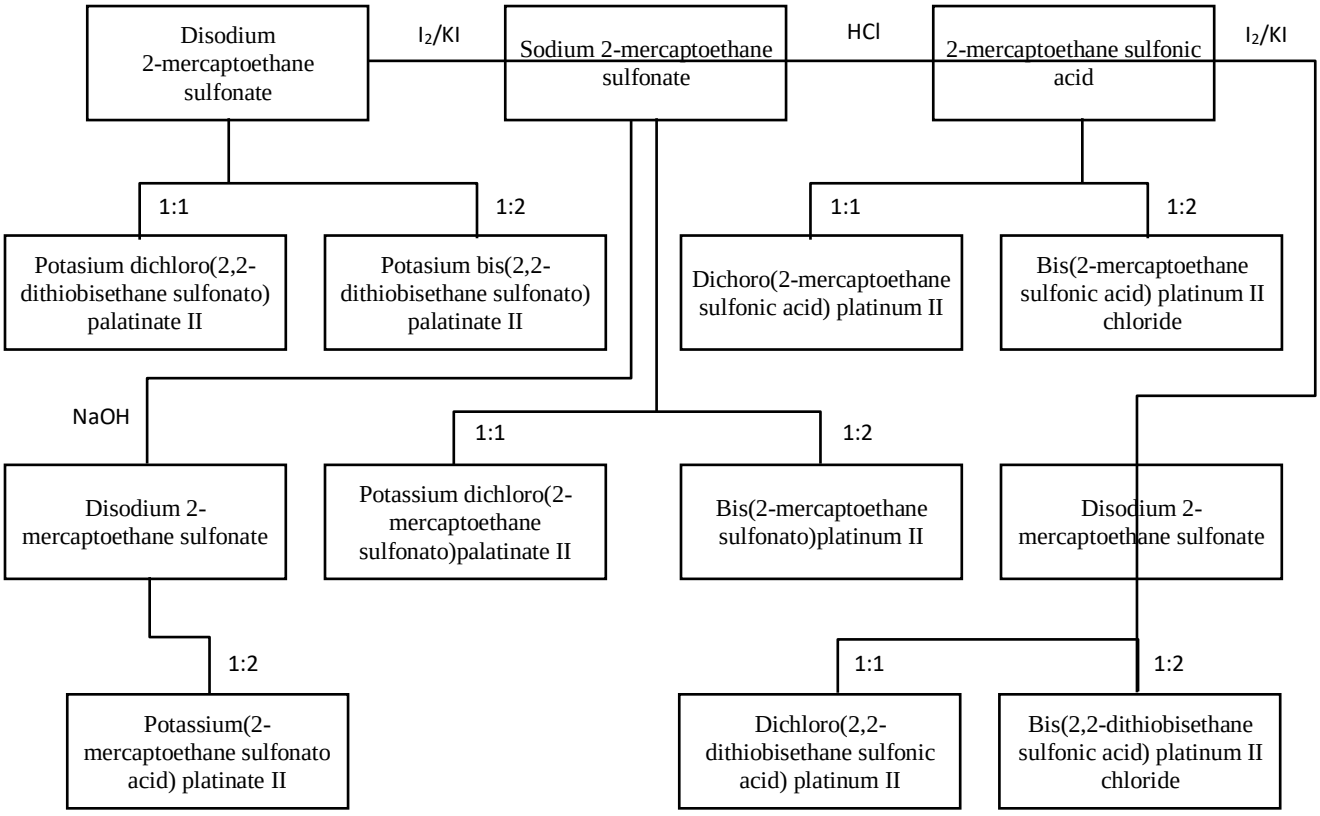


Figure 8. Outline scheme of all prepared compounds.

This study was done in the Department of Pharmaceutical Sciences, Biochemical Sciences and Health Section, University of Perugia, Italy. Using the ATP bioluminescent cell viability assay.

II METHOD

2.1 Materials

Chemicals

Via Light Plus Kit Lonza, a high sensitivity cell proliferation /cytotoxicity kit with extended signal stability, USA, was used in this study. The kit contains ATP monitoring reagent plus (AMR); lyophilized, assay buffer, cell lysis reagent. The culture medium consisted of RPMI 1640 with 2 mM glutamine, 10% FBS (fetal bovine serum), and 100 U (units) penicillin and 100 μg streptomycin/mL, referred as cRPMI. Confluent cultures were split using 0.25% trypsin/EDTA. Monolayers were incubated at room temperature for 5–10 min until cell detachment. Fresh

medium was added to disperse cells and suspensions were then centrifuged and adjusted at the desired concentration in culture medium.

The three cell line used were A549 (human lung adenocarcinoma epithelial cell line, ATCC CCL-185), BEAS-2B cells (derived from normal bronchial epithelium obtained from autopsy of noncancerous individuals and infected with a replication-defective SV40/adenovirus 12 hybrid, ATCC CRL-9609), HeLa cells (human cervix adenocarcinoma epithelial cell line, ATCC CCL-2). White opaque walled 96-well plates (Corning®, Coaster®, NY, USA).

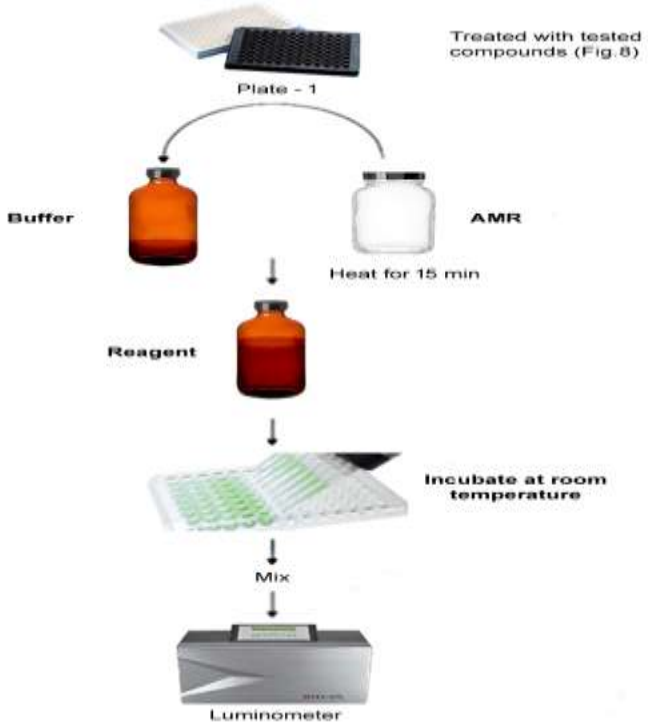
A photon emitted from the complexes after incubation with cell line were analyzed by Infinite pro200 (TECAN)

2.2. Procedure

A density of 1×104 /mL of HeLa, BEAS-2B or A549 cell per well were plated and incubated to have a monolayer in each well. Monolayer were then treated with different concentrations of compounds C1, C2, C3, C4, C5, C6, C7, C8, C9, L1, L2, L3, L4, L5 (0, 7.8, 15.6, 31.25, 62.5, 125, 250 µg/ mL) for 24 hours as illustrated in figure 8. Reconstitute ATP monitoring reagent (AMR) in tris-acetate buffer, leave for 15 minute at room temperature to ensure complete rehydration. Remove the culture plate from the incubator and allow cooling to room temperature for at least 15 minute. Program the luminometer to take a 1 second integrated reading of each appropriate well.

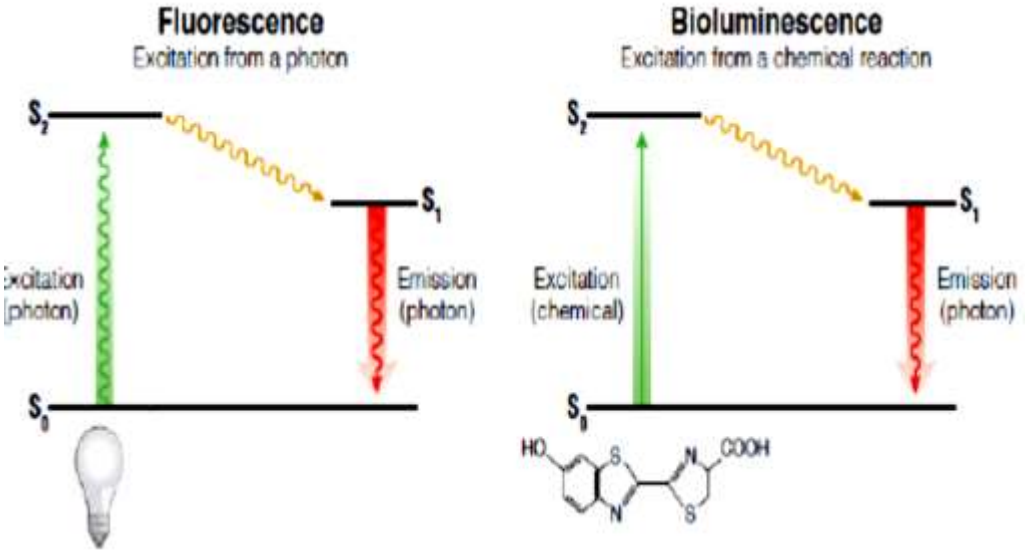
Add 50 µL of cell lysis reagent to each well and wait at least 10 minute. Add 100µL of AMR to each appropriate well and incubate for 2 minutes at room temperature. Place the plate in luminometer and initiate the program.

Results are expressed as 50% cytotoxic concentration (CC50) in µg/ml. The CC50 correspond the quantity of drug generating 50% of cell viability, compared to the control (cisplatin and 5-florouracil).



Figuer.9 Schematic outlining the protocol for the cytotoxic experiment

Modern drug programs are increasingly dependent upon cell-based assays as a means of screening compound libraries. These assays allow the researcher to address the potential global effects resulting from cytostatic or cytotoxic events that may affect the validity of specific assay measure (Worzella et al., 2008). The bioluminescence provides a unique technology for accurately quantifying physiological parameters within the cell. The advantages of bioluminescence technology is that it does not require an input light (photon), which eliminate problems with florescence interference (Promega, 2007) as seen in figure 10.



Figuer.10 Differences between florescence and bioluminescence adopted from (Worzella et al., 2008).

The bioluminescence assay using the adenosine triphosphate (ATP) - depended luciferin -luciferace system (eq. 2 and eq.3)become a popular method for ATP determination due to its high sensitivity and specificity (He et al., 2009).

Studies on cell lines and primary culture from ovarian and gastric cultures indicated that this assay is able to exhibit high sensitivity even at very low cell number (Dawson et al., 2011). It's a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells (Promega, 2007). Cells were lysed in the wells to extract ATP, and luciferase was added to catalyze the formation of light from ATP and luciferin (Crump et al., 2012). Light is proportionally emitted to metabolic activity and is quantified with a luminometer. A decrease in ATP indicate drug sensitivity, whereas no loss of ATP suggests that the tumor is resistant to the agent of interest (Kreahling and Altiok, 2015).

$$\text{ATP + Luciferin} \xrightarrow{\text{Luciferase / Mg+2}} \text{Adenyl- luciferin + PPi} \qquad \text{eq. 2}$$

$$\text{Adenyl- luciferin} + O_2 \longrightarrow \text{Oxyluciferin + AMP} + CO_2 + \text{Light} \qquad \text{eq.3}$$

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Luminescence measurements were determined for drug treated and control cell populations. The point at which the luminescence gave 50% of the value obtained for the non-drug treated cell population was determined and extrapolated to give the drug concentration which killed 50% of the tested cell population. The drug concentration was taken as CC50 in µg/ml and these values were represented as bar histograms. If no drug concentration killed at least 50% of a cell population, that population was deemed "resistant" to that drug (Dawson et al., 2011).

III RESULTS

In this work, the viability of cell lines (HeLa, BEAS-2B and A549) treated with the ligands (L1, L2, L3, L4, L5) and the complexes (C1, C2, C3, C4, C5, C6, C7, C8, C9) measured using luminescent cell viability assay using cisplatin and 5-florouracil as a control. Moreover, cytotoxicity was evaluated on fresh human PBMC. All in vitro experiments were repeated in triplicate. The concentration 250µg/ml was the highest tested one .Different cell lines showed a different susceptibility as expressed in histogram figures 11 (A, B, C).

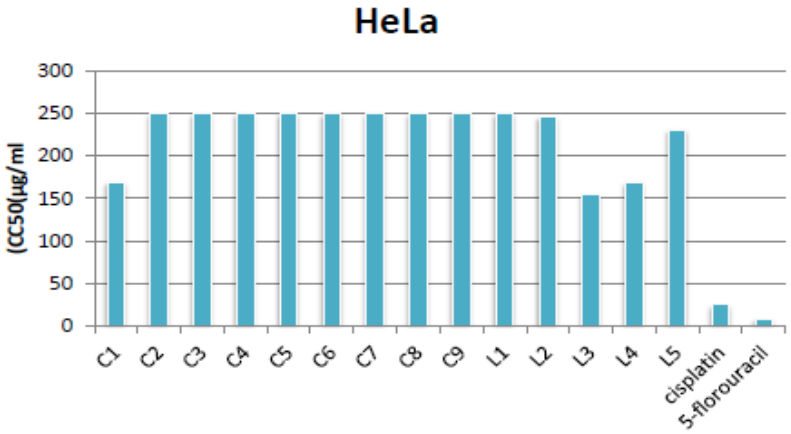

Figure 11A. The response of HeLa cell line to the ligands and the complexes.

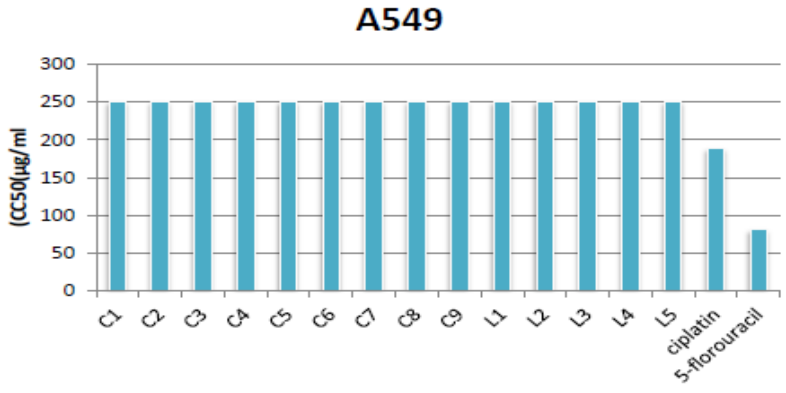

Figure 11B. The response of A549 cell line to the ligands and the complexes.

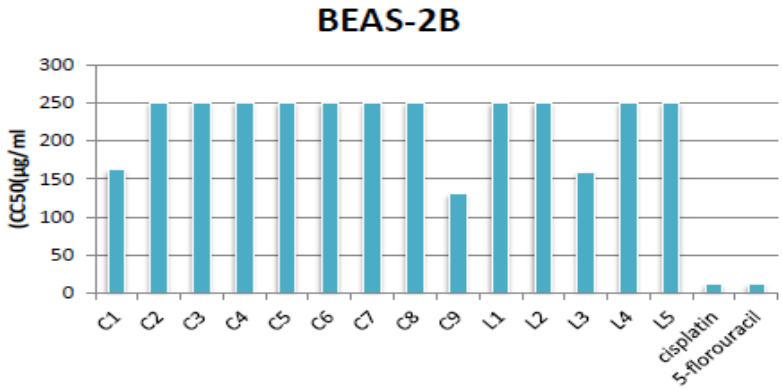

Figure 11C. The response of BEAS-2B cell line to the ligands and the complexes.

The best cyto-protection results were obtained with A549 cell line that were close to the used control cisplatin, indicating the possibility of producing a chemoprotection with acceptable cytotoxicity. All three cell lines showed the same viability's response towards complexes C2-C8 (seven out of nine); C1 showed variant CC50 µg/ mL, either parallel to other tested complexes as in A549 or nearly the same responses towards both HeLa and BEAS-2B, in other site C9 had the same cytotoxic level with other complexes against A549 and HeLa, but with less CC50 µg/ mL towards BEAS-2B. All Ligands under cytotoxicity screening

Showed either same histographs to the S-donor Platinum based complexes or less than. General speaking all complexes and their S- donor ligands were of pronounced lower toxicities to the cell panel under investigation compared to both positive control cisplatin and 5- Fluorouracil. The results showed that the complexes cytotoxicity is lower than cisplatin and 5-florouracil and this is in harmony with the previous literatures, it is probably due to the complexes with sulfur or as H2S and polysulfide can serve as an effective combination therapy with cisplatin, which reduced the adverse effect and the cytotoxicity (Xu et al., 2019; Cao et al., 2018).

IV CONCLUSIONS

Platinum complexes with sulfur donor ligand were prepared in an attempt to reduce the toxic effect of platinum compounds on the kidney by reducing or reversing the interaction of platinum atom with sulfur containing biomolecules in the body. The mesna, which is used as rescue agent for platinum and ifosfamide was used as a ligand. Cytotoxicity study by ATP-bioluminescence assay on HeLa, BEAS-2B and A549 cell line gave indication that the activity of these complexes is lower than cisplatin except on A549, it was not largely different from that of cisplatin , this give a positive correlation with S- donar Platinum complex activities and their cytotoxicity as a potential cytoprotective explored for platinum based therapy . Enzymatic study to determine the effect of complexes on urinary tubular enzymes like Glutathion-S-transferase (GST) and Gamma glutamyl transpeptidas (GGT) for the

detection of kidney injury are recommended for further study , in addition the synthesis and cytoprotection study of more types of S- donors platinum –based therapy might be more warranted in the future.

V Acknowledgment

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