Effect of cisplatin on glutathione redox status in isolated plasma and cytosolic fraction

Kifayatullah Shah1*, Muhammad Farid Khan1, Amir Badshah2, Syed Umer Jan3, Arshad Farid1, Hadia Bibi2 and Haroon Khan1

1Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gomal University, D. I. Khan, Khyber-Pakhtoonkha, Pakistan.
2Department of Pharmacy, University of Peshawar, Peshawar, Khyber-Pakhtoonkhwa, Pakistan.
3Department of Pharmacy, University of Baluchistan, Quetta, Pakistan.

Accepted 2 October, 2012

Cisplatin has been used therapeutically in the treatment of malignant tumors. Meanwhile, the major limitations associated with cisplatin are its side effects in the form of nephrotoxicity, neurotoxicity, emetogenesis and emerging resistance. Most of these problems are due to its adverse effects on the body’s endogenous cytoprotective molecules like glutathione (GSH). In current study, the effect of glutathione on the improvement of cisplatin therapy along with control on its growing problem of resistance was emphasized. The effect of cisplatin on the chemical and metabolic status of glutathione was evaluated in human venous blood after its separation into plasma and cellular fraction using ultraviolet (UV)-visible spectrophotometer. The glutathione in isolated plasma and cellular fractions of the blood was exposed to different concentrations of cisplatin. It was found that there was a gradual depletion in the concentration of reduced glutathione. Similarly, time-dependent effect of cisplatin was also evaluated on the status of glutathione, in which positive correlation was found between exposure of glutathione to the given concentrations of cisplatin and the depletion of reduced glutathione as the time passed from 0 to 5 h. This depletion in the concentration of reduced GSH is either due to formation of Pt-SG complex or due to the conversion of this multifunctional molecule (glutathione) to its physiologically inactive disulfide form (GSSG). This study was carried out in vitro, which in principle depicts a model of in vivo reaction. This decrease in blood GSH levels after cisplatin treatment will result in decreased antioxidant capacity of the blood, which in turn will result in numerous pathological conditions.

Key words: Reduced glutathione (GSH), plasma and cellular fractions of blood, Ellman’s method, cisplatin.

INTRODUCTION

Glutathione is the most abundant cytoprotective thiol that maintains redox environment of the cell, and thus helps in the vitality of the body cells (Guoyao et al., 2004). In the human body, glutathione mostly exist in its reduced form (GSH), but it can be oxidized by many factors including free radicals and during intoxication reactions. When GSH/GSSG ratio shifts toward the oxidizing state, it results in the activation of several signaling pathways, thereby reducing cell proliferation and increasing apoptosis (Sen, 2000). Thus, oxidative stress acts as key factor in the pathogenesis of many diseases, including cancer, inflammation and diabetes mellitus (Turrens, 2003), which can be minimized by glutathione. Glutathione is formed from three constituent’s amino acids namely glutamic acid, cysteine and glycine. Most of the cellular GSH (85-90%) is present in the cytosol, with the remainder being in many organelles (including the mitochondria, nuclear matrix, and peroxisomes). With the

*Corresponding author. E-mail: kifayatrph@gmail.com.
exception of bile acid, which may contain up to 10 mmol/L GSH, extracellular concentrations of GSH are relatively low (e.g., 2-20 µmol/L in plasma (Jones, 2002). There are three major thiol-containing molecules in the erythrocytes namely hemoglobin, glutathione and ergothione. The concentration of GSH found in erythrocytes is about 2-3 mM. In leukocytes, the concentration of GSH is 5-10 mM and maximum concentration exists in hepatocytes (7-14 mM) because metabolism and most of other cellular reaction occurs in the liver. Many investigators (Bashandy et al., 2011; Suhair and Hamdi, 2011; Etuk et al., 2009) have proven the protective role of GSH in poisoning caused by plant extracts.

In view of the fact that a vast majority of cytotoxic metal-containing compounds including cisplatin are administered intravenously, special consideration should therefore be given to interactions of these metal drugs with macromolecular blood components like glutathione that can be taken up and get accumulated in tumor tissue (Kratz, 1993). cis-Dichlorodiamineplatinum(II) or cisplatin is a frequently used and very effective chemo-therapeutic drug for the treatment of various malignancies (Rosenberg, 1985; Prasad and Giri, 1994). However, high-doses administered to patients produce dose-dependent nephrotoxic and hepatotoxic side effects (Jordan and Carmo-Fonseca, 2000; Yoshida et al., 2000; Pratibha et al., 2006). Formation of free radicals, leading to oxidative stress, has been shown to be one of the pathogenic mechanisms of these side effects (Jordan and Carmo-Fonseca, 2000). The treatment of tumor cells with cisplatin provokes several responses, including membrane peroxidation, dysfunction of mitochondria, inhibition of protein synthesis and DNA damage (Cohen and Lippard, 2001; Sadowitz et al., 2002). But the ability of cisplatin to react with DNA and the formation of cisplatin-DNA adducts are thought to be the main mechanisms underlying its cytotoxic action (Pinto and Lippard, 1985; Zamble and Lippard, 1995). Therefore, in current study the effect of cisplatin on the redox status of glutathione is emphasized because there is an increasing amount of evidence that cisplatin-induced cytotoxicity is due to oxidative damage resulting from free radical generation and that the administration of antioxidants is efficient in inhibiting these side effects.

**MATERIALS AND METHODS**

The followings were used: L-Glutathione (GSH) (Fluka), cisplatin or cis-dichlorodiamineplatinum(II) (Korea United Pharm.Inc), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma), sodium hydroxide (Fluka AG), potassium dihydrogen phosphate (Merck), HCl 35% (Kolchigh AG), disodium edetate (Riedel Dehean AG Sleeze Hannover), sodium chloride (Merck), chloroform (Merck), ethanol (Merck), distilled water (double distilled), pH 7.6 buffer solution. The instruments used included: ultraviolet (UV)/visible 1601 spectrophotometer (Shimadzu), pH meter: NOV-210 (Nova scientific company Ltd. Korea), oven: Memmert Model U-30.854 (Schwabach, Germany), magnetic stirrer, hot plate: 400 (England), sensitive Sartorius weighing balance.

**Preparation of stock solutions**

Briefly, 100 ml of 0.9% NaCl solution was prepared by dissolving 90 mg of a pharmaceutical grade sodium chloride in sufficient quantity of water. Cisplatin injection (UNISTIN 10 mg/20 ml), containing 1.7 mM of cisplatin base was diluted to 1.0 mM by adding 14.483 ml of water for injection to make 35 ml of cisplatin (1.0 mM) isotonic solution. Glutathione (1.0 mM) standard solution was prepared by dissolving 30.75 mg of GSH in 100 ml of 0.1 N HCl. Also, 200 ml of phosphate buffer (0.2 M) having pH of 7.6 was prepared by mixing 50 ml of monobasic potassium phosphate (KH2PO4) solution (0.2 M) with 42.2 ml of NaOH (0.2 M) and making the volume up to 200 ml with distilled water. Finally, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (1.0 mM) was prepared by dissolving 39.6 mg of DTNB in 0.2 M phosphate buffer (pH 7.6) to make 100 ml of DTNB solution.

**Preparation of biological samples**

**Isolation of plasma**

Venous blood was taken from a healthy human volunteer in a heparinized plastic bag. About 1.8 ml of this blood was then transferred by means of a disposable syringe (dipped in 0.5 M disodium edetate solution) to each of 2.0 ml Eppendorf’s test tubes. Subsequently, the blood was centrifuged at 3600 rpm for 15 min, resulting in the precipitation of red blood cells. The supernatant layer of plasma, about 0.5 ml from each of the Eppendorf’s tubes was taken and mixed with 50 µL of 5 mM disodium edetate solution and then placed in refrigerator at 4°C till use.

**Isolation of cytosolic fraction**

The red blood cell fractions left after isolation of plasma in the test tubes earlier mentioned was taken and washed three times with 0.9% NaCl solution. This fraction of blood was centrifuged at 3000 rpm softly for the next 5 min. The supernatant layer was discarded and 0.5 ml of red blood cell fraction thus obtained was then mixed with 0.5 ml of distilled water. It was then placed in a refrigerator at 4°C for 1 h to induce lyses of red blood cells. Afterward, 0.6 ml of chloroform: ethanol (3:5) mixture was added to each of the above test tubes, mixed thoroughly to precipitate hemoglobin, followed by the addition of 0.1 ml of water. These mixtures were then centrifuged hard for 10 min at 10000-12000 rpm. The pale yellow supernatant layer (cytosolic fraction) from each test tube was collected and kept in refrigerator at 4°C till use.

**Determination of glutathione content after treatment with cisplatin**

Both the extracellular (plasma) and intracellular (Lysate) glutathione content estimation after treatment with different concentration of cisplatin were carried out using modified Ellman’s (DTNB) method (Ellman’s, 1959). To 1 ml (1000 µL) of plasma and/or cytosolic fraction taken in five separate test tubes, 1 ml (1000 µL) of 0.2, 0.4, 0.6, 0.8 and 1.0 mM isotonic solutions of cisplatin were added separately and shaken to obtain cisplatin plus plasma and/or cytosolic fraction stock mixtures. The final concentrations of cisplatin in these stock mixtures were 0.1 mM (100 µM), 0.2 mM (200 µM), 0.3 mM (300 µM), 0.4 mM (400 µM) and 0.5 mM (500 µM). Next, the test samples were prepared for "0" time readings by taking 0.2 ml (200 µL) of cisplatin plus plasma and/or cytosolic fraction stock mixture from each of the previously prepared test
tubes, and they were separately diluted with 2.3 ml (2300 µL) of phosphate buffer pH 7.6 followed by the addition of 0.5 ml (500 µL) of 1 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) stock solution into each of the test samples. The final concentrations of cisplatin were 0.00666 mM (06.66 µM), 0.01333 mM (13.33 µM), 0.02000 mM (20.00 µM), 0.02666 mM (26.66 µM) and 0.03333 mM (33.33 µM), respectively in each of the test samples. Finally, the mixtures were shaken and incubated in room temperature for 5 min after which the absorbances were recorded at fixed wavelength of 412 nm.

Similarly, for taking readings of plasma and/or cytosolic fraction glutathione (GSH) control solution (Blank solution) at 0 h, 1.0 ml (1000 µL) of plasma and/or cytosolic fraction were taken in a test tube and diluted with 1.0 ml (1000 µL) of phosphate buffer pH 7.6.

Then, 0.2 ml of this plasma and/or cytosolic fraction were added to 2.3 ml of phosphate buffer (pH 7.6), followed by the addition of 0.5 ml of 1.0 mM DTNB stock solution in a test tube. This mixture was shaken thoroughly and incubated at room temperature for 5 min. The absorbances were then recorded at 00, 01, 02, 03, 04 and 05 h intervals after repeating the same steps for preparation of GSH blank solution of plasma and/or cytosolic fraction. As earlier mentioned for 0 h reading, test samples were prepared from each of the cisplatin plus plasma and/or cytosolic fraction stock mixtures by the same procedure as aforementioned after every one hour for five hours, and the absorbances were recorded at 412 nm (Amax for thiolate anion TNB) using UV-Visible spectrophotometer after preparing final mixture. Thus, the effect of cisplatin on the chemical status of glutathione in plasma and/or cytosolic fraction was studied in terms of determination of the absorbencies, which were then converted into concentration of GSH in mixtures by Elman’s method.

\[ Y = mx + b \] (specifically: \[ y = 0.172x \cdot 0.025 \])

The correlation coefficient (R²) with a value of 0.999 indicates a good regression within the given range of concentrations that will be analyzed in this study. The absorption of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) blank solution was also obtained at fixed wavelength of 412 nm.

### RESULTS

#### Interaction of cisplatin with plasma glutathione level

Extracellular GSH content was measured after exposure to different concentrations of cisplatin for different period of time. Glutathione content in isolated plasma fraction of blood showed gradual decrease in GSH content as it was exposed to increasing concentrations of cisplatin as shown in Table 1. Similarly, GSH content was also found to be proportionally decreased as it was exposed to cisplatin for increased period of time (0 to 5 h). Therefore, the change in GSH content was statistically significant (p < 0.05) as shown in Figures 1 to 5.

### Interaction of cisplatin with intracellular GSH

The intracellular (cytosolic fraction) GSH content was measured after exposure to different concentrations of cisplatin for varying period of time. Glutathione content in isolated cytosolic fraction of blood showed gradual decrease in GSH content as it was exposed to increasing concentrations of cisplatin as shown in Table 2. Similarly, GSH content was also found to be proportionally decreased as it is exposed to Cisplatin for increased period of time (0 to 5h). Hence, the change in GSH content was statistically significant (p <0.05) as shown in Figures 6 to 10.

### DISCUSSION

Biological thiols are gaining increasing interest of researchers because of their emerging use as

<table>
<thead>
<tr>
<th>S/N</th>
<th>Conc. of cisplatin (µM)</th>
<th>Real abs/ GSH conc. at 0 h</th>
<th>Real abs/ GSH conc. at 1 h</th>
<th>Real abs/ GSH conc. at 2 h</th>
<th>Real abs/ GSH conc. at 3 h</th>
<th>Real abs/ GSH conc. at 4 h</th>
<th>Real abs/ GSH conc. at 5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.67</td>
<td>0.434</td>
<td>2.67</td>
<td>0.441</td>
<td>2.57</td>
<td>0.396</td>
<td>2.45</td>
</tr>
<tr>
<td>2</td>
<td>13.34</td>
<td>0.392</td>
<td>2.42</td>
<td>0.367</td>
<td>2.28</td>
<td>0.342</td>
<td>2.13</td>
</tr>
<tr>
<td>3</td>
<td>20.00</td>
<td>0.351</td>
<td>2.19</td>
<td>0.334</td>
<td>2.09</td>
<td>0.321</td>
<td>2.01</td>
</tr>
<tr>
<td>4</td>
<td>26.67</td>
<td>0.311</td>
<td>1.95</td>
<td>0.286</td>
<td>1.81</td>
<td>0.266</td>
<td>1.69</td>
</tr>
<tr>
<td>5</td>
<td>33.33</td>
<td>0.277</td>
<td>2.67</td>
<td>0.256</td>
<td>1.63</td>
<td>0.233</td>
<td>1.50</td>
</tr>
</tbody>
</table>
Table 2. Effect of different concentrations of cisplatin on the chemical status of glutathione (GSH) with time in cytosolic fraction after separation of blood.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Conc. of cisplatin (mM)</th>
<th>Real abs*/ Conc. at 0 h</th>
<th>Real abs*/ Conc. at 1 h</th>
<th>Real abs*/ Conc. at 2 h</th>
<th>Real abs*/ Conc. at 3 h</th>
<th>Real abs*/ Conc. at 4 h</th>
<th>Real abs*/ Conc. at 5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.67</td>
<td>0.475</td>
<td>2.91</td>
<td>0.454</td>
<td>2.78</td>
<td>0.438</td>
<td>2.69</td>
</tr>
<tr>
<td>2</td>
<td>13.34</td>
<td>0.421</td>
<td>2.59</td>
<td>0.396</td>
<td>2.45</td>
<td>0.383</td>
<td>2.37</td>
</tr>
<tr>
<td>3</td>
<td>20.00</td>
<td>0.384</td>
<td>2.38</td>
<td>0.361</td>
<td>2.24</td>
<td>0.343</td>
<td>2.14</td>
</tr>
<tr>
<td>4</td>
<td>26.67</td>
<td>0.347</td>
<td>2.16</td>
<td>0.323</td>
<td>2.02</td>
<td>0.311</td>
<td>1.95</td>
</tr>
<tr>
<td>5</td>
<td>33.3</td>
<td>0.296</td>
<td>1.87</td>
<td>0.272</td>
<td>1.73</td>
<td>0.254</td>
<td>1.62</td>
</tr>
</tbody>
</table>

Real abs./conc. for blank GSH: 0.566, 3.44, 0.562, 3.41, 0.563, 3.42, 0.558, 3.39, 0.555, 3.37, 0.554, 3.37

Abs.: Absorbance; Conc.: concentration. Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank solution was 0.064 at 412nm. *Real Absorbance = Absorbance of mixture - Absorbance of DTNB blank solution.

Figure 1. Effect of cisplatin (6.67 µM) with time on isolated Plasma GSH. ■ Control Plasma GSH; ♦ Cisplatin + GSH. Results are the mean ± SEM of 3 experiments of plasma GSH.

Biomarkers of disease status. Besides their vital role in antioxidant biochemistry, thiols have functions such as: synthesis and maintenance of proteins structure and activity, redox sensitive signal transduction and receptor modification, cell growth and proliferation, apoptosis, xenobiotic
metabolism, and immune regulation (Sen, 1998). Glutathione (GSH), an endogenous intracellular thiol containing tripeptide is an important thiol that is mainly the center of concern in cancer therapy (Arrick and Nathan, 1984). In the present study, our main interest was to ascertain the interaction of cisplatin with important biomolecule, glutathione at ex. vivo level as a model of in vivo reaction to depict the picture of its acute toxicity. Glutathione-S-transferase is the focal point of the detoxification system that protect cells from oxidative and chemicals induced toxicity by promoting the conjugation between the thiol (SH) group of glutathione and the electrophilic moiety of toxic substrates, including cisplatin (Welters et al., 2001).

According to our findings, when glutathione, the most abundant thiol in the cell that is maintained in reduced form by NADPH-dependent glutathione reductase (Wang and Ballatori, 1998) in the plasma and cellular fraction of the blood was exposed to various concentrations of cisplatin, there was significant decrease in the concentration of reduced glutathione. This shows that cisplatin causes an increased deployment of reduced glutathione.

Figure 2. Effect of cisplatin (13.33 µM) with time on isolated Plasma GSH. ■ Control plasma GSH; ♦ Cisplatin + GSH. Results are the mean ± SEM of 3 experiments of plasma GSH.

Figure 3. Effect of cisplatin (20.00 µM) with time on isolated Plasma GSH. ■ Control plasma GSH; ♦ Cisplatin + GSH. Results are the mean ± SEM of 3 experiments of plasma GSH.
(GSH) and converts this multifunctional molecule to its oxidized or disulfide form (GSSG). The concentration of GSH in the samples after treatment with varying concentration of cisplatin was determined using $\lambda_{max}$ as 412 nm according to a well known Elman's method (Ellman, 1959). The concentrations of cisplatin that were used during the experiments range from 6.67 - 33.34 $\mu$M. The pH was maintained at 7.6 by phosphate buffer, which is nearly equal to body pH and was proposed to be suitable for performing the *in vitro* experiments on thiols by Evans (1975).

Our results confirmed the relationship between cisplatin-mediated toxicity and decreased GSH levels, which has been previously observed in Dalton's lymphoma cells. When glutathione-S-transferase activity was assayed in Dalton's lymphoma cells, it was found to be decreased by 60-80% after cisplatin treatment. However, the low activity of glutathione-S-transferase along with lower Glutathione concentration in Dalton's lymphoma cells suggests the possibility of a reduced conjugation of GSH with cisplatin because it is known that cisplatin-GSH conjugates can be formed directly or
catalyzed by GST (Ishikawa and Osman, 1993). This may also suggest reduced elimination of the drug through export pumps and availability of more drugs in tumor cells causing cytotoxic effects. Glutathione-S-transferase is the focal point of the detoxification system that protects cells from oxidative and chemical-induced toxicity by promoting the conjugation between the thiol (SH) group of glutathione and the electrophilic moiety of toxic substrates, including cisplatin (Welters et al., 2001). Previous studies have shown that resistance to cisplatin develop due to: (1) reduction in the uptake of the drug; (2) increased detoxification and excretion by conjugation of cisplatin by thiols; and (3) changes in the capability of the cell to recognize and process cisplatin - DNA adducts which in turn triggers apoptosis (Fink et al., 1998). If any of the aforementioned processes fail to function properly, then it may lead to resistance to cisplatin.

During this study, time-dependent effect of different concentrations of cisplatin on the chemical status of glutathione was also observed and it was found that there was a gradual depletion of reduced glutathione as the time passed from 0 to 5 h, showing that cisplatin is responsible for depletion of glutathione. The decreased level of glutathione in the body then contributes to
nephrotoxicity, mutagenicity and defects in immune response. Indeed, when GSH levels were increased in the hosts, the nephrotoxic as well as mutagenic effects of cisplatin treatment in cancer were found to be decreased (Della Rovere et al., 2000). It is depicted from the data obtained herein that the cytotoxicity of cisplatin is due to cisplatin-induced biochemical changes in plasma and cellular fraction of blood, effecting buffering capacity of glutathione and metabolic processes within the cell.

**Conclusion**

The interaction of cisplatin with glutathione causes reduction in the level of glutathione in the body due to the formation of Pt-SG complex or conversion of reduced GSH to its disulfide form (GSSG), which in turn predisposes us to numerous pathological conditions. It is thus concluded that antioxidant supplementation would be beneficial to increase the concentration of GSH that is reduced due to anticancer therapy of cisplatin.

**ACKNOWLEDGEMENTS**

We are thankful to Prof. Dr. Gul Majeed Khan, Dean, Faculty of Pharmacy and Mr. Nusratullah Khan, Chairman, Department of Pharmaceutical Chemistry,
Gomal University D. I. Khan, for providing necessary work place and essential instruments/equipments. They were kind enough to provide all the chemicals required during the research work.

REFERENCES


