Pure Appl. Chem., Vol. 85, No. 2, pp. 365–375, 2013.
http://dx.doi.org/10.1351/PAC-CON-12-06-10
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The role of metal coordination complexes in cytosolic cellular defense*

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Abstract: The metal coordination complexes are known to induce cytotoxic effects on various cell lines and shown to have great potential for therapeutic interventions. Their main mechanism of action is through the mediation of enzyme activities in signaling pathways essential for cellular functioning. The overall cellular responses are dose-dependent and require high exposure levels and duration to overcome cellular defense against external toxicants. However, their effect through signal transduction components is limited due to the conferred drug resistance associated with glutathione transferase (GST)-mediated mechanisms. The GST family of enzymes is not only related to anticancer drug resistance, but also associated with cancer development where they may also contribute kinase signaling events including non-receptor protein tyrosine kinase (PTK)-related pathways. In the current study, we evaluated the effect of symmetrical and mononuclear complexes of Pd(II), Pt(II), and Ni(II) with organic ligands on cytosolic targets involved in glutathione utilization, antioxidant defense, and kinase signaling by virtue of acellular in vitro analyses.

Keywords: anticancer activity; antioxidant activity; bioactive molecules; biomolecular chemistry; biotechnology; bioinorganic chemistry; bioanalytical methods; drug resistance; enzyme inhibitors; glutathione metabolizing enzymes; protein tyrosine kinase; symmetrical metal complexes.

INTRODUCTION

The use of metal coordination complexes in drug discovery research begins with the effective use of cisplatin for cancer chemotherapy [1–3]. Besides the fact that cisplatin is the most successful metal complex with anticancer activity, its clinical use is limited due to severe side effects, such as myelotoxicity, nephrotoxicity, neurotoxicity, and peripheral neuropathy [1,2]. Then, the continuous efforts in the search for biologically active metal complexes resulted in the synthesis of many structures with different metal centers, and followed by their evaluation for cytotoxicity on various cancer cells. Among those, some of the platinum complexes have shown promise for therapeutic intervention. However, carboplatin and oxaliplatin are amongst very few that have found a role in clinical treatment of solid tumors, usually in combination with other chemotherapeutic agents [1–5]. In addition, numerous non-platinum complexes were found to be ineffective by comparison with their platinum-based reference structures [5,6]. The successors of cisplatin, namely, carboplatin and oxaliplatin, are shown to have similar therapeutic efficacies on solid tumors, but with lower side effects than cisplatin. Structure–activity

Pure Appl. Chem. **85**, 315–462 (2013). A collection of invited papers based on presentations at the 12th Eurasia Conference on Chemical Sciences, Corfu, Greece, 16–21 April 2012.
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studies with cisplatin, carboplatin, and oxaliplatin revealed that these drugs exert antitumor activity by inducing apoptosis through similar mechanisms, where DNA or cellular enzymes are drug targets. In these mechanisms, drugs that interact with DNA are shown to cause DNA strand break or DNA-complex adduct formation [7,8]. For drug–enzyme interaction, on the other hand, the outcome is the altered enzyme activities important in signaling pathways, which are essential for cellular functioning [9,10]. Of these drug targets, DNA is proven to be the main biological target of the metal complexes. Under normal circumstances, the increased metabolic rate of cancer cells facilitates the uptake of complexes, and the achieved complex concentration facilitates their interaction with DNA [11]. Therefore, to develop complexes with tolerable toxicity and enhanced efficacy, the researchers in the field are recently more focused on the interactions of metal complexes with biomolecules inside the cells. Actually, the cellular defense provided by the detoxification system against internal and external toxicants, including metal complexes, is shown to result in drug resistance by increased DNA repair activity and reduced DNA-drug complex formation [12–14]. This resistance prevents cells from accumulating drugs in nucleus where the drug's main biological target (DNA) resides, and may occur in different cell types by different cellular mechanisms (Fig. 1, as reviewed in ref. [12]). Furthermore, sulfhydryl molecules (thiols), such as cellular glutathione (GSH), can interact with metal centers and inhibit their biological effectiveness. This may arise from the accumulation of thiols, such as GSH, or increased thiol reflux through the reaction cycles of antioxidant defense and detoxification system enzymes that employ thiols. In this context, overactive glutathione utilizing enzymes, such as glutathione transferases (GSTs), are directly linked with platinum-based resistance [9,12,14–17]. GSTs are the member of the phase II detoxification system enzymes that catalyze the nucleophilic addition of GSH to diverse molecules, including metal complexes, and interfere with drug transport into nucleus [12,16,18–20]. They have critical role in cellular defense against toxic agents from internal and external sources, while exhibiting both transferase and peroxidase activities [20,21]. On the other hand, distinct from GSTs, glutathione peroxidases (GPXs) also utilize GSH while removing the accumulated peroxides in cytosol.

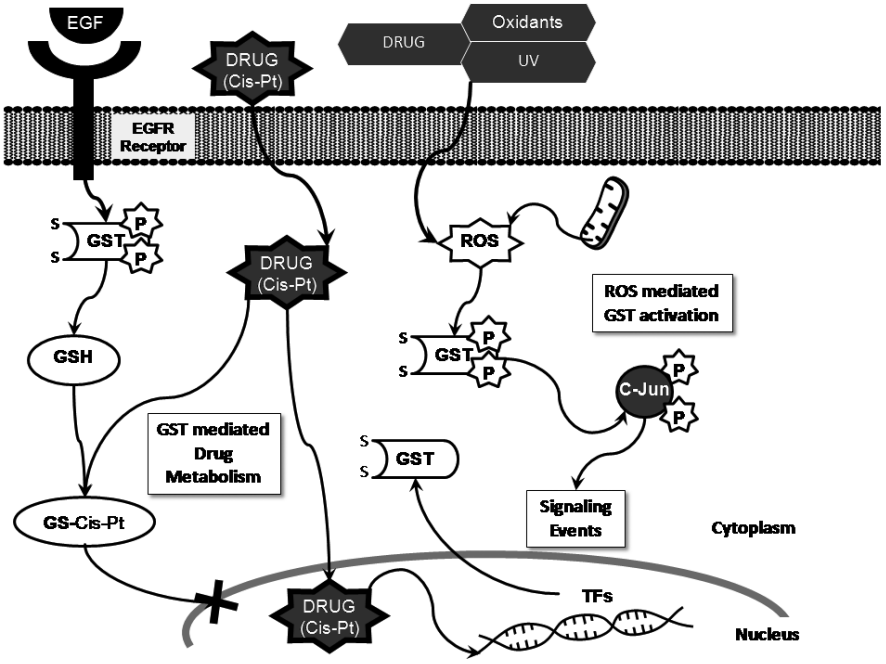

Fig. 1 The cellular mechanisms involved in GST-mediated drug resistance and relation with kinase-activated mechanisms and antioxidant defense (ref. [12]).

Both GST and GPX are critical components of antioxidant defense and play a protective role against oxidative stress-induced apoptosis. Therefore, their altered activities are related to either oxidative DNA damage or excessive apoptosis prevention, and both are a very strong reason for cancer susceptibility [21,22]. In addition to GPX, catalase (CAT), as another antioxidant system component, is also employed in peroxide removal, but located in a different cellular compartment than GPX. Similar to GPX and GST, CAT is also implicated in the growth and spread of cancer cells, and is a factor in the development of drug resistance [23]. The varying tissue level of CAT in different cancer types is defined as a possible cause for resistance to metal complex-based drugs, particularly in tissues with elevated CAT activities [24,25]. Another mechanism that reduces the possible interaction between DNA and metal complex involves the regulatory proteins or enzymes. Depending on the tissue level and activity of regulatory proteins in signaling pathways, these proteins, such as kinases, may prevent metal complexes to reach DNA [9,12]. Enzymes of the protein tyrosine kinase (PTK) family are one of the largest groups of regulatory proteins that participate in that kind of drug resistance. These enzymes represent approximately 17 % of human kinome, and constitute more than 60 % of oncogenes and proto-oncogenes of human genome. Their improper function is not only the characteristics of numerous diseases, but also the reason for lessened anticancer drug action [12,26,27]. PTK-involved signal transduction pathways are also affected by altered cellular detoxification and antioxidant defense systems and cause the reduced effectiveness of therapeutics [12,28]. Moreover, the deregulations in PTK signaling, as reported recently, are one of the possible causes for GST over activity and related drug resistance [12,26,27]. Owing to the fact that cytoplasm is rich in CAT, GPX, GST, and PTK, these enzymes may provide the main barrier for drugs, such as metal coordination complexes, to pass through to reach the nucleus. Therefore, it may be promising to enhance therapeutic efficacy of metal complexes with capacity to modulate activities of CAT, GPX, GSTs, and PTK, by introducing suitable ligands.

Amongst many metals investigated in the search for novel non-platinum complexes, Pd(II) has been extensively studied, whereas more limited studies have been undertaken on Ni(II), owing to toxicity associated with excessive exposure in industrial environments. Nickel is responsible for the essential oxidation–reduction reactions in various plants and microorganisms, by forming metal-centered proteins [28]. It has also been known for decades as DNA interfering agent in its cationic form, as similarly observed with other metal cations, and this interaction can be reversed by increased cellular GSH levels [28,29]. In a recent study, Ni(II) coordination complexes were developed as novel anticancer agents by using naphthoquinone-based thiosemicarbazone and semicarbazone ligands [30]. Then, methoxy-substituted Ni(II) derivatives with salophene ligands were reported as apoptosis inducers in vitro [31]. In these studies, the analyzed complexes were effectively altering the activities of enzymes in kinase signaling and antioxidant defense, but in a dose-dependent manner. These and several other Ni(II) complexes, including nickel sulfide, were tested on different human tumor cell lines, and their effect on DNA interacting proteins, DNA itself, and signaling components related to DNA transcription were evaluated. Although the limited use of Ni(II) coordination complexes in anticancer drug discovery, palladium, another palladium group element with strong similar coordination chemistry of Pt(II), was extensively studied and reviewed by several scientific groups [32,33]. Since it is the second most studied metal after platinum, the disadvantages in using the certain ligands were also successfully identified. Such that, phosphine, mono- and bidentate nitrogen ligands, peptide-like structures are a few examples, some of which were shown to be more tissue targeting capacity than the platinum derivatives but with closer cytotoxicity [34]. Considering that the Pt(II) and Pd(II) can easily interact with every nucleophilic biomolecule in cells, their chance to enter the nuclear compartment and interact with DNA appears to be less likely. However, under physiological conditions, it was shown that less labile dithiocarbamate ligands may enhance DNA targeting of these complexes. Then, very recently both Pt(II) and Pd(II) complexes with octyldithiocarbamate and 2,2'-bipyridine ligands were shown to induce cytotoxicity [35]. The common approach of these studies were, in terms of cellular defense, the cellular GSH maintenance and its role in apoptosis upon exposure to Ni(II), Pt(II), and Pd(II) complexes. However, none of these studies involved the possible role of those complexes on antioxidant defense

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system components. In addition to these complexes, numerous compounds with variety of ligands, from basic organic molecules to amino acids, and to peptide-like structures are not mentioned since they fall outside the scope of the study.

Since its first use in cancer, cisplatin is still part of certain chemotherapy regimens, even though there are brutal side effects. It may be beneficial to evaluate all available metal coordination complexes synthesized for different purposes than therapeutic use, and by this way, the efforts in finding successful anticancer drugs may be facilitated. In this concept, we synthesized and analyzed not only new metal coordination complexes, but also the complexes that have been synthesized and characterized almost 10–30 years ago [36–42]. In our attempts to search for complexes with full biological activity, we evaluated the effect of mononuclear and symmetrical complexes of Ni(II), Pd(II), and Pt(II) on cytosolic targets involved in drug metabolism, antioxidant defense, and kinase signaling by virtue of acellular and cellular in vitro analyses.

METHODS

The evaluation of metal coordination complexes against various cytosolic targets were performed by our biochemistry research group (Atilim University, Ankara, Turkey) using the Pt(II) and Pd(II) complexes with diethyl dithiocarbamate [43] ligands (**1** and **2**, respectively), Pt(II) complexes with tris- (**3**) and bis- (**4**) substituted dimethyl sulfide (DMSO) [40], and Ni(II) complex with diethylxanthate [37] ligands, as shown in Fig. 2. These complexes were provided by the inorganic chemistry research group at the same institution, where their synthesis and characterization were completed as previously reported [36–42]. For all assays, the complexes were prepared from 10 mM stocks in DMSO, and diluted with assay medium or buffer for final concentrations of 96 nM to 5 mM, and all measurements were made within this range.

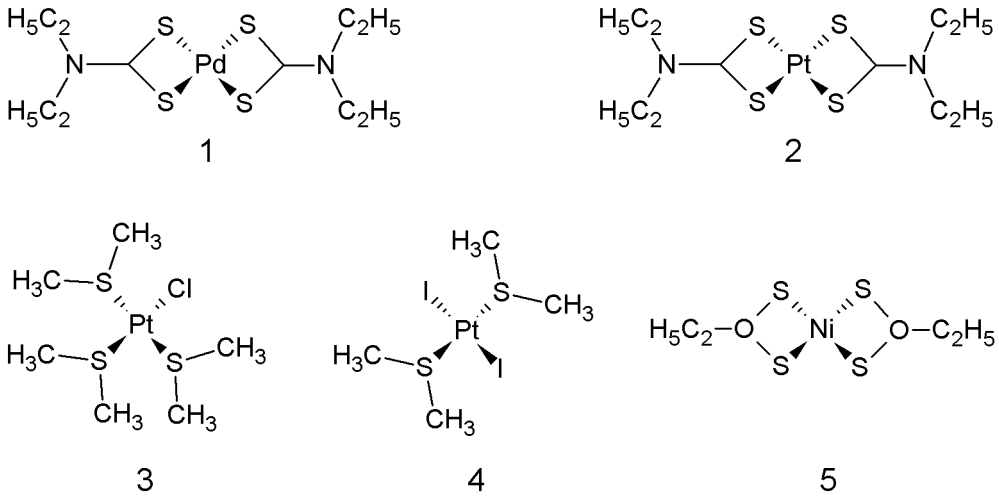

Fig. 2 Mononuclear and symmetrical complexes of Ni(II), Pd(II), and Pt(II) evaluated against cytosolic targets.

The effect of complexes on enzymes, namely, GST, GPX, PTK, and CAT were evaluated by employing methods which were miniaturized and optimized by our biochemistry research group.

The change in total GST activities was measured against the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), by monitoring the thioether (GSH-CDNB conjugate) formation at 340 nm. The measurements were performed in a 100 mM potassium phosphate buffer at pH 6.5 with 2.4 mM CDNB and 3.2 mM GSH, using optimized microplate application protocol [19].

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The GPX activity was measured against the substrate, tertiary butyl hydroperoxides (*t*-BuOOH), and the decrease in nicotinamide adenine dinucleotide phosphate (NADPH) was monitored at 340 nm. The GPX activity changes were measured by using purified GPX (37.5×10^{-3} unit/ml), 2 mM GSH, 0.25 mM NADPH, GSH-reductase (GR, 0.5 unit/ml) and 0.3 mM *t*-BuOOH, in 0.2 ml of 50 mM Tris HCl (pH 8.0).

The CAT activity was measured by the miniaturized version of the previously described protocol [44], using purified CAT (20 unit/ml) from bovine liver cytosol (Sigma) as enzyme source, against 5 mM hydrogen peroxide substrate, in 50 mM potassium phosphate buffer (pH 7.0). The change in CAT activity was monitored at 520 nm, after addition of chromogen solution supplemented with horse radish peroxidase (HRP).

The PTK activity was measured using ProFluor Src-Family Kinase Assay for microplate application [45] with some modifications [19]. The kinase activity was monitored with Src-family kinase R110 substrate (λex/λem: 485/530 nm), where the decrease in fluorescence of each microplate well inversely relates to kinase activity of the enzyme within the wells.

The cytotoxicity was evaluated using K562 human leukemia cell line maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 5 % FBS. The growth inhibition of cells was determined by SulfoRhodamine B (SRB) assay and Trypan Blue (TB) dye exclusion methods [46], at certain time points after exposure to metal complexes. The growth inhibition was determined as previously described [47], and reported as the concentration required to induce 50 % growth inhibition of cells (GI_{50}).

The enzyme calibration and the dose response curves were constructed using 3–4 independent experiments in 96 well microplates, each in triplicates. The cell counts and cytotoxicity assays were performed both in 6 well and 96 well plates, using microscopic evaluations and spectroscopic analysis (colorimetric).

The inhibitory activities of complexes against enzyme targets were calculated as 50 % inhibitory concentration, or IC_{50} value, which is defined as complex concentration that exerts 50 % inhibition on target enzyme activity. Both IC_{50} and GI_{50} values were determined by nonlinear regression analysis, with sigmoidal dose–response 4-parameter logistic equation, GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA. For experiments performed in 96 well microplates, Spectramax M2e, Multi-Mode Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) was used.

RESULTS

The coordination complexes of platinum group of metals have a typical tendency to interact with purified bacterial and genomic DNA, but such affinity does not always result in cytotoxic effects. Since a typical coordination complex can easily lose its ligands, due to the weak nature of coordination bond with respect to any covalent bond between carbon, nitrogen, or oxygen atoms, the metal center becomes more available to interact with any reactive biomolecules inside a cell, and in turn, their interaction with nuclear targets may not seem likely to occur. Therefore, instead of showing DNA-complex interaction, it may be beneficial to begin with identifying the possible cytotoxicity of complexes on model cells, since such an approach will also show if the complex causes any effects on the essential functioning of cells. In this context, we analyzed the cytotoxic effects of complexes on K562 human leukemia cells under physiological conditions after 24, 48, and 72 h exposure. The results showed that, of the five complexes analyzed, **1**, **4**, and **5** exerted a reasonable effect on cell viability. The GI_{50} values calculated for complexes were in the range of 6.309–7.943 μM, showing the similar inhibition pattern observed with cisplatin control.

In our attempts to search for complexes with full biological activity, which is the inhibitory activities of complexes, we also evaluated the effect of mononuclear and symmetrical complexes of Ni(II),

Pd(II), Pt(II) on drug metabolizing and antioxidant defense system enzymes, CAT, GPX, GST and PTK, by virtue of acellular in vitro analyses. Since Pt(II) is known to have a strong affinity toward thiols, such as GSH, the effects of complexes were evaluated against the GST family of enzymes. The members of this family are not only the main thiol utilizing enzymes, but also the most active detoxification system components that form conjugates with reactive agents, solubilize them, and facilitate their discharge. Here, for complexes **1–5**, 50 % inhibition of enzyme activity was observed, where 70 % active bovine liver cytosol preparations were used as total GST source to achieve the enzyme inhibition profiles at linear range of the complexes analyzed. The best GST inhibition profile was observed for **2** with IC_{50} of 134.8 nM, followed by **1** with IC_{50} of 1.18 μM. For complexes **3**, **4**, and **5**, GST inhibition was observed with IC_{50} of 27.67, 8.09, and 40.22 μM, respectively. These values were calculated after normalization of raw data to 100 % enzymatic activity (data not shown). Another critical property of GSTs is their peroxidase activity under physiological conditions. This property is important in elimination of organic hydroperoxides produced in cellular redox reactions, in addition to the capacity of CAT to remove hydrogen peroxide, and GPX to remove both hydrogen peroxide and organic hydroperoxides present in cytosol. Although metals can undergo redox processes, each of these enzymes participates to maintain the reducing environment of cell cytosol, the condition that generally limits the occurrence of several oxidation states of transition-metal ions. Thus, under normal physiological conditions, most of the oxidation products are conjugated with suitable biomolecules to be neutralized. For cancer cells, the metabolism rate is much higher than the normal cells. Therefore, upon exposure to coordination complexes; the accumulation of metal ions emerges more pronounced than their detoxification. If the resulting disturbance of the cellular redox homeostasis is controlled by GPX, GSH was used excessively to produce the glutathione disulfide (reduced GSH, GS-SG), while the organic hydroperoxides were reduced to corresponding alcohol and free hydrogen peroxide to water. By providing reasonable amount of thiols to improve redox balance, GSH reductase completes the cellular redox cycling by converting accumulated GS-SG to GSH. In many cancer types, GPX activity is either unchanged or slightly modified. That is why the inhibition of GPX may be beneficial to induce apoptosis in cancer cells that refrain from using mitochondria for energetic purposes, and so the cancer cells escape from apoptotic control of this organelle. In this study, of the complexes evaluated, only two of them were found to be active inhibitors of GPX with IC_{50} values of 13.430 μM for **2**, and 3.061 μM for **5** (Fig. 3). Another mechanism that induces apoptosis by hydrogen peroxide accumulation is through CAT inhibition; but this enzyme was shown to exhibit varying activity levels in tumors of the same and different tissue origins. Therefore, for specified cases, CAT-mediated apoptosis induction may provide benefits over general GPX inhibition approaches, since overactive CAT may show tumor preference and cause improved target specificity. In this context, among the complexes analyzed, only complex **5** showed CAT inhibition with IC_{50} of 36.69 nM (Fig. 4). Since CAT is located in cytosol and more likely to interact with complex before it is accumulated and located into nucleus, the antitumor effect of this complex may be facilitated at this IC_{50}.

Although several metal coordination complexes have been shown to modulate serine/threonine kinases, some of which are directly related with GST-mediated signaling components. On the other hand, tyrosine kinases are identified as either the reason or the outcome of many carcinogenic processes, and for this reason, they are universally accepted drug targets for specific hemolytic malignancies and solid tumors. In this context, PTK inhibition may provide enhanced antitumor activity of the metal coordination complexes, in addition to their effect on DNA and other nuclear enzymes, such as topoisomerases. However, there are only a few examples of PTK-related studies of the metal complexes. Since the first kinase activity was detected with src family tyrosine kinase “c-src”, the prototype member of all tyrosine kinases located in cytosol, c-src is a universally accepted PTK model for drug-targeting studies. Therefore, to evaluate overall PTK inhibition profile, we analyzed the complexes against recombinant c-src, and only the complexes **1** and **5** were found to be active PTK inhibitors with IC_{50} of 38.41 and 40.54 μM, respectively (Fig. 5).

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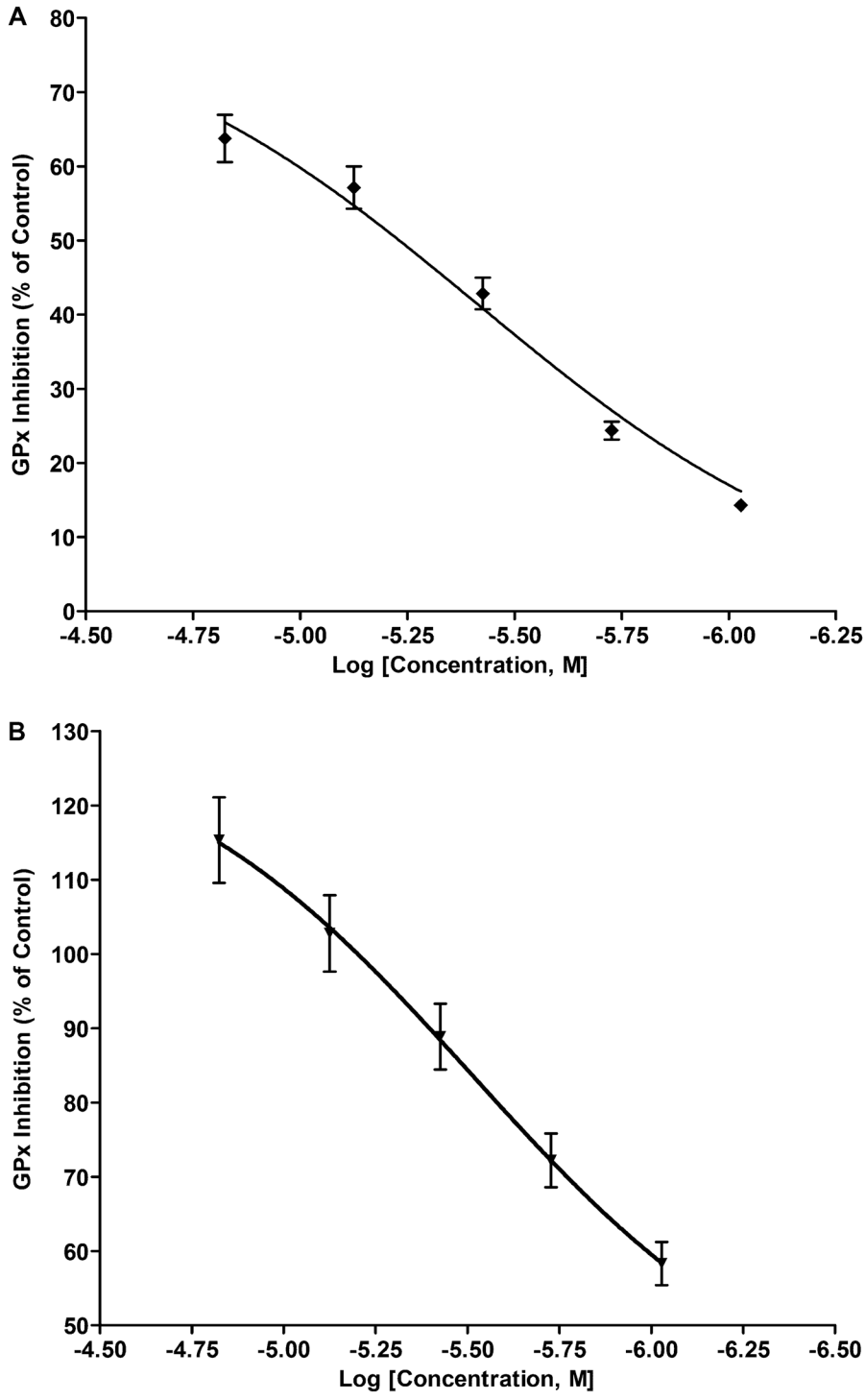

Fig. 3 GPX inhibition profile of complexes **2** (A) and **5** (B).

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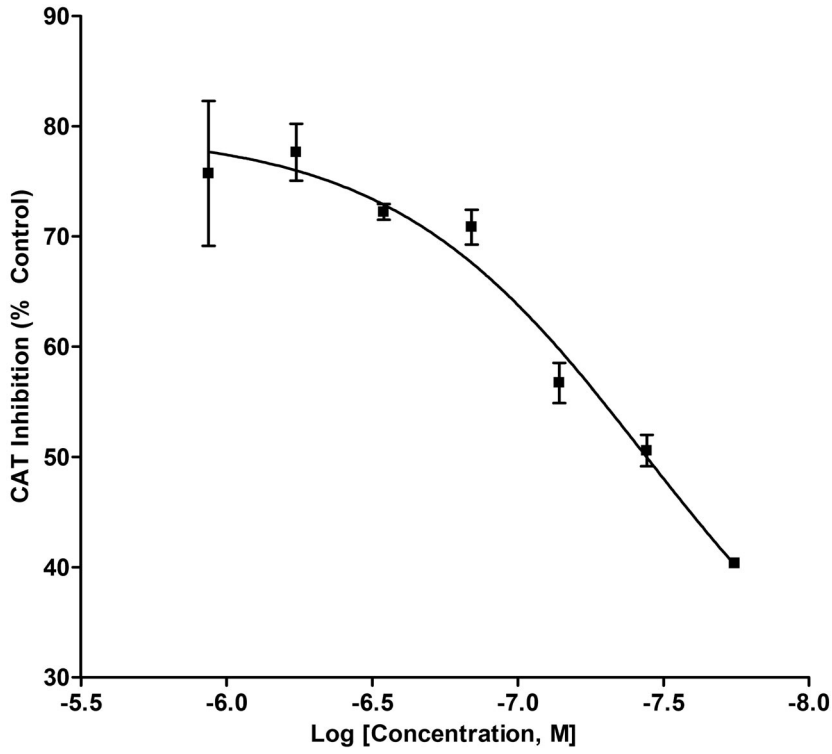

Fig. 4 CAT inhibition profile of complex **5**.

In this study, complexes determined as active inhibitors for CAT, GPX, and PTK showed 80–100 % inhibition of target enzymes at their highest possible doses used in assays. These concentrations are closely related with in vivo doses of representative drug cisplatin, hence, revealing their strong affinity toward the biological targets under study. Overall results showed that, among the complexes analyzed, complex **1** exhibits the best antioxidant and antitumor activity, and may provide potential use for the tumors with selectively high CAT activity. Since Ni(II) is already known to form DNA-adduct, these results may show its enhanced cytotoxic property even before its localization to nucleus. This complex may also become a functional tool in research efforts to understand the mechanisms controlling the translocation of complexes from cytosol to nucleus. Moreover, for research purposes, the complexes **1**, **2**, and **5** can be used as strong GPX inhibitors, **3** and **4** as mild GST inhibitors, and **1** for evaluating PTK inhibitors acting at low micromolar ranges. Also, complex **5**, as CAT inhibitor with low nanomolar activity, can be used as a potential research tool to dissect antioxidant mechanisms related to CAT, in addition to be developed for chemotherapeutic agent. As a result of the complexes evaluated, complexes **2** and **5** may provide full biological response in vitro, and hence, further analysis may provide opportunities for these complexes to be developed as therapeutics.

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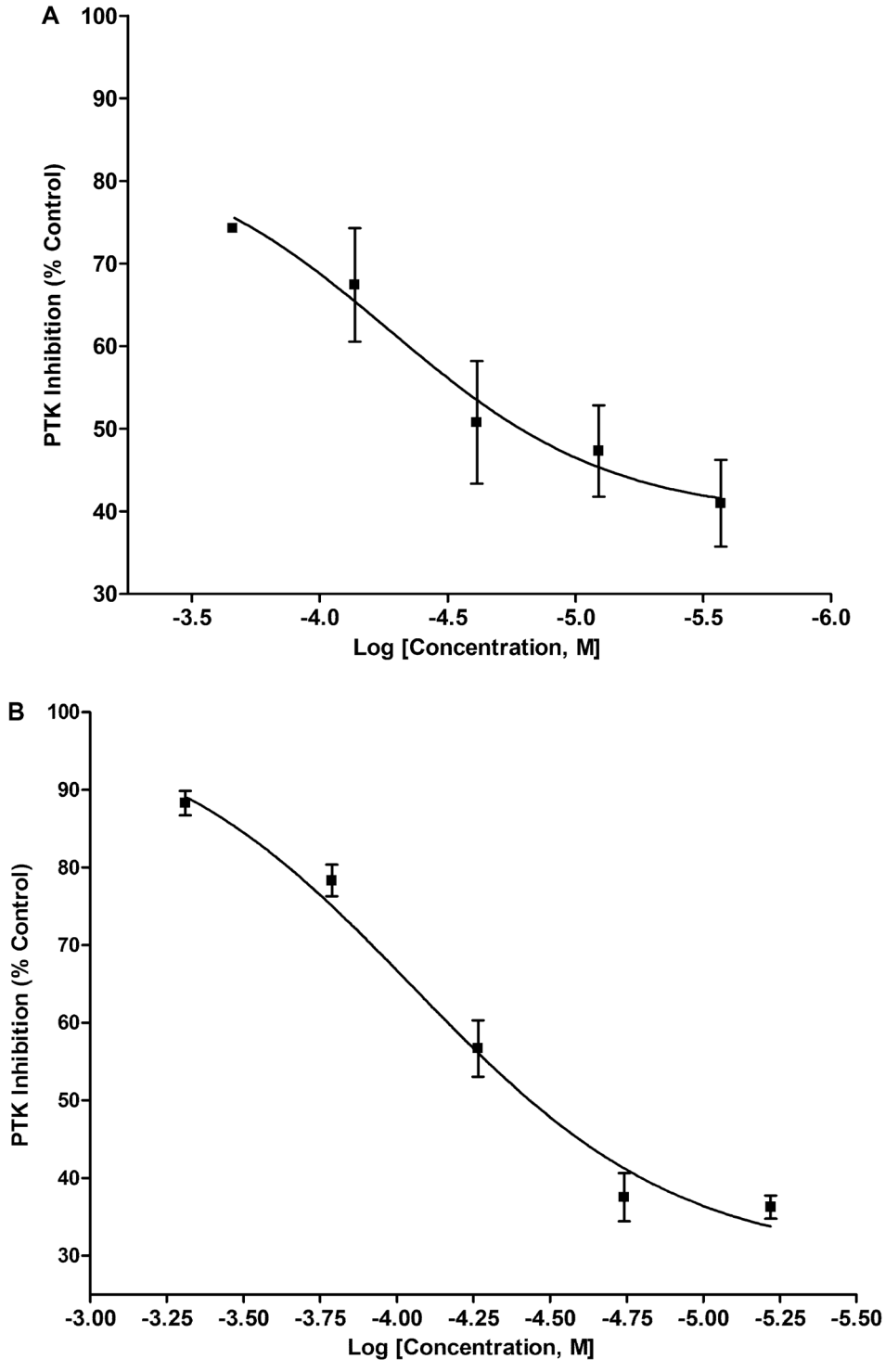

Fig. 5 PTK inhibition profiles of complex **1** (A) and complex **5** (B).

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