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CLUJ-NAPOCA

Faculty of Chemistry and Chemical Engineering

Mechanism of action involving metalloproteins, oxidative and nitrosative stress and compounds with anticancer activity

Summary

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Chapter 1.

A briefly introduction on oxidative and nitrosative stress related with hemoproteins (hemoglobin and mioglobin) free radical chemistry was presented in the first part of chapter 1, followed by the structural characterization of the interaction of Pt based anticancer drugs with proteins.

Cisplatin and related compounds are known to exert much of their useful therapeutic effects via binding to DNA.[1] The need for more effective drugs as well as the wide range of side-effects (nausea, progressive peripheral sensory neuropathy, fatigue, vomiting, alopecia, hematological suppression, renal damage) have, for several decades now, fuelled interest into understanding the complex mechanisms of interaction of cisplatin and related compounds with various biomolecules.[2, 3] One notable observation in this respect has been that cisplatin-derived platinum can bind to a range of proteins, as demonstrated by elemental analyses, chromatography and mass spectrometry. Indeed, it has been estimated that less than 5% of the cisplatin that has entered a cell will be found bound to DNA; the rest will bind to proteins and small peptides.[4] As expected, cisplatin has a preference for binding thiol groups, with glutathione and metallothionein as important targets;[5] in fact, cisplatin induces a rise in the level of thiol groups in cells which is at least in part responsible for the resistance developed against this drug. Thiol-blocking reagents favor cisplatin-DNA binding in vivo. The thioether sulfur in methionine is also an important target, as witnessed among others by the fact that Pt-methionine adducts were detected in the urine of cisplatin-treated patients. [6]

Cisplatin–Hb complexes were shown to be formed using clinically relevant concentrations of cisplatin and Hb; heme release was a noted side effect of platinum binding. Hb is present in high concentrations in blood and is particularly sensitive to changes in redox status, to the extent that under stress conditions such as physical effort or certain pathological conditions it engages in toxic reactions with oxidative stress agents- primarily peroxide – yielding free radicals and highly-oxidizing states at the iron (ferryl, Compound II).[7, 8, 9, 10] Mass spectrometry was employed to show that myoglobin can bind cisplatin at residue His116, Ser117, Lys118, and His 119.[11] As such, it may be expected that Hb an Mb might be sensitive to the stress imposed by cisplatin in patients.[12]

Serious side effects of chemotherapy such as cisplatin-induced toxicity are, in part, the result of the formation of free radical such as superoxide anion and hydroxyl radical. These

highly reactive oxygen species can cause extensive tissue damage through reactions with all biological molecules - lipids, proteins and nucleic acids, leading to the formation of oxidized substances. Also, free radicals may deplete glutathion (GSH) levels and inhibit the activity of antioxidant enzymes. Enzymatic (gluatathione, thioredoxin) and molecular defense mechanism are present in the cell to prevent the integrity of biological membranes from oxidative processes caused by free radicals. The administration of antioxidants such as Vitamin E, Vitamin C, selenium and carotenoids, before or after treatment with platinum drug has been used to protect or ameliorate against nephrotoxicity in human and animals, without compromising the anti-tumor activity.[13]

Chapter 2

In chapter II, cisplatin is shown to modulate some physiological parameters of hemoglobin and myoglobin (most of which may be linked to free radical reactivity), such as dioxygen affinity, autooxidation and pro-oxidative reactivity with hydrogen peroxide and nitrate – as measured by UV-vis spectroscopy.

These changes are also observable in whole erythrocytes, and they are alleviated by antioxidant compounds. Computational tools for the analysis of cell shape was used to quantify the similarity or difference between images of red blood cells either exposed to cisplatin or exposed to control solutions containing no cisplatin. Thus, morphometric methods and fractal analysis using the standard box-counting method for shape characterization of digital images of cisplatin-treated individual red blood cells, support the spectroscopic findings.

Also, electron paramagnetic resonance (EPR) spectra are shown illustrating for the first time direct detection of a free radical in E coli cell exposed to cisplatin, as well as indirect detection of such radicals using a spin trap. These directly support platinum-containing drugs as a source of imbalance in free radical metabolism within living cells – which may have implication in the side-effects incurred by such drugs in patients.

It is tempting to then speculate that at least part of the side-effects of cisplatin can be explained by this oxidative stress pathway; it remains to be tested to what extent the therapeutically beneficial effects of cisplatin, which are well-known to involve DNA chemistry, are affected by such oxidative stress interactions.

Chapter 3

Since 1883, when Berthsen first described the synthesis of phenothiazine, derivatives containing these heterocyclic core constantly attracted the scientific interest and over the years, major applications were developed in the chemistry of dyes, pharmaceuticals, additives for lubricants, polymers and others. [14, 15] Pharmacological properties of these compounds was mainly due to radical stable cation formed on the heterocyclic units.[16]

Different dyes with 3-formyl-10-alkyl-phenothiazine salts were recently synthesized in the organic chemistry laboratory of the Faculty of Chemistry and Chemical Engineering, UBB, Cluj-Napoca, by condensation of formyl derivatives with methylpiridine in basic media. Compounds were investigated by spectroscopic analysis: UV-Vis, fluorescence, NMR, FTIR and mass spectrometry and will be tested for their ability to photoinactivate viruses in red cell suspensions due to the formation of singlet oxygen species.

Also it was described the first microwave assisted preparation of primary phenothiazinyl-amines by catalytic amination of halogeno-phenothiazines, together with their use as a starting materials for new phenothiazine analogues of Troger's Bases. The newly described PTBs are considered as promising molecular tweezers shaped precursors for new functional materials. Molecular biology studies as DNA probes are currently under investigation.

An inorganic Sn compound was synthesized in the inorganic laboratory and characterized by NMR and GS-Ms. The purpose of synthesis was to investigate the biological activity of the compound.

In chapter III it was described the influence of these new experimental compounds on the autooxidation rates of Hb and Mb. Both types of proteins can be taken to relate to free radical stress, as free radicals (superoxide, peroxyl, peroxides) are known to be involved in accelerating protein autooxidation.

Dyes have a measurable effect on the autooxidation rates of hemoglobin and myoglobin, accelerating this process in qualitatively similar manner in both proteins. Moreover, the effect seen on solutions of purified proteins is mirrored in a similar effect measured directly on blood, monitoring again hemoglobin. Since the magnitude and trends of the effect does not mirror those seen on pure hemoglobin, it is reasonable to expect that the compounds have the ability to interact with more than one protein, and thus modulate prooxidant reactivity on more than one pathway. Using UV-vis and EPR spectroscopy and mass spectrometry a reaction intermediate

(radical cation) was demonstrated to be formed during oxidation of phenothiazine units dyes in the presence of a hemoprotein with peroxidatic activity.

Amine derivatives have a strong effect on the autooxidation rates of hemoglobin and myoglobin, accelerating this process in qualitatively similar manner in both proteins. On the other hand, the corresponding Troger bases show much smaller, or even negligible effects.

Sn compound have a smaller, or even neglijable effect upon the autooxidation rates of hemoglobin and myoglobin. A little prooxidant effect was induced by this compound on hemoglobin. On the other hand it slowly inhibits the autooxidation of myoglobin, acting like an antioxidant.

Chapter 4

Beyond its essential role in the electron transport chain, cytochrome *c* is known to be involved in apoptosis, where its release from the mitochondria is a key step. Free radical reactivity, and in particular interaction with peroxides, has been shown for cytochrome *c* and proposed to be relevant for apoptosis. It has been shown that the sixth ligand of the heme iron in this protein, a methionine, can transiently dissociate under various conditions, including changes in pH or temperature, so that binding of peroxides to the iron is conceivable The result of the interaction of cytochrome *c* with the mitochondria-specific phospholipid cardiolipin is a complex which acts as a specific and potent oxidant. Binding to anionic phospholipids causes conformational changes in the protein and disruption of the S-Fe coordination bound between Met_{80} and heme iron, transforming cytochrome *c* from a protein carrier in to a prooxidant-peroxidase which have an important role in the apoptosis process. [17]

In chapter IV it is shown that treatments favoring liberation of the sixth coordination position at the cytochrome *c* heme iron and therefore increased accessibility for peroxide (by partial denaturation with guanidinium hydrochloride) lead to a drastic increase in the reactivity of cytochrome *c* towards hydrogen peroxide, to the point where the ascorbate peroxidase activity demonstrated here for native cytochrome *c* is drastically improved (both in terms of K_m and V_{max}) upon partial denaturation with guanidinium hydrochloride. Moreover, a reaction intermediate is detected by stopped-flow UV-vis spectroscopy upon treatment of guanidine-treated cytochrome *c* with peroxide, which resembles the spectrum of globin Compound II^7 and is thus proposed to be

a high-valent ferryl species – the first of its kind to be directly detected in a cytochrome *c* at room temperature.[18]

Accordingly, these agents (such as drugs that we studied) that alter protein structure could increase accessibility to the iron. Therefore, the last part of this chapter is focused on the interaction of cisplatin and experimental compounds described in the previous section with cytochrome *c*.

Similarly to what was previously shown for a number of other heme-containing proteins[19, 20, 21] cytochrome *c* induces autooxidation of lipids in a liposome system and that this process is affected by cisplatin in a concentration-dependent manner.

The mechanism of this autooxidation is known to involve lipid hydroperoxides and free-radical chemistry; it has been proposed that the protonated form of ferryl (Fe^{4+}-OH^{-})[7, 22] is the reactive species regulating the peroxidasic activity of heme-containing proteins. The antioxidant behavior of Sn compound has also been detected in the liposomes experiment where it inhibit lipid peroxidation induced by cytochrome *c*. Some of the amines and Troger bases also inhibit lipid peroxidation by cytochrome *c*.

Chapter 5

In conclusion, the toxicity of metal based anticancer compounds is, in part, the result of oxidative and nitrosative stress induced by several specific ways. In this thesis, it has been shown how cisplatin and some potential anticancer drugs can modulate the behavior of three hemoproteins (hemoglobin, myoglobin and cytochrome *c*). These proteins are sensitive to changes in redox status, to the extent that under stress conditions such as physical effort or certain pathological conditions they engage in toxic reactions with oxidative stress agents-primarily peroxide – yielding free radicals and highly oxidizing states at the iron. Binding of the metal compounds on the protein surface causes changes in protein chains that can block, or conversely, allows the access of peroxides to the hem. Some natural antioxidants were found to protect against prooxidant effect of cisplatin.

The reaction intermediate detected in some conditions upon treatment of cytochrome *c* with peroxides, brings further evidence that the initial step in apoptosis, a phenomenon in which it is involved, is represented by a peroxidase mechanism where the ascorbate has an important role.

Moreover, some experiments *in vitro* have shown how the metabolism of free radical of a human cell (red cell) and of the symbiotic cell *E. coli* may be affected by cisplatin.

Thus, using UV-vis and EPR spectroscopy, stopped-flow techniques, electron microscopy, mass spectrometry and in vitro cell culture have brought some additional evidence about the ways in which some anticancer agents can affect the body. These investigation methods may be useful in the future to predict the toxicity of biologically active compounds.

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