Cellular targets of metal-based anticancer drugs: Is electron microscopy a forgotten method?

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Cisplatin is a successful anticancer metallo-drug but displays dose-limiting side effects and resistance. Efforts have been made to synthesize novel compounds that can potentially improve cisplatin’s clinical benefits. However, the mechanisms of action are not well understood and need to be investigated. Molecular and cell biology studies point to intracellular targets like the DNA, the mitochondria or to global effects like disruption of the cell redox balance. Electron microscopy is the only method able to visualize directly cellular detail down to macromolecular level and to detect unexpected cellular changes related to the mechanism of action. Combined with Electron Probe X-Ray Microanalysis, the precise location of metals inside the cells can be mapped. In spite of its high value as a tool for understanding the mechanism of action of metal-based drugs, in this context electron microscopy has not been used extensively. Current research has detected alterations of several organelles such as the nucleus, mitochondria, endomembranar system and lisosomes thus shedding more light onto the underlying mechanisms.

Keywords: cancer; metal-based compounds; mechanisms of action; cellular targets

1. Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. According to recent World Health Organization (WHO) projections, cancer in a near future will be the overall leading cause of death worldwide [1]. The increase of the survival rates will be due to better cancer treatment in particular to more efficient anticancer drugs.

Transition metals have been considered for decades to have medicinal value. The unique electronic structure of transition metals offers great versatility, in terms of the ability to tune the properties of a given molecule. With the advent of modern medicine, many metal-based drugs have proven to be highly effective in the clinic. Classical, well-known examples include silver compounds (skin protection after burning), technetium compounds (diagnostic tools), bismuth salts (treatment of diarrhea and stomach ulcers), gold complexes and copper salts (treatment of arthritis) and platinum compounds (antitumor drugs).

1.1 Platinum-based cancer chemotherapy

The platinum drugs represent a unique and important class of antitumor agents. The development of cisplatin, serendipitously discovered by Rosenberg and colleagues (1965), paved the way to a new area of anticancer research based on metallo-drugs [2,3]. Cisplatin prove effective against several cancers particularly in the treatment of germ-cell tumors. However, high toxicity and acquired resistance are fundamental obstacles in its clinical application [4,5]. Systematic approaches to the development of new analogues (Fig. 1) have produced agents with less toxicity and novel mechanisms of action. To date such approaches have not achieved more cures than can be achieved with the parent compound, cisplatin [6].

Fig. 1 Currently approved platinum drugs for cancer therapy. Cisplatin was followed by carboplatin and most recently by oxaliplatin.
1.2 Basic mechanisms involving platinum drugs
The uptake of platinum drugs has been attributed to a combination of mechanisms, passive diffusion and facilitated transport via the copper transporter-1 (CTR1) \[5,7\]. The antitumor properties of cisplatin are attributed to the displacement reactions of the chloride ligand leading to DNA crosslinking activities. In addition to DNA, amino acids, peptides, proteins and thiol biomolecules (glutathione) are involved in its mechanism of action \[8,9\]. A number of additional properties of cisplatin are now emerging including mitochondrial alterations leading to the activation of the intrinsic pathways of apoptosis and other signaling mechanisms leading to cell death \[10,11\].

The binding of cisplatin and other analogues to proteins and enzymes is generally believed to be the cause of several severe side effects such as ototoxicity and nephrotoxicity. The interference of cisplatin with glucose metabolism is also a cause of its relevant toxicities \[12,13\].

1.3 Ruthenium drugs candidates
In the search for new metal based anticancer agents ruthenium complexes have raised great interest. Many of their properties can justify the great expectations posed on these entities. These properties can be resumed in i) ability to adopt numerous oxidation states (most commonly II, III, and IV); ii) octahedral geometry, an improvement over the platinum drugs, allowing for more intensive tuning of the complexes electronic and steric properties; iii) facility to exchange with O- and N- donor molecules in a way similar to platinum drugs; iv) slow rate of ligand exchange in comparison with other transition metal complexes; v) transport by binding to serum albumin and transferrin, as ruthenium mimic iron in binding to proteins \[2,14\].

Ruthenium complexes are thought to have a distinct mode of action from that of platinum compounds. Whereas for cisplatin DNA is believed to be the primary molecular target, for ruthenium complexes the targets and the exact mechanism of action are still not well understood.

Extracellular or intracellular proteins may play an important role in the cell death mechanisms. Interaction with biologically relevant thiols resulted in the inhibition of enzymes such as thioredoxin reductase or cathepsin B which have been identified as alternative novel targets for these metal-based drugs \[10,15-17\].

1.3.1 Coordination compounds
A broad spectrum of ruthenium compounds have been shown to exhibit potential anticancer properties, emerging as promising alternatives to platinum-based cancer therapy. As a result of intense research focused on their cytotoxic effects and also their mechanism of action, two Ru(III) compounds, NAMI-A and KP1019, have already entered clinical trials \[18-20\].

Fig. 2 Ruthenium drug candidates in phase II clinical trials: NAMI-A (left) and KP1019 (right).

1.3.2 Organometallics
An organometallic complex is defined as a molecule with a distinct metal-carbon bond. In the frame of ruthenium compounds, the instability and the complicated ligand exchange chemistry of inorganic ruthenium complexes present some drawbacks that can be overcome by the more stable organoruthenium complexes in terms of providing better drug candidates. Moreover, although the general reputation as being unstable, some ruthenium organometallics have displayed high water- and air- stability and an interesting spectrum of anticancer activity. Ru(II)(η⁶-arene) compounds and a few others, are currently undergoing preclinical evaluation \[2,21\].
2. Strategies for biological evaluation of potential metal-based anti-cancer drugs

The biological evaluation strategy should consider an understanding of the molecular properties of the compounds under research related to their behavior in a biological environment, their activity against a panel of tumor cells, identification of the uptake mechanisms and cellular accumulation, the mechanisms of action and potential targets. Aiming the assessment of pharmacokinetic and toxicological properties, the in vivo activity in relevant tumor models should also be considered. This strategy will be exemplified with some Pt(II) and Ru(II) complexes in the search for novel metal-based anticancer drugs. The importance of mechanistic studies which have led to the identification of new targets are emphasized with the help of innovative analytical approaches that provide a basis for a deeper understanding of the biological profile of these metal-based drugs [22].

3. Design strategies, structure activity relationship and mechanistic insights for anticancer compounds

It has been established that the properties of metal-coordinated compounds are largely determined by the nature of ligands and donor atoms bound to the metal. The achievements in the design and development of some Pt(II) and Ru(II) complexes as antitumor agents are summarized. Special emphasis has been focused on the recognition of structure-activity relationships as well as cellular morphological alterations induced by the compounds aiming the identification of the mechanisms of cell death.

Electron microscopy was used as a particularly suitable method for probing unknown and unsuspected alterations in cellular organelles, allowing the high resolution visualization of samples that can give decisive information concerning the target cellular structures affected by the compounds [23]. The application of X-ray microanalysis in transmission and scanning electron microscopy, eventually coupled with new improved preservation methods to avoid extraction of the compounds by the preparation methods, can allow the visualization of the binding sites of the metal-containing compounds [24,25]. Altogether these methods can answer a very important set of questions pertaining to the interaction of the compounds with cells and their effects in cellular physiology.

With the aim of designing compounds that would display a synergistic effect between platination and DNA intercalation, a series of Pt(II) complexes anchored by pyrazolyl-diamine chelators bearing anthracenyl (L²Pt) or acridine orange (AO) (L³Pt) as DNA-binding groups was prepared (Fig.3) [26,27].

![Figure 3](image_url)  
**Fig. 3** [PtCl(pz²NN)]⁺ complexes anchored by pyrazolyl-diamine (pz²NN) ligands incorporating anthracenyl or acridine orange DNA-binding groups.

The anthracenyl derivative L²Pt showed very strong cytotoxic effects in comparison with the AO derivative L³Pt and cisplatin on the ovarian carcinoma A2780 cells. The results of the ultrastructural analysis seem to be in agreement with the viability and cellular uptake studies, revealing that the high cytotoxic potency found for L²Pt can be justified in terms of a different mode of action from L³Pt or cisplatin. L²Pt accumulates more in the cytoplasm than in the nuclei of ovarian cancer cells and shows a lower affinity for DNA with a less probable involvement of intercalative binding. The enhanced cytotoxicity of L²Pt might therefore be due to interaction with biological target(s) other than nuclear DNA.

Representative TEM images obtained for L²Pt are shown in Fig. 4. L²Pt induced extensive necrotic changes with overall extraction of the cytoplasmic matrix contents and densification of the mitochondrial matrix. In addition, the organelles are often found clustering near the cell center, the nucleus edematous and the chromatin dispersed. In
contrast, cells treated with L3Pt presented much more conserved morphologies, with most cells having nearly normal ultrastructure (results not shown).

Fig. 4 TEM images showing the ultrastuctures of A2780 cells: a) untreated cells (control); b) cells after treatment with L3Pt (20 μM, 2 h). N- Nucleus; G – Golgi apparatus; M - Mitochondria.

Another group of prospective anticancer drugs were developed with the aim of designing ruthenium complexes with strong biological activity and studied their behavior under a broad variety of relevant chemical and biological assays. The Ru(η5-C5H5) fragment was selected as the organometallic pharmacophore, which has been intensively and successfully studied [28-33]. The IUPAC defines a pharmacophore to be "an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response".

Special emphasis has been focused on the recognition of structure-activity relationships for two related complexes with aromatic bidentate N,N´-heteroaromatic ligands (Fig. 5).

Fig. 5 Chemical structures of related Ru(η5-C5H5) complexes with bidentate N,N´-heteroaromatic ligands

It was found for [Ru(η5-C5H5)(PPh3)(2,2'-bipyridyl)][CF3SO3] (TM34) (PPh3 = triphenylphosphane) high cytotoxic activity against human tumor cell lines, in particular A2780 ovarian cancer cells and MDAMB231 breast cancer cells. The results obtained for TM34 revealed fast antiproliferative effects even at short incubation times for both cell lines; preferential localization at the cell membranes (by ICP-MS); cellular uptake by an energy-dependent process, probably endocytosis; disruption and vesiculation of the Golgi apparatus (TEM) which suggest the endosomal/lysosomal system as a possible target [31,32]. A similar effect, but at a higher dose (100 μM, 3 h), was found for its water-soluble analogue [Ru(η5-C5H5)(mTPPMSNa)(2,2'-bipy)][CF3SO3] (TM85) (mTPPMS = m-diphenylphosphane benzene-3-sulfonate) in the MDAMB231 cells, confirming the effect of the complex on the endomembranar system [32]. This result appeared to be related to the differences in the cytotoxic activity of both complexes.

As can be depicted in Fig. 6, TM85 (100 μM, 3 h) showed reduced and vesiculated Golgi apparatus, which suggests the involvement of the endomembranar system as a target for its mode of action. A similar effect, vesiculation and disruption of the Golgi apparatus, was also found for its analog TM34 in the MDAMB231 cells but at a lower dose, 10 μM and 3 h treatment (Fig. 7). This result appeared to be related with the differences in the cytotoxic activity for both
complexes. Increasing the time of exposure (100 μM, 24 h), TM85 treated cells display a paucity of cytoplasmic membranes, small Golgi apparatus, confirming the effects of the drug on the endomembranar system. Enlarged mitochondria and abnormal nucleoli were observed at 24 h suggesting additional effects on these organelles or alternatively a cellular reaction to cellular damage leading to inhibition of cell survival and proliferation.

Fig. 6 Representative TEM images showing the morphology and ultrastructure of MDAMB231 cells treated with TM85: a) control, cells without treatment showing well developed Golgi apparatus; b) cells after treatment with TM85 (100 μM, 3 h) showing reduced and vesiculated Golgi apparatus; c) and d) cells after treatment with TM85 (100 μM, 24 h) showing enlarged mitochondria with crystals and cytoplasmic clefts (c); abnormal nucleoli suggesting inhibition of function (d). N – Nucleus; Ni – Nucleolus; G – Golgi apparatus; M – Mitochondria; C – Cytoplasmic clefts.

TM34 has a higher cellular accumulation in the MDAMB231 cells. However, the complex is less cytotoxic in these cell lines. The complex inhibits by a dose-dependent process the activity of a lysosomal enzyme. By TEM, we could detect the enzyme localization and evaluate the effect of the complex on the morphology of both cell types [31]. The complex induced disruption and vesiculation of the Golgi apparatus, which suggest the endosomal/lysosomal system as a possible target (Fig. 7). In the A2780 cells, complex treatment also provoked alterations in the mitochondria. The higher cytotoxic effect observed for TM34 in the ovarian cancer cells may be explained by this additional effect on the mitochondria despite having a lower complex cellular accumulation [31].
Results demonstrate the usefulness of transmission electron microscopy for the evaluation of the effects of Pt(II) and Ru(II) based complexes in tumor cells. The method is a “catch all” one, being able to disclose the entire set of alterations induced by the drugs upon cell structure and is therefore able to detect otherwise unsuspected effects that the drugs may display.

References


