A study on the development of novel polymeric micelles incorporating anticancer drugs designed to improve tumor targeting
(腫瘍組織への選択的集積性を有する制癌剤内包高分子ミセルの構築)

カブラル　ロレンゾ　オラシオ　アマデオ
Cabral Lorenzo Horacio Amadeo

Colloidal carriers have been used for many years mainly as the modified formulation of drug molecules exhibiting low aqueous solubility. The discovery of new therapeutic agents has facilitated a demand for more sophisticated carrier systems which are able to protect agents from inactivation due to chemical or enzymatic degradation, migrate and selectively accumulate at target sites in the body, thus enhancing the performance of the delivered agents. The recent progress in polymer science and nanotechnology certainly lend a strong basis to develop such colloidal carriers with high performance and modulated targetability.

Among these colloidal carriers, polymeric micelles have received significant attention as a promising supramolecular carrier system due to their small size and stability which lead to a prolonged blood circulation with reduced non-specific accumulation in normal tissues and preferential accumulation in solid tumors by the EPR effect (Enhanced Permeability and Retention effect). In addition to these exceptional properties, a high loading capacity of hydrophobic drug in the micelle core establishes polymeric micelle as a unique anticancer drug delivery system.

Previously, cis-dichlorodiammineplatinum(II) (cisplatin, CDDP, figure 1), a widely used anticancer drug, was incorporated into poly(ethylene glycol)-poly(amino acid) block copolymers [poly(aspartic acid) or poly(glutamic acid)] forming polymeric micelles. The physicochemical and biological properties of this micelle were extensively studied indicating that CDDP-loaded micelles are an effective delivery system for CDDP complexes.

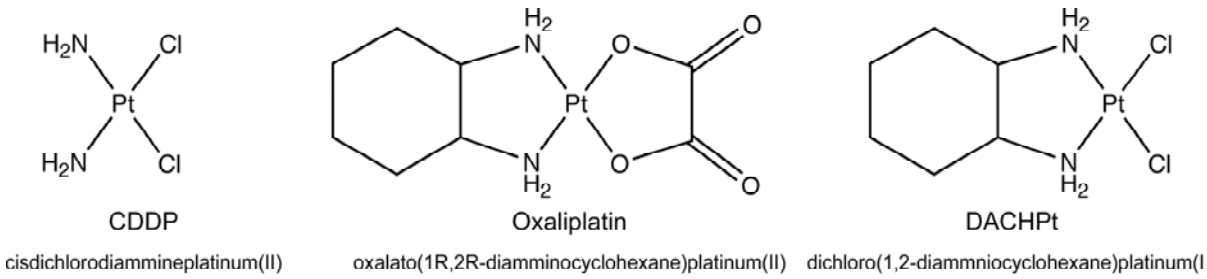

Figure 1. Platinous anticancer drugs. CDDP and Oxaliplatin have been clinically approved

CDDP shows acute dose-related side effects (such as nephrotoxicity, ototoxicity, neurotoxicity, nausea, vomiting and mielosupression) and the appearance of intrinsic and acquired resistance. Thus, since the discovery of CDDP in the mid 1960s, enormous efforts had been devoted to developing improved CDDP analogs. Nevertheless, only two of these analogs reached final approval, cis-diammin(cyclobutane-dicarboxylato1,1(2-0)-0,0)platinum(II) (carboplatin) and oxalate 1,2-diaminocyclohexane platinum(II) (oxaliplatin, figure 1). Oxaliplatin is a derivative of the highly active dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt, figure 1). DACHPt has shown a wide and markedly different spectrum of activity than CDDP, such as lower toxicity than CDDP and no cross-resistance with CDDP in many CDDP resistant cancers. However, the solubility of DACHPt in water is much lower than CDDP (0.25mg/ml for DACHPt vs. 1.2mg/ml for CDDP). Oxaliplatin is a third generation platinum drug approved by the Food and Drug Administration in U.S.A. (FDA) in 2004. Stability, solubility, formulation and safety issues were more promising for oxaliplatin than for other DACH-platinum compounds initially selected for preclinical testing and evaluated in early clinical trials. Oxaliplatin possesses an oxalate group, which is displaced by water and nucleophiles in biological media to activate the drug, and it also has a non-hydrolysable diaminocyclohexane (DACH) ligand, which is maintained in the final active cytotoxic metabolites of the drug.

Even though oxaliplatin shows better relative tolerability compared to other platinum compounds, a small number of side effects (like cumulative peripheral distal neurotoxicity and acute dysesthesias)

restrains the range of working doses. Consequently, enormous effort has been dedicated to develop drug delivery systems that increase the blood residence time of oxaliplatin, and target it to solid tumors by taking advantage of the enhanced permeability and retention (EPR) effect. Liposomes and macromolecular carriers (water soluble polymer–drug conjugates) were the first to be attempted However, successful formulations have not been developed yet due to the unfavorable properties of platinum drugs.

The development of polymeric micelles loaded with DACHPt could lead to an oxaliplatin carrier with superior properties, such as prolonged blood circulation or higher tumor accumulation. Thus, in the present study, novel polymeric micelles entrapping DACHPt in their core were prepared through the polymer metal-complex formation between DACHPt and poly(ethylene glycol)-poly(glutamic acid) [PEG-P(Glu)] or poly(ethylene glycol)-poly(aspartic acid) [PEG-P(Asp)] block copolymers in distilled water (Figure 2).

The DACHPt-loaded micelle size ranged from 25 to 50nm with narrow distribution determined by dynamic light scattering measurement. The release of platinum complexes from the micelles cores was measured in phosphate buffer saline (pH 7.4; 150mM NaCl) at 37°C. DACHPt-loaded micelle prepared with PEG-P(Glu) showed a sustained release rate of platinum after an induction period of 12 hours whereas DACHPt-loaded micelles prepared with PEG-P(Asp) released the drug rapidly during the first 12h and reached a plateau of 30% drug released at 48h . In the same conditions, the kinetic stability of DACHPt-loaded micelle prepared with PEG-P(Glu) was found to be very high, keeping the initial size, for 240 hours. Conversely, DACHPt-loaded micelles prepared from PEG-P (Asp) showed much lower kinetic stability, maintaining the initial size for only 36h in phosphate buffered saline. Therefore, PEG-P(Glu) seems to be a promising platform for the construction of DACHPt-loaded micelles since prolonged blood circulation and increased tumor accumulation are strongly associated with micellar stability in the bloodstream.

DACHPt-loaded micelle activity was studied *in vitro* against human and murine cancer cell lines

with the objective of establishing its anticancer potential and also to analyze whether or not the way of supplying the drug affected the drug efficacy.

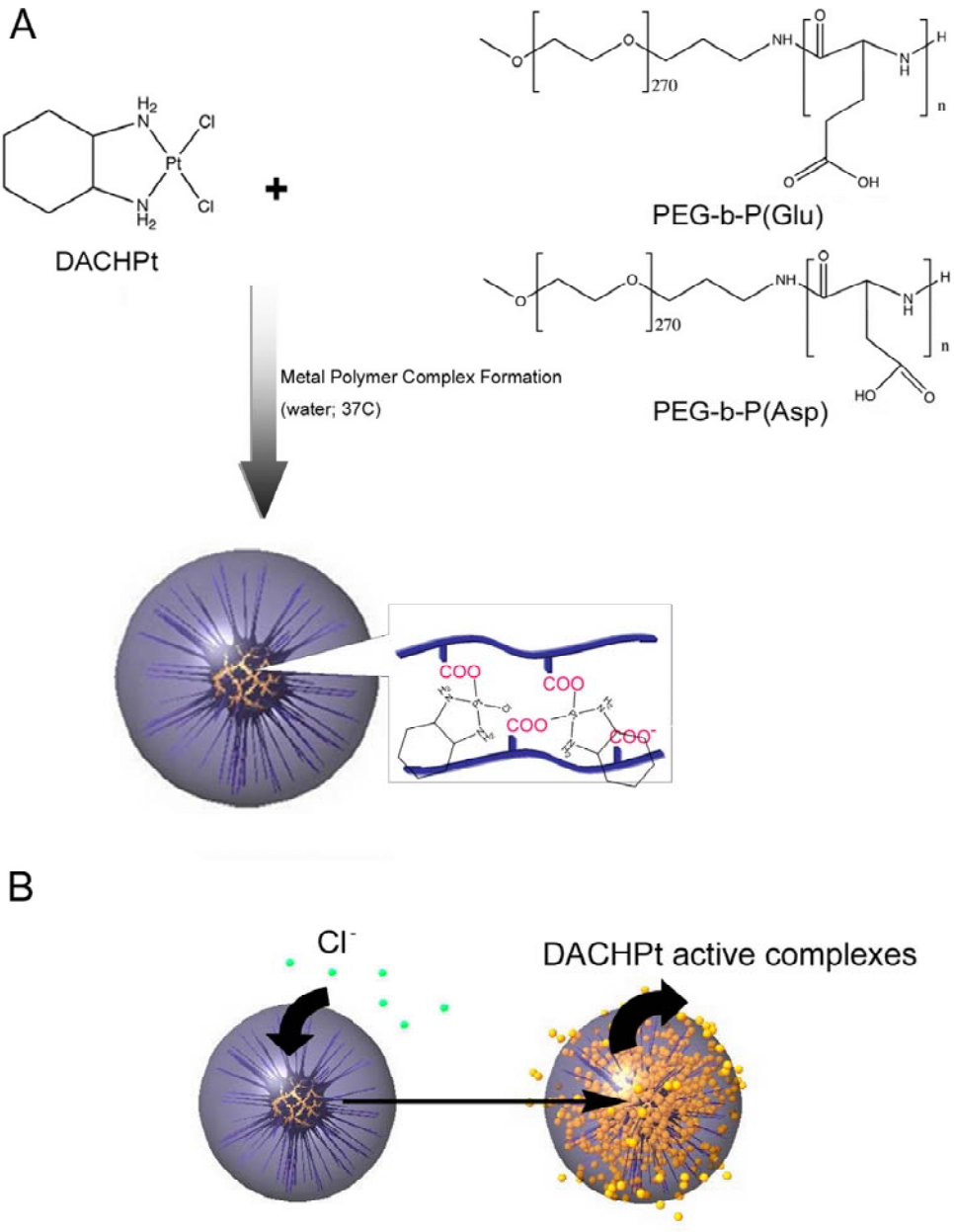

Figure 2. A. Preparation of DACHPt-loaded micelles from PEG-P(Asp) or PEG-P(Glu) by polymer complex formation. B. In chloride containing media, the chloride ions diffuse into the micelle core and cleave the metal-polymer complex producing the DACHPt release

DACHPt-loaded micelles exhibited considerable *in vitro* cytotoxicity, lower than oxaliplatin but

increasing with exposure time as a result of the release of platinum complexes from the micelle. The *in vitro* cytotoxity of DACHPt-loaded micelles was also studied on CDDP-resistant human non-small cell lung cancer (PC-9/CDDP) and CDDP-resistant human small lung cell cancer (SBC-3/CDDP). Free CDDP and free oxaliplatin presented a decreased activity against the CDDP-resistant cancer cells. Meanwhile, CDDP-loaded micelles and DACHPt-loaded micelles maintained their cytotoxic activity on SBC-3/CDDP. The intracellular accumulation of platinum after drug exposure was studied by ICP-MS in the same cancer cell lines. The accumulation was much higher for free drugs than micelles in non-resistant cancer cells. However, for SBC-3/CDDP cell free drugs accumulated ten times less. DACHPt-loaded micelles presented similar accumulation level in both resistant and non-resistant cancer cells. Moreover, the DNA of these cancer cells was isolated and the DNA platination level was measured. DACHPt-loaded micelles and oxaliplatin showed similar DNA-bound platinum at equitoxic doses after 24h of incubation. However, after 48h of incubation, the Pt-DNA-adducts were much lower for DACHPt-loaded micelles than for oxaliplatin at equitoxic doses, suggesting that DACHPt-loaded micelles generate different cytotoxic mechanisms or increase the toxicity of the Pt-adducts. The *in vitro* cytotoxicity was also evaluated against the human tumor cell panel of the Japanese Foundation for Cancer Research. DACHPt-loaded micelles and oxaliplatin showed a similar fingerprint, however, DACHPt-loaded micelles showed higher activity in some tumor cell lines. From these results, DACHPt-loaded micelles have been proven to have potent *in vitro* anticancer activity, at times higher than free oxaliplatin suggesting that DACHPt-loaded micelles can enhance the efficiency of the active platinum complexes.

In vivo studies were performed to characterize the biological behavior of the micelles as well as to optimize the micelle formulation. Therefore, biodistribution studies of DACHPt-loaded micelles prepared with PEG-P(Glu) 12-40 (PEG-P(Glu) 12-40: Mw_{PEG}: 12,000; p(Glu) units: 40) were performed at different times on mice bearing subcutaneous C-26 tumors. This DACHPt-loaded

micelles have long circulation in blood and showed 20-fold greater accumulation in tumor even after 48h. Morevover, the biodistribution of DACHPt-loaded micelles prepared with PEG-P(Glu) block copolymer having different p(Glu) lengths [p(Glu): 20, 40, and 70 units] was studied with the aim of optimizing the system's biological performance. The micelles prepared from PEG-*b*-P(Glu) with 20 units of P(Glu) exhibited the lowest non-specific accumulation in the liver and spleen to critically reduce non-specific accumulation, resulting in higher specificity to solid tumors. In addition, DACHPt-loaded micelles prepared with PEG-P(Glu) 12-20 or 12-40 presented extremely high antitumor activity against a subcutaneous murine colon adenocarcinoma 26 while toxicity was considerably reduced for the PEG-P(Glu) 12-20 formulation. Furthermore, DACHPt-loaded micelles presented extremely high antitumor activity against a series of intractable tumor models: a subcutaneous BxPC3 pancreatic tumor model that is refractive to established therapies and hypovascular, a melanoma lung metastasis model and a HeLa intraperitoneal metastasis. The outstanding results obtained in all the tumor models suggest that DACHPt-loaded micelles could be an outstanding drug delivery system for oxaliplatin in the treatment of solid tumors.

Nevertheless, the activity of most long circulating drug carriers decreases due to their poor cellular uptake, which is often a disadvantage for exerting drug efficacy compared to free drugs that rapidly move into the interior of the cells. Therefore, it is likely that if a macromolecular drug carrier is actively incorporated by cancer cells, the specificity and bioavailability of those drug delivery systems should increase more than a drug delivery system that just takes advantage of the EPR effect to target the tumor site. There are many examples for utilizing the existing endocytosis pathways (Figure 3) for specific delivery of drugs. The conjugation of normally endocytosed ligands with macromolecular carriers frequently improves intracellular accumulation of the prodrug. Among these ligands, the folic acid has been intensively investigated as a means for tumor-specific delivery of a broad range of experimental therapies including several conceptually new treatments. Herein, folic acid was conjugated on the surface of polymeric micelles incorporating the active

complexes of Cisplatin or DACHPt in order to obtain folate-bound polymeric micelles directed to the Folate Binding Protein (FBP) overexpressed on cancer cell surfaces (Figure 3).

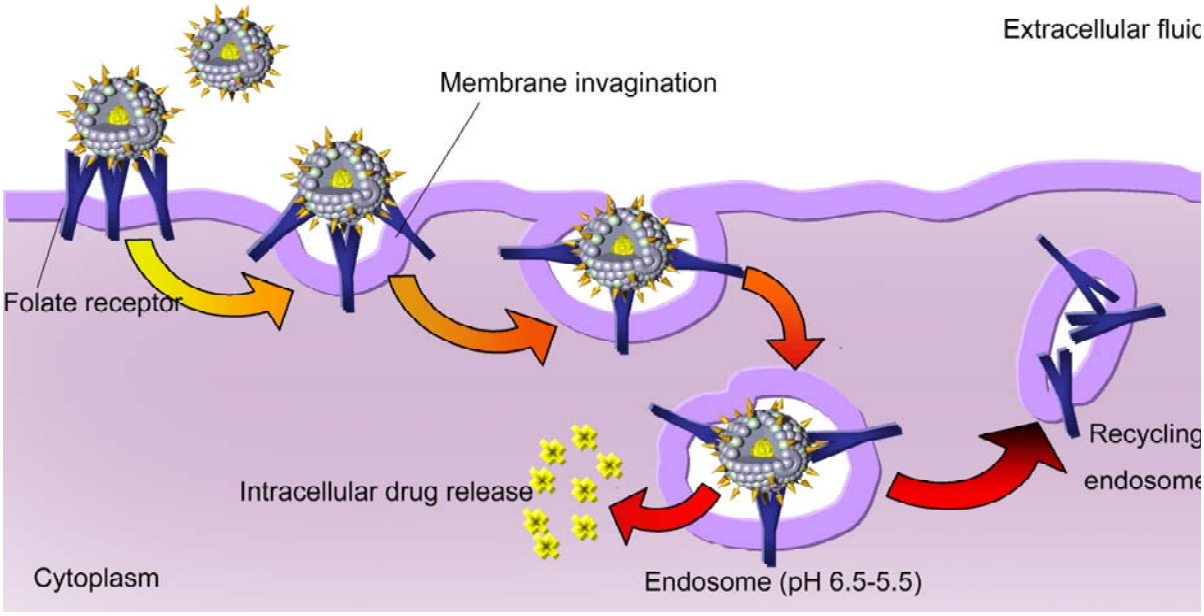

Figure 3. Folate receptor-mediated endocytosis of folate-drug conjugates. Exogenously added folate-drug conjugates bind specifically to the folate binding protein with high affinity. The plasma membrane invaginates around the conjugate/folate receptor complex to form an intracellular vesicle (endosome). As the lumen of the maturing endosome acidifies up to pH 5.5, the receptor changes conformation and releases the conjugate. Eventually, the fates of the folate ligand, attached drug cargo and the folate receptor are determined during a sorting process within late endosomal elements.

Platinum drug-loaded micelles were prepared through metal-polymer complex formation with Acetal-Benzyl-poly(ethylene glycol)-b-poly(glutamic acid) (Ac-Bz-PEG-b-P(Glu)). A hydrazide group was introduced to folic acid to obtain folate-hydrazide. Then, the folate-hydrazide was bounded to the surface of the micelles to obtain folate-conjugated platinum drug-loaded micelles. The folate-conjugated platinum drug-loaded micelles presented narrowly distributed diameters of approximately 40nm. The zetapotential of folate-conjugated platinum drug-loaded micelles at pH 7.4 decreased as the percentage of conjugated folate on the micelle surface increased. The interaction of folate-bound platinum drug-loaded micelles with the folate binding protein (FBP) was determined to be very strong by surface plasmon resonance (SPR) studies. Moreover,

folate-conjugated platinum drug-loaded micelles showed enhanced *in vitro* growth inhibitory activity against the pharyngeal cancer KB cells after short incubation much higher than non-targeted platinum drug-loaded micelles and comparable to that of free drugs. These results suggest that the folate-targeting method for platinum drug-loaded micelles seems to be a very effective way to increase the specificity and activity of platinum drug-loaded micelles to cancer cells.

From the results attained in this study it can be concluded that the DACHPt-loaded micelle offers an exceptional platform for the delivery of the active complexes of oxaliplatin specifically targeting the tumor tissue and enhancing the drug activity. Furthermore, since polymeric micelles can be tuned relatively easily, the future perspectives for this micellar system are enormous.