論文の内容の要旨

論文題目　**A study on the intracellular behavior and pharmacological effect of polymeric micelle incorporating platinum anticancer drug**
(白金錯体制癌剤を内包する高分子ミセルの細胞内挙動および
薬理作用に関する研究**)**

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Platinum-based chemotherapy is widely used for the treatment of many malignancies [1]. Oxaliplatin, a diaminocyclohexane-containing platinum, is a member of the family of Pt-containing chemotherapeutic agents that also include cisplatin and carboplatin. Oxaliplatin is the only Pt-containing drug to have been shown to be effective in the treatment of colorectal cancer, and even though discovered 25 year ago, still remains standard of care for this particular disease. The prognosis for these patients, however, is poor because of frequent chemoresistance acquired during treatment [2-3].

The use of polymeric drug delivery systems can have enhanced anticancer effects compared with the therapeutic entities they contain due to more specific targeting of tumor tissues. The increased permeability of the tumor vasculature allows the tumor to accumulate large molecules more efficiently than normal tissues. At the same time, the poor lymphatic drainage of tumors allows high concentrations of polymeric drug to be retained in these tissues. This has been referred to as the enhanced permeability and retention (EPR) effect [4].

Previously, we have successfully prepared polymeric micelles incorporating (1,2-diaminocyclohexane)platinum(II) (DACHPt/m) [5-6], the parent complex of oxaliplatin. This system is at present being evaluated in phase I clinical trials. In preclinical experiments, DACHPt/m resulted in a high and selective accumulation of platinum at tumor tissue, and they showed to be effective in murine colon adenocarcinoma 26 tumors, which are not sensitive to oxaliplatin treatment. Moreover, DACHPt/m showed a high antitumor activity against intraperitoneal HeLa metastases,

while oxaliplatin failed to suppress metastatic growth [6]. Although these appear encouraging results, the cellular activity of DACHPt/m remains to be characterized.

Recently, several studies have indicated that macromolecular drugs may have improved cellular pharmacology, or even overcome multidrug resistance (MDR) to mainly natural-product-based drugs which is caused by the overexpression of ATP-dependent efflux pumps such as P-glycoprotein (P-gp). Since macromolecular drugs enter cells by endocytic internalization, these drugs are able to penetrate cells without being recognized by P-gp leading to high intracellular drug concentration that can overcome efflux-pump mediated drug resistance [7]. Unlike the other chemotherapeutic agents, platinum drugs are not affected by P-gp expression [8]. The major mechanisms of platinum drugs are decreased membrane transport, increased cytoplasmic detoxification, increased DNA repair, and increased tolerance to platinum-induced DNA damage [9].

In the present work, the cytotoxicity of DACHPt/m against HT29 human colon cancer cells was studied. It was demonstrated that DACHPt/m has a greater cytotoxic effect compared with oxaliplatin. Moreover, the cell growth inhibition profile of DACHPt/m was examined using a human cancer cell panel. DACHPt/m seemed to be more effective against several cancer cell lines such as melanoma LOXIM VI, colon cancer HT29 and Breast cancer BSY-1 cell lines probably due to different cellular internalization or distribution of DACHPt/m from that of oxaliplatin.

In this study, a new method to assess cellular distribution and processing pathways of DACHPt/m in living cells by time lapse imaging using 2 different fluorescence-labeled DACHPt/m (F-DACHPt/m) was developed (Figure 1). During micelle state, only the dye conjugated to the micelle shell produces fluorescence while the core-conjugated dye remains quenched. In chloride containing media, the DACHPt is released from the micelle core, and as the platinum density at the core decreases, the core-conjugated dye is dequenched. Using this method, I could follow the cellular uptake of the micelles and their intracellular fate by time-lapse and confocal microscopy. The release rate of the platinum drug from the micelles was consistent with the fluorescence dequenching profile of the core-conjugated dye.

Moreover, the release of DACHPt from DACHPt/m under different intracellular conditions mimicking early endosome: 10 mM phosphate buffer, pH6.9, plus 20 mM NaCl, and late endosome: 10 mM phosphate buffer, pH5.5, plus 70 mM NaCl [10], was evaluated by the dialysis method. The release rate of platinum complexes in the environment of late endosomes accelerated up to 24 hours and was found to be faster than the release rate at early endosomal conditions, suggesting that DACHPt/m may mainly release DACHPt after reaching the late endosomal or lysosomal compartments. In addition, the release rates of F-DACHPt/m at endosomal conditions were consistent with those of DACHPt/m while the changes in the fluorescence intensity corresponded to the DACHPt release profiles.

To study the intracellular trafficking of F-DACHPt/m, HT29 cells were incubated with F-DACHPt/m and analyzed by time-lapse microscopic observation. After 6 h incubation, HT29 cells treated with F-DACHPt/m emitted the fluorescence only from the shell-conjugated dye (Bodipy-FL) while the core-conjugated dye remained quenched (Bodipy-TR) indicating that F-DACHPt/m might enter the cells in micelle form. After 24 h incubation, F-DACHPt/m displayed the fluorescence from the core-conjugated dye at the perinuclear areas.

To verify whether or not the F-DACHPt/m is located in the acidic late endosomal and lysosomal compartments, confocal microscopy studies were performed by incubation with LysoTracker. After 6h incubation, F-DACHPt/m accumulation in the late endosomal and lysosomal compartments became clear. After 24 h incubation, the accumulation of F-DACHPt/m in the late endosome and lysosome was significantly increased, and after 55 h, the core fluorescence (Bodipy TR) became evident and showed colocalization with the fluorescence from the shell and LysoTracker at the perinuclear regions.

At the previous study, the profile of DACHPt release was comparable to the fluorescence profile of the core fluorescence. Thus, the specific accumulation of F-DACHPt/m in the late endosome and lysosome, and the preferential release of DACHPt in those environments suggest that DACHPt/m may enter cancer cells by endocytosis and deliver the platinum complexes at the perinuclear region.

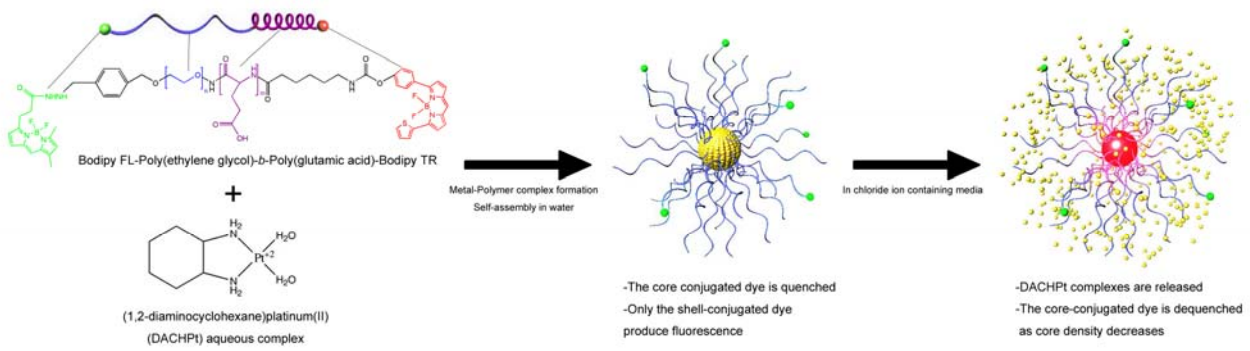

Figure 1 Fluorescence tagged block copolymer micelles, containing dichloro(1,2-diaminocyclohexane) platinum(II)(DACHPt, the oxaliplatin parent complex, self-assembled through polymer-metal complex formation of DACHPt with Bodipy FL –poly(ethylene glycol)-*b*-poly(glutamic acid)[PEG-*b*-P(Glu)]-Bodipy TR in distilled water. During micelle formation, only the shell-conjugated dye produces fluorescence while the core-conjugated dye remains quenched. As DACHPt is released from the micelle in chloride ion containing media, the core-conjugated dye is dequenched and emits fluorescence.

Additionally, the total cellular Pt levels and the amount of Pt bound to DNA after drug exposure (10μM) were measured by ICP-MS. In both intracellular Pt accumulation and amount of Pt-DNA adducts, exposure to oxaliplatin and DACHPt/m resulted in a time-dependent increase in Pt levels. For the total Pt levels, the exposure of HT29 cells to oxaliplatin resulted in 2-fold higher Pt accumulation compared to DACHPt/m. In contrast, no significant differences in DNA platination were observed between oxaliplatin and DACHPt/m exposure. Moreover, the ratio between platinum adducts and intracellular accumulation presented a much higher value for micelle being consistent with the hypothesis that DACHPt/m probably enhance platinum delivery to the nucleus. It should be noted that in the late endosome condition at 24 hours only 28% of the drug loaded in the micelles is released, suggesting that the DNA binding efficiency is markedly high, since the drug bound to the polymer might not bind to DNA.

Since DACHPt/m showed differences in cellular uptake, selective release at perinucleus, and efficient platinum delivery to DNA, the possibility that DACHPt/m retain antitumor activity against oxaliplatin resistant cell lines (HT29/ox) was studied. Thus, HT29/ox cells were prepared by exposure to oxaliplatin for 1 hour every other day. The level of resistance to oxaliplatin was evaluated by MTT

assay. Thus, HT29/ox exhibited 10-fold resistance to oxaliplatin as compared to the sensitive parental HT29 cells while DACHPt/m overcame acquired resistance to oxaliplatin in HT29/ox cells.

To further confirm the enhancement of *in vivo* antitumor activity by DACHPt/m, Balb/c nu/nu mice bearing subcutaneous HT29 cells or HT29/ox cells were treated i.v. three times at 2-day intervals with oxaliplatin at doses of 8 mg/kg or DACHPt/m at doses of 4 mg/kg on a Pt basis which is the maximum tolerated value for each drug. The tumor volume in mice treated by DACHPt/m was approximately 10 times smaller than non-treatment group ($p<0.01$), and approximately 3 times smaller than those treated with oxaliplatin after 3 weeks (Figure 2A). Importantly, a similar high antitumor activity of DACHPt/m was observed against HT29/ox, while the change in tumor size after treatment with 8 mg/kg of oxaliplatin injected by the i.v. route was not different from control ($P>0.1$) (Figure 2B).

26 genes were selected among those available in the complete NCI database as potentially related to sensitivity or resistance to platinum compounds from the data available in the literature [11-23]. The coefficients of correlation were obtained between the cytotoxicity of two drugs (i.e.,oxaliplatin and DACHPt/m) or relative GI_{50} (the ratio of GI_{50} of DACHPt/m and GI_{50} of oxaliplatin) against a human cancer cell panel, and the level of expression of those markers. Metallothionein (MT1Q) and methionine synthase (MTR) were found positively correlated to GI_{50} of oxaliplatin, while there was no correlation with DACHPt/m. Thus, it was of interest to establish whether or not MT1Q and MTR expression was also upregulated in oxaliplatin resistant cells. The confirmation of the mRNA expression was determined by quantitative real-time RT-PCR. The expression of MT1Q and MTR protein in HT29 and HT29/ox were determined by Western blotting. Thus, MT1Q and MTR expression were found to be significantly higher in oxaliplatin resistant cells compared to parent cells.

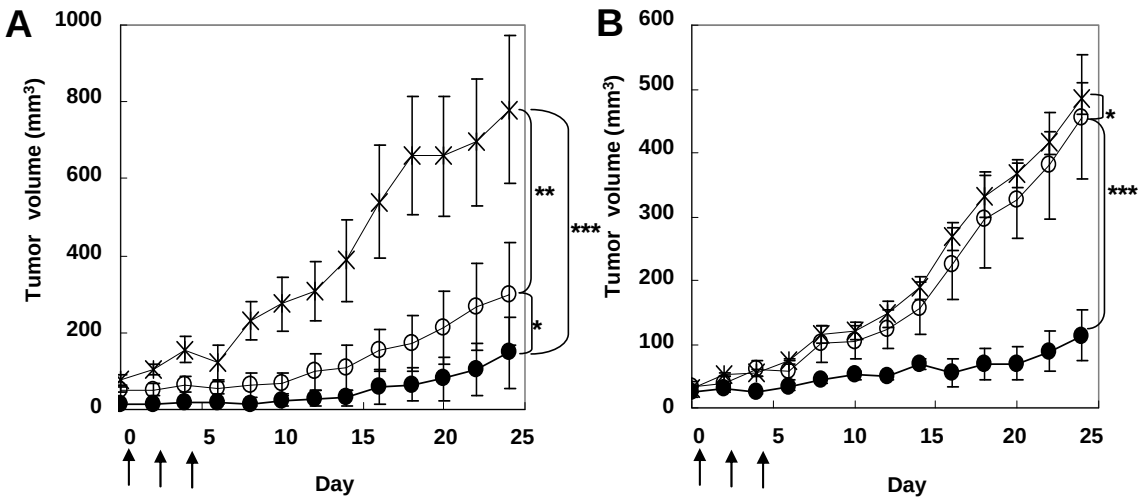

Figure 2. Antitumor activity of DACHPt/m against s.c.HT29 tumor model. Saline(crosses); oxaliplatin at 8 mg/kg(open circles);DACHPt/m at 4mg/kg (filled circles). A. Tumor volume against HT29 model; B. Tumor volume against HT29/ox model. Data are expressed as mean. Error bars show s.e.m.; n=4. *P>0.1, **P<0.05, ***P<0.01

Similar to other platinum drugs, the biological activity of oxaliplatin is based on its ability to form lethal DNA lesions, including interstrand DNA crosslinks and DNA-protein crosslinks. Once inside cells, oxaliplatin is activated by the addition of water molecules to form chemically reactive DACHPt aqua species. This is facilitated by the relatively low chloride concentrations that are found within cells [24]. Since DACHPt aqueous complexes easily react with other organelles and proteins leading to inactivation of drug, and activation of cellular defense mechanisms, it is important for platinum drugs to sneak up into the nucleus to avoid recognition by the cellular detoxification system. On the contrary, DACHPt/m probably enter via endocytosis pathway and might protect their cargo from interaction with high sulphur species, such as methionine and metallothioneins, which reacts with the activated aqua species and effectively export DACHPt form the cells, during the intracellular trafficking (Figure 3), resulting in the enhancement of drug delivery to the nucleus and the avoidance of certain cellular defense mechanisms in cytoplasm.

In summary, it has been proved that DACHPt/m was internalized by endocytosis, accumulated at perinuclear region and efficiently deliver DACHPt to DNA. This unique intracellular behavior of

DACHPt/m probably has responsibility for overcoming resistance in HT29/ox. This mechanism might be different from previously described polymeric drug conjugates overcoming certain kinds of multidrug resistance. Thus, this research provides new aspects for cancer therapy using drug delivery systems.

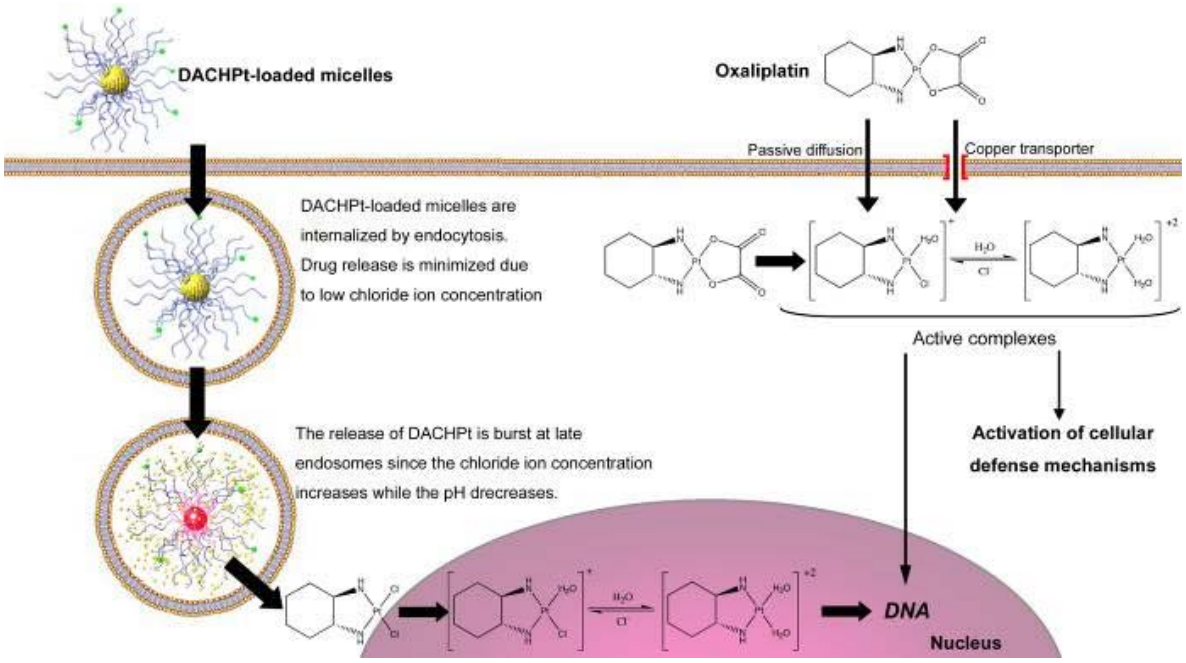

Figure 3. Micelle mechanisms. Scheme depicting the possible reaction pathway of oxaliplatin and DACHPt/m in the cell. Oxaliplatin might enter cells by passive or through the copper transporter CTR1. Once oxaliplatin is in the cytoplasm, most of the activated aqua species are eliminated by detoxification system, while a small fraction binds to DNA. DACHPt/m enter tumor cells by endocytosis, which is accompanied by an increase of acidity and chloride concentration inside the vesicle as it matures into late endosomes. The release of platinum from DACHPt/m is 7 fold higher at late endosome environment than early endosome. Thus, DACHPt/m may release DACHPt at perinucleus resulting in enhanced delivery to the nucleus.

Refereces:

[1] Wong E, Giantomenico CM. *Chem Rev* 1999;99:2451-66.

[2] Kelland L. *Nat Rev Cancer* 2007;7:573-84.

[3] Rabik CA, Dolan ME. *Cancer Treat Rev* 2007;33:9-23.

[4] Matsumura Y, Maeda H. *Cancer Res* 1986;46:6387-92.

[5] Cabral H, Nishiyama N, Okazaki S, et al. *J Control Release* 2005;101:223-32.

[6] Cabral H, Nishiyama N, Kataoka K. *J Control Release* 2007;121:146-55.

[7] Davis ME, Chen Z, Shin DM. *Nature Rev Drug Discov* 2008;7:771-82.

[8] Szakacs G, Paterson JK, Ludwig JA, et al. *Nature Rev Drug Discov* 2006;5:219-34.

[9] Kelland L. *Nat Rev Cancer* 2007;7:573-84.

[10] Sonawane ND, Thiagarajah JR, Verkman AS. *J Biol Chem* 2002;277:5506-13.

[11] Perez RP. *Eur J Cancer* 1998;34:1534-42.

[12] Niedner H, Christen R, Lin X, et al. *Mol Pharmacol* 2001;60:1153-60.

[13] Fink D, Nebel S, Aebi S, et al. *Cancer Res* 1996;56:4881-6.

[14] Godwin AK, Meister A, O'Dwyer PJ, et al. *Proc Natl Acad Sci USA* 1992;89:3070-4.

[15] Mistry P, Kelland LR, Abel G, et al. *Br J Cancer* 1991;64:215-20.

[16] Kelley SL, Basu A, Teicher BA, et al. *Science (Wash. DC)* 1988;241:1813-5.

[17] Chen ZS, Mutoh M, Sumizaw T, et al. *Exp Cell Res* 1998;240:312-20.

[18] Eliopoulos AG, Kerr DJ, Herod J, et al. *Oncogene* 1995;11:1217-28.

[19] Miyake H, Hanada N, Nakamura H, et al. *Oncogene* 1998;16:933-43.

[20] Perego P, Giarola M, Righetti SC, et al. *Cancer Res* 1996;56:556-62.

[21] Arnould S, Hennebelle I, Canal P, et al. *Eur J Cancer* 2003;39:112-9.

[22] Shirota Y, Stoehlmacher J, Brabender J, et al. *J Clin Oncol* 2001;19:4298-304.

[23] Marth C, Widschwendter M, Kærn J, et al. *Br J Cancer* 1997;76:1328-32.

[24] Jamieson ER, Lippard SJ. *Chem Rev* 1999;99:2467-98.