Efficacy of the Platinum Analog {Pt(cis-dach)(DPPE) - $2NO_3$} on Histocultured Human Patient Bladder Tumors and Cancer Cell Lines

SUNG-GOO CHANG[1], JEE-CHANG JUNG[2], YOUNG-SOO RHO[3], JUNG-SIK HUH[1], JIN-IL KIM[1] and ROBERT M. HOFFMAN[4]

[1]Department of Urology, [2]Department of Pharmacology, School of Medicine, [3]Department of Medicinal Chemistry, School of Pharmacy, Kyung Hee University Seoul, 130-702 Korea; [4]AntiCancer Inc., 7917 Ostrow Street, San Diego CA 92111, U.S.A.

Abstract. *Cisplatinum is currently used as a front line agent in many important tumors, but its dose-limiting nephrotoxicity prevents potential efficacy. There is therefore great interest in developing new platinum agents that have less toxicity. We have synthesized new platinum analogues containing DACH as a carrier ligand and DPPE as a leaving group. Previously we showed that these new platinum complexes have much less nephrotoxicity than cisplatinum. In the present study, the efficacy of one new platinum complex was evaluated with human patient bladder tumor specimens in three-dimensional histoculture as well as with monolayer cultures of cancer cell lines. The efficacy end points used were glucose consumption and thymidine incorporation on the histocultured specimens and MTT reduction on monolayer cell cultures. Our results showed that the new platinum complex was more effective at high concentration (10^{-3}M) but less effective at low concentration (10^{-4}M) compared to cisplatinum on histocultured bladder tumor specimens. The compound demonstrated higher efficacy than cisplatinum on P-388, and L-1210 leukemic cell lines. The new analog demonstrated similar efficacy to cisplatinum on the MKN-45 human stomach cancer cell line. The PC-14 human lung cancer cell line, MH_1C_1 rat hepatoma cell line, NIH-OV3, SKOV-3 ovarian cancer cell lines were as sensitive to the new analog as to cisplatinum at high concentrations of the new platinum analogue. The cisplatinum-resistant M-14 melanoma cell line was not sensitive to either the new analog or cisplatinum. Based on these results, this novel platinum compound appears to be a valuable lead compound with high efficacy and low nephrotoxicity.*

Correspondence to: Prof. Sung-Goo Chang M.D. Ph.D., Dept. of Urology, Kyung Hee University Medical Center, # 1 Hoegidong Tongdaemun-Ku, Seoul 130-702 Korea. Tel: 82-2-958-8533; Fax: 82-2-959-6048.

Key Words: Platinum analog, antitumor activity, histoculture, human bladder tumor.

Cisplatinum is currently used as a first line chemotherapeutic agent for the treatment of testicular cancer, bladder cancer and other cancers. However, the efficacy of cisplatinum is compromised by its propensity to cause several types of dose-limiting toxicity, including nephrotoxicity, nausea, neurotoxicity and myelosuppression. The nephrotoxicity is due to the fact that the kidney accumulates and retains platinum to a greater extent than other organs and is the prominent excretory organ for platinum complexes.

Consequently, there is much interest in obtaining agents that have less toxicity and have more favorable therapeutic indices. To accomplish this goal, we synthesized new platinum analog containing 1,2-diaminocyclohexane (dach) as a carrier ligand and diphenylphosphinoethane (DPPE) as a leaving group. Previously, we have shown that this new platinum complex has much less nephrotoxicity than cisplatinum.

In the present study we report on the synthesis of a new platinum complex and the efficacy of the new platinum complex evaluated on human patient bladder tumor specimens in three dimensional histoculture as well as on monolayer cultures of several different types of cancer cell lines.

Materials and Methods

Synthesis of platinum(II) complexes. (cis-1,2-diaminocyclo-hexane) dichloroplatinum(II)- [Pt(cis-dach) Cl_2] was added to a solution of K_2PtCl_4 (2.5 g, 6.02 mM) in water (80 ml), to which a solution of cis-dach 2HCl (1.13 g, 6.02 mM) in water (20 ml) was added. The mixture was adjusted to pH 6.5 by titration with 5 % NaOH and stirred for 30 min. at room temperature. The yellow crystals were formed and filtered. The yellow crystals were dried by vacuum evaporation.

(cis-1,2-diaminocyclohexane) dinitrate platinum(II)-[Pt(cis-dach) $(NO_3)_2$] was added to a suspension of Pt(cis-dach)Cl_2 (1 g, 6.02 mM), to which a solution of $AgNO_3$ (890 mg, 5.26 mM) was added stepwise in distilled water (10 ml). The reaction mixture was stirred for 24 hours at room temperature. The reaction product of AgCl was filtered off. The filtrate was concentrated under reduced pressure and dried by lyopholization.

{1,2-Bis (diphenylphosphino) ethane}(cis-1,2-diaminocyclohexane)-Pt(II) nitrate-[Pt(cis-dach)(DPPE)]-$(NO_3)_2$. H_2O was added to a solution of Pt(cis-dach)$(NO3)_2$ (500 mg, 1.15 mM) in 15 ml of water, to which a solution of DPPE (460 mg, 1.15 mM) was added in 20 ml of acetone. The product was recrystalized from water.

Tissue specimens. Human bladder superficial transitional cell carcinoma tissue, identified by frozen section at the time of transurethral resection in patients with bladder cancer, was transported in a sterile container to the laboratory located near the operating room.

Cell culture. Monolayer cultures of cancer cell lines were grown with PC-14 (human lung cancer), MKN-45 (human stomach cancer), MH_1C_1 (rat hepatoma), P-388 (rat leukemia), L-1210 (rat leukemia), M-14 (human melanoma), NIH-OV3 (human ovarian cancer) and SKOV-3 (human ovarian cancer).

Collagen-sponge-gel histoculture of human superficial bladder tumor tissue. Bladder tumor tissues were divided into 2 to 3 mm diameter pieces and five pieces were placed on top of previously hydrated Spongostan gels (1 × 1 × 1 cm) (Health Design Indust. Rochester, NY). Each gel occupied one well of a six-well plate. Three milliliters of Eagle's Minimal Essential Medium (MEM)(GIBCO Grand Island, NY) supplemented with 10 % fetal bovine serum (GIBCO), 50 μg/ml gentamicin final concentration, and cefotaxime (Hoechst, Somerville, NY) at a final concentration of 1 μg/ml were added. The final volume of medium was sufficient to reach the upper gel surface without immersing it. Covered culture plates were maintained in a humidified, 5 % CO_2 incubator at 37°C. The cultures underwent sterile media changes every 72 hours. Histoculture was continued up to 11 weeks after explantation.

Drug treatment. Histocultured specimens were incubated in medium with 10^{-3}M, 10^{-4}M or 10^{-5}M of the new platinum complex or cisplatinum for 72 hours. Cancer cell lines were incubated for 48 hours with various experimental drug concentrations that corresponded to concentration of reported cisplatinum used in our previous experiments (1). After drug treatment, the specimens were washed with phosphate buffered saline and fresh medium.

MTT assay on cancer cell lines. This assay was performed essentially as described previously(2). Briefly, the confluent cancer cells were disaggregated using 0.002 % EDTA in 0.05 % trypsin. A single- cell suspension was made by centrifugation (1000 rpm, 10 min.), and resuspension in DME/F12 (10^6 cell/ml). This suspension was seeded at 2 × 10^6 cells *per* well in 96-well plates with 100 μl of medium *per* well. Drugs were added at various concentration and cultures were then incubated for 48 hours in an incubator at 37°C with a highly humidified atmosphere, 5 % CO_2 and 95 % air. After the treatment period, 50 μl of medium containing MTT (5 mg/ml) was added to each well.

After 4 hours of exposure, the medium was removed and the wells were washed with PBS, and then 50 μl of DMSO was added to each well to solubilize the precipitates. The plates were transferred to an ELISA reader to measure the extracted dye at 630 nm. All experiments were performed at least 3 times, with 6 wells for each concentration of the tested agents.

Glucose consumption from histocultured specimens. This study was performed essentially as described by Chang *et al* (3). Briefly, fifty μl culture medium were removed every 24 hours for determination of medium glucose content in triplicate using the HK 20 assay kit from Sigma (St Louis MO). Measurements were made by monitoring the change in optical density at 340 nm due to the reduction of NAD catalized by hexokinase with the glucose substrate before and after treatment. The glucose content of the medium was plotted in semilog form *versus* time using the Sigma plot program (Jandel Scienfic, Corte, Madera CA). A simple exponential model of glucose consumption was then fitted to the data with the Systat program (Systat. Inc. Evanston, IL). The half-life of glucose was calculated from the slope parameter of this model using the equation $t_{1/2}$= 0.693/s where s= slope of the best fit linear regression line of the natural log of the glucose concentration plotted *versus* time. The glucose content of the medium was measured daily for 3 days. The log values over 3 days were plotted vs time and the slope of the best-fit line was taken as the glucose consumption rate during the 3-day measurement period (one period).

DNA precursor uptake. Autoradiography was carried out after the cultures were exposed to medium containing 4 uCi/ml [^{3}H] thymidine for 72 hours. Fixation, embedding, sectioning and deparaffinization were subsequently carried out. Specimen slides were exposed to Kodak NTB2 liquid emulsion (Kodak Rochester NY) for 10 days at 4°C, followed by standard development in D19 Kodak developer. The slides were then stained with hematoxylin and eosin. The DNA labeling index was defined as the number of cells labeled *per* total cells evaluated on microscopic field. An effort was made to evaluate only the most active area of all slides to avoid false-positive scoring.

Results

Histocultures. Transurethral resectioned specimens of human bladder tumor tissues were histocultured for up to 11-weeks after explantation. The histoculture specimens grew by expansion and infiltration of the collagen gels (Figure 9). The histocultured explants in each culture grew from the small pieces originally explanted into a larger tissue mass. From these results, we conclude that the long-term histoculture of human bladder transitional cell carcinoma tissue is possible and should be useful for long term studies of drug responsiveness.

Efficacy of the new platinum complex on cancer cell lines in monolayer culture. Several cancer cell lines were treated with various concentrations of new platinum complex (KHPC-006) and cisplatinum. The concentrations were determined by previously reported cisplatinum effects on each cell line (1). The cytotoxicity of the new complex on the P-388 leukemic cell line and L-1210 leukemic cell line was more effective than that of cisplatinum as determined by the MTT assay (Figure 1). The cytotoxicity of the new complex on the MKN-45 human stomach cancer cell line was very similar to that of cisplatinum (Figure 2). The cytotoxicity of the new complex on the PC-14 human lung cancer cell line, MH_1C_1 rat hepatoma cell line, NIH-OV3 ovarian cancer cell line, SKOV-3 ovarian cancer cell line was similar to that of cisplatinum at high concentration (Figures 3, 4). The M-14 melanoma cell line was cisplatinum resistant. The cytotoxicity of the new complex on the M-14 melanoma cell line was found to be very low (Figure 5).

Efficacy of the new platinum complex on human bladder tumor

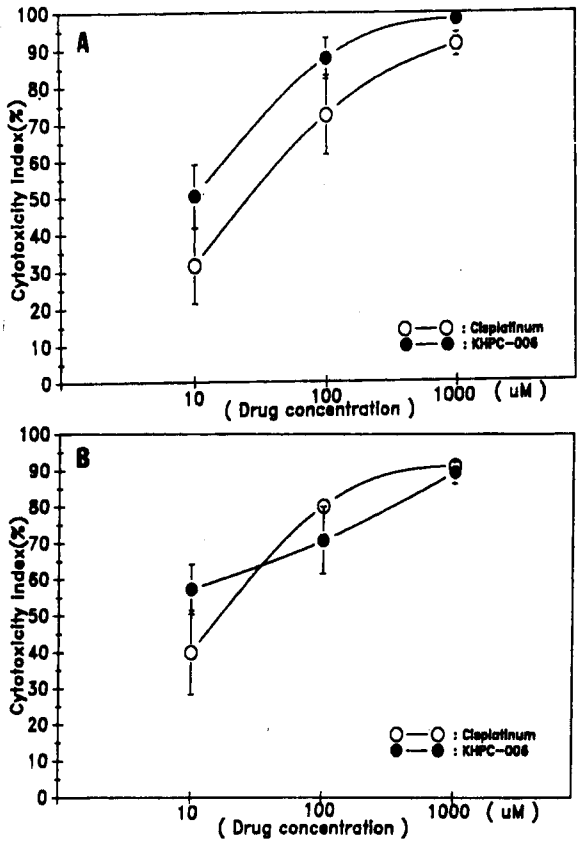

Figure 1. *A) The P-388 leukemic cell line treated by cisplatinum and the new complex (KHPC-006). The new complex had more cytotoxic effects than cisplatinum. B) The L-1210 leukemic cell line was sensitive to cisplatinum and the new complex, but the new complex had more cytotoxic effects at lower concentration than did cisplatinum. 2 × 10^6 cells were treated in monolayer culture with 48 hours exposure.*

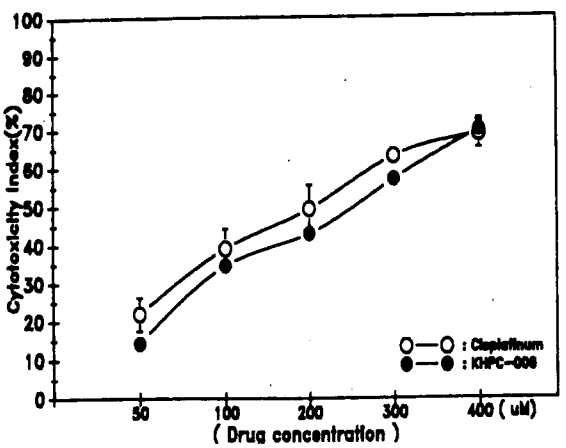

Figure 2. *The MKN-45 human stomach cancer cell line treated with cisplatinum and KHPC-006. The cytotoxic effects of cisplatinum and KHPC-006 were similar, particulary at high concentration.*

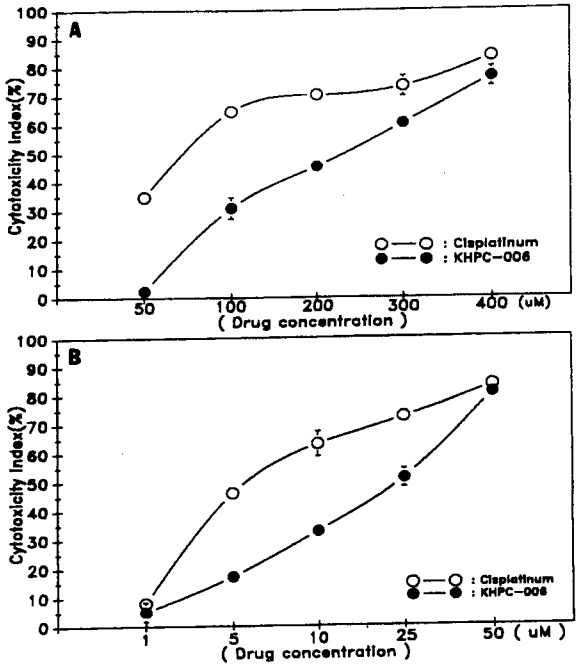

Figure 3. *A) The cytotoxic effect of the new complex on the PC-14 human lung cancer cell line was gradually increased by increasing the concentration until it was similar to cisplatinum at 400 μM. B) The cytotoxic effect of the new complex on the MH_1C_1 rat hepatoma cell line was approximately 80 % at 50 μM. The error bars indicate a 95 % confidence interval.*

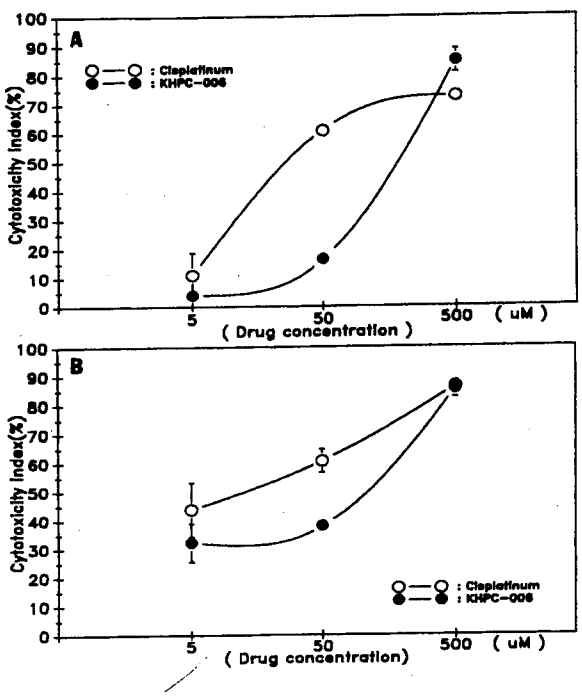

Figure 4. *A) The NIH-OV3 ovarian cancer cell line was sensitive at high concentrations of cisplatinum and KHPC-006. There were no cytotoxic effects of KHPC-006 at 10 and 100 μM. B) The SKOV-3 ovarian cancer cell line was also sensitive to both cisplatinum and KHPC-006 at high concentration.*

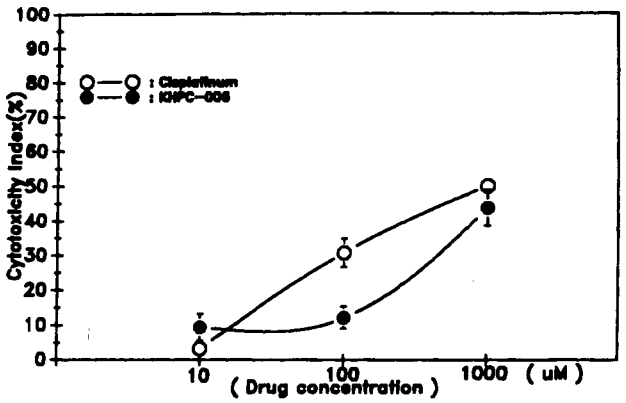

Figure 5. *The M-14 melanoma cell line was resistant to both cisplatinum and KHPC-006. There was no cytotoxic effect even at high concentration.*

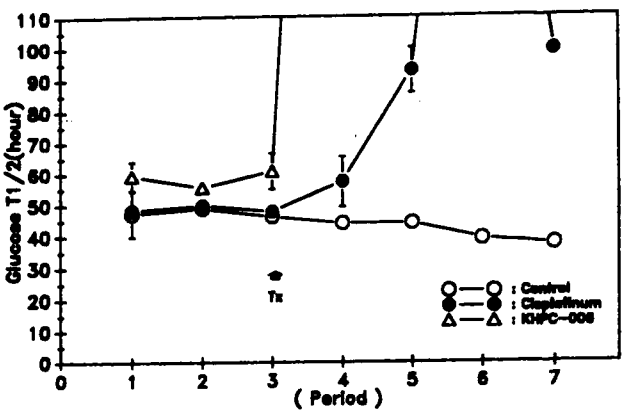

Figure 6. *The histocultured human bladder transitional cell carcinoma treated with 10^{-3} M cisplatinum and 10^{-3} M KHPC-006 with 72 hours exposure. All experimental histocultures had their own control (period 1,2,3). The glucose $T_{1/2}$ was abruptly delayed after treatment with KHPC-006 and did not recover, contrast the glucose $T_{1/2}$ gradually increased and recovered in the case of cisplatinum-treated histocultures. Tx: treatment.*

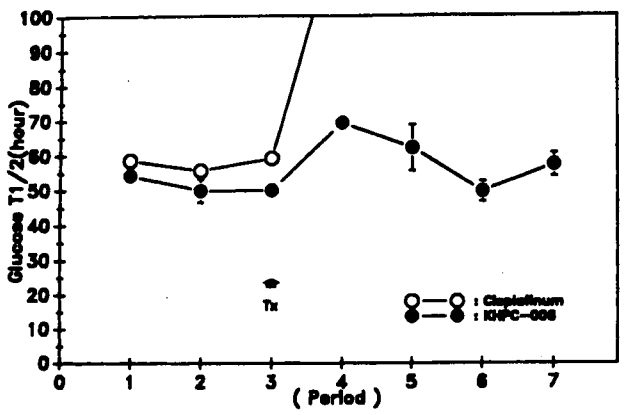

Figure 7. *Eight-week histocultured human bladder tumor tissues treated with 10^{-4} M cisplatinum and KHPC-006: The histocultures were exposed for 72 hours to either drug. One period constituted 3 days. Each well had its own control. Cisplatinum-treated histocultures revealed markedly delayed glucose $T_{1/2}$. However the glucose $T_{1/2}$ was only slightly delayed and then recovered in the KHPC-006-treated histocultures.*

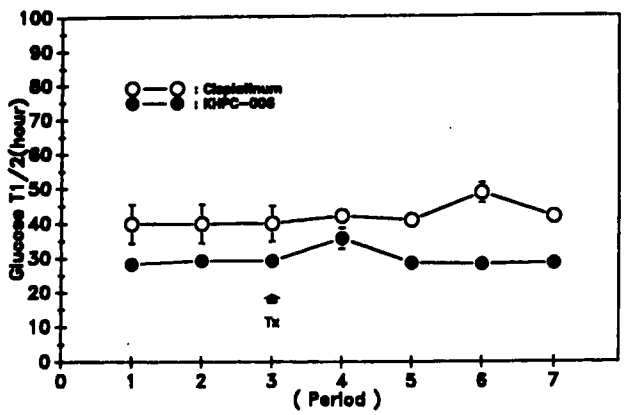

Figure 8. *Histocultures of human bladder tumors treated with 10^{-5} M cisplatinum or 10^{-5} M KHPC-006: There was no evidence of definitive delayed glucose $T_{1/2}$ after treatment with either drug. Error bars indicate 95% confidence interval.*

specimens in histoculture. The efficacy of the new platinum complex was determined by the glucose consumption test carried out on 8-week histocultured bladder tumor specimens. The medium glucose half-life varied with the different drug concentrations tested. Figure 6 shows the medium glucose content half-life from histocultures at a concentration 10^{-3} M. The results demonstrated that the glucose consumption rate in the control histocultures remained constant over 7 periods of measurement covering 8 weeks of histoculture. This long term constancy of metabolism of the human bladder transitional cell carcinoma histoculture allows the long-term measurement of drug-response and recovery with this end point.

The glucose half-life was abruptly elongated after treatment with a concentration of 10^{-3} M of the new platinum complex, and did not recover (Figure 6). In contrast the glucose half-life was gradually increased and recovered in the 10^{-3} M cisplatinum-treated histocultures. The glucose half-life in the histocultures treated with a 10^{-4} M concentration of the drugs was more variable. The medium glucose content half-life was longer in cisplatinum-treated histocultures than in those with the new complex (Figure 7). Figure 8 shows experimental groups treated with a 10^{-5} M concentration of

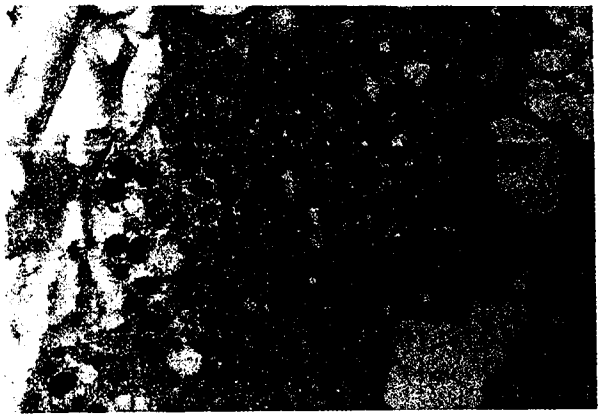

Figure 9. *Autoradiographic findings on 11-week histocultured human bladder tumor control specimens revealed well proliferating tumor cells and high uptake thymidine. The glucose $T_{1/2}$ of this group was approximately 45 hours and thymidine labeling index was 0.205 (X200).*

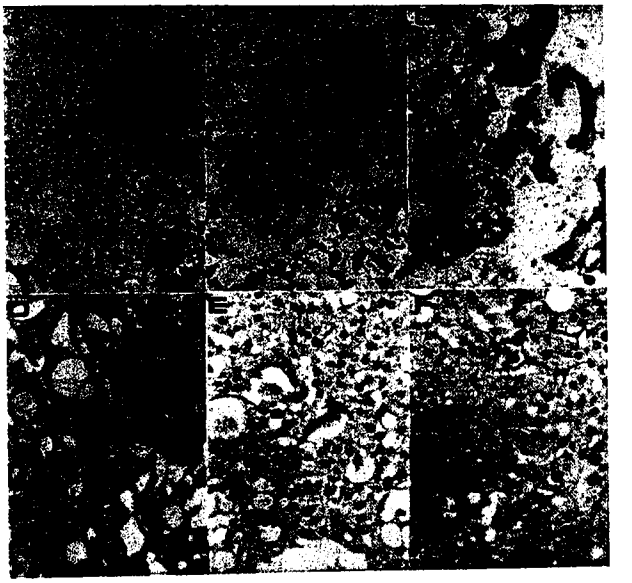

Figure 10. *Autoradiography carried out on 11-week histocultured human bladder tumor specimens after [^{3}H]thymidine uptake. Autoradiography was carried out after completion of the glucose consumption test in the histocultures. A, B and C were treated with cisplatinum at 10^{-3} M, 10^{-4} M and 10^{-5} M, respectively and D, E and F were treated with KHPC-006 at 10^{-3} M, 10^{-4} M and 10^{-5} M, respectively. The cytotoxic effect was more potent at high concentration of both drugs than at the lower drug concentrations. Necrosis occured more often at the high dose of the cisplatinum-treated group (A,B) than the KHPC-006-treated group (D,E). The thymidine labeling index was follows: A,B, no uptake; C, 0.02; D, 0.01; E, 0.03; and F, 0.04. (X200).*

the new complex or cisplatinum. There was no evidence of delayed glucose half-life after treatment with either drug.

Efficacy of the new platinum complex on histocultures determined by microautoradiography of [^{3}H]thymidine uptake. Autoradiography carried out on 11-week histocultured specimens was carried out after glucose consumption was determined in the histocultures. As can be seen in Figure 10, the effects of the new complex and cisplatinum had parallel effects on glucose consumption and [^{3}H]thymidine uptake on histocultured human bladder transitional cell carcinoma. The efficacy was higher with both the new complex and cisplatinum at higher concentration than the lower concentrations. However, necrosis was seen more often in the high dose cisplatinum-treated group than in the high dose of the new platinum-treated group.

Discussion

Since the antitumor activity of cisplatinum was described by Rosenberg (4,5,6), it has become one of the most important anticancer chemotherapeutic agents for human cancers. Approximately 75 % of patients with disseminated germ cell tumors are curable with cisplatinum-based chemotherapy (7,8).

Platinum coordination complexes consist of a platinum leaving group and a carrier ligand. The carrier ligand is responsible for anticancer activity. The chemical structure of the amine as a carrier ligand is an important factor influencing antitumor activity. The anticancer activity of cisplatinum is attributed to the preferential reaction of the carrier ligand with the N-7 atom on the guanidine base in DNA. Such reactions ultimately form compounds in which both chlorides are replaced by nucleic acid groups. The anticancer activity of cisplatinum complexes also depends on the stereoselectivity of the reaction. We have reported that the 1,2-diaminocyclohexane (dach) (9,10) carrier ligand shows particular promise because of its excellent antitumor activity, low toxicity and lack of cross resistance with cisplatinum (11,12). The leaving group determines stability, reactivity and water solubility.

Pt(II) complexes appear to enter the cells by diffusion. The hydrolysis of the leaving group is responsible for the formation of the activated species of the drug, which reacts with DNA, resulting in inhibition of DNA replication. This explanation suggests that anticancer activity of Pt(II) complexes is closely correlated with the replacement rate of the leaving group *in vivo*.

Our platinum-based drug development program is aimed at developing drugs capable of broadening the anticancer effect and decreasing side effects. To reach these objectives we have recently synthesized [Pt(II) (cis-dach)(DPPE)] · $(NO_3)_2$. We previously reported that this new complex has very low nephrotoxicity (13).

Collagen-sponge-gel-supported histoculture has been shown to support the growth and native three-dimensional architecture of both tumors and various normal tissues,

thereby allowing the histocultured tissue to resemble the *in vivo* situation (14,15,16,17).

Chang *et al* (18,19) have reported that histocultured normal renal cortical tissues evaluated with the glucose consumption test provide a good assay for cisplatinum toxicity. Furthermore, it was shown that glucose consumption measurements in histocultured human bladder tumors were more sensitive than the thymidine incorporation end point for measuring drug efficacy (20).

In this study the anticancer effect of the new complex was evaluated by glucose-consumption measurement in histocultured human bladder superficial transitional cell carcinoma. MTT reduction measurements in cancer cell lines in monolayer culture and autoradiography on histocultured specimens were also used for the determination of the efficacy of the new complex. The results revealed that the newly-developed platinum complex has similar or greater anticancer efficacy than cisplatinum, particulary at high concentrations. As mentioned above, however, this new complex has very low nephrotoxicity. Therefore, this new complex may possibly be useful clinically for high dose chemotherapy with reduced side effects.

Based on these results, this novel platinum complex represents a valuable lead and justifies clinical studies in the development of a new anticancer chemotherapeutic agent capable of improving anticancer efficacy with low toxicity.

Acknowledgements

These studies were supported in part by Research Grant for Development of New Drug (1995) from the Ministry of Health and Social Affairs of Korea, Research Fund of Kyung Hee University Medical Center Seoul, Korea and Han Hwa Pharmaceutical Company Seoul, Korea.

References

1 Jung J-C, Lee M-H, Chang S-G, and Rho Y-S: Antitumor activity and nephrotoxicity of the novel platinum(II) coordination complex. J Korean Pharmacol *31:* 103-114, 1995.

2 Bosanquet AG *et al:* An assessment of a short term tumor chemosensitivity assay in chronic lymphocytic leukemia. Br J Cancer *47:* 781-789, 1983.

3 Chang S-G, Perrapato SD, Slocum HK, Huben RP and Hoffman RM: Glucose consumption end point in primary histoculture indicates recovery of human tumor from drug treatment. *In Vitro* Cell Dev Biol *28A:* 585-587, 1992.

4 Rosenberg B, VanCamp L, Grimley EB and Thomson AJ: The inhibition of growth or cell division in *Escherichia coli* by different ionic species of platinum(IV) complexes. J Biol Chem *242:* 1347-1352, 1967.

5 Rosenberg B, VanCamp L and Kligas T: Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature *205:* 698-699, 1965.

6 Rosenberg B, VanCamp L, Krosko JE and Mansour VH: Platinum compounds: a new class of potent anticancer agents. Nature *222:* 385-386, 1969.

7 Einhorn LH: Treatment of testicular cancer: A new and improved model. J Clin Oncol *8:* 1777-1781, 1990.

8 Osanto S, Bukman A, VanHoek F *et al:* Long term effect of chemotherapy in patient with testicular cancer. J Clin Oncol *10:* 574-579, 1992.

9 Ridgway HJ, Speer RJ and Hall JM: Analogs of sulfato 1,2-diaminocyclohexane platinum(I): Modification in leaving ligands. J Clin Hematol Oncol *7:* 220-225, 1977.

10 Inagaki K and Kidani Y: Difference in binding of (1,2-cyclohexane diammine) platinum(II) isomer with d(GPG). Inorg Chemi *25:* 1-7, 1986.

11 Kelsen DP, Scher H, Alcock N, *et al:* Phase I clinical trial and pharmacokinetics of 4'-carboxyphthalato (1,2 -diaminocyclohexane) platinum(II). Cancer Res *42:* 4831-4835, 1982.

12 Burchenal JH, Kalaher K, O'Toole T and Chisholm J: Lack of cross-resistance between certain platinum coordination compounds in mouse leukemia. Cancer Res *37:* 3455-3457, 1977.

13 Chang S-G, Kwon D-H, Kim J-I, Jung J-C, Rho Y-S and Hoffman RM: New platinurn complex compounds with reduced nephrotoxicity discovered in long-term histoculture of human renal cortical tissue. Anticancer Res *15:* 675-682, 1995.

14 Leighton J: A sponge matrix method for tissue culture. Formation of organized aggregates of cells *in vitro.* J Natl Cancer Inst *12:* 545-561, 1951.

15 Hoffman RM: To do tissue culture in two or three dimension? That is the question. Stem cells *11:* 105-111, 1993.

16 Freeman AE and Hoffman RM: *In vivo*-like growth of human tumors *in vitro.* Proc Natl Acad Sci *83:* 2694-2698, 1986.

17 Vescio RA, Redfen CH, Nelson TJ, *et al: In vivo*-like drug response of human tumor growing in three dimensional, gel-supported primary culture. Proc Natl Acad Sci *84:* 5029-5033, 1987.

18 Chang S-G, Toth K, Black JD *et al:* Growth of human renal cortical tissues on collagen gel. *In Vitro* Cell Dev Biol *28A:* 128-135, 1992.

19 Chang S-G, Chai S-E, Kim E-S, Yoon C, *et al:* The measurement of glucose consumption in histoculture to determine effects of doxorubicin and cisplatinum on gastric carcinoma. Anticancer Res *13:* 1303-1310, 1993.

20 Chang S-G, Lee JH, Hong DH, Lee HL, Chai S-E and Hoffman RM: Comparison of glucose consumption and thymidine-incorporation endpoints on histocultured human superficial bladder tumor. Anticancer Res *14:* 77-84, 1994.

Received May 10, 1996
Accepted July 16, 1996