Anti-tumor activity of heptaplatin in combination with 5-fluorouracil or paclitaxel against human head and neck cancer cells *in vitro*

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Heptaplatin (HTP), a newly developed platinum analog, has been approved for the treatment of gastric cancers in South Korea. In this study we explored the potential of HTP for the treatment of head and neck squamous cell cancers (HNSCC). The anti-proliferative activity of HTP was evaluated in FaDu, a human HNSCC cell line. Combinations of HTP with 5-fluorouracil (5-FU) or paclitaxel (PTX) were determined using combination indexes, and were compared with combinations of cisplatin and 5-FU or PTX. In order to evaluate the transport of HTP into tumor tissue, its penetration through multicell layers (MCLs) of cancer cells was measured. Cisplatin + 5-FU and HTP + 5-FU showed additive to antagonistic interactions. In terms of the HTP + paclitaxel combination, HTP showed antagonism and additivity at the 50 and 80% growth inhibition levels, respectively. An additive interaction was obtained and apoptosis was increased by 2-fold at both inhibition levels when the combinatorial PTX dose was reduced to 1/10. HTP, but not cisplatin or oxaliplatin (L-OHP), maintained its anti-proliferative activity after MCL penetration at clinically relevant concentrations, which can be attributed to lower protein binding of HTP. In conclusion, the present study suggests that low-dose PTX may sensitize tumor cells to HTP. HTP also showed greater penetration through multilayers of tumor cells compared to cisplatin and L-OHP, which may be an important characteristic for solid tumor treatment. Overall, the present study supports the clinical development of HTP in combination with low-dose PTX against HNSCC. *Anti-Cancer Drugs* **17:377–384 © 2006 Lippincott Williams & Wilkins.**

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Introduction

Head and neck squamous cell cancer (HNSCC) is often associated with a prolonged history of tobacco and alcohol abuse, although it also occurs in people who have never smoked and in those that consume relatively little alcohol. Chemotherapy is usually used to treat local or disseminated recurrent-metastatic HNSCC [1]. The importance of chemotherapy has increased, and the most active agents currently used are methotrexate, cisplatin, 5-fluorouracil (5-FU) and paclitaxel (PTX) [2]. Combinations of these agents have been used to improve clinical outcomes, e.g. cisplatin and PTX combination trials addressed therapeutic benefits in HNSCC patients [3,4]. Although cisplatin is one of the most effective anti-cancer agents for HNSCC, primary or acquired resistance [5], and severe side-effects such as nephrotoxicity, emetogenicity and neurotoxicity limit its clinical usefulness [6–8].

Heptaplatin (HTP; *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]platinum(II), SKI-2053R, Sunpla) is a new anti-cancer agent. As a platinum analog, the anti-tumor activity of HTP has been shown to be superior or comparable to that of cisplatin against various cancer cell lines, including cisplatin-resistant cell lines [9–12]. HTP in combination with 5-FU was approved for the treatment of advanced, metastatic or recurrent gastric cancers by the Korean Food and Drug Administration in 1999. A preclinical study indicated that HTP has a favorable toxicity profile by general pharmacological evaluation and less nephrotoxicity than cisplatin, which was also demonstrated in phase I and II clinical trials [13–15]. The maximum tolerated dose of HTP was 480 mg/m^2, and the major dose-limiting toxicities were hepatotoxicity, nephrotoxicity and myelosuppression [15]. No patients in a phase II study (360 mg/m^2) showed grade 3 or 4 toxicity, and protenuria, the most frequent toxicity, was reported to be mild and transient [14].

In the present study, we studied the potential of HTP for the treatment of HNSCC when combined with other

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anti-cancer agents, i.e. 5-FU or PTX, and compared results with that of cisplatin in FaDu, a human HNSCC cell line. We also evaluated the activity of HTP after penetration through a multilayer model of tumor cells and compared it with cisplatin and oxaliplatin (L-OHP). We found that HTP in combination with 5-FU (molar ratio 1 : 2) or PTX (molar ratio $1 : 0.02 \times 10^{-3}$) had cytotoxicities comparable with combinations of cisplatin with 5-FU or PTX against FaDu cells. The activity of HTP was not reduced after penetration through multilayers of tumor cells for 24 h, whereas significant decreases in activity were found for cisplatin and L-OHP.

Materials and methods

Chemicals

HTP and L-OHP were kindly donated by SK Chemicals Life Science Research Center (Suwon, South Korea) and Sanofi-Synthelabo (Malvern, Pennsylvania, USA), respectively. The chemical structures of HTP and cisplatin are shown in Fig. 1. 5-FU and PTX were provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, Maryland, USA). Cisplatin and other reagents, unless stated otherwise, were purchased from Sigma (St Louis, Missouri, USA). MEM, RPMI 1640 and FBS were purchased from Gibco/BRL (Grand Island, New York, USA).

Cell lines and cell culture conditions

The human pharyngeal squamous cell carcinoma cell line FaDu and a human colorectal squamous cancer cell line DLD-1 were obtained from the Korean Cell Line Bank (Seoul, South Korea). These cell lines were maintained in MEM and RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

Culture of multicell layers (MCLs)

DLD-1 cells were grown on collagen-coated microporous (0.4-μm) membranes in Transwell inserts (Corning Costar, Acton, Massachusetts, USA) at a plating density of 2.5×10^5/100 μl. MCLs were grown for up to 8 days with daily medium replenishment in both top and bottom chambers. For histological examinations, MCL inserts were embedded in paraffin, cut into 5-μm sections using a cytotome, placed on microscope slides and H & E stained.

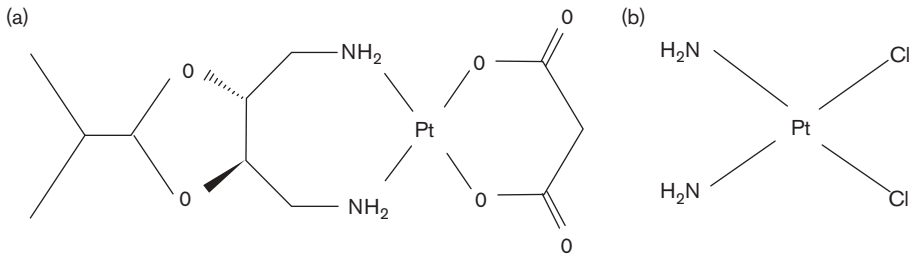
Cytotoxicity assay

The sulforodamine B (SRB) assay was conducted as previously described [16]. Cells in the log phase were harvested and plated in 96-well plates at a density of 1800 cells/well. After a 24-h incubation, cells were exposed to drug-containing media for 72 h. For cytotoxicity assay after MCL penetration, 24-h conditioned media in the bottom chamber of the Transwell insert was used. Cells were then fixed with 10% TCA (ChemExper Chem, Lancaster, UK) and then with 0.4% SRB for 15 min. After five washes with 1% acetic acid, protein-bound dye was extracted with 10 mmol/l unbuffered Tris (Amresco, Solon, Ohio, USA) and absorbance was measured.

Cell cycle effects and apoptosis induction

To analyze cell cycle distributions, cells were collected and fixed with 10 ml of 70% cold ethanol while vortexing. The cells were then kept at 4°C for 1 h and stored at −20°C until analysis. Upon analysis, fixed cells were washed and resuspended in 1 ml of PBS containing 50 μg/ml RNase A and 50 μg/ml ethidium bromide. After 20 min of incubation at 37°C, cells were analyzed for DNA content by flow cytometry (FACSVantage; Becton Dickinson Immunocytometry Systems, San Jose, California, USA). For each sample, 10 000 events were acquired. Cell cycle distributions were determined using cell cycle analysis software (Modfit; Verity, Topsham, Maine, USA). For the simultaneous determination of cell cycle distribution and apoptosis, we used Apo-Direct kits (Pharmingen, San Diego, California, USA). Flow cytometric analysis was performed using FL1 (FITC) and FL2 (propidium iodide), and data acquisition and analysis were done using CellQuest software. For each sample, 10 000 events were recorded.

Fig. 1



Chemical structure of HTP (a) and cisplatin (b).

Data analysis

IC_x defined as the drug concentration required to reduce the absorbance to $(100-x)\%$ of the control in each test was determined using an Emax model (1):

$$\%\,\text{Cell viability} = (100-R)\times\left(1-\frac{[D]^m}{K_d^m+[D]^m}\right)+R \quad (1)$$

where [D] is the drug concentration, K_d is the concentration of drug that produces a 50% reduction in absorbance (i.e. IC_{50}), m is a Hill-type coefficient and R is the residual unaffected fraction (the resistance fraction).

The nature of the interactions between two drugs was determined by using median-effect analysis [17]. Median-effect analysis permits the calculation of the combination index (CI) at various levels of cell kill (2). CI was calculated for a cell death range of 50–80%, i.e. CI_{50}–CI_{80}:

$$CI_x = \frac{[D]_A}{[D_x]_A}+\frac{[D]_B}{[D_x]_B}+\alpha\frac{[D]_A[D]_B}{[D_x]_A[D_x]_B} \quad (2)$$

where CI_x is CI for a fixed effect x for a combination of drug A and drug B. $[D_x]_A$ and $[D_x]_B$ and are the concentrations of drug A or B alone giving an effect x, $[D]_A$ and $[D]_B$ are the concentrations of drug A or B in combination A + B giving an effect x, and α is 0 when A and B are mutually exclusive and 1 when A and B are mutually non-exclusive. A $CI_x = 0.8$–1.2 was defined as additive, $CI_x \le 0.8$ as synergistic and $CI_x \ge 1.2$ as antagonistic. Statistical analyses were conducted using the S-Link (S-Link, Seoul, South Korea) statistics program.

Results

We evaluated the anti-proliferative activity of HTP and cisplatin when given alone and in combination with 5-FU or PTX against FaDu human HNSCC cells. IC_{50}'s showed a wide range from 1.25 nmol/l to 7.59 μmol/l, i.e. 5.20 μmol/l for HTP, 1.02 μmol/l for cisplatin, 7.59 μmol/l for 5-FU and 1.25 nmol/l for PTX (Table 1). Resistance fractions (R), which represent the fraction of cells that were insensitive to drug treatment, were 25.4% for 5-FU and 11.8% for PTX, but 0.63 and 5.3% for the two platinum agents, respectively (Table 1).

Anti-proliferative effects of cisplatin or HTP combined with 5-FU or PTX were examined at fixed molar ratios based on approximate equitoxic concentrations at the level of 50% growth inhibition (Table 2). Hence, combinations of platinum drugs with 5-FU or PTX were examined at molar ratios of 1 : 2 and $1:0.2\times10^{-3}$, respectively. For HTP and PTX combination, a 10-fold lower dose of PTX was also tested to determine cytotoxic interactions, i.e. HTP:PTX = $1:0.02\times10^{-3}$ molar ratio. At the 50% level of cytotoxicity, the CI (CI_{50}) for the HTP + 5-FU combination was 1.09–1.35, implying additive to antagonistic interaction, and a similar interaction (CI 0.98–1.12) was also obtained for the cisplatin + 5-FU combination (Table 2). The fraction resistant to 5-FU (25%, Table 1) was abrogated upon its combination with either platinum agent. In the case of the HTP + PTX combination ($1:0.2\times10^{-3}$), the CI_{50} was 1.56–2.16, indicating antagonism. At the 80% level of cell kill, however, the CI_{80} was 1.10–1.33, indicating an additive effect (Table 2). These data indicate that the antagonistic interaction between the two drugs could be overcome at a higher dosing level causing an 80% cell kill. However, the cisplatin + PTX combination showed an additive to antagonistic effect with a CI_{50} of 1.16–1.26 and this interaction did not change at a higher dosing level, i.e. CI_{80} 1.16–1.24 (Table 2). Furthermore, when the relative dose of PTX was reduced by 10-fold, CI_{50} and CI_{80} for HTP + PTX decreased to 1.02–1.13 and 0.94–0.99, indicating a more favorable interaction.

Table 1 Anti-proliferative activity parameters of HTP, cisplatin, 5-FU and PTX against FaDu, a human pharyngeal cell line (dose–response curves of these agents were analyzed using the Emax model)

Parameter	HTP	Cisplatin	5-FU	PTX[a]
IC_{50} (μmol/l)	5.20 ± 0.36	1.02 ± 0.15	7.59 ± 1.15	1.25 ± 0.57
IC_{80} (μmol/l)	10.6 ± 0.54	2.55 ± 0.81	ND[b]	9.37 ± 7.12
R[c]	0.63 ± 0.28	5.30 ± 1.49	25.4 ± 1.07	11.8 ± 2.27

Each value indicates the mean of three independent experiments.
[a]Concentration of paclitaxel in nmol/l.
[b]Not determined due to $R > 20$.
[c]R is the residual unaffected fraction (the resistance fraction).

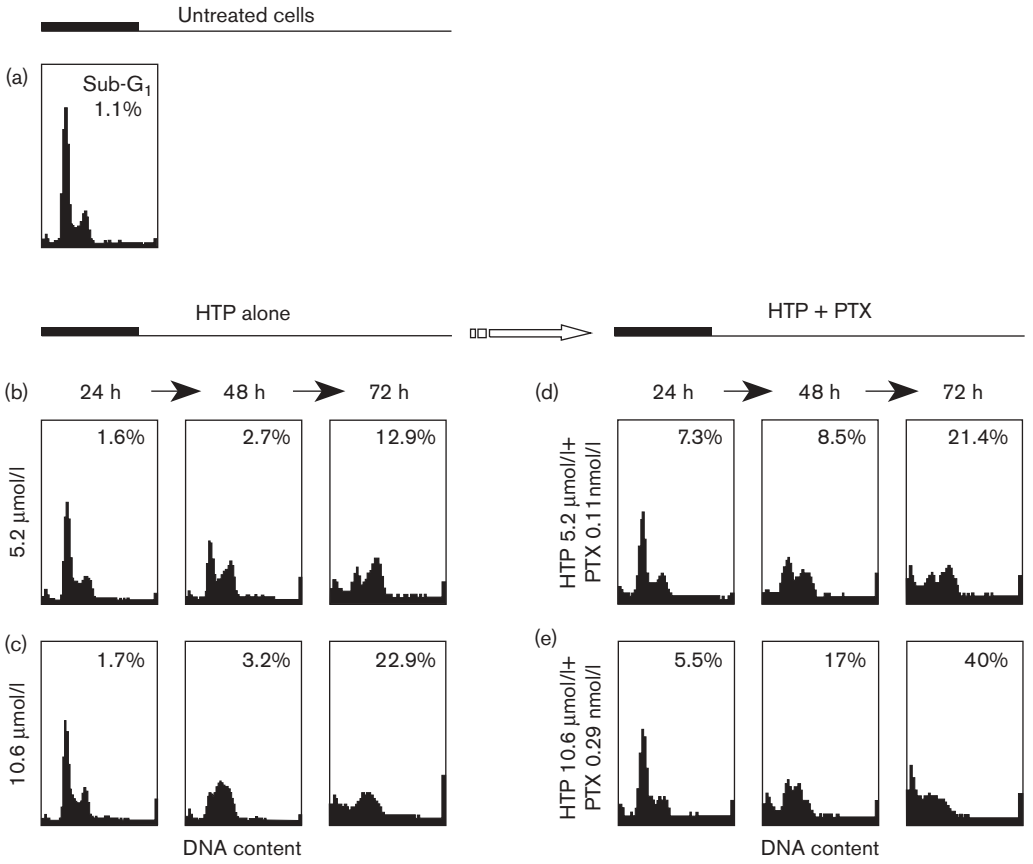
Since HTP showed the most favorable interaction with PTX at $1:0.02\times10^{-3}$ molar ratio (Table 2), we evaluated their interaction with respect to cell cycle distribution and apoptosis induction. The cell cycle arrest effects of HTP and PTX were assessed alone and in combination at two different concentration levels, i.e. around IC_{50} and IC_{80}. Cells exposed to 5.20 and 10.6 μmol/l HTP (IC_{50} and IC_{80} level concentrations, respectively) did not induce significant changes in the cell cycle distribution of FaDu cells until 48 h (Fig. 2b and c). When exposed to 5.2 μmol/l HTP for 72 h, 56.1% of the cells were arrested in the G_2/M phase (*cf.* untreated control: 13.8%) (Fig. 2b). In contrast, exposure to 10.6 μmol/l HTP for 72 h induced S phase arrest (76.7 versus 32.7% in untreated control) along with G_1 and G_2/M phase abrogation (Fig. 2c). For PTX, treatment at 1.25 nmol/l (IC_{50} level) induced no significant changes, but at 9.37 nmol/l (IC_{80} level) G_2/M arrest (41.6 versus 13.8% in untreated control) was induced after 48 h (data not shown). These data indicate that the cell cycle effect was concentration and time dependent for both agents. When HTP was combined with PTX at a molar ratio of $1:0.02\times10^{-3}$, the pattern of changes in the cell cycle distribution was not significantly different from that of HTP alone (Fig. 2b and c versus d and e). Nonetheless,

Table 2 Combinatorial effects of HTP or cisplatin with 5-FU or PTX

		5-FU		PTX		
		HTP (1 : 2)[a]	Cisplatin (1 : 2)	HTP (I) (1 : 0.2 × 10^{-3})[b]	HTP (II) (1 : 0.02 × 10^{-3})	Cisplatin (1 : 0.2 × 10^{-3})[b]
CI_{50}	$\alpha=0$[c]	1.09 ± 0.31	0.98 ± 0.16	1.56 ± 0.22*	1.02 ± 0.05*	1.16 ± 0.12
	$\alpha=1$[c]	1.35 ± 0.42	1.12 ± 0.20	2.16 ± 0.39**	1.13 ± 0.07**	1.26 ± 0.16
CI_{80}	$\alpha=0$[c]	ND[a]	ND[a]	1.10 ± 0.16	0.94 ± 0.08	1.18 ± 0.22
	$\alpha=1$[c]	ND[a]	ND[a]	1.33 ± 0.28	0.99 ± 0.12	1.24 ± 0.24
R		3.06 ± 2.67	1.38 ± 0.82	2.57 ± 2.61	0.93 ± 1.31	4.53 ± 1.74

CIs were calculated at the 50 and 80% growth inhibition levels, i.e. CI_{50} and CI_{80}. Drugs were given simultaneously for 72 h. Ratios of administered agents were based on equitoxic concentrations of HTP with 5-FU or PTX. For the HTP + PTX combination, PTX was administered at two concentration levels.
[a]Combination ratio based on IC_{50} ratio of HTP:5-FU (5.20 μmol/l : 7.59 μmol/l).
[b]Combination ratio based on IC_{50} ratio of HTP:PTX (5.20 μmol/l : 1.25 nmol/l).
[c]$\alpha=0$ when two agents are mutually exclusive and $\alpha=1$ when two agents are mutually non-exclusive.
[d]Not determined due to $R>20$=[see (1) and (2)].
*$P<0.05$;
**$P<0.01$.

Fig. 2

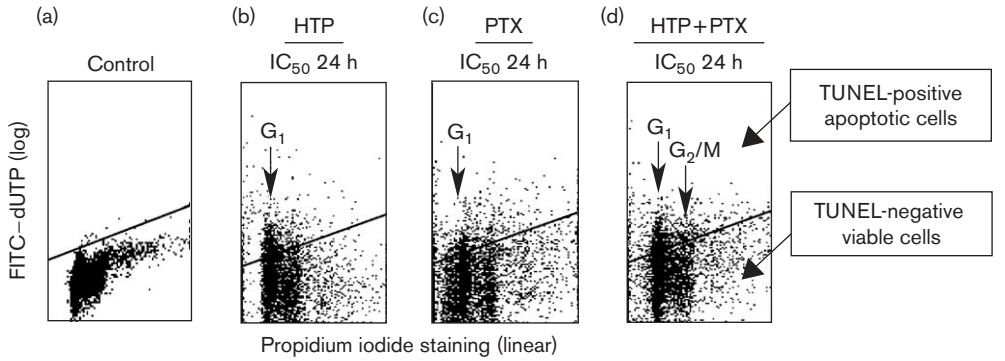


Representative DNA histograms showing the cell cycle effects induced by HTP alone (b and c) or by HTP + PTX (d and e). HTP concentration levels were 0 (a), 5.2 (b and d) and 10.6 μmol/l (c and e); PTX concentrations were 0.11 (d) or 0.29 nmol/l (e). DNA content histograms were obtained at 24, 48 and 72 h post-treatment as indicated. Percentages of sub-G_1 fractions are indicated in each histogram.

about a 2-fold increase in the hypodiploid population was observed at both concentration levels after 72 h (Fig. 2d and e). Similar results were observed when the TUNEL assay was used to detect apoptotic cells (data not shown).

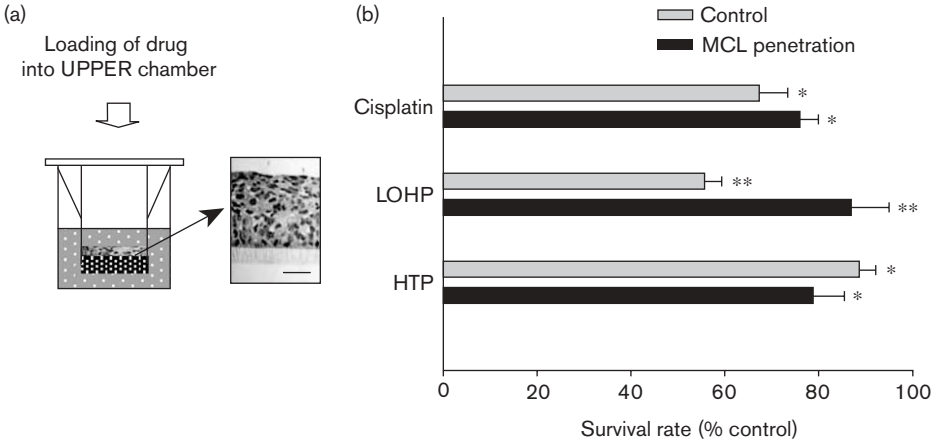
In order to evaluate the association between specific cell cycle phases and apoptosis induction, we used bivariate analysis using the TUNEL method when cells were exposed to HTP or PTX alone or in combination (molar ratio of 1 : 0.02 × 10^{-3}) for 24 h. When cells were exposed

Fig. 3



Induction of cell cycle-specific apoptosis in FaDu cells exposed to 0 (a), 5.20 μmol/l of HTP (b), 1.25 nmol/l of PTX (c) or HTP (5.2 μmol/l) + PTX (0.11 nmol/l) (d). Cells were harvested after 24 h of treatment and processed for bivariate analysis of TUNEL/DNA content by flow cytometry.

Fig. 4



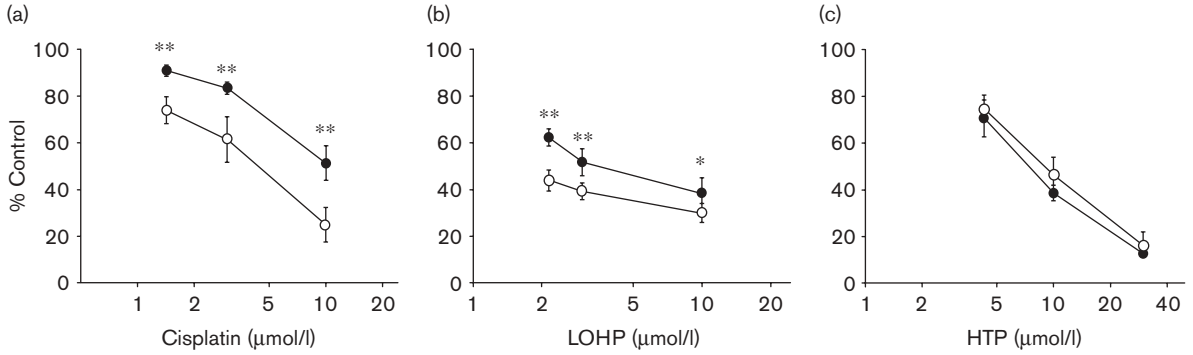
Anti-proliferative activity of platinum agents after 24 h of penetration through a MCL of DLD-1 cells grown on a Transwell insert. (a) A diagram of the method used for the penetration experiments. The MCL was a 45-μm thick 14 cell layer (bar indicates 20 μm). (b) The anti-proliferative activity of each drug with or without MCL penetration. FaDu cell viabilities were assessed after 72 h of drug exposure using SRB assays. The loaded drug concentrations in the top chamber were 20 μmol/l for cisplatin, 30 μmol/l for L-OHP and 60 μmol/l for HTP. Note that drug concentrations were diluted 7-fold upon penetration into the bottom chamber. *$P<0.05$; **$P<0.01$.

to 5.2 μmol/l HTP or 1.25 nmol/l PTX, most TUNEL-positive cells showed G_1 phase DNA content (Fig. 3b and c). When cells were treated with HTP + PTX in combination (5.2 μmol/l + 0.11 nmol/l), TUNEL-positive cells showed G_2/M phase and G_1 phase DNA contents (Fig. 3d). These data indicate that PTX contributed to the induction of cell cycle-specific apoptosis, i.e. apoptotic induction of G_2 phase cells without affecting the cell cycle distribution (Fig. 3d).

To compare penetration abilities of the three platinum drugs (i.e. cisplatin, L-OHP and HTP), we used a MCL model of DLD-1 cells, instead of FaDu cells due to their inability to form MCLs. The drug concentrations loaded in top chambers (100 μl) were 20, 30 and 60 μmol/l for cisplatin, L-OHP and HTP, respectively, which were maximum plasma levels observed in clinical studies. For cisplatin and L-OHP, 11.5 and 35.9% decreases in anti-proliferative activity were observed after MCL penetration, i.e. survival rates increasing from 78.9 to 88.8% and from 55.8 to 87.0%, respectively (Fig. 4b). The anti-proliferative activity of HTP, however, increased by 13.1%, i.e. the survival rate decreased from 76.0 to 67.2% (Fig. 4b). These data indicate that the activities of cisplatin and L-OHP, but not of HTP, are decreased by 24-h penetration through cancer cell layers.

A decrease in activity was observed for cisplatin and L-OHP when incubated in cell-free Transwell inserts for 24 h (data not shown). In order to assess the contribution

Fig. 5



Changes in anti-proliferative activities of platinum agents after 24 h incubation in culture medium. FaDu cells were exposed to conditioned drug-containing media (closed circles) or control media (open circles). Cell viabilities were assessed using SRB assays after a 72-h exposure. The drug concentrations used were 1.43, 3.0 and 10 μmol/l for cisplatin (a), 2.15, 3.0 and 10 μmol/l L-OHP (b), and 4.3, 10 and 30 μmol/l for HTP (c). *$P<0.05$; **$P<0.01$. Note that the comparison was made at each concentration.

made by membrane binding and irreversible protein binding to partial activity loss, we determined changes in anti-proliferative activities of these platinum agents against FaDu cells after incubation in culture medium for 24 h at 37°C. For cisplatin and L-OHP, significant changes in anti-proliferative activity were observed, but not for HTP (Fig. 5). This loss of activity of cisplatin and L-OHP in culture medium prior to penetration might contribute to their reduced activities observed after penetration through MCLs of DLD-1 cells (Fig. 4b).

Discussion

Previous studies reported that the IC_{50}'s of HTP against human gastric cancer cells were 12.09 μmol/l for MKN-45 and 12.52 μmol/l for KATOIII cells [18]. The activities of HTP against human lung cancer cells, such as PC-9 and PC-14, have been reported as 13.58 and 15.70 μmol/l, respectively [12]. The IC_{50} of HTP (5.2 μmol/l) against FaDu human HNSCC cells, as determined in the present study, was lower than in human gastric and lung cancer cells. Cisplatin and carboplatin have been used to treat head and neck cancers. Hence, the main purpose of our study was to evaluate the potential of HTP for the treatment of human HNSCC by examining its combinatorial interactions with 5-FU or PTX and its penetration through cancer cell layers. The IC_{50} of HTP in FaDu cells was higher than that of cisplatin (5.2 versus 1.02 μmol/l); however, the IC_{50} of cisplatin against various head and neck cancer cell lines ranged between 2.2 (in CAL60) and 12.6 μmol/l (in Detroit 562), and HTP also lies in this range [19].

Cisplatin and 5-FU are frequently used in combination against head and neck cancers. It has been reported that cisplatin enhances the cytotoxicity of 5-FU by inhibiting methionine transport, increasing ternary complex expression and inhibiting DNA synthesis [20–22]. A recent study reported that the combination of cisplatin and 5-FU increases susceptibility to apoptosis, in which p53 may play an important role [23]. A point mutation of p53 (codon 248, CGG→CTG) has been reported in FaDu cells [24,25], which may explain why the cisplatin + 5-FU combination showed only an additive effect (Table 2). Cytotoxicity was synergistic when cisplatin was given with 5-FU independently of administration sequence in human breast cancer cells [26]. The optimal combination schedule, however, remains controversial because other reports have suggested that greater cytotoxic activity is obtained when 5-FU is administered before cisplatin [27–29]. The combination of cisplatin and PTX has, however, been used to treat advanced recurrent diseases [30]. This is noteworthy because a number of head and neck cancers harbor p53 mutations [31] and PTX can induce p53-independent apoptosis in tumor cells [32]. An additive interaction was shown for the cisplatin + PTX combination in FaDu cells independently of treatment sequence and combination ratios [33]. Although many studies reported conflicting results [33], e.g. antagonistic interaction in human melanoma cells [34], cisplatin + PTX has generally shown greater synergy when cisplatin is administered after, rather than before, PTX [35].

HTP showed a dose-dependent cell cycle arrest effect in FaDu cells, i.e. the G_2/M phase arrest at 5.2 μmol/l (IC_{50} level) and the S phase arrest at 10.6 μmol/l (IC_{80} level) (Fig. 2b and c). Cisplatin is known to induce cell cycle arrest at the G_2 phase [36]. It has also been shown, however, in human pancreatic cells exposed to cisplatin that an accumulation of cells in the S phase precedes G_2/M phase arrest and that progression into the G_2/M phase is concentration dependent, i.e. slower progression at a higher drug concentration. Apparent dose-dependent

cell cycle arrest can result from this slower progression in cells exposed to cisplatin at a higher drug concentration and a similar explanation can be applied to HTP.

The combination HTP + PTX in the present study showed only additive to antagonistic results at the equitoxic ratio. However, a more favorable interaction was observed and apoptosis induction increased by 2-fold when the relative dose of PTX was decreased to 1/10 (Fig. 2d and e, and Table 2). The pattern of cell cycle distribution was not changed when HTP was combined with PTX, suggesting that no significant effect on the cell cycle was induced by low concentrations of PTX (Fig 2d and e). Since neither significant cytotoxic nor cell cycle effects were induced by low concentrations of PTX (data not shown), the increased apoptosis induced by adding PTX indicates that PTX has a sensitization effect. In addition, our data show that the apoptotic cells induced by HTP or PTX alone were from the G_1 phase, but when the drugs were combined they were from the G_2/M and G_1 phases. These data suggest that low concentrations of PTX when combined with HTP induce apoptosis via some additional mechanism, which warrants further investigation.

Factors controlling DNA platination include drug uptake, rate of platinum adduct formation/repair and concentration of cellular thiols [37]. With respect to drug uptake, especially in solid tumors, penetration through MCLs is important after drug delivery to the tumor site by the systemic circulation. Hence, using MCLs, we evaluated the penetration ability of HTP and compared it to that of other platinum agents (Fig. 4). We evaluated the penetration abilities of HTP, cisplatin and L-OHP by measuring remaining cytotoxicity after MCL penetration. The loading concentration was selected based on the plasma concentrations in patients, which were 12- to 20-fold higher than the IC_{50}'s in monolayers. The expected survival rates were 78.9, 55.8 and 76.0% for cisplatin, L-OHP and HTP, respectively, after dilution in penetration wells. It can be assumed for small molecules such as these platinum compounds that concentrations in the extravascular space surrounding the tumor site are similar to systemic plasma concentrations. Of the three agents, HTP maintained cytotoxicity after 24-h penetration through MCLs (Fig. 4b), but cisplatin and L-OHP showed reduced activity; the poor tissue penetration of cisplatin has been reported elsewhere [38].

DLD-1 cells were relatively easy to form MCLs compared to FaDu cells; hence, drug penetration data were obtained using MCLs of DLD-1 cells. This should not impose any problem on data interpretation since only minimal influence of cell type on drug penetration through MCLs has been demonstrated [38]. In addition, the relative sensitivities of these cell lines were similar, i.e. IC_{50} for cisplatin and HTP was 1.02 and 5.2 μmol/l in FaDu cells and 3.6 and 10.7 μmol/l in DLD-1 cells, respectively.

We also evaluated the activity of platinum agents in culture medium. HTP maintained its cytotoxicity after 24 h in culture medium, whereas cisplatin and L-OHP showed significant decreases in cytotoxicity (Fig. 5). Protein-free platinum species have been considered to be biologically active [39]. The protein binding of HTP has been reported to be 12–32% in dog plasma [40]. However, L-OHP and cisplatin bind to plasma proteins irreversibly at the 85–88% level [39,41]. Hence, the reduced activity of cisplatin and L-OHP in cell culture media may be attributed to irreversible protein binding.

The present study demonstrated anti-proliferative activity of HTP against human HNSCC cells. Sub-additive interaction between HTP and PTX was observed when combined at equitoxic levels. More synergistic interaction was obtained by sensitizing FaDu cells to HTP with 1/10 dose of PTX, which may be related to induction of cell cycle-specific apoptosis. HTP, but neither cisplatin nor L-OHP, maintained activity after penetration through multilayers of tumor cells, indicating the greater penetration ability of HTP into solid tumor tissue compared to cisplatin and L-OHP. The present study provides useful preclinical data for the development of combination regimen of HTP with low-dose PTX against human HNSCC.

References

1 Seiwert TY, Cohen EE. State-of-the-art management of locally advanced head and neck cancer. *Br J Cancer* 2005; **92**:1341–1348.
2 Hoffman HT, Robinson RA, Spiess JL, Buatti J. Update in management of head and neck sarcoma. *Curr Opin Oncol* 2004; **16**:333–341.
3 Forastiere AA. Current and future trials of taxol (paclitaxel) in head and neck cancer. *Ann Oncol* 1994; **5**(**Suppl**):S51–S54.
4 Forastiere AA. Paclitaxel (Taxol) for the treatment of head and neck cancer. *Semin Oncol* 1994; **21**:49–52.
5 Fram RJ. Cisplatin and platinum analogues: recent advances. *Curr Opin Oncol* 1992; **4**:1073–1079.
6 Loeher PJ, Einhorn LH. Drugs five years later. Cisplatin. *Ann Intern Med* 1984; **100**:704–713.
7 Muggia FM, Norris Jr K. Future of cancer chemotherapy with cisplatin. *Semin Oncol* 1989; **16**:123–128.
8 Reed E, Dabholkar M, Chabner BA. Platinum analogues. In: Chabner BA, Longo DI (editors): *Cancer chemotherapy and biotherapy: principles and practice*. Philadelphia: Lippincott-Raven; 1996. pp. 357–378.
9 Kim DK, Kim G, Gam J, Cho YB, Kim HT, Tai JH, *et al.* Synthesis and antitumor activity of a series of [2-substituted-4,5-bis(aminomethyl)-1, 3-dioxolane]-platinum(II) complexes. *J Med Chem* 1994; **37**:1471–1485.
10 Kim DK, Kim HT, Cho YB, Tai JH, Ahn JS, Kim TS, *et al.* Pharmacokinetics and antitumor activity of a new platinum compound, *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]platinum(II), as determined by *ex vivo* pharmacodynamics. *Cancer Chemother Pharmacol* 1995; **37**:1–6.
11 Kim DK, Kim HT, Cho YB, Tai JH, Ahn JS, Kim TS, *et al.* Antitumor activity of *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]platinum(II), a new platinum analogue, as an anticancer agent. *Cancer Chemother Pharmacol* 1995; **35**:441–445.
12 Hong WS, Kim HT, Kim KH, Kim DK. *In vitro* antitumor activity of a new platinum complex, *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]platinum(II) (SKI-2053R), against human lung and stomach cancer cell lines. *Anticancer Res* 1995; **15**:51–54.

13 Lee YS, Kang KS, Shin DJ, Cho JJ, Kim HO, Kim BH, *et al.* Subacute toxicity of *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]-platinum(II) (SKI-2053R) in beagle dogs. *Korean J Toxicol* 1992; **8**: 235–254.

14 Kim NK, Im SA, Kim DW, Lee MH, Jung CW, Cho EK, *et al.* Phase II clinical trial of SKI-2053R, a new platinum analogue, in the treatment of patients with advanced gastric adenocarcinoma. *Cancer* 1999; **86**:1109–1115.

15 Kim NK, Kim TY, Shin SG, Park YL, Lee JA, Cho YB, *et al.* A phase I study of *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-1,3-dioxolane]platinum(II) in patients with advanced malignancies. *Cancer* 2001; **91**:1549–1556.

16 Au JL, Li D, Gan Y, Gao X, Johnson AL, Johnston J, *et al.* Pharmacodynamics of immediate and delayed effects of paclitaxel: role of slow apoptosis and intracellular drug retention. *Cancer Res* 1998; **58**:2141–2148.

17 Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**:27–55.

18 Kim HT, Kim DK, Cho YB, Kim TS, Jung I, Kim KH, *et al.* Influence of exposure and infusion times on the cytotoxicity and pharmacokinetics of *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2-isopropyl-1,3-dioxolane]-platinum(II). *Cancer Chemother Pharmacol* 1998; **41**:109–116.

19 Magne N, Fischel JL, Dubreuil A, Formentom P, Marcie S, Lagrange JL, *et al.* Sequence-dependent effects of ZD1839® ('Iressa') in combination with cytotoxic treatment in human head and neck cancer. *Br J Cancer* 2002; **86**:819–827.

20 Shirasaka T, Shimamoto Y, Ohshimo H, Kimura A, Fukushima M. Mechanism for synergistic antitumor effect in the combination of 5-fluorouracil with cisplatin *in vivo* tumor models: from the view of biochemical modulation of 5-fluorouracil. *Gan To Kagaku Ryoho* 1991; **18**:403–409.

21 Shirasaka T, Shimamoto Y, Ohshimo H, Saito H, Fukushima M. Metabolic basis of the synergistic antitumor activities of 5-fluorouracil and cisplatin in rodent tumor models *in vivo*. *Cancer Chemother Pharmacol* 1993; **32**: 167–172.

22 Araki H, Fukushima M, Kamiyama Y, Shirasaka T. Effect of consecutive lower-dose cisplatin in enhancement of 5-fluorouracil cytotoxicity in experimental tumor cells *in vivo*. *Cancer Lett* 2000; **160**:185–191.

23 Matsuhashi N, Matsuo A, Kawaguchi Y, Sugiyama Y, Saji S. Relation between p53 expression of human colon cancer cell lines and induction of apoptosis by anticancer drugs. *Gan To Kagaku Ryoho* 2003; **30**: 1855–1858.

24 Kim MS, Li SL, Bertolami CN, Cherrick HM, Park NH. State of p53, Rb and DCC tumor suppressor genes in human oral cancer cell lines. *Anticancer Res* 1993; **13**:1405–1413.

25 Min BM, Baek JH, Shin KH, Gujuluva CN, Cherrick HM, Park NH. Inactivation of the p53 gene by either mutation or HPV infection is extremely frequent in human oral squamous cell carcinoma cell lines. *Eur J Cancer B Oral Oncol* 1994; **30**:338–345.

26 Barberi-Heyob M, Griffon G, Merlin JL, Weber B. Sequence-dependent growth-inhibitory effects of the *in vitro* combination of fluorouracil, cisplatin, and dipyridamole. *Cancer Chemother Pharmacol* 1993; **33**:163–170.

27 Cho H, Imada T, Oshima T, Shiozawa M, Rino Y, Takanashi Y. In-vitro effect of a combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) on human gastric cancer cell lines: timing of cisplatin treatment. *Gastric Cancer* 2002; **5**:43–46.

28 Tanaka T, Masuda H, Naito M, Tamai H. Pretreatment with 5-fluorouracil enhances cytotoxicity and retention of DNA-bound platinum in a cisplatin resistant human ovarian cancer cell line. *Anticancer Res* 2001; **21**: 2463–2469.

29 Palmeri S, Trave F, Russello O, Rustum YM. The role of drug sequence in therapeutic selectivity of the combination of 5-fluorouracil and *cis*-platin. *Sel Cancer Ther* 1989; **5**:169–177.

30 Sandercock J, Parmar MK, Torri V, Qian W. First-line treatment for advanced ovarian cancer: paclitaxel, platinum and the evidence. *Br J Cancer* 2002; **87**:815–824.

31 Brachman DG, Graves D, Vokes E, Beckett M, Haraf D, Montag A, *et al.* Occurrence of p53 gene deletions and human papilloma virus infection in human head and neck cancer. *Cancer Res* 1992; **52**:4832–4836.

32 Wahl AF, Donaldson KL, Fairchild C, Lee FY, Foster SA, Demers GW, *et al.* Loss of normal p53 function confers sensitization to Taxol by increasing G_2/M arrest and apoptosis. *Nat Med* 1996; **2**:72–79.

33 Levasseur LM, Greco WR, Rustum YM, Slocum HK. Combined action of paclitaxel and cisplatin against wildtype and resistant human ovarian carcinoma cells. *Cancer Chemother Pharmacol* 1997; **40**: 495–505.

34 Mohith A, Photiou A, Retsas S. The combination of paclitaxel with cisplatin exhibits antagonism *in vitro* against human melanoma. *Anticancer Drugs* 1996; **7**:493–498.

35 Parker RJ, Dabholkar MD, Lee KB, Bostick-Bruton F, Reed E. Taxol effect on cisplatin sensitivity and cisplatin cellular accumulation in human ovarian cancer cells. *J Natl Cancer Inst Monogr* 1993; **15**:83–88.

36 Ando T, Kawabe T, Ohara H, Ducommun B, Itoh M, Okamoto T. Involvement of the interaction between p21 and proliferating cell nuclear antigen for the maintenance of G_2/M arrest after DNA damage. *J Biol Chem* 2001; **276**:42971–42977.

37 Sadowitz PD, Hubbard BA, Dabrowiak JC, Goodisman J, Tacka KA, Aktas MK, *et al.* Kinetics of cisplatin binding to cellular DNA and modulations by thiol-blocking agents and thiol drugs. *Drug Metab Dispos* 2002; **30**: 183–190.

38 Tannock IF, Lee CM, Tunggal JK, Cowan DS, Egorin MJ. Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. *Clin Cancer Res* 2002; **8**:878–884.

39 Cole WC, Wolf W. Preparation and metabolism of a cisplatin/serum protein complex. *Chem-Biol Interact* 1980; **30**:223–235.

40 Cho YB, Kim DK, Kim KH, Miyamoto G. Pharmacokinetics of *cis*-malonato[(4*R*,5*R*)-4,5-bis(aminomethyl)-2- isopropyl-1,3-dioxolane]-platinum(II) in dogs. *Drug Metab Dispos* 1995; **23**:1280–1285.

41 Pendyala L, Creaven PJ. *In vitro* cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. *Cancer Res* 1993; **53**:5970–5976.