with oxaliplatin alone, although a slight decrease in the G_0/G_1 population and an increase in the G_2/M population were observed compared with cells treated with oxaliplatin alone. These findings indicate that cell cycle distribution patterns with the sequential combinations were mostly influenced by the initial drug administered. Interestingly, simultaneous exposure led to accumulation of cells in the G_2/M phase, a pattern similar to that caused by paclitaxel alone, indicating that paclitaxel might have a dominant effect in cell cycle progression as compared to oxaliplatin, or that oxaliplatin might take more time to exhibit its activity than paclitaxel.

To confirm the activities of sequential combinations, the apoptotic activity was investigated after treatment of AZ-521 cells by measuring the sub-G_1 population by FACS analysis. The presence of hypodiploid DNA (sub-G_1) is associated with cells undergoing apoptosis. As shown in Table 2, paclitaxel followed by oxaliplatin induced a G_2/M block, with substantial induction of apoptosis in the majority of the treated cells (75%). The induction rate of apoptosis by this sequential treatment was greater than that of paclitaxel alone (56–66%) or oxaliplatin alone (35–38%). By contrast, the reverse sequence caused G_1 block, and the apoptotic population was 38–41%, that is less than that induced by paclitaxel alone (56–66%), and similar to that induced by oxaliplatin alone (35–38%). These findings indicate the sequence of oxaliplatin followed by paclitaxel is antagonistic in inducing apoptosis.

Discussion

In this study, we examined in vitro the sequence dependency of the paclitaxel and oxaliplatin combination in three human cancer cell lines derived from tongue, esophagus, and stomach. Simultaneous treatment with these two drugs resulted in mostly additive effects. With the sequence paclitaxel followed by oxaliplatin, either synergism or additivity was observed in all three cell lines, indicating that this sequence would be the most effective schedule. By contrast, a clear antagonism was observed with the sequence oxaliplatin followed by paclitaxel in all of the cell lines.

To explain the possible mechanism underlying the synergistic interaction of paclitaxel followed by oxaliplatin, we further analyzed the perturbations induced in cell cycle by flow cytometric analysis using AZ-521 cells. First, we found that a 24-h treatment with paclitaxel markedly affected the cell cycle distribution, producing a relevant accumulation in the G_2/M phase, and induced apoptosis in 56% of treated cells. Oxaliplatin alone induced apoptosis (35%) by arresting cells in the G_1 phase. Exposure to oxaliplatin immediately after treatment with paclitaxel led to apoptosis in the majority of cells (75%) without affecting cell cycle distribution induced by paclitaxel. These results suggest that oxaliplatin may kill the cells recovering from the mitotic block produced by paclitaxel as they progress into S phase, accounting for the synergistic interaction. By contrast, oxaliplatin followed by paclitaxel had an antagonistic effect, reducing the rate of apoptosis to 39%. This would probably be explained by the decrease in the G_2 population targeted by paclitaxel, because pretreatment with oxaliplatin caused accumulation of cells at G_1/S boundary, thereby reducing the number of cells entering G_1 phase.

Unlike cisplatin, oxaliplatin, a new type of platinum derivative containing a diaminocyclohexane carrier ligand, appears to arrest the cells mainly at G_1 phase, suggesting an action distinct from that of cisplatin causing accumulation of cells in the G_2/M phase [16, 29]. It has been consistently demonstrated that oxaliplatin exhibits activity in cell lines with acquired cisplatin resistance and is active even in tumor types that are intrinsically resistant to cisplatin as well as carboplatin [6, 9, 25]. Therefore, this non-cross-resistance might be due to the differential patterns of DNA damage induced [21] and distinct cell cycle perturbations between oxaliplatin and other platinum compounds. With regard to a synergistic or additive interaction observed when paclitaxel preceded oxaliplatin, a similar sequence-dependent interaction has been reported with the combination of paclitaxel and CDDP. Synergistic or additive effects have been observed when paclitaxel precedes cisplatin [4, 18, 22, 27], whereas antagonistic interactions have been observed with the reverse sequence [15, 30]. There are several explanations for the increased activity of the sequence paclitaxel followed by cisplatin: cisplatin hastens the exit from mitosis in paclitaxel-treated cells

Table 2 Cell cycle perturbation (%) and apoptosis induced by paclitaxel and oxaliplatin in AZ-521 cells. The apoptotic population percentages were determined by measuring the sub-G_1 phase by FACS analysis after collecting floating and trypsinized adherent cells at various times following drug exposure. The data presented are the mean percentage values from three independent experiments

	24 h				36 h				48 h			
	G_0/G_1	S	G_2/M	Apoptosis	G_0/G_1	S	G_2/M	Apoptosis	G_0/G_1	S	G_2/M	Apoptosis
Control	52.64	31.75	15.61	3.52								
Paclitaxel	15.02	16.66	68.32	39.07	20.09	19.88	60.02	66.61	23.53	25.07	51.40	56.47
Oxaliplatin	71.87	3.12	25.01	11.01	76.96	9.51	13.53	37.80	86.89	5.22	7.89	35.12
Oxaliplatin + paclitaxel	21.56	12.05	66.39	38.57	24.93	8.05	67.02	57.70	29.34	5.31	65.35	65.32
Paclitaxel → oxaliplatin					28.32	26.01	45.67	75.83	15.02	34.13	50.85	74.91
Oxaliplatin → paclitaxel					68.68	3.42	27.91	40.78	81.16	2.57	16.27	37.93

[18]; paclitaxel induces an increase in intracellular uptake of cisplatin [4]; and paclitaxel inhibits repair of cisplatin-induced DNA damage [22]. Therefore, we hypothesize that similar, if not identical, mechanisms to those demonstrated for the interaction between cisplatin and paclitaxel may operate for the combination of oxaliplatin and paclitaxel.

Clinically, oxaliplatin is frequently used in combination to improve its efficacy. Over the past years, oxaliplatin combinations have been explored preclinically and clinically, mainly with thymidylate synthase inhibitors [24], other platinum compounds [25], and topoisomerase I inhibitors [31]. Based on the fact that paclitaxel exhibits synergism with cisplatin, combinations of oxaliplatin and paclitaxel would be expected to show potent activity similar to that of paclitaxel and cisplatin. Recently, clinical activity of the oxaliplatin and paclitaxel combination has been shown in platinum-pretreated patients with ovarian cancer [8]. The combination of oxaliplatin and docetaxel has also been reported to be a feasible and well-tolerated outpatient regimen as front-line chemotherapy in patients with non-small-cell lung cancer or advanced breast cancer [14]. Although the biochemical basis for their interaction remains unknown, the clear sequence-dependent activity of the combination of oxaliplatin and paclitaxel should be incorporated into the design of a clinical trial.

References

1. Becouarn D, Ychou M, Ducreux M, et al (1998) Phase II trial of oxaliplatin as first-line chemotherapy in metastatic colorectal cancer patients: Digestive Group of French Federation of Cancer Centers. J Clin Oncol 16:2739–2744
2. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
3. Chou TC, Motzer RJ, Tong V, Bosl GJ (1994) Computerized quantitation of synergism and antagonism of Taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst 86:1517–1524
4. Christen RD, Jekunen AP, Jones JA, Thiebaut F, Shalinsky DR, Howell SB (1993) In vitro modulation of cisplatin accumulation in human ovarian carcinoma cells by pharmacologic alteration of microtubules. J Clin Invest 92:431–440
5. Donehower RC, Rowinsky EK, Grochow LB, Lomgnecker SM, Ettinger DS (1987) Phase I trial of taxol in patients with advanced cancer. Cancer Treat Rep 71:1171–1177
6. Dunn T, Schmoll HJ, Grunwald V, Bokemeyer C, Casper J (1997) Comparative cytotoxicity of oxaliplatin and cisplatin in non-seminomatous germ cell cancer cell lines. Invest New Drugs 15:109–114
7. Extra JM, Espie M, Calvo F, et al (1990) Phase I study of oxaliplatin in patients with advanced cancer. Cancer Chemother Pharmacol 25:299–303
8. Faivre S, Kalla S, Cvitkovic E, et al (1999) Oxaliplatin and paclitaxel combination in patients with platinum-pretreated ovarian carcinoma: an investigator-originated compassionate-use experience. Ann Oncol 10:1125–1128
9. Fukuda M, Ohe Y, Kanzawa F, Oka M, Hara K, Saijo N (1995) Evaluation of novel platinum complexes, inhibitors of topoisomerase I and II in non-small cell lung cancer (NSCLC) sublines resistant to cisplatin. Anticancer Res 15:393–398
10. Garufi C, Nistico C, Brienza S, et al (2001) Single-agent oxaliplatin in pretreated advanced breast cancer patients: a phase II study. Ann Oncol 12:179–182
11. Ishiyama M, Shiga M, Sasamoto K, Mizogichi M, He P (1993) A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull 41:1118
12. Jennerwein MM, Eastman A, Khokhar A (1989) Characterization of adducts produced in DNA by isomeric 1,2-diaminocyclohexane platinum (II) complexes. Chem Biol Interact 70:39–49
13. Kano Y, Akutsu M, Tsunoda S, Suzuki K, Yazawa Y (1996) In vitro schedule-dependent interaction between paclitaxel and cisplatin in human carcinoma cell lines. Cancer Chemother Pharmacol 37:525–530
14. Kouroussis C, Agelaki S, Mavroudis D, Kakolyris S, Androulakis N, Kalbakis K, Souglakos J, Mallas K, Bozionelou V, Pallis A, Adamtziki H, Georgoulias V (2003) A dose escalation study of docetaxel and oxaliplatin combination in patients with metastatic breast and non-small cell lung cancer. Anticancer Res 23:785–791
15. Liebmann JE, Fisher J, Teague D, Cook JA (1994) Sequence dependence of paclitaxel (Taxol) combined with cisplatin or alkylators in human cancer cells. Oncol Res 6:25–31
16. Ma J, Maliepaard M, Nooter K, Boersma AW, Verweij J, Stoter G, Schellens JH (1998) Synergistic cytotoxicity of cisplatin and topotecan or SN-38 in a panel of eight solid-tumor cell lines in vitro. Cancer Chemother Pharmacol 41:307–316
17. Matsuoka H, Sugimachi K, Ueo H, Kuwano H, Nakano S, Nakayama M (1987) Sex hormone response of a newly established squamous cell line derived from clinical esophageal carcinoma. Cancer Res 47:4134–4140
18. Milross CG, Peters LJ, Hunter NR, Mason KA, Milas L (1995) Sequence-dependent antitumor activity of paclitaxel (Taxol) and cisplatin in vivo. Int J Cancer 62:599–604
19. Monnet I, Brienza S, Hugret F, et al (1998) Phase II study of oxaliplatin in poor-prognosis non-small cell lung cancer (NSCLC): Association pour le Traitement des Tumeurs Intra-thoraciques. Eur J Cancer 34:1124–1127
20. Nakano S, Nakayama M, Ichinose I, Mitsugi K, Nagafuchi S, Niho Y (1989) Characterization of a newly established, TA-4-producing squamous carcinoma cell line derived from metastatic tongue carcinoma. Int J Cancer 44:301–306
21. Page JD, Husain I, Sancar A, Chaney SG (1990) Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality. Biochemistry 29:1016–1024
22. Parker RJ, Lee KB, Dabholkar M, Bostick-Bruton F, Simmis M, Reed E (1993) Influence of Taxol: cisplatin sequencing on cisplatin-DNA adduct repair in human ovarian cancer cells. Proc Am Assoc Cancer Res 34:356
23. Piccart MJ, Green JA, Lacave AJ, et al (2000) Oxaliplatin or paclitaxel in patients with platinum-pretreated advanced ovarian cancer: a randomized phase II study of the European Organization for Research and Treatment of Cancer Gynecology Group. J Clin Oncol 18:1193–1202
24. Raymond E, Buquet-Fagot C, Djelloul S, Mester J, Cvitkovic E, Allain P, Louvet C, Gespach C (1997) Antitumor activity of oxaliplatin in combination with 5-fluorouracil and the thymidylate synthase inhibitor AG337 in human colon, breast and ovarian cancers. Anticancer Drugs 8:876–885
25. Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K, Fojo T (1996) Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. Biochem Pharmacol 52:1855–1865
26. Rowinsky EK (1997) The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48:353–374
27. Rowinsky EK, Citardi MJ, Noe DA, Donehower RC (1993) Sequence-dependent cytotoxic effects due to combinations of cisplatin and the antimicrotubule agents Taxol and vincristine. J Cancer Res Clin Oncol 119:727–733

28. Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly in vitro by taxol. Nature 277:665–667

29. Sorenson CM, Eastman A (1988) Mechanism of cis-diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double-strand breaks. Cancer Res 48:4484–4488

30. Vanhoefer U, Harstrick A, Wilke H, Schleucher N, Walles H, Schroder J, Seeber S (1995) Schedule-dependent antagonism of paclitaxel and cisplatin in human gastric and ovarian carcinoma cell lines in vitro. Eur J Cancer 31A:92–97

31. Zeghari-Squalli N, Raymond E, Cvitkovic E, Goldwasser F (1999) Cellular pharmacology of the combination of the DNA topoisomerase I inhibitor SN-38 and the diaminocyclohexane platinum derivative oxaliplatin. Clin Cancer Res 5:1189–1196

Synergistic interaction between oxaliplatin and SN-38 in human gastric cancer cell lines *in vitro*

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Abstract. The interaction between CPT-11 and oxaliplatin, a new platinum derivative that has a great antitumor activity against colon cancer, has not been determined in gastric cancer cells. In this study, we investigated *in vitro* cytotoxic activity of oxaliplatin alone or in combination with SN-38, an active metabolite of CPT-11, using different exposure schedules in three human gastric cancer cell lines (AZ-521, MKN-45, and NUGC-4). Cytotoxicity was determined by WST-1 assay. Different treatment schedules of the two drugs were compared and evaluated for synergism, additivity, or antagonism with a quantitative method based on the median-effect principle of Chou and Talalay. Cell cycle perturbation was evaluated by flow cytometry. In 24-h exposure, simultaneous administration of oxaliplatin and SN-38 showed a synergistic effect in AZ-521 and NUGC-4 cells, and an additive effect in MKN-45 cells. Greater than additive effects were observed in all of the cell lines when cells were treated with oxaliplatin followed by SN-38, whereas such effects were observed only in NUGC-4 cells in the reverse sequence. Flow cytometric analyses at IC_{50} indicated that apoptosis was most prominent in simultaneous exposures with accumulation of cells in both G_0/G_1 and S phases. These results suggest that SN-38 may kill the cells recovering from the G_1 block produced by oxaliplatin as they progress into the S phase. Simultaneous administration appears most active in gastric cancer cell lines. These results may provide important information for a clinical trial of oxaliplatin and CPT-11 combination for patients with gastric cancer.

Introduction

Gastric cancer remains one of the leading causes of cancer death worldwide and the prognosis of patients with un-resectable and recurrent gastric cancer is extremely poor. Although complete resection is the only curative approach, randomized trials demonstrated that fluorouracil-based chemotherapy improves survival and quality of life in patients with advanced gastric cancer (1-3). Nevertheless, none of these regimens can be regarded as standard treatment because of their low activity (4). Therefore, it is of extreme importance to develop new strategies with better clinical efficacy in the treatment of advanced gastric cancer.

Oxaliplatin is a new platinum analogue that exhibits a wide spectrum of antitumor activity against tumors resistant to cisplatin as well as carboplatin (5-7), and has shown to be more active in preclinical models compared with cisplatin, in that it requires fewer DNA adducts to achieve an equal level of cytotoxicity (8). In comparison to other platinum compounds, oxaliplatin lacks the nephrotoxicity of cisplatin and myelosuppression of carboplatin, but it produces a reversible cold-sensitive peripheral neuropathy (9). Oxaliplatin has shown antitumor activity against colon cancer both *in vitro* and *in vivo*, and is now used in the chemotherapeutic treatment of metastatic colorectal cancer (10). Recently, it has been demonstrated that adding oxaliplatin to a standard adjuvant treatment (fluorouracil and leucovorin) improves the efficacy of adjuvant treatment of colon cancer (11). It has also been demonstrated that oxaliplatin is active in gastric cancer. In phase II studies in patients with advanced gastric cancer, the combination of oxaliplatin and 5-fluorouracil had a significant activity with a favorable toxicity profile (12,13).

Irinotecan hydrochloride (CPT-11) is rapidly converted *in vivo* to its active metabolites, SN-38, by carboxylesterase and shows the potent antitumor activity against various solid tumors, including colorectal, lung and ovarian cancers (14-17), through the inhibition of DNA topoisomerase-I (18,19). Recently, CPT-11 has also been demonstrated to be active against gastric cancer, with the response rate of 18% in a phase II study (20), and has produced a much higher response rate than conventional chemotherapy when combined with cisplatin (21). Moreover, we previously reported that maximal synergy is achieved *in vitro* when cisplatin and SN-38 is applied simultaneously (22,23).

Because of a different mechanism of action and non-overlapping toxicity profile, the combination of oxaliplatin and CPT-11 is currently under clinical investigation in patients with colorectal cancer (24). Although preclinical studies have shown a schedule-dependent additivity and synergism

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Schedule A

Schedule B

Schedule C

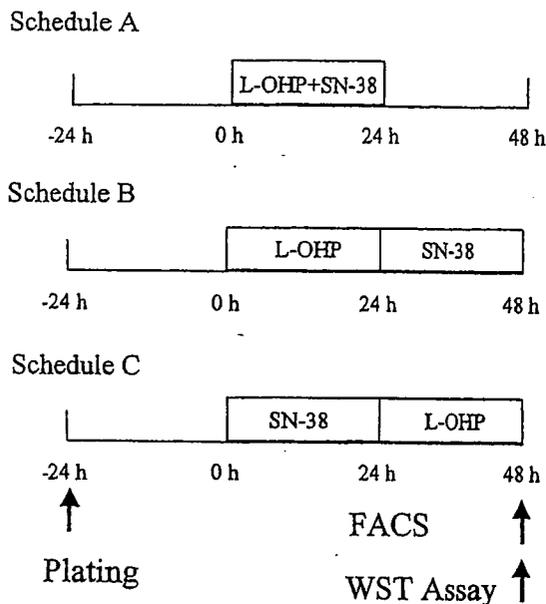Figure 1. Description of the three combination schedules.

Table I. IC_{50} values of oxaliplatin and SN-38 in a panel of three cell lines.

	AZ-521	MKN45	NUGC-4
Oxaliplatin (µg/ml)	0.95±0.4	1.7±0.9	1.49±0.9
SN-38 (ng/ml)	14.2±0.4	19.5±0.5	17.83±0.9

Cells were treated for 24 h with various concentrations of SN-38 (0.3-1000 ng/ml) and oxaliplatin (0.3-1000 µg/ml). Results are expressed as the concentration that inhibits 50% of growth in comparison with controls (IC_{50}). The values are mean ± SD of three independent experiments.

between these drugs in colon cancer cell lines (25,26), such studies have yet to be determined in gastric cancer cells where both drugs are also active. In this study, we investigated *in vitro* cytotoxic activity of oxaliplatin alone or in combination with SN-38, an active metabolite of CPT-11, using different exposure schedules in three human gastric cancer cell lines, including AZ-521, MKN-45 and NUGC-4.

Material and methods

Cell line and culture. The three human gastric cancer cell lines, AZ-521, MKN-45 and NUGC-4, were purchased from Japanese Cell Resource Bank. The cells were propagated in D-MEM supplemented with 10% heat-inactivated FCS in an incubator at 37°C and 100% humidity with 5% CO_2 and air.

Drugs. SN-38 and oxaliplatin were a gift from Yakult (Tokyo, Japan). Stock solutions of SN-38 were prepared in DMSO and stored at -4°C prior to use. Oxaliplatin was prepared in distilled water. The final concentration of DMSO for all experiments and treatments was maintained at <0.02%. These conditions were found to be non-cytotoxic.

Growth inhibition assay. Cytotoxic activity was measured using the WST-1 assay (Wako-chemical Co. Japan) according to the manufacturer's instructions (27). The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of viable cells. Briefly, AZ-521, MKN-45 and NUGC-4 cells were plated into 96-well microtiter plates at a density of $5x10^3$ cells/well. After overnight incubation, the cells were treated for 24 h with graded concentrations of SN-38 (0.3-1000 ng/ml), or oxaliplatin (0.3-1000 µg/ml). To define the best schedule for the combination, either simultaneous or sequential 24-h exposures to the two agents were tested. Forty-eight hours after the beginning of the treatment, cells were washed with PBS and 100 µl medium, and the plates were incubated at

37°C for another 3 h after addition of 10 µl WST solution. Absorbance at 480 and 640 nm was measured using a Delta Soft ELISA analysis program for Macintosh computer interfaced with Bio-Tek microtiter reader (immuno Mini NJ-2300). Wells containing only DMEM and WST solution were used as controls. Each experiment was performed using six replicated wells for each drug concentration and carried out independently at least three times. The IC_{50} was defined as the concentration that reduced the absorbance in each test by 50%. For the combination experiments, three different schemes were used to investigate the interaction of SN-38 and oxaliplatin, as shown in Fig. 1; (A) oxalipatin and SN-38 were exposed simultaneously for 24 h and incubated for an additional 24 h with drug-free medium, (B) oxaliplatin was administered for 24 h followed by SN-38 for 24 h, or (C) SN-38 was administered for 24 h followed by oxaliplatin. Immediately after these treatments, the cytotoxic effects were evaluated by WST-1 assay.

Analysis of combination effects. Combination analysis was performed using the method described by Chou and Talalay (28,29), using the Calcusyn software program for automated analysis (Bio-soft, Ferguson, MO). The influence on the combination of the two drugs was evaluated by comparing the sequential assays with assays involving oxaliplatin or SN-38 exposures alone. The combination effect was evaluated from isoeffect analysis (CIs), calculated as follows: $CI=C_{oxaliplatin}/Cx_{oxaliplatin}+C_{SN-38}/Cx_{SN-38}$, where $Cx_{oxaliplatin}$ and Cx_{SN-38} are the concentrations of oxaliplatin and SN-38 alone, respectively, needed to achieve a given effect (x%), and $C_{oxaliplatin}$ and C_{SN-38} are the combined concentrations of oxaliplatin and SN-38 needed for the same effect (x%). These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The CIs were calculated under the assumption of a mutually exclusive drug interaction, i.e. that the effect of SN-38 may influence the effect of oxaliplatin and vice versa. The combination is considered as synergistic when the CI is <1, and antagonistic when it is >1, whereas a value of 1 indicates additivity.

Cell cycle determination. Human gastric cancer cell line, AZ-521, cells were cultured at $1x10^5$ cells per 60 mm dish. The same protocols as described in the growth inhibition assay were used. After treatment, the cells were harvested,
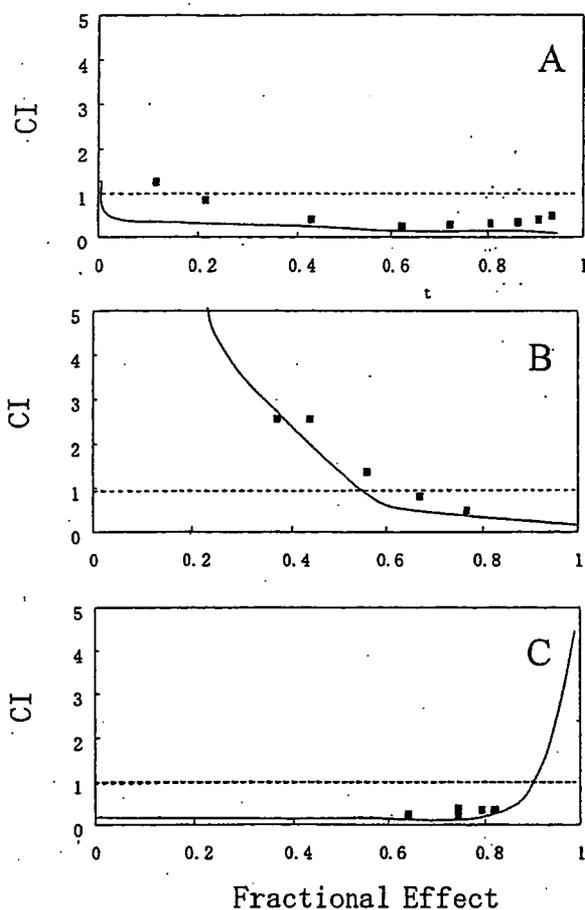
Figure 2. Combination index (CI) plots obtained from three gastric cancer cell lines exposed simultaneously to oxaliplatin and SN-38 for 24 h. (A) AZ-521, (B) MKN-45, (C) NUGC-4.
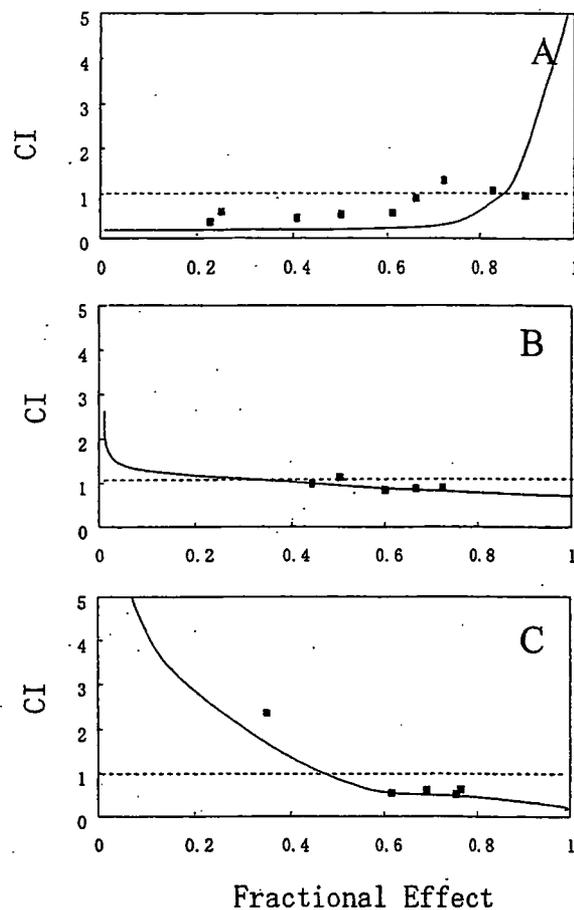
Figure 3. Combination index (CI) plots obtained from three gastric cell lines exposed to oxaliplatin for 24 h followed by SN-38 for 24 h. (A) AZ-521, (B) MKN-45, (C) NUGC-4.

washed twice in ice-cold PBS (pH 7.4), fixed in 100% ethanol and stored at 4°C for up to 3 days, prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were washed with PBS and stained with a solution containing propidium iodide and RNase (Sigma-Aldrich, St. Louis, MO, USA) on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELLQuest or ModFit 3.0 software packages (Becton Dickinson, San Jose, CA, USA), and the percentages of apoptotic populations were determined by measuring the sub-G_1 phase using FACS analysis at various times following drug exposure. Each experiment was performed in triplicate.

Results

Determination of IC_{50} value. Three gastric cancer cell lines were exposed to oxaliplatin or SN-38, an active metabolite of CPT-11, for 24 h, and assayed for growth using the WST-1 assay. The IC_{50} for each drug and each cell line are shown in Table I. For oxaliplatin, the IC_{50} for 24-h exposure ranged from 0.95 µg/ml (2.39 µM) for AZ-521 cells to 1.7 µg/ml (4.3 µM) for MKN45 cells. The AZ-521 cell line was 2-fold more sensitive than MKN45 or NUGC-4 cell lines. AZ-521 cells were the most sensitive to SN-38 (14.2 ng/ml; 36 nM) among the three tumor cell lines, MKN45 cells being the

least sensitive (19.5 ng/ml; 49 nM). These IC_{50} values were used to select concentration ranges for the combination studies.

Median-effect analysis of oxaliplatin and SN-38 combination in vitro. Oxaliplatin and SN-38 were tested in different combinations to define the most effective schedule. Three different schedules were tested, simultaneous or sequential drug exposures, as shown in Fig. 1, and exposure time to each drug was 24 h. Fig. 2 illustrates the CI plot obtained from three gastric cancer cell lines exposed simultaneously to oxaliplatin and SN-38 for 24 h. When either AZ-521 or NUGC-4 cells were treated with oxaliplatin and SN-38 simultaneously, the CI values were <1 at all levels of killed cell fraction, indicating a marked synergistic effect. This did not apply to MKN45 cells, where greater than additive effects were seen at the ranges corresponding to >50% inhibition of cell growth. A similar effect was observed when cells were treated with oxaliplatin for 24 h followed by SN-38 for 24 h and showed a greater than additive effect in all of the cell lines (Fig. 3), although the cytotoxic effects of this sequential administration appear less than those of simultaneous treatment. As shown in Fig. 4, when cells were treated with SN-38 followed by oxaliplatin, a greater than additive effect was observed only in NUGC-4 at all levels of killed cell fraction. However, in the MKN45 cell line, a greater than additive effect was observed at higher levels of
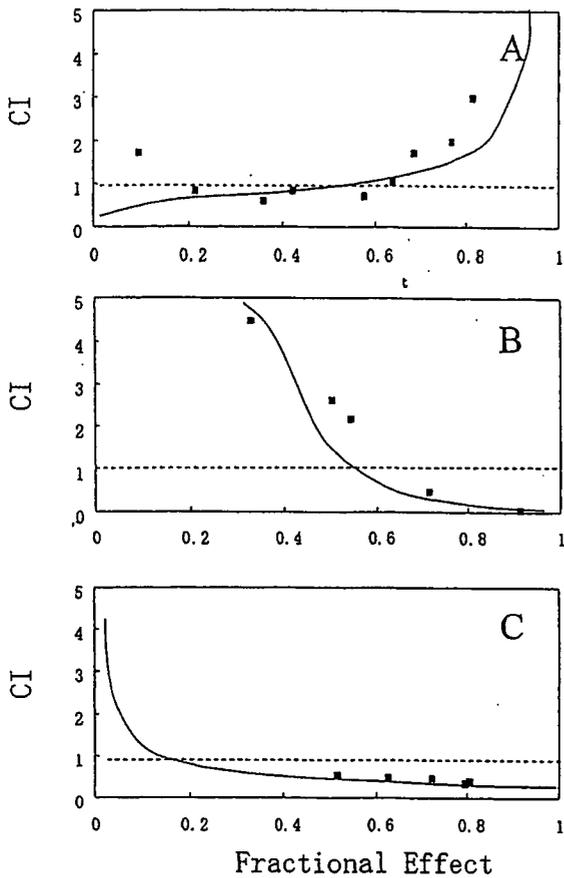
Figure 4. Combination index (CI) plots obtained from three gastric cell lines exposed to SN-38 for 24 h followed by oxaliplatin for 24 h. (A) AZ-521, (B) MKN-45, (C) NUGC-4.

beginning of the treatment, using flow-cytometric analysis. SN-38 alone induced the accumulation of cells in the S phase and markedly decreased the population of G_0/G_1 and G_2/M phases. By contrast, oxaliplatin alone caused an increase in the G_0/G_1 and G_2/M population and a decrease in the population of the S-phase. Oxaliplatin prior to SN-38 caused almost identical distribution patterns to those observed with oxaliplatin alone, indicating that the activity of oxaliplatin is unaffected by SN-38 in this treatment period. In contrast, the treatment with SN-38, prior to oxaliplatin, induced the accumulation of cells in the S phase as well as the reduction of the G_2/M cell population, showing similar distribution patterns to those observed in the cells treated with SN-38 alone, although a slight increase in G_0/G_1 and decrease in S population were observed as compared to those treated with SN-38 alone, indicating a modest influence of oxaliplatin. Therefore, in sequential combination, cell cycle distribution patterns for the initial drug appear mostly unaffected by the second drug administered, indicating a dominant effect of the initial drug. Interestingly, simultaneous exposures led to the accumulation of cells in both G_0/G_1 and S phases as well as a marked reduction of G_2/M population, indicating that the activities of individual drugs were exhibited with a substantial interaction.

To confirm the activities of these combinations, the apoptotic activity was investigated after treatment of AZ-521 cells by measuring the population of the sub-G_1 phase using FACS analyses. The presence of hypodiploid DNA (sub-G_1) is associated with cells undergoing apoptosis. As shown in Table II, SN-38 followed by oxaliplatin and the reverse sequence induced apoptosis in 12 and 8% of treated cells, respectively, being comparable to the rates of apoptosis induced by SN-38 (7.5%) or oxaliplatin alone (5.9%). By contrast, the simultaneous treatment induced apoptosis in 20.9% of treated cells.

killed cell fraction. Conversely, this sequence is antagonistic in AZ521 cells at the range corresponding to >70% killed cell fraction (Fig. 4).

Cell cycle perturbation and apoptosis. In an attempt to explain the mechanisms underlying the different types of interaction, the effects of SN-38 and oxaliplatin on cell cycle distribution and apoptosis were studied in AZ-521 cells (Table II). The cells were treated with these drugs at the IC_{50} concentration, either alone or in combination with different schedules, and cell cycle distribution was analyzed at 36 and 48 h after the

Discussion

Cisplatin and CPT-11 are widely used anticancer drugs that act against gastric cancer (21), and a significant synergy was observed experimentally when combining them *in vitro* (23,30-32). As oxaliplatin has a markedly different spectrum of activity to cisplatin (7), we examined the interaction of oxaliplatin and SN-38 in a panel of gastric cancer cell lines *in vitro*,

Table II. Cell cycle perturbation (%) and apoptosis induced by L-OHP and SN-38 in the AZ-521 cell line.[a]

	36-h				48-h			
	G_0/G_1 (%)	S (%)	G_2/M (%)	sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)	sub-G_1 (%)
Control					57.57	21.10	21.32	
SN-38	33.08	66.75	0.18	4.98	14.04	85.48	0.48	7.49
L-OHP	60.37	10.85	28.75	5.23	59.55	15.58	24.86	5.89
SN-38+L-OHP	56.31	41.06	2.69	11.85	55.86	44.14	0.00	20.87
L-OHP→SN-38	59.38	12.89	27.73	5.07	62.23	13.84	23.93	7.58
SN-38→L-OHP	21.64	73.81	4.54	6.82	26.12	64.19	9.69	12.14

[a]Data represent mean percentage values from three independent eperiments. L-OHP, oxaliplatin.

with special reference to cell- and schedule-dependency of this combination. Simultaneous exposure to oxaliplatin and SN-38 for 24 h produced synergistic interaction in AZ-521 and NUGC-4 cell lines, whereas only an additive effect was observed in MKN-45. A greater than additive effect was observed in all of the cell lines when cells were treated with oxaliplatin followed by SN-38. However, a greater than additive effect was observed only in NUGC-4 cells when treated with SN-38 followed by oxaliplatin. Either sequence produced no more than additive effects in MKN-45 cell lines. Therefore, simultaneous treatment appears most active at least in these three gastric cancer cell lines.

It has been shown *in vitro* that the combination of oxaliplatin and SN-38 produces a strong synergism in the HT-29 human colon cancer cell line, regardless of the sequence of administration or the exposure time of drugs (26). Among them, 1-h exposure of oxaliplatin followed by SN-38 with a 3-h interval was most cytotoxic. Based on these results, clinical trials were studied by the schedule of sequential administration of oxaliplatin followed by CPT-11. As CPT-11 administered by 90-min infusion resulted in a long-term half-life of SN-38 in humans (33) and oxaliplatin is a highly time-dependent drug (26), 24-h exposure rather than 2-h exposure seems to be better for translating these results into the clinical setting. Arnould and coworkers reported, when using the HT-29 colon cancer cell line, that a synergism was observed when cells were simultaneously exposed to oxaliplatin and CPT-11 for 24 h or when cells were first exposed to CPT-11 for 24 h and then oxaliplatin for 24 h, whereas the reverse sequence showed only an additive effect (26). Our data support their results in the gastric cancer cell line, in that simultaneous treatment is most active. However, as opposed to their sequential results, we found that oxaliplatin followed by SN-38 was much more active than the reverse sequence. Correspondingly, we reported that synergistic interactions were mostly exhibited by concurrent and sequential schedules in which CDDP precedes SN-38 in HST-1 human squamous carcinoma cells (23). The discrepancy in the activities of the sequential combinations may be caused by the difference of pharmacokinetics *in vitro* between SN-38 and CPT-11, a prodrug needed to be converted to SN-38 by carboxylesterase.

To explain the possible mechanism underlying the synergistic interaction of oxaliplatin and SN-38, we further analyzed the cell cycle perturbations using the AZ-521 human gastric cancer cell line. We found that a 24-h treatment with SN-38 markedly affected the cell cycle distribution, producing a relevant accumulation in the S phase, and induced apoptosis in 7% of treated cells. Oxaliplatin alone induced apoptosis (6%) by arresting cells in the G_0/G_1 and G_2/M phases. In sequential combinations, distribution patterns of the cell cycle for the initial drug were unaffected by the second drug administered, with comparable rates of apoptosis (8-12%). By contrast, simultaneous exposures led to the accumulation of cells into both G_0/G_1 and S phases and a marked reduction of G_2/M population, with subsequent apoptosis in 21% of treated cells, indicating that the activities of individual drugs were exhibited with a substantial interaction. These results suggested that SN-38 may kill the cells recovering from the G_1 block produced by oxaliplatin as they progress into S phase, accounting for a synergistic interaction.

The exact mechanism for the synergistic interaction remains unclear. Previously, we demonstrated that synergistic interaction of CDDP and SN-38 would be due to the inhibition by SN-38 of the repair of CDDP-induced DNA interstrand cross-links (22). Since a great synergy between oxaliplatin and SN-38 has been shown in the same treatment schedules as in the CDDP and SN-38 combination, the inhibition by SN-38 of the repair of oxaliplatin-induced DNA interstrand cross-links may cause the synergy. However, the synergistic mechanism may vary from cell to cell, depending on the cell type, because it has been reported that clearly opposite interactions exist among human colon cancer cell lines in the combination of oxaliplatin and CPT-11 (25). Moreover, as shown in the present study, simultaneous combination of oxaliplatin and SN-38 at IC_{50} for 24 h accumulates AZ-521 cells almost exclusively into G_0/G_1 and S phases 24 h after treatment, whereas simultaneous combination of oxaliplatin and CPT-11 at IC_{50} for 24 h has been reported to accumulate HT-29 cells into S and G_2/M phases in the same period of time (26). Further experiments would be necessary for clarifying this discrepancy. Nonetheless, the present study highlights the importance of a treatment schedule for the combination of oxaliplatin and CPT-11. These *in vitro* findings might provide important information for a future clinical trial of the combination of oxaliplatin and CPT-11 for gastrointestinal cancer.

References

1. Glimelius B, Hoffman K and Haglund U: Initial or delayed chemotherapy with best supportive care in advanced gastric cancer. Ann Oncol 5: 189-190, 1994.
2. Murad AM, Santiago FF, Petroianu A, Rocha PR, Rodrigues MA and Rausch M: Modified therapy with 5-fluorouracil, doxorubicin, and methotrexate in advanced gastric cancer. Cancer 72: 37-41, 1993.
3. Pyrhonen S, Kuitunen T, Nyandoto P and Kouri M: Randomized comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus supportive care with alone in patients with non-resectable gastric cancer. Br J Cancer 71: 587-591, 1995.
4. Vanhoefer U, Rougier P, Wilke H, Ducreux MP, Lacave AJ, van Cutsem E, Planker M, Santos JG, Piedbois P, Paillot B, Bodenstein H, Schmoll HJ, Bleiberg H, Nordlinger B, Couvreur ML, Baron B and Wils JA: Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus etoposide, leucovorin, and fluorouracil versus infusional fluorouracil and cisplatin in advanced gastric cancer: A Trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group. J Clin Oncol 18: 2648-2657, 2000.
5. Dunn TA, Schmoll HJ, Grunwald V, Bokemeyer C and Casper J: Comparative cytotoxicity of oxaliplatin and cisplatin in non-seminomatous germ-cell cancer cell lines. Invest New Drugs 15: 109-114, 1997.
6. Kidani Y, Noji M and Tashiro T: Antitumor activity of platinum(II) complexes of 1,2-diamino-cyclohexane isomers. Gann 71: 637-643, 1980.
7. Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K and Fojo T: Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines of the National Cancer Institute's Anticancer Drug Screening panel. Biochem Pharmacol 52: 1855-1865, 1996.
8. Fukuda M, Ohe Y, Kanzawa F, Oka M, Hara K and Saijo N: Evaluation of novel platinum complexes, inhibitors of topo-isomerase I and II in non-small cell lung cancer (NSCLC) sublines resistant to cisplatin. Anticancer Res 15: 393-398, 1995.
9. Extra JM, Espie M, Calvo F, Ferme C, Mignot L and Marty M: Phase I study of oxaliplatin in patients with advanced cancer. Cancer Chemother Pharmacol 25: 299-303, 1990.

10. Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC and Alberts SR: A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 22: 23-30, 2004.
11. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I and De Gramont A: Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) Investigators. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 350: 2343-2351, 2004.
12. Kim DY, Kim JH, Lee SH, Kim TY, Heo DS, Bang YJ and Kim NK: Phase II study of oxaliplatin, 5-fluorouracil and leucovorin in previously platinum-treated patients with advanced gastric cancer. Ann Oncol 14: 383-387, 2003.
13. Louvet C, Andre T, Tigaud JM, Gamelin E, Douillard JY, Brunet R, Francois E, Jacob JH, Levoir D, Taamma A, Rougier P, Cvitkovic E and De Gramont A: Phase II study of oxaliplatin, fluorouracil, and folinic acid in locally advanced or metastatic gastric cancer patients. J Clin Oncol 20: 4543-4548, 2002.
14. Bleiberg H: CPT-11 in the gastrointestinal cancer. Eur J Cancer 35: 371-379, 1999.
15. Fukuoka M, Niitani H, Suzuki A, Motomiya M, Hasegawa K, Nishiwaki Y, Kuriyama T, Ariyoshi Y, Negoro S, Masuda N, et al: A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. J Clin Oncol 10: 16-20, 1992.
16. Rosen LS: Irinotecan in lymphoma, leukemia, and breast, pancreatic, ovarian, and small-cell lung cancers. Oncology 12: 103-109, 1998.
17. Sugiyama T, Yakushiji M, Ochiai K and Noda K: Japanese ovarian trials: focus on irinotecan. Oncology 17: 29-33, 2003.
18. Kaneda N, Nagata H, Furuta T and Yokokura T: Metabolism and pharmacokinetics of the camptothesin analogue CPT-11 in the mouse. Cancer Res 50: 1715-1720, 1990.
19. Liu LF, Desai SD, Li TK, Mao Y, Sun M and Sim SP: Mechanism of action of camptothesin, Ann NY Acad Sci 922: 1-10, 2000.
20. Bugat R: Irinotecan in the treatment of gastric cancer. Ann Oncol 14: 37-40, 2003.
21. Boku N, Ohtsu A, Shimada Y, Shirao K, Seki S, Saito H, Sakata Y and Hyodo I: Phase II study of a combination of irinotecan and cisplatin against metastatic gastric cancer. J Clin Oncol 17: 319-323, 1999.
22. Masumoto N, Nakano S, Esaki T, Fujishima H, Tatsumoto T and Niho Y: Inhibition of cis-diamminedichloroplatinum (II)-induced DNA interstrand cross-link removal by 7-ethyl-10-hydroxy-camptothecin in HST-1 human squamous-carcinoma cells. Int J Cancer 4: 70-75, 1995.
23. Masumoto N, Nakano S, Esaki T, Tatsumoto T, Fujishima H, Baba E, Nakamura M and Niho Y: Sequence-dependent modulation of anticancer drug activities by 7-ethyl-10-hydroxy-camptothecin in an HST-1 human squamous carcinoma cell line. Anticancer Res 15: 405-409, 1995.
24. Garufi C, Bria E, Vanni B, Zappala AM, Sperduti I and Terzoli E: A phase II study of irinotecan plus chronomodulated oxaliplatin, 5-fluorouracil and folinic acid in advanced colorectal cancer patients. Br J Cancer 17: 1870-1875, 2003.
25. Guichard S, Arnould S, Hennebelle I, Bugat R and Canal P: Combination of oxaliplatin and irinotecan on human colon cancer cell lines: activity *in vitro* and *in vivo*. Anticancer Drugs 12: 741-751, 2001.
26. Arnould S, Guichard S, Hennebelle I, Cassar G, Bugat R and Canal P: Contribution of apoptosis in the cytotoxycity of the oxaliplatin-irinotecan combination in the HT-29 human colon adenocarcinoma cell line. Biochem Pharm 64: 1215-1226, 2002.
27. Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y and Ueno K: A combined assay of cell viability and *in vitro* cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19: 1518-1520, 1996.
28. Chou TC and Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27-55, 1984.
29. Chou TC, Motzer RJ, Tong V and Bosl GJ: Computerized quantitation of synergism and antagonism of Taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst 86: 1517-1524, 1994.
30. Fukuda M, Nishio K, Kanzawa F, Ogasawara H, Ishida T, Arioka H, Bojanowski K, Oka M and Saijo N: Synergism between cisplatin and topoisomerase I inhibitors, NB-506 and SN-38, in human small cell lung cancer cells. Cancer Res 56: 789-793, 1996.
31. Janss AJ, Cnaan A, Zhao H, Shpilsky A, Levow C, Sutton L and Phillips PC: Synergistic cytotoxicity of topoisomerase I inhibitors with alkylating agents and etoposide in human brain tumor cell lines. Anticancer Drugs 9: 641-652, 1998.
32. Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S and Miura Y: Effects of CPT-11 in combination with other anti-cancer agents in culture. Int J Cancer 50: 604-610, 1992.
33. Sasaki Y, Ohtsu A, Shimada Y, Ono K and Saijo N: Simultaneous administration of CPT-11 and 5-fluorouracil: alteration of the pharmacokinetics of CPT-11 and SN-38 in patients with advanced colorectal cancer. J Natl Cancer Inst 86: 1096-1098, 1994.

ORIGINAL ARTICLE

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In vitro sequence-dependent interaction between nedaplatin and paclitaxel in human cancer cell lines

Abstract *Purpose*: To define the most effective combination schedule of paclitaxel and nedaplatin, a new platinum derivative, we investigated the in vitro interaction between these drugs in AZ-521 and NUGC-4 gastric adenocarcinoma and KSE-1 esophageal squamous carcinoma cell lines. *Materials and methods*: Cytotoxic activity was determined by the WST-1 assay. Different treatment schedules of the two drugs were compared and evaluated for synergism, additivity, or antagonism using a quantitative method based on the median-effect principle of Chou and Talalay. Cell-cycle perturbation and apoptosis were evaluated by means of flow cytometry. *Results*: Upon 24-h sequential exposure, the sequence paclitaxel followed by nedaplatin induced greater than additive effects in all of the cell lines, with synergistic interactions in NUGC-4 and KSE-1 cells. By contrast, antagonistic effects were observed with the reverse sequence. Simultaneous treatment resulted in either a synergistic or antagonistic effect, depending on the cell line. Therefore, the sequence paclitaxel followed by nedaplatin appears most active, at least in these three cell lines. Flow cytometric analyses at IC_{50} indicated that paclitaxel induced G2/M arrest with subsequent induction of apoptosis (56%) in the sub-G1 phase. When paclitaxel preceded nedaplatin, apoptosis was most prominent (70%) with pronounced G2/M arrest. By contrast, the reverse sequence yielded only 28% induction of apoptotic cells, with almost identical cell-cycle distribution patterns to those observed with nedaplatin alone, indicating that the activity of paclitaxel is abolished by pretreatment with nedaplatin. *Conclusions*: Our

findings suggest that the interaction of nedaplatin and paclitaxel is highly schedule dependent and that the sequential administration of paclitaxel followed by nedaplatin should be thus incorporated into the design of a clinical trial.

Keywords Nedaplatin · Paclitaxel · Sequence dependence · Drug interaction

Introduction

Cisplatin has played a major role in the chemotherapy of a variety of solid tumors over the past two decades. However, the clinical usefulness of cisplatin is limited due to its toxicity to many normal tissues, such as kidney. Nedaplatin is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical antitumor activity against various solid tumors with lower nephrotoxicity [10]. Preclinical studies indicate that nedaplatin has an antitumor activity comparable to cisplatin [2, 12] and has been shown experimentally to overcome cisplatin resistance in a cisplatin-resistant K562 cell line [12]. Clinically, single-agent nedaplatin has shown a wide spectrum of antitumor activity, producing the favorable response rates in head and neck [9], esophagus [24], non-small cell lung [7], and cervical cancers [18]. The activity of nedaplatin against gastric cancer, however, still remains unclear, despite the fact that nedaplatin has a spectrum of antitumor activity similar to that of cisplatin in phase-I and phase-II studies.

Paclitaxel has demonstrated broad clinical efficacy in a variety of malignancies including ovarian, non-small-cell lung [22], esophageal [1], head and neck [6], gastric [21] and cervical [16] cancers. Paclitaxel in combination with cisplatin is well known for its sequence-dependent synergy in vitro and in vivo [11, 17], and the sequence of paclitaxel followed by cisplatin has been recommended

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for clinical studies. Although combination of paclitaxel and nedaplatin is expected to have a potent activity similar to that of paclitaxel and cisplatin combination, few preclinical data for the interaction between these drugs are currently available. Moreover, the efficacy of nedaplatin against gastric cancer cell lines has yet to be determined in vitro. In order to obtain the clinical rationale for the optimal administration schedule of this combination for the treatment of gastric and esophageal cancers, we investigated the interaction between nedaplatin and paclitaxel using an in vitro model of human cancer cell lines derived from esophagus and stomach, using a quantitative method that assesses the synergism or antagonism between these two agents.

Materials and methods

Cell lines and culture

The human AZ-521 and NUGC4 gastric adenocarcinoma cell lines were kindly provided by JCRB Cell Bank (Tokyo, Japan) and maintained in Dulbecco's minimum essential medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Ireland, NY, USA) in an incubator at 37°C and 100% humidity with 5% CO_2 and air. The human KSE-1 esophageal squamous carcinoma cell line [15] was established in our laboratory and maintained under the same conditions as were AZ-521 cells.

Drugs

Nedaplatin was a gift from Shionogi (Osaka, Japan) and paclitaxel was a gift from Bristol-Myers (Tokyo, Japan). Stock solutions of nedaplatin were prepared in distilled water and those of paclitaxel were prepared in dimethylsulfoxide (DMSO). Both solutions were stored at −4°C prior to use. The final concentration of DMSO for all experiments and treatments was maintained at less than 0.02%. These conditions were found to be noncytotoxic.

Cytotoxicity assay

Cytotoxic activity was measured by means of the WST-1 assay (Wako Chemicals, Osaka, Japan) using manufacturer's instructions [8]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, cells were plated into 96-well microtiter plates at a density of 5×10^3 cells/well, and incubated for 24 h for sufficient cell growth. Cells were then treated with graded concentrations of nedaplatin (0.3–1,000 µg/ml) or paclitaxel (0.3–1,000 ng/ml) alone for 24 h, and were incubated with drug-free medium for an additional 24 h. Cells were washed with PBS, and 100 µl medium and

10 µl WST-1 solution were added to each well; then the plates were incubated at 37°C for another 3 h. Absorbances at 450 nm and 620 nm were measured using a Delta Soft ELISA analysis program for Macintosh computer interfaced with a Bio-Tek microplate reader (Immuno-Mini NJ-2300). Wells containing only DMEM and WST-1 were used as controls. Each experiment was performed using six replicated wells for each drug concentration and carried out independently at least three times. The IC_{50} was defined as the concentration that reduced the absorbance in each test by 50%.

For the combination experiments, three different schemes were used to investigate the interaction of paclitaxel and oxaliplatin as shown in Fig. 1: (a) nedapaltin and paclitaxel were exposed simultaneously for 24 h and incubated for an additional 24 h with drug-free medium, (b) nedaplatin was administered for 24 h followed by paclitaxel for 24 h, or (c) paclitaxel was administered for 24 h followed by nedaplatin. Immediately after these treatments, the cytotoxic effects were evaluated by WST-1 assay.

Analysis of combination effects

On the basis of the growth inhibition curve for each single drug, we analyzed the effects of the drug combinations using the method described by Chou and Talalay and a Calcusyn software program for automated analysis (Biosoft, Cambridge, UK) [3, 4]. The influence on the combination of the two drugs was evaluated by comparing the sequential assays with assays involving nedaplatin or paclitaxel exposure alone. The combination effect was evaluated from isoeffect analysis (Cis), calculated as follows: $CI = C_{nedaplatin}/Cx_{nedaplatin} + C_{paclitaxel}/Cx_{paclitaxel}$ where $Cx_{paclitaxel}$ and $Cx_{paclitaxel}$ are
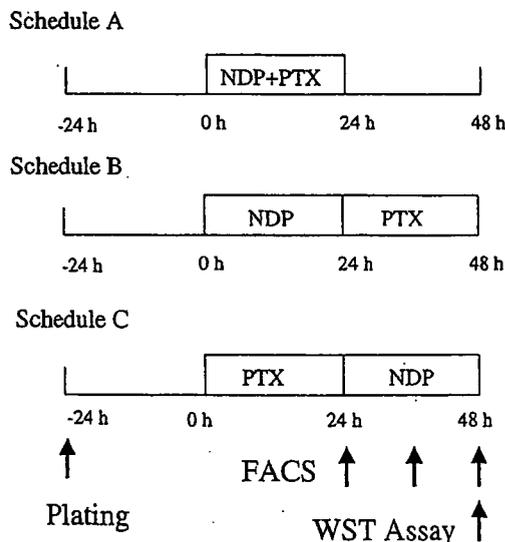
Schedule A

Schedule B

Schedule C

Fig. 1 Description of the three combination schedules. NDP nedaplatin, PTX paclitaxel

the concentrations of nedaplatin and paclitaxel alone, respectively, needed to achieve a given effect ($x\%$) and $C_{nedaplatin}$ and $C_{paclitaxel}$ are the concentrations of nedaplatin and paclitaxel needed for the same effect ($x\%$) when the drugs were combined. These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The combination was considered as positive (synergistic) when the combination index was < 1 and negative (antagonistic) when it was > 1.

Cell-cycle determination

Human gastric cancer cell line, AZ-521 cells were cultured at 1×10^5 cells per 60-mm dish. The same protocols as described in the growth inhibition assay were used. After treatment, the cells were harvested, washed twice in ice-cold PBS (pH 7.4), and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell-cycle analysis. After the removal of ethanol by centrifugation, cells were then washed with PBS and stained with a solution containing propidium iodide and RNase (Sigma-Aldrich, St. Louis, MO, USA) on ice for 30 min. Cell-cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELL-Quest or ModFit 3.0 software packages (Becton Dickinson, San Jose, CA, USA), and the percentages of apoptotic populations were determined by measuring the sub-G1 phase using FACS analysis after collecting floating and trypsinized adherent cells at various times following drug exposure. Each experiment was performed in triplicate.

Results

Single-agent experiments

The cytotoxic activities of nedaplatin and paclitaxel were tested individually on the three tumor cell lines. Each drug was exposed to the cells for 24 h. The IC$_{50}$ values ($\pm$ SD) are summarized in Table 1. The IC$_{50}$ value of nedaplatin for KSE-1 (3.4 μg/ml) was not significantly different from those for AZ-521 (3.8 μg/ml) and NUGC-4 (4.6 μg/ml) gastric cancer cells, indicating that nedaplatin appears to be equally effective against these

Table 1 IC$_{50}$ values of nedaplatin and paclitaxel in a panel of three cell lines. Cells were treated with various concentrations of nedaplatin or paclitaxel for 24 h. Results are expressed as the concentration that inhibits 50% of growth in comparison with controls (IC$_{50}$). The values are mean $\pm$ SD of three independent experiments

	AZ-521	NUGC-4	KSE-1
Nedaplatin (μg/ml)	3.8 ± 1.3	4.6 ± 1.3	3.4 ± 0.3
Paclitaxel (ng/ml)	14.0 ± 1.9	26.0 ± 2.6	18.9 ± 0.9

esophageal and gastric cancer cell lines. By contrast, the IC$_{50}$ of paclitaxel for these cell lines varied, depending on the cell type. AZ-521 gastric cancer cells were most sensitive to paclitaxel (14 ng/ml) among the three tumor cell lines, NUGC-4 gastric cancer cells being least sensitive (26 ng/ml).

Median-effect analysis of paclitaxel and oxaliplatin combination in vitro

Nedaplatin and paclitaxel were tested in different combinations to define the most effective schedule. Three different schedules were tested (simultaneous exposure or sequential drug exposures) as shown in Fig. 1. When cells were treated with nedaplatin and paclitaxel simultaneously (Fig. 2), the CI values were below 1 at all levels of killed cell fraction in NUGC-4 and at higher levels of killed cell fraction in KSE-1 cells (Fig. 2b, c), indicating a marked synergistic effect, while moderately antagonistic effects (CI > 1) were seen in AZ-521 cells at the ranges corresponding to less than 70% killed cell fraction (Fig. 2a). When cells were treated with paclitaxel followed by nedaplatin, greater than additive effects were obtained in all of the cell lines, with synergistic interactions observed in NUGC-4 and KSE-1 cells at all ranges (Fig. 3). By contrast, largely antagonistic effects were seen in all of the cell lines when cells were treated with the reverse sequence (Fig. 4), although this sequence appeared to be synergistic in KSE-1 cells at the higher cytotoxic ranges (Fig. 4c). Therefore, the sequence paclitaxel followed by nedaplatin appears most active at least in these three cell lines.

Cell-cycle perturbation and apoptosis

In an attempt to explain the mechanisms underlying the different types of interaction, the effects of paclitaxel and nedaplatin on cell-cycle distribution and apoptosis were studied in AZ-521 cells (Table 2). The cells were treated with these drugs either alone or in combination, with different schedules, and cell-cycle distribution was analyzed 36 h and 48 h after the beginning of treatment, using flow cytometric analysis. Paclitaxel alone at a dose of 12.5 ng/ml induced the accumulation of cells in the G2/M phase. At a concentration of 5 μg/ml, nedaplatin alone caused an increase in the S population and a decrease in the G0/G1 population without affecting the population of G2/M, indicating that it inhibited both S to G2 and G2/M to G1 progression. The simultaneous exposure led to the accumulation of cells in the S and G2/M phase and a decrease in the population of G1, showing the combined activity of both drugs. The treatment with paclitaxel prior to nedaplatin led to the accumulation of cells more exclusively into the G2/M phase, showing similar distribution patterns to those observed in the cells treated with paclitaxel alone, although a significant increase in G2/M population was
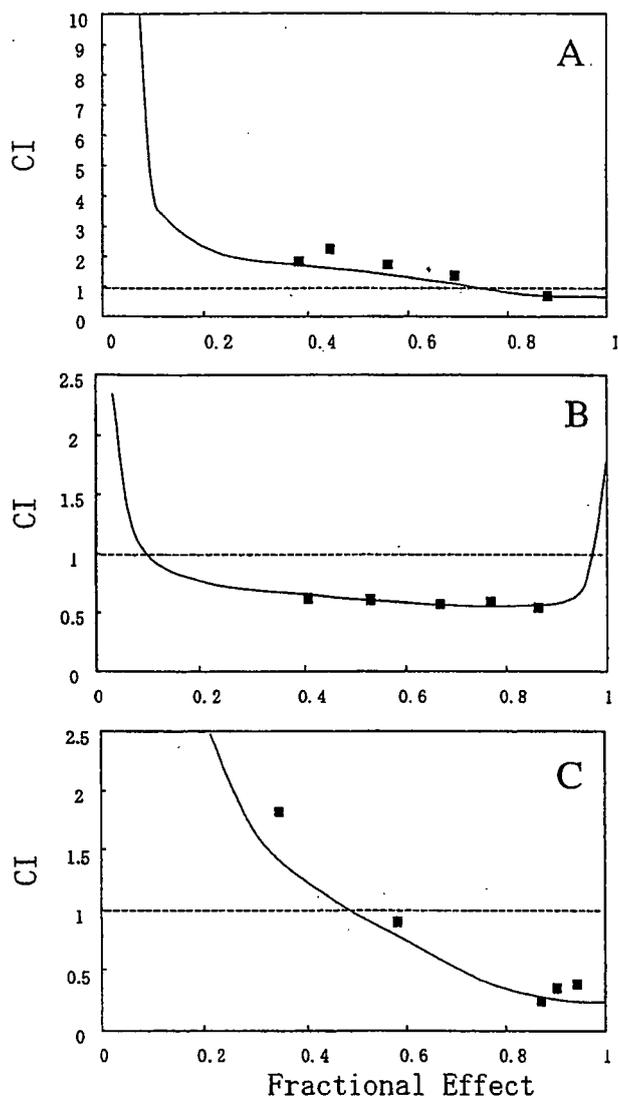
Fig. 2 Combination index (*CI*) plots obtained from three cancer cell lines exposed simultaneously to nedaplatin and paclitaxel for 24 h. a AZ-521; b NUGC-3; c KSE-1
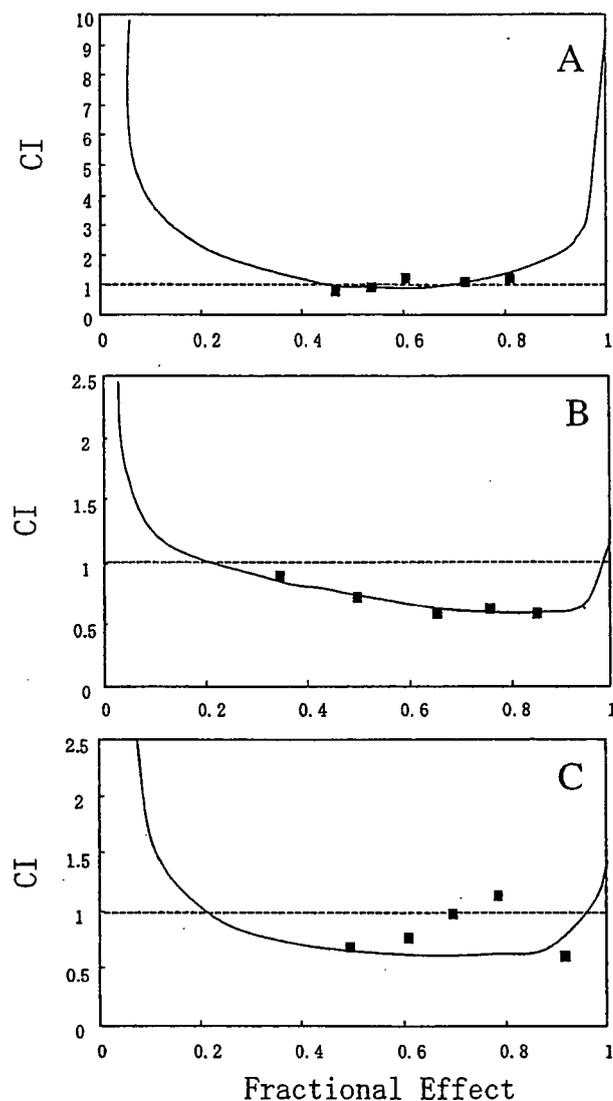

Fig. 3 Combination index (*CI*) plots obtained from three gastric cell lines exposed to paclitaxel for 24 h followed by nedaplatin for 24 h. a AZ-521, b NUGC-3, c KSE-1

observed when compared with treatment with paclitaxel alone. In contrast, nedaplatin prior to paclitaxel caused almost identical distribution patterns to those observed with nedaplatin alone. These data indicate that cell-cycle distribution patterns in sequential combination were mostly influenced by the initial drug administered.

To confirm the activities of sequential combination, the apoptotic activity was investigated after treatment of AZ-521 cells by measuring the population of sub-G1 phase using FACS analyses. The presence of hypodiploid DNA (sub-G1) is associated with cells undergoing apoptosis. As shown in Table 2, paclitaxel followed by nedaplatin induced the G2/M block, with substantial induction of apoptosis in the majority of the treated cells (70%). The induction rate of apoptosis by this sequential administration was greater than those of

paclitaxel alone (56–66%) or nedaplatin alone (14–17%). By contrast, the reverse sequence caused S-phase block, and apoptotic population was 24–28%, being less than those induced by paclitaxel alone (56–66%), and slightly more than those induced by nedaplatin alone (14–17%). These data indicate that sequence nedaplatin followed by paclitaxel is antagonistic in inducing apoptosis.

Discussion

In this study, we examined the schedule-dependent interaction of nedaplatin and paclitaxel in a panel of three human cancer cell lines derived from stomach and esophagus in vitro. First, we compared the sensitivity of
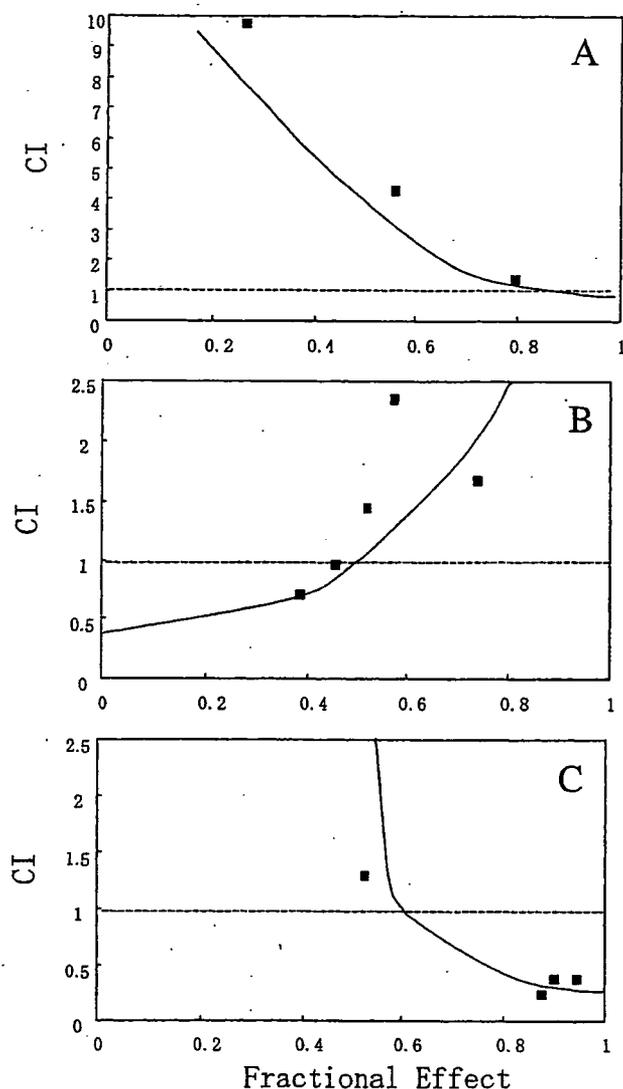
Fig. 4 Combination index (*CI*) plots obtained from three cell lines exposed to nedaplatin for 24 h followed by paclitaxel for 24 h. a AZ-521, b NUGC-3, c KSE-1

nedaplatin between these esophageal and gastric cancer cell lines, because nedaplatin has been clinically shown to be active against esophageal cancer [24], but its clinical efficacy against gastric cancer has not been determined. We have found that the IC_{50} of nedaplatin is not significantly different between these gastric and esophageal cancer cell lines, indicating that nedaplatin may be potentially effective against gastric cancer. Second, we found that the sequence paclitaxel followed by nedaplatin resulted in either synergism or additivity in all three cell lines. By contrast, a clear antagonism was observed in the reverse sequence in two of three cell lines. Simultaneous treatment with these two drugs resulted in either synergistic or antagonistic effects, depending on the cell line. Therefore, the sequence paclitaxel followed by nedaplatin appears to be the most active, at least in these three cell lines. A similar sequence-dependent antitumor activity of this combination was demonstrated in a preclinical in vivo mouse tumor model [26].

To explain the possible mechanism underlying the synergistic interaction of paclitaxel following nedaplatin sequence, we further analyzed the perturbations induced on cell cycle by flow cytometric analyses using the AZ-521 human gastric cancer cell line. We found that a 24-h treatment with paclitaxel markedly affected the cell-cycle distribution, producing a relevant accumulation in the G2/M phase with subsequent induction of apoptosis (56%) in the sub-G1 phase. Nedaplatin alone caused an increase in the S population and a decrease in the G0/G1 population without affecting the population of G2/M, suggesting that it inhibited both S to G2/M and G2/M to G1 progression. The treatment with paclitaxel prior to nedaplatin accumulated cells almost exclusively into the G2/M phase with prominent apoptosis (70%). By contrast, the reverse sequence yielded only 28% induction of apoptotic cells with almost identical cell-cycle distribution patterns to those observed with nedaplatin alone, indicating that the activity of paclitaxel is abolished by pretreatment of nedaplatin, accounting for an antagonistic interaction. The inhibition of paclitaxel-induced cytotoxicity by nedaplatin would probably be explained by the decrease in the number of G2 population targeted by paclitaxel, because pretreatment with

Table 2 Cell-cycle perturbation (%) and apoptosis induced by nedaplatin and paclitaxel in the AZ-521 cell line. Data are presented as mean percentage values from three independent experiments. *NDP* nedaplatin, *PTX* paclitaxel

Treatment	36-h				48-h			
	G0/G1(%)	S(%)	G2/M(%)	Apoptosis[a]	G0/G1(%)	S(%)	G2/M(%)	Apoptosis[a]
Control					58.34	19.91	21.76	3.52
NDP	40.85	31.89	27.26	14.52	37.81	39.91	22.28	17.05
PTX	16.33	24.65	58.42	66.61	19.45	18.02	62.53	56.47
NDP+PTX	12.85	41.48	45.66	49.53	16.98	38.93	44.09	54.76
PTX → NDP	10.91	20.28	68.81	66.10	11.70	13.88	74.42	70.14
NDP → PTX	37.31	44.97	17.72	24.05	36.99	40.40	22.60	28.46

[a] The percentages of apoptotic populations were assessed by measuring sub-G1 phase using FACS analyses after collecting floating and trypsinized adherent cells at various times following drug exposure

nedaplatin accumulates cells mostly in the phases before G2/M, thereby reducing the number of cells entering G2/M phase.

We have found that nedaplatin can arrest the cells mainly at both G1 and S phases, suggesting a distinct action from that of cisplatin or oxaliplatin, because cisplatin and oxaliplatin mainly accumulate cells into G2/M [14, 23] and G1 phases [25], respectively. However, sequence-dependent interactions between paclitaxel and these three platinum compounds did not differ; a synergistic or additive interaction was observed when paclitaxel precedes CDDP [13, 17, 20, 27] or oxaliplatin [25], whereas there were antagonistic interactions in the reverse sequence [13, 25, 27]. Several explanations for increased activity of the sequence paclitaxel followed by cisplatin are shown: cisplatin hastens the exit from mitosis in paclitaxel-treated cells [17]; paclitaxel induces an increase in intracellular uptake of cisplatin [5]; and paclitaxel inhibits repair of cisplatin-induced DNA damage [19]. Therefore, we hypothesize that the similar mechanisms, if not identical, to those as demonstrated in the interaction between CDDP and paclitaxel may also operate in the combination of nedaplatin and paclitaxel.

Clinically, single-agent nedaplatin produced promising response rates in phase-II trials for the treatment of head and neck, lung, esophagus and cervical cancers [7, 9, 18, 24]. In this study, we have shown that nedaplatin is effective against gastric cancer cells and exhibits a significant synergy with paclitaxel. Since these drugs have an overlapping spectrum of clinical efficacies, this combination is a promising chemotherapeutic regimen for the treatment of patients with these cancers. Although the biochemical basis for their interaction remains unknown, a clear sequence-dependent activity of nedaplatin and paclitaxel combination should be thus incorporated into the design of a clinical trial.

References

1. Ajani JA, Ilson DH, Daugherty K, Pazdur R, Lynch PM, Kelsen DP (1994) Activity of taxol in patients with squamous cell carcinoma and adenocarcinoma of the esophagus. J Natl Cancer Inst 86:1086–1091
2. Alberts DS, Fanta PT, Running KL, Adair LP Jr, Garcia DJ, Liu-Stevens R, Salmon SE (1997) In vitro phase II comparison of the cytotoxicity of a novel platinum analog, nedaplatin (254-S), with that of cisplatin and carboplatin against fresh, human ovarian cancers. Cancer Chemother Pharmacol 39:493–497
3. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
4. Chou TC, Motzer RJ, Tong V, Bosl GJ (1994) Computerized quantitation of synergism and antagonism of Taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst (Bethesda) 86:1517–1524
5. Christen RD, Jekunen AP, Jones JA, Thiebaut F, Shalinsky DR, Howell SB (1993) In vitro modulation of cisplatin accumulation in human ovarian carcinoma cells by pharmacologic alteration of microtubules. J Clin Invest 92:431–440
6. Forastiere AA, Shank D, Neuberg D, Taylor SG IV, DeConti RC, Adams G (1998) Final report of a phase II evaluation of paclitaxel in patients with advanced squamous cell carcinoma of the head and neck: an Eastern Cooperative Oncology Group trial (PA390). Cancer 82:2270–2274
7. Fukuda M, Shinkai T, Eguchi K, Sasaki Y, Tamura T, Ohe Y, Kojima A, Oshita F, Hara K, Saijo N (1990) Phase II study of (glycolate-O,O') diammineplatinum(II), a novel platinum complex, in the treatment of non-small-cell lung cancer. Cancer Chemother Pharmacol 26:393–396
8. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He P (1993) A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull 41:1118
9. Inuyama Y, Miyake H, Horiuchi M, Hayasaki K, Komiyama S, Ota K (1992) A late phase II clinical study of cis-diammine glycolato platinum, 254-S, for head and neck cancers. Japanese Gan To Kagaku Ryoho 19:871–877
10. Kameyama Y, Okazaki N, Nakagawa M, Koshida H, Nakamura M, Gemba M. (1990) Nephrotoxicity of a new platinum compound, 254-S, evaluated with rat kidney cortical slices. Toxicol Lett 52:15–24
11. Kano Y, Akutsu M, Tsunoda S, Suzuki K, Yazawa Y (1996) In vitro schedule-dependent interaction between paclitaxel and cisplatin in human carcinoma cell lines. Cancer Chemother Pharmacol 37:525–530
12. Kobayashi H, Takemura Y, Miyachi H, Ogawa T (1991) Antitumor activities of new platinum compounds, DWA2114R, NK121 and 254-S, against human leukemia cells sensitive or resistant to cisplatin. Invest New Drugs 9:313–319
13. Liebmann JE, Fisher J, Teague D, Cook JA (1994) Sequence dependence of paclitaxel (Taxol) combined with cisplatin or alkylators in human cancer cells. Oncol Res 6:25–31
14. Ma J, Maliepaard M, Nooter K, Boersma AW, Verweij J, Stoter G, Schellens JH (1998) Synergistic cytotoxicity of cisplatin and topotecan or SN-38 in a panel of eight solid-tumor cell lines in vitro. Cancer Chemother Pharmacol 41:307–316
15. Matsuoka H, Sugimachi K, Ueo H, Kuwano H, Nakano S, Nakayama M (1987) Sex hormone response of a newly established squamous cell line derived from clinical esophageal carcinoma. Cancer Res 47:4134–4140
16. McGuire WP, Blessing JA, Moore D, Lentz SS, Photopulos G (1996) Paclitaxel has moderate activity in squamous cervix cancer. a gynecologic oncology group study. J Clin Oncol 14:792–795
17. Milross CG, Peters LJ, Hunter NR, Mason KA, Milas L (1995) Sequence-dependent antitumor activity of paclitaxel (taxol) and cisplatin in vivo. Int J Cancer 62:599–604
18. Noda K, Ikeda M, Yakushiji M, Nishimura H, Terashima Y, Sasaki H, Hata T, Kuramoto H, Tanaka K, Takahashi T et al (1992) A phase II clinical study of cis-diammine glycolato platinum, 254-S, for cervical cancer of the uterus. Japanese Gan To Kagaku Ryoho 19:885–892
19. Parker RJ, Lee KB, Dabholkar M, Bostick-Bruton F, Simmis M, Reed E (1993) Influence of taxol: cisplatin sequencing on cisplatin-DNA adduct repair in human ovarian cancer cells. Proc Am Assoc Cancer Res 34:356
20. Rowinsky EK, Citardi MJ, Noe DA, Donehower RC (1993) Sequence-dependent cytotoxic effects due to combinations of cisplatin and the antimicrotubule agents taxol and vincristine. J Cancer Res Clin Oncol 119:727–733
21. Sakamoto J, Morita S, Yumiba T, Narahara H, Kinoshita K, Nakane Y, Imamoto H, Shiozaki H (2003) Ascitic Gastric Cancer Study Group of the Japan South West Oncology Group. A phase II clinical trial to evaluate the effect of paclitaxel in patients with ascites caused by advanced or recurrent gastric carcinoma: a new concept of clinical benefit response for non-measurable type of gastric cancer. Jpn J Clin Oncol 33:238–240
22. Sekine I, Nishiwaki Y, Watanabe K, Yoneda S, Saijo N (1996) Phase II study of 3-hour infusion of paclitaxel in previously untreated non-small cell lung cancer. Clin Cancer Res 2:941–945

23. Sorenson CM, Eastman A (1988) Mechanism of cis-diammin-edichloroplatinum (II)-induced cytotoxicity: role of G2 arrest and DNA double-strand breaks. Cancer Res 48:4484–4488

24. Taguchi T, Wakui A, Nabeya K, Kurihara M, Isono K, Kakegawa T, Ota K (1992) A phase II clinical study of cis-diammine glycolato platinum, 254-S, for gastrointestinal cancers. 254-S Gastrointestinal Cancer Study Group. Japanese Gan To Kagaku Ryoho 19:483–488

25. Tanaka R, Ariyama H, Qin B, Takii Y, Baba E, Mitsugi K, Harada M, Nakano S (2005) In vitro schedule-dependent interaction between Paclitaxel and Oxaliplatin in human cancer cell lines. Cancer Chemother Pharmacol (in press)

26. Yamada H, Uchida N, Maekawa R, Yoshioka T (2001) Sequence-dependent antitumor efficacy of combination chemotherapy with nedaplatin, a newly developed platinum, and paclitaxel. Cancer Lett 172:17–25

27. Vanhoefer U, Harstrick A, Wilke H, Schleucher N, Walles H, Schroder J, Seeber S (1995) Schedule-dependent antagonism of paclitaxel and cisplatin in human gastric and ovarian carcinoma cell lines in vitro. Eur J Cancer 31A:92–97

Comparison of *HER2* gene amplification assessed by fluorescence *in situ* hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer

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Abstract. A monoclonal antibody to HER2 protein is widely used in the treatment of patients with HER2-overexpressing breast cancer and has also been found to exhibit antitumor activity in human gastric cancer cells that overexpress HER2. The purpose of this study was to evaluate the frequency of HER2 overexpression and concordance between the results for protein expression and gene amplification in both surgical and biopsy specimens of gastric cancer as assessed with two commercial kits, one for immunohistochemistry (IHC) and the other for fluorescence *in situ* hybridization (FISH). The specimens consisted of formalin-fixed, paraffin-embedded sections of biopsy specimens and surgically resected tumors from 200 cases of invasive gastric cancer that had been treated surgically at the National Cancer Center Hospital East. The lesions were analyzed with the IHC kit, and expression was graded by the United States Food and Drug Administration (FDA)-approved grading system. Gene amplification was evaluated by FISH. IHC revealed HER2 overexpression in 46 of the 200 (23%) cases. The FISH assay was technically successful in 199 cases (99.5%), and gene amplification was observed in 54 cases (27.1%). The concordance rate between the results obtained by IHC and FISH was 86.9%. The concordance rate between the findings in the surgically resected tumors and the 200 pre-treatment biopsy specimens was 88.7%. HER2 expression can be assessed in gastric cancer with a commercial kit as previously reported in breast cancer. Even small biopsy specimens were found to be suitable for evaluating gastric cancer for HER2 overexpression.

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Introduction

The *HER2* (also called c-erbB2) is a proto-oncogene and is located on chromosome 17q21 (1). *HER2* encodes a M_r 185,000 transmembrane glycoprotein, which is a member of the HER receptor family and possesses tyrosine kinase activity. Overexpression of HER2 protein has been described in approximately 25-30% of invasive breast cancers, and it has been used as a marker of resistance to various therapeutic modalities, and short disease-free survival (2,3). Trastuzumab (Herceptin, Genentech, Inc., South San Francisco, CA), a monoclonal antibody to the HER2 protein, is a promising agent for the treatment of breast cancer patients with a poor prognosis, and Slamon *et al* have reported that addition of Trastuzumab to the chemotherapy regimen yields a significantly higher response and prolongs time to progression and overall survival of a breast cancer patients with HER2 overexpression (4).

Various methods are available to determine the HER2 status of breast cancer, however, many of them require fresh tissue, involve a complicated procedure, and are costly. Immunohistochemistry (IHC) is widely used to evaluate protein expression in formalin-fixed, paraffin-embedded specimens, and, Southern blot hybridization is recognized as the standard method for analysis of *HER2* gene amplification, but the procedure requires a large, fresh specimen (5). Fluorescence *in situ* hybridization (FISH) can be used to analyze small formalin-fixed, paraffin-embedded specimens for gene amplification, and Press *et al* have evaluated FISH as a mean of assessing *HER2* amplification in breast cancer (3). In their study, FISH was used to test for *HER2* amplification in 140 breast cancers in which gene amplification had already been demonstrated by Southern hybridization and it was found to have a sensitivity of 98% and a specificity of 100%. IHC and FISH are widely used methods for evaluating HER2 status for breast cancer, furthermore, Food and Drug Administration (FDA) in the United States approved IHC and FISH tests to determine HER2 status for breast cancer patients: Hercep test kit (DakoCytomation Denmark A/S, Glostrup, Denmark), and PathVysion HER2 DNA probe kit (Vysis Inc., Downers Grove, IL). Many investigators have

compared IHC and FISH as means of evaluating HER2 in breast cancer (5-7). In general, correlation level between IHC and FISH results approximately 90% in IHC strong positive cases (3+), however weakly positive cases (2+) obtain only a minor association. Therefore, National Compresive Cancer Network (NCCN) guidelines for treatment of breast cancer recommend that HER2 testing should be done using IHC and/ or FISH, an IHC results of 2+ should be confirmed with FISH (8).

Many investigators subsequently evaluated HER2 status in gastric cancer by IHC, and the frequency of HER2 overexpression varied widely, from 8 to 31% (9-14). The consensus of almost all reports is that the majority of positive cases are the intestinal type histologically. Methods of IHC which evaluate for HER2 status in gastric cancer have not been standardized and there is a wide range in the frequency of overexpression, furthermore, there have been few reports claiming to have demonstrated *HER2* gene amplification in gastric cancer (15-17).

In xenograft experiments, Trastuzumab has also shown antitumor activity in gastric cancer cell lines with HER2 overexpression, and synergy has been demonstrated with some cytotoxic agents (18). Results of that study encourage the clinical investigation of Trastuzumab for patients with HER2-overexpressing gastric cancer.

We usually collect small biopsy specimens endoscopically from advanced gastric cancers before initiating chemotherapy. In unresectable cases, tumor behavior before treatment has to be evaluated on the basis of these small specimens alone. However, since gastric cancer is considered to be a heterogeneous tumor, small biopsy specimens may not reflect its behavior.

Before using Trastuzumab in the treatment of gastric cancer, concordance between protein expression and gene amplification of *HER2* has to be confirmed with commercial kits as examined for breast cancer, and the feasibility of using biopsy specimens to evaluate HER2 status must also be confirmed.

The purpose of this study was to evaluate the frequency of HER2 overexpression in gastric cancer and the concordance between protein expression and gene amplification in both surgical and endoscopic biopsy specimens with two commercial kits, an IHC and a FISH.

Materials and methods

A total of 1,254 patients with primary gastric cancer underwent surgery at the National Cancer Center Hospital East (Kashiwa, Japan) between July 1992 and March 2000. Of the 1,254 patients, 261 cases were invasive intestinal-type gastric cancer. We selected 200 of 261 cases in which preoperative endoscopic biopsy samples were completely preserved in our hospital. The specimens consisted of formalin-fixed, paraffin-embedded sections of preoperative endoscopic biopsy specimens and surgically resected tumors.

All tissues were fixed with 10% buffered formalin, generally for 24 and 48 h, and paraffin-embedded. Sections 4-μm thick were cut from a paraffin block of each specimen and applied to slides for IHC, and 5-μm thick sections were cut and applied to slides for FISH.

The IHC analysis was performed with the Hercep test kit (DakoCytomation Denmark A/S) at Dako Cytomation Co. Ltd, Kyoto, Japan. FISH for *HER2* gene amplification was performed with the PathVysion HER2 DNA probe kit (Vysis Inc.) at FALCO Biosystems, Kyoto, Japan.

IHC for HER2 protein expression. The immunohistochemical analysis with the Hercep test was performed according to the manufacturer's guidelines. All reagents were included in the kit. Briefly, heat-induced epitope retrieval was performed on the deparaffinized sections in advance by immersing the slides in Epitope Retrieval Solution (10 mM citrate buffer; pH 6.0), which had been preheated to 95˚C. They were then placed in a 95˚C water bath for 40 min, followed by 20-min at room temperature, then endogenous peroxidase was quenched with Peroxidase Blocking Reagent. Next, the slides were incubated at room temperature for 30 min with ready-to-use rabbit polyclonal antibody to HER2 oncoprotein, and the primary antibody was detected by incubation at room temperature for 30 min with Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulins). After washing, slides were developed with Substrate Chromogen Solution at room temperature for 10 min. The expression grading and evaluation were performed in accordance with the FDA-approved system for breast cancer (19). Only membrane staining intensity and pattern were evaluated using the 0 to 3+ scale. Scores of 0 or 1+ were considered negative, a score of 2+ was weakly positive when >10% of the tumor cells showed weak to moderate complete membrane staining, and a score of 3+ was strongly positive when a strong complete membrane staining was observed in >10% of the tumor cells (Fig. 1). In the present study, we defined HER2 expression positive when tumor cell staining was evaluated as 2+ or greater with Hercep test.

FISH for HER2 gene amplification. The results of FISH for *HER2* were evaluated using a PathVysion HER2 DNA probe kit which uses a dual-color probe to determine the number of copies of both *HER2/neu* (SpectrumOrange) and CEP17 (chromosome enumeration probe 17) (SpectrumGreen). The kit was used according to the manufacturer's protocol. Briefly, an appropriate formalin-fixed paraffin-embedded tissue block from each case was selected by the pathologists, cut into 5-μm thick sections, and mounted on silane-coated slides (Dako A/S). One of the sections was stained with H&E and used for the microscopic confirmation of the invasive part of the carcinoma tissue, and other sections were used for the FISH assay. The slide for FISH was deparaffinized in Hemo-De for 10 min three times, and then dehydrated in 100% ethanol for 5 min twice. Air-dried tissue sections were treated in 0.2 N hydrochloric acid for 20 min, then washed in distilled water for 3 min. After immersion in Vysis wash buffer for 1 min, they were immersed in pre-treatment solution for 30 min at 80˚C. After washes in distilled water for 3 min and immersion in Vysis wash buffer for 5 min twice, the slides were exposed to protease solution at 37˚C for 20-30 min, then immersed in Vysis wash buffer for 5 min twice, and dried in air for 20 min at room temperature. The slides were subsequently immersed in 10% buffered formalin for 10 min, and then immersed in Vysis wash buffer for 5 min, twice, and dried in air for 15 min
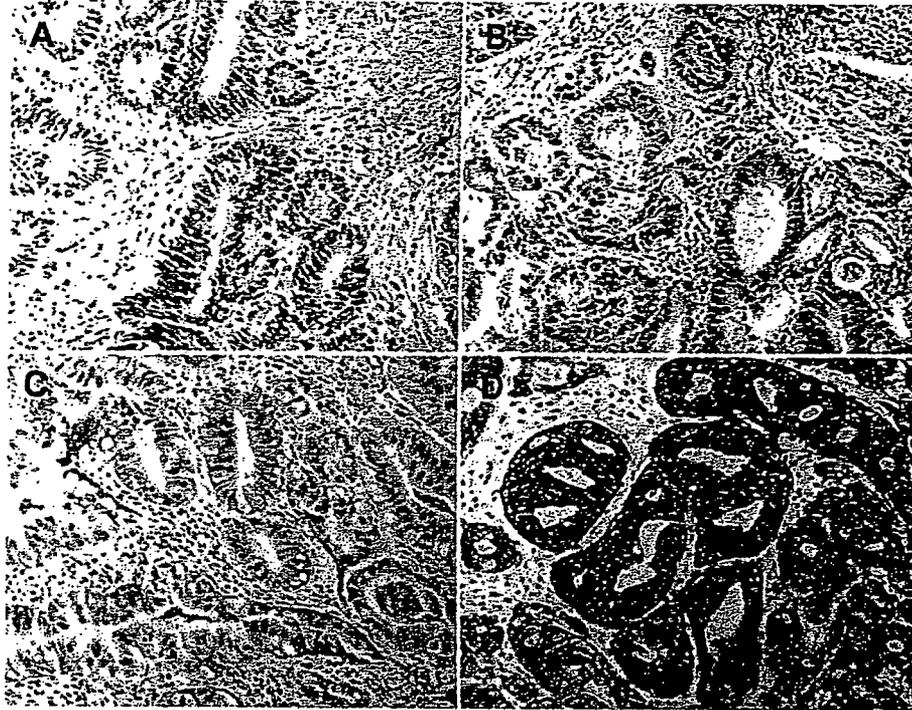
Figure 1. The expression grading and evaluation were estimated in accordance with the FDA-approved system, using the 0 to 3+ scale, score of 0 (A) or 1+ (B) were considered negative, 2+ (C) was weak positive and 3+ (D) was strong positive for HER2 overexpression.
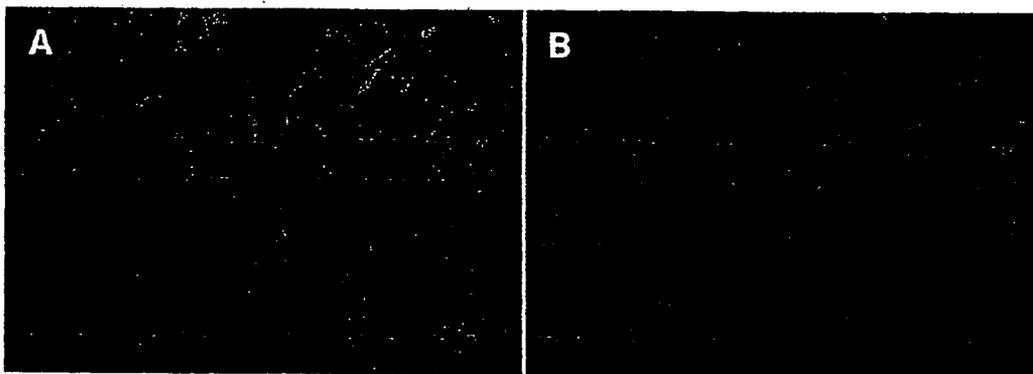

Figure 2. The ratio of HER2 signals to CEP17 signals was determined, with ratios of <2.0 considered non-amplified (A, negative) and those ≥2.0 amplified (B, positive).

at room temperature. Hybridization was performed at 37°C for 14-18 h with a denatured DNA probe followed by immersion in pre-warmed post-wash solution at 72±1°C for 2 min. Finally, the slides were air dried in the dark and counterstained with 4,6-diamidino-2-phenylindole (DAPI).

The total numbers of the HER2 and CEP17 signals were counted in 60 interphase tumor cell nuclei examined with a fluorescent microscopes and appropriate filters. The ratios of HER2 signals to CEP17 signals were calculated as follows: when the ratio was <2.0, the gene was considered non-amplified and when it was ≥2.0, the gene was considered to be amplified (Fig. 2).

Statistical analysis. To assess the concordance rate between the results for protein overexpression and gene amplification and between the results for surgically resected tumor and biopsy specimens, we compared the positive and negative rates

calculated for each examination. Confidence intervals were computed using the normal approximation to the binomial distribution. Positive predictive value (PPV) was calculated as the number of positive in biopsy specimens divided by the number of positive surgically resected tumors. Negative predictive value (NPV) was calculated as the number of negative biopsy specimens divided by the number of negative surgically resected tumors.

Results

Patient and lesion characteristics are shown in Table I. Median age was 66 years (range 39-88 years), and the male/female ratio was 155/45. There were 194 tubular adenocarcinomas and 6 papillary adenocarcinomas. The pathological stage was: T2/3 in 99/101, N0/1/2/3 in 54/84/47/15, and stage I/II/III/IV in 42/55/67/36 patients.

Table I. Patient and lesion characteristics.

Gender	
Male	155
Female	45
Age	
Median	66
Range	33-88
Histology	
Tubular (tub)	194
Papillary (pap)	6
Pathological TNM classification	
T-stage	
T2	99
T3	101
N-stage	
N0	54
N1	84
N2	47
N3	15
M-stage	
M0	176
M1	24
I	42
II	55
III	67
IV	36

Table II. Her2 overexpression in surgically resected tumors.

IHC score	No.			%
	Tub	Pap	Total	
0	126	0	126	63
1+	28	0	28	14
2+	12	0	12	6
3+	28	6	34	17
Total	193	6	200	
Positive rate (%)	20.6	100		23

Table III. *HER2* gene amplification in surgically resected tumors.

HER2/CEP17[a] ratio	Tub	Pap	Total
Median	1.4	6.8	1.4
Range	1.0-12.3	1.5-9.7	1.0-12.3
<2	144	1	155
≥2	49	5	54
Positive rate (%)	25.3	83.3	27.1

FISH assay was technically successful in 199 (99.7%) of the 200 cases. [a]CEP17, chromosome enumeration probe 17.

HER2 overexpression in surgically resected tumors. The results for HER2 overexpression in the surgically resected tumors are shown in Table II. All 200 cases could be evaluated by IHC. Hercep test score was 0 in 126 cases (63%), 1+ in 28 (14%), 2+ in 12 (6%), and 3+ in 34 cases (17%), respectively. All 6 of papillary adenocarcinomas were 3+ (100%). Of the 200 surgically resected tumor specimens, 46 (23%) of the tumors were found to exhibit HER2 protein overexpression [95% confidence interval (CI): 17-28%].

HER2 gene amplification in surgically resected tumors. The results for *HER2* gene amplification in the surgically resected tumors are shown in Table III. FISH assay was technically successful in 199 (99.7%) of the 200 cases and the *HER2* gene was judged to have been amplified in 54 of 199 cases (27.1%, 95% CI 21-33.2%). The median number of signals per nucleus was 1.4 (range 1.0-12.3) with the median number of signals per nucleus in 54 amplified cases of 5.55. In 194 cases with tubular adenocarcinoma, FISH assay was unsuccessful in 1 case and 49 of the 193 cases (25.3%) were determined to be amplified. Five of the 6 cases (83.3%) of papillary adenocarcinoma were evaluated as amplified with the median number of signals per nucleus of 6.8 (range 1.5-9.7).

Concordance between the results of IHC and FISH in surgically resected tumors. The concordance rate between the

Table IV. Concordance between the results of IHC and FISH in surgically resected tumors.

IHC score	FISH		Concordance rate (%)
	Positive	Negative	
0	12	113	90.4
1+	5	23	82.1
2+	7	5	58.3
3+	30	4	88.2
			86.9

results of IHC and FISH in the HER2-protein overexpression cases was 86.7% (58.3% for 2+, 88.2% for 3+) (Table IV). Of the 153 cases that were HER2 protein-negative by IHC, 136 cases were not showed amplification with FISH and its concordance rate was 88.8% (90.4% for 0, 82.1% for 1+). The results of the two assays were concordant in 173 of the 199 cases (86.9%, 95% CI 82.2-91.6%).

Concordance between the results for HER2 overexpression determined by IHC in surgically resected tumors and biopsy specimens. The IHC method was technically successful in all