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Effects of Experimental Chemoendocrine Therapy With a Combination of a Pure Antiestrogen and 5-Fluorouracil on Human Breast Cancer Cells Implanted in Nude Mice

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Abstract: The antitumor effects of an experimental chemoendocrine therapy combining a new pure antiestrogen ICI 182780 and 5-fluorouracil (5-FU) were studied on MCF-7 human breast cancer cells implanted in nude mice. ICI 182780 had a dose-dependent antitumor activity, which was potentiated by the concomitant use of 5-FU. When compared with the control group, the estrogen receptor (ER) level in the ICI 182780 group was lower and that in the combination group was markedly lower. Cell cycle analysis by flow cytometry (FCM) resulted in a lower percentage of S-phase cells (%S) in the treated mice. No significant difference was observed in the 5-FU concentrations in tumor cells, while the 5-FU content in RNA was significantly higher in the combination group. The changes in free thymidylate synthetase (TS) concentration indicated TS synthesis after the administration of 5-FU to be more greatly suppressed in the combination group than in the 5-FU group. These results suggest that ICI 182780 and 5-FU exert their combination effect mainly on ER-positive cells, and that the suppression of TS synthesis in tumor cells and the potentiation of the 5-FU-induced metabolic dysfunction of RNA are thus involved in the mode of action of this combination therapy.

Key Words: chemoendocrine therapy, pure antiestrogen, 5-fluorouracil, nude mouse, breast cancer

Introduction

Breast cancer is a hormone-dependent cancer which is generally known to respond to chemoendocrine therapy. Evidence has also been obtained that estrogen receptor (ER)-positive breast cancer is sensitive to endocrine therapy while ER-negative cancer is not very sensitive.[1] Meanwhile, even apparently ER-positive breast cancer is intermingled with ER-negative cells, so that a combination of endocrine therapy with chemotherapy which has a different mode of action is required to improve the response rate. In addition, a possible reduction of adverse reactions of chemotherapy by concomitant endocrine therapy is of clinical significance.[2]

Among the various forms of endocrine therapy for breast cancer, antiestrogens, especially tamoxifen (TAM), have been the most widely used. TAM, although an antiestrogen, also has estrogenic activity[3–5] and has recently been reported to be associated with the occurrence of endometrial cancer.[6,7] Among the newly developed antiestrogens, ICI 182780, a pure antiestrogen free from estrogenic activity, is expected to be particularly useful in the treatment of breast cancer.[8,9]

In this experiment, the antitumor effects of a combination of ICI 182780 and a widely used fluorinated pyrimidine, 5-fluorouracil (5-FU) were studied, and the mode of action of this chemoendocrine therapy on human breast cancer implanted in nude mice was evaluated.

Materials and Methods

The animals used were BABL/c nu/nu female nude mice aged 7–8 weeks and weighing 19–22 g, obtained from Nippon Crea, Tokyo, Japan. The cell line used was MCF-7, a nude mouse transplantable human breast cancer. It was a hormone-dependent, human breast cancer cell line originally established by Soule et al.[10] in 1970 from cancerous pleural effusion of a postmenopausal breast cancer patient who was 69 years old, donated 3 years ago by Dr T. Kubota, Department of Surgery, Keio University Medical School, and since then has been serially cultivated by the anthors.

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InnoPharma Exhibit 1103.0001

The tumor was cut into fragments measuring about 8 mm^3 which were implanted into the dorsal subcutaneous tissue of the mice by a trocar. For the tumor to grow in the exponential growth phase, immediately after the implantation 5 mg/kg estradiol dipropionate and 250 mg/kg progesterone caproate were intramuscularly injected into the mice.[11] The nude mice bearing tumors weighing 40–90 mg were then assigned, 14–16 days after implantation, to receive either one of the trial medications. The tumor weight was estimated by the formula [long axis $\times$ (short axis)^2 $\times$ 1/2], which has been reported to correlate with the actual tumor weight.[12]

The drugs used were pure antiestrogen ICI 182780 and 5-FU. ICI 182780 was formulated as a 50 mg/ml solution in castor oil and 5-FU was dissolved in distilled water by an ultrasonic cleaner to make a 12 mg/ml concentration. The nude mice in the ICI 182780 group were administered a single dose of 0.5, 1, 3, or 5 mg subcutaneously on the first day of treatment. Those in the 5-FU group were administered intraperitoneally a 60 mg/kg dose of 5-FU three times at 4-day intervals on and after the first day of treatment. The combination group received 1 mg of ICI 182780 and 60 mg/kg of 5-FU in the same manner as that for the individual monotherapy groups.

Assessment of the Antitumor Effects

The long and short axes of each tumor were measured by a sliding caliper twice a week over the period from day 1 to day 21 to estimate the tumor weight and the mean tumor weight for each group. The mean tumor weight on Day X (W) was divided by the initial mean weight (W_0) and the obtained values (the relative mean tumor weight ratio: W/W_0) were plotted against the time (days) after the start of the treatment so as to produce a tumor growth curve for each group. The ratio of W/W_0 of each treated group to W/W_0 of the control group was also calculated (T/C). For the ER measurements and cell cycle analysis by flow cytometry (FCM), the tumors were resected on day 21, cut in half to remove any necrotized tissue, and then frozen until use.

ER Measurements

ERs in the frozen tissues were determined by the dextran-coated charcoal (DCC) method and the results were analyzed by Scatchard plots to determine the number of binding sites (B_{max}).

Cell Cycle Analysis by FCM

The frozen tissues were minced, mixed with 0.1% ribonuclease and 0.1% polyoxyethylene(10)octylphenyl ether, and filtered with a 40-μm nylon mesh. The filtrate was mixed with the same volume of propidium iodine solution (to make the final concentration of 50 μg/ml). A DNA histogram was prepared using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA) and the percentage of cells in S phase (%S) and G phases ($\%G_0G_1$) were determined by the software Sum of Broaded Rectangles (SOBR). The tumor 5-FU and thymidylate synthetase (TS) concentrations, and 5-FU contents in tumor cell RNA were determined in the 5-FU monotherapy and the combination groups. Nude mice bearing MCF-7 intramuscularly received 5 mg/kg estradiol dipropionate and 250 mg progesterone caproate immediately and 14 days after implantation. At 28 days after implantation, treatment with 5-FU alone or in combination with ICI 182780 was started in the same manner as mentioned before. The tumors were resected at either 1 to 12 h after the last administration of 5-FU, cut in half and frozen within 5 min after the necrotized tissue was removed. The tumors resected at 1 h after the last administration of 5-FU were used for the tissue 5-FU assay, those taken at 1 and 12 h after the last administration of 5-FU for the TS assay, and those taken at 12 h after the last administration of 5-FU for 5-FU contents in RNA.

Tumor 5-FU Assay

The tumor tissue specimens were mixed with silica gel and cold acetonitorile, homogenized, and centrifuged. The obtained supernatant was concentrated to dryness, reconstituted with ethanol, passed through a silica gel column to be adsorbed by silica gel, eluted by acetone, then concentrated to dryness. The resulting solid was reconstituted with a solvent and subjected to high-performance liquid chromatography (HPLC).[13]

Tumor TS Assay

In accordance with the method reported by Spears et al.[14] the tumor tissues were homogenized and centrifuged. Sufficient amounts of ^{3}H-labeled fluorodeoxyuridine monophosphate ([^{3}H]FdUMP) and methylenetetrahydrofolate (CH_2FH_4) were added to the resulting cytosol fraction to form a ternary complex. Free [^{3}H]FdUMP was removed by DDC for TS and radioactivity was determined by a liquid scintillation counter. Buffer, pH 8.0, was added to the cytosol fraction to cut the TS-FdUMP bond and, as in the free TS measurement, sufficient amounts of [^{3}H]FdUMP and CH_2FH_4 were added, and radioactivity was counted by scintillation counter to determine the total TS. The TS inhibition rate (TSIR) was calculated by the equation [(total TS — free TS)/(total TS) $\times$ 100%].

5-FU Assay in Tumor Cell RNA

Gas chromatography–mass spectrometry (GC-MS) was used.[15] The tumor tissue specimens were homogenized in distilled water. Ice-cold trichloroacetic acid was added, mixed, and centrifuged to remove the supernatant. The precipitate was rinsed, hydrolyzed by potas-

InnoPharma Exhibit 1103.0002

sium hydroxide, and then perchloric acid was added. After centrifugation, the supernatant was neutralized by potassium hydroxide, and newly appearing precipitate was removed to obtain a mononucleotide solution. This solution was used to determine the concentrations of RNA and 5-FU by GC-MS, and the 5-FU content in RNA was also calculated.

Criteria for Antitumor Effects

The treatment was considered to be effective when the minimum T/C during the treatment was not higher than 0.42.[12] The significance of the intergroup difference in the mean tumor weight was tested by two-way repeated measures ANOVA (analysis of variance). The combination effect was considered to be synergetic when T/C in the combination group was smaller than the product of T/Cs in the two forms of monotherapy. The two-way repeated measures ANOVA was used to test the significance of intergroup differences in the nude mouse body weight change, and Student's *t*-test and the Mann-Whitney *U*-test were used for ER, %S, $\%G_0G_1$, tumor 5-FU concentrations, TS concentrations, and 5-FU content in RNA. A difference with *P*-value less than 0.05 was considered to be significant.

Results

Antitumor Effects on MCF-7

ICI 182780 exhibited a dose-dependent antitumor activity which was statistically greater at 3 and 5 mg as compared with the control (Fig. 1). As the minimum T/C was as low as 0.35 (≦0.42), 5 mg was assessed to be effective (Table 1).

Although neither 1 mg of ICI 182780 alone nor 5-FU alone was significantly different from the control with regard to the antitumor activity, the difference between the combination and the control was significant (Fig. 2). The combination, however, failed to show a synergetic effect with a minimum T/C of 0.42 marginally exceeding the product of T/C of the two forms of monotherapy ($0.61 \times 0.68 = 0.41$) (Table 1).

Changes in the Body Weights of Nude Mice

The body weights of the nude mice decreased slightly in those treated with both 5 mg of ICI 182780 alone and with the combination. There was a significant difference between 1 mg and 5 mg of ICI 182780, but not between the control and any of the treatment groups (Table 2).

ER Values at the Time of Tumor Resection

The mean ER value at the time of tumor resection (B_{max}) was 605.5 fmol/mg protein in the control group,

Table 1. Tumor response to trial medication

Treatment		Minimum T/C
Control	(n = 14)	1.00 (on day 21)
ICI 182780 0.5 mg	(n = 7)	0.75 (on day 21)
ICI 182780 1 mg	(n = 11)	0.61 (on day 21)
ICI 182780 3 mg	(n = 10)	0.51 (on day 21)
ICI 182780 5 mg	(n = 14)	0.35 (on day 21)
5-FU	(n = 10)	0.68 (on day 21)
Combination	(n = 10)	0.42 (on day 21)

T/C, mean ratio of the estimated tumor weight to the baseline in the treated group/that in the control group
5-FU, 5-fluorouracil

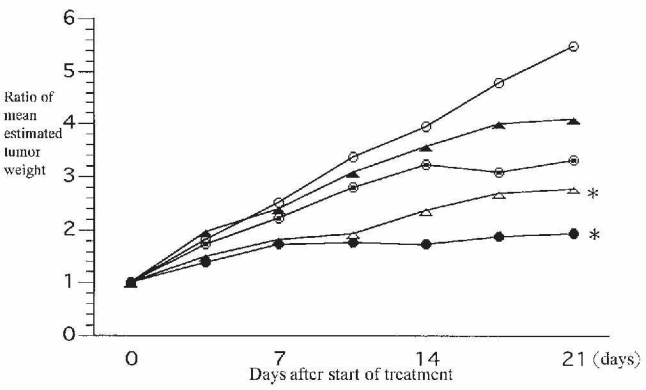

Fig. 1. Tumor response of MCF-7 to ICI 182780. *Vertical axis*, mean estimated tumor weight on a given day/that on day 0. *Open circles*, control (n = 14); *closed triangles*, 0.5 mg (n = 7); *dotted circles*, 1 mg (n = 11); *open triangles*, 3 mg (n = 10); *closed circles*, 5 mg (n = 14). *$P < 0.05$ compared with control

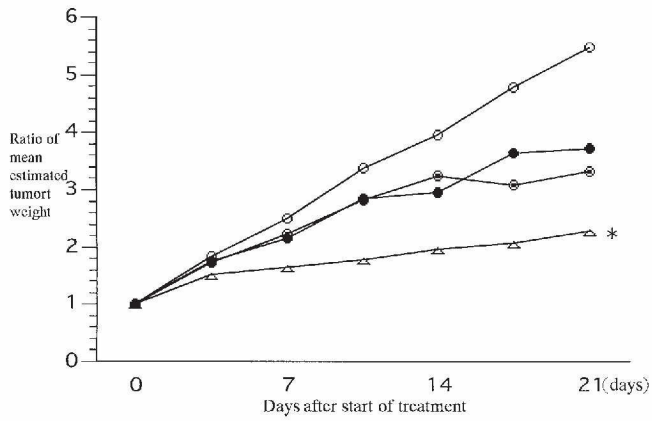

Fig. 2. Tumor response of MCF-7 to the combination of ICI 182780 and 5-fluorouracil. *Vertical axis*, mean estimated tumor weight on a given day/that on day 0. *Open circles*, control (n = 14); *dotted circles*, 1 mg ICI 182780 (n = 11); *closed circles*, 5-fluorouracil (n = 10); *triangles*, combination (n = 10). *$P < 0.05$ compared with control

InnoPharma Exhibit 1103.0003

Table 2. Changes in body weights (BW) of the nude mice

Treatment		BW at baseline (g)	BW on day 21 of treatment (g)
Control	($n = 7$)	20.51 ± 1.43	21.40 ± 2.17
ICI 182780 1 mg	($n = 4$)	22.00 ± 1.20	23.70 ± 1.42 *
ICI 182780 5 mg	($n = 6$)	20.85 ± 1.13	20.78 ± 1.24 *
5-FU	($n = 5$)	20.38 ± 1.88	21.02 ± 3.16
Combination	($n = 6$)	23.03 ± 1.28	21.37 ± 2.27

* $P < 0.05$

Table 3. Estrogen receptor (ER) count on day 21 of treatment (by dextran-coated charcoal method)

Treatment		ER content (fmol/mg protein)
Control	($n = 4$)	605.5 ± 48.3 *
ICI 182780 1 mg	($n = 5$)	198.5 ± 83.2 * *
5-FU	($n = 4$)	418.1 ± 164.7 *
Combination	($n = 4$)	52.3 ± 76.8 *

* $P < 0.05$

while the mean ER decreased to 198.5, 418.1, and 52.3 fmol/mg protein after the administration of 1 mg of ICI 182780, 5-FU, and the combination, respectively. The fall of ER was significantly greater with 1 mg of ICI 182780 than with 5-FU, and with the combination than with either monotherapy (Table 3).

Cell Cycle Analysis by Flow Cytometry

The mean %S in the control group was 35.6%, while it was 14.2% for 1 mg of ICI 182780, 19.2% for 5-FU, and 19.8% for the combination therapy. The mean $\%G_0G_1$ was 55.4% for the control, while the values in the treated groups were 82.8%, 76.4%, and 76.8%, respectively (Table 4). The treated groups showed a significant reduction in the %S and a significant increase in the $\%G_0G_1$ as compared with the control group. ICI 182780 at 1 mg resulted in a greatest reduction of the %S and the greatest elevation of the $\%G_0G_1$, with a significant difference in the %S from that of the combination therapy, and a significant difference in the $\%G_0G_1$ as compared with 5-FU alone and the combination therapy.

5-FU and TS Concentrations in Tumors and 5-FU Contents in Tumor Cell RNA

The mean 5-FU concentration in the tumors was 302.0 ng/g in the 5-FU group, which was not significantly different from that of the combination group, 201.9 ng/g (Table 5). On the other hand, the mean 5-FU content in RNA was significantly higher in the combination group (102.6 ng/mg RNA) than in the 5-FU group (65.2 ng/mg RNA) (Table 6).

The total TS at 1 h after the last administration of 5-FU was 30.8 pmol/g tissue in the 5-FU group and 30.5 pmol/g tissue in the combination group, with no significant difference in TSIR (94.1% and 97.2%, respectively). At 12 h after the last administration of 5-FU, however, both the total and free TS (61.9 and 24.1 pmol/g tissue) in the 5-FU group were significantly higher than those in the combination group (51.3 and 17.5 pmol/g tissue, respectively) (Table 7).

Discussion

Breast cancer is a hormone-dependent tumor for which endocrine therapy is widely accepted as being effective. TAM is one of the most commonly used antiestrogens. However, TAM is reported to have partial estrogenic activity, and it has also been reported to produce estrogenic effects on some targets and antiestrogenic effects on others.[3–5]

ICI 182780, a new pure antiestrogen free from estrogenic activity, is a promising new therapy modality. One of the features of the drug is the lack of effect on gonadotropins.[8] This means that the drug does not block cerebral ERs and therefore does not interfere with the hypothalamus-pituitary-ovary axis in breast cancer patients. In this sense, the drug is expected to be especially beneficial to premenopausal patients.[8] Unlike TAM, in which the uterotropic activity has been recently reported to be associated with a risk of endometrial cancer,[6,7] ICI 182780 is devoid of uterotropic activity and therefore free from this concern.

Meanwhile, even apparently ER-positive breast cancer heterogeneously consists of both ER-positive and ER-negative tumor cells. Antiestrogens are not so effective for ER-negative tumor cells.[1] Therefore, a combination of hormone therapy with chemotherapy, which has a different mode of action, can be expected to improve the response rate. There are a number of clinical reports using randomized controlled trials to compare chemotherapy alone and chemoendocrine therapy in recurrent breast cancer[16–29] and some studies comparing

InnoPharma Exhibit 1103.0004

Table 4. Cell cycle analysis on day 21 of treatment (by flow cytometry)

Treatment		%S	%G_0G_1
Control	(n = 5)	35.6 ± 2.4	55.4 ± 2.3
ICI 182780 1 mg	(n = 4)	14.2 ± 3.3	82.8 ± 2.9
5-FU	(n = 5)	19.2 ± 4.3	76.4 ± 4.6
Combination	(n = 4)	19.8 ± 2.4	76.8 ± 2.6

%S, percentage of cells in S phase; % G_0G_1, percentage of cells in G phases
* $P < 0.05$

Table 5. 5-FU concentrations in the tumor[a]

Treatment		5-FU concentration (ng/g)
5-FU	(n = 6)	302.0 ± 154.6
Combination	(n = 8)	201.9 ± 83.7

NS, not significant
[a]Tumors resected at 1 h after the last administration of 5-FU

Table 6. 5-FU contents in tumor cell RNA[a]

Treatment		5-FU content in RNA (ng/mg RNA)
5-FU	(n = 6)	65.2 ± 18.0
Combination	(n = 5)	102.6 ± 34.6

* $P < 0.05$
[a]Tumors resected at 12 h after the last administration of 5-FU

Table 7. Concentrations of thymidylate synthetase (TS)

Treatment		TS total (pmol/g tissue)	TS free (pmol/g tissue)	TSIR (%)
5-FU at 1 h[a]	(n = 9)	30.8 ± 13.4	2.2 ± 2.6	94.1 ± 4.8
Combination at 1 h	(n = 11)	30.5 ± 7.1	0.9 ± 0.7	97.2 ± 2.2
5-FU at 12 h[b]	(n = 6)	61.9 ± 6.9	24.1 ± 2.3	61.0 ± 2.1
Combination at 12 h	(n = 5)	51.3 ± 8.1	17.5 ± 2.1	65.2 ± 7.2

TSIR, TS inhibition rate
* $P < 0.05$
[a]Tumors resected at 1 h after the last administration of 5-FU
[b]Tumors resected at 12 h after the last administration of 5-FU

endocrine therapy alone and chemoendocrine therapy.[20–23] Most of these reports show higher response rates to the combination therapy. However, the expected combination effect of the chemoendocrine therapy has yet to be clinically demonstrated.

On the other hand, experimentally, Watanabe[24] reported that the chemoendocrine therapy combining TAM, the most widely used drug in clinical practice, and the fluorinated pyrimidine 5-FU, which is relatively widely used, exerted a synergetic effect on R-27 in vivo, while Kubota et al.[25] failed to demonstrate a synergic effect of a combination of TAM and 1 M tegafur—4 M uracil (UFT) on Br-10. In vitro, Benz et al.[26,27] reported a synergetic effect of the combination therapy on MCF-7. Sato[28] reported that dimethylbenzanthracene-induced rat mammary tumors responded to the combination therapy, while Yamamoto et al.[29] failed to demonstrate any synergetic effect. Although differences in the cell lines, dosages, and the types of administration tested prevent us from making a simple comparison across these studies, there is still no agreement on the efficacy of this combination therapy. Under these circumstances, the authors investigated the antitumor effects and mode of action of the experimental chemoendocrine therapy combining pure antiestrogen and 5-FU.

A single administration of ICI 182780 at 0.5, 1, 3, and 5 mg showed a dose-dependent antitumor activity with a significant difference between 3 or 5 mg and the control. At 5 mg, the drug was assessed to be "effective" with a minimum T/C as low as 0.35. The toxic effect of the drug was considered to be minimal, as there were neither any deaths of the treated animals nor toxic symptoms other than a reduction of the mean body weight in the 5-mg group, which was not as significant as compared with the control.

Referring to the report of Kondo et al.[30] who reported the sensitivity of 5-FU on the tumor implanted in nude mice, the mice in the 5-FU group were administered

InnoPharma Exhibit 1103.0005

60 mg/kg of 5-FU three times at 4-day intervals on and after the first day of treatment. The 5-FU group failed to exert an antitumor effect with the minimum T/C of 0.68, and the effect was not significantly different from that of the control group. These findings agree with those of Koh[31] who reported that MCF-7 did not respond to 5-FU in vivo.

In contrast to the minimum T/C of 0.61 for 1 mg ICI 182780 alone and 0.68 for 60 mg/kg 5-FU alone which were both assessed to be ineffective, the combination was assessed to be effective with a minimum T/C of 0.42, which was significantly different from that of the control but fell marginally short of the synergic effect. The combination therapy was associated with a reduction in the mean body weight, which was not significant as compared with the control. There were also no deaths seen in the treated mice. These results thus suggest the usefulness of this combination therapy.

A comparison of the ER values showed a significantly lower B_{max} in the 1-mg ICI 182780 group than that of the control. This may be accounted for by the antitumor effect of ICI 182780 on ER-positive cells, as antiestrogen is expected to work through its effect on ER. The ER value in the 5-FU group decreased slightly but not significantly from that of the control. Judging from the observed mild antitumor effect of 5-FU and its mode of action, 5-FU seems to exert its effect directly on tumor cells regardless of the ER status. In contrast, the authors observed a marked reduction of the ER value in the combination group, which closely agreed with the findings of Watanabe who reported a marked reduction in the ER value in R-27 treated with a combination of TAM and 5-FU.[24] These findings as well as the increased antitumor response to the combination therapy thus suggest a potentiation of the effects on the ER-positive cells using this therapy.

The cell cycle analysis by FCM resulted in a decrease in the %S and an increase in the $\%G_0G_1$ by ICI 182780. TAM was reported to reduce the ratio of the cells in the S phase and accumulate cells in the G_0G_1 phase.[32] Like TAM, ICI 182780 also seems to slow down the cycle progression. On the other hand, 5-FU at a low dose is known to prolong the S phase and at a high dose to accumulate cells as in the G_1 phase.[33] Our findings of the decreased %S and the increased $\%G_0G_1$ support Watanabe[24] who reported a reduction of the %S in R-27 treated with 5-FU.

An earlier report that DNA is synthesized in the S phase and the %S is inversely proportional to the tumor doubling time[34] is consistent with our findings that the %S was significantly lower in the groups which responded to the treatment than that of the control. The FCM used, however, failed to explain the combination effect from the results of the cell cycle analysis, as the greatest %S reduction was not related to the greatest tumor response, which was seen in the combination group.

It is said that 5-FU may have two major modes of action. First, 5-FU is phosphorylated to FdUMP in the body, which formulates a ternary complex with methylenetetrahydrofolate and TS to inactivate TS and consequently inhibit the synthesis of DNA. Secondly, 5-FU is phosphorylated to fluorouridine triphosphate (FUTP), which is taken up by RNA instead of UTP and consequently interferes with the metabolism of RNA. Although much controversy remains as to which makes a greater contribution to the antitumor effect of the drug,[14,35–37] the authors tried to elucidate the mode of action of the combination therapy from both possible modes of action of 5-FU, that is, the inhibition of the DNA synthesis and the metabolic dysfunction of RNA, by determining the 5-FU and TS concentrations in tumor cells and 5-FU contents in RNA for the 5-FU and the combination groups.

There was no significant difference in the 5-FU concentrations in tumor cells, although they were slightly higher in the 5-FU group (mean of 302.0 ng/g) than in the combination group (201.9 ng/g). Antiestrogen ICI 182780 is, therefore, unlikely to interfere with the uptake of 5-FU by tumor cells.

In contrast, the 5-FU content in RNA was significantly higher in the combination group than in the 5-FU group. The metabolic dysfunction of RNA was thus suggested to contribute to the increased tumor response of the combination. In addition to ICI 182780, 5-FU also slowed down cell cycle progression and accumulated cells in the G_0G_1 phase, as indicated by FCM. Ueki reported a decreased %S and increased $\%G_0G_1$ of SC-115, an androgen-responsive mouse mammary tumor, at 24 h after administration of 5-FU, and a reincrease of %S at 1 week after administration.[38] The action of ICI 182780 to decrease %S and increase $\%G_0G_1$ seems to last for some time, while that of 5-FU may disappear shortly after administration. 5-FU is said to work on RNA in the G_1 phase.[39] In our study, 5-FU was administered in the combination group, when the $\%G_0G_1$ level was maintained at a high level by ICI 182780 and, as a result, the uptake of 5-FU by RNA may be facilitated.

There was no significant difference between 5-FU alone and the combination group with regard to the total TS, free TS, or TSIR at 1 h after the last administration of 5-FU. The TSIR approximated 100% in both groups. At 12 h after the last administration of 5-FU, the TSIR was of the order of 60% in both groups without any significant difference. The TSIR in both groups was as high as near 100% shortly after the administration of 5-FU and remained at a relatively high level of approximately 60% even at 12 h after the last administration of 5-FU. These findings suggest the possible contribution

InnoPharma Exhibit 1103.0006

of the inhibition of DNA synthesis by 5-FU to the antitumor effect in both groups. On the other hand, no difference in the TSIR between the groups suggests that a potentiation of the inhibition of DNA synthesis by 5-FU is not attributable to the increase in the antitumor response to the combination. The total TS and free TS in both groups at 12 h after the last administration of 5-FU were higher than those at 1 h after the last administration of 5-FU. These parameters were not significantly different between the groups at 1 h after the last administration of 5-FU, but were significantly lower in the combination group than in the 5-FU group at 12 h after the last administration of 5-FU. These findings thus suggested that the TS synthesis observed in the tumor cells in order to produce 5-FU induced a rapid decrease in free TS to be suppressed by the combination therapy. As the antitumor response is reported to correlate with the free TS concentration,[40] TS synthesis was more greatly suppressed by the combination therapy based on the higher antitumor response to the combination than to the 5-FU alone.

These results suggest the antitumor effect of ICI 182780 in ER-positive breast cancer cells, which was potentiated by the concomitant administration of 5-FU.

Conclusion

(1) ICI 182780 showed a dose-dependent activity against MCF-7, which was potentiated by the concomitant administration of 5-FU. (2) The observed marked reduction in the ER value in the combination therapy group of ICI 182780 and 5-FU suggests that the combination therapy may mainly work on ER-positive tumor cells. (3) ICI 182780 seems to slow down the cycle progression, as indicated by the decrease in the %S and the increase in the $\%G_0G_1$. (4) In the combination group, the synthesis of TS after the administration of 5-FU was suppressed and the 5-FU-induced metabolic dysfunction of RNA was potentiated.

References

1. Furr BJ, Patterson JS, Richardson DN, Slater SR, Wakeling AE (1979) Tamoxifen. Pharmacological and biochemical properties of drug substances 2. American pharmaceutical association academy of pharmaceutical sciences, Washington, pp 355–399
2. Ashizawa T, Ishida H, Okabe M, Gomi K (1993) Effect of medroxyprogesterone acetate on antitumor efficacies and side effect on 5-fluorouracil (in Japanese with English abstract). Gan To Kagaku Ryoho 20:941–947
3. Harper MJK, Walpole AL (1966) Contrasting endocrine activities of cis and trans isomers in a series of substituted triphenylethylenes. Nature 212:87
4. Boccardo F, Guarneri D, Rubagotti A, Casertelli GL, Bentivoglio G, Conte N, Campanella G, Gaggero G, Comelli G, Zanardi S, Nicolo G (1984) Endocrine effects of tamoxifen in postmenopausal breast cancer patients. Tumori 70:61–68
5. Fornander T, Rutqvist LE, Wilking N, Carstrom K, Schoultz BV (1993) Oestrogenic effects of adjuvant tamoxifen in postmenopausal breast cancer. Eur J Cancer 29A:497–500
6. Nayfield SG, Karp JE, Ford LG, Dorr FA, Kramer BS (1991) Potential role of tamoxifen in prevention of breast cancer. J Natl Cancer Inst 83:1450–1459
7. Jaiyesimi IA, Buzdar AU, Decker DA, Hortobagyi GN (1995) Use of tamoxifen for breast cancer: twenty-eight years later. J Clin Oncol 13:513–529
8. Wakeling AE (1993) The future of new pure antiestrogens in clinical breast cancer. Breast Cancer Res Treat 25:1–9
9. Parker MG (1993) Action of "pure" antiestrogens in inhibiting estrogen receptor action. Breast Cancer Res Treat 26:131–137
10. Soule HD, Vazquez J, Long A, Albert S, Brennan M (1973) A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409–1413
11. Takeuchi T (1995) Chemo- and endocrino-therapy of breast carcinoma xenografts in the dormant or exponential growth phase (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 96:379–387
12. Hanatani Y, Kubota T, Yamada Y, Tsuyuki K, Nakada M, Matsumoto S, Kumai K, Yoshino K, Ishibiki K, Abe O (1980) Experimental chemotherapy of human carcinomas serially transplanted to nude mice (in Japanese with English abstract). Nippon Gan Chiryo Gakkai Shi (J Jap Soc Cancer Ther) 15:1114–1120
13. Masuike T, Watanabe I, Takemoto Y (1985) Quantitative method of 5-fluorouracil and its metabolites in biological samples using high performance liquid chromatography (in Japanese with English abstract). Yakugaku Zasshi 105:1058–1064
14. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH (1982) In vivo kinetics of thymidylate synthetase inhibition in 5-fluorouracil-sensitive and- resistant murine colon adenocarcinoma. Cancer Res 42:450–456
15. Masuike T, Kikuchi K, Saito A, Hashimoto T, Takemoto Y (1995) Determination of 5-fluorouracil incorporated into RNA in tissue using gas chromatography-mass spectrometry (in Japanese with English abstract). Antibiot Chemother 11:162–167
16. Cocconi G, De Lisi V, Boni C, Mori P, Malacarne P, Amadori D, Giovanelli E (1983) Chemotherapy versus combination of chemotherapy and endocrine therapy in advanced breast cancer. Cancer 51:581–588
17. Kardinal CG, Perry MC, Weinberg V, Wood W, Ginsberg S, Raju RN (1983) Chemoendocrine therapy *vs* chemotherapy alone for advanced breast cancer in postmenopausal women: preliminary report of a randomized study. Breast Cancer Res Treat 3:365–371
18. Mouridsen HT, Rose C, Engelsman E, Sylvester R, Rotmensz N (1985) Combined cytotoxic and endocrine therapy in postmenopausal patients with advanced breast cancer. A randomized study of CMF *vs* CMF plus tamoxifen. Eur J Cancer Clin Oncol 21:291–299
19. Viladiu P, Alonso MC, Avella A, Beltran M, Borras J, Ojeda B, Bosch FX (1985) Chemotherapy versus chemotherapy plus hormonotherapy in postmenopausal advanced breast cancer patients. A randomized trial. Cancer 56:2745–2750
20. Falkson G, Falkson HC, Glidewell O, Weinberg V, Leone L, Holland JF (1979) Improved remission rates and remission duration in young women with metastatic breast cancer following combined oophorectomy and chemotherapy. Cancer 43:2215–2222
21. Cavalli F, Beer M, Marts G, Jungi WF, Alberto P, Obrecht JP, Mermillod B, Brunner KW (1983) Concurrent or sequential use of cytotoxic chemotherapy and hormone treatment in advanced breast cancer: report of the Swiss group for clinical cancer research. Br Med J 286:5–8
22. Australian and New Zealand Breast Cancer Trials Group, Clinical Oncological Society of Australia (1986) A randomized

InnoPharma Exhibit 1103.0007

trial in postmenopausal patients with advanced breast cancer comparing endocrine and cytotoxic therapy given sequentially or in combination. J Clin Oncol 4:186–193
23. Kiang DT, Gay J, Goldman A, Kennedy BJ (1985) A randomized trial of chemotherapy and hormonal therapy in advanced breast cancer. N Engl J Med 313:1241–1246
24. Watanabe O (1994) Experimental chemoendocrine therapy of human breast carcinoma xenograft serially transplanted into nude mice (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 95:263–270
25. Kubota T, Josui K, Ishibiki K, Abe O, Yamada Y, Asanuma F, Kawamura E, Kou J, Shiina E (1990) Experimental combined chemo- and endocrino-therapy of UFT and tamoxifen on human breast carcinoma xenografts serially transplanted into nude mice (in Japanese with English abstract). Nippon Gan Chiryo Gakkai Shi (J Jap Soc Cancer Ther) 25:2767–2773
26. Benz C, Santos G, Cadman ED (1983) Tamoxifen and 5-fluorouracil in breast cancer: modulation of cellular RNA. Cancer Res 43:5304–5308
27. Benz C, Cadman ED, Gwin J, Wu T, Amara J, Eisenfeld A, Dannies P (1983) Tamoxifen and 5-fluorouracil in breast cancer: cytotoxic synergism in vitro. Cancer Res 43:5298–5303
28. Sato Y (1989) Experimental studies on the effect of chemo-endocrine therapy on DMBA induced rat mammary cancer (in Japanese with English abstract). J Nihon Univ Med Assoc 48:413–421
29. Yamanoto H, Morimoto T, Monden Y (1989) Effect of chemo-endocrine therapy on DMBA-induced rat mammary carcinoma (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 90:1786–1792
30. Kondo T, Imaizumi M, Taguchi, T, Abe O, Hattori T, Wakui A, Kitano M (1987) A model for sensitivity determination of anticancer agents against human cancer using nude mice (in Japanese with English abstract). Gan To Kagaku Ryoho 14:680–686
31. Koh J (1988) Experimental chemo- and chemoendocrine therapy of human breast carcinomas serially transplanted into nude mice (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 89:91–102
32. Suzuki M (1992) Flow cytometric analysis of effects of chem-ohormonal agents on the cell cycle distribution of MCF-7 cells (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 93:71–80
33. Today RA, Crissman HA (1972) Use of flow microflurometry in detailed analysis of effects of chemical agents on cell cycle progression. Cancer Res 32:2726–2732
34. Nakamura A (1988) Experimental and clinical study on flow cytometric DNA analysis of human breast carcinoma (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 89:737–746
35. Darnenberg PV (1977) Thymidylate synthetase — a target engyme in cancer chemotherapy. Biochim Biophys Acta 473:73–92
36. Cory JG, Breland JC, Carter GL (1979) Effect of 5-fluorouracil on RNA metabolism in Novikoff hepatoma cells. Cancer Res 39:4905–4913
37. Ardalan B, Cooney DA, Jayaram HN, Carrico CK, Glazer RI, Macdonald J, Schein PS (1980) Mechanisms of sensitivity and resistance of murine tumors to 5-fluorouracil. Cancer Res 40:1431–1437
38. Ueki H (1987) Synergistic effect of cell kinetics-directed chemo-endocrine therapy on experimental mammary tumors (in Japanese with English abstract). Nippon Geka Gakkai Zasshi (J Jpn Surg Soc) 88:1576–1583
39. Maybaum J, Ullman B, Mandel HG, Day JL, Sadee W (1980) Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. Cancer Res 40:4209–4215
40. Kubota T, Fujita S, Kodaira S, Yamamoto T, Josui K, Arisawa Y, Suto A, Ishibiki K, Abe O, Mabuchi K, Fuse M (1991) Antitumor activity of fluoropyrimidines and thymidylate synthetase inhibition. Jpn J Cancer Res 82:476–482

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