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Enhancement of antitumor activity of mitomycin C in vitro and in vivo by UCN-01, a selective inhibitor of protein kinase C

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Abstract. UCN-01 (7-hydroxy-staurosporine) is a potent and selective inhibitor of protein kinase C (PKC), one of several protein kinases examined. UCN-01 itself was shown to exhibit antitumor activity in vitro and in vivo in oncogene-activated human and murine tumor cell lines. Since the mechanism(s) of action of UCN-01 is thought to be different from those of alkylating agents, including mitomycin C (MMC), we tested the combined effect of UCN-01 with MMC on human epidermoid carcinoma A431 cells. UCN-01 potentiated the antiproliferative activity of MMC and yet it did not affect the growth of the cells in vitro. However, other nonselective protein kinase inhibitors, such as staurosporine, K-252a, KT6124 (a derivative of K-252a) and H7, did not enhance the activity of MMC. Isobologram analysis revealed that the interaction of UCN-01 with MMC was synergistic in its antiproliferative activity. A DNA histogram of A431 cells treated with both UCN-01 and MMC showed a block in the cell cycle at the G_1/S phase. However, a histogram of cells treated with UCN-01 or MMC alone showed a G_1 or a G_2M block, respectively. The combined effect of UCN-01 with MMC was further examined in vivo in xenografted A431 cells in nude mice. The combination of both drugs in a single i.v. injection exhibited greater antitumor activity than MMC and UCN-01 alone ($P < 0.01$). This synergistic antitumor effect was also confirmed in two other solid tumor cell lines, i.e. human xenografted colon carcinoma Co-3 and murine sarcoma 180. The same was observed in the i.v.-inoculated P388 leukemia model, in which we saw an increased lifespan of mice when UCN-01 was combined with MMC. These results suggests the feasibility of using UCN-01 in clinical oncology, especially in combination with alkylating agents such as MMC. In addition, this combination therapy might be a novel chemotherapeutic approach to MMC-insensitive tumors in clinical trials.

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Introduction

UCN-01 (7-hydroxy-staurosporine) (Fig. 1) was isolated from the culture broth of *Streptomyces* spf. It has a potent and selective inhibitory activity against Ca^{2+} and phospholipid-dependent protein kinase C (PKC) among several protein kinases examined [20]. Additionally we have found that UCN-01 has the ability to inhibit the growth of human and murine oncogene-activated tumor cell lines in vitro and in vivo in animal models [1]. In contrast, the nonselective protein kinase inhibitor, staurosporine, did not exhibit any antitumor activity in vivo although it has much greater antiproliferative activity than UCN-01 in vitro [1, 22].

UCN-01 was reported to inhibit the activity of PKC, interacting with the latter's catalytic domain in a manner similar to staurosporine [21] and K-252a [18]. In addition, UCN-01 inhibited several biological effects of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on intact human tumor cell lines [1, 21], suggesting that this compound could act as an inhibitor of PKC in living cells.

Several structurally related compounds such as rebeccamycin [9] and KT6124 [2] have been reported to interact with DNA both in cell-free [2] and cellular [2, 9] systems. It should be noted that UCN-01 did not interact with DNA in both cell-free and cellular systems [1]. These results suggests that UCN-01 exhibits its antitumor activity by the inhibition of PKC and/or other protein kinases, not by interaction with DNA, although the exact mechanism(s) of action remains unclear.

Recently, PKC and other protein kinases have been reported to play a part in the sensitivity of the cells to several anti-cancer agents in vitro [4–7, 11, 15–17, 24]. Protein kinase inhibitors such as staurosporine, quercetin, and tamoxifen enhanced the antiproliferative activity of cis-diamminedichloroplatinum(II) (CDDP) in vitro [15]. The antiproliferative activity of another alkylating agent, nitrogen mustard, was also enhanced by quercetin [15]. In addition, ether lipid analogue BM41440, which was believed to inhibit PKC, was also reported to enhance the antiproliferative activity of CDDP [16] in vitro.

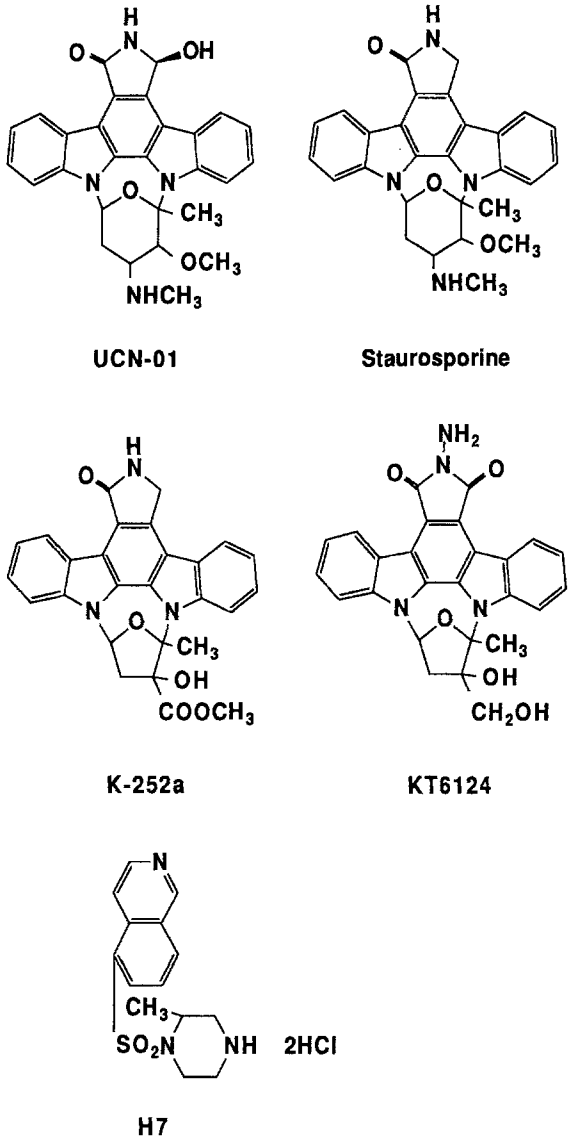

Fig. 1. Structure of protein kinase inhibitors

In contrast, several studies indicated that activators of PKC such as bryostatin [5], TPA [6], and lyngbyatoxin [7] could sensitize the cells to the cytotoxic effect of CDDP. It was also reported that the activation of PKC by TPA [11] and the excessive number of PKC-α molecules by gene transfer [24] could reduce the antiproliferative activity of adriamycin. Thus, it is unclear whether the activation or the inhibition of protein kinase(s), including PKC, can sensitize the cells to the anticancer agents.

Recently, we have examined the effect of UCN-01 on the antiproliferative activities of several clinically useful anticancer agents on human epidermoid carcinoma A431 cells in vitro [3]. UCN-01 enhanced the antiproliferative activity of alkylating agents [mitomycin C (MMC) and CDDP], antimetabolites (5-fluorouracil and cytosine arabinoside), and interferons β and γ. However, UCN-01 did not affect the antiproliferative activity of topoisomerase II inhibitors (adriamycin and etoposide), a mitotic poison (vindesine), a dihydrofolate reductase inhibitor (methotrexate), an inhibitor of RNA synthesis (actinomycin D), or bleomycin.

In this report, we examine the combined cytotoxic effect of UCN-01 and MMC in vitro and their combined antitumor activity in vivo, thus exploring a novel approach to tumor therapy.

Materials and methods

Drugs. UCN-01, staurosporine and K-252a were produced by fermentation in our laboratories as previously described [18, 21]. KT6124 was synthesized from K-252a as previously described [2]. H7 [14] was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Structures and inhibitory activities against protein kinases of these compounds are summarized in Fig. 1 and Table 1. MMC was produced by Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan.

Cell culture. Human epidermoid carcinoma A431 [13] was obtained from the American Type Culture Collection through Dainippon Pharmaceutical Co., Osaka, Japan. The cell cultures were performed at 37° C in a humidified atmosphere of 5% CO_2.

Animals and tumors. Murine lymphocytic leukemia P388 cells were passaged in adult male DBA/2 mice and used for the experiments in adult male BALB/c × DBA/2 F_1 (CDF_1) mice weighing 20–25 g. Sarcoma

Table 1. Antiproliferative activity against human epidermoid carcinoma A431 cells and inhibitory activity against protein kinases of various protein kinase inhibitors

	IC_{50} (μM)				Source
	Antiproliferative activity A431 cells	Inhibition of protein kinase			
		PKC	PKA	$pp60^{v\text{-}src}$	
UCN-01	0.21	0.0041	0.041	0.042	[20]
Staurosporine	0.0030	0.0027	0.0082	0.0064	[22]
K-252a	0.11	0.025	0.016	NT	[18]
KT6124	0.28	0.47	0.47	NT	[2]
H7	100	6.0	3.0	NT	[14]

Cells were cultured in 24-well plates on day 0, treated with various compounds on day 1, and then cultured for 96 h. The antiproliferative activity was determined as described in Materials and methods

PKC, Protein kinase C; PKA, cyclic-AMP-dependent protein kinase; $pp60^{v\text{-}src}$, v-src tyrosine kinase

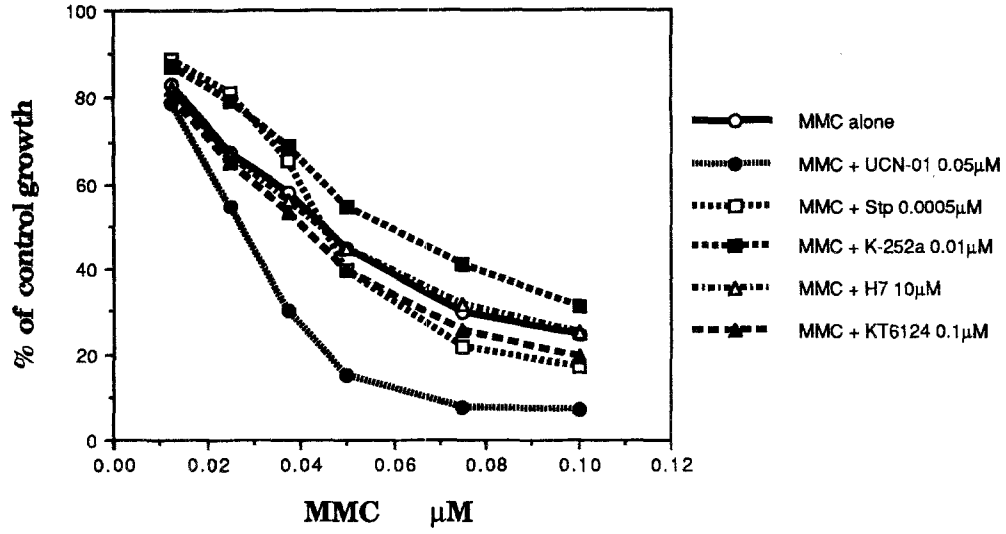

Fig. 2. Effect of UCN-01, staurosporine, K-252a, KT6124, and H7 on the antiproliferative activity of mitomycin C against A431 cells by 96 h continuous exposure. The cells (1.5×10^4/well) were cultured in 24-well plates on day 0, treated with mitomycin alone (○), mitomycin C + UCN-01 0.05 μM (●), mitomycin C + staurosporine 0.0005 μM (□), mitomycin C + K-252a 0.01 μM (■), mitomycin C + H7 10 μM (△) or mitomyin C + KT6124 0.1 μM (▲) on day 1, and then cultured for 96 h. The antiproliferative activity as determined as described in Materials and methods

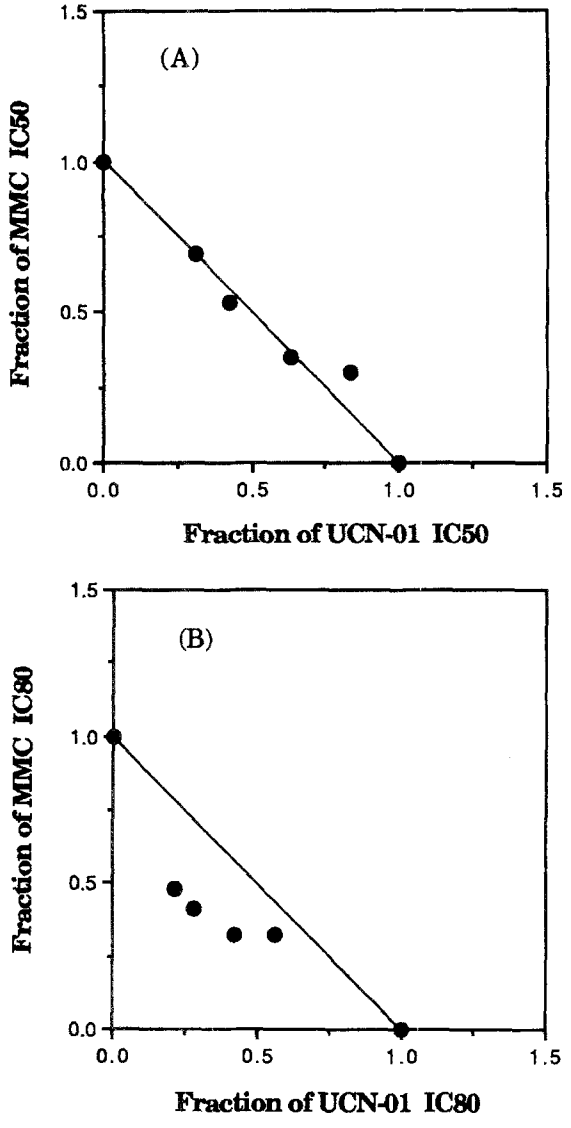

Fig. 3 A, B. Isobologram analysis of the combined effect of UCN-01 with mitomycin C on A431 cells by 96 h continuous exposure. The cells (1.5×10^4/well) were cultured in 24-well plates on day 0, treated with UCN-01, mitomycin C, or both drugs on day 1, and then cultured for 96 h. The isobologram analysis was carried out according to the method of Berenbaum [8]. **A** IC_{50}; **B** IC_{80}

180 cells were passaged and used for the experiments in adult male ddY mice weighing 20–25 g. These animals were obtained from SLC. Shizuoka, Japan. Human tumor cell xenografts were passaged and used in adult male BALB/c nude mice weighing 20–25 g (Nihon Clea Co., Tokyo, Japan). P388 leukemia was supplied by the National Cancer Institute, Bethesda, Md. through the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Sarcoma 180 was kindly supplied by the National Cancer Center. Tokyo, Japan. Co-3 (well-differentiated adenocarcinoma of colon) was supplied by Dr. Kubota, Keio University, Tokyo. A431 xenograft line was established by inoculation of the cultured cells into adult male BALB/c nude mice.

In vitro antiproliferative activity. A431 cells (1.5×10^4/1 ml per well) were precultured in Dulbecco's minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco, Grand Island, N. Y.) for 24 h in Nunclon 24-well multidishes (No 167008; Nunc, Rosklide, Denmark). Drugs were added to the plates (n = 3) in serival dilutions, and the plates were incubated for another 96 h. The total cell number was counted by a micro-cell counter (Toa Medical Electronics Co. Ltd., Kobe, Japan) after separating the cells using 0.05% trypsin –0.02% ethylene diammine tetraacetic acid (EDTA) (trypsin from Difco Laboratories, Detroit, Mich.; EDTA from Wako Pure Chemical Industries Co. Ltd., Osaka, Japan).

Evaluation of antitumor activity. Sarcoma 180 cells were inoculated s.c. in the axillary region of ddY mice. Other human solid tumors were inoculated s.c. into the flank of BALB/c nude mice. For evaluation of antitumor activity, tumor volumes were were calculated using the formula devised by the National Cancer Institute [12]:

$$\text{volume (mm}^3\text{)} = \frac{\text{length (mm)} \times [\text{width (mm)}]^2}{2}$$

The criterion for effectiveness was a ratio of the treated vs control (T/C) tumor volumes of 0.5 and less. Statistical significance was assessed using the Mann-Whitney U-test. In the case of the xenografted human tumor models A431 and Co-3, drug efficacy was expressed as the ratio of the mean V/V_0 value to that in the control group, where V is the tumor volume at the day of evaluation and V_0 is the tumor volume at the day of the initial treatment with the drug.

The antitumor activity for i.v.-inoculated tumors was evaluated by calculating the percentage increase in lifespan (ILS). The observation period was 30 days.

Evaluation of combined effect. The combined effect of UCN-01 with MMC in vivo was deemed "synergistic" if the maximum antitumor effect obtained by the combination of both drugs was significantly greater than that obtained by UCN-01 or MMC alone at the maximum tolerable dose.

Table 2. Combined effect of UCN-01 and MMC, administered a single injection, against xenografted human epidermoid carcinoma A431 cells in nude mice

Drug	Dose (mg/kg)	T/C_{min} (day)	Deaths
MMC	6.0	0.48 (14)[a]	0/5
MMC	4.0	0.56 (14)	0/5
UCN-01	14.5	0.58 (4)	0/5
UCN-01	12.1	0.64 (4)	0/5
MMC+UCN-01	6.0+14.5	0.13 (7)	5/5
MMC+UCN-01	6.0+12.1	0.24 (7)	5/5
MMC+UCN-01	4.0+14.5	0.17 (12)[a, b, c]	0/5
MMC+UCN-01	4.0+12.1	0.45 (7)[a, c]	0/5

A431 cells were inoculated s.c. on day −14, and the drugs were injected i.v. on day 0. Tumor volume was measured on days 0, 3, 7, 12, and 14 as described in Materials and methods, and T/C values were calculated. The sizes of untreated control tumors (mean ± SD) on days 0, 3, 7, 12, and 14 were 99.5 ± 18.0, 245 ± 68, 442 ± 139, 761 ± 214, and 1043 ± 222 mm^3, respectively.

T/C_{min}, minimum treated vs control value

[a] $P < 0.01$ against untreated control

[b] $P < 0.01$ against MMC 6.0 mg/kg

[c] $P < 0.01$ against MMC 4.0 mg/kg (Mann-Whitney U-test)

Table 3. Combined effect of UCN-01 with MMC administered via a single i.v. injection against xenografted human colon carcinoma Co-3 cells in nude mice

Drug	Dose (mg/kg)	T/C_{min} (day)	Deaths
MMC	6.0	0.45 (14)[b]	0/5
MMC	4.0	0.49 (14)[b]	0/5
UCN-01	14.5	0.61 (4)	0/5
UCN-01	12.1	0.59 (8)	0/5
MMC+UCN-01	6.0+14.5	0.15 (14)	2/5
MMC+UCN-01	6.0+12.1	0.17 (14)	4/5
MMC+UCN-01	4.0+14.5	0.20 (14)[a, c, e]	0/5
MMC+UCN-01	4.0+12.1	0.25 (14)[a, d, f]	0/5

Co-3 cells were inoculated s.c. on day −16 and the drugs were injected i.v. on day 0. Tumor volume was measured on days 0, 4, 8, 11, and 14 as described in Materials and methods, and T/C values were calculated. The sizes of untreated control tumors on days 0, 4, 8, 11, and 14 were 187 ± 81.1, 518 ± 153, 836 ± 201, 1172 ± 318, and 1529 ± 592 mm^3, respectively.

[a] $P < 0.01$, [b] $P < 0.05$ against untreated control

[c] $P < 0.01$, [d] $P < 0.05$ against MMC 6.0 mg/kg

[e] $P < 0.01$, [f] $P < 0.05$ against MMC 4.0 mg/kg (Mann-Whitney U-test)

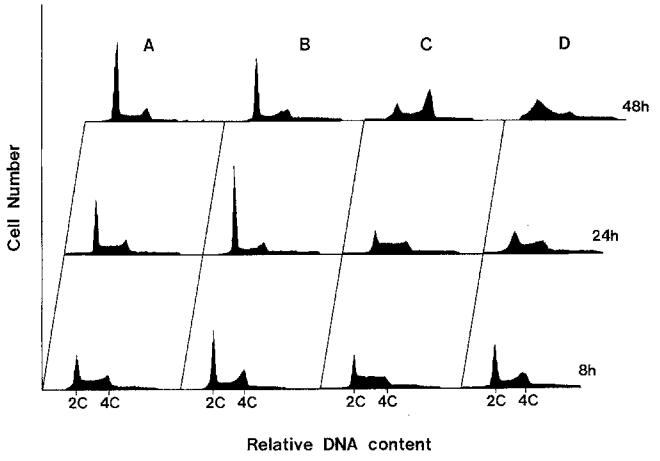

Fig. 4 A – D. Cell cycle distribution of A431 cells treated with **A** no drug, **B** UCN-01, **C** mitomycin C, and **D** both drugs at 8, 24 and 48 h after the addition of drugs. The drugs were administered 1 day after the seeding of the cells in 100-mm dishes. The DNA histograms were obtained by flow cytometry as described in Materials and methods. *2C* and *4C* refer to the DNA content

The combined effect of UCN-01 and MMC in vitro was assessed by means of isobologram analysis [8].

Cell cycle analysis. 6×10^5 A431 cells in 20 ml culture medium were precultured for 20 h in a 100-mm culture dish (Falcon 3003; Becton Dickinson, Lincoln Park, N. J.). After the addition of drugs, the cells were incubated for the time indicated. Cellular DNA content was measured by flow cytometry as follows: The cells were fixed in 70% ethanol, washed with phosphate buffered saline without calcium [PBS(−)] and then incubated with 250 μg/ml of RNase (Sigma) containing 0.1% Triton X-100 (Wako Pure Chemical Industries) in PBS(−) for 20 min at 37° C. Then the cells were stained with propidium iodide (Sigma) at a final concentration of 50 μg/ml for 20 min on ice. Fluorescence of individual cells was measured with a flow cytometer (Epics Elite, Coulter, Hialeah, FL). The cell cycle distribution was calculated using a Multicycle program (Coulter).

Results

Effect of various protein kinase inhibitors on the antiproliferative activity of MMC on A431 cells

The antiproliferative activities of protein kinase inhibitors (Fig. 1) against human epidermoid carcinoma A431 cells are shown in Table 1. To test the combined effect of these inhibitors on the antiproliferative activity of MMC, the following concentrations were used: 50 nM for UCN-01, 0.5 nM for staurosporine, 10 nM for K-252a, 100 nM for KT6124, and 10 μM for H7. UCN-01, K-252a, KT6124, and H7 did not exhibit any significant inhibition of the growth of the cells at these concentrations. Staurosporine inhibited the growth of the cells by 10–15%. As shown in Fig. 2, only UCN-01 enhanced the antiproliferative activity of MMC on A431 cells.

Isobologram analysis of the combined effect of UCN-01 and MMC

To determine the combined effect of UCN-01 and MMC more precisely, classical isobologram analysis was performed using the IC_{50} (cytostatic condition) and IC_{80} (cytotoxic condition) values for A431 cells. As shown in Fig. 3 A, the effect of UCN-01 with MMC on the cytostatic condition was additive. When IC_{80} values were used, the combined effect was revealed to be synergistic (Fig. 3 B).

Cell cycle analysis

DNA histograms of A431 cells treated with UCN-01, MMC and the combination of both drugs are shown in Fig. 4. UCN-01, at the dose of 0.05 μM which minimally affected the growth of the cells, arrested the cell cycle

Table 4. Combined effect of UCN-01 and MMC administered via a single i. v. injection on s. c.-inoculated sarcoma 180 in ddY mice

Dose of UCN-01 (mg/kg)	Tumor size ±SD (mm^3) [T/C]				
	MMC 0 mg/kg	MMC 6 mg/kg	MMC 4 mg/kg	MMC 2.67 mg/kg	MMC 1.78 mg/kg
0	2052±793 [1.0]	754±388[a] [0.37]	1266±173 [0.62]	1446±169 [0.70]	1648±303 [0.80]
14.5	1593±190 [0.78]	219±121[a, c] [0.11]	699±126[a, d] [0.34]	787±252[a, e, f] [0.38]	795±385[a, e, f, g] [0.39]
12.1	1397± 53 [0.68]	271± 90[a, c] [0.13]	697±348[a, e] [0.34]	1269±146 [0.62]	1329±207 [0.65]
10.1	1529±386 [0.75]	427±189[a, b] [0.21]	977± 92[a, e] [0.48]	1316±673 [0.64]	1476±203 [0.72]

Sarcoma 180 (5×10^6 cells/mouse) was inoculated s. c. into ddY mice on day 0, and the drugs were injected i. v. on day 1. Tumor volume was measured on day 7 as described in Materials and methods, and T/C ratios were calculated

[a] $P < 0.01$ against untreated control

[b] $P < 0.01$, [c] $P < 0.05$ against MMC 6.0 mg/kg

[d] $P < 0.01$, [e] $P < 0.05$ against MMC 4.0 mg/kg

[f] $P < 0.01$ against MMC 2.67 mg/kg

[g] $P < 0.01$ against MMC 1.78 mg/kg (Mann-Whitney U-test)

Table 5. Combined effect of UCN-01 with MMC by a single i. v. injection on i. v.-inoculated P388 leukemia in CDF1 mice

Dose of UCN-01 (mg/kg)	Mean survival ±SD (days) [ILS %]					
	MMC 0 mg/kg	MMC 6 mg/kg	MMC 4 mg/kg	MMC 2.67 mg/kg	MMC 1.78 mg/kg	MMC 1.19 mg/kg
0	8.2±0.4 [0]	13.8±0.4[a] [68]	12.4±0.5[a] [51]	11.6±0.5[a] [41]	10.8±0.4[a] [32]	10.0± [22]
14.5	9.0±0 [10]	9.0±1.4 [10]	16.8±1.3[a, b, c] [105]	13.4±1.1[a, e] [63]	12.8±0.8[a, e, f] [56]	12.0±0.7[a, g, h] [46]
12.1	8.8±0.8 [7]	14.6±6.3[a] [78]	16.6±1.5[a, b, c] [102]	14.6±0.9[a, c, d] [78]	12.4±0.5[a, f] [51]	12.0±0[a, f, h] [46]

P388 (1×10^6) cells were inoculated i. v. on day 0, and the drugs were injected i. v. on day 1.

[a] $P < 0.01$ against untreated control

[b] $P < 0.01$ against MMC 6.0 mg/kg

[c] $P < 0.01$ against MMC 4.0 mg/kg

[d] $P < 0.01$, [e] $P < 0.05$ against MMC 2.67 mg/kg

[f] $P < 0.01$, [g] $P < 0.05$ against MMC 1.78 mg/kg

[h] $P < 0.01$ against MMC 1.19 mg/kg (Mann-Whitney U-test)

transiently at the G_1 phase by 24 h (Fig. 4B). MMC alone (at 0.45 μM) retarded S phase progression of the cells by 24 h and blocked the cell cycle at G_2M phase by 48 h (Fig. 4C). In contrast, the combination of both drugs caused a S phase prolongation of 48 h (Fig. 4D).

Combined effect of UCN-01 and MMC on xenografted A431 cells in nude mice

The combined effect of UCN-01 and MMC on xenografted A431 tumor in nude mice was also examined. The maximum tolerable doses of MMC and UCN-01 in nude mice by single i. v. injection were 6.0 and 14.5 mg/kg, respectively. UCN-01 alone was inactive even at the maximum tolerable dose. MMC was inactive at the lower dose of 4.0 mg/kg, but exhibited weak but significant antitumor activity at the maximum tolerable dose (Table 2). In contrast, the combination of UCN-01 and MMC achieved a minimum T/C ratio of less than 0.5 at all doses examined (Table 2). Significant antitumor activity without toxic death was obtained at the lower dose of MMC combined with both UCN-01 doses. However, all the mice died at the higher dose of MMC administered in combination with UCN-01 (Table 2). It should also be noted that the antitumor activity of the combination was significantly ($P < 0.01$) stronger than that of MMC at the maximum tolerable dose.

Combined effect of UCN-01 and MMC in other solid tumor models

Since the combination of UCN-01 and MMC demonstrated a tumor-reducing effect in vivo, we tested it in other solid tumor models which were less sensitive to MMC. In the human colon carcinoma Co-3 xenograft model, the combination of UCN-01 (14.5 mg/kg and 12.1 mg/kg) and MMC (4.0 mg/kg) exerted significant ($P < 0.01$) antitumor activities without any toxic death, and these effects were superior to that of MMC alone. But again, the combination of UCN-01 with a higher dose of MMC (6.0 mg/kg) resulted in toxic death in some animals (Table 3).

The combined effect of both drugs was also assessed in a murine solid tumor model, sarcoma 180. In this system, full doses of both drugs, i.e. MMC 6.0 mg/kg plus

UCN-01 at 14.5 mg/kg, could be combined without toxic death in mice (Table 4). The combinations of MMC 6.0 mg/kg plus UCN-01 14.5, 12.1 or 10.1 mg/kg were apparently more effective than MMC alone at the same dose (P <0.01). In addition, combinations of MMC 4.0, 2.67 and 1.78 mg/kg plus UCN-01 at 14.5 mg/kg and MMC 4.0 mg/kg plus UCN-01 12.1 mg/kg caused antitumor effects similar to those obtained with the maximum tolerable dose of MMC.

Combined effect of UCN-01 and MMC in the i. v.-inoculated P388 leukemia model

Finally we tested the combined effect of UCN-01 and MMC in the i. v.-inoculated P388 leukemia model. As shown in Table 5, a single i. v. injection of UCN-01 did not exert any protective effect, while a single i. v. injection of MMC increased the lifespan of the mice by 62%. The combination of MMC 4.0 mg/kg plus UCN-01 14.5 or 12.1 mg/kg yielded a significantly (P <0.01) greater ILS of 105% and 102%, respectively. In addition, at the doses of MMC 2.67, 1.78 or 1.19 mg/kg plus UCN-01 14.5 or 12.1 mg/kg, the antitumor effects of the combinations were significantly more pronounced than those of either drug alone.

Discussion

In this study we explored a novel chemotherapeutic approach to tumor therapy by examining the combined effect of two drugs from widely divergent classes, UCN-01 [1, 20, 21] and MMC [10, 23].

As shown in Fig. 2, UCN-01 potentiated the antiproliferative activity of MMC at a concentration as low as 50 nM. At this concentration, UCN-01 did not affect the growth of A431 cells. However, it did affect the cell cycle transiently (Fig. 4) and inhibited PKC activity of the cells (S. Akinaga et al., unpublished observation). Interestingly, other nonselective inhibitors such as staurosporine, K-252a and H7 did not exhibit any effect on the antiproliferative activity of MMC (Fig. 2). These results suggest that the selective inhibition of PKC by UCN-01 might contribute to the enhancement of the antiproliferative activity of MMC. KT6124, a derivative of K-252a, was shown to exert its antitumor activity through the possible DNA fragmentation and was not shown to interact with PKC and/or other protein kinases in the intact cells [2]. The fact that this compound did not enhance the antiproliferative activity of MMC (Fig. 2) suggests that the direct interaction of UCN-01 with DNA may be negligible in its mode of action.

Since UCN-01 itself is a potent antiproliferative agent (Table 1), we examined in more detail its effect in combination with MMC, using the isobologram method [8], which is commonly employed for the evaluation of drug combinations. On the basis of the data in Fig. 3, we conclude that UCN-01 and MMC exert an additive cytostatic effect and a synergistic cytotoxic effect.

Recent studies have shown that several protein kinase inhibitors potentiate the antiproliferative activity of alkylating agents such as CDDP and nitrogen mustard in vitro [15, 16]. Hoffman et al. [15] reported that quercetin, a nonselective inhibitor of protein kinases, potentiated the antiproliferative activity of CDDP and/or nitrogen mustard. They also showed that other protein kinase inhibitors, such as staurosporine and tamoxifen, potentiated the activity of CDDP [15]. The same group has reported that an ether lipid analogue and a probable PKC inhibitor, BM 41440, can enhance the activity of CDDP [16]. These findings, taken together with our results, suggest that combination of protein kinase inhibitors with alkylating agents can result in higher antiproliferative activity. In contrast, several activators of PKC, e. g. bryastatin 1 [5], TPA [6] and lyngbyatoxin [7], have been reported to potentiate the activity of CDDP in vitro. It is presently unclear whether the activation or the inhibition of protein kinases is more important for the cytotoxicity of DNA-reactive anticancer agents. Nevertheless, the results suggest that protein kinase(s), including PKC, may be a target for the potentiation of the antiproliferative activity as well as antitumor activity of alkylating agents and other DNA-reactive agents.

Cell cycle analysis by FCM revealed that UCN-01 and MMC exhibit quite different effects on the progression of the cell cycle of A431 cells. As reported previously [4], MMC caused the delay of S phase at the early stage and later blocked the cell cycle at S/G_2M phase (Fig. 4C). In contrast, UCN-01 exerted transient G_1 phase arrest (Fig. 4B). At higher concentrations, UCN-01 blocked the cell cycle progression of the cells at G_1 phase (data not shown). These findings can be taken as a strong indication for combining both drugs. Interestingly, the DNA histogram pattern of the cells treated with combined UCN-01 and MMC was quite different from that of the cells treated with UCN-01 or MMC alone (Fig. 4D vs B and C). However, the true mechanism(s) for the cell cycle block around S phase caused by UCN-01 and MMC in combination is unknown. Further studies are in progress.

The considerable antitumor activity of UCN-01 combined with MMC in vitro, prompted us to examine this phenomenon in vivo in animal models. The xenografted A431 human epidermoid carcinoma model was selected and, as predicted from the results of the in vitro study, the combination of UCN-01 plus MMC exhibited marked antitumor activity against xenografted A431 carcinoma, significantly superior to that of MMC or UCN-01 alone (P <0.01; Table 2). To further study the significance of this combination chemotherapy in vivo, two other solid tumors, human xenografted colon carcinoma Co-3 and murine syngeneic solid tumor sarcoma 180, the former reported to be less sensitive to MMC [19], were used. In both of these solid tumor models, the combination therapies exhibited synergistic antitumor effects which were significantly (P <0.01) greater than those of the maximum tolerable dose (6.0 mg/kg) of MMC alone (Tables 3, 4). In addition, the combination modality increased the lifespan of the mice inoculated i. v. with P388 leukemia to a greater extent than MMC alone (P <0.01; Table 5). The data from sarcoma 180 and P388 (Tables 4, 5) also suggested that UCN-01 could be used to reduce the dose of MMC in vivo to minimize its toxicity. However, at higher doses of MMC

(6.0 mg/kg) in combination with UCN-01 a careful toxicological examination should be conducted, since the toxic effects of the two agents were found to be additive in xenograft models (Tables 2, 3) and in the P388 leukemia model (Table 5).

To our knowledge this is the first report that an inhibitor of PKC and/or other protein kinases can enhance the antitumor activity of MMC in vivo as well as in vitro. Although little is known about the mechanism(s) of the combined effect of UCN-01 and MMC, the results of our in vivo studies strongly suggest that this novel combination chemotherapy may merit clinical trials in cancer patients.

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