Multiform Combination Effects of Smooth Muscle Relaxants with Antitumor Agents in Rat Ascites Hepatoma AH66 Cells

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Abstract—At non-cytotoxic concentrations, actions of smooth muscle relaxants except for the action of isoproterenol (IPN) on the effect of vinblastine (VBL) and mitomycin C (MMC) in rat ascites hepatoma AH66 cells resistant to these antitumor agents clearly separated into two groups. IPN hardly influenced the effects of both VBL and MMC. Although verapamil, a calcium-antagonist, and W-7, a calmodulin inhibitor, enhanced the growth-inhibitory effect and uptake of VBL by inhibiting the VBL efflux, these drugs did not influence the effect and uptake of MMC. In contrast, forskolin, an adenylate cyclase activator, db-cAMP, a cAMP analog, and theophylline, a cyclic nucleotide phosphodiesterase inhibitor, potentiated the effect of MMC, but did not influence the effect of VBL. The combination effect of forskolin and db-cAMP might be elucidated from the increase of inward transport of MMC through the action of the intracellular cAMP elevated by these drugs. Theophylline, however, only slightly increased both intracellular cAMP level and MMC uptake into the cells, similar to the action of IPN. We thought that the combination effect of theophylline was effected through its other activity of repair inhibition against AH66 cells, which are resistant to MMC due to their high capacity to repair impaired DNA. Thus, the smooth muscle relaxants used in this study enhanced the growth-inhibitory effect of a distinct antitumor agent through their individual activity against tumor cells.

It is important to determine how to increase sensitivity and how to overcome resistance of tumor cells to antitumor agents. Many investigators have attempted to enhance the effect of antitumor agents by non-antitumor drugs (1-5). Recently, some drugs showing smooth muscle relaxation have been introduced as candidates for antitumor combined drugs (6-12). Tsuruo et al. (7, 8, 11) showed that calcium-antagonists and calmodulin inhibitors increased the sensitivity of acquired resistance of mouse leukemia cells to Vinca alkaloids to a level similar to that of sensitive cells. We have indicated that β-adrenergic stimulants enhanced the antitumor effect of mitomycin C in both in vitro and in vivo experiments (9, 10, 12). It is necessary to examine the combination effect of smooth muscle relaxants on multiple resistant cells to antitumor agents. There are many rat ascites hepatoma cell lines possessing characteristic sensitivities to antitumor agents. Among them, the AH66 cell line is known to be naturally resistant to alkylating agents and Vinca alkaloids (13). In this study using the AH66 cell line, we investigated the combination effect of several smooth muscle relaxants with vinblastine and mitomycin C and discussed the synergisms with their biochemical action mechanisms.
Materials and Methods

Agents: Antitumor agents used were vinblastine (VBL, Shionogi) and mitomycin C (MMC, Sankyo). dl-Isoproterenol hydrochloride (IPN, Nakai), N\(^6\),O\(^2\)-dibutyl cyclic AMP (db-cAMP, Sigma), theophylline (Wako) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, Seikagaku Kogyo) were also purchased from commercial sources. Forskolin was kindly donated by Nihon Kayaku Co., Ltd., Tokyo. Verapamil was kindly provided by Eisai Co., Ltd., Tokyo. Agents except forskolin were dissolved in phosphate-buffered saline (PBS) immediately before use. Forskolin was dissolved in ethanol and used at a final concentration of 0.5% ethanol which did not influence the effect of VBL and MMC and did not effect intracellular cAMP level.

Cytotoxicity test of antitumor agents combined with smooth muscle relaxants: Rat ascites hepatoma AH66 cells were maintained serially by the intraperitoneal passage at weekly intervals in female Donryu rats (Shizuoka Laboratory Animal Center). The cells were withdrawn from the abdominal cavity of rat at 5 to 7 days after the cell inoculation, washed with Hanks’ solution, suspended in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) to a density of 2 \(\times\) 10\(^5\) cells/ml and cultured in the presence of an antitumor agent and a smooth muscle relaxant for 2 days at 37°C in a CO\(_2\) incubator. After the cultivation, the cells were counted microscopically after they were detached from the plastic dish by trypsinization.

Examination on the uptake of VBL by AH66 cells: Cells (5 \(\times\) 10\(^5\)/ml) were incubated with a non-antitumor drug, 0.1 \(\mu\)Ci \(^3\)H-VBL (9.9 Ci/mmol, Amersham) and 10\(^{-7}\) M unlabeled VBL in DMEM supplemented with 10% fetal calf serum, at 37°C for designated periods. After the incubation, the treated cells were centrifuged (3,000 rpm for 5 min at 4°C) and washed twice with cold PBS. The cells were solubilized with protosol (New England Nuclear), and the intracellular radioactivity was counted in a liquid scintillation counter.

Determination of MMC amount in AH66 cells: MMC is rapidly metabolized in the cells to several metabolites (14, 15). Therefore, the amounts of MMC taken up into the cells were determined by measuring the decrease in the absorbance of the incubation medium at 360 nm for the direct spectrophotometry method and at 363 nm for analytical high-performance liquid chromatography (HPLC) according to our previous report (16). Cells (10\(^7\)/ml) were incubated with a non-antitumor drug and MMC (3 \(\times\) 10\(^{-5}\) M) in PBS at 37°C for designated periods. After the incubation, the treated cells were chilled and centrifuged at 3,000 rpm for 5 min. The supernatant fluid was used for MMC determination.

Examination on the efflux of antitumor agents from AH66 cells: The cells incubated with \(^3\)H-VBL or MMC for 30 min at 37°C were collected by centrifugation, washed and resuspended in fresh medium. The cells were incubated for 30 min, and the retained intracellular radioactive VBL was counted, or the culture supernatant was applied to HPLC for determination of MMC and its metabolites.

Determination of cAMP contents in AH66 cells: Cells (3 \(\times\) 10\(^6\)/ml) were treated with the test materials at 37°C for designated periods. After the treatment, the cells were chilled and collected by centrifugation. The intracellular cAMP was extracted by homogenizing in 6% trichloroacetic acid and assayed using a cAMP assay kit (Yamasa Shoyu) following the procedure developed by Honma et al. (17).

Results are expressed as the mean±S.E. Statistical significance was evaluated using Student’s \(t\)-test.

Results

Influence of smooth muscle relaxants on the in vitro effect of VBL and MMC: Smooth muscle relaxants were used at non-cytotoxic concentrations by themselves. As shown in Figs. 1 and 2, the actions of drugs except for that of IPN clearly separated into two groups. IPN did not influence the effects of both VBL and MMC. Verapamil and W-7 synergistically potentiated the effect of VBL, while it did not influence the effect of MMC. As W-7 was cytotoxic at concentrations over 10\(^{-5}\) M, the combination effect of
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Fig. 1. Combined growth-inhibitory effect of smooth muscle relaxants with VBL. A) AH66 cells were incubated with $2 \times 10^{-6}$ M VBL in the absence (○) or presence of various concentrations of each drug for 2 days. B) AH66 cells were incubated with various concentrations of VBL in the absence (○) or presence of a constant concentration of each drug ($10^{-5}$ M verapamil or W-7) for 2 days. Each point represents the mean±S.E. (bar) of 3 experiments done in triplicate. Symbols of combined drugs: ●, verapamil; ▲, W-7; □, IPN; ▼, forskolin; △, db-cAMP; ■, theophylline. *Significantly different from VBL alone at $P<0.01$.

verapamil was more potent than that of W-7 in the concentration ranges used in this study. At $10^{-5}$ M of each drug, the growth-inhibitory effect of VBL was increased about 20- and 3-fold by verapamil and W-7, respectively. In contrast, forskolin, db-cAMP and theophylline significantly enhanced the effect of MMC in a dose-dependent manner, but they were all ineffective on the effect of VBL. Among them, theophylline only at high concentration ($10^{-3}$ M) showed the strongest combination effect and increased the effect of MMC by about 10-fold. At lower concentrations, however, db-cAMP and forskolin was more effective than theophylline.

Influence of smooth muscle relaxants on the uptake of VBL and MMC: VBL contents in AH66 cells reached a plateau level after about 20 min of incubation, and verapamil and W-7 dose-dependently increased the uptake of VBL; and at $10^{-5}$ M of each drug, each elevated the plateau level about 3- and 2-fold that of the non-treated state, respectively (Fig. 3). While forskolin and db-cAMP did not influence the uptake of VBL, these drugs accelerated the progressive uptake of MMC (Fig. 4). Theophylline showed only a slight increase of MMC uptake even at $10^{-3}$ M (Fig. 4). Verapamil and W-7 did not increase the uptake of MMC in the cells at the range of concentrations used. IPN hardly affected both VBL uptake and MMC uptake.

On the other hand, we have indicated that the intracellular accumulation of VBL was lower in resistant rat ascites hepatoma cells including AH66 cells than in sensitive cells and that the capacity to extrude VBL was higher in resistant cells than in sensitive cells (5). Tsuruo et al. (7) reported that the increase of uptake of vincristine by calcium channel inhibitors such as verapamil was
Fig. 2. Combined growth-inhibitory effect of smooth muscle relaxants with MMC. A) AH66 cells were incubated with 10^{-7} M MMC in the absence (○) or presence of various concentrations of each drug for 2 days. B) AH66 cells were incubated with various concentrations of MMC in the absence (○) or presence of a constant concentration of each drug (10^{-4} M forskolin, 10^{-4} M db-cAMP or 10^{-3} M theophylline) for 2 days. Each point represents the mean±S.E. (bar) of 3 experiments done in triplicate. Symbols of combined drugs are the same as those in the legend of Fig. 1. *Significantly different from MMC alone at P<0.01.

Table 1. Influence of smooth muscle relaxants on the efflux of VBL and MMC

| Antitumor agent | Drug            | Amount extruded (% of initial amount in the cells) |
|-----------------|-----------------|---------------------------------------------------|
| VBL             | None            | 77.6±2.1                                          |
|                 | Verapamil (10^{-5} M) | 48.8±1.9*                                        |
|                 | W-7 (5×10^{-6} M) | 56.5±2.7*                                        |
| MMC             | None            | 0                                                 |
|                 | Forskolin (10^{-4} M) | 0                                                 |
|                 | Db-cAMP (10^{-4} M) | 0                                                 |

The cells were loaded with VBL or MMC for 30 min, washed, resuspended in fresh medium, and incubated in the absence (none) or presence of each drug for 30 min. The amount of VBL extruded from the cells was calculated from the decreased amount of VBL in the cells. The amounts of MMC and its metabolites contained in the culture supernatant were determined by HPLC. Each value represents the mean±S.E. of 3 experiments done in triplicate. *Significantly different from the control at P<0.01.

Attained by the inhibition of the efflux of the antitumor agent from vincristine resistant P388 cells, but not by acceleration of the influx. Next, the influence of the drugs on the efflux of the antitumor agents was examined. Table 1 shows the amounts of VBL and MMC extruded from the cells for 30 min after washing out extracellular antitumor agents. While about 80% of the initial amount of VBL in the cells was extruded, the extrusion was significantly suppressed by verapamil and W-7. MMC and any its metabolites could not be detected either in the absence or presence of drugs in the culture.
Influence of smooth muscle relaxants on the uptake of VBL. A) The cells were incubated with 1.1 x 10^{-7} M VBL in the absence (○) or presence of various concentrations of each drug for 30 min. B) The cells were incubated with 1.1 x 10^{-7} M VBL in the absence (○) or presence of 10^{-5} M verapamil or W-7 for the indicated periods. Each point represents the mean±S.E. (bar) of 3 experiments done in triplicate. Symbols of drugs are the same as those in the legend of Fig. 1. *Significantly different from the control at P<0.01.

Fig. 3. Effect of smooth muscle relaxants on the uptake of VBL. A) The cells were incubated with 1.1 x 10^{-7} M VBL in the absence (○) or presence of various concentrations of each drug for 30 min. B) The cells were incubated with 1.1 x 10^{-7} M VBL in the absence (○) or presence of 10^{-5} M verapamil or W-7 for the indicated periods. Each point represents the mean±S.E. (bar) of 3 experiments done in triplicate. Symbols of drugs are the same as those in the legend of Fig. 1. *Significantly different from the control at P<0.01.

Influence of smooth muscle relaxants on the intracellular cAMP level: Since the antibody of cAMP in the radioimmunoassay system used in this study showed a weak cross-reactivity with db-cAMP, the intracellular cAMP, after the treatment with db-cAMP was not estimated. Forskolin significantly increased cAMP level in AH66 cells in a dose-dependent manner with a peak at 15 min. IPN and theophylline increased only about 50% of non-treated level at the maximum level. Verapamil and W-7 did not influence the level at all (Fig. 5).

Discussion

Although the drugs examined in this study did not enhance the cytotoxic effects of both VBL and MMC on AH66 cells, the drugs, except for IPN, synergistically potentiated either of the two antitumor agents. Verapamil, a slow calcium channel inhibitor (18), prominently enhanced the effect and uptake of VBL in a dose-dependent manner and inhibited the efflux of VBL from the hepatoma cells. W-7, a calmodulin inhibitor (19), also potentiated the effect of VBL in a manner similar to verapamil, but its efficacy was less than that of verapamil at the range of non-cytotoxic concentrations. Tsuruo et al. (7-9) also reported that calcium-antagonists and calmodulin inhibitors inhibited the efflux of Vinca alkaloids from vincristine resistant P388 leukemia cells. However, the mechanisms involved in the inhibition of the efflux of the antitumor agents by cellular calcium modifiers are not yet clear. We have shown that VBL was taken up into rat ascites hepatoma cells by a passive transport and extruded by an active system (20). Furthermore, we have indicated that the activities of ATPases in the plasma membrane of AH66 cells were significantly higher than those of other VBL sensitive cells and suggested the involvement of Ca^{2+}-ATPase and/or Mg^{2+}-ATPase with the active efflux of VBL (21). From these evidence, we can speculate that VBL is extruded through some carrier systems activated by calcium-mediated phosphoryla-
Fig. 4. Effect of smooth muscle relaxants on the uptake of MMC. A) The cells were incubated with $3 \times 10^{-5}$ M MMC in the absence (O) or presence of various concentrations of each drug for 30 min. B) The cells were incubated with $3 \times 10^{-5}$ M MMC in the absence (O) or presence of $10^{-4}$ M forskolin or db-cAMP for the indicated periods. Each point represents the mean±S.E. (bar) of 3 experiments done in triplicate. Symbols of drugs are the same as those in the legend of Fig. 1. *Significantly different from the control at $P<0.01$.

In the cells, and verapamil and W-7 inhibit the activation pathway.

On the other hand, verapamil and W-7 did not have any influence on the effect of MMC. In contrast, although forskolin, db-cAMP and theophylline hardly affected the effect and uptake of VBL, they synergistically enhanced the growth-inhibitory effect of MMC on AH66 cells. IPN enhanced neither the effect of VBL nor the effect of MMC. Recently, we reported using AH130 cells that cAMP, which was continuously elevated in the cells by the stimulation with β-adrenergic drugs and additional action of MMC on the cellular membrane (11, 22, 23), accelerated the activation of MMC in the cells (24), and this was followed by an increase in the inward passive transport of the agent (25). In this study, forskolin, which has been reported to activate adenylate cyclase by modifying the interaction between the guanine nucleotide-binding regulatory protein and the catalytic unit (26), significantly elevated the intracellular cAMP level and increased the uptake of MMC in AH66 cells to an extent similar to that by db-cAMP. Because MMC taken up was not extruded from the cells, the accumulation of MMC in the cells by forskolin and db-cAMP should be explained as an increase of the passive influx of the agent through the action of cAMP in the cells. On the other hand, IPN, a strong β-adrenergic stimulant, increased the intracellular cAMP only by about 50% of the non-treated level at the maximum. AH66 cells were thought to show low responsiveness to IPN like AH13 cells reported in our previous paper (11). It seems that cAMP produced in the cells by IPN is too low to cause the uptake of MMC. While theophyl-
Fig. 5. Influence of smooth muscle relaxants on the intracellular cAMP level. A) The cells were incubated in the absence (--) or presence of various concentrations of each drug for 15 min. B) The cells were incubated in the absence (--) or presence of 10^-4 M forskolin, 10^-4 M IPN or 10^-3 M theophylline for the indicated periods. Each point represents the mean±S.E. (bar) of 3 experiments done in triplicate. Symbols of drugs are the same as those in the legend of Fig. 1. *Significantly different from the control at P<0.01.

line also increased the intracellular cAMP only a little and did not increase the MMC uptake, this drug at a high concentration (10^-3 M) showed the strongest potentiation of the effect of MMC among the drugs used in this study. Therefore, the combination effect of theophylline with MMC is difficult to elucidate from participation of cAMP. Methylxanthine derivatives are known to be potent DNA repair inhibitors (27), together with cyclic nucleotide phosphodiesterase inhibitors (28). The major antitumor mechanism of MMC is the polyfunctional alkylation of DNA by its active metabolites (14, 15, 29). The sensitivities of the cells to alkylating agents have been elucidated from the difference in their repair capacity of the impaired DNA, and when the rat ascites hepatoma cells were exposed to ultraviolet light, the viability of resistant cells to alkylating agents decreased in the presence of caffeine more than that of sensitive cells (30). Consequently, the potentiation of the effect of MMC by theophylline seems to be led by its repair-inhibitory action.

Recently, it has been shown that W-7 inhibited by itself cell growth in vitro (31) and in vivo (32). Furthermore, forskolin and other agents, which elevate the intracellular cAMP level, have been reported to suppress the tumor cell growth and tumor metastasis (33–36). This study indicates that smooth muscle relaxants including W-7 and forskolin at non-cytotoxic concentrations potentiate the growth-inhibitory effect of antitumor agents on AH66 cells. Consequently, these relaxants are considered to be available for applied cancer chemotherapy. We may be able to select the pertinent combination therapy for various types of tumors and
symptoms of patients.

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