Potentiation of Adriamycin Cytotoxicity by Dipyridamole against HeLa Cells 

in Vitro and Sarcoma 180 Cells in Vivo

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ABSTRACT

Dipyridamole (DP) is clinically prescribed for its vasodilating and antiplatelet effects. DP also inhibits nucleoside transport and enhances cytostatic activity of antimetabolites. We obtained evidence for augmentation of the cytotoxic effect of Adriamycin (ADM) by DP, both in vitro and in vivo. Nontoxic levels of DP enhanced the cytotoxicity of ADM against HeLa cells, and the 50% effective concentration of ADM was decreased 2.4-fold by DP. DP also increased the activity of ADM in clonogenic assays. Intracellular levels of ADM in the case of concomitant exposure to ADM and DP were 1.5-fold higher than in the case of exposure to ADM alone, determined using high-performance liquid chromatography. Incorporation of ADM into the cells pretreated with DP was also increased (1.4-fold), while the efflux was little affected. The growth of Sarcoma 180 tumors was prominently suppressed by the combination of ADM and DP, compared to findings with ADM alone. DP also prolonged the survival of Sarcoma 180 tumor-bearing mice, when given in combination with ADM.

While the enhancement of cytostatic activity of antimetabolites has been attributed to a decrease in utilization of the salvage pathway by DP, our data show that the synergic effects of DP with ADM were the result of increased intracellular levels of ADM.

INTRODUCTION

DP,1 a drug described clinically as a vasodilator and antiplatelet agent, is a potent inhibitor of membrane nucleoside transport (1–3). An enhanced cytostatic effect of the antimetabolites acivicin (4, 5), methotrexate (6), PALA (7), and 5-FU (8) has been noted in cultured cells lines, when DP was given in combination. Despite its poor effect in vivo, clinical studies of combination chemotherapy have been done (9, 10). The mechanism of this effect by DP has been attributed to a synergism between the blockade of the de novo pathway of nucleoside synthesis by antimetabolites and the inhibition of the salvage pathway by DP, as a result of a decrease in nucleoside uptake (4–7). Little is known of the synergic effects of DP on other anticaner drugs, the mechanisms of which differ from those of antimetabolites.

ADM is a anthracycline antibiotic, which seems to act through an intercalation with DNA and interference with the template activity necessary for DNA replication and RNA synthesis (11–13), the damage or breakage of DNA by bioreductive alkylation (14), or the generation of oxygen free radicals (15–17). It has been reported that ADM exerts cytotoxic activity, without entering the cells; hence the cell membrane may be one of the targets of the cytotoxic activity (18–20).

We have now obtained evidence that DP enhances the cytotoxic effect of ADM, both in vitro and in vivo.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: DP, dipyridamole; PALA, N-phosphonacetyl-L-aspartate; 5-FU, 5-fluourouracil; ADM, Adriamycin; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; ODS, octadecyl silane; S-180, Sarcoma 180.

MATERIALS AND METHODS

Drugs. Dipyridamole was purchased from Boehringer Ingelheim Japan Co. (Japan). Adriamycin was obtained from Kyowa Hakko Co. (Japan).

Cell Culture. HeLa cells were cultured in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Japan) containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY), 2 x 10^-4 M L-glutamine, 40 μg/ml of gentamycin, 100 μg/ml of streptomycin, and 100 units per ml of penicillin G.

Inhibition of Cell Growth. Two x 10^5 exponentially growing HeLa cells plated in 60-mm culture dishes and maintained for 24 h at 37°C in a humidified 5% CO₂ atmosphere. The cells were then exposed to various concentrations of ADM and/or DP, trypsinized, and harvested at 24, 48, and 72 h after administration. The number of viable cells was determined by the dye exclusion method (21). Each experiment was performed in triplicate.

Clonogenic Assay. Two hundred cells were seeded in 60-mm dishes with the medium containing various concentrations of ADM and/or 5 x 10^-4 M DP and maintained under the condition described above. Two wk later, colonies were determined. Clusters of more than 0.5 mm in diameter were counted as one colony, and usually the control dishes contained 130 to 150 colonies.

Adriamycin Uptake to HeLa Cells. HeLa cells (2.0 x 10^5) were exposed simultaneously to ADM (0.74 x 10^-7 M; 0.4 μg/ml) and DP (5 x 10^-4 M) for 1 to 24 h, under the same conditions described above. After the indicated time, the cells were harvested, washed 3 times with cold PBS, and centrifuged. The pellet was dissolved in 1 ml of PBS, and the intracellular level of ADM was determined, using HPLC (22). The cell suspension was sonicated for 5 s and extracted with a mixture of butanol and toluene (1/1, v/v). The extract was evaporated and the residue dissolved with a mixture of PBS and methanol (1/1, v/v). An aliquot of this solution was injected onto a reversed-phase ODS column filled with TSK gel:ODS-120A (Toyo Soda Co., Japan) and eluted with a mixture of 1 N formic acid and methanol (55/45, v/v). The fluorescence signal was monitored at 470 nm of excitation and 585 nm of emission (Model 204 fluorometer; Hitachi, Japan). Each experiment was performed in triplicate.

Incorporation of ADM into the cells pretreated with DP was also investigated. After incubation with DP (5 x 10^-4 M) for 24 h, the cells were washed 3 times with PBS, fresh medium containing ADM was added, and the preparation was incubated for a further 24 h prior to harvest. Treatment of the cells and analysis with HPLC were done in the same way as described above.

ADM Excretion from HeLa Cells. After exposure to ADM at the concentration of 0.74 x 10^-7 M (0.4 μg/ml) for 3 h, the cells were rinsed 3 times and incubated in fresh medium, with or without 5 x 10^-4 M DP, for 24 h. Intracellular levels of ADM were determined using HPLC as described above.

Effects on S-180 Solid Tumor. Six-wk-old ddY mice were housed under conditions of constant temperature and humidity. To investigate the effect of ADM and DP on an experimental tumor, 5 x 10^4 murine S-180 ascites cells were implanted s.c. into these mice on Day 0, and the mice were treated with various doses of ADM s.c. and/or DP i.p. from Days 1 to 8. On Day 9, the mice were killed, and tumor and body weights were recorded. Ten mice were included in one group.

Effects on Survival. Five x 10^3 S-180 cells were implanted i.p. into ddY mice on Day 0. ADM (0.5 mg/kg) and/or DP (100 mg/kg) was given i.p. on Days 3, 6, and 9. Ten mice were included in one group, and the survival time was recorded.

Statistical Analysis. Differences in tumor weights between the groups on combination therapy and those of single use of ADM were analyzed.
by Student's t test. The mean survival periods of S-180-bearing mice were compared using the generalized Wilcoxon test.

RESULTS

Inhibition of Cell Growth. When the cytotoxic effect of ADM combined with DP against HeLa cells was assessed using the dye exclusion method, ADM inhibited cell growth in a dose- and time-dependent fashion. DP (0.05, 0.5, or 5 × 10^{-6} M) did not affect cell growth, but these doses did enhance the cytotoxicity of ADM in a dose-dependent manner. At the ADM concentration of 185 × 10^{-6} M, the cell number decreased from 4.5 × 10^{4} to 0.4 × 10^{4} in the presence of DP at 5 × 10^{-6} M for 72 h (Fig. 1). The enhancement of ADM by DP was evident with all concentrations of ADM examined, and the 50% effective concentration of ADM, the concentration which killed 50% of the cells, decreased from 22.1 to 9.2 × 10^{-6} M by DP of 5 × 10^{-6} M (Fig. 2).

Clonogenic Assay. The effect of ADM on colonies of HeLa cells was determined. ADM inhibited the colony formation dose dependently. DP of 5 × 10^{-6} M augmented the inhibitory effect of ADM logarithmically, and the percentage of survival of colonies was decreased 10-fold lower than that seen with ADM alone, at the ADM concentration of 120 × 10^{-6} M (Fig. 3).

Adriamycin Uptake and Excretion of HeLa Cells. The uptake of ADM into HeLa cells determined with HPLC is shown in Fig. 4. The ADM concentration (0.74 × 10^{-6} M) corresponds to the peak human plasma concentration (23). ADM was incorporated into the HeLa cells time dependently. Combined with DP (5 × 10^{-6} M) the ADM uptake was increased 1.5-fold higher than that of ADM alone during the initial 24 h after administration (Fig. 4). The increase of ADM uptake was also observed in the case of fresh medium following pretreatment with DP for 24 h. The effect of DP was seen at both 0.74 and 7.4 × 10^{-6} M ADM (Fig. 5). As the medium was fresh, the change in membrane transport of ADM was due to the pretreated DP and not to the combined effect of ADM and DP on the cell membrane. On the other hand, the efflux of ADM was so slow that the 65% initial intracellular level of ADM was preserved at 24 h in the fresh medium. Although a larger amount of ADM tended
Effects of a combination of ADM and DP on S-180 solid tumors

Five \(10^4\) S-180 ascites cells were implanted s.c. in ddY mice, and ADM and/or DP was given for 8 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor wt (mg)</th>
<th>Body wt change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>660 ± 260°</td>
<td>111</td>
</tr>
<tr>
<td>DP (75 mg/kg)</td>
<td>665 ± 230</td>
<td>109</td>
</tr>
<tr>
<td>DP (100 mg/kg)</td>
<td>640 ± 290</td>
<td>105</td>
</tr>
<tr>
<td>ADM (0.5 mg/kg)</td>
<td>410 ± 150°</td>
<td>112</td>
</tr>
<tr>
<td>ADM (0.5 mg/kg) + DP (100 mg/kg)</td>
<td>240 ± 90°</td>
<td>103</td>
</tr>
<tr>
<td>ADM (1.0 mg/kg)</td>
<td>385 ± 110°</td>
<td>91</td>
</tr>
<tr>
<td>ADM (1.0 mg/kg) + DP (75 mg/kg)</td>
<td>280 ± 40°</td>
<td>98</td>
</tr>
<tr>
<td>ADM (1.0 mg/kg) + DP (100 mg/kg)</td>
<td>290 ± 75°</td>
<td>95</td>
</tr>
</tbody>
</table>

The mean body weights on Day 9, expressed as the percentage of that on Day 0 in each group.

The mean ± SD of tumor weights in each group on Day 9.

*°* P < 0.05 between each treatment group.

Table 2: Effects of a combination of ADM and DP on S-180 solid tumors

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</tbody>
</table>

The mean body weights on Day 9, expressed as the percentage of that on Day 0 in each group.

The mean ± SD of tumor weights in each group on Day 9.

*°* P < 0.05 between each treatment group.

Fig. 5. Uptake of ADM into HeLa cells after pretreatment with DP. Following pretreatment with DP for 24 h, the medium was replaced with fresh medium, and incorporation of ADM into the HeLa cells was determined, as described in Fig. 4. □, ADM (0.74 × 10\(^{-4}\) m) without pretreatment of DP (5 × 10\(^{-4}\) m); △, ADM (0.74 × 10\(^{-4}\) m) with pretreatment of DP (5 × 10\(^{-4}\) m); ○, ADM (7.4 × 10\(^{-4}\) m) without pretreatment of DP (5 × 10\(^{-4}\) m); ◇, ADM (7.4 × 10\(^{-4}\) m) with pretreatment of DP (5 × 10\(^{-4}\) m); bars, SE.

Fig. 6. Intracellular retention of ADM in HeLa cells. After exposure to ADM (0.74 × 10\(^{-4}\) m) for 3 h, the cells were washed, and fresh medium, with or without DP (5 × 10\(^{-4}\) m), was added for a further 24 h. The intracellular level of ADM was determined as described in Fig. 4. ○, without 5 × 10\(^{-4}\) m DP; ◇, with 5 × 10\(^{-4}\) m DP; bars, SE.

Table 1: Relationship between the extracellular concentration of DP and intracellular level of ADM in uptake and efflux studies

<table>
<thead>
<tr>
<th>ADM level (10(^{-11}) g/cell)</th>
<th>DP × 10(^{-4}) m</th>
<th>0</th>
<th>0.05</th>
<th>0.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake</td>
<td>9.8 ± 1.3°</td>
<td>10.7 ± 0.8</td>
<td>13.2 ± 0.6</td>
<td>14.7 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Efflux</td>
<td>6.3 ± 0.5</td>
<td>6.1 ± 0.4</td>
<td>6.2 ± 0.7</td>
<td>6.1 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

*°* Intracellular level of ADM determined with the HPLC method.

The relationship between the extracellular concentration of DP and cellular uptake or efflux of ADM is shown in Table 1. After 5-h incubation with DP and ADM (0.74 × 10\(^{-4}\) m), the ADM uptake was increased in proportion to the extracellular concentration of DP. However, the intracellular level of ADM was not influenced by the concentration of DP, when determined at 5 h after exchanging the medium.

Fig. 7. Effects on survival of ddY mice bearing S-180 ascites tumor. Five \(10^4\) S-180 cells were implanted i.p. into ddY mice on Day 0. ADM (0.5 mg/kg) and/or DP (100 mg/kg) was given i.p. on Days 3, 6, and 9 (1). a, control; b, 100 mg/kg DP; c, 0.5 mg/kg ADM; d, 0.5 mg/kg ADM plus 100 mg/kg DP.

Fig. 8. Survival curves of ddY mice bearing S-180 solid tumors. Survival curves are presented for mice treated with ADM and/or DP. a, control; b, DP (100 mg/kg); c, ADM (0.5 mg/kg); d, ADM (0.5 mg/kg) + DP (100 mg/kg). Bars, SE.

DISCUSSION

We found that the nontoxic level of DP (5 × 10\(^{-4}\) m) enhanced the cytotoxicity of ADM, in all concentrations tested, against...
Potentiation of ADM cytotoxicity by DP in vitro and in vivo

HeLa cells in vitro. The possible mechanisms of this synergistic effect are: (a) inhibition of nucleoside salvage by DP; (b) enhancement of cytotoxicity of ADM with increased uptake of the drug by DP; and (c) interaction of ADM and DP on the cell membrane.

(a) DP is an inhibitor of membrane nucleoside transport in a variety of cultured cell lines (1–3), and this property has been utilized to enhance the cytostatic action of antimetabolites by preventing nucleoside salvage and depletion of nucleoside pools (4–7). This inhibition of nucleoside salvage by DP may also be operative in the case of ADM. As uridine uptake into HeLa cells is decreased in the presence of DP (1), this possibility cannot be ruled out. According to Grem and Fischer (8), however, nucleoside salvage in vitro did not play an important role in modulating the cytotoxicity of 5-FU, although the incorporation of thymidine or uridine was prevented by DP. They found that the output of fluorodeoxyuridine monophosphate, an active metabolite of 5-FU, was decreased by DP (24). From the point of view of the cytoidal mechanism of ADM, de novo synthesis of nucleosides tends to be preserved. Johnston and Glazer (25) found no correlation between the cytotoxicity of anthracyclines and the inhibition of nucleic acid synthesis or the efflux of nucleosides. Even if DP does block nucleoside transport, the cytoidal effect of anthracyclines might not be influenced significantly because of the preserved de novo pathway. The mechanism of synergism can hardly be explained only from the standpoint of blockade of the salvage pathway.

(b) While the increase of ADM uptake by DP was apparent, the efflux rate was not significantly influenced. The mechanism of action of ADM has been attributed to the intercalation with DNA and the interference with the template activity necessary for DNA replication and RNA synthesis (11–13), the damage or breakage of DNA by bioreductive alkylation (14), or the generation of reductive drugs and oxygen free radicals (15–17). It may be that the increased intracellular levels of ADM, as induced by DP, may enhance these mechanisms. ADM-resistant cells exhibited an enhanced active efflux and a lower intracellular retention of ADM (26, 27). On the contrary, it has been reported that the cytotoxicity of anthracyclines does not directly correlate with influx, efflux, or intracellular accumulation (28–30). Thus, while the relationship between intracellular levels and cytoidal effect of ADM is not clear, our findings do suggest that there is an enhancement of the cytotoxicity of ADM with increase in uptake of the drug. Since the uptake of ADM correlates with its passive diffusion (28, 31), DP might affect the structure of the membrane and alter the permeability to ADM.

(c) It has been reported that ADM could be cytotoxic without entering cells and that the cell membrane is one of the targets of its cytoidal effect (18–20). As DP can also alter membrane permeability to various substances, such as phosphate and sugars (32), it is feasible that the synergism between ADM and DP is due to a combined damage of the cellular surface. However, according to the ADM uptake in fresh medium after preincubation with DP (Fig. 5), this change of membrane might be induced nontoxicly by DP without the coordination of ADM and be preserved for several hours. Our findings show little correlation between the synergism and the membrane toxicity of ADM and DP.

We observed an enhanced inhibition of tumor growth and prolonged survival when combining ADM and DP. Despite its dramatic effect in vitro, there have been few reports on the usefulness of the combination of antimetabolites and DP in vivo (6). This may relate to the insufficient blockade of nucleoside salvage by DP in vivo. According to Moyer et al. (33), DP (100 mg/kg) did not significantly reduce the salvage of uridine by the liver or kidney of mice and produced only a small, transient reduction of salvage of uridine by L1210 tumors. In a clinical study of PALA and DP (9), the human plasma uridine concentration decreased after DP treatment. If DP had blocked the cellular uptake of uridine, the plasma uridine might have increased. Thus, it is necessary to consider effects of DP on the somatic metabolism of nucleosides, especially in the liver.

On the other hand, it is feasible that DP enhances the cytotoxicity of ADM in vivo as a result of increased cellular uptake of ADM as well as in vitro. From our data and those of others (28, 29), the cellular uptake of ADM is rapid, despite a long retention. If ADM is administered together with DP, an earlier accumulation and a longer retention can be obtained. A rapid incorporation of ADM by DP might prevent an excess load of ADM, necessary for its cytoidal effect, and side effects would be minimized. There was little effect of the combination therapy on the body weight of mice. The side effects in vivo and clinically are being given attention.

Acknowledgments

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References

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