A Nelarabine-resistant T-Lymphoblastic Leukemia CCRF-CEM Variant Cell Line Is Cross-resistant to the Purine Nucleoside Phosphorylase Inhibitor Forodesine

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Abstract. Background/Aim: Forodesine inhibits purine nucleoside phosphorylase, resulting in an accumulation of intracellular dGTP and consequently cell death. 9-β-D-Arabinofuranosylguanine (ara-G) is an active compound of nelarabine that is intracellularly phosphorylated to a triphosphate form, which inhibits DNA synthesis. Both agents show cytotoxicity toward T-cell malignancies. In the present study, we investigated the cytotoxicity of forodesine in vitro using ara-G-resistant leukemia cells. Materials and Methods: T-Lymphoblastic leukemia cell line CCRF-CEM and ara-G-resistant CEM variant cell line CEM/ara-G that we had previously established were used. Results: A growth-inhibition assay demonstrated that CEM cells were insensitive to single-agent forodesine treatment. The cells were also insensitive to deoxyguanosine at a maximal concentration of 10 μM. CEM/ara-G cells were 80-fold more resistant to ara-G than were CEM cells, and the mode of sensitivity to forodesine and deoxyguanosine was similar to that of CEM cells. In the presence of 10 μM deoxyguanosine, forodesine effectively inhibited the growth of CEM cells but not that of CEM/ara-G cells. Flow cytometric analyses showed that combination of forodesine and deoxyguanosine induced apoptosis of CEM cells but not of CEM/ara-G cells. The addition of ara-G did not augment the cytotoxicity of the forodesine/deoxyguanosine combination towards CEM cells or CEM/ara-G cells. The combination index revealed antagonism between forodesine and ara-G. The intracellular production of ara-G triphosphate was reduced in the presence of forodesine. Conclusion: Nelarabine-resistant CEM/ara-G cells are insensitive to forodesine.

Key Words: Forodesine, deoxyguanosine, nelarabine, ara-G, ara-GTP, leukemia.

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Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) catalyzes the phosphorolysis of 2-ribonucleosides and 2-deoxyribonucleosides (inosine, deoxyinosine, xanthosine, deoxyxanthosine, guanosine, and deoxyguanosine) to the corresponding base and sugar-1-phosphate (1-3). When PNP is inhibited, deoxyguanosine accumulates in the cells. Deoxyguanosine is then phosphorylated to deoxyguanosine monophosphate by deoxycytidine kinase and deoxyguanosine kinase and is finally phosphorylated to deoxyguanosine triphosphate (dGTP). The overproduction of dGTP in cells perturbs the deoxyribonucleotide pool, inhibits DNA synthesis, and ultimately induces cell death (1-6). The T-cell population is markedly reduced in patients with congenital PNP deficiency syndrome, suggesting that inhibition of PNP may be selectively cytotoxic toward T-cell malignancies (1-6). Forodesine (immucilin H, BCX-1777) is a highly specific and potent inhibitor of PNP (7-9). This agent has been investigated in clinical trials for patients with relapsed or refractory T-cell malignancies, including peripheral T-cell lymphoma (10).

Nelarabine, 2-amino-9-β-D-arabinofuranosyl-6-methoxy-9H-purine, is a relatively new purine nucleoside analog used for the treatment of T-cell malignancies, including T-lymphoblastic leukemia and lymphoma (11). Nelarabine is de-methylated to the active compound 9-β-D-arabinofuranosylguanine (ara-G) in the plasma and is taken up by leukemia cells via the nitrobenzylthioinosine-sensitive nucleoside membrane transporter equilibrative nucleoside transporter 1 (12). Ara-G is then phosphorylated to ara-G monophosphate by the deoxycytidine kinase and deoxyguanosine kinase. It is then further phosphorylated to its triphosphate form, ara-G triphosphate (ara-GTP). Ara-GTP competes with dGTP for DNA polymerase and is subsequently incorporated into DNA, resulting in the termination of DNA synthesis (13-16).

Both forodesine and nelarabine are used in the treatment of refractory or relapsed T-cell malignancies (17, 18). The present study was conducted to investigate the cytotoxic effect of forodesine on cultured T-lymphoblastic leukemia cells in the context of cellular sensitivity to nelarabine in
vitro. For this purpose, the T-lymphoblastic leukemia cell line CCRF-CEM and an ara-G-resistant CEM variant cell line that had previously been established in our laboratory were used (19). The effects of forodesine were further examined in other cell lines including myeloid and B-lymphoblastic cells (20).

Materials and Methods

Chemicals and reagents. Forodesine was kindly supplied by Mundipharma Research Limited (Cambridge, UK) and was dissolved in water. Ara-G was purchased from R.I. Chemicals (Orange, CA, USA) and was dissolved in dimethyl sulfoxide. Deoxyguanosine was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide. All other chemicals were of analytical grade.

Cell culture and drug treatment. Human T-lymphoblastic leukemia CCRF-CEM cells, ara-G-resistant CEM variant cells (CEM/ara-G) developed previously in our laboratory (19), myeloid leukemia HL-60 cells, cytarabine-resistant HL-60 variant cells (HL/ara-C20) (20), and B-lymphoma DB cells were cultured in RPMI-1640 media supplemented with 10% fetal calf serum. The cells (2×10^6 cells/ml, 10 ml) were incubated at 37°C with different concentrations of drugs for different time periods (10 nM – 1 mM). The cells were then washed twice with PBS and centrifuged (500 × g, 5 min, 4°C) to collect the cell pellet.

Proliferation assay. Growth-inhibitory effects were determined by the sodium 3-(1-[(phenylamino)-carbonyl-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. The assay was performed according to the manufacturer’s instructions (Roche, Indianapolis, IN, USA) with slight modifications (20, 21).

Quantitation of apoptotic cell death. To evaluate cytotoxicity, propidium iodide (Beckman Coulter, Fullerton, CA, USA) and flow cytometry were performed after 96-h treatment with a cytotoxic agent. Apoptotic cell death was defined as the sub-G1 cell population.

Calculation of the combination index (CI). CI analysis provides quantitative information on the nature of drug interactions, especially on the combined effects that occur with the use of two different agents. The CI method is based on that described by Chou and Talalay (23), and the CI values were determined using CalcuSyn (version 2.0) computer software (Biosoft, Great Shelford, Cambridge, UK). CI values of less than 1, 1, and more than 1 indicate synergy, additivity, and antagonism, respectively (23).

Determination of intracellular ara-GTP concentrations. The concentration of the intracellular active metabolite of ara-G, ara-GTP, was determined using high-performance liquid chromatography (HPLC) as described previously (24). Briefly, ara-G-treated cells were centrifuged to collect the cell pellet (400 x g, 10 min, 4°C). The acid-soluble fraction, which contains the nucleotide pool, was extracted from the sample, and subjected to HPLC using a TSK gel DEAE-25W column (length, 250 mm; internal diameter, 4.6 mm; Tosoh, Tokyo, Japan) and a 0.06 M Na_2HPO_4 (pH 6.9)–20% acetonitrile buffer. The elution was performed at a constant flow rate of 0.7 ml/min at ambient temperature. The ara-GTP peak was identified by its retention time and was quantitated from its peak area at an absorbance of 254 nm.

Statistical analyses. Statistical analyses and graph generation were performed with GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA, USA).

Results

Forodesine is cytotoxic to CEM cells but not to ara-G-resistant CEM/ara-G cells. CEM/ara-G cells were 80-fold more resistant to ara-G than were CEM cells (Table I) (Figure 1A). The growth-inhibition tests demonstrated that CEM cells were insensitive to forodesine as a single-agent (Figure 1B). The cells were also insensitive to the maximum concentration of deoxyguanosine, which was 10 μM (Figure 1C). The mode of the drug sensitivity in CEM/ara-G cells was similar to that in CEM cells (Figures 1B and C). In the presence of 10 μM deoxyguanosine, which mimics the physiological deoxyguanosine concentration, forodesine effectively inhibited the growth of CEM cells (Figure 1D). However, the combination of forodesine and deoxyguanosine was unable to inhibit the growth of CEM/ara-G cells (Figure 1D).

Forodesine is not cytotoxic to myeloid leukemia HL-60 cells or B-cell lymphoma cells. The anti-leukemic effect of forodesine in combination with deoxyguanosine was also evaluated in non-T-cell malignant cell lines, including myeloid leukemia HL-60 cells, cytarabine-resistant HL-60 variant cells (HL/ara-C20) (20), and B-cell lymphoma DB cells. Overall, neither forodesine-alone nor its combination with deoxyguanosine was effective at inhibiting cell growth of myeloid cells (Figures 2A-D) and B-cell malignant cells (Figures 3A-C), suggesting a T-cell-specific action for forodesine.

The combined effect of forodesine and ara-G. Because both forodesine and nelarabine target T-cell malignancies, the effect of a combined treatment with forodesine and ara-G was evaluated in CEM cells and CEM/ara-G cells. CEM cells were treated with forodesine and deoxyguanosine with and without ara-G. We found that the growth-inhibitory effects of forodesine and deoxyguanosine were not augmented by the addition of ara-G in CEM cells (Figure 4A). CEM/ara-G cells were still insensitive to forodesine plus ara-G (Figure 4B). The cytotoxic effects were determined by examining apoptotic cell death in response to each treatment condition. When CEM cells were treated with forodesine in the presence of deoxyguanosine, the induction of apoptosis was greater than the one observed with forodesine alone (Figure 4C). The further addition of ara-G did not increase apoptotic cell death (Figure 4C). Apoptosis was not induced under these same treatment conditions in CEM/ara-G cells (Figure 4D). To further investigate the
effect of the forodesine and ara-G combination, the CI was calculated. This was done by incubating CEM cells with both forodesine and ara-G at equimolar concentrations for 72 h followed by the XTT assay. The 50%-growth inhibitory concentration (IC50) value was 2.8 μM, and the CI value was 1.355 (Figure 5). This suggested that forodesine and ara-G have antagonistic effects on growth inhibition.

Intracellular ara-GTP production in the presence of forodesine. The cytotoxicity of ara-G depends on the intracellular concentration of ara-GTP. To determine if forodesine treatment modulates the intracellular production of ara-GTP, CEM cells were incubated with ara-G in the presence or absence of forodesine and deoxyguanosine (Figure 6). The ara-GTP production was significantly reduced by the addition of forodesine (74.0 pmol/10^7 cells without forodesine, 37.1 pmol/10^7 cells with forodesine) \((p=0.003\text{, Mann–Whitney})\), which might be responsible for the antagonism between ara-G and forodesine. The mechanisms of this reduced ara-GTP production were not elucidated in the present study.

Discussion

Both forodesine and ara-G are chemotherapeutic agents that target T-cell malignancies. Both agents use the purine salvage pathway to exert their cytotoxic effects. Therefore, it is important to compare the cytotoxicity of these agents, to determine if any cross-resistance exists, and to examine the effects of the combined treatment with forodesine and
The present study was conducted to determine the anti-leukemic effects of forodesine using T-cell leukemia cells in the context of ara-G resistance. This study demonstrated the T-cell-specific cytotoxicity of forodesine and ara-G (Figures 2-4). Forodesine and ara-G were equally cytotoxic to CEM cells, and cross-resistance was observed between these two agents (Table I) (Figure 1). Moreover, the combined treatment growth inhibition experiments revealed that forodesine and ara-G had antagonistic effects (Figure 5). This might be due to the reduced production of ara-GTP in the presence of forodesine (Figure 6).

Bantia et al. reported that forodesine in the presence of deoxyguanosine inhibited the proliferation of CEM cells with an IC50 of 0.015 μM (9). This inhibition was accompanied by elevated dGTP and dATP levels. Deoxycytidine and lamivudine completely and partially reversed this inhibition, respectively. Importantly, deoxycytidine rescued the dGTP and dATP levels, dATP levels, but not dGTP levels, remained increased upon lamivudine treatment, suggesting that the accumulation of dGTP is critical to the cytotoxic activity of forodesine. Furthermore, when CEM cells were treated with the combination of forodesine (1 μM) and deoxyguanosine (10 μM), annexin V staining revealed significant apoptosis. In contrast, both agents alone did not induce apoptosis. Compared to our results, the IC50 value was low in this previous study. However, these authors determined cell

Figure 2. The growth-inhibitory effects of forodesine (A), deoxyguanosine (dGuo) (B) and 1 μM or 10 μM forodesine in the presence of 10 μM deoxyguanosine (C, D) on HL-60 cells and HL/ara-C20 cells. Proliferation was determined using the XTT assay after a 72-h incubation. The values are the means ± SD of at least three independent experiments. The IC50 values for dGuo were 360 μM in HL-60 cells and 330 μM in HL/ara-C20 cells.
proliferation using a Coulter counter and tritiated thymidine incorporation in contrast to the XTT assay, which was employed here. For the analysis of apoptosis, the experimental conditions and the results obtained were comparable to ours. Huang et al. investigated the mechanisms behind cell sensitivity and resistance to forodesine and deoxyguanosine (25). Tritiated forodesine transport assays demonstrated that the equilibrative nucleoside transporters facilitated the uptake of forodesine in CEM cells, whereas tritiated deoxyguanosine uptake was primarily dependent upon concentrative nucleoside transporters. It was also suggested that deoxycytidine kinase, not deoxyguanosine kinase, was the rate-limiting enzyme for the phosphorylation of deoxyguanosine. Because ara-G is transported into leukemic cells via equilibrative nucleoside transporter 1 and is subsequently phosphorylated to ara-GTP by deoxycytidine kinase and deoxyguanosine kinase, it would be reasonable to expect cross-resistance between ara-G and forodesine. Homminga et al. investigated the sensitivity to forodesine and ara-G in vitro in diagnostic samples of 96 patients with pediatric leukemia (26). The mRNA expression levels of the membrane transporters and kinases that are involved in nucleoside metabolism were also examined (26). The cytotoxic effects of forodesine and ara-G were higher in T-cell acute lymphoblastic leukemia samples than in B-cell precursor lymphoblastic leukemia and acute myeloid leukemia samples. However, no correlation was found between forodesine-deoxyguanosine cytotoxicity and ara-G cytotoxicity (26). The sensitivity of leukemia cells to forodesine/deoxyguanosine was associated with higher deoxyguanosine kinase levels and an increased accumulation of intracellular dGTP (26). Equilibrative nucleoside transporters were not associated with forodesine sensitivity. The sensitivity of leukemic cells

Table I. Drug sensitivity.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CEM IC50 (μM)</th>
<th>CEM/ara-G IC50 (μM)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-G</td>
<td>2.5</td>
<td>200</td>
<td>80</td>
</tr>
<tr>
<td>Forodesine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dGuo</td>
<td>35</td>
<td>47</td>
<td>1.3</td>
</tr>
<tr>
<td>Forodesine (+10 μM dGuo)</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Forodesine+ara-G (+10 μM dGuo)</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

CEM and CEM/ara-G cells were incubated with different concentrations of ara-G (9-β-D-arabinofuranosylguanine), forodesine, deoxyguanosine (dGuo), alone and in combination for 72 h. The IC50 (50%-growth inhibitory concentration) was then determined with the XTT assay. The relative resistance (RR) was obtained by dividing the IC50 value for CEM/ara-G cells by that for CEM cells. -: The IC50 value was not determined due to the concentration being beyond the upper detection limit (1 mM).

Figure 3. The growth-inhibitory effects of forodesine (A), deoxyguanosine (dGuo) (B) and 1 μM or 10 μM forodesine in the presence of 10 μM deoxyguanosine (C) on DB cells. Proliferation was determined using the XTT assay after a 72-h incubation. The values are the means±SD of at least three independent experiments. The 50%-growth inhibitory concentration value for dGuo was 440 μM in DB cells.

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to ara-G was associated with levels of nucleoside transporters (26). Moreover, forodesine and ara-G were not antagonistic with regard to cytotoxicity (26). The findings reported by Homminga et al. are not in accordance with our results or those of Huang et al. There are several explanations for this discrepancy. Some differences might be due, in part, to the inclusion of non-T-cell malignant samples in Homminga et al.'s study. The discrepancy might also be attributable to the differences between cultured cell lines and primary samples. Ara-G resistance in cultured CEM/ara-G cells might be higher than in clinical samples. Additionally, it may be difficult to assess the cytotoxicity of cell cycle-specific anti-metabolites in patients' leukemia cells in vitro, as these cells are unlikely to proliferate readily in culture after harvesting.

In conclusion, the present study revealed that forodesine was specifically cytotoxic against malignant T-cells; however, cells that were resistant to another T-cell specific anti-metabolite, ara-G, were insensitive to forodesine. Forodesine and ara-G co-treatment resulted in antagonistic effects on the cell viability. Although these findings were obtained from cultured cell lines and cannot directly be applied to the clinic,
the benefits of forodesine should be carefully considered when treating patients with T-cell malignancies that are resistant to nelarabine-based chemotherapy.

Disclosure Statement

The Authors have nothing to disclose concerning any of the drugs or agents used in this study.

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References


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