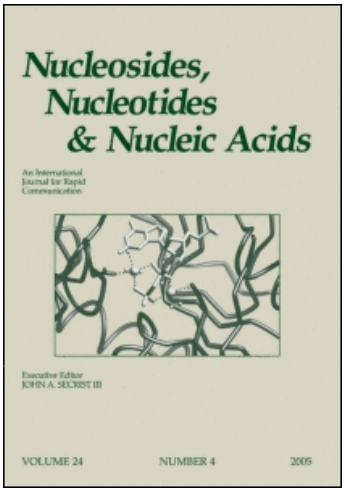

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Increased Cytotoxicity of 2′,2′-Difluoro-2′-Deoxycytidine in Human Leukemic Cell-Lines After a Preincubation with Cyclopentenyl Cytosine

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ABSTRACT

The in vitro modulating effect of Cyclopentenyl cytosine (CPEC) on the metabolism of gemcitabine was studied in lymphocytic and myeloid leukemic cell-lines. In MOLT-3 cells, that were pretreated with CPEC, the incorporation of 2′,2′-difluoro-2′-deoxycytidine triphosphate (dFdCTP) into DNA was significantly increased by 57–99% in comparison with cells that were only treated with gemcitabine. The increased incorporation of dFdCTP into DNA in CPEC pretreated cells was paralleled by an increase in apoptotic and necrotic cells of 17–34%. In HL-60 cells that were preincubated with CPEC, increased concentrations of the mono-/di- and triphosphate form of gemcitabine were observed, as well as an increased incorporation of dFdCTP into DNA (+773%). This increased incorporation was paralleled by a significant increase in apoptosis and necrosis. We conclude that CPEC enhances the

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incorporation of dFdCTP into DNA and thus increases the cytotoxicity of gemcitabine in lymphocytic and myeloid leukemic cell-lines.

Key Words: Cyclopentenyl cytosine; CPEC; Gemcitabine; Leukemia; CTP synthetase.

INTRODUCTION

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC) proved to have in vivo cytotoxic activity against solid tumours and hematological malignancies.[1–4] The cytotoxicity of gemcitabine depends amongst others on the phosphorylation to dFdCTP and on the incorporation of dFdCTP into DNA. dCTP may inhibit the phosphorylation of dFdC by feedback inhibition of deoxycytidine kinase (dCK) and may decrease the incorporation of dFdCTP into DNA by competition for DNA polymerase. Cyclopentenyl cytosine (CPEC) is a cytidine analogue that inhibits CTP synthetase inducing a depletion of CTP and dCTP in leukemic cell-lines and in bone marrow samples of children with acute leukemia.[5] CPEC is currently being studied in a phase II single drug clinical trial in patients with relapsed acute myeloid and lymphocytic leukemia and may be useful in future combination regimens. The combinations of CPEC and various other nucleoside analogues have been studied in vitro in our laboratory on leukemic and neuroblastoma cell-lines. We previously showed in leukemic cell-lines that CPEC could enhance the phosphorylation of arabinofuranosyl cytosine (araC), the incorporation of araCTP into DNA, as well as the cytotoxicity of araC.[6,7] We hypothesized that in leukemic cell-lines the cytotoxicity of dFdC could be enhanced by a preincubation with CPEC.

MATERIALS AND METHODS

MOLT-3 and HL-60 cells were preincubated for 24 h with 6.25 and 12.5 nM of CPEC (kindly provided by the National Cancer Institute, Bethesda, U.S.A.). Subsequently, 0–50 nM [^{3}H]dFdC was added for 4 h. [^{3}H]dFdC metabolites were quantified by anionic exchange HPLC after neutralization of perchloric acid extraction and in DNA of harvested leukemic cells as reported previously.[6] DNA synthesis was assessed by analyzing the [^{14}C]thymidine incorporation into DNA.[6] Apoptosis and necrosis were assessed by flowcytometry with FITC-labeled-Annexin V and propidium iodide[6] after a 24 h preincubation with 12.5–37.5 nM of CPEC and a subsequent coincubation with 3–12 nM of gemcitabine for 24 h.

The differences in concentrations of dFdC-metabolites, in incorporation of dFdCTP into DNA and in percentages of apoptotic and necrotic cells caused by a pre-treatment with CPEC were analyzed by the t-test for paired samples, using SPSS 10.0.7 software.

RESULTS

In MOLT-3 cells no significant alterations were observed in concentrations of dFdCMP, dFdCDP or dFdCTP after a preincubation with 12.5 nM of CPEC and a

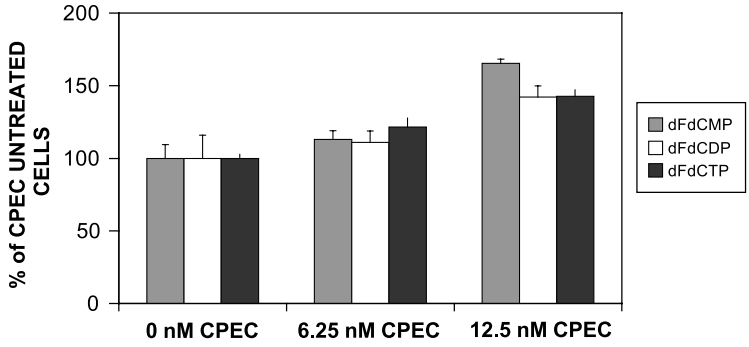

Figure 1. dFdC metabolites in HL-60 after CPEC preincubation and 3 nM dFdC coincubation.

coincubation with 3 and 32 nM of dFdC. However, the incorporation of dFdCTP into DNA was significantly increased in the CPEC pretreated samples by 99 ± 4% (mean relative increase ± SD) and 57 ± 2% using 3 and 32 nM of dFdC, respectively ($p = 0.001$). The percentage of apoptotic and necrotic cells increased relatively by 17, 34 and 27% after 37.5 nM of CPEC and 3, 6 and 12 nM of dFdC respectively compared to the cumulative effect of both single drugs. In HL-60 cells significantly increased concentrations of dFdCMP and dFdCTP of 65 and 43% were observed in the samples (pre)treated with 12.5 nM of CPEC and 3 nM of dFdC as compared with dFdC treated samples (Fig. 1). The increase in dFdCDP concentrations (+42%) was not significant. Under these conditions, the incorporation of dFdCTP into DNA was significantly increased by 773 ± 38%, reducing the synthesis of DNA to 30% of the DNA synthesis in the samples that were not pretreated with CPEC. The synthesis of DNA was also more decreased than in the samples that were only treated with CPEC.

The percentage of apoptotic and necrotic cells increased relatively by 278 and 67% after a preincubation with 12.5 nM of CPEC using 6 and 12 nM of dFdC (Fig. 2, $P < 0.01$). Moreover, the 16% of apoptotic/necrotic cells that were observed after a pre/coincubation with 12.5 nM of CPEC and 6 nM of dFdC (which as single drugs hardly induced any apoptosis) was more than the apoptosis/necrosis that was observed

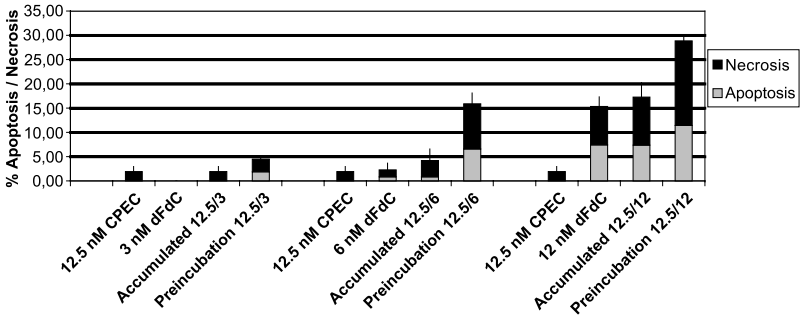

Figure 2. FACS analysis of HL-60 after 24 hours preincubation with CPEC and 24 hours with dFdC.

using 12 nM of dFdC as a single drug (Fig. 2), suggesting a synergistic effect of CPEC and dFdC.

DISCUSSION

A preincubation with low concentrations of CPEC increases the incorporation of dFdCTP into DNA in T-lymphocytic or myeloid leukemic cells, which is paralleled by a decreased synthesis of DNA. Moreover, a significant increase of apoptosis and/or necrosis was observed in the samples that were treated with CPEC and dFdC as compared with the samples that were treated with single drugs. The extent of this increase in apoptosis/necrosis is in favour of a synergistic effect between CPEC and gemcitabine. We observed a stronger effect of CPEC on gemcitabine cytotoxicity in the myeloid HL-60 leukemic cell-line, as compared with the lymphocytic MOLT-3 leukemic cell-line.

Our results form a rationale for a combination therapy of CPEC and gemcitabine in (relapsed) leukemia and warrant further (pre)clinical developments of this concept, which might be especially interesting for acute myeloid leukemia, for which gemcitabine has entered into clinical trials.[8]

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