Abstract

Background and Objective. Fludarabine has shown a definite clinical activity in B-cell chronic lymphocytic leukemia (CLL). If the effects of this drug could be potentiated, it could be useful in order to obtain complete remissions. In this study we evaluated the effects of the combination of fludarabine and gemcitabine, a deoxycytidine analog that has shown both in vitro and in vivo activity against a variety of solid tumors.

Design and Methods. CLL cells from 10 patients were cultured in vitro in the presence of fludarabine (0.5-1,000 µg/mL) and gemcitabine (0.1-5,000 µg/mL), both alone and in different combinations. Cytotoxic activity was tested by the XTT colorimetric assay. Furthermore we evaluated BCL-2 protein expression and, subsequently, the induction of apoptosis at baseline and after exposing cells to different concentrations of fludarabine and gemcitabine.

Results. The IC50 of fludarabine and gemcitabine on CLL cells was 550 and 1,100 µg/mL, respectively, in our series of samples; the cytotoxicity of either drug was not influenced by the percentage of BCL-2 positive cells in the same sample. The addition of gemcitabine increased fludarabine-induced cytotoxicity; however, isobologram analysis of the data showed synergism only when lower doses of gemcitabine were combined to fludarabine. Induction of apoptosis reflected this pattern of activity.

Interpretation and Conclusions. Gemcitabine was able to increase the activity of fludarabine only when low doses of the former were employed. As both compounds incorporate into DNA blocking chain elongation, our results could be explained by the drugs interfering at that level. The possibility of potentiating the effects of fludarabine with low doses of gemcitabine renders this combination promising in view of an in vivo use.

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Key words: fludarabine, gemcitabine, CLL, apoptosis
sedimentation on Ficoll Hypaque (Lymphoprep, Nycomed Pharma, Oslo, Norway), adherent cells were eliminated by 1 hour adherence on plastic flasks. After purity testing with monoclonal antibodies (anti CD2, CD4, CD5, CD19, CD20), non-adherent cells were resuspended in RPMI1640 medium (Gibco Europe, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco).

Drugs

Fludarabine was purchased from Inveresk Clinical Research (Edinburgh, Scotland); the drug was dissolved in normal saline and subsequently diluted in RPMI1640 medium (Gibco Europe, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco).

Evaluation of cytotoxicity

Two for 10^5 CLL cells were resuspended in 200 µL RPMI1640 +10%FCS and were seeded in triplicate in 96 well microtiter plates. Drugs were added, either alone or in various combinations, at concentrations ranging from 0.5 to 1,000 µg/mL (fludarabine) and from 0.1 to 5,000 µg/mL (gemcitabine). These concentrations were chosen on the basis of previously reported data. Control cultures were carried out with same amount of media but without drugs. After 72 hours of incubation at 37°C, 50 µg of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylaminocarbonyl)-2H-tetrazolium hydroxide (XTT, Sigma Chemicals, St Louis, MO, USA) and 0.38 µg of phenazine methosulfate (PM S, Sigma), were added to each well, as previously described. After 4 hours of incubation at 37°C the plates were mixed on a mechanical plate shaker and absorbance at 450 nM was measured by an EIA microwell reader (Sigma Diagnostics). Growth inhibition was calculated as percent of control; the cytotoxic activity of the drug combination was evaluated by isobologram analysis of the data.

Results

Cytotoxic effect

Figure 1 shows the cytotoxic activity, as measured by XTT microculture assay, of gemcitabine on CLL cells of our series of patients. Under our experimental conditions, the IC50 of the drug was 1,100 µg/mL. The dose-effect relationship was determined by presenting the percentage of cytotoxicity as a function of drug concentration on a log scale.

Table 1. Patient characteristics.

<table>
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<tr>
<th>Pt.</th>
<th>Sex/age</th>
<th>previous therapy</th>
<th>disease status</th>
<th>Lymph. Hb (g/dL)</th>
<th>Plt. (x10^9/L)</th>
<th>BCL-2 (% cells)</th>
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<tr>
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<td>none</td>
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<td>11.6</td>
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<td>stable</td>
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</table>

Figure 1. Cytotoxic effect of gemcitabine on CLL cells. Data are expressed as mean ± SD of 10 different cases.
response curves of the combination of fludarabine (IC50 = 550 µg/mL) and gemcitabine are reported in Figure 2. Gemcitabine enhanced fludarabine-induced cytotoxicity on CLL cells (23% and 28% increase when gemcitabine 1 µg/mL was added to fludarabine 5 or 50 µg/mL, respectively). However, isobologram analysis of the data (Figure 3), demonstrated that the two drugs acted synergistically only when low doses of gemcitabine were combined with fludarabine, while with higher doses of gemcitabine the drug combination appeared to have merely additive effects.

**Apoptosis**

Both fludarabine and gemcitabine are known to induce apoptosis. Here we tested whether the combination of the two drugs could act synergistically on this phenomenon. As shown in Figure 4, a low percentage of apoptotic cells was detectable even in untreated samples, this percentage increased significantly upon incubation with either fludarabine or gemcitabine alone. The combination of fludarabine 5 µg/mL with a low dose of gemcitabine (10 µg/mL) induced a significantly higher amount of apoptosis than that induced by each drug alone (p=0.02 and p=0.007 compared to fludarabine 5 µg/mL and to gemcitabine 10 µg/mL, respectively). Increasing the concentration of gemcitabine, however, apoptosis failed to increase by more than the amount observed after exposure to the single drug.

**BCL-2 protein expression**

BCL-2 protein was detected in all the samples in which the assay was performed (Table 1). The mean percentage of positive cells was 68.5±10.3 (range 53-85%). There was no relationship between the percentage of BCL-2 cells and different disease status, or sensitivity to Fludarabine and to gemcitabine.

**Discussion**

In recent years, the use of fludarabine has significantly changed the therapeutic options that can be offered to CLL patients. In fact, the possibility of achieving complete remissions even at the molecular level makes it feasible to employ intensive consolidation therapies, such as autologous stem cell transplantation, at least in younger patients. With the aim of eradicating the disease, it could be useful to potentiate the effects of Fludarabine by adding drugs that could exert a synergistic activity. The addition of cyclophosphamide ± mitoxantrone improved the activity of fludarabine in vitro and in vivo, in low-grade non-Hodgkin’s lymphomas; fludarabine has shown to inhibit cellular repair of cisplatin-induced DNA damage in vitro; combination with Ara-C resulted in a synergistic effect as fludarabine enhances Ara-C metabolism and incorporation into DNA.

In this study we evaluated the effects of the combination of fludarabine with gemcitabine, another pyrimidine deoxyribonucleoside analog, that has shown activity, both in vitro in a variety of solid tumors and leukemia models, and in vivo as monotherapy for non-small cell lung cancer, pancreatic cancer, peripheral T-cell lymphomas, Hodgkin’s disease and non-Hodgkin’s lymphomas. In accordance with results reported by Morabito et al., our data show that BCL-2 protein expression is not correlated to in vitro sensitivity to fludarabine and no relation-
citabine is worth testing in the efficacy of fludarabine with a low dose gemitabine at the level of DNA, the possibility of enhancing the relationship between fludarabine and gemcitabine incorporates preferentially into repairing DNA, both drugs are used in high doses. This was indirectly demonstrated by Iwasaki et al., who examined the different patterns of incorporation into DNA of various nucleoside analogs. At variance to Ara-C, which incorporates preferentially into repairing DNA, both fludarabine and gemcitabine incorporate within replicating DNA. So fludarabine and Ara-C could cooperate at different DNA sites, while fludarabine and gemcitabine, when both are used in high doses, could cause an intracellular accumulation of false substrates to replicating DNA that cells are not able to use. This effect would be further enhanced in CLL by the slow proliferating activity of the cells.

Although studies would be useful in order to clarify the relationship between fludarabine and gemcitabine at the level of DNA, the possibility of enhancing the efficacy of fludarabine with a low dose gemcitabine is worth testing in vivo in CLL patients.

**Contributions and Acknowledgments**

PT and PLZ designed the study, analyzed the data and wrote the paper with the collaboration of MM; AP performed all the experiments; GV and ST revised the manuscript and gave their final approval.

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**Disclosures**

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