THE IN VITRO CYTOTOXIC EFFECT OF MITOXANTRONE IN COMBINATION WITH FLUDARABINE OR PENTOSTATIN IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT

Background and Objective. Clinical studies indicate that combination chemotherapy with mitoxantrone (Mitox) and a purine analog can improve the response rate in indolent lymphoproliferative disorders. We explored the in vitro Mitox- fludarabine (FAMP)- and pentostatin (Pento)-induced cytotoxicity and their interactions in CLL.

Methods. The peripheral lymphocytes of 24 CLL patients were tested at different drug concentrations, with Mitox, FAMP or their combinations in 22 cases, and with Mitox, Pento or their combinations in 20 cases, 18 of which were the same from the FAMP group. The MTT assay was chosen for the drug-induced cell cytotoxicity and flow cytometry analysis of the DNA hypodiploid peak for the study of the apoptotic process. Drug interactions were calculated in the MTT assay according to both multiplicative and maximum models.

Results. According to the lethal dose (LD) 50 values, when the three drugs were tested alone, 11 out of 22 and 8 out of 20 samples were sensitive to Mitox in the FAMP and Pento groups, respectively; on the other hand, 2 out of 22 and 0 out of 20 samples appeared sensitive to FAMP or Pento alone, respectively. Analyzing the MTT assay data with the multiplicative and maximum model, the combinations of Mitox+FAMP and Mitox+Pento at different drug concentrations were synergistic in 28.2% and 39.3%, respectively. At leukemic cell survival ≤50%, 11.7% and 11.1% of all combinations were synergistic in the Pento and FAMP group, respectively. The number of synergistic interactions at a therapeutically achievable plasma-drug concentration was an inverse function of the Mitox concentration. In the FAMP group, a direct correlation was found between the LD50 values of both FAMP and Mitox and the number of synergistic interactions, while the Pearson correlation coefficient was not significant in the Pento group. Finally, as measured by the DNA hypodiploid peak, Mitox (0.25 µg/mL) plus Pento (0.16 µg/mL) showed a significantly enhanced apoptosis in comparison to each single drug, while Mitox failed to demonstrate an additive effect with FAMP (1 µg/mL).

Interpretation and Conclusions. This experience demonstrates the extent of the in vitro synergism of Mitox with FAMP and Pento in inducing cell cytotoxicity; it also shows an adjunctive apoptotic effect for the Mitox-Pento association only. ©1997, Ferrata Storti Foundation

Key words: chronic lymphocytic leukemia, mitoxantrone, fludarabine, pentostatin, MTT assay, apoptosis

Mitoxantrone (Mitox) is an anthracenedione which exerts its anti-tumor activity by interacting with DNA and causing DNA strand breakage.1 Mitox is active apart from cell-cycle, inhibiting both resting and dividing neoplastic cells and thus resulting effective in both acute leukemia4 and lymphoma.5 In particular, a high response rate was reported in low-grade lymphoma either as a single agent6 or in combination with purine analogs.7,8

B-cell chronic lymphocytic leukemia (CLL) is a clonal proliferation of malignant B-lymphocytes that is considered incurable with conventional chemotherapy and is generally palliated with chlorambucil.9 Other conventional therapeutic options consist of anthracycline-containing regimens and, more recently, purine analogs and high-dose chlorambucil which have resulted in a higher remission rate.9,10

Apoptosis has increasing significance considering that it represents an important and ubiquitous mode of action by anti-tumor drugs.11 CLL cells constitute resistance genes12 and show a poor propensity to apoptosis. A combined use of Mitox

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with either fludarabine (FAMP) or pentostatin (Pento) might be effective in CLL patients. This prompted us to examine CLL cell chemosensitivity to in vitro exposure to Mitox either alone or in combination with FAMP or Pento. The non-clonogenic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, repeatedly used for the in vitro study of a single-drug or drug-combination chemosensitivity of CLL cells, was employed. We also examined whether the anthracycline-induced apoptotic profile, evaluated by flow-cytometry analysis, might be modified by the addition of either FAMP or Pento. Our results indicate that Mitox may synergize in vitro with FAMP and Pento, although in a low percentage of cases, while additive or antagonistic effects have been reported more frequently.

**Materials and Methods**

**Patients and cell separation**

Samples were taken from 24 previously untreated (14 cases) and previously treated CLL patients who had not undergone therapy from at least one month. Three patients were in Rai stage 0, 7 were in stage I, 8 were in stage II, 2 were in stage III and 4 were in stage IV. The diagnosis was based on the clinical picture and on the typical morphological and immunological findings. Peripheral blood mononuclear cells were separated by centrifugation onto a Ficoll-Hypaque gradient (Lymphoprep, Nycomed AS, Oslo, Norway). After isolation, cells were washed twice and re-suspended in RPMI 1640 medium (Flow Laboratories, Opera, Italy) supplemented with 15% heat-inactivated FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone, 2 mmol/L L-glutamine (Flow). Moreover, 5 µg/mL of insulin and transferrin and 5 ng/mL of sodium selenite (Sigma) were added to the medium in order to improve CLL cell survival. All samples contained 80-90% leukemic cells.

**Drug preparation and MTT assay**

Commercially available Mitox (Novantrone, Wyeth-Lederle, Milan, Italy), FAMP (Fludara, Schering AR, Germany) and Pento (Nipent, Wyeth-Lederle, Milan, Italy), were diluted in medium. MTT assay for cell viability was used to estimate drug chemosensitivity as previously described. In brief, 1x10^6 CLL cells were incubated in 96 round-bottom wells (Nunc, Roskilde, Denmark) with different concentrations of Mitox, FAMP and Pento in quadruplicate in a humidified atmosphere for four days at 37°C in 5% CO2. Fifty µL of 5 mg/mL MTT (Sigma) in PBS were added to every well and the plates were re-incubated for an additional four hours. Fifty µL of 100% dimethylsulfoxide (DMSO) (Calbiochem, La Jolla, CA, USA) were added to each well to solubilize the MTT formazan. The plates were kept in the incubator for 1 hour and then their spectrophotometric absorbance at 540 nm was determined using a multi-well scanning spectrophotometer (EL 310, Biotek Instruments, Burlington, VT, USA). Leukemic cell survival (LCS) was calculated by the formula: (mean OD treated wells/mean OD control wells) x 100%. The higher the cytotoxicity induced by a drug or by a drug combination, the lower the LCS. The dose-dependent cytotoxic effects of the drugs were studied in each sample by culturing CLL cells with 2.5-0.0025 µg/mL Mitox, 100-0.01 µg/mL FAMP and 16-0.0016 µg/mL Pento. Drug dose-response curves were drawn and their lethal dose (LD)50 values (i.e. the drug dose capable of killing 50% of the cells) were determined by using our own customized computer software.

**Assessment of in vitro drug interactions**

For the calculations of drug interactions, the multiplicative model and the maximum model were followed as previously described. The multiplicative model predicts the effect of a drug combination as the product of the effect of each single drug, as is expected LCS (drug A + drug B)=LCS (A) x LCS (B). The maximum model predicts that the effect of a drug combination is similar to that of the most active single drug (Dmax). Consequently, we defined the drug combination as synergistic when the observed LCS for a drug combination was lower than the product of the effect of each single drug: observed LCS (A+B) < expected LCS (A) x LCS (B). For the definition of additivity the following formula was applied: expected LCS (A) x LCS (B) < observed LCS (A+B) < LCS (Dmax), where Dmax is the lowest LCS value between A and B. Likewise, the definition of antagonism was satisfied when expected LCS (A) x LCS (B) < observed LCS (A + B) > LCS (Dmax). Sensitivity, additivity and antagonism calculation were performed by using our own software.

For the study of the interactions between Mitox with either FAMP or Pento, four Mitox (2.5, 0.25, 0.025 and 0.0025 µg/mL), five FAMP (100, 10, 1, 0.1 and 0.01 µg/mL) and five Pento (16, 1.6, 0.16, 0.016 and 0.0016 µg/mL) concentrations were tested for a total of 20 drug combinations.

**Flow cytometry analysis of apoptotic nuclei**

Freshly isolated CLL cells that had been incubated for 4 days with either no drug, Mitox 0.025 µg/mL, FAMP 1 µg/mL or Pento 0.16 µg/mL, or combinations thereof, were used for the flow cytometry determination of the apoptotic nuclei. CLL cells were then re-suspended in a 1 µL hypotonic solution of propidium iodide (Sigma) (PI 50 µg/mL in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma) and the tubes were left at 4°C overnight in the dark. The PI-fluorescence of the nuclei was measured by an EPICS Profile II flow
cytometer (Coulter Electronics, Hialeah, FL, USA). The flow cytometer was aligned using the highest quality 10 µm fluorospheres (Coulter EPICS, DNA-check beads) that had a population % HPCV of less than 2% for FL2 and FS. The nuclei traversed the light beam at 488 nm laser. A 600 nm dichroic mirror and a 635 band pass filter (bandwidth 20 nm) were used for collecting the PI red fluorescence, and data of the hypodiploid DNA peak was acquired on a logarithmic scale.

Statistical analysis

Descriptive statistics, Pearson correlation coefficient and Wilcoxon matched-pairs signed-ranks test were performed using the SAS/STAT software package, release 6.06 of SAS Institute Inc., 1993.

Results

Mitox effect on apoptotic cell death induced by FAMP and Pento

The flow cytometry analysis results of apoptotic nuclei after a 4-day culture with either medium, each single drug or drug combination is depicted in Figure 1. In the first set of experiments carried out in 22 samples by using a single drug concentration, Mitox and FAMP significantly enhanced the percentage of spontaneous apoptosis (Mitox versus medium, p=0.04; FAMP versus medium, p=0.0042), while the addition of the anthrancenedione to FAMP did not further improve the apoptosis induced by the purine analog (Figure 1A). Similar results were obtained in a second set of experiments using Mitox and Pento (Mitox versus medium, p=0.01372; Pento versus medium, p=0.038), but in this case Mitox-Pento combination significantly enhanced the apoptotic effect of each single drug (Mitox+Pento versus Mitox, p = 0.0007; Mitox+Pento versus Pento, p=0.0008) (Figure 1B).

Mitox-, FAMP- and Pento-induced cytotoxicity

The drug effect on cell survival was also analyzed by the MTT colorimetric assay. CLL cell samples were cultured with several 1-log increased concentrations of Mitox, FAMP and Pento, and the LD50 values were calculated. Table 1 shows the LD50 values obtained in two groups of 22 and 20 patients studied for the combinations of Mitox with either FAMP or Pento, respectively. We arbitrarily defined a sample as sensitive when its LD50 was below a threshold representing the drug plasma level after standard therapy, namely Mitox ≤ 0.25 µg/mL,22 Pento ≤ 1.6 µg/mL23 and FAMP ≤ 1 µg/mL.24 Thus, 11 out of 22 and 8 out of 20 samples were sensitive to Mitox in the FAMP and Pento groups, respectively; on the other hand, 2 out of 22 and 0 out of 20 samples appeared sensitive to FAMP and Pento, respectively.

In vitro cytotoxicity induced by Mitox+FAMP and Mitox+Pento combinations and drug interaction evaluation

In the combination study, both FAMP- (Figure 2A) and Pento-LD50 (Figure 2B) values were significantly reduced by the addition of Mitox 2.5 µg/mL (p=0.0007 and p=0.0015, respectively) and Mitox 0.25 µg/mL (p=0.0010 and p=0.0015, respectively). Table 2 shows the in vitro effect of drug interactions in CLL samples. The interactions between Mitox and FAMP or Pento were tested in 20 different combinations in 22 and 20 samples, respectively, resulting in 440 and 400 interactions for Mitox+FAMP and Mitox+Pento, respectively. In particular, 124 combinations (28.2% of the total) and 157 combinations (39.3%) were synergistic in the FAMP and Pento group, respectively. A comparable percentage of antagonistic interactions (46.1% versus 41.2%) was observed in the two groups, while a slightly higher percentage of additive interactions was detected in the Mitox+FAMP interactions (25.7% versus 19.5%). Lastly, the synergistic interactions at LCS ≤ 50% were 11.1% and 11.7% in the FAMP and Pento groups, respectively.
In vitro effect of mitoxantrone in CLL

Enumeration and efficacy of synergistic interactions

The distribution of drug interactions of Mitox with Pento and FAMP, at dose combinations below the patients’ plasma concentrations achieved after standard dose therapy,22-24 are shown in Table 3. It is worth noting that the number of synergistic interactions inversely correlates with the Mitox concentrations. Table 4 shows the correlation between the Mitox-, FAMP-, and Pento-LD50 values with the number of synergistic, additive and antagonistic interactions. A significant direct correlation was demonstrated between the number of synergistic interactions and the Mitox-LD50 values in the Mitox+FAMP group, while the number of additive interactions inversely correlated with the Mitox-LD50 values in both drug combination groups.

Discussion

Treatment strategies for CLL vary from the watch-and-wait theory to aggressive combination chemotherapy.6 Standard-dose chlorambucil has long been considered the treatment of choice in CLL.
Subsequent clinical trials demonstrated enhanced response rates and a significant improvement in survival with high-dose chlorambucil in comparison with anthracycline-containing regimens. The standard treatment recommendations were again challenged with the advent of the nucleoside analogs,6,9 In CLL, FAMP is apparently the most promising and the most extensively used purine analog, considering that several randomized trials comparing this agent with chlorambucil are still ongoing.25,26 The remaining analogs, 2′-chlorodeoxyadenosine and Pento, although significantly fewer investigations have been done on them, are able to produce responses in patients with CLL.27 Considering that few new agents with in vitro evidence of a potential anti-leukemic effect for CLL are under investigation,28 as an alternative to experimenting with new drugs, new combinations of effective drugs could have an important role in the therapeutic strategy of CLL. In this respect, the therapeutic option of purine analogs in combination with Mitox is more and more frequent for the treatment of lymphoproliferative disorders.4,5

Few in vitro drug combination studies have been reported in CLL.15,19,20 Synergy was observed between chlorambucil and Pento in CLL cells,20 while an additive effect on B neoplastic cell apoptosis was described with a combination of chlorambucil and FAMP.20 More recently, the phenomenon of synergism between methylxanthine derivatives and chlorambucil was proven in vitro.31 Furthermore, we recently demonstrated that the in vitro combination between chlorambucil and either methylprednisone or deflazacort resulted in a synergistic effect.17 Finally, we demonstrated an in vitro synergism of chlorambucil with FAMP and 2′-chlorodeoxyadenosine in inducing CLL cell cytotoxicity (in preparation). In the present in vitro study, the MTT assay results, evaluated by the maximum and multiplicative models,17,19,20 indicated that the phenomena of additivity and synergism, when pooled together, were found in 53.9% and 58.8% of cases for the Mitox+FAMP and Mitox+Pento groups, respectively. It is worth noting that evaluating synergistic and additive interactions at the therapeutically achievable plasma concentration of each drug, the percentage reduced to 21.1% and 21.4% for the Pento and FAMP group, respectively. It should also be noted that the number of synergistic interactions increased with the lowering of Mitox concentrations. Lastly, a correlation analysis demonstrated that the lower the drug cytotoxicity induced by Mitox, the higher the probability obtaining synergism between the anthracenedione and the purine analog. Considering that the concern for in vivo clinical toxicity may represent the major reason for combining Mitox with FAMP or Pento, these results should be considered in view of the optimal drug interaction employing the concentration of the two drugs useful in achieving a synergistic effect. However, it is also evident that synergistic interactions are effective if the LCS values achieved by the combinations of the drugs are at least less than 50%. Clinically, preliminary data demonstrated that Mitox may be successfully associated with acceptable toxicity to purine analogs.4

At flow-cytometry analysis, the Mitox+Pento combination was able to significantly improve each drug cytotoxic effect, while the Mitox+FAMP interaction was unable to increase any further the apoptotic effect of Mitox and FAMP taken as single drugs. This latter report is apparently in contrast with the MTT assay results. It should be emphasized, however, that a single drug concentration was used in the flow cytometry test while dose-response curves were performed in the MTT assay. In addition, the apoptotic phenomenon explored by flow cytometry does not provide a real-time analysis of the apoptotic process, but merely uses an end-point analysis. Lastly, assays of apoptosis do not take into consideration the overall cell survival which is a crucial point in determining drug efficacy. Considering that the MTT assay is an easy, semi-automated method for analyzing multiple dose-response curves and defining synergism and additivity by the multiplicative and additive models, it could be a better choice than flow cytometry in evaluating drug synergism.

This study was performed in both treated and untreated cases. Although the aim was not the comparison of the two groups of patients, we found that there were no statistically significant differences in terms of drug interactions, while the mean values of FAMP-LD50 significantly differed

### Table 4. Pearson correlation between the number of synergistic, additive and antagonistic interactions of Mitox+FAMP and Mitox+Pento drug combinations and the Mitox-, FAMP- and Pento-LD50 values

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<th>Drug-LD50</th>
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<td>FAMP</td>
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<td>Sy</td>
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<td>Drug-LD50</td>
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<td>Sy</td>
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<td>p=0.003</td>
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<td>Mitox</td>
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<td>Sy</td>
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between the group of untreated (7.2±3.6 s.e.m) and treated (21.9±11.2 s.e.m) (p=0.023) cases, but no difference was found for the other drugs. On the other hand, flow cytometry results showed that significantly higher mean values of apoptotic cells were counted among samples obtained from treated patients, as compared to untreated cases, both in the absence of drugs (27.8±4.3 s.e.m. versus 17.9±3.7 s.e.m., p=0.0317) and after incubation with the combination Mitox+FAMP (39.6±5.6 s.e.m versus 27.3±3.3 s.e.m., p=0.0475). However, the relatively low number of cases compared in each group requires further specific studies on this aspect.

In conclusion, these in vitro results support the clinical finding that the combination of Mitox with FAMP or Pento is an effective treatment in CLL. Further clinical investigations are required to optimize the concentration of each single drug in order to obtain in vivo synergistic effects with the lowest clinical toxicity. We demonstrated that the in vitro MTT assay substantiated with models measuring the effect of interactions of drugs \(^{19,20}\) is a useful method in studying synergism in CLL. An in vitro/in vivo controlled study on the use of the therapeutic combination of Mitox with FAMP or Pento, considering also biological parameters of disease,\(^{33}\) would be of interest.

**References**