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# APOPTOTIC AND ANTIPROLIFERATIVE EFFECTS OF GEMCITABINE AND GEMCITABINE PLUS ARA-C ON BLAST CELLS FROM PATIENTS WITH BLAST CRISIS CHRONIC MYELOPROLIFERATIVE DISORDERS

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**ABSTRACT**

**Background.** Blast phase of chronic myeloid leukemia (CML) as well as the rare acute transformation of other chronic myeloproliferative disorders constitute forms of leukemia that are particularly refractory, even to aggressive chemotherapy. Many attempts have thus been made to identify new drugs that could be active in these diseases. We wanted to evaluate whether gemcitabine (dFdC), a pyrimidine analogue widely employed in lung cancer chemotherapy, was able to block in vitro proliferation of bcr/abl-positive and bcr/abl-negative blast cells in primary culture. We already showed that gemcitabine is active in inhibiting proliferation and inducing apoptosis of HL60 cells.

**Methods.** We studied the influence of dFdC on the proliferative potential of blasts by means of tritiated thymidine uptake, colony formation in semisolid medium and cell cycle parameters at flow cytometry. The efficacy of dFdC in inducing apoptosis was evaluated by flow cytometry ($A_0$ peak) and by DNA agarose gel electrophoresis.

**Results.** We demonstrated that dFdC already inhibits tritiated thymidine uptake at doses of 10 μM after 72 hours of culture, and that this effect is dose dependent. The addition of Ara-C 5 μM in the culture medium to dFdC provoked a synergistic inhibitory effect. Consistent results were obtained when cell cycle distribution was studied. In fact, cell incubation in the presence of dFdC resulted in a significant decrease of cells in S phase, although with a certain heterogeneity among cases. The antileukemic activity of dFdC appeared to be specific since it was mediated through apoptosis. We could demonstrate the appearance of the pre-G1 apoptotic peak at cytofluorimetric analysis, and the characteristic DNA fragmentation pattern at agarose electrophoresis in all 10 cases after treatment with different doses of dFdC. Induction of apoptosis was maximal for the highest doses of dFdC (100 mM) and for the combination of dFdC and Ara-C.

**Interpretation and Conclusions.** Following incubation with Gemcitabine leukemic blasts from chronic myeloproliferative disorders are induced to accumulate intracytoplasmatic and nuclear Ara-C and undergo apoptosis. These observations suggest that gemcitabine could be considered a candidate drug, capable of being used in polychemotherapy of refractory acute phase chronic myeloproliferative disorders.


*Key words: Gemcitabine, Ara-C, chronic myeloproliferative disorders blast crisis, apoptosis*



Combination chemotherapy that includes pyrimidine analogs, in particular fludarabine (F-Ara) and arabinosylcytosine (Ara-C), has been successfully applied to the treatment of refractory acute myeloid leukemias[1] and chronic myeloid leukemia (CML) in blast crisis.[2]

The pyrimidine analog Gemcitabine (dFdC) has been shown to be extremely active, as a single drug or in combination with Ara-C in inhibiting the *in vitro* growth of acute and chronic myeloid leukemia blast cells.[3-5] The synergistic cytotoxic effect demonstrated in the sequential usage of dFdC and Ara-C is due to an increase in Ara-C uptake and retention, which peaks 24 hours after dFdC incubation.[6] dFdC acts by self-locking in the penultimate position of the DNA elongating chain, thereby blocking any further cell replication. Potentiation of Ara-C cytotoxicity to leukemic blasts, together with the intrinsic efficacy of dFdC prompted us to verify whether the activity of this molecule persisted in primary cultures of immature cells from blast crisis both $Ph^1$-positive and negative of chronic myeloproliferative disorders. These pathologies are particularly resistant to chemotherapeutic regimens and are characterized by an extremely poor prognosis.[7] In fact, it has been shown that the product of the bcr/abl fusion gene is a tyrosin kinase that acts by endowing cells with resistance to apoptotic cell death.[8] Moreover, other mutations and oncogenic events can precipitate chronic myeloid

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leukemia, as well as other myeloproliferative disorders, into accelerated phase of the disease, which is especially refractory to chemotherapy.[9] We therefore evaluated whether dFdC alone or in combination with Ara-C was able to inhibit proliferation and, more interestingly, induce apoptosis in primary cultured blasts obtained from myeloproliferative diseases.

## Materials and Methods

*Cells*

Mononuclear cells obtained from 10 patients affected by chronic myeloproliferative disorders in blast crisis were separated on Ficoll-Isopaque 1.077 g/L density gradient centrifugation; adherent cells were depleted by plastic adherence (1 hour at 37°C) and T cells by E-rosetting. Seven cases were bcr/abl positive (cases #2, 4, 5, 6, 7, 9, 10) and 3 bcr/abl negative (cases #1 and 3 were diagnosed as myelofibrosis, #8 as essential thrombocytosis) at RT-PCR, performed as previously reported.[10] All cases showed more than 30% immature cells in peripheral blood smears, either of myeloid or lymphoid origin, as ascertained by immunophenotype analysis.

*Chemicals*

Gemcitabine (dFdC) was kindly supplied by Eli Lilly (Indianapolis, IN, USA); cytosine arabinoside (Ara-C) was purchased from Upjohn (Kalamazoo, MI, USA).

*Tritiated thymidine uptake*

Blast cells were cultured in RPMI 1640 10% fetal calf serum (FCS) with the addition of 10 ng/mL of IL-3 and 10 ng/mL of GM-CSF. Cells were plated onto 96-microwell plates at a concentration of $1\times10^5$/mL in the presence of dFdC 1 μM, 10 μM, 100 μM, and Ara-C 5 μM with or without dFdC at 1 and 10 μM. Control cultures were carried out in the absence of any drug. After 48 hours of culture in these conditions, cells were exposed for 18 hours to tritiated thymidine ([$^3$H]-TdR, 5 μCi/well, specific activity 2 Ci/mmol, Amersham, UK). Pulsed cells were harvested by an automated cell harvester (Skatron, Norway) and incorporation of [$^3$H]-TdR was evaluated by β scintillation counting.

*Colony formation*

In parallel to [$^3$H]-TdR uptake, blast cells were plated in RPMI 10% FCS ($0.1\times10^6$ cells/dish) in the presence of GM-CSF and IL-3 10 ng/mL in 0.9% methylcellulose (1 mL) and incubated at 37°C in a fully humidified atmosphere (5% $CO_2$). Cultures were carried out in the absence or in the presence of drugs: dFdC 10 μM, 100 μM and Ara-C 5 μM with or without dFdC at 10 and 100 μM. Clusters of 10 to 50 cells and colonies of 50 cells or more were scored at day 14 of cultures. Colony numbers represent the means of triplicate cultures.

*Cell kinetics and apoptosis*

Cells were cultured in 75 cm$^2$ area flasks (Greiner, Germany) with dFdC at 1 and 10 μM alone or in combination with Ara-C 5 μM, in RPMI 10% FCS additionated with IL-3 and GM-CSF as mentioned above. After incubation, cells were washed thoroughly, resuspended in PBS/ethanol 1:3 vol/vol and kept overnight at 4°C. After fixation, cells were incubated in propidium iodide (PI) 50 mg/mL plus Nonidet 0.01%, RNAse 62 μg/mL. DNA content and cell cycle parameters were measured in a FACScan flow cytometer (Becton Dickinson, San Josè, CA). Analysis of data was carried out with the CellFit program, applying the MANL statistic program, which quantifies the amount of the apoptotic peak *pre-G1* present in the region of channel 100.

*DNA fragmentation*

Cellular DNA was obtained from blast cells cultured in the same conditions with or without drugs as for cell cycle determination (see above). DNA was obtained by lysing the nuclear membrane and then extracting it with phenol/chloroform. Equal amounts of purified DNA were then loaded onto 2% agarose gel and electrophoresis was performed at low voltage (60 V) for 5 hours. The appearance of the characteristic 200 base pairs DNA digestion fragments or multiples thereof and the ladder-like DNA smear were considered an indication of apoptosis.

*Statistical analysis*

The Student's t-test for grouped data ($p< 0.05$) was employed for statistical analysis. Standard deviations were evaluated for at least 2 experiments carried out with cells from the same case. All experiments were performed in triplicate.

## Results

*[$^3$H]-TdR uptake*

Tritiated thymidine uptake was consistently inhibited in primary blast cell cultures performed in the presence of dFdC at dosages of 10 μM and 100 μM (Table 1). In the majority of cases, inhibition was dose dependent, although in two cases (#4 and 6) this effect was already maximal at the lowest dose, indicating an extreme sensitivity of blast cells to dFdC. Incubation of cells in the presence of Ara-C lead to inhibition of [$^3$H]-TdR uptake. Combination of dFdC and Ara-C induced a profound blockage of leukemic cell proliferation, indicating a potentiating effect in almost the totality of cases.

*Colony formation*

In four out of the five cases studied, colony and cluster numbers decreased significantly ($p<0.05$) in cultures supplemented with dFdC or Ara-C (Figure

Table 1. [$^3$H]-TdR uptake after dFdC and Ara-C treatment. Cells obtained from PB or BM of patients affected by myeloproliferative disorders in blast crisis were purified by Ficoll gradient centrifugation, and adherent cell and T-lymphocyte depleted. Cells were incubated in the absence or presence of dFdC and Ara-C, as single drug and in association. Cultures were carried out for 72 hours in 96-microwell titer trays. Cells were pulsed with 0.5 μCi [$^3$H]-TdR per well. After 18 hours of incubation cells were harvested and thymidine incorporation was evaluated by β-scintillation counting. Results are expressed as percentage of incorporation. Control untreated cells are consider as having 100% incorporation.

| # | bcr/abl | CB phen | dFdC 10 μM | dFdC 100 μM | Ara-C 5 μM | Ara-C 5 μM dFdC 10 μM | Ara-C 5 μM dFdC 100 μM |
|---|---|---|---|---|---|---|---|
| 1 | neg | M | 14 | 11 | 7 | 11 | 9 |
| 2 | pos | L | 70 | 40 | 33 | 23 | 20 |
| 3 | neg | M | 60 | 52 | 54 | 45 | 36 |
| 4 | pos | M | 0.5 | 0.5 | 0 | 0 | 0 |
| 5 | pos | L | 62 | 47 | 60 | 39 | 45 |
| 6 | pos | M | 0 | 0 | 0 | 0 | 0 |
| 7 | pos | M | 4 | 2 | 0 | 0 | 0 |
| 8 | neg | M | 13 | 3.8 | 11 | 2.8 | 0 |
| 9 | pos | M | 38 | 16 | 55 | 32 | 28 |
| 10 | pos | M | 21 | 18 | 7 | 0 | 0 |

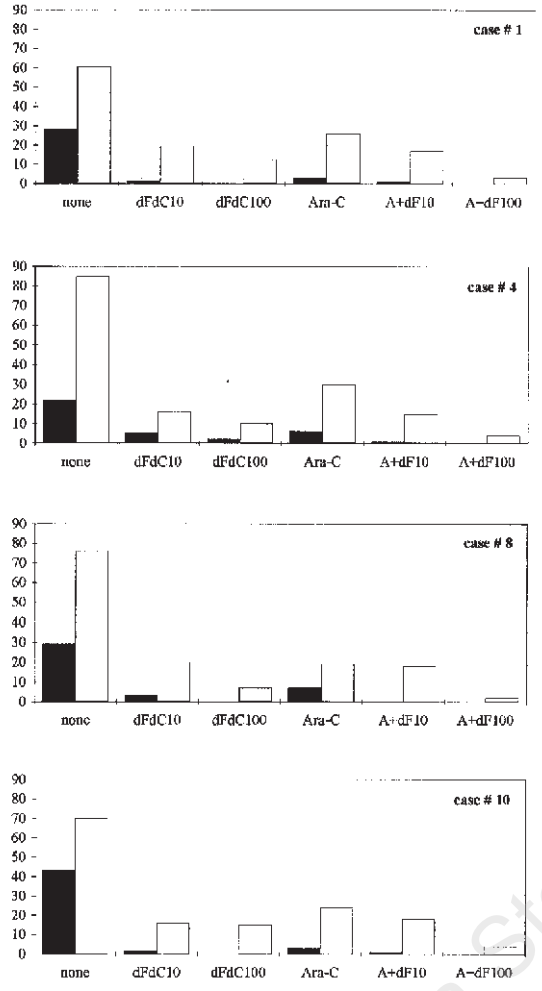


Figure 1. Colony formation after cell treatment with dFdC alone or in association with Ara-C. Blast cells were exposed to dFdC at 10 and 100 μM with or without Ara-C 5 μM in semisolid culture. Conditions of culture were as reported in *Materials and Methods*. The number of colonies (dark bars) and clusters (white bars) was evaluated after 14 days of culture using an inverted microscope. Results from triplicate experiments are given.

1). The inhibition of colony and cluster formation was clearly dFdC dose dependent, with a complete disappearance of colonies in cultures supplemented with dFdC 100 mM. Only a few clusters were detectable in cells exposed to the combination of Ara-C and maximal dosage of dFdC. Case #2 did not show any significant reduction in the number of colonies.

*Cell cycle*

It was not possible to evaluate the cell cycle distribution of blasts from the entire patient cohort because in some instances the number of cells in S phase of the cycle was negligible. When cell cycle parameters were analyzable, data were consistent with inhibition of cellular proliferation (Table 2). dFdC constantly and significantly ($p < 0.05$) diminished the percentage of cells entering into S phase of the cycle and a similar effect was shown by Ara-C; the combination of the two drugs yielded the strongest inhibition of progression through cycle, except for case #5, in which this effect was absent. In fact, although dFdC and Ara-C were active as single drugs, the combination of them did not induce a further decrease in the number of cells in S phase.

*Apoptosis*

We used a FACSscan flow cytometer to analyze DNA content and the appearance of the *pre-G1* $A_0$ apoptotic peak. Only 9 of the cases were fully evaluable for cell cycle parameters and DNA quality (Table 3). The percentages of apoptotic cells ranged from 2 (case #3 at 10 μM dFdC) to 93 (case #1 at Ara-C 5 μM plus dFdC 100 μM) and paralleled the sensitivity to the drugs shown in [$^3$H]-TdR uptake inhibition and cell cycle modifications, except for case #5. In case #7 the highest rate of apoptotic death was determined by Ara-C as single drug and in association with dFdC, but not by dFdC alone.



| *Case #* | | *2* | *3* | *5* | *7* | *8* | *10* |
|---|---|---|---|---|---|---|---|
| **none** | **$G_0/G_1$** | 83.4±0.02 | 95.6±0.01 | 50.8±0.06 | 92.3±0.01 | 56±0.04 | 94.6 |
| | **S** | 15.7±0.1 | 4.1± 0.1 | 43.5± 0 | 6.0± 0.06 | 41.1±0.07 | 4.3±1.2 |
| | **$G_2$/M** | 0.9±0.05 | 0.1± 0.54 | 5.7±0.09 | 1.7± 0.43 | 2.9±0.9 | 1.1±0.9 |
| **dFdC 100 μM** | **$G_0/G_1$** | 91.1± 0.3 | 96.8±0.13 | 86.4±0 | 96.2±0.02 | 81.6±0.01 | 96.3±0.03 |
| | **S** | 7.9± 0.3 | 3.0±0.0 | 10.2±0.01 | 3.1±0.4 | 18.3±0.5 | 2.0±0.08 |
| | **$G_2$/M** | 1.0±0.07 | 0.2±0.3 | 3.4± 0.02 | 0.8±0.05 | 0.1±0.9 | 1.7±1.0 |
| **Ara-C 5 μM** | **$G_0/G_1$** | 94.5±0.2 | 95.5±0.02 | 88.4±0.04 | 95.5±0.32 | nd | 95.5± 0.3 |
| | **S** | 4.0± 0.5 | 3.3± 0.13 | 9.0± 0.01 | 3.4± 0.35 | nd | 3.0±0.012 |
| | **$G_2$/M** | 1.5±0.03 | 1.1± 0.23 | 2.6±0.03 | 1.0± 0.01 | nd | 1.4±0.08 |
| **Ara-C + dFdC** | **$G_0/G_1$** | 95.6± 3 | 96.0±0.01 | 75.5±0.27 | 96.7±0.12 | nd | 95.6± 0.2 |
| | **S** | 3.1±0.04 | 3.6± 0.54 | 23.0±0.07 | 3.0 ± 0.8 | nd | 3.2±0.07 |
| | **$G_2$/M** | 1.3±0.04 | 0.4±0.01 | 1.5±0.3 | 0.3± 0.2 | nd | 1.2±0.04 |

Table 2. Cell cycle distribution of patient leukemic cells exposed to Ara-A and dFdC. Cells were treated as specified in Table 1. Cultures were carried out for 72 hours in standard conditions. DNA content of cells and cell cycle parameters were evaluated in a FACSScan flow cytometer after propidium iodide (PI) staining. Results are expressed as percentage of cells per phase ± % standard deviations.

Table 3. Percentage of apoptotic cells as evaluated by cytofluorimetric analysis. After culture in the presence of dFdC and Ara-C alone and combined, propidium iodide (PI)-stained cells were analyzed at cytofluorimeter for DNA content. The percentage of events present in the aneuploid AO apoptotic peak is reported here.

| *Case* | *none* | *dFdC 10 µM* | *dFdC 100 µM* | *Ara-C 5 µM* | *Ara-C 5 µM dFdC 10 µM* | *Ara-C 5 µM dFdC 100 µM* |
|---|---|---|---|---|---|---|
| 1 | 7 | 25 | 70 | 75 | 88 | 93 |
| 2 | 5 | 31 | 45 | 60 | 88 | 88 |
| 3 | 2 | 2 | 9 | 5 | 9 | 19 |
| 4 | 5 | 15 | 21 | 25 | 34 | 38 |
| 5 | 4 | 34 | 48 | 62 | 89 | 91 |
| 6 | 4 | 10 | 10 | 45 | 47 | 65 |
| 7 | 0 | 5 | 15 | 32 | 60 | 90 |
| 8 | 3 | 19 | 25 | 30 | 35 | 36 |
| 9 | 0 | 8 | 20 | 30 | 67 | 70 |

*DNA fragmentation*

DNA agarose gel electrophoresis demonstrated in all cases significant DNA fragmentation in cells treated with dFdC and the combination of dFdC and Ara-C, with a dose-dependent increase in the rate of digested DNA, paralleling the data obtained at FACSscan analysis. Figure 2 illustrates the DNA electrophoresis of case #5, in which, notwithstanding a moderate response to dFdC in terms of thymidine uptake and reduction of S phase cells, apoptotic cell death was strongly induced.

## *Discussion*

In this study we analyzed the capability of dFdC, a pyrimidine analog, to inhibit the *in vitro* growth of chronic myeloproliferative disorder blast cells. In order to evaluate the efficacy of this drug, we determined its potential for inhibiting [$^3$H]-TdR uptake of primary cultures of blast cells obtained from 10 cases of chronic myeloproliferative disorders, both bcr/abl positive (CML) and negative (2 myelofibroses and one thrombocythemia). In parallel, we analyzed the ability to form colonies in semisolid medium as well as cell cycle parameters after liquid culture in the presence of dFdC, and determined the percentage of apoptotic cells and the rate of endonucleosomic DNA cleavage. It has been shown that dFdC is taken up and accumulates at a higher rate (at least 3 times) in tumor cells than in lymphocytes, in which no dose-response incorporation has been demonstrated.[11,12]

According to our data thorugh scheduling with dFdC[13] it is also possible to enhance Ara-C efficacy *in vitro* in a subset of cases in which chemotherapy is extremely ineffective *in vivo*. We analyzed the sensitivity of leukemic blast cells in the ultimate, acute phase of chronic myeloproliferative disorders. The complex pathogenesis of this heterogeneous group of diseases results in clinical behavior which differs substantially from that of acute leukemias. For these reasons, we considered it extremely relevant that a new chemotherapeutic agent such as dFdC was identified; dFdC is endowed with strong cytotoxic activity, both alone and in combination with Ara-C, and exhibits limited side effects when used in appropriate scheduling.[13,14] We have previously shown that acute myeloid leukemia blasts are induced to accumulate Ara-C in the cytoplasm and nucleus following dFdC preincubation.[2] We demonstrated here that this effect is also attainable in leukemic blasts from chronic myeloproliferative disorders. Moreover, in all cases presented here, dose-dependent apoptosis was observed in blasts of both myeloid and lymphoid phenotype. Apoptosis is a physiological phenomenon determining cell death via activation of interleukin-1 converting enzyme (ICE) and ICE-like proteases, through several transduction pathways.[16,17] Quantification of *in vitro* apoptosis in cell populations treated with different chemotherapeutic drugs has recently been labeled an essential tool for evaluating their efficacy.[18] The apoptotic effect shown by dFdC may be due to its extreme activity against quiescent $G_0$ cells like lymphoid cells.[19] dFdC is widely used in clinics for the treatment of non-small cell lung (NSCL) and ovarian tumors; its antineoplastic activity is apparently not accompanied by general toxicity and has selective effects on leukemic cells.[14,15] These observations as a whole encourage further and enlarged investigations to verify whether dFdC could be considered a candidate drug for alternative treatment of blast phase CML and chronic myeloproliferative disorders.

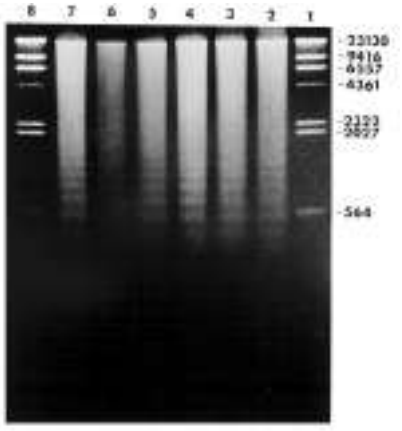


Figure 2. DNA fragmentation pattern of CML case #5. Lane 1 and lane 8: molecular weight marker II, Boeringher Manneheim. Lane 2: DNA obtained from untreated blast cells. Lane 3: DNA after cell treatment with dFdC 10 µM; lane 4 after dFdC 100 µM; lane 5: after Ara-C 5 µM ; lane 6: after Ara-C plus dFdC 10 µM; lane 7: Ara-C plus dFdC 100 µM. Equal amounts of purified DNA were loaded on each lane.

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