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Communication

Cytotoxic effects of cladribine and tezacitabine toward HL-60

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The aim of the study was to determine the relation between the cytotoxic and cytostatic effects of tezacitabine and cladribine on a HL-60 cell line and the time of exposure of cells to these drugs. Cell viability and induction of apoptosis were assessed using flow cytometry methods. Apoptosis was confirmed by direct microscopic observation. Growth inhibition was examined by cell counting. After 24 h incubation tezacitabine was equally or less toxic compared to cladribine. However, toxicity of tezacitabine strongly rose after 48 h incubation leading to massive cell death at doses much lower than those of cladribine. Assessment of the effect of increased exposure time on the clinical efficacy of tezacitabine is indicated.

Keywords: cladribine, tezacitabine, leukemia, HL-60 cells, nucleoside analogs, flow cytometry

Several cytotoxic purine and pyrimidine nucleosides have proved useful for chemotherapy of various hematological and epithelial malignacies (Galmarini *et al*., 2001; Faderl *et al*., 2002). Development of these drugs has usually been empirical. *In vitro* assays of cytotoxicity toward a panel of established malignant cell lines and phase I clinical studies have been employed to select tumor types susceptible to a drug and to determine its maximal tolerated dose (MTD), respectively. The aforementioned types of studies are rarely sufficient to establish an optimal dosing schedule, which later undergoes modifications according to the results of pharmacokinetic studies and clinical evaluations of safety and efficacy.

The development of the purine analog cladribine (2-chloro-2′-deoxyadenosine, 2CdA) may serve as an example of such modifications of dosing schedule. The drug is particularly effective in HCL where more than 90% of complete remissions have been reported repeatedly (Piro *et al*., 1990; Robak *et al*., 1999). It also displays a considerable clinical activity in some other lympho- and myeloproliferative diseases including B-CLL (Karlsson *et al*., 2002), low-grade non-Hodgkin's lymphoma (Robak *et al*., 1997) and chronic myelogenous leukemia (Gollard *et al*., 1997). The MTD of cladribine in patients with leukemias and lymphomas has been established as 0.7 mg/kg per treatment course, courses repeated at monthly intervals (Saven & Piro, 1994). In the first clinical trial cladribine was given as 7-day long continuous infusion because it was thought that a week-long exposure of malignant lymphocytes to the drug was a prerequisite of optimal cytotoxic effect (Piro *et al*., 1990). However, pharmacokinetic studies provided evidence that the drug enters lymphocytes within 1–2 h and accumulates intracellularly in the form of relatively stable phosphates (Liliemark, 1997). Treatment courses consisting of 5 daily doses given either as 2 hour-long i.v. or s.c. infusions (0.14 mg/kg per day), or orally (0.28 mg/kg per day) were found to be similarly effective as 7 day-long continuous infusions (Liliemark & Juliusson, 1995). Subsequent trials have shown that courses consisting of three daily doses of cladribine repeated every third week for up to 10 courses in B-CLL (Karlsson *et al*., 2002) and doses 0.15 mg/kg given at weekly intervals in HCL (Lauria *et al*., 1999) are similarly effective and accompanied by less severe side effects. Furthermore, when cladribine was

Abbreviations: B-CELL, B-cell chronic lymphocytic leukemia; 2CdA, cladribine, 2-chloro-2′-deoxyadenosine; dCK, deoxycytidine kinase; DAPI, 4′,6-diamidino-2-phenylindole; FDA, fluorescein diacetate; FMdC, tezacitabine, ((*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine; HCL, hairy cell leukemia; i.v., intravenous; PBS, phosphate buffered saline; PI, propidium iodine; s.c., subcutaneous.

used as an immunosuppressant to treat multiple sclerosis, the same cumulative dose was markedly more effective in reducing the number of circulating lymphocytes when given once a week than when given in courses consisting of five consecutive daily doses and repeated monthly (Grieb *et al*., 2001). It has been suggested that when the injections are repeated daily, the entry of cladribine to the lymphocytes may be blocked by deoxycytidine which is liberated from dying cells and competes with the drug for its phosphorylating enzyme, deoxycytidine kinase (dCK) (the evidence for such competition has been found *in vitro* (Cohen *et al*., 1997)).

Tezacitabine ((*E*)-2′-deoxy-2′-(fluoromethylene)-cytidine, FMdC) is a novel pirimidine derivative undergoing clinical development as a treatment for both hematological malignancies (Faderl *et al*., 2002) and solid tumors (Seley, 2000). Its MTD has been established at 7.5 mg/m^2/day for 5 days (Faderl *et al*., 2001). Various dosing schedules were employed in the studies reported to date. Patients with solid tumors were treated with 30-minute-long i.v. infusions repeated either every three weeks, or twice a week for three weeks (Rodriguez *et al*., 2002; Flaherty *et al*., 2003). In a preliminary study with hematological malignancies the drug was also given as 30-min i.v. infusions, and the doses were repeated daily for 5 consecutive days (Faderl *et al*., 2001). In an on-going study tezacitabine is given as continuous i.v. infusion (Faderl *et al*., 2002). The question which type of dosing schedule (short or longer exposure of malignant cells to the drug) will yield optimal cytotoxic effect remains open.

To find out whether a prolonged exposure of leukemic cells to tezacitabine will increase the cytotoxicity of the drug, in the present study we compared the effects of continuous exposure of HL-60 leukemia cells to various concentrations of FMdC and 2CdA for 24 and 48 h.

MATERIALS AND METHODS

Chemicals. FMdC was synthesized by Drs. M. Bretner and K. Felczak (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland) according to the modified method of McCarthy *et al*. (1991). Its chemical identity and purity was confirmed by proton NMR and HPLC, respectively. 2CdA (pharmaceutical grade, >99% by HPLC) was a gift from the Foundation for Development of Diagnostics and Therapy (Warszawa, Poland). Both substances were prepared as 1 mM stock solution in PBS (Institute of Immunology and Experimental Therapy, Poland). Annexin V kit from Caltag (USA), fluorescein diacetate (FDA) from Sigma (USA), 4′,6-diamidino-2-phenylindole (DAPI), sulforhodamine 101 and propidium iodide (PI) from Molecular Probes (USA), and Isoton II from Beckman Coulter (USA) were used.

Cell culture and drug exposure. HL-60 human promyelocytic leukemia cells (ATCC, USA) were cultured in RPMI 1640 medium (Institute of Immunology and Experimental Therapy, Poland) supplemented with 20% fetal bovine serum, L-glutamine and antibiotic–antimycotic solution (Gibco, USA) in a humidified 5% CO_2/95% air atmosphere at 37°C. Cell cultures were routinely checked for mycoplasma contamination. For experiments 3×10^5 cells/ml in 2 ml medium were seeded in a 12-well plate (Nunc, Denmark). HL-60 cells were exposed continuously to the same concentrations of 2CdA or FMdC, namely 10, 25, 50, 100 or 200 nM in final volume of the medium, for either 24 or 48 h. Cells were subsequently collected, spinned down, rinsed with cold PBS and prepared for labeling.

Flow cytometry. Cytometric data were collected using a FACSVantage flow cytometer and analyzed by CellQuest software (BD Biosciences, USA).

Apoptosis assay by Annexin V/PI labeling. After washing with PBS 10^6 cells per ml were suspended in binding buffer on ice. Aliquots of 100 μl of the cell suspension were labeled using AnnexinV kit, according to manufacturer's procedure. Briefly, 5 μl of Annexin V-FITC and 10 μl of 50 μg/ml PI were added and incubated for 15 min at room temperature in the dark, then 400 μl of cold binding buffer was added and cells were mildly vortexed and kept on ice. Flow cytometry measurements were performed 1 h after labeling.

Viability assay by FDA/PI labeling. After washing with PBS 10^6 cells per ml were suspended in cold PBS and 100 μl aliquots of the cell suspension were labeled with a slightly modified method of Ross *et al*. (1989). Briefly, 10 μl of 0.5 μg/ml FDA and 10 μl of 50 μg/ml PI were added for 5 min at room temperature, then 400 μl of cold PBS was added and cells were mildly vortexed and kept on ice. Flow cytometry measurements were performed within 1 h after labeling.

Growth inhibition. Cells were collected directly from the culture after exposure to the drugs, suspended in cold Isoton II buffer and counted in a Coulter Counter Z2 (Beckman Coulter, USA). Growth inhibition was calculated from the formula: $[(C_k–C_p)/(C_{KON}–C_p)] \times 100\%$, where C_p – cell count at the beginning of the experiment; C_k – cell count after incubation with medium containing the tested drug; C_{KON} – cell count after incubation with medium containing no drug (Monks *et al*., 1991).

Morphological evaluation. Cells were collected after exposure to the drugs, washed in cold PBS and fixed in ice cold 70% ethanol for at least 24 h, then washed out from alcohol and stained with 1.0 μg/ml DAPI and 10 μg/ml sulforhodamine 101 for 1 h. Cell morphology was evaluated using BX60 fluo-

rescence microscope equipped with digital camera DP50 (Olympus, Japan), under Nomarsky contrast and fluorescence.

Statistics. All experiments were repeated at least 4 times. The results are presented as arithmetic means ± standard deviations. Statistical analysis was performed using the Statistica for Windows software (StatSoft, USA), version 6.0. Three-way ANOVA was used to compare data from 2CdA and FMdC treatments. To assess the significance of differences between the untreated group and each of the actively treated groups, Tukey *post-hoc* test was used, *P* values less than 0.05 (marked with asterisks on graphs) were considered as statistically significant.

RESULTS

The effects of drug treatment at variant concentrations and exposure times on cell viability, induction of apoptosis and growth inhibition were highly significant ($P < 10^{-6}$). The interactions between these three independent variables according to the viability and apoptosis results were also highly significant ($P < 10^{-6}$).

A dose- and time-dependent decrease of cell viability was observed during incubation with both substances. However, at 24 h 2CdA at the highest dose (200 nM) was significantly more cytotoxic (Fig. 1a), whereas at 48 h it was significantly less cytotoxic than FMdC (Fig. 1b).

Both drugs induced dose- and time-dependent cell apoptosis as detected by phosphatidylserine translocation. After 24 h exposure their effects were of comparable magnitude (Fig. 2a). However, after 48 h of exposure the proapoptotic effects of FMdC were significantly stronger, except for the higher concentrations (100 and 200 nM) at which virtually all cells were in apoptosis (Fig. 2b).

Both substances appeared to be efficient in growth inhibition at both exposure times, but FMdC suppressed the growth of cells at significantly lower doses than 2CdA (GI_{50} for FMdC at 24 h and 48 h < 25 nM and < 10 nM, respectively, compared to > 50 nM and > 25 nM for 2CdA (Fig. 3). No differences were observed at the highest doses, at both

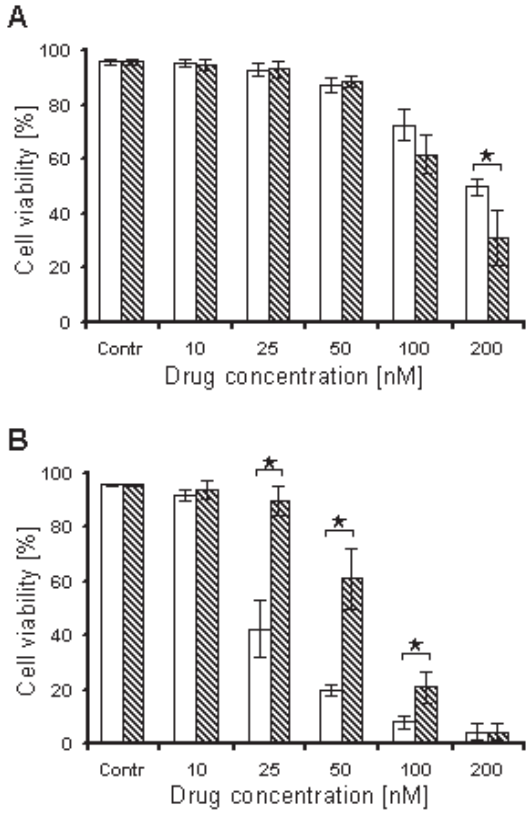

Figure 1. Decrease in viability of HL-60 cells after 2CdA or FMdC treatment.

Cells were incubated for: A) 24 h or B) 48 h, respectively, with various concentrations of FMdC (empty bars) or 2CdA (dashed bars). Cells were washed and stained with FDA/PI. Viability was assessed by flow cytometry. Asterix denotes results statistically significant at $P < 0.05$.

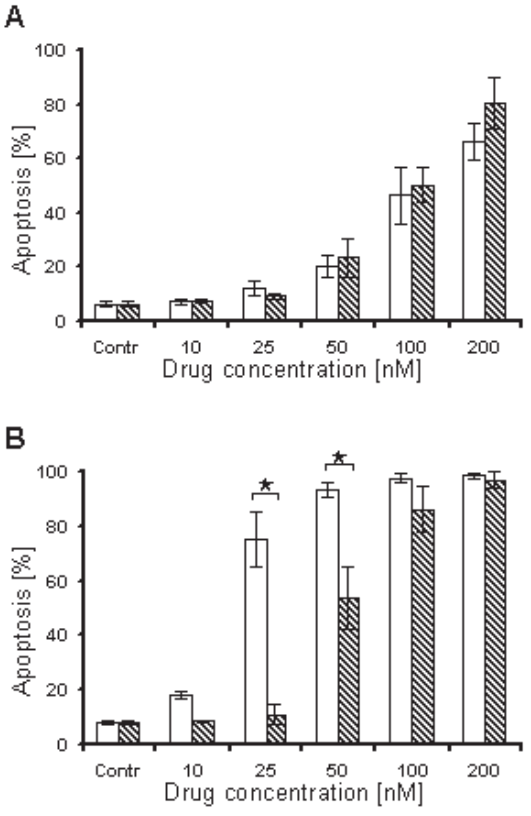

Figure 2. Induction of apoptosis of HL-60 cells after 2CdA or FMdC treatment.

Various concentrations of FMdC (empty bars) or 2CdA (dashed bars), were added to cultured cell for: A) 24 h or B) 48 h incubation, respectively. Treated cells were washed and stained with Annexin V/PI. Apoptosis occurrence was tested by flow cytometry. Asterix denotes results statistically significant at $P < 0.05$.

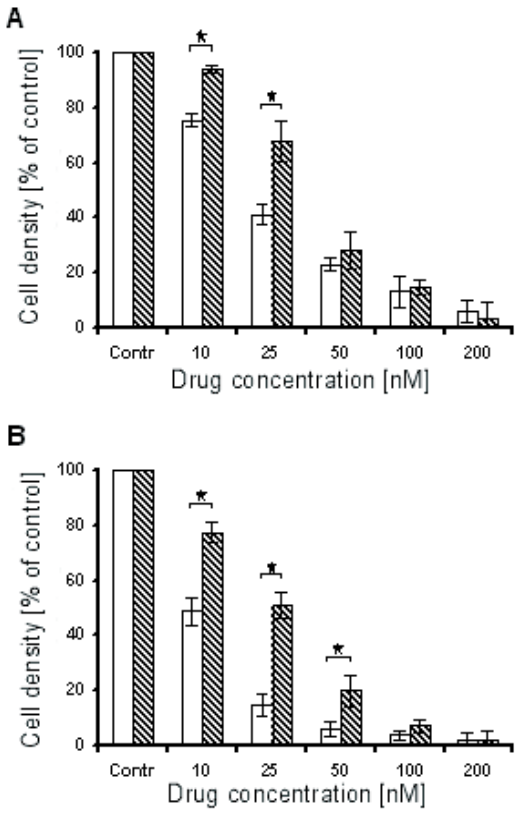

Figure 3. Growth inhibition of HL-60 cells after drug treatment.

Cells were incubated for: A) 24 h or B) 48 h, respectively, with various concentrations of FMdC (empty bars) or 2CdA (dashed bars). Cells were counted using Coulter Counter. Asterix denotes results statistically significant at $P < 0.05$.

exposure times the growth inhibition was very efficient.

The apoptotic death of cells was confirmed by morphological examination (Fig. 4). The shrinkage of cells, chromatin condensation and formation of apoptotic bodies were found at higher concentrations and longer exposure times.

DISCUSSION

The finding that both tezacitabine and cladribine are significantly toxic toward HL-60 leukemia cells *in vitro* confirms results reported previously (Skierski *et al*., 1999; Grieb *et al*., 2000). The novel observation of the present study is that the increase in the cytotoxic effects of FMdC over time is much more pronounced than that of 2CdA. Since we were unable to find any data concerning intracellular pharmacokinetics of FMdC, we may only speculate on the reasons of this difference.

Cladribine and tezacitabine display somewhat different mechanisms of cell killing. At clinically achievable doses inhibition of ribonucleotide reductase is believed to be the principal mediator of tezacitabine cytotoxicity (Takahashi *et al*., 1998), whereas it seems to play a minor role in cladribine toxicity (Griffig *et al*., 1989). Cladribine cytotoxicity is highly selective toward leukemic cells, whereas tezacitabine is active also toward a range of solid tumors. On the other hand, both nucleoside analogs share the requirement of intracellular activation by cytidine kinase-dependent phosphorylation to exert their cytotoxic effects, both are resistant to deamination by respective deaminases and accumulate as phosphates in the target cells (Liliemark, 1997; Takahashi *et al*., 1998).

There are at least two possible explanations of the increase of FMdC (but not 2CdA) cytotoxicity over time. One may consider the possibility that in HL-60 cells the system of transport and intracellular activation of 2CdA is for some reasons much more efficient, relative to the intracellular accumulation of the active form of the drug required for cytotoxicity, than that for FMdC. If FMdC phosphates accumulate in leukemic cells at a significantly lower relative rate than 2CdA phosphates, longer exposure time will be required to achieve maximal cytotoxicity of the former drug. Also, our experiments do not exclude the possibility that both FMdC and 2CdA phosphates quickly accumulate in the target cells but for some other reasons the cellular cytotoxic re-

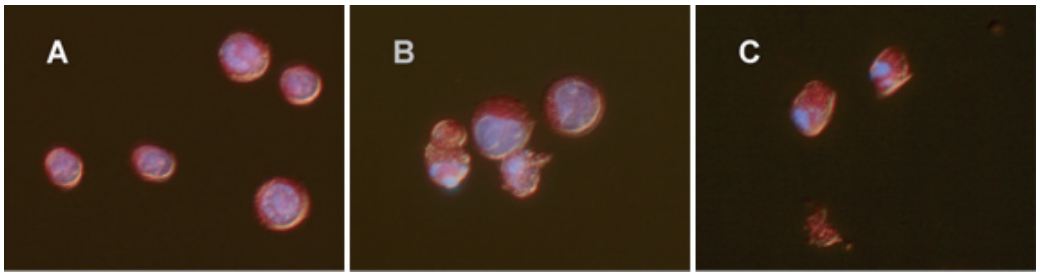

Figure 4. Morphological examination of cells stained with DAPI/sulforhodamine 101.

HL-60 cells were incubated for 48 h with 2CdA or FMdC, respectively. Morphology of cells was observed using fluorescence microscope. No changes were observed for untreated cells (A). Typical changes, like chromatin condensation and formation of apoptotic bodies were observed for cells treated with 50 nM 2CdA (B) and cells treated with 100 nM FMdC (C).

sponse develops much more slowly in the case of FMdC.

Whatever the mechanism responsible for the relatively slow development of cytotoxic effects of tezacitabine toward HL-60 leukemia cells, there is an obvious need to study the intracellular pharmacokinetics of this drug in different types of target tissues, i.e., in solid tumors as well as in leukemias. Until such data become available, continuous infusion of tezacitabine over at least two days may be a rational approach in clinical efficacy studies of this drug in hematological malignancies.

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