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Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2′-*arabino*-fluoro-2′-deoxyadenosine

(chemotherapy/immunosuppression/*scid* mice)

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ABSTRACT **2-Chlorodeoxyadenosine (CdA) is active in chronic lymphocytic leukemia, hairy-cell leukemia, and low-grade lymphomas. In part, this spectrum of activity may be attributable to the selective toxicity of CdA to nondividing lymphocytes and monocytes. However, CdA is unstable at acidic pH and is degraded by bacterial nucleoside phosphorylases. The present experiments demonstrate that the 2′-*arabino*-fluoro derivative of CdA, designated CAFdA, is also directly toxic to quiescent lymphocytes and macrophages. Unlike CdA, CAFdA was stable at pH 2 and resisted degradation by *Escherichia coli* nucleoside phosphorylase. Cell killing was preceded by the formation of DNA strand breaks and could be prevented by supplementation of the medium with deoxycytidine. The initial DNA damage initiated the pattern of oligonucleosomal DNA fragmentation characteristic of apoptosis. Mutant lymphoblasts, deficient in deoxycytidine kinase, with elevated cytoplasmic 5′-nucleotidase, or with expanded deoxynucleotide pools secondary to increased ribonucleotide reductase activity, were cross-resistant to both CAFdA and CdA toxicity. One-week oral treatment with CAFdA (1 mg/ml in drinking water) achieved an average plasma concentration of 0.56 μM and eliminated 90% of chronic lymphocytic leukemia cells transplanted into severe combined immunodeficiency (*scid*) mice. Under the same conditions, CdA was much less active. Collectively, these results suggest that CAFdA could be effective as an oral agent in indolent lymphoproliferative diseases and in autoimmune diseases where lymphocyte and monocyte depletion is desirable.**

2-Chlorodeoxyadenosine (CdA) is an adenosine deaminase-resistant deoxyadenosine analog that has proven useful in the treatment of hairy-cell leukemia, chronic lymphocytic leukemia (CLL), low-grade lymphomas, cutaneous T-cell lymphomas, and autoimmune hemolytic anemia (refs. 1–4 and D.A.C. and C.J.C., unpublished data). CdA differs from most other purine and pyrimidine antimetabolites because it is directly toxic to nondividing peripheral blood lymphocytes and monocytes (5–7). *In vivo* infusions of CdA have been shown to cause progressive lymphopenia and reversible but profound monocytopenia (7). The toxicity of CdA to lymphocytes and monocytes is augmented by continual exposure to the drug for several days. Because CdA is unstable in acid and is degraded rapidly by bacterial phosphorylases (H.B.C., D.B.W., and D.A.C., unpublished data), it is typically given as a continuous intravenous infusion. This route of administration is not ideal for most patients with CLL and is unsuitable for subjects with chronic autoimmune diseases. Therefore, it would be useful to have a compound with the properties of CdA but that resisted acid and enzymatic degradation.

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Marquez *et al.* (8) reported that the introduction of a fluorine at the 2′-*arabino* (up) position of 2′,3′-dideoxyadenosine increased the acid stability of the compound. Parker *et al.* (9) found that the 2′-*arabino*-fluoro derivative of CdA, termed 2-chloro-2′-*arabino*-fluoro-2′-deoxyadenosine or 6-amino-2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-9*H*-purine (CAFdA; Fig. 1), inhibited the growth of human K562 cells by blocking both ribonucleotide reduction and DNA polymerization (9). Therefore, it was of interest to determine if CAFdA shared with CdA the ability to kill nondividing lymphocytes and monocytes *in vitro* and *in vivo*.

CdA and related adenine deoxynucleosides are generally much less toxic to mouse than to human lymphocytes (10, 11). Thus, there is no simple method to test the *in vivo* ability of these compounds to eliminate quiescent lymphocytes and monocytes. In the present experiments, we have taken advantage of a severe combined immunodeficiency (*scid*) mouse model to maintain nondividing CLL cells for prolonged periods (12, 13, 31). This system permitted us to compare the *in vivo* efficacy of orally administered CAFdA and CdA. Our results show CAFdA is toxic at nanomolar concentrations to nondividing lymphocytes and monocytes and is effective orally for the treatment of CLL transplanted into *scid* mice.

MATERIALS AND METHODS

Preparation and Stability of CdA and CAFdA. CdA was synthesized by transfer of the deoxyribose moiety from thymidine to 2-chloroadenine utilizing *Lactobacillus helveticus* transdeoxyribosylase, as described (1). CAFdA was prepared (H.B.C., D.B.W., and D.A.C., unpublished data) from 2,6-dichloropurine and 2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranosyl bromide by the sodium salt glycosylation procedure (14), followed by treatment of the resulting blocked nucleoside with methanolic ammonia.

To assess the acid stability of the congener drugs, 1 mM CdA or 1 mM CAFdA was incubated at 37°C in 0.1 M sodium acetate buffer adjusted to pH 2 or 3 with HCl. From 1 to 24 h later, samples of the mixtures were diluted 1:50 into 20 mM sodium phosphate (pH 6.5; buffer A) and injected onto a C_{18} μBondapak reverse-phase HPLC column that was eluted with a 0–50% (vol/vol) linear gradient of acetonitrile over 30 min. The UV-absorbing peaks corresponding to CdA and CAFdA were identified, and the percentages of the starting nucleosides were calculated by comparisons of the relative peak sizes. To measure the stabilities of the two nucleosides to bacterial degradation, a crude preparation of *Escherichia*

Abbreviations: CdA, 2-chlorodeoxyadenosine; CAFdA, 2-chloro-2′-*arabino*-fluoro-2′-deoxyadenosine; CLL, chronic lymphocytic leukemia.

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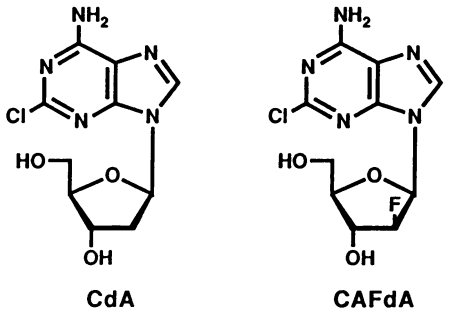

FIG. 1. Chemical structures of CdA and CAFdA. Substitution with a chlorine atom at position 2 of the purine ring renders the compounds resistant to adenosine deaminase. Substitution of a fluorine atom at the 2'-*arabino* (up) position of the deoxyribose moiety confers acid stability to the glycosidic linkage in CAFdA.

coli purine nucleoside phosphorylase was prepared as follows: *E. coli* paste (strain B, Calbiochem) was sonically disrupted in 5 mM potassium phosphate (pH 8), and the supernatant after centrifugation was fractionated by precipitation between 30% and 60% saturated ammonium sulfate. The resulting enzyme preparation was heated at 56°C for 30 min to inactivate other phosphorylases, as described by Krenitsky *et al.* (15). CAFdA or CdA was incubated with the bacterial phosphorylase (1 mg/ml) in 0.1 M sodium phosphate (pH 6.5) for various times, the reactions were stopped by boiling, and the nucleosides and bases were quantitated after separation by HPLC, as described above.

Toxicity to Cell Lines and to Normal Lymphocytes and Monocytes. We compared the antiproliferative effects of CdA and CAFdA to the human CEM T-lymphoblastoid cell line and to CEM mutants deficient in deoxycytidine kinase (16) or with elevated cytoplasmic 5'-nucleotidase (17). The DHL9 human histiocytic lymphoma cell line and a deoxyadenosine-resistant variant with elevated ribonucleotide reductase activity were also studied (18). The cell lines were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% (vol/vol) fetal bovine serum (complete medium). The cells were suspended at an initial density of 1×10^5 cells per ml in complete medium containing the indicated concentrations of CAFdA or CdA. After 3 days in culture, viable cells in the drug-treated and control cultures were compared by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction assay, as described by Mosmann (19).

Lymphocytes and monocytes were purified from human peripheral venous blood exactly as described (7). Briefly, the standard procedure involved sedimentation through Histopaque (Sigma) and two cycles of monocyte adherence to gelatin-coated plastic flasks. This procedure routinely gave 90% pure monocytes as determined by nonspecific esterase staining. For toxicity studies, the nondividing lymphocytes and monocytes were suspended at a density of 1×10^6 cells per ml in RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 10% (vol/vol) pooled human AB serum. Samples (200 μl) were dispersed among the wells of microtiter trays and either CAFdA or CdA was added to the indicated final concentrations. Five days later, viable lymphocytes were enumerated microscopically after addition of erythrosin B. Drug toxicity to nondividing monocytes was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction assay (19). In each case, the results are presented as percentage of the control value in cultures lacking added nucleosides.

DNA Strand Breaks. The formation of DNA strand breaks in CAFdA-treated peripheral blood lymphocytes was measured by the alkali-unwinding method of Birnboim and Jevcak (20), as described for CdA (6, 7). The results are expressed as the percent control double-stranded DNA.

Apoptosis Assay. Leukemic lymphocytes were isolated from the peripheral blood of a patient with hairy-cell leukemia and leukocytosis by Histopaque density-gradient centrifugation. Release of oligonucleosomal DNA fragments in the leukemic cells exposed *in vitro* to CdA or CAFdA was measured by a modification of the diphenylamine reaction (21). DNA fragments were visualized directly by ethidium bromide staining after electrophoresis in 2% agarose gels (22).

Radioimmunoassay. The radioimmunoassay for CdA was performed as described (1). Briefly, the assay measures the ability of unlabeled CdA or cross-reactive material to inhibit the binding of [8-^{3}H]CdA (Moravek Biochemicals, Brea, CA) to a high-titer rabbit antibody. Free and bound antigen were separated by precipitation in 50% ammonium sulfate and radioactivity was quantitated by scintillation counting. The results are expressed as the percentage of antibody-bound [8-^{3}H]CdA.

Treatment of *scid* Mice. *scid* mice from the Medical Biology Institute (La Jolla, CA) were maintained in microisolator cages. CLL cells from two patients were isolated by Histopaque sedimentation, washed in minimal essential medium (MEM), and suspended in MEM at 1×10^8 cells per ml at 37°C. The CLL cells had viabilities of ≥90%, as assessed by erythrosin B dye exclusion. Groups of mice received 5×10^7 CLL cells by intraperitoneal injection. One week after the cell transfers either CdA or CAFdA at 1 mg/ml was added to the water bottles of the treated animals. One week later, venous blood samples from treatment groups were pooled separately for the measurement of plasma CdA or CAFdA levels. Then the animals were sacrificed by cervical dislocation, and the CLL cells in the peritoneal cavities were collected by washing twice with 3 ml of warm Hanks' balanced salt solution. Viable cells were determined by erythrosin B dye exclusion. In one experiment, the residual CLL cells were further distinguished from mouse cells by immunofluorescent analysis using anti-CD20 and anti-CD5 antibodies (31).

RESULTS

Stability of CAFdA and CdA. Compared to CdA, CAFdA was much more stable at pH 2 and was more resistant to phosphorolysis by *E. coli* (Fig. 2). However, prolonged incubation of CAFdA with *E. coli* extracts led to the eventual formation of 2-chloroadenine. Thus, two potential barriers that might impede the oral bioavailability of CdA (i.e., gastric acid instability and enzymatic catabolism) were partially overcome by substitution of fluorine at the 2' up position of the deoxyribose moiety.

Toxicity Studies. The comparative toxicities of CAFdA and CdA to the seven cell types are summarized in Table 1. The CAFdA was 2- to 3-fold less toxic than CdA to proliferating CEM and DHL9 lymphoblasts and to nondividing peripheral blood lymphocytes and monocytes. The deoxycytidine kinase-deficient CEM mutant was impervious to growth inhibition by CAFdA, demonstrating that the antiproliferative actions of the drug required phosphorylation by this enzyme. Similarly, supplementation of the medium with exogenous deoxycytidine protected nondividing lymphocytes and monocytes from CAFdA toxicity (Fig. 3). The CEM mutant with increased cytoplasmic 5'-nucleotidase activity was 2- to 3-fold cross-resistant to CAFdA and CdA, when compared to wild-type T lymphoblasts. The DHL9 variant with increased ribonucleotide reductase activity was highly resistant to both CAFdA and CdA. These biochemical genetic studies strongly suggest that the metabolic pathways that govern susceptibility to CAFdA and CdA toxicities are similar.

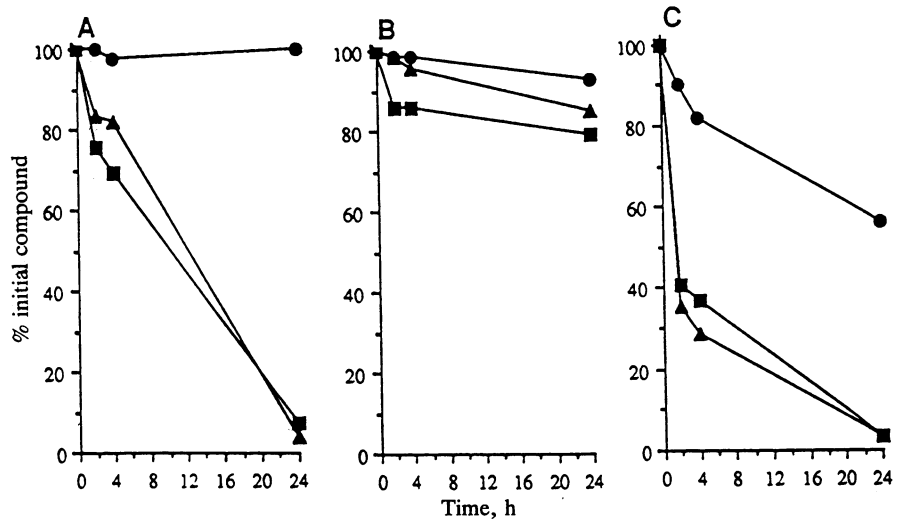

FIG. 2. Degradation of CAFdA and CdA. CAFdA (●), CdA (▲), or deoxyadenosine (■), at 1 mM, was incubated at 37°C at pH 2 (*A*) or pH 3 (*B*) or with *E. coli* nucleoside phosphorylase in 0.1 M sodium phosphate (pH 6.5) (*C*). At the indicated times, the nucleosides and bases were separated by HPLC and quantitated.

DNA Strand Break Formation. Exposure of nondividing lymphocytes, monocytes, or CLL cells to CAFdA induced the prompt formation of DNA strand breaks, or alkali-labile sites, as measured by the fluorescent unwinding technique (Fig. 4). DNA damage was detectable 4 h after drug exposure in monocytes and by 8 h in normal or leukemic lymphocytes. At the time DNA damage was first detected, the cells were still viable as measured by dye exclusion. As expected, supplementation of the medium with deoxycytidine markedly reduced DNA strand break formation.

Apoptosis in Leukemic Cells Exposed to CAFdA or CdA. Incubation of hairy-cell leukemia cells *in vitro* with CdA or CAFdA at 1 μM led to the release of low molecular weight DNA fragments. By 48 h, treatment with CdA or CAFdA resulted in the fragmentation of a substantial portion of the leukemic cell DNA (22% and 12%, respectively). DNA electrophoresis revealed the characteristic "ladder" pattern of the released DNA fragments migrating as integer multiples of 190-base-pair nucleosomal units (Fig. 5). At the time internucleosomal DNA cleavage became evident in the CAFdA-treated cells, we observed morphologic changes of nuclear condensation and cytoplasmic compaction that are considered hallmarks of apoptosis (23).

Table 1. Comparative toxicities of CdA and CAFdA to human lymphocytes and monocytes

Cell type	ID_{50}, nM		
	CdA	CAFdA	CAFdA/ CdA ratio
CEM (wild type)	21	67	3.1
CEM (deoxycytidine kinase deficient)	>16,000	>16,000	—
CEM (increased 5'-nucleotidase)	60	126	2.1
DHL9 (wild type)	80	150	1.9
DHL9 (increased ribonucleotide reductase)	4,000	6,000	1.5
Peripheral blood lymphocytes	15	28	1.9
Monocytes	22	47	2.1

ID_{50} values of CAFdA and CdA were compared in CEM T lymphoblasts, CEM mutants deficient in deoxycytidine kinase or with increased 5'-nucleotidase, DHL9 lymphoblasts, and DHL9 cells with increased ribonucleotide reductase. For nondividing lymphocytes and monocytes, the ID_{50} refers to the concentration that produced a 50% reduction in cell numbers after 5 days. Note that the toxicity profiles of CAFdA and CdA are remarkably similar.

Radioimmunoassay. CAFdA and CdA reacted nearly equivalently in the standard competition radioimmunoassay, using radioactive CdA as the ligand. In contrast, chloroadenine was at least 10-fold less effective in inhibiting ligand binding. This made it possible for us to compare CAFdA and CdA concentrations in the plasmas of mice after oral administration of either drug. Both CAFdA and CdA remained intact at a concentration of 1 mg/ml during a 1-week incubation in the drinking water at room temperature, at which time plasmas from 4 or 5 treated mice were pooled. After precipitation of proteins with 0.4 M perchloric acid, the supernatants were fractionated by reverse-phase HPLC. Those fractions corresponding to CAFdA or CdA, respectively, were combined and analyzed by competition radioimmunoassay. As indicated in Table 2, the average blood level of CAFdA (562 nM) was substantially higher than CdA (48 nM).

Oral Activity of CAFdA Against CLL. Two weeks after transplantation into the peritoneal cavities of *scid* mice, most CLL cells were recovered in a viable state (Table 2). Cytofluorometric analysis showed that these cells were small, relatively uniform in size, and still coexpressed surface antigens CD20 and CD5. Approximately 50% of the peritoneal mononuclear cells in the CAFdA-treated animals were presumably of murine origin because they did not stain with anti-CD20 or anti-CD5 antibodies. Oral administration of CAFdA eliminated 90% of the transplanted CLL populations from two patients (Table 2). Under the same conditions, CdA was less effective, eliminating approximately half of the CLL cells.

DISCUSSION

Nucleoside analogs that inhibit cell proliferation have achieved an important place in the management of acute leukemias, certain solid tumors, and autoimmune diseases. CdA and related adenine deoxynucleosides differ from most other purine and pyrimidine antimetabolites, because they are exquisitely toxic to nondividing human lymphocytes and monocytes and to rapidly proliferating cells. In part, this may explain the potent activity of CdA (2–4), deoxycoformycin (24, 25), and fluro-*arabino*-AMP (fludarabine phosphate) (26) in indolent lymphoproliferative diseases.

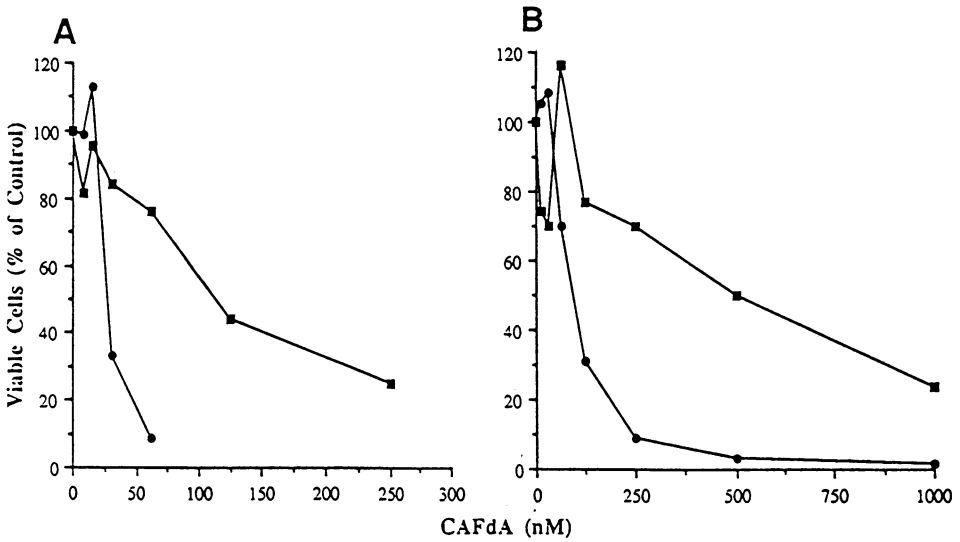

FIG. 3. Toxicities of CAFdA to normal lymphocytes and monocytes. Nondividing lymphocytes (*A*) or monocytes (*B*) were incubated for 5 days in complete medium with (■) or without (●) 100 μM deoxycytidine.

Cell-cycle-nonspecific antimetabolites would be expected to have harmful side effects to normal tissues. This is not the case with CdA, because the toxicity of the drug depends upon biochemical pathways that differ substantially among cell types. Sensitivity to CdA killing is principally a function of the ratio of deoxycytidine kinase to cytoplasmic 5′-nucleotidase (5, 16, 17). Long-lived cells that have higher deoxycytidine kinase activity than cytoplasmic 5′-nucleotidase activity progressively accumulate CdA phosphates. The exact mechanism by which CdA 5′-triphosphate kills nondividing lymphocytes and monocytes has not been established. However, the formation of single-strand breaks in DNA is the earliest biochemical change detected in CdA-treated cells and may, therefore, be related to drug toxicity (6). As a consequence of the early DNA strand breaks, normal and leukemic lymphocytes (27) and monocytes (28) undergo apoptosis with characteristic internucleosomal cleavage of DNA.

CdA is unstable in acid and is a substrate for bacterial nucleoside phosphorylases and *trans*-deoxyribosylases (1). The introduction of a fluorine at the 2′-*arabino* position of 2′,3′-dideoxyadenosine has been reported to increase its stability in acid (8). As shown above, the 2′-*arabino*-fluoro derivative of CdA, designated CAFdA, is also stable at pH 2 and is degraded slowly by *E. coli* extracts. When compared to CdA, the oral administration of CAFdA to mice achieved 10-fold higher concentrations in the plasma.

The introduction of a small chemical change in a nucleoside can have profound and unexpected effects on its metabolism and toxicity. It was therefore critically important to demonstrate that CAFdA and CdA are metabolized similarly in human cells. For this purpose, the three classes of CdA-resistant mutant lymphoblasts provided an instructive test system. The fact that human lymphocytes deficient in deoxycytidine kinase (16), with elevated 5′-nucleotidase activity (17), or with increased deoxynucleotide pools secondary to augmented ribonucleotide reductase activity (18) were equally cross-resistant to CdA and CAFdA strongly suggests that the two deoxynucleosides are metabolized similarly in these cells. In further support of this hypothesis, the toxicity of CAFdA toward nondividing lymphocytes and monocytes was prevented by supplementation of the medium with deoxycytidine and was preceded by the formation of single-strand breaks in DNA. Based upon these results, it seems likely that the biological activities of CAFdA and CdA in humans will be similar.

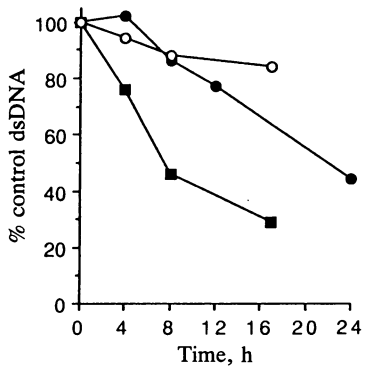

FIG. 4. DNA strand breaks in CAFdA-treated lymphocytes, monocytes, and CLL cells. Normal peripheral blood lymphocytes (●), monocytes (■), and CLL cells (○) were exposed to 1 μM CAFdA for 4–24 h at which times DNA strand breaks were estimated by the alkali fluorescent unwinding method of Birnboim and Jevcak (20). The results are expressed as percent control double-stranded DNA (dsDNA).

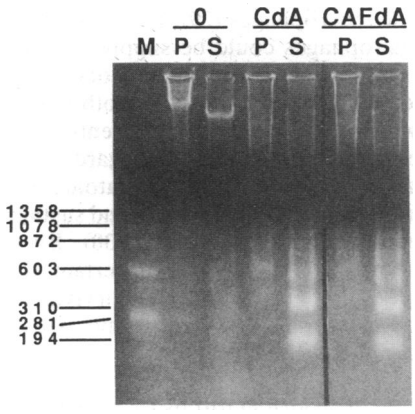

FIG. 5. Oligonucleosomal DNA fragmentation in leukemic lymphocytes exposed to CdA or CAFdA. Hairy-cell leukemia cells were treated *in vitro* with CdA or CAFdA at 1 μM for 48 h. Soluble low molecular weight DNA fragments (lanes S) were separated from high molecular weight chromatin (lanes P) by gentle lysis with 0.2% Triton X-100. Electrophoresis was performed in a 2% agarose gel in Tris acetate/EDTA buffer (pH 8.0) for 3 h. DNA bands were visualized under UV after ethidium bromide staining. ΦX174 plasmid cut with *Hae* III served as fragment size markers (lane M); positions in bases are shown to the left.

Table 2. Oral activity of CAFdA and CdA

Treatment group	Plasma drug level, nM	% of CLL cells surviving
CAFdA	562	8.9 ± 6.6 (9)
CdA	48	46.9 ± 20 (9)
Control	0	83.0 ± 28 (8)

Groups of *scid* mice were transplanted intraperitoneally with 5×10^7 CLL cells in two experiments. One week later treatment with CAFdA or CdA (1 mg/ml in the drinking water) was begun. In one experiment, pooled plasma samples from 4 or 5 mice per group were obtained for drug levels by radioimmunoassay. In both experiments, the percentages of surviving CLL cells were determined individually, and the results shown are the mean ± SEM for all animals; the number in each treatment group is shown in parentheses.

Previously, two major obstacles have hampered animal studies of CdA and related adenine deoxynucleosides as therapeutic agents for the treatment of indolent lymphoproliferative diseases. The first problem was the lack of a well-characterized mouse model for hairy-cell leukemia or for CLL. Most murine transplantable leukemias and lymphomas have a sizable growth fraction and do not mimic the slowly evolving course of lymphoproliferative diseases in humans. The second problem was that purine deoxynucleosides are generally much more toxic to human lymphocytes than to mouse lymphocytes. As examples, deoxycoformycin is not very active in murine leukemias (24), whereas CdA displays antileukemic activity in mice at dosages nearly 100-fold higher than are used in humans (1).

The use of *scid* mice transplanted with human lymphocytes offers one potential approach to overcome these problems. *scid* mice may be reconstituted for prolonged periods with normal human lymphocytes or with lymphoid progenitors (12, 13). CLL cells may also be maintained for weeks after adoptive transfer into *scid* mice (31). In this model, administration of CAFdA in drinking water was more effective than CdA in eliminating the transplanted CLL cells. The increased efficacy correlated with the higher plasma levels of CAFdA, as determined by a specific radioimmunoassay.

In the long run, one of the most promising uses of CAFdA and related compounds may be for the treatment of autoimmune diseases. These illnesses are probably maintained by expanded populations of long-lived memory T lymphocytes that react with self components and release cytokines that activate tissue macrophages (29). If autoreactive T cells and activated macrophages could be suppressed, with the preservation of normal lymphocyte precursors, clinical improvement should ensue. As shown here, both CAFdA and CdA are toxic *in vitro* at nanomolar concentrations to normal lymphocytes and monocytes. In this regard, it is also relevant that synovial lymphocytes from rheumatoid arthritis patients have been transplanted into *scid* mice and sustained autoantibody production has been achieved (30). By using such a model, it should be possible to ascertain the effects of CAFdA on the *in vivo* function and survival of tissue lymphocytes and macrophages from patients with arthritis and related autoimmune diseases.

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