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Disposition and metabolism in mice of the potential antitumor and anti-human immunodeficiency virus-1 agent, 2-chloro-2′,3′-dideoxyadenosine*

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Summary. A high-performance liquid chromatographic (HPLC) procedure was developed to examine the preclinical pharmacology and pharmacokinetics of 2-chloro-2′,3′-dideoxyadenosine (CldAd). The HPLC assay for CldAd in human plasma was linear from 0.25 to 500 μg CldAd/ml. Coefficients of variation for the measurement of CldAd in human plasma were 9.7%, 4.1%, and 2.7% at 2.5, 25, and 250 μg/ml, respectively. Binding of CldAd to human and mouse plasma proteins was determined by filtration to be 26.9% and 34.4%, respectively. CldAd concentrations decreased by <5% when CldAd was stored for 126 h at 37° C in 0.9% NaCl or 0.1 M NaH_2PO_4 (pH 7.4) or when CldAd was stored for 24 h at 37° C in citrate-buffered human blood or plasma. Estimates of the lethal dose for 50% (LD_{50}) and 10% (LD_{10}) of male $CD2F_1$ mice that received a single i.v. dose of CldAd were 27 and 24 mg/kg, respectively. Elimination of a 24-mg/kg i.v. bolus dose of CldAd from mouse plasma was biphasic, with half-lives of 0.73 and 14.7 min. The apparent volume of distribution of CldAd was 215 ml/kg and the total body clearance was 20 ml min^{-1} kg^{-1}. No CldAd metabolites were detected in mouse plasma after in vivo exposure or in human whole blood or plasma after in vitro incubation. CldAd was detected in the urine of mice within 2 min after exposure, and the total urinary excretion of unchanged CldAd for 24 h after exposure to 24 mg/kg was 3.4% of the delivered dose. At least two possible CldAd metabolites were detected in mouse urine; they did not co-elute with 2-chloro-2′,3′-dideoxyinosine, 2-chloroadenine, or 2-chlorohypoxanthine.

Introduction

The 2′,3′-dideoxy derivatives of adenosine, guanosine, inosine, cytidine, and thymidine inhibit the infectivity of human immunodeficiency virus-1 (HIV) toward human T-lymphoblasts in vitro [18]. Several studies indicate that these 2′,3′-dideoxynucleosides are phosphorylated to their respective 5′-triphosphates, which then serve as substrates for retroviral reverse transcriptase [8, 16]. Incorporation of the 2′,3′-dideoxynucleotides into retroviral DNA results in chain termination [1]. The 2′,3′-dideoxynucleotides are poor substrates for mammalian DNA polymerase α [25], which may explain the low host-cell toxicity and selective anti-HIV activity exhibited by these compounds [14, 19].

2′,3′-dideoxyadenosine is one of the least cytotoxic and, in vitro, the most effective of the anti-HIV dideoxynucleosides [18]. However, in vivo, 2′,3′-dideoxyadenosine is catabolized by adenosine deaminase to 2′,3′-dideoxyinosine, which may be further catabolized by purine nucleoside phosphorylase to the inactive hypoxanthine and dideoxysugar [24]. Modification of 2′,3′-dideoxyadenosine by inclusion of a halogen atom on the C-2 carbon of the purine base inhibits deamination by adenosine deaminase but does not significantly decrease the uptake or phosphorylation by human T-lymphoblasts [13]. The 2-halo-2′,3′-dideoxyadenosines, including 2-chloro-2′,3′-dideoxyadenosine (CldAd), are as effective as in vitro anti-HIV agents as is 2′,3′-dideoxyadenosine [13, 19].

In addition to anti-HIV activity, the dideoxynucleosides may possess significant antitumor activity, as suggested by studies using structurally related compounds. Although the 2′,3′-dideoxynucleotides are poor substrates for mammalian DNA polymerase α, they are recognized as substrate analogues by the terminal deoxynucleotidyl transferase (TdT) DNA polymerase of primitive lymphocytes [3]. The selective toxicity of 2′,3′-dideoxyadenosine toward TdT-positive lymphoblastic leukemia cells in culture suggests that the use of 2′,3′-dideoxynucleotides to treat TdT-positive leukemic cells in vivo may be clinically relevant [23]. The 2-halo analogues of deoxyadenosine are also effective inhibitors of DNA synthesis in human lym-

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phoblastic cells [12]. 2-Bromo-deoxyadenosine, alone or in combination with other drugs, significantly increases the life span of L1210 leukemia-bearing mice [2].

In this paper we report the development of a high-performance liquid chromatographic (HPLC) assay for CldAd in biological fluids. This assay was used to study the stability of CldAd in human and mouse blood and plasma as well as the pharmacokinetic disposition of CldAd in male $CD2F_1$ mice.

Materials and methods

Chemicals. CldAd (NSC 619531) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.); its purity was >97% as determined by HPLC. CldAd was only slightly soluble in 0.9% NaCl or 0.1 M NaH_2PO_4 buffer (pH 7.4) but was very soluble in dimethylsulfoxide (DMSO). Therefore, CldAd was first dissolved in a minimal volume of DMSO before its addition to 0.9% NaCl or 0.1 M NaH_2PO_4 buffer. The maximal final DMSO concentration was 10%, and the limit of CldAd solubility in 10% DMSO/saline was approximately 3 mg/ml.

Several derivatives of CldAd were prepared for comparison with CldAd metabolites detected in mouse urine. 2-Chloroadenine, the free base of CldAd, was prepared by treating CldAd with 3 M HCl at 60° C [9]. The conversion of CldAd to 2-chloroadenine was determined by HPLC analysis to be complete within 10 min. CldAd was deaminated to 2-chloro-2′,3′-dideoxyadenosine by incubation of 0.2 mg/ml CldAd with 10 units/ml adenosine deaminase in a 10 mM NaH_2PO_4 buffer (pH 7.4) at 20° C [13]. In all, 25% of the conversion of CldAd to 2-chloro-2′,3′-dideoxyadenosine was determined by HPLC analysis to be complete within 24 h. 2-Chloro-2′,3′-dideoxyadenosine was collected from the HPLC column effluent, and 2-chlorohypoxanthine, the free base of 2-chloro-2′,3′-dideoxyadenosine, was prepared by treating 2-chloro-2′,3′-dideoxyadenosine with 3 M HCl at 60° C for 10 min.

Caffeine, adenosine deaminase (Type IV), β-glucuronidase (Type VII) and arylsulfatase (Type H-1) were obtained from Sigma Chemical Co. (St. Louis, Mo). Tetrabutyl ammonium phosphate (PIC-A) was obtained from Millipore Corporation (Milford, Mass). All other chemicals were reagent-grade or better and were purchased from commercial sources.

Animals. Male $CD2F_1$ mice weighing 23–25 g were used in all experiments. Mouse plasma was obtained from animals that were lightly anesthetized with diethyl ether. As soon as rapid limb movements had ceased, the animals were exsanguinated by bleeding from the retro-orbital venous plexus into chilled, heparinized 1.5-ml centrifuge tubes. Heparin-treated blood was centrifuged at 10,700 *g* for 3 min to obtain plasma.

Sample preparation. Specimens of human whole blood, human and mouse plasma, or mouse urine were treated with 1.5 vol. acetonitrile, and 10–20 µg caffeine/ml was added as an internal standard. Samples were stored overnight at 4° C and subsequently centrifuged at 10,700 *g* for 10 min. The supernate was removed and assayed for CldAd. The acetonitrile-treated supernates from mouse urine samples were diluted with 4 vol. 0.1 M KH_2PO_4 (pH 7.4) prior to assay.

Assay. HPLC analysis was performed using a 25-cm Hibar-II RP-18 5-µm column (Merck, Darmstadt, FRG) preceded by a 1-cm RP-18 precolumn (Altech Associates Inc., Deerfield, Ill.). Sample injection volumes were typically 10 or 25 µl. Two solvent systems were used to elute CldAd: elution system A consisted of a 20-min linear gradient of 10%–30% methanol in a 10-mM KH_2PO_4 buffer (pH 6.5); solvent system B consisted of a 30-min linear gradient of 10%–30% methanol in a 2.5-mM PIC-A, 10-mM KH_2PO_4 buffer (pH 6.5). The flow rate for both solvent systems was 1.5 ml/min. Eluting compounds were detected at 254 nm with a variable wavelength detector (798 575A Hewlett-Packard, Avondale, Pa.), which was also used to record the UV absorption spectra from 210 to 400 nm of CldAd and CldAd derivatives.

In vitro studies. The stability of CldAd in 0.9% NaCl, 0.1 M NaH_2PO_4 buffer (pH 7.4), and fresh citrate-buffered human blood or plasma was determined. CldAd stability studies in 0.9% NaCl and 0.1 M NaH_2PO_4 buffer were conducted at 4°, 20°, and 37° C and at initial CldAd concentrations of 2.2, 22, and 220 µg/ml. Stability studies in human blood and plasma were conducted at the same temperatures and at initial CldAd concentrations of 7.5, 25, and 250 µg/ml.

Binding of CldAd to human or mouse plasma proteins was determined by filtration of plasma through Centrifree micropartition filters using YMT membranes with a molecular weight cutoff of 30 kDa (Amicon, Danvers, Mass.). CldAd was added at initial concentrations of 7.5, 25, and 250 µg/ml to citrate-buffered human plasma or heparin-treated mouse plasma and then incubated at 20° C for 10 min before filtration. Binding of CldAd to the micropartition filter was minimal, and its recovery from filtered 0.9% NaCl was 99% ± 3.4% (mean ± SD of the three CldAd concentrations tested).

Uptake of CldAd by human blood cells was determined after the addition of 25 µg CldAd/ml to citrate-buffered human blood, followed by incubation for 30 min at 20° C. Blood was centrifuged at 10,700 *g* for 1 min, plasma was separated from packed cells, and CldAd concentrations were determined in the plasma.

Toxicity studies. CldAd was injected i.v. into the tail vein of male $CD2F_1$ mice. Doses of 19, 24, 30, and 40 mg/kg were used, and five to eight mice were exposed at each dose. Animals were observed for 30 days post-exposure. Estimates of the lethal dose for 50% of the animals (LD_{50}) were obtained by probit analysis [11] of the mortality observed after CldAd injection.

Pharmacokinetic studies. CldAd was given at a dose of 24 mg/kg (72 mg/m^2) by rapid (<30 s) i.v. injection into the tail vein of male $CD2F_1$ mice. Blood was collected from three or four mice at each time point from 0 to 90 min postinjection. The collected blood was treated with heparin and immediately centrifuged, and CldAd was measured in the plasma as described above. Pharmacokinetic parameters were estimated by nonlinear regression analysis of plasma drug concentrations (PCNONLIN, Statistical Consultants, Inc., Lexington, Ky.) using a weighting factor of $1/y^2$.

Mouse urine was collected from the bladder of animals sacrificed at various times after CldAd administration. The urine was centrifuged at 10,700 *g* for 3 min and CldAd was measured in the supernate. The total urinary excretion of CldAd for 24 h after drug administration was also determined. Three groups of five mice were given 24 mg/kg CldAd/kg by i.v. tail-vein injection and immediately placed in metabolism cages. Urine was collected at 4° C for 24 h, and the metabolism cage was washed with 20 ml 0.1 M NaH_2PO_4 buffer (pH 7.4) at the end of the experiment. CldAd concentrations were measured in the collected urine and the washes.

Results

HPLC assay

Reversed-phase HPLC of CldAd in solvent system A resulted in the separation of CldAd from normal human plasma constituents (Fig. 1 A). The unambiguous identification of CldAd in biological samples that contained substantial amounts of normal phosphorylated adenine nucleotides [i.e. adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP)] required the addition of 2.5 mM PIC-A to the mobile phase (solvent system B; Fig. 1 B). Biological samples, such as whole blood or plasma obtained after in vivo CldAd exposure, in which phosphorylated derivatives of CldAd may have been present were chromatographed in solvent systems A and B. The assay for CldAd in human plasma was linear up to a concentration of at least

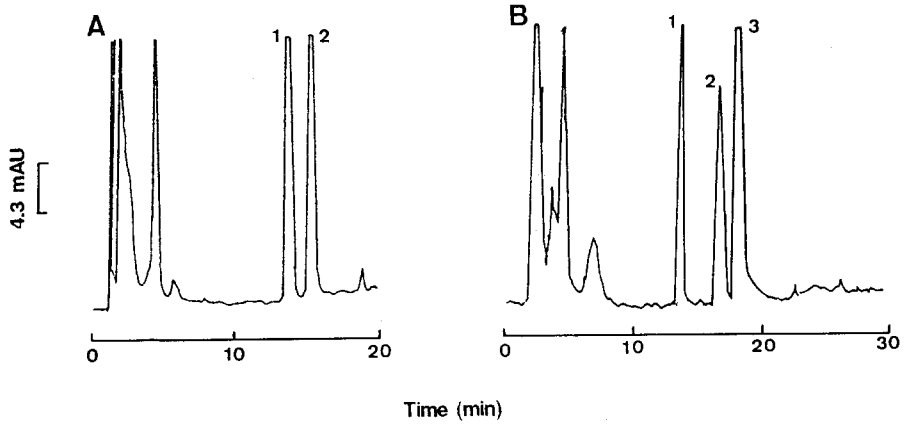

Fig. 1. HPLC chromatograms of CldcAd added to **A** human plasma and **B** human blood and incubated at 37° C for 24 h. CldcAd (*peak 2*) was separated from normal plasma constituents and caffeine (*peak 1*), added as an internal standard, by solvent system A. CldcAd (*peak 2*) in whole blood was separated from normal blood constituents and caffeine (*peak 1*) by solvent system B, which included 2.5 mM PIC-A. *Peak 3* in chromatogram **B** co-eluted with adenosine triphosphate

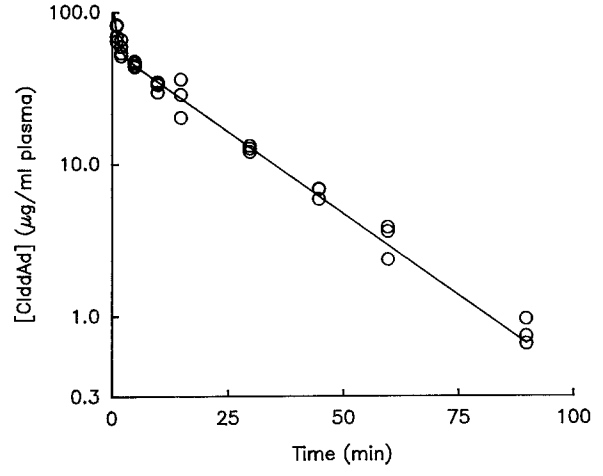

Fig. 2. Plasma CldcAd in male $CD2F_1$ mice following i. v. bolus administration of 24 mg/kg CldcAd. Each point represents one animal. The *continuous line* shows the nonlinear regression analysis of the experimental data

500 μg/ml, with a lower limit of detectability of 0.25 μg/ml. Recoveries of 25 μg/ml CldcAd and of 25 μg/ml caffeine (internal standard) from human plasma after acetonitrile precipitation of plasma proteins were 87% and 91%, respectively. The coefficients of variation of ten repeated assays for CldcAd in human plasma were 9.7% at 2.5 μg/ml, 4.1% at 25 μg/ml, and 2.7% at 250 μg/ml.

CldcAd stability and plasma protein binding

CldcAd concentrations decreased by <5% when the substance was stored at 4°, 20°, or 37° C for 126 h in 0.9% NaCl or 0.1 M NaH_2PO_4 buffer (pH 7.4). Concentrations also decreased by <5% when CldcAd was stored at 4°, 22°, or 37° C for 24 h in human citrate-buffered blood or plasma.

CldcAd binding to plasma proteins at 20° C was determined by filtration to be 26.9% ± 2.2% and 34.4% ± 9.1% for human and mouse plasma (mean ± SD of three concentrations), respectively. CldcAd was distributed equally between plasma and the red blood cells in human blood. The percentage of CldcAd added to human whole blood that was found in the plasma was 54.5% ± 2.6% (mean ± SD of seven replicates), and the hematocrit of the blood used in these studies was 47%.

Toxicity studies

A single i.v. dose of CldcAd in male $CD2F_1$ mice produced significant mortality at doses of >20 mg/kg. CldcAd exposure and the resulting responses, in number of deaths/number of animals dosed, were: 40 mg/kg, 5/5; 30 mg/kg, 1/5; 24 mg/kg, 2/5; 19 mg/kg, 0/8; and 15 mg/kg, 0/3. Probit analysis of this data provided LD_{50} and LD_{10} estimates of 27 mg/kg (81 mg/m^2) and 24 mg/kg (72 mg/m^2), respectively. No immediate effects of CldcAd were observed at these doses. Animals were not moribund until shortly before death, which usually occurred 16–24 h post-exposure. All deaths were observed within 72 h after CldcAd exposure. The injection of an equal volume of DMSO, at a concentration of 10% DMSO in 0.9% NaCl, produced no signs of toxicity.

Pharmacokinetic studies

The elimination of an i.v. bolus dose of 24 mg/kg CldcAd from mouse plasma was biphasic (Fig. 2). Plasma CldcAd concentrations (C) vs time post-exposure (t) were best fit by a two-compartment model ($C = Ae^{-\alpha t} + Be^{-\beta t}$) Estimates of the apparent first-order rate constants obtained by nonlinear regression for this model were: α, 0.942 min^{-1}; and β, 0.047 min^{-1}. Estimates of the corresponding zero-time intercepts for this model were: A 56.7 μg/ml; and B 53.7 μg/ml. The half-life for the initial phase of CldcAd elimination was 0.73 min, and that for the final phase was 14.7 min. The apparent volume of distribution of CldcAd was 215 ml/kg and the total body clearance was 20 ml/min/kg. No CldcAd metabolites were detected in mouse plasma.

CldcAd was detected in mouse urine within 2 min after an i.v. bolus dose of 24 mg/kg, and maximal urine concentrations of 170 μg CldcAd/ml were obtained within 30 min post-exposure. The total urinary excretion of unmetabolized CldcAd over a 24-h period after an i.v. bolus dose of 24 mg/kg was 3.4% ± 0.6% (mean ± SD of three deter-

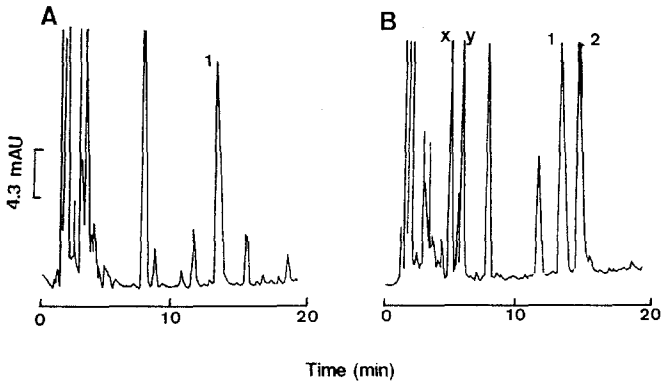

Fig. 3. HPLC chromatograms of mouse urine **A** before and **B** 45 min after administration of an i.v. bolus dose of 24 mg/kg CldAd. Caffeine (*peak 1*) was added to the urine as an internal standard; *peak 2* co-eluted with CldAd. Putative but unidentified CldAd metabolites are labeled *x* and *y*

minations) of the delivered dose. In addition to the parent drug, at least two possible CldAd metabolites were detected in mouse urine (Fig. 3). These metabolites exhibited UV absorption spectra characteristic of adenine nucleosides, with absorption maxima occurring at 261–269 nm (results not shown). Elution times for these metabolites, relative to CldAd, were 0.33 and 0.39 in solvent system A (Fig. 3B) and 0.40 and 1.21 in solvent system B. 2-Chloroadenine, 2-chloro-2′,3′-dideoxyadenosine, and 2-chlorohypoxanthine added to mouse urine did not co-elute with the unidentified CldAd metabolites. HPLC retention times for the prepared CldAd derivatives, relative to CldAd in solvent system A, were: 2-chloroadenine, 0.43; 2-chloro-2′,3′-dideoxyadenosine, 0.30; and 2-chlorohypoxanthine, 0.19. Incubation of mouse urine with β-glucuronidase or arylsulfatase as previously described [7] did not affect the chromatographic behavior of the unidentified CldAd metabolites (results not shown).

Discussion

The in vivo antiretroviral activity of 2′,3′-dideoxyadenosine is limited by its deamination to 2′,3′-dideoxyinosine [8] and the subsequent conversion of 2′,3′-dideoxyinosine to hypoxanthine [24]. 2′,3′-Dideoxyadenosine is rapidly deaminated in vitro by human or mouse plasma to 2′,3′-dideoxyinosine [4], and attempts to regulate the in vivo degradation of 2′,3′-dideoxyadenosine have included co-administration of the adenosine deaminase inhibitor 2′-deoxycoformycin [6] or the synthesis of 2′,3′-dideoxyadenosine analogues, e.g., CldAd, that are resistant to deamination [13]. Previous studies indicate that the relative efficiency of purified adenosine deaminase for the deamination of 2′,3′-dideoxyadenosine is 88 times greater than that for the deamination of CldAd [13]. Our inability to detect 2-chloro-2′,3′-dideoxyinosine in human plasma or whole blood in vitro or in mouse plasma or urine in vivo is consistent with the resistance of CldAd to deamination.

Activation of 2′,3′-dideoxynucleosides requires their phosphorylation to the corresponding nucleotides, and previous studies indicate that CldAd is phosphorylated by human T-lymphocyte deoxycytidine kinase to the mono-, di-, and triphosphates [13]. In the present studies, CldAd concentrations did not change and its nucleotides could not be detected in human whole blood after incubation for 24 h. However, based on the reported rate of CldAd phosphorylated in human T-lymphocytes [13], the expected decrease in whole-blood concentrations at 7.5 μg CldAd/ml would be <0.3% in 24 h [13]. This decrease in CldAd concentration is within the experimental variability of the assay and would not have been detected. Since active nucleotide phosphatases are present in plasma and CldAd nucleotides may be rapidly dephosphorylated to the nucleoside, the absence of CldAd nucleotides in mouse plasma after in vivo CldAd exposure is not unexpected. Human plasma phosphatase is sufficiently active to dephosphorylate 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate completely to the nucleoside within 1 min after cessation of the infusion [15].

CldAd concentrations in mouse plasma decreased rapidly after a single i.v. bolus dose, with a terminal elimination half-life of 14.5 min. No metabolites were observed in mouse plasma after i.v. administration of CldAd, although 99% of the delivered dose was eliminated from mouse plasma within 1.5 h. However, two unidentified metabolites, which did not co-elute with 2-chloro-2′,3′-dideoxyinosine, 2-chlorohypoxanthine, or 2-chloroadenine, were detected in mouse urine within 10 min after CldAd administration. Assuming that these metabolites exhibit an extinction coefficient comparable with that of CldAd, 9.5% of the total CldAd dose was excreted as either metabolite or parent drug within 24 h after exposure. Only 3.4% of the delivered CldAd dose was excreted unchanged in the urine over 24 h. These results suggest that the rapid clearance of CldAd from mouse plasma does not result from urinary excretion of a metabolite or the parent drug. Alternative routes of CldAd clearance from plasma may include excretion of the parent drug or its metabolites in the bile or metabolism of CldAd to a metabolite, such as a phosphorylated nucleotide, which is trapped intracellularly. 2-Chlorodeoxyadenosine and 9-β-D-arabinofuranosyl-2-fluoroadenine (2-F-ara) are converted by human lymphoblastoid cells in culture to the mono-, di-, and trinucleotides, and these nucleotides accumulate intracellularly to concentrations 70- to 100-fold those of the extracellular nucleosides [2, 20].

The urinary excretion of CldAd appears to be substantially lower than that of other nucleoside analogues. Mice dosed i.v. with 51.2 mg/kg [^{3}H]-dideoxyinosine excrete 32% of the delivered dose unchanged in the urine within 24 h [10]. The 24-h urinary excretion of [^{3}H]-dideoxycytidine in mice given 100 mg/kg i.v. dideoxycytidine is 80% [17]. The reasons for the relatively low urinary excretion of CldAd in the present studies are unknown. It is possible that the saturation of capacity-limited processes, such as the tissue uptake of CldAd or CldAd binding to plasma proteins, is required before substantial amounts of the parent drug are excreted in the urine. The 24-mg/kg dose of CldAd given in these studies is somewhat lower than the doses of nucleoside analogues that have been given in previous studies, and urinary excretion of CldAd may be more pronounced at higher doses.

Although CldAd is resistant to deamination, its half-life in mouse plasma is only slightly longer than the 5.9 min reported for 2′,3′-dideoxyadenosine [21]. The difficulties inherent in the use of the mouse as a model for humans in the in vivo testing of adenosine analogues have been discussed previously [15]. Differences between humans and mice include a more broadly distributed ability to phosphorylate deoxyadenosine in mouse tissues [5] and a greater rate of adenosine deamination in human tissue [22]. Both phosphorylation and deamination are expected to contribute to the clearance of adenine derivatives from plasma. The resistance of CldAd to deamination suggests that its clearance is primarily dependent on the rate of phosphorylation. These considerations suggest that adenine deaminase-resistant derivatives of adenine, such as CldAd, may exhibit substantially longer plasma half-lives in humans than in mice. For example, the adenine deaminase-resistant nucleoside 9-β-D-arabinofuranosyl-2-fluoroadenine exhibits biphasic kinetics in human plasma in vivo with elimination half-lives of 0.60 and 9.3 h [20]. These elimination half-lives are substantially longer than the 0.73 and 14.7 min half-lives for CldAd elimination from mouse plasma.

In summary, an HPLC assay was developed for CldAd and its metabolites in blood and urine. CldAd concentrations decreased by <5% after 24 h in saline, phosphate buffer, and human blood at 37° C. Elimination of CldAd from mouse plasma following an i.v. bolus dose was biphasic, with a terminal half-life of 14.7 min. The total excretion of unchanged drug in mouse urine after 24 h was 3% of the delivered i.v. bolus dose, and a further 6.5% of the delivered dose was excreted as two unidentified metabolites. This suggests that CldAd is either eliminated by another route or trapped within tissues by phosphorylation.

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