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Interspecies differences in the kinetic properties of deoxycytidine kinase elucidate the poor utility of a phase I pharmacologically directed dose-escalation concept for 2-chloro-2′-deoxyadenosine

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Abstract 2-Chloro-2′-deoxyadenosine (CdA, Cladribine), is a purine antimetabolite currently under investigation in phase II clinical trials for the treatment of lymphoid malignancies. Significant differences in CdA toxicity between mice and humans were observed during phase I clinical evaluation. For the elucidation of interspecies differences in drug toxicity the pharmacokinetics of CdA after subcutaneous injection and the kinetic properties of the CdA-phosphorylating enzyme, deoxycytidine kinase (dCK), were compared in mice and humans. The ratio of the dose lethal to 10% of mice (LD_{10}) to the maximum tolerated dose (MTD) in humans was 50 and the ratio of the area under the curve obtained at approximately one-half the LD_{10} ($AUC_{approx.\ one\text{-}half\ the\ LD_{10}}$)/$AUC_{MTD}$ was 49. A significant interspecies difference was observed in the kinetic properties of dCK, the main CdA-activating enzyme. With CdA as a substrate, the Michaelis constant (K_m) of dCK in crude extracts of mouse thymus was 10 times higher than that in human thymus. An approximately 9-fold interspecies difference in maximum velocity (V_{max})/K_m indicated a higher efficiency of dCK for CdA in humans than in mice. The peak plasma concentration was 210 times higher and exceeded the K_m in mice. Initial and terminal half-lives were approximately 7 times shorter in mice and trough levels were similar in mice and humans. Thus, the differences in AUCs at equitoxic doses are largely explained by differences in the target enzyme properties and the pharmacokinetic pattern. The observed lower tolerance for CdA in humans as compared with mice confirms the view that antimetabolites may not be good candidates for pharmacokinetically guided dose-escalation schemes unless detailed information on interspecies variability in drug bioactivation is available.

Keywords Chlorodeoxyadenosine · Deoxycytidine kinase · Pharmacokinetics · Human· Mouse

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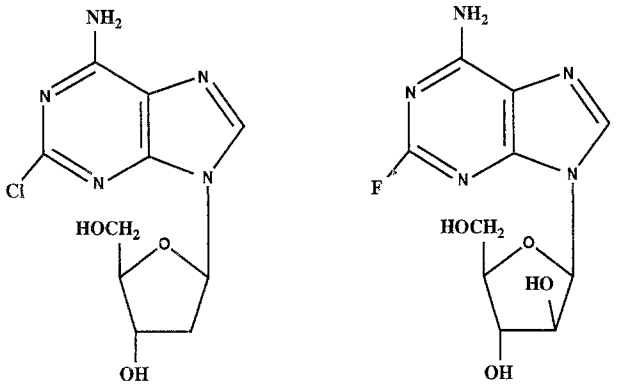
Introduction

Clinical testing of new anticancer drugs is based on the initial evaluation of drug toxicity in phase I clinical trials, the main goal of which is to determine the maximum tolerated dose (MTD). This information is then used in subsequent phase II studies of therapeutic activity. As based on preclinical toxicology data, usually one-tenth of the dose lethal to 10% of drug-treated mice (LD_{10}) is the phase I starting dose and the rate of escalation until the MTD is reached is governed empirically by the "modified Fibonacci" scheme [7].

The concept of using preclinical pharmacology for controlling the rate of dose escalation was introduced by Collins et al. [7] and is generally termed "pharmacologically guided dose escalation" [10]. The main assumption was that similar biological effects (e.g., toxicity) would occur at similar plasma levels in animals and humans. The area under the concentration versus time curve (AUC or CxT) is the most appropriate measure of plasma exposure and, according to the above concept, is often poorly correlated to the delivered dose, particularly when different species are being compared. Therefore, the ratio of the total AUC values (in units of concentration versus time) at the human MTD and mouse LD_{10} should be closer to



CdA F-ara-A

Fig. 1 Structural formulae of 2-chloro-2′-deoxyadenosine (*CdA*) and 2-fluoroadenine arabinoside (*F-ara-A*)

unity than the corresponding ratio of the doses based on milligrams per square meter of body surface area.

Although a great deal of evidence has been collected thus far to support this hypothesis [8, 12, 14, 16], four antimetabolites (*N*-phosphonoacetyl-L-aspartate-PALA, dihydroazacytidine, fludarabine, and dideoxycytidine) have been found to be outliers of this pharmacodynamic concept [8]. Interspecies differences in target-organ sensitivities would therefore limit the utility of this hypothesis.

The purine antimetabolite 2-chloro-2′-deoxyadenosine (CdA, Cladribine, Fig. 1) is currently under investigation in phase II clinical trials for the treatment of lymphoid malignancies. It shows very high activity in hairy-cell leukemia (HCL) and substantial activity in chronic lymphocytic leukemia (CLL), low-grade non-Hodgkin's lymphoma, and cutaneous T-cell lymphoma as well as in the treatment of childhood acute myelogenous leukemia (AML) [5].

The MTD determined in phase I studies of CdA in hematologic and nonhematologic malignancies was 0.09 mg/kg per day when the drug was given as a continuous infusion over 7 days [6, 25]. The dose of 0.12 mg/kg per day given as a 2-h infusion on 5 successive days gave results equivalent to those observed after a continuous 7-day infusion [4]. The dose-limiting toxicity is bone marrow suppression.

The subcutaneous injection used in humans yields a bioavailability of 100% and the pharmacokinetic profile is very similar to that observed after a 2-h intravenous infusion [22]. Furthermore, the toxicity and efficacy of subcutaneously injected CdA are similar to those of CdA given intravenously at the same dose [18].

To exert its cytotoxic effect, CdA, as a prodrug, requires intracellular phosphorylation by deoxycytidine kinase (dCK). There is a large variability of dCK activity between various organs in humans. Hematopoietic tissues, in particular the thymus, have very high levels [2]. The same enzyme activates fludarabine and dideoxycytidine, two of four antimetabolites which represent outliers of the pharmacokinetically guided dose-escalation concept. Due to this similarity and the observation that the toxicity of most antimetabolites is not well predicted by either dose or AUC [10] it can be suspected that CdA is another exception.

Since to our knowledge, data on CdA have not yet been reported, the objective of our retrospective study was to compare the pharmacokinetics of CdA in mice with data previously obtained in patients after subcutaneous injection and to elucidate the biochemical basis for interspecies differences in drug sensitivity.

Materials and methods

Chemicals and reagents

All chemicals and solvents used were of either analytical reagent grade or high-performance liquid chromatography (HPLC) grade. CdA was synthesized by Dr. Z. Kazimierczuk (The Foundation for Diagnosis and Therapy, Warsaw, Poland) and a sterile, pyrogen-free solution (2 mg/ml) in saline was prepared. [8-^{3}H]- CdA (20 Ci/mmole) was purchased from Moravek Biochemicals (Brea, Calif., USA).

Toxicity studies

Animal studies were carried out in random-bred female NMRI mice with a weight of 23–30 g (B&K Universal, Sollentuna, Sweden). The LD_{10} value for CdA was determined after the administration of subcutaneous injections for 5 successive days. CdA was tested over a range of five doses—12.6, 17, 42, 75, and 100 mg/kg per day. For each dose level, groups of five mice were used. Mice were observed for at least 4 weeks and were given food and water ad libitum.

Pharmacokinetics studies in mice

Female NMRI mice (23–30 g) were given 42 mg/kg of CdA (approximately one-half the LD_{10}) subcutaneously. Three mice for each time point were killed while under anesthesia with diethyl ether, and blood samples were collected from the carotid arteries into Eppendorf vials at 15, 30, 60, and 90 min as well as 2, 4, 6, 15, and 24 h after drug administration. Each sample was processed separately.

All blood samples were centrifuged immediately, and the plasma was removed and assayed for CdA by a reversed-phase HPLC method as previously described [21]. Aliquots of 100 µl of mouse plasma spiked with 50 pmol of internal standard Guaneran (6-nitroimidazole-6-thioguanine, a generous gift from Dr. G. Elion, Wellcome Foundation) were extracted with 2.5 ml of ethyl acetate in silanized glass tubes. After centrifugation the organic phase was evaporated to dryness, dissolved in the mobile phase, and analyzed. Each sample was run in triplicate.

Pharmacokinetic calculations

Mouse-plasma CdA concentration-versus-time data were analyzed by extended nonlinear least-squares regression using a commercially

available program (SIPHAR; Societe Simed, Creteil, France). The AUC was calculated using the pharmacokinetics model fitted by means of weighted least-squares analysis to two-compartmental modeling from zero to infinity. A weighting factor of 1/concentration was used. The area extrapolated from the last point to infinity was less than 1%. Plasma clearance (CL) was calculated as follows: CL = dose/AUC. The pharmacokinetic data obtained in humans after subcutaneous injection of CdA were taken from a previously published report [22].

In vitro plasma protein binding

Equilibrium dialysis was used to determine the binding of CdA to mouse plasma proteins. The apparatus consisted of Lucite cells using SPECTRA/POR molecular porous membranes (mol. wt. cutoff, 3500 Da). Mouse plasma (200 µl) spiked with 50, 5, and 0.5 µ*M* CdA was introduced on one side of the dialysis membrane and an equal volume of saline solution (pH 7.4), to the other side. Dialysis was performed at 37°C in a dark environment under continuous agitation overnight (16 h). Equally sized samples (100 µl) were taken from both sides of the dialysis membrane and the CdA concentration was determined by the HPLC method.

The percentage of protein-bound drug was calculated according to the following equation:

$$\% \text{ Bound} = \frac{C_{pl} - C_{bu}}{C_{pl}} \times 100,$$

where C_{pl} and C_{bu} are the CdA concentrations in plasma and buffer, respectively, at the end of the dialysis. Nonspecific binding to the filter was assessed by comparing the concentrations of CdA measured in a saline buffer alone before and after dialysis and was consistently less than 2%.

Enzyme assay

A selective assay for dCK activity [2] was utilized for the determination of apparent Michaelis constant (K_m) and maximum velocity V_{max} values for dCK using CdA as the substrate in crude extracts of murine, rat, and human thymocytes. Since the highest levels of dCK activity detected in lymphoid tissues in the mouse and rat were observed in the thymus [9], this was used as the source of the enzyme in our study. Also, in comparison with bone marrow, another dCK-rich source, the samples of thymus from all three species were much easier to obtain.

NMRI mice aged 1–3 months were killed, and thymuses pooled from two mice were used for isolation of thymocytes for each independent sample. Rat thymocytes were isolated from the thymus of a 1-month-old Lewis rat. Human thymocytes were prepared from healthy thymuses of four 1 to 2-year-old children obtained at cardiac surgery.

dCK activity was assayed radiochemically by the method of Ives and Wang [17] with [8-^{3}H]-CdA (0.25 µ Ci/sample). Six different concentrations of CdA (1–50 µ*M* for human and 10–450 µ*M* for murine and rat enzyme extracts) were used for the determination of apparent K_m and V_{max} values. There was no degradation of [8-^{3}H]-CdA under enzyme assay conditions as determined by HPLC. V_{max} and K_m values were calculated by least-squares fitting to the Michaelis-Menten equation with the ENZFITTER program (Elsevier Biosoft, UK).

To show that dCK was the main phosphorylating enzyme, we performed a competitive study in which deoxycytidine (dCyd), a known competitive inhibitor of other substrates, was added in a 10-fold excess of the CdA concentration.

Results

Toxicity experiments

Since a dose of 100 mg/kg per day given for 5 successive days was lethal to 40% of the treated mice (LD_{40}) within the 1st week, whereas no death was observed in five mice treated at 75 mg/kg per day, the LD_{10} was assumed to be between 75 and 100 mg/kg per day in our experiment. The former dose is equivalent to 240 mg/m^2 per day using a conversion factor of 3.2 [11]. The MTD found in humans evaluated in phase I clinical trials was 0.12–0.17 mg/kg per day when the drug was given for 5 successive days (Beutler, personal communication; [23]). The equivalent dose of 4.8–7.0 mg/m^2 per day was calculated using a conversion factor of 40.

HPLC analysis

The HPLC assay for the determination of CdA in mouse plasma demonstrated linearity over the range of 10–1000 n*M* (2.86–286 ng/ml, $r = 0.99$). Plasma samples taken at less than 1.5 h after CdA dosing were appropriately diluted (5–50 times). The intra-assay coefficient of variation was 6%–8% at concentrations of 100–750 n*M* and the interassay variability was < 10% (3%–7.8%) in the same range. The limit of detection in mouse plasma was 6 n*M* (1.7 ng/ml) as compared with 1 n*M* in human plasma [21].

Pharmacokinetics studies

CdA was undetectable in mouse plasma at 24 h after dosing whereas it was detectable in human plasma due to the higher sensitivity of the assay and to the greater amount of samples available (1 ml of human plasma versus 100 µl of mouse plasma used).

A semilogarithmic plot of CdA concentration in plasma versus time after a subcutaneous injection of approximately one-half the LD_{10} (135 mg/m^2) in mice and the MTD (4.8 mg/m^2) in humans is shown in Fig. 2. At each time point the mean concentration ± SD obtained in three mice and/or in ten patients is indicated. The plasma concentration versus time curve in mice could be fitted to an open two-compartmental model, as could that in humans [20, 22], except that the slopes are much steeper in mice (Fig. 2). The half-lives of the two phases of drug disposition and elimination in mice were 11.4 and 150 min, respectively. The corresponding values in humans were much longer—1.47 and 13.3 h, respectively (Table 1).

The maximal concentration of CdA in plasma was reached after approximately 15 min in mice and within 20 min in patients (Fig. 2). However, the peak was much higher in mice than in patients as summarized in

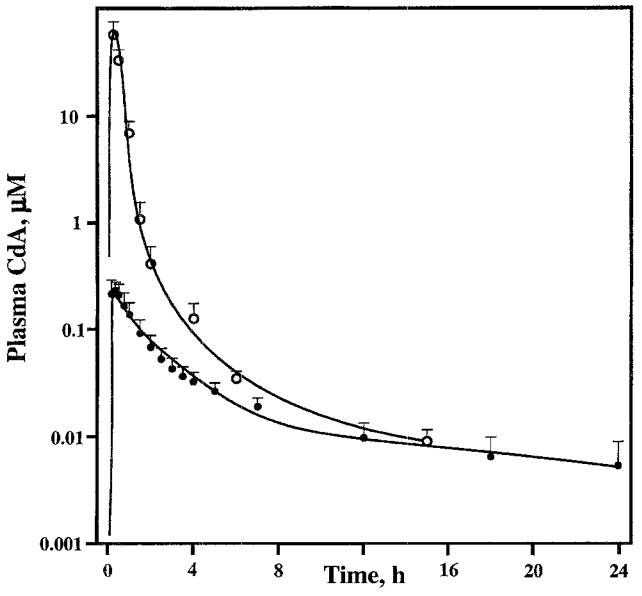


Fig. 2. CdA plasma concentration (at approximately one-half the LD_{10} in mice and at the MTD in humans) versus time curve generated after subcutaneous injection. At each time point the mean concentration ± SD calculated either for 3 mice (○) or from the data of 10 patients (●) is indicated

Table 1. The AUC determined in mice at approximately one-half the LD_{10} was about 49 times higher than the AUC found in patients at the MTD (32.8 and 0.67 μ*M* h, respectively).

Plasma protein binding

The mean plasma protein binding (± SD) of CdA in mice as evaluated at 50 μ*M* was 5.7% ± 1.4% ($n = 6$) and at the lower concentrations of 5 and 0.5 μ*M* it was in the range of 5.7%–10%. As compared with the percentage bound to human plasma (25% ± 12.5%) at relevant concentrations [1], CdA was bound approximately 4 times less extensively in mouse plasma.

Kinetic parameters of deoxycytidine kinase

The apparent K_m and V_{max} values obtained for dCK in crude extracts of mouse and human thymocytes are shown in Table 2. No significant difference was observed between the apparent V_{max} values recorded for mice (820 pmol mg^{-1} min^{-1}) and humans (752 pmol mg^{-1} min^{-1}). However, there were distinct and reproducible differences in the apparent K_m values and the V_{max}/K_m ratio. The apparent K_m of dCK was 9 times higher in mice than in humans. In the case of rat thymocyte extracts differences in the K_m (41 μ*M* for rat versus 5 μ*M* for human) and V_{max} values (1018 pmol mg^{-1} min^{-1} for rat versus 752 pmol mg^{-1} min^{-1} for human) were observed.

Table 1 Pharmacokinetic parameters of CdA for mice and humans after subcutaneous administration. Data represent mean values obtained in 3 mice and in 10 patients

Parameter	Mice (mean)	Human[a] (mean ± SD)
$t1/2\alpha$(h)	0.19	1.47 ± 0.80
$t1/2\beta$(h)	2.50	13.30 ± 7.1
Peak concentration (μ*M*)	54.80	0.26 ± 0.08
Time of peak concentration (h)	0.25	0.34 ± 0.13
CL ($l\,h^{-1}\,m^2$)	14.40	25.9 ± 7.8
AUC (μ*M*)	32.80	0.67 ± 0.18

[a] From Liliemark et al. [22]

Table 2 Kinetic parameters obtained for dCK using CdA as the substrate in crude extracts of mouse and human thymocytes. Data represent mean values ± SD for 4–5 independent samples run in duplicate

Thymocytes	K_m (μ*M*)	V_{max} (pmol mg^{-1} min^{-1})	V_{max}/K_m	Samples (n)
Mouse	43.5 ± 18.4	820 ± 172	18.9	5
Human	4.5 ± 1.1	751.6 ± 80	167	4

Table 3 Specific activity of CdA kinase in mouse and human thymocytes. Data represent average values for 2 experiments

Substrate	Mice (pmol mg^{-1} min^{-1})	Humans (pmol mg^{-1} min^{-1})
CdA[a]	675	816
CdA[a] + 500 μ*M* dCyd		11
CdA[a] + 2 m*M* dCyd	11	
CdA[a] + 5 m*M* dCyd	27	

[a] 450 μ*M* for murine and 50 μ*M* for human enzyme extracts

The specific activities found for CdA-phosphorylating enzyme in extracts of mouse and human thymocytes are summarized in Table 3. An excess of deoxycytidine decreased the specific activity to less than 5% of its original value, thus strongly suggesting that dCK is the main CdA-phosphorylating enzyme in mouse and human thymocytes.

Discussion

The original description of the pharmacologically guided dose-escalation (PGDE) hypothesis proposed the following explanations for variation in toxicity between mice and humans: differences in drug metabolism, elimination, and binding properties; exposure time differences; and species differences in target-cell sensi-

tivity. It was recognized that antimetabolites might not be good candidates for PGDE. Fludarabine was the most remarkable example [7, 8]. It seemed probable that drugs with a metabolism resembling that of fludarabine might represent outliers of the proposed concept.

Significant differences in CdA toxicity between mice and humans were observed when the phase I clinical trial was started. The dose used in the first patient was 0.9 mg/kg per day, which was about 1% of the mouse LD_{50}. However, the therapy had to be discontinued due to unacceptable toxicity. Subsequent patients were treated with only 0.09 mg/kg per day for 7 days [3, 4]. This dose, associated with a good therapeutic response and with acceptable toxicity, was regarded as the MTD. A similar MTD was later found using intermittent intravenous infusion in patients with solid tumor [23]. It was unknown whether these large interspecies differences in toxicity were due to interspecies differences in pharmacokinetics or to other factors. Although it is obvious that PGDE would not have been appropriate for CdA, no evaluation of species differences in CdA toxicity in relation to its pharmacokinetics has previously been reported. The aim of our study was therefore to compare the metabolism, pharmacokinetics, and toxicity of CdA in mice and humans so as to elucidate the mechanism behind the interspecies differences in sensitivity to CdA treatment.

In the evaluation of differences in drug pharmacokinetics between mice and humans, checking for nonlinearity at the LD_{10}, one-half the LD_{10}, and one-tenth the LD_{10} is proposed among some basic principles [13]. When approximately one-half the LD_{10} was chosen as the dose for mouse pharmacokinetics, a 49-fold difference between the AUCs determined at this dose in mice and at the human MTD was observed. Since it was obvious that the ratio of the AUCs obtained at equitoxic doses (LD_{10}/MTD) would be even higher and significantly different from unity, in our retrospective comparison we used the AUC value obtained at approximately one-half the LD_{10} instead of that determined at the LD_{10}.

Since CdA is not the active species, in evaluating the usefulness of the PGDE hypothesis for CdA it would be appropriate to compare the AUCs of the active metabolites (triphosphates) in both mice and humans. However, no reliable and sensitive method for the determination of intracellular CdA metabolites has yet been developed. There will also be difficulties in obtaining enough sample for monitoring of intracellular metabolites in mice.

An alternative possibility, i.e., to model the intracellular AUC for CdA monophosphate (CdAMP), is offered by the approach recently reported by Zaharko et al. [26], considering that the V_{max}/K_m ratio itself represents an in vitro cellular clearance (expressed in liters per minute per milligram of protein). Although it would be interesting to study whether better interspecies-toxicity correlations could be obtained, it was beyond the scope of our investigation to apply this model concept.

The large differences (49-fold) observed in AUCs between mice at a dose near one-half the LD_{10} and humans at the MTD suggest that human cells are much more sensitive than murine cells to the toxic effects of CdA. The 9-fold difference observed in the apparent K_m and in the V_{max}/K_m ratio for dCK between mouse and human thymocytes using CdA as the substrate thus indicates a higher efficiency for the enzyme in humans as compared with mice. These results are in accordance with previously observed species differences in substrate specificity between partially purified mouse and human dCK [15] and pure recombinant mouse and human dCK expressed in *Escherichia coli* [19].

This partly explains the 50- to 100-fold difference in sensitivity to CdA observed between humans and mice. There are also large differences in the plasma half-lives (8-fold for $t_{1/2\alpha}$ and 5-fold for $t_{1/2\beta}$) of CdA between humans and mice (Table 1). A more rapid distribution of CdA to tissues is probably the reason for the shorter initial half-life recorded for mice.

The peak concentration of CdA measured at approximately one-half the LD_{10} in mice (Table 1) is above the K_m for dCK (Table 2), indicating that the phosphorylation of CdA might be approaching saturation at these high plasma levels. In contrast, the trough concentration is similar or may be even lower in mice as compared with humans. A prolonged exposure to CdA has been shown to be of major importance for its cytotoxic effects [24]. It is therefore probable that these pharmacokinetic differences augment the interspecies differences in sensitivity to CdA.

To elucidate further the species differences in pharmacokinetics, determination of the plasma protein binding of drugs has been recommended by the European Organization for Research and Treatment of Cancer (EORTC PAM) guidelines [10]. Lower protein binding of CdA in mouse plasma indicates that more pharmacologically active drug is available in mice than in humans. However, this small difference in the free fraction (94% versus 75%) is probably of insufficient importance to explain large differences in pharmacokinetics.

Our results show large differences in the kinetic properties of dCK between the most commonly used experimental animals (rat and mouse) and humans when CdA is used as the substrate. These data are of major importance since mice or rats are usually the first-choice animals for preclinical experiments on CdA activity in other disorders such as rejection of transplanted organs and autoimmune diseases such as multiple sclerosis. In conclusion, these results show that a thorough knowledge about differences in the bioactivation of antimetabolites between humans and animals is a prerequisite for the use of phase I PGDE.

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