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ORIGINAL ARTICLE

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Plasma and cellular pharmacology of 8-chloro-adenosine in mice and rats

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Abstract *Purpose:* The nucleoside 8-chloro-adenosine (8-Cl-Ado) is currently being developed for treatment of multiple myeloma and leukemias. Although accumulation of the phosphorylated drug product is known to occur within cell lines, its metabolic fate in plasma or circulating cells in animals is unclear. The purpose of the present study was to determine the pharmacology of 8-Cl-Ado in rodents through examination of plasma and cellular levels of parent drug and metabolites. In addition, we sought to determine whether an inhibitor of adenosine deaminase, 2′-deoxycoformycin (dCF), could enhance intracellular formation of 8-Cl-ATP by preventing degradation of 8-Cl-Ado to 8-Cl-inosine (8-Cl-Ino). *Methods:* A validated HPLC assay permitted simultaneous determination of 8-Cl-Ado, 8-Cl-adenine (8-Cl-Ade), dCF, and 8-Cl-Ino. Radiolabeled cellular nucleotides were obtained from peripheral blood mononuclear cells (PBMC) of both mice and rats using a perchloric acid extraction procedure and were separated by HPLC. *Results:* Stability of 8-Cl-Ado in the presence or absence of dCF was examined in fresh plasma from mice, rats and humans. Conversion of 8-Cl-Ado to 8-Cl-Ino was only marginally affected by coincubation with dCF. In CD_2F_1 mice given 8-Cl-Ado i.p. at 100 mg/kg, there was rapid appearance in plasma of both 8-Cl-Ade and 8-Cl-Ino. The identities of the metabolites were confirmed by mass spectrometry. The plasma [^{3}H]8-Cl-Ado concentration 1 h after drug injection was 1.3 μ*M* in mice while the intracellular levels of [^{3}H]8-Cl-AMP and [^{3}H]8-Cl-ATP were 1 m*M* and 350 μ*M*, respectively. Mice that had received dCF (2 mg/ml) 30 min prior to [^{3}H]8-Cl-Ado had 27% less intracellular [^{3}H]8-Cl-ATP in PBMC compared to mice without dCF pretreatment. The pharmacokinetics of 8-Cl-Ado were examined in greater detail in Sprague-Dawley rats. Animals were given [^{3}H]8-Cl-Ado (42.5 mg/kg, i.v.) by itself or 30 min following injection of dCF (4 mg/kg). Mononuclear cells in mice accumulated 350 or 1200 μ*M* [^{3}H]8-Cl-ATP 1 h after injection of either 50 or 100 mg [^{3}H]8-Cl-Ado, respectively. The major metabolite in these cells was the monophosphate, which was four- to sevenfold higher in concentration than the triphosphate metabolite. In rats, [^{3}H]8-Cl-AMP concentrations in PBMC were similar to those of the triphosphate metabolite which achieved a peak of 90 μ*M* 2 h after a bolus injection of 8-Cl-Ado (40 mg/kg). Cellular clearance of 8-Cl-ATP appeared to be slow: 24 h after injection of 8-Cl-Ado the cellular concentration of 8-Cl-ATP was still 40 μ*M*. *Conclusions:* The use of dCF did not significantly alter 8-Cl-ATP levels in PBMC and is not considered to be a useful therapeutic strategy. Even though a portion of 8-Cl-Ado is metabolically inactivated in plasma, high levels of cytotoxic 8-Cl-ATP accumulated intracellularly in these animals and were retained for a considerable length of time. Further development of 8-Cl-Ado is recommended.

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Abbreviations *8-Cl-Ade:* 8-chloro-adenine · *8-Cl-Ado:* 8-chloro-adenosine · *8-Cl-cAMP:* 8-chloro-cyclic adenosine monophosphate · *8-Cl-IMP:* 8-Cl-inosine monophosphate · *8-Cl-Ino:* 8-chloro-inosine · *ADA:* adenosine deaminase · *dCF:* deoxycoformycin · *HPLC:* high pressure liquid chromatography · *LC/MS/MS:* liquid chromatography tandem mass spectrometry · *PBMC:* peripheral blood mononuclear cell · *PCA:* perchloric acid

Introduction

Initial in vitro studies evaluating the growth-inhibitory actions of various site-selective cyclic adenosine monophosphate (cAMP) analogs have revealed a beneficial pharmacologic activity of 8-Cl-cAMP [1]. Additional investigations have demonstrated that this cAMP analog exhibits cytotoxicity in multiple tumor cell types, including gliomas, leukemias, and breast, colon, gastric, and lung cancers [2, 3, 4, 5]. Extension of the in vitro studies to animal tumor models has suggested that 8-Cl-cAMP is efficacious against mouse epithelial carcinomas and human breast and colon xenografts [6, 7]. Studies of 8-Cl-cAMP in recent clinical trials have further indicated its usefulness against a variety of human carcinomas [7, 8]. While these investigations have provided evidence of consistent cytotoxicity of 8-Cl-cAMP, metabolism and mechanistic studies suggest that it may act as a prodrug through conversion to 8-Cl-adenosine (8-Cl-Ado) [9, 10] (Fig. 1). Several investigations have now provided direct evidence that 8-Cl-cAMP-mediated tumor cell cytotoxicity is, in fact, due to 8-Cl-Ado [11]. This compound may work as a conventional antimetabolite agent acting through inhibition of RNA polymerases [10, 11, 12, 13, 14]. Studies in which the metabolic fate of 8-Cl-Ado has been investigated have demonstrated that this analog is phosphorylated intracellularly by adenosine kinase to its monophosphate form which is then converted to di-, and triphosphates presumably by using cellular mono- and diphosphokinases [10]. These observations provide a rationale for the use of 8-Cl-Ado rather than 8-Cl-cAMP as an anticancer drug.

In addition to intracellular phosphorylation of 8-Cl-Ado, the drug can be metabolized by adenosine deaminase (ADA) to 8-Cl-Ino or by AMP deaminase to 8-Cl-inosine monophosphate (8-Cl-IMP; see Fig. 2). Because of the abundance and high specific activity of these enzymes in large body organs [15, 16], the drug may be extensively metabolized in whole body systems. Additionally, phosphorolysis of 8-Cl-Ado may occur to form the free base, 8-Cl-adenine (8-Cl-Ade) [17]. These reactions may serve to lower the efficacy of the drug since they can decrease the opportunity for phosphorylation to the active triphosphate form (8-Cl-ATP) in cells. Preclinical studies in rodents were conducted to address these issues. Here we report analytical methods for isolation and quantitation of 8-Cl-Ado and metabolites such as 8-Cl-Ino and 8-Cl-Ade in plasma. In addition, the blood mononuclear cellular pharmacology of 8-Cl-Ado was determined after administration of the drug to mice and rats. We also evaluated the ability of a potent ADA inhibitor, deoxycoformycin (dCF) [18], to block metabolism of 8-Cl-Ado in vivo and thereby possibly affect intracellular levels of 8-Cl-ATP.

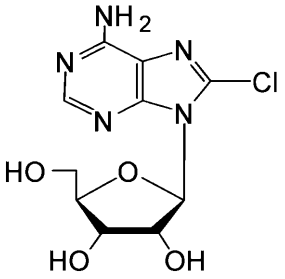

Fig. 1. Structure of 8-Cl-Ado

Materials and methods

Animals

Female CD_2F_1 mice (20–25 g) and male Sprague-Dawley rats (150–175 g) were provided by the National Cancer Institute (Bethesda, Md.) and maintained in a specific pathogen-free environment. All experiments involving animals were performed in accordance with the guidelines of the institution's Animal Care and Use Committee in strict accordance with Federal regulations.

Drugs and other chemicals

8-Cl-Ado and dCF were obtained from the Developmental Therapeutics Program, National Cancer Institute. Radiolabeled 8-Cl-Ado ([2-^{3}H]8-Cl-Ado; specific activity 11.6 Ci/mmol, purity

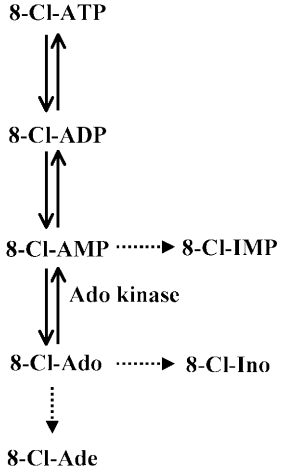

Fig. 2. Metabolism of 8-Cl-Ado. The conversion of 8-Cl-Ado to the initial phosphorylated intracellular metabolite 8-Cl-AMP is produced by adenosine kinase. Further phosphorylation steps lead to 8-Cl-ATP, the presumed active intracellular metabolite. Extracellular metabolism of 8-Cl-Ado can occur through conversion to either the inosine or adenine forms of this drug

>98.5%) was purchased from Moravek Biochemicals (Brea, Calif.). For analysis of 8-Cl-Ado cellular metabolism, 8-Cl-Ado mono-, di-, and triphosphates were custom-synthesized by BioLog (La Jolla, Calif.). All other chemicals were of analytical grade.

Analytical methods

Compounds were extracted from rodent plasma (250 µl) using perchloric acid (PCA) precipitation (100 µl of 20% PCA). The samples were mixed and then placed on ice for 15 min prior to centrifugation (5 min at 8000 *g*). To this was added 30 µl 10 *N* KOH. The pH was then checked and adjusted to about pH 6.5 if necessary. The samples were centrifuged again (8000 *g* for 15 min) prior to analysis. Supernatants were filtered then injected directly into the HPLC system. An HPLC-based analytical assay suitable for the simultaneous separation and quantitation of dCF, 8-Cl-Ino, and 8-Cl-Ado was established (see Fig. 3). Samples prepared from plasma precipitates were applied to a Waters Xterra MS C18, 3.5 µm column 100×4.6 mm (Waters Corporation, Milford, Mass.). The compounds were separated using a linear gradient consisting of (A) 98% 80 m*M* ammonium acetate/2% acetic acid and (B) 90% methanol/8% ammonium acetate/2% acetic acid run at a flow rate of 0.8 ml/min. The gradient went from 100% A to 100% B over 20 min. Compounds were detected at a wavelength of 265 nm. The assay was validated for determination of 8-Cl-Ado and 8-Cl-Ino with respect to reproducibility (within-day and between-day), accuracy and precision. The relative error for determination of 8-Cl-Ado in both mouse and rat plasma was less than 12.5% at 100 and 1000 ng/ml. Assays were linear over the range 66 to 1000 ng/ml with correlation coefficients (r^2) consistently greater than 0.998. Validation of the assay for 8-Cl-Ade was not possible due to lack of an authentic reference standard; the identity of this compound, however, was verified using mass spectrometry.

The HPLC assay was modified to run in conjunction with an LC/MS instrument to confirm the identity of compounds of extracted plasma samples. The equipment consisted of a Micromass VG Platform LC/ESI-MS instrument (Micromass, Beverly, Mass.). The electrospray source was run in a positive mode. The lower limit of quantitation for 8-Cl-Ado using LC/MS analysis was 1.56 ng/ml.

Stability studies

Solutions of 8-Cl-Ado (in the presence or absence of dCF) were incubated (37°C) with either plasma or heparinized whole blood samples obtained from human volunteers, rats or mice. Samples were removed from the water bath at selected times. Plasma samples were processed as described above and then quantified with the HPLC/UV method.

Plasma pharmacology of 8-Cl-Ado in rodents

CD_2F_1 mice were given 8-Cl-Ado (100 mg/kg) by either intraperitoneal (i.p.) or intravenous (i.v.) (tail vein) routes of administration. Groups of mice (four per group) were killed at 2, 5, 10, 20, 30, 40, 60, 90 and 120 min following drug injection. Later time-points were not collected as preliminary experiments indicated that the HPLC/UV assay might not be able to quantify drug levels beyond 2 h. Sprague-Dawley rats were used to assess the relative effect of dCF on 8-Cl-Ado in plasma and within peripheral blood mononuclear cells (PBMC). A group of 12 rats received 8-Cl-Ado alone while 12 additional rats were first given 4 mg dCF/kg (i.p.) 30 min prior to i.v. administration of radiolabeled 8-Cl-Ado (42.5 mg/kg). Groups of four rats each were killed at 2, 5, and 24 h following injection of 8-Cl-Ado.

Cellular pharmacology

For cellular pharmacology, blood samples were collected 1 h after injection of 8-Cl-Ado in mice and at 1, 2, 5, and 24 h after drug injection in rats. To obtain an adequate number of PBMC, blood samples were pooled from ten mice. Enough blood volume was obtained from a single rat, however, to isolate $>1\times10^7$ cells per sample. PBMC were isolated from blood samples and processed with Fico/Lite (for mice) or Fico/Lite LR (for rats) according to the manufacturer's instructions (Atlanta Biologicals, Norcross, Ga.). The PBMC collected from the interface were washed and counted using a Coulter Counter (Coulter Electronics, Hialeah, Fl.) coupled with a channelizer. The mean volume of cells in a given population was calculated and used to quantify nucleotide concentration.

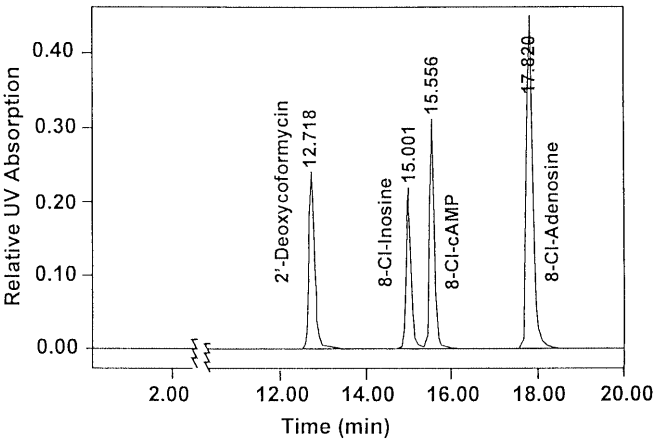

Fig. 3. HPLC separation of 8-Cl-Ino, 8-Cl-Ado, 8-Cl-cAMP and dCF

Measurement of intracellular nucleotides

Radiolabeled nucleotides were extracted using PCA as described previously [19] and were neutralized with KOH and stored at –20°C. An aliquot (100 µl) of neutralized extract was applied to an anion-exchange Partisil-10 SAX column (Waters) and eluted at a flow rate of 1.5 ml/min [20]. A 50-min concave gradient (curve no. 7, Waters 600E System Controller) from 60% 0.005 *M* $NH_4H_2PO_4$ (pH 2.8) and 40% 0.75 *M* $NH_4H_2PO_4$ (pH 3.6) to 100% 0.75 *M* $NH_4H_2PO_4$ (pH 3.6) was used. The eluate was detected with an automatic radiometric detector along with liquid scintillation fluid using a Radiomatic Flow-through HPLC system (Packard, Downers Grove, Ill.). Tritium counts were recorded for each radioactive peak and the mono-, di-, and triphosphates formed from 8-Cl-Ado were identified through comparison of retention profiles and absorption spectrums with those of authentic standards. The intracellular concentration of nucleotides contained in the extract was calculated from a given number of cells of a determined mean volume. This calculation assumed that the nucleotides were uniformly distributed in the total cell volume.

Statistical analyses

Student's two-tailed paired *t*-test was used to determine the significance of differences in 8-Cl-ATP accumulation in PBMC obtained from mice and rats between cells from dCF-treated and untreated animals. The same test was used for 8-Cl-Ino formation in mouse plasma after 8-Cl-Ado infusion in animals with or without dCF.

Results

Stability of drug in plasma

Whether 8-Cl-Ado undergoes deamination in plasma or whole blood and whether dCF can be used to inhibit this

metabolism was investigated ex vivo. Studies performed with 8-Cl-Ado using freshly obtained human, mouse and rat plasma with or without the addition of dCF indicated that the ADA inhibitor had no effect on the relative stability of 8-Cl-Ado (data not shown). Incubation of 8-Cl-Ado in whole blood (mouse, rat and human) resulted in rapid uptake of drug into red blood cells. The presence of dCF did not affect the extent of uptake (data not shown).

Plasma pharmacology in mice

The relative formation of [^{3}H]8-Cl-Ino following administration of [^{3}H]8-Cl-Ado to mice was determined. As shown in Fig. 4, preadministration of dCF produced a significant decrease in the relative formation of 8-Cl-Ino at early time-points (i.e. 5 and 10 min). At later time-points through 2 h, the relative levels of 8-Cl-Ino were similar to or even higher than those in mice without dCF treatment. A more thorough understanding of the relationship of 8-Cl-Ino formation to 8-Cl-Ado plasma levels was achieved after administration of non-radiolabeled 8-Cl-Ado to mice. Levels of 8-Cl-Ado and 8-Cl-Ino were determined using HPLC/UV analysis (Fig. 5). As expected, pretreatment with dCF had no effect on plasma levels of 8-Cl-Ado but did seem to lower the relative extent of formation of 8-Cl-Ino across all time-points measured following injection of 8-Cl-Ado (data not shown). The greatest apparent decrease in 8-Cl-Ino formation due to dCF was seen at early time-points (5 and 10 min).

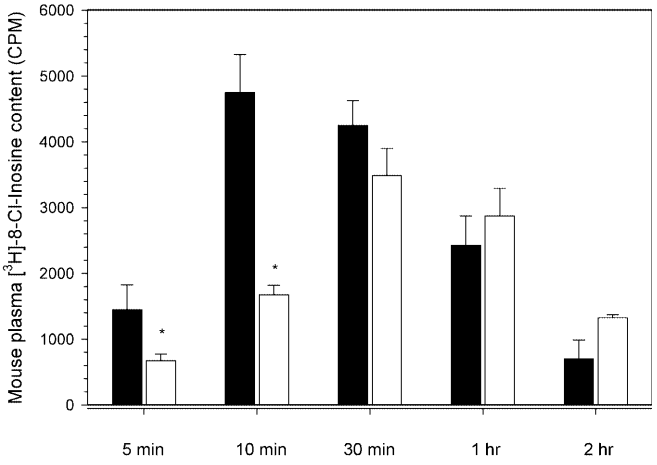

Fig. 4. Formation of [^{3}H]8-Cl-Ino from [^{3}H]8-Cl-Ado (with or without coadministration of dCF) in mice. In an effort to determine whether administration of dCF to mice could alter the relative formation of [^{3}H]8-Cl-Ino from [^{3}H]8-Cl-Ado, plasma was obtained from mice and subjected to HPLC analysis. Only relative values for 8-Cl-Ino are indicated, as no authentic radiolabeled standard [^{3}H-8-Cl-Ino] was available. Data are presented as means ± SD (four or five mice per time-point, $*P < 0.05$ by Student's *t*-test). *Solid bars* formation of [^{3}H]8-Cl-Ino in absence of dCF; *open bars* formation of [^{3}H]8-Cl-Ino following prior administration of dCF as described in Methods1

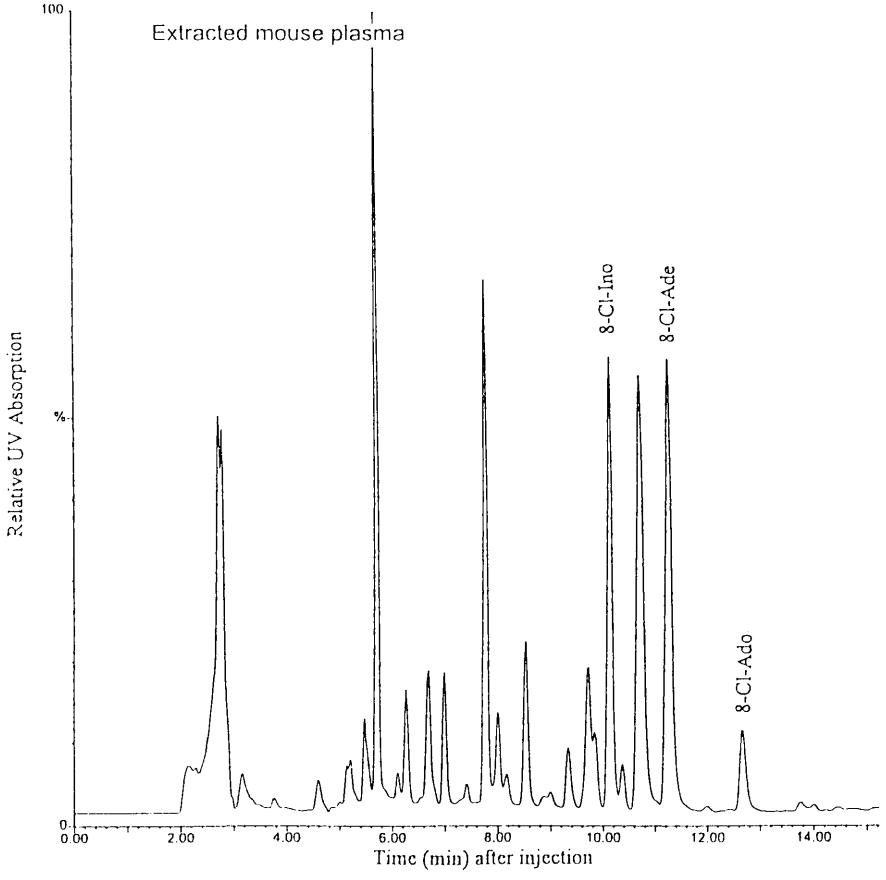

Fig. 5. HPLC chromatogram of extracted mouse plasma following administration of 8-Cl-Ado (100 mg/kg). 8-Cl-Ino and 8-Cl-Ado were readily detected. An additional drug-derived peak was subsequently identified as 8-Cl-Ade

During analyses of samples for relative content of 8-Cl-Ado and 8-Cl-Ino, an additional drug-related HPLC peak was consistently noted in the mouse plasma chromatogram (see Fig. 5). Subsequent isolation and analysis of this peak using LC/MS showed it to be 8-Cl-Ade. As shown in Fig. 6, 8-Cl-Ino, 8-Cl-Ade and 8-Cl-Ado were identified with m/z ratios of 302.83, 169.73 and 301.96, respectively. Explorations for this 8-Ado-derived metabolite were undertaken in subsequent analyses of the relative effect of dCF on 8-Cl-Ado metabolism in mice and rats. Administration of 8-Cl-Ado to mice by either the i.p. or i.v. route of administration resulted in the rapid formation of 8-Cl-Ade (Fig. 7). Although no authentic standard for 8-Cl-Ade was available, HPLC peaks were collected and submitted to analyses using the LC/MS/MS method described above. Both routes of administration produced 8-Cl-Ade concentrations in plasma that quickly exceeded concentrations of parent 8-Cl-Ado although concentrations of 8-Cl-Ade had returned to below those of 8-Cl-Ado by 40 to 60 min.

Plasma pharmacology in rat

The plasma concentration-time profile of 8-Cl-Ado and metabolites following i.v. injection of radiolabeled drug to rats is shown in Fig. 8. Plasma concentrations of 8-Cl-Ado could only be detected for 30 min indicating rapid clearance of this compound in this species. The estimated plasma half-life for 8-Cl-Ado was < 10 min. There was rapid formation of 8-Cl-Ade that was unaffected by prior injection of dCF (Fig. 8B). The estimated

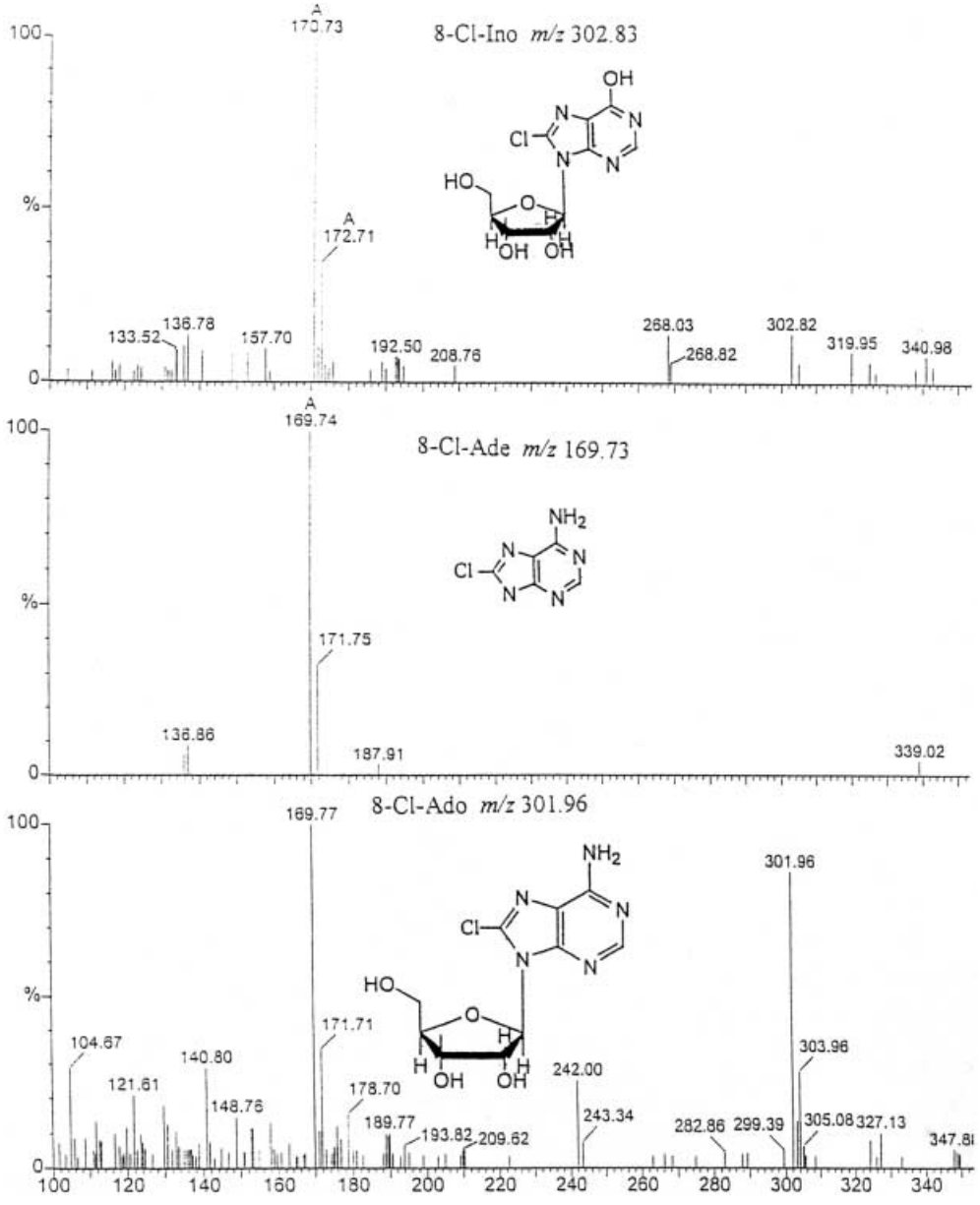

Fig. 6. Electrospray LC/MS/MS spectra demonstrating the identity of 8-Cl-Ado-derived peaks extracted from mouse plasma after administration of 100 mg/kg. The compounds 8-Cl-Ino, 8-Cl-Ade and 8-Cl-Ado were identified with m/z ratios of 302.83, 169.73 and 301.96, respectively

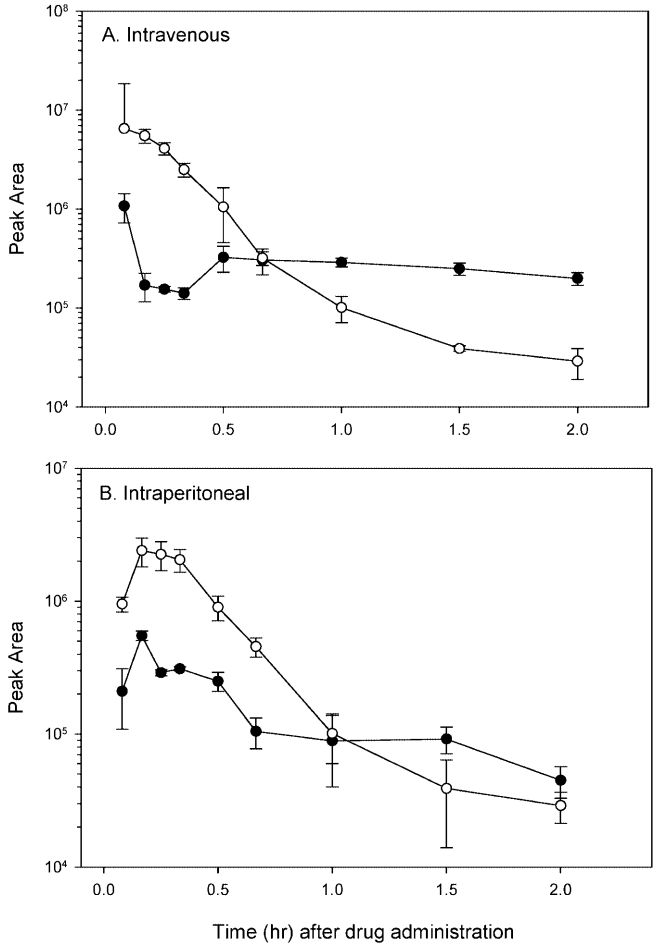

Fig. 7A, B. Formation of 8-Cl-Ade following administration of 8-Cl-Ado by either the i.v. or i.p. route. Female CD_2F_1 mice were injected with either (**A**) 85 mg/kg 8-Cl-Ado i.v. or (**B**) 100 mg/kg 8-Cl-Ado i.p. 8-Cl-Ade was identified by isolation of the chromatographic peak followed by analyses using LC/MS/MS as described in Methods. Data are presented as means ± SD from four mice per time-point (*open circles* 8-Cl-Ade, *closed circles* 8-Cl-Ado)

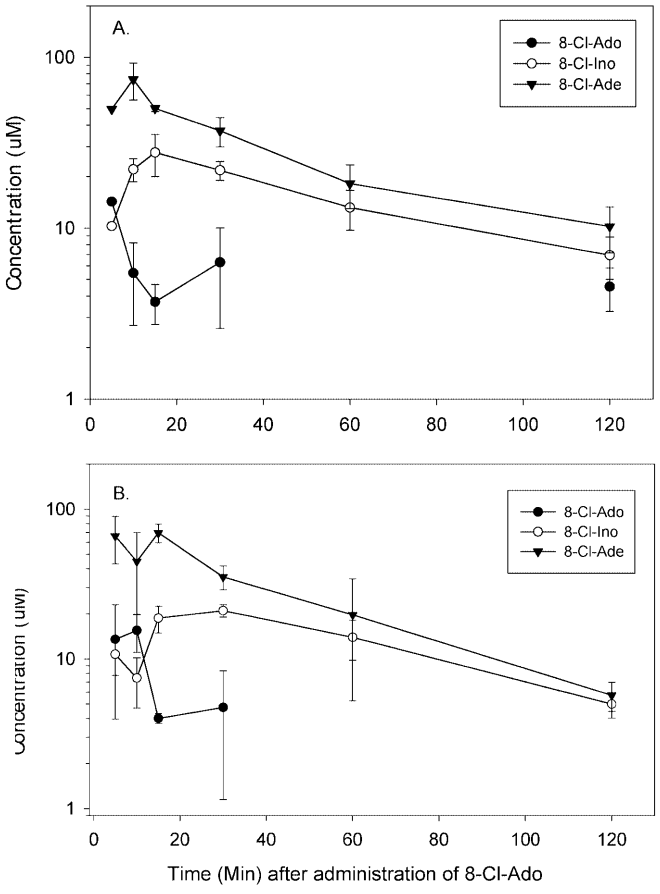

Fig. 8A, B. Plasma concentration-time profiles for 8-Cl-Ado and metabolites following i.v. administration of [^{3}H]8-Cl-Ado at 42.5 mg/kg to Sprague-Dawley rats. The relative effect of administration of dCF (4 mg/kg; i.p. administration) on the conversion of 8-Cl-Ado to 8-Cl-Ade and 8-Cl-Ino was determined (**A** without dCF pretreatment, **B** treatment with dCF 30 min prior to 8-Cl-Ado). None of these compounds could be detected beyond the 2-h time-point. Data are presented as means ± SD ($n = 3$–5)

plasma half-life for 8-Cl-Ade was approximately 40 min, while for 8-Cl-Ino it was slightly more than 1 h. Similar to what was observed with dCF treatment in mice, administration of dCF to rats decreased the relative formation of 8-Cl-Ino only during the first 20 min following drug administration.

Cellular pharmacology in mice

Peripheral blood samples were collected from ten mice and pooled for pharmacology studies. At 1 h after infusion of 8-Cl-Ado at 50 and 100 mg/kg, the major intracellular metabolite detected was 8-Cl-AMP which was approximately 1200 and 9000 μ*M*, respectively (Table 1). Sizeable levels of cytotoxic 8-Cl-ATP (350 and 1200 μ*M*, respectively) accumulated following both the 50 and 100 mg/kg doses. 8-Cl-ADP was the lowest of all three phosphorylated products with concentrations between 80 and 170 μ*M*. No deaminated products, i.e. mono-, di-, or triphosphates, of 8-Cl-Ino were detectable in these PBMC. Prior administration to mice of dCF resulted in a 27–38% decrease in the relative formation of all intracellular phosphorylated metabolites of 8-Cl-Ado.

Cellular pharmacology in rats

In contrast to the study in mice, enough blood was obtained from each rat to provide $> 1 \times 10^7$ cells for cellular pharmacology investigations. To investigate whether the enzyme ADA was important in clearance of 8-Cl-Ado, the pharmacology investigation was done with or without dCF. For illustrative purposes, all data from one time-point (2 h) are presented in Fig. 9. Blood samples were obtained from four rats that constituted four data points for each arm, i.e. without (open symbols) or with (closed symbols) ADA inhibitor, dCF.

Table 1. Relative formation of phosphorylated metabolites of 8-Cl-Ado in PBMC obtained from mice with or without coadministration of dCF. The values shown represent single determinations of pooled samples from ten mice obtained 1 h after administration of 50 mg/kg [^{3}H]8-Cl-Ado i.v. with or without pretreatment with dCF (2 mg/kg, i.p.). Numbers in parentheses represent the decrease in 8-Cl-Ado metabolite formation in mice pretreated with dCF relative to those that received 8-Cl-Ado only

dCF	8-Cl-Ado (mg/kg)	Phosphorylated product (μ*M*)		
		8-Cl-AMP	8-Cl-ADP	8-Cl-ATP
Without	50	1200	76	347
	100	8973	172	1157
With	100	5597 (38%)	119 (31%)	846 (27%)

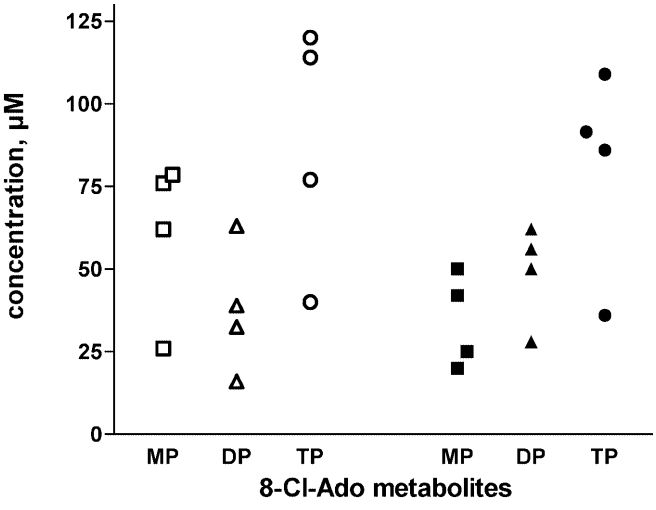

Fig. 9. Cellular pharmacology of 8-Cl-Ado in rat PBMC. Blood samples were obtained 2 h after infusion of 42 mg/kg of 8-Cl-Ado with (*closed symbols*) or without (*open symbols*) dCF. PBMC were separated and nucleotides were extracted as described in Methods. Mono-, di-, and triphosphates of 8-Cl-Ado were quantitated as described. The four data points for each phosphorylated product represent four separate experiments

These data suggest that for each phosphorylated product, there was heterogeneity among individuals, which was expected. Similar data were obtained at other time-points and are presented in Table 2.

In the absence of dCF in rats, 8-Cl-ATP was the major metabolite, but at early time-points (1 and 2 h after infusion), the concentration of 8-Cl-AMP was similar to that of 8-Cl-ATP (Table 2). The concentration of 8-Cl-AMP ranged between 50 and 60 μ*M* during the first 5 h. At 24 h after infusion, 10 μ*M* 8-Cl-AMP was retained in these cells. Among the phosphorylated products, 8-Cl-ADP was the lowest, but a detectable concentration was present 24 h after the end of infusion. The concentration of 8-Cl-ATP reached a peak 2 h after infusion. A similar level of triphosphate was measured at 5 h after infusion. Greater than 40% of the 8-Cl-ATP peak was retained 24 h after infusion, suggesting slow elimination of this cytotoxic metabolite.

To determine whether the pattern of triphosphate accumulation was changed by infusing an ADA inhibitor (dCF), 8-Cl-ATP values were compared in each arm. Although the data suggest a trend toward lower triphosphate values in the dCF arm, especially at late time-points, statistical analysis suggested no significant difference in 8-Cl-ATP concentration. The *P*-values were 0.75, 0.80, 0.11, and 0.17 at 1, 2, 5, and 24 h after infusion, respectively.

Discussion

The metabolic pathways for 8-Cl-Ado are expected to be similar to those for other Ado or dAdo analogs. For example, for metabolic activation, the analog is phosphorylated by nucleoside kinase to its monophosphate form, which then would be converted to di-, and triphosphates [10, 21]. Both free nucleoside and monophosphate could also be metabolically inactivated by deaminase to form 8-Cl-Ino or 8-Cl-IMP, respectively (Fig. 2). With the potential for numerous anabolic and catabolic routes, it is essential to understand in vivo

Table 2. Relative formation of phosphorylated metabolites of 8-Cl-Ado in PBMC obtained from rats with or without coadministration of dCF. Values are means ± SD from four separate experiments. Numbers in parentheses represent the decrease in 8-Cl-Ado metabolite formation in rats pretreated with 4 mg dCF/kg i.p. relative to those that received 8-Cl-Ado only (42.5 mg [^{3}H]8-Cl-Ado/kg, i.v.)

dCF	Time (h)	Phosphorylated product (μ*M*)		
		8-Cl-AMP	8-Cl-ADP	8-Cl-ATP
Without	1	51 ± 30	22 ± 5	51 ± 33
	2	60 ± 24	37 ± 19	88 ± 37
	5	50 ± 21	37 ± 13	100 ± 50
	24	11 ± 7	18 ± 5	41 ± 20
With	1	59 ± 9	9 ± 5	60 ± 14
	2	34 ± 14	49 ± 15	81 ± 31
	5	16 ± 11 (68%)	21 ± 15 (43%)	52 ± 7 (48%)
	24	6 ± 2 (45%)	10 ± 3 (44%)	21 ± 7 (49%)

pharmacokinetics and metabolism of the drug prior to launching phase I clinical studies.

Although metabolic inactivation would be expected to occur, to be effective as a therapeutic agent, phosphorylation of 8-Cl-Ado is a necessary step. The majority of clinically active and useful dAdo analogs such as fludarabine nucleoside, cladribine, and clofarabine are phosphorylated by dCyd kinase [20, 22, 23, 24, 25]. Because this is the rate-limiting step for accumulation of the triphosphate form of the drug, the effectiveness of the analog is basically dependent on the relative affinity of the kinase to the substrate and the specific activity of enzyme in the target tumor tissue. The affinity of kinase to these analogs varies (Km 10 μM, 50–80 μM, and 100–300 μM, respectively) making clofarabine the best substrate and fludarabine the worst [22, 23, 24]. To some extent, these affinities are reflected in the clinical usefulness of these agents. In addition, the specific activity of the kinase dictates the accumulation of phosphorylated analog in the target tissue and the success of these agents in the setting of indolent leukemia may represent the presence of high activity of dCyd kinase in chronic lymphocytic leukemia cells [26, 27].

For both these aspects, the affinity of the substrate to kinase and the prevalence of enzyme activity, 8-Cl-Ado should be considered a unique analog. First, and in contrast to other conventional nucleoside analogs, 8-Cl-Ado is phosphorylated to the monophosphate, 8-Cl-AMP, with Ado kinase and not dCyd kinase. In vitro investigation using purified enzymes has identified the enzyme as Ado kinase [21]. Consistent with the in vitro studies, a mutant cell line lacking Ado kinase has been shown to be resistant to 8-Cl-Ado and does not accumulate 8-Cl-ATP [10]. Second, a previous study has indicated that 8-Cl-Ado is a favorable substrate for phosphorylation by Ado kinase (K_m 7 μM versus 1.7 μM for the natural substrate, Ado, with a 50% V_{max} compared to that of Ado [21]). Third, the distribution and activity of Ado kinase in the target tissue is high indicating that the effectiveness of this agent would not be limited or selective [28, 29]. The accumulation of high levels of mono-, and especially triphosphate forms of 8-Cl-Ado in mice and rat PBMC (Fig. 9, Tables 1 and 2) and in multiple myeloma or leukemia cell lines [10] supports this statement. Our metabolite analyses demonstrated that even though free nucleoside, mono-, di-, and triphosphates accumulated, the major metabolites are mono- and triphosphates. This is consistent with our previous studies in cell lines in vitro [10] and other adenosine and deoxyadenosine analogs, especially chlorinated analogs such as cladribine and clofarabine [20, 30]; however, it differs from arabinosylguanine, cytarabine, and gemcitabine, which accumulate primarily as triphosphates [31, 32, 33].

Purine nucleoside phosphorylase (PNPase) catalyzes the cleavage of Ado, dAdo, and their analogs to produce the base [34]. Although this enzyme prefers natural substrates over congeners, phosphorolysis of the analogs is still commonly observed during in vivo investigations. Extensive investigations of PNPase from different sources have demonstrated that bacterial enzyme cleaves 2-fluoro-ara-A to form 2-fluoroadenine which is then phosphorylated to 2-fluoro-ATP [35]. Similar to fludarabine, cleavage of cladribine to form 2-chloroadenine and phosphorylation to accumulate 2-chloro-ATP has been reported [35]. Because of either arabinose or deoxyribose sugars in these analogs, the liberation of halogenated bases, followed by the formation of halogenated ribose analog is identifiable. In contrast, with 8-Cl-Ado, even though formation of 8-Cl-Ade has been observed in rodents [17], it would be difficult to determine whether this base eventually gets converted to form 8-Cl-ATP. Interestingly, the concentration of 8-Cl-Ade was much higher compared to parent drug and was maintained for a longer time (Figs. 7 and 8). Metabolism of 8-Cl-Ado to the primary metabolite, 8-Cl-Ade, has been also noted in dogs [16]. If 8-Cl-ATP is being synthesized from 8-Cl-Ade in the circulating cells, the formation of 8-Cl-Ade may provide a benefit for sustained transformation to 8-Cl-ATP. Based on the findings in previous investigations with fludarabine and cladribine [35, 36], it would be expected that the cleaved 8-Cl-Ade would be phosphorylated to make 8-Cl-ATP.

The third route in the metabolic conversion of 8-Cl-Ado is through deamination of nucleoside analog and/or its monophosphate. In fact, the deamination of arabinosyl adenine and its low solubility in water are the major reasons for its failure as a chemotherapeutic agent [37]. To circumvent these limitations, halogenated dAdo analogs such as fluoro-ara-A and cladribine were synthesized [38]. To facilitate the use of fluoro-ara-A as a therapeutic agent, a phosphate group was added to produce a soluble prodrug, fludarabine (F-ara-A monophosphate).

The kinetic value of ADA for metabolic inactivation of 8-Cl-Ado suggests that phosphorylation may be a preferred route of 8-Cl-Ado metabolism. This is based on the fact that that 8-Cl-Ado is a poor substrate for deamination by ADA (K_m 830 μM versus 29 μM for Ado, with a V_{max} of 1.8% [20]). With these kinetic values, it could be expected that 8-Cl-Ado would be quickly converted to its monophosphate without major metabolism by ADA. dCF is a potent inhibitor of ADA, with a Ki value of 0.0025 nM [17] and is a relatively weak inhibitor of AMP deaminase [39, 40]. The observation that the presence or absence of dCF did not make any difference in the accumulation of 8-Cl-ATP in the in vitro cell line experiments [10] is consistent with this postulate.

As an extension of these studies, we tested the stability of 8-Cl-Ado in human, mouse, and rat plasma. Although ADA is present in the plasma of each species, the addition of the ADA inhibitor, dCF, did not result in any change in drug stability (data not shown but described in the Results). These data suggest that either the activity of the enzyme was low in plasma or the affinity of the enzyme for 8-Cl-Ado is low. When whole blood was used in place of plasma, there was a rapid

uptake of the drug in the PBMC and the addition of dCF did not affect the concentration of drug. Although these cell-free enzyme assays, whole cell metabolic studies, and in vitro plasma analyses suggest that phosphorylation of 8-Cl-Ado may be a preferred route of metabolism, the high specific activity of ADA may result in inactivation of the analog through deamination. For this purpose, mice and rats were treated with 8-Cl-Ado, with or without dCF, an inhibitor of ADA (Figs. 4 and 8). Although dCF has been shown before to inhibit ADA [18], pretreatment of rodents with dCF did not affect significantly the production of 8-Cl-Ino. Consistent with these findings, cellular accumulation of phosphorylated 8-Cl-Ado did not increase with dCF infusion. Rather, there appeared to be a decrease in the mono, di, and triphosphates of 8-Cl-Ado in PBMC obtained from both mice (Table 1) and rats (Table 2).

These paradoxical results may be due to inhibition of Ado and dAdo deamination that is otherwise present at low concentrations in circulating plasma. For example, it has been shown that pediatric patients with ADA deficiency have higher levels of plasma Ado and dAdo [41, 42, 43] and that the clearance of Ado is slower than that of dAdo. Similarly, in mice injected with 5 mg/kg dCF, the plasma concentrations of dAdo were 1–2 μM and of Ado were 10–20 μM. In the untreated mice the levels of these nucleosides were below the level of detection [41]. Because the same enzyme is responsible for the phosphorylation of both Ado [44] and 8-Cl-Ado, and because the affinity of this enzyme (Ado kinase) is higher for the natural substrate, Ado [21], phosphorylation of injected 8-Cl-Ado may be inhibited in such a scenario. In addition, increased levels of Ado have been shown to cause substrate inhibition of Ado kinase [45, 46] and a decrease in the phosphoribosylation of Ade [45]. Under these conditions, increased Ado would compete with 8-Cl-Ado for phosphorylation; it will inhibit the phosphorylation of 8-Cl-Ado by Ado kinase, and also phosphoribosylation of 8-Cl-Ade. Such postulates need to be tested to explain these results.

The encouraging results of this study are shown in Tables 1 and 2. In both mice and rats, the accumulation of phosphorylated metabolites in PBMC was high and persistent, even while the plasma concentrations of 8-Cl-Ado were quite low (< 38 μM in mice and 20 μM in rats) and detectable for only 30 min. For example, using approximately equivalent doses of 8-Cl-Ado in mice and rats, intracellular levels of more than 1000 and 100 μM 8-Cl-ATP were measured in PBMC in mice and rats, respectively. Additionally, the concentration of 8-Cl-Ado metabolites, which was measured in rats only, increased 2 h after infusion, while at the same time the plasma concentration of 8-Cl-Ado was below the limit of detection. This may be explained by the slow phosphorylation rate of 8-Cl-Ado in the cell or more probably due to phosphorylation of cleaved 8-Cl-Ade, the levels of which were maintained for 2 h (Fig. 8).

In conclusion, these in vivo plasma and cellular pharmacokinetic studies suggest that, in a similar manner to other Ado or dAdo analogs, 8-Cl-Ado is rapidly catabolized in the plasma leading to low circulating concentrations of 8-Cl-Ado which are maintained for a very short time. Nonetheless, 8-Cl-Ado reaches PBMC, and phosphorylated products including cytotoxic 8-Cl-ATP are formed. These data suggest that even with bolus administration of 8-Cl-Ado, 8-Cl-ATP is formed in cells and retained for a long time. If persistent exposure to increased 8-Cl-Ado plasma concentrations results in higher accumulation of 8-Cl-ATP in cells, a strategy employing a continuous or prolonged infusion may be beneficial.

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