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Protection against fludarabine neurotoxicity in leukemic mice by the nucleoside transport inhibitor nitrobenzylthioinosine*

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Summary. Fludarabine phosphate (F-ara-AMP, Fludara) is rapidly converted in the circulation to fludarabine (F-ara-A) and is among the most effective single agents in the treatment of chronic lymphocytic leukemia. Although current treatment protocols are well tolerated, severe neurotoxicity was a consequence of high-dose F-ara-AMP regimens used in early phase I trials against adult acute leukemia. The present study showed that in mice implanted with leukemia L1210, fatal neurotoxicity, which initially manifested as hind-limb paralysis, was a consequence of high-dose F-ara-AMP treatment. However, the incidence of neurotoxicity was reduced by the coadministration of NBMPR-P, the 5′-phosphate of nitrobenzylthioinosine, a potent inhibitor of the *es* equilibrative nucleoside transport (NT) system. NBTGR-P, the 5′-phosphate of nitrobenzylthioguanosine (also a potent NT inhibitor) similarly prevented F-ara-AMP neurotoxicity in this experimental system. Treatment with F-ara-AMP/NBMPR-P combinations was more effective with respect to the fractional yield of "cured" mice than were the same treatment regimens without NBMPR-P.

Introduction

Clinical trials have shown that F-ara-AMP (fludarabine phosphate) has considerable activity against chronic lymphocytic leukemia (CLL), indolent forms of non-Hodgkin's lymphoma and other lymphoproliferative malignancies (for reviews see [6, 32]). F-ara-AMP is given intravenously, and in the circulation, the agent is dephosphorylated (within 2–4 min [21]), with plasma concentration-time relationships for the dephosphorylation product showing a triexponential decay pattern in patients [21]. Because F-ara-A is a poor substrate for adenosine deaminase as compared with ara-A [4], the 2-fluoro group evidently contributes to the stability of the nucleoside drug.

The entry of F-ara-A into L1210 mouse leukemia cells appears to be transporter-mediated [28], a property that was also evident in the present study of the modulation of F-ara-A cytotoxicity by the nucleoside transport (NT) inhibitor nitrobenzylthioinosine (NBMPR). The relative contributions of the several NT systems expressed in L1210 cells to F-ara-A uptake have yet to be defined. Coexpressed in cultured L1210 cells are (a) a concentrative, Na^+/nucleoside cotransport system [8–12] and (b) two equilibrative NT systems, one of which, the *es* NT system, shows a distinctive, high sensitivity to NBMPR [3, 9]. The other NT system in L1210 cells, the *ei* system (named for its relative insensitivity to NBMPR [9]), appears to be more sensitive to dipyridamole than to NBMPR [10].

Intracellular F-ara-A in converted to the triphosphate in several cell types, including L1210 cells [1, 2]. F-ara-ATP inhibits DNA polymerases and ribonucleotide reductase [24, 31], and in CCRF-CEM cells it is incorporated into DNA at 3′-termini, resulting in blockade of DNA-strand elongation [16]. The incorporation of F-ara-AMP into DNA correlates with loss of clonogenicity and may therefore contribute to F-ara-A cytotoxicity [16].

F-ara-AMP is well tolerated in current low-dose schedules (for example, 15–25 mg/m^2 daily [29]), which have mostly employed daily treatment for 5 days, with courses repeated at 4-week intervals [15, 29]. In the treatment of CLL, response rates of 50%–60% have been obtained in previously treated patients [17]. Although low doses of F-ara-AMP have been relatively safe, treatment of leukemia patients with high F-ara-AMP doses (for example, >96 mg/m^2 daily for 5–7 days [29]) has induced severe

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Abbreviations: ara-A, 9-β-D-arabinofuranosyladenine; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine); F-ara-AMP, fludarabine 5′-monophosphate (Fludara); NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBMPR-P, NBMPR 5′-monophosphate; NBTGR, 2-amino-6[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBTGR-P, NBTGR 5′-monophosphate; NBdAdo-P, N^6-(4-nitrobenzyl)-9-β-D-2′-deoxyribofuranosyladenine 5′-monophosphate

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Table 1. Treatment of leukemic mice with F-ara-AMP and NBMPR-P

F-ara-AMP (mg/kg)	NBMPR-P (mg/kg)	ILS[a] (%)	60-day survivors	Paralysis
100	0	63	0/20	4/20
100	25	136	5/20	2/20
125	0	75	0/20	5/30
125	25	150	25/40	4/40
150	0	88	1/35	24/35
150	25	217	18/35	10/35
175	0	100	2/30	7/30
175	25	325	21/30	2/30
200	0	125	11/49	11/49
200	25	200	30/50	0/50
225	0	131	2/20	4/20
225	25	194	12/20	1/20
250	0	125	3/20	6/20
250	25	163	8/20	0/20

Female B6D2F$_1$ mice were implanted i.p. with 10^6 L1210/C2 cells, and treatments (daily × 5) with F-ara-AMP and NBMPR-P at the doses indicated were begun 24 h later

[a] ILS (increase in life span) represents the difference between median survival times of treated and untreated (control) animals that died leukemic deaths, expressed as a percentage of the control group survival time. The median survival times of the control groups were 7–9 days

Table 2. Paralysis in leukemic mice treated with F-ara-AMP

Treatment	Fraction of treated mice paralysed (%)	Median onset of paralysis (days)	Median time of death after onset of paralysis (days)
F-ara-AMP	35	17	2
F-ara-AMP plus 25 mg/kg NBMPR-P	8.8	21	3

Paralytic responses in mice treated with F-ara-AMP with and without coadministration of NBMPR-B were recorded. The responses shown are summarised from Table 1

Table 3. Effect of the NBMPR dose on the toxicity and efficacy of F-ara-AMP

F-ara-AMP (mg/kg)	NBMPR-P (mg/kg)	ILS[a] (%)	60-day survivors	Paralysis
200	0	150	4/30	8/30
200	15	175	13/30	4/30
200	25	192	15/30	0/30
200	35	200	3/20	1/20
200	50	212	20/30	0/30
200	75	206	2/20	2/20

Female BD2F$_1$ mice were implanted i.p. with 10^6 L1210/C2 cells, and treatments (daily × 5) with F-ara-AMP together with graded doses of NBMPR-P were begun 24 h later

[a] See definition in Table 1

neurotoxicity (dementia with blindness, coma and death) [22, 29, 30]. Neurological toxicity appears to be a function of both F-ara-AMP dose and the rate of administration [15].

In the present study, mice that had received intraperitoneal (i.p.) implants of L1210 leukemia cells were treated with combinations of F-ara-AMP and NBMPR-P, a prodrug form of NBMPR of high aqueous solubility that is rapidly dephosphorylated in vivo [23]. A substantial enhancement in leukemic cell kill resulted from the coadministration of the two agents. We also report that fatal neurotoxicity was evident in leukemic mice treated with F-ara-AMP alone and that NBMPR-P protected mice against this neurotoxicity.

Materials and methods

Female B6D2F$_1$ mice (C57BL6J × DBA2J f$_1$ hybrids) were obtained from the Health Sciences Laboratory Animal Services, University of Alberta. Mouse leukemia L1210/C2 cells, a clonal line established in culture, were maintained in B6D2F$_1$ mice by weekly passage of 10^5 cells from ascitic fluid. For chemotherapy experiments, mice (20–22 g) were implanted i.p. with 10^6 L1210/C2 cells, and treatments (daily × 5) were begun 24 h after implantation. Therapeutic agents were dissolved in 0.15 M NaCl and were injected i.p. in volumes proportional to 0.2 ml/20 g body weight (group average weight at tumor implantation), and drug pairs were dissolved in the same solution. Control mice received injections of 0.15 M NaCl. Survivors were scored twice daily for 60 days, and when neurotoxicity had become evident, mice were caged individually.

F-ara-AMP was provided by the Drug Synthesis and Chemistry Branch, DTP, DCT, National Cancer Institute (Bethesda, Md.). NBMPR was prepared in our laboratory [26]. Dilazep was a gift from F. Hoffmann La Roche & Co. Ltd. (Basel, Switzerland). NBMPR-P, nitrobenzyldeoxyadenosine 5′-phosphate (NBdAdo-P) and nitrobenzylthioguanosine 5′-phosphate (NBTGR-P) were prepared as disodium salts by the Research Laboratory, Yamasa Shoyu Ltd. (Chosi, Japan).

Results

The possibility that coadministration of NBMPR-P and F-ara-AMP might increase the in vivo leukemic cell kill relative to that achieved by F-ara-AMP alone was addressed in the experiments summarized in Tables 1, 3 and 4. Mice implanted with 10^6 L1210/C2 cells were treated with graded doses of F-ara-AMP given with or

Table 4. Treatment of leukemic mice with F-ara-AMP and other NT inhibitors

F-ara-AMP (mg/kg)	NT inhibitor (mg/kg)	ILS[a] (%)	60-day survivors	Paralysis
200	0	169	2/20	9/20
	NBdAdo-P:			
200	50	186	3/20	2/20
200	100	225	1/10	3/10
	NBTGR-P:			
200	25	256	11/20	0/20
200	50	31	2/20	0/20
	Dilazep:			
200	50	206	4/20	3/20
200	75	188	0/10	1/10

Female B6D2F$_1$ mice were implanted i.p. with 10^6 L1210 cells, and treatments (daily × 5) with F-ara-AMP, given with or without NT inhibitors, were begun 24 h later

[a] See definition in Table 1

without NBMPR-P (25 mg/kg) by the i.p. route on a daily × 5 schedule. As shown in Table 1, at all F-ara-AMP doses tested, coadministration of NBMPR-P increased both the proportion of long-term survivors ("cures") in the treatment groups and the life span of mice that died leukemic deaths following F-ara-AMP treatment at doses of 200 mg/kg or less.

Neurological toxicity, which initially manifested as hind-limb paralysis, occurred in F-ara-AMP-treated mice, and its incidence was reduced by coadministration of NBMPR-P. Treatment with F-ara-AMP alone at 200 mg/kg yielded 22% cures and a 22% incidence of neurotoxicity. The addition of NBMPR-P to the treatment yielded 60% cures, and fatal neurotoxicity did not occur. The neurological toxicity first became evident as paresis of the hind limbs and progressed to ascending paralysis, which was usually bilateral and uniformly fatal. Paralysed mice were isolated, were provided with food and water on cage floors, and were observed frequently. The paralysis appeared at about 12 days after the end of treatment (Table 1). In all, 35% of all mice treated with F-ara-AMP alone became paralysed, whereas the incidence of paralysis in mice that received NBMPR-P together with F-ara-AMP was 9%. In mice that received NBMPR-P, the onset of paralysis was delayed for 3–4 days, and the median survival was increased by about 1 day (Table 2). The choice of a modulatory dose of NBMPR-P was based on previous experience in this laboratory, in which mice were protected against potentially lethal doses of toxic nucleosides by coadministration of NT inhibitors [19, 20, 25]. In the experiments reported in Table 3, F-ara-AMP was injected at the optimal dose of 200 mg/kg together with graded NBMPR-P doses in an attempt to optimise the modulatory activity of the latter. Cure rates of 60% were obtained at the most effective NBMPR-P dose level. Among the three other potent NT inhibitors studied, 25 mg/kg NBTGR-P was as effective as the same dose of NBMPR-P, and higher doses were toxic to the mice (data not shown). At equieffective doses, dilazep and NBdAdo-P slightly improved the therapeutic index of F-ara-AMP but were inferior to NBTGR-P (Table 4) and NBMPR-P.

Discussion

Investigations conducted in our laboratory have shown that rodents may be protected against otherwise lethal doses of particular nucleoside analogs by the coadministration of NT inhibitors including NBMPR, NBTGR, N^6-nitrobenzyl-2′-deoxyadenosine, dipyridamole and dilazep [18–20, 25]. In the present study, the nitrobenzyl-substituted nucleosides were given as the 5′-monophosphate esters, which have higher aqueous solubility than the corresponding free nucleosides, but which act in the latter form. Potentially lethal doses of tubercidin or nebularine given together with host-protective doses of NT inhibitors have been active in the treatment of some rodent neoplasms [19, 20, 25] and of rodent models of some hemoparasitic diseases [13, 14]. Host protection in these instances appears to derive from reduction by NT inhibitors of the access of the toxic nucleoside(s) to sensitive, dose-limiting tissues of the host [18]. The present study provides a clear example of protection by NBMPR against fluarabine neurotoxicity in leukemic mice.

Pre-clinical studies of F-ara-AMP toxicity in mice did not recognise the neurotoxicity of F-ara-AMP, perhaps because that toxicity is expressed in a delayed fashion, as the present study indicates. The median onset of paralysis in the present experiments was 17 days after tumor inoculation and was initially apparent as hind-limb paralysis. Hemiplegia occurred in some instances, and two cases of bilateral fore-limb paralysis were noted. In animals treated with NT inhibitors, the onset of F-ara-AMP-induced neurotoxicity was somewhat later than that in mice that did not receive such treatment, suggesting that the NT inhibitors may have reduced the access of F-ara-A to sensitive nervous tissue. Nervous system lesions underlying these abnormalities in mice have not been reported, but demyelination has been observed in the brain and spinal cord of humans who have developed F-ara-AMP toxicity [7]. In both mice (present study) and humans [30], the onset of F-ara-AMP neurotoxicity was delayed, and the symptoms were progressive and fatal. Instances of paresis and paralysis in F-ara-AMP-treated humans have been reported [30].

The 60-day survivors in the chemotherapy experiments were mice in which the leukemic cell population had been virtually eliminated by the treatment regimen [27]. Although substantial kill of leukemia cells was achieved in experiments using F-ara-AMP alone (as indicated by the increased survival times of mice that eventually died leukemic deaths and by the numbers of 60-day survivors), it is clear that combinations of NBMPR-P and F-ara-AMP were more effective than F-ara-AMP as a single agent (Tables 1, 3). As shown in Table 1, protection by NBMPR-P against the neurotoxicity of F-ara-AMP at 200 mg/kg (daily × 5) revealed the full effect of this particular therapeutic regimen, which yielded 30/50 long-term survivors. The pharmacological basis for the enhanced therapeutic effect of the combined agents relative to that of F-ara-AMP alone is not known, but it is possible that NBMPR may enhance the uptake and retention of F-ara-A by the leukemia L1210 cells in vivo in the same manner in which NT inhibitors have enhanced the ara-A content of leukemic L1210 cells in culture [10]. In the latter study, NT inhibitors appeared to impair transporter-mediated ara-A efflux processes without inhibiting a Na^+-linked influx process. 6-Thiopurine metabolites derived from NBMPR [5] might also contribute to the cytotoxicity of the combined agents.

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