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Investigation of antiviral activity of 1-β-D-arabinofuranosylthymine (ara-T) and 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-ara-U) in monkeys infected with simian varicella virus

Kenneth F. Soike*, Gary Baskin, Connie Cantrell and Peter Gerone

Delta Regional Primate Research Center, Tulane University, Three Rivers Road, Covington, LA 70433, U.S.A.

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Summary

1-β-D-Arabinofuranosylthymine (ara-T) and 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-ara-U) were shown to have antiviral activity in vitro and in vivo against simian varicella virus. Both compounds successfully prevented clinical disease caused by inoculation of African green monkeys with simian varicella virus, eliminating the development of rash and substantially suppressing viremia. Ara-T treatment was effective by either intraperitoneal or oral routes of administration and BV-ara-U was active by both oral and intramuscular routes. Ara-T, however, was associated with the appearance of marked signs of neurotoxicity. Histologic examination of brain tissue demonstrated chromatolysis and pyknosis of neurons and pyknotic nuclei in glial cells. The neurologic impairment persisted in affected monkeys. This observation of central nervous system toxicity in monkeys is in contrast to studies in mice and rats where high doses of ara-T by multiple routes of administration were nontoxic. No apparent toxicity was observed in monkeys treated with BV-ara-U.

antiviral; simian varicella virus; monkeys; 1-β-D-arabinofuranosylthymine; 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil; neurotoxicity

Introduction

Antiviral activity of 1-β-D-arabinofuranosylthymine (ara-T) has been reported with in vitro inhibition of herpes simplex (HSV) types 1 and 2, varicella-zoster (VZV) and

* To whom correspondence should be addressed. Telephone: (504) 892-2040, ext. 231.

Epstein–Barr (EB) viruses [1,6,9,13–15,22]. In vivo activity was shown by both ara-T and its 5'-monophosphate (ara-TMP) in mice inoculated intracerebrally with either HSV type 1 or 2 [7,12]. Ara-T treatment was effective whether administered intravenously, intraperitoneally, subcutaneously or orally, resulting in increased survivors and/or increased survival time. Toxicity in mice was not a problem following oral doses of 3 g/kg or intraperitoneal doses of 1 g/kg twice daily for 4.5 days [7]. No significant illness or pathology was observed with ara-T treatment at either of these doses. Toxicity in rats also was not observed, nor was any teratogenicity seen at high doses of ara-T [16].

Analogs of ara-T including alkyl, alkenyl and halogenovinyl derivatives also have shown varying antiherpesvirus activity [8,10,11]. 1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-ara-U) and 1-β-D-arabinofuranosyl-*E*-(2-chlorovinyl)uracil (CV-ara-U) were more active than ara-T against HSV type 1 but much less active against HSV type 2 [11]. Descamps et al. [4] compared BV-ara-U-with *E*-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and reported BV-ara-U to be approximately 10 times less active than BVDU in the inhibition of HSV type 1 in Vero cells. While both BV-ara-U and BVDU were less effective in inhibition of type 2 HSV than type 1, BVDU was the more active of the two compounds. This data was in contrast with the report of Machida et al. [11] who used a different HSV-1 strain and human embryonic lung fibroblast cells for virus cultivation. One potential explanation is that the cell culture systems used to support replication of the herpesvirus have an effect upon the relative potency of antiviral drugs, as has been shown by De Clercq [2].

In vitro studies have demonstrated susceptibility of varicella-zoster virus (VZV) to various antiherpesviral compounds including BVDU and BV-ara-U [3,9,17]. BV-ara-U appeared to be slightly more active than BVDU tested against strains of VZV [9,17]. While no satisfactory animal model exists for testing antiviral efficacy against human varicella virus, we have employed infection of monkeys with simian varicella virus as a means of assessing activity with some success [5,18,19]. We have therefore examined ara-T and BV-ara-U for antiviral activity against simian varicella infection in the African green monkeys. While antiviral effects were demonstrated in this system we have also observed a marked neurotoxicity of ara-T upon oral and intraperitoneal administration. Toxic signs were seen in monkeys treated orally with daily total doses as low as 25 mg/kg per day. The results of these studies are presented in this report.

Materials and Methods

In vitro studies

The antiviral activity of ara-T and BV-ara-U was compared with other antiherpesviral thymidine analogs in an in vitro assay. Vero cell cultures in 60 mm petri dishes were inoculated with a dilution of simian varicella virus to give a countable number of plaques in a volume of 2 ml. Following a 4 h period for virus adsorption, dilutions of the respective antiviral compounds were added in additional 2-ml volumes giving final concentrations of each compound in 3.3-fold steps within the expected range of activity. Each drug concentration was tested in triplicate.

Following sufficient incubation to permit the development of plaques, the cultures were fixed in methanol, stained with Giemsa–basic fuchsin and the number of plaques counted. The end point for antiviral activity of each compound was the concentration inhibiting 75% of the plaques present in control cultures determined by plotting the number of plaques versus compound concentration.

Monkeys

African green monkeys (*Cercopithecus aethiops*) were purchased as wild-caught animals from a commercial supplier. Upon arrival at this Center all animals were held in quarantine and stabilized for a minimum period of 90 days. Blood specimens were taken on two occasions for baseline hematology and clinical chemistry tests and sera tested for presence of antibody to simian varicella virus. None of the monkeys were found to have antibody to this virus.

Virus inoculation

Virus infection was induced by inoculation of 1.5 ml of a stock simian varicella virus preparation intratracheally by insertion of a French number 5 catheter into the trachea as well as by injection of 1.5 ml subcutaneously into the abdominal area. The virus was titrated at the time of inoculation and the administered number of plaque forming units (PFU) of virus was determined.

Preparation of drugs

Ara-T and BV-ara-U were provided by Dr. Maureen Myers of the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases and were received originally from Yamasa Shoyu Company Ltd., Choshi, Japan. Each of the drugs was dissolved in sterile distilled water at concentrations to give the desired dose in volumes of 5 ml/kg administered orally or 1.0 ml/kg administered intramuscularly or intraperitoneally. The pH of the drug solutions was near neutrality and the drug solutions were prepared fresh daily.

Experimental protocol

Baseline values for hematology and clinical chemistry were obtained twice prior to virus inoculation. Clinical chemistry tests included blood urea nitrogen, creatinine, albumin, globulin, total proteins, bilirubin and aspartate and alanine aminotransferases. Treatment with ara-T or BV-ara-U was initiated 48 h after virus inoculation at the indicated doses and routes of administration. Blood was taken from each monkey at 3, 5, 7, 9, and 11 days following virus inoculation for hematology and clinical chemistry tests and for inoculation of tissue culture for detection of viremia. For isolation of virus from the blood, 3 ml of blood were collected in heparin and the lymphocytes in the sample separated on a Ficoll–Hypaque gradient. The separated lymphocytes were washed twice and suspended in 10 ml of tissue culture medium (RPMI-1640 with 15% fetal bovine serum) which was divided as the inoculum for two 25-cm^2 tissue culture flasks seeded with Vero cells. After sufficient incubation at 37°C in 5% CO_2 and air to allow for virus replication, the cultures were fixed in methanol and the cell sheets stained with methylene blue–basic fuchsin and the number of

plaques counted. The number of plaques was reported as the mean of the two flasks.

In addition all monkeys were observed daily for the development of clinical symptoms and the appearance of rash. Other clinical signs such as appetite, activity and general responsiveness were noted.

Histopathology

Upon death of any monkey a complete necropsy was performed by a veterinary pathologist (G.B.). During gross examination tissue samples from all major organs including the brain and spinal cord were taken and fixed in 10% neutral formalin. Each of the tissues was subsequently processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The slides were examined for virus or drug related histopathologic changes.

Results

In vitro studies comparing ara-T and BV-ara-U with other known antiviral compounds for inhibition of simian varicella virus showed that the antiviral activity of ara-T was intermediate between acyclovir and adenine arabinoside-5'-monophosphate (ara-AMP) and the bromovinyl uracil analogs (Table 1). BV-ara-U was the most active of the five compounds studied inhibiting replication of simian varicella virus at a dose of 0.0014 μg/ml.

In an initial experiment with ara-T, 12 African green monkeys were infected with 1.4×10^4 PFU of simian varicella virus. 48 h after virus inoculation, 4 monkeys were treated with ara-T given as an oral gavage at 100 mg/kg twice daily or a total dose of 200 mg/kg per day. Four additional monkeys were treated by intraperitoneal injection of 50 mg/kg twice daily (100 mg/kg per day). Four infected control monkeys received PBS orally by stomach tube as infection control animals.

After 5 days of ara-T treatment, signs of neurologic toxicity appeared in two monkeys (Table 2), one in each of the ara-T treatment groups (B263 and B027). Treatment of all monkeys was terminated at that time after 5 days of ara-T administration. Neurologic signs appeared in three monkeys the following day (B024, B028 and B261) and in one more (B262) two days later. Ultimately, three of the four monkeys in each of the treatment groups developed symptoms of neurologic toxicity. The two monkeys which were the first to develop symptoms died on post-infection days 11 and 14 (9 and 12 days after the initiation of ara-T treatment). Of the four surviving monkeys displaying neurotoxicity, three still showed residual signs of neurologic impairment one year later.

The neurologic signs appeared as uncoordinated muscular activity. The monkeys appeared aware of their surroundings and when stimulated responded with vigorous thrashing uncoordinated convulsive movements. Treatment of the monkeys with Valium® or pentobarbital was marginally successful. In the two monkeys that died (B263 and B027), the brain and spinal cords were examined. The postmortem intervals were overnight and 2 h. Grossly, one brain appeared normal but the cervical spinal cord of the other was slightly swollen.

TABLE 1

In vitro inhibitory activity of antiherpesviral thymidine analogs against simian varicella virus

Drug	Concentration (μg/ml)	Mean no. plaques (% reduction for control)	Concentration for 75% inhibition (μg/ml)
None	0	394	-
Acyclovir	1.0	>200	
	3.3	139 (65%)	6.2
	10.0	46 (88%)	
	33.3	0 (100%)	
Ara-AMP	1.0	>200	
	3.3	115 (71%)	4.7
	10.0	4 (99%)	
Ara-T	0.1	>200	
	0.33	174 (56%)	
	1.0	70 (82%)	0.83
	3.3	37 (91%)	
	10.0	12 (97%)	
BVDU	0.0033	300 (24%)	
	0.01	16 (96%)	0.0073
	0.033	0 (100%)	
BV-ara-U	0.001	127 (68%)	
	0.0033	26 (93%)	0.0014
	0.01	3 (99%)	
	0.033	1 (100%)	

Acyclovir = 9-(2-hydroxyethoxymethyl)guanine; ara-AMP = adenine arabinoside-5'-monophosphate; ara-T = 1-β-D-arabinofuranosylthymine; BVDU = *E*-5-(2-bromovinyl)-2'-deoxyuridine; BV-ara-U = 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil.

Histologically the changes were minimal to mild and were limited to neurons and oligodendroglia (Fig. 1–3). The changes were most apparent in the thalamus, brain stem, and spinal cord, but rare affected cells could be found elsewhere as well. Scattered neurons in the affected areas were swollen and had an eosinophilic cytoplasm. Occasional neurons contained a single large clear cytoplasmic vacuole. Nuclei of affected neurons were often marginated, pale, and swollen. Some were fragmented. A few nuclei were pyknotic. Scattered oligodendroglial nuclei were pyknotic or fragmented. Numerous normal cells remained even in the most severely affected areas.

In spite of the adverse neurotoxicity of ara-T by both oral and intraperitoneal routes of administration, the compound inhibited the development of viremia and the appearance of rash (Table 2). Viremia was essentially eliminated in each of the monkeys treated with ara-T and no rash appeared in the treated monkeys. Each of the four untreated control monkeys developed viremia and rash detectable over an extended period of time.

TABLE 2

Effect of ara-T administered by gavage or by intraperitoneal injection on the course of simian varicella virus infection in the monkey

Ara-T treatment (mg/kg per day)	Monkey no.	Viremia*				Rash (days post-infection)	Neurotoxicity (days post-infection)
		3	5	7	9		
Control	B029	0	7	171	126	9–15	None
	B266	3.5	140	TNTC	TNTC	7–14	None
	B260	0	1	73	518	9–15	None
	B255	2	48	19.5	14	9–15	None
Ara-T (200 mg/kg per day, p.o.)	B025	0	0	0	0	None	Slight (8)
	B026	0	0	0	1.5	None	None
	B262	1	0	0	0	None	Marked (10)
	B263	4.5	0	0	0	None	Marked (7)
Ara-T (100 mg/kg per day, i.p.)	B027	1	0	0	0	None	Marked (7)
	B028	0	0	0	0	None	Slight (8)
	B261	0	0	1.5	1.5	None	Marked (8)
	B259	1	0	0	0	None	None

* Mean PFU per 25-cm^2 flask inoculated with lymphocytes from 3 ml of heparinized blood (see text).

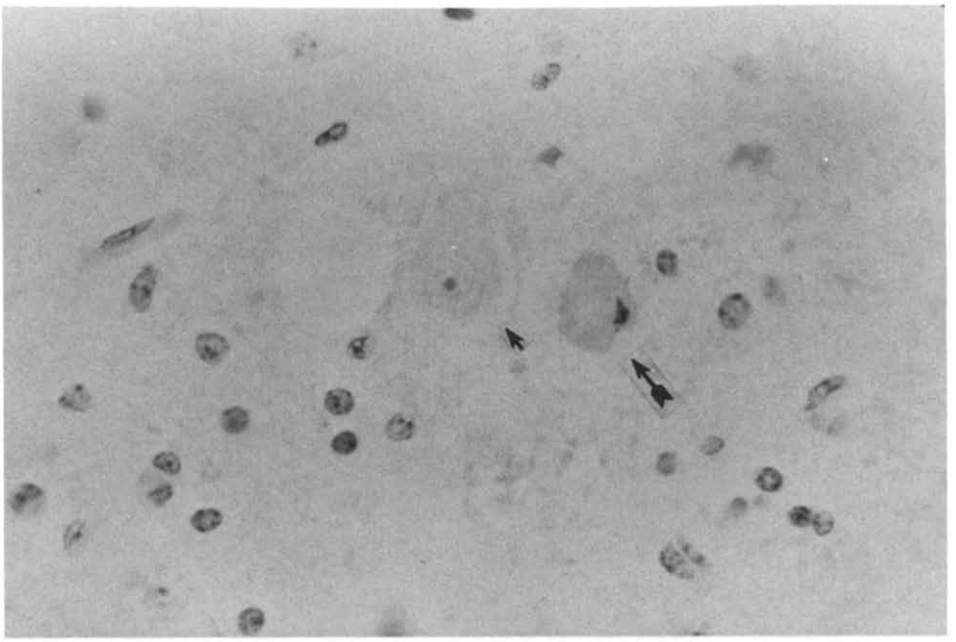

Fig. 1. Damaged neuron undergoing chromatolysis (arrow) – brain stem. Hematoxylin-eosin stain.

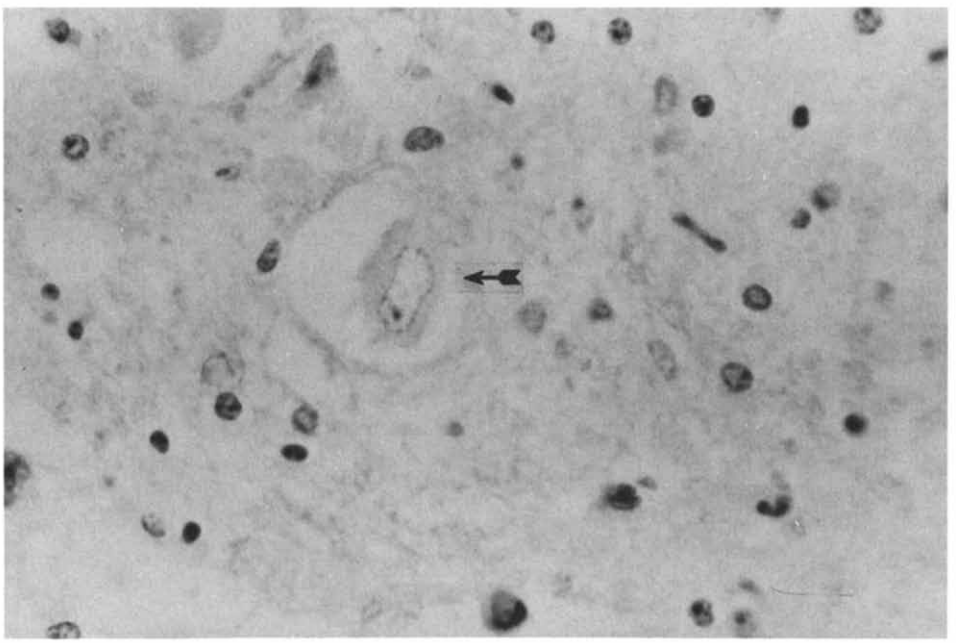

Fig. 2. Damaged neuron with pyknotic nucleus and hypereosinophilic cytoplasm (arrow); note adjacent unaffected neuron (arrowhead) – brain stem. Hematoxylin-eosin stain.

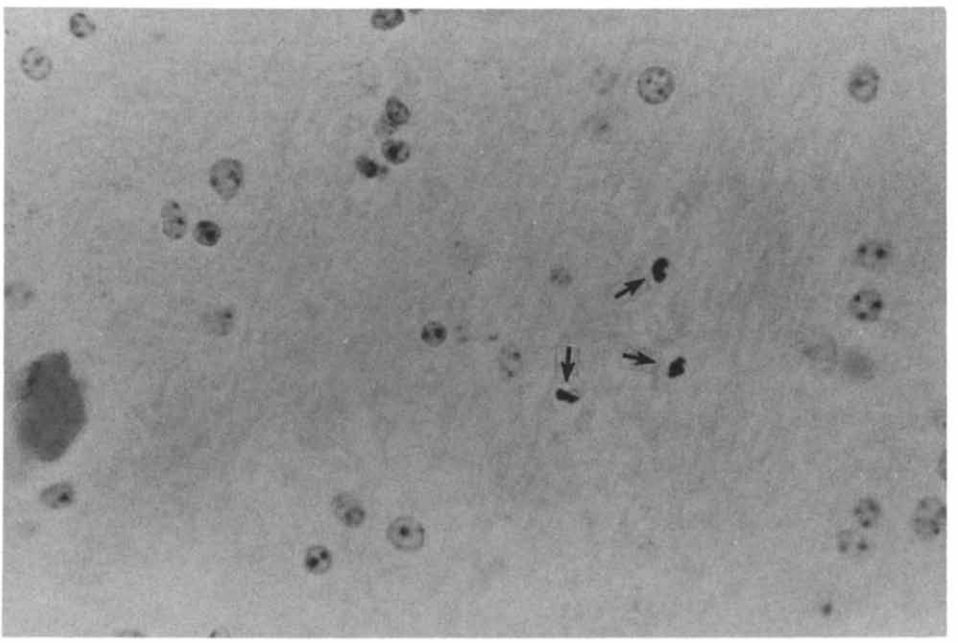

Fig. 3. Glial cells with pyknotic nuclei (arrows) – hippocampus. Hematoxylin-eosin stain.

In a second experiment, 12 African green monkeys were infected with simian varicella virus and treated orally at two dose levels of ara-T. Four monkeys received ara-T by gavage at a dose of 100 mg/kg per day in divided daily doses. A second group of four monkeys received ara-T by stomach tube at 25 mg/kg per day also given twice daily. The four untreated control monkeys received phosphate buffered saline by gavage at the same treatment schedule.

Seven days after virus inoculation or after five days of ara-T treatment tremors were observed in one monkey receiving the 100 mg/kg per day dose (Table 3). The following day one additional monkey in the group receiving 100 mg/kg per day and two treated with ara-T at 25 mg/kg per day developed neurologic signs each reflecting the toxicity previously seen. A third monkey in the 100 mg/kg per day group showed obvious neurologic symptoms at 10 days post-infection. The neurotoxicity persisted in monkeys C125 and C126 which received the high ara-T dose and disappeared in the other three monkeys. Ara-T treatment did inhibit simian varicella virus infection. Viremia was markedly suppressed by both 100 and 25 mg/kg per day of oral ara-T administration and rash was completely inhibited. Clinical disease with rash and viremia was observed in each of the four untreated control monkeys. Two of the four control monkeys died as a consequence of disseminated simian varicella infection apparent on necropsy.

In an experiment to evaluate BV-ara-U for antiviral efficacy in simian varicella virus infection of the African green monkey as well as its potential neurotoxicity, 12 monkeys were infected by inoculation of 5.4×10^4 PFU of virus. On the second day

TABLE 3

Effect of ara-T given as oral gavage at 100 or at 25 mg/kg per day on the course of simian varicella virus infection in the monkey

Ara-T treatment (mg/kg per day)	Monkey no.	Viremia*				Rash (days post-infection)	Neurotoxicity (day of appearance)
		3	5	7	9		
Controls	C118	41.5	149	TNTC	57	8–14	None
	C119	20	205.5	313.5	16.5	8–14	None
	C120	53.5	251.5	Dead	Dead	7–Death	N.D.
	C121	9.5	592.5	TNTC	TNTC	8–Death	N.D.
Ara-T (100 mg/kg per day, p.o.)	C122	1.5	0	0	0	None	Slight (10)
	C124	5.5	0	0	0	None	None
	C125	0	0	0	0	None	Marked (7)
	C126	0	0	0	0	None	Marked (8)
Ara-T (25 mg/kg per day, p.o.)	C127	1	1.5	2.5	0	None	None
	C128	0	3.5	6	0	None	None
	C129	1.5	0	3	2	None	Slight (8)
	C130	2	1	0	0	None	Slight (8)

* Mean PFU per 25-cm^2 flask inoculated with lymphocytes from 3 ml of heparinized blood (see text).
N.D. = no data.

TABLE 4

Effect of BV-ara-U treatment given by gavage or by intramuscular injection on the course of simian varicella infection in the monkey

BV-ara-U treatment (mg/kg per day)	Monkey no.	Viremia*					Rash (days post-infection)
		3	5	7	9	11	
Control	C224	0	0	4.5	19	12.5	9–14
	C225	0	18	TNTC	TNTC	TNTC	8–11 (died)
	C226	0	0	3.5	5	5	11–12
	C228	0	0	1	7	11.5	11–14
BV-ara-U (100 mg/kg per day, p.o.)	C229	0	0	0	0	0	None
	C232	0	Died - day	3			-
	C233	0	0	0	0	0	None
	C234	0	0	0	0	0	None
BV-ara-U (20 mg/kg per day, i.m.)	C235	0	0	0	0	0	None
	C236	0	0	0	0	0	None
	C238	0	0	2	0	0	None
	C239	0	0	2	9	17	None

* Mean PFU per 25-cm^2 flask inoculated with lymphocytes from 3 ml of heparinized blood (see text).

post-infection, four monkeys were treated twice daily with BV-ara-U at 100 mg/kg per day given by gastric tube. Another four monkeys received BV-ara-U intramuscularly at 20 mg/kg per day also as two injections daily.

Viremia was observed in repeated blood specimens taken from the four infected control monkeys (Table 4). One monkey (C225) died on the eleventh day post-infection from simian varicella infection, confirmed on necropsy. The vesicular rash characteristic of simian varicella infection appeared in each of the four control monkeys. In monkeys treated with BV-ara-U, viremia and development of rash were prevented by BV-ara-U at 100 mg/kg per day orally. One monkey in this group (C232) died on the third day post-infection with an unrelated hemorrhagic colitis. The monkeys treated intramuscularly with BV-ara-U at 20 mg/kg per day did not develop rash either but a minimal viremia appeared in two monkeys on the seventh day post-infection which persisted in one monkey through two additional sampling days. No signs of neurotoxicity were observed during a 21-day observation period.

No abnormalities were observed in hematology or clinical chemistry parameters which were monitored during the course of treatment with either ara-T or BV-ara-U.

Discussion

Studies in African green monkeys infected with simian varicella virus have confirmed the antiviral activity of both ara-T and BV-ara-U. Oral administration of ara-T at doses as low as 25 mg/kg per day or intramuscular injection of BV-ara-U at 20 mg/kg per day suppressed the development of viremia and the appearance of rash associated with this infection in African green monkeys. Treatment with either drug was effective even when delayed as long as 48 h after virus inoculation.

Treatment of monkeys infected with simian varicella virus with ara-T would appear to be more effective than treatment with either acyclovir, adenine arabinoside or ara-AMP [19–21]. Oral treatment with ara-T at doses as low as 25 mg/kg per day prevented the appearance of rash and reduced viremia which was not possible with adenine arabinoside or ara-AMP at doses of 15 mg/kg per day given intramuscularly [20]. Similarly, while acyclovir given intravenously at 20 – 45 mg/kg per day inhibited the development of rash, no effect was seen on viremia [21]. Doses of acyclovir of 100 mg/kg per day prevented both rash and viremia [19]. Both BV-ara-U and BVDU [11] were highly active against simian varicella virus infection but studies employing comparable doses and routes of administration of these two compounds were not performed.

Of major concern was the neurotoxicity observed with ara-T in monkeys treated orally with daily doses between 25 and 200 mg/kg per day. Intraperitoneal injection at 100 mg/kg per day resulted in similar neurotoxic signs and death in one monkey. Histopathologic examination showed a mild multifocal neuronal involvement in the thalamus, brain stem and spinal cord with chromatolysis and pyknosis of affected neurons. The signs of neurologic damage persisted in some monkeys for longer than one year.

Similar neurotoxicity was not produced in mice given acute oral doses of ara-T at 15

g/kg or twice daily at 3 g/kg for 4.5 days [7]. Intraperitoneal injections of 1 g/kg twice daily for 4.5 days also were not lethal; however, higher doses did cause death of some mice. Similar high doses of ara-T-AMP were shown to be relatively non toxic in mice [12].

Studies in rats for acute toxicity gave an LD_{50} of greater than 8 g/kg when given intraperitoneally [16]. Death appeared to be related to crystallization of ara-T in the urinary bladder with urinary retention. Death was not observed with oral doses of 10 g/kg, or subcutaneous doses of 5 g/kg. Subacute toxicity studies in rats with intraperitoneal injections of 1.0 g/kg for 3 months showed no effect on food consumption or weight gain. No significant effects were seen when hematology and clinical chemistry parameters were monitored. No teratogenic effects occurred in litters of pregnant rats treated with ara-T at doses of 300 or 1000 mg/kg.

It would appear that in the monkey ara-T has a potential toxicity. The toxic manifestation directed toward the central nervous system resulted in relatively severe and persistent neurologic impairment. Although these symptoms did not appear in rodent species, this demonstration in a nonhuman primate would predict potential danger in its application to humans. Similar neurotoxic signs did not appear in monkeys treated with BV-ara-U and may not be a consequence of treatment with the halogenovinyl analogs.

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