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Antimalarial and Toxic Effects of the Acyclic Nucleoside Phosphonate (S)-9-(3-Hydroxy-2-Phosphonylmethoxypropyl)Adenine in *Plasmodium berghei*-Infected Mice

LOUIS J. J. W. SMEIJSTERS,¹ HANS NIEUWENHUIJS,¹ ROB C. HERMSEN,² GERRY M. DORRESTEIN,³ FRITS F. J. FRANSSEN,¹ and J. PROSPER OVERDULVE¹*

Institute of Infectious Diseases and Immunology, Department of Parasitology,¹ and Department of Pathology,³ Faculty of Veterinary Medicine, University of Utrecht, Utrecht, and Department of Medical Microbiology, University of Nijmegen, Nijmegen,² The Netherlands

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Plasmodium berghei-infected mice died with low levels of parasitemia after repeated intraperitoneal administration (five times at 15 mg kg of body weight⁻¹ every other day) of the in vitro active antimalarial acyclic nucleoside phosphonate (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA]. Toxicological studies showed that the main cause of death resulted from (S)-HPMPA-induced nephrotoxicity. Although concomitant intraperitoneal administration of the tubular epithelium transport blocker probenecid prevented (S)-HPMPA-induced toxicity, mice eventually died with a high level of parasitemia, despite repeated administration of high doses of (S)-HPMPA. The short half-life of (S)-HPMPA in plasma combined with the insusceptibility of the nonreplicative stages of the parasite to (S)-HPMPA could explain this failure to eradicate all parasites. Indeed, a low but sustained (calculated) level of 200 nM (S)-HPMPA in plasma completely cured *P. berghei*-infected mice. However, these mice, which received a total dose of only 28 mg kg⁻¹ administered via osmotic pumps for 7 days, died because of the toxicity of the drug. These findings indicate that nephrotoxicity hinders the use of (S)-HPMPA as a drug against blood stage parasites. An alternative application of (S)-HPMPA as a potent prophylactic drug is discussed.

Infection with species of the protozoan parasite *Plasmodium* threatens approximately one-fourth of the world's population. A rapidly developing resistance of the parasite to formerly effective drugs stresses the need for the development of new antimalarial compounds. Previous work showed that the acyclic nucleoside phosphonate (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(S)-HPMPA], the prodrug of the diphosphorylated active compound, is a potent inhibitor of mid-schizont stages of both P. falciparum and P. berghei (21). Repeated intraperitoneal administration (four times) of 20 mg of (S)-HPMPA kg of body weight⁻¹ (estimated initial level in plasma, 0.1 mM, or $\approx 1,200$ times the 50% inhibitory concentration [IC₅₀] for in vitro-cultured P. berghei parasites) strongly suppressed parasitemia in P. berghei-infected BALB/c mice but did not eliminate all parasites (9). Although this treatment resulted in the almost complete eradication of parasites, some of the mice which remained infected with small numbers of parasites (<0.1%) died, while the surviving mice developed increasing levels of parasitemia after treatment was stopped. Two conditions in concert may account for the failure of radical treatment: (i) the short half-life in plasma (5 to 10 min) of acyclic nucleoside phosphonates (4, 15, 16) and (ii) a narrow window of susceptibility of erythrocytic parasites to (S)-HPMPA, with schizonts, which constitute a minor part of an asynchronous parasite population, being inhibited only by (S)-HPMPA (21).Death after repeated (S)-HPMPA administration, despite minimal parasitemia, suggests toxicity. The nephrotoxicity of acyclic nucleoside phosphonates has been described in mice (6) and humans (12-14), especially when it is administered

repeatedly (15). (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(S)-HPMPC]-induced nephrotoxicity has been attributed to the active uptake of acyclic nucleotide analogs by organic anion transporters present at both the basolateral and the brush border membranes of the proximal convoluted tubule cell (6). Cyclic derivation of (S)-HPMPC, resulting in a modification of its charge, elevated the therapeutic index (6). Inhibition of (S)-HPMPC uptake by the organic anion transport blocker probenecid also reduced nephrotoxicity (6, 12, 14). Depending on the ratio between tubular secretion and reabsorption of a drug in the presence of probenecid, concomitant administration of probenecid can result in an elevation of the level of the drug in plasma and the half-lives of several drugs in plasma (10), including nucleoside and nucleotide analogs like zidovudine (17) and (S)-HPMPC (8). Therefore, in the case of P. berghei cotreatment with probenecid not only could reduce or prevent (S)-HPMPA-induced nephrotoxicity but also could increase the effectiveness of (S)-HPMPA on asynchronous infections. This report describes the toxicological effects and antimalarial efficacies of different regimens of

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* Corresponding author. Mailing address: P.O. Box 80165, 3508 TD Utrecht, The Netherlands. Phone: 30-2532559. Fax: 30-2540784.

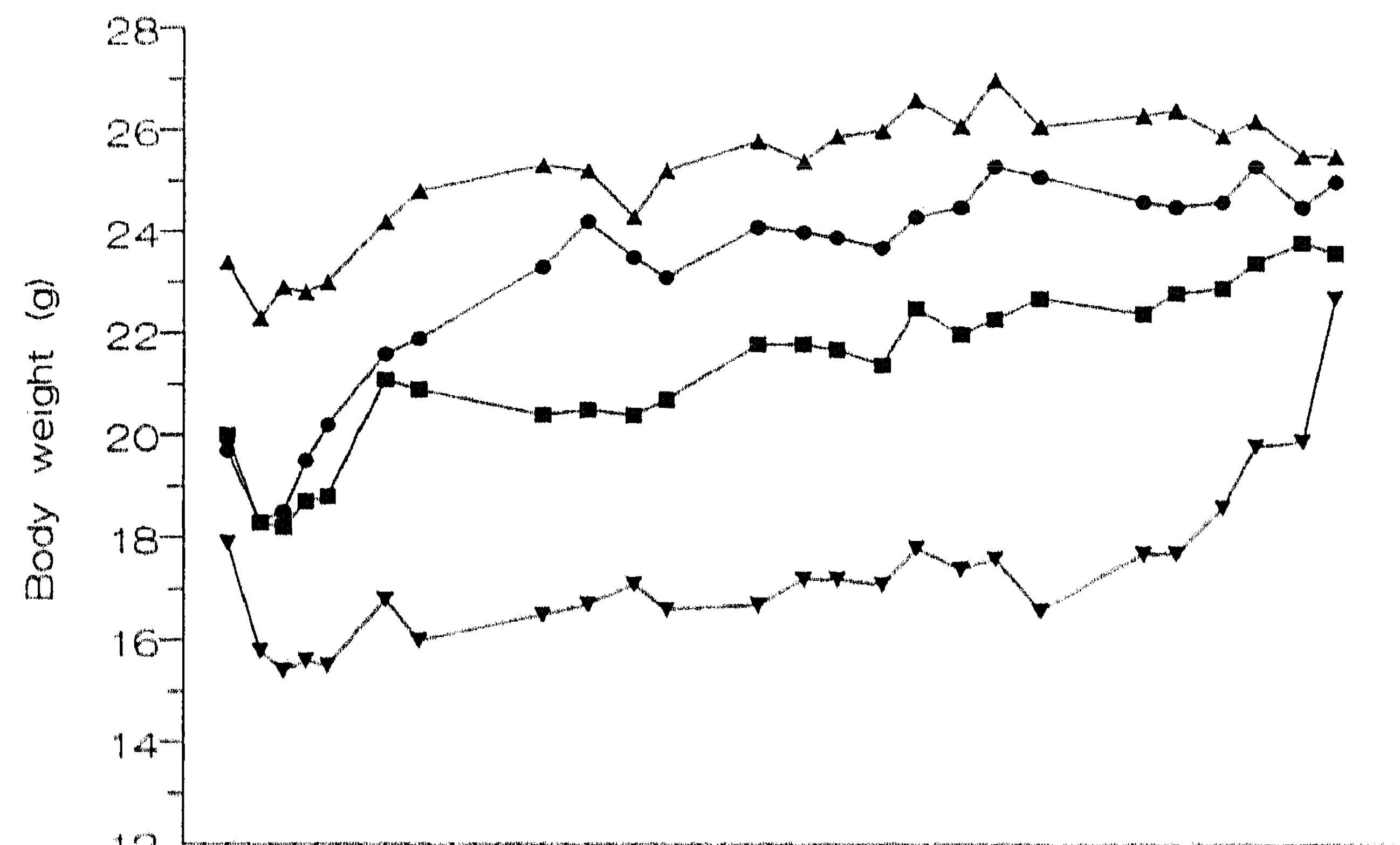
(S)-HPMPA in the presence and absence of probenecid.

MATERIALS AND METHODS

(S)-HPMPA and (S)-cHPMPA. (S)-HPMPA and (S)-cyclic-HPMPA [(S)cHPMPA] were synthesized by A. Holý (Academy of Science, Prague, Czech Republic) and were obtained directly from him or from E. de Clercq (Rega Institute, Leuven, Belgium). For the structure of cyclic derivation, see the report by Bischofberger et al. (6).

Toxicological studies. BALB/c mice were exposed to different doses of intraperitoneally (i.p.) administered (S)-HPMPA on the days indicated in the legend to Fig. 1. Mice were observed daily for clinical signs, and their body weights were determined on the indicated days. Two mice exposed to four times 80 mg of

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In the second experiment, BALB/c mice were infected with 10° parasites on day 0, and sustained (S)-HPMPA treatment [effective volume, 87 μ]; release, 1 μ I h⁻¹ of 15 or 30 mM (S)-HPMPA for 3.5 days; model 1003D; Alza Corporation] was started on day 2.

RESULTS

Toxicology studies. To investigate the cause of death of *P*. berghei-infected mice which occurred during (S)-HPMPA treatment, despite low levels of parasitemia, the toxicological effects of the drug were studied in noninfected mice. Repeated i.p. (S)-HPMPA administration caused an acute and doserelated loss of body weight (Fig. 1). Weight loss was dramatic after the administration of doses of 20 mg kg⁻¹ and greater. Body weight started to increase within 1 week after the cessation of drug administration except in the group treated four times with 80 mg kg⁻¹. In this latter group, a substantial increase in weight occurred only after a 3-month lag period. A similar slow increase in weight was observed after an initial rapid increase in the group treated with 40 mg kg⁻¹. To analyze the cause of the (S)-HPMPA-induced toxicity further, two mice from the group receiving 80 mg kg⁻¹ were sacrificed for histopathological evaluation. Histopathological examination of the kidneys revealed severe degeneration of kidney tubule epithelium. The tubular lumina were enlarged and contained proteinaceous material. No alterations were observed in the glomeruli. Both mice showed cataract of the eyes. The livers showed parenchymal necrosis as well as megalocytosis and karyomegatosis, indicating the restoration of degeneration. Hepatotoxicity was confirmed by the elevated levels of liverspecific enzymes (alkaline phosphatases, alanine aminotransferase, and aspartate aminotransferase) in the plasma (Table 1). The small and large intestines, spleen, and bone marrow showed no abnormalities. Effect of concomitant (S)-HPMPA and probenecid administration. The effect of probenecid on both (S)-HPMPA-induced toxicity and antiplasmodial efficacy was analyzed in P. bergheiinfected mice. The administration of (S)-HPMPA every other day suppressed parasite replication, but as has been observed previously, all mice died early during treatment with very low levels of parasitemia (< 0.1%) (Fig. 2). Although infected mice were not examined histopathologically after death, the observed weight loss indicates severe (S)-HPMPA-induced toxicity. Noninfected mice receiving the same regimen of (S)-HPMPA or even twofold higher doses survived, despite considerable loss of weight (data not shown). Treatment of infected mice with probenecid 30 min prior to (S)-HPMPA administration reduced (S)-HPMPA-induced toxicity, as indicated by increased survival (Fig. 2) and reduced loss of weight (body weights on day 10 postinfection with and without co-

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FIG. 1. Effect of (S)-HPMPA on body weights of BALB/c mice (10 mice per group) after i.p. administration of 10 (\blacktriangle), 20 (\odot), 40 (\blacksquare) and 80 (\forall) mg of (S)-HPMPA kg⁻¹ on days 0, 4, 7, and 11. The range of the standard deviation (not shown) was 0.9 to 2.5 g. The body weights of nontreated mice were determined up to day 21. Their average body weight (25.9 g) did not increase between day 14 and day 21.

HPMPA kg⁻¹ (Fig. 1) as well as two control mice were bled on day 21 after the start of treatment for analysis of enzyme and biochemical parameters (Table 1), and immediately afterward, these mice were sacrificed for general pathological and histopathological examination.

Infection and determination of parasitemia. Mice were infected by i.p. injection of P. berghei ANKA blood stages. Parasitemia was determined by microscopic examination of Giemsa-stained blood smears.

i.p. drug treatment. BALB/c mice (six mice per group) infected with 10⁶ parasites on day 0 were treated every other day, starting on day 2, by i.p. injection of (S)-HPMPA, (S)-cHPMPA (both at 15 mg kg⁻¹), and probenecid (0.285 g kg^{-1}). Separate groups of mice received probenecid (0.285 g kg^{-1}) 30 min prior to (S)-HPMPA or (S)-cHPMPA administration. A control group of six infected mice was not exposed to any treatment (previous experiments had shown that i.p. administration of phosphate-buffered saline [PBS] had no effect on the course of infection). Mice were observed for clinical signs and were weighed every day.

Sustained administration of (S)-HPMPA. Osmotic pumps (effective volume, 170 µl; release, 1 µl h⁻¹ for 7 days; model 2001; Alza Corporation, Palo Alto, Calif.) containing 7.5 or 15 mM (S)-HPMPA were implanted i.p. in anesthetized C57Black/6J (weight, 26 to 28 g) mice on day -1. Mice were infected on day () with 10⁵ parasites. Nontreated control mice did not receive pumps, because previous experiments had shown that implanted pumps containing PBS do not affect the course of infection. Mice were observed for clinical signs and were weighed daily. The release (R_{pump}) of (S)-HPMPA producing the levels in plasma indicated in Table 2 was calculated by the following equation: R_{pump} (in moles kilogram⁻¹ hour⁻¹) = C_{m} (in moles liter⁻¹) × V (in liters kg⁻¹) × (ln 2 $\times I_{1/2}$ (h 1), where C_{ss} is the steady-state concentration in plasma, V is the volume of distribution, and $t_{1/2}$ is the half-life. The half-life of (S)-HPMPA in plasma (10 min) was determined in rats (4). The volume of distribution (V) was assumed to be 0.7 liters kg⁻¹. A release of 1 μ l of 15 mM (S)-HPMPA h⁻¹ therefore results in a steady-state concentration in plasma of 0.2×10^{-6} mol liter¹, which equals ± 2.5 times the IC_{so} for *P. berghei* in vitro.

TABLE 1. Levels of various components in plasma at day 10 after the end of repeated i.p. treatment of BALB/c mice with (S)-HPMPA^e

Grouph	Urea (mmol liter ¹)	Creatinine (µmol liter ⁻¹)	Total protein (g liter ⁺)	Albumin (g liter ¹)	Alkaline phosphatase (U liter ⁻¹)	Alanine aminotransferase (U liter ⁻¹)	Aspartate aminotransferase (Ulliter 1)	
Controls	nin and a factor of factor for the second for a factor of the	ĸĸĸĸĸĊĸĊĸĸĸĸŦĸŦĸŦĸŎġĸĸĸĸĸĸĔĸĸŔĸĸŔĸĸŔĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸĸ	nanda hina ala hariking kananya	ĨĸIJĨġĸĬġĸĔġĸĔġĸĬĸĬĸĬĸĸĸĸĸĔĸĊĸŎĸĸĸĸĸĸĸĸĸĸĸŔġĸĸŔġĸĸŔġĸĸŔġĸĸŔġĸĸĔġĸĔġĸĔġĸĔġĸĔġĸĔġĸĔġĸĔġĸĸĸĸĸĸĸĸ	₩₽₩₩₽₽₽₩₽₩₽₽₩₽₩₽₽₩₽₽₩₽₽₩₽₽₩₽₽₩₽₽₩₽₽₩₽₽₩	militadedatestatestatestatestatestatestatest	алыйалантын ракиналалдагын аларын каларын каларын каларын түркен каларын каларын каларын каларын каларын калар Тамийалантын ракиналарын каларын каларын каларын каларын каларын түркен каларын каларын каларын каларын каларын	
M1	5.6		5()	31	86	16		
M2	5.2	37	51	31	70			
(S)-HPMPA-treated mice								
					980			
LI2	6.9	, 和 新知识的社会和学习教育和	31					

" The mice (H1 and H2) were treated four times with 80 mg of (S)-HPMPA kg⁻¹. Experiment was conducted as described in Materials and Methods, ^b M1, M2, H1, and H2, mice selected from the experiment depicted in Fig. 1.

' ---, the creatinine level could not be determined in the interior blood samples of (S)-HPMPA-treated mice.

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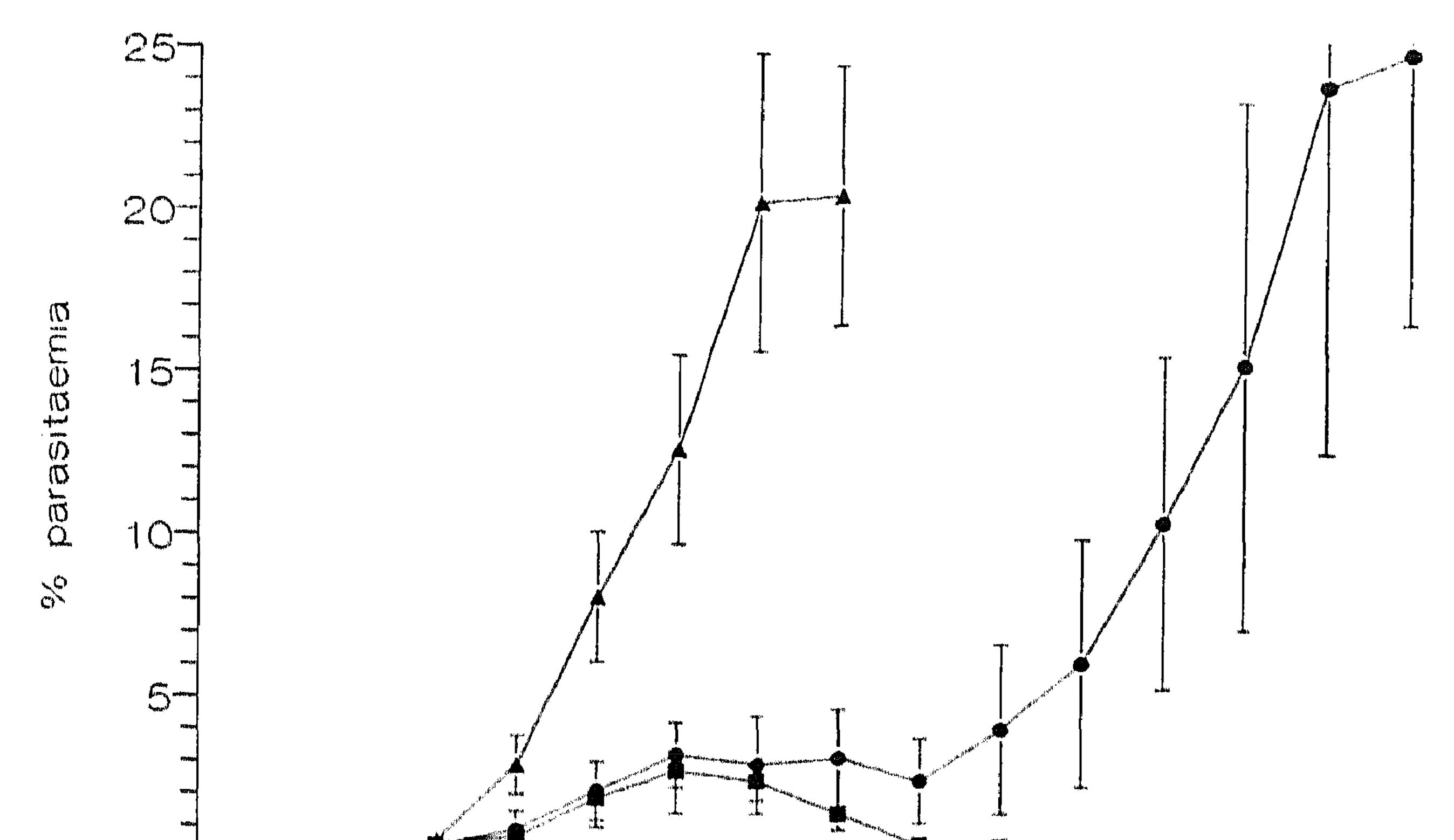
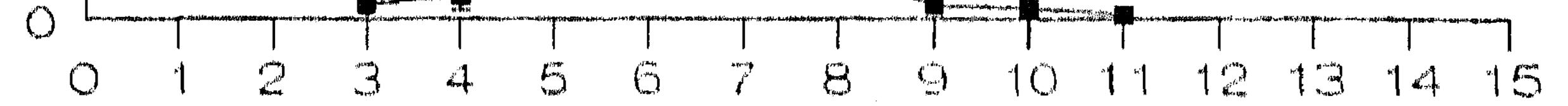


TABLE 2. Effect of sustained release of (S)-HPMPA on P. berghei infection in mice"

Animal	Calculated (S)-HPMPA level in plasma (nM) ^b	% Parasitemia on the following day postinfection:							Day
	level in plasma (nivi)"	4	5	6	7	8	9	19	-
M1	200	< 0.1	0	()	0	0	0	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	13
M2	200	< 0.1	0	()	0	0	0		14
M3	200	0	0	0	0	0	0		16
M4	100	< 0.1	0.1	0.1	0.1	0.1	<0.1	10	28
M5	100	(0.1)	0.2	0.2	1	1		17	27
M6	100	0.1	0.2	0.3	0.5	0.5	Ö	7	22
M7	None	0.3		4	9	9	9	50	25



Day post infection

FIG. 2. Effect of repeated i.p. administration of (S)-HPMPA alone and (S)-HPMPA plus probenecid on *P. berghei* parasitemia in BALB/c mice. The experiment was conducted as described in Materials and Methods. Data are for nontreated infected mice (\blacktriangle), mice treated with (S)-HPMPA (15 mg kg⁻¹), (\blacksquare), and mice treated with (S)-HPMPA (15 mg kg⁻¹) and probenecid 0.285 g kg⁻¹) (\bullet). The course of infection in mice treated with (S)-cHPMPA (15 mg kg⁻¹), probenecid (0.285 g kg⁻¹), and (S)-cHPMPA plus probenecid did not differ from that in nontreated infected mice. Treatment of mice receiving (S)-HPMPA plus probenecid was stopped at day 18 postinfection. Bars indicate standard deviations. The mean day of death in mice treated with (S)-cHPMPA, probenecid, and (S)-cHPMPA plus probenecid and nontreated mice was day 8 postinfection (range, days 6 to 9). The mean days of death in mice treated with (S)-HPMPA and (S)-HPMPA plus probenecid were days 11 (all mice) and 24 (range, days 23 to 26) postinfection, respectively. All differences were highly significant (for all, P = 0.004 by the nonparametric Mann-Whitney test).

treatment with probenecid, 24.7 \pm 1.7 and 19.2 \pm 1.0 g, re-

	INONC	U)	1		مرد به 19			DU		
M8	None	Ľ	3	12	13	11	8	60	21	
M9	None	0.5	3	14	15	16			O	

" Experiment was conducted as described in Material and Methods.

^b Calculated as described in Material and Methods.

^e Day of death postinfection.

DISCUSSION

Repeated i.p. administration of the acyclic nucleoside phosphonate (S)-HPMPA, which strongly inhibits the growth of cultured *Plasmodium* parasites, induces a dose-related loss of body weight in noninfected mice. The acute loss of weight observed in all treated groups and the fast (10 and 20 mg kg^{-1}), biphasic (40 mg kg^{-1}), and slow (80 mg kg^{-1}) recovery of body weight afterward indicate that (S)-HPMPA induces acute as well as long-term adverse physiological effects. Histopathological examination of mice treated four times with 80 mg kg⁻¹ every third or fourth day demonstrated that (S)-HPMPA induced hepatotoxicity and severe nephrotoxicity. The degeneration of tubular epithelial cells in the kidneys must be the major cause of weight loss, because (S)-HPMPA-induced hepatotoxicity was less severe and the regeneration of liver cells could already be detected 10 days after the last day of treatment. Tubular malfunction impairs not only the reabsorption of glucose, amino acids, and ions but also that of water, causing an acute loss of weight, as was observed in the present study. Except for one death in the group of 10 mice treated four times with 80 mg kg⁻¹, no lethal toxicity of (S)-HPMPA was observed in noninfected mice. In contrast, mortality did occur in mice with minimal levels of parasitemia treated five times with a total of 75 mg kg⁻¹ (15 mg kg⁻¹ every other day (Fig. 2), while control (noninfected) mice, although also showing a considerable loss of weight, survived (data not shown). The deaths among the former group of mice should be attributed at least in part to (S)-HPMPA-induced nephrotoxicity rather than malaria, because inhibition of (S)-HPMPA uptake by correctment of infected mice with the tubular transport blocker probenecid prevented the observed early deaths (Fig. 2) and reduced the amount of weight loss. Lethality was also observed in mice, despite effective elimination of the parasite, which were exposed for 7 days to a sustained release of (S)-HPMPA (in order to kill all parasites as soon as they passed through schizogony) of a total of only 28 mg kg⁻¹ (Table 2). The repeated administration of acyclic nucleotides at short intervals compared with the administration of the same amount as a single dose elevates toxicity (15, 20a). Constant (or more extended) exposure of the renal tubular epithelium to acyclic nucleotide analogs, even when the dose is only modest, is apparently more toxic than a high-dose but short-term exposure. Surprisingly, however, the administration of probenecid,

spectively).

In the presence of probenecid treatment, the level of parasitemia again started to increase from day 9 postinfection, despite concomitant (S)-HPMPA treatment, while in mice treated with (S)-HPMPA alone, the level of parasitemia decreased until the mice died. Because the concomitant administration of probenecid elevates the levels of several drugs in plasma, these findings suggest that the decrease in the level of parasitemia following (S)-HPMPA treatment alone might partly be due to an indirect mechanism different from the inhibition of parasite replication (see Discussion).

Effect of sustained release of (S)-HPMPA. The ineffectivity of the regimen of (S)-HPMPA and probenecid combined (Fig. 2) indicates that (S)-HPMPA must have an extremely short half-life in plasma, because the initial level in plasma after administration was estimated to be 900 times the in vitro IC₅₀ for P. berghei. Therefore, we investigated whether constant low levels of (S)-HPMPA, administered via implanted osmotically driven pumps, in plasma could eradicate parasites from P. berghei-infected mice. The data in Table 2 indicate that the sustained release of (S)-HPMPA (total dose over 7 days, 14 mg kg⁻¹) resulting in an estimated level in plasma of 100 nM $(\pm 1.25 \text{ times the IC}_{50} \text{ for in vitro-cultured P. berghei parasites})$ suppresses parasite multiplication only temporarily. However, only a twofold increase in the constant level in plasma caused radical cure. Despite this effectiveness, mice did not survive but died 15 days, on average, after the start of treatment (Table 2). An additional experiment showed that a twofold reduction in the period of sustained release under otherwise identical conditions suppressed parasitemia only modestly and that an elevated (S)-HPMPA level of 400 nM in plasma for 3.5 days, although strongly suppressing parasitemia but not eradicating all parasites, caused fatal toxicity (data not shown).

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which reduces the nephrotoxicity of acyclic nucleoside phosphonates (see Results) (12, 14) and which is known to elevate the levels of several drugs in plasma (see the introduction), seems to have no additional inhibitory effect on malaria parasite growth. The less effective antiplasmodial efficacy of (S)-HPMPA and probenecid combined compared with that of (S)-HPMPA alone (Fig. 2) most likely does not result from a possible reduction in the antiplasmodial efficacy of (S)-HPMPA in the presence of probenecid, because in vitro experiments showed that although high concentrations of probenecid inhibit the growth of *P. falciparum*, neither synergistic nor antagonistic effects were observed with probenecid in combination with (S)-HPMPA (data not shown). Neither were such effects observed in *P. berghei*-infected mice during the first week of infection (Fig. 2). One may therefore question whether the near complete suppression of parasitemia seen in vivo from day 9 postinfection after (S)-HPMPA treatment (Fig. 2) is due to the direct inhibition of the parasite by (S)-HPMPA, particularly in view of the very short-term effective level of the drug in plasma (see below). The decrease in the level of parasitemia could be induced indirectly, e.g., as a result of disturbance of plasma homeostasis caused by (S)-HPMPAinduced nephrotoxicity. Blood smears of these mice indeed suggested an osmotic deviation, because nearly all erythrocytes were crenated. The cause of the sudden rise in the level of parasitemia seen from day 9 postinfection onward in mice treated simultaneously with (S)-HPMPA and probenecid (Fig. 2) remains to be elucidated. An infection-induced increase in the relative amount of reticulocytes, for which P. berghei parasites show a preference (11) and in which they multiply more efficiently, may play a role, but other explanations, including the selection of (S)-HPMPA-resistant parasites, which has been demonstrated in in vitro cultures of *P. falciparum* (22) cannot yet be excluded. The inability to eradicate P. berghei infections from mice by i.p. administration of (S)-HPMPA does not result from a lower level of susceptibility of P. berghei parasites to (S)-HPMPA, because previous in vitro studies showed that the susceptibilities of P. berghei and P. falciparum isolates to (S)-HPMPA are similar, which was also confirmed to be the case in vivo (see below). The relative ineffectiveness of repeated injections of large amounts of (S)-HPMPA against P. berghei blood stages in vivo is apparently due to its short half-life in plasma and the in insusceptibility of nonreplicative parasite stages to (S)-HPMPA (21). Indeed, preliminary results indicate that in rats, less than 50% of the intravenously administered (S)-HPMPA can be detected in plasma after 10 min (4). In contrast, a prolonged sustained (S)-HPMPA level of only 200 nM (2.5 times the in vitro IC_{50}) administered for 7 days eradicates all blood-stage forms but causes fatal toxicity. Toxicity could not be prevented by a twofold decrease in the administration period because a minimal level of more than 400 nM in plasma during such a period is needed to eradicate all parasites, and under those circumstances toxicity is fatal. Whether other regimens of sustained release of (S)-HPMPA, with or without concomitant probenecid administration, were effective against blood-stage forms and lacked toxicity was not further investigated. Such complex administration schedules, if successful, seem devoid of practical applicability, particularly in the case of treatment of *P. falciparum* infection, of which the nonreplicative (S)-HPMPA-insusceptible blood-stage forms (rings and trophozoites) take twice as long to develop as P. berghei blood-stage forms. Can the toxicity of (S)-HPMPA be reduced in another way without the loss of antimalarial activity? To answer this question, derivatives of (S)-HPMPA with a modified charge of the

phosphonyl group, which plays a pivotal role in the organic anion-dependent uptake by the tubular epithelium of the kidney, were examined for their antimalarial activities. Repeated i.p. administration of 15 mg of (S)-cHPMPA kg⁻¹, however, did not have any effect on the course of a P. berghei infection (see legend to Fig. 2), and different noncharged bis(pivaloyloxymethyl) derivatives (23) of (S)-HPMPA lack antimalarial activity in vitro (20). Recently, Cihlar et al. (7) described a protein-mediated and phosphate (phosphonate)-specific transport of the acyclic nucleotide analog 9-(2-phosphonylmethoxyethyl)adenine. If (S)-HPMPA uptake in malaria parasites also depends on the presence of the phosphonate group, attempts to circumvent anion-dependent nephrotoxicity by alteration or deletion of this group would also abolish the effectiveness of the drug's antiplasmodial activity. The prospect of applying (S)-HPMPA or closely related derivatives [e.g., (S)-3-deaza-HPMPA] as antimalarial agents against blood stages in vivo seems limited. Recent findings, however, point to a promising alternative application of these potent in vitro inhibitors as antimalarial agents. Short-term sustained release of a duration and level that caused no apparent toxicity during liver stage development of P. berghei prevented blood infections (19). Moreover, recent research showed that nucleoside analogs (18) and the antimalarial drug primaguine (5) can be selectively targeted toward the liver. We are developing a similarly efficient delivery method for (S)-HPMPA, allowing the use of even much lower doses. Such an approach opens the way to the application of acyclic nucleotide analogs like (S)-HPMPA as effective prophylactic drugs, even if they are administered on an infrequent basis since, in contrast to their short half-lives in plasma, the intracellular halflives of activated acyclic nucleoside phosphonates exceed many hours (1-3).

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