Anticancer activity of stabilized palifosfamide *in vivo*: schedule effects, oral bioavailability, and enhanced activity with docetaxel and doxorubicin

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Palifosfamide, the DNA-alkylating metabolite of ifosfamide (IFOS), has been synthesized as a stabilized tris or lysine salt and found to have preclinical and clinical antitumor activity. Stabilized palifosfamide overcomes limitations of IFOS because of patient-to-patient variability in response resulting from variable prodrug activation, resistance and toxicities of metabolic byproducts, acrolein and chloroacetaldehyde. Palifosfamide represents an effective alternative to IFOS and other DNA-alkylating prodrugs. The antitumor activities of stabilized palifosfamide were investigated *in vivo*. Dose response, route and schedule of administration, and interaction with docetaxel or doxorubicin were investigated in NCr-*nu/nu* mice bearing established orthotopic mammary MX-1 tumor xenografts. Oral activity was investigated in P388-1 leukemia in CD2F1 mice. Oral and intraperitoneal bioavailabilities were compared in Sprague–Dawley rats. Stabilized palifosfamide administered by optimized regimens suppressed MX-1 tumor growth ($P<0.05$) by greater than 80% with 17% complete antitumor responses and up to three-fold increase in time to three tumor doublings over controls. Median survival in the P388-1 ($P<0.001$) model was increased by 9 days over controls. Oral bioavailability in rats was 48–73% of parenteral administration, and antitumor activity in mice was equivalent by both routes. Treatment with palifosfamide-tris combined with docetaxel or doxorubicin at optimal regimens resulted in complete tumor regression in 62–75% of mice. These studies support investigation of stabilized palifosfamide in human cancers by parenteral or oral administration as a single agent and in combination with other approved drugs. The potential for clinical translation of the cooperative interaction of palifosfamide-tris with doxorubicin by intravenous administration is supported by results from a recent randomized Phase-II study in unresectable or metastatic soft-tissue sarcoma. *Anti-Cancer Drugs* 23:173–184 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Palifosfamide, which is also called isophosphoramide mustard (IPM), is the DNA alkylating metabolite of the anticancer agent ifosfamide (IFOS) [1]. The mechanism of action and antitumor activity of palifosfamide in comparison with IFOS, cyclophosphamide (CPA), and its alkylating metabolite, phosphoramide mustard, have been described previously [2,3]. The clinical rationale for palifosfamide as an anticancer agent has been recognized; however, its chemical instability has limited its pharmaceutical development. The stabilization of palifosfamide [4] has now enabled its advancement into the clinic [5,6].

IFOS and chemically related CPA are widely used cytotoxic anticancer drugs. Both are prodrugs and are activated by 4-hydroxylation by cytochrome P450 oxidases—typically, CYP2B6 and CYP3A4—in the liver to produce the bifunctional alkylating agents, IPM and phosphoramide mustard, which cross-link DNA at guanine N-7 to form covalent interstrand and intrastrand linkages that prevent DNA replication and cause cell death [7–9].

The therapeutic activity of IFOS and CPA is limited by the requirement for metabolic activation and by the production of metabolites with toxicities unrelated to DNA alkylation. Interpatient variability in clinical responses appears to result from genetic and environmental factors [10] that alter the activity of the cytochrome P450s required to metabolically activate the prodrugs [11,12]. Resistance to IFOS and CPA is thought to arise because of increased expression by tumors of aldehyde dehydrogenases that convert intermediates in the pathway of prodrug activation into inactive metabolites: carboxy-ifosfamide and carboxy-phosphamide in the metabolism of IFOS and CPA, respectively [1]. Toxic byproducts of the metabolism of the prodrugs include acrolein and chloroacetaldehyde [1]. Acrolein is produced during the conversion of IFOS and CPA into active mustards. Chloroacetaldehyde is produced in an alternative dechloroethylation reaction that can inactivate CPA and

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IFOS. Whereas CPA is almost completely metabolized by the pathway leading to the active mustard, it is estimated that 20–60% of IFOS undergoes dechloroethylation to produce chloroacetaldehyde *in vivo* [13–16]. Acrolein is responsible for hemorrhagic cystitis [17] and chloroacetaldehyde for nephrotoxicity and neurotoxicity [18].

In the USA, IFOS is approved for testicular cancer and is the standard of care for sarcoma, head and neck cancer, and some forms of non-Hodgkin's lymphoma. It is also sometimes used for small cell lung cancer, breast cancer, and prostate cancer. In the treatment of cancer with IFOS, hemorrhagic cystitis can be reduced by comedication with mercaptoethane sulfonate sodium (mesna), which is a thiol compound capable of detoxifying acrolein locally in the bladder [17]. However, no therapeutic intervention is available to prevent the renal toxicity caused by chloroacetaldehyde. As a result, the clinical efficacy of high-dose IFOS, which is often used in the refractory/resistant setting [19–21], is limited by the risk of renal failure resulting from tubular necrosis within days after administration [22].

The potential advantages of using the active metabolite palifosfamide itself as the cytotoxic agent in cancer in order to overcome the limitations imposed by the requirement for prodrug activation have been well recognized [2,23,24]; however, its pharmaceutical development was precluded because of its chemical instability. In an aqueous environment, palifosfamide is rapidly hydrolyzed and inactivated [23,25]. We have developed two salt forms of palifosfamide that have greater stability than the free acid and are suited for clinical development: palifosfamide formulated as a salt with lysine (palifosfamide-Lys) [26] or as a tris/mannitol salt (palifosfamide-tris) [27]. Both salt forms have similar activities when tested in mouse models and similar pharmacokinetics in humans. Previously, the free acid, palifosfamide, was shown to have broad antitumor activity against a panel of cancer cell lines and human tumor xenografts in mice [2], and the activity of stabilized palifosfamide was similar or greater in these models. Palifosfamide-Lys demonstrated antitumor activity against sarcoma cell lines *in vitro* and against human osteosarcoma and rhabdomyosarcoma tumor xenografts in mice *in vivo* [28]. Interestingly, palifosfamide-Lys has been shown to be active against CPA-resistant xenografts that overexpress aldehyde dehydrogenase 3A1 in mice, suggesting that stabilized palifosfamide might be effective in treatment of resistant or relapsed patients with sarcoma [28]. In Phase-I and Phase-II clinical studies, neither central nervous system toxicity nor hemorrhagic cystitis associated with IFOS treatment was observed following intravenous administration of palifosfamide-tris or palifosfamide-Lys [29].

The studies reported here have examined the effect of dose, schedule and route of administration of stabilized palifosfamide on tumor growth. Stabilized palifosfamide administered parenterally or orally to mice bearing established orthotopic breast tumors or inoculated with an aggressive leukemia cell line produced statistically significant antitumor activity. An initial pharmacokinetic study in rats indicated oral bioavailability in the range of 48–73% for palifosfamide-tris. When palifosfamide-tris was combined with docetaxel or doxorubicin in the treatment of mice bearing established human breast tumor xenografts, significantly ($P < 0.05$) more potent antitumor effects were observed than with the agents individually. Combination treatments also produced tumor regression in up to 75% of the mice and tumor-free survival in up to 38%, whereas the only single agent to produce tumor regression was docetaxel, which did so in 13% of the mice. Significant cooperative interactions could be obtained at dose levels of palifosfamide-tris and docetaxel or doxorubicin that were well tolerated in the mouse model. The results suggest that the drug combinations might be applied safely in the clinic. The feasibility of this approach appears to be borne out by the safety and efficacy of palifosfamide-tris in combination with doxorubicin in a randomized Phase-II study in patients with advanced soft-tissue sarcoma [5].

Materials and methods

Reagents

The active drug substance, IPM, is referred to as palifosfamide. Palifosfamide-tris is a stabilized form of palifosfamide complexed with tromethamine (tris), and the chemical name is *N*,*N*′-bis(2-chloroethyl) phosphorodiamidic acid tris(hydroxymethyl)amino methane salt. In palifosfamide-tris, the molar ratio of palifosfamide to tris is 1 : 1. For the preparation of the lysine salt, 2 molar equivalents of L-lysine were added to a saline solution (0.91% NaCl w/v) of palifosfamide (9 mg/ml). Palifosfamide-tris and palifosfamide-Lys were diluted in saline to obtain treatment dosage concentrations. Docetaxel (40 mg/ml in 100% Tween 80) was formulated in 5% ethanol/7.5% tween 80/87.5 D5W). Doxorubicin (Ben Venue Laboratories, Bedford, Ohio, USA) was formulated as a stock solution of 2 mg/ml in saline and diluted in saline to obtain treatment dosage concentrations.

Animals

Six-week-old female athymic (NCr-*nu*/*nu*) mice were purchased from Taconic Farms (Germantown, New York, USA), and 5–6-week-old male (BALB/c x DBA/2)F1 (CD2F1) mice, from Frederick Cancer Research and Development Center (Frederick, Maryland, USA). Mice were acclimatized in the laboratory for 1 week before experimentation. The animals were housed in microisolator cages, up to five per cage, on a 12-h light/dark cycle. The animals received filtered tap water and sterilizable rodent diet (TD8656; Harlan-Teklad, Madison, Wisconsin, USA) *ad libitum*. Mice were observed daily and clinical signs were noted. All experimental procedures were

approved by the Institutional Animal Care and Use Committee of Southern Research Institute.

MX-1 human mammary orthotopic tumor xenograft model

Thirty to forty milligram fragments of MX-1 human mammary tumors maintained in an in-vivo passage were implanted subcutaneously in NCr-*nu/nu* in the mammary fat pad using a 12-gauge trocar. Tumors were allowed to reach 75–245 mm^3 in size (range for each experiment is described in Results) before the start of treatment on day 0 when animals were assigned to control ($n = 10$) or drug-treatment groups ($n = 6$ for dose and schedule studies and $n = 8$ for drug combination studies as described in Results). The subcutaneous tumors were measured and the mice were weighed twice weekly. Tumor volume was determined by caliper measurements (mm) using the formula for an ellipsoid sphere: volume (mm^3) $= L \times W^2/2$, where L and W refer, respectively, to the longer and shorter perpendicular dimensions. Mice were euthanized when they became moribund or their tumors became ulcerated or reached 4000–5000 mm^3. Deaths unrelated to tumor burden, tumor regressions, number of tumor-free surviving mice, and time to reach three tumor-mass doublings in individual animals were determined. Death or euthanasia according to the above criteria defined the endpoint in survival studies.

P388-1 mouse leukemia model

The P388-1 cell line was maintained as an in-vivo passage. CD2F1 mice were injected intraperitoneally with 1×10^6 P388-1 cells on day 0. On day 1, mice were administered vehicle ($n = 10$) or varying doses of palifosfamide-Lys ($n = 8$/dose level) either by gavage or intraperitoneal injection. Mortality data were collected daily and any mouse that became moribund was euthanized.

Agent treatments

Agents were administered on the basis of exact body weight: 0.2 ml/10 g body weight for stabilized palifosfamide and 0.1 ml/10 g body weight for docetaxel and doxorubicin.

Rat pharmacokinetic study

Female Sprague–Dawley rats were dosed once by gavage or intravenous injection. Palifosfamide-tris was administered in 20, 30, and 40 mg/kg doses. Blood samples from the retro-orbital sinus were obtained from animals predose and at 0.5, 1, 2, 4, 6, 8, 12, and 24 h postdose. Samples were collected from three animals per dose level at each time point. Plasma was frozen at $-20^{\circ}C$ before organic extraction and assay of palifosfamide by gas chromatography with mass spectroscopy (reproducibility $> 85\%$, limit of quantification 0.5 μg/ml palifosfamide). Standard pharmacokinetic parameters for palifosfamide were calculated using WinNonlin Pro v.3.1 software (Pharsight, Sunnyvale, California, USA) and noncompartmental analysis for oral and intravenous administration.

Statistical analysis

The significance of differences between mean tumor volumes in control and experimental groups of mice was determine by unpaired, two-tailed Student's *t*-test. Significance of tumor-size differences was calculated at time points when at least 50% of control mice were surviving. The individual animal's time to reach three tumor-mass doublings for schedule studies and four tumor-mass doublings for the combination studies was used as an endpoint in a Student's *t*-test or life table analysis to compare growth data between groups. A stratified Kaplan–Meier estimation followed by the Mantel–Haenszel log-rank test was used to compare mouse survival between groups.

Results

Response of orthotopic MX-1 xenografts to single and multiple dose administration of palifosfamide-Lys

To determine whether dose splitting allowed a higher total dose to be better tolerated and elicit higher antitumor activity than the same dose in a single bolus, the effect of a single high-dose administration was compared with the same total dose administered in five split doses. MX-1 tumor fragments were implanted in mammary fat pads of NCr-*nu/nu* mice and palifosfamide-Lys treatment was started on day 9 when tumors had grown to 75–198 mm^3. A dose of 180 mg/kg administered as a single bolus injection (q1dx1) elicited antitumor responses but was toxic, resulting in the deaths of 30% of animals. Repeated administrations of 180 mg/kg were not tolerated (data not shown). The maximal tolerated dose (MTD) for a single administration was determined to be 120 mg/kg. However, repeated administrations of 120 mg/kg were toxic, whereas a single dose and multiple doses of 80 mg/kg were well tolerated but not effective (Table 1). To determine whether the same total dose of 180, 120 or 80 mg/kg administered at smaller doses by repeated injections was

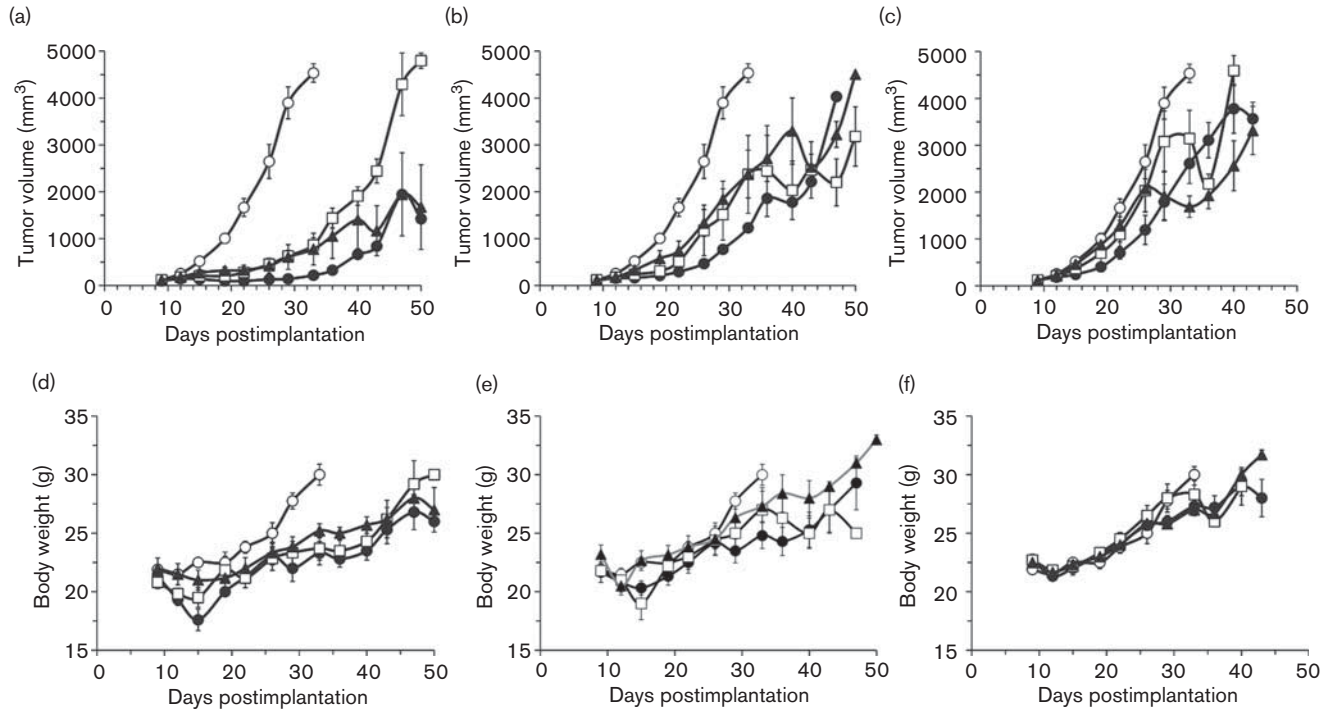
Table 1 **Dose and schedule-dependent antitumor effects of palifosfamide on MX-1 tumors in mice**

Total dose (mg/kg)	Split dose (mg/kg/day)	Schedule	TTD (days)	TGI (%)	CR	IMS (days)	TFS
0	–	–	10.1	0	0		0/10
180	–	q1dx1	35.8	97	2	23	1/6
	36	q1dx5	25	85	0	18	0/6
	36	q2dx5	33.4	89	2	25	0/6
120	–	q1dx1	24.7	87	1	18	1/6
	24	q1dx5	20	78	0	14	0/6
	24	q2dx5	15.8	55	0	11	0/6
80	–	q1dx1	17.2	61	0	11	0/6
	16	q1dx5	12.7	17	0	–	0/6
	16	q2dx5	11.5	61	0	–	0/6

CR, complete response; IMS, increase in median survival of test compared with control animals (survival is death or euthanasia according to criteria of the Institutional Animal Care and Use Committee of Southern Research Institute); TFS, tumor-free survival at end of study (day 57) expressed as a proportion of treated animals that survive; TGI, tumor growth inhibition expressed as percent of control [(Control – Test × 100)/Control] on day 29; TTD, time to three doublings in tumor size.

better tolerated than a single administration and still elicited measurable antitumor responses, mice were treated with a single high dose administration or with one-fifth of the high dose (36; 24 and 16 mg/kg, respectively) for five consecutive days from days 9 to 13 (q1dx5), or five times every other day between days 9 and 17 (q2dx5). A control group received vehicle q1dx5. In the control group, mean time to three doublings (TTD) was 10.1 days and only one out of 10 mice survived until day 36; therefore day 29 was the last time point at which tumor volume and body weight could be compared statistically between control and test mice. Palifosfamide-Lys inhibited tumor growth in a dose-dependent and schedule-dependent manner (Fig. 1a–c and Table 1). Compared with control, inhibition of tumor growth (TGI) was significant ($P < 0.05$) at single and cumulative doses of 180 mg/kg. For 180 mg/kg single administration, TGI was 97% and TTD was 35.8 days. Two mice had a complete response (CR) and were tumor free at study termination (day 57). For 36 mg/kg split doses administered for five consecutive days, TGI was 85% of control and TTD 25 days, and for administration for five times every other day, TGI was 89%, TTD was 33.4 days, and two mice had a CR lasting until study termination (Fig. 1a and Table 1). Similarly, TGI was significant ($P < 0.05$) at single and cumulative doses of 120 mg/kg: TGI was 87% and TTD was 24.7 days for a single dose with one CR and tumor-free survival at study termination; TGI was 78%, and TTD was 20 days for 24 mg/kg doses administered on five consecutive days; and TGI was 55% and TTD was 15.8 days for five times every other day (Fig. 1b and Table 1). At the dose of 80 mg/kg, significant TGI was only obtained with the single bolus administration: TTD was 17.2 days, and TGI was 61% (Fig. 1c and Table 1). Toxicity occurred in mice administered the highest palifosfamide-Lys dose of 180 mg/kg q1dx1 as indicated by early death on day 15 unrelated to tumor burden in two out of six mice. However, by splitting the 180 mg/kg dose into five doses administered q1dx5 or q2dx5, early death was avoided while increased survival of the mice was maintained (Fig. 1a). Palifosfamide-Lys administered at 180 mg/kg, either q1dx1 or q1dx5, and at 24 mg/kg, q1dx5, caused weight loss relative to baseline on day 9 that reached a nadir on day 15 of greater than 10% compared with control [Fig. 1d and e; ($P < 0.05$)]. When palifosfamide-Lys was administered by the q2dx5 schedule, no significant weight loss occurred at any of the dose levels on day 15 (Fig. 1d–f), whereas significant ($P < 0.05$)

Fig. 1



Effect of dose and administration schedule of palifosfamide-Lys on tumor growth and body weight in NCr-*nu/nu* mice xenografted orthotopically with MX-1 human mammary tumors. Palifosfamide-Lys at total cumulative doses of 180 (a and d), 120 (b and e), and 80 (c and f) mg/kg was administered to mice by q1dx1 (●), q1dx5 (□), or q2dx5 (▲) schedules. Vehicle (○) was administered q1dx5. In comparison to vehicle, palifosfamide-Lys produced significant ($P < 0.05$) reductions in tumor growth when administered q1dx1 at doses of 180 (a), 120 (b), or 80 (c) mg/kg, and q1dx5 or q2dx5 at doses of 36 (a) or 24 (b) mg/kg. Palifosfamide-Lys at doses of 180 mg/kg q1dx1 (d), and 36 (d) or 24 mg/kg (e) q1dx5, caused significant ($P < 0.05$) body-weight loss. Maximum weight losses relative to baseline on day 9 were observed on day 15, at which time, losses were greater than 10% with palifosfamide-Lys at 180 mg/kg q1dx1 (d), and with 24 mg, q1dx5 (e); but less than 10% with the other doses and dosing schedules.

antitumor activity continued to be obtained at the 180 mg/kg (Fig. 1a) and 120 mg/kg (Fig. 1b) total dose levels. Body weights were significantly lower in mice administered 180 or 120 mg/kg palifosfamide-Lys by all three schedules than in vehicle-treated mice on day 29 (Fig. 1d and e); however, it is possible that reduced tumor weight in the drug-treated versus control mice (Fig. 1a and b) accounted for some of these differences at the later time point.

Comparison of bioavailability of palifosfamide-tris administered by systemic or oral routes

Palifosfamide-tris at doses of 20, 30, and 40 mg/kg was administered in a single gavage or intravenous injection to female Sprague–Dawley rats. The pharmacokinetic parameters are shown in Table 2. Plasma drug–concentration/time profiles indicated a general linear trend of increasing palifosfamide plasma concentrations with increasing dose levels (Fig. 2). T_{max} occurred within 0.5 h when palifosfamide-tris was administered by either route of administration. However, 0.5 h was the first sampling time point, and possible differences in T_{max} occurring earlier could not be detected in this experiment. C_{max} and AUC tended to increase linearly with increasing dose, and estimates of $t_{1/2}$ for palifosfamide ranged from 1.0 to 1.5 h (Table 1). At each dose level, $AUC_{0-\infty}$ values were used to calculate bioequivalence of the oral dose relative to the intravenous dose, and these values indicated oral bioavailability of 48, 65, and 73% at the respective dose levels of palifosfamide-tris of 20, 30, and 40 mg/kg in female rats.

Comparison of antitumor effects of palifosfamide-tris administered by systemic and oral routes

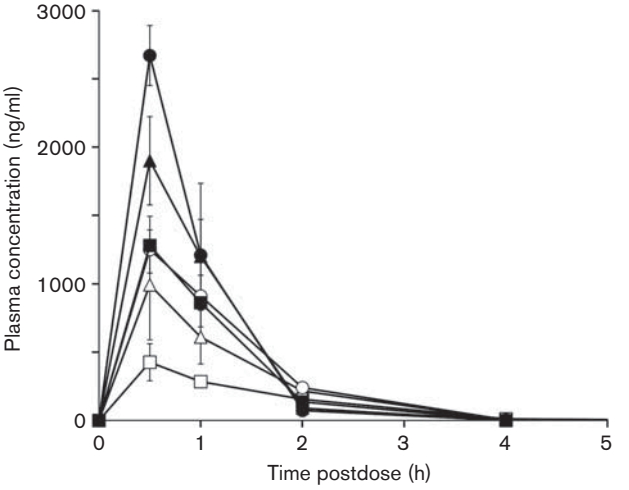
The efficacy of palifosfamide-tris administered by parenteral and oral routes was compared in NCr-*nu/nu* mice implanted with MX-1 tumors. Palifosfamide-tris was administered q1dx5 at doses up to and including the MTDs to mice bearing 144–197 mm^3 orthotopic MX-1 tumors on day 10 after tumor implantation. At doses of 36 or 24 mg/kg/day administered intraperitoneally or doses of 120 or 81 mg/kg/day administered by gavage, palifosfamide-tris produced significant reductions in tumor size compared with control treatment (Fig. 3a).

Table 2 Pharmacokinetic parameters of palifosfamide-tris following a single administration intravenously or by gavage to female Sprague–Dawley rats

	Dose (mg/kg)					
	20		30		40	
Parameter	Intravenous	Oral	Intravenous	Oral	Intravenous	Oral
C_{max} (ng/ml)	1280	426	1900	992	2670	1250
AUC_{0-t} (ng.h/ml)	1354	670	1987	1281	2279	1672
$AUC_{0-\infty}$ (ng.h/ml)	1427	681	1987	1283	2305	1674
$t_{1/2}$ (h)	1.25	1.25	1.50	1.25	1.00	1.25

$AUC_{0-\infty}$, area under the plasma concentration–time curve extrapolated to infinity; AUC_{0-t}, area under the plasma concentration–time curve to the last measurable concentration during the sampling interval; C_{max}, maximum observed plasma concentration; $t_{1/2}$, plasma elimination half-life.

Fig. 2



Mean plasma concentration (±SE) versus time profiles for palifosfamide for female Sprague–Dawley rats following a single oral dose of 20 (□), 30 (△), or 40 (○) mg/kg or a single intravenous dose of 20 (■), 30 (▲), or 40 (●) mg/kg.

There was no statistically significant difference between the antitumor effects of palifosfamide-tris obtained at the individual dose levels, and no significant difference was apparent between activities obtained with oral or parenteral administration in this experiment.

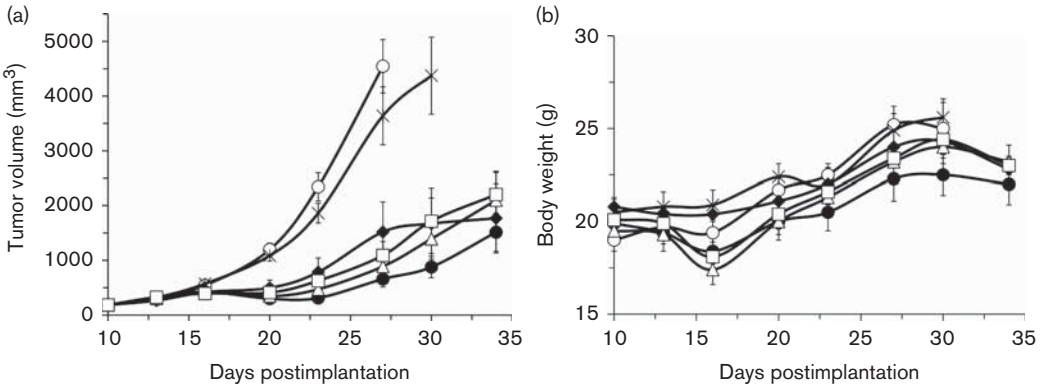
Relative to baseline body weight, 120 and 81 mg/kg palifosfamide-tris administered by gavage caused significant loss of body weight on day 16; however, weight was completely regained by day 20 (Fig. 3b). Neither of the doses of palifosfamide-tris administered intraperitoneally caused significant change in body weight.

Effects on survival in syngeneic P388-1 leukemia of continuous oral administration of palifosfamide-Lys

Male CD2F1 mice were injected intraperitoneally with P388-1 leukemia cells on day 0. Starting on day 1, treatment groups were administered palifosfamide-Lys by gavage at 280 mg/kg/day, q1dx1, q1dx5, or for 10 days (q1dx10). Control mice received vehicle q1dx10. Administration of a single dose of palifosfamide-Lys increased median survival by 1 day compared with vehicle treatment, whereas continuous daily administration for 5 and 10 days increased median survival by 6 and 9 days, respectively, compared with control (Fig. 4a $P < 0.05$). The progressively increased survival obtained by increasing the duration of administration of palifosfamide-Lys occurred in a significant manner: 10-day duration more than 5-day duration, $P < 0.005$; and 5-day duration more than 1-day duration, $P < 0.005$.

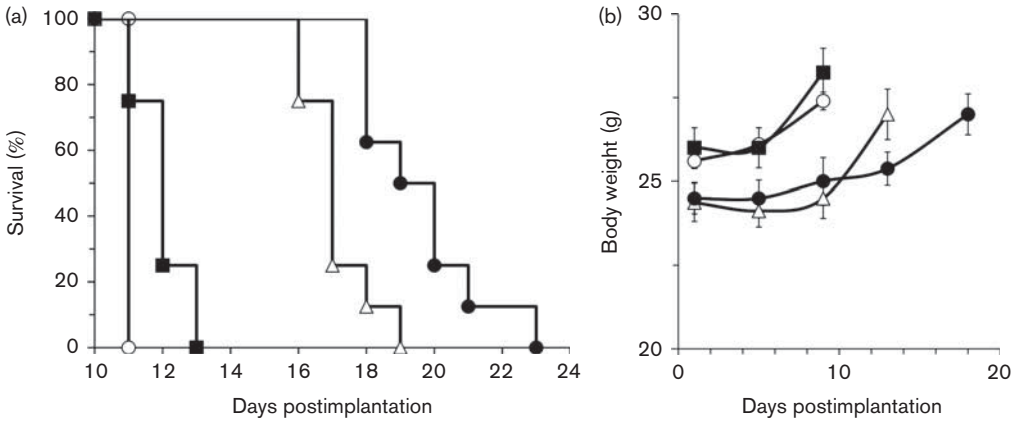
Palifosfamide-Lys was well tolerated at the 280 mg/kg/day level when administered orally by each of the three regimens tested. No early deaths unrelated to tumor burden were observed (Fig. 4a), and no significant losses

Fig. 3



Comparison of antitumor activity of palifosfamide-tris administered by oral and parenteral routes in NCr-*nu/nu* mice xenografted orthotopically with MX-1 human mammary tumors. Palifosfamide-tris was administered q1d x5 from days 10 to 14 after tumor implantation as follows: 36 mg/kg/dose intraperitoneally (●), 24 mg/kg/dose intraperitoneally (◆), 120 mg/kg/dose orally (△), and 81 mg/kg/dose orally (□). Control mice received vehicle q1dx5 intraperitoneally (○) or orally (×). Comparison of tumor size between treatment and control groups indicated that all palifosfamide-tris treatments produced significant antitumor effects on days 20 ($P<0.0005$), 23 ($P<0.0005$), 27 ($P<0.005$), and 30 ($P<0.05$) (a). Palifosfamide-tris administered orally at 120 and 81 mg/kg doses caused significant body-weight loss of 17 and 13%, respectively, on day 16 compared with baseline, whereas intraperitoneal administration at either dose had no significant effect on body weight (b).

Fig. 4



Effect of palifosfamide-Lys orally on survival of mice transplanted with P388-1 mouse leukemia cells. CD2F1 mice were injected intraperitoneally with 1×10^6 P388-1 cells on day 0. Palifosfamide-Lys was administered orally at a dose of 280 mg/kg/day q1dx1 (■), q1dx5 (△), or q1dx10 (●), starting on day 1. Control mice received vehicle orally q1d x10 (○). Mouse survival (a) was significantly ($P<0.05$) increased by palifosfamide-Lys administered by all regimens in comparison to vehicle treatment. No significant loss of body weight (b) relative to baseline on day 1 occurred when palifosfamide-Lys was administered at 280 mg/kg/day by any of the dosing regimens.

in body weight occurred relative to the weight at the start of dosing on day 1 (Fig. 4b). The results indicate that continuous oral administration of stabilized palifosfamide at doses that are well tolerated could translate into an effective clinical treatment for cancers and be developed as a maintenance therapy.

Response of orthotopic MX-1 xenografts to treatment with palifosfamide-tris in combination with docetaxel

NCr-*nu/nu* mice bearing 100–245 mm^3 MX-1 mammary tumors on day 10 after tumor implantation were administered palifosfamide-tris or its vehicle intraperitoneally, q1dx5, at 54, 24, or 12 mg/kg/day. Docetaxel or its vehicle was administered intravenously every sixth day for three cycles (q6dx3) at 10, 5, and 2.5 mg/kg/cycle. For combination treatments, palifosfamide-tris at each dose level was combined with docetaxel at each dose level using the respective regimens. Control mice received saline q1dx5 and the docetaxel vehicle q6dx3.

In comparison to single-agent treatment, the combination of 54 mg/kg/day palifosfamide-tris and 10 mg/kg/cycle

Table 3 Antitumor effects of palifosfamide-tris in combination with docetaxel or doxorubicin in mice

	Chemotherapy (mg/kg/cycle)					
Palifosfamide (mg/kg/day)	Docetaxel	Doxorubicin	TFD	Day 31 tumor volume (mm^3)	TGI	CR
0	0	0	14.3	4868		
54	0	0	33.4	728	86	0
24	0	0	16.2	4131	19	0
12	0	0	14.5	3888	20	0
0	10	0	32.6	427	92	1
0	5	0	16.7	4363	11	0
0	2.5	0	15.1	4800	2	0
54	10	0	70	0	100	6
54	5	0	27.6	1825	63	0
54	2.5	0	26.6	1024	78	0
24	10	0	55.2	72	98	4
24	5	0	20.6	3309	33	0
24	2.5	0	13.6	4536	7	0
12	10	0	25.8	1717	65	0
12	5	0	16.7	4050	17	0
12	2.5	0	17.4	3035	28	0
0	0	8	23.5	3002	39	0
0	0	5.3	17	3726	24	0
0	0	3.5	14.1	4050	17	0
54	0	8	64	20	100	5
54	0	5.3	45	270	95	3
54	0	3.5	32	878	82	0
24	0	8	45	294	94	0
24	0	5.3	24	2162	56	0
24	0	3.5	20	3524	28	0
12	0	8	53	219	96	4
12	0	5.3	22	3111	37	0
12	0	3.5	20	4212	14	0

CR, complete response; TFD, Time to four tumor doublings; and as defined in Table 1; TGI, inhibition of tumor growth.

docetaxel exhibited significantly ($P < 0.05$) greater antitumor activity than either agent alone as indicated by TGI (Table 3 and Fig. 5a). Only three of ten control mice survived to day 34; therefore, day 31 was the last day at which tumor volume and body weight could be compared statistically between control and test groups. On day 31, tumors in combination-treated mice were undetectable with TGI of 100% as compared with 86 and 92% with single agent treatment of palifosfamide or docetaxel, respectively. Mammary tumor mass in five of the six surviving mice regressed to an undetectable level by day 24, and three of the mice remained tumor free until study termination on day 81. In contrast, 54 mg/kg/day palifosfamide-tris as a single agent did not produce tumor regression, and 10 mg/kg/cycle docetaxel alone only caused regression in one of eight mice by day 20 with mammary tumor regrowth detectable on day 45. Palifosfamide-tris at 54 mg/kg/day in combination with docetaxel at 10 mg/kg/cycle appeared to exhibit some toxicity as indicated by early death unrelated to tumor burden in two out of eight mice; nevertheless it resulted in significantly ($P < 0.05$) increased median survival compared with either agent alone.

When the dose of palifosfamide-tris was reduced to 24 mg/kg/day, it produced no statistically significant effect on tumor growth (Fig. 5e), and in mice treated with 24 mg/kg/day palifosfamide-tris combined with 10 mg/kg/cycle docetaxel, tumor growth was not inhibited significantly more than by docetaxel alone (Fig. 5e). However, median survival was increased by 24 days in mice treated with the combination over that in mice treated with docetaxel alone ($P < 0.05$). At 5 mg/kg/cycle, docetaxel did not have a statistically significant antitumor effect by itself, and, in combination with palifosfamide-tris at this dose level, docetaxel did not increase antitumor activity over that obtained with palifosfamide-tris by itself at either 54 or 24 mg/kg/day (Fig. 5c and g). Similarly, at 2.5 mg/kg/cycle, docetaxel had no antitumor activity by itself and did not enhance the antitumor activity of palifosfamide-tris. At the lowest dose tested, 12 mg/kg/day, palifosfamide-tris was inactive, either as single agent or with docetaxel at 10 mg/kg/cycle (Table 3).The combination of 24 mg/kg/day palifosfamide-tris and 10 mg/kg/cycle docetaxel also increased the regression of mammary tumors compared with the respective agents alone, but without the early mortality observed with 54 mg/kg/day palifosfamide-tris. In four out of eight mice, tumor mass declined to an undetectable level by day 31 (Table 3). Mammary tumor regrowth occurred in two mice by day 48 and in one mouse by day 69, resulting in one mouse remaining apparently tumor-free at study termination.

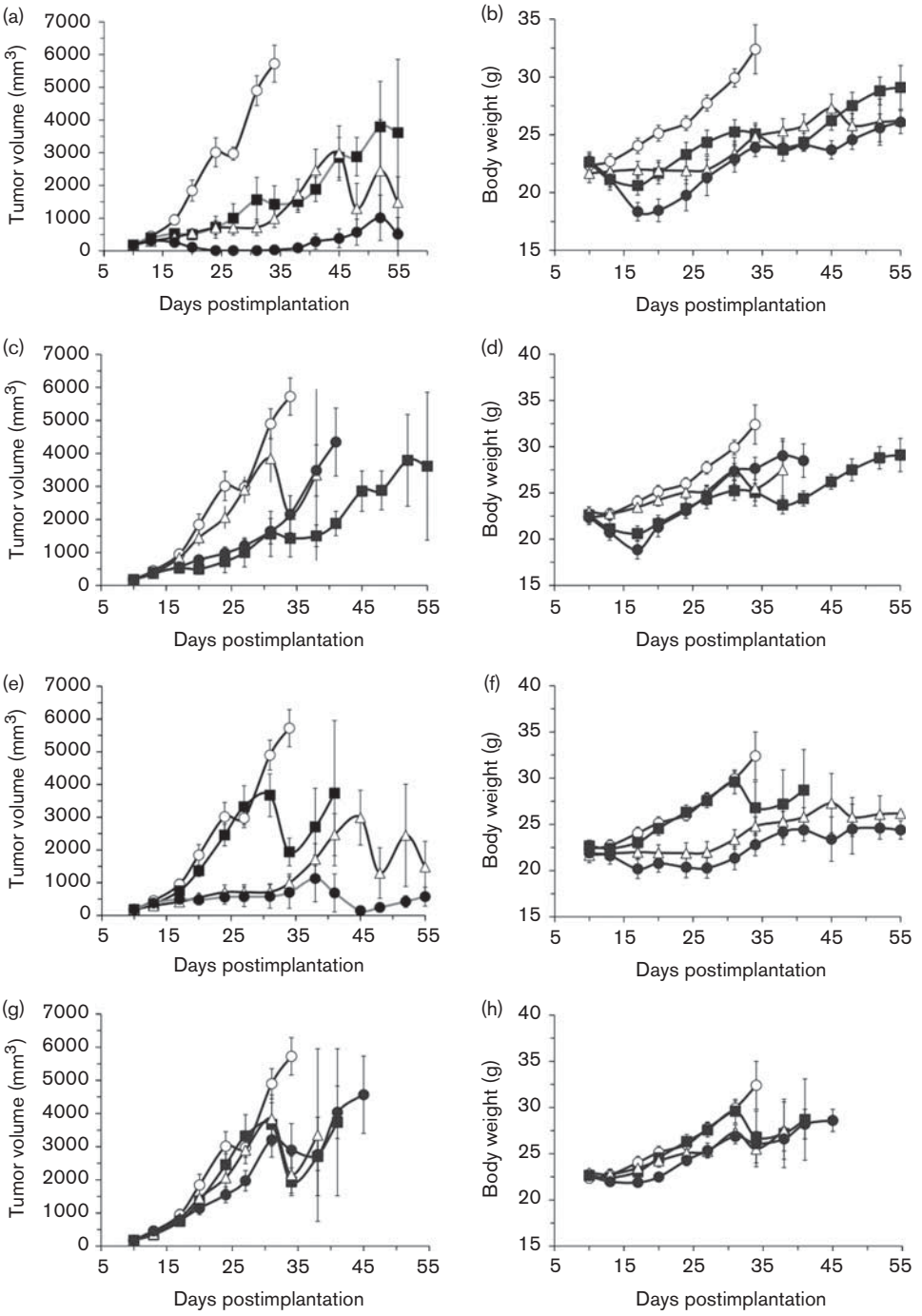
Treatment of mice with 54 or 24 mg/kg/day palifosfamide-tris as a single agent did not cause significant weight loss compared with baseline at day 9 (Fig. 5b and f). The combination of 54 mg/kg/day palifosfamide-tris with 10 or 5 mg/kg/cycle docetaxel produced maximum weight losses that were significant ($P < 0.05$) and exceeded 10% (Fig. 5a and d); however, after a nadir in body weight between days 15 and 20, weight was progressively regained. Mice treated with 24 mg/kg/day palifosfamide-tris combined with 10 mg/kg/cycle docetaxel or with this dose of docetaxel alone did not exhibit significant weight loss relative to baseline at day 9; but they did not gain weight over the course of the experiment (Fig. 5f). Mice treated with 24 mg/kg/day palifosfamide-tris and 5 mg/kg/cycle docetaxel, either individually or in combination, did not exhibit significant weight loss compared with controls (Fig. 5g and h).

Response of orthotopic MX-1 xenografts to treatment with palifosfamide-tris in combination with doxorubicin

The antitumor effects of 54, 24, or 12 mg/kg/day palifosfamide-tris combined with doxorubicin were investigated in the same study as that described above for the combination with docetaxel. In place of docetaxel, doxorubicin or its vehicle was administered intravenously every fourth day for three cycles (q4dx3) at 8, 5.3, and 3.5 mg/kg/cycle.

Treatment with the combination of 54 mg/kg/day palifosfamide-tris with either 8 or 5.3 mg/kg/cycle doxorubicin reduced tumor growth to a significantly ($P < 0.05$) greater extent than the agents did individually at the

Fig. 5

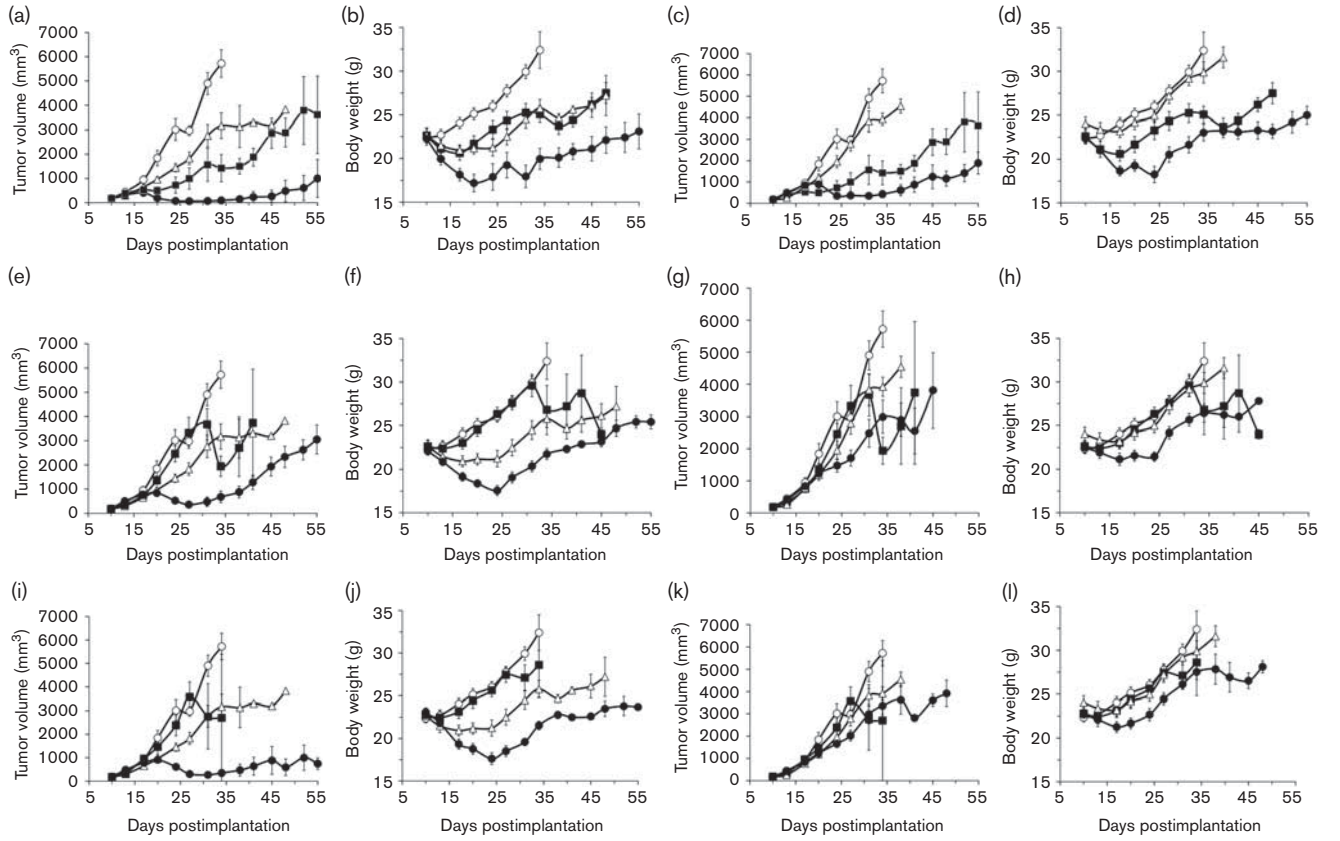


Antitumor effects of palifosfamide-tris and docetaxel in combination in NCr-*nu/nu* mice xenografted orthotopically with MX-1 human mammary tumors. Starting on day 10 after tumor implantation in mammary fat pads, palifosfamide-tris was administered intraperitoneally q1dx5 at 54 mg/kg/day (a–d) or 24 mg/kg/day (e–h). Docetaxel was administered intravenously q6dx3 at 10 mg/kg/cycle (a, b, e, and f) or 5 mg/kg/cycle (c, d, g, and h). Control mice were administered vehicles by the same regimens. Each dosage of palifosfamide-tris was combined with docetaxel, and each agent was administered individually. Mean tumor volumes and mean mouse body weights are shown for control (○), palifosfamide-tris (■), docetaxel (△), and palifosfamide-tris combined with docetaxel (●). In comparison to either single agent treatment, 54 mg/kg/day palifosfamide-tris with 10 mg/kg/cycle docetaxel produced a significantly ($P<0.05$) greater reduction in tumor growth (a). Compared with docetaxel alone, the combination of 24 mg/kg/dose palifosfamide-tris with 10 mg/kg/cycle did not produce significantly greater tumor inhibition (e). Palifosfamide-tris at 54 mg/kg/day with 5 mg/kg/cycle docetaxel had no significantly greater antitumor effects than palifosfamide-tris by itself (c). Palifosfamide-tris at 24 mg/kg/day with 5 mg/kg/cycle docetaxel produced no significant antitumor effects compared with control (g) when compared on day 31. Maximum body weight losses relative to baseline on day 10 were observed on day 17, but these only reached significance ($P<0.05$) for 54 mg/kg/day palifosfamide-tris combined with 10 mg/kg/cycle docetaxel (b; 19% loss) or combined with 5 mg/kg/cycle docetaxel (d; 16% loss). Relative to controls, mice treated with 10 mg/kg/cycle docetaxel alone or in combination with 24 mg/kg/day palifosfamide-tris failed to gain weight (f, $P<0.05$). 24 mg/kg/day palifosfamide-tris and 5 mg/kg/cycle docetaxel did not significantly affect body weight, either individually or in combination (h).

corresponding dose levels (Fig. 6a and c). In mice treated with the combination of 54 mg/kg/day palifosfamide and 8 mg/kg/cycle doxorubicin, TGI was 100% compared with 86 and 39% for single agent palifosfamide or doxorubicin, respectively (Fig. 6a and Table 3). Using 5.3 mg/kg/cycle doxorubicin, tumors in combination-treated mice were inhibited by 95% relative to control versus 86% in palifosfamide-tris-treated mice, and 24% in doxorubicin-treated mice. Combination treatment at these dose levels caused toxicity as indicated by early death unrelated to tumor burden in one out of eight mice and significant body weight loss of greater than 10% relative to baseline at day 10 (Fig. 6b and d and Table 3). At termination of the experiment on day 81, complete tumor regression was observed in two out of eight mice and one out of eight mice treated with 54 mg/kg/day palifosfamide combined with, respectively, 8 and 5.3 mg/kg/cycle doxorubicin, whereas regression did not occur in mice treated with the agents individually. The combination of 54 mg/kg/day palifosfamide-tris with 3.5 mg/kg/cycle doxorubicin did not achieve antitumor effects that were significantly greater than those of palifosfamide-tris alone (Table 3).

Palifosfamide-tris as a single agent had no significant effect on tumor growth at dose levels of 24 or 12 mg/kg/day (Fig. 6e and i and Table 3). However, palifosfamide-tris

Fig. 6



Antitumor effects of palifosfamide-tris and doxorubicin in combination in NCr-*nu/nu* mice xenografted orthotopically with MX-1 human mammary tumors. In the same experiment as that of Fig. 5, palifosfamide-tris and doxorubicin were administered in combination or individually, and the appropriate vehicles served as controls. Palifosfamide-tris was administered at 54 mg/kg/day (a–d), 24 mg/kg/day (e–h), or 12 mg/kg/day (i–l). Doxorubicin was administered intravenously q4dx3 at 8 mg/kg/cycle (a, b, e, f, i, and j) or 5.3 mg/kg/cycle (c, d, g, h, k, and l). Mean tumor volumes and mouse body weights are shown for control (○), palifosfamide-tris (■), doxorubicin (△), and palifosfamide-tris combined with doxorubicin (●) (b, d, f, h, j, and l). In comparison to either single-agent treatment, 54, 24 or 12 mg/kg/day doses of palifosfamide-tris combined with 8 mg/kg/cycle doxorubicin produced significantly ($P<0.05$) greater reductions in tumor growth (a, e, and i). Palifosfamide-tris of 54 mg/kg/day combined with 5.3 mg/kg/cycle doxorubicin reduced tumor growth to a significantly ($P<0.05$) greater extent than single agent treatment (c). Compared with single agents, 24 mg/kg/day palifosfamide-tris combined with 5.3 mg/kg/cycle doxorubicin reduced tumor growth to a significantly ($P<0.05$) greater extent until day 34 (g). Palifosfamide-tris of 12 mg/kg/day with 5.3 mg/kg/cycle doxorubicin produced no significant antitumor effect compared with control (k). Maximum weight losses relative to baseline on day 10 were observed on days 17, 20, or 24. Significant ($P<0.05$) maximum weight losses resulted from treatment with 54 mg/kg/day palifosfamide-tris combined with 8 mg/kg/cycle (b; 23%) or 5.3 mg/kg/cycle (d; 19%) doxorubicin, 24 mg/kg/day palifosfamide-tris combined with 8 mg/kg/cycle doxorubicin (f; 20%), and 12 mg/kg/day palifosfamide-tris combined with 8 mg/kg/cycle doxorubicin (j; 24%). Single agent treatments and combinations at other dose levels did not cause significant weight loss relative to baseline on day 10.

combined at either of these dose levels with 8 mg/kg/cycle doxorubicin produced significantly ($P < 0.005$) greater reductions of tumor growth than doxorubicin by itself. Day 31 tumors were 10-fold smaller in combination-treated mice using 24 mg/kg/day palifosfamide-tris than in doxorubicin-treated mice (Fig. 6e and Table 3), and 13-fold smaller using 12 mg/kg/day palifosfamide-tris (Fig. 6i and Table 3). At these dose levels, the combination did not cause early mortality; but, unlike the agents individually at the respective doses, the combination did cause significant maximum loss of body weight of greater than 10% relative to baseline (Fig. 6f and j). The combination of 12 mg/kg/day palifosfamide-tris with 8 mg/kg/cycle doxorubicin resulted in TGI of 96% and a loss of detectable mammary tumor mass in four of the eight mice treated by day 45. However, mammary tumor regrowth occurred in the regressor mice between days 48 and 55. The combination of 24 or 12 mg/kg/day palifosfamide-tris with 5.3 mg/kg/cycle doxorubicin produced significant antitumor effects compared with vehicle treatment (Fig. 6g and k and Table 3); but only the combination using 24 mg/kg/day palifosfamide-tris reduced tumor growth significantly ($P < 0.05$) more than doxorubicin by itself; however, the effect was small – a 1.5-fold reduction in tumor size on day 31 in combination-treated versus doxorubicin-treated mice. Combination treatment at these dose levels did not cause significant loss of body weight relative to baseline (Fig. 6h and l and Table 3). Combination of palifosfamide-tris with 3.5 mg/kg/cycle doxorubicin produced no significant antitumor effects compared with vehicle treatment, regardless of the dose level of palifosfamide-tris used (Table 3).

As noted above, in these experiments, palifosfamide-tris by itself at the dose level of the 54 mg/kg/day produced significantly greater antitumor effects ($P < 0.05$) than the 24 or 12 mg/kg/day. However, in combination with 8 mg/kg/cycle doxorubicin, all three doses of palifosfamide-tris produced very similar and statistically significant TGI and increases in survival compared with controls. The tumor growth inhibitions produced by combining 8 mg/kg/cycle doxorubicin with 54, 24, or 12 mg/kg/day palifosfamide-tris were statistically indistinguishable (Fig. 6a, e and i and Table 3). As observed in the combination of palifosfamide-tris and 10 mg/kg/cycle docetaxel, reducing the dose of palifosfamide-tris used in combination with 8 mg/kg/cycle doxorubicin decreased toxicity while maintaining efficacy. In contrast, when the dose of doxorubicin was reduced to 5.3 mg/kg/cycle, only the combination with palifosfamide-tris at 54 mg/kg/day produced a significantly greater antitumor effect than palifosfamide-tris alone (Fig. 6c and Table 3). Despite body-weight losses exceeding 10% in mice treated with palifosfamide-tris and doxorubicin combined at dose levels required for significantly enhanced efficacy compared with single-agent activity, body weight was recovered during the course of treatment (Fig. 6b, f and j).

Discussion

Stabilization of palifosfamide [4] has made it possible to explore its clinical potential [5,6]. In a series of preclinical studies, stabilized palifosfamide was shown to suppress the growth of human and mouse tumors in mice [4,26,27,30] and human sarcoma cells *in vitro* [28]. In the present studies, stabilized palifosfamide suppressed the growth of established orthotopic xenografts of human MX-1 breast carcinoma. Palifosfamide also increased survival in the P388-1 mouse leukemia model. In combination with docetaxel or doxorubicin, palifosfamide produced enhanced antitumor effects compared with the agents used individually in the MX-1 model.

In the MX-1 tumor model, toxicity of high-dose (180 mg/kg) palifosfamide could be avoided by splitting the total dose into multiple daily doses. When administered by well-tolerated multidose schedules, palifosfamide at the total dose of 180 mg/kg suppressed tumor growth by 89% compared with 97% when administered as single bolus. A lower total dose (120 mg/kg) of palifosfamide was shown to have equivalent efficacy whether administered as a single bolus or as multiple split doses, although repeated bolus doses of 120 mg/kg were toxic. Pharmacokinetics of palifosfamide-tris indicated oral bioavailability of 48–73% in female rats. In MX-1 tumor-bearing mice, the antitumor activities of a parenteral MTD dose of 24 mg/kg and an oral MTD dose of 81 mg/kg of palifosfamide-tris were statistically equivalent. The preclinical efficacy data indicate that stabilized palifosfamide is suitable for use in multiple dose regimens administered either parenterally or orally with low toxicity.

In the P388-1 leukemia model in CD2F1 mice, palifosfamide-Lys significantly increased survival of mice in a schedule-dependent manner. P388-1 tumor progression is rapid, resulting in 100% mortality by day 11 after tumor inoculation. Compared with the NCr-*nu/nu* immunodeficient mice bearing MX-1 xenografts, CD2F1 mice were relatively resistant to palifosfamide toxicity, and oral administration of multiple doses of palifosfamide-Lys at a dose level of 280 mg/kg was well tolerated. Increasing the duration of daily administration at this dose level from 1 to 10 days was shown to progressively prolong mouse survival. The significant antitumor activity of orally administered palifosfamide in the P388-1 model reported here extends earlier results demonstrating activity by parenteral administration against a CPA resistant subline of P338 leukemia [2].

Chemotherapeutic agents are usually administered at high doses in cycles interspersed with breaks. Continuous administration of chemotherapeutic agents (metronomic chemotherapy) has recently been recognized as a potentially safer and more effective clinical treatment, due to lengthened exposure of cancer cells to drug, than high-dose cyclical treatment [31,32]. The ability of stabilized palifosfamide to suppress tumor growth when

administered as multiple low doses and its oral bioavailability suggests its suitability for periods of administration required in metronomic regimens, possibly in combination with other anticancer drugs. One potential combination, which is currently being tested clinically, is with doxorubicin [5,6]; an agent which intercalates with DNA [33] to inhibit DNA replication and transcription by interference with topoisomerase II [34,35]. As demonstrated here, palifosfamide at doses that are suboptimal when the agent is used alone can produce significant cooperative antitumor effects in combination with doxorubicin or docetaxel in the MX-1 breast carcinoma xenograft model. These preclinical results support the ongoing clinical development of palifosfamide-tris. The significantly enhanced antitumor activity obtained by administration of suboptimal doses of palifosfamide-tris in combination with doxorubicin provides a strong rationale for the current clinical study of palifosfamide-tris in combination with doxorubicin and future clinical investigation in regimens incorporating other anticancer agents.

In the 1980s and 1990s, treatment with IFOS, either as a single agent or in combination with other agents, was refined for sarcoma [36–39]. Results of these studies led to the current combination of IFOS with doxorubicin as a regimen in metastatic sarcoma [21]. Although this regimen produces significantly higher objective responses, it does not translate into improved overall survival of patients because of high toxicity [40–42]. IFOS has excellent bioavailability when administered orally; but the occurrence of encephalopathy and nephrotoxicity because of its metabolite, chloroacetaldehyde, limits its clinical usefulness [13,14,18]. Recently reported results of a randomized Phase-II trial evaluating the combination of palifosfamide-tris and doxorubicin in patients with unresectable or metastatic soft tissue sarcoma in the front-line and second-line setting indicate that the preclinical activity of palifosfamide-tris in combination with doxorubicin will successfully translate to the clinic and afford therapeutic benefit for treatment of sarcoma [5]. The combination of palifosfamide-tris with doxorubicin in patients with sarcoma appeared to be well tolerated and showed significant increase in the time to progression compared with the effect of doxorubicin alone [5,6].

Palifosfamide is a nitrogen mustard belonging to the same class of DNA cross-linking agents as classical alkylating agents, such as bendamustin, IFOS, and CPA, and alkylating-like platinums, such as cisplatin and satraplatin [43]. On the basis of its broad antitumor activity preclinically, *in vivo* in mice and *in vitro* against tumor cell lines [4,26–28,30], and the clinical activity observed to date in sarcoma [5,6], palifosfamide-tris is a candidate for clinical development as an improved treatment for other cancers responsive to DNA-alkylating agents. In particular, the oral activity of palifosfamide-tris demonstrated in the current preclinical studies together with the improved safety compared with IFOS positions palifosfamide-tris as a particularly versatile anticancer agent.

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Conflicts of interest

There are no conflicts of interest.

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