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ORIGINAL ARTICLE

Preclinical activity of palifosfamide lysine (ZIO-201) in pediatric sarcomas including oxazaphosphorine-resistant osteosarcoma

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Abstract
Purpose Oxazaphosphorines, such as ifosfamide (IFA), are frequently used in the treatment of pediatric sarcomas. They are pro-drugs and undergo hepatic metabolism into the active moiety and potentially toxic by-products such as acrolein and chloracetaldehyde, which may cause hemorrhagic cystitis and encephalopathy, respectively. In addition, resistance to oxazaphosphorines can be mediated by overexpression of enzymes involved in their catabolism. Isophosphoramide mustard (IPM, palifosfamide) is the active moiety of IFA. In the current study, the activity of palifosfamide lysine (ZIO-201), a stable form of palifosfamide, was evaluated in a panel of sarcoma cell lines and tumor xenografts including oxazaphosphorine-resistant xenografts.
Methods The cytotoxic effect of palifosfamide lysine was studied in osteosarcoma (OS), Ewing's sarcoma (ES) and rhabdomyosarcoma (RMS) cell lines using the MTT assay. In vivo, the maximum tolerated dose (MTD) of palifosfamide lysine was determined in SCID mice based on a 3-day intravenous (IV) administration schedule. The effect on tumor growth and event-free survival was assessed at the MTD in all three sarcoma xenografts. In OS, cyclophosphamide (CPA)-resistant and -sensitive xenografts (OS31 and OS33, respectively) were evaluated for palifosfamide lysine activity. ALDH1A1 and ALDH3A1 gene expression data for the OS xenografts were mined from the Pediatric Preclinical Testing Program gene expression data. ALDH3A1 enzyme levels were compared between the CPA-resistant and -sensitive xenografts.
Results Palifosfamide lysine was cytotoxic against all the cell lines tested with the IC_{50} ranging from 0.5 to 1.5 μg/ml except for OS222, which had an IC_{50} of 7 μg/ml. The IV MTD of palifosfamide lysine in mice was 100 mg/kg per day for three consecutive days. Tumor growth inhibition was seen in both OS31 and OS33 xenografts and the RMS xenograft resulting in a significant difference in event-free survival between the control and the treated groups. Differential gene expression of ALDH3A1 but not ALDH1A1 was noted in the OS31 xenograft. This was confirmed by RT-PCR and the ALDH3A1 enzyme assay. ALDH3A1 enzyme activity was measured at 100 mIU/mg of protein in OS31 xenograft but no significant activity was seen in the OS33 xenograft.
Conclusions We conclude that palifosfamide lysine has broad activity in a panel of sarcoma cell lines. It inhibits tumor growth in OS and RMS xenografts. Furthermore, it is active against the CPA-resistant, ALDH3A1 overexpressing, OS xenograft suggesting that it might have the potential of overcoming this resistance mechanism against oxazaphosphorines and may be an active agent in resistant/relapsed sarcomas in patients.

Keywords Isophosphoramide mustard · IPM · Palifosfamide lysine · Pediatric sarcomas · Oxazaphosphorine

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Introduction

Oxazaphosphorines such as ifosfamide (IFA) and cyclophosphamide (CPA) are alkylating agents which are active against a variety of pediatric sarcomas such as rhabdomyosarcoma (RMS), Ewing's sarcoma (ES), osteosarcoma (OS) and other undifferentiated soft tissue sarcomas. One or both of these agents are the standard of care for patients with these sarcomas. IFA and CPA are pro-drugs that undergo Cytochrome-P450-dependent hepatic metabolism to active metabolites [1]. IFA, for instance, gets converted to 4-hydroxyifosfamide in the liver. 4-Hydroxyifosfamide then undergoes further metabolism by the aldehyde dehydrogenase (ALDH) class of enzymes to either the active metabolite, isophosphoramide mustard (IPM, palifosfamide), or the inactive metabolite carboxyphosphamide (Fig. 1). Similarly, CPA gets converted to 4-hydroxycyclophosphamide in the liver which undergoes further metabolism by the ALDHs to either the active metabolite, phosphoramide mustard or the inactive metabolite, carboxyphosphamide. Thus, both IFA and CPA have very similar metabolic pathways. Overexpression of certain ALDHs, such as ALDH1A1 and ALDH3A1, are thought to mediate resistance to oxazaphosphorines. In breast cancer, ALDH1A1 expression has been linked to response to CPA [2, 3]. In lung cancer cells, both ALDH1A1 and 3A1 have been shown to equally contribute to the resistance to 4-hydroperoxycyclophosphamide (4-HC) [4]. Additionally, toxic metabolites such as acrolein and chloracetaldehyde are formed as byproducts of this metabolic pathway. Accumulation of acrolein in the bladder may result in hemorrhagic cystitis, while chloracetaldehyde may cause encephalopathy [1].

Palifosfamide is a bi-functional alkylator cross-linking guanine–guanine sequences causing irreversible DNA damage [5]. It is the active agent of IFA [6]. Palifosfamide does not need to undergo the metabolic activation and hence has two potential advantages: (1) it does not generate acrolein and chloracetaldehyde as toxic metabolites and potentially may have a favorable toxicity profile compared to IFA and (2) it bypasses one of the known resistance mechanisms mediated by ALDHs. Palifosfamide, by itself, is too unstable to be effectively administered clinically. It has a short half-life after reconstitution. The addition of lysine residues stabilizes palifosfamide and extends its half-life, thus making clinical administration feasible.

We hypothesized that palifosfamide lysine should be an active agent against pediatric sarcomas and that it may be able to overcome ALDH-mediated resistance to oxazaphosphorines. We studied the activity of palifosfamide lysine in a variety of sarcoma cell lines and mouse tumor models. To test the activity of palifosfamide lysine in CPA-resistant tumors, the CPA-resistant and -sensitive OS xenografts (OS31 and OS33, respectively) were chosen based on the Pediatric Preclinical Testing Program data [7].

Materials and methods

Cells and cell culture

Human OS cell lines SaOS-2 (ATCC, Manassas, VA), OS222, OS229 and OS230 were cultured in modified Eagle's medium containing 10% fetal bovine serum, 0.5% penicillin–streptomycin and 1% glutamine at 37°C with 5% CO_2. Human RMS cell lines RH30 and RD (ATCC, Manassas, VA) were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 0.5% penicillin–streptomycin and 1% glutamine at 37°C with 5% CO_2. ES cell lines SKES-1 and SK-PNDW (ATCC, Manassas, VA) were cultured in DMEM containing 10% fetal bovine serum, 0.5% penicillin–streptomycin and 1% glutamine at 37°C with 5% CO_2. The OS31 and OS33 xenograft lines (a gift of Peter J. Houghton, St. Jude Children's Research Center, Memphis, TN) are tumor lines maintained by serial passage in mice [7].

Drug

Palifosfamide lysine was kindly provided by ZIOPHARM Oncology, Inc. (New York, NY). It was dissolved in phosphate buffered saline (PBS) and used immediately after reconstitution.

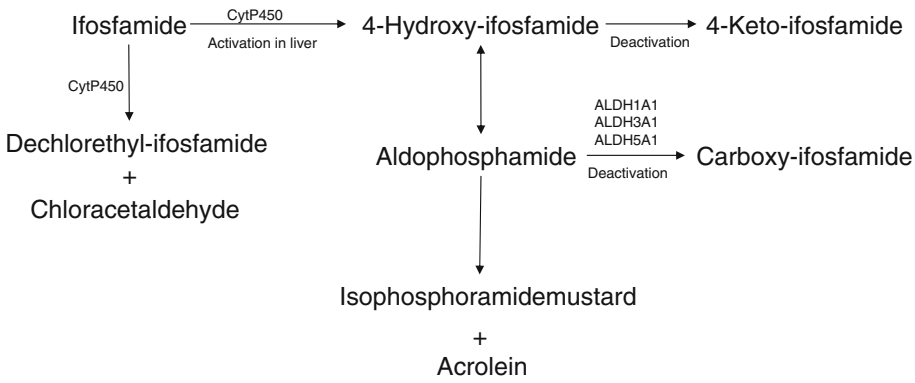

Fig. 1 Metabolic pathway of ifosfamide

Cytotoxicity assay

Cells were plated in 96-well flat-bottomed microtiter plates with approximately 500 cells per well in 100 µl of media. After 24 h of incubation at 37°C, cells were treated with increasing concentrations of palifosfamide lysine in separate plates either as a single-day treatment or three consecutive days of treatment, with fresh drug being added each day. The plates were incubated for 72 h at 37°C with 5% CO_2. After 72 h, 250 µg of 3-(4,5)-dimethylthiazol-2yl-2,5-diphenylterazalium bromide (MTT, Sigma, St. Louis, MO) was added to each well and incubated at 37°C for 6 h. MTT was converted to formazine crystals by mitochondria of viable cells, which were then dissolved in 100 µl of dimethyl sulfoxide. Optical density was measured at 595 nm using a VERSAmax spectrophotometer (Molecular Devices, Sunnyvale, CA). Viability was calculated as the absorbance of drug-treated cells divided by the absorbance of vehicle-treated cells. The IC_{50} was defined as the drug concentration at which absorbance was 50% of that of the controls.

Animal experiments

Five- to 6-week-old CB17 female SCID mice (Taconic Farms, Germantown, NY) were used for the in vivo experiments. Palifosfamide lysine was freshly dissolved in PBS and injected intravenously via the tail vein at doses ranging from 50 to 250 mg/kg of body weight daily for three consecutive days to determine the maximum tolerated dose (MTD). Mice were treated in groups of 3. A toxic event was defined as weight loss greater than or equal to 20% of the animal's starting weight, or death. The MTD was defined as the highest dose at which no toxicity occurred in three of three mice. After the determination of MTD, two OS tumors (OS31 and OS33) from the established PPTP panel were subcutaneously transplanted in the flanks of the SCID mice. For RMS, RH30 cells and for ES, SKES-1 cells were harvested and suspended in matrigel at a concentration of 1×10^7 cells/ml. A total of 0.2 ml of this suspension was injected in the flank of each mouse. Mice were monitored closely for tumor growth. Tumor volumes were determined by the formula: $mm^3 = \pi/6 \times D \times d^2$ where D is the maximal diameter and d is the diameter perpendicular to D. Once the tumors reached 50–150 mm^3, mice were randomized into control and treatment groups (5-8 mice/group) for each tumor line. Cyclophosphamide was administered at the dose of 150 mg/kg intraperitoneally once a week for 6 weeks [7]. Palifosfamide lysine was administered intravenously at the MTD of 100 mg/kg for three consecutive days as a one-time treatment and serial tumor volumes were determined over the next 6 weeks. Mice were sacrificed at the end of the experiment. All experiments were carried out in accordance with an approved animal institute protocol at the Albert Einstein College of Medicine.

Microarray

The available PPTP Affymetrix HG-U133 Plus 2.0 gene expression data on the OS31 and OS33 xenografts were mined, with permission from the PPTP investigators, to look for differential expression of ALDH1A1 and ALDH3A1.

Real time quantitative polymerase chain reaction (RT-PCR)

ALDH3A1 expression was assessed using RT-PCR. The A549 lung carcinoma cell line was used as a positive control as it has been previously shown to highly express ALDH3A1 [4, 8]. Briefly, mRNA was extracted from the two OS xenografts and the A549 cells. A volume of 100 ng of mRNA was converted into cDNA using reverse transcriptase enzyme and random hexamers. The following PCR primer sequences were designed for the experiment: ALDH3A1 5′-TGTAGAGCTCGTCCTGCTGA-3′ and 5′-GCAGACCTGCACAAGAATGA-3′. Primer specificities were confirmed by BLAST Internet software-assisted search. Real time quantitative PCR was performed on all samples using 7500 real time PCR system (Applied Biosystems, Foster City, CA).

ALDH3A1 quantification in cells and tissues

ALDH3A1 enzyme activity was determined in A549 cells and tissues using benzaldehyde as substrate and NADP as the electron acceptor as previously described [9]. Briefly, protein was harvested from cells and tissues using a lysis buffer and protein concentration was determined by measuring absorbance at 595 nm. The reaction mixture was prepared using 32 mM sodium pyrophosphate, 1 mM EDTA (Fisher Scientific, Pittsburgh, PA), 4 mM NADP (Axxora, San Diego, CA), 5 mM reduced glutathione, 0.1 mM pyrazole (Sigma, St. Louis, MO), and the test preparation. Benzaldehyde (Sigma, St. Louis, MO) was prepared at a concentration of 80 mM. The reaction mixture and the substrate were preincubated for 5 min at 37°C. A total of 50 µl of substrate was added to 950 µl of the reaction mixture and a change in absorbance due to conversion of NADP to NADPH was measured at 340 nm using a kinetics assay for 20 min. Rate of reaction was calculated from the linear portion of the curve and the final enzyme activity was calculated in mIU/mg of protein. Each experiment was done three times to calculate mean enzyme activity and standard deviations.

Statistical analyses

For the in vivo experiments, relative tumor volumes (RTV), defined as tumor volume at a particular day divided by tumor volume at day 0, were used for statistical analyses. An event was defined as RTV greater than four times the original RTV or death. RTV greater than four times the original RTV has been used as a standard event by the PPTP for its entire in vivo drug testing models [7]. RTV at event and at day 22 were compared between the control and treated groups using the Wilcoxon-test. Event-free survival (EFS) between the two groups was compared using the Log-rank test.

Results

Cytotoxicity assay

Palifosfamide lysine has broad activity in sarcoma lines in vitro (Fig. 2). The IC_{50} ranged from 0.5 to 1.5 μg/ml (2.25–6.75 μM) for most cell lines except OS222, which had the IC_{50} of 7 μg/ml (31.5 μM). For most cell lines, the IC_{50} was similar for the single-day and the 3-day treatment schedules (Table 1). These results imply that there is rapid uptake of the drug in vitro, with a saturating toxic effect after 24 h.

MTD in mice

In vivo, the MTD was determined to be 100 mg/kg of palifosfamide lysine administered intravenously for three consecutive days. At this dose, the mean weight loss was less than 15%, with complete recovery to baseline within 4 weeks of treatment. Doses higher than 100 mg/kg for three consecutive days were prohibitively toxic leading to either greater than 20% loss of body weight or death. This dosing schedule was used only once during the entire experiment.

Effect on in vivo tumor growth

Palifosfamide lysine inhibits the growth of both CPA-resistant and -sensitive human OS xenografts in SCID mice. Palifosfamide lysine showed significant anti-tumor activity in both OS31 and OS33 xenografts as compared to the untreated controls (Figs. 3c, d, 4c, d). Statistically significant differences in the RTV at day 22 between the control and the treated groups were seen in both tumor lines (P value = 0.002 for OS31 and 0.001 for OS33). The difference in EFS between the treated and the untreated groups was also statistically significant in both tumor lines (P value = 0.0009 for OS31 and 0.01 for OS33). This is significant in comparison to the activity of CPA in these xenografts whereby mice with OS31 demonstrated progressive disease when treated with CPA (Figs. 3a, b, 4a, b). This is in concordance with the previously published PPTP data on CPA activity in these xenografts [7].

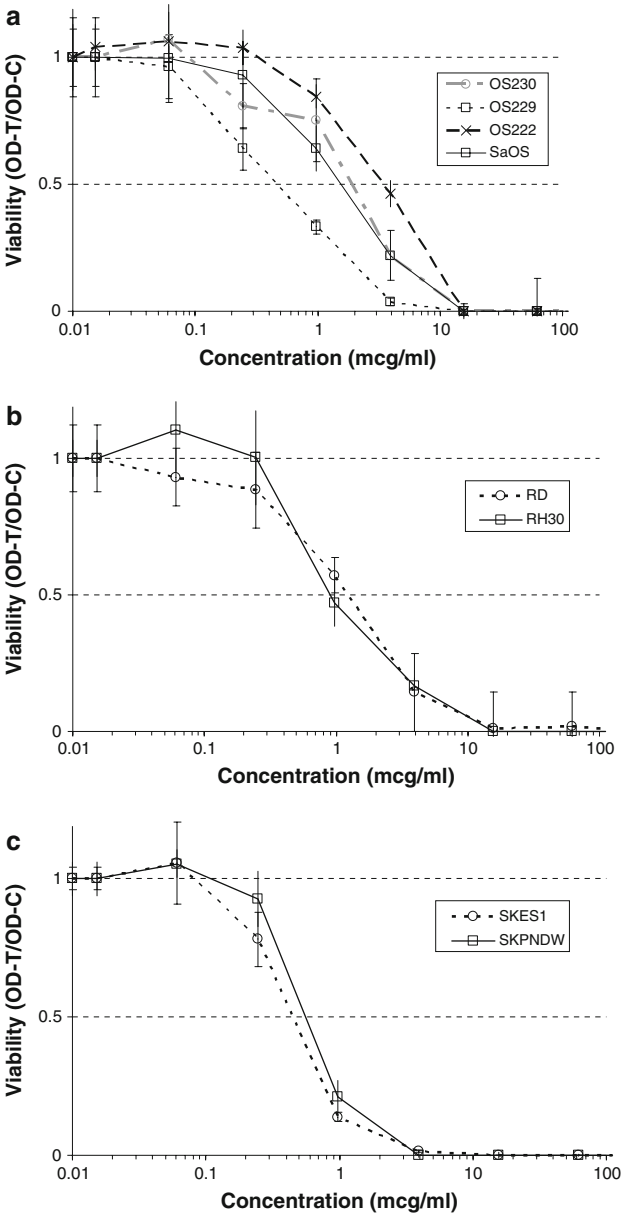

Fig. 2 Activity of IPM-lysine in vitro. **a** OS, **b** RMS, and **c** ES cell lines were incubated with increasing concentrations of IPM-lysine for 72 h. At the end of incubation, cell viability was assessed by using MTT. Viability is depicted on the graphs as a measure of absorbance (OD) of treated cells/controls

In the RH30 tumors there was a statistically significant difference in EFS between the control and the treated groups (P value = 0.03) (Fig. 5). However, in the SKES-1 tumors, at the dosing schedule used in this study, although there was a brief early response (up to day 7) of decreased tumor growth compared to the

Table 1 In vitro cytotoxicity of palifosfamide lysine in sarcoma cell lines with daily × 1 and daily × 3 dosing showing similar IC_{50} for most cell lines with either schedule

Italicized values represent the ones where the IC_{50} is similar in both treatment groups (daily × 1 and daily × 3). The unitalicized ones are different in the two groups

Cell line	Histology	Daily × 3 IC_{50} (μg/ml)	Daily × 1 IC_{50} (μg/ml)
SaOS-2	Osteosarcoma	*1.08*	*1.23*
OS222	Osteosarcoma	1.21	7.0
OS229	Osteosarcoma	*0.31*	*0.50*
OS230	Osteosarcoma	*1.09*	*1.50*
SK-PNDW	Ewing's sarcoma	0.44	1.13
SKES-1	Ewing's sarcoma	0.31	1.01
RH30	Alveolar rhabdomyosarcoma	*0.85*	*0.86*
RD	Embryonal rhabdomyosarcoma	*1.02*	*0.99*

control group, it was not sustained and the tumors in the treated group grew similar to the control group for the remainder of the experiment. Hence, overall the difference in RTV or EFS was not statistically significant (data not shown).

Gene expression in OS xenografts

Analysis of the PPTP Affymetrix HG-U133 Plus 2.0 microarray data showed that neither OS31 nor OS33 xenografts expressed ALDH1A1. ALDH3A1 expression was observed in the OS31 xenograft but not in the OS33 xenograft (data not shown).

RT-PCR

ALDH3A1 mRNA expression was present in the OS31 xenograft but not in the OS33 xenograft consistent with the previously published microarray data (Fig. 6). The degree of expression was much less in comparison to the A549 cell line, which was used as the positive control for the experiment.

ALDH3A1 enzyme activity

OS31 tumors had a significantly higher ALDH3A1 enzyme activity as compared to the OS33 xenograft (Fig. 7). A549

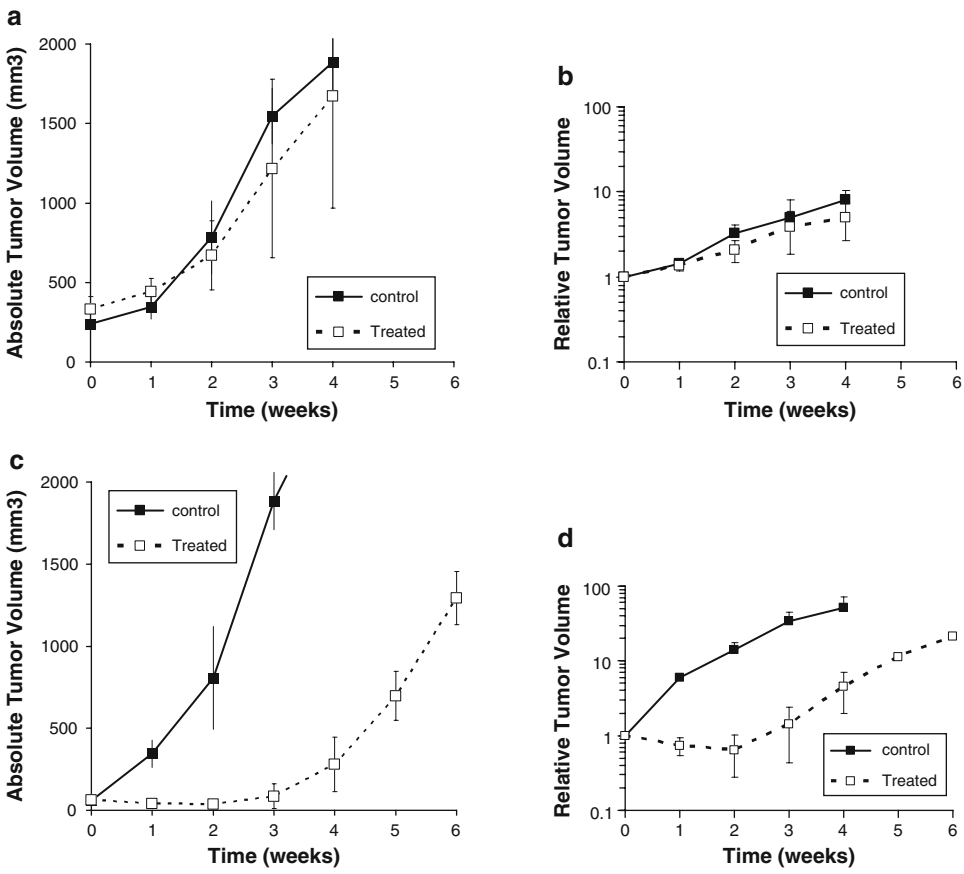

Fig. 3 Comparison of activities of cyclophosphamide (**a**, **b**) and palifosfamide lysine (**c**, **d**) in OS31 xenografts. As depicted by the absolute and relative tumor volumes in each group, OS31 is resistant to cyclophosphamide but sensitive to palifosfamide lysine

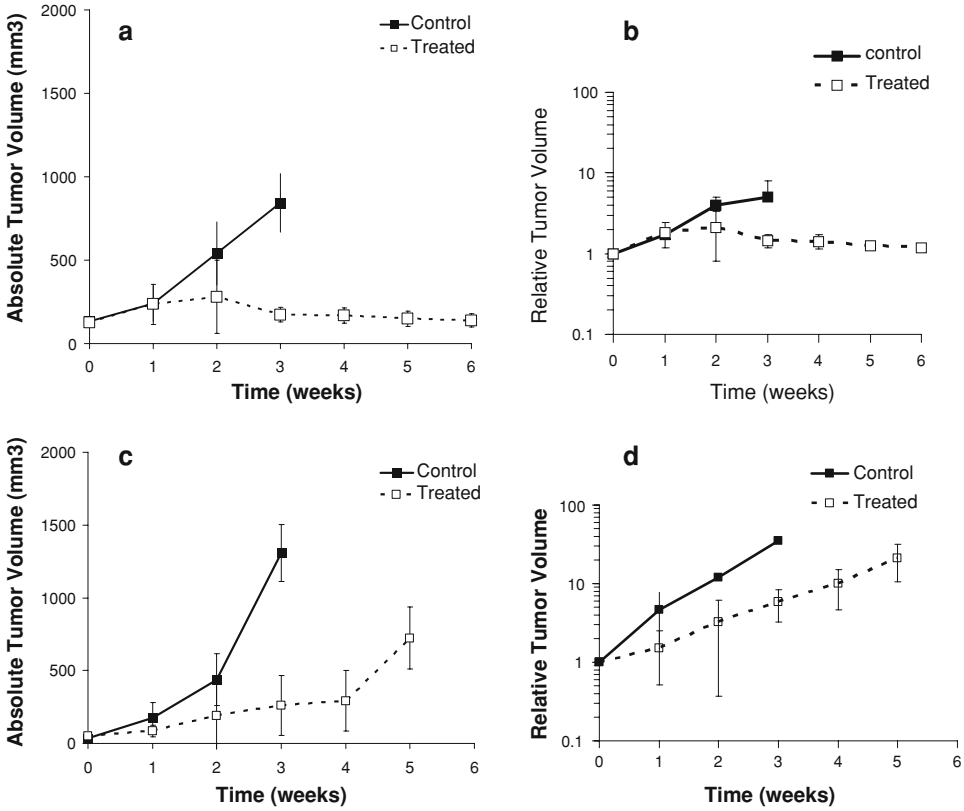

Fig. 4 Comparison of activities of cyclophosphamide (**a**, **b**) and palifosfamide lysine (**c**, **d**) in OS33 xenografts. As depicted by the absolute and relative tumor volumes in each group, OS33 is sensitive to both cyclophosphamide and palifosfamide lysine

cells were used as a control for the experiment as they have previously been shown to have high levels of ALDH3A1 enzyme activity [2, 4]. The mean enzyme activity in OS31 xenografts was 100 ± 20 mIU/mg of protein and in OS33 xenografts was 3 ± 0.6 mIU/mg of protein. The mean enzyme activity in the control A549 cells was 300 ± 36 mIU/mg of protein.

Discussion

We herein demonstrate that palifosfamide lysine has broad activity in a variety of pediatric sarcoma cell lines and xenografts. In addition, it has activity against CPA-resistant OS xenografts. Significant tumor growth delay was seen in both OS31 and OS33 tumors suggesting that palifosfamide lysine was able to overcome CPA resistance in the OS31 xenograft. The CPA resistance in the OS31 xenograft may in part be mediated by the overexpression of ALDH3A1, as demonstrated by the increased enzyme activity in these tumors.

Ifosfamide is a pro-drug that undergoes hepatic activation via the Cytochrome-P450 pathway to 4-hydoxyifosfamide (4-HI). 4-HI is further converted either into the active metabolite, isophosphoramide mustard, or the inactive metabolite, carboxyphosphamide, by the ALDHs 1A1, 3A1

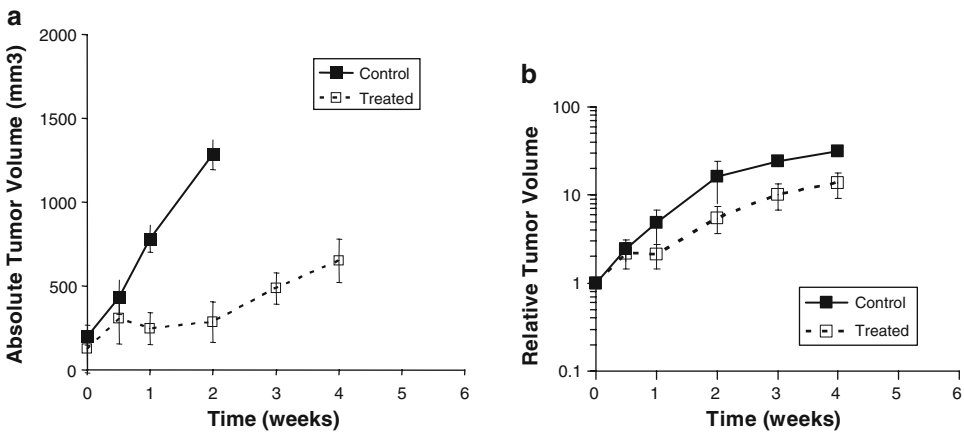

Fig. 5 Activity of palifosfamide lysine in rhabdomyosarcoma xenografts showing difference in (**a**) absolute tumor volume and (**b**) relative tumor volume, respectively, between the control and the treated groups

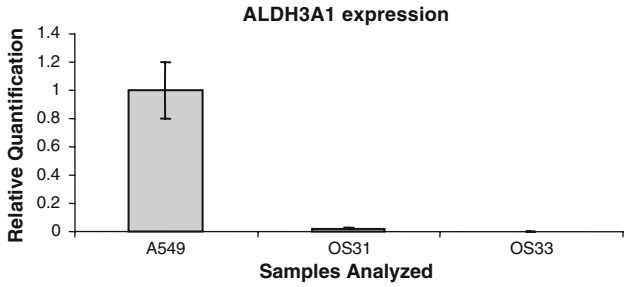

Fig. 6 RT-PCR analysis showing ALDH3A1 mRNA expression in OS31 and OS33 using A549 cells as a standard

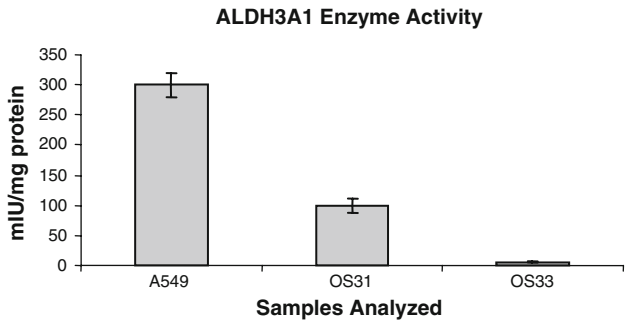

Fig. 7 ALDH3A1 enzyme activity in OS31 and OS33 xenografts using A549 cells as a control

and 5A1. Overexpression of ALDH1A1 and ALDH3A1 has been linked to resistance to oxazaphosphorines such as CPA in a variety of human cancers, presumably by directing the metabolism of 4-HI to the inactive metabolite. Sladek et al. demonstrated that overexpression of ALDH1A1 predicted resistance to chemotherapy in breast cancer [2, 3]. ALDH3A1 overexpression has also been linked to resistance to oxazphosphorines in human colon carcinoma cell lines [10]. Elevated levels of ALDH mediate resistance to CPA in medulloblastoma cell lines [11]. ALDH1A1 and ALDH3A1 are overexpressed in the A549 lung adenocarcinoma line and have been shown to contribute equally to the resistance to 4-HC [4]. One of the ways to potentially circumvent ALDH-mediated oxazaphosphorine resistance is to use active metabolites such as palifosfamide, which are not detoxified by ALDHs [12, 13].

Palifosfamide is a bi-functional alkylator that causes irreversible G:C base pairing, thus causing DNA damage. In addition to having the potential advantage of bypassing resistance mechanisms, it also has a theoretically favorable toxicity profile compared to ifosfamide since it does not generate toxic metabolites such as acrolein and chloracetaldehyde, which may cause hemorrhagic cystitis and encephalopathy, respectively. Its major toxicity is myelosuppression and renal tubular damage [6, 14]. IPM is too unstable to be administered clinically, but the addition of a lysine residue makes it stable for clinical administration.

Our data in the current preclinical models suggests that palifosfamide lysine (stabilized palifosfamide) is an active agent in OS, ES and RMS cell lines in vitro. It was cytotoxic against all tested cell lines with the IC_{50} ranging from 0.5 to 1.5 μg/ml except for OS222, which had an IC_{50} of 7 μg/ml. The cytotoxic effect of palifosfamide lysine against these sarcoma cell lines is thought to be due to its alkylating effect, causing irreversible DNA crosslinking and cell death [5]. In vivo, it has significant anti-tumor activity against OS and RMS xenografts, including the CPA-resistant OS xenograft. We determined the MTD of palifosfamide lysine in SCID mice to be 100 mg/kg when administered intravenously for three consecutive days. The 3-day dosing schedule is similar to that being used in current clinical trials [14]. Significant delay in tumor growth and increase in EFS was noted in OS31, OS33 and RMS xenografts. Its activity against CPA-resistant OS31 xenograft suggests that palifosfamide lysine might be an effective therapeutic agent in tumors that are resistant to oxazaphosphorines.

Although our data suggests that the proposed resistance mechanism in this tumor model may be mediated via the differential expression of ALDH3A1, overexpression of ALDH3A1 is just one of the many proposed mechanisms of oxazaphosphorine resistance and it may or may not play a role in the activity of palifosfamide in these tumor models.

The data presented above suggest that palifosfamide lysine is active in a broad range of pediatric sarcomas in vitro and in vivo including oxazaphosphorine-resistant sarcomas. By bypassing a primary mode of oxazaphosphorine resistance and toxicity, palifosfamide lysine may prove to have a therapeutic advantage over cyclophosphamide and ifosfamide. Clinical trials are underway to evaluate the safety and efficacy of palifosfamide lysine.

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