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CD10 Is a Key Enzyme Involved in the Activation of Tumor-activated Peptide Prodrug CPI-0004Na and Novel Analogues: Implications for the Design of Novel Peptide Prodrugs for the Therapy of CD10$^+$ Tumors

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ABSTRACT

Traditional chemotherapeutic drugs are often restricted by severe side effects and lack of tumor specificity. Peptide prodrugs cleavable by peptidases present in the tumor environment have been explored to improve the therapeutic index of cytotoxic drugs. One such prodrug of doxorubicin (Dox), CPI-0004Na [*N*-succinyl-β-alanyl-L-leucyl-L-alanyl-L-leucyl-Dox (sALAL-Dox)] has been shown to have an improved antitumor efficacy profile with reduced toxicity compared with Dox in tumor xenograft models (V. Dubois *et al.*, Cancer Res., *62:* 2327–2331, 2002). In this study, we demonstrate that CD10, a cell surface metalloprotease expressed on a variety of tumor cell types, is capable of cleaving CPI-0004Na and related peptide prodrugs such as *N*-succinyl-β-alanyl-L-isoleucyl-L-alanyl-L-leucyl-Dox (sAIAL-Dox). This proteolytic cleavage generates leucyl-Dox, which is capable of entering cells and generating intracellular Dox. In a [^{3}H]thymidine proliferation assay, analogues of CPI-0004Na showed a 100–300-fold increase in potency on CD10$^+$ cells compared with CD10$^-$ cells. Cytotoxicity of CPI-0004Na was inhibited by phosphoramidon, a known inhibitor of CD10 enzymatic activity. Furthermore, Chinese hamster ovary CHO-S cells, which are resistant to CPI-0004Na, could be sensitized to the cytotoxic effect of the prodrug by transfection of a CD10 cDNA. Tumor xenograft studies using LNCaP prostate tumor cells support the important role of CD10 in the antitumor efficacy of these prodrugs against tumors expressing CD10. CPI-0004Na and sAIAL-Dox achieved statistically significant 70% tumor growth inhibition at day 22. CD10 is expressed on many types of human tumors including B-cell lymphoma, leukemia, and prostate, breast, colorectal, and lung carcinomas; therefore, CD10-cleavable prodrugs may be effective in a range of different tumor types.

INTRODUCTION

Use of traditional chemotherapeutic drugs is restricted by severe side effects and lack of tumor specificity of these cytotoxic agents. Less toxic prodrug forms that can be selectively activated in tumor tissue (TAPs)[4] have been explored in attempts to improve the therapeutic index. Some approaches to the development of TAPs take advantage of inherent properties of the tumor, for example, selective enzyme expression, hypoxia, or low extracellular pH in the vicinity of the tumor. Others activate prodrugs by exogenous enzymes delivered to tumor cells via monoclonal antibodies or generated in tumor cells by gene transfection (reviewed in Ref. 1). Peptides that are cleaved by tumor-associated enzymes, such as plasmin and prostate-specific antigen, have been widely investigated when conjugated to cytotoxics (2–5). For example, a peptide-Dox conjugate targeting prostate-specific antigen is under clinical evaluation for prostate cancer (6).

In one approach to search for novel peptides that are selectively cleaved by tumor cells, a series of peptide conjugates of Dox were made and screened for their stability in whole blood and their activation by enzymes released by several cancer cell lines (7–9). A tetrapeptide derivative, sALAL-Dox (CPI-0004Na), was identified as a candidate TAP (7–9). This prototype compound was shown to be stable in blood and body fluids due to β-alanyl and *N*-cap succinyl groups and is unable to enter cells, possibly due to its large size. Extracellular enzymatic cleavage of the prodrug yields a product, L-Dox, that can be absorbed into cells. Once inside the cell, an intracellular aminopeptidase releases Dox, which is then able to exert its cytotoxic effects through interaction with DNA. sALAL-Dox is up to 4.6 times less toxic than Dox when administered i.v. in normal animals, and tumor xenograft studies in nude mice indicated a higher antitumor efficacy against human breast (MCF-7/6) and colon (LS-174-T and CXF-280/10) xenografts at equitoxic doses when compared with Dox (7). Tissue distribution studies in MCF-7/6 tumor-bearing nude mice confirmed that the improved efficacy of the prodrug is the result of selective generation and uptake of Dox at the tumor site (7). In addition, mice treated with equimolar sALAL-Dox compared with Dox alone accumulated 2-fold higher Dox in tumor tissue and 1.4–29-fold reduced levels of Dox in normal tissue (7).

Until recently, the enzyme(s) responsible for cleavage of sALAL-Dox and similar prodrugs has been unknown. A prodrug-activating endopeptidase activity has been characterized by Dubois *et al.* (10) in human cancer cell extracts as well as media from overgrown cultures of cancer cell lines and identified as the TOP (EC 3.4.24.15).[5] TOP is a ubiquitous thiol-dependent mammalian cell cytoplasmic metalloendopeptidase (11), which may be released from necrotic cells in a tumor mass. TOP cleaves sALAL-Dox to AL-Dox, which is then rapidly cleaved to L-Dox by extracellular aminopeptidases, in blood or in cell growth media (10).[5] TOP typically cleaves oligopeptides on the carboxyl side (P1) of a hydrophobic residue such as leucine; however, it does not cleave peptides that contain isoleucine at the P1 position (12).

In vitro analysis of the sensitivity of cultured human cell lines to sALAL-Dox showed that a number of cell lines were sensitive to the effects of the prodrug, even under conditions in which TOP was not released. This prompted a search for additional endopeptidases that might activate sALAL-Dox and also the related prodrug sAIAL-Dox, which is not a TOP substrate (12). In this study, we present data showing that CD10 (CALLA or neprilysin) is also able to cleave

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[4] The abbreviations used are: TAP, tumor-activated prodrug; Dox, doxorubicin; sAIAL-Dox, *N*-succinyl-β-alanyl-L-isoleucyl-L-alanyl-L-leucyl-doxorubicin; sALAL-Dox, *N*-succinyl-β-alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin; sLAG-Dox, N-succinyl-L-leucyl-L-alanyl-L-glycyl-doxorubicin; L-Dox, leucyl-doxorubicin; TOP, thimet oligopeptidase; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; MTD, maximum tolerated dose; SD-MTD, single dose maximum tolerated dose; RD-MTD, repeat-dose maximum tolerated dose; TGI, tumor growth inhibition.

[5] V. Dubois *et al.* TOP (EC 3.4.24.15) activates CPI-0004Na, an extracellularly TAP of Dox, manuscript in preparation.

TAP-Dox prodrugs (for example, sALAL-Dox and sAIAL-Dox) both *in vitro* and *in vivo*. *In vivo* experiments suggest that CD10 is an important enzyme for cleavage of prodrugs, resulting in potent antitumor effects at well-tolerated doses. The potential clinical advantages of prodrugs cleavable by CD10 will be discussed.

MATERIALS AND METHODS

Synthesis of Suc-β-Ala-Leu-Ala-Leu-Dox, Suc-β-Ala-Ile-Ala-Leu-Dox, and Suc-Leu-Ala-Gly-Dox. Dox was supplied by Meiji, HATU was supplied by Aldrich (Milwaukee, WI); all resins and amino acids were supplied by either ABI (Foster City, CA), Novabiochem (San Diego, CA), Advanced ChemTech (Louisville, KY), Peptide International (Louisville, KY), or SynPep (Dublin, CA). Tetrapeptides (Fmoc-β-Ala-Leu-Ala-Leu-OH, Fmoc-β-Ala-Ileu-Ala-Leu-OH) and a tripeptide (Fmoc-Leu-Ala-Gly-OH) were synthesized using solid-phase approach with standard Fmoc chemistry (13, 14), and the products were purified using preparative HPLC. Suc-Leu-Ala-Gly-Dox, Suc-β-Ala-Ile-Ala-Leu-Dox, and Suc-β-Ala-Leu-Ala-Leu-Dox were synthesized and characterized using a procedure described previously (15). The compounds were purified by preparative HPLC, giving a purity on the analytical HPLC of 98%. Impurities consisted predominantly of alternative enantiomers. No free Dox was detectable.

CD10 Transfection of CHO-S Cells. CD10 cDNA was cloned from a human fetal cDNA library by PCR using three sets of overlapping primers: a5HindCD10 (AAGCTTGCCGCCACCATGGGCAAGTCAGAAAGTCAGATG); a3XbaCD10 (TCTAGAAGGGAGGCCAAGTCGAGGTTGGTC); b5XbaCD10 (TCTAGAGATTACTATGAATGCACTGGAATC); b3XhoCD10 (CTCGAGGTACTCATTATTCAGTTTGTTATC); c5XhoCD10 (CTCGAGTTGAACTACAAAGAAGATGAATAC); and c3PacCD10 (TTAATTAATCACCAAACCCGGCACTTCTTTTC). The fully assembled coding region was subcloned into a mammalian expression vector pCDNA3.1/Hygro (Invitrogen) downstream of the hCMV-MIE promoter. DNA sequence analysis confirmed sequence identity with the coding sequence of human CD10 (GenBank accession number Y00811). The expression plasmid was linearized and stably transfected into CHO-S cells (Invitrogen) by electroporation; 10^7 CHO-S cells were resuspended in 1 ml of PBS with 20 μg of linearized DNA and placed in a 0.4-cm cuvette. The cells were pulsed twice at 1500 V and 3 μF and allowed to recover in standard CD-CHO-S media for 2 days. CD10-expressing transfectant clones were selected under 500 μg/ml hygromycin and screened by flow cytometry for cell surface CD10 expression using a phycoerythrin-conjugated mouse antihuman CD10 clone B-E3 (Biosource International, Inc., Camarillo, CA).

[^{3}H]Thymidine Proliferation Assay. All cells used were purchased from American Type Culture Collection, except for BALL-1 cells, which were obtained from Coulter Corp. (Miami, FL). Suspension cells HL-60, BALL-1, or Ramos cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS. On the day of the study, the cells were collected, washed, and resuspended at a concentration of 0.5×10^6 cells/ml in culture media. Cell suspension (100 μl) was added to 96-well plates. Serial dilutions (3-fold increments) of Dox or test compounds were made, and 100 μl of compounds were added per well. Finally, 10 μl of 100 μCi/ml [^{3}H]thymidine were added per well, and the plates were incubated for 24 h. The plates were harvested using a 96-well Harvester (Packard Instruments) and counted on a Packard Top Count counter. Four parameter logistic curves were fitted to the [^{3}H]thymidine incorporation as a function of drug molarity using Prism software to determine IC_{50} values. For adherent cells, PC3 and LNCaP (prostate carcinoma) were cultured in DMEM plus 10% FCS. On the day of the study, the cells were detached from the plate with a trypsin-EDTA solution (0.05% trypsin and 0.53 mM EDTA; Life Technologies, Inc.). The collected cells were washed and resuspended at a concentration of 0.25×10^6 cells/ml in culture media. Cell suspension (100 μl) was added to 96-well plates, and the plates were incubated for 3 h to allow the cells to adhere. The proliferation assays were performed as described for the suspension cells, except that cells were harvested using EDTA according to standard methods.

Prodrug Cleavage by Purified CD10. CD10 from porcine kidney (100 ng; Elastin Products, Owensville, MO) was combined with 80 μM prodrug in 8 μl of 125 mM 2-morpholinoethanesulfonate (pH 6.5) buffer and incubated for 4 and 8 h at 37°C. The reactions were stopped by 4-fold dilution into acetonitrile, and after centrifugation to remove precipitated protein, the supernatants were diluted 4-fold into water and analyzed by reverse-phase HPLC. HPLC was carried out on a TSK Super-ODS column run in 20 mM ammonium formate (pH 4.5) with a 26–68% linear gradient of acetonitrile. Dox-linked peptides were detected by fluorescence (235 nm excitation, 560 nm emission) and identified by comparison of the retention time of each peak with known standards. The percentage of hydrolysis was calculated from area of product peaks (Agilent Chemstation) divided by the sum of product plus substrate peak areas. The percentage of substrate hydrolyzed after 4 h was approximately half that hydrolyzed after 8 h.

Prodrug Cleavage by CD10-expressing Cells. The prodrugs sALAL-Dox, sAIAL-Dox, and sLAG-Dox were tested by incubation with either CHO-S cells or CHO-S cells stably transfected with a CD10 cDNA and shown to express CD10 on the cell surface. Time points were taken after 1, 4 and 24 h incubation at 37°C, and after centrifugation to remove cells, the samples were analyzed by reverse-phase HPLC as described above. Conversion rates were calculated using a $1/Y^2$ weighted linear least squares fit to percentage of product *versus* time where percentage of product was determined as integrated peak areas for product over substrate plus product. The percentage of converted/unit time was expressed in μM/unit time by multiplying by the zero time substrate concentration.

Immunohistochemical Analysis of CD10 Expression on LNCaP Tumor Xenografts. LNCaP xenografts grown in nude mice were fixed in formalin. Formalin-fixed tumors were embedded in paraffin blocks according to standard procedures. Five-μm-thick tissue sections were cut using a microtome and applied to slides. Slides were deparaffinized by passage through xylene and graded ethanol. Steam heat-induced antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) was used for antigen unmasking. Slides were washed with PBS, and endogenous peroxidase was quenched with 0.3% hydrogen peroxide for 10 min. Slides were washed with PBS followed by overnight incubation at 4°C with either mouse antihuman CD10 monoclonal antibody clone 56C6 (Novocastra Laboratories Ltd.) at a 1:40 dilution or mouse IgG1 isotype control immunoglobulin clone MOPC-21 (Becton Dickinson). Slides were washed with PBS and then incubated for 30 min with horseradish peroxidase-conjugated goat antimouse immunoglobulins (DAKO) followed by incubation with fresh 3,3′-diaminobenzidine chromagen (DAKO). Slides were then counterstained with hematoxylin, dehydrated, and mounted with mounting media.

***In Vivo* Dose-ranging Safety Studies.** In a MTD toxicity study in normal female ICR mice, a single, i.v. dose of sALAL-Dox or sAIAL-Dox at either 50, 75, or 100 mg/kg (equivalent to 28, 42, or 56 mg/kg of Dox on a molar basis) or sLAG-Dox at 110, 132, 154, 176, 198, or 220 mg/kg (equivalent to 70, 84, 98, 112, 126 or 140 mg/kg of Dox on a molar basis) was administered to groups of five mice. A control group of five mice was given vehicle [sterile Dulbecco's sodium PBS (pH 7.2)]. Mice were weighed and observed daily for signs of toxicity (abnormal appearance and behavior) for 7 weeks (day 49). Mice were terminated either at the conclusion of the study (day 49), or when signs of mortality (body weight loss exceeding 25% of their individual initial weight) or morbidity (in particular, paralysis or uncoordinated gait) were observed. Group mean MTD was determined as the highest dose group in which body weight loss did not exceed 10% and there was no incidence of mortality.

LNCaP Tumor Xenograft Study. LNCaP cells (7.5 million cells) resuspended in 0.1 ml of PBS and 0.1 ml of Matrigel (Becton Dickinson) were injected s.c. into the side of male nude mice fed on a high-fat diet. Dosing was initiated when the mean tumor weight reached approximately 200 mg. Mice were weighed, measured for tumors, and checked for health status twice a week for 36 days after the initial dosing. Mice with >25% body weight loss after the initiation of dosing were euthanized as a toxic end point. Mice with tumors of >1000 mg were euthanized as a cancer end point. The weight of a tumor (mg) was estimated by length × width × width/2 (mm). All animals were euthanized at the end of the study (36 days after initial dosing). Parameters evaluated include mean tumor weight at day 22, the last time point before the first mouse reaching cancer and/or toxic end point; TGI (TGI at day 22, percentage of mean tumor weight inhibition over control); survivors at day 36, tumors reaching the predetermined cutoff size of 1000 mg (cancer end point), and tolerability of the dosing regimen, by number of mice exhibiting toxic end point (>25% body weight loss).

RESULTS

CD10 Expression on Cells Correlates with the Enzyme Capable of Cleaving sALAL-Dox and sAIAL-Dox. A panel of human-tumor derived cell lines was tested for sensitivity to the prodrug sALAL-Dox

in a cell proliferation assay (Table 1). There was wide variability between cell lines in the sensitivity to sALAL-Dox, despite the fact that all of the cell lines tested were similarly sensitive to Dox, the active metabolite of sALAL-Dox. Two cell lines, LNCaP and Ramos, were particularly sensitive to the effects of the prodrug. Cell proliferation of LNCaP and Ramos cells was inhibited, despite the fact that cells were maintained in high viability cultures before administration of the prodrug. Intracellular peptidases, such as TOP,[5] which has been described to cleave sALAL-Dox (10), are not expected to be released into the medium under these conditions.

LNCaP and Ramos cells were also equally sensitive to the related prodrug sAIAL-Dox. Dox-peptides containing isoleucine are poor substrates for TOP (12), again suggesting that TOP cannot fully account for activation of the peptide prodrugs in this assay. This prompted a search for additional secreted or cell surface endopeptidases that could be candidates for peptide prodrug-activating enzymes. One such enzyme, which is present on a number of B-cell and prostate-derived tumor cell lines and has an appropriate substrate specificity, is CD10 (16). Flow cytometric analysis was therefore carried out to determine the levels of CD10 present on prodrug-sensitive and -resistant cell lines. Fig. 1 shows that there is a positive correlation between cell lines in the panel that express detectable levels of CD10 and those that are sensitive to sALAL-Dox and sAIAL-Dox.

CD10 Enzyme Cleaves sALAL-Dox and sAIAL-Dox but not sLAG-Dox. *In vitro* experiments using enzymatically active porcine kidney CD10 with detection by fluorescence HPLC indicated that both sALAL-Dox and sAIAL-Dox are cleaved by CD10 to form only L-Dox as Dox adduct. Conversely, sLAG-Dox was not hydrolyzed in these tests (Table 2). The amount of enzyme used in these experiments cleaves about 400 times more glutaryl-L-alanyl-L-alanyl-phenylalanyl-*para* nitrophenylaniline (Elastin Products) than sAIAL-Dox, based on an assay run under similar incubation conditions but using the aminopeptidase and spectrophotometric detection method recommended by the manufacturer (data not shown). sAIAL-Dox is a better substrate of CD10 than sALAL-Dox, which may be the reason that LNCaP and Ramos cells are less sensitive to sALAL-Dox than sAIAL-Dox. Ramos cells are comparatively more resistant to sALAL-Dox than LNCaP cells, which may reflect differences in enzymatic activity of CD10 expressed on the surface of different cells or differences in enzymatic activity other than CD10 contributing partially to cleavage of sALAL-Dox.

Table 1 *Activity of TAP-Dox prodrugs on LNCaP, PC-3, Ramos, and BALL-1 cells*

	IC_{50} (μM)			
	LNCaP	PC-3	Ramos	BALL-1
CD10 expression	+	−	+	−
Dox	0.02	0.08	0.01	0.02
L-Dox	0.11	0.09	0.08	ND[a]
sALAL-Dox	0.87	28	7	17
sAIAL-Dox	0.19	57	0.42	50
sLAG-Dox	15	39	23	23

[a] ND, not determined.

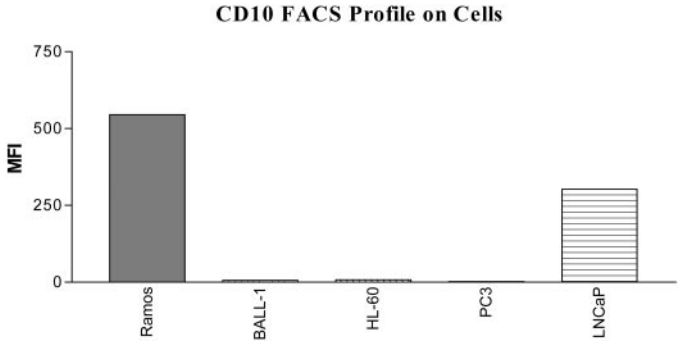

Fig. 1. CD10 expression on cultured tumor cells.

Table 2 *Percentage of hydrolysis of TAP-Dox prodrugs after 8 h by purified CD10 enzyme*

Substrate	% Hydrolysis	Fluorescent products
sALAL-Dox	10.9	L-Dox
sAIAL-Dox	22.1	L-Dox
sLAG-Dox	0	None

Table 3 *Effect of a CD10 inhibitor on activity of sAIAL-Dox on different cells*

Phosphoramidon concentration (nM)	BALL-1[a] ($CD10^-$) IC_{50} (μM)	RAMOS[a] ($CD10^+$) IC_{50} (μM)	LNCaP[a] ($CD10^+$) IC_{50} (μM)
0	22	0.51	0.24
30	20	2.1	1.3
100	21	5.3	3.3
300	24	8.1	7.7
1000	25	9.7	11.3
3000	26	10.5	15.4

[a] Inhibition of cell proliferation was determined by [^{3}H]thymidine incorporation.

CD10 Inhibitor Inhibits Cleavage of sAIAL-Dox by $CD10^+$ Cells. CD10-selective inhibitors were used to further investigate the role of CD10 in cleavage of sALAL-Dox and sAIAL-Dox. Table 3 showed that sAIAL-Dox has the highest degree of selectivity for $CD10^{hi}$ LNCaP and Ramos cells, with an IC_{50} of 0.24 and 0.51 μM, respectively. In the presence of increasing concentrations of the CD10 inhibitor phosphoramidon, sAIAL-Dox became less active at inhibiting proliferation of LNCaP and Ramos cells (Table 3). In contrast, this CD10 inhibitor had no effect on the activity of sAIAL-Dox on $CD10^-$ BALL-1 cells, supporting that CD10 cleaved sAIAL-Dox.

CD10-transfected CHO-S Cells Cleaved sALAL-Dox and sAIAL-Dox Much More Efficiently than $CD10^-$ Parental Cells. To provide direct evidence that CD10 can cleave peptide prodrugs, CHO-S cells were transfected with human CD10 cDNA expressed from a cytomegalovirus promoter, and stable transfectant clones were isolated. Stable CHO-S CD10 Clone 8 was shown by flow cytometry to express a high level of CD10, around 3-fold higher than that of LNCaP cells (data not shown), and was used in the following studies. 10 μM sALAL-Dox, sAIAL-Dox, and sLAG-Dox were separately incubated at 37°C with parental and CD10-transfected (Clone 8) CHO-S cells. At 1, 4, and 24 h, an aliquot of each supernatant was analyzed by reversed-phase HPLC for cleavage of TAP-Dox prodrugs. The results are summarized in Table 4. When incubated with sALAL-Dox, there was little metabolism to L-Dox observed with the parental CHO-S cells, whereas the metabolism with CD10-transfected (Clone 8) CHO-S cells was over 3% per hour, approximately 8 times faster. Metabolism rates for sLAG-Dox were similarly low in both cell lines. With sAIAL-Dox as a substrate, Clone 8 CHO-S cells again had a faster metabolism rate (2% per hour), which was 4 times faster than that of the parental cells.

Relative Antiproliferative Potency of TAP-Dox Compounds on Parental and CD10-transfected CHO-S Cell Lines. To test whether CD10 expression confers sensitivity to killing by sALAL-Dox and sAIAL-Dox, CD10-transfected CHO-S cells and parental cells were tested in a [^{3}H]thymidine proliferation assay in the presence of Dox, sALAL-Dox, or sAIAL-Dox. The results are summarized in Table 5. CD10-transfected Clone 8 cells were more sensitive to killing by sALAL-Dox and sAIAL-Dox (2-fold and 6-fold, respectively) than the parental cells, supporting that CD10 contributes to cleavage of sALAL-Dox and sAIAL-Dox. However, CD10-transfected CHO-S cells were about 10–20-fold less sensitive to sALAL-Dox and sAIAL-

Table 4 *Metabolic conversion rates of TAP-Dox prodrugs by CHO-S and CD10-transfected cells*

	sALAL-Dox		sLAG-Dox		sAIAL-Dox	
Substrate	Parental	CD10 transfectant	Parental	CD10 transfectant	Parental	CD10 transfectant
% Conversion/hour	0.4	3.2	0.6	0.6	0.5	2.1
μM/min	0.04	0.32	0.06	0.06	0.05	0.21
Metabolic products	L-Dox	L-Dox	G-Dox[a]	G-Dox	L-Dox	L-Dox

[a] G-Dox, Glycyl-doxorubicin.

Table 5 *Activity of TAP-Dox prodrugs on CHO-S parental and CD10-transfected cells*

Cells	Dox IC_{50} (μM)	L-Dox IC_{50} (μM)	sALAL-Dox IC_{50} (μM)	sAIAL-Dox IC_{50} (μM)	sAIAL-Dox/Dox IC_{50}
Parental	0.51	11	17	49	96
CD10 transfectant	0.57	12	10	8.4	15

Dox than $CD10^+$ LNCaP and Ramos cells. This may be due to the fact that CHO-S cells have reduced levels of leucine aminopeptidase because L-Dox is 100 times more potent on LNCaP/Ramos when compared with CHO-S cells. In addition, CHO-S cells are less sensitive to Dox (Table 1 and Table 5).

Because CHO-S cells were not sensitive to L-Dox, supernatants were collected 24 h after exposure of CHO-S parental cells or CD10-transfected Clone 8 cells to each of the following compounds: Dox; sALAL-Dox; sAIAL-Dox; and sLAG-Dox. The supernatants were then transferred to L-Dox-sensitive $CD10^-$ HL-60 cells (IC_{50}, 0.18 μM), and cell proliferation was measured 24 h later. [^{3}H]thymidine proliferation data show that while supernatants from parental CHO-S cells did not inhibit HL-60 proliferation in the presence of sALAL-Dox and sAIAL-Dox (Fig. 2*B*), supernatants from $CD10^+$ Clone 8 cells did (Fig. 2*C*). Supernatants from $CD10^+$ Clone 8 cells incubated with sLAG-Dox did not inhibit HL-60 proliferation, which was consistent with the fact that sLAG-Dox is a poor substrate for CD10. As an additional control (Fig. 2*A*), fresh Dox, sALAL-Dox, sAIAL-Dox, and sLAG-Dox were added to HL-60, and IC_{50} measurements were made 24 h later. When fresh compounds are added to HL-60 cells (CD10 negative), cleavage of sALAL-Dox and sAIAL-Dox does not occur, again indicating that CD10 is required for cleavage. In summary, the *in vitro* data shown support the hypothesis that CD10 cleaves the prodrug sALAL-Dox or sAIAL-Dox to release Dox/L-Dox, thereby inhibiting cellular proliferation.

***In Vivo* Dose-ranging Safety Studies on sALAL-Dox, sAIAL-Dox, and sLAG-Dox.** *In vitro* stability studies of these TAP compounds showed that they were relatively stable in mouse and human whole blood. sALAL-Dox had <2% hydrolysis/h and a $t_{1/2}$ of >90 h (data not shown) in mouse and human whole blood. Similarly, <2% hydrolysis was observed for sAIAL-daunorubicin or sALAG-daunorubicin after incubation for 2 h in human whole blood. To evaluate TAP-Dox compounds *in vivo*, we first did a dose-ranging safety study in mice on compounds dosed i.v. singly. The SD-MTD in normal mice for sALAL-Dox, a substrate of both TOP and CD10, was determined to be >28 and <42 mg/kg Dox equivalents (Fig. 3). The SD-MTD for sAIAL-Dox, a substrate of CD10 but not TOP, was >42 and <56 mg/kg Dox equivalents (Fig. 3). Additional experiments with smaller dosing intervals will be necessary to determine the SD-MTDs more precisely for sALAL-Dox and sAIAL-Dox. sLAG-Dox, a substrate for TOP but not CD10, was found to have a much higher SD-MTD, which was >140 mg/kg Dox equivalents, the highest dose tested (Fig. 3).

Comparison of TGI by TAP-Dox Compounds and Dox in the $CD10^+$ LNCaP Prostate Carcinoma Model. Immunohistochemical analysis of LNCaP prostate tumor xenografts showed that CD10 had an intense cell surface membrane-associated pattern of staining and was relatively evenly distributed around the periphery of all cells in the tumor tissue sample shown in Fig. 4. To test whether CD10 contributes to cleavage of TAP-Dox compounds *in vivo*, an LNCaP prostate carcinoma xenograft model was set up in athymic mice to evaluate the antitumor efficacy of Dox, sALAL-Dox, sAIAL-Dox, and sLAG-Dox at approximately equitoxic doses. LNCaP cells were implanted s.c. in athymic mice, and treatment was initiated on established tumors with a mean tumor weight of approximately 200 mg. The dosing regimen and results are summarized in Table 6. All compounds were dosed i.v. weekly for a total of three doses. Dox was dosed at 4 mg/kg, which was close to its RD-MTD. sALAL-Dox and sAIAL-Dox were dosed on an equimolar basis at 22 mg/kg Dox, about 20% below their RD-MTD. sLAG-Dox was dosed at 70 mg/kg Dox, a level that is 3 times greater than the molar concentration of sALAL-Dox and sAIAL-Dox.

sALAL-Dox, sAIAL-Dox, and sLAG-Dox were very well tolerated, with no toxic end points at the end of the study (day 36) and with

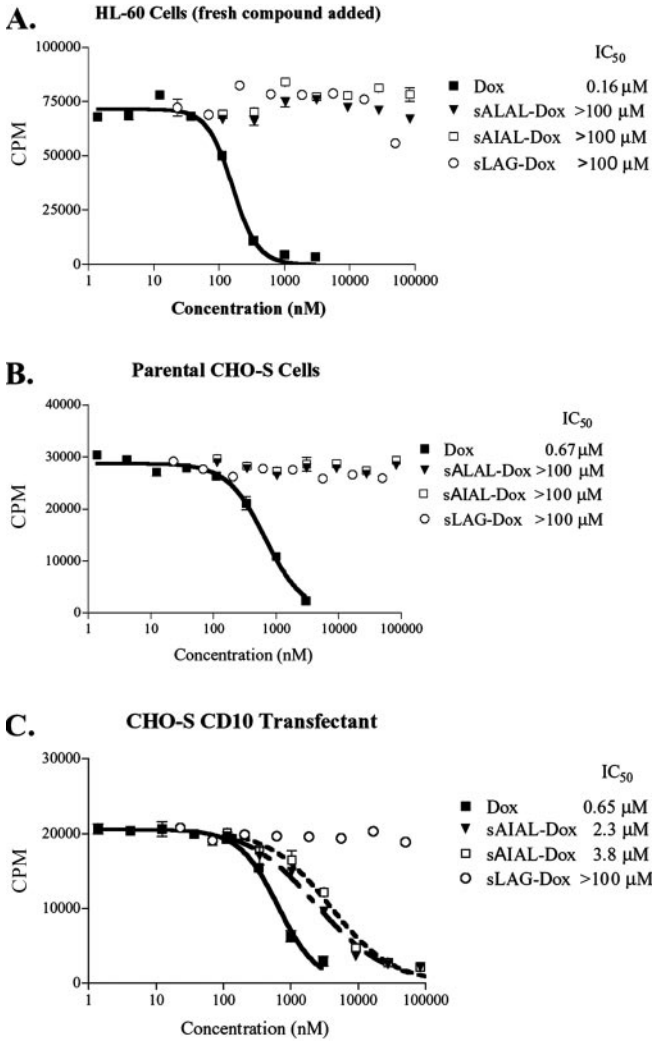

Fig. 2. Proliferation of HL-60 cells in the presence of supernatant collected from CHO-S and CD10-transfected CHO-S cells incubated with TAP-Dox compounds.

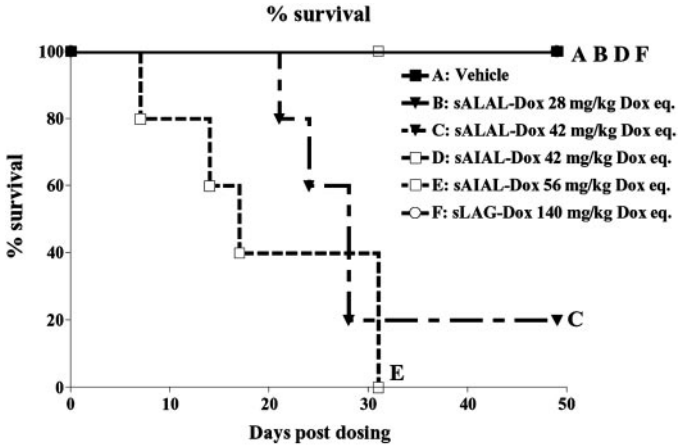

Fig. 3. *In vivo* dose-ranging safety studies on sALAL-Dox, sAIAL-Dox and sLAG-Dox.

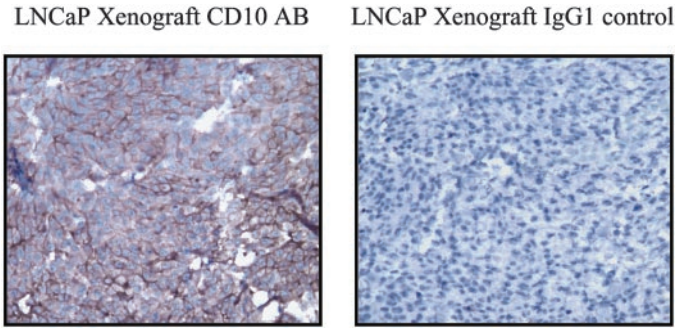

Fig. 4. CD10 expression on LNCaP tumor xenografts grown in athymic mice.

maximal 5% loss of group mean adjusted body weight (tumor weight subtracted; Fig. 5). Mice receiving 4 mg/kg Dox had one toxic end point (>25% weight loss) on day 22 and about 7% loss of group mean adjusted body weight on day 22. Control group mice also had slight weight loss (up to 5% loss of group mean adjusted body weight) and had two toxic end points that could be a result of tumor growth.

sALAL-Dox and sAIAL-Dox had similar efficacy: both greatly inhibited tumor growth (Fig. 5) and achieved statistically significant 70% TGI on day 22 compared with the control group (Table 6). sLAG-Dox dosed at 3 times the molar concentration of sALAL-Dox and sAIAL-Dox, inhibited tumor growth to a lesser extent than sALAL-Dox or sAIAL-Dox (TGI at day 22: 40 ± 30% *versus* 73–78 ± 28%), and failed to achieve statistical significance from control (Table 6). Compared with the Dox alone treatment, sALAL-Dox and sAIAL-Dox achieved statistically significant 40–50% TGI on day 19 and day 22 (data not shown). In addition, no tumors reached the >1000 mg predetermined cancer end point in both sALAL-Dox and sAIAL-Dox groups (Fig. 5), compared with 2, 4, and 4 mice reaching cancer end point in the sLAG-Dox, Dox, and control groups, respectively. Therefore, sALAL-Dox and sAIAL-Dox were more effective and safer than Dox alone. The dose-efficacy response differentiation of sALAL-Dox and sAIAL-Dox *versus* sLAG-Dox supports the critical *in vivo* role of CD10 in TAP compound cleavage in $CD10^+$ LNCaP tumors for sAIAL-Dox and sALAL-Dox. The observation that sALAL-Dox and sAIAL-Dox have similar efficacy on LNCaP xenografts suggests that CD10 plays a more dominant role than TOP in sALAL-Dox cleavage in $CD10^+$ LNCaP tumors.

DISCUSSION

In this study, we demonstrated *in vitro* that CD10 is able to cleave the prodrugs sALAL-Dox and sAIAL-Dox. Previous data had suggested that TOP (EC 3.4.24.15)[5] was a candidate enzyme responsible for cleavage of sALAL-Dox (10). TOP is primarily an intracellular enzyme and is found in a wide variety of tumor and normal cells. TOP may be released from tumor cells and thus be prevalent in tumor extracellular fluid as a result of tumor-associated necrosis or during angiogenesis, which is frequently associated with tumor growth. Interestingly, this enzyme is activated by reduction and is expressed as an inactive multimer under oxidizing conditions[5] (10); therefore, the hypoxic environment of tumors may favor activation. However, other enzymes may also play a role in the cleavage of peptide prodrugs, and data from a series of peptide-dox prodrugs suggested that cleavage took place even under conditions in which TOP was not released from the cell (10).[5] In addition, sAIAL-Dox is not a substrate for TOP (12), yet it was readily cleaved in our cellular assays.

Sensitivity to sALAL-Dox correlated with CD10 expression, suggesting that the enzyme could be relevant in the activation of sALAL-Dox and sAIAL-Dox. CD10, a neutral endopeptidase (EC 3.4.24.11), is a 90–110-kDa zinc-dependent cell surface metallopeptidase that cleaves small peptides on the amino side of hydrophobic amino acids (16). The range of substrates known to be cleaved by CD10 is broad and includes bombesin-like peptides, bradykinin, substance P, atrial naturetic peptide, enkephalins, endothelin, and the oxidized chain of insulin (16).

The relevance of CD10 to *in vitro* cleavage of TAPs has been demonstrated here by several lines of evidence including a direct comparison of the same cell line with or without CD10 through the use of transfected CHO-S cells. *In vivo* relevance was examined through comparison of prodrugs that are efficiently cleaved by CD10 but not TOP (sAIAL-Dox), cleaved by CD10 and TOP (sALAL-Dox), and cleaved by TOP but not CD10 (sLAG-Dox). The prodrugs sALAL-Dox and sAIAL-Dox showed increased antitumor efficacy compared with Dox at well-tolerated doses against $CD10^+$ LNCaP prostate carcinoma xenografts grown in nude mice, indicating that both prodrugs may have clinical benefit in treating $CD10^+$ tumors. In addition, both tetrapeptide prodrugs appeared more efficacious than the TOP-cleaved prodrug, sLAG-Dox, at approximately equitoxic doses. CD10 cleavage also appeared to contribute to the *in vivo*

Table 6 *In vivo antitumor efficacy of TAP-Dox in LNCaP prostate carcinoma model*

Parameters evaluated include mean tumor weight at day 22, the last time point before the first mouse reaching cancer and/or toxic end point; TGI (at Day 22, % mean tumor weight inhibition over control); survivors at day 36, tumors reaching the predetermined cutoff size of 1000 mg (cancer end point), and tolerability of the dosing regimen, by number of mice exhibiting toxic end point (>25% body weight loss).

Group	Compound[a]	SD-MTD (mg/kg)	RD-MTD (mg/kg)	Dose# (mg/kg)	*N*	Tumor weight (mg) at day 22	TGI at day 22	Survivor at day 36	Cancer end point	Toxic end point
A	Saline				8	677 ± 185		2	4	2
B	Doxorubicin	8–16	4	4	8	324 ± 61	52 ± 29%	3	4	1
C	sALAL-Dox	>50; <75 (>28; <42 Dox equi)[b]	50 (28 Dox equi)	40 (22 Dox equi)	8	180 ± 20	73 ± 28%[c]	8	0	0
D	sAIAL-Dox	>75; <100 (>42; <56 Dox equi)	53 (30 Dox equi)	40 (22 Dox equi)	8	152 ± 19	78 ± 28%[c]	8	0	0
E	sLAG-Dox	>220 (140 Dox equi)	>110 (70 Dox equi)	110 (70 Dox equi)	8	409 ± 76	40 ± 30%	6	2	0

[a] All compounds were dosed intravenously Q7Dx3, 3 weekly dosing.
[b] Dox equi, Dox equivalents.
[c] Statistically different from the control and Dox group at the $P = 0.05$ (two-tailed unpaired *t* test with Welch's correction).

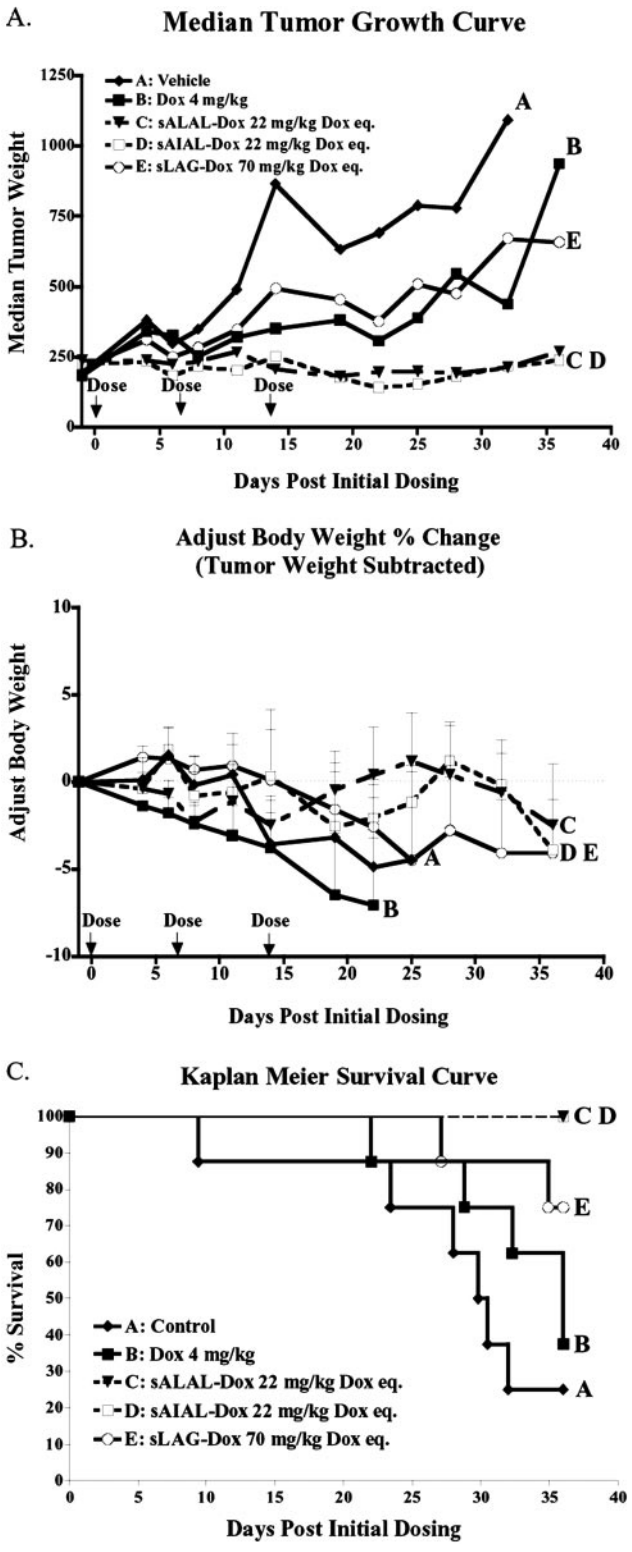

Fig. 5. Summary of the effects of TAP-Dox compared with Dox (4 mg/kg) in the LNCaP human prostate xenografts in male mice (Q7Dx3). *A*, median tumor weight curves; *B*, percentage mean adjusted body weight (tumor weight subtracted) change until the first mouse reached toxic end point. *C*, Kaplan-Meier survival curves for mice, death included both toxic and cancer end points.

systemic toxicity, as evidenced by sLAG-Dox being much better tolerated in mice than sALAL-Dox and sAIAL-Dox. Expression of CD10 on normal tissues may contribute to the toxicity because CD10 has been reported to be present on a number of different cell types including precursor B cells, activated B cells, and epithelial cells in lung, colon, and kidney (17). Nevertheless, the lower doses required for potent antitumor effects suggest that CD10-cleavable prodrugs may be preferable to those cleaved only by TOP. Significantly, CD10 is expressed on the proximal convoluted tubular epithelial cells of the kidney, which may represent an important site of elimination and metabolism of the peptide prodrugs.

CD10 is frequently expressed on many tumor types, such as non-Hodgkin's and Hodgkin's lymphoma, leukemia, multiple myeloma, renal cell carcinoma, prostate carcinoma, endometrial stromal sarcomas, pancreatic adenocarcinoma, and malignant melanoma (18). In addition, CD10 is selectively overexpressed by stromal cells in invasive colon and breast tumors (19, 20). The identification of this mechanism of prodrug activation therefore suggests appropriate clinical indications for evaluating CPI-0004Na and related prodrugs.

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