HYDROLYSIS OF DINITROBENZAMIDE PHOSPHATE PRODRUGS: THE ROLE OF ALKALINE PHOSPHATASE

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SUMMARY

Phosphate prodrugs which undergo hydrolysis *in vivo* have been used to improve the solubility and pharmacokinetic properties of a number of drugs. Dinitrobenzamide mustards (DNBM) are examples of such drugs. We investigated the ability of purified alkaline phosphatase isoforms to dephosphorylate three DNBM phosphate prodrugs. In addition, the relative rate of dephosphorylation of these phosphate prodrugs in a number of tissues was determined. These phosphate prodrugs are indeed substrates for alkaline phosphatase, with time dependent formation of the hydrolysis product. Intestinal alkaline phosphatase (IAP) and placental alkaline phosphatase (PLAP) had the highest activity for these substrates and compound P2 was the most rapidly metabolised. Similarly, compound P2 had the shortest half life in mouse serum ($t_{½}$ = 1.15 h) compared with P1 ($t_{½}$ = 13.34 h) and P3 ($t_{½}$ = 4.4 h). However, serum has very low dephosphorylase activity for these substrates compared with intestine and liver homogenates. In addition, there is little or no difference in the relative rate of dephosphorylation of each of the three compounds in mouse tissues in contrast to the pattern observed with purified alkaline phosphatase and mouse serum. Hence additional phosphatase enzymes may be involved in the metabolism of phosphate prodrugs *in vivo*.

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KEY WORDS

phosphate prodrugs, alkaline phosphatase, metabolism

INTRODUCTION

Dinitrobenzamide mustards (DNBM) are nitroaromatic anticancer prodrugs that can be selectively bioactivated within the tumour tissue by endogenous nitroreductases /1/ or by *E coli* nitroreductase in gene-directed enzyme prodrug therapy (GDEPT) /2-4/. To enhance drug penetration into poorly vascularised and hypoxic regions of the tumours, highly lipophilic compounds have been developed. However, this approach partially limits the solubility of the compounds and in particular the ability to administer these compounds by intravenous injection. One strategy to overcome the poor solubility of these compounds is to attach a phosphate moiety /5,6/. This approach has already been proven to be effective in improving the pharmacokinetic profile of drugs, such as fosphenytoin /7/ and etoposide-phosphate /8/.

Alkaline phosphatase (AP) is an enzyme that is associated with the hydrolysis of phosphate prodrugs. AP (E.C.3.1.3.1) is an ecto-metallo-enzyme which is catalytically active on the external surface of the cell membrane /9-11/. A number of different isozymes of alkaline phosphatase (AP) have been identified, including the tissue specific placental (PLAP), intestinal (IAP) and germ-cell (GCAP) isoforms, as well as the tissue non-specific isoform (TNAP) which is expressed in bone ($_B$TNAP) kidney ($_K$TNAP), liver ($_L$TNAP) and neutrophils ($_N$TNAP). These isoforms arise from the existence of multiple gene loci; i.e. 1p36.1-34 for the tissue non-specific (TNAP) isoform and three genes at 2q34-37 for the tissue specific isoforms /12/. In addition, TNAP also undergoes post-translational modification resulting in tissue selective expression /12/. In certain cancers there is an increased expression of the carcino-placental (Regan) isoform of AP /13/. This isoform, which is normally not found in adults, is indistinguishable from the placental isoform and is believed to be derived from the de-repression of the placental phosphatase gene in malignant cells /13, 14/.

Abbreviations: AP = alkaline phosphatase; IAP = intestinal alkaline phosphatase isoform; PLAP = placental alkaline phosphatase isoform; TNAP = tissue non-specific alkaline phosphatase isoform; DNBM = dinitrobenzamide mustard.

In the present study we examined the potential role of purified AP to dephosphorylate a series of DNBM phosphate prodrugs (Fig. 1) at physiological pH. The results provide an insight into the contributions of the different AP isoforms, as well as the relative importance of various tissues, in this dephosphorylation process.

MATERIALS AND METHODS

Materials

The dinitrobenzamide (DNBM) hydroxyethyl phosphate prodrugs 3-[[5-[bis(2-bromoethyl)amino]-2,4-dinitrobenzoyl]amino]propyl dihydrogen phosphate (P1), 2-[[2-[bis(2-bromoethyl)amino]-3,5-dinitrobenzoyl]amino]ethyl dihydrogen phosphate (P2) and 3-({3-[bis(2-bromoethyl)amino]-2,6-dinitrobenzoyl}amino)propyl dihydrogen phosphate (P3) (Fig. 1), the respective hydrolysis products 5-[bis(2-bromoethyl)amino]-*N*-(3-hydroxypropyl)-2,4-dinitrobenzamide (1), /15/, 2-[bis(2-bromoethyl)amino]-*N*-(3-hydroxyethyl)-3,5-dinitrobenzamide (2), 3-[bis(2-bromoethyl)amino]-*N*-(3-hydroxypropyl)-2,6-dinitrobenzamide (3) and 2-[*N,N*-bis(2-bromoethyl)amino]-3,5-dinitrobenzamide, used as an internal standard, were kindly supplied by the Auckland Cancer Society Research Centre, University of Auckland, New Zealand, and synthesised as described previously /6/.

TNAP (kidney, porcine), PLAP (placenta, human) and IAP (intestine, bovine calf) isoforms were purchased from Sigma-Aldrich Co. (Germany). Ammonium formate was obtained from Acros Organics (USA) and acetonitrile from Scharlau Chemie S.A. (Spain). All other chemicals used were of analytical grade.

Tissue homogenate preparation

Mouse (CD-1 *nu/nu*) liver, intestine and blood were collected under the appropriate institutional ethical guidelines and stored frozen. Frozen WiDr tumour xenografts were kindly provided by the Auckland Cancer Society Research Centre. Tissues were defrosted, wet weight recorded and a 25% (w/v) homogenate was prepared in ice-cold Tris buffer (0.05 M, pH 7.4) with an automatic homogeniser (Ika Labrotechnik, Germany). The homogenate was then centrifuged (Thermo Electron Corporation, Centra CL3R refrigerated centrifuge)

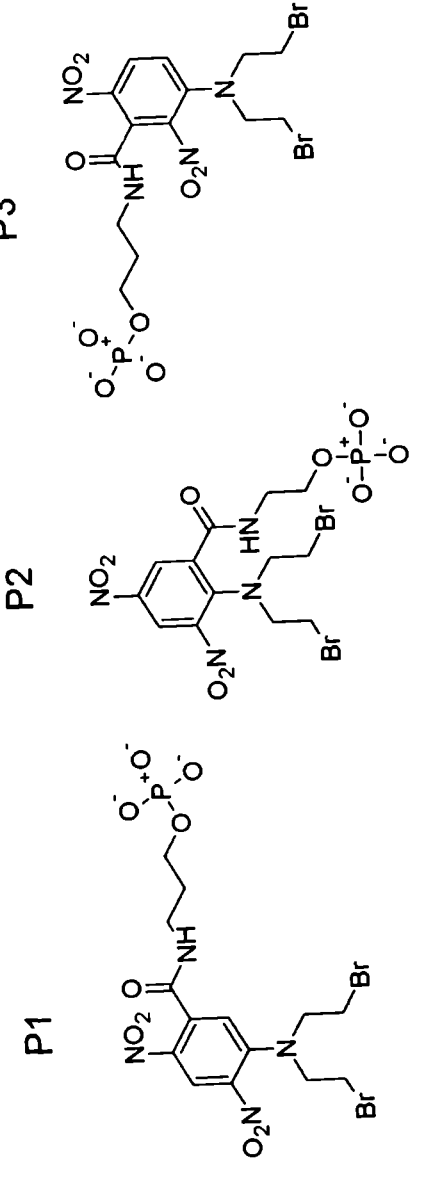

Fig. 1: Structures of the dinitrobenzamide mustard (DNBM) phosphate prodrugs studied.

at 1,000 *g* at 4°C for 30 minutes, and the supernatant frozen at -80°C until use. Blood was collected into heparinised tubes and diluted (1:4 v/v) in Tris buffer (0.05 M, pH 7.4) prior to use. Serum was prepared from mouse whole blood collected in non-heparinised tubes and allowed to clot overnight at 4°C. After centrifugation at 1,500 *g* for 10 minutes, the supernatant was removed and diluted (1:4 v/v) in Tris buffer (0.05 M, pH 7.4) prior to use.

Dephosphorylation of DNBM phosphate prodrugs by purified AP

Following preliminary studies to determine appropriate time and enzyme concentration for optimal catalytic conditions, the phosphate prodrugs (300 μM) were incubated in Tris buffer (0.05 M, pH 7.4) in the presence and absence of purified AP isoforms (0.05 U/ml; equivalent to 6.8, 360 and 8.9 ng for TNAP, IAP and PLAP, respectively) at room temperature. Sequential samples were removed every 30 minutes to determine the loss of phosphate prodrug and the formation of the corresponding alcohol metabolite. The rate of dephosphorylation was measured for each isoform until at least 50% loss of the substrate was observed (2-24 hours depending on isoform). To compare the activity of the isoforms at pH 7.4, the rate of reaction was calculated as nmol per μg of AP protein.

Time-dependent dephosphorylation of DNBM phosphate prodrugs by mouse serum

Each DNBM phosphate prodrug (100 μM) was incubated with mouse serum (1.5 ml) at 37°C in a shaking water bath. Samples (0.1 ml) were collected at the following time points: 0, 2, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150 and 180 minutes. Ice-cold methanol (200 μl) was added immediately with the addition of internal standard (5 μl of 500 μM). The solution was vortexed and proteins precipitated (-80°C, overnight) and centrifuged at 9,300 *g* for 20 minutes. The supernatant was then evaporated to dryness under vacuum and the resulting residue was resuspended in 100 μl mobile phase (45 mM ammonium formate, pH 4.5, and 80% acetonitrile, 50:50 v/v) and 50 μl injected onto the HPLC. Samples were then analysed by HPLC as described below.

Dephosphorylation of DNBM phosphate prodrugs by mouse tissue homogenates

Each DNBM phosphate prodrug (100 μM) was incubated with 100 μl of tissue homogenate (25% w/v), blood or serum (25% v/v) in triplicate for 5 minutes at 37°C in a shaking water bath. Samples were processed and analysed as described above. Relative dephosphorylation of each phosphate prodrug was determined following normalisation of activity based on the total wet weight of the organ or total blood volume.

Analysis

HPLC analysis was carried out using an Agilent 1200 system with an Alltech Altima C8 5μ (3.2 × 150 mm) column. The mobile phase comprised (A) 45 mM ammonium formate (pH 4.5) and (B) 80% (v/v) acetonitrile in water at a flow rate of 0.5 ml/min. Initial conditions were 20% B (0-4 min), increasing to 90% (4-22 min) and returning to 20% (22-30 min). Detection was via diode array absorbance at 370 nm with a bandwidth of 4 nm (reference at 550 nm with bandwidth of 50 nm). To quantify the DNBM phosphate prodrugs and the corresponding alcohol metabolite, standard curves were prepared using authentic standards. Mouse serum samples (0.1 ml) containing drug standards (0-100 μM) with the addition of ice-cold methanol (200 μl) and internal standard (5 μl of 500 μM) were processed in the same manner as the incubations described above prior to injection onto HPLC. Additional standard curves were constructed from sequential injections (5-80 μl) of a 300 μM drug stock solution directly onto the HPLC.

RESULTS

To determine the role of AP in the dephosphorylation of the DNBM phosphate prodrugs, compounds P1, P2 and P3 were incubated with purified TNAP, IAP and PLAP isoforms at physiological pH. HPLC analysis of the incubate indicated loss of DNBM phosphate prodrug and formation of a single more hydrophobic peak with retention time and absorbance spectra the same as the respective alcohol metabolite (Fig. 2). This confirmed that the DNBM phosphate

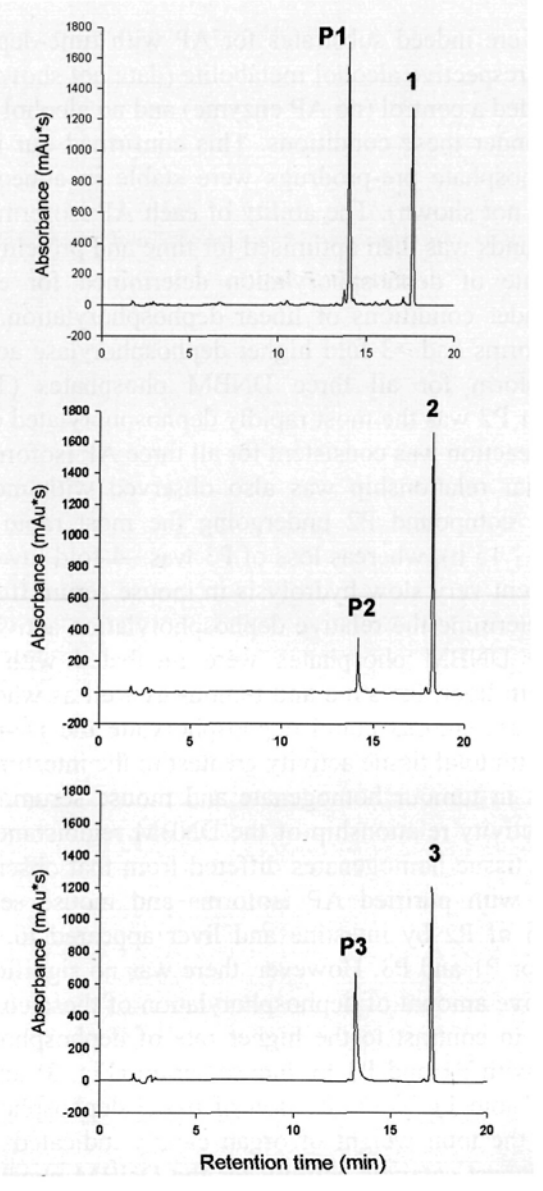

Fig. 2: Formation of the hydrolysis products of the dinitrobenzamide mustard (DNBM) phosphate prodrugs following incubation with purified alkaline phosphatase. HPLC chromatograms of incubations of the DNBM phosphate prodrugs (a) P1, (b) P2 and (c) P3 with intestinal alkaline phosphatase (IAP). The respective hydrolysis products (1, 2, 3) formed were confirmed by comparison with authentic samples.

prodrugs were indeed substrates for AP with time-dependent formation of the respective alcohol metabolite (data not shown). All incubations included a control (no AP enzyme) and no alcohol formation was observed under these conditions. This confirmed our initial findings that the phosphate pre-prodrugs were stable in aqueous solution at 37°C (data not shown). The ability of each AP isoform to metabolise the compounds was then optimised for time and protein concentration, and the rate of dephosphorylation determined for each drug and isoform under conditions of linear dephosphorylation. The IAP and PLAP isoforms had >3-fold higher dephosphorylase activity than the TNAP isoform for all three DNBM phosphates (Table 1). The regioisomer P2 was the most rapidly dephosphorylated compound and this rapid reaction was consistent for all three AP isoforms studied.

A similar relationship was also observed with mouse serum *in vitro*, with compound P2 undergoing the most rapid dephosphorylation ($t_{½}$ =1.15 h), whereas loss of P3 was ~4-fold lower ($t_{½}$ = 4.4 h). P1 underwent very slow hydrolysis in mouse serum ($t_{½}$ =13.34 h). In order to determine the relative dephosphorylation activity of different tissues the DNBM phosphates were incubated with tissue homogenates from liver, intestine and tumour as well as whole blood (Fig. 3). All tissues studied could dephosphorylate the DNBM phosphate prodrugs with total tissue activity greatest in the intestinal homogenate and lowest in tumour homogenate and mouse serum. However, the structure-activity relationship of the DNBM regioisomers when incubated with tissue homogenates differed from that observed following incubation with purified AP isoforms and mouse serum. Dephosphorylation of P2 by intestine and liver appeared to be lower than observed for P1 and P3. However, there was no significant difference in the relative amount of dephosphorylation of these compounds (Fig. 3). This is in contrast to the higher rate of dephosphorylation of P2 compared with P1 and P3 by mouse serum (Fig. 3) and purified AP isoforms (Table 1). Normalisation of tissue dephosphorylase activity relative to the total weight of organ clearly indicated that the tissue with the highest catalytic activity for the DNBM phosphates was the intestine, with a relatively high activity also observed with liver homogenate. In contrast, whole blood and tumour had relatively low catalytic activity for the dephosphorylation of the DNBM phosphates and serum activity was minimal.

TABLE 1

Rate of dephosphorylation of the DNBM phosphate prodrugs by purified alkaline phosphatase isoforms

DNBM phosphate prodrug	Alkaline phosphatase isoform Dephosphorylation activity (nmol/μg)		
	TNAP	IAP	PLAP
P1	0.016 ± 0.003	0.206 ± 0.046	0.188 ± 0.028
P2	0.046 ± 0.009	0.258 ± 0.073	0.214 ± 0.057
P3	0.046 ± 0.021	0.208 ± 0.023	0.140 ± 0.015

Data are means ± standard deviation of triplicate incubations. Formation of the hydrolysis product of the DNBM phosphate prodrug is normalised to the total amount of enzyme in the incubation (nmol/μg).
DNBM = dinitrobenzamide mustard; TNAP = tissue non-specific alkaline phosphatase; IAP = intestinal alkaline phosphatase; PLAP = placental alkaline phosphatase.

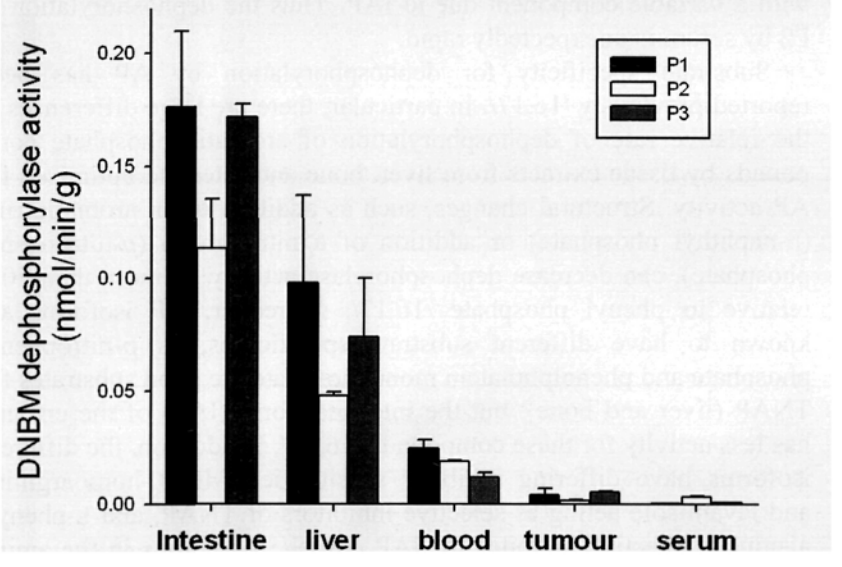

Fig. 3: Comparison of the relative dephosphorylation rate of the dinitrobenzamide mustard (DNBM) phosphate prodrugs normalised to the total wet weight of organ. Data are shown as formation of the hydrolysis product and normalised to the total wet weight of the organ (g) or total volume (ml) for blood and serum. The data are means ± standard deviation of triplicate incubations.

DISCUSSION

The results of this study indicate that purified forms of AP can dephosphorylate DNBM phosphate prodrugs *in vitro,* thus confirming the presumed role of this enzyme in this process *in vivo.* The IAP and PLAP isoforms appear to be the most catalytically active for dephosphorylation of the DNBM phosphate prodrugs. In mouse serum there appears to be a substrate specificity for this reaction, as within the regioisomers studied, P2 was more rapidly dephosphorylated, and overall this regioisomer appears to be a slightly better substrate for purified AP isoforms than P1 or P3.

In non-pathological states, although serum AP activity is relatively low, detectable levels of TNAP shed from liver and bone are observed /13/. However, IAP can also be detected in serum particularly after meals, although this accounts for less than 25% of total AP activity in serum /13/. Additional TNAP may also be present in serum due to contamination by neutrophils. Hence in normal serum the major isoform responsible for dephosphorylase activity is probably TNAP with a variable component due to IAP. Thus the dephoshorylation of P2 by serum is unexpectedly rapid.

Substrate specificity for dephosphorylation by AP has been reported previously /16,17/. In particular, there are large differences in the relative rate of dephosphorylation of aromatic phosphate compounds by tissue extracts from liver, bone and intestine optimised for AP activity. Structural changes, such as addition of an aromatic ring (β-naphthyl phosphate) or addition of a nitro group (*p*-nitrophenyl phosphate), can decrease dephosphorylase activity by more than 70% relative to phenyl phosphate /16,17/. Moreover, AP isoforms are known to have different substrate specificities, as *p*-nitrophenyl phosphate and phenolphthalein monophosphate are good substrates for TNAP (liver and bone), but the intestinal form (IAP) of the enzyme has less activity for these compounds /16,17/. In addition, the different isoforms have differing inhibitor sensitivities with L-homoarginine and levamisole acting as selective inhibitors of TNAP, and L-phenylalanine a selective inhibitor for IAP /18,19/. Variations in the amino acid sequence of the active site of AP isoforms have been reported /20/ and are likely to result in the different substrate and inhibitor profiles of these isoforms.

Surprisingly, the rapid dephosphorylation of P2 compared with P1 and P3 observed in mouse serum was not seen in other tissues such as intestine and liver. Indeed administration of DNBM phosphate prodrugs to mice indicates that there is no overall difference in the dephosphorylation of these compounds /21,22/.

Of the tissues studied the intestine has the highest total catalytic activity for the dephosphorylation of the DNBM phosphate compounds. However, *in vivo* IAP is located predominantly on the brush border of epithelial cells lining the small intestine /23/ and hence the intestine may only be an important site of dephosphorylation following an oral dose of phosphate prodrugs. Interestingly, the chemotherapy agent cisplatin is associated with gastrointestinal toxicity and damage to the brush border membrane of the GI tract /24/ and a subsequent decrease in AP activity. However, cisplatin is also a direct inhibitor of IAP *in vitro* /24/; the effect of co-adminstration of cisplatin with DNBM phosphate prodrugs on the rate of dephosphorylation of these compounds is not known. Similarly, levamisole is given as adjuvant therapy in 5-flurouracil treatment of colon cancer /25/ and this compound along with theophylline and lidocaine are known pharmacological inhibitors of AP /18,19/ and as such may also effect dephosphorylation *in vivo*.

The liver also has very high total tissue phosphatase activity and this was more than two-fold higher than whole blood. A similar high activity of liver compared with blood has been reported for the dephosphorylation of combretastatin /26/. As AP is an ecto-enzyme that is catalytically active on the surface of the cell membrane the liver is likely to be a major site of dephosphorylation of the DNBM compounds with systemic (blood) dephosphorylation playing a lesser role. However, it is important to note that the activity of AP in whole blood will be affected by pathological states and also treatment with granulocyte colony-stimulating factor (G-CSF). TNAP is expressed in neutrophils and activity can be induced by G-CSF /27/ which is used as adjuvant therapy in some chemotherapy schedules. Expression of this isoform is also elevated in bacterial infections and Hodgkin's disease but significantly decreased in chronic myeloid leukaemia /27/. Hence the relative role of liver versus systemic (blood) dephosphorylation of DNBM phosphate prodrugs may differ in a clinical setting.

Expression of the carcino-placental isoform is increased in some cancers and this is believed to be due to de-repression of the PLAP

gene in malignant cells /28/. The DNBM phosphate prodrugs were shown to be good substrates for purified PLAP but the role of the carcino-placental isoform is currently not known. Phosphate prodrugs of aniline mustards, which have structural similarity to the DNBM compounds, have however been shown to be substrates for the carcino-placental isoform in HeLa cells /29/. A sample of WiDr human tumour xenograft was used in the present study as a preliminary assessment of the relative ability of neoplastic cells to dephosphorylate DNBM phosphate prodrugs compared with AP expressed in normal tissue. WiDr tumour had relatively low DNBM phosphatase activity compared with normal tissues. However, other tumour types may overexpress AP, and whether a difference in phosphatase activity in neoplasia has any influence on DNBM dephosphorylation requires further study.

Although we have demonstrated that DNBM phosphate prodrugs are substrates for AP the relationship between dephosphorylation by purified isoforms, serum and tissues is not clear. AP exists in tissues as a tetramer but when shed into the circulation it is found as a mixture of dimers and tetramers /30/. Both forms are active /31/; however, differences in the number of phosphate binding sites are observed in the tetramer /32/ and hence substrate specificity may also be altered.

Alkaline phosphatase shows maximal biochemical activity *in vitro* at alkaline pH (pH 8-10) and the physiological role of this enzyme is not clear. However, a number of other orthophosphoric monoester phosphohydrolases also exist, including acid phosphatase which has maximal biochemical activity *in vitro* at acid pH (pH 4-6) /33/. Two types of acid phosphatase exist: a high molecular weight protein (>100 kDa) localised to the lysosomal fraction of the cell, and a low molecular weight protein (<20 kDa) localised in the cytosol /34/. Multiple isoforms of acid phosphatase also exist, including the tartrate-resistant acid phosphatase (TRAP) found mainly in bone and macrophages. TRAP has catalytic specificity for a wide range of natural and synthetic substrates, including phosphoric esters of aromatic alcohols, such as *p*-nitrophenyl phosphate, α-naphthyl phosphate and phosphotyrosine /35/. The dephosphorylation of the DNBM phosphate prodrugs was determined at physiological pH (pH 7.4) and hence other phosphatase enzymes, such as acid phosphatase, either working alone or in conjunction with alkaline phosphatase, may also dephosphorylate

the DNBM phosphate prodrugs in whole tissue homogenates and *in vivo*. Indeed, dephosphorylation of the MRI contrast agent Teslascan is catalysed by both alkaline and acid phosphatases /36/ and this compound is more rapidly dephosphorylated *in vivo* than *in vitro* /36/. Acid phosphatase activity in normal serum is ~50-fold lower than alkaline phosphatase /36/, and this may explain the relative selectivity of serum for P2, a selectivity which was not observed in whole blood or tissues.

A number of phosphate prodrugs have been proposed, or are used, in the cancer chemotherapy field, and include amifostine, an organic thiophosphate prodrug (used as an adjuvant to prevent chemotherapy related toxicity) /37/; etoposide-phosphate /8/; combretastain A4 phosphate /26/; cyclic phosphate prodrugs for targeted delivery to the liver in hepatocellular carcinoma /38/; and radioidoniated quinazolinone derivatives for AP enzyme mediated diagnosis and therapy /39/. A number of these approaches assume a selective catalytic role for either systemic or tumour AP. However, the importance of other phosphatases, and the involvement of tissues such as liver, in the dephosphorylation of such compounds should not be overlooked, as the site of these phosphatases may influence the biodistribution of the active compounds.

ACKNOWLEDGEMENTS

Funding for this work was provided by the University of Auckland Staff Research Fund.

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