Biochemical Pharmacology, Vol. 27, pp. 199–202. Pergamon Press, 1978. Printed in Great Britain.

ENZYME ACTIVATED ANTI-TUMOR AGENTS—IV

COMPARATIVE KINETICS OF *N,N-p*-DI-2-CHLOROETHYLAMINOPHENYL PHOSPHATE HYDROLYSIS CATALYSED BY PHOSPHATASES OF NORMAL AND NEOPLASTIC TISSUES

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(*Received* 20 *May* 1977; *accepted* 17 *June* 1977)

Abstract—Values of the Michaelis constant (K_m) and maximum velocity (V) have been determined for the hydrolytic conversion of *N,N-p*-di-2-chloroethylaminophenyl phosphate (AMPh) to *N,N-p*-di-2-chloroethylaminophenol (AMOH) catalysed by acid and alkaline phosphatases present in mouse serum and in whole homogenates of mouse bone marrow, small intestine mucosa and the Adj-PC6A plasmacytoma. K_m values were generally similar. Alkaline phosphatase of the small intestine mucosa and acid phosphatase of the bone marrow catalysed the reaction more rapidly that the corresponding tumour enzymes. Serum phosphatases were also active. The results suggest that the greater selectivity of AMPh compared to that of AMOH may not be due to a localised conversion of AMPh to AMOH in the tumor.

N,N-p-di-2-Chloroethylaminophenol(*p*-hydroxyaniline mustard, AMOH) is a potent but unselective cytotoxic drug [1]. Connors and Whisson [2] proposed that the preferential liberation of AMOH from its *O*-phosphate derivative (*N,N-p*-di-2-chloroethylaminophenyl phosphate, AMPh) in neoplastic tissues containing high levels of phosphatases would result in a selective antineoplastic effect. Following the synthesis of AMPh [3] it was shown that this agent was similar in chemical reactivity to AMOH [4]. It was proposed, however, that the ionic character of AMPh would retard its penetration through cell membranes; thus the expression of cytotoxic activity would be dependent upon catalytic activation by phosphatases [3]. Enzyme kinetics studies showed that the hydrolytic conversion of AMPh to AMOH was catalysed by acid and alkaline phosphatases [5, 6].

Bukhari *et al.* have shown that the therapeutic index (LD_{50}/ED_{90}) for AMPh against the Adj-PC6A mouse tumour was greater than the corresponding value for AMOH [3]. The aim of the present investigation was to determine whether the selective activity of AMPh is due to preferential conversion to AMOH by tumour enzymes. Values of the Michaelis constant (K_m) and the maximum velocity (V) were determined for the hydrolysis of AMPh catalysed by acid and alkaline phosphatases present in whole homogenates of the Adj-PC6A tumour. These were compared with values for mouse bone marrow and intestinal mucosa which are rapidly proliferating host tissues particularly sensitive to alkylating agent toxicity [7]. The ability of serum enzymes to catalyse the reaction was also investigated.

MATERIALS AND METHODS

Reagents

AMPh (dicyclohexylamine salt) and AMOH (hydrochloride) were kindly supplied as a gift from the Chester Beatty Research Institute, London. Other reagents were of analytical reagent grade or of the highest grade available commercially.

Animals and Adj-PC6A tumour

Balb/c female mice were obtained from an inbred pure strain colony maintained in this department. They were allowed Oxoid 41B (Oxo Limited, London) and water *ad lib.* The Adj-PC6A plasmacytoma, obtained originally from the Chester Beatty Research Institute, London, was passaged routinely in the ascites form as described previously [8]. Solid tumours were obtained by subcutaneous inoculation of 2×10^6 cells in the flank region.

Antineoplastic assay

Antineoplastic assays were carried out using methods described previously [3, 8, 9]. AMOH was administered by intraperitoneal injection in 10% dimethyl sulphoxide/arachis oil and AMPh was given in water by the same route. Values of the ED_{90} (dose required to reduce mean tumour weight to 10 per cent of the control) and LD_{50} (dose required to reduce mean survival to 50 per cent of the control) were obtained directly from dose–response curves. The chemotherapeutic index was given by LD_{50}/ED_{90}. Data presented are the mean values of two determinations.

Tissue preparation

Mice were killed by cervical dislocation and exsanguinated by cardiac puncture. Tumours, of the same size as those used for the antineoplastic assay (*ca* 0.5 g wet weight), were dissected out and rinsed thoroughly in ice-cold distilled water. The small intestine was removed and washed several times with ice-cold saline (0.95% w/v) to expel the luminal contents; it was then placed on an ice-cold glass plate and the mucosa was removed by applying gentle strokes to the serosal surface. Bone marrow was collected by forcing ice-cold saline through the femur. Bone mar-

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row cells and intestinal scrapings were washed twice in ice-cold saline and then sedimented by centrifugation (400 *g*, 5 min). Whole tissue homogenates (1–2% w/v) were prepared by homogenisation of the tissues in ice-cold distilled water using an Ultra-Turrax Homogeniser (Janke and Kunkel, KG). Blood serum was obtained by centrifugation of whole blood (400 *g*, 5 min).

Enzyme assays

Enzyme assays were carried out in a shaking water-bath at 37° using an incubation period of 10–20 min. The AMPh concentration in the assay was varied over the range 0.2–2.0 mM, the maximum value of which was limited by the solubility of the drug.

1. *Acid phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2).* The reaction mixtures contained acetic acid–sodium acetate buffer (66 mM), AMPh and tissue homogenate or serum (0.2 ml) in a total volume of 0.6 ml. The optimum pH was determined for each tissue. Values obtained were: small intestine mucosa, 4.9; bone marrow, 5.7; Adj-PC6A tumour, 5.5; serum, 5.0.

2. *Alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC: 3.1.3.1.).* Conditions employed in the assay of alkaline phosphatase activity were identical to those described above except that the buffer was sodium carbonate–sodium bicarbonate (33 mM). Each of the alkaline phosphatases studied exhibited a dependence of the pH optimum upon AMPh concentration, higher values being obtained at increasing substrate concentrations. Thus it was necessary to determine the pH optimum at each substrate concentration used.

The enzyme reactions were terminated by the addition of sulphuric acid (5 N, 0.5 ml) and the inorganic phosphate ion released during the incubation was determined using methods reported previously [5, 6]. Under the conditions described the total amount of inorganic phosphate released was directly proportional to both the length of the incubation period and the amount of enzyme solution added.

Protein concentration was determined by the method of Lowry *et al.* [10] using bovine serum albumin (Sigma Chemical Co.) as standard. Enzyme activities were expressed as μmoles inorganic phosphate released/min/g protein for tissues and as μmoles/min/l for serum.

Determination of kinetic constants

Values of the Michaelis constant (K_m) and the maximum velocity (V) for the hydrolysis of AMPh were determined from double-reciprocal (Lineweaver–Burk) plots of initial velocity against substrate concentration [11]. The line of best fit was computed by the method of least squares regression analysis using a Multi-8 computer. Data shown are mean values of two experimental determinations.

RESULTS

Antineoplastic assays

The results of the antineoplastic assays using the Adj-PC6A tumour are given in Table 1. It may be seen that the chemotherapeutic index for AMPh was greater than the corresponding value for AMOH, thus confirming previous findings [3].

Table 1. Values of the ED_{90}, LD_{50} and chemotherapeutic index for AMPh and AMOH against the solid Adj-PC6A plasmacytoma

Drug	ED_{90} (μmoles/kg)	LD_{50} (μmoles/kg)	Chemotherapeutic index
AMPh	4.3	221	51.4
AMOH	6.0	52	8.7

Enzyme kinetics

The acid and alkaline phosphatases of mouse bone marrow, small intestine mucosa, serum and the Adj-PC6A plasmacytoma were all able to catalyse the hydrolysis of AMPh. Plots of initial velocity against substrate concentration were rectangular hyperbolas. Double-reciprocal plots of these data were linear in all instances indicating that the reaction kinetics were of Michaelis–Menten type [12]. A typical plot is shown in Fig. 1.

Values of K_m and V for the hydrolysis of AMPh catalysed by acid and alkaline phosphatase in whole homogenates of small intestine mucosa, bone marrow, serum and Adj-PC6A solid tumour are given in Tables 2 and 3. It may be noted that the K_m value for the Adj-PC6A tumour alkaline phosphatase was higher than the corresponding value for the small intestine mucosa and bone marrow enzymes but lower than that of the serum.

The value of the maximum velocity for the tumour enzyme was greater than that observed for the bone marrow enzyme but lower than that of the small intestine mucosa.

It may also be seen that K_m values for the hydrolysis of AMPh by various acid phosphatases were generally similar. The values were of the same order as those observed for the alkaline phosphatases. However, the value of the maximum velocity for the Adj-PC6A tumour enzyme was greater than the corresponding value for the small intestine mucosa enzyme but lower than that of the bone marrow.

By calculating the arithmetic sum of the maximum velocities for the acid and alkaline phosphatases of

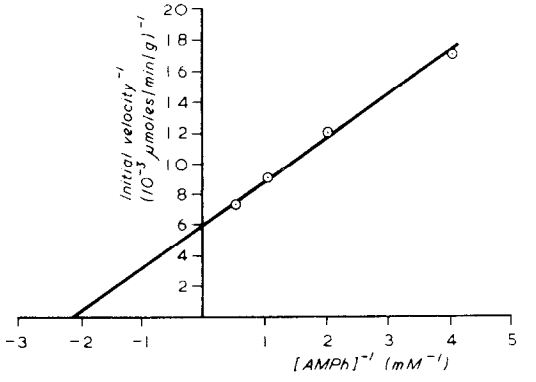

Fig. 1. Double-reciprocal plot of initial velocity against substrate concentration for the hydrolysis of AMPh catalysed by alkaline phosphatase present in whole homogenate of the solid Adj-PC6A plasmacytoma.

Table 2. Values of K_m and V for the hydrolysis of AMPh catalysed by alkaline phosphatases*

Tissue	K_m	V
Small intestine mucosa	0.41	269
Bone marrow	0.27	135
Serum	1.21	42
Adj-PC6A tumour	0.50	176

* Units of V are μmoles inorganic phosphate released/min/g tissue protein or/l serum; units of K_m are mM.

Table 3. Values of K_m and V for the hydrolysis of AMPh catalysed by acid phosphatases*

Tissue	K_m	V
Small intestine mucosa	0.36	27
Bone marrow	0.50	71
Serum	0.35	16
Adj-PC6A tumour	0.33	50

* For other details see legend to Table 2.

each of the various tissues a comparative estimate of their total phosphatase activities may be obtained. The order of activity was found to be: small intestine mucosa > tumour ~ bone marrow. Maximum velocity values for serum enzymes are expressed in different units and are not directly comparable with those of the tissue enzymes. However, the values obtained did indicate a considerable level of catalytic activity.

DISCUSSION

The present studies have confirmed in our own laboratory the finding of Bukhari *et al.* [3] that AMPh is more selective than AMOH against the Adj-PC6A plasmacytoma *in vivo*. According to the proposed hypothesis [2] the antineoplastic activity of AMPh would be dependent upon its more rapid conversion to AMOH in the tumour than elsewhere. In particular, a selective effect would require a preferential hydrolysis in the tumour compared to the rapidly proliferating hematopoetic cells of the bone marrow and epithelial cells of the intestinal mucosa which are acutely sensitive to alkylating agent toxicity [7].

The enzyme kinetics studies, reported in the present communication, have shown that the acid and alkaline phosphatases of the bone marrow, small intestine mucosa, serum and solid Adj-PC6A tumour all have the ability to catalyse the hydrolytic conversion of AMPh to AMOH. The affinities of these various enzymes for AMPh, as determined by their respective K_m values, are generally similar. It should be pointed out that the K_m values for AMPh are of the same order as the concentration which would be expected, on the basis of a uniform distribution, following an LD_{50} dose of the drug *in vivo* (see Table 1).

Important differences were noted in the values of the maximum velocity of AMPh hydrolysis catalysed by phosphatases of normal and neoplastic tissues. Alkaline phosphatase of the small intestine mucosa and acid phosphatase of the bone marrow catalysed the hydrolysis of AMPh more rapidly than the corresponding tumour enzymes. Moreover, the total phosphatase levels present in these tissues would suggest that a preferential activation of AMPh would occur in the small intestine mucosa and, in addition, that the rate of AMPh hydrolysis in the Adj-PC6A tumour would be similar to that in the bone marrow.

It may be concluded, therefore, that the greater selectivity of AMPh as compared to that shown by AMOH against the Adj-PC6A tumour cannot be explained by the comparative kinetic properties of phosphatases present in whole homogenates of the tumour and normal tissues *in vitro*. Similar results have also been obtained for a different tumour line (HT 67)*.

The behaviour of enzymes *in vivo* may be quite different from that observed *in vitro* (see Youdim and Woods [14]); thus the comparative activation of AMPh may differ in these two situations. On the other hand, the subcellular localisation of the phosphatases may be important in determining the site of AMOH production. Previous studies have shown that the location of β-glucuronidase may be involved in the selective activation of the *O*-glucuronide derivative of AMOH [8, 15]. Differences in the solubility and pharmacokinetic properties of AMOH and AMPh may also contribute towards their disparate cancerostatic activities. Although the two compounds are similar in chemical reactivity [4] AMOH is considerably more lipophilic than AMPh*.

The present studies have demonstrated the ability of mouse serum phosphatases to catalyse the rapid hydrolysis of AMPh. These findings support previous results which have shown that AMPh is considerably more toxic towards cells in culture in the presence of active serum phosphatases [6]. In view of the observed catalytic activity of serum phosphatases it would be expected that AMOH would be liberated rapidly in the blood and distributed throughout the animal body. Previous studies have demonstrated the hydrolysis *in vivo* of drugs conjugated with phosphate groups [16–18]. Moreover, recent experiments have shown that phenyl phosphate, related in structure to AMPh and exhibiting markedly similar kinetic constants for serum phosphatases, is hydrolysed extensively in the mouse [19].

Chemotherapy screening studies in our own laboratory [13] and elsewhere [3] have shown that AMPh, though more effective than AMOH, is less selective than the parent compound aniline mustard. However, it has not been possible to assess the effect of AMPh against neoplastic tissues exhibiting phosphatase levels in excess of those of the sensitive host tissues. A number of transplantable mouse tumours have been screened for phosphatase activity and of these the Adj-PC6A plasmacytoma was the most active*.

The results of the present study have indicated that in view of the ubiquitous nature of the phosphatases and the unfavourable distribution of their total activities among normal and neoplastic tissues a truly localised conversion of AMPh to AMOH would not be expected. The greater selectivity of AMPh compared to that of AMOH may indicate the involvement of additional factors.

Acknowledgements—The authors wish to thank Professor W. C. J. Ross, Mr. J. L. Everett and Dr. D. E. V. Wilman

* P. Workman and J. A. Double, unpublished results.

of the Chester Beatty Research Institute, London, and Dr. T. A. Connors of the MRC Toxicology Unit, Carshalton, for providing the alkylating agents used in this study, and also Mrs. M. Hutchinson for the animal breeding. Financial support was provided by the Medical Research Council, The Yorkshire Cancer Research Campaign and the National Large Bowel Cancer Project, Grant No. 5R26 CA 18126-02. This work will be submitted by one of us (P.W.) in part fulfillment of the Ph.D. degree in the University of Leeds.

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