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ENZYME ACTIVATED ANTI-TUMOUR AGENTS

CONJUGATES OF *p*-HYDROXYANILINE MUSTARD AS SUBSTRATES FOR HYDROLYTIC ENZYMES

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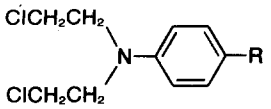
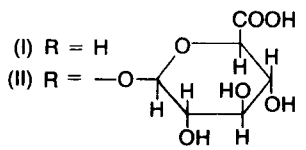
Abstract— The *O*-phosphate, *O*-sulphate and *O*-glucuronide of *p*-hydroxyaniline mustard were synthesised with the objective that they might be selective anti-tumour agents for tumours with high levels of phosphatase, sulphatase and glucuronidase respectively. This paper shows that the *O*-phosphate and *O*-glucuronide can be deconjugated by the appropriate enzymes *in vitro* to release the rapidly reacting *p*-hydroxyaniline mustard. The *O*-sulphate however proved refractory to hydrolysis by all aryl sulphatases tested.

IN 1965 Connors and Whisson[1,2] observed that a mouse plasma cell tumour (Adj-PC5) which was generally resistant to alkylating agent therapy was remarkably sensitive and readily cured by aniline mustard (I). In subsequent work[3,4] they established a correlation between aniline mustard sensitivity and tumour glucuronidase levels and proposed that the drug was active in this situation because it was metabolised by the liver to the *O*-glucuronide (II) and subsequently hydrolysed selectively in the high glucuronidase environment of the tumour with consequent release of the rapidly hydrolysing and potently cytotoxic compound—*p*-hydroxyaniline mustard (III). The metabolic steps they proposed have recently been confirmed in the rat by the same group of workers.[5] Preliminary clinical trials indicate that there is a correlation between high glucuronidase levels and response to aniline mustard in tumours of the prostate.[6]

A corollary of these observations was that (a) the *O*-glucuronide of *p*-hydroxyaniline mustard (AMGI, II) might be a more selective drug than aniline mustard itself and (b) that the *O*-phosphate (AMPh., IV) and *O*-sulphate (AMSu, V) of *p*-hydroxyaniline mustard could be conceived as possible selective drugs for tumours of high phosphatase and sulphatase respectively.

The three conjugates (IV, V, II) have recently been synthesised[7] and we present in this paper in full, an investigation into their conversion to *p*-hydroxyaniline mustard by the relevant hydrolytic enzymes, which was previously reported in abstract form.[8]





(III) R = OH
(IV) R = -O-PO(OH)$_2$
(V) R = -O-SO$_2$OH

MATERIALS AND METHODS

Drugs

All the alkylating agents used in this study were a gift from the Chester Beatty Research Institute to whom we wish to express our sincere thanks.

AMPh (*p*-di-2-chloroethylaminophenyl phosphate) was used as either a dicyclohexylamine salt or a sodium salt, AMSu (*p*-di-2-chloroethylaminophenyl sulphate) as either a lutidine salt or a sodium salt and AMGl (*p*-di-2-chloroethylaminophenyl-β-D-glucopyranosiduronic acid) as its *tert*-butyl ammonium salt. The organic base salts of AMPh and AMSu were not readily soluble in water and limited the maximum concentrations which could be used in this study. All drug solutions were used immediately so that mustard hydrolysis was minimal during an experiment.

Enzyme methods

(i) *Glucuronidase.* The ability of purified beef liver β-glucuronidase (Sigma Chemical Co., Ltd) to utilise AMGl as a substrate was compared to *p*-nitrophenol glucuronide (PNPG). Glucuronidase activity was measured by the estimation of glucuronic acid released by the method of Wagner,[9] which measures glucuronic acid in the presence of excess glucuronides. Under the conditions used in these experiments no colour was produced by AMGl or PNPG. Buffer solution (0·5 ml) (pH 4·9, 0·2 M acetate) plus purified beef liver glucuronidase solution (200–500 μg/ml in water, 0·5 ml) were incubated at 37° and reaction started by the addition of substrate solution (0·5 ml) at various concentrations (0·75–3 mM). The reaction was terminated at 10 min by the addition of phosphoric acid (8 ml). A 0·2 per cent solution of naphthoresorcinol in acetic acid (6 ml) was added and the reaction mixture heated at 70° for 75 min. The cooled solution was extracted with toluene (3 ml), the toluene dried over sodium sulphate and its absorbance at 570 nm recorded against a toluene blank on a Unicam SP1800 spectrophotometer. The results were plotted by the method of Lineweaver and Burk and line of best fit computed by the method of least squares using a Multi-8 computer. At least two enzyme concentrations were used in each experiment and K_m values reported are the mean of at least three experiments.

(ii) *Sulphatase.* In order to follow the enzymatic breakdown of AMSu a colorimetric estimation of sulphate ion was developed from a method reported by Morgan.[10] The method depends on the formation of a complex at pH 12 between Methyl thymol blue (MTB, Sigma Chemical Co.) and barium ion. The reagent contains equimolar amounts of Ba^{2+} and MTB. At pH 2 no complex is formed and in the presence of sulphate ion barium ion is precipitated from solution as $BaSO_4$. The pH is adjusted to 12 and formation of the complex occurs. MTB is however in excess due to the removal of Ba^{2+}, can be measured at 440 nm and is equivalent to the sulphate ion present in the system.

MTB (0·296 g/100 ml), $BaCl_2H_2O$ (0·381 g/250 ml) and HCl solutions (0·1 N) were mixed in a ratio of 40:25:4 and 51 parts water and 380 parts ethanol added. One volume of this MTB reagent solution was added to one volume of sample containing sulphate ion and after 5 min at room temperature one volume of 0·880 s.g. ammonia solution added. Optical density relative to an appropriate blank was measured at 440 nm and referred to a calibration curve of sodium sulphate (5–100 μg/ml). Phosphate and carbonate ions but not borate interfered with this colorimetric method.

Lysosomal and microsomal fractions of rat liver were prepared by standard methods. A 25 per cent homogenate of rat liver in 0·25 M sucrose was prepared using a Teflon-glass homogeniser, filtered through gauze and centrifuged at 600 *g* for 10 min to remove nuclei. The supernatant was then centrifuged at 15,000 *g* for 15 min and the crude pellet containing mitochondria and lysosomes used as a source of lysosomal enzymes. Microsomes were pelleted from the supernatant of the 15,000 *g* spin by centrifugation at 108,000 *g* for 1 hr and resuspended to give the microsomal enzyme preparation. Each preparation was homogenised with an Ultra-Turrax homogeniser before use.

Three sources of sulphatase were used (1) purified limpet arylsulphatase (Sigma Chemical Co., Ltd), (2) rat liver lysosomes and (3) rat liver microsomes. In each case equal volumes of buffer, enzyme preparation and substrate (0·75–3 mM) were incubated at 37° for 10 min. Where nitrocatechol sulphate (NCS) or *p*-nitrophenol sulphate (PNPS) were substrates the reaction was terminated by addition of 1% sodium dodecyl sulphate in 0·2 M glycine buffer pH 10·4 and absorption of the released nitrocatechol or *p*-nitrophenol measured at 510 and 403 nm respectively. When AMSu was the substrate, reaction was terminated by addition of MTB reagent or, when sub-cellular fractions were the enzyme source, by addition of two volumes of ethanol. In the latter case, sulphate concentration was measured in the supernatant after centrifugation. The buffers used were pH 5·6 acetate (0·2 M) for enzyme preparations (1) and (2) and pH 8·0 phosphate or Tris (0·2 M) for preparation (3). K_m values were calculated as above.

(iii) *Phosphatases.* The substrate specificities of purified calf intestinal mucosa alkaline phosphatase and wheat germ acid phosphatase (Sigma Chemical Co. Ltd) for AMPh and *p*-nitrophenol phosphate (PNPP) were compared by measuring the release of inorganic phosphate. A total volume of 0·6 ml containing equal parts of enzyme (500–1000 μg/ml) buffer and substrate (0·75–6 mM) solutions were incubated for 10 min at 37°. Sulphuric acid (5 N, 0·6 ml) was then added followed by 2·5% ammonium molybdate solution (0·6 ml), reducing agent (0·1 ml), and water (5·0 ml). The reducing agent solution (100 ml) contained 1-amino-2-naphthol-4-sulphonic acid (0·16 g), sodium bisulphite (0·8 g) and sodium sulphite (1·6 g).

The blue colour produced was recorded at 660 nm against an appropriate blank and referred to a calibration curve of KH_2PO_4 in water. In some experiments *p*-nitrophenol was measured at 403 nm after addition of SDS glycine buffer [as in (ii) above].

RESULTS

AMPh as a substrate for phosphatases. The K_m values PNPP and AMPh as substrates of wheat germ acid phosphatase and calf-intestinal alkaline phosphatase were measured by release of inorganic phosphate. The results are presented in Table 1. The human serum enzymes (data not shown) were also able to hydrolyse AMPh.

AMGl as a substrate for glucuronidase. Hydrolysis of AMGl was estimated by release of glucuronic acid (see Methods) and its ability to act as a substrate for glucuronidase compared to PNPGl. The K_m values (presented in Table 1) were closely similar for the two substrates.

TABLE 1. K_m VALUES FOR ACID AND ALKALINE PHOSPHATASE AND β-GLUCURONIDASE

Enzyme	K_m (mM) PNPP	AMPh
Alkaline phosphatase	1·01	1·13
Acid phosphatase	0·85	2·30
	PNPG	AMGl
β-Glucuronidase	0·26	0·29

AMSu as a substrate for sulphatase. Because of the negative results obtained in attempts to hydrolyse AMSu with sulphatase a larger number of enzyme sources were used. Limpet aryl sulphatase and rat liver microsomes and lysosomes were used and their activity against AMSu, NCS, and PNPS compared. The results are presented in Table 2.

TABLE 2. K_m VALUES FOR SULPHATASES

Enzyme	K_m (mM) NCS	PNPS	AMSu
Limpet sulphatase	0·45	*	*
Rat liver lysosomes	1·54	*	*
Rat liver microsomes	*	0·9	*

* No detectable activity in substrate range studied (0·25–2 mM)

DISCUSSION

The objective of the work described in this paper was to establish whether or not the three drug conjugates under consideration were substrates for the enzymes for which they were designed before investigating them in more depth. For simplicity purified commercially available enzymes were used where possible and in all instances K_m values of the drugs were compared with substrates commonly used for assay of the appropriate enzyme. All substrates were compared in a limited solubility range, the upper limit of which was determined by the poor solubility of the drugs in water. Short incubation times were used in all experiments so that mustard hydrolysis did not become a significant complicating factor. Once adequate methodology had been established it was readily shown (Table 1) that AMPh was a good substrate for both acid (wheat germ) and alkaline (calf intestinal) phosphatases and K_m values were of a similar order to those for *p*-nitrophenyl phosphate. Equally, β-glucuronidase from beef liver hydrolysed AMGl with equal efficiency to *p*-nitrophenyl glucuronide (Table 1).

The situation with aryl sulphatase was somewhat more complex due to the differing substrate specificities of different aryl sulphatase types (see review by Roy[11]). Purified mammalian sulphatases are not available commercially and we therefore initially tested limpet aryl sulphatase which is a Type II (see Roy) sulphatase with considerably greater affinity for NCS than simpler substrates such as PNPS. As shown in Table 2 it hydrolyses NCS but has no measurable activity (at the substrate concentrations used) against PNPS or AMSu. Crude rat liver microsomal fractions were

used as sources of mammalian sulphatases. Rat liver lysosomes contain sulphatases A and B which are also of Type II and equally failed to deconjugate AMSu. Sulphatase C of rat liver microsomes has relatively low substrate specificity but with preference of the simpler substrates such as PNPS over NCS. Again no activity was found against AMSu.

The ability of even the relatively non-specific aryl sulphatase C of mammalian tissue to hydrolyse AMSu is not too surprising in the light of the observations on the substrate specificity of similar bacterial enzymes in which it is found that the introduction of electrophilic substituents into the ring promotes affinity for the enzyme whereas substrates with nucleophilic substituents have the opposite effect. Aminoaryl sulphates are thus poor substrates.[12] The biological results of Bukhari *et al.*[7] surprisingly show that AMSu, which like the other conjugates is unlikely to pass through cellular membranes, because it is largely in an ionised state at physiological pH, should be as toxic to mice as AMPh or AMGl. The respective LD_{50} figures for the sodium salts of AMGl, AMPh and AMSu in Balb C mice are 270, 113 and 230 mg/kg. If AMSu largely fails to enter cells one would expect it to be rapidly excreted and relatively non-toxic. The possibility remains open that some unspecified sulphatase activity may activate the drug *in vivo*.

This paper has therefore shown that AMGl and AMPh are substrates for the enzymes for which they were designed but that AMSu is not. The preliminary tumour screening data of Bukhari *et al.*[7] against the alkylating agent sensitive Adj-PC6A tumour showed both AMGl and AMPh to be therapeutically effective in this system. It remains to establish whether the agents will be selectively active against tumours with high levels of the appropriate enzymes, to determine whether enzymic activation occurs extracellularly or at the membrane as suggested for Synkavit by Morley and Dendy,[13] and to establish which isoenzymes are likely to be responsible for the activation process *in vivo*.

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REFERENCES

1. T. A. Connors and M. E. Whisson, *Nature, Lond.* **205,** 406 (1965).
2. T. A. Connors and M. E. Whisson, *Nature, Lond.* **206,** 689 (1965).
3. T. A. Connors and M. E. Whisson, *Nature, Lond.* **210,** 866 (1966).
4. M. E. Whisson, T. A. Connors and A. Jeney, *Archs Immunologica Ther. Experimentalis* **14,** 825 (1966).
5. T. A. Connors, P. B. Farmer, A. B. Foster, A. M. Gilsenan, M. Jarman and M. J. Tisdale, *Biochem. Pharmac.* **21,** 1373 (1972).
6. C. Young, A. Yagoda and E. Bittar, *Abstracts of the Seventh Annual Meeting of the Am. Assoc. of Clin. Oncologists* (1971).
7. M. A. Bukhari, J. L. Everett and W. C. J. Ross, *Biochem. Pharmac.* **21,** 963 (1972).
8. C. R. Ball and J. A. Double, Proc. Brit. Assoc. for Cancer Research, *Br. J. Cancer* **28,** 81 (1973).
9. W. Wagner, *Anal. Chim. Acta* **29,** 227 (1963).
10. G. B. Morgan, E. C. Tabor, C. Golden and H. Clements, *Technicon Symposium,* 1966, Vol. I, p. 534. Mediad, New York (1967).
11. R. G. Nichols and A. B. Roy, In *The Enzymes,* (Ed. P. D. Boyer), Vol. V, p. 21. Academic Press, New York (1971).
12. E. Boyland, D. Manson, P. Sims and D. C. Williams, *Biochem. J.* **62,** 68 (1958).
13. T. G. Morley and P. P. Dendy, *Br. J. Cancer* **28,** 55 (1973).