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SYNTHESIS AND PRELIMINARY CHARACTERIZATION OF A NOVEL SUBSTRATE FOR γ-GLUTAMYL TRANSFERASE

A POTENTIAL ANTI-HEPATOMA DRUG

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Abstract—The synthesis and chemical characterization of the γ-glutamyl adduct of phenylenediamine mustard is reported. The activity of this compound, γ-[*N*,*N*-bis(2-chloroethyl)-*p*-phenylenediamine]-glutamate, as a substrate for γ-glutamyl transferase is demonstrated and compared with the activity of glutathione. The possible use of this material as a directed anti-hepatoma agent is discussed.

γ-Glutamyl transferase (EC 2.3.2.2) levels in the serum have long been used as indicators of hepatobiliary dysfunction [1, 2]. More recently it has been reported that hepatic γ-glutamyl transferase is significantly increased in hepatoma tissue [3]. Studies on experimentally induced hepatoma in the rat and in rat hepatoma cells in culture have indicated that it is specifically the transformed cells that show the increased levels of γ-glutamyl transferase activity [4–6].

γ-Glutamyl transferase shows a relatively broad substrate specificity towards the chemical nature of the γ-glutamyl donor [7]. The possibility therefore exists for the synthesis of adducts of glutamate linked via a peptide bond to various cytotoxic agents. These compounds may then prove to be substrates for γ-glutamyl transferase and thus be activated at sites of high γ-glutamyl transferase activity. This paper reports the synthesis of the γ-glutamyl adduct of *N*,*N*-bis(2-chloroethyl)-*p*-phenylenediamine (phenylenediamine mustard), and its preliminary characterization as a substrate with respect to γ-glutamyl transferase. A preliminary investigation of the anti-hepatoma cell activity of this compound has already been reported [8].

MATERIALS AND METHODS

Synthesis. N^{α}-*t*-Butyloxycarbonyl-α-*t*-butyl glutamate was prepared from L-glutamic acid according to the procedure described previously [9]. *N*,*N*-Bis(2-chloroethyl)-*p*-phenylenediamine hydrochloride (phenylenediamine mustard) was prepared as described in the literature [10]. Isobutylchloroformate and triethylamine were purchased from Aldrich Chemical Co. and purified by distillation. Column chromatography was performed on a medium pressure liquid chromatograph (MPLC) system [11]. Proton nuclear magnetic resonance spectra were recorded on a Varian Associates spectrometer EM-390, and are expressed as δ units (ppm) relative to tetramethylsilane as internal standard. Mass spectra were recorded on a Varian Associates MAT731 spectrometer.

N^{α}-t-*Butyloxycarbonyl*-α-t-*butyl*-γ-N,N-*bis*(2-*chloroethyl*)-p-*phenylenediamine glutamate.* N^{α}-*t*-Butyloxycarbonyl-α-*t*-butyl glutamate (0.6 g, 2 mmole) was dissolved in dry tetrahydrofuran (6 ml) and reacted with isobutyl chloroformate (0.26 ml, 2 mmole) in the presence of triethylamine (0.28 ml, 2 mmole) at −15°. The reaction mixture was stirred at that temperature for 15 min and a pre-cooled solution of *N*,*N*-bis(2-chloroethyl)-*p*-phenylenediamine hydrochloride (0.54 g, 2 mmole) and triethylamine (0.28 ml, 2 mmole) in dry dimethylformamide (5 ml) was added. After stirring the reaction mixture for 1 hr at −15° and for 6 hr at 25°, the solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate (100 ml). The organic phase was washed with cold 1 N HCl, water, saturated $NaHCO_3$ and water. Removal of the solvent (after drying over anhydrous $MgSO_4$) gave the crude product as an oil, which was purified by medium pressure silica gel column chromatography using 5% ethyl acetate in methylene chloride. The pure product was obtained as a foam (0.42 g, 42%). ^{1}H NMR ($CDCl_3$): δ 1.42 (*s*, 9H, *t*-butyl), 1.45 (*s*, 9H, *t*-butyl), 2.12 (*m*, 2H, α CH and NH), 5.25 (*m*, 1H, NH), 7.4 (AA'BB' pattern, J = 8.5 Hz, 4H, aromatic). Mass spectra (field desorption): *m*/*z* 519 [MH^+]. Analysis calculated for $C_{24}H_{37}N_3O_5Cl_2$: C, 55.60; H, 7.14; N, 8.11; Cl, 13.71. Found: C, 55.49; H, 7.24; N, 8.23; Cl, 13.63%.

γ-[N,N-*Bis*(2-*chloroethyl*)-p-*phenylenediamine*]-*glutamic acid hydrochloride (GPDM).* N^{α}-*t*-Butyloxycarbonyl-α-*t*-butyl-γ-*N*,*N*-bis(2-chloroethyl)-*p*-phenylenediamine glutamate (0.52 g, 1 mmole) was dissolved in a solution of anhydrous HCl in dioxane (4 N, 3 ml), and the solution was

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stirred at 25° for 2 hr. The solvent was removed *in vacuo* and the residue was triturated with dry ether. The solid product thus obtained was crystallized from ethanol–ether, giving pure product as a white, hygroscopic solid (0.3 g, 77%). R_f 0.27 in 2-butanone–acetone–water (8:1:1). ^{1}H NMR (DMSO-d_6): δ 2.2–2.45 (*m*, 4H, methylenes), 3.65 (*t*, J = 6 Hz, 8H, methylenes), 4.18 (*m*, 1H, α CH) 7.42 (AA'BB' pattern, J = 8.5 Hz, 4H, aromatic). Mass spectra (field desorption): *m/z* 363 [MH$^+$]. Analysis calculated for $C_{15}H_{21}N_3O_3Cl_2 \cdot HCl$: C, 45.17; H, 5.52; N, 10.54. Found: C, 45.03; H, 5.67; N, 10.47%.

Enzyme kinetics. The activity of GPDM as a substrate for γ-glutamyl transferase was estimated by a modification of the method of McIntyre and Curthoys [12]. GPDM (0.2 ml of 0.12–2.50 mM) in 0.1 M 2-amino-2-methyl-1,3-propandiol buffer, pH 8.5, containing 5 mM alanine and 4.4×10^3 Bq [^{14}C]alanine (Amersham International), was incubated at 37° with 12 μg γ-glutamyl transferase (Sigma London Ltd.) in a total volume of 0.2 ml. The reaction was terminated by the addition of 0.5 ml 0.05 M acetic acid. An aliquot (0.5 ml) of this reaction mixture was added to a small column (3 × 0.5 cm) of AG1-X2 resin (Biorad Laboratories) that had been pre-equilibrated with 0.05 M acetic acid. The unreacted substrate was eluted from the column with 4.0 ml 0.05 M acetic acid. The product was eluted from the column with 2 ml 3 M acetic acid directly into 10 ml scintillant (PCS, Amersham International). Where indicated, reduced glutathione (1 mM) was substituted in place of GPDM into the reaction mixture. Activity is expressed as nmole product formed per min per mg protein.

Inhibition of γ-glutamyl transferase activity was measured fluorometrically according to Smith *et al.* [13], using γ-glutamyl 7-amino-4-methyl coumarin as the substrate.

RESULTS

The activity of GPDM as a substrate for γ-glutamyl transferase was investigated with alanine as an acceptor, and compared with the behaviour shown by glutathione, the probable natural substrate for the enzyme. The results are shown as plots of [S]/v vs [S] in Fig. 1. It is apparent that GPDM is an effective substrate for γ-glutamyl transferase, with a K_m six times higher than that of glutathione. However, the V_{max} (slope^{-1}) for each substrate appears to be identical.

The ability of GPDM to interact with γ-glutamyl transferase was confirmed by the observation of the apparent inhibition in the presence of GPDM of γ-glutamyl transferase activity, as measured by the release of 7-amino-4-methyl coumarin from the γ-glutamyl adduct. The effect of various concentrations of GPDM on the initial velocity as the substrate concentration was varied is shown in Fig. 2. The near parallel nature of the lines is indicative of competitive inhibition. The inset shows a secondary plot of the apparent K_m vs inhibitor concentration. This gives a K_i value of 0.8 mM compared with a K_m for γ-glutamyl 7-amino-4-methyl coumarin of 0.4 mM [13].

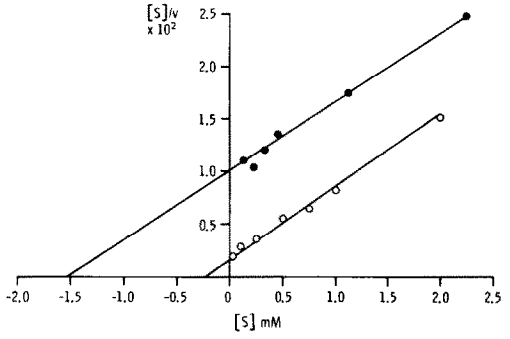

Fig. 1. Comparison of the substrate activities of reduced glutathione and GPDM. [S]/v vs [S] plots of the variation in the initial rate of transpeptidation at constant alanine (5 mM) as glutathione (○—○) and GPDM (●—●) were varied. The assay was carried out as described in Materials and Methods and the lines were fitted to the points using kinetic parameters obtained from direct linear plots [14] of the data. K_m values of 0.25 and 1.51 mM were obtained for glutathione and GPDM, respectively.

DISCUSSION

The present study demonstrates that GPDM is an effective substrate for γ-glutamyl transferase. The K_m value of 1.5 mM, although 6-fold greater than that of glutathione, is of the same order as that shown for other substrate analogues [15]. The fact that the maximal velocity obtainable with either donor substrate is the same indicates that the rate-limiting step in the enzyme reaction is the same for both substrates. This is not unreasonable since the reaction is thought to proceed via a ping-pong mechanism [16], and therefore formation and release of the γ-glutamyl product should be independent of the formation of the γ-glutamyl–enzyme complex.

Use of GPDM as an 'inhibitor' of γ-glutamyl transferase confirmed the substrate activity of this compound. As expected, a primary plot of the data

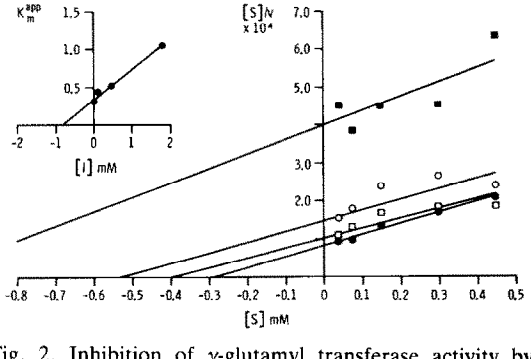

Fig. 2. Inhibition of γ-glutamyl transferase activity by GPDM. [S]/v vs [S] plots showing the variation of initial rate with γ-glutamyl 7-amino-4-methyl coumarin concentration at fixed glycyl glycine concentration (15 mM) and different fixed inhibitor (GPDM) concentrations: ●—●, 0 mM; □—□, 0.09 mM; ○—○, 0.45 mM; ■—■, 1.8 mM. The lines were fitted as for Fig. 1. The inset shows a secondary plot of the K_m^{app} vs inhibitor concentration, and was fitted by linear regression. The x-axis intercept gives a K_i of 0.8 mM.

indicated competitive inhibition. The derivative plot gave a K_i similar in value to the K_m obtained for substrate activity. This suggests that the K_m reflects the dissociation constant for the γ-glutamyl donor–enzyme complex, and indicates that the higher K_m (compared with glutathione) is a result of a decreased affinity for this compound.

At higher concentrations (5 mM) than reported in this study, GPDM showed substrate inhibition. This may be due to alkylation by this compound but is more likely to be a result of autotransfer activity, i.e. acting as an acceptor as well as a donor substrate [13].

These findings show that GPDM is a good substrate for γ-glutamyl transferase and thus the observed increased sensitivity of JB1 cells to GPDM [8] can be directly ascribed to the high γ-glutamyl transferase activity of this cell line. This study has confirmed the possibility of synthesis of γ-glutamyl adducts for use as selective cytotoxic agents capable of activation at sites of high γ-glutamyl transferase activity.

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