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SHORT COMMUNICATIONS

A direct link between LY83583, a selective repressor of cyclic GMP formation, and glutathione metabolism

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Abstract—LY83583 (6-anilino-5,8-quinolinedione), considered to be a relatively specific repressor of cyclic GMP formation, is shown in the present study to inhibit ($K_i = 3\ \mu M$) glutathione reductase from bovine intestinal mucosa. As glutathione disulphide has been reported to inhibit guanylate cyclase irreversibly [Braughler, *Biochem Pharmacol* **32**: 811–818, 1983], the inhibition of glutathione reductase should affect the activity of guanylate cyclase and may thus have physiological implications in the action of endothelium-derived relaxation factor and the design of muscle relaxants. LY83583 is reduced by NADPH and glutathione reductase in aerobic media and this may offer a route to the metabolic activation of LY83583. These results may have significant implications for the design of heart-regulating drugs (e.g. those used in angina), such as glyceryl trinitrate, which act via guanylate cyclase.

The search for new inhibitors of antigen-induced leukotriene release led to the discovery of 6-anilino-5,8-quinolinedione (LY83583*) [1] (Scheme 1), subsequently found to lower selectively cyclic GMP but not cyclic AMP levels [1–6], making LY83583 a useful physiological tool [7]. The effect of LY83583 on cyclic GMP levels appears to reflect its ability to inhibit endothelium-derived relaxing factor and block the activation of soluble guanylate cyclase [8]. In designing inhibitors of glutathione reductase and of trypanothione reductase [9], we were stimulated by the report that menadione binds to human erythrocyte glutathione reductase, a system studied in detail by X-ray diffraction methods [10]. Molecular graphics analysis of this system led us to test LY83583 as an inhibitor of glutathione reductase.

Materials and Methods

Glutathione reductase (EC 1.6.4.2), Type IV from baker's yeast, type VII from bovine intestinal mucosa, NADPH and cytochrome *c* were obtained from the Sigma Chemical Co. (Poole, U.K.) and LY83583 from Calbiochem (La Jolla, CA, U.S.A.). Enzymes were shown to be homogeneous by the criterion of PHAST SDS–PAGE. Glutathione disulphide was from Fluka Feinchemikalien (Dorset, U.K.). All other reagents were of the highest quality available. Water was distilled and Milli-Q purified before use. Enzyme concentrations were determined [11] and glutathione reductase assayed [12] as described. When

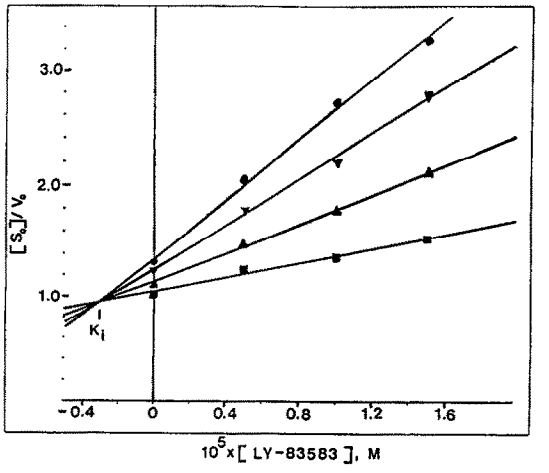
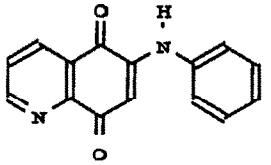


Fig. 1. Cornish-Bowden plot ($[S_o]$/initial velocity versus [I]) obtained with glutathione reductase from bovine intestinal mucosa (8 nM) at 25° with varying concentrations of GSSG (0.25 to 2 K_m), NADPH (0.1 mM) at a series of concentrations of LY83583 (5, 10 and 15 μM). Points are experimental: lines are theoretical for linear, uncompetitive inhibition with $K_i = 3\ \mu M$, $K_m = 200\ \mu M$ (for GSSG) and $V_{max} = 2.3\ \mu M$/sec. GSSG concentrations: (●) 93, (▼) 70, (▲) 46, (■) 23 μM.



Scheme 1.

LY83583 was tested as a substrate for GR, oxidation of NADPH was measured at 340 nm. In addition, enzyme activity was monitored by coupling radical formation to reduction of cytochrome *c* (20 μM) and measuring absorbance changes ($\varepsilon_{550} = 21{,}000$) [13]. Anaerobic measurements were performed in rubber-stoppered cuvettes that were flushed repeatedly with argon. All buffers and solutions used in the assays were treated in this manner. Data were analysed by non-linear least squares regression analysis using the Enzfitter programme of L.J. Leatherbarrow, distributed by Elsevier Biosoft.

Results and Discussion

LY83583 was found to inhibit strongly glutathione

* Abbreviations: LY83583, 6-anilino-5,8-quinolinedione; GSG, reduced glutathione; GSSG, oxidized glutathione.

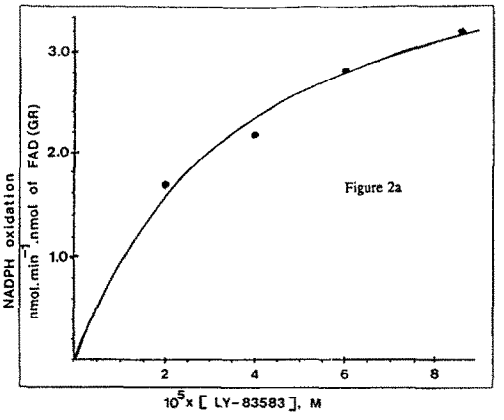
reductase from bovine intestinal mucosa by pure, uncompetitive inhibition [14], diagnosed using the combination of Lineweaver–Burk ($1/V_o$ versus $1/[S_o]$), Dixon ($1/V_o$ versus [I]) and Cornish-Bowden ($[S_o]/V_o$ versus $[I_o]$) plots, the last of which is shown in Fig. 1. The value of K_i obtained from the Cornish-Bowden plot (Fig. 1) was $3.0 \pm 0.4\ \mu M$. With glutathione reductase from yeast, the inhibition type was found to be partial uncompetitive (data not shown) [14]. For this type of inhibition, the yeast glutathione reductase K_i value was evaluated from a secondary replot of $1/\Delta_{intercept}$ versus $1/[I_o]$ where $\Delta_{intercept}$ is the value of the Lineweaver–Burk intercept (on the $1/V_o$ axis) corrected by subtraction of the value of that intercept at $[I_o] = 0$. The value of K_i thus obtained for LY83583 with the yeast enzyme was $14.0 \pm 2.5\ \mu M$ and an α value of 0.14, giving a value of 14×0.14 as K_i' ($2\ \mu M$). Although the ping-pong mechanism used by glutathione reductase complicates matters, these kinetics are most easily explained by the classical uncompetitive model [14] for the bovine intestinal enzyme, in which scheme the inhibitor binds only to the ES complex and not to free E. The partial inhibition model (Eqn 1) is better for the yeast enzyme, with $\alpha = 0.14$.

$$\begin{array}{ccccc} E & +S & \overset{K_s}{\rightleftharpoons} & ES & \overset{K_p}{\rightleftharpoons} E + P \\ K_i \downarrow\uparrow & & & \alpha K_i \downarrow\uparrow & \\ EI & +S & \underset{\alpha K_s}{\rightleftharpoons} & EIS & \underset{\beta K_p}{\rightleftharpoons} EI + P. \end{array} \quad (1)$$

When LY83583 was tested as a substrate for glutathione reductase [with LY83583 replacing oxidized glutathione (GSSG)] under aerobic conditions, NADPH oxidation was linear with time and several molar equivalents of NADPH were consumed per mole of LY83583 (Fig. 2a). Thus, LY83583 probably undergoes enzyme-catalysed reduction with subsequent reoxidation by O_2 and concomitant production of superoxide ion ("redox cycling"), as observed for some quinones with trypanothione reductase [13]. This was confirmed by monitoring cytochrome *c* reduction by the superoxide ion produced in the coupled assay described above (Fig. 2b). Under these conditions LY83583 had an apparent K_m of $79.9\ \mu M$. When LY83583 ($10\ \mu M$) was incubated with glutathione reductase ($0.7\ \mu M$) and NADPH (0.1 mM) for 30 min under aerobic and anaerobic conditions, respectively, and then thoroughly dialysed, no loss of enzyme activity was observed compared to a control without LY83583.

LY83583, regarded as a specific repressor of cyclic GMP formation, acts by inhibiting endothelium-derived relaxing factor production or release and blocks activation of soluble guanylate cyclase [8]. Both inhibitory actions are thought to require intracellular reduction of the compound by endogenous thiols. In addition, the activation of guanylate cyclase is associated with its partial oxidation to form specific enzymic disulphide bonds induced by low concentrations of nitrovasodilators or GSSG [15]. However, *in vitro* higher concentrations of GSSG (1 mM) are known to inhibit irreversibly guanylate cyclase [16]. Therefore, a direct activation/inhibition of guanylate cyclase should be sensitive to the intracellular ratio of reduced glutathione (GSH):GSSG. The potent *in vitro* inhibition of glutathione reductase by LY83583 ($K_i = 3\ \mu M$) reported here should lead to accumulation of GSSG and therefore possibly expose guanylate cyclase to such irreversible inhibition. Some of the physiological effects of LY83583 may reflect its potential ability to alter the GSH:GSSG ratio or to cause build-up of GSSG.

The concentration of LY83583 required *in vitro* to inhibit guanylate cyclase by 50% (IC_{50}) varies according to the conditions but is in the range 1–10 μM, depending on whether the guanylate cyclase is unstimulated or stimulated by sodium nitroprusside and whether dithiothreitol is



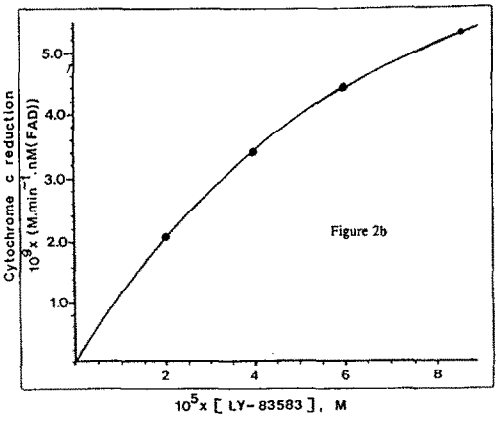


Fig. 2. (a) Reduction of LY83583 by bovine intestinal glutathione reductase ($0.7\ \mu M$) as a function of LY83583 concentration, measured at 340 nm in 1 mM EDTA, 0.1 M potassium phosphate buffer (pH 7.0) in the presence of NADPH (0.1 mM) at 25°. (b) Enzymatic reduction of LY83583 as a function of LY83583 concentration. Rates were measured under aerobic conditions at 25° in 1 mM EDTA, 0.1 M potassium phosphate buffer (pH 7.0) in the presence of glutathione reductase ($0.7\ \mu M$), NADPH (0.1 mM), cytochrome *c* ($20\ \mu M$) and various LY83583 concentrations coupling superoxide production to cytochrome *c* reduction. The points are experimental: the line was obtained by non-linear least squares regression analysis to the Michaelis–Menten equation using a maximal rate of cytochrome *c* reduction ($V_{max} = 1.03 \times 10^{-8}$ M (cytochrome reduced)/min/nmol of FAD (i.e. glutathione reductase).

present [17]. Clearly, these results are in the region of the K_i we observe for LY83583 with glutathione reductase.

There has been a report that superoxide ion ($O_2^{\cdot}$) can directly activate guanylate cyclase [18]. As we have found that the action of glutathione reductase on LY83583 is to produce $O_2^{\cdot}$ (Fig. 2b), LY83583 may have such an activating effect on guanylate cyclase. This could act to counter the activation of guanylate cyclase by the changes in GSSG levels expected of LY83583 from our work. The balance of these opposing effects offers a possible seat of side-effects of LY83583.

It is possible that the mode of action of nitro-compounds used to treat heart problems (e.g. glyceryl trinitrate) may be linked to the GSH:GSSG ratio through the relationship of GSSG and guanylate cyclase. The recognition of

glutathione reductase as an alternative molecular target for LY83583 represents an opportunity to design novel physiological tools, possibly with pharmacological use, and also points to potential side-effects of LY83583.

In addition, there have been suggestions that LY83583 must be metabolically reduced for activity [8]. It is possible that the efficient redox cycling that we have found of LY83583 by glutathione reductase provides this metabolic reduction by means of reduction of molecular oxygen.

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