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Highly potent inhibition of prostaglandin 15-hydroxydehydrogenase in-vitro and of prostaglandin inactivation in perfused lung by the new azobenzene analogue, Ph CL 28A

C. N. BERRY*, J. R. S. HOULT†, J. A. PHILLIPS, T. M. MCCARTHY AND H. AGBACK‡

Department of Pharmacology, King's College, Strand, London WC2R 2LS, UK and ‡Department of Chemical Research, Pharmacia AB, S-751 82, Uppsala, Sweden

The new azobenzene analogue Ph CL 28A (2-hydroxy-5-(3,5-dimethoxycarbonyl-benzoyl)-benzene acetic acid) potently inhibited prostaglandin 15-hydroxydehydrogenase (PGDH) in the nanomolar range in-vitro and inhibited prostaglandin inactivation in rat perfused lung at similar concentrations, and is the most potent PGDH inhibitor yet available. It was synthesized because electronegative substituents in the B ring of homosalazine enhance PGDH inhibitory potency. Ph CL 28A inhibited human placental PGDH non-competitively with regard both to substrate $PGF_{2\alpha}$ (K_i = 18·7 ± 0·9 nM) and the NAD^+ cofactor (K_i = 57·6 ± 2·9 nM); inhibition was greatly reduced at pH ≥ 8·0. PhCL 28A hydrolyses spontaneously in alkali ($t_{\frac{1}{2}}$ at pH 9·0 = 45 h) to the less active dicarboxylic acid (IC50 human placental PGDH 1·6 μM versus 0·028 μM for Ph CL 28A). The new analogue is 1000 × more active than the parent compound sulphasalazine from which it derives. The IC50 values for five azobenzene analogues in rat perfused lung (Ph CL 28A = 72 nM) correlated strongly with those obtained using purified PGDH (r = 0·99), suggesting that they inhibit pulmonary prostaglandin degradation at the enzyme step rather than at the hypothetical carrier. The new compound will be a useful probe for PGDH structure and function.

Recent studies have shown that the anticolitic drug sulphasalazine inhibits the inactivation of prostaglandins both in-vitro (Hoult & Moore 1978, 1980) by a direct non-competitive interaction with the enzyme prostaglandin 15-hydroxydehydrogenase, PGDH (Berry et al 1983), and in the intact lung (Hoult & Moore 1980). Based on this information a large series of azobenzene analogues lacking the pyridylsulphamoyl ring system of sulphasalazine was prepared in order to investigate the structure-activity relations for PGDH inhibition (Berry et al 1983). This revealed that optimal activity occurred in compounds containing an additional methylene group interpolated at the salicylcarboxyl function, such as in the prototype compound homosalazine (Fig. 1), and that increased activity could be obtained by incorporation of highly electronegative substituents in the B ring.

The present paper shows that the introduction of two electronegative ester groups into this B ring gives a compound of vastly increased potency which inhibits PGDH in the nanomolar range. We compared the new compound (Ph CL 28A; 2-hydroxy-5-(3,5-dimethoxycarbonyl-benzoyl)-benzene acetic acid¶) with other analogues both in-vitro and in the rat perfused lung to establish whether activities for PGDH inhibition obtained in cell-free systems can be of predictive value for effects in intact organs.

MATERIALS AND METHODS

Materials

The [9β-^{3}H]prostaglandin $F_{2\alpha}$ (sp. act. 16·2 Ci mmol^{-1}) and [1-^{14}C]arachidonic acid (sp. act. 58 mCi mmol^{-1}) were obtained from Amersham International, UK; prostaglandin $F_{2\alpha}$ tromethamine salt, arachidonic acid, reduced glutathione and NAD^+ were purchased from Sigma London Ltd, Poole, Dorset, UK. Sulphasalazine and the four azobenzene analogues were prepared in the Department of Chemical Research, Pharmacia AB, Uppsala, Sweden. Waters C_{18} reverse phase chromatography Sep-Pak cartridges were purchased from Millipore (UK) Ltd, Harrow, Middlesex, UK. Puri-

† Correspondence.
* Present address: Department of Biology, L.E.R.S., 23–25 Avenue Morane Saulnier, Meudon, 92360, France.

¶ Name conforms to IUPAC nomenclature; alternative: 3,5-dicarbomethoxy-3′-carboxymethyl-4′-hydroxy-azobenzene.

fied human placental type I NAD^+-dependent prostaglandin 15-hydroxydehydrogenase (200 mU ml^{-1}) was a kind gift from Dr J. Jeffery, Department of Biochemistry, Marischal College, Aberdeen, UK. One mU is defined as that amount of enzyme which oxidizes one nanomole of prostaglandin E_1 per minute at 37 °C, pH 7·4.

Preparation of rat colon 100 000g supernatants

Colons taken from male Sprague-Dawley rats weighing 175–250 g were homogenized in 4 vol. 50 mM phosphate buffer pH 7·5 (containing 1 mM EDTA and cysteine) and 100 000*g* supernatants prepared as described by Hoult & Moore (1977).

Inhibition of prostaglandin breakdown in purified PGDH and rat colon cytosolic supernatants

This was assayed radiochemically using methods which have been fully described (Hoult & Moore 1977, 1980). In brief, incubation tubes contained 100–200 μU PGDH or 0·18 ml rat colon supernatant, 0·01 ml NAD^+ (final concentration 5 mM), 0·01 ml vehicle or various concentrations of inhibitor drug and 2 μg (5·7 nmol) prostaglandin $F_{2\alpha}$, containing 0·1 μCi radiolabel (=2·4 ng) to a final volume of 0·2 ml, and were incubated at 37 °C for 15–60 min, as appropriate, so as to give 60–75% inactivation of $PGF_{2\alpha}$. After extraction, the metabolites were localized by reference to authentic standards and by radio-TLC scanning and separated by thin layer chromatography in a solvent containing ethyl acetate–acetone–glacial acetic acid (90 : 10 : 1·5 by volume) and the extent of breakdown was calculated by comparing the proportion of counts in the relevant zones of the chromatogram after correcting for any impurities of the radioactive substrate found in the metabolite zone (usually 4–9%). This was determined in each experiment by incubating radiolabelled $PGF_{2\alpha}$ alone in the absence of any enzyme preparation.

Inhibition of prostaglandin breakdown in the rat isolated perfused lung

Rat isolated lungs were set up and perfused with warm well-oxygenated Krebs solution at 6 ml min^{-1} via a cannula inserted into the pulmonary artery, as described by Hoult & Moore (1980). Prostaglandin inactivation was determined by injecting 100 ng doses of prostaglandin $F_{2\alpha}$ containing 0·125 μCi radiolabel via a side line. The perfusates were collected for 90 s post-infusion, acidified to pH 3·0 with concentrated hydrochloric acid and loaded onto C_{18} Sep-Paks which had been pre-wetted with 3 ml acetonitrile followed by 5 ml distilled water. The loaded Sep-Paks were washed with 5 ml distilled water and the prostaglandins eluted with 5 ml ethyl acetate. After evaporation to dryness, the eluates were resuspended in acetone (20 μl) and 7 μl aliquots were subjected to radio-TLC as described above. Each lung was tested with a maximum of 16 challenges, 4 control and 12 in the presence of various doses of inhibitor. Control experiments established that PG inactivation in the absence of inhibitor is sustained for up to 4 h after the lungs have been set up under these conditions (Berry 1981), as shown also for guinea-pig isolated perfused lungs (Crutchley & Piper 1974).

Inhibition of prostaglandin biosynthesis in sheep seminal vesicle microsome preparations

Portions of sheep seminal vesicles weighing 1–4 g were cut from frozen whole glands (Northeast Biochemical Laboratories Ltd, Uxbridge, Middlesex, UK), allowed to thaw and homogenized in 4 vol. pH 7·4 50 mM Tris-HCl buffer. After preparation of microsomes by differential centrifugation at 10 000*g* and 100 000*g* at 4 °C (for 15 and 45 min, respectively), the microsomal pellets were resuspended in the same buffer to give a protein concentration of 2·5–6 mg ml^{-1} and incubated at 37 °C for 60 min in a final volume of 0·4 ml with 10 μg ml^{-1} arachidonic acid containing 0·02 μCi [1-^{14}C]arachidonic acid (0·1 μg) and 3 mM reduced glutathione. After ethyl acetate extraction, the extent of conversion of arachidonic acid to prostaglandins (mainly PGE_2 on account of the addition of glutathione) was measured by radiochromatography using the upper phase of the solvent ethyl acetate–iso-octane–acetic acid–water (90 : 50 : 20 : 100 by volume).

Data analysis

Results are expressed as mean values ± s.e.m. K_i values were computed by linear regression analysis.

RESULTS

The structures of Ph CL 28A and the other azobenzene analogues used in these experiments are given in Fig. 1. This illustrates the general resemblance of the new compounds to the parent molecule sulphasalazine, but shows that they lack the pyridylsulphamoyl moiety. Several of them also contain an extra methylene group interpolated at the salicyl carboxyl function. The prototype compound, homosalazine, is shown, and its activity as a PGDH inhibitor has been described previously (Berry et al 1983). Also illustrated is Ph CL 32A which is formed when the

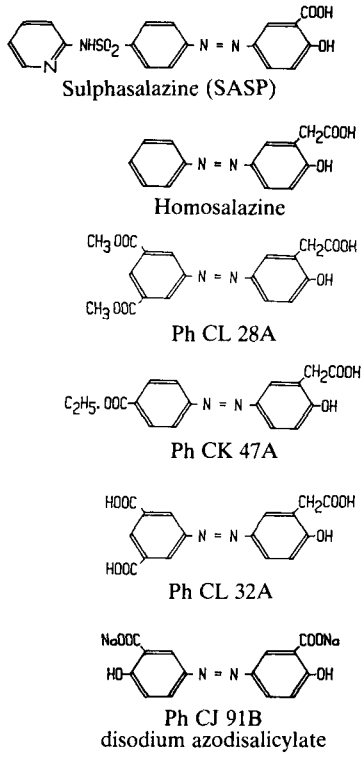

FIG. 1. Structures of azobenzene analogues.

3,5-ester groups of Ph CL 28A are spontaneously hydrolysed in alkaline solution. One of the azobenzene analogues, Ph CJ 91B (disodium azodisalicylate), does not contain the extra methylene group at the salicyl carboxyl function.

Fig. 2 shows that Ph CL 28A caused concentration-dependent inhibition of the inactivation of prostaglandin $F_{2\alpha}$ by purified human placental PGDH, and in this experiment was almost 10 000 times more active than the parent compound sulphasalazine. The calculated IC50 values for the two compounds were 11·4 nM and 105 μM, respectively.

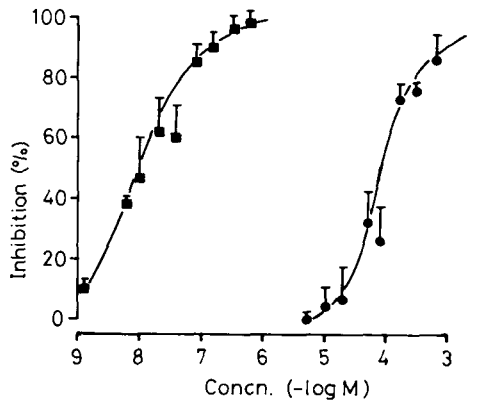

FIG. 2. Inhibition of prostaglandin $F_{2\alpha}$ inactivation by human placental PGDH incubated with Ph CL 28A (■) or sulphasalazine (●). Points show mean ± s.e.m., 4 tests at each concentration.

Table 1 summarizes values for the IC50 values for Ph CL 28A and four other azobenzene analogues against PGDH in both the human placental PGDH and rat colon 100 000*g* supernatant systems, and also shows the extent of inhibition obtained when each compound was tested at 5 μM on the human placental enzyme. These experiments were performed with a different placental enzyme preparation from that used in Fig. 2. The data emphasize the high potency of Ph CL 28A, which is active in the nanomolar range, and show that it is at least ten times more active than both Ph CK 47A (the most active compound identified in our earlier series, Berry et al 1983) and Ph CL 32A (its hydrolysis product). Furthermore, as found in previous experiments with other sulphasalazine-like drugs, inhibition of PGDH activity by Ph CL 28A or other analogues is independent of the tissue or species from which the enzyme derives.

However, unlike other sulphasalazine analogues, the esters Ph CL 28A and Ph CK 47A are not stable

Table 1. Effects of azobenzenes on prostaglandin inactivation and biosynthesis.

	t_R (min)*	IC50 (μM) Human placental PGDH	IC50 (μM) Rat colon HSSN	% inhib. at 5 μM on human placental PGDH	PG biosynthesis in sheep seminal vesicle microsomes, 10–1000 μM
Ph CL 28A	21·9	0·08	0·056	97·8 ± 1·2	No effect
Ph CK 47A	24·7	0·29	2·9	96·5 ± 1·7	No effect
Ph CL 32A	3·3	1·6	0·47	82·9 ± 3·2	No effect
Sulphasalazine	5·1	21·0	214	26·3 ± 9·9	No effect
Ph CJ 91B	6·0	70·0	275	9·3 ± 3·9	Potentiation at 1000 μM

* Retention times by HPLC on C-8 reverse phase Radial-Pak 10 μ cartridges using a solvent of 33% acetonitrile in 0·046 M orthophosphoric acid, pH 2·8, 2 ml min^{-1}. HSSN = 100 000*g* supernatant.

when made up as stock solutions in 0·5% (w/v) sodium carbonate solution (to facilitate solubility of these poorly soluble weak carboxylic acids). In the case of Ph CL 28A there is gradual hydrolysis to the corresponding 3,5-dicarboxylic acid Ph CL 32A (see Fig. 1) with an approximate $t_{\frac{1}{2}}$ at 4 °C of ~2 days. This was verified by separate HPLC experiments showing a $t_{\frac{1}{2}}$ of 45 h for a 0·5 mM solution at pH 9·0, whereas hydrolysis at pH 7·0 was less than 5% in 140 h. Indeed, solutions of Ph CL 28A prepared from stocks more than one day old exhibit reduced capacity to inhibit PGDH. In an experiment on the human placental PGDH preparation, freshly prepared Ph CL 28A had an IC50 value of 0·02 μM, whereas a >2 week old solution was much less active (IC50 = 2·5 μM), as was Ph CL 32A (IC50 = 1·4 μM). Further, the 'old' solution of Ph CL 28A was found to consist largely of material with identical HPLC retention time to that of Ph CL 32A (see Table 1). This is evidence that the chemical composition and biological potency of Ph CL 28A solutions vary in parallel, according to the extent of hydrolysis to the less active 3,5-dicarboxylic acid.

We investigated the mechanism of PGDH inhibition by Ph CL 28A in terms of possible interactions with the substrate ($PGF_{2\alpha}$) or cofactor (NAD^+) sites on the enzyme. Analysis by Dixon plots showed that the drug is a non-competitive inhibitor, in terms of both the substrate and cofactor (Fig. 3). The K_i values were 18·7 ± 0·9 nM (n = 4) versus substrate and 57·6 ± 2·0 nM (n = 4) versus cofactor. We have previously shown that sulphasalazine and related drugs interact non-competitively with the substrate, but the K_i values for the less active compounds were correspondingly larger (Berry et al 1983).

Further experiments using Ph CL 28A showed that its inhibitory activity was markedly dependent upon pH. Fig. 4 shows the broad pH optimum of PGDH in rat colon 100 000*g* supernatant. Over the range pH 6·9 to 8·6 there is very little difference in enzyme activity, but at more acidic values (e.g., pH 6·6) activity is considerably reduced, perhaps because of an increasing contribution from the reverse reaction (NADH-dependent reduction of the 15-keto metabolite, see Yamazaki & Sasaki 1975). By contrast, although 50 nM Ph CL 28A was an effective inhibitor at pH 6·6 and pH 6·9, under more alkaline conditions (>pH 8·0) its activity as an inhibitor was progressively lost (Fig. 4). Similar results were found for Ph CL 28A at 10 nM, as well as for other azobenzene analogues.

As well as inhibiting prostaglandin $F_{2\alpha}$ metabolism in cell-free systems, the five compounds also in-

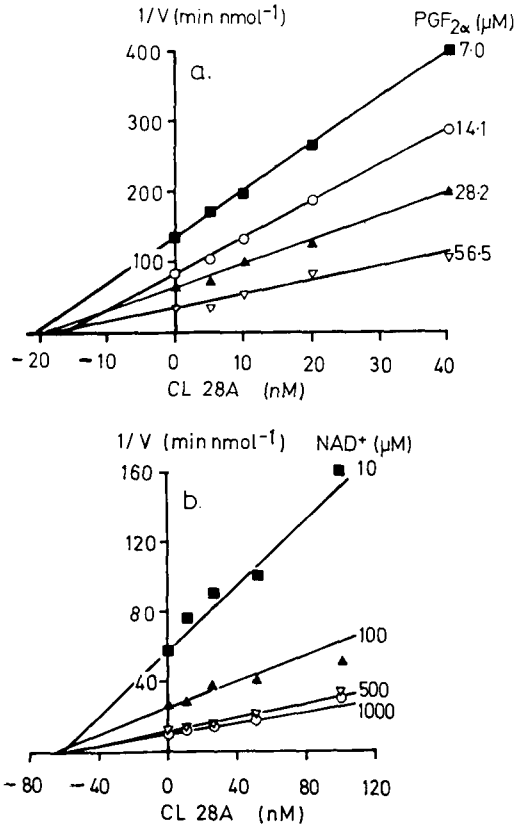

FIG. 3. Dixon plots of the inhibition of human placental PGDH by Ph CL 28A at varying substrate (panel a) or NAD^+ cofactor (panel b) concentrations. The points show the mean values from 4 tests at each concentration. Lines and K_i values in text computed by linear regression analysis.

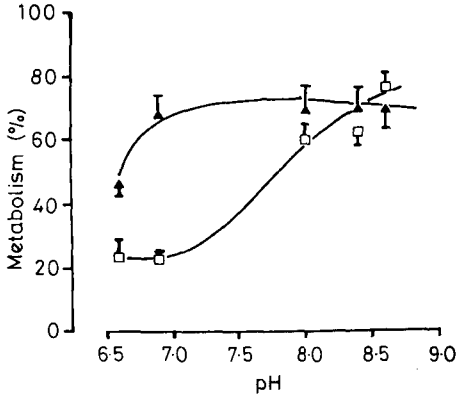

FIG. 4. Activity of PGDH in rat colon 100 000*g* supernatant in the presence (□) and absence (▲) of 50 nM Ph CL 28A as a function of pH. Points show mean ± s.e.m., 4 determinations each.

hibited its breakdown in the rat isolated perfused lung (Fig. 5). The order of potency was Ph CL 28A (IC50 = 72 nM) ≫ Ph CK 47A (IC50 = 1·4 μM) > Ph CL 32A (IC50 = 18 μM) > sulphasalazine (IC50 = 76 μM) > Ph CJ 91B (IC50 = 330 μM). This order of potency is identical to that observed in the tests on the purified human placental PGDH summarized in Table 1, and there is a very strong correlation between the two, r = 0·99, $P < 0·001$, as shown in the inset to Fig. 5. In both systems, Ph CL 28A was 1000 times more potent than the parent compound, sulphasalazine.

Finally, the five compounds were tested as possible inhibitors of the biosynthesis of prostaglandins from arachidonic acid in the sheep seminal vesicle microsome preparation. With the exception of Ph CJ 91B, which demonstrated a small but significant capacity to enhance prostaglandin generation when added at 100 or 1000 μM (e.g., conversion to prostaglandins at 1000 μM Ph CJ 91B was 160·1 ± 12·0% of control $P < 0·05$), none of the azobenzene derivatives had any effects at concentrations up to 1000 μM. This is summarized in Table 1.

DISCUSSION

These studies show that Ph CL 28A is a highly potent inhibitor at nanomolar concentrations of prostaglandin inactivation both in cell-free systems and in the rat intact perfused lung. In fact, Ph CL 28A is the most potent inhibitor of PGDH yet reported in the literature, and it will be interesting to find whether it has any effect on other dehydrogenase enzymes. Most other known PGDH inhibitors (apart from other azobenzene analogues which we have recently been testing) such as carbenoxolone (Peskar et al 1976; Moore & Hoult 1982), nafazatrom (Wong et al 1982), diphloretin phosphate (Crutchley & Piper 1974; Hoult & Moore 1980), non-steroidal anti-inflammatory drugs (Moore & Hoult 1982) as well as various other compounds (reviewed by Hansen 1976) are much less active, in the micromolar-millimolar range, and many also have other prominent pharmacological actions. Noteworthy exceptions are certain 13,14-methylene-15-ketoprostanoate compounds, of which the 13β,14β-isomer was the most active with an IC50 value of 0·14 μM against partially purified swine lung PGDH, that

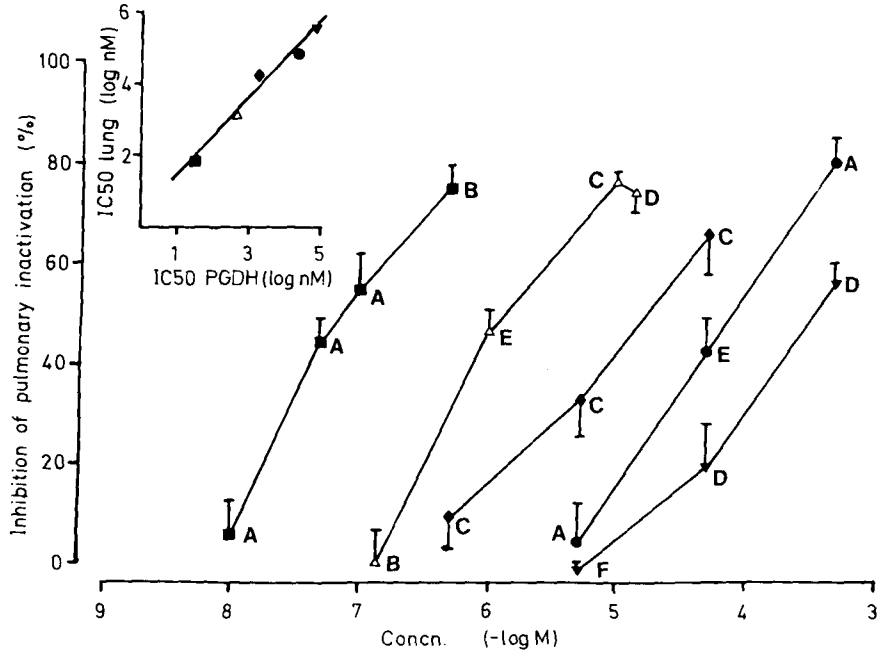

FIG. 5. Inhibition by Ph CL 28A, sulphasalazine and other azobenzene analogues of the inactivation of prostaglandin $F_{2\alpha}$ in rat isolated perfused lungs. Results show mean ± s.e.m. for (m, n) determinations, where m is the number of tests and n is the number of lungs on which they were performed A = (24, 6), B = (8, 2), C = (16, 4), D = (6, 2), E = (20, 5), F = (14, 4). Inset shows correlations between lung IC50 values taken from this data (ordinate, scale as $\log_{10}$ nM), and IC50 values for human placental PGDH from Table 1 (abscissa, scale as $\log_{10}$ nM). Key: (■), Ph Cl 28A; (△), Ph CK 47A; (◆), Ph CL 32A; (●), sulphasalazine; (▼), Ph CJ 91B.

inhibit PGDH at sub-micromolar concentrations (Yamazaki et al 1981), and even these are at least 5 times less active than Ph CL 28A.

The synthesis of Ph CL 28A came about as a rational extension of our previous finding that electronegative substituents capable of withdrawing electrons from the conjugated B ring system of homosalazine enhance the inhibitory activity against PGDH (Berry et al 1983). This was formalized in terms of a correlation between activity and the Hammett σ substituent constant, and it was logical to develop compounds such as Ph CL 28A in which two electronegative substituents with large σ values ($2 \times 0{\cdot}37 = 0{\cdot}74$) are inserted. Nevertheless, high σ values are not an indispensable condition for extreme potency because p-NO_2 ($\sigma = 0{\cdot}78$) and p-SO_2NH_2 ($\sigma = 0{\cdot}57$) rather disappointingly did not achieve the very high activity of the present compound. This suggests that other electronic and steric factors also play a crucial part in determining effective interactions at the enzyme surface. Another example of the sensitive effects of small variations in structure is given by Ph CJ 91B (disodium azodisalicylate), which lacks the methylene group of the homosalazine series and is a weak PGDH inhibitor (Table 1, Fig. 5) as expected.

We also observed that the inhibitory effect of Ph CL 28A is strongly pH-dependent, and is prevented at alkaline pH. This pH dependence for inhibition is common to all analogues so far tested and does not appear to be related to instability of the analogue itself, as even stable compounds show similar pH dependence. This indicates that the binding of the azobenzene to the allosteric site is pH dependent, but we have not yet investigated whether this reflects chemical effects in the drug molecule itself (e.g., lactone formation) or changes in the binding site on the enzyme itself (e.g., ionization of residues with pK values in the range tested).

There are two reasons why it is interesting that Ph CL 28A is a highly potent inhibitor of prostaglandin breakdown in the rat isolated lung and that the other four compounds also inhibit this process in direct relation to their in-vitro potency on the human enzyme (Fig. 5). First, it demonstrates that these compounds are active in intact cell systems and that the straightforward and inexpensive in-vitro test has considerable predictive value for such activity. Second, it may provide further insight into the nature of prostaglandin inactivation in the lung.

In a previous paper (Hoult & Moore 1980) it was suggested that the inhibitory activity of sulphasalazine in the perfused lung was probably exerted by an interaction with the cytosolic PGDH enzyme rather than with the (hypothetical) carrier which is postulated to transport the prostaglandin into the cells in which inactivation takes place. This interpretation has been challenged by Bahkle (1980) and by Hellewell & Pearson (1982) who confirmed the ability of sulphasalazine to inhibit pulmonary prostaglandin inactivation but opted for the carrier as the primary target. This was on the basis of the slowing of both the efflux profile of tritiated prostaglandins (Bahkle 1980; Hellewell & Pearson 1982) and of the 'inactivation' (i.e., apparent sequestration) of the non-metabolizable analogue 16,16-dimethyl prostaglandin E_2 (Bahkle 1980). Although persuasive, these experiments do not prove conclusively that the inhibitor acts on the carrier. Indeed, our data in Fig. 5 showing an almost perfect correlation of the capacity of the five compounds to inhibit PGDH in-vitro and pulmonary prostaglandin degradation points strongly to the enzyme as the probable site of action in the intact tissue. The alternative explanation—that the compounds act on the transport/carrier molecule—would require that from a structural point of view it bears a remarkable resemblance to PGDH itself, especially as the inhibitors act non-competitively (i.e., at an allosteric site) on PGDH.

Regardless of the outcome of this interesting dilemma, it can be concluded that Ph CL 28A has strikingly high potency as inhibitor of PGDH and should be useful as a probe for investigating the structure and function of this enzyme. It may also be possible to use this compound to evaluate the biological consequences of PGDH inhibition in-vivo. Finally, Ph CL 28A could assist in elucidating the mechanism of action of sulphasalazine for the prevention of relapse in ulcerative colitis, as this is still contentious (see Hoult & Moore 1982, for review). This is because in Ph CL 28A there is a much higher degree of separation between actions to inhibit PGDH and actions on prostaglandin biosynthesis.

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