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Enzymatic oxidation of phthalazine with guinea pig liver aldehyde oxidase and liver slices: inhibition by isovanillin

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The enzymes aldehyde oxidase and xanthine oxidase catalyze the oxidation of a wide range of N-heterocycles and aldehydes. These enzymes are widely known for their role in the metabolism of N-heterocyclic xenobiotics where they provide a protective barrier by aiding in the detoxification of ingested nitrogen-containing heterocycles. Isovanillin has been shown to inhibit the metabolism of aromatic aldehydes by aldehyde oxidase, but its inhibition towards the heterocyclic compounds has not been studied.

The present investigation examines the oxidation of phthalazine in the absence and in the presence of the inhibitor isovanillin by partially purified aldehyde oxidase from guinea pig liver. In addition, the interaction of phthalazine with freshly prepared guinea pig liver slices, both in the absence and presence of specific inhibitors of several liver oxidizing enzymes, was investigated.

Aldehyde oxidase rapidly converted phthalazine into 1-phthalazinone, which was completely inhibited in the presence of isovanillin (a specific inhibitor of aldehyde oxidase). In freshly prepared liver slices, phthalazine was also rapidly converted to 1-phthalazinone. The formation of 1-phthalazinone was completely inhibited by isovanillin, whereas disulfiram (a specific inhibitor of aldehyde dehydrogenase) only inhibited 1-phthalazinone formation by 24% and allopurinol (a specific inhibitor of xanthine oxidase) had little effect. Therefore, isovanillin has been proved as an inhibitor of the metabolism of heterocyclic substrates, such as phthalazine, by guinea pig liver aldehyde oxidase, since it had not been tested before.

Thus it would appear from the inhibitor results that aldehyde oxidase is the predominant enzyme in the oxidation of phthalazine to 1-phthalazinone in freshly prepared guinea pig liver slices, whereas xanthine oxidase only contributes to a small extent and aldehyde dehydrogenase does not take any part.

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Precision-cut liver slices are now extensively used for *in vitro* metabolic and toxicological studies (Ekins, 1996a; De Kanter *et al.*, 1999; Lerche-Langrand & Toutain, 2000; Panoutsopoulos *et al.*, 2004a; Price *et al.*, 2004). The main advantage of liver slices is that they can be easily prepared from different animal models, including man, using the same method. They closely resemble the organ from which they are derived and all cell types are present maintaining the architecture of the tissue as in the whole organ *in vivo* (Ekins, 1996b; Toutain *et al.*, 1998). Liver slices retain their normal liver hepatocyte population and their physiological and biochemical functions for many hours (Smith *et al.*, 1986; Sipes *et al.*, 1987). The critical factors for maintaining optimal viability of slices include slice thickness, composition of the culture medium and the incubation time used. Freshly prepared rat liver slices are able to retain high viability of cells for up to 48 h of incubation.

Compounds containing aromatic nitrogen heterocyclic rings, such as purines, pyrimidines, pteridines, quinolines and diazanaphthalenes, are widely distributed throughout nature and often play an important role in biochemical processes. In addition, a large number of drugs in use today contain such ring systems. Most studies on the metabolism of foreign compounds have been directed at the cytochrome P450 mixed function oxidase system but in the last two decades there is an increased awareness of the role that the molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase, play in the oxidation of this type of drugs.

Both aldehyde oxidase and xanthine oxidase catalyze the oxidation of a wide range of N-heterocycles and aldehydes (Pelsy & Klibanov, 1983; Beedham, 1985; Beedham *et al.*, 1995a; Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004b). Aldehyde oxidase activity is predominant in the liver (Sasaki *et al.*, 1983; Beedham *et al.*, 1987), whereas xanthine oxidase activity is present at high levels in the lactating mammary gland (Bruder *et al.*, 1982) and primarily in cow's milk. Although these enzymes are similar in structure and molecular properties, they differ in substrate specificity (Krenitsky *et al.*, 1972; Pelsy & Klibanov, 1983; Panoutsopoulos & Beedham, 2004).

The most active substrates of aldehyde oxidase are aromatic heterocycles containing two fused six-membered rings. Phthalazine (2,3-diazanaphthalene) is oxidized to 1-phthalazinone by both aldehyde oxidase (AO) and xanthine oxidase (XO) (Fig. 1) although it is a

Figure 1. The metabolic pathway of phthalazine oxidation.

far better substrate for aldehyde oxidase with a K_m value in the region of 5×10^{-5} M for the guinea pig liver enzyme and 1×10^{-4} M for the rabbit enzyme (Stubley *et al.*, 1979; Beedham *et al.*, 1990). The K_m value with bovine milk xanthine oxidase is some 30 times higher. In contrast, 3-hydroxy-4-methoxybenzaldehyde (isovanillin) has been shown to be a reasonable substrate of xanthine oxidase (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004b), but a potent competitive inhibitor of aldehyde oxidase (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004a; 2004b).

The present study examines the oxidation of phthalazine with guinea pig liver aldehyde oxidase as this enzyme has similar substrate specificity to human aldehyde oxidase (Beedham *et al.*, 1987). Isovanillin has been shown to inhibit the metabolism of aromatic aldehydes by aldehyde oxidase (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004a; 2004b) but its inhibition towards the heterocyclic compounds has not been studied. Therefore, the oxidation of phtha-

lazine with guinea pig liver aldehyde oxidase was also assayed in the presence of isovanillin. In addition, the interaction of the heterocycle phthalazine with guinea pig liver slices, both in the absence and presence of specific inhibitors for each oxidizing enzyme (disulfiram for aldehyde dehydrogenase, isovanillin for aldehyde oxidase and allopurinol for xanthine oxidase) were investigated.

MATERIALS AND METHODS

Animals. Dunkin-Hartley guinea pigs (450–950 g) were used in this study. Animals were fed with FD1 pellets supplemented with ascorbic acid and received hay three times weekly. All the animals were allowed food and water *ad libitum* and maintained in a strictly controlled temperature (18 ± 1°C), humidity (50–55%) and a lighting cycle (07.00–19.00 h light and 19.00–07.00 h dark). Animals were handled with humane care in accordance with the National Institutes of Health guidelines.

Chemicals. The following compounds were supplied from the manufacturers indicated: 3,4-dihydroxybenzoic acid (protocatechuic acid), 3-hydroxy-4-methoxybenzaldehyde (isovanillin), 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), tetraethylthiuram disulfide (disulfiram), allopurinol, diethylamine (Sigma Chemical Co. Ltd, Poole, Dorset, U.K.), phthalazine (Aldrich Chemical Co. Ltd, Gillingham, Dorset, U.K.), perchloric acid (BDH Chemicals Ltd, Poole, U.K.), acetonitrile HPLC grade (Rathburn Chemicals Ltd, Walkeburn, Scotland). 1-Phthalazinone was synthesized according to published procedures (Dennis *et al.*, 1975).

Preparation of partially purified aldehyde oxidase from guinea pig liver. Centrifugation was carried out using an MSE High Speed 18 centrifuge fitted with an angled rotor. Guinea pigs were killed by cervical dislocation, their livers were placed in 3–4 volumes of ice-cold isotonic potassium chloride solution (1.15% KCl, w/v) and they were homogenized in a Tri-R stir-R Model S63C variable speed homogenizer fitted with a Teflon pestle. Partially purified aldehyde oxidase was then prepared according to the method of Beedham *et al.* (1990). The protein content estimation in aldehyde oxidase was determined using Coomasie Blue dye reagent and bovine serum albumin as the standard, measured at 595 nm, according to the method of Bradford (1976).

HPLC analysis of phthalazine and its metabolites. Reversed-phase high performance liquid chromatography (HPLC) was performed using a system from Waters Associates (Northwich, Cheshire, U.K.), which consisted of a single piston reciprocating 501 pump, a WISP 710B auto-injector, a Lambda-Max 481 LC spectrophotometer (with variable wavelength) and a Data Module 740. Compounds were separated using a stainless steel Hypersil ODS column 5 μm (25 cm × 4.6 mm internal diameter). A Waters Guard Pak pre-column with a C18 insert was fitted to the HPLC system for the analysis of incubation extracts, to avoid contamination of the column with any biological materials. All solvents were maintained at 4°C and 20 μl samples were injected for each analysis. The wavelength used for the HPLC analysis of phthalazine and its metabolites was 285 nm.

HPLC separation of the heterocycle phthalazine and its possible metabolites was achieved with a mobile phase containing 15% of HPLC grade acetonitrile and 85% (v/v) 2.2 × 10^{-1} M orthophosphate buffer, pH 2.9, containing 1.1 × 10^{-1} M diethylamine at a flow rate of 1.5 ml/min. The mobile phase was filtered and degassed for 10 min under vacuum before use. The addition of diethylamine to the mobile phase was to ensure that the retention times were not influenced by the silica matrix of the packing material.

Incubation conditions for the production of 1-phthalazinone from phthalazine by aldehyde oxidase. In a total volume of 3 ml, phthalazine (1 × 10^{-3} M) was incubated at 37°C in a shaking water bath with 60 μl

guinea pig liver aldehyde oxidase (24 mg/ml) and Krebs-Henseleit buffer, pH 7.4, containing 2.4×10^{-2} M bicarbonate. Aliquots (0.2 ml) were removed at 0 min and at various time intervals, mixed with 0.1 ml 3.6% perchloric acid and centrifuged for 2.5 min at maximum speed on a Beckman microfuge B. The supernatant was then analyzed by HPLC. Standard concentrations of phthalazine and its corresponding metabolites were also analyzed by HPLC, to quantitate their concentrations in incubation mixtures. Control incubations without enzyme were also performed. Incubations were also carried out in the presence of isovanillin, which is a potent inhibitor of aldehyde oxidase (Beedham *et al.*, 1995b; Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004a; 2004b).

Preparation of guinea pig liver slices. Guinea pigs were killed by cervical dislocation, their livers were immediately excised and placed in ice-cold Krebs-Henseleit solution, pH 7.4, containing 2.4×10^{-2} M bicarbonate, which was continuously oxygenated with 95% O_2/5% CO_2.

Liver slices were obtained according to the method of Panoutsopoulos *et al.* (2004a), which was developed to mimic the Krumdieck liver slicer (Krumdieck *et al.*, 1980). The prepared liver slices were stored in oxygenated Krebs- Henseleit buffer pH 7.4 containing 2.4×10^{-2} M bicarbonate at 4°C until required.

Incubation conditions with guinea pig liver slices. Phthalazine (1×10^{-3} M) was incubated in 20 ml vials containing four liver slices in a total volume of 3 ml of Krebs-Henseleit buffer pH 7.4, the addition of the liver slices indicated the beginning of the experiment (t = 0 min).

The incubation mixtures were maintained at 37°C in a shaking water bath to facilitate mixing. The medium was oxygenated with 95%O_2/5% CO_2 initially and every subsequent hour for 5 min. Preliminary experiments determined that this amount of oxygenation was sufficient to maintain optimum metabolite production. Incubations with liver slices were also performed in the presence of inhibitors. The inhibitors used were isovanillin for aldehyde oxidase (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004a; 2004b), allopurinol for xanthine oxidase (Peterson *et al.*, 1990) and disulfiram for aldehyde dehydrogenase activity (Deitrich & Erwin, 1971; Lipsky *et al.*, 2001).

Aliquots (0.2 ml) were removed at t = 0 min and at various time intervals, added to 0.1 ml of 3.6% perchloric acid and centrifuged for 2.5 min at maximum speed on a Beckman microfuge B. The supernatant was then analyzed by HPLC. Control incubations without liver slices were also performed. Standard solutions of phthalazine and its possible metabolites were also analyzed by HPLC. After analysis, the slices were blotted dry and weighed to determine the total weight of liver used for each incubation.

RESULTS

Oxidation of phthalazine with guinea pig liver aldehyde oxidase

Incubations of phthalazine (1×10^{-3} M) with partially purified guinea pig liver aldehyde oxidase resulted in an almost complete conversion of phthalazine (retention time, Rt = 5.6 min) into its metabolite, 1-phthalazinone (Rt = 8.6 min), 120 min after the incubation was initiated (Fig. 2). Control phthalazine incubations, in the absence of aldehyde oxidase, did not show any decrease in phthalazine concentration and no metabolites were formed over the 120 min incubation period.

Effect of isovanillin on phthalazine oxidation catalyzed by guinea pig liver aldehyde oxidase

In the presence of isovanillin (1×10^{-3} M), a small decrease in phtalazine concentration was observed and very little metabolite was

produced during the 120 min incubation period (Fig. 2). Therefore, isovanillin inhibited phthalazine metabolism by about 96%.

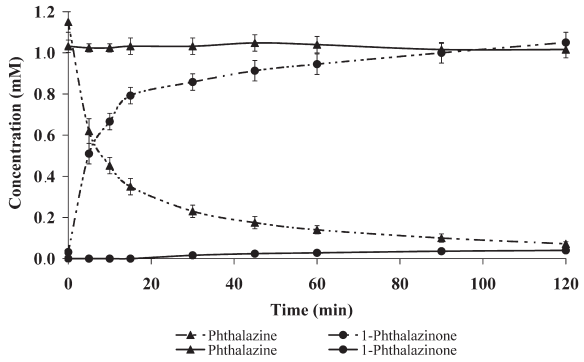

Figure 2. Inhibition of phthalazine oxidation catalyzed by guinea pig liver aldehyde oxidase.

Phthalazine (1×10^{-3} M) was incubated in the absence (broken lines) and presence (solid lines) of isovanillin (1×10^{-3} M) with 60 μl of guinea pig liver aldehyde oxidase (24 mg/ml) in 3 ml of Krebs-Henseleit buffer, pH 7.4, at 37°C. Each point is the mean of six determinations. Values are expressed as means ± S.E.

Metabolism of phthalazine by guinea pig liver slices

Incubations of phthalazine with guinea pig liver slices resulted in an 85% decrease in phthalazine concentration during the 180 min incubation time (Fig. 3). A single metabolite was formed which accounted for 71% (t = 180 min) of phthalazine conversion and co-chromatographed with 1-phthalazinone. Control incubations of phthalazine, in the absence of guinea pig liver slices, showed no such decrease in the concentration of phthalazine and, likewise, no metabolites were formed.

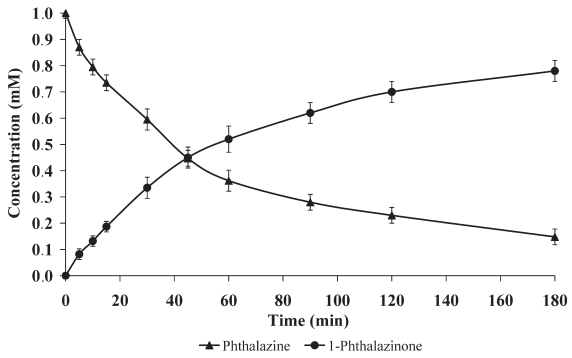

Figure 3. Metabolism of phthalazine by guinea pig liver slices.

Phthalazine (1×10^{-3} M) was incubated with four guinea pig liver slices (133 mg) in 3 ml of Krebs-Heinseleit buffer, pH 7.4, at 37°C. Each point is the mean of six determinations. Values are expressed as means ± S.E.

Effect of inhibitors on phthalazine metabolism by guinea pig liver slices

Isovanillin. In the presence of isovanillin (1×10^{-3} M), phthalazine metabolism by guinea pig liver slices was significantly reduced with only low amounts of 1-phthalazinone produced (approx. 7%) in 90 min. It was noted that during the first 90 min of incubation, isovanillin acted as a good inhibitor showing maximum inhibition. However, after 90 min most of the isovanillin had been metabolized into isovanillic acid and protocatechuic acid. After this, less inhibition was observed and therefore phthalazine was more rapidly metabolized to 1-phthalazinone (Fig. 4).

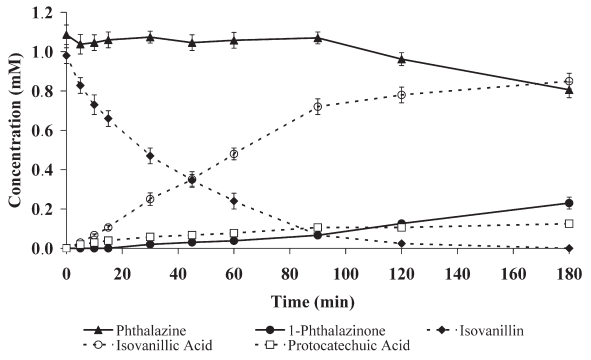

Figure 4. Effect of isovanillin on phthalazine metabolism by guinea pig liver slices.

Phthalazine (1×10^{-3} M) was incubated in the presence of isovanillin (1×10^{-3} M) with four guinea pig liver slices (140 mg) in 3 ml of Krebs-Heinseleit buffer, pH 7.4, at 37°C. The metabolism of the inhibitor isovanillin by the liver slices into isovanillic acid and protocatechuic acid is also shown in the figure. Each point is the mean of five determinations. Values are expressed as means ± S.E.

Allopurinol. The presence of allopurinol (5×10^{-5} M) resulted in a 7% inhibition in phthalazine conversion and a 5% inhibition in 1-phthalazinone formation over the 90 min incubation period (not shown). A higher concentration of allopurinol (1×10^{-4} M) resulted in a similar inhibition in phthalazine conversion (8%) and it was only slightly more effective as an inhibitor (8%) in the formation of 1-phthalazinone under similar conditions (not shown).

Disulfiram. The presence of disulfiram (1×10^{-4} M) during incubation of phthalazine with the guinea pig liver slices resulted in a 24% inhibition of the production of 1-phthalazinone at t = 90 min. Thus, disulfiram only inhibits aldehyde oxidase activity in liver slices to a small extent (not shown).

DISCUSSION

The molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase, are more widely known for their role in the metabolism of heterocyclic compounds, such as purines, pteridines and diazanaphthalines (Beedham, 1987; Critchley *et al.*, 1992) rather than the metabolism of aldehydes (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004b). It has been suggested that aldehyde oxidase and xanthine oxidase provide a protective barrier for the organism by detoxifying ingested nitrogen-containing heterocycles since high concentrations of these enzymes are found in the liver (Krenitsky, 1978; Beedham, 1987).

Guinea pig liver aldehyde oxidase enzyme was chosen in this study as it has similar substrate specificity to human liver aldehyde oxidase (Beedham *et al.*, 1990; Beedham *et al.*, 1987). The partially purified aldehyde oxidase would not contain contaminating aldehyde dehydrogenase, since the mitochondrial enzyme is removed by centrifugation and both cytosolic and mitochondrial isoenzymes are heat labile. In addition, aldehyde dehydrogenase requires NAD^+ as a cofactor, but this was not added to the incubations as aldehyde oxidase utilizes molecular oxygen as an electron acceptor (Beedham, 1987; Panoutsopoulos & Beedham, 2004). As phthalazine does not contain an aldehyde group it is not a substrate for aldehyde dehydrogenase.

Incubation of phthalazine with partially purified guinea pig liver aldehyde oxidase led to its being rapidly metabolized into 1-phthalazinone. Since isovanillin has been shown to have a potent inhibitory effect upon the metabolism of phenylacetaldehyde (Panoutsopoulos *et al.*, 2004a) and other aldehydes (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004b) by guinea pig aldehyde oxidase, it was also tested against a heterocyclic substrate, phthalazine. Consequently, aldehyde oxidase incubations were performed in the presence of isovanillin using phthalazine as substrate. As phthalazine metabolism is inhibited by about 96%, this indicates that isovanillin is not only an inhibitor of aldehyde oxidation (Panoutsopoulos & Beedham, 2004; Panoutsopoulos *et al.*, 2004a; 2004b) but also of the metabolism of heterocyclic substrates by guinea pig liver aldehyde oxidase.

In order to confirm that aldehyde oxidase is active in guinea pig liver slices, phthalazine was incubated under similar conditions to those for phenylacetaldehyde (Panoutsopoulos *et al.*, 2004a). Phthalazine is not a substrate for aldehyde dehydrogenase and only a relatively poor substrate for xanthine oxidase (Stubley *et al.*, 1979; Beedham *et al.*, 1990). Thus, any 1-phthalazinone formed will be predominantly due to the action of aldehyde oxidase.

In freshly prepared precision-cut liver slice incubations, phthalazine was rapidly converted to a single metabolite, 1-phthalazinone. As phthalazine is a substrate for both aldehyde oxidase and xanthine oxidase *in vitro* (Stubley *et al.*, 1979; Beedham *et al.*, 1990) and *in vivo* (Johnson *et al.*, 1984) it is conceivable that 1-phthalazinone may be produced in guinea

pig liver slices by either or both enzymes. Liver slice incubations with phthalazine showed a similar metabolic profile to that observed with vanillin over the 180 min incubation period (Panoutsopoulos & Beedham, 2005). In addition, there is also a close relationship between the profiles of vanillin and phthalazine when incubated with partially purified aldehyde oxidase (Panoutsopoulos *et al.*, 2004b).

The effect of isovanillin on phthalazine oxidation by guinea pig liver slices up to 90 min is similar to that obtained with partially purified aldehyde oxidase. This indicates that aldehyde oxidase is solely responsible for the production of 1-phthalazinone in liver slices and that isovanillin also acts as a potent inhibitor of phthalazine oxidation under these conditions. As the oxidation of isovanillin to isovanillic acid is not greatly altered by the presence of phthalazine it would appear that, in liver slices, isovanillin is metabolized by an enzyme other than aldehyde oxidase. In fact, isovanillin is metabolized to isovanillic acid and protocatechuic acid by aldehyde dehydrogenase (Panoutsopoulos *et al.*, 2004b). It is known that disulfiram is a specific inhibitor of the aldehyde dehydrogenase activity (Deitrich & Erwin, 1971; Lipsky *et al.*, 2001). Since phthalazine does not contain an aldehyde group, it is unlikely that phthalazine is a substrate of aldehyde dehydrogenase. Therefore, the unexpected inhibition by disulfiram suggests that disulfiram only inhibits aldehyde oxidase activity in liver slices to a small extent.

Phthalazine, which is rapidly oxidized by aldehyde oxidase, is also a poor substrate for xanthine oxidase (Stubley *et al.*, 1979; Beedham *et al.*, 1990). Therefore, the small decrease in the production of 1-phthalazinone caused by allopurinol may be the result of inhibited xanthine oxidase activity. Alternatively, allopurinol is also a substrate for aldehyde oxidase (Hille & Massey, 1981; Moriwaki *et al.*, 1993) and would be expected to compete for the active site. This may also be a contributing factor in allopurinol inhibition of phthalazine oxidation in liver slices.

All three inhibitors have similar effects on phthalazine and vanillin oxidation in liver slices and, therefore, it is likely that the same enzyme is responsible for their oxidation. This enzyme appears to be aldehyde oxidase. This is further reinforced by studies performed with liver slice incubations of phthalazine in the presence of menadione, another specific aldehyde oxidase inhibitor (Rajagopalan & Handler, 1964). The results showed that menadione (1×10^{-4} M) inhibited the oxidation of phthalazine to 1-phthalazinone by 79% within 15 min (unpublished observations).

In conclusion, phthalazine is metabolized by freshly prepared precision-cut liver slices to 1-phthalazinone mainly by aldehyde oxidase, whereas xanthine oxidase activity only contributes to a small extent and aldehyde dehydrogenase does not take part in the metabolism of phthalazine.

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