KINETICS OF DRUG METABOLISM IN RAT LIVER SLICES: IV. COMPARISON OF ETHOXYCOUMARIN CLEARANCE BY LIVER SLICES, ISOLATED HEPATOCYTES, AND HEPATIC MICROSOMES FROM RATS PRETREATED WITH KNOWN MODIFIERS OF CYTOCHROME P-450 ACTIVITY

DAVID J. CARLILE, NANCY HAKOOZ, AND J. BRIAN HOUSTON

School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom

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ABSTRACT:

To evaluate the theory that within precision-cut liver slices intercellular transport occurs in parallel with cellular metabolism and to illustrate the constraints these places on clearance predictions, the kinetics of ethoxycoumarin O-deethylation have been determined under varying conditions of hepatic cytochrome P-450 activity. Liver slices, isolated hepatocytes, and microsomes were obtained from rats treated with the inducers phenobarbital (PB) and β-naphthoflavone (βNF) and the inhibitor aminobenzotriazole (ABT). In hepatocytes and microsomes, a two-site kinetic model with a high-affinity, low-capacity site and an unsaturated low-affinity, high-capacity site described the hydroxycoumarin formation data. There were marked increases in Vmax (2- to 5-fold and 50- to 70-fold for PB and βNF, respectively) in both systems and in CLint (3- and 9-fold for PB and βNF, respectively) in hepatocytes and substantial decreases in both parameters (3–8 and 12–23% of control, respectively) in ABT hepatocytes and microsomes. A qualitatively similar response was evident in slices obtained from livers of rats treated with phenobarbital and ABT, but although slices from βNF livers produced high metabolic rates (comparable to slices obtained from livers of rats treated with phenobarbital), these showed a linear increase with substrate concentration without indication of a high-affinity site. The intrinsic clearance parameters were scaled to full liver capacity using hepatocellularities and microsomal recovery indices to allow direct comparison of these responses. The slice system consistently underestimated the effects of the modifiers. When compared with hepatocytes, estimates of 30, 15, and 1% for ABT, PB, and βNF, respectively, were observed and the degree of underestimation was dependent on the magnitude of intrinsic clearance and was consistent with the above theory.

Precision-cut liver slices offer an alternative in vitro approach to the use of intact cells or subcellular fractions for investigating drug metabolism. There is a growing body of literature indicating the value of this system for identifying routes of metabolism (Harris et al., 1994; Prueksaritanont et al., 1995; Bach et al., 1996; Ekins, 1996), and there is good accord, on a qualitative level, between the metabolites formed by slices and those formed by other in vitro systems as well as in vivo. Recently we have explored another potential application of liver slices, namely, their utility in predicting rates of metabolism (Worboys et al., 1995, 1996a,b, 1997). Freshly isolated hepatocytes in suspension and, to a lesser extent, hepatic microsomes provide accurate predictions of in vivo intrinsic clearance (CLint) and hepatic clearance (Houston, 1994; Houston and Carlile, 1997). However, there are a number of additional advantages in the use of precision-cut liver slices for prediction work. In addition to maintaining intact architecture, their preparation is simpler, quicker, and does not involve the use of proteolytic enzymes.

We have found that liver slices, in comparison with isolated hepatocytes in suspension, consistently underpredict CLint when both parameters are normalized for their respective hepatocellularity (Worboys et al., 1996a). Thus, either there are only a limited number of hepatocytes in the slice that contribute to clearance or not all hepatocytes are operating at their full metabolic potential. Furthermore, the degree of this underprediction appears to be related to the magnitude of the CLint value; high-clearance drugs are underpredicted to a larger degree than low-clearance drugs. Direct evidence is available that indicates that this phenomenon is dependent on the extent to which the drug distributes within the slice during the incubation period (Worboys et al., 1997). Thus, there is a need to consider rates of intercellular transport and intracellular metabolism as parallel processes when interpreting metabolite production by liver slices.

These conclusions were formulated from data on six drugs selected for study on the basis of differences in both physicochemical properties and clearance values (Worboys et al., 1997). Therefore, it is important to confirm that these experimental findings reflect the general properties of precision-cut slices rather than originating from physicochemical properties of the drugs under investigation. To establish that the above conclusions are not compound-dependent, we have undertaken a series of studies with one particular drug substrate,
7-ethoxycoumarin (EC), using slices from rats treated with certain well documented modifiers of cytochrome P-450 (CYP) activity to generate a range of $C_{\text{int}}$ values. EC is used frequently as a probe substrate (Carlile et al., 1998), its clearance in rat liver slices is intermediate (Worboys et al., 1995), and, at least in microsomes, it responds to the classic inducers phenobarbital (PB) and β-naphthoflavone (βNF) (Boobis et al., 1986; Fry et al., 1992) and the suicide inhibitor aminobenzotriazole (ABT) (Ervine and Houston, 1990). In addition, analogous studies have been carried out in freshly isolated hepatocytes and hepatic microsomes to allow a detailed comparison of the quantitative responses seen in the three in vitro preparations obtained from rats treated with known modifiers of CYP activity. To ensure that valid comparisons are made, based solely on changes in CYP activity, in vitro parameters have been scaled to in vivo units (Carlile et al., 1997) using hepatocellularity and microsomal recovery factors specifically determined for PB, βNF, and ABT treatments.

Materials and Methods

Chemicals. EC, 7-hydroxycoumarin (HC), Earle's balanced salt solution, Krebs-Henseleit buffer, and β-glucuronidase were purchased from Sigma Chemical Company (Poole, Dorset, UK). All other chemicals were purchased from either BDH (Lutterworth, Leicester, UK) or Sigma.

Animals. Male Sprague-Dawley rats (225–270 g) were obtained from the Biological Services Unit at the University of Manchester. They were housed two to four per cage on sawdust bedding in rooms maintained at a temperature of 20 ± 2°C and allowed free access to water and Chow Rat and Mouse diet. Rats were either untreated (UT) or administered i.p. injections of either PB (80 mg/kg in 0.9% saline, for 3 days), βNF (100 mg/kg in corn oil, for 3 days), or ABT (50 mg/kg in 0.9% saline, one dose). Induced rats were sacrificed on the fourth day, and ABT rats were sacrificed 3 h after treatment. In each case the animals were divided into three groups for either hepatocyte ($N = 4$), microsomal ($N = 4$), or liver slice ($N = 4$) studies.

Slice Preparation and Incubation. Animals were sacrificed by cervical dislocation, and the liver was quickly excised into ice-cold Earle's balanced salt solution. Circular columns of tissue were prepared from the liver using two 10-mm diameter). Slices were prepared in ice-cold Earle’s balanced salt solution using a Krumdieck Tissue Slicer (Alabama R & D, Munford, AL).

Slices were transferred into Krebs-Henseleit buffer (pH 7.4, room temperature) and floated onto stainless steel wire mesh inserts. Slices were supported by a motor-driven borer (180 rpm, 10-mm diameter). Slices were prepared in Krebs-Henseleit buffer, and β-glucuronidase was added to incubation (Carlile et al., 1997). After a preincubation for 10 min with buffer (Worboys et al., 1995), its clearance in rat liver slices is intermediate (Worboys et al., 1995), and, at least in microsomes, it responds to the classic inducers phenobarbital (PB) and β-naphthoflavone (βNF) (Boobis et al., 1986; Fry et al., 1992) and the suicide inhibitor aminobenzotriazole (ABT) (Ervine and Houston, 1990). In addition, analogous studies have been carried out in freshly isolated hepatocytes and hepatic microsomes to allow a detailed comparison of the quantitative responses seen in the three in vitro preparations obtained from rats treated with known modifiers of CYP activity. To ensure that valid comparisons are made, based solely on changes in CYP activity, in vitro parameters have been scaled to in vivo units (Carlile et al., 1997) using hepatocellularity and microsomal recovery factors specifically determined for PB, βNF, and ABT treatments.

In Vitro Kinetic Studies. The formation of HC was determined in slices, isolated hepatocytes, and microsomes from rats treated with either PB, βNF, or ABT. As illustrated for the PB (Fig. 1A) and ABT (Fig. 1B) cases, these profiles could be best described by a two-site model with a high-affinity, low-capacity site and a low-affinity, high-capacity site that was not saturated over the concentration range studied. The $C_{\text{int}}$ was determined from the sum of $C_{L1}$ and $C_{L2}$. In the case of the data from βNF slices, no saturation was observed and $C_{L1}$ was obtained from the slope of the linear relationship between HC formation rate and EC concentration.

Hepatocellular was used to scale the parameters from slice and isolated hepatocyte studies to in vivo units denoted by SRW. The microsomal recovery index was used in a similar manner for microsomal data (Carlile et al., 1997).

Results

In Vitro Kinetic Studies. The formation of HC was determined over a wide concentration range (0.5–100 μM) in slices, isolated hepatocytes, and microsomes from rats treated with either PB, βNF, or ABT. As illustrated for the PB (Fig. 1A) and ABT (Fig. 1B) cases, these profiles could be best described by a two-site model with a high-affinity, low-capacity site and a low-affinity, high-capacity site that was not saturated over the concentration range under investigation. In cells and microsomes from βNF-treated rats an identical model was appropriate, whereas in slices, although rates of HC formation were similar to the PB rates, a linear increase in rate with substrate concentration (Fig. 1C) was seen. In general, there was consistency in the trend for the changes in kinetic parameters across the three in vitro systems (Tables 1–3).

Marked changes in both $V_{\text{max}}$ and clearance terms ($CL_1$ and $CL_2$) were observed between slices and microsomes.
were observed in hepatocytes from treated livers when compared with the corresponding parameters from UT rats (Table 1). The anticipated increases in the induced livers (5- and 70-fold for \( V_{\text{max}} \), 3- and 9-fold for \( CL_{\text{int}} \) for PB and \( \beta \)NF, respectively) and decreases in the inhibited livers (8 and 23% of UT for \( V_{\text{max}} \) and \( CL_{\text{int}} \), respectively) for these parameters were observed. Thus, the induction effects observed in cells from \( \beta \)NF rats were more marked than those observed for PB cells. Also, the changes in \( K_m \) were minor with the exception of \( \beta \)NF treatment, which resulted in a 5-fold increase.

Similar responses were evident in the microsomal preparations from the \( \beta \)NF and ABT livers (Table 2): \( V_{\text{max}} \) increased 126-fold and decreased to 3%, \( CL_{\text{int}} \) increased 9-fold and decreased to 12% when compared with UT. Both \( CL_1 \) and \( CL_2 \) were markedly influenced by \( \beta \)NF and ABT treatment whereas only \( CL_2 \) was increased in response to PB, despite the increase in \( V_{\text{max}} \). As was the case with hepatocytes, there was a statistical significant increase in \( K_m \) in the \( \beta \)NF case when compared with UT animals.

In \( \beta \)NF slices only one clearance term could be determined, and this was of a value similar to the UT \( CL_{\text{int}} \) (Table 3). Both PB and ABT treatments gave the expected changes in slices for the \( V_{\text{max}} \) (7-fold

**FIG. 1.** Relationship between rate of hydroxycoumarin formation and ethoxycoumarin concentration for slices (■), isolated hepatocytes (▲), and microsomes (×) prepared from PB-treated (A), ABT-treated (B), and \( \beta \)NF-treated (C) rats.

The inset in A–C represents the formation rate data over the ethoxycoumarin concentration range to 10 \( \mu \)M to demonstrate the high-affinity, low-capacity component.

A, for these particular preparations, \( V_{\text{max}} = 37.7 \) and 96.3 nmol/min/10^6 cells for slices and hepatocytes, respectively, and 174.1 nmol/min/mg for microsomes, \( K_m = 11.1, 2.4, \) and 5.6 \( \mu \)M for slices, hepatocytes, and microsomes, respectively, and \( CL_2 = 0.6 \) and 13.0 \( \mu \)l/min/10^6 cells for slices and hepatocytes, respectively, and 17.0 \( \mu \)l/min/mg for microsomes. B, for these particular preparations, \( V_{\text{max}} = 0.88 \) and 4.40 nmol/min/10^6 cells for slices and hepatocytes, respectively, and 9.93 nmol/min/mg for microsomes, \( K_m = 0.7, 0.2, \) and 3.6 \( \mu \)M for slices, hepatocytes, and microsomes, respectively, and \( CL_2 = 0.1 \) and 0.6 \( \mu \)l/min/10^6 cells for slices and hepatocytes, respectively, and 0.8 \( \mu \)l/min/mg for microsomes. C, for these particular preparations, \( V_{\text{max}} = 1095 \) nmol/min/10^6 cells for hepatocytes and 4996 nmol/min/mg for microsomes, \( K_m = 4.0 \) and 10.2 \( \mu \)M for hepatocytes and microsomes, respectively, \( CL_2 = 9.4 \) \( \mu \)l/min/10^6 cells for hepatocytes and 19.1 \( \mu \)l/min/mg for microsomes, and \( CL_{\text{int}} = 3.2 \) \( \mu \)l/min/10^6 cells for slices.
increase and 67% decrease, respectively) and clearance terms (3-fold increase and 50% decrease, respectively). There were no statistical differences in the $K_{int}$ values from slices obtained from the UT and PB livers. The effects of the three modifiers on HC production, as measured by $V_{max}$ and/or $CL_{int}$ in slices, hepatocytes, and microsomes are summarized in Fig. 2.

Sealed In Vitro Parameters. To compare the absolute parameters from each in vitro system in the PB-, βNF-, and ABT-treated states, the parameters $V_{max}$ and $CL_{int}$ are scaled to in vivo units using the respective hepatocellularities and microsomal protein indices.

The hepatocellularity of livers from PB-, βNF-, and ABT-treated rats based on protein determination in both freshly isolated hepatocytes and liver homogenate are similar to UT (Table 4). There was no statistical difference between the induced and UT groups for either protein measurement. For the ABT group the protein concentration in the homogenate was reduced when compared to UT; however, this trend was also reflected in the cellular measurement. Therefore, for all treatments the hepatocellularity was maintained at approximately 1.2 x 10^6 SRW. Also presented in Table 4 are the microsomal protein recovery indices determined from the ratio of the liver homogenate/microsomal CYP. ABT treatment results in a substantial decrease in CYP content in both homogenate and microsomes of approximately 3-fold. This produces a modest increase in the protein recovery index compared with UT. Although βNF is an inducer of CYP 1A isoforms, there is no overall change in CYP content with this compound, demonstrating that the levels of other CYP isoforms decline. Moreover, there is no change in the microsomal protein yield compared with UT. Also shown in Table 4 are the corresponding data for PB and UT livers from an earlier study (Carlile et al., 1997).

It is instructive to consider the ratio of the slice/cell parameters as a measure of the consistency between these two in vitro systems (Table 5). For PB the clearance ratio (0.15) is similar to UT (0.14), and for ABT the ratio is higher (0.3), indicating that slice metabolic activity is 15–30% of the isolated cells. For βNF, however, the very low ratio of 0.01 is striking, reflecting the severe underestimation by the slice system of the effects of this inducer. In contrast, the $V_{max}$ ratios for PB and UT are similar (0.3 and 0.4, respectively), which contrasts with the ABT ratio of approximately 1. Therefore, on the basis of this latter parameter an identical response is seen in both in vitro systems for treatment with the suicide inhibitor but not for treatment with the inducers. Also shown in Table 5 are the ratios of the slice/microsomal parameters. For $V_{max}$ and $CL_{int}$, trends are seen that are similar to the above-discussed slice/cell ratios with the exception of the PB case. The poor response to this inducer seen in microsomes in comparison to cells explains the higher values observed for the slice/microsome ratios.

Figure 3 illustrates the relationship between the $CL_{int}$ for HC formation from EC in isolated hepatocytes and in precision-cut liver slices from PB-, βNF-, and ABT-treated as well as UT livers. Also shown in this figure are previously published $CL_{int}$ values for caffeine, phenytoin, tolbutamide, diazepam (4'-hydroxylation, 3'-hydroxylation and N-demethylation pathways), and ondansetron (hydroxylation and N-demethylation pathways). In all cases clearance is expressed per million cells.

Discussion

There are several advantages to the use of precision-cut liver slices over that of freshly isolated hepatocytes: in particular, the ease of preparation, the avoidance of proteolytic enzymes, and the maintenance of intact hepatic architecture (Parrish et al., 1995; Bach et al., 1996). The latter feature, however, may also be problematic because it has substantial impact on the interpretation of kinetic studies on drug metabolism. The accessibility of substrate within the slice is controlled by two parallel processes: intercellular transport and intracellular metabolism. Thus, a gradient in drug concentrations will exist between the cells situated on the surface and those in the core of the slice. Under these conditions the rate of metabolism achieved will be an average value arising from this concentration gradient, and the clearance by the slice is lower than that seen for the corresponding number of isolated hepatocytes (Worboys et al., 1996a).

Reduced accessibility of substrate and the existence of concentration gradients within the slice (Worboys et al., 1997) limit the interpretation of the term slice clearance; therefore, an alternative measure of the ability of the slice to metabolize drugs in the absence of any distribution limitation is required. A valuable parameter for this purpose is $V_{max}$, because this rate measurement is concentration-independent and refers to the inherent metabolic activity of the slice. Comparison of this parameter for six pathways of metabolism, which undergo saturation in both slices and isolated cells, demonstrated that liver slices (of 260-μm thickness) show 35% of full metabolic activity (Worboys et al., 1997). To elucidate further the relationship between metabolic rates by slices and by intact cells, we investigated the kinetics of one particular substrate, EC, in livers from animals treated with various modifiers of CYP activity, specifically, PB, βNF, and ABT. This allows the comparison of parameter values across the three in vitro systems plus an assessment of the sensitivity of each system to a defined change in CYP activity.

Each of these modifiers has been established to markedly alter the rate of O-deethylation of EC in hepatic microsomes (Boobis et al., 1986; Ervine and Houston, 1990). The use of both βNF and PB is a classic treatment for induction of CYP isoforms of the 1A and 2B families (Guengerich et al., 1982), and both isoforms are known to be important in HC formation from EC (Ryan and Levin, 1990). ABT generally is regarded as a nonspecific inhibitor of CYP activity, at least at high doses. We have confirmed these observations in microsomes and report similar responses to these modifiers in isolated hepatocytes. Both $V_{max}$ and $CL_{int}$ are affected in the rank order $βNF > PB > UT > ABT$, and the range of values for these parameters in hepatocyte suspension are 1000- and 40-fold, respectively. There was no change in $K_{int}$ when either PB or ABT was compared with UT; however $βNF$, surprisingly, showed an increase. It should be remembered that this parameter, particularly in the case of EC, which displays two-site enzyme kinetics, is an empirical parameter describing the data rather than reflecting a specific interaction with a given isozyme.

For slices, the response to the modifiers was not totally consistent with the response noted in isolated hepatocytes and microsomes (see Fig. 2). In the case of PB similar effects were seen in slices as in the cells, although in absolute terms the $V_{max}$ and $CL_{int}$ values were lower in slices than in cells. Because this trend is also seen with UT, the use of the PB/UT ratio as a measure of induction shows that both systems
give the same quantitative effect (3-fold increase). In contrast, microsomal CL showed a minimal response to PB induction despite an increase in V_{\text{max}}, the latter being consistent with a previous report (Boobis et al., 1986).

The action of ABT as a suicide inhibitor is well documented and is clearly reflected in the CL for each in vitro system. The response (as gauged by the ABT/UT clearance ratio) is greater both in isolated cells and microsomes, presumably for reasons linked to limited accessibility of substrate. Whereas the slice CL is only 15% of the corresponding hepatocellular value in UT (as is the case for PB), this value increases to 30% after ABT treatment. This observation is consistent with the previously reported trend that, for lower clearance drugs, there is closer agreement between the two systems. Slower metabolism is associated with deeper tissue penetration, and, consequently, a higher percentage of cells are able to contribute to the clearance process (Worboys et al., 1997). Thus, the use of the ratio of clearances as a measure of inhibition results in an underestimation of the degree of effect resulting from the ABT treatment.

The V_{\text{max}} value for HC formation after ABT treatment is essentially identical in slices and in isolated cells. This observation is surprising considering earlier studies discussed above showed a consistent difference between the two systems. However, in the ABT case there appears to be full participation from all hepatocytes in the slice, and V_{\text{max}} observed is substantially lower (by 5-fold) than the V_{\text{max}} values reported in the previous study. Further studies are required to establish whether there is a link between low metabolic demands, which occur under ABT inhibition, and the percentage of activity manifest in these particular slices. However, these findings with ABT slices would indicate that the 35% average activity, previously reported for slices under V_{\text{max}} conditions, results from incomplete metabolic activity by all cells rather than full activity from a reduced number of hepatocytes.

The most dramatic difference between slice and isolated cell kinetics is seen after βNF treatment. In hepatocytes, as in microsomes, the relationship between rate of HC formation and EC concentration can vary with substrate concentration.
A linear dependence between rate of production and substrate concentration explains the observed behavior. The rate of metabolism will be extremely fast and the rate of metabolism is governed by the rate of delivery into the cell, and there is essentially no passage into the deeper layers of cells in the slice. Thus, the rate of metabolism will be a result of a smaller number of cells in the βNF case than in the UT case, and the apparent clearance underestimates the true activity of the βNF-treated slice. An alternative explanation would be the complete loss of the activity of the high-affinity site in the slice preparation, but a mechanism that would allow this to occur in slices but not isolated hepatocytes is difficult to comprehend.

In general, slices would appear to be less sensitive in reflecting changes in CYP activity than isolated hepatocytes because of the limited range of clearance values that are apparent in the former in vitro system. As shown in Fig. 3 the range of activity in hepatocytes, based on data from the present study and that published previously for five substrates metabolized by eight pathways of metabolism, covers three orders of magnitude whereas the corresponding range of activity in slices, for the same drugs under the same conditions, is only 50-fold. Furthermore, whereas clearance by both in vitro systems ultimately will be limited by rate of substrate access (either transport into the cell for the hepatocyte and/or between cells for the slice), this limitation occurs far more readily in slices. The present studies, using the same substrate in liver preparations that display a range of CYP activity, confirm the relationship previously demonstrated with six different substrates. Thus, the phenomenon of underprediction by slices relative to isolated hepatocytes is an intrinsic characteristic of this in vitro system and not related to the differing physicochemical properties of the drugs under investigation.

In conclusion, these studies have confirmed the importance of considering substrate accessibility within slices when interpreting metabolite kinetic studies. The changes seen in the rate of HC formation by slices from rats treated with different modifiers of CYP activity can be rationalized, although they are not always predictable from the responses evident in isolated hepatocytes and microsomes. The lower CYP activity evident in ABT-treated livers results in greater accessibility than in the control slice as well as a larger percentage of hepatocytes contributing to slice clearance. In contrast,
the higher metabolizing activity resulting from treatment with the CYP inducer βNF results in less accessibility than in the control slice and a lower percentage of contributing hepatocytes. The overall consequences of such changes is to underestimate the degree of effect of these modifiers when only slice data are available. In the case of PB, the effects are more moderate, and the full response of this inducer is manifest in both in vitro systems. These findings will have implications in testing for induction by the approach adopted here, that is, using slices from treated animals. However, they may be of much lesser concern in studies involving the maintenance culture of control slices in the presence of the putative inducing agent (Lake et al., 1996).

References


