In our previous work we confirmed previous reports that the clearance of midazolam, but not desloratadine, in cryopreserved human hepatocytes (n = 50) was an order of magnitude less than that in pooled human liver microsomes (n = 200). We also demonstrated that this was not due to low levels or activity of CYP3A4 in human hepatocytes because CYP3A4 activity in microsomes isolated from the pooled hepatocytes was comparable to that in pooled human liver microsomes (within 20%). Previous investigators have proposed that the restricted clearance of midazolam (and other high clearance drugs metabolized by CYP3A4) in hepatocytes may be due to a limitation imposed by membrane permeability or the availability of cofactor (NADPH), two factors that would not impact midazolam clearance by human liver microsomes. In the present study, we investigated if these factors were involved in the greatly reduced clearance of midazolam in human hepatocytes relative to human liver microsomes. Furthermore, the influence of ionic strength on CYP3A4 activity was also investigated, and was identified as a potential cause for the observed differences in CYP3A4 activity in microsomes versus hepatocytes.

Materials and Methods

In vitro metabolism of midazolam in hepatocytes with membrane permeabilization and cofactor supplementation

The in vitro metabolism of midazolam was assessed with pooled human hepatocytes (n = 50) at 1 μM. Briefly, midazolam (1 mM) was incubated for 0, 15, 30, 60, and 120 min at 37°C (5% CO2) with human hepatocytes. To determine the effect of cofactor supplementation or membrane permeabilization on midazolam metabolism, the following treatments were performed: (A) intact cells (control), (B) intact cells + exogenous NADPH (0.1 mM), (C) cells disrupted by sonication for 60 sec, (D) cells sonicated for 60 sec + exogenous NADPH (0.1 mM), (E) cells treated with saponin (0.01% w/v) for 5 min, and (F) cells treated with 0.01% saponin for 5 min + exogenous NADPH (0.1 mM). At the end of each incubation time, the reaction was stopped by adding an equal volume of acetonitrile containing internal standard (d4-1'-hydroxymidazolam). Protein bound protein was removed by centrifugation (3200 RCF for 10 min). The supernatant fractions were analyzed by LC/MS/MS to determine the rate of disappearance of midazolam and the rate of formation of 1'-hydroxymidazolam (the major metabolite formed by CYP3A4). An AB Sciex API2000 was used with Shimadzu HPLC pumps and autosampler systems to quantify midazolam with calibration curves spanning 0.05–100 μM.

Conclusions

The studies described here were intended to examine why human hepatocytes metabolize midazolam at roughly 1/10 the rate observed in human liver microsomes, which has been proposed to reflect restrictions imposed by membrane permeability or cofactor availability in hepatocytes, restrictions that would not apply to human liver microsomes. Metabolizing the hepatocyte plasma membrane by sonication or saponin treatment did not increase the rate of metabolism of midazolam. This suggests that membrane permeability does not restrict the rate of metabolism of midazolam by human hepatocytes. This conclusion is further supported by the observation that the rate of uptake of midazolam into hepatocytes (based on medium loss) is substantially greater than its rate of metabolism (whole system loss).

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The addition of exogenous NADPH to intact hepatocytes or hepatocytes permeabilized by sonication or saponin treatment did not increase the rate of metabolism of midazolam, indicating that cofactor availability does not restrict the metabolism of midazolam in human hepatocytes.

An evaluation of the effects of incubation conditions on CYP3A4 activity in human liver microsomes established that CYP3A4 activity increases with increasing ionic strength over the range of phosphate buffer concentrations from 10 to 200 mM. When human liver microsomes were incubated in various cell culture media, CYP3A4 activity decreased by a factor of 5 or more relative to 50 mM phosphate buffer (our standard incubation buffer). These results suggest that the relatively low ionic strength of cell culture medium is potentially responsible for the greatly restricted metabolism of midazolam in human hepatocytes (relative to that in human liver microsomes).

Further studies are required to explore this interesting possibility, which has implications for the conduct of studies that compare the in vitro metabolism of CYP3A4 substrates (and metabolism-dependent inhibitors) in human liver microsomes and hepatocytes.

References