Midazolam Clearance in Human Hepatocytes is Restricted Compared with Human Liver Microsomes but not by Cell Permeability or Cofactor Availability

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Introduction

In our previous work we confirmed previous reports that the clearance of midazolam, but not dextromethorphan, 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 CYP3A4/5 in human hepatocytes because CYP3A4/5 activity in microsomes isolated from the pooled human 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 a limitation imposed by membrane permeability or the availability of cofactor (NADPH), two factors that would not impact midazolam clearance by human liver microsomes.[1-2] 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 CYP3A4/5 activity was also investigated, and was identified as a potential cause for the observed differences in CYP3A4/5 activity in microsomes versus hepatocytes.

Materials and Methods

Chemicals and test system
Midazolam, β-NADPH, saponin and Waymouth's medium were purchased from Sigma-Aldrich (St. Louis, MO); Dulbecco's modified Eagle medium (DMEM) + HEPES was purchased from Gibco (Grand Island, NY); Krebs-Henseleit buffer (KHB) and modified Chee's medium with supplementation (MCM+) were prepared in house at XenoTech LLC (Lenexa, KS); Cryopreserved human hepatocytes (n=50; mixed gender) were isolated from non-transplantable livers and cryopreserved in house. The sources of all other chemicals and reagents have been described previously.[3-4]

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 1x10^6 cells/mL. Briefly, midazolam (1 µM) was incubated for 0, 15, 30, 60, 90 and 120 min at 37°C (5% CO_2) with human hepatocytes. To determine the effect of cofactor supplementation or membrane permeability 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 (d_4-1'-hydroxymidazolam). Precipitated protein was removed by centrifugation (920 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 CYP3A4/5). An API2000 (AB Sciex) was used with Shimadzu HPLC pumps and autosampler systems to quantify midazolam with calibration curves spanning 0.05 – 1.2 µM, respectively. MRM transitions for the detection of midazolam were 326/291. 1'-Hydroxymidazolam was quantified as described previously.[3]

Whole system and media loss of midazolam in cryopreserved human hepatocytes
The contribution of midazolam metabolism and hepatocellular uptake was assessed by comparing the whole system loss of midazolam (which reflects metabolism) versus medium loss (which reflects metabolism and cell uptake)[5]. Briefly, 1 µM midazolam was incubated with 1x10^6 cells/mL pooled human hepatocytes (n=50) at 37°C for 0, 5, 1, 2, 4, 6, 15, 30, 45 and 60 min. Reactions were started with the addition of hepatocytes. For the assessment of whole system loss, reactions were stopped at each time point with the addition of an equal volume of acetonitrile containing internal standard (d_4-1'-hydroxymidazolam). For the assessment of medium loss, at the end of each incubation period, samples were transferred to Eppendorf tubes (Fisher Scientific, Pittsburgh, PA) and rapidly centrifuged (5 sec) followed by transfer of the supernatant fraction to an equal volume of stop reagent (acetonitrile with internal standard). Samples were processed and analyzed as outlined above.

The effect of buffer ionic strength and cell culture media on CYP3A4/5 activity in human liver microsomes
CYP3A4/5 activity (midazolam 1'-hydroxylation) in human liver microsomes was assessed in phosphate buffer preparations of varying ionic strength (10 – 300 mM phosphate) as well as commonly used cell culture media (KHB, MCM+, Waymouth, and DMEM+HEPES). Briefly, 1 µM midazolam was incubated at 37°C for 5 min with 0.1 mg/mL human liver microsomes (n = 200) in a wide range (10-300 mM) of concentrations of phosphate buffer (containing 3 mM $MgCl_2$ and 1 mM EDTA at pH 7.4) or in different cell culture media. Reactions were initiated with NADPH and stopped after 5 min with an equal volume of stop reagent (acetonitrile with internal standard). The samples were processed and analyzed as described above.

Results

Figure 1 shows the effect of disrupting cell membrane permeability and cofactor (NADPH) supplementation on midazolam metabolism by human hepatocytes. Sonication or treatment with saponin had little or no effect on the rate of disappearance of midazolam or the rate of formation of 1'-hydroxymidazolam (shown for the 60-min time point). Likewise, the addition of NADPH to intact hepatocytes or hepatocytes permeabilized by sonication or saponin treatment failed to stimulate the metabolism of midazolam.

The rate of hepatocellular uptake + metabolism (medium loss) versus the rate of metabolism of midazolam (whole-system loss) is shown in **Figure 2**. In the whole system loss assay (cells + medium), drug loss can occur only by metabolism; whereas in the medium loss assay the loss of drug can occur by metabolism and uptake (which represents non-specific binding to the plasma membrane, passive diffusion into hepatocytes and/or transporter-mediated uptake). The data in **Figure 2** indicate that there is an initial, rapid uptake of midazolam into cryopreserved human hepatocytes. The rapid uptake likely reflects rapid passive diffusion of midazolam into hepatocytes and non-specific binding of midazolam to intracellular and plasma membrane lipids and proteins.

Figure 3 shows the effect of buffer ionic strength and various cell culture media on CYP3A4/5 activity in human liver microsomes. A phosphate concentration-dependent increase in CYP3A4/5 activity was observed from 10 mM (30% activity of standard buffering conditions, i.e., 50 mM phosphate) to 200 mM phosphate (140% activity of standard buffering conditions). When human liver microsomes were incubated in cell culture media, CYP3A4/5 activity declined markedly (by at least 80%) relative to 50 mM phosphate buffer. Microsomal CYP3A4/5 activity was highest in KHB medium, followed by Waymouth's medium, DMEM+HEPES and MCM+.

Figures

Figure 1. The effects of membrane permeabilization (with sonication or saponin) and cofactor supplementation on in vitro metabolism midazolam in human hepatocytes (1x10^6 cells/mL)

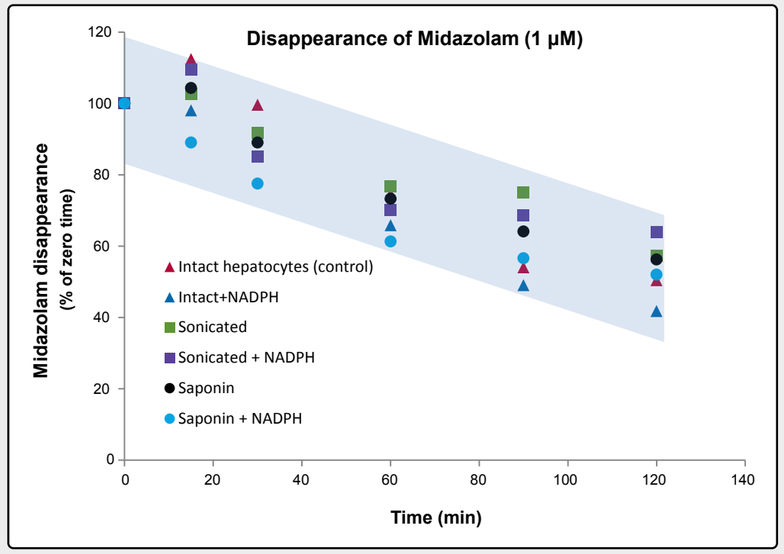


Figure 2. The contribution of metabolism and uptake as determined by whole system loss versus medium loss of midazolam (1 µM) with human hepatocytes (1x10^6 cells/mL)

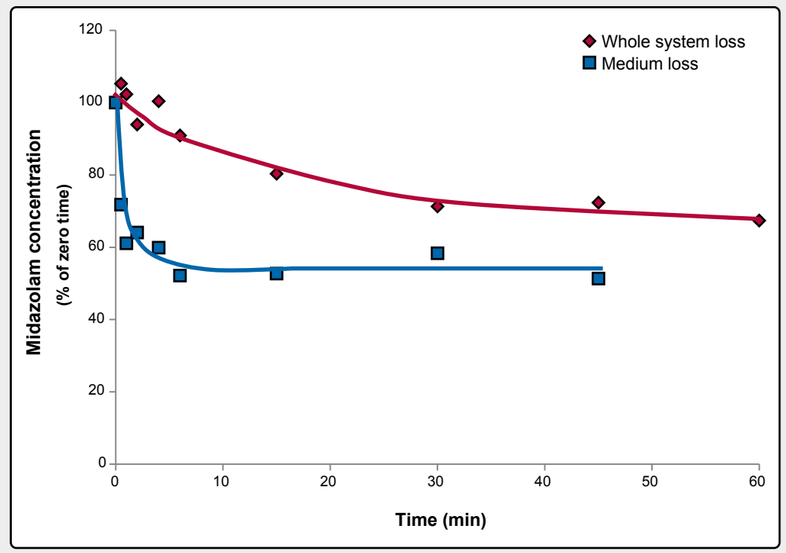
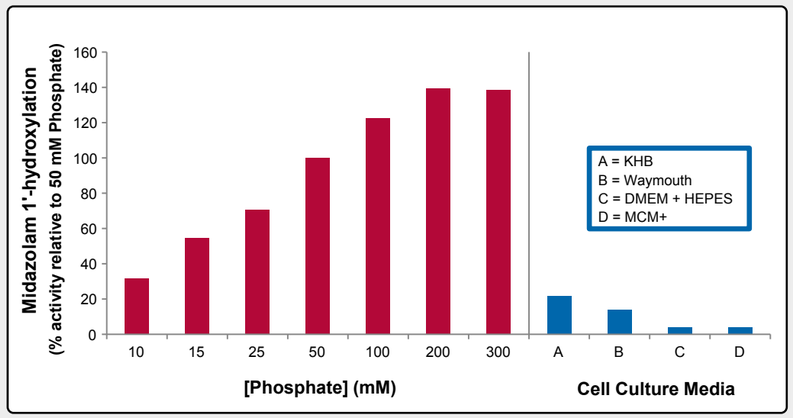


Figure 3. The effect of buffer ionic strength and cell culture media on CYP3A4/5 activity in human liver microsomes (0.1 mg/mL)



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[2] or cofactor availability in hepatocytes[1]; restrictions that would not apply to human liver microsomes.
- Permeabilizing 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 midazolam metabolism 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).
- The addition of exogenous NADPH to intact human 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 CYP3A4/5 activity in human liver microsomes established that CYP3A4/5 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, CYP3A4/5 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 CYP3A4/5 substrates (and metabolism-dependent inhibitors) in human liver microsomes and hepatocytes.

References

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