Effects of Using Different Assay Buffers on the Activity of Recombinant Human CYP3A4 Co-Expressed in *E. coli* with Human NADPH P450 Reductase.

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Abstract

Bacterial membrane preparations containing recombinant human cytochrome P450s (CYP) co-expressed with human NADPH P450 reductase (reductase) are widely used in the in vitro analysis of drug metabolism. Although the effect of using different buffer components on the activity of CYPs has been investigated to some extent in systems using purified CYP reconstituted in an artificial phospholipid mix together with purified reductase it has not been investigated in the bacterial membrane preparations described above. The assay buffer components used can vary widely between laboratories so we have studied the effects of changing the buffer components on the activity of recombinant human CYP3A4 co-expressed with human NADPH P450 reductase in E. coli.

The CYP3A4 activity as measured by testosterone and nifedipine turnover was much lower in 50 mM HEPES, 50 mM Tris and 25 mM phosphate buffer than in 50 and 100 mM phosphate buffer. We found that the effect of including $MgCl_2$ in the assay was buffer dependent, increasing turnover of both substrates in 50 mM HEPES, 50 mM Tris and, to a much lesser extent, in 25 mM and 50 mM phosphate buffers. $MgCl_2$ did not affect the turnover of either substrate in 100 mM phosphate buffer. Altering the ratio of reductase : CYP is known to affect the activity of CYP3A4 and, by using different expression systems, we are able to produce "low" and "high" reductase membrane preparations. Using these preparations, we demonstrated that the buffer dependent effects of $MgCl_2$ are independent of the reductase : CYP ratio. We also showed that the inhibitory effect of α-naphthoflavone is buffer and $MgCl_2$ dependent. Significant inhibition of testosterone turnover by CYP3A4 by 10 µM α-naphthoflavone was only seen in HEPES, Tris and the lower concentrations of phosphate buffer in the presence of $MgCl_2$. Very little inhibition was observed in the absence of $MgCl_2$ in those buffers. 10 µM α-naphthoflavone was inhibitory in 100 mM phosphate buffer regardless of whether $MgCl_2$ was present or not.

Clearly the effects associated with using different assay buffer components must be borne in mind when designing in vitro metabolism assay protocols and interpreting the results of those assays.

Introduction

New chemical entities (NCEs) are routinely screened *in vitro* for metabolism by, and inhibition of, cytochrome P450s, the principal enzyme family involved in the oxidation of xenobiotics. Although Cypex Ltd uses either 50 mM or 100 mM potassium phosphate buffer for all of the QC assays carried out on its recombinant P450 products (Bactosomes) end users may have established their assays using other buffer systems. There is evidence that the activity of recombinant cytochrome P450s can vary depending on the buffer used in the assay. For example Crespi, C. (1998) showed that the activity of insect cell expressed recombinant human cytochrome P450s can be affected by the concentration of the phosphate buffer used and Yamazaki, H. *et al* (1995) demonstrated that the activity of recombinant human CYP3A4 used in reconstituted systems can be affected by the choice of assay buffer. Here we have investigated the effects of using different buffers and different concentrations of potassium phosphate buffer on the activity of recombinant human CYP3A4 co-expressed with human NADPH P450 reductase in *E. coli*.

Materials & Methods

All chemicals were obtained from Sigma-Aldrich or VWR unless otherwise indicated. Recombinant human CYP3A4 co-expressed in *E. coli* with human NADPH P450 reductase (Bactosomes) and ketoconazole were supplied by Cypex Ltd.

CYP3A4 activity was assayed by measuring testosterone or nifedipine turnover at 37°C for 5 minutes in the buffer specified (total assay volume 0.2 ml). The buffers used were, 25, 50, 100 and 150 mM potassium phosphate pH 7.4, 50 mM potassium HEPES pH 7.4 and Bactosome storage buffer, 50 mM Tris acetate pH 7.6, 250 mM sucrose, 0.25 mM EDTA (1 x TSE). The substrate concentration in the assay was 32 µM (testosterone) or 100 µM (nifedipine). The reactions were started by the addition of 40 µl 5x NADPH generating system (5 mM NADPH, 25 mM glucose-6-phosphate, 5 U/ml glucose-6-phosphate dehydrogenase). Reactions were stopped by the addition of 25 µl 1 M HCl . Samples were centrifuged at 13,000 rpm in a microfuge and the supernatants analysed by reverse phase HPLC with UV detection. All assays were performed on at least three different preparations of *E. coli* membranes.

Results

As the phosphate concentration in the assay is increased, the activity of *E. coli* expressed CYP3A4 increases (Fig. 1), testosterone turnover was 23 fold higher in 150 mM potassium phosphate than in 25 mM potassium phosphate. This is similar to the increase in activity seen in insect cell expressed CYP3A4 with increasing phosphate concentration (Crespi, C. (1998)). There is a concomitant decrease in the time for which testosterone turnover is linear as the activity increases. Testosterone turnover by CYP3A4 in 50 mM HEPES buffer and 50 mM Tris based buffer was significantly lower than that seen in 50 mM phosphate buffer (Fig. 2). In 25 and 50 mM phosphate, 50 mM HEPES and 50 mM Tris buffers the inclusion of $MgCl_2$ resulted in a significant increase in the activity of CYP3A4 when either testosterone or nifedipine were used as substrate (Figs. 2, 3 and 4). Surprisingly, the inclusion of $MgCl_2$ had no effect in 100 mM phosphate buffer. The same was seen when using *E. coli* membranes containing CYP3A4 with a lower level of co-expressed NADPH P450 reductase (low reductase CYP3A4 Bactosomes) (Fig. 5). The activating effect of $MgCl_2$ was greatest in Tris and HEPES buffer (Fig. 3b) with testosterone turnover increasing over 12 fold on the addition of $MgCl_2$. The effect was less marked in 25 mM phosphate buffer (approx 5 fold increase in activity and 50 mM phosphate buffer (2.5 fold increase in activity).

The effect of using different assay buffers on the inhibition of testosterone turnover by CYP3A4 by α-naphthoflavone and ketoconazole was investigated. In Tris, HEPES and 25 mM phosphate buffers 10 µM α-naphthoflavone was only inhibitory in the presence of $MgCl_2$. In 100 mM phosphate buffer α-naphthoflavone was inhibitory regardless of whether $MgCl_2$ was included or not (Fig. 5). 0.5 µM Ketoconazole was inhibitory to the same degree in all of the buffers tested and in both the presence and absence of $MgCl_2$.

Fig 1. Effect of phosphate concentration on testosterone turnover by CYP3A4R Bactosomes.

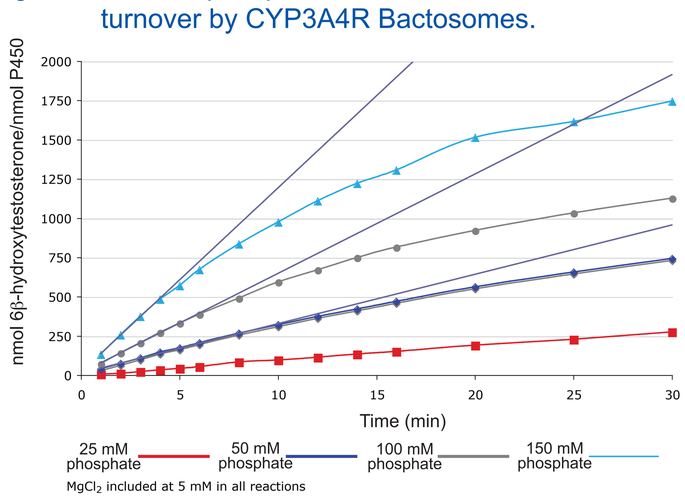


Fig 2. Effect of 20 mM $MgCl_2$ on testosterone turnover by CYP3A4R Bactosomes in different assay buffers.

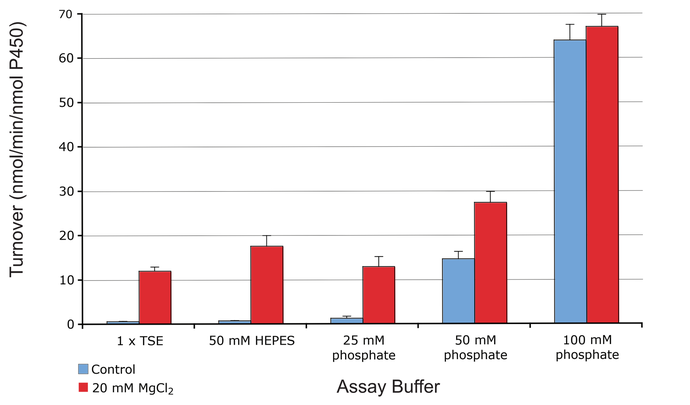


Fig 3a. Effect of $MgCl_2$ on testosterone turnover by CYP3A4R Bactosomes.

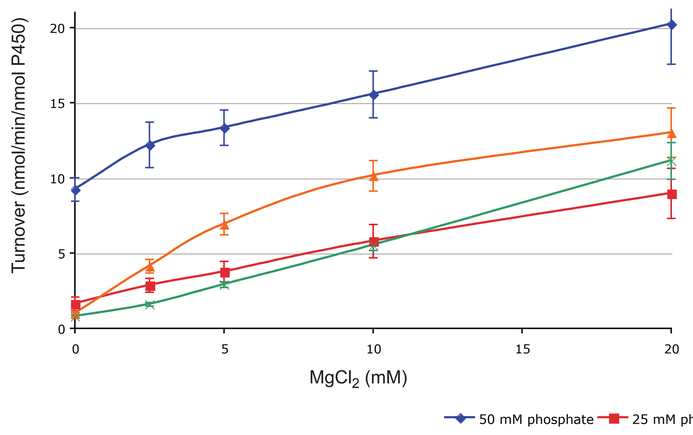


Fig 3b. Effect of $MgCl_2$ on testosterone turnover by CYP3A4R Bactosomes.

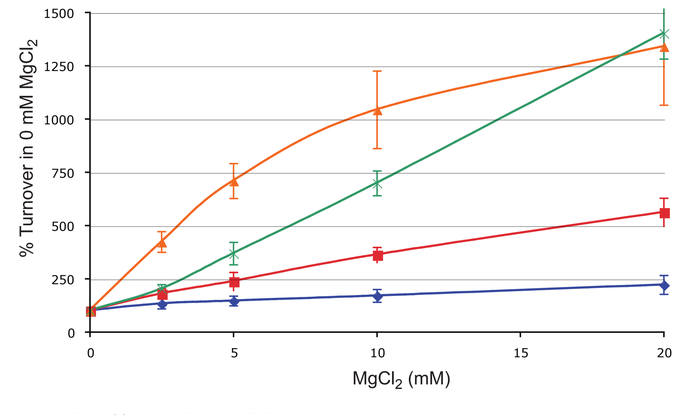


Fig 4. Effect of 20 mM $MgCl_2$ on nifedipine turnover by CYP3A4R Bactosomes in different assay buffers.

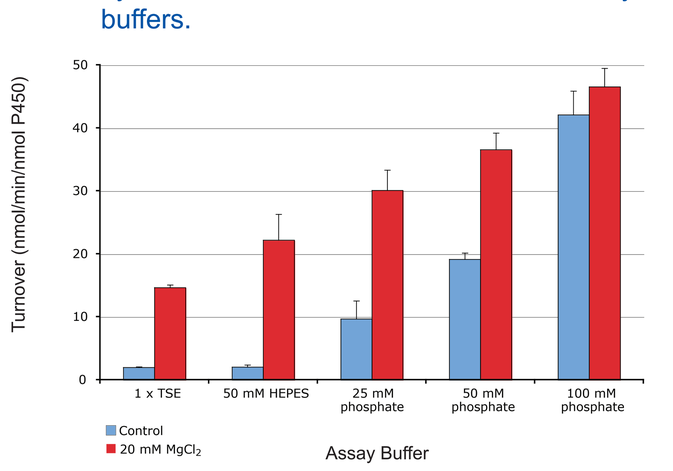


Fig 5. Effect of 20 mM $MgCl_2$ on testosterone turnover by CYP3A4LR Bactosomes in different assay buffers.

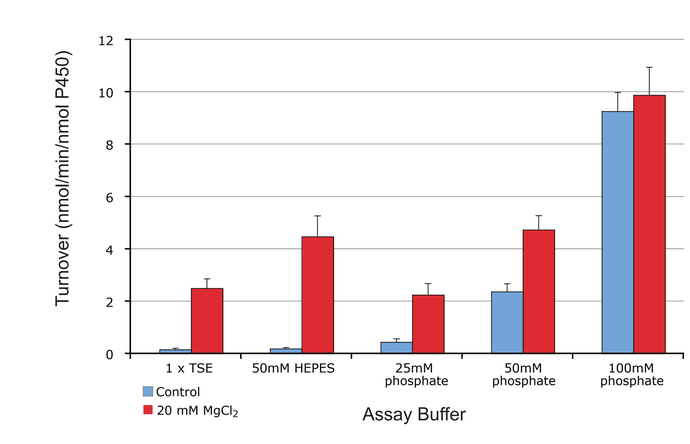


Fig 6. Effect of 20 mM $MgCl_2$ on inhibition of testosterone turnover by CYP3A4LR Bactosomes by 10 µM α-naphthoflavone in different assay buffers.

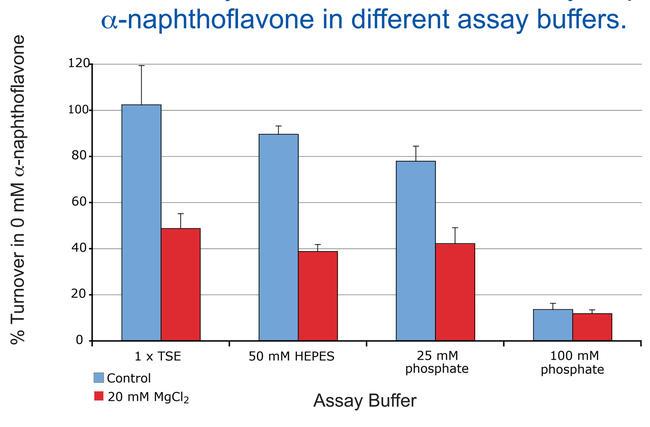
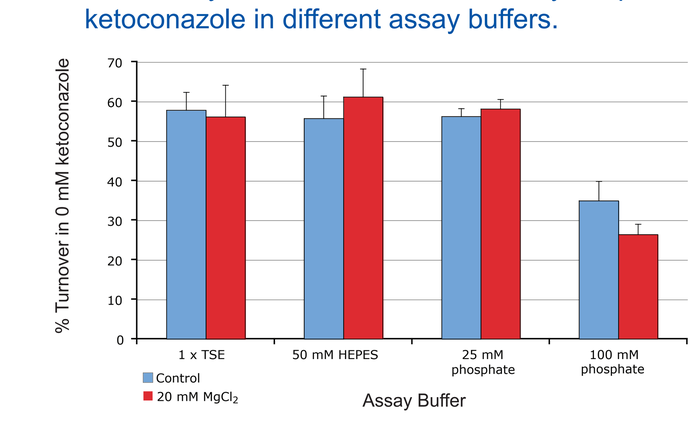


Fig 7. Effect of 20 mM $MgCl_2$ on inhibition of testosterone turnover by CYP3A4LR Bactosomes by 0.5 µM ketoconazole in different assay buffers.



Conclusions

- The buffer used in the assay system to determine CYP3A4 activity can significantly affect the activity of the enzyme; both testosterone and nifedipine turnover by CYP3A4 is highest in potassium phosphate buffer.

- The activity of CYP3A4 increases with potassium phosphate concentration.

- The stimulatory effect of $MgCl_2$ is buffer dependent, it is greatest in assay systems using Tris, HEPES and low concentration potassium phosphate buffers. The inclusion of $MgCl_2$ in the assay system using 100 mM potassium phosphate has no effect on the activity of CYP3A4.

- The inhibitory action of α-naphthoflavone on testosterone turnover by CYP3A4 is dependent on the presence of $MgCl_2$ in Tris, HEPES and low concentration phosphate buffer. It is $MgCl_2$ independent in high concentration phosphate buffer.

- The inhibitory action of ketoconazole is $MgCl_2$ independent regardless of the assay buffer used

- These buffer and $MgCl_2$ effects must be borne in mind when developing *in vitro* assays using cytochrome P450s.

References

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