In situ evaluation of CYP1A2, CYP2B6 and CYP3A4/5 induction in cultured human hepatocytes with a cocktail of probe substrates: A comparison of percent positive control values.

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ABSTRACT

It has previously been demonstrated that a cocktail of probe substrates can be effectively used to screen simultaneously for induction of CYP1A2, CYP2B6 and CYP3A4/5 with *in situ* (cell-based) incubations based on the magnitude of induction relative to appropriate positive controls. Industry guidelines recommend that a compound be considered an inducer *in vitro* and therefore warrant further investigation *in vivo* if it elicits an increase in cytochrome P450 (CYP) enzyme activity that is equal to or greater than 40% of that caused by a suitable enzyme inducer (percent positive control). The objective of this study was to determine the percent of positive control value of known CYP inducers (at varying concentrations) compared with the US FDA-preferred positive controls, omeprazole (100 µM, CYP1A2), phenobarbital (750 µM, CYP2B6) and rifampin (10 µM, CYP3A4/5) based on *in situ* incubations of primary cultures of human hepatocytes with three marker substrates (namely, 100 µM phenacetin, 500 µM bupropion, and 100 µM midazolam) either individually or as a cocktail. Freshly isolated human hepatocytes (n=3) were cultured in a sandwich configuration and treated for three days with vehicle control (dimethyl sulfoxide, DMSO; 0.1%, v/v), the aforementioned positive controls or one of the following enzyme inducers: β-naphthoflavone (1-100 µM), lansoprazole (1-100 µM), dexamethasone (1-100 µM), phenytoin (1-100 µM), carbamazepine (1-100 µM) , and efavirenz (0.25-25 µM). CYP induction was measured by LC/MS/MS based on phenacetin *O*-dealkylation (CYP1A2), bupropion hydroxylation (CYP2B6) or midazolam 1´-hydroxylation (CYP3A4/5). The results indicate that, when calculated as percent of the applicable positive control, the cocktail approach is equal to individual substrate approach in terms of assessing the enzyme-inducing potential of drugs. When CYP inducers exceeded the 40% threshold relative to the appropriate positive control, they did so in both the individual and cocktail substrate incubations at similar concentrations. These results further support the use of a cocktail of probe substrates to screen simultaneously for the induction of CYP1A2, CYP2B6 and CYP3A4/5 in cultured human hepatocytes.

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INTRODUCTION

Induction of cytochrome P450 (CYP) activity is one of the principal mechanisms of drug-drug interactions. For this reason, in their most recent draft guidance documents (EMA, 2010; US FDA, 2006), the EMA and US FDA both recommend the evaluation of new drug candidates for their ability to induce CYP enzymes in three preparations of human hepatocytes. Currently, the EMA and US FDA recommend the evaluation of a minimum of three CYP enzymes, namely CYP1A2, CYP2B6 and CYP3A4 (EMA, 2010; US FDA, 2006) as these enzymes largely reflect the activation of three major xenosensors, namely the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (Parkinson and Ogilvie, 2008). Furthermore, the EMA and US FDA recommend that a drug candidate be considered an inducer *in vitro* and therefore warrant further investigation *in vivo* if it elicits an increase in enzyme activity that is equal to or greater than 20% (EMA) and 40% (US FDA) of that caused by the appropriate positive control.

The objective of this study was to determine the percent of positive control value of known CYP inducers (at varying concentrations) compared with the FDA-preferred positive controls, omeprazole (1-100 µM, CYP1A2), phenobarbital (750 µM, CYP2B6) and rifampin (10 µM, CYP3A4/5) based on *in situ* incubations of primary cultures of human hepatocytes with three marker substrates (namely, 100 µM phenacetin, 500 µM bupropion and 100 µM midazolam) either individually or as a cocktail.

MATERIALS & METHODS

Freshly plated human hepatocytes from three donors were prepared at XenoTech, LLC (Lenexa, KS). The sources of the other reagents used in the study have been described previously (Madan *et al.*, 2003; Paris *et al.*, 2009).

Human hepatocytes were cultured and treated with known CYP inducers essentially as described by Robertson *et al.*, 2000, Amunom *et al.*, 2009 and Madan *et al.*, 2003. In brief, freshly isolated hepatocytes were plated in 48-well plates in a sandwich configuration (collagen substratum with Matrigel overlay) and allowed to adapt to the culture conditions for three days. The hepatocyte cultures were treated once daily for three consecutive days with media containing DMSO (0.1% v/v; vehicle control) or one of the following inducers: omeprazole (100 µM), phenobarbital (750 µM), rifampin (10 µM), β-naphthoflavone (1-100 µM), lansoprazole (1-100 µM), dexamethasone (1-100 µM), phenytoin (1-100 µM), carbamazepine (1-100 µM), and efavirenz (0.25-25 µM).

Twenty-four hours after the final treatment, the hepatocytes were incubated with 100 µM phenacetin, 500 µM bupropion and 30 µM midazolam either individually or as a cocktail to measure CYP1A2, CYP2B6 and CYP3A4/5 activity, respectively. Reactions were terminated after 30 minutes with the addition of a predetermined volume of acetonitrile containing an appropriate internal standard (deuterated metabolite). The metabolites, namely acetaminophen, hydroxybupropion and 1´-hydroxymidazolam, and the corresponding internal standards were quantitated by HPLC-MS/MS, essentially as described by Amunom *et al.*, 2009.

RESULTS

Table 1 compares CYP1A2, CYP2B6 and CYP3A4/5 fold induction in individual human hepatocytes which were incubated with phenacetin, bupropion and midazolam either individually or as a cocktail.

- On average, CYP1A2 fold induction decreased from 53-fold to 30-fold, or 45%, when human hepatocytes treated with100 µM omeprazole were incubated *in situ* with a cocktail substrate solution versus phenacetin alone.
- On average, CYP2B6 fold induction increased from 8.2-fold to 9.8-fold, or 18%, when human hepatocytes treated with 750 µM phenobarbital were incubated *in situ* with a cocktail substrate solution versus bupropion alone.
- On average, CYP3A4/5 fold induction decreased from 3.18-fold to 2.7-fold, or 14%, when human hepatocytes treated with 10 µM rifampin were incubated *in situ* with a cocktail substrate solution versus midazolam alone.

Figure 1 shows the percent of positive control of CYP1A2 (based on the fold induction with 100 µM omeprazole) when human hepatocytes (n = 3) were incubated with phenacetin alone or with a cocktail of phenacetin, bupropion and midazolam.

- The 20% and 40% of positive control thresholds were exceeded by β-naphthoflavone (at ~5 µM and ~20 µM, respectively), omeprazole (at ~10 µM and ~30 µM, respectively) and lansoprazole (at 35-55 µM and 75-90 µM, respectively) at similar concentrations regardless of whether substrate incubations were conducted with phenacetin either alone or as part of the cocktail.

Figure 2 shows the percent of positive control of CYP2B6 (based on the fold induction with 750 µM phenobarbital) when human hepatocytes (n = 3) were incubated with bupropion alone or with a cocktail of phenacetin, bupropion and midazolam.

- The 20% and 40% of positive control thresholds were exceeded by β-naphthoflavone (at ~8 µM and ~37 µM, respectively), omeprazole (at ~10 µM and ~30 µM, respectively), lansoprazole (at 35-55 µM and 75-90 µM, respectively), and carbamazepine (~3 and ~8 µM, respectively) at similar concentrations regardless of whether substrate incubations were conducted with bupropion either alone or as part of the cocktail.
- Phenytoin exceeded both the 20% and 40% thresholds at less than 10 µM regardless of whether substrate incubations were conducted with bupropion either alone or as part of the cocktail.
- Dexamethasone exceeded the 20% threshold at 20-30 µM but did not, on average, exceed the 40% threshold in either group.

Table 1.

Fold induction of CYP1A2, CYP2B6 and CYP3A4/5 activity in induced hepatocytes incubated with phenacetin, bupropion and midazolam either individually or as a cocktail.

	Phenacetin *O*-dealkylation (Fold induction by 100 µM omeprazole)		Bupropion hydroxylation (Fold induction by 750 µM phenobarbital)		Midazolam 1´-hydroxylation (Fold induction by 10 µM rifampin)	
Donor	Single Substrate	Cocktail	Single Substrate	Cocktail	Single Substrate	Cocktail
1	26	24	7.1	6.8	3.3	2.7
2	58	28	5.1	5.3	2.7	2.7
3	78	37	13	17	3.5	2.8

Figure 3 shows the percent of positive control of CYP3A4/5 (based on the fold induction with 10 µM rifampin) when human hepatocytes (n = 3) were incubated with midazolam alone or with a cocktail of phenacetin, bupropion and midazolam.

- The 20% and 40% of positive control thresholds were exceeded by lansoprazole (at ~4 µM and ~9 µM, respectively), dexamethasone (at ~9 µM and ~17 µM, respectively), phenytoin (at ~4 µM and ~10 µM, respectively), efavirenz (at ~14 µM and ~44 µM, respectively), and carbamazepine (at ~6 and ~11, respectively) at similar concentrations regardless of whether substrate incubations were conducted with phenacetin alone or as part of the cocktail.

Figure 1.

CYP1A2 induction in human hepatocytes: Effect of incubating phenacetin either alone or as a cocktail (together with bupropion and midazolam). Values are $\bar{x} \pm SD$ (n=3).

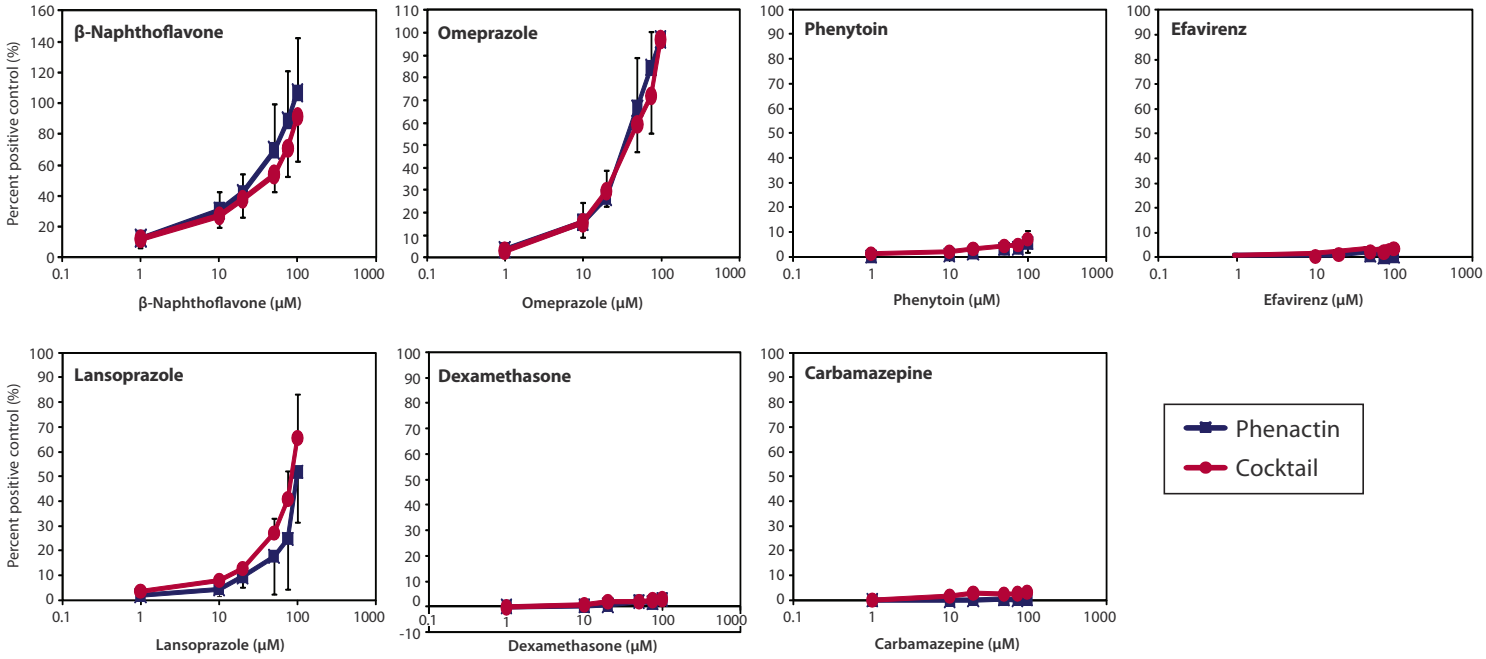


Figure 2.

CYP2B6 induction in human hepatocytes: Effect of incubating bupropion either alone or as a cocktail (together with phenacetin and midazolam). Values are $\bar{x} \pm SD$ (n=3).

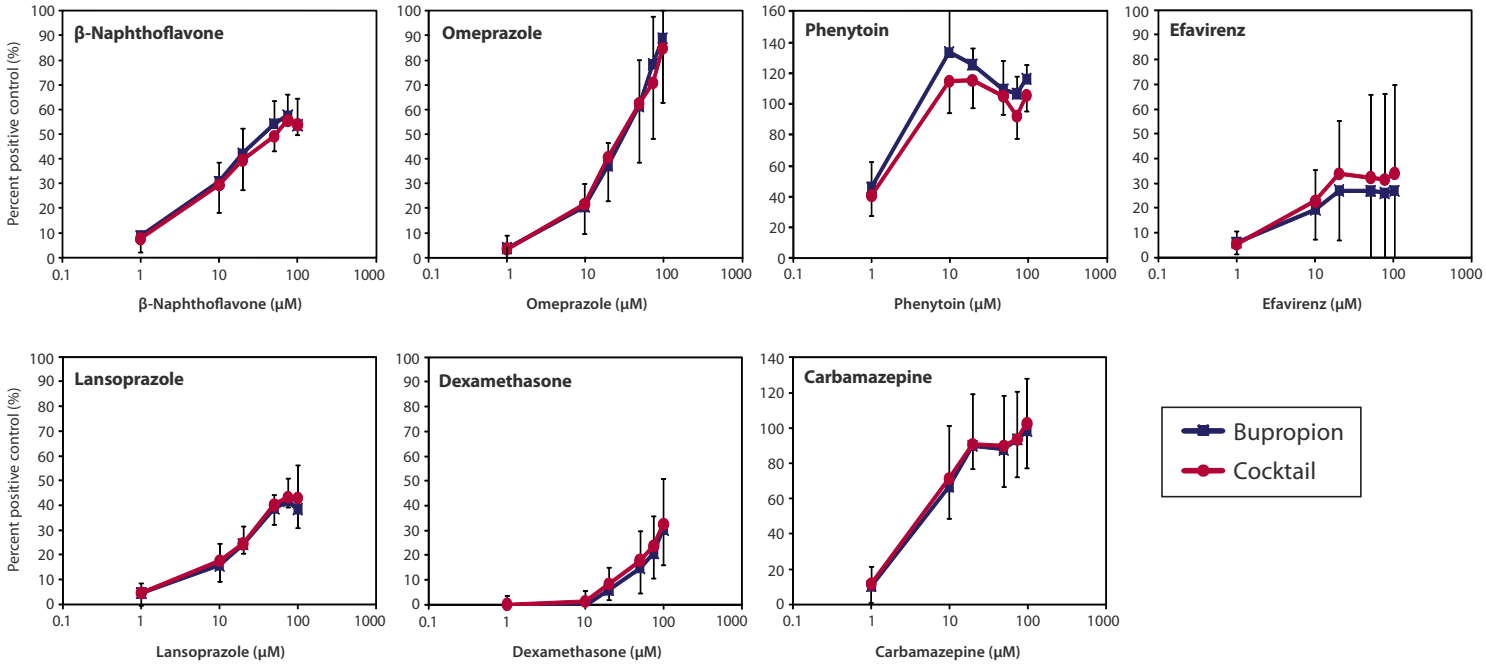
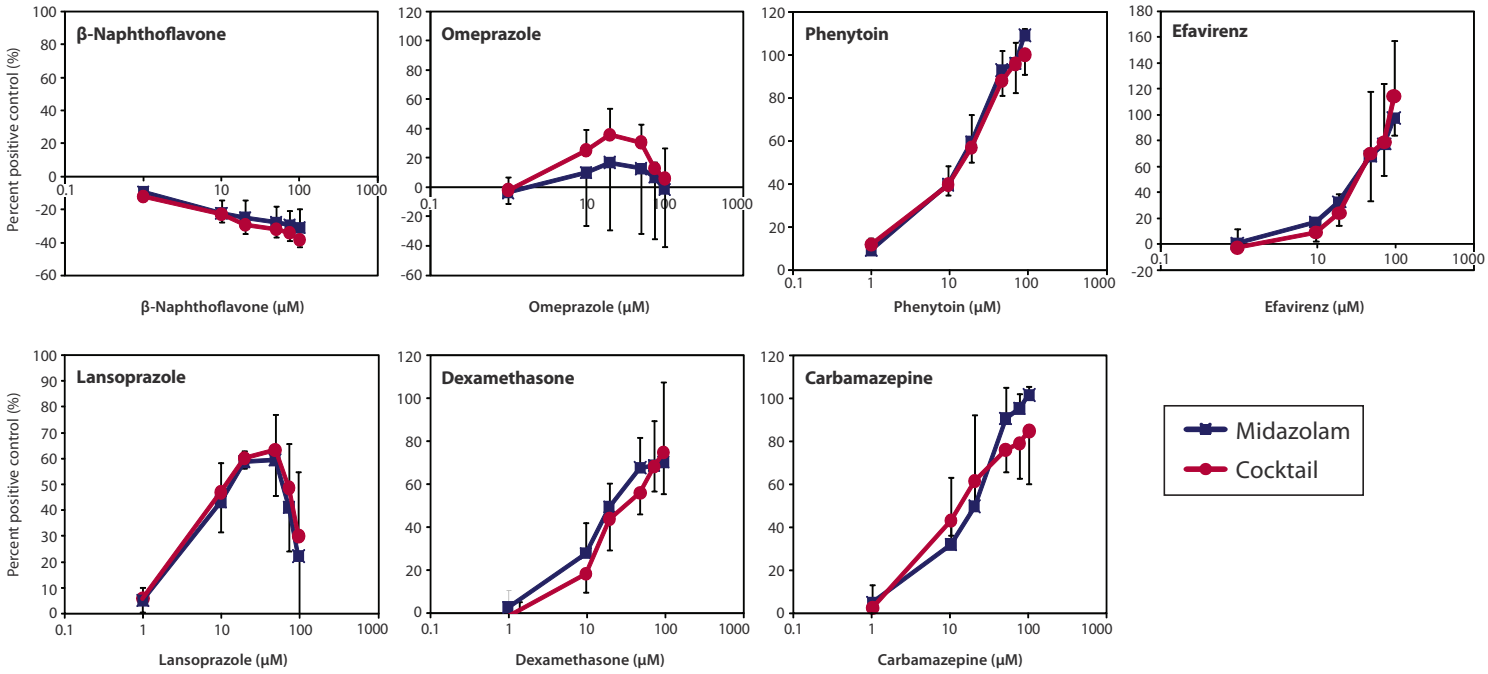


Figure 3.

CYP3A4/5 induction in human hepatocytes: Effect of incubating midazolam either alone or as a cocktail (together with phenacetin and bupropion). Values are $\bar{x}$ ± SD (n=3).



CONCLUSIONS

- CYP induction studies performed with a cocktail of probe substrates (phenacetin, bupropion and midazolam) resulted in a decrease in CYP1A2 and CYP3A4/5 fold induction and an increase in CYP2B6 fold induction when compared to incubations with a single probe substrate.
- When calculated as percent of the applicable positive control, the cocktail approach is equal to the individual substrate approach in terms of assessing the enzyme-inducing potential of drugs.
- When CYP inducers exceeded either the 20% or 40% threshold relative to the appropriate positive control, they did so in both the individual and cocktail substrate incubations at similar concentrations.
- These results further support the use of a cocktail of probe substrates to screen simultaneously for the induction of CYP1A2, CYP2B6 and CYP3A4/5 in cultured human hepatocytes.

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