

# CHARACTERIZATION OF ANTIBODIES WITH SELECTIVE RECOGNITION OF CYTOCHROME P450S ON IMMUNOBLOTS

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## ABSTRACT

We have developed antipeptide antibodies to human cytochrome P450 CYP3A and rat cytochrome P450 1B1. The anti-human CYP3A antisera (WB-3A4) specifically detects human CYP3A4 and 3A7 but not 3A5 on immunoblots. This antisera recognizes a single band in human liver microsomes which comigrates with cDNA-expressed human CYP3A4 microsomes. The anti-rat CYP1B1 antisera (WB-R1B1) recognizes a single band in methylcholanthrene treated rat liver microsomes which comigrates with rat CYP1B1 microsome standard. No crossreactivity is evident in lymphoblast control (Con), phenobarbital (PB), clofibrate (CF), untreated (UT; male Sprague Dawley) rat liver microsomes, or available cDNA-expressed enzymes. These reagents are useful for characterizing the levels of specific enzymes *in vivo*.

## INTRODUCTION

The cytochrome P450 superfamily of mixed function oxidases are among the most important drug metabolizing enzymes (Spatzenegger and Jaeger (1995) Drug. Metab. Rev. 27: 397-417). These enzymes are expressed in almost every tissue with the highest levels found in liver. In mammals, the cytochrome P450 superfamily has been separated into 14 families and 26 subfamilies based on similarity of sequence. Within a subfamily, each member is usually more than 55% identical. Consequently, many antibodies designed to recognize a specific family member results in an antibody that recognizes an entire subfamily. Since tissue distribution of subfamily members can substantially overlap, there is a need for immunochemical reagents with selective recognition of specific cytochrome P450 forms.

In humans, the cytochrome P450 3A subfamily is composed of three enzymes: CYP3A4, CYP3A5, and CYP3A7. CYP3A4 represents the majority of P450 found in liver. CYP3A5 is also expressed in liver although generally at a lower level. CYP3A7 is expressed in the placenta but not in the liver (Hakkola *et al.* (1998) Crit. Rev. Toxicol. 28: 35-72). Because tissue expression of CYP3A overlap and isoform specific enzyme assays are not available, isoform-selective immunochemical reagents are needed.

The CYP1 family contains three members: CYP1A1, CYP1A2, and CYP1B1. All three family members are induced by dioxin and methylcholanthrene in livers of female Sprague Dawley rats (Walker *et al.* (1998) Carcinogenesis 19:395-402). CYP1B1 is of current interest because of its potential role in estradiol metabolism and its expression in some tumors (Hayes *et al.* (1996) Proc. Natl. Acad. Sci. 93:9776-81; Dohr *et al.* (1995) Arch. Biochem. Biophys. 321:405-412). To study the role of CYP1B1 in metabolism, a specific immunochemical reagent is necessary.

This poster presents characterization of two anti-peptide antibodies (WB-3A4 and WB-R1B1) which recognize both cDNA-expressed and liver derived enzyme on immunoblots.

## MATERIALS & METHODS

### Materials

WB-3A4 and WB-R1B1, whole serum preparations from rabbits immunized with peptides specific for human CYP3A4/7 and rat CYP1B1, respectively, were provided by GENTEST Corp. Antibodies were developed using approaches described by Edwards *et al.* (Biochem. Pharm. (1994) 49: 39-47). Human liver microsomes, cDNA expressed cytochrome P450 (human and rat) containing microsomes (baculovirus-expressed or lymphoblast-derived) were commercially available from GENTEST Corp. Untreated male Sprague Dawley, methylcholanthrene-, phenobarbital- and clofibrate-treated rat liver microsomes were purchased from Daiichi Pure Chemical. Ten percent acrylamide gels were purchased from Novex. Nitrocellulose (0.45 µm pore) was purchased from Schleicher & Schuell. Chemiluminescent substrate (LumiGLO) was purchased from Kirkegaard & Perry Laboratories. All other reagents were Sigma products.

### Immunoblotting Conditions

#### Alkaline Phosphatase Detection

- (1) Samples were prepared by adding each to an equal volume of 2X SDS sample buffer (60 mM Tris pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue) then boiling for 5 minutes.
- (2) Samples were loaded on an SDS-PAGE and electrophoresis was performed according to manufacturer's instructions. For example, electrophoresis on a 14 X 16 cm 10% acrylamide gel at 60V overnight (16-18 hrs) in a Hoefer electrophoresis apparatus.
- (3) Proteins were transferred to nitrocellulose by electroblotting at room temperature for 1 hour at 0.8 mA per cm<sup>2</sup> nitrocellulose in 192 mM glycine, 25 mM Tris, 20% methanol with a Hoefer Semiphor™ semidry transfer unit.
- (4) The nitrocellulose was blocked in 5% powdered nonfat milk, 25 mM Tris (pH 7.5), 150 mM NaCl for 1 hour at room temperature.
- (5) The nitrocellulose was incubated with primary antisera at 1:500 in 0.5% powdered nonfat milk, 25 mM Tris (pH 7.5), 150 mM NaCl for 1 hour at room temperature.
- (6) The nitrocellulose was rinsed 3 times with 0.1% tween 20, 25 mM Tris (pH 7.5), 150 mM NaCl for 5 minutes each.
- (7) The nitrocellulose was incubated with alkaline phosphatase conjugated goat a-rabbit IgG at 1:500 in 0.5% powdered nonfat milk, 25 mM Tris (pH 7.5), 150 mM NaCl for 1 hour at room temperature.
- (8) The nitrocellulose was rinsed 3 times with 0.1% Tween 20, 25 mM Tris (pH 7.5), 150 mM NaCl.
- (9) The nitrocellulose was developed with NBT/BCIP developing solution at room temperature until bands appeared (5 to 15 minutes).

#### Enhanced Chemiluminescent Detection

- (1) Follow steps 1 through 4 above.
- (2) The nitrocellulose was incubated with primary antisera (at 1:500 for WB-3A4 and 1:1500 for WB-R1B1) in 0.5% powdered nonfat milk, 25 mM Tris (pH 7.5), 150 mM NaCl for 1 hour at room temperature.
- (3) The nitrocellulose was rinsed 3 times with 0.1% tween 20, 25 mM Tris (pH 7.5), 150 mM NaCl for 5 minutes each.
- (4) The nitrocellulose was incubated with HRP-conjugated goat a-rabbit IgG at 1:500 in 0.5% powdered nonfat milk, 25mM Tris (pH 7.5), 150mM NaCl for 1 hour at room temperature.
- (5) The nitrocellulose was rinsed 3 times with 0.1% tween 20, 25 mM Tris (pH 7.5), 150 mM NaCl for 5 minutes each.
- (6) The nitrocellulose was developed and exposed to film according to the manufacturer of the chemiluminescent substrate.

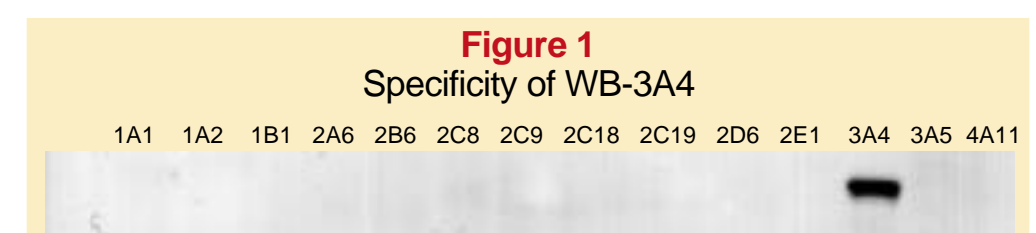
## CONCLUSIONS

- WB-3A4 shows sensitivity and specificity for human CYP3A4 and CYP3A7 using a panel of cDNA-expressed enzymes.
- WB-R1B1 is sensitive and specific for rat CYP1B1 using a panel of cDNA-expressed enzymes and induced rat liver microsomes. No cross reactivity with other rat CYP1A enzymes was observed.
- WB-3A4 and WB-R1B1 are useful reagents for *in vitro* determination of the levels of human CYP3A and rat CYP1B1, respectively.
- Work is underway to extend this approach to develop anti-peptide antibodies against phase II enzymes (initially human UGTs).

## RESULTS

### Anti-human CYP3A4/7

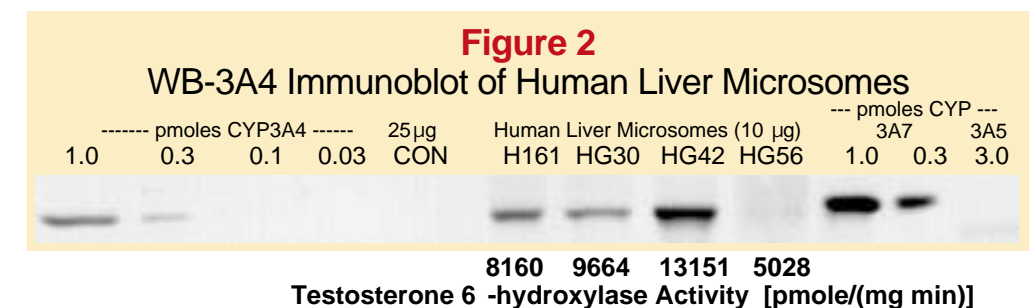
WB-3A4 is a human CYP3A4 and 3A7 selective reagent for immunoblotting. While we have not rigorously evaluated the relative affinity for CYP3A4 and CYP3A7, the amino acid sequence is identical in the region of the peptide. No cross reactivity was observed with cDNA-expressed CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A5, or CYP4A11 (3 pmole baculovirus-expressed enzyme per lane, Figure 1 below).



WB-3A4 recognizes a single band in human liver microsomes which comigrates with human CYP3A4 microsome standard (figure 2). The intensity of the immunoreactive band in the human liver microsomes is highly correlated with the levels of CYP3A4 catalytic activity as measured by testosterone 6β-hydroxylation ( $r^2 = 0.93$ ).

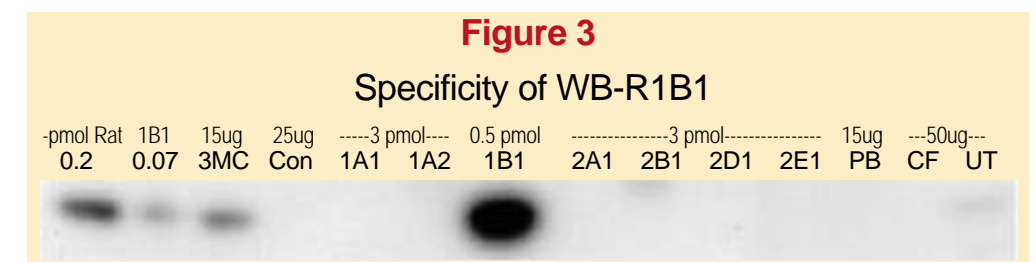
The CYP3A7 microsome standard migrates very closely (slower mobility) with CYP3A4 (figure 2). Therefore care should be taken when a sample is used which may contain both CYP3A4 and CYP3A7.

CYP3A4 is detected to a limit of 0.1 pmole when using alkaline phosphatase conjugated secondary antibody. This level of sensitivity should be adequate for most applications. WB-3A4 complements WB-3A5, an antisera specific for human CYP3A5 on immunoblots. This material is commercially available.



### Anti-rat CYP1B1

WB-R1B1 specificity was determined by western blotting of a panel of cDNA-expressed rat cytochrome P450 enzymes (3 pmole/lane) and rat liver microsomes. No crossreactivity is evident in lymphoblast control (Con), phenobarbital (PB), clofibrate (CF), untreated (UT; male Sprague Dawley) rat liver microsomes, or available cDNA-expressed enzymes (figure 3). The combination of cDNA-expressed enzymes and induced microsomes represents a relatively comprehensive test for specificity. The testing will be expanded as more cDNA-expressed enzymes become available. WB-R1B1 recognizes a single band in methylcholanthrene treated (3MC) rat liver microsomes which comigrates with rat CYP1B1 microsome standard (figures 3 and 4).



WB-R1B1 detects rat CYP1B1 to a limit of 0.1 pmole with alkaline phosphatase detection and 0.05 pmole with enhanced chemiluminescence detection. This level of sensitivity should be adequate for most applications.

