# 2P-20 Evaluation of interspecies metabolic activities for Non-CYP enzymes and into a suitable incubation condition for human

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## Introduction

Recently, lead compound screenings has become widely used in drug development. This has led to the development of increasing range of lead compound structures with metabolic stability for Cytochrom P450 (CYP). However, because of the unpredictable metabolic reaction and species differences by Non-CYP enzymes, there has been cases that the test compound's blood concentration was found to be significantly lower than expected in the clinical phase, resulting in the discontinuation in its development. Therefore, it is necessary to establish an *in vitro* evaluation system of metabolic activities for Non-CYP enzymes.

We evaluated interspecies differences in metabolic activities for Non-CYP enzymes (aldehyde oxidase (AO), aldo-keto reductase (AKR) and carbonyl reductase (CR)) in liver cytosol (human, monkey, rat, mouse and dog).

We also evaluated the inhibitory effect of typical inhibitor for each Non-CYP enzyme (AO, xanthine oxidase (XO), AKR and CR). And, we tested the metabolic activities of Non-CYP enzymes in anaerobic conditions using human liver hepatocytes.

In this presentation, we have established an evaluation method for metabolic activities of Non-CYP enzymes (AO･XO･AKR･CR).

## Method

**Table 1 Model substrate, model substrate metabolite, and typical inhibitor for each Non-CYP enzyme-mediated metabolism.**

| Enzyme | Model substrate | Model substrate metabolite | Typical inhibitor |
|---|---|---|---|
| AO | Carbazeran | 4-Hydroxycarbazeran | Hydralazine |
| XO | 6-Mercaptoprine | Thiouric acid | Allopurinol |
| AKR | Naltrexone | Naltrexol | Phenolphthalein |
| CR | Doxorubicin | Doxorubicinol | Quercetin |

Model substrates, model substrate metabolites and typical inhibitors were selected based on References.

**<Experimental method>**

Reaction samples contained potassium phosphate buffer (100 mmol/L, pH 7.4) , liver cytosol (Human: H0610.C, mixed gender pool of 50, Monkey: P2000.C, male pool of 10, Dog: D1000.C, male pool of 11, Mouse: M1000.C, male pool of 1000, Rat: R1000.C, male pool of 454, Sekisui Xeno Tech, LLC), NADPH generating system (Corning) (AKR and CR) and each model substrate. Reactions were terminated by adding acetonitrile solution containing internal standard (tolbutamide).

The model substrate metabolites were measured by LC-MS/MS (HPLC system : LC-10ADvp, Shimadzu, MS/MS system :API4000 QTRAP, SCIEX).

**<Calculation of velocity, $K_m$, $V_{max}$ >**

$$\text{Velocity (pmol/min/mg protein)} = \frac{\text{Model substrate metabolite concentration(pmol/mL)}}{\text{Liver cytosol concentration (mg protein/mL)} \times \text{Incubation time (min)}}$$

$K_m$ and $V_{max}$ values were calculated from the relationship between the velocity and model substrate concentration according to the equation shown below (Michaels-Menten model).

$$V = V_{max} \times S / (K_m + S)$$

$V_{max}$: Maximum velocity (pmol/min/mg protein), $K_m$: Michaels constant (μmol/L), S: model substrate concentration (μmol/L)

**<Calculation of % of control>**

Remaining activity (% of control) of each Non-CYP enzyme was calculated to the following equation.

$$\text{\% of control} = \frac{\text{Peak area ratio in typical inhibitor group}}{\text{Peak area ratio in control group}} \times 100$$

## References

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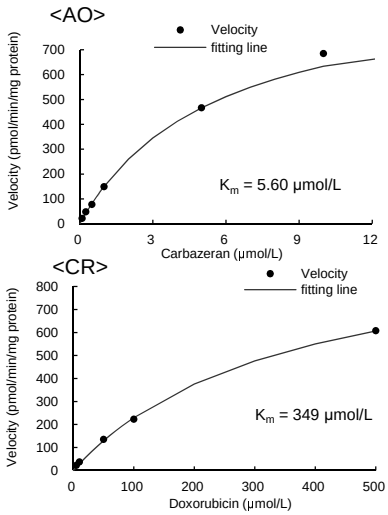
## Results



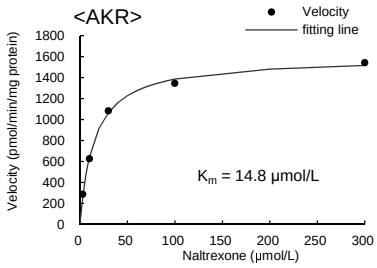




**Figure 1 Kinetics for each Non-CYP enzyme in human liver cytosol.**
Assay was performed in duplicate. Each model substrate (0.1 to 10 μmol/L carbazeran for AO activity, 3 to 300 μmol/L naltrexone for AKR activity and 5 to 500 μmol/L doxorubicin for CR activity) was incubated in 0.1 mg protein/mL of human liver cytosol at 37 ˚ C for 5 min (AO and AKR) or 10 min (CR), respectively.
The $K_m$ values for carbazeran, naltrexone and doxorubicin were 5.60, 14.8 and 349 μmol/L, respectively.

<AO>

| Species | $K_m$ (μmol/L) | $V_{max}$ (pmol/min/mg protein) | $CL_{int}$ (μL/min/mg protein) |
|---|---|---|---|
| Human | 5.60 | 986 | 176 |
| Monkey | 6.04 | 7130 | 1180 |
| Dog | ND | ND | – |
| Mouse | 55.6 | 101 | 1.82 |
| Rat | 5.79 | 91.8 | 15.9 |

ND: Not determined due to low metabolic clearance.

<AKR>

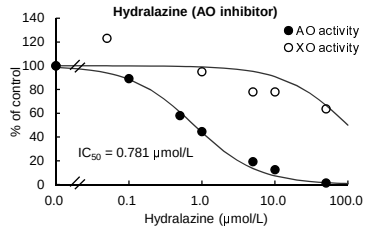
| Species | $K_m$ (μmol/L) | $V_{max}$ (pmol/min/mg protein) | $CL_{int}$ (μL/min/mg protein) |
|---|---|---|---|
| Human | 14.8 | 1590 | 107 |
| Monkey | 14.2 | 260 | 18.3 |
| Dog | >100 | NC | 0.0457* |
| Mouse | >100 | NC | 0.185* |
| Rat | 615 | 181 | 0.294 |

NC: Not calculated because no saturation was observed within the substrate concentration investigated.
*: These values were calculated from the velocity at 10 μmol/L naltrexone.

<CR>

| Species | $K_m$ (μmol/L) | $V_{max}$ (pmol/min/mg protein) | $CL_{int}$ (μL/min/mg protein) |
|---|---|---|---|
| Human | 349 | 1030 | 2.95 |
| Monkey | 451 | 1850 | 4.10 |
| Dog | 51.2 | 1540 | 30.1 |
| Mouse | 411 | 571 | 1.39 |
| Rat | 205 | 491 | 2.40 |

**Table 2 Interspecies differences (human, monkey, dog, mouse and rat) of kinetics for each Non-CYP enzyme in liver cytosol.**
$CL_{int}$ were calculated using the following equation "$CL_{int} = V_{max} / K_m$". AO, AKR and CR were observed to show inter species difference of $CL_{int}$ (AO: monkey> human> rat> mouse>>dog, AKR: human> monkey>>rat, mouse and dog, CR: dog> >monkey, human, rat and mouse).



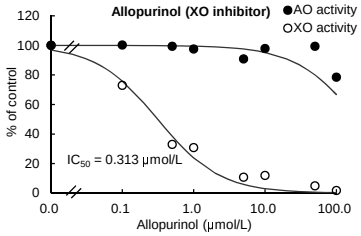
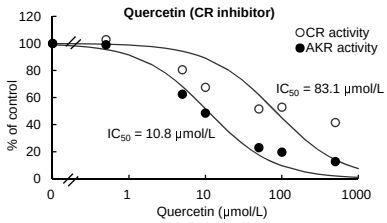




**Figure 2 Inhibitory effect of typical inhibitor for each oxidase in human liver cytosol.**
Assay was performed in duplicate. Each model substrate (5 μmol/L carbazeran for AO activity and 100 μmol/L 6-mercaptoprine for XO activity) was incubated in 0.5 mg protein/mL of human liver cytosol at 37 ˚ C for 5 min (AO), or 60 min (XO), respectively.
$IC_{50}$ values were calculated using the following equation "% of control = ($IC_{50}$ / ($IC_{50}$ + typical inhibitor concentration (μmol/L)) ) × 100".



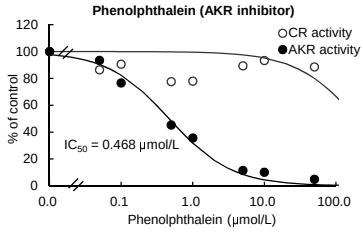




**Figure 3 Inhibitory effect of typical inhibitor for each reductase in human liver cytosol.**
Assay was performed in duplicate. Each model substrate (300 μmol/L doxorubicin for CR activity and 10 μmol/L naltrexone for AKR activity) was incubated in 0.1 mg protein/mL of human liver cytosol at 37 ˚ C for 10 min.
$IC_{50}$ values were calculated using the following equation "% of control = ($IC_{50}$ / ($IC_{50}$ + typical inhibitor concentration (μmol/L)) ) × 100".

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## Discussion

This study characterized the interspecies differences in metabolic activities of Non-CYP enzymes (AO, AKR and CR) in liver cytosol (human, monkey, rat, mouse and dog). AO, AKR and CR were observed to show inter species difference for $CL_{int}$ (Figure 1 and Table 2). AO and AKR showed high $CL_{int}$ in human, indicating that the metabolic activity of the compounds metabolized by these enzymes is underestimated using only *in vitro* studies with CYP enzymes or *in vivo* studies in rodents. Suggesting that we need to evaluate the metabolic activities of Non-CYP enzymes.These results may help us to predict the compound's pharmacokinetics in human. We compared the metabolic activities of Non-CYP enzymes in anaerobic and aerobic conditions using human liver hepatocytes, but no clear difference was observed under both conditions (Data not shown).

We also evaluated the inhibitory effect of typical inhibitor for each Non-CYP enzyme. Hydralazine (AO inhibitor) and allopurinol (XO inhibitor) showed specificity as a each typical inhibitor for oxidase (Figure 2). Phenolphthalein (AKR inhibitor) showed specific inhibition for AKR. However, quercetin (CR inhibitor) inhibited both CR and AKR activities (Figure 3). These results indicated that using only quercetin (CR inhibitor) is not sufficient for the evaluation of CR substrate, and both CR and AKR inhibitors should be used for the evaluation of CR or AKR substrate.

In this presentation, We have established an evaluation method for metabolic activities of Non-CYP enzymes (AO･XO･AKR･CR). Therefore, these results will help us to identify the metabolic enzymes for the compounds metabolized by Non-CYP enzymes.