Aldehyde oxidase (AO) is a cytosolic enzyme present in the liver of humans and other mammals that catalyzes various oxidation and reduction reactions. Its biotransformation by AO is an important clearance mechanism for many drugs and drug candidates, with increasing emphasis in certain chemical spaces, and in some cases, such as zaleplon, AO metabolism leads to rapid in vivo clearance.

Several publications have demonstrated the under-prediction of in vivo human clearance from in vitro clearance data, which are typically conducted with human liver subcellular fractions, such as S9 or cytosol. Zientek and colleagues (2010) described a rank order approach, or ‘yardstick’ approach, to categorize known AO substrates into low, medium or high clearance categories based on in vitro CL<sub>AO</sub>. With this approach, new drugs candidates may be ranked in vivo S9 or cytosol and the predicted in vivo clearance can be qualitatively evaluated. These subcellular fractions, S9 and cytosol, are commercially available from multiple suppliers and in many formats (individuals and pools of various sizes), which leads to variation in AO activity.

Because of the necessity to scale AO clearance with a rank-order approach, the present study set forth to determine which human liver S9 and cytosol lots (individual or pooled) can be utilized to predict AO clearance once thresholds values are determined with appropriate probe drugs. Therefore, this study evaluates the impact of low versus high AO activity in human liver S9 and cytosol preparations on the prediction of scaled clearance for AO substrates with the ‘yardstick’ approach.

**Methods**

Individual and pooled human liver cytosol (n = 1 or n = 50) and S9 (n = 1 or n = 200) fractions were prepared internally. Phthalazine, zaleplon, 4-epibenzylguanine, zaleplon and methotrexate were purchased from Sigma-Aldrich (St. Louis, MO). Carbazeran and deoxypenciclovir were purchased from Toronto Research Chemicals (Toronto, Ontario).

Metabolic clearance was evaluated in human liver S9 and cytosol fractions selected from five individuals that spanned >10-fold range in AO activity based on previous individual characterization. Each S9 and cytosol AO activity was previously characterized by measurement of phthalazine (20 µM, 10 min) oxidation (incubation conditions similar to those described below). Enzyme activity was evaluated by monitoring the disappearance (parent loss) of four drugs at <1 µM with a validated AO assay, namely carbazeran, deoxypenciclovir, zoniporide, benzylguanine, zaleplon and methotrexate. Briefly, incubations (200 µL, 37°C) were conducted in duplicate with human liver S9 (2.5 µg/mL) or cytosol (1 µg/mL) (phosphate buffer 90 mM, pH 7.4) for up to 4 hours in the presence of one of the selected drugs. Time points were adjusted to account for any expected half-life standards. Parent drug was monitored by LC-MS/MS and remaining drug was quantified using a standard curve prepared for each compound in the appropriate matrix. Disappearance at each time point was determined by comparison to the zero time control (% remaining).

Half-life (t<sub>1/2</sub>) was determined by log transformation of the parent loss data with point was determined by comparison to the zero time control (% remaining).

Metabolic stability (clearance) of the AO substrates carbazeran, zoniporide and zaleplon in individual (five) and pooled (n = 50) human liver cytosol fractions

Metabolic stability (clearance) of the AO substrates carbazeran, zoniporide and zaleplon in individual (five) and pooled (n = 200) human liver S9 fractions

**Results**

• Figure 1 illustrates the metabolic clearance of carbazeran, zoniporide and zaleplon in human liver S9 (2.5 mg/mL) or cytosol (1 mg/mL) in phosphate buffer (50 mM, pH 7) in microsomal or reconstituted S9 fractions with an appropriate internal standard. Parent drug was monitored by LC-MS/MS and remaining drug was quantified using a standard curve prepared for each compound in the appropriate matrix. Disappearance at each time point was determined by comparison to the zero time control (% remaining).

• Table 1 provides the half-life of carbazeran, zoniporide and zaleplon in individual (five) and pooled (n = 50) human liver cytosol fractions. The in vitro clearance of carbazeran (Figure 1A), a high AO substrate, increased in accordance to the rank order previously determined for phthalazine oxidation (Table 1). Across the range of individuals, the half-life of carbazeran ranged from 0 to 5 minutes. In pooled cytosol, the observed half-life of carbazeran was four minutes which was similar to the median individual sample.

• In a similar manner, the in vitro clearance of zoniporide (Figure 1B), a moderate AO substrate, increased in accordance to the rank order previously determined for phthalazine oxidation (Table 1). Across the range of individuals, the half-life of zoniporide ranged from 19 to >240 minutes. In pooled cytosol, the observed half-life of zoniporide was 89 minutes which was similar to the median individual sample.

• High, moderate and low clearance drugs (Table 1) could be segregated based on the observed half-life in all cytosol samples (individual and pooled) with the exception of the lowest activity individual (H1).

• Figure 2 illustrates the metabolic clearance of carbazeran, zoniporide and zaleplon in individual (five) and pooled (n = 200) human liver S9 fractions. The in vitro clearance of carbazeran (Figure 2A), a high AO substrate, increased in accordance to the rank order previously determined for phthalazine oxidation (Table 1). Across the range of individuals, the half-life of carbazeran ranged from 108 to >240 minutes. In pooled S9, the observed half-life of carbazeran was 5 minutes which was similar to the H2 individual sample.

• In a similar manner, the in vitro clearance of zoniporide (Figure 2B), a moderate AO substrate, increased in accordance to the rank order previously determined for phthalazine oxidation (Table 1). Across the range of individuals, the half-life of zoniporide ranged from 108 to >240 minutes. In pooled S9, the observed half-life of zoniporide was 74 minutes which was similar to the H2 individual sample.

• The in vitro clearance of zaleplon (Figure 2C), a low AO substrate, increased in accordance to the rank order previously determined for phthalazine oxidation (Table 1). Across the range of individuals, the half-life of zaleplon ranged from 90 to >240 minutes. In pooled S9, the observed half-life of zaleplon was >240 minutes which was similar to the highest individual sample.

• High, moderate and low clearance drugs (Table 2) could be segregated based on the observed half-life in all S9 samples (individual and pooled).

**Conclusions**

• In human liver S9, half-life and scaled clearance could be calculated from all five individuals, including the lowest activity sample. In each case, the ‘yardstick’ approach could be applied to all samples tested with reasonable thresholds, including the lowest and highest activity donors representing over a 10-fold range in AO activity.

• In human liver cytosol, half-life and scaled clearance could be calculated from four of five individuals, the exception being the lowest activity AO sample. Similarly, the ‘yardstick’ approach could be applied with reasonable thresholds for all samples tested, except for the lowest AO activity sample.

• These data indicate that, because of the necessity to scale AO clearance with a rank-order approach, nearly all human liver S9 and cytosol (individual or pooled) can be utilized to predict AO CL/MS, once threshold values are determined with appropriate probe drugs.

**References**

1. Zientek et al., Drug Metab Dispos 38:1322-1327, 2010