Introduction

Mechanism-based inhibition (MBI) is associated with irreversible or quasi-reversible loss of enzyme activity. This type of inhibition may be a cause of particularly profound and long-lasting drug-drug interactions, requiring synthesis of new enzyme for return of normal enzyme function. Description of the effects of MBI is based on the in vitro estimation of two key parameters, namely $k_{\text{inact}}$ (the maximum rate constant for inactivation) and $K_i$ (the concentration of inhibitor which produces half the maximal rate of inactivation). The experimental procedure involves two steps (Silverman, 1995):

1. pre-incubation of enzyme with inhibitor (enzyme inactivation),
2. incubation with a probe substrate (measurement of residual enzyme activity).

To prevent further inactivation during step 2, enzyme and inhibitor should be diluted into a solution containing a high concentration of probe substrate. In this preliminary study we have investigated the impact of dilution and 3 different probe substrate concentrations on estimates of $k_{\text{inact}}$ and $K_i$ for 3,4-methylenedioxyamphetamine (MDMA), an inhibitor of CYP2D6. Although recommendations have been made for the optimal design of MBI studies (Silverman, 1995), to our knowledge, the quantitative effects of experimental variables on $k_{\text{inact}}$ and $K_i$ have not been investigated systematically.

Experimental Methods

Details of the MBI experiments are given elsewhere (Heydari et al. 2004). In brief, the pre-incubation mixture contained MDMA (2 – 40 µM and 0.625 – 12.5 µM for experiments with and without dilution, respectively), KCL, an NADPH-generating system, potassium phosphate buffer (pH 7.4). All reactions were started by addition of yeast microsomes expressing CYP2D6 (20 pmol), and were carried out at 37°C. In the first experimental scheme, the CYP2D6 probe, dextromethorphan (DEX) (5, 10 or 20 µM), was added directly to the pre-incubation mixture after different pre-incubation times (1 – 5 min) (no dilution). In the second scheme, aliquots (62 µl) were taken from the pre-incubation mixture at different times (1 – 5 min) and added to incubation tubes containing DEX solution (final DEX concentration 20 µM; final volume 250 µl; a 4 fold dilution of the MDMA in the pre-incubation mixture). The reactions were stopped after 5 min by the addition of perchloric acid.

The inhibition of CYP2D6 was determined by the conversion of DEX to dextrorphan in the presence and absence of MDMA.

Data Analysis

Inactivation constants ($k_{\text{obs}}$) were calculated from the % inhibition of the conversion of DEX to dextrorphan (Ito et al. 1998). Hence, $k_{\text{inact}}$ and $K_i$ values were estimated using the following equation (Heydari et al. 2004) and non-linear regression.

$$k_{\text{obs}} = \frac{k_{\text{inact}} 	imes [I]}{K_i + [I]}$$

Statistical inferences were made using the z-test.

Results

Figure 1 shows the model fits to the relationship between $k_{\text{obs}}$ and MDMA concentration obtained from experiments with no dilution and using different DEX concentrations. Increasing concentrations of DEX were not associated with statistically significant differences in estimates of $K_i$ or $k_{\text{inact}}$ values ($p > 0.05$).

![Figure 1](image)

A 4-fold dilution of the pre-incubation mixture resulted in higher values of $k_{\text{inact}}$ and $K_i$ relative to those observed without dilution (0.29 ± 0.03 SE vs 0.21 ± 0.02 min⁻¹; $p < 0.02$ and 12.9 ± 3.6 vs 3.5 ± 0.8 µM; $p < 0.01$; respectively).

Discussion

The observations indicate that probe substrate concentrations in excess of $K_m$ (= 1.7 µM for DEX) will not have a major impact on $K_i$ and $k_{\text{inact}}$ values. Dilution after pre-incubation minimises any additional MBI that may occur during the subsequent incubation period and increases the value of $K_i$. However, the fold dilution that fully prevents inactivation during the incubation stage requires further evaluation as the literature is inconsistent in this regard (Ghanbari et al. in preparation).

These preliminary data emphasise the importance of study design in the accurate characterisation of the kinetics of MBI, with implications for in vitro-in vivo extrapolation of the extent of MBI-based drug-drug interactions.

References