Synergistic *In Vitro* Hepatotoxicity of Ketoconazole and Cyclophosphamide in Primary Cultured Human Hepatocytes

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**Introduction**

We report here an unexpected observation of synergistic hepatotoxicity between cyclophosphamide (CP) and ketoconazole (KZ). As both CP and KZ are known to be hepatotoxic in humans in vivo, the observed synergism may be one of the exacerbating factors for their potential to cause liver damage. Our observation may have significance in the care of cancer patients as antifungals such as ketoconazole are often co-prescribed with anticancer agents such as cyclophosphamide. The observed synergistic toxicity may also be relevant to the occurrence of idiosyncratic drug induced liver failures.

**Methods**

- Cryopreserved human hepatocytes: 999Elite™ plateable cryopreserved human hepatocytes (In Vitro ADMET Laboratories Inc., Columbia, MD) were used in the study. The study was performed with hepatocytes from six different donors with similar results (manuscript in preparation). Results obtained from a single donor, HEK293, are presented in this poster.
- The hepatocytes were thawed and recovered in Universal Cryopreservation Recovery Medium (In Vitro ADMET Laboratories), and resuspended in Universal Cryopreservation Plating Medium (In Vitro ADMET Laboratories) for quantification of cell viability and concentration. The hepatocytes were plated in 384-well collagen-coated plates for 4 hrs., followed by drug treatment.
- The cells were treated with 0, 0.31, 0.63, 1.25, 2.5, 5, 10, and 20 mM of CP. At each CP concentration, cells were also co-treated with 0, 1.25, 2.5, 5, and 10 µM of KZ for 24 hrs. The KZ concentrations chosen were noncytotoxic, while the CP concentrations chosen yielded dose-dependent cytotoxicity.
- Viability was determined via quantification of cellular ATP contents based on luminescence (ATPLite™, Perkin-Elmer). Results are expressed as Relative Viability using the following equation:

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\text{Relative Viability} \% = \frac{\text{ATP (treatment)}}{\text{ATP (solvent control)}} \times 100
\]

**Results**

- At the chosen concentrations, cyclophosphamide (CP) yielded dose-dependent cytotoxicity (Fig 1), with its cytotoxicity enhanced by noncytotoxic concentrations of ketoconazole (KZ).
- At 1.25 µM KZ, CP cytotoxicity was reduced (Fig. 2), an observation consistent with the inhibition of CYP3A4 activity by KZ. This leads to a decreased level of activation of CP to cytotoxic metabolites.

- An unexpected observation was made at the higher concentrations of 5 and 10 µM of KZ, where the apparent CP cytotoxicity was increased rather than decreased (Figs 1 and 2). For instance, in one of the experiments, the relative cytotoxicity values upon treatment of the hepatocytes with 5 mM CP in the presence of 0, 1.25, 2.5, 5 and 10 µM of KZ were 60.61%, 118.7%, 70.5%, 32.6%, and 14.4%, respectively. The apparent enhancement of CP cytotoxicity by KZ at 5 and 10 µM was reproduced in multiple independent experiments using human hepatocytes from six donors.
- No synergistic cytotoxicity was observed when HEK293 cells were used by treatment with CP and KZ (Fig 3).

**Summary**

Synergistic toxicity was observed between two hepatotoxic drugs, cyclophosphamide and ketoconazole.

1. Noncytotoxic concentrations of ketoconazole were found to increase the cytotoxicity of cyclophosphamide. At each of the concentrations of cyclophosphamide evaluated, ketoconazole was found to elicit dose-dependent increases in cytotoxicity (decrease in viability).
2. No additional cytotoxicity was observed when HEK293 cells were used, which suggests that synergism may require competent drug metabolizing enzymes that are present in human hepatocytes but not in HEK293 cells.

**Conclusions**

Idiosyncratic drug induced liver injuries continue to be a challenge for drug development. Drugs found to be nonhepatotoxic as single entities in preclinical and clinical trials have been found to cause severe liver injuries when prescribed with other drugs, leading to fatalities or a need for liver transplantation.

Our observation that the combination of noncytotoxic concentrations of two hepatotoxic drugs, cyclophosphamide and ketoconazole, could lead to significantly higher cytotoxicity in human hepatocytes suggests that a similar phenomenon may be responsible for the rare cases of idiosyncratic drug induced liver injuries.

The observed synergistic toxicity of ketoconazole and cyclophosphamide suggests that exposure of an individual to multiple hepatotoxins, even with each drug at nontoxic levels, may result in significant hepatotoxicity. Investigations to define the mechanism and physiological relevance of this observation may elucidate environmental factors crucial to the prevention of idiosyncratic drug-induced liver injuries.

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