

EFFECTS OF InVitroGold™ ON SELECTED CYP ENZYME ACTIVITY LEVELS AND CELLULAR APOPTOSIS AND VIABILITY IN HUMAN HEPATOCYTES

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Abstract

Human hepatocytes constitute a physiologically relevant experimental model for the evaluation of hepatocellular apoptosis, necrosis, and drug-drug interactions related to the induction of CYP enzyme activities. Traditionally, human hepatocytes have been cultured on collagen-coated plates with or without an overlay ("sandwich") of collagen or some other extracellular matrix. We report here the utilization of a proprietary ("InVitroGold™") system that maintains optimal conditions to support the viability and responsiveness of cultured hepatocytes. The effects of this system on constitutive enzyme activity levels of selected CYPs (CYP1A2, CYP2B6, CYP2C9, and CYP3A4) as well as on the responsiveness of these CYPs to the treatment of known inducers (50 μM omeprazole for CYP1A2, 1 mM phenobarbital for CYP2B6, and 25 μM for CYP2C9 and CYP3A4) in freshly isolated or plateable cryopreserved human hepatocytes were investigated. Hepatocytes were cultured in 48-well plates in traditional collagen-sandwich or InVitroGold™ culture conditions. Cells were then treated with either vehicle or known inducers for 48 hours before dosing with selective CYP substrates. Cryopreserved hepatocytes cultured in the InVitroGold™ condition had higher constitutive enzyme activity levels for all CYPs except CYP1A2. The magnitude of induction was also greatly increased when cultured in InVitroGold™ compared with hepatocytes cultured in a collagen-sandwich. Notably, InVitroGold™ did not increase constitutive enzyme activity levels for all tested CYPs nor the responsiveness to inducer treatment in freshly isolated hepatocytes. Furthermore, hepatocytes cultured in the InVitroGold™ condition responded in a similar manner to collagen-sandwiched hepatocytes when treated with apoptosis- or necrosis-inducing chemicals. These data demonstrate that the InVitroGold™ system of culturing hepatocytes is preferable to traditional collagen-sandwich culture for studying the potential for new chemical entities to induce CYPs, especially in cryopreserved human hepatocytes.

Introduction

Human hepatocytes have been increasingly utilized in a broad range of *in vitro* studies including drug metabolism, drug-drug interactions, and cytotoxicity. These results are then correlated with *in vivo* data to help predict undesirable side effects with new drugs. The advent of plateable cryopreserved human hepatocytes (PCHH) has markedly facilitated the conduct of these studies by overcoming limitations in human liver availability, and by allowing the use of a given hepatocyte preparation repeatedly in multiple *in vitro* studies. PCHH can form monolayers with greater than 70% confluence when plated on collagen-coated plates, and sandwiching with an overlay further optimizes the culture environment. In this study, we demonstrate the use of a new proprietary culture system, InVitroGold™. The effects of this system on the constitutive and induced expression of several CYPs are compared with the traditional collagen-sandwich system. Further, the suitability of using the InVitroGold™ system in cytotoxicity studies is evaluated in comparison to the collagen-sandwich system.

Materials and Methods

Hepatocyte Cultures. Fresh human hepatocytes and cryopreserved human hepatocytes were obtained from In Vitro Technologies, Inc.

Plating of Fresh and Plateable Cryopreserved Human Hepatocytes. Vials of PCHH were thawed in a 37°C water bath, and cells were diluted in InVitroGold™ CP medium. Fresh human hepatocytes were isolated using a two-step perfusion method. Cell counts and viability were determined by Trypan blue exclusion. The cell suspensions were then diluted to 700,000 viable cells per ml with InVitroGold™ CP medium, and transferred to collagen coated 48-well plates (140,000 cells per well). Plates were incubated overnight in a 37°C, 5% CO₂ humidified incubator to allow attachment of the hepatocytes, then plating medium was replaced with sandwich medium, containing either collagen or InVitroGold™, and the hepatocytes were incubated until use.

Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4. Sandwich medium was removed and replaced with InVitroGold™ HI medium containing 50 μM omeprazole for CYP1A2; 1 mM phenobarbital for CYP2B6; 25 μM rifampin for CYP2C9 and CYP3A4; or vehicle. After 24 hours, new medium containing inducers or vehicle controls was added to the plated hepatocytes and cells were incubated an additional 24 hr.

Determination of CYP Activities. Medium was removed and replaced with modified Krebs-Henseleit buffer containing the following CYP substrates: 100 μM phenacetin for CYP1A2; 1 mM S-mephenytoin for CYP2B6; 50 μM tolbutamide for CYP2C9; 125 μM testosterone for CYP3A4. Incubations were performed in a 37°C, 5% CO₂ humidified incubator for four hours, and stopped by addition of an equal volume of ice-cold methanol. The amount of metabolite formed was determined by LC-MS or LC-UV.

MTT Assay. Cell necrosis was evaluated by measuring MTT reduction. MTT was added to a final concentration of 0.5 mg/ml to each well containing the positive control, 500 μM chlorpromazine, or the vehicle. Plates were incubated in a 37°C, 5% CO₂ humidified tissue culture incubator for three hours, then medium from all wells was removed, and 0.2 ml of acidified isopropanol was added to each well to dissolve the MTT formazan. Absorbance of MTT formazan was measured at 572 nm and 690 nm on a Wallac Victor³ multilabel counter, and the corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value.

Caspase 3/7 Assay. Apoptosis was evaluated by measuring the activation of caspases 3 and 7 using the Apo-ONE® Homogeneous Caspase 3/7 Assay (Promega Corp.). This kit allows the determination of caspase 3/7 catalytic activity by measuring the formation of a fluorescent product using a plate reader (excitation wavelength 485 nm, emission wavelength 535 nm).

Results

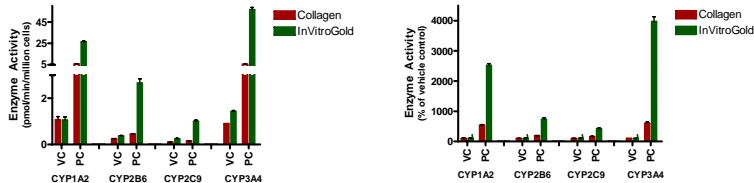


Figure 1. Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 in plateable cryopreserved human hepatocytes. Expressed as specific activity (left) or percent of vehicle control (right)

• Representative results show that InVitroGold slightly increased constitutive CYP expression but had a marked effect on induced levels relative to collagen-sandwich

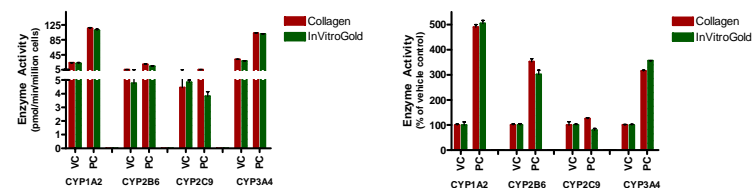


Figure 2. Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4 in fresh human hepatocytes. Expressed as specific activity (left) or percent of vehicle control (right).

• Representative results show that InVitroGold had little or no effect on constitutive or induced CYP expression relative to collagen-sandwich.

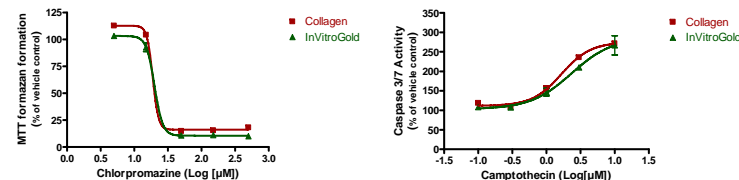


Figure 3. Cytotoxicity of chlorpromazine (left) or camptothecin (right) in plateable cryopreserved human hepatocytes.

• The InVitroGold system did not modulate the cytotoxic response to a necrotic agent, chlorpromazine, or an apoptotic agent, camptothecin.

Conclusions

Plateable cryopreserved human hepatocytes represent a valuable experimental system useful for investigating hepatic metabolism, drug-drug interactions, and cytotoxicity.

Plateable cryopreserved human hepatocytes expressed higher constitutive and induced levels of selected CYP activities with the InVitroGold™ system than with the collagen sandwich system. The magnitude of the induction response was consistently larger with the InVitroGold™ system. Fresh hepatocytes did not show these effects.

Cytotoxic responses to chlorpromazine or camptothecin were appropriate in the InVitroGold™ system and did not differ from the collagen sandwich system.

The InVitroGold™ system appears to provide an improved culture environment for plateable cryopreserved human hepatocytes.