

# #1624 Determination of Apoptosis in Plateable Cryopreserved Human Hepatocytes

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## Abstract

Cryopreservation of human hepatocytes has provided the opportunity for study of toxicity and drug metabolism of compounds without the need to wait for fresh tissue. Some cryopreserved hepatocytes have the ability to form an adherent monolayer when thawed and plated on collagen-coated tissue culture plates. Previous work with these plateable cryopreserved human hepatocytes (PCHH) has demonstrated their usefulness for long-term (4 day) toxicity studies for necrosis. In this work we present data on the use of PCHH for evaluating chemically-induced apoptosis. PCHH were plated on collagen coated 48-well flat bottom plates and incubated for 24 hours at 37°C, 5% CO<sub>2</sub> in a humidified incubator. After monolayers were established, they were treated with camptothecin (100 nM, 1 µM, 10 µM and 100 µM) in serum-free medium for 4, 24 and 48 hours. Three different endpoints were determined; MTT assay for necrosis, and caspase 3/7 and DNA fragmentation for apoptosis. After 4 hours, no necrosis or apoptosis was identified by any of the assays. However, a significant increase in apoptotic markers was identified at 24 hours in both caspase 3/7 and DNA fragmentation assays. After 24 hours, at camptothecin levels of 10 and 100 µM, caspase 3/7 levels increased 4 fold, and DNA fragmentation levels increased 3 – 4 fold. At 48 hours, caspase 3/7 levels increased 7 – 8 fold, while DNA fragmentation results increased 2.5 fold. Small increases in caspase 3/7 and DNA fragmentation were seen at 1 µM camptothecin, with little or no increase at 100 nM. Viability, as measured by MTT, demonstrated little or no decrease after 48 hours at camptothecin levels of 100 nM and 1 µM, but decreased with 10 and 100 µM concentrations after 24 hours (50% viable) and 48 hours (<25% viable). Camptothecin induced apoptosis in a time-dependent and concentration-dependent manner as indicated by both caspase 3/7 and DNA fragmentation assays. These results indicate that PCHH is a useful system for evaluating the ability of unknown compounds to initiate apoptosis in human hepatocytes.

## Introduction

Cell death can occur by several different pathways. Necrosis has traditionally been the mechanism by which cytotoxicity was measured. However, apoptosis may be the more common event. A necrotic event is defined as a loss of cell membrane integrity, which results in the release of the intracellular components in an uncontrolled sequence of events. In contrast, apoptosis is a series of energy driven events, which result in the degradation of intracellular components. Some of the hallmark apoptotic events are zeiosis (membrane blebbing), activation of caspases, mitochondrial dysfunction and formation of nucleosomes by DNA fragmentation. Apoptotic events can be identified using various methods including annexin V staining, caspase cleavage of fluorometric substrates, mitochondrial cytochrome C release, and DNA fragmentation staining. When such assays are run along with more common cell viability assays like MTT conversion, LDH release or ATP measurements, both apoptotic and necrotic events can be identified. Identifying the potential of new drug candidates to cause necrosis and/or apoptosis, and distinguishing between these two effects, is increasingly important in pre-clinical drug development.

## Materials and Methods

**Plated human cryopreserved hepatocytes.** Plateable human cryopreserved hepatocyte Lot QKR was obtained from In Vitro Technologies, and thawed in a 37°C water bath. The cells were diluted in Plating Medium, then mixed and centrifuged at 100 x g for five minutes. The supernatant was removed and the cell pellet resuspended in Plating Medium. Viability and cell counts were determined by Trypan blue exclusion. The cell suspension was diluted to 700,000 viable cells per ml with Plating Medium. A volume of 0.2 ml of the cell suspension was transferred to a collagen coated 48-well plate. Plates were incubated overnight in a 37°C, 5% CO<sub>2</sub>, humidified incubator to allow attachment of the hepatocytes. Each well was washed one time with Incubation Medium prior to the addition of drug compounds.

**Preparation of stock solutions.** Camptothecin was dissolved in DMSO to prepare 100X stock solutions.

**Dosing solutions for plated hepatocytes.** Solutions were prepared by diluting the drug stocks 1:100 in Incubation Medium. A vehicle control (VC) solution was formed by adding DMSO to Incubation Medium to a final concentration of 1%. Medium was removed from the 48-well plates, and dosing solutions (1:100) and VC solutions were added. The plates were returned to the incubator. Cells were incubated for 4, 12, 24, or 48 hours, and then assessed for the MTT reduction, caspase 3/7 activity and DNA fragmentation.

**MTT Assay.** Thiazolyl blue tetrazolium bromide (MTT) was dissolved in deionized H<sub>2</sub>O at a concentration of 5 mg/ml to form a 10X solution. Acidified isopropanol was prepared by diluting hydrochloric acid (HCl) into isopropanol to a final concentration of 0.04 M HCl. At the time of the assay, a background blank was prepared by removing medium from three VC wells and adding 0.2 ml of methanol to the wells. After five minutes at room temperature, the methanol was removed and the VC incubation medium returned to the wells. 10X MTT was added 1:10 to the dosing solution for each drug, VC and background blank. Plates were incubated in a 37°C, 5% CO<sub>2</sub>, 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. The absorbance of MTT formazan was measured at 572 nm and 690 nm on a Wallac Victor<sup>2</sup> multilabel counter. The corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value. The average background blank was subtracted from the average of the other experimental groups to derive the adjusted absorbance. The dosing groups were compared to VC by dividing adjusted absorbance of the dosing group by the adjusted absorbance of the VC and multiplying by 100 to get the percentage of VC.

**Caspase 3/7 Assay.** The caspase activity was determined by using Apo-ONE<sup>®</sup> Homogenous caspase 3/7 Assay (Promega, Madison, WI). The assay was performed as described in the assay instructions. The plate was analyzed on the Wallac Victor<sup>2</sup> multilabel counter with excitation at 485 nm emission at 535 nm. The average background blank was subtracted from the average of the other experimental groups to derive the adjusted fluorescence. The dosing groups were compared to VC by dividing the adjusted fluorescence of the dosing group by the adjusted fluorescence of the VC and multiplying by 100 to get the percentage of VC.

**DNA Fragmentation Assay.** The DNA fragmentation was determined by using Cell Death Detection ELISA<sup>plus</sup> (Roche Diagnostics, Mannheim, Germany). The assay was performed as described in the assay instructions. The plate was analyzed on the Wallac Victor<sup>2</sup> multilabel counter for the absorbance at 405 nm. The average background blank was subtracted from the average of the other experimental groups to derive the adjusted absorbance. The dosing groups were compared to VC by dividing the adjusted absorbance of the dosing group by the adjusted absorbance of the VC and multiplying by 100 to get the percentage of VC.

## Results

**MTT Assay.** Viability of hepatocyte monolayers was assessed using the reduction of MTT to MTT formazan. Cultures maintained viability throughout the experiment, as illustrated by VC samples at each time point (Fig. 1). No discernable toxicity was observed at the four- (Fig. 2) and 12-hour (Fig. 3) time points in all dosed conditions. The LD<sub>50</sub> of the camptothecin was 10 µM and 5.5 µM at 24- (Fig. 4) and 48-hours (Fig. 5), respectively. Minimum viability at 48-hours was measured at 100 µM camptothecin, and was 9% as compared to the VC (Fig. 5).

**Caspase 3/7 Assay.** Caspase 3/7 activity was not significant at the four- (Fig. 2) and 12-hour (Fig. 3) time points but did increase at the 24- (Fig. 4) and 48-hour (Fig. 5) time points. The activity plateaued at 10mM camptothecin for the latter two time points and continued to increase from the 24- to 48-hour time points, 473% and 774% at the 100 µM camptothecin, respectively.

**DNA Fragmentation Assay.** DNA fragmentation was the initial indicator for camptothecin toxicity. The four-hour (Fig. 2) time point did not show any increase in nucleosome formation, however a 40% increase was detected at the 12-hour (Fig. 3) time point. The 24-hour time point had the largest induction at 418% for the 100 µM camptothecin as compared to the VC (Fig. 4). At 48-hour time point, the DNA fragmentation diminished to a maximum of 250% at the concentration of 10 µM camptothecin (Fig. 5).

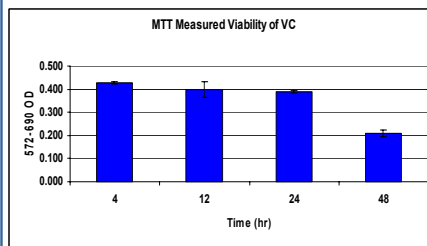


Figure 1. Viability of hepatocytes in vehicle control medium as measured by MTT conversion at 4-, 12-, 24-, and 48-hour time points.

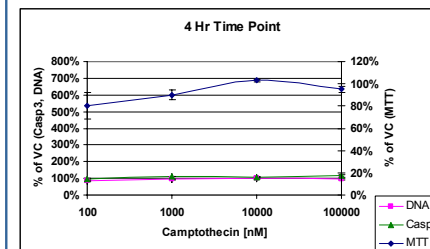


Figure 2. Results at four-hours. All data is referenced to the vehicle control for each time point and is plotted as a percentage of the vehicle control.

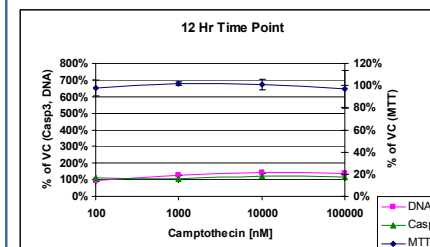


Figure 3. Results at 12-hours. All data is referenced to the vehicle control for each time point and is plotted as a percentage of the vehicle control.

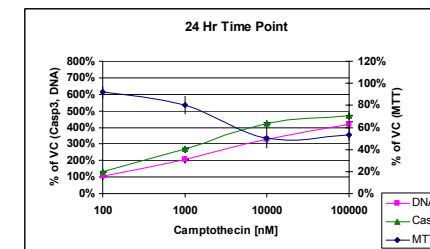


Figure 4. Results at 24-hours. All data is referenced to the vehicle control for each time point and is plotted as a percentage of the vehicle control.

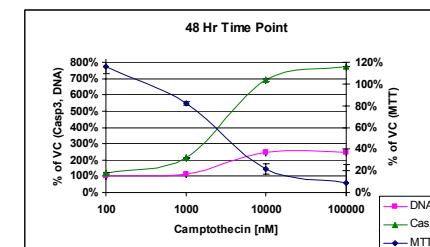


Figure 5. Results at 48-hours. All data is referenced to the vehicle control for each time point and is plotted as a percentage of the vehicle control.

## Conclusion

- PCHH present a robust cell-based system to evaluate chemically-induced apoptosis in human hepatocytes.
- An increase in both caspase 3/7 activity and DNA fragmentation was observed when camptothecin was used to induce apoptosis.
- PCHH maintain apoptotic “machinery” and therefore, can be used to distinguish between apoptotic or necrotic events.
- PCHH allow for high throughput screening of test compounds at multiple concentrations and for multiple time periods.