Evaluation of a Novel Anti-Tumor Drug Using In Vitro Toxicity Screening in Rat Hepatoma (H4IIE) Cells, Normal Rat Kidney (NRK) Cells, and Rat Primary Hepatocytes

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ABSTRACT

Cytoreg® is a novel drug candidate shown to have cytotoxic activity against cancer cells. The purpose of this study was to determine whether Cytoreg® was cytotoxic to normal rat cells and non-tumor cells in order to differentiate tumor from non-tumor cells. A series of experiments were conducted in order to investigate the effects of Cytoreg® on mitochondrial function, oxidative stress, and apoptosis. Dosing was based on a series of sponsor-requested dilutions, including pH values at 2.0, 7.2, 7.5, 8.0, 9.0, 10.0, and 11.0. The highest dilution evaluated was the lowest exposure concentration. The effect of pH in the absence of drug was also determined. Cells were exposed to Cytoreg® and pH in a series of experiments to determine the cytotoxicity of the compound at various dilutions and pH values. Cytoreg® was not cytotoxic up to and including the 1:500 exposure concentration. The pH experiments showed the cytotoxicity of Cytoreg® to be due to the extracellular pH of the compound. A series of sponsor-requested experiments were performed using a tumor cell line and a normal liver cell line. Cytoreg® was tested over a range of dilutions from very high (1:10 to very low (1:500)).

RESULTS

FIGURE 1: Effects of Cytoreg® and pH on H4IIE Cells at 6 and 24 Hours Exposures

Following exposure to Cytoreg® (Fig. 1A, 1B), toxicity was measured at 6 and 24 hr in the H4IIE cells. Cell death was measured at pH 6.8 at 1.95% which was near physiologic pH (pH = 7.4). The effect of pH on cell death at 6 and 24 hr (Fig. 1C, 1D) showed no toxicity at or above pH 6.8. Effects on the mitochondrial markers, MTT and ATP, were observed at pH < 6.8 only in time points, with the effect being more pronounced at pH < 6.5 (Fig. 1C, 1D). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

FIGURE 2: Comparison of Cell Death Markers in Three Cell Types at 24 Hours Exposures

Following exposure to Cytoreg® (Fig. 2A, 2B), toxicity was measured at 6 and 24 hr in rat primary hepatocytes. Cell death was not detected at the 1:500 dilution (data shown in Table 1). However, there were substantial reductions in the ATP and MTT markers at pH < 6.8. The effect of pH on cell death at 6 and 24 hr (Fig. 2C, 2D) showed no toxicity at or above pH 6.5. Effects on mitochondrial markers, MTT and ATP, were observed at pH < 6.5 in time points, with the effect being more pronounced at pH < 6.0 (Fig. 2C, 2D). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

Following exposure to Cytoreg® (Fig. 3A, 3B), toxicity was measured at 6 and 24 hr in the NRK cells. Cell death was not detected at the 1:500 dilution (data shown in Table 1) and toxicity profiles were similar to both the tumor and non-tumor cell types. The effect of pH on cell death at 6 and 24 hr (Fig. 3C, 3D) showed no toxicity at or above pH 6.5. Effects on mitochondrial markers, MTT and ATP, were observed at pH < 6.5 in time points, with the effect being more pronounced at pH < 6.0 (Fig. 3C, 3D). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

METHODS

Cell Culture Conditions

For the H4IIE cells, several culture plates were seeded with cells in 10% of media to a density of 60,000 cells per plate. Cells were cultured at 37°C, 5% CO₂ in a humidified atmosphere. On the third day after seeding, test compounds (pre-intact or intact) were added to the plates. For the primary hepatocytes, collagen-coated 96-well plates were seeded with cells in 10% of medium, allowed to plate in 37°C for 24 hr, and then analyzed for MTT activity and ATP levels as well as cell number and membrane leakage.

Test Compound

The test compound was used to prepare starting dilutions of 1:110, 1:1,100, 1:1,100, 1:1,000, and 1:1,000 in cell culture medium buffered with 35 mm HEPES. The pH of the dosing solutions was measured at each exposure concentration tested. The lowest exposure concentration (1:500) was near physiologic pH (pH = 7.2; see table below). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

Membrane Leakage

Cell death was determined by monitoring membrane leakage from cells using either lucifer yellow or calcein dye. The results of these experiments indicate that the Cytoreg® had a high specificity and sensitivity in the detection of tumor cells.

Mitochondrial Function

Adenosine Triphosphate (ATP)

Cellular Adenosine triphosphate (ATP), a marker for mitochondrial respiration and extramitochondrial reductase activity. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

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MTT Assay (Tetrazolium Dye Reduction)

Cells in 96-well plates were evaluated for their ability to reduce solution MTT (pH=7.4) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan MTT (pH=10). Substrate was used to measure mitochondrial respiration and extramitochondrial reductase activity. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

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REFERENCES