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 compound is acidic in nature and has the potential of being an effective first line treatment for various cancer indications. This study was designed to identify potential cytotoxicity not related to the pharmacology and to determine whether the drug can distinguish tumor from non-tumor cells. Rat primary hepatocytes, normal rat kidney (NRK) cells and rat hepatoma (H4IIE) cells were used as the test systems. *In vitro* toxicity assays were used to measure membrane leakage, cell number, mitochondrial function, oxidative stress, and apoptosis. Dosing was based on a series of sponsor-requested dilutions, including dilutions of 2000, 1000, 500, 100, 50, 10, and 1. The highest dilution represented the lowest exposure concentration. The effect of pH in the absence of drug was also determined. Cells were cultured in 96-well plates and exposed for 6 and 24 hr. In H4IIE cells, the compound was lethal to more than 90% of the cells at the 500 dilution at 24 hr. These effects occurred prior to any cytotoxic effect related to low pH. Dilutions of 100 and lower showed cytotoxicity that could not be distinguished from effects due to low pH. Rat primary hepatocytes were used to investigate cytotoxic effects on non-tumor cells. Exposure dilutions of 2000, 1000, and 500 did not produce any acute cytotoxicity in these cells, as was observed in H4IIE cells at the 500 dilution, but did produce effects on the mitochondria. NRK cells were evaluated to investigate specificity and selectivity in another non-tumor cell line. No cytotoxic effects were observed up to and including the 500 dilution after 6 and 24 hr. Significant effects were observed on the mitochondria at dilutions of 100 and lower, consistent with effects due to low pH. These data indicate that the tumor cell line under conditions of near physiologic pH is considerably more sensitive to the cytotoxic effects of Cytoreg® than are non-tumor cells based on cell number and membrane integrity.

INTRODUCTION

The anti-tumor drug known as Cytoreg® was evaluated in three cell types for cytotoxicity and the ability to distinguish tumor from non-tumor cells. Rat hepatoma-derived cells (H4IIE), normal rat primary hepatocytes, and a non-tumor cell line derived from normal rat kidney tissue (NRK) were evaluated.

In the first set of experiments, the test compound was evaluated for toxicity using the H4IIE cell line. In order to separate out toxicity related to the acidic nature of the compound from specific effects related to the compound itself, a series of experiments were done to evaluate the effects of pH on the test system. The second set of experiments was done using cryopreserved rat primary hepatocytes to evaluate the toxicity of the test compound on cells derived from normal tissue. The third experiment was performed using cells derived from normal rat kidney (NRK) and was done to evaluate the specificity and selectivity of the test compound in another non-tumor cell line. Cytoreg® was tested over a range of dilutions from very high (1:1) to very low (1: 2000).

METHODS

Cell Culture Conditions

For H4IIE and NRK cells, flat bottom 96-well plates were seeded with cells in 100 µL of media 48 hr prior to dosing. Cells were cultured at 37°C, 5% CO_2 in Modified Eagle Media supplemented with serum. On the third day after seeding, test compounds prepared in medium were added to the plates. For rat primary hepatocytes, collagen-coated 96-well plates were seeded with cells in 100 uL of medium, allowed to plate down for 6 hr, and dosed with test compound. Cells were exposed for 6 and 24 hrs and then analyzed for MTT activity and ATP levels as well as cell number and membrane leakage.

Test Compound

The test compound stock was used to prepare dosing dilutions of 1:1, 1:10, 1:50, 1:100, 1:500, 1:1000, and 1:2000 in cell culture medium buffered with 25 mM HEPES.

Cell Number

Cell number was determined in a separate plate using a specific nucleic acid binding dye that fluoresces upon interaction with DNA and RNA. There is a direct correlation between intracellular RNA/DNA levels and cell number. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

Membrane Leakage

Cell death was determined by monitoring membrane leakage from cells using either ELISA or activity assays. The marker enzymes were specific for the tissue. Calculations were expressed as percent change relative to cell death as determined by complete cell lysis. Treatment values were divided by cell death values and subtracted from 100 to determine percent live cells.

MTT Assay (Tetrazolium Dye Reduction)

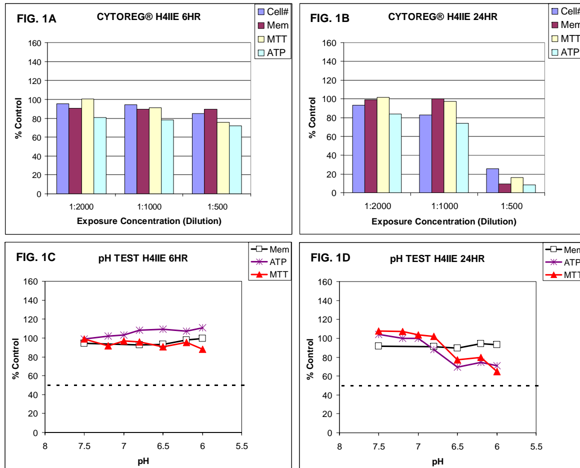
Cells in 96-well plates were evaluated for their ability to reduce soluble-MTT (yellow), (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide) to formazan-MTT (purple). Viable cells have the greatest amount of MTT reduction and hence the highest absorbance readings. The reduction of MTT has been linked to 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.

Adenosine Triphosphate (ATP)

Cellular Adenosine triphosphate (ATP) was determined using a luciferase-based assay. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

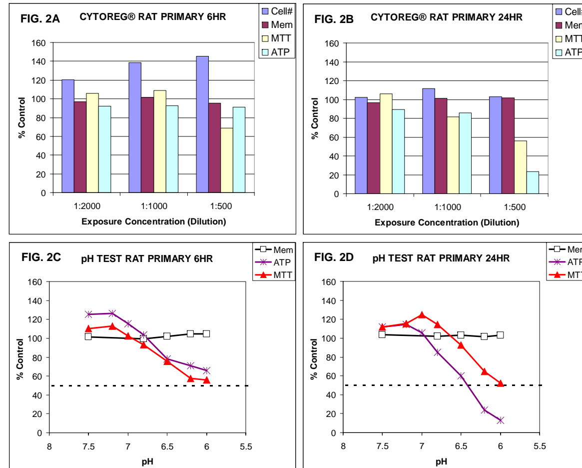
RESULTS

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



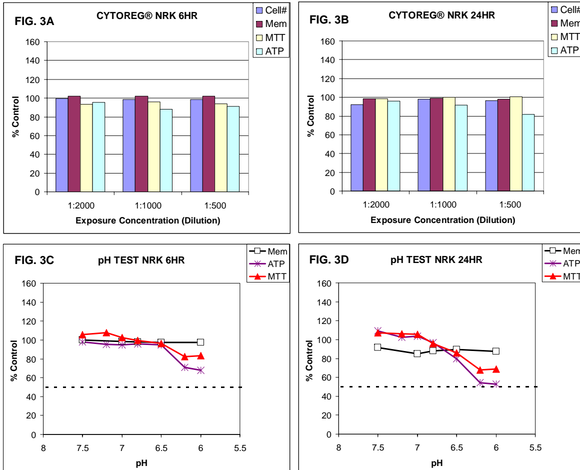
Following exposure to Cytoreg® (Fig. 1A, 1B), toxicity was measured at 6 and 24 hr in the H4IIE cells. Cell death was observed at 24 hr at the 1:500 dilution which was near physiologic pH (pH=7.2). The effect of pH on cell health at 6 and 24 hr (Fig. 1C, 1D) showed no toxicity at or above pH 6.8. Effects on the mitochondrial markers, MTT and ATP, were observed at pH ≤ 6.5 at 24 hr (Fig. 1D). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

FIGURE 2: Effects of Cytoreg® and pH on Rat Primary Hepatocytes at 6 and 24 Hr Exposures



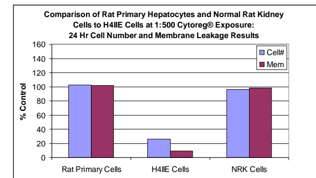
Following exposure to Cytoreg® (Fig. 2A, 2B), toxicity was measured at 6 and 24 hr in rat primary hepatocytes. Cell death was not observed at the 1:500 dilution (as observed in the H4IIE cells), however there were substantial reductions in the ATP and MTT markers at 24 hr. The effect of pH on cell health at 6 and 24 hr (Fig. 2C, 2D) showed no toxicity at or above pH 6.8. Effects on the mitochondrial markers, MTT and ATP, were observed at pH ≤ 6.5 at both time points, with the effects being more pronounced at 24 hr (Fig. 2D). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

FIGURE 3: Effects of Cytoreg® and pH on NRK Cells at 6 and 24 Hr Exposures



Following exposure to Cytoreg® (Fig. 3A, 3B), toxicity was measured at 6 and 24 hr in the NRK cells. Cell death was not observed at the 1:500 dilution (as observed in the H4IIE cells) and toxicity profiles were similar at both time points. The effect of pH on cell health 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 ≤ 6.2 at both time points, with the effects being more pronounced at 24 hr (Fig. 3D). Error bars not shown for clarity (CV% < 20%; n=3-7 replicates per data point).

FIGURE 4: Comparison of Cell Death Markers in Three Cell Types at 24 Hr Exposures



Following exposure to Cytoreg®, toxicity was measured at 24 hr using the cell viability markers, cell proliferation and membrane leakage. At the 1:500 dilution, the immortalized tumor cell line (H4IIE) showed a marked decrease in cell viability. No toxicity was observed in the rat primary hepatocytes and normal rat kidney (NRK) cells at the same exposure concentration. This 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).

Table 1: pH of Dosing Solutions in Cell Culture Medium Buffered with 25 mM HEPES

Exposure Conc. Dilution	Compound Concentration	pH
1:1	High	< 3
1:10		< 3
1:50		< 3
1:100		3.7
1:500		7.2
1:1000		7.2
1:2000	Low	7.3

The pH of the dosing solutions was measured at each exposure concentration tested. The lowest exposure concentrations (1:2000, 1:1000, 1:500) were very close to physiologic pH.

SUMMARY

Rat Hepatoma Derived Cells (H4IIE): Tumor Cell Line

- Cytoreg® was cytotoxic (>90%) at the 1:500 exposure concentration.
- Apoptosis was observed at the 1:500 dilution at 24 hr (data not shown).
- Near-physiologic pH (7.2) was maintained at low exposures (up to 1:500 dilution).

Normal Rat Primary Hepatocytes: Non-Tumor Cells

- Cytoreg® was not cytotoxic up to and including the 1:500 exposure concentration but did show a decrease in the mitochondrial markers, MTT and ATP at the 1:500 dilution.
- Slight apoptosis was observed at the 1:500 dilution at 24 hr (data not shown).
- Near-physiologic pH (7.2) was maintained at low exposures (up to 1:500 dilution).

Normal Rat Kidney (NRK): Non-Tumor, Non-Liver Cell Line

- Cytoreg® was not cytotoxic up to and including the 1:500 exposure concentration.
- No effects were observed on the mitochondrial markers, MTT and ATP.
- Apoptosis was observed at higher exposure concentrations at 24 hr (data not shown).
- Near-physiologic pH (7.2) was maintained at low exposures (up to 1:500 dilution).

Comparison of Cell Death Markers between Cell Types

- Cytoreg® was cytotoxic at 24 hr to the tumor cell line at a physiological-relevant pH of 7.2 and at an exposure concentration of 1:500 dilution. The non-tumor cells (both liver and kidney) did not demonstrate the same cytotoxicity. These effects occurred prior to any cytotoxic effect related to low pH .

CONCLUSIONS

The anti-tumor drug known as Cytoreg® was evaluated for cytotoxicity using three cell types: a rat hepatoma-derived cell line (H4IIE), a normal non-tumor liver cell type (rat primary hepatocyte), and an immortalized cell line not derived tumor cells (Normal Rat Kidney, NRK).

The Cytoreg® was found to be cytotoxic to the tumor cells at the 1:500 exposure dilution at 24 hr exposure. This was at near-physiologic pH of 7.2 and showed almost maximal reduction in the markers of cell viability.

The Cytoreg® and pH experiments showed the cytotoxicity of Cytoreg® to be due to the chemical mixture of the drug and not to the physical chemical changes conferred onto the test system. At higher exposures, the pH dropped to levels below 4 and therefore the effect on the cells at this extremely acidic pH could not be differentiated from the desired effect of the drug itself (data not shown).

In contrast, the non-tumor cells did not produce cytotoxicity at the 1:500 exposure dilution, and the pH (7.2) was physiologically relevant.

Summary

- These data indicate that the tumor cell line under conditions of near-physiologic pH was considerably more sensitive to the cytotoxic effects of Cytoreg® than were the non-tumor cells.
- The results of these experiments indicate that the Cytoreg® had a high specificity and was selective for tumor cells at exposure dilutions within range of physiologic pH.
- These data demonstrate the value of running *in vitro* toxicity assays on new oncology drug candidates in multiple cell types with multiple biochemical endpoints.

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