Wortmannin Blockade of the Lethal Effects of 5-Fluoro-2'-Deoxyuridine: The Effects of the Addition Time of Wortmannin

Dahl Clark

Department of Pharmacology

University of North Carolina at Chapel Hill School of Medicine

School of Science and Mathematics, Durham, NC

Research Apprenticeship Program

August 1, 1998

Advisor: Dr. Barry Goz, Professor, Department of Pharmacology

University of North Carolina at Chapel Hill

Introduction

Anticancer drugs function by inhibiting a crucial step in the metabolism or reproduction of cancer cells. The anticancer drug 5-fluoro-2'-deoxyuridine (FdUrd) kills cancer cells through several pathways, but the one most documented is the inhibition of the thymidylate synthase enzyme which prevents the formation of thymidylate, an essential precursor for DNA. Through an unknown process, most cancer cells recognize that they can no longer produce DNA and undergo programmed cell death, or apoptosis, as a result. If another drug, wortmannin, is administered to FdUrd-treated Chinese Hamster ovary (CHO) AA8 cells at certain early time periods, it has been shown to decrease the frequency of apoptotic cells. At other later times, wortmannin has little or no effect of apoptosis. Because of this, we believe that wortmannin may be inhibiting the cell's death signal at a point in the apoptotic pathway that is sensitive to wortmannin. If the steps of the apoptotic pathway are known, a greater understanding of the processes that occur to bring about cancer cell death and how it may be more easily killed will be gained.

The goal of these experiments was to determine how the antiapoptotic effects of wortmannin on FdUrd-treated cells change over a 24-hour period. The experiments began with adding wortmannin to flasks at 0, 2, 4, 8, 16, or 20 hours after the start of FdUrd treatment. All flasks were incubated for 24 hours before they were removed and prepared for counting. Control, FdUrd-only, and wortmannin-only flasks were incubated in parallel. At this time, the cells, for measurement of viability, were transferred to 75 cm^2 flasks. At 7-9 days, the flasks were removed and the percent control plating efficiencies were determined for each of the addition times of wortmannin. Wortmannin decreased the frequency of apoptotic cells up until approximately four hours after the start of FdUrd treatment. The time at which wortmannin ceases to inhibit the wortmannin-sensitive apoptotic step was somewhere within the time interval of 4-8 hours after the start of FdUrd treatment.

Materials and Methods

Cell Line

The AA8 line of Chinese Hamster ovary (CHO) cells were used. They were grown in 25 cm^2 culture flasks and later placed in 75 cm^2 flasks to form colonies.

Preparing growth medium

Growth medium was made by adding 50 mL of 10x Minimum Essential Medium (GIBCO BRL, NY) to 450 mL of distilled H_2O) to make 500 mL of total solution. Added to H_2O and MEM solution was 25 mL of fetal bovine serum and 14.5 mL of 7.5% $NaHCO_3$ solution. Glutamine, non-essential amino acids, and sodium pyruvate were added immediately prior to use to a bottle of solution heated in a 37°C water bath for 5 minutes.

Preparing cells for counting

A 25 cm^2 flask was removed from the incubator, and under sterile conditions, the used medium was poured off into a sterile waste bottle. 5 mL of phosphate-buffered solution (PBS) was pipetted into the flask with a 10 mL pipette, the cells were rinsed with the PBS to remove remaining used medium, and the PBS was poured off into a waste bottle. Next, 2 mL ethylenediaminetetraacetate (EDTA) in PBS was pipetted into the flask using a 5 mL pipette to chelate magnesium and calcium, causing cells to detach from the flask. The cells were incubated for 4-5 minutes to aid chelation, then the flask was tapped vigorously to detach cells into suspension. The suspension was pipetted up and down to break cell clumps and was pipetted into a 100 mL bottle containing 5 mL medium. To distribute cells in the medium and to wash cells from the pipette, the suspension was pipetted up and down. About 0.1 mL of the suspension was removed for counting with a hemacytometer.

To determine cell count after treatment with FdUrd and/or wortmannin, small sterile tubes containing 0.5 mL medium each were appropriately labeled. After used medium had been poured off, 5 mL PBS added, 2.5 mL EDTA added sequentially, the flasks were put into the incubator for 4-5 minutes. The cells were tapped off into the flask. The suspension was pipetted up and down to mix the cells and 0.5 mL cell suspension was pipetted into the corresponding labeled tube.

Seeding the 25 cm^2 Flasks

After the cells have been counted, enough of the cell suspension that was needed to reach seeding density (5 x 10^3 cells per flask) was pipetted out of the bottle under sterile conditions and placed into 100 mL of medium that had been heated to 37°C in a water bath. Five mL of cell suspension was pipetted into each flask. The flasks were then incubated overnight.

Adding FdUrd to Cells

A day after placing the cells in the incubator, 0.1 mL of 10 μM FdUrd was added to each flask for a final concentration of 200 nm. The flasks were put into the incubator for 24 hours. After 24 hours, the cells were taken out, counted, and plated for colony formation. The concentration of FdUrd present in each flask was calculated to be

$$\frac{0.1 \text{ mL FdUrd in flask} \times 10\mu\text{M FdUrd}}{5 \text{ mL cell suspension}} = 0.2\ \mu\text{M} = 200 \text{ nM}$$

Adding Wortmannin to Cells

5 μL of 10 mM wortmannin solution was pipetted with a micropipet into each flask requiring the addition of wortmannin at a certain time. The concentration of wortmannin in each of the flasks was calculated to be

$$\frac{5\ \mu L \times 10\ mM}{5\ mL} = 1 \times 10^{-5}\ M = 10 \mu M$$

Preparing 75 cm^2 Flasks for Colonies

For each 25 cm^2 flask, a 75 cm^2 flask was prepared by adding 25 mL of medium. An appropriate number of cells was added to the flasks which were incubated for 7-9 days to form colonies.

Discussion

The goal of these experiments was to determine how the antiapoptotic effects of wortmannin changes over a 24-hour period, for the purpose of determining when the step inhibited by wortmannin occurs. It had been shown earlier (Goz, unpublished data) that wortmannin's effects begin to decrease sometime between 4 and 16 hours in SV28 cells. The results of these experiments have narrowed the time frame to somewhere between approximately 4 and 8 hours, and further experiments using time points in between will be needed to determine the exact time when wortmannin's antiapoptotic effects begin to drop sharply. The greatest drop in percent control plating efficiency was found during the time interval of 4-8 hours, which leads us to believe there may be a point between 4 and 8 hours after the start of FdUrd treatment when the step in the apoptotic pathway that is sensitive to wortmannin ceases to be inhibited by the drug because it has already taken place.