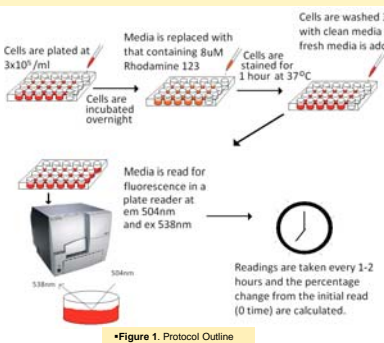


## Introduction

Multiple drug resistance is characterized by overexpression of membrane transport proteins ABCG2 and ABCB1 that induce efflux of anticancer drugs and subsequent tumor cell resistance. Overcoming MDR has become a major challenge for chemotherapy protocols. We have developed a high-throughput assay system based upon measurement of efflux of the fluorescent dye rhodamine 123 (R123) that is known to bind to the MDR transporters ABCG2 and ABCB1 in a manner similar to many chemotherapeutic drugs. A protocol for monitoring MDR in tumor cell lines has been developed using the MES-SA (control) and MES-SA/DX5 (MDR) human uterine sarcoma cell lines in which the cells are loaded with the fluorescent dye (R123) and the active transport of the dye out of the cells (efflux) is measured.

To stain cells, R123 (M0542)(50 to 200 ng/mL) was added to cell media, where it accumulates in the cytosol and mitochondria. Once cells reached a steady state level of R123, verapamil or cyclosporin A (known efflux inhibitors) were added, the cells washed, lysed and fluorescence measured using excitation at 505 nm and reading emission at 534nm. Cells exhibiting multi-drug resistance showed rapid loss of R123, while control cells exhibited higher retention of the dye. The rate of loss from individual cells was examined using flow cytometry and automated microplate systems suitable for high throughput operation. This kinetic data was also compared with efflux rates for the calcein AM and Hoechst 33342 probes as well as with data from previous studies using various tumor cell types available in the literature.



## Methods

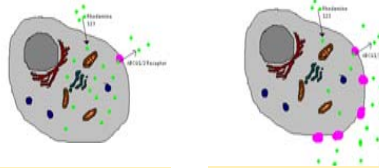
### Cell Preparation

- MES-SA and Mes-SA/DX5 cells were cultured in 100mm tissue culture dishes until 80-90% confluent.
- The cells were harvested using EDTA solution.
- The plates was then returned to the incubator for 1 hour.
- After an hour the staining media was aspirated and the cells washed 3 times with media. 500ul of fresh media was added to the cells.

### Rhodamine 123 Efflux Assay

- Staining media was prepared by diluting 1mg/ml stock solution of Rhodamine 123 in media to a final concentration of 8uM
- The media was aspirated from the cells and replaced with 500ul/well of staining media, with 3 wells per cell line left unstained as blanks.
- The plates was then returned to the incubator for 1 hour.
- After an hour the staining media was aspirated and the cells washed 3 times with media. 500ul of fresh media was added to the cells.
- The fluorescence of the media was immediately read on a Biotek Synergy MX plate reader at em 504, ex 538 to give a reading at zero time
- The plate was then incubated in the reader at 37°C and the fluorescence read at 1-2 hour intervals for 8-24 hours.
- The percentage increase in efflux was calculated for each timepoint using the formula:  $[(\text{[n-time well-time blank]} / (\text{[0-time well]-time blank})) \times 100]$
- The data was plotted on a bar graph and differenced between Mes-SA and Mes-SA/DX5 observed.

## Mechanism of Multiple Drug Resistance



## Structures

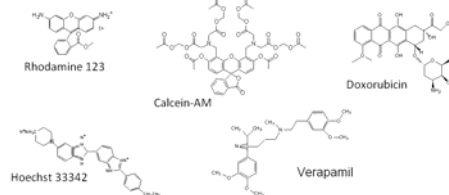


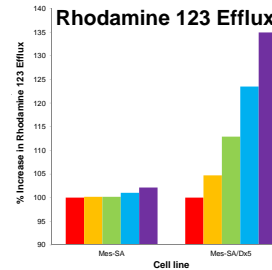
Figure 4. Structures of Compounds used in assay

## Effect of Doxorubicin on cells



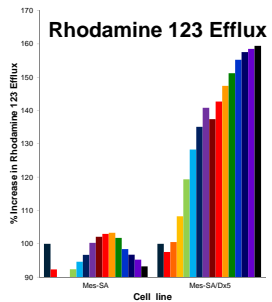
- Figure 5. To ensure the resistant cell line was indeed resistant the cells were treated with the drug Doxorubicin at varying concentrations
- The Doxorubicin was applied to the cells for 2 hours then removed the cells washed and fresh media added.
- After 48 hours the cells were examined and photographed
- The parental cells show a high level of cell death where the resistant line shows virtually no effect from the drug.

## Measuring the Efflux of Rhodamine 123 over time



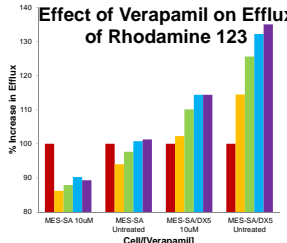
**Figure 6.** The assay was carried out as described in the method with readings taken every hour for 4 hours. At this time the difference in the amount of efflux between the 2 cell lines was already marked. The Mes-SA/DX5 cells pumped over 30% more Rhodamine 123 out compared to the Mes-SA cells.

## Measuring the Efflux of Rhodamine 123 over increased time



**Figure 7.** The experiment was then repeated but allowed to run for a longer period, with the measurement intervals being increased to 2 hours after 7 hours of reading. The longer time period shows an even greater difference between the levels of efflux of the two cell lines.

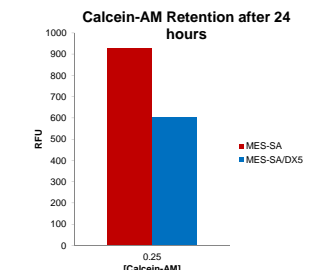
## Determining the Effect of Verapamil on Rhodamine 123 efflux into Culture Media



**Figure 8.** Cells were stained with 8uM Rhodamine 123 for 1 hour

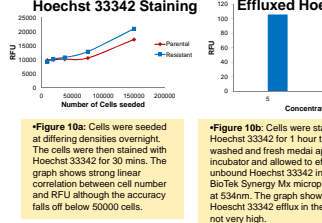
- The staining media was then washed off and replaced with either normal media or that containing 10uM Verapamil
- The fluorescence was then immediately read and the cells allowed to efflux with regular fluorescence readings being taken
- The increase in efflux for each was then calculated and plotted on the graph.
- The data shows that treatment with verapamil does reduce the efflux of Rhodamine 123 out of the MES-SA/DX5 cells although there is still a greater level of efflux than the Mes-SA cells.
- This shows that this assay is useful in measuring the effects of modulators of MDR.

## Retention of Calcein-AM



**Figure 9.** The cells were stained with Calcein-AM at 0.25uM for 15 minutes. The staining media was removed, the cells washed and fresh media added. The cells were then returned to the incubator for 24 hours. The amount of Calcein-AM retained in the cells was measured by bottom reading fluorescence at ex 494nm/em 517nm on a Biotek MX Synergy microplate reader. The difference in the amount retained between the 2 cell lines was noticeable but less than a 2 fold difference.

## Efflux of Hoechst 33342



## Discussion and Conclusions

In order to develop a high-throughput microtiter assay for multiple drug resistance (MDR), we developed a protocol for monitoring MDR in tumor cell lines using the MES-SA (parental) and MES-SA/DX5 (resistant) human uterine sarcoma cell lines in which the cells are loaded with the fluorescent dye (R123) and the active transport of the dye out of the cells (efflux) is measured.

Loading efficiency for the two cell lines, and monitoring cell numbers after loading were found to be important parameters for quantitation of efflux mechanisms. This was overcome by taking a reading of each well at the beginning of the measurement period and using this as the reference against which other measurements were compared. This meant that any slight differences in seeding between wells or differences in Rhodamine 123 uptake were removed and a true comparison of the two cell lines was possible.

The efflux of the two cell lines is compared the MES-SA cells show practically no increase in efflux over the course of the measurement whereas the MES-SA/DX5 cells show more than 60% increase in efflux compared to the start of the experiment. This increase shows that these cells do indeed have an overexpression of membrane transport proteins ABCG2 and ABCB1 that induce efflux of anticancer drugs and subsequent tumor cell resistance and that this can be easily determined by a high throughput assay. The use of Rhodamine as a measure of MDR holds up well when compared with Calcein-AM and Hoechst 33342.

The assay can also be used to measure the effect of compounds as modulators of Multiple drug resistance as effects of drug resistance in cells would have major clinical impact.

This assay has many advantages over assays involving drug treatment as it has a short, labor minimal setup and can be run overnight, equipment permitting, to allow rapid generation of results in a busy high throughput format.

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