

Abstract

Malignant mesothelioma (MM) is a form of cancer originating in the mesothelium that is almost always caused by exposure to asbestos. Microarrays have previously demonstrated that MM cells had changes in gene expression indicative of enhanced production of reactive oxygen species, such as NADPH Oxidase 4 (Nox4). The continual presence of ROS results in the increase and duration of pro-proliferative pathways, which lead to the up regulation of cell cycle genes. Different MM cell lines expressed isoforms of FoxM1 necessary for cell cycle transit in the absence of growth factors, yet were responsive to inhibitors of Nox4. Thiostrepton (TS) is a natural antibiotic, which has been shown to inhibit the oncogenic transcription factor FoxM1 and is cytotoxic to MM cells in a dose dependant fashion. Drugs elicit cytotoxic effects through several mechanisms. Drug accumulation in the mitochondria can result in a compromised membrane potential with a resultant disruption in electron transport. Lysosome perturbation has been shown to cause the formation of autophagosomes and autophagic cytopathology. Numerous compounds have been shown to initiate the apoptotic process in cells. Using CELLestial™ assay kits we have profiled the response of cells to thiostrepton. MM cells lines were exposed to TS along with agents known to perturb lysosomes, mitochondria, or induce apoptosis and their responses compared to normal LP9 cells. TS does not appear to induce apoptosis as measured by nuclear condensation in either normal or malignant cells. Interestingly marked differences between the normal LP9 cells and MM cells in the lysosomal response to thiostrepton are observed. To facilitate the process of cytotoxicity profiling of TS on mesothelioma cells, we have automated all of the fluid handling steps of these assays. These data and process steps serve as an example of how the cytotoxicity profile of other anticancer agents or potential drug compounds can be streamlined. Assay work flow along with performance results will be provided.

Introduction

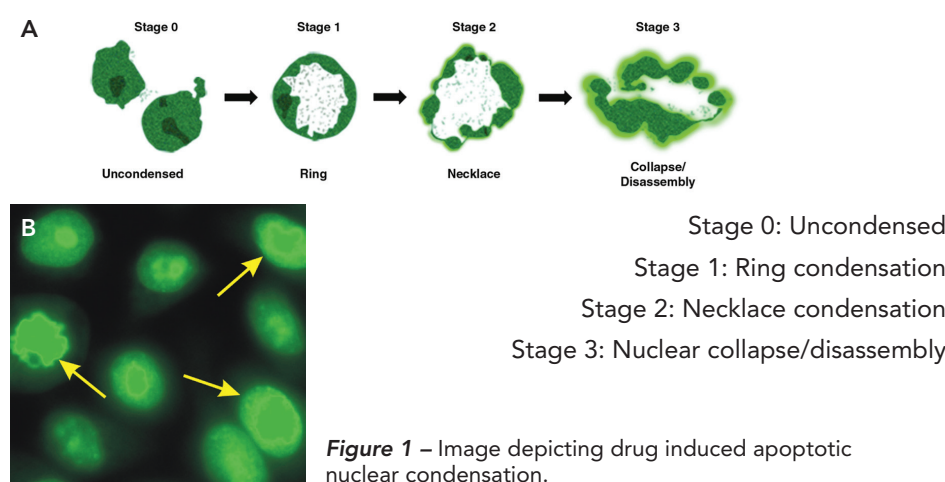
The identification of cytotoxic agents specific to tumor cells is a critical element of the drug discovery process for cancer chemotherapy. Once potential agents have been identified, the determination of their toxicity profile provides critical information in regards to their mechanism of action as well as indications of potential side effects of the drugs. Cytotoxic effects of drug molecules are often first observed as perturbations of normal cellular organelle functionality. For example, mitochondria, which play a central role in cellular oxidative respiration, can have a compromised membrane potential resulting from drug accumulation. This toxicity has been shown to contribute to the toxicity of various organs.

Cationic amphiphilic drug compounds can be absorbed by cells by simple diffusion and accumulate inside the acidic cellular organelles, a process referred to as lysosomotropism. Drug accumulation into subcellular organelles can lead to many undesirable effects [Ikeda et. al: *BBRC* **377**:268-274 (2008)]. Numerous cationic amphiphilic drugs are known to trigger phospholipidosis, which is typified by excessive intracellular accumulation of phospholipids as lamellar bodies [Anderson and Borlak; *FEBS Lett.* **580**:5533-5540 (2006)]. While the origins of these lamellar bodies remain unknown, this adaptive response appears to be generated by an autophagic or heterophagic processes.

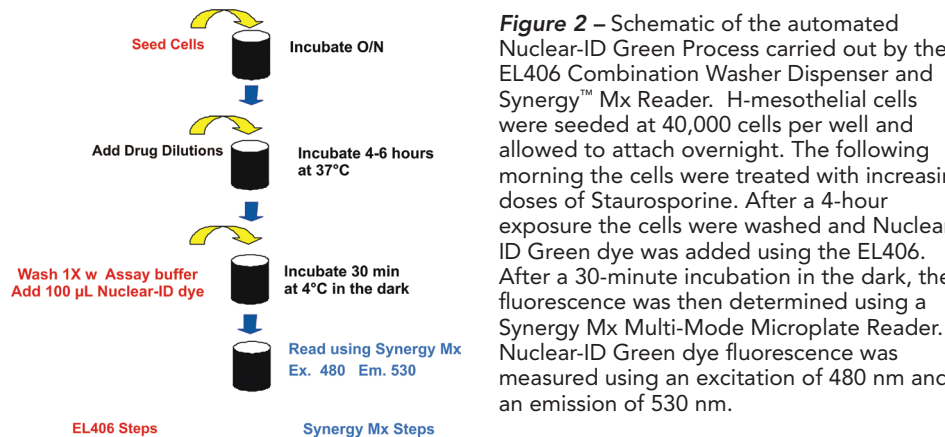
Apoptosis or programmed cell death can be caused by a number of different factors involving two basic pathways. The extrinsic pathway involves the “death” ligand binding to cell surface receptors or the induction by granzyme released from T-lymphocytes. [Stennicke and Salvesen; *BBA*. **1477**:299-306 (2000)]. The intrinsic pathway is initiated by cellular stress and generally involves changes to the mitochondria which results in an active apoptosome complex that activates caspase-9. [Stennicke and Salvesen; *Cell Death and Dif.* **6**:1054-1059 (1999)] During the process chromatin undergoes a phase change from a heterogeneous genetically active network to a highly condensed form that is subsequently fragmented and packaged into apoptotic bodies.

Using three different CELLestial cell cytotoxicity assays we profile the cytotoxic effects of thiostrepton on mesothelioma cells. In addition we will demonstrate the utility of the EL406™ Combination Washer Dispenser to automate the liquid handling steps required to perform routine compound toxicity testing.

CELLestial Nuclear-ID™ Green Assay



Apoptosis or programmed cell death can be caused by a number of different factors. During the process chromatin undergoes a phase change from a heterogeneous genetically active network to a highly condensed form that is subsequently fragmented and packaged into apoptotic bodies. The Nuclear-ID Green assay kit identifies cells in late stage apoptosis. The basis of which is that cells with condensed compacted chromatin will bind greater amounts of the dye as compared to healthy cells.



Nuclear Condensation

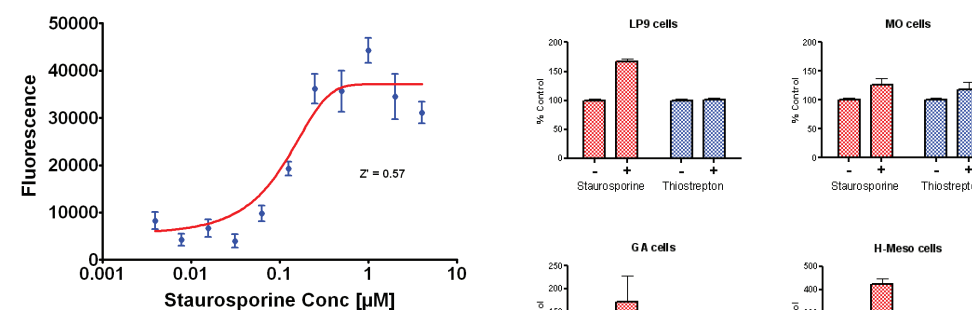
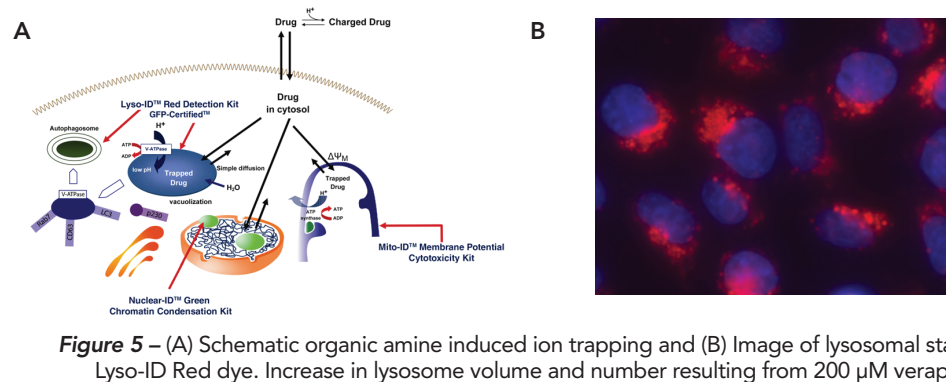


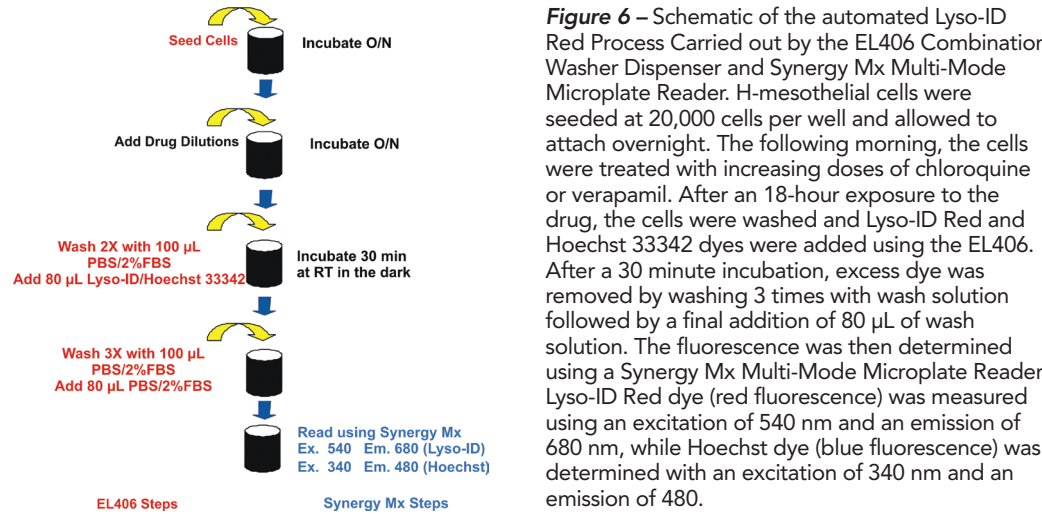
Figure 3 – Dose responsive change in nuclear condensation caused by staurosporine.

Figure 4 – Effect of staurosporine and thiostrepton on nuclear condensation in different mesothelioma cell lines.

CELLestial Lyso-ID® Red Assay



Many drugs cause an accumulation of phospholipids and lysosomes in the cytoplasm. For example, amioderone induces an abnormal accumulation of phospholipids that appear as vacuoles with multilamellar inclusions often referred to a autophagosomes. Other organic amines cause vacuolar-ATPase driven ion trapping, which has been associated with vacuolar and autophagic cytopathology. Lyso-ID Red is a fluorescent dye that accumulates in lysosomes. An increase in signal is indicative of an increase in the number or size of cellular lysosomes. In addition to the lysosomal specific dye the assay also uses Hoechst 33342 nuclear stain. A decrease of 30% or greater of the Hoechst signal is indicative of generalized cytotoxicity.



Increase in Lysosome Content

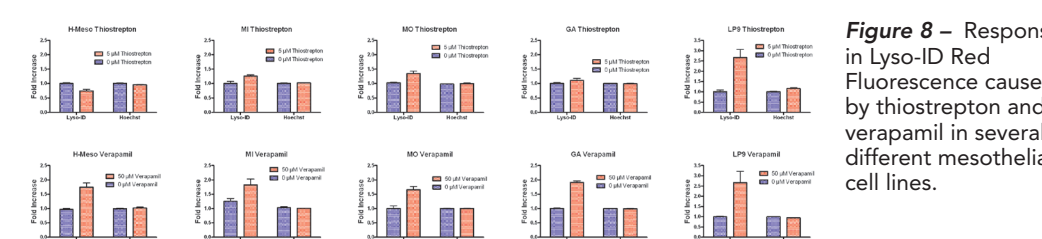
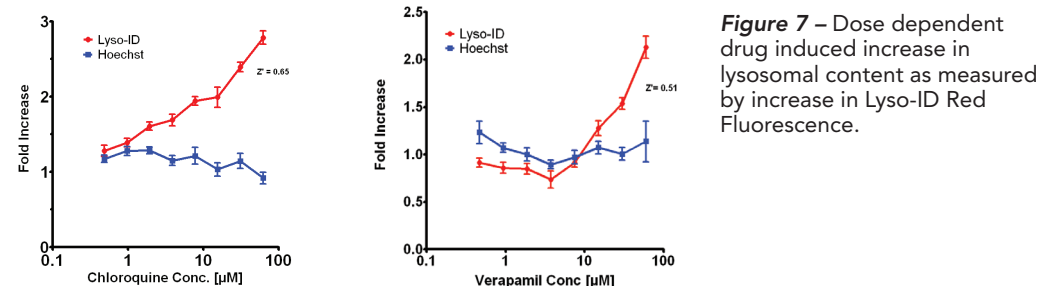
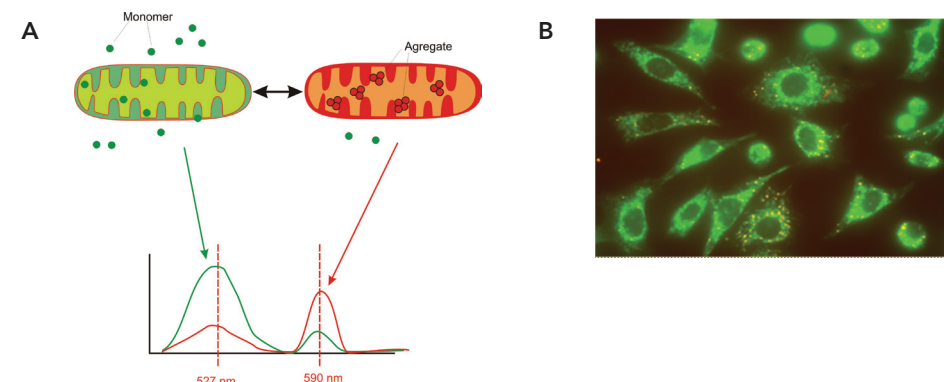
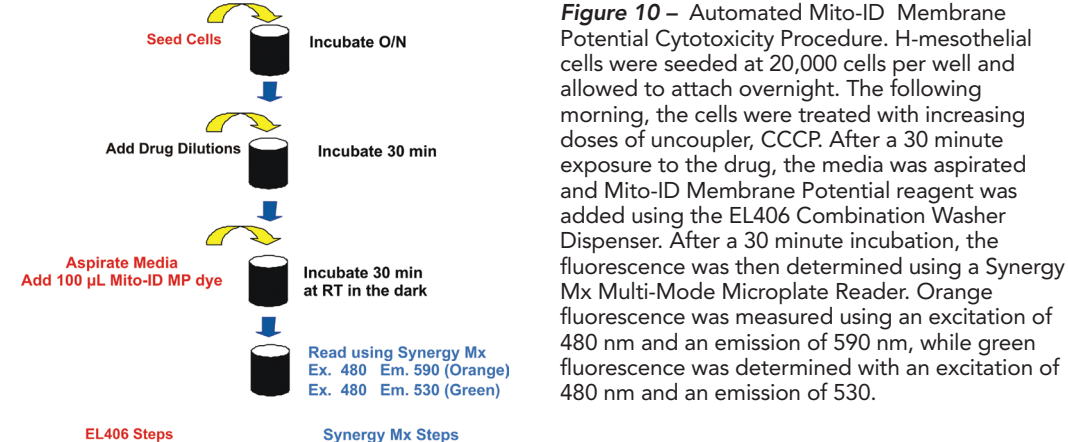


Figure 8 – Response in Lyso-ID Red Fluorescence caused by thiostrepton and verapamil in several different mesothelial cell lines.

CELLestial Mito-ID™ Membrane Potential Assay



Mitochondria play a central role in cellular oxidative respiration. Recently it has been discovered that compromised membrane potential caused by drug accumulation contributes to the toxicity of various organs. The Mito-ID Membrane Potential assay uses a cationic dye that accumulates in the cell cytosol as a monomer which primarily emits green fluorescence. In normal energized cells, the dye can also accumulate in the mitochondria as orange fluorescent J-aggregates. Mitochondrial damage or loss of membrane potential is indicated by a loss of orange fluorescence.



Loss of Membrane Potential

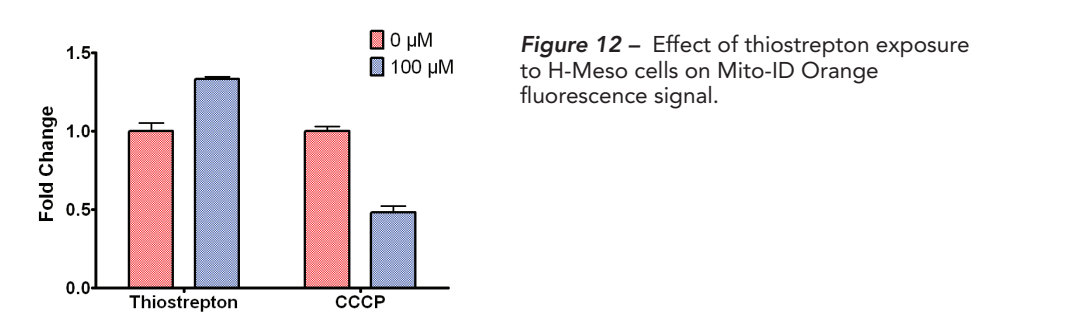
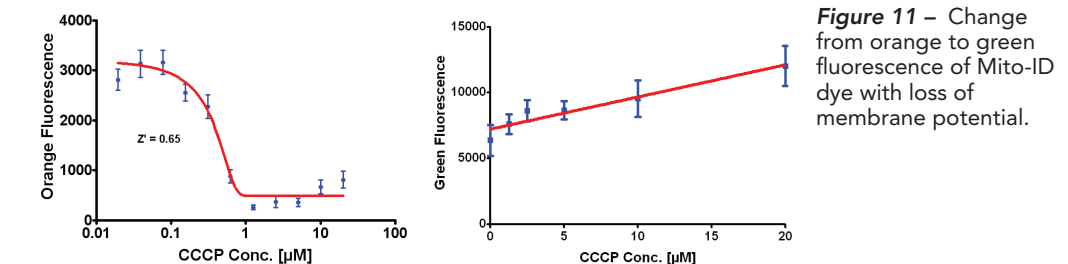


Figure 12 – Effect of thiostrepton exposure to H-Meso cells on Mito-ID Orange fluorescence signal.

BioTek Instrumentation



Figure 13 – EL406 Combination Washer Dispenser



Figure 14 – Synergy Mx Multi-Mode Microplate Reader

Conclusions

- Thiostrepton does not induce apoptosis as measured by nuclear condensation.
- Thiostrepton increases lysosomal volume in normal LP9 cells, but not malignant mesothelioma cell lines.
- Thiostrepton causes a slight increase in mitochondrial membrane potential in mesothelioma cell lines.
- CELLestial kits provide an easy means to screen compounds for potential cytotoxic effects.
- The described assays are rapid, sensitive and specific. They are also compatible with standard high-throughput microplate-based screening workflows.
- The EL406 Combination Washer Dispenser is capable of automating the different fluid handling steps of several different CELLestial assays.
- The Synergy Mx Multi-Mode Microplate Reader offers ease of use and wavelength flexibility for use in multiple assay determinations.
- Significant Z' values indicate excellent assay performance.