Application Note

Automated Drug-Induced Chromatin Condensation Detection

Detection of End-Stage Apoptosis Using the Nuclear-ID™ Kit from Enzo Life Sciences

Paul Held, BioTek Instruments, Winooski, VT
Kheng Newick, University of Vermont, Burlington, VT
Dee Shen and Wayne Patton, Enzo Life Sciences, Farmingdale, NY

Anticancer agents typically exhibit cytotoxic effects on tumor cells as a primary function. This occurs through several different mechanisms, such as apoptosis or programmed cell death. The specific mechanism through which they act can have important consequences in terms of efficacy, side effects and normal cell toxicity and can be discerned by a variety of assays. In this report we have examined thiostrepton and staurosporine with regards to the induction of apoptosis using the Nuclear-ID™ Green Chromatin Condensation Kit from Enzo Life Sciences. An automated procedure using the EL406™ Combination Washer Dispenser to automatically aspirate media, wash cells and dispense reagents has been developed.

Introduction

Apoptosis or programmed cell death can be caused by a number of different factors involving two basic pathways. The extrinsic pathway involves the binding of "death inducing ligands" to cell surface receptors or the induction by cytotoxic T-lymphocytes by granzyme. This pathway results in the activation of Caspase-8 [1]. The intrinsic pathway is initiated by cellular stress and generally involves changes to the mitochondria that release Cytochrome c, which interacts with Apaf-1, dATP and multiple molecules of pro-caspase-9 to generate an active apoptosome complex that activates caspase-9. [2] 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.

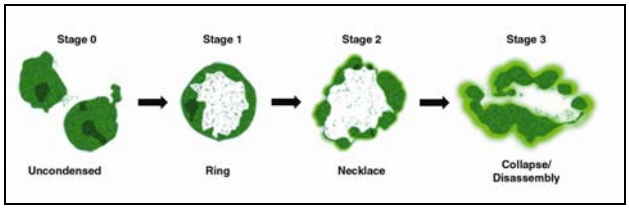

Figure 1. Stages of Apoptotic Nuclear Condensation.

Using different cell extracts in a cell-free system, three stages of apoptotic chromatin condensation have been defined based upon morphological and biochemical criteria such as: Stage 1, Ring condensation; stage 2, Necklace condensation; and stage 3, nuclear collapse/disassembly [3].

Interestingly, electron microscopy has revealed that neither chromatin nor subnuclear structures were present in the ring-condensed structures. Stage 2 or necklace condensation required DNase activity, while stage 1 did not. Stage 3 required hydrolyzable ATP, while the other stages did not [3].

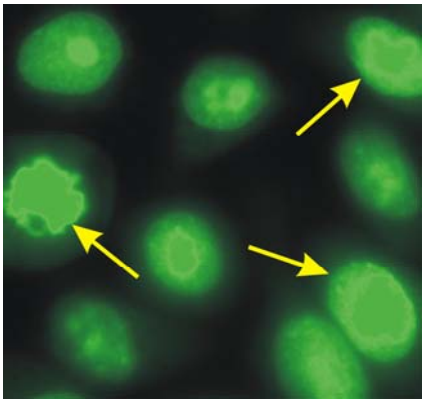

Figure 2. Image depicting drug induced apoptotic nuclear condensation. Arrows indicate cells with condensed nuclei.

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 (Figure 2).

Materials and Methods

H-Mesothelioma (H-Meso) cells were seeded at 40,000 cells per well and allowed to attach overnight. The following morning the cells were treated with increasing doses of staurosporine or thiostrepton. After a 4-hour exposure the cells were washed and Nuclear-ID™ Green dye was added using the EL406. After a 30-minute incubation in the dark, the fluorescence was then determined using a Synergy Mx reader. Nuclear-ID™ Green dye fluorescence was measured using a Synergy Mx (BioTek Instruments) with an excitation of 480 nm and an emission of 530 nm.

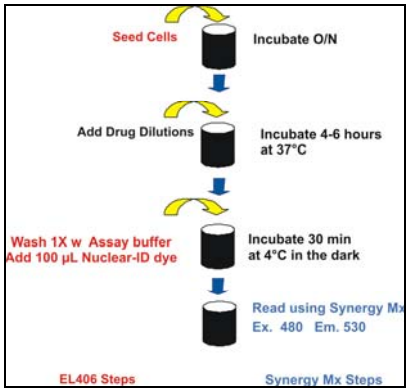

Figure 3. Schematic of the Automated Nuclear-ID™ Green process carried out by the EL406 Combination Washer Dispenser and Synergy Mx Reader.

Instrumentation

The EL406 offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used to remove media, as well as dispense reagents to the 96-well cell plates.

The Synergy™ Mx incorporates a quadruple monochromator system which selects wavelengths with a repeatability of plus or minus 0.2 nm. The optical head can focus up and down on samples with a 100 µm resolution. It also uses a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. The ultra low noise digital photon integration system and high-quality optics ensure the highest sensitivity.

Results

Initial experiments tested staurosporine as a positive control for the induction of apoptosis in mesothelioma cells. Staurosporine is a non-specific protein kinase inhibitor capable of triggering both the morphological and intranucleosomal DNA fragmentation typical of apoptosis [4]. As seen in Figure 4, Staurosporine elicits a greater than 5-fold increase in Nuclear-ID green fluorescence with increasing dosages. A semi-log plot of the data demonstrates a classic sigmoidal dose response to the drug. The calculated EC_{50} for staurosporine with H-Meso cells was 0.13 µM. This compound can be used as a positive control to test the effect that the antibiotic thiostrepton may or may not have towards eliciting a similar response.

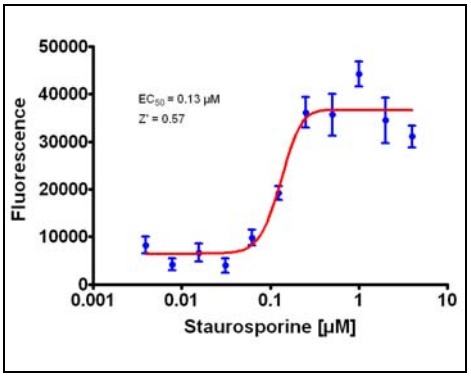

Figure 4. Dose responsive change in nuclear condensation caused by staurosporine.

When different mesothelioma cell lines are treated with 1 µM staurosporine similar, but less profound, responses as with h-meso cells are observed (Figure 5). Because the related compound Siomycin A has been shown to induce apoptosis in melanoma cancer cells we were interested in what if any effect thiostrepton would have on mesothelioma cells [6]. Unlike staurosporine, when cells are treated with 10 µM thiostrepton virtually no increase in Nuclear-ID™ staining was observed in 3 of 4 cell lines tested, while the remaining cell line showed a very modest statistically insignificant increase (Figure 5).

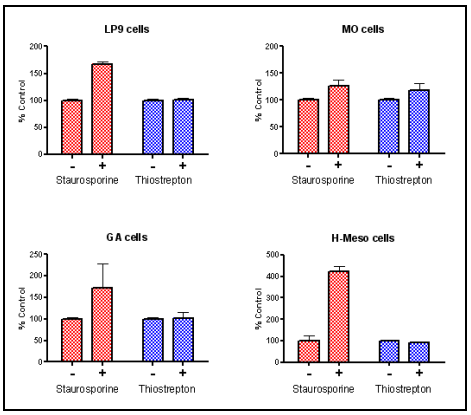

Figure 5. Comparison of thiostrepton and staurosporine induction of nuclear condensation as measured by Nuclear-ID™ staining.

Discussion

The determination of the mechanism by which anticancer compounds exhibit their cytoxicity tests provides important information regarding not only the drug in question, but also the biology of the cancer. These data indicate that the compound thiostrepton does not induce apoptotic nuclear condensation in mesothelioma cells. Several different tumor cell lines were tested with similar results in regard to thiostrepton. The mesothelioma cells are tumor cell lines that exhibit markedly different cellular growth patterns and gene expression profiles, but show little differences in their response to thiostrepton, but do respond as expected to staurosporine. Thiostrepton first isolated from bacteria in 1955 as a bacterial antibiotic has been reported to exhibit activity against breast cancer cells through targeting the transcription factor forkhead box M1 [5], but its exact mechanism of action is not completely understood.

Cell-based fluorescence assays typically involve the removal of media, the addition and removal of reagents, and the washing of cells. The EL406 Combination Washer Dispenser is an ideal platform to automate these tasks. The dual-action manifold is capable of rapid removal of media or wash buffer, while the peristaltic pump is capable of accurate delivery of sterile fluids, with relatively low dead volume. Both of these features were used in combination for these experiments.

The Nuclear-ID Chromatin Condensation kit is one of the CELLestial™ assays available from Enzo Life Sciences. These cell based assays allow drug compounds to be profiled in regards to their cytotoxicity; the ability to induce apoptosis being one of the parameters.

References

1. Stennicke, H.R. and G.S. Salvesen (2000) Caspases-Controlling Intracellular Signals by Protease Zymogen Activation, *Biochimica Biophysica Acta*, **1477**:299-306.

2. Stennicke, H.R. and G.S. Salvesen (1999) Catalytic Properties of the Caspases, *Cell Death and Differentiation*, **6:**1054-1059.

3. Toné S, Sugimoto K, Tanda K, Suda T, Uehira K, Kanouchi H, Samejima K, Minatogawa Y, Earnshaw WC. (2007) Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. Exp Cell Res. 2007 Oct 1; 313(16):3635-44.

4. Bertrand, R. E. Solary, P. O'Connor, K. W. Kohn and Y. Pommier (1994) Induction of a Common Pathway of Apoptosis by Staurosporine. Exp. Cell Res. 211:314-321.

5. Kwok JM, S.S. Myatt, C.M. Marson, R.C. Coombes, D. Constantinidou and E.W. Lam EW (2008). "Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression". *Mol. Cancer Ther.* **7** (7): 2022–32.

6. Bhat, U.G., P.A. Zipfel, D.S. Tyler, and A. L.Gartel (2008) Novel Anticancer Compounds Induce Apoptosis in Melanoma cells. Cell Cycle, 7:1851-1855.

Rev. 2/16/10, AN021610_04

RA44420.502650463

5994-2613EN
September 1, 2021