Multiparametric evaluation of apoptosis: Effects of standard cytotoxic agents and the cyanoguanidine CHS 828

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Abstract

A multiparametric high-content screening assay for measurement of apoptosis was developed. HeLa cells and lymphoma U-937 cells were exposed to cytotoxic drugs in flat-bottomed optical microtiter plates. After incubation, the DNA-binding dye Hoechst 33342, fluorescein-tagged probes that covalently bind active caspases and chloromethyl-X-rosamine to detect mitochondrial membrane potential (MMP) were added. Image acquisition and quantitative measurement of fluorescence in a defined number of cells per well was performed using the automated image capture and analysis instrument ArrayScan. The usefulness of the assay was tested in cells exposed to standard cytotoxic drugs as well as in experimental cytotoxic cyanoguanidine CHS 828. A time- and dose-dependent activation of caspase-3, decrease in MMP, and increase in nuclear fragmentation and condensation were observed for the standard drugs, with the ability to correlate the parameters on a single cell basis. CHS 828 induced caspase-3 activation and reduction in MMP with modest changes in nuclear morphology. The method described was considered to be a rapid and information-rich apoptosis assay suitable both for correlating morphological and biochemical apoptotic events in single cells as well as for screening and evaluation of novel substances with apoptosis-inducing capabilities. [Mol Cancer Ther 2004;3(5):521–6]

Introduction

The majority of anticancer drugs presently used in clinical setting have been described to induce cell death by apoptosis (1). Apoptosis has been subject to intense study and was originally described by Kerr *et al*. (2) as cell death with certain morphological characteristics. However, besides the morphological criteria, subsequent studies have described important biochemical hallmarks of apoptosis. One of the most important groups of proteins involved in apoptosis are the cystein aspartate-specific proteases also called caspases (3). Caspases are activated by different toxic stimuli via mainly two routes. Stimulation of death receptors can directly activate a cascade of caspase activation and subsequent activation of nucleases important for ordered nuclear degradation. Alternatively, caspases can be activated by changes in mitochondrial membrane integrity, with release of factors activating caspases (4). One method to study caspase activation in cells is to use fluorochrome-labeled inhibitors of caspases (FLICA) that covalently bind to the active site of activated caspases (5). Changes in nuclear morphology, mitochondrial membrane potential (MMP), and caspase activation are thus important markers for an ongoing apoptotic process in the cells.

The current focus of drug discovery in the area of oncology is to identify molecules important for apoptosis regulation in tumor cells (6). Thus, in the process of identifying and evaluating promising new anticancer agents, it is important to rapidly and easily identify substances with apoptosis-inducing properties. One approach to identify and evaluate substances that induce apoptosis would be to use automated information-rich image-based analysis, often called high-content screening (7). With this approach, one is able to measure fluorescence intensity and localization on a cell-by-cell basis. In this work, we describe a rapid and information-rich method for identifying individual cells or subpopulation of cells expressing one or more features attributable to apoptosis. We present evaluation of caspase-3 activity, MMP, and nuclear morphology in cells exposed to the novel cytotoxic drug CHS 828 (8) and a number of experimental and clinically used drugs.

Materials and Methods

Cell Culture and Growth Conditions

The human U-937 lymphoma cell line (9) and the cervical adenocarcinoma HeLa cell line (10) were grown in RPMI 1640 and MEM Eagle, respectively. Both media were supplemented with 10% heat inactivated FCS, 2 mM glutamine, 50 μg/ml streptomycin, and 60 μg/ml penicillin (all from Sigma Aldrich, St. Louis, MO). Cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells in log growing phase were used for all experiments. The cell lines were subcultivated and checked for growth characteristics and morphology twice a week.

Chemicals

CHS 828 was obtained from LEO Pharmaceutical Products, Ballerup, Denmark, and a stock solution of 10 mM was prepared in DMSO. Staurosporine (Sigma Aldrich) was dissolved in DMSO. Standard drugs were obtained

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from commercial sources and were dissolved according to the manufacturers' instructions. The FLICA probes, FAM-VAD-FMK for measurement of pan-caspase activity and FAM-DEVD-FMK for measurement of caspase-3 and partly caspase-7, were supplied as part of the CaspaTag kit (Serologicals) dissolved in DMSO and diluted in PBS. Chloromethyl-X-rosamine (MitoTracker Red CMXRos, Molecular Probes, Eugene, OR) was dissolved in DMSO and further diluted in +PBS. Hoechst 33342 (Sigma Aldrich) was dissolved in water.

Apoptosis Induction

HeLa cells were plated in 96-well plates with flat optical bottom (Perkin-Elmer Inc., Wellesley, MA), and left to attach overnight before addition of drugs. For U-937 cells, drugs were added immediately after plating. Number of cells seeded per well was determined by the proliferation rate and the optimal number of cells per well for apoptosis analysis. Plates were incubated at the culture conditions indicated above.

Cell Staining

One hour before the end of drug exposure, FAM-VAD-FMK or FAM-DEVD-FMK was added at a final concentration of 20 μM to stain cells with activated caspases. MitoTracker Red was added during the last 30 min of exposure at a final concentration of 100 nM to evaluate MMP. The staining solutions were removed and the plates were washed twice with PBS followed by 10 min fixation with 1% paraformaldehyde and 10 μM Hoechst 33342. Plates were then washed twice. All plate washing was performed with a Multiwash plate washer (Dynatec Laboratories, Chantilly, VA). Plates were centrifuged before each aspiration to avoid loss of cells growing in suspension or cells detached due to toxic stimuli. Processed plates were kept at +4°C for up to 48 h before analysis.

Image Acquisition and Cytometric Analysis

Plates with stained and fixed cells were analyzed using the ArrayScan high-content screening system (Cellomics Inc., Pittsburgh, PA). This system is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. Images were acquired for each fluorescence channel, Hoechst 33342, FLICA, and MitoTracker Red using suitable filters with a 10× (for screening runs) or 20× objective as indicated. In each well, 500–1000 cells were analyzed unless otherwise indicated. Automatic focusing was performed in the nuclear channel to ensure focusing regardless of staining intensities in the other channels. The value for characterizing morphology is influenced both by increased Hoechst 33342 staining intensity as well as the variability in the staining. A condensed or fragmented nuclei thus results in a high value. Images and data regarding intensity and texture of the fluorescence within each cell, as well as the average fluorescence of the cell population within the well, were stored in a Microsoft SQL database for easy retrieval.

Statistics

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Log EC_{50} calculations were performed using the built-in algorithms for dose-response curves with variable slope. A fixed maximum value of the dose-response curve was set to the maximum obtained value for each drug. To evaluate the usefulness of assay in a screening setting, the Z'-value was calculated according to Zhang *et al*. (11) using the following equation:

$$Z' = 1 - [(3SD_{poscontrol} + 3SD_{negcontrol})/(Mean_{poscontrol} - Mean_{negcontrol})]$$

Results

Method Development

HeLa cells exposed to vehicle (PBS) or 0.4 μM staurosporine, chosen as a model substance, for 6 h are shown in Fig. 1, A and B, respectively. Images acquired in the nuclear channel (*left*) and the FAM-VAD-FMK channel (*right*) are shown. On the basis of the nuclear staining, the area of interest in the second channel, FAM-VAD-FMK, was identified by expanding the area by two pixels. This expansion was necessary to cover parts of the cytoplasmic regions of the cells. As an estimate of nuclear fragmentation and condensation, the value of Hoechst 33342 staining variability within each nuclei was calculated. As a measure of pan-caspase activity within cells, the average fluorescence intensity within the object's mask was calculated. It should be noted that only cells with condensed or fragmented nuclei stain positive for FAM-VAD-FMK. Single cell plots are shown in Fig. 1C. In cells exposed to vehicle, there was a low proportion of spontaneous apoptosis, that is, cells with fragmented or condensed nuclei and FAM-VAD-FMK-positive staining, whereas in cells exposed to staurosporine for 6 h, there was a dose-dependent increase in morphological changes as well as an increase in pan-caspase activity. Data can also be presented on the whole cell population within the wells. In Fig. 1D, the mean average FAM-VAD-FMK intensity of 1000 cells/well is expressed. Exposure of HeLa cells to 10 μM staurosporine for 4 and 6 h produced a rapid and pronounced pan-caspase activation. The pan-caspase inhibitor z-VAD-FMK reduced the FAM-VAD-FMK staining of the cells substantially. Dose-response curves generated for pan-caspase activation and changes in nuclear morphology in HeLa cells after exposure to staurosporine are presented in Fig. 1E. The log EC_{50} value for pan-caspase activation in this setting was 0.31 ± 0.2 μM (SE). Intra-day variability between multiple wells was generally low. Analysis of triplicate wells of HeLa cells exposed to 10 μM staurosporine for 6 h resulted in an average intra-day coefficient of variation (CV) of 16% ($n = 5$). Inter-day CV with the same conditions was 18% ($n = 5$).

Suitability for Screening for Caspase Activity

The dynamic range of the assay signal and the variability of the signal in negative and positive controls are important for the ability to identify hits in a screening assay. HeLa cells exposed to vehicle or positive control, staurosporine

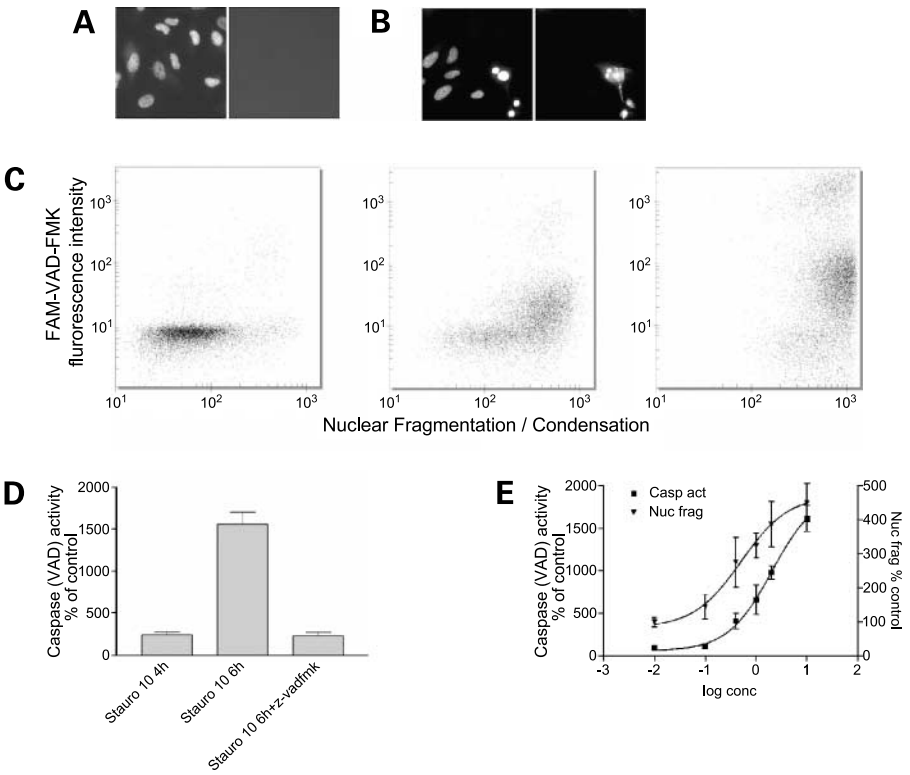

Figure 1. Measurement of caspase activation and nuclear fragmentation/condensation in HeLa cells. Cells exposed to vehicle (**A**) or 0.4 μM staurosporine for 6 h (**B**) are shown as an example of images acquired by the ArrayScan high-content screening system using 20× objective. Nuclear channel (*left*) and the FAM-VAD-FMK channel (*right*). **C,** single cell plots (10,000 cells per condition) of caspase activity and nuclear fragmentation/condensation for cells exposed to vehicle (*left*), staurosporine 0.4 μM (*middle*) or 10 μM (*right*). **D,** time-dependent activation of caspase activity in cells exposed to 10 μM staurosporine for 4 and 6 h as well as cells co-incubated with 50 μM z-VAD-FMK as percentage of untreated control. **E,** dose-dependent activation of caspase activity (*left axis*) and nuclear fragmentation/condensation (*right axis*). *Points,* mean of three independent experiments; *bars,* SE.

10 μM for 6 h, were stained with FAM-VAD-FMK and Hoechst 33342. Analysis of average FAM-VAD-FMK intensity in 1000 cells per well using 10× objective was performed. The average *Z′*-value was calculated to 0.56, indicating a high dynamic range with low variability. A plot of individual well signals from both negative and positive control (25 samples each) is shown in Fig. 2. Image acquisition and analysis was performed in 45 min per plate. With use of automated plate loading, this would correspond to a throughput of about 3000 substances per day using 96-well plates.

Multiparametric Apoptosis Analysis of CHS 828 and Some Standard Drugs

A number of clinically well-established and experimental anticancer drugs were analyzed for all three apoptosis parameters. U-937 cells exposed to etoposide showed a dose-dependent increase in fragmentation/condensation, caspase-3 activity, and a concomitant reduction in MMP (Fig. 3).

In Fig. 4, A–E, dose-response plots are shown for caspase-3 activity, MMP, and nuclear fragmentation and condensation. All standard drugs tested induced a high level of caspase-3 activity and changes in nuclear morphology. Exposure to staurosporine (Fig. 4A) and etoposide (Fig. 4B) induced a large reduction in MMP at 6 h. The modest reduction of MMP induced by vincristine (Fig. 4C) and melphalan (Fig. 4D) can be explained by disappearance of many late apoptotic cells that has taken place during the 24-h incubation. Apoptotic parameters are analyzed exclusively in cells with nuclei that are identifiable using the image analysis algorithm. Cells in very late stages of apoptosis are thus not included in the analysis.

The novel cyanoguanidine CHS 828 induced induction of caspase-3 activity, reduction in MMP, and changes in nuclear morphology at 72 h as indicated in Fig. 4E. The induction of caspase-3 activity occurred at very low concentrations with a log EC_{50} of -3.95 ± 0.79 μM (Table 1). In wells where cells were exposed to 1–10 μM CHS 828, the cell number was reduced by 80% compared to control (not shown).

Discussion

High-content screening is a tool for generating data on multiple parameters in single cells as well as in populations

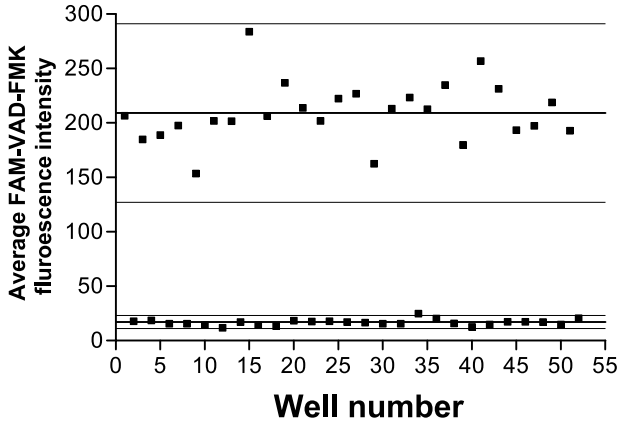

Figure 2. HeLa cells exposed to 10 μM staurosporine for 6 h and stained with Hoechst 33342 (10 μM) and FAM-VAD-FMK (20 μM). Analysis of average FAM-VAD-FMK intensity in 1000 cells per well using 10× objective. *Points,* wells. The *thin lines* enclose the 99% confidence interval for each population.

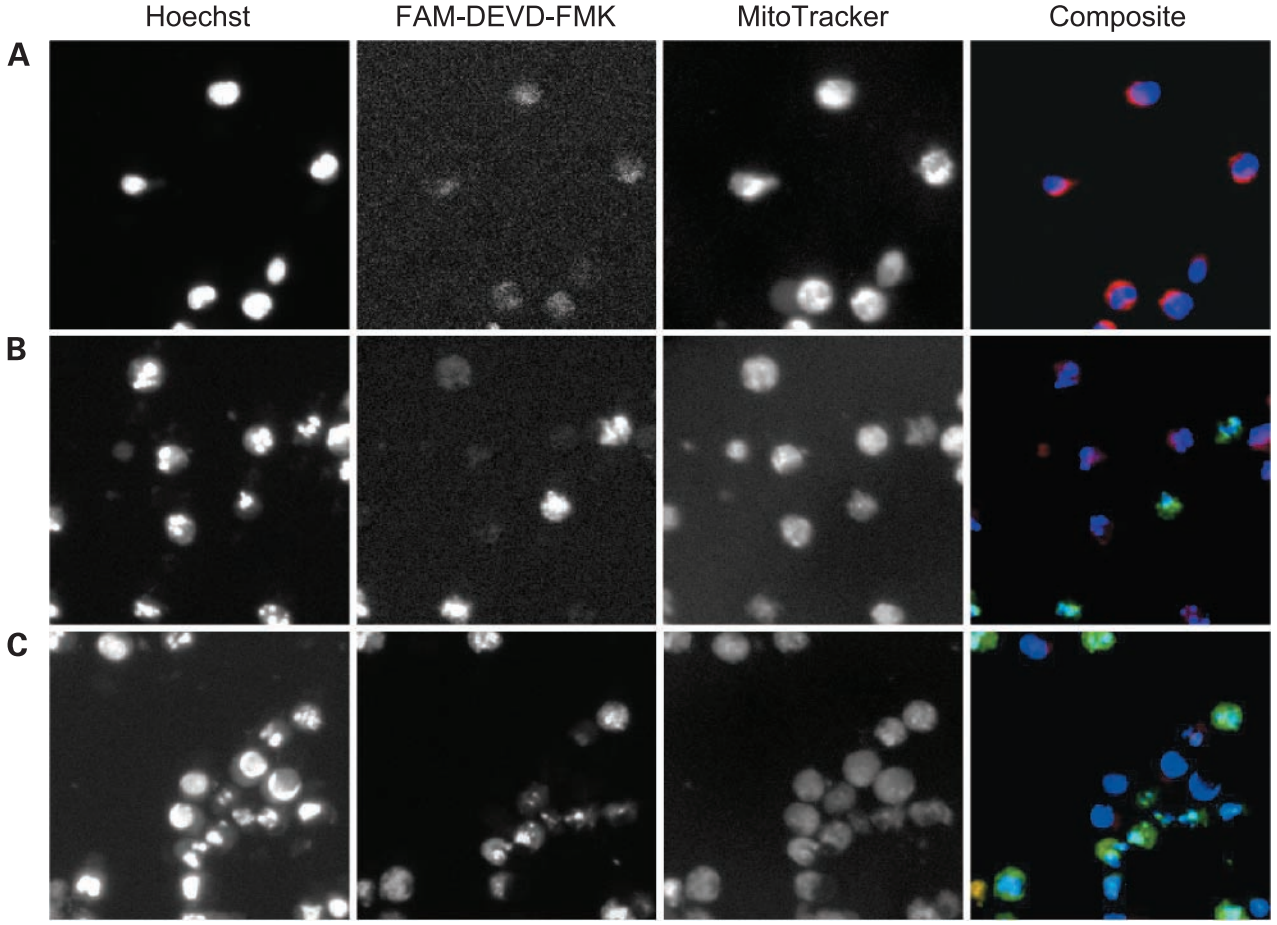

Figure 3. Images acquired in three different channels for assessment of nuclear fragmentation/condensation (*Hoechst*), caspase-3 activity (*FAM-DEVD-FMK*), and MMP (*MitoTracker*) in U-937 cells. Staining as indicated in Fig. 2 with addition of 100 nM MitoTracker Red CMXRos. Cells were exposed to vehicle (**A**), etoposide 60 μM (**B**) or 300 μM (**C**) for 24 h.

of cells (7, 12). In this work, we present the development and evaluation of a high-content screening assay for measuring apoptosis. Apoptosis is a complex process controlled by multiple molecules located in different subcellular locations (4, 6). Therefore, it is of great importance to mechanistic studies of anticancer drugs to be able to simultaneously analyze multiple targets and morphological changes within single cells and cell populations. The assay described here allows for measurement of three fundamental aspects of apoptosis, putatively important in evaluation of novel anticancer drugs.

Image analysis of cells exposed to staurosporine for 6 h showed a correlation between nuclear morphological changes and pan-caspase activation. The observation that cells staining positive for FLICA also have condensed or fragmented nuclei clearly indicates that the method accurately measures apoptosis. A number of observations (5, 13, 14) have indeed suggested that FLICA staining reflects caspase activation, and may therefore be used as an early marker of apoptotic cells. An alternative approach to study caspase-3 activation would be to use antibodies directed against the active form of caspase-3. However, immunostaining requires multiple washing steps after antibody addition, and staining of cells in suspension would be difficult and tedious. The current method only requires four washing steps in total, making it feasible to centrifuge microtiter plates after each step and allowing for analysis of suspension cells with high throughput.

The non-specific protein kinase inhibitor staurosporine induced a marked increase in caspase-3 activity already after 6 h of incubation. The same concentrations that induced caspase activation also reduced MMP as well as inducing changes in nuclear morphology. The reduction in MMP was limited to 67% of control at the time point studied, while changes in nuclear morphology were extensive. Staurosporine is known to induce apoptosis in a number of cell systems (15), and our data on HeLa cells support this observations. To evaluate and compare the quality of screening assays, Zhang *et al*. (11) developed a simple dimensionless statistical parameter. By evaluating the dynamic range of the assay and the SD of negative and positive controls, one can calculate the Z′-value. The value ranges from 0 to 1 and a value above 0.5 indicates that the assay can identify a hit with great confidence. Our assay

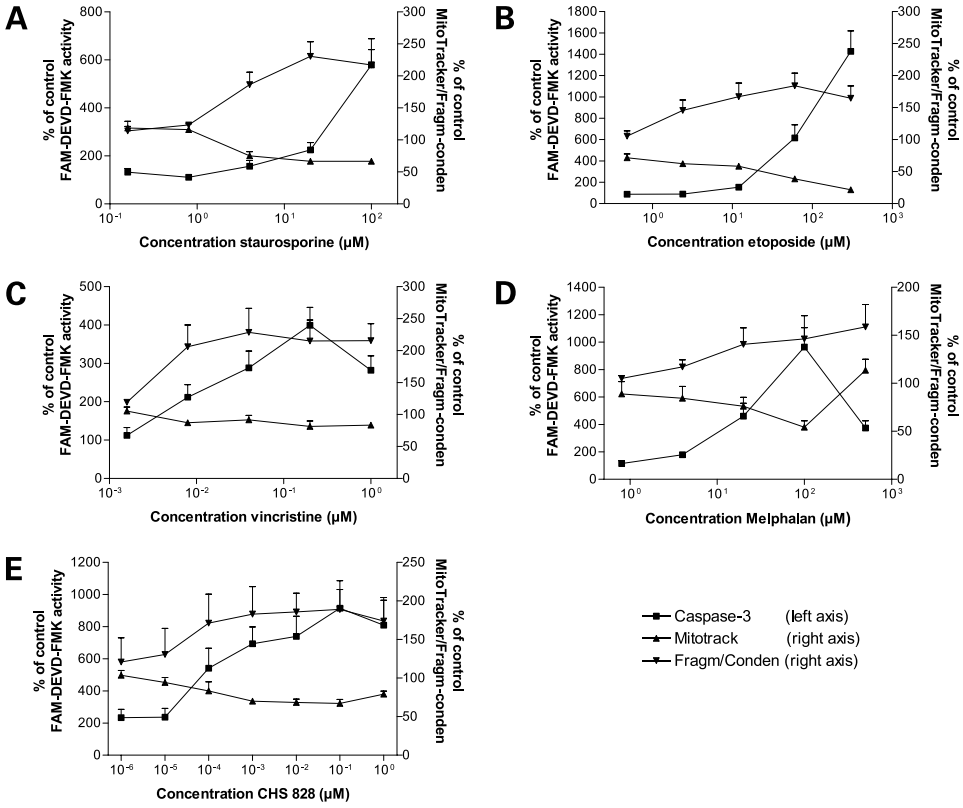

Figure 4. Dose-response curves for caspase-3 activity (*left axis*), nuclear fragmentation/condensation (*right axis*), and MMP (*right axis*) as percentage of untreated control. U-937 cells were exposed to staurosporine (**A**) or etoposide (**B**) for 6 h, vincristine (**C**) or melphalan (**D**) for 24 h, or CHS 828 (**E**) for 72 h. Staining as indicated in Fig. 3. *Points,* mean of three to five independent experiments; *bars,* SE.

showed a Z′-value of 0.56 for caspase activity measurement, indicating that the assay is well suited for screening purposes. Some of the variability can be attributed to loss of cells in late stages of apoptosis. It is possible that an alternative positive control or time point would give an increased dynamic range, thereby further increasing the Z′-value. Time for staining and analysis is less than 3 h post-apoptosis induction and the assay is suitable for automation. The method provides a number of apoptotic parameters presented on single cells or populations. Here, the FAM-DEVD-FMK or FAM-VAD-FMK was chosen to measure the activation of caspases with preference to cleave the DEVD or VAD peptides, respectively. The choice of probe for measuring MMP has been based on biological as well as practical considerations. MitoTracker Red accumulates to different degreed in mitochondria depending on the MMP (16). The total cellular staining is thus dependent both on the number and membrane potential of the mitochondria. The covalent attachment of the probe to sulfhydryl groups in the mitochondria makes it useful in assays requiring fixation of cells (16). It would not be possible to use probes that are not fixable, because differences in time after apoptosis induction would differ between the first and the last well scanned. The mitochondrial specificity of the probe is debated because it can theoretically bind covalently to sulfydryl groups in the cytoplasm (17). However, a number of publications have demonstrated the usefulness of the probe for measuring MMP in aldehyde-fixed cells (16, 18).

Table 1. Log EC_{50} for caspase-3 activity in U-937 cells exposed and stained as in Fig. 4

Drug	Log EC_{50} ± SE (μM)
Staurosporine	1.52 ± 0.14
Etoposide	1.98 ± 0.36
Vincristine	−1.76 ± 0.15
Melphalan	1.36 ± 0.08
CHS 828	−3.95 ± 0.79

Note: Calculations were performed using algorithms for dose-response curves with variable slope. A fixed maximum value of the dose-response curve was set to the maximum obtained value for each drug.

Exposure to the clinically well-established podophyllotoxin derivative etoposide for 6 h produced pronounced caspase-3 activity as well as MMP reduction. Etoposide-induced apoptosis is known to involve caspase activation and nuclear fragmentation (19). Our data support these observations. The alkylating agent melphalan and, especially, the microtubule stabilizing agent vincristine induced caspase-3 activity in U-937 cells. However, at high concentrations, both drugs reduced the activity. It has been described that apoptosis-inducing drugs tend to change the mode of cell death to necrosis at suprapharmacological concentrations (20, 21). The modest reduction in MMP seen with both drugs can most likely be explained by the long incubation time (24 h) before staining of the cells. At this time point, one can expect degradation of many of the fragile apoptotic cells during plate processing, leaving only cells in early or mid-phase apoptosis and with

identifiable nuclei. For drugs inducing cell death within a shorter time frame (*e.g.*, etoposide and staurosporine) a clear reduction in MMP was observed. The nuclei of these cells were still identifiable, although fragmented, allowing measurement of MMP on all cells progressing through apoptosis during these 6 h.

The cytotoxic cyanoguanidine CHS 828 has previously been described to induce active cell death associated with a modest increase in caspase-3 activity, DNA fragmentation, measured by TUNEL staining, and reduction in MMP (22–24). Here we describe a modest increase in caspase-3 activity at 48 h (not shown) followed by a more pronounced activation at 72 h. CHS 828 was extremely potent in induction of caspase-3 activity, with a log EC_{50} of -3.95 ± 0.79 μM. Reduction in MMP was modest as well as nuclear morphological changes. Studies of individual nuclei indicated that condensation was the primary morphological alteration (not shown). The caspase-3 activity observed was higher (22), whereas morphological changes resemble those observed previously (23–25). The present results thus confirm the mode of cell death described previously for CHS 828.

The method described is not limited to studies of the three parameters presented here. By altering the selection of probes, we can study the activity of different enzymes or organelles (7). Image-based cell analysis can also provide information on cell density and thus give information about proliferation in the cell culture. It would also be possible to extract information about the cell cycle phase for each cell based on the total intensity of the Hoechst 33342 staining (26). In conclusion, we have presented a multiparametric high-content screening assay reporting multiple apoptotic parameters on both adherent and suspension cells. The assay characterized apoptosis induction using standard drugs as well as the novel cyanoguanidine CHS 828. The assay conveniently evaluates time- and dose-dependent induction of apoptosis and correlate important parameters in individual cells.

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