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Synthesis & Pharmacological Activity of Flow Cytometry Evaluation of Apoptosis

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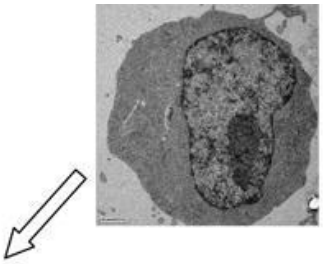
Abstract**—** *The specific morphological and biochemical hallmarks of an apoptosing cell are very context- and stimulus-dependent. Flow and image cytometry can quickly analyze the vast majority of the conventional apoptotic markers. As a result, cytometry evolved became a go-to method for several investigations of cell death. Over the last two decades, several cytometric techniques have been developed to detect apoptotic cells and investigate the processes involved in this kind of cell death. Treatment of UACC62 (Melanoma) cells with the synthesized coumarin was followed by analysis of morphological alterations, membrane change, mitochondria membrane potential, and caspase-3 activity using a flow cytometer and Annexin V-PI staining, JC-1, and caspase-3 enzyme kits. Doxorubicin, a common chemotherapy drug, was used as a benchmark.*

Keywords**— *flow cytometry, apoptosis, single cell analysis, mitochondria, caspases, Annexin V, DNA fragmentation***

I. INTRODUCTION

Over the last decade, researchers from a wide range of biomedical disciplines have begun to concentrate on the processes behind cell death. A variety of signaling cascades control the beginning, the middle, and the end of these processes. Important morphological and molecular differences between typical caspase-dependent apoptosis and unintentional cell death (hence referred to as necrosis) are shown in Figure 1. Many markers for use in microscopy, cytometry, and molecular biology have been developed based on the tinkering with the parameters shown in Figure 1. However, it's worth noting that there might be a huge discrepancy in the prevalence of several apoptotic markers. Growing evidence also suggests other, potentially crucial mechanisms are at work when it comes to the disposal of many cells throughout healthy and pathological processes. Therefore, only the death program with all "hallmarks of apoptotic cell death" should be referred to as "apoptosis." These hallmarks include (i) activation of caspases as an absolute marker of cell death; (ii) tight (geometric) compaction of chromatin; (iii) activation of endonucleases(s) causing internucleosomal DNA cleavage and extensive DNA fragmentation; (iv)



CLASSICAL APOPTOSIS	ACCIDENTAL CELL DEATH (NECROSIS)
Morphological features	
Cell dehydratation & shrinkage	Cell swelling
Loss of pseudopodia or microvilli	Nuclear and mitochondrial swelling
Detachment from the surface (anoikis)	Vacuolization of cytoplasm
"Blebbing" of plasma membrane	Rupture of plasma membrane
Chromatin condensation	Dissolution of chromatin
Nucleolar segregation	Dissolution of cell constituents and attraction of inflammatory cells
Nuclear fragmentation	Scar formation
Preservation of mitochondria structure	
Disassembly of the Golgi apparatus	
Formation of apoptotic bodies	
Shedding of apoptotic bodies	
Engulfment and elimination by phagocytes/neighboring cells	
Biochemical & molecular features	
Preservation of ATP levels	Mitochondrial dysfunction
Loss of mitochondrial membrane potential	Rapid depletion of intracellular ATP
Oxidative stress (ROS generation)	Lack of caspase activation
Cardiolipin peroxidation	Random degradation of DNA
Release of cytochrome c from mitochondria	Rapid loss of plasma membrane integrity
Release of AIF, EndoG, Smac/Diablo and HtrA2 from mitochondria	Uncontrolled release of cytoplasmic constituents
Mobilization of intracellular Ca^{2+} (Ca^{2+} flux)	
Activation of caspases	
Activation of serine proteases (serpases)	
Activation of calpains	
Activation of endonucleases	
PARP cleavage	
DNA fragmentation	
Loss of DNA double helix stability (susceptibility to denaturation)	
Extensive phosphorylation of histone H2AX	
Endonucleolytic DNA degradation	
Separate packaging of DNA and RNA into apoptotic bodies	
General preservation of plasma membrane integrity (increased permeability only for very small cationic probes e.g. YO-PRO1)	
Externalization of phosphatidylserine on the outer leaflet of plasma membrane	
Activation of transglutaminase (TGase2)	

Fig.1 Morphological and biochemical hallmarks of apoptosis and accidental cell death (necrosis). Note that some features characterizing apoptosis may not be present as they heavily depend on particular cell type, stimuli and cellular microenvironment.

Flow cytometry, the primary tool for fast evaluation of various cellular features at the single cell level, allows for quantitative examination of the vast majority of conventional apoptotic characteristics in this context. Flow cytometry's primary benefits lie in the fact that it can analyze single cells, assess several parameters at once, and provide results quickly. Consequently, the sensitivity issues of conventional bulk methods like fluorimetry, spectrophotometry, and gel techniques are resolved by flow cytometry. We only cover a small subset of the available cytometric assays in this chapter, including those that measure mitochondrial transmembrane potential (m loss), caspase activation, plasma membrane remodeling, and DNA fragmentation.

II. FLOW CYTOMETRY EVALUATION OF APOPTOSIS

Apoptotic cells were detected to determine whether the coumarin chemicals used in the research induced apoptosis. In this study, we employed the Annexin V kit, the JC-1 test, and the caspase-3 kit to look for membrane potential changes in the mitochondria, the plasma membrane, and caspase activation. The potential for the UACC-62 (Melanoma) cancer cell line to trigger apoptosis was investigated in more detail.

Morphological Examination

As a kind of programmed cell death, apoptosis is characterized by changes in cell size and shape as well as chromatin condensation and fragmentation (Vermeulen et al., 2005).

At 50 g/ml, UACC-62 (Melanoma) cells treated with SVCM1, SVCM2, SVCM4, SVCM5, and Doxorubicin showed increased cell death under the microscope. Apoptosis hallmarks are visible in cells treated with synthetic coumarin compounds and the doxorubicin control (Figure 2) but not in untreated UACC-62 (Melanoma) cells or cells treated with DMSO (Figure 2). The cells get smaller, more distinct, and take on a spherical structure.

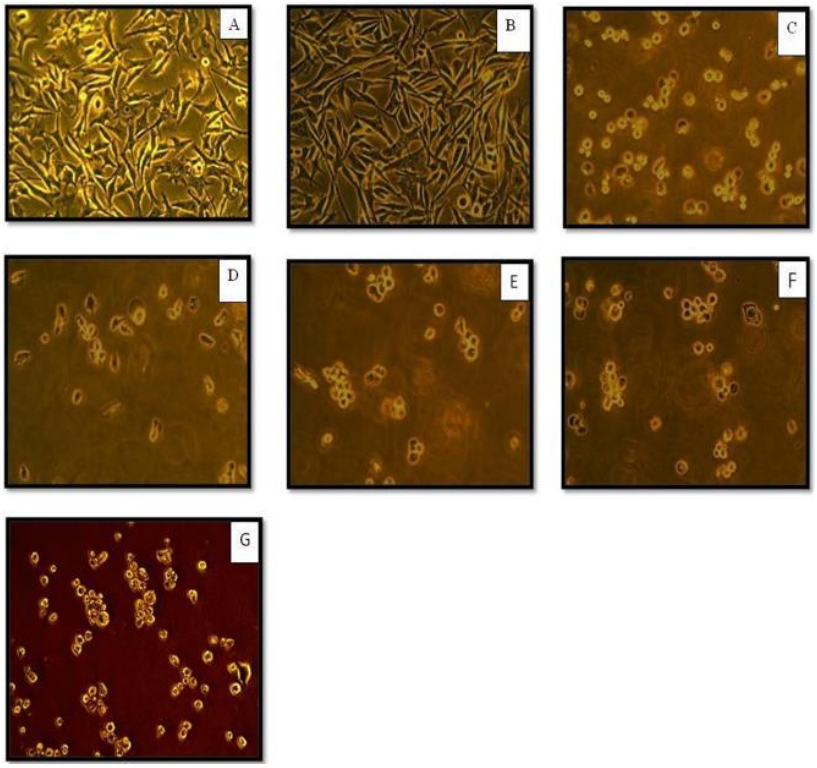


Fig.2: Microscopic observation of the morphology of UACC-62(Melanoma) cells: (A) In the absent of the tested compounds, (B) Treated with 50 µg/ml of DMSO, treated with compounds (C)SVCM1; (D) SVCM2; (E) SVCM4; (F) SVCM5 and (G) Doxorubicin 6 µg/ml respectively.

Membrane changes

We used the Apoptotic system in accordance with the manufacturer's (BD Biosciences) guidelines. Using FITC, the percentage of cells in a population that are undergoing programmed cell death might be determined. At least 10,000 events were recorded per

sample using FL2 channel (565 to 605 nm) flow cytometry to identify cell fluorescence.

Annexin positive cells were counted and their percentage is shown in Table 1. Compounds SVCM1, SVCM2, and SVCM5 triggered early apoptosis (13.1, 12.1, and 15.1%) in UACC-62 cells. After removing the 7.75% DMSO concentration, these results appeared. Late apoptosis was also induced by the chemicals SVCM1, SVCM2, SVCM4, and SVCM5. About 25.4 percent, 28.5 percent, 68.1 percent, and 27.3 percent of the samples showed apoptosis. A result of 10.4 percent was obtained after deducting the apoptotic response to DMSO. These results suggest that coumarins compounds have the potential to trigger apoptosis in UACC-62 (Melanoma) cell lines. The UACC-62 (Melanoma) cell line showed acceptable annexin V results due to doxorubicin (6 g/ml) inducing a 46% increase in late apoptosis.

Table 1: UACC-62 (Melanoma) treated with coumarin and stained with FITC/PI

Treatment	Viable (%)	Apoptosis (%)	Late apoptosis (%)	Necrotic (%)
Untreated Cells unstained	100	0	0	**0**
Untreated stained cells Annexin/PI	51.9	0	23.9	**7.98**
Annexin only	58.3	33.45	0	**0.**
DMSO	80.5	7.75	10.4	**3.62**
Doxorubicin	3.18	0	46	**39.9**
SVCM1	28.9	13.1	25.4	**14.5**
SVCM2	27.7	12.1	28.5	**17.5**
SVCM4	14	0	67.9	**2**
SVCM5	**23.6**	**15.1**	**27.3**	**15.9**

Mitochondria membrane potential

Changes in the mitochondrial membrane are a precursor to apoptosis. JC-1 monomers and aggregates emit light at different wavelengths and have different mitochondrial membrane potentials, according to BD Bioscience. Cell fluorescence was determined by comparing the ratio of fluorescence between the FL1 and FL2 channels, and at least 10,000 events were collected per sample using the flow cytometer.

Table 2 Table of the mitochondrial membrane potential results

Treatment	Stained Viable cell (%)	Apoptosis (%)
Untreated unstained Cells	100	0
Untreated stained cells	93.6	4.58
DMSO	92.3	6.50
Doxorubicin	27.6	49.5
SVCM1	65.6	27.8
SVCM2	77.2	16.2
SVCM4	84.9	8.5

SVCM5	67.5	25.8

Staining for living cells is shown in the upper right quadrant (Q2), whereas apoptotic cells (percent) are located in the third quadrant from the bottom. Cells treated with 6 g/mL doxorubicin (A), 50 g/mL DMSO (B), 50 g/mL SVCM1 (E), SVCM2 (F), SVCM4 (G), and SVCM5 (H), respectively, are shown.

Apoptosis detection using caspase-3 kit

The caspase-3 kit was used successfully to detect apoptosis in treated UACC-62 (Melanoma) cells. These caspases break a number of proteins, which affects the cell's structure and function and eventually leads to cell death. Caspase-3 activity is the effector molecule that is common to most apoptotic pathways (Jeong et al., 2007).

UACC-62 (melanoma) cells were initially successfully treated and stained with PE-conjugate polyclonal active caspase-3 antibody utilizing the caspase-3 kit. The treatment of cells with coumarin compounds resulted in a significant activation of caspase-3. Table 3 shows that SVCM4 had the greatest caspase-3 activity at 97.64%, but that SVCM1, SVCM2, and SVCM5 also showed considerable activity at 70.6%, 74.2%, and 77.84%, respectively. The natural caspase-3 activity in DMSO was accounted for, and the findings were adjusted accordingly. When doxorubicin (6 g/mL) was administered as a placebo, caspase-3 activity was at 97.74 percent. The results for caspase-3 in every sample were so confirmed.

Table 3 Caspase-3 activity of coumarins SVCM1, SVCM2, SVCM4, SVCM5

Treatment	Caspase negative PE-A-	Caspase positive PE-A+
Negative control	97.9	2.06
Doxorubicin	0.21	97.74
SVCM1	27.3	70.64
SVCM2	23.7	74.24
SVCM4	0.25	97.64
SVCM5	20.1	77.84

III. CONCLUSION

In this study, compounds SVCM1, SVCM2, SVCM4, SVCM5, and SVCM7 were shown to have potential as larvicidal medicines. Although the compounds were successful in killing mosquitoes after two minutes of treatment, the insects recovered, and the chemicals did not show significant adulticidal efficiency.

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