a. What will be the estimated IC_{50} of the test compound? (1 mark)

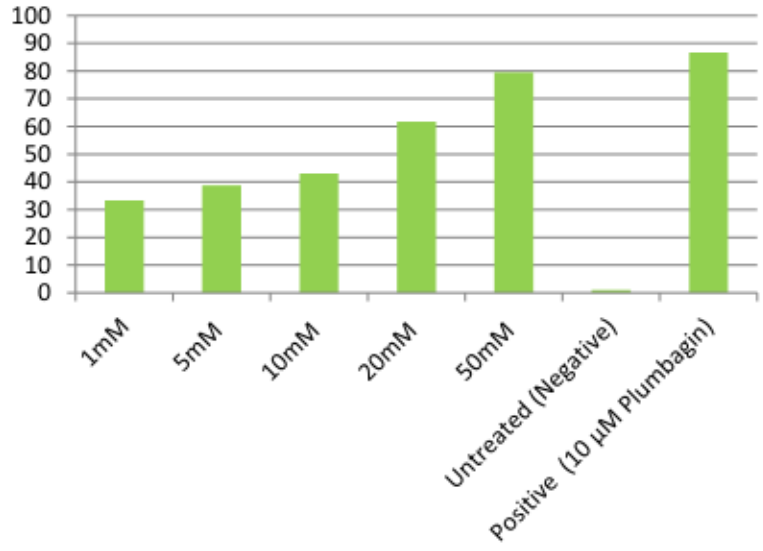
b. Do you think that the test compound can be a better lead molecule than a known anticancer compound 'Plumabgin' knowing that the latter is also toxic to normal cells? Justify your answer according to concentrations used and toxicity profiles shown in the figure. (2 marks)

7. Explain why apoptotic mechanism of cell death is a good target for drug development? **(2 marks)**

4. Explain the results you have obtained in this experiment by analysing the histogram that you have generated. **(4 marks)**

5. List three hallmarks of apoptosis (except phosphatidylserine exposure), which can be targeted using various assays to confirm the apoptotic cell death. **(3 marks)**

6. Various concentrations of a test compound were given to MCF-7 cells for 24 hours. Plumbagin was used as a positive control. The percentage change of apoptotic cell death is presented on the Y-axis. Answer following questions based on the data presented in the figure below: **(3 marks)**



Practical Report 3 (MCBG 2033/2029, Drug Discovery)

Measuring cell death using trypan blue exclusion method in response to treatment with a compound in a time-course experiment.

Lecturer: Prof Mandeep Kaur Total Marks: 20

Person Number_____ TA_____ Date:

1. Draw a labelled histogram using Percentage change in cell death on Y-axis and drug concentrations used in experiment on the X-axis. **(4 marks)**

Paste histogram here

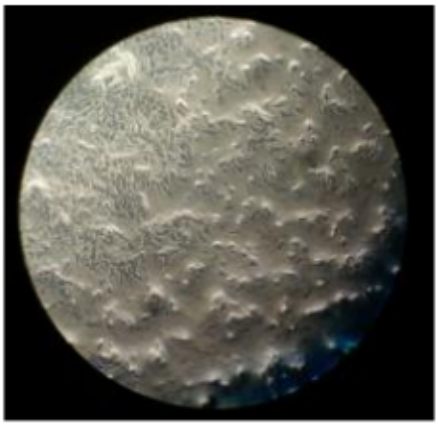
2. How APOPercentage dye detects cells undergoing apoptosis? **(2 marks)**

3. Some cells in untreated sample may take up dye and are coloured pink? Give your reasons for following scenarios: **(2 marks)**

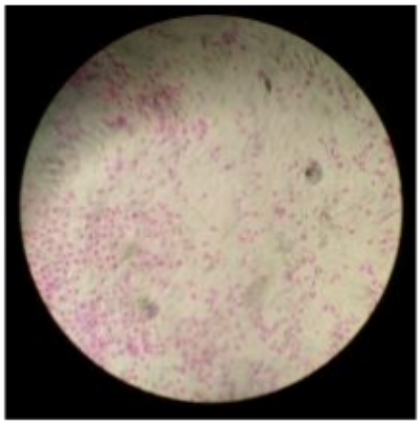
a. About 10% cells are stained pink in the untreated sample.

b. About 50% cells are stained pink in the untreated sample.

Sample pictures



Untreated



Treated (10 μM Plumbagin for 24 hours)

3. Calculations

1. Take mean of three OD values for each sample, blank and untreated.

2. Subtract mean of blank from mean of samples and untreated. Result is the final mean.

3. Calculate percent cell death using final mean values

Percentage change in cell death in treated as compared to untreated:

$$\frac{(\text{Sample} - \text{Untreated})}{\text{Untreated}} \times 100$$

4. Draw a histogram using Percentage change in cell death on Y-axis and time points used in experiment on the X-axis.

Column1	2	3	4	5	6	7	8	9	10	11	12
Blank	PBS only	PBS only	PBS only								
Untreated	0	0	0								
10 mM H_2O_2	-	-	-								
50 mM H_2O_2	-	-	-								
50 µM AP	-	-	-								
15 mM EDTA	-	-	-								

Blank- Contains solution only that will be used for absorbance measurement, in this case it is PBS.
Untreated- Contains cells which have not been treated with any chemical.
Treated- Four sample concentrations of compounds shown above in the table (final concentrations).

a. The cells were grown in 45 µl of media, incubated overnight at 37 °C in a CO_2 incubator.

b. Add 5 µl apoptotic inducer drug/compound (H_2O_2 and AP only) as shown in table (by touching the tip on the walls of the well) in columns 2, 3 and 4. For EDTA, remove media from wells and add 50 µl of EDTA provided.

Do not add the chemical to wells for 'Untreated'. Makeup the required volume by adding 5 µl PBS (total in the well should be 50 µl).

c. Incubate at 37 °C for one hour.

d. Discard the medium.

e. Add 65 µl of dye (0.5 µl of dye diluted in 64.5 µl PBS) and cover the plate with foil.

f. Incubate (37°C, 5% CO_2) for 15 minutes and check under microscope if cells in EDTA treated wells are pink. If cells are not pink, then leave for another 5-10 minutes.

g. Discard the reagent mixture from all wells.

h. 1 x 100 µl PBS wash (**Gently by the walls of each well as cells may be loose**).

i. Discard PBS

j. Add 100 µl PBS into each well (prevent drying out of the cells).

Look the plate under the microscope. Visual analysis of the cultures will reveal an admixture of live (clear cells) and apoptotic (pink dye stained) cells in each experimental condition. Notice the difference in the number of cells stained pink in each sample.

k. Take OD (optical density) spectrophotometrically using a plate reader at 550 nm.

features associated with apoptosis can also be observed in other types of cell death such as necrosis or necroptosis. Therefore, it is recommended to analyse more than one parameter to identify apoptosis as a cause of death in the studied population.

APOPercentage Aassay

In viable cells, the distribution of phospholipids between the inner and outer leaflet of the plasma membrane is asymmetric. In this asymmetric distribution, phosphatidylcholine and sphingomyelin are predominantly located in the outer leaflet, while phosphatidyl-thanolamine and phosphatidylserine (PS) are found in the inner plasma membrane leaflet. However, during apoptosis PS translocates to the outer plasma membrane leaflet and can thus be used as a biomarker to identify apoptotic cells.

The APOPercentage assay (Biocolor Ltd., UK), detects apoptosis at the stage when PS is externalized. The APOPercentage assay makes use of an anionic halogenated fluorescein dye, the disodium salt of 3, 4, 5, 6,-tetrachloro-2′, 4′, 5′, 7′-tetraiodofluorescein (TCTF), which is taken up by apoptotic cells, which gives such cells a purple-red appearance. Dye uptake can be visualized by bright-field microscopy and quantified by spectrophotometric measurement at 550 nm.

Safety Precautions

1. Good laboratory techniques are to be used at all times.
2. Any material that has been in contact with human, murine, or GM cell lines is treated as potentially infectious.
3. Wear gloves and lab coat at all times**.**
4. Do not touch any body part with hands while having gloves on.

MATERIALS

1. APOPercentage dye (light sensitive).
2. 1x PBS.
3. Compounds: Hydrogen Peroxide (H_2O_2), Acetyl Plumbagin (AP), Ethylenediaminetetraacetic acid (EDTA)
4. MCF-7 (estrogen receptor positive (ER+) breast cancer) cells grown in a 96-well plate.
5. Aluminium foil.
6. Micropipettes and tips.
7. Inverted microscope.
8. Spectrophotometer- plate reader

PROCEDURE

1. Visualize cells under microscope

Each group will receive a 96-well plate with 5000 cells cultured in it. You will go in groups to visualize the plate under a microscope. Focus the microscope using knobs and see cells under 10X. Take note of the distribution and morphology of cells in each plate. Note down what you have observed and answer the questions in the assignment.

2. Treating the cells with a chemical and performing the assay

The cells will be treated with different concentrations of the various compounds. Following is the chemical treatment plan in a 96-well plate:

this cell death process is initiated are the intrinsic and extrinsic cell death pathways, both converging on caspase activation.

The **intrinsic cell death pathway** is governed by the Bcl-2 family of proteins, which regulate commitment to cell death through the mitochondria. A myriad of intracellular death signals are communicated through the intrinsic cell death pathway, such as DNA damage, oncogene activation, growth factor deprivation, ER stress and microtubule disruption. The key step in the intrinsic cell death pathway is the permeabilization of the mitochondrial outer membrane, which has been identified as a 'point of no return' after which cells are committed to cell death. Following permeabilization, release of various proteins from the mitochondrial intermembrane space promotes caspase activation and apoptosis. Cytochrome C binds APAF-1 (apoptosis protease-activating factor 1), inducing its oligomerization and thereby forming a structure called the apoptosome that recruits and activates an initiator caspase, caspase 9. Caspase 9 cleaves and activates the executioner caspases, caspase 3 and 7, leading to apoptosis.

Activation of the **extrinsic cell death pathway** occurs following the binding on the cell surface of "death receptors" to their corresponding ligands such as Fas, TNFR1 or TRAIL. These death receptors have two distinct signalling motifs, death domains (DD) and death effector domains (DED), that allow them to interact and recruit other adaptor molecules such as Fas-associated death domain protein (FADD) and caspase 8, which can then directly cleave and activate the executioner caspases, caspase 3 and caspase 7, leading to apoptosis.

Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8 cleavage and activation of the BH3-only protein BID (BH3-interacting domain death agonist), the product of which (truncated BID, known as tBID) is required in some cell types for death receptor-induced apoptosis.

Parameters of apoptosis

Apoptosis occurs via a complex signalling cascade that is tightly regulated at multiple points, providing many opportunities to evaluate the proteins involved. The image below (Fig 2) shows the main parameters of apoptosis and the approximate relative time when they are likely to be detected.

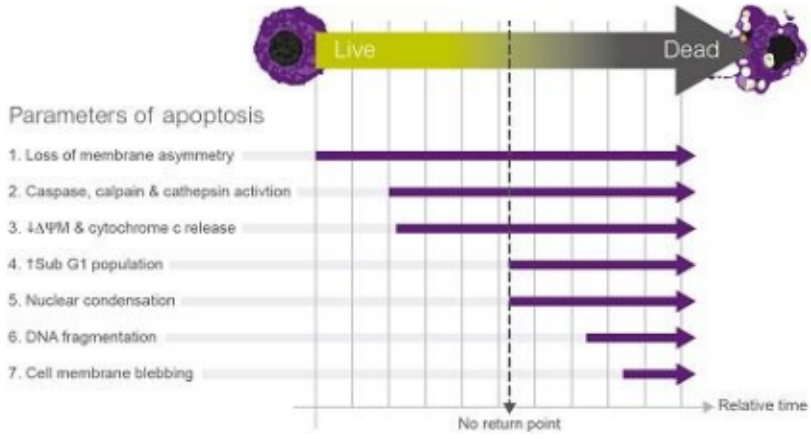


Fig 2

These parameters do not happen in a sequential order, and many of them will overlap and occur at the same time. Loss of membrane asymmetry or initiation of caspase cascade are biochemical features of apoptosis which do not necessarily lead to cell death. However, other downstream features such as decrease of the mitochondrial membrane potential ($\Delta\Psi m$) and concomitant release of cytochrome C into the cytosol are generally considered points of no return, after which it is very unlikely the cell will survive. Some of these biochemical and morphological

Practical 3: Quantifying apoptotic death in cancer cells using APOPercentage assay after treatment with various compounds.

Purpose

This document describes the procedure to measure apoptotic cell death in the culture using the APOPercentage dye.

Introduction

Following information has been taken from freely available resources listed below:
https://www.ncbi.nlm.nih.gov/books/NBK26873/
http://www.abcam.com/kits/introduction-to-apoptosis
http://www.biotechniques.com/BiotechniquesJournal/2008/September/A-low-cost-flow-cytometric-assay-for-the-detection-and-quantification-of-apoptosis-using-an-anionic-halogenated-fluorescein-dye/biotechniques-45310.html

The cells of a multicellular organism are members of a highly organized community. The number of cells in this community is tightly regulated—not simply by controlling the rate of cell division, but also by controlling the rate of cell death. If cells are no longer needed, they commit suicide by activating an intracellular death program. This process is therefore called **programmed cell death**, although it is more commonly called **apoptosis** (from a Greek word meaning "falling off," as leaves from a tree).

Apoptosis (Fig 1) is a form of cell death, also known as programmed cell death, in which a 'suicide' program is activated within the cell, leading to fragmentation of the DNA, shrinkage of the cytoplasm, membrane changes and cell death without lysis or damage to neighbouring cells as cells are engulfed by phagocytes (macrophages). It is a normal phenomenon, occurring frequently in a multicellular organism.

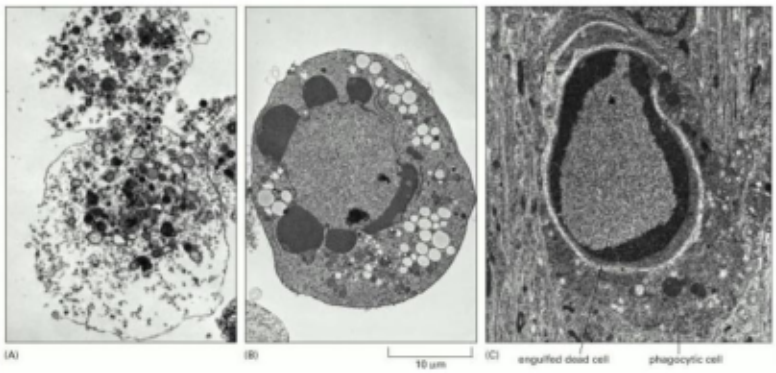


Fig 1: These electron micrographs show cells that have died by (A) necrosis or (B and C) apoptosis. The cells in (A) and (B) died in a culture dish, whereas the cell in (C) died in a developing tissue and has been engulfed by a neighbouring cell. Note that the cell in (A) seems to have exploded, whereas those in (B) and (C) have condensed but seem relatively intact. The large vacuoles visible in the cytoplasm of the cell in (B) are a variable feature of apoptosis. (Courtesy of Julia Burne.) From: Programmed Cell Death (Apoptosis), Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002.

Apoptotic mechanism

During apoptosis, a family of cysteine-aspartate proteases known as caspases accelerates cell death through restricted proteolysis of over 400 proteins. The two main pathways through which