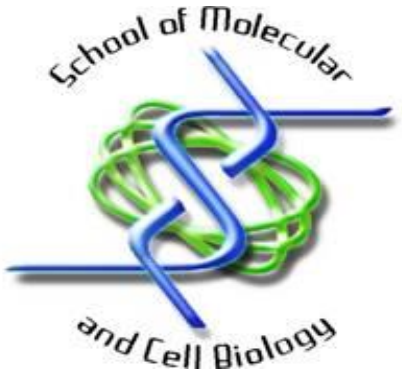
University of the Witwatersrand

School of Molecular and Cell Biology



MCBG 2033/2029

Drug Discovery

Practical Course Manual

Prof. Mandeep Kaur

2018

General Information:

The practical course is compulsory. Students have to confirm their attendance by signature. In case of sick leave, a Medical Certificate has to be provided and submitted to the MCB Office, Gate House, Ground Floor.

Good Laboratory Practice:

A lab coat must be worn during the entire practical session. Protective goggles must be worn when handling dangerous chemicals (follow the advice of the senior technicians and TAs). No open shoes (e.g. sandals) are allowed. Laboratory Gloves must be worn during all practical work. The advice of senior technicians and TAs must be followed. In case of any misconduct, the student will immediately be removed from the lab.

Senior Technicians: Sabeeha Mahomed, Carol Pilane

Laboratory Working Groups

The work will be performed in working groups. One TA is responsible for one group.
Each working group will consist of 2-3 students.

Assessment

Each student has to submit three Practical Course Reports. The Practical Course Reports will be marked by your TA.

The Practical Course Reports need to be either handed in to the School of Molecular and Cell biology (Shutter Box Biochemistry and Cell Biology II) by due date or to be handed over to your group's TA before leaving the practical lab, as announced by the lecturer.

Timetable

Practical 1: Development of Gleevec: a successful drug for treating Chronic Myeloid Leukemia (CML)

(17th September, 2018, Venue FNB105 lab, 13:45)

Practical 2: Measuring cell death using trypan blue exclusion method in response to treatment with a compound in a time-course experiment.

(1st October, 2018, Venue OLS lab, 13:45)

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

(8th October, 2018, Venue OLS lab, 13:45)

Practical 1: Development of Gleevec: a successful drug for treating Chronic Myeloid Leukemia (CML)

The material presented here has been adapted from following:

http://www.historyofnimr.org.uk/mill-hill-essays/essays-yearly-volumes/2003-2/Gleevec-cml-abl-and-targeted-molecular-therapy-for-cancer/ by Qiling Xu (2003) Mill Hill Essays.

https://www.hindawi.com/journals/cherp/2014/357027/

Nida Iqbal and Naveed Iqbal. Imatinib: A Breakthrough of Targeted Therapy in Cancer. Chemotherapy Research and Practice. 2014, Article ID 357027, 9 pages, http://dx.doi.org/10.1155/2014/357027.

What is Gleevec? How was it discovered?

In Basel, Switzerland, 3 January 2000, the pharmaceutical company Novartis announced that the European Commission approved Gleevec (also known as Imatinib, a 2-phenyl amino pyrimidine derivative) as a first-line treatment for adult and child patients with CML, enabling doctors to provide the drug to newly diagnosed patients. The approval was based on information collected over twelve months in a large study comparing Gleevec with the best previous treatment for CML. Patients treated with Gleevec were nine times more likely to achieve a response in which no cancer cells remained. In other patients Gleevec significantly delayed the time to progression of the more advanced stages of CML. Gleevec was invented in the late 1990s by biochemist Nicholas Lyndon then working for Ciba-Geigy (now Novartis), and its use to treat CML was driven by Brian Druker, an oncologist at the Dana-Farber Institute. The first clinical trial of Imatinib took place in 1998 and the drug received FDA approval in May 2001. Lyndon, Druker, and the other colleagues were awarded the Lasker-DeBakey Clinical Medical Research Award in 2009 for "converting a fatal cancer into a manageable condition" and the Japan Prize in 2012 for their part in "the development of a new therapeutic drug targeting cancer-specific molecules."

Normal cells produced in the bone marrow develop to maturity in a step-by-step process, until they leave the bone marrow and enter into the blood stream. Among immature bone marrow cells are stem cells that will become red blood cells and different kinds of white blood cells. Leukemia is an uncontrolled multiplication of the white blood cells. As in many other forms of cancer, the leukemic cells are descended from a single cell that lost its ability to maintain normal control over cell division and growth. There are a number of types of leukemia, corresponding to the many types of white blood cells and the stages they go through as they mature. CML, arises in a bone marrow cell, which normally forms two types of cells called granulocytes and platelets. So, CML patients have abnormally high numbers of these cells in their blood. Clinically there are three phases of CML depending on the number of immature blood cells in the blood and bone marrow. During the initial chronic phase the number of immature stem cells is increased but they can still mature to form different white blood cells that function normally. At this stage the patients have mild symptoms and respond to treatment. After an average of four to five years, the CML typically progresses to the accelerated phase, in which there are more immature cells in the blood and the disease is not as responsive to treatment. The final, often fatal stage, is the "blast crisis", where immature cells dominate and chronic leukemia has become an aggressive acute leukemia.

There are three major treatments for CML. Firstly, chemotherapy and radiotherapy can be used to kill cancerous cells but this has the disadvantage that normal cells in the blood are also killed. The treatment offers some relief rather than a cure since the methods are poorly targeted and highly toxic. A second treatment is aimed at improving the body's immune system to fight the cancer. Interferon alpha is a protein naturally produced in our bodies to fight viruses. In the 1980s interferon alpha was introduced to treat CML in combination with chemotherapy. This treatment has achieved long-lasting remissions in 30% of patients compared with 5% of those using conventional chemotherapy. It does not, however, completely remove the cancer cells. The final treatment option is high-dose chemotherapy and total body irradiation followed by bone marrow transplantation. This is the only treatment capable of disease eradication, but is restricted to patients who can identify a suitable bone marrow donor and who are medically fit to undergo the procedure. The most effective therapy would be one that specifically eliminates the cancer cells. However, such a treatment was not available before scientists had a molecular understanding of the disease.

Human cells have 46 chromosomes, 23 pairs in females, 22 pairs plus 2 unpaired in males. In 1960 two researchers in Philadelphia noticed that an abnormally small chromosome was consistently present in the cells of CML patients. This was given the name of the Philadelphia (Ph) chromosome. This was the first time that a chromosomal abnormality had been associated with a malignant disease. In 1973 the Ph chromosome was found to be the result of swapping parts of chromosome 9 and 22, producing a longer chromosome 9 and a shorter chromosome 22. Two genes flanking the points in the DNA where this swap over occurs were identified in the early 1980s. One is the abl (ablation) gene from chromosome 9 that was already known as the human counterpart of a mouse virus gene. The other gene from chromosome 22 was given the name of bcr (short for breakpoint cluster region). At that time the function of Bcr was unknown, but the abl gene was widely studied. The Abl protein is an enzyme, known as a protein kinase that attaches a phosphate group to specific proteins. When this happens it triggers a cascade of events in the cells that regulate the division of cells and cell death. These are both common mechanisms used to control the number of cells in the body. Cell death is a protection mechanism used to eliminate excess or abnormal cells. Disruption of either of these mechanisms can result in uncontrolled cell division manifested as cancer. Abl protein in normal cells is only enzymatically active when the cell is stimulated by a growth factor. The effect of the Ph chromosome translocation is to bring the bcr and abl genes together resulting in a fused bcr-abl gene. A major breakthrough in understanding the molecular nature of CML came when it was discovered that the fused Bcr-Abl protein produced from the fused gene is a continuously active protein kinase. This unrestrained activation of the kinase activity leads to an increase in the rate of cell division and the protection of the cells from cell death. Consequently a large number of Ph chromosome-containing cells is found in CML patients.

How Gleevec works?

Tyrosine kinases are important mediators of the signaling cascade, determining key roles in diverse biological processes like growth, differentiation, metabolism, and apoptosis in response to external and internal stimuli. Deregulation of protein kinase activity has been shown to play a central role in the pathogenesis of human cancers. In 1995 and 1996 a few such compounds that specifically inhibited the kinase activity of Bcr-Abl were identified. Gleevec, is a tyrosine kinase inhibitor with activity against Abl, Bcr-Abl, PDGFRA, and c-KIT. The active sites of tyrosine kinases each have a binding site for ATP. The enzymatic activity catalyzed by a tyrosine kinase is the transfer of the terminal phosphate from ATP to tyrosine residues on its substrates, a process known as protein tyrosine phosphorylation. Gleevec works by binding close to the ATP binding site, locking it in a closed or self-inhibited conformation, therefore

inhibiting the enzyme activity of the protein semi-competitively. This process ultimately results in "switching-off" the downstream signaling pathways that promote leukemogenesis. Gleevec also inhibits the Abl protein of noncancer cells, but cells normally have additional redundant tyrosine kinases which allow them to continue to function even if Abl tyrosine kinase is inhibited. Some tumour cells, however, have a dependence on Bcr-Abl. Inhibition of the Bcr-Abl tyrosine kinase also stimulates its entry into the nucleus, where it is unable to perform any of its normal antiapoptotic functions. Gleevec is well absorbed after oral administration with a bioavailability exceeding 90%. It is extensively metabolized, principally by cytochrome P450 CYP3A4 and CYP3A5, and can competitively inhibit the metabolism of drugs that are CYP3A4 or CYP3A5 substrates. Gleevec is generally well tolerated. Common side effects include fluid retention, headache, diarrhea, loss of appetite, weakness, nausea and vomiting, abdominal distention, edema, rash, dizziness, and muscle cramps. Serious side effects may include myelosuppression, heart failure, and liver function abnormalities.

Gleevec was referred as a "wonder drug". However, CML comes in many ugly faces and Gleevec cannot cure them all. When treated during the more aggressive stage of blast crisis, most CML patients ultimately develop a drug-resistant disease. The majority of relapsed patients have mutations within the Bcr-Abl kinase region that reduce or abolish drug binding. The key regions of the kinase required both for regulation of Abl under normal circumstances, and for inhibition of Bcr-Abl by Gleevec, have now been identified. It is of great importance to understand the underlying mechanisms of drug resistance so that a new generation of targeted anti-cancer agents can be designed. The resistance to Gleevec has also led to a renewed interest in other drugs that may be helpful in this situation. These are targeted at the cellular events following the activation of Bcr-Abl, the stability of the Bcr-Abl proteins and alternative inhibitors of Abl kinase.

We are undergoing a revolution in medicine in which a better understanding of mechanisms of disease, such as cancer, and of pathways at the molecular and genetic levels has provided new opportunities for rational therapies and novel approaches for diagnosis. Molecular targets include molecules and pathways that are involved in cell proliferation, programmed cell death, DNA repair and secondary tumour formation. Through genetic testing and gene-based treatment we can anticipate dramatic changes in disease prevention, diagnosis and treatment. As each individual has a unique composition in our genetic make-up, a person-based medical treatment will be a reality in the not too distant future. It is noteworthy that these recent and future advances in our understanding of human diseases are deeply rooted in knowledge of genetics, molecules and pathways that come from basic research. Progress in understanding the molecular biology of different types of leukemia is the key to the development of accurate diagnosis and targeted treatment. Gleevec has set a precedent for the approach of molecularly targeted therapy and has demonstrated that it is pivotal to identify the right target for the right group of patients.

Practical exercise:

Go to website: https://www.learner.org/courses/biology/casestudy/cancer.html

Click on "Launch case study" to start the exercise and follow the instructions on the screen. Complete the exercise and answer the questions in the assignment below. Hand it over to your TA before leaving the lab.

Practical Report 1 (MCBG 2033/2029, Drug Discovery)
Development of Gleevec: a successful drug for treating Chronic Myeloid Leukemia (CML)

Lecturer: Prof Mandeep Kaur Total Marks: 20

Person Number_____ TA_____ Date:

1. Draw the structure of Gleevec? **(1 mark)**

2. Which chromosomal mutation is responsible for CML? In how many patients this mutation is detected? **(3 marks)**

3. What is IC_{50} in terms of enzyme activity? **(1 mark)**

4. What are the IC_{50}'s of compound 3 against Abl enzyme and mutant Bcr-Abl, when tyrosine mimic is selected as a lead compound? **(2 marks)**

5. Out of phosphate, methypiperizine and isoprene, which group was used modify ATP lead compound and why? **(2 marks)**

6. During development of Gleevec, it was found to be active against Bcr-Abl at lower concentrations but inhibited PKA kinases at higher concentrations. Would this observation make Gleevec a good drug candidate? Answer in 'yes' or 'no' and justify your answer.
(3 marks)

7. Gleevec is a very successful drug against CML. But it has been observed that many CML patients do not respond to Gleevec. **(8 marks)**

a) At what disease stage and why some patients do not respond to Gleevec? Describe molecular mechanisms involved. (5 marks)

b) Which cellular processes can further be targeted to develop new drugs for CML? (3 marks)

Practical 2: Measuring cell death using trypan blue exclusion method in response to treatment with a compound in a time-course experiment.

Purpose

This document describes the procedure to measure cell death in the culture using the Trypan blue exclusion method.

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**.**

Introduction

The dye exclusion method is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium. Trypan blue is a diazo dye, synthesized by the German scientist Paul Ehrlich in 1904, selectively colour dead cells blue. It is excluded from live but accumulates in dead cells, is one of the most commonly used methods for assessment of viability in a given cell population. It traditionally requires time-consuming cell counting, a method prone to investigator bias and sampling error. By automating this method using spectrophotometry, it provides an attractive method to rapidly quantify dying cells in the cell culture.

EXPERIMENT

The purpose of this laboratory exercise is to acquaint you with some of the fundamentals of culturing cells *in vitro*.

(1) What do cell cultures look like?
(2) Where do the cells grow?
(3) What do the cells in the culture look like?

In this exercise you will:

(1) Observe the cell culture provided;
(2) Determine the percentage of cell death.

MATERIALS

1. 0.4% Trypan Blue stain (fresh & filtered) in phosphate buffered saline.
2. 1% SDS.
3. 1x PBS.
4. 10 mM H_2O_2
5. MCF-7 (estrogen receptor positive (ER+) breast cancer) cells grown in a 96-well plate.
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 one concentration of the given chemical over time-intervals. We are performing a time-course experiment to see how a particular concentration of a chemical affects living cells over time. This will also help us to identify time-frame in which drug starts to kill cancer cells and in how much time it can kill approximately 50% of the cells.
Following is the chemical treatment plan in a 96-well plate:

Column1	2	3	4	5	6	7	8	9	10	11	12
Blank	SDS only	SDS only	SDS only								
Untreated	0	0	0								
90 minutes	-	-	-								
60 minutes	-	-	-								
30 minutes	-	-	-								
10 minutes	-	-	-								

Blank- Contains solution only that will be used for absorbance measurement, in this case it is SDS.
Untreated- Contains cells which have not been treated with any chemical.
90 minutes- cells that have been treated with the chemical for 90 minutes.
60 minutes- cells that have been treated with the chemical for 60 minutes.
30 minutes- cells that have been treated with the chemical for 30 minutes.
10 minutes- cells that have been treated with the chemical for 10 minutes.

a. The cells were grown in 50 µl of media, incubated overnight at 37 °C in a CO_2 incubator.

b. Add 10 µl of provided chemical (by touching the tip on the walls of the well) in columns 2, 3 and 4. First add chemical in ’90 minutes’ sample, start incubation at 37 °C, after 30 minutes add chemical in ’60 minutes’ sample and repeat the process of incubation and treating again till you finish all chemical treatments (10 minutes sample).

Do not add the chemical to wells labelled ‘Untreated’. Makeup the required volume by adding 10 µl PBS (total in the well should be 60 µl).

c. Incubate at 37 °C for required time and visualize under the microscope to see the change in morphology, if any.

d. Add 60 µl of sterile 0.4% trypan blue solution to all the samples and untreated wells (except blank) and incubate the in the incubator (37 °C) for 15 minutes.

e. Remove dye containing media via gentle but thorough washing (2-3×100 µl) with (0.01 M PBS). A slow, steady wash is necessary to prevent loss of dead cells which may result from mechanical handling.

f. Look the plate under the microscope. Visual analysis of the cultures will reveal an admixture of live (trypan blue negative) and dead (trypan blue positive) cells in each experimental condition.

Notice the difference in the number of cells stained blue at each time-point.

g. Add 100 µl of sodium dodecyl sulphate (SDS; 1% w/v) to all the sample wells, untreated wells and blank wells, and mix the contents gently **taking care not to introduce air bubbles**.

h. Take OD (optical density) spectrophotometrically using a plate reader at 590 nm.

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.

Practical Report 2 (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 time points used in experiment on the X-axis. **(7 marks)**

a. Paste histogram here (4 marks)

b. How would you interpret the results presented in this histogram? (3 marks)

2. What is the principle behind trypan blue exclusion Test of cell viability? **(1 mark)**

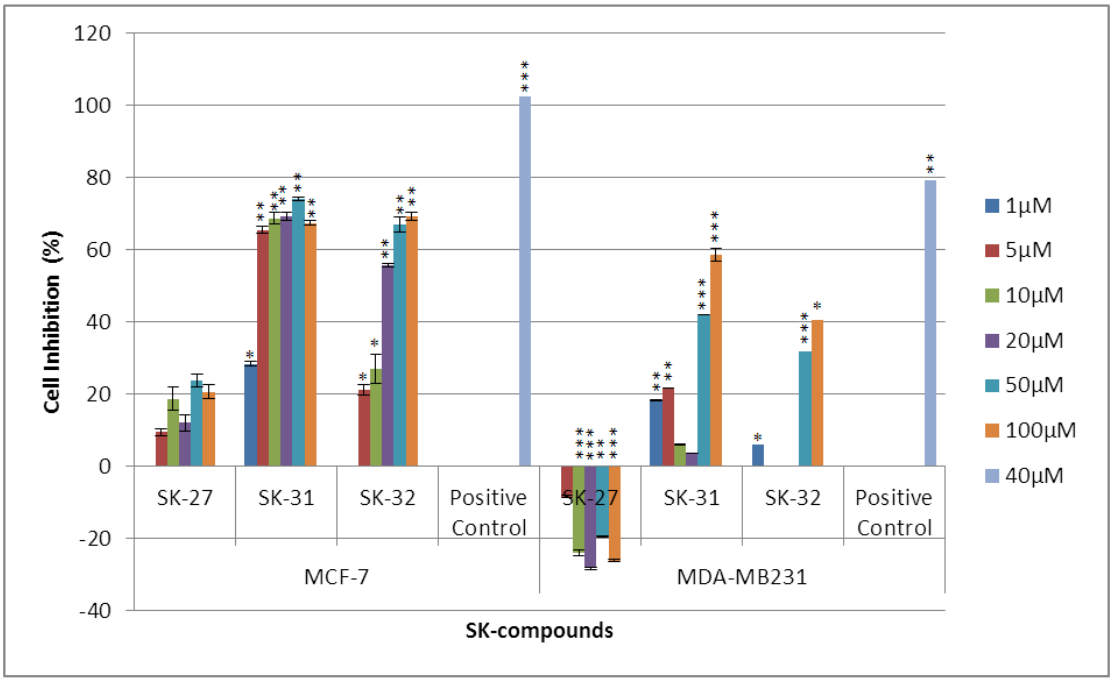
3. You have seen cells under microscope. Answer the following using your observation: **(2 marks)**

a. What is the confluency of cells in the plate? Write your answer in percentage.

b. What is the morphology of the cells that you have observed under the microscope?

4. Why do we need to use a blank and a negative control (untreated sample)? **(2 marks)**

5. In the following diagram, a comparison of different compounds 'SK' compounds is presented in two different cell lines. The cytotoxicity of 'SK' compounds were tested on two different breast cancer cell lines, namely; estrogen receptor positive (ER+) MCF-7 cells and triple negative MDA-MB-231 cells (ER-) using an MTT (3-(4,5-Dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (assay that helps to quantify alive cells). Y-axis shows the percentage of cells inhibited (growth inhibition) and X-axis shows the compounds and the cell lines. The legend on the side shows different concentrations of the each compound tested. **(8 marks)**



a. In which cell line the compounds were most active in general? (1 mark)

b. Compare the activity of each compound in two different cell lines and explain which compound is most active in which cell line? Keep into account the concentrations used while explaining your answers. (2 marks)

c. Out of the three compounds tested, which compound do you think is the most promising lead for further development for treating estrogen-positive breast cancer? How activity of this compound can be increased? Justify your answer. (3 marks)

d. The compound SK-27 is only active in ER+ MCF-7 cells while MDA-MD-231 cells do not respond to SK-27. Based on this observation, explain the possible mechanism of action of this compound in both types of cells. (2 marks)

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.

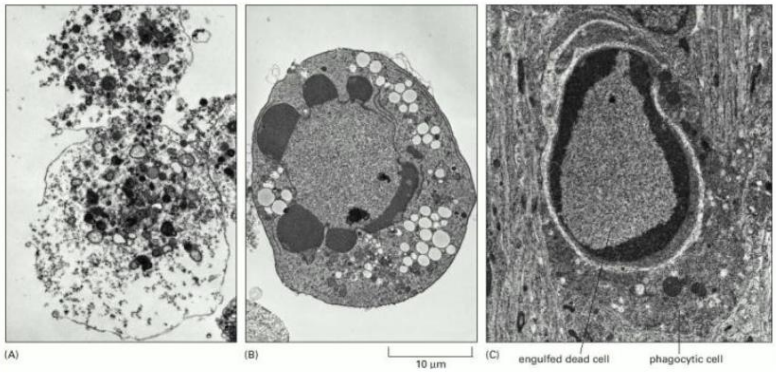


Fig 1: These electron micrographs show cells that have died by (A) necrosis or (B and C) apoptosis. The cells in (A) and (B) died in a culture dish, whereas the cell in (C) died in a developing tissue and has been engulfed by a neighbouring cell. Note that the cell in (A) seems to have exploded, whereas 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

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.

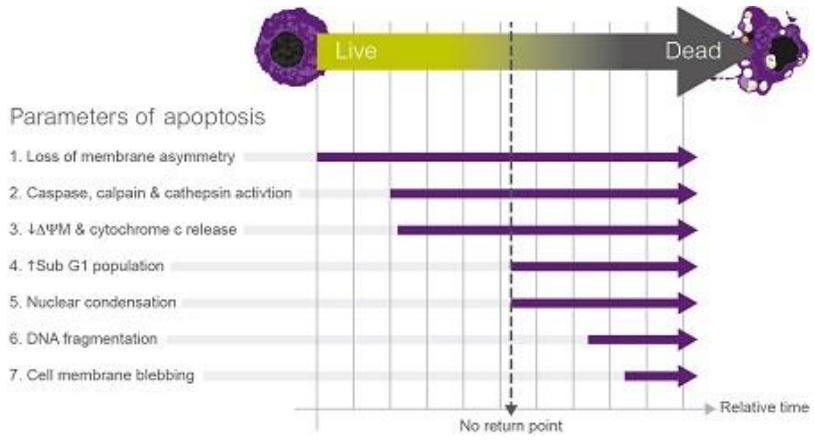


Fig 2

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

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:

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**).

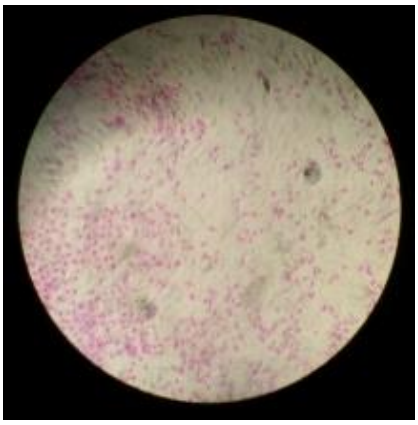
i. Discard PBS

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.

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.

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)**

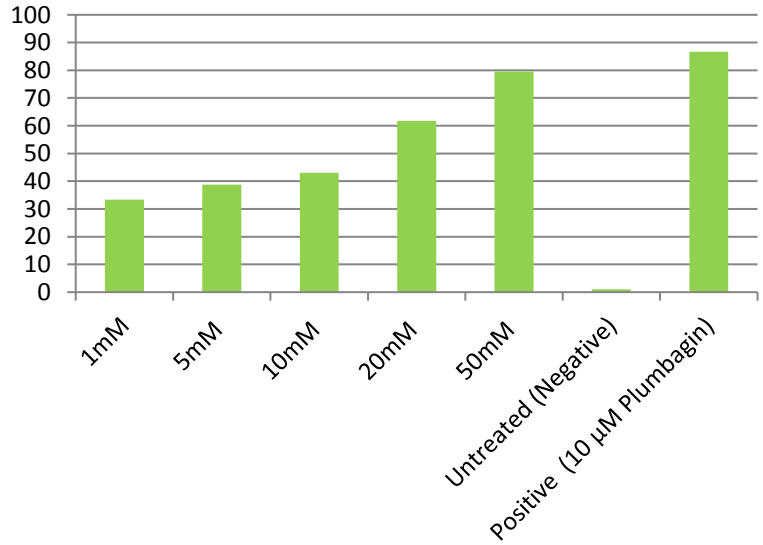
a. About 10% cells are stained pink in the untreated sample.

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

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)**



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)**