Chapter

Cell-Based Assays in Cancer Research

Biba Vikas and Sukumaran Anil

Abstract

Cell growth is referred to as cell proliferation, that is, the increase in cell numbers during repeated cell division. Cell growth can be defined as the enlargement of cell volume, which might take place in the absence of cell division. Growth and reproduction are features of cells in all living organisms. All cells reproduce by dividing into two, with each parental cell giving rise to two daughter cells each time they divide. Various genes are involved in the control of cell division and growth. Reproduction in unicellular organisms are referred to cell division and in multicellular organisms it is tissue growth and maintenance. Survival of the eukaryotes depends upon interactions between various cell types, that helps in the balanced distribution. This is achieved through the highly regulated process of cell proliferation. Knowledge in cell cycle is necessary to determine the best time to collect cells, to harvest cell products, or to move cells to a new growth environment. Cancer cells do not die at the natural point in a cell's life cycle. Cancer cells occur as the results of cellular changes caused by the uncontrolled growth and division of cells. The chapter focuses on cancer cell maintenance, apoptosis, and its detection assays.

Keywords: cancer cell maintenance, apoptosis, cytotoxicity, cell-based assays

1. Introduction

Cell division and the increase in cell quantity is called cell growth. Cell growth happens in favorable nutrient conditions. It is the process by which cells accumulate mass and increase in physical size. The growth of tissues that are not self-renewing occurs by a combination of increase in number and increase in size of the component cells. Cells will progress unimpeded through the cell cycle and divide; one cell will become two, two will become four, four will become eight, and so on. Cellular growth is ensured by the alternation of DNA duplication and cell division cycles [1]. When the cell reaches maximum size, the important point is that the surface area to the volume ratio becomes smaller as the cell gets larger. When the cell grows beyond a certain limit, inadequate material will be able to cross the membrane sufficient to accommodate the increased cellular volume. Increase of size and change in shape of a developing organism depend on the increase in the number and size of cells. The increase in cell number is due to cellular reproductive mechanism called mitosis. Cells are limited to their programmed size because the cell membrane must transport oxygen and food into the cell, as well as transport waste like CO_2 and H_2O out of the cell. As the cell grows, the inside grows faster than the outside. Cellular growth is ensured by the alternation of DNA duplication and cell division cycles. As the cell grows, the inside grows faster than the outside. Cellular growth is ensured

by the alternation of DNA duplication and cell division cycles; through transcription the coupling of cell divisions is taking place in metabolic pathways [2].

Cancer cell growth: cancer is a condition where cells grow uncontrollably in a specific part of the body. Cancerous cells can invade and destroy surrounding healthy tissue and organs. Cancer cells divide relentlessly, forming solid tumors or flooding the blood with abnormal cells. Cell division is a normal process for growth and repair. A parent cell divides to form two daughter cells and is used to build new tissue, or it is used to replace cells that have died because of aging or damage. Healthy cells stop dividing when there is no longer a need for more daughter cells, but cancer cells continue the divisions. They are capable of spreading from one part of the body to another in a process known as metastasis [3]. Cell proliferation and cell death are such diametrically opposed cellular fates that are linked and interdependent processes [4, 5].

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a suitable artificial environment. In vitro assays are performed to check the proliferation that reflects cellular responses to various stimuli. These techniques help to observe cell division and quantity of cells. Cell culture technology shows a good progress in biology and is heavily dependent on cell culture technology. Chemotherapeutic agent phenoxodiol, a synthetic analog of daidzein, a well-known isoflavone from soybean (*Glycine max*) was developed as a therapeutic agent against cervical, ovarian, prostate, renal, and vaginal cancers. They induce apoptosis through the inhibition of antiapoptotic proteins [6]. Apoptotic shrinkage, disassembly into apoptotic bodies, and engulfment of individual cells characteristically occur without associated inflammation. This could then be the consequence of releasing intracellular contents into tissues. This could be the consequence of releasing intracellular contents into tissues; the mitochondria remain unchanged morphologically [7].

1.1 The intrinsic pathway for programmed cell death

The signaling pathway for programmed cell death involves non-receptor-mediated intracellular signals inducing activities in the mitochondria that initiate apoptosis. Stimuli for the intrinsic pathway are caused by viral infections or damage to the cell by toxins, free radicals, or radiation. Damage to the cellular DNA can also induce the activation of the intrinsic pathway for programmed cell death [8]. Proapoptotic proteins activate caspases to mediate the destruction of the cell through different pathways. These proteins translocate to the nucleus of cells, thereby inducing DNA fragmentation which is a hallmark of apoptosis. The members of the Bcl-2 family of proteins and the tumor suppressor protein p53 regulate proapoptotic event in the mitochondria (**Figure 1a**). The Bcl-2 family members of proteins may be pro- or antiapoptotic. Bcl-2, Bcl-x, Bcl-xL, Bcl-XS, Bcl-w, and BAG are the antiapoptotic proteins. These proteins are currently under investigation as potential targets for anticancer therapy. Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk are proapoptotic proteins. The upregulation of these proteins or their increased activation may offer an approach for cancer therapy [9]. Cellular pathways that modulate the activities of the p53 protein are also currently being evaluated as targets for potential anticancer therapies [10].

1.2 The extrinsic pathway for programmed cell death

The extrinsic signaling pathway leads to apoptosis that is through transmembrane death receptors, members of the tumor necrosis factor (TNF) receptor gene super family. Members of this receptor family bind to extrinsic ligands and

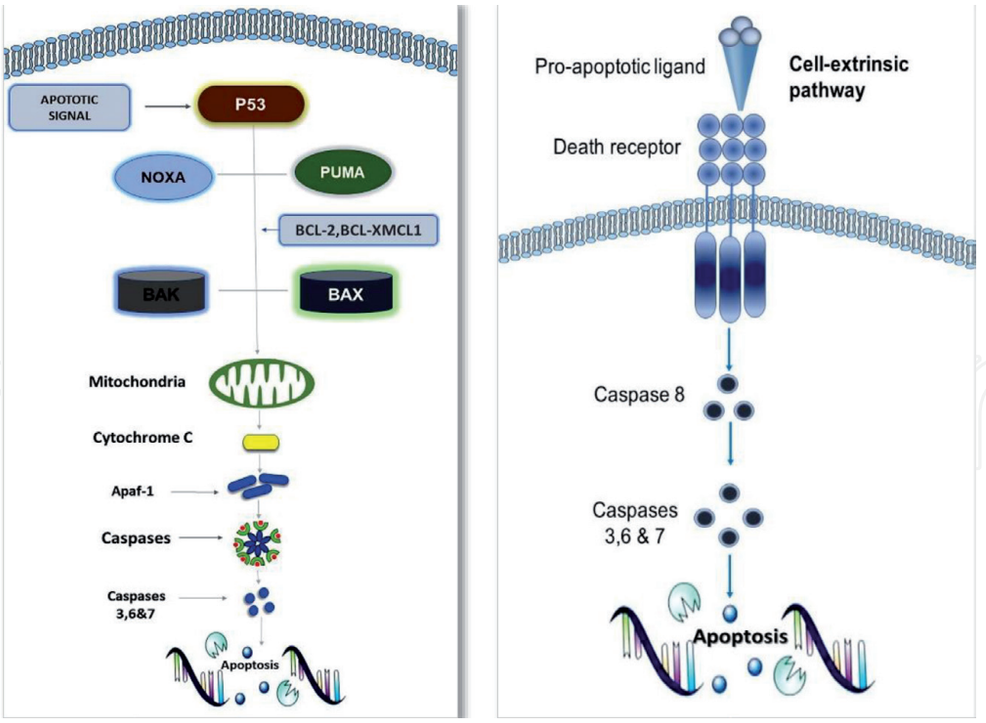

Figure 1.
(a) The intrinsic pathway for programmed cell death. (b) The extrinsic pathway for programmed cell death.

transduce intracellular signals that result in the destruction of the cell [11]. The most well-characterized ligands of these receptors are FasL, TNF-alpha, Apo3L, and Apo2L, and its corresponding receptors are FasR, TNFR1, DR3, and DR4/DR5, respectively [8, 9, 12]. Molecules that stimulate the activity of these proapoptotic proteins or activate these receptors are currently under the evaluation for their therapeutic potential in the treatment of cancer, including hematologic malignancies. The signal transduction of extrinsic pathway involves various caspases which are proteases with specific cellular targets. Once activated, the caspases affect several cellular functions as part of a process that results in the death of the cells [8]. This visible transformation of apoptosis is accompanied by biochemical changes. Those at the cell surface include the externalization of phosphatidyl serine and other alterations that promote recognition by phagocytes. Intracellular changes include the degradation of the chromosomes of the chromosomal DNA into high molecular weight and oligonucleosomal fragments and cleavage of a specific subset of cellular polypeptide [13, 14]. These cleavages are accompanied by a family of intracellular proteases called caspases (**Figure 1b**).

2. 2D and 3D cell culture systems

Cell culture is used in vitro in cell biology, tissue morphology, and mechanisms of diseases, drug action, protein production, and the development of tissue engineering. The stage of the culture after the cells can be isolated from the tissue and proliferates under favorable conditions until they reach confluency. In this stage, the cells have to be passaged or subcultured by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. Various researches in cancer biology are based on experiments using two-dimensional cell culture by growing cells in flat dishes, made of plastic. The dish culture system is mainly used for developing adherent two-dimensional cell monolayers. 3D cell culture applications are usually beneficial in tissue engineering and regenerative

medicine. 3D cell culture is an artificially created environment in which biological cells are allowed to grow and interact with their surroundings in all three dimensions. These three-dimensional cultures are grown in bioreactors, small capsules in which the cells can grow into spheroids, otherwise 3D cell colonies. Approximately 300 spheroids are usually cultured per bioreactor 3D cell culture which allows cells in vitro to grow in all directions, similar to how they would in vivo [15]. An increasing shift in research is occurring, where 3D cell culture systems are replacing 2D cell culture systems and translating 2D in vitro research to 3D before or as an alternative to testing using in vivo animal models [16, 17].

2.1 Preparation of culture media

Culture media provide artificial environment to grow the cancer cells in vitro. Culture media can be prepared by mixing DMEM powder (glucose, L-glutamine, pyridoxine, HCl, without pyruvate) in autoclaved triple distilled water. To this 1.95 g of HEPES buffer and 3.75 g sodium bicarbonate can be added. Antibiotics such as penicillin (500 μl) and streptomycin (500 μl) and fungicide-amphotericin-B (750 μl) can also be added. The volume can then be made up to 1000 ml, and the pH will adjust to 7.2–7.4. The medium will then filter under negative pressure using 0.22 μm cellulose filter. Sterility of the medium can be tested before use. Ten percent FCS can be mixed with the medium prior to culture.

2.2 Maintenance of adherent cancer cell lines

Adherent cell lines will grow in vitro until they form a monolayer over surface area available or medium depleted of nutrients. Adherent cells human oral cancer cells (KB), lung adenocarcinoma (A-549), and breast cancer (MCF-7) can be cultured in tissue culture flasks. The cells were disaggregated by trypsinization and subcultured when the monolayer reaches about 70% confluency. The cells will be cryopreserved at −80°C. With an inverted microscope, the degree of confluency of the cell monolayer can be assessed, and the absence of bacterial and fungal contaminants can be confirmed. Spent medium can be removed. Cells can be washed with PBS-EDTA for removing the traces of serum. Trypsin/EDTA (500 μl) will be applied onto the cell monolayer, and the flask is swirled to cover the monolayer with trypsin. The flask will be incubated at 37°C for 2–3 min. The flask can be examined under the inverted microscope to ensure uniform detachment of the cells. 1–2 ml of medium can be added to the flask as fast as possible to lessen the trypsin-induced stress, and the contents of the flask can be transferred to a centrifuge tube. Cells should be centrifuged at 1500 rpm, for 10 min. The supernatant will be discarded, and the cells were resuspended in minimum volume of medium. Cells can be counted using a hemocytometer, and the required numbers of cells can be subcultured in a new flask containing fresh DMEM with 10% FCS. This process can be repeated as demanded by the growth characteristics of the cell line.

2.3 Maintenance of cancer suspension cell lines

In general terms cultures derived from blood (e.g., lymphocytes grow in suspension) cells may be seen as single cells or clumps. For these types of cell lines, subculturing is done by dilution in small volume of media before counting. The culture can be viewed using an inverted phase contrast microscope; cells growing in exponential phase should be bright, round, and refractive. Cell suspension can be mixed well and dispersed uniformly by repeated pipetting in order to make single-cell suspension. The cells can be counted, and 1×10^6 cells can be seeded to a fresh

bottle containing 10 ml of DMEM medium with 10% FBS antibiotics and incubated at 37°C and subcultured every third day. On the day of the experiment, single-cell suspension will be prepared. The cells will be counted and the viability can be checked. The concentration can be adjusted with the medium containing 10% FBS and antibiotics.

2.4 Establishing cell cultures from frozen cells

About 10 ml of growth medium placed in a 15-ml conical tube. Thaw the frozen cryovial of cells for 40–60 s by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath, and decontaminate the cryovial by immersing it in 70% (v/v) ethanol at room temperature (RT). Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium. Cells can be collected by centrifugation at 2000 rpm for 5 min at RT. Remove the growth medium by aspiration, and then resuspend the cells in the conical tube in 5 ml of fresh growth medium. Add 10 ml of growth medium to a 75-cm^2 tissue culture flask, and transfer 5 ml of cell suspension to the same. Place the cells in a 37°C incubator at 5% CO_2. Monitor cell density daily. Cells can be passaged when it will attain 50% confluency.

3. Determination of cell viability by trypan blue exclusion method

Trypan blue is a dye used to determine the viability of a cell. Living cells exclude the dye, and dead cells will take up the blue dye. The blue stain is easily visible, and cells can be counted using a light microscope. The reactivity is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. When the cells are confluent, remove the cell media through aspiration, and add 5 ml of PBS swirl and aspirate. Then add 2 ml of trypsin/EDTA, and swirl to cover the monolayer of cells. Incubate for few minutes at 37°C. To remove the cells, strike the side of the plate or the flask with the palm. Check under a microscope to ensure that all the cells are dislodged. Add 8 ml of cell media containing fetal calf serum (FCS) to the cells containing the culture flask. The FCS will neutralize the action of trypsin. Transfer the cell suspension to a sterile centrifuge tube, and centrifuge the cell suspension at 1000*g* to pellet the cells. Wash the cell pellet twice with PBS. Resuspend the cell pellet in appropriate volume of PBS or cell media. Dilute 10 μl of cell suspension, and place 10 μl on a hemocytometer, and count the cells under a microscope. There are grid markings on the hemocytometer that can be seen under magnification. Count the cells in all four other quadrants of the grid. Divide this number by four to determine the average number of cells in one quadrant. To calculate the number of cells, multiply the average number of cells per quadrant by dilution factor. Multiply this number by 10,000 to calculate the number of cells in 1 ml of suspension. The equation is as follows: average number of cells per quadrant C dilution factor C 10,000 = number of cells/ml. To calculate the total number of cells, multiply the number of cells per ml by the volume (ml) of the cell suspension.

Calculating the % of viable cells: The cells (10,000) are suspended in 500 μl media and treated with varying concentrations of drug and incubate for 24 h. Centrifuge at 1500 rpm for 10 min. Discard 400 μl medium. Resuspended the pellet in the remaining medium. Mix 0.5 mg of trypan blue in 1 ml PBS. Take 10 μl of cell suspension and mix with trypan blue solution. Incubate for 5 min at room temperature. Count the numbers of unstained cells on the hemocytometer under a microscope. As mentioned above the dead cells will take up the trypan blue stain. First count the blue cells in the field and then white cells. Count the total number of

cells. The percentage of viable cells can be determined by dividing the number of unstained cells by the total number of cells and multiplying by 100. The equation is as follows: % of Cytotoxicity = [No. of blue cells/Total no. of cells] × 10.

3.1 Evaluation of cytotoxicity using MTT assay

The assay detects the living cells, and it be used to measure cytotoxicity, proliferation, or activation [18]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay is based on the principle that mitochondrial dehydrogenase enzyme forms viable cells to cleave the tetrazolium rings of the pale yellow MTT and forms a dark blue formazan crystal which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The solubilization of the cells is by the addition of a detergent that cause in the liberation of the crystals, which are solubilized. The color can be quantified using a multi-well plate reader at 570 nm. The cells can be maintained in DMEM medium, supplement with 10% FCS. Briefly, cells in the log phase of growth can be harvested, counted, and seed (5×10^3 cells/well in 100 μl) in 96 well titer plates (Axygen), and add PBS to the outer wells (200 μl/well). After 24 h of incubation at 37°C in 5% CO_2 to allow cell attachment, media will be removed; cultures will be treated with various concentration of compounds diluted with medium. Cells and media are used as the negative controls. The plates are further incubated for 24, 48, and 72 h. On completion of incubation, with the extract, media can be removed without disturbing the adherent cells. In the case of suspension cells lines, the media can be removed after the plates are centrifuge at 2000 rpm for 15 min. To each well, 100 μl of 5 mg/ml stock solution of MTT dye will be added, and plates can be incubated for 2 h in the dark at 37°C in a CO_2 incubator. About 100 μl of lysis buffer can be added to each well, and the plates can be incubated for 4 h in the dark in a CO_2 incubator and absorbance can be read using ELISA plate reader. Three replicates are set up for each concentration. The concentration required to reduce absorbance by 50% (IC_{50}) in comparison to control cells is determined. In MTT assay colorless well indicates the cytotoxicity of KB human oral cancer cells (**Figure 2**)

$$1\ \%\ \text{of Growth inhibition} = 100 - \frac{\text{Absorbance of treated cells} \times 100}{\text{Absorbance of control cells}} \quad (1)$$

3.2 WST-1 assay

This method is used to detect the cytotoxicity of compound towards various cancer cell lines. The mitochondrial dehydrogenase enzyme cleaves the tetrazolium salt to formazan. The amount of the dye generated by the activity of

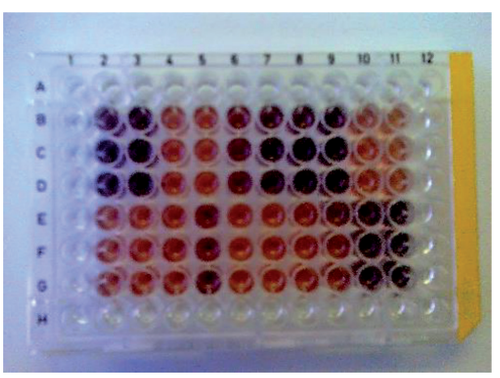

Figure 2.
MTT assay: purple color indicate the viability of cells.

dehydrogenase is directly proportional to the number of living cells. In the WST-1 assay protocol, add the WST-1 assay reagent to the cell culture media, and incubate for between 0.5 and 4 h, shake well. The formazan crystals produced by viable cells can be quantified and can be read in microplate reader at 440 nm. Cellular proliferation represents the ability of healthy cells to divide and create progeny [19]. Therefore, cell viability assays and cell proliferation assays are used to calculate the number of healthy cells in a population or the rate of growth of a population of cells [20].

3.3 MTS assay

MTS is a colorimetric method used to quantify viable cells in proliferation assay. The NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells reduce the MTS tetrazolium compound and form colored formazan product that is soluble in cell culture media. It can be used to test cell proliferation, cell viability, and cytotoxicity. The formazan dye can be quantified by measuring the absorbance at 490–500 nm.

4. Viability assay in normal cells

Attempts are pursued to develop drugs that are nontoxic to normal cells; meanwhile toxic to cancer cells can be considered as good anticancer agent.

4.1 Isolation of lymphocytes from whole blood

In 1968, Boyum [21] described methods for the isolation of mononuclear cells from circulating blood and bone marrow. The solution contains Ficoll and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001. This medium facilitates rapid recovery of viable lymphocytes from small volumes of blood on centrifugation. It is usually employed as the initial isolation step prior to enumeration of T, B, and null lymphocytes. In brief, 3 ml of blood will be taken in heparinized test tube. About 5 ml of PBS will be added and mixed well by inversion. About 3 ml of Ficoll Hypaque solution was added in a conical centrifuge tube. Carefully layered 8 ml of blood-PBS mixture on to the Ficoll Hypaque, keeping the tube in a slanting position. Centrifuged at 2000 rpm for 30 min. The opaque interface containing mononuclear cells was aspirated and transferred into a clean conical centrifuge tube with a Pasteur pipette and discard the upper layer. About 10 ml of PBS solution was added and mixed by inversion. Centrifuged at 1500 rpm for 10 min and the supernatant was discarded. Resuspend the pellet with 5 ml PBS and centrifuge at 1500 rpm for 10 min. Repeat the step thrice and resuspend the lymphocyte pellet in 500 μl PBS.

4.2 Lymphocyte viability assay

In vitro experiments with compounds can be tested in lymphocyte using lymphocyte viability assay. The lymphocytes were aspirated from the gradient plasma interfaces and washed twice in PBS, and then the final cell pellets will be resuspended in RPMI-1640 medium containing 10% FCS and 100 μl streptomycin, and 100 μl fungicide can be added to avoid contamination; pH 7.4 is ideal. Cells can be harvested, counted, and seeded (5×10^3 cells/well in 100 μl) in 96 well titer plates (Axygen). PBS will be added to the outer wells (200 μl/well). After 24 h of incubation at 37°C in 5% CO_2 to allow cell attachment, the media will be

removed. Cells will be treated with varying concentration of compounds diluted with medium. The plates can be incubated for 72 h. After incubation, the media will be removed without disturbing the cells, and to each well, 100 μl of 5 mg/ml stock solution of MTT will be added, and the plates can be incubated for 2 h in the dark at 37°C in a CO_2 incubator. About 100 μl of lysis buffer will be added to each well, and the plates can be further incubated for 4 h in the dark in a CO_2 incubator, and absorbance can be read using ELISA plate. Three replicates will set up for each concentration.

5. Detection of apoptosis by comet assay

Comet assay can be done for the quantitation of low levels of DNA damage in individual [22]. Cancer cells except the control cells can be treated with various drugs. Then it can be centrifuged to get the pellets, and pour 1% NMPA agar on the base slides using filler pipette and allow to solidify. Then the slides were kept in a polar ice pack. The pellets thus obtained will be taken and is mixed with 200 μl of 0.5% LMPA agar. About 15 0 μl cells from the above tubes were taken and poured over the base slides, and allow to solidify after placing a cover slip over it, and then remove the cover slip and pour a layer of 1% LMPA agar over it, and allow to solidify and keep these slides in a coupling jar containing lysing solution. It will be kept in refrigerator for 1:30 h, and subject it to electrophoresis for 20–30 min in electrophoresis apparatus at 25 V. Then the slides were taken and washed with neutralizing buffer solution for three times. Pour ethidium bromide stain over the slides, and view through fluorescent microscope. The figure shows the comet assay on KB human oral cancer cells after treatment with compound for 24 h (**Figure 3**).

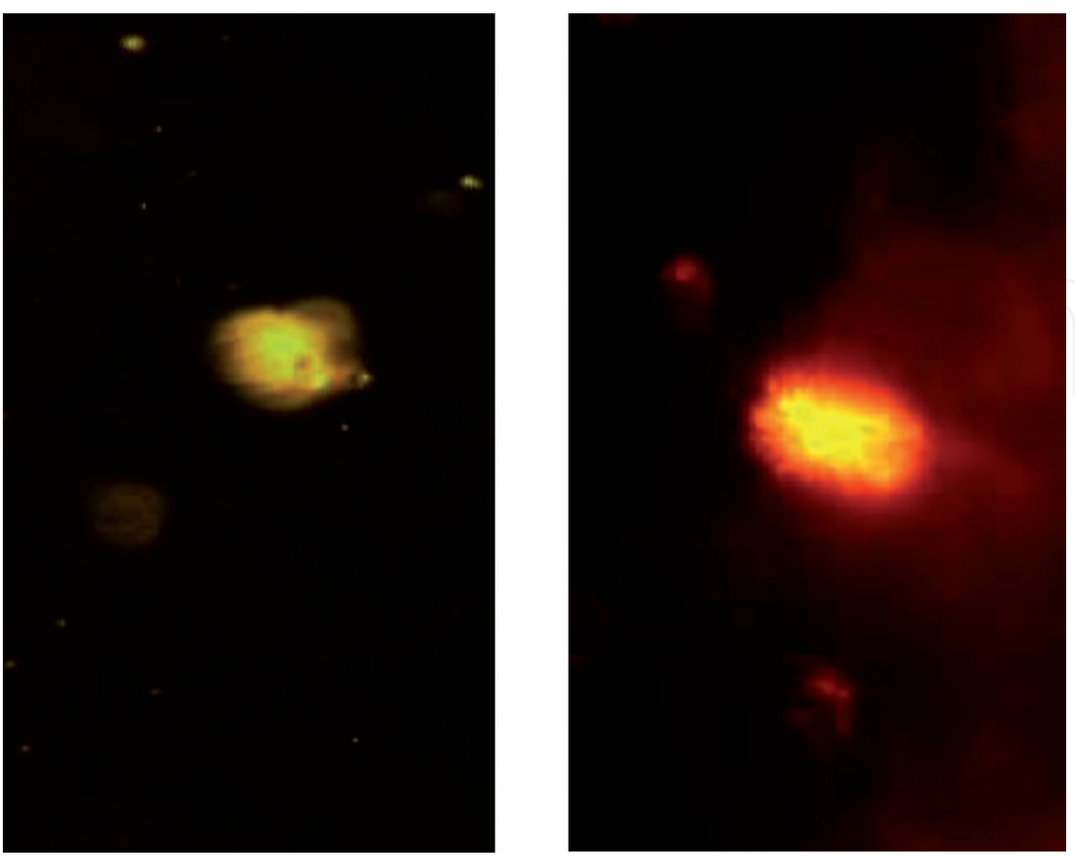

Figure 3.
Comet assay: (a) non-apoptotic cells, (b) comet-shaped apoptotic cells.

5.1 DNA ladder assay

Breakdown of genomic DNA into multiples of approximately 180 bp is considered to be a hallmark of apoptosis [14]. This cleavage of chromosomes produces a large number of DNA breaks, and subsequently a simultaneous amount of new 3′-OH DNA ends. In normal living cells, only an insignificant number of 3′-ends are present; this helps to detect apoptosis. The enzyme terminal deoxynucleotidyl transferase (TdT) has the capability to incorporate individual deoxyribonucleotide triphosphates to the 3′-end of double- or single-stranded DNA. This quality can be detected using 3′-ends with nucleotides that have been labeled with radioactive, fluorescent, or digoxigenin labels. Apoptosis can be measured quantitatively by using gel electrophoresis; here the apoptotic DNA is organized into a typical ladder pattern of multiples of 180 bp. In situ labeling of 3′-ends can be used to detect qualitatively apoptotic cells (**Figure 4**).

5.2 Detection of morphological features of apoptotic cells by acridine orange-ethidium bromide dual staining

The morphological features of apoptosis induced by the compounds will be evaluated by acridine orange-ethidium bromide dual (AO/EtBr) staining [23]. Briefly, cells

Figure 4.
Apoptotic cells can be seen as fragmented DNA in smear appearance.

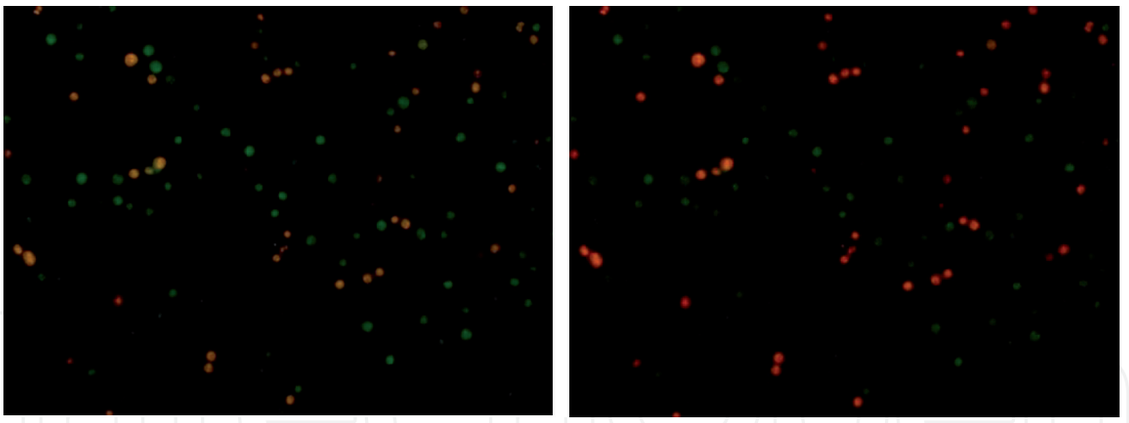

Figure 5.
Acridine orange-ethidium bromide dual staining: (a) and (b) apoptotic cells in orange color, live cells in green color.

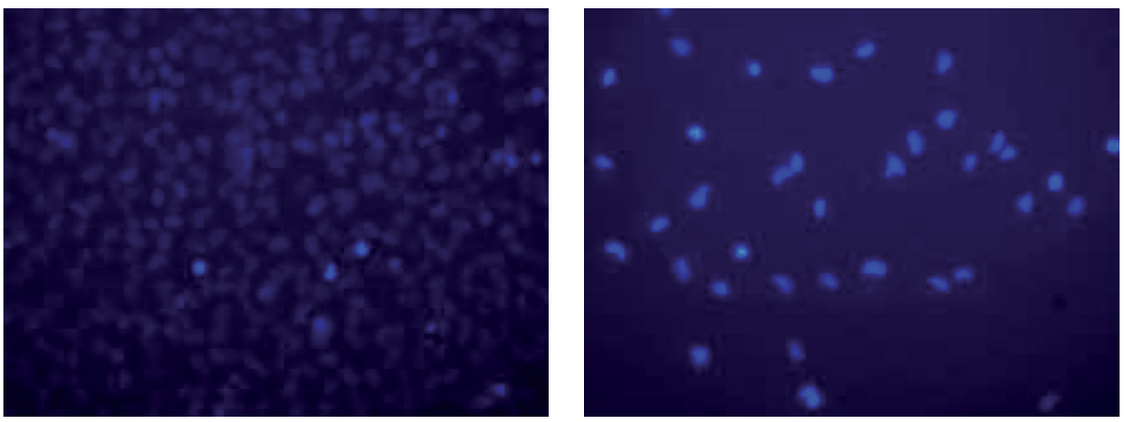

Figure 6.
Hoechst 33342: (a) non-apoptotic cells in blue color, (b) apoptosis cells in fluorescent blue color.

will be seeded in a 96 well plate at a density of 5×10^5 cells and treat with different concentrations of compound for 24 h. After washing once with PBS, the cells can be stained with 100 μl of a mixture (1:1) of acridine orange-ethidium bromide (4 μg/ml) solutions. The cells can be immediately washed with PBS and observed under fluorescence microscope at 450–490 nm. The effects of the compound treated on human oral cancer cells (KB) for 24 h can be visualize using fluorescent microscope (**Figure 5**).

5.3 Analysis of cell death by Hoechst 33342 staining

Chromatin condensation will be assessed by nuclear staining with Hoechst 33342 [24]. Briefly, cells will be seeded in a 96 well plate at a density of 5×10^5 cells and then treated with different concentrations of compounds for 24 h. After washing once with PBS, the cells will be stained with 100 μl of Hoechst 33342 (10 mg/ml stock) and incubate at room temperature for 5 min. Stained cells can be imaged by fluorescence microscope at 350–460 nm compound induced in KB human oral cancer cells for 24 h, stained with Hoechst 33342 (**Figure 6**).

5.4 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

After treatment with the compound apoptosis, induction can be detected by TUNEL assay using the DeadEnd apoptosis detection kit (dUTP nick-end labeling

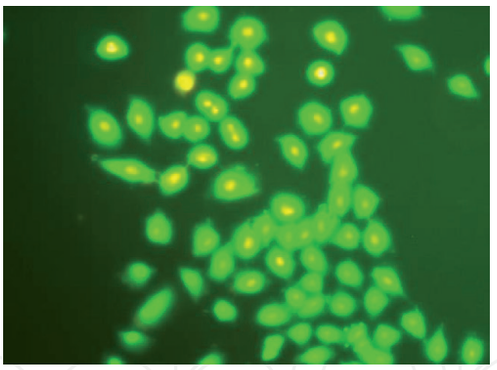

Figure 7.
TUNEL assay.

TUNEL assay) from Promega (Madison, USA). Briefly, the cells will be grown in coverslips and treated with the compound for 24 h. The cells can be washed with phosphate-buffered saline and fix by immersing the slides in 4% paraformaldehyde for 25 min at room temperature, washed twice with PBS for 5 min. The cells will be permeabilized with 0.2% Triton X-100 solution in PBS for 5 min, washed twice in PBS, and then covered with 100 μl of equilibration buffer and kept for 5–10 min. The equilibrated areas will be blotted around with tissue paper, and 100 μl of terminal deoxynucleotidyl transferase (Tdt) reaction mix will be added to the sections on the slide and incubated at 37°C for 60 min inside a humidified chamber for the end labeling reaction to occur. Immersing the slides in 2× sodium chloride-sodium citrate buffer for 15 min terminated the reactions. The slides will be washed thrice by immersing in fresh PBS for 5 min to remove the unincorporated biotinylated nucleotides. The endogenous peroxidase activity was blocked by immersing the slides in 0.3% hydrogen peroxide. After washing, horse radish peroxidase-labeled streptavidin solution was applied, and the slides incubated for 30 min. After incubation, the color will be developed with the peroxidase substrate (hydrogen peroxide) and the stable chromogen (diaminobenzidine). The slides will be mounted and examined in a light microscope. The apoptotic index (AI) can be calculated as follows: AI = (number of apoptotic cells/total number) × 100%. TUNEL can also be combined with annexin V to comprise a more robust assay that is capable of distinguishing apoptosis and necrosis going on in the cells. Since annexin V binding is reported to occur prior to DNA fragmentation, it is capable of detecting necrotic or early apoptotic cells that exhibit a negative response from TUNEL [25]. In KB human oral cancer cells treated with compound for 24 h, apoptotic cells can be seen in green color (**Figure 7**).

5.5 Annexin V staining by flow cytometry

In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. Annexin V is a 36 KDa phospholipid-binding protein and has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}). Apoptotic cells which are otherwise undetectable by staining with propidium iodide (PI) can be directly detected through the staining with fluorochrome-conjugated annexin V. Dead cells are stained with both annexin V and PI, whereas viable cells cannot be stained with either annexin V or PI. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid. Phosphatidyl serine from the cytoplasmic interphase translocated to the extracellular surface. This loss of membrane asymmetry was detected using the binding properties of annexin V to phosphatidyl serine. Annexin V FITC is a sensitive probe for identifying the

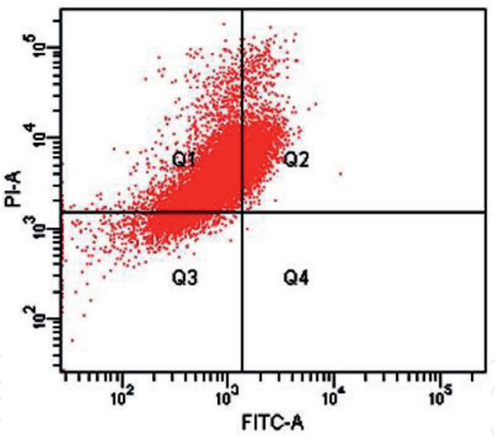

Figure 8.
Apoptotic KB human oral cancer cells after treatment with compound showing apoptosis.

apoptotic cells which binds to negatively charged phospholipid surfaces. Annexin V FITC staining precedes the loss of membrane integrity which accompanies the later stages of cell death resulting from apoptotic or rather necrotic process. Therefore, staining with annexin V FITC is conjugated to vital dye propidium iodide. The assay distinguishes between viable cells (annexin V –ve, PI –ve), early apoptotic cells (annexin +ve PI –ve), late apoptotic cells (annexin +ve PI +ve), and necrotic cells (annexin –ve PI +ve) (**Figure 8**). Annexin V is represented in red. An apoptotic cell stained with annexin V (green) is surrounded by potential phagocytes. The PSR (orange) might bind to exposed PS that is configured in a recognizable "eat-me" form by annexin I (bottom) (**Figure 8**). Alternatively, PSR might bind to a ligand composed of PS and annexin I (upper left) or PSR might bind annexin I, which serves as a bridge between exposed PS on the dying cell and PSR on the phagocyte (upper right) [26].

5.6 Assessment of caspase 3 expression by flow cytometry

Caspase-3 is a key protease that is activated during apoptosis. Briefly, the cells will be treated with various concentrations of compound. After 24 h of incubation, the cells will be washed twice with cold PBS and prepared for acquisition using FITC conjugated monoclonal active caspase-3 antibody apoptosis detection kit. The cells can be fixed in cytofix solution at a concentration of 1×10^6 cells/0.5 ml. The cells will be fixed in ice for 30 min, resuspended in perm wash buffer containing antibody, and incubated for 30 min at room temperature. Analyses by flow cytometry. 10,000 cells can be acquired, and the results can be interpreted using DIVA software analysis (**Figure 9**).

5.7 Determination of caspase activity by fluorimetry

The ApoAlert caspase assay plates contain the fluorogenic substrates specific for different caspases immobilized in the wells of a 96-well plate. When cell lysate containing the active caspase is applied to the wells, caspase will cleave its substrate, and a fluorescent product will be released that can be detected with a standard fluorescence plate reader. Caspase assay plates enable the analysis of the apoptotic caspase response. This assay design is ideal for studies involving multiple cell types or multiple cell treatments. These plates are provided in two formats: a single caspase format for studies that focus on a specific caspase or a profiling format for analyzing several different caspases simultaneously (caspase-3, caspase-8, caspase-9, and caspase-2).

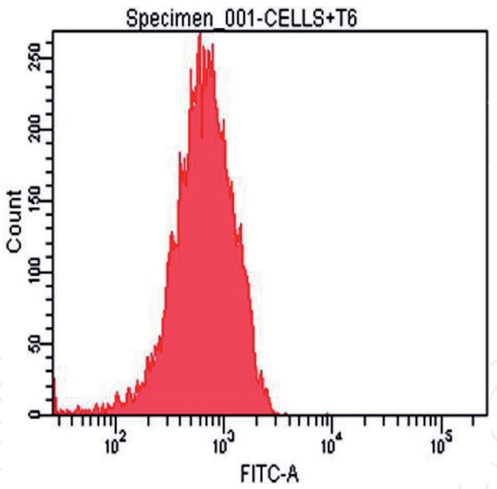

Figure 9.
Caspase 3 cleavage of KB human oral cancer cells.

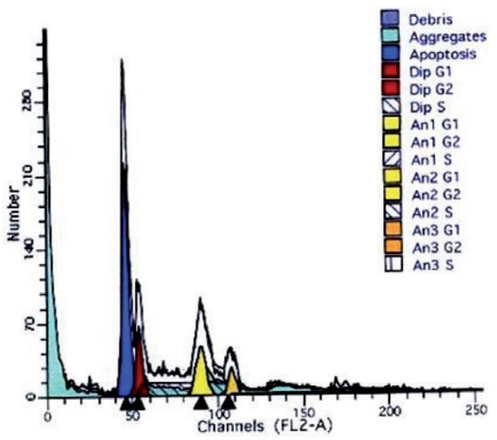

Figure 10.
Cell cycle analysis showing KB human oral cancer cells.

5.8 Detection of apoptosis by cell cycle analysis

The phase of the cell cycle at which compounds treated cancer cells got arrested can be determined using a fluorescent-activated cell sorter (FACS—Becton Dickinson). The cells will be grown in tissue culture flasks and treat with different concentrations of compounds. After 24 h of treatment, the cells can be harvested and spun down at 3500 rpm for 7 min. The cells can be then fixed in 70% ethanol for 30 min. After centrifugation, the pellet can be dissolved in PBS, and 5 μl of RNAse A (10 mg/ml) will be added and incubated for 30 min at 37°C. About 10 μg/ml of propidium iodide will be added, and after 15 min of incubation in the dark, the cells can filtered through 0.75 μm filter and analyzed by flow cytometry. Thirty thousand cells can be acquired, and the results can be interpreted using CellQuest Pro software analysis (**Figure 10**).

6. Conclusions

An ideal compound should possess no toxic effects on normal human lymphocyte but at the same time exhibited cytotoxic activity on tumor cell lines. The time- and dose-dependent cytotoxic effect of the compound can be tested through

the various viability assays discussed in this chapter. The compound might have the potential to induce programmed cell death in cancer cells and can be confirmed through apoptotic studies. Induction of apoptosis in cancer cells is recognized as an efficient strategy for cancer chemotherapy. Apoptosis also seems to be a reliable marker for the evaluation of potential agents to bring out for cancer prevention. Cell-based assays are useful for the assessment of live cells and apoptotic cells after treatment with therapeutic agents. The efficacy of compounds in vitro testing before entering into the clinical trials helps to bring out potent drugs into the limelight for the treatment of diseases. Cell-based assays are useful for evaluating therapeutic potency of the developing approved drugs and biologics for the clinical management of cancer.

Conflict of interest

There are no conflicts of interests.

Acronyms and abbreviations

AO/EtBr	acridine orange-ethidium bromide dual staining
KB	human oral cancer cell lines
KDa	kilodalton
FACS	Fluorescent-activated cell sorter

Author details

Biba Vikas[1*] and Sukumaran Anil[2]

1 Jawaharlal Nehru Tropic Botanical Garden and Research Institute, Trivandrum, India

2 Department of Dentistry, Hamad Medical Corporation, Doha, Qatar

*Address all correspondence to: bibavikas@gmail.com

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