

Cytotoxic assays for Lung and Colon cancer

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Summary

Cancer is difficult to conquer in all living systems. Despite decades of work at the clinical and the research level, a cure for certain types of cancer is still years away and cancer incidence continues to grow. Colorectal cancer and Lung cancers are the leading cause of cancer death in both men and women, and its annual incidence and mortality rates have both risen over the past 25 years. Despite the availability of so many treatment option including chemotherapy, radiotherapy, surgery, or use of medicines the side effects of the therapy remains unavoids. Hence in such cases the treatment should be without side effects. For that whatever new entity is discovered should be checked for their potency against cancer. Natural product represents reservoir of diverse templates and are being tapped to outsource novel anticancer agent. Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. It helps to screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical. This review summarizes the methods of screening of newly discovered agent having anticancerous potential so that they can be used for targeting the more specific colon and lung cancers.

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Introduction

Cancer causes significant morbidity and mortality and is a major health problem worldwide. Colon cancer and lung cancer are the most frequently diagnosed malignancies in the world. The yearly incidence of colon cancer is estimated to be 1.0 million, and approximately 500,000 people die as a result of colon cancer worldwide. The American Cancer Society estimates that 219,440 new cases of lung cancer in the U.S. will be diagnosed and 159,390 deaths due to lung cancer will occur in 2009. These chronic cancers are leading cause of cancer death. Thus; these cancers are worldwide disease and needs to be addressed seriously. [1, 2, 3, 4.] Medicines derived from plants have played a pivotal role in health care of ancient and modern cultures. Almost 60% of drugs approved for cancer treatment are of natural origin. [5] Therefore there is an urgent need to develop alternative therapeutic measures against this deadly disease. There is always the hope that the search among the traditional medicinal plants may provide potent and safe medicines. Significant steps have taken by the WHO to carry out research on plants that work having greater activity and few or no side effects. [6] Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. It helps to screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical. [7] Classical cancer invasion assays have been carried out in tissue culture for decades, primarily to generate information about the relationship between cell motility and invasion. However, a number of these techniques are encumbered with problems of quantification, reproducibility, and flexibility. Not surprisingly, such assays often produce crude quantitative data, since they are typically difficult to standardize and reproduce. Thus there is an increasing need for a standardized, flexible, objective invasion assay with high-resolution for inspection of individual cells that can provide quantitative spatial information in a timely manner. [8]

Materials and Methods

Cytotoxicity Assay's of lung Cancer

1) Lipid Peroxidation Assay^[9]

Aim-Arsenic trioxide mediated cytotoxicity and oxidative stress, lung carcinoma cell lines.

Cell line used-Human lung carcinoma cell line (A549).

Chemical-fetal bovine serum (FBS), penicillin/streptomycin/fungizone, phosphate buffered saline (PBS) and trypsin versene.

Cell Culture-

Cells were maintained in F12-K medium supplemented in 10 % FBS and 1% penicillin, streptomycin and fungizone mixture, as adherent cells. The cells were grown in a humidified incubator under an atmosphere of 95% air and 5% CO₂ at 37°C to sub-confluence (80-95%). The culture medium for each cell line was replaced every 48 hours. After growing to 80-95% confluence, the medium was aspirated off and the cell monolayer was washed three times with sterile phosphate buffered saline (PBS). The cell monolayer was treated with 1 ml trypsin versene per plate and incubated briefly at 37°C. The cells were then viewed microscopically to ensure a complete cell detachment.

Cells were re-suspended in F-12K complete medium for A549, stained with 4% trypan blue (1 to 2 minutes), and counted with a hemocytometer. The cells were seeded at a density of 5×10^5 cells in 13 x 100 mm tissue culture plates, prior to arsenic trioxide treatment.

Assay-

Cell lines were seeded at a density of 3×10^5 cells in 13 x 100 mm tissue culture plates. The cells were then grown in a humidified incubator under an atmosphere of 95% and 5% CO₂ at 37°C, to 75% confluence. The medium was aspirated from the cell monolayer and treated with 4 ml of various doses of arsenic trioxide (0, 4, 6, 8, 10 µg/ml). The experiment was carried out in triplicates. The control was grown in the absence of As₂O₃. After 48 hr exposure time, the treatments were removed and the cells were removed from the plate by scraping. The cells were collected and resuspended in 1 ml of PBS, and were washed three times with PBS pH 7.4. The cell lysis was by homogenization and sonication. The lipid peroxidation assay was performed according to the manufacture protocol; Optical densities were read at 586 nm on a Varian Cary 300 Bio UV-Visible spectrophotometer.

Conclusion

In this study, Arsenic Trioxide is acutely toxic to Lung carcinoma cells (A549). There was a time and dose-dependent response with regard to arsenic trioxide toxicity to the two cell lines. A biphasic response was obtained showing a slight increase in cell viability within the dose range of 0-0.4 µg/ml in cell, followed by a gradual decline. The median lethal doses (LD50s) of Arsenic trioxide for MCF-7 cells were 21.5, 18.4, 15.3 µg/ml upon 18, 24, and 48 hr treatment, respectively. LD50 values for cells were 32.24, 25.79 and 11.5 µg/ml upon 18, 24, 48 hr treatment, respectively. This study demonstrated that cytotoxicity differs in human cell lines, indicating that some cell types are moresensitive than others. The cytotoxicity occurred at higher levels of exposure whereas proliferation occurred at lower doses.

2) MTT Assay^[10]

Aim- To determine Cytotoxic activity of extract of *Marchantia Convolutia* on human lung cancer cell lines

Cell line used- Human non-small cell lung carcinoma cell lines H1299.

Chemical- phosphate buffered saline (PBS), 0.025% trypsin-EDTA,

Preparation of plant extracts-

The powdered plant material (500 g) was extracted three times with 50% ethanol (v/v) (3000 ml). The accumulated alcoholic extract was concentrated to dryness under reduced pressure and extracted with petroleum ether (3×100 ml). The residue was extracted three times with ethyl acetate (3 ×100 ml). Then the residue was extracted three times with n-butanol (100 ml). The filtrate was concentrated to dryness under reduced pressure. The residues were dissolved in ethanol to form stock solutions sterile filtered with filters (0.2 µm) before testing.

Assay-

Cells were washed with phosphate buffered saline (PBS) free of Magnesium and Calcium. The PBS was decanted and cells detached with 0.025% trypsin-EDTA (Sigma)

and PBS was added to a volume of 50 ml. The cell pellet, obtained by centrifugation was suspended in 10 ml of medium to make a single cell suspension, viable cells density being counted by trypan blue exclusion in a haemocytometer and then diluted with medium to give the previously-determined optimal plating densities for H1299. 100 μ l/well of these cell suspensions were seeded in 96-well microtiter plates and incubated at 37°C to allow for cell attachment. After 24 h the cells were treated with the extracts. Each extract (500 μ g/ml) was tested initially against cancer cell lines. The active extracts were considered to be those which gave less than 50% survival at 72 h. The active extracts were further diluted in medium to produce 8 concentrations of 15, 30, 40, 50, 100, 200 μ g/ml of each extract. 100 μ l/well of each concentration was added to the plates in six replicates. At the end of exposure time, the medium was removed and then 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were performed by the cell titer kit TM (Promega) following the standard procedure. 20ml of MTT (5 mg/ml) in PBS was incubated with cells in a 96-well plate for 2 h at 37°C. Subsequently, the medium containing MTT was removed, and 100 μ l of acidified isopropanol (0.04 N HCl) added. Spectrophotometric absorbance of each sample was measured at 470 nm using a microplate reader (Bio-Rad, model 3550). The data's were normalized (A570 nm). The mean absorbance was plotted against drug concentration. Three replicate plates were used to determine the cytotoxicity of each extract. The concentration of each extracts reduced cell survival by 50% (IC50) was determined from cell survival curves. The effect of all extracts on different cell lines was studied by measuring cell numbers by MTT assay after treatment of the cultures with each extract for 72 h. The treatment of all cell lines with ethyl acetate extract decreased cell numbers. However, both petroleum ether and n-butanol extracts did not inhibit cell proliferation. As indicated in treatment of H1299 and HepG2 cells with ethyl acetate extract resulted in loss of cell viability. However, H1299 and HepG2 cells more resistant to Petroleum ether and n-butanol extracts, when compared to their corresponding IC50 of H1299.

Conclusion

The treatment of all cell lines with ethyl acetate extract decreased cell numbers. The results obtained in this study indicated that extracts of *Marchantia Convoluta* was preferentially active against cancer cells. The ethyl acetate extract of *Marchantia Convoluta* were the most cytotoxic against human non-small cell lung carcinoma cell.

3) SULFORHODAMINE –B ASSAY (SRB ASSAY)

i) Aim- To determine cytotoxic activity of *Pulsatilla koreana* on human lung cancer cell lines^[11]

Cell line used- Lewis lung carcinoma (LLC) cells.

Chemical- fetal bovine serum, sodium bicarbonate, penicillin G, and streptomycin at 37°C under a humidified atmosphere of 5% CO₂.

Preparation of plant extracts-

The powdered roots of *Pulsatilla koreana* (50 g) were extracted three times with 50 % aqueous EtOH (500 mL) and the resulting extracts were combined and concentrated in vacuo to yield a light brown residue (22 g). The residue was suspended in acetone (300 mL), and successively centrifuged (3,000 rpm), and the resulting supernatant was

removed to give a brown precipitate. The precipitate was poured into water (100 mL) and filtered to remove the insoluble part. The filtrate was concentrated to give a brown mass.

Cell Culture-

The cell lines were maintained as a monolayer in RPMI1640 media supplemented with 10% fetal bovine serum, sodium bicarbonate, penicillin G, and streptomycin at 37°C under a humidified atmosphere of 5% CO₂.

Assay-

Viable cells were seeded in the growth medium into 96 well micro titer plates (3-4×10⁴ cells per each well) and allowed to attach overnight. The test sample was dissolved in DMSO and adjusted to a final sample concentration ranging from 0.3 µg/mL to 10µg/mL by a dilution with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was then adjusted to <0.1%. After 72 h incubation, the medium was removed and the remaining cells were fixed with 10% trichloroacetic acid (TCA) for 1 h at 4°C. The TCA-treated cells were washed extensively with water and dried in air. Subsequently, 50 µL of the SRB solution (0.4% in AcOH) was added to each well at room temperature. After standing for 1 h, the wells were washed 3-4 times with 1 % AcOH and dried in air. The bound dye was dissolved in Tris base (100 µL of 10 mM). The absorbance of the Tris solution was measured using a micro-plate reader at 520 nm. The ED₅₀ value was defined as the *Pulsatilla saponin D* concentration needed to reduce the absorbance relative to the vehicle-treated controls by 50%.

Conclusion

The *Pulsatilla saponin -D* showed moderate cytotoxic activity (ED₅₀, 6.3 µg/mL to >10 µg/mL) against the cancer cell lines. It was found to be a main antitumor component of the roots of *P. koreana*.

ii) **Aim-** To determine cytotoxic activity *Thai medicinal plants* of on human lung cancer cell lines^[12]

Cell line used- large cell lung carcinoma (CORL-23).

Preparation of plant extracts-

Plant materials were dried at 50°C, powdered and extracts obtained by methods similar to those practised by Thai traditional doctors, e.g. water extraction and ethanolic extraction. In brief, for For ethanolic extraction, dried ground plant material (100 g) was percolated with 95 %ethanol for 3 days. The ethanolic extracts were then filtered and concentrated to dryness under reduced pressure. The ethanolic extracts were dissolved in DMSO to make a stock solution, sterilized by filtration (pore size as 0.2 µm) before testing.

Assay-

Cells were fixed by layering 100 ml of ice-cold 40% trichloroacetic acid on top of the growth medium. Cells were incubated at 4°C for 1 hour, after which plates were washed five times with cold water, excess water drained off and the plates left to dry in air. SRBstain (50 µl; 0.4% in 1% acetic acid) was added to each well and allowed to be in contact with the cell for 30 minutes. Subsequently, to remove excess dye, they were washed with 1% acetic acid, rinsed 4 times until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base [tris (hydroxy methyl) aminomethane, pH 10.5] (Sigma,) was added to each well to solubilise the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (6 replicate) was read on a Power Wave X plate reader at 492 nm. Cell survival

was measured as the percentage of the absorbance compared with the control (non-treated cells). The IC₅₀ values were calculated from the Prism program obtained by plotting the percentage of surviving cells versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines the extracts with IC₅₀ values < 20 µg/ml were considered “active”. The IC₅₀ of active plants against cancer cells were compared with IC₅₀ of normal cells and calculated by the student t-test using InStat program.

Conclusion

The result obtained in this work indicated that a third of the studied plants, which were the ingredients of Thai folk medicine to treat cancer patients, were active against cancer cells. The ethanolic extracts of *Dioscorea membranacea* rhizomes are the most cytotoxic against lung cancer cell line and showed no cytotoxicity against the normal cell line.

4) MTT COLORIMETRIC ASSAY ^[13]

Aim- To determine cytotoxic activity of Functionalized N-(2-oxyiminoethyl) Piperazinyl Quinolones on human lung cancer cell lines.

Cell line used - Human lung carcinoma cell line (A549).

Chemical- Dimethyl sulfoxide (DMSO), MTT Solution.

Cell Culture- The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 100 µg/mL streptomycin and 100 µg/mL penicillin. Each well and the plate were incubated for another 1h. After incubation, the culture medium was replaced with 100 µL of DMSO.

Assay- cultures in the exponential growth phase were trypsinized and diluted in complete growth medium to give a total cell count of 5×10^4 cells/mL. One hundred microliter of suspension was added to wells of sterile 96-well plates. After plating, 50 µL of a serial dilution of every agent was added. Each compound dilution was assessed in triplicate. Three wells containing only tumor cells suspended in 150 µL of complete medium were used as controls for cell viability. The plates were then incubated for 72 h. After incubation, 30 µL of a 5 mg/mL solution of MTT was added to each well and the plate was incubated for another 1h. After incubation, the culture medium was replaced with 100 µL of DMSO. Then, the absorbance of each well was measured by using a microplate reader at 492 nm wavelengths. For each compound, dose-response curves for each cell line were measured with different drug concentrations, and the concentration causing 50% cell growth inhibition (IC₅₀, equating to cytostatic activity) compared with the control were calculated. For each agent, the overall mean IC₅₀ was determined, and that was the mean of the values for all cell lines.

Conclusion

The IC₅₀ values of compounds against tumor cell lines indicate that all these compounds possessed poor activity.

5) MTS ASSAY ^[14]

Aim- To determine cytotoxic activity of airborne particulates sampled roadside in Rodent on human lung cancer cell lines.

Cell line used - Chinese hamster lung fibroblasts.

Chemical- Earle's salt, 10% fetal bovine serum (FBS), DMSO, Phosphate buffered saline.

Cell Culture Cell line were grown in a minimum essential medium with Earle's salt (MEM-E) supplemented with 10% inactivated calf serum (CS) and in MEME supplemented with 10% fetal bovine serum (FBS), respectively. Cultures were incubated at 37°C in 5% CO₂ in air. Growing cultures were suspended by trypsinization when the monolayer was 80% confluent. The cellular viability was estimated by the trypan blue dye exclusion test.

Assay-

Cultures were suspended by trypsinization and 2 or 3 × 10³ cells in 100 µl of medium were seeded on a 96-well microtiter culture plate, respectively. One day after seeding, cultures were exposed in triplicate to various concentrations of airborne particulate extracts for 24 hr. Samples were dissolved in DMSO, which did not exceed 1.0% (v/v) of the final incubation medium. Control and positive control cultures were treated with DMSO and cycloheximide (0.1 µg/well). After 24 hr treatment, assay was performed. cytotoxicity, monitored as an increase in lactate dehydrogenase (LDH) leakage, was evaluated with LDH Cytotoxic Test. Cultures were seeded on microtiter plates at a density of 5 × 10³ cells/0.1 ml of medium per well. After 24 hr, the medium was removed and the cultures washed with phosphate buffered saline, then exposed to various concentrations of airborne particulate extracts and positive control for 2 hour.

Conclusion

The Airborne particulates of roadside atmosphere have shown that crude extract was the most cytotoxic in assay and cell proliferation. The assay is based upon the mitochondrial bio-reduction of a tetrazolium salt and can detect reversible functional cell damage.

6) METHYLENE BLUE ASSAY^[15]

Aim- To determine cytotoxic activity of *Carissa Carandas* extract on human lung cancer cell lines.

Cell line used-NCI (lung cancer cell line).

Chemical- Trypsin-EDTA, n-hexane, Chloroform, Glutaraldehyde, 0.15M Sodium Chloride, 0.05% (w/v) Methylene blue solution.

Assay- Cells were harvested with 0.05% (v/v) Trypsin-EDTA and plated on 96-well plate at cell density of approximately 6000 cells/well. Cell viability before plating was determined by Trypan blue exclusion test. The cells were allowed to attach and incubated for 24-48 hours. When the cells reached confluency between 80-90%, the medium was replaced with fresh medium containing only 0.5% (v/v) FBS. The cells were incubated for another 4 hours, for the cells to achieve quiescent state. The cells were treated with different concentration of n-hexane, chloroform and methanol extract from *Carissa Carandas*. Control cells were cultured in 0.5% (v/v) FBS containing medium. Vincristine sulphate was used at positive control. After treatment, the plates were incubated for 72 hours. Cell survival was determined by methylene blue staining method. Glutaraldehyde was added to each well to a final concentration of 2.5% (v/v) and surviving cell is fixed for 15 min. After washing with 0.15M Sodium Chloride and removing the dead cells, the fixed cells were stained with 0.1 ml of 0.05% (w/v) methylene blue solution for 15 minutes. After washing off the excess dye 0.15M Sodium Chloride solution.

Conclusion

The study was shown that the n-hexane extract of *Carissa Carandas* exhibited cytotoxicity on the cell line.

7) COLONY FORMATION ASSAY^[16]

Aim- To determine cytotoxic activity of Cisplatin in non-small cell lung cancer cell lines.

Cell line used- The human NSCLC cell lines H460, A549, and Calu-1

Preparation of Adenoviral Vectors-

The preparation of recombinant (E1- and E3-deleted)adenoviral vectors expressing the Fhit protein was performed in fetal kidney 293 cells using standard techniques, For Ad-Fhit, a 707-bp fragment of FHIT cDNA was amplified by reverse transcription-polymerase chain reaction(RT-PCR) from human placental cDNA and cloned into an adenoviral shuttle vector (pQBI-AdCMV5). The adenovirus expressingLacZ (Ad-LacZ) protein was used as a control vector.

Chemical- Cisplatin.

Assay- Cells were transduced with Ad-Fhit and Ad-LacZ constructs at a multiplicity of infection (MOI) = 5. Because Ad-Fhit infection alone resulted in a reduced number of outgrowing colonies compared with Ad-LacZ infection, 24 hours later, cells were harvested and seeded in 60-mm plates in triplicate (500 cells/dish for Ad-LacZ, and 1000 cells/dish for Ad-Fhit-transduced cells). After 24 hours, cells were exposed to 0.1 to 10 Mm cisplatin for 1 hour or 24 hours. When the drug was removed, the cells were washed with saline and then incubated in drug free medium until colonies in control samples have become evident (approximately 10 days later). Samples were stained with 1% crystal violet in methanol for 1 hour, and colonies of at least 30 cells were counted using an inverse microscope. IC₅₀ is defined as the drug concentration producing a 50% decrease in cell survival.

Conclusion

To evaluate whether the combined effect of Ad-Fhit and cisplatin results in an increased apoptotic response.

8) IHC ASSAY^[17]

Aim- To determine cytotoxic activity of Acutiaporberine a novel bisalkaloid derived *Thalictrum acutifolium* Boivin in non-small cell lung cancer cell lines.

Cell line used—PLA-801 NSCLC cell lines

Chemical-Acetone, Formaldehyde, Phosphate, Phosphate Buffer Solution (PBS).

Cell Culture-The human NSCLC cell lines was derived from a male patient diagnosed with adenocarcinoma and previously treated with chemotherapy. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. 100 u/ml penicillin and 100 u/ml of streptomycin at 37⁰C in a humidified atmosphere of 5% CO₂/95% air.

Assay- This assay was carried out to detect the protein products of some apoptosis-associated genes. The cells, cultured on glass coverslips, were treated acutiaporberine for different times, fixed in solution of 45% acetone, 25% formaldehyde, 30% 1 Mm phosphate, pH 6.1-6.2 and then transferred to PBS. A fresh solution of 0.3% H₂O₂ in methanol was used to treat the cells for 30 min at room temperature to inhibit the endogenous peroxidase activity. Then, after blocking with 1.5% serum, the cells were

mixed with Bcl-2 mouse monoclonal antibody (1:100), Bax mouse monoclonal antibody (1:100), c-Myc mouse monoclonal antibody (1:100) or p53 mouse monoclonal antibody (1:100) and incubated at 4^o overnight. After washing with PBS, the cells were incubated with goat anti-mouse IgG (15 ug/ml) and then with a horseradish peroxidase streptavidin solution (5ug/ml) was used for development and cell were counterstained with Mayer's hematoxylin solution.

Conclusion

Acutiaporberine on the expression in PLA-801 cells of several apoptosis associated genes, namely c-myc, bax, bcl-2 and p53. A high expression level c-myc gene product might increase the sensitivity of cells to apoptosis-inducing factor. A high expression of the bcl-2 gene significantly protects cells from apoptosis induced by various factor, whereas the bax protein reverse this process.

Cytotoxicity Assay's of colon cancer:

1) CELL SURVIVAL ASSAY^[18]

Aim- To determine cytotoxic activity of Phortress against colon cancer cell lines

Cell line used - HT29, SW480, SW620 cell line

Chemical- 1% Crystal violet.

Cell Culture- Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by the collagenase perfusion technique, detailed in all experiments with HUVEC were performed on tissue culture plates/petri dishes etc. that had been pre-coated with 0.2% gelatin.

Assay- Petri dishes (100 mm) were plated with 5x10⁵ cells in 10 ml of media or 60 mm Petri dishes were plated with 1.8x10⁵ cells in 3.5 ml of media. The cells were allowed to attach for 24 h. Media was aspirated off and the exponentially growing cells were then exposed to drug for 72 h, following which they were trypsinised and plated out for colony formation. Incubation time was 4 weeks for all tumour cell lines except SW620 (3 weeks), 2 weeks for fibroblasts and 10 days for HUVEC. Finally, colonies were fixed with methanol and stained with 1% crystal violet (Sigma) vide a protocol modified from Freshney. Colonies were counted by eye and confirmation by microscopy carried out as necessary. Any cluster of cells greater than 50 in number was counted as a colony. All survival points were in triplicate and experiments repeated at least twice.

Conclusion- It shows that the control drug decreases the proliferation of endothelial as well as colorectal cell lines.

2) SULFORHODAMINE –B ASSAY (SRB ASSAY)^[19]

Aim- To determine cytotoxic activity of Oxitan and Eloxatin in Colon Cancer Cell Line

Cell line used - SW620 cell lines.

Chemical- 0.25% trypsin-EDTA.

Assay- This assay was used in this study to estimate cell numbers indirectly by staining total cellular protein with the SRB. The protocol was based on that originally described by with some modifications. In brief, cells at the exponential growth phase were detached with 0.25% trypsin-EDTA to make single cell suspensions. The viable cells were counted by trypan blue exclusion using a haemocytometer and diluted with medium to give a final

concentration of 1×10^4 cells/ml. Cell suspension was then seeded in 96-well microtiter plates and treated with various concentrations of drugs ranging from 1 to 1000 ng/ml. The plates were incubated for 72 hours at 37°C in a 5% CO₂ humidified incubator. The number of viable cells was indirectly estimated by staining total cellular protein with the SRB. The bound dye was solubilized with Tris buffer. The absorbance of each well (three replicates for each concentration) was measured using a Sunrise Tecan ELISA plate reader International Diagnostic Systems at 510 nm. The number of viable cells was determined by measuring the intensity of color in each well. The percentage of cell survival was calculated using equation I. IC₅₀ value was expressed as the concentration of drugs in nanograms per milliliter that caused a 50% growth inhibition compared with controls.

Conclusion

This assay has been widely used to indirectly estimate cell numbers by measuring the cellular protein content of adherent and suspension culture. The treatment of an SW620 colon cancer cell line with the test formulation (Oxitan®) and the reference formulation (Eloxatin®) markedly decreased cell viability in a dose-dependent manner.

3) COLONY FORMATION ASSAY [20]

Aim- To determine cytotoxic activity of the *Gleditsiae Semen* Extracts on Colon Cancer Cell Line.

Cell line used- HT-29 Cells.

Preparation of the methanolic extract from Gleditsiae Semen- Each 5 g of GS was extracted with 100 ml of methanol for 3 days at room temperature and filter through Whatman No. filter paper. The methanol was then removed by evaporation in vacuo, and a dried methanol extract was obtained. Methanolic extract from *Gleditsiae Semen* was called GSE. The GSE was then dissolved in dimethyl sulfoxide (DMSO) at concentration 5 mg/ml for experiments.

Chemical- Streptomycin, 10% formaldehyde, 0.01% Crystal violet.

Cell Culture- The cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 mg/ml NaHCO₃ in a humidified 37°C incubator gassed with 5% CO₂. GSE were dissolved in DMSO and added the culture medium so that final concentration of DMSO was less than 1%.

Assay- The Cells were seeded at 5.0×10^4 cells/well in 24-well plates, incubated overnight, and treated with different concentrations of GSE for 24 hr. The cells were diluted in new medium, replated at 1.0×10^4 cells/well in 6-well plates, cultured under normal growth condition for 7 or 8 days at 37°C in humidified atmosphere containing 5% CO₂ to form colonies. The colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Cell survival was calculated by normalizing the survival of control cells as 100%. IC values were determined from the dose-response curves of colony formation inhibition.

Conclusion

Result clearly demonstrated that *Gleditsiae Semen* Extracts significantly inhibit cell proliferation in cell lines of cancer. These result the potential of *Gleditsiae Semen*, a natural product, as an agent of chemotherapeutic activity.

4) CLONOGENIC SURVIVAL ASSAY ^[21]

Aim- To determine cytotoxic activity of Cisplatin on Colon Cancer Cell Line

Cell line used- HCT116 Cells.

Chemical- 10% (v/v) fetal bovine serum, Cisplatin, Phosphate-buffered saline (PBS) Solution.

Cell Culture- Cells were routinely cultured as monolayer's in 299s DMEM/F12 nutrient medium supplemented with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, 100 IU/mL penicillin G and 100 µg/mL streptomycin sulfate in a 37 °C chamber incubator providing a humidified atmosphere of 5% CO₂ in air. All cultures were free of Mycoplasma contamination. Exposure to ⁶⁰Co γ radiation was performed in a Gamma cell 220 unit as described. Treatment with cisplatin was performed by incubating cells in growth medium containing the indicated concentration of the drug for 2 h at 37 °C. Following incubation, the medium was replaced with fresh medium lacking cisplatin. The PI3K inhibitor wortmannin and the CaMKII inhibitor 1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) were purchased from. Stock solutions of wortmannin and KN62 (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -70 °C. To determine the effects of these protein kinase inhibitors on the radio sensitivity of HCT116 cells, cultures were treated with each inhibitor for 1 hour prior to irradiation and for 24 h post-irradiation. Control cultures were incubated in medium containing 0.1% (v/v) DMSO.

Assay-

Cells of an exponentially-growing monolayer culture were harvested by the use of 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.53 mM EDTA (3 min incubation at 37 °C) and suspended in ~5 mL of PBS. Using a 5-mL pipette, the cells were pipetted up and down several times, forcing them through the tip of the pipette to break up the clumps. One mL of this suspension was diluted in ~20 mL of medium and immediately the cells were counted using a Coulter counter. After microscopic examination to ensure a reasonable quality of single-cell suspension, the cells were diluted in a volume of medium to yield ~60 cells/mL. Five-mL samples of the resultant single-cell suspension were then pipetted in 60-mm dishes. Using the same protocol, normal human fibroblasts (strain GM38) were plated out at 300 cells per dish in 100-mm dishes (10 mL medium/dish). After plating, the cells were incubated for ~4 h and then exposed to different doses of γ rays (between 0 and 8 Gy) or treated for 2 h in growth medium with different concentrations of cisplatin (between 0 and 4 µg/mL). Cultures were incubated for 18 days with one medium renewal at day 7 (fibroblasts) or for 10 days without medium renewal (tumor cells). The cells were then fixed and stained with crystal violet, and the number of survivors (i.e., colonies containing >50 cells) scored. Survival curves were constructed by plotting CFA (expressed as a percentage of the sham-irradiated control cultures) on a logarithmic scale as a function of the radiation dose or cisplatin concentration administered on a linear scale.

Conclusion

It measures the sum of all modes of cell death, encompassing both early and late events such as delayed growth arrest. In this assay, however, the impact of cell-to-cell communication is disregarded because the cells are plated out at very low densities.

5) MTT ASSAY ^[22]

Aim- To determine cytotoxic activity of β -Glucan enhanced apoptosis in human colon cancer cells.

Cell line used- SNU-C4 Cells.

Chemical- fetal bovine serum (FBS).

Cell Culture-

Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37. in an atmosphere of 5% CO₂ and 95% air.

Assay-

Cells were cultured in each well of a 96-well plate. The β -glucan was added to the cells with the concentrations of 1, 5, 10, 20, 25, 50, 100, and 200 μ g/mL at 24 hr. Control was the cells without addition of β -glucan. After a further 24 hrs incubation, 20 μ L of MTT was added to each well and incubated for 4 hr. After the plate was incubated in the dark for 4 hr, the medium was discarded and 150 μ L of DMSO was added to each well and incubated for 20 min. The absorbance at a test wavelength of 540 nm with a reference wavelength of 690 nm was measured using a micro titer plate reader. The optical density (O.D.) was calculated as the difference between the absorbance from reference wavelength and from test wavelength. Percent viability was calculated as (O.D. of β -glucan treated sample / O.D. of none treated sample) \times 100.

Conclusion

The induction of apoptosis by β -glucan in colon cancer cells may be related with modulation of Bcl-2 family resulting in the activation of caspase-3 expression. It may be a beneficial natural agent for colon cancer treatment and chemoprevention.

6) HIGH DENSITY SURVIVAL ASSAY^[23]

Aim- To determine cytotoxic activity of Cisplatin on Colon Cancer Cell Line

Cell line used- HCT116 Cells.

Chemical- Cisplatin, EDTA

Cell Culture- Cells were routinely cultured as monolayer's in 299s DMEM/F12 nutrient medium supplemented with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, 100 IU/mL penicillin G and 100 μ g/mL streptomycin sulfate in a 37 °C chamber incubator providing a humidified atmosphere of 5% CO₂ in air. All cultures were free of Mycoplasma contamination. Exposure to ⁶⁰Co γ radiation was performed in a Gamma cell 220 unit as described. Treatment with cisplatin was performed by incubating cells in growth medium containing the indicated concentration of the drug for 2 h at 37 °C. Following incubation, the medium was replaced with fresh medium lacking cisplatin. The PI3K inhibitor wortmannin and the CaMKII inhibitor 1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) were purchased from. Stock solutions of wortmannin and KN62 (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -70 °C. To determine the effects of these protein kinase inhibitors on the radio sensitivity of HCT116 cells, cultures were treated with each inhibitor for 1 hour prior to irradiation and for 24 h post-irradiation. Control cultures were incubated in medium containing 0.1% (v/v) DMSO.

Assay-

Cells of an exponentially-growing monolayer culture were harvested by the use of trypsin/EDTA, counted, plated in 35-mm dishes at 5×10^5 /dish, and incubated for 24 h. The cells were then exposed to various doses of Gamma rays, or were treated with various concentrations of cisplatin for 2h) and incubated for another 24 h. The cells of each dish were then detached by the use of trypsin/EDTA, and 1/10 of the content of each dish was seeded into a 100-mm dish (containing 10 mL fresh medium) and incubated for 5 days. The cells of each dish were again detached and 1/10 of the content of each dish was seeded into 60-mm dishes (3 dishes for each time point). The Cells were incubated further 24 h in medium containing either 0.01 μ Ci/mL of [methyl- 14 C]-thymidine or 0.01 μ Ci/mL of [methyl- 3 H]-thymidine). The radiolabeled nucleosides were purchased. The amount of radioactivity incorporated into the cells of each dish was then determined as described. The degree of cell killing by a particular radiation/cisplatin treatment was determined from the amount of radioactivity incorporated in cells of treated dishes compared to sham-treated control dishes. Survival curves were constructed by plotting the numbers of [14 C]-thymidine or [3 H]-thymidine counts (expressed as a percentage of control cultures) on a logarithmic scale as a function of the radiation dose or cisplatin concentration administered on a linear scale.

Conclusion

This assay measure the sum of all modes of cytotoxic events, it is not influenced by artifacts such as poor quality of the single-cell preparations and poor cloning efficiencies often associated with the conventional

7) TRYPAN BLUE EXCLUSION ASSAY ^[24]

Aim- To determine cytotoxic activity of low-dose 10-hydroxycamptothecin in human colon cancer

Cell Culture- The human colon cancer cell line Colo 205 was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. Cells were seeded in a 6-well plate and incubated with various concentrations of 10-HCPT. After 48 h, the treated cells were washed twice with phosphate-buffered saline (PBS), flash frozen in liquid nitrogen, and stored at -80°C. The harvested cells were lysed in ice-cold report lysis buffer. After cleaning the resulting lysates by centrifugation, protein in the clear lysate. Lysate protein (15 μ g) was resolved by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with antibodies against the following cellular proteins:

DNA topoisomerase I, p53, p27, phosphorylated p27, PCNA, p21 (Upstate) and α -tubulin (Sigma). The protein content was visualized using a BM chemiluminescence blotting kit. The blots were exposed to X-ray film for various times.

Chemical- fetal bovine serum (FBS), trypan blue.

Cell line used- Human colon cancer cell line Colo 205.

Assay-

Cells (5×10^5) were seeded in 25T flasks overnight and then treated without (control) and with 5, 10, 15 or 20 nM of HCPT, respectively. After treatment for 24-120 h, cells were harvested by trypsin-EDTA and then centrifuged at 1,500 rpm for 5 min at 4°C. The cell

pellet was resuspended in culture medium containing 0.04% trypan blue and the viable cells were enumerated by a hemocytometer

10-HCPT inhibits the cell growth and reduces the cell viability of human colon cancer cell line.

Conclusion

It conclude that 10-HCPT can be administered orally and a long-term low maintenance dose of 10-HCPT is the best administration strategy to achieve maximal anticancer effects. The results of this study should be a useful reference for future clinical human colon cancer therapy.

8) APOPTOSIS ASSAY ^[25]

Aim- To determine cytotoxic activity of Pioglitazone, in RB-deficient human colorectal cancer cells

Cell Culture- Human CRC cell lines SNU-C4 and SNU-C2A were established from poorly differentiated colorectal adenocarcinoma, and cultured in RPMI1640medium supplemented with 10% fetal bovine serum (FBS),100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95% air/5%CO₂ at 37.C.

Chemical- Pioglitazone.

Cell line used- Human colorectal cancer cell lines SNU-C4 and SNU-C2A

Assay- The extent of apoptosis was evaluated by annexin V-FITCand flow cytometry. The annexin V assay was used, in whichannexin was conjugated with FITC. PI was used as counter stain.Briefly, cells were treated with 50 µM pioglitazoneor 0.1% DMSO for the indicated times. After incubation,cells were harvested, washed with PBS (pH 7.4),centrifuged, and stained with annexin V-FITC and 2 µg/ml PI in binding buffer (10 mM Hepes, Ph 7.4/140mMNaCl/2.5mMCACl₂) for 15 min at 37.C in the dark. The samples were analyzed by flow cytometry using aFACScan flow cytometer. Data analysis was performed using CellQuest software.

Conclusion

It shows that PPAR γ is expressed in SNUC4 and SNU-C2A cells and that treatment of these cells with pioglitazone inhibits cell growth through a G2/M phase block and apoptosis. Furthermore, down-regulation of cyclin B1 and cdc2 and up-regulation of p21 may be involved in the G2/M phase block by pioglitazone. Pioglitazone induces apoptosis via caspase-dependent and caspase-independent pathways, accompanied by the decreased expression of Bcl-2, XIAP, and COX-2 and increased expression of Bax. These results suggest that pioglitazone may be of therapeutic importance in the treatment of human CRC. However, further studies of the effects of pioglitazone on tumor growth in vivo and investigations of the molecular mechanism involved in pioglitazone-induced apoptosis are necessary.

9) MTT COLORIMETRIC ASSAY ^[26]

Aim- To determine cytotoxic activity of Halogenated Monoterpenes from Plocamium cartilagineum

Chemical- Dimethyl sulfoxide(DMSO)

Cell line used- human colon adenocarcinoma (SW480)

Assay- Cells in the logarithmic growth phase were added to 96-well flat-bottom microtiter plates cells and incubated for 6 d with different concentrations of the compounds dissolved in absolute ethanol or dimethyl sulfoxide(DMSO) for compound 1 and lindane. This prolonged time of incubation was used to predict possible adverse cytotoxic effects of compounds on CHO cells. In all cases, the viability of the cells treated under the same conditions with the residual concentration of solvents was \geq 95%. The relative potency of the active compounds (IC₅₀, effective inhibitory concentration to give 50% cell viability) was determined as described For reversibility experiments, cells were incubated with the minimal cytotoxic concentration of each compound, washed three times with fresh culture medium and cultured in compound-free medium for different periods of time. Three independent experiments were carried out in duplicate.

Conclusion-These Assay is used for to determine whether the cytotoxic effect was reversible, cells were incubated for 6 d with compounds.

10) Cell Proliferation ASSAY [27]

Aim- To determine cytotoxic activity of *Garlic* in Rat Colon.

Experimental Design-

The experiment was designed in four sets, each with thirty rats, according to the experimental parameters used. Each set was divided in three groups, viz., Normal, Carcinogen control and *Garlic*-treated. The Carcinogen control and the *Garlic*-treated groups were initiated with AOM by three weekly subcutaneous injections (15mg/kg. b.w.) administered between 11 am – 12 noon. Each rat in the *Garlic* treated group received an oral administration of a 2.5% (w/v) aqueous suspension of *garlic* at a dose of 1ml/rat/day continuously starting from the 1st day of AOM injection for twelve weeks. The total observation period was 12 weeks. The Normal group received no treatment with AOM or the garlic suspension.

Chemical-Nitroblue Tetrazolium.

Assay-Rats were sacrificed and the colons were sliced into several parts and placed into BrDU labelling medium when the DNA of proliferated S-phase cells was labelled. The tissue slices were fixed and processed under normal histological procedures and the sections were subsequently incubated with an anti-BrDU Monoclonal antibody (at 37oC for 30min.). Binding was detected with an alkaline phosphataseconjugated- antimouse-immunoglobulin antibody (antimouse-Ig-AP). The bound anti-mouse-Ig-AP was visualized using nitroblue tetrazolium (NBT), an AP-substrate solution. The BrDU Labelling Index (BrDU LI) was determined by dividing the number of labelled cells by the total cells counted and multiplying by 100.

Conclusion- Significant reduction in BrDU LI and significant increase in the Apoptotic Index (AI) were observed in garlic treated rat colons.

Conclusion

Colorectal cancer and Lung cancers are the leading cause of cancer death. Natural product represents reservoir of diverse templates and are being tapped to outsource novel anticancer agent. It is very necessary to validate the potential of new entity against these chronic cancers. There are various screening methods for assessing the potential against

cancer. This review summarizes the possible assays for screening the new entity against colon and lung cancers. The resulting assays are suitable for screening of large compound libraries to identify cell cycle interfering chemotherapeutics targeting preferentially proliferating cells.

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