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Original Research Article

Fluorescence resonance energy transfer (FRET) based high throughput drug screening for the determination of antineoplastic activity of natural compounds from herbs

Sharmistha Gupta[1] and Mithun Mukherjee[2]

[1]West Bengal State Council of Science and Technology, West Bengal-India
[2]Ramakrishna Vivekananda Mission Institute of Advanced Studies, West Bengal-India

A B S T R A C T

Objectives: In the present experiment a very useful cell-based high throughput screening method that uses a novel bio-sensor which specifically identifies apoptosis based on fluorescence resonance energy transfer technology.

Methodology: A stable HeLa cell line expressing a FRET-based bio-sensor protein was produced. Cells undergoing apoptosis have an activated enzyme 'caspase-3' which is a protease this activated enzyme breaks sensor protein and causes the fluorescence emission spectra to shift from 590 nm to 512 nm changing color from yellowish green to blue. A reduction in the green/blue emission ratio indicated apoptosis. After growing the sensor cells in 96-well plates different chemical compounds are added to the wells wherein the fluorescent profile was measured using fluorescent plate reader.

Results: It is a novel cell based HTS method and was proved to be highly effective and sensitive process for detecting anti-neoplastic compounds which induce apoptosis. In this experiment we screened compounds obtained from two plants used in Indian medicines.

Keywords: Drug discovery, HTS, FRET, fluorescent protein, apoptosis, caspase, anticancer drugs; herbal medicine, tannins, quercetins.

A R T I C L E I N F O

Contents

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***Corresponding Author**
Sharmistha Gupta
West Bengal State Council of Science and Technology, West Bengal-India
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1. Introduction

Now a day's one of the major cancer treatments is chemotherapy. Most chemotherapy drugs such as vincristine, taxol, and etoposide and others, exert their anticancer effects by arresting cells at different stages of the cell cycle and further promoting them into apoptotic cell death (Gidding et al., 1999; Sleiman and Stewart, 2000). Since these anticancer drugs fail to discriminate between cancer cells and normal cells, during chemotherapy many normal cells are also killed, thereby damaging patient's immune system resulting in peripheral neurotoxicity, (Einzig et al., 1991). Developing new anticancer drugs with high efficacy and specifically targeting cancer cells is the focus of medical research now a days. The initial step to drug discovery is to screen for drug leads from a huge pool of compounds. The commonly used in vitro caspase activity assay utilizes cell extracts from a large population of cells. There are methods which are time consuming, laborious and costly methodologies and therefore, will be difficult for a high throughput drug screening like Annexin V require the use of a fluorescent microscope or fluorescence-activated cell sorting analysis, the TdT-mediated dUTP nick end labeling method is only applicable to fixed cells but not living cells. In this paper, a high throughput screening method has been developed for detecting caspase-3 activation during the process of apoptosis in living cells grown in a 96-well plate (Tian et al., 2007). Caspase-3 is a cysteine protease which is activated by apoptotic inducers. Upon activation it breaks cellular proteins at specific cleavage sites (Nicholson, 1999; Wolf and Green, 1999), leading to cell death. Here high throughput screen uses a stable cell line (HeLa-C3) expressing a caspase biosensor, which is an engineered protein consisting of three parts: a donor cyan fluorescent protein (CFP), a 16-amino-acid peptide linker containing the caspase-3 cleavage site and an acceptor yellow fluorescent protein (YFP) (Luo et al., 2001).

The method of identification is on the basis of the effects of fluorescence resonance energy transfer (FRET) (Cubitt et al., 1995; Heim and Tsien, 1996). In the absence of activated caspase-3, the fluorescent emission energy of the excited donor fluorescent protein CFP can be transferred to the acceptor molecule YFP, leading to the emission of yellow fluorescent light. When caspase-3 is activated during apoptosis, it will cleave the biosensor (CFP-linker-YFP) at the linker peptide containing the recognition sequence for caspase-3, DEVD. Cleavage of the sensor protein terminates the effect of FRET, resulting in a change in the emission profile of the sensor protein because fluorescent light is emitted from CFP instead of YFP. Thus, by measuring the fluorescence emission ratio between YFP and CFP, we detect the amount of activation of caspase-3 in living cells during the process of apoptosis. We examined this system using a few known anticancer drugs, including, Quercetins, tannins, etoposide and vincristine. Furthermore, we have used this screening system to detect the potential anticancer activity of a few compounds from *Caesalpinia pulcherrima* (L.)Sw and three compounds from *Cocculus hirsutus* (L.)Diels. Leaves of *Caesalpinia pulcherrima* (L.)Sw has been shown to possess antitumor activity (Chiang et al., 2003). The leaves of *Caesalpinia pulcherrima* (L.) Sw are rich in flavonoids and tannins. Moreover in the district of Murshidabad in West Bengal, Leaves are smashed and taken once a day before meal to treat for colon cancer. Further leaf extracts of Cocculus hirsutus (L.)Diels showed significant anti-tumor activity on Carrot Disc Diffusion Bioassay, in which after 3 weeks of incubation of *Agrobacterium tumefaciens* on carrot discs, only the negative control showed tumors developing from the meristematic tissue around the central vascular system but no tumors were detected in the discs that were treated with 1000 ppm of *Cocculus hirsutus* (L.) diels extracts (Mon et al., 2011).

2. Materials and methods

Materials

Vincristine and Etoposide were dissolved in dimethyl sulfoxide (DMSO). The stock concentrations of TNF-α (50µg $ml^{-1)}$, cycloheximide (50µg ml^{-1}), were prepared in water. 25% H_2O_2 was diluted to 5 M with double-distilled H_2O as a stock solution and then added to the cells at 1:1000 dilutions. Quercetin, and tannins were dissolved in DMSO at a 5 mM and trilobine and isotrilobine were also dissolved in DMSO at 100 mg ml^{-1} each and then further diluted to make 50 µM stock concentrations with DMSO. Stock solutions were sterilized by filtration prior to their use using filters with a pore size of 0.22 mm.

Methodolgy:

1. Generation of stable HeLa-C3 cell line

The mammalian material was fused with the CFP-DEVD-YFP fusion gene coding the sensor C3 protein was previously generated and characterized. The plasmid DNA of sensor C3 was introduced into HeLa cells by electro oration method (Luo and Chang, 2004). Transfected cells were seeded in a 90 mm culture dish, and 48 h after electro oration, cells were trypsinized and seeded into two six-well plates in a medium having 500 µg ml^{-1} geneticin. About 14-15 days of transfection, GFP-positive clones were first selected under a fluorescence microscope, then trypsinized individually and seeded into 90 mm plates. GFP-positive clones with high purity and strong fluorescence were further selected by this 'local trypsinization' and 'subculture' method. At the end, four clones were chosen for further examination to confirm the induction of apoptosis.

2. Drug addition to Cultured Cells

HeLa-C3 cells were cultured in minimum essential medium (MEM) having 10% fetal bovine serum, 100 U ml^{-1}

penicillin, 100 mg ml^{-1} streptomycin and 500 mg ml^{-1} geneticin, in a 5% CO_2 humidity incubator at 37^0 C. In order to test the effects of different compounds or drugs, 25000 HeLa-C3 cells were seeded into each well of a 96-well plate. Medium was removed and replaced with 100 ml of fresh medium containing compounds to be tested were added. The emission was measured at 520 + 8 nm for CFP and 590s + 8 nm for YFP by Perkin-Elmer Victor plate reader.

3. Caspase-3 activity assay

After treating the cells with different stimuli, they were collected and lysed in lysis buffer containing protease inhibitors of aprotinin, leupeptin, pepstatin A, phenyl methyl sulfonyl fluoride and ethylene diamine tetraacetic acid (EDTA). The cell lysates were centrifuged for 30 min at 4^0C. Caspase-3 activity assay was performed in a 96-well plate culture. For each assay, 10 ml of the cell lysates with about 100 µg of proteins was first mixed with 100 ml of the assay buffer plus 2 ml of the caspase-3 substrate. Then the mixture was incubated at 37^0C in darkness for 1 h. The fluorescent intensity was measured using a Perkin–Elmer Victor plate reader with excitation wave-length at 316 nm and emission wavelength at 460 nm. Caspase-3 activity was presented in units of 'activity per µg protein'. The protein concentration was measured by the Bradford method.

4. Western blot analysis

100 micrograms (µg) of protein from the already used cell lysate as used for caspase-3 activity assay was separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 10% fatless milk, the membranes were incubated with different antibodies. Further the membranes were incubated and developed using the ECL Western-blotting analysis system.

5. Imaging techniques

HeLa-C3 cells were grown overnight on a 25-mm round coverslip mounted onto a 35-mm Petri dish with an observation window and treated with various compounds for different amounts of time. The fluorescent images of living cells were obtained using an inverted fluorescence microscope equipped with a FRET filter set. The cells were excited using an excitation filter along with a diachronic mirror of 455 nm. The emission images of YFP and CFP were recorded using a computer-controlled digital camera. Inverted fluorescence microscope was used to obtain the image of the phased cells.

6. Y/C emission ratio measurement and calculation

Cells cultured in 96-well plates were treated with various compounds. At specific time points, the emission signals of YFP (590±5nm) and CFP (512±5nm) were respectively measured. The background fluorescence was then measured from the wells containing only medium. After subtracting the background fluorescence from the signals thus recorded, net YFP and CFP readings were deduced. In the present paper, FRET is identified with Y/C emission ratio that is net YFP reading divided by the net CFP reading from the same well.

3. Results & Discussion

Activation of caspase-3 during apoptosis was detected from HeLa-C3 cells using a fluorescent plate reader. Under normal growth conditions most HeLa-C3 cells emit green fluorescent light owing to the energy transfer from CFP to YFP. When these HeLa-C3 cells were treated with different apoptotic stimuli (including Vincristine, TNF-α and UV-irradiation) for various periods of time, the fluorescent light emitted changed from a yellowish green to a blue color, indicating that the fluorescent energy can no longer be transferred from CFP to YFP. When we measured the changes in fluorescent intensity emitted from HeLa-C3 cells seeded in a 96-well plate using a fluorescent plate reader after 9 h of UV treatment, the Y/C emission ratio reduced from 4.10 to 2.05, with a net reduction value of 2.05. An even greater reduction in Y/C emission ratio of 2.40 was detected 36 h after treatment with vincristine. Moreover, the reductions in the Y/C emission ratio after UV irradiation can be prevented by pre-treatment with the pan-caspase inhibitor (20 mM) for $2\text{-}2^{1/2}$ h. This result suggests that these fluorescent changes were caused by caspase-3 activation. Consistent with this finding, we also found that the reduction of Y/C emission ratio was correlated with the increase in caspase-3 activity.

Specificity of HeLa-C3 cells for detecting apoptosis but not necrosis:

The biosensor in the HeLa-C3 cells was made in such a way so as to detect caspase-3 activation during apoptosis in living cells. In principle, this detection should be specific only to apoptotic cell death during which caspase-3 is activated and insensitive to necrotic cell death when caspase-3 is not activated. In order to verify this, we compared differential responses of HeLa-C3 cells to apoptotic stimulus (UV-irradiation for 5 min) and necrotic stimulus (continued treatment with 5 mM H_2O_2). Results of Western blotting have shown that caspase-3 was cleaved 5 h after UV-irradiation into its active form. At the same time, the fusion protein of sensor C3 was also cleaved into two polypeptides: YFP-DEVD and CFP. For cells treated with a necrotic inducer (5 mM H_2O_2) for 2–12 h, however, no cleavage of caspase-3 or sensor protein was observed even though most of the cells were undergoing cell death. Fragmented DNA, was seen indicating apoptotic cell death, was clearly observable in HeLa-C3 cells when treated with UV-irradiation. Intact DNA was observable in cells continually treated with 5 mM H_2O_2, indicating that apoptotic cell death did not occur. Suggesting that HeLa-C3 cells can selectively detect apoptotic, but not necrotic, cell deaths. HeLa-C3 cells can detect caspase-3 activation occuring during apoptosis induced by multiple anticancer drugs for which we tested this system with two of the known anticancer compounds, including vincristine and ET. Vincristine can block cells during mitosis and then induce them into apoptosis (Takano et al., 1993; Mollinedo and Gajate, 2003). In this study, three concentrations of vincristine (1, 50 and 100 nM) were added to HeLa-C3

cells. Screening results showed that 50 nM of vincristine reduced the Y/C emission ratio to about 6, and a higher concentration of vincristine at 100 nM further reduced the Y/C ratio to less than 5 within 48 h.We found that about 40% of the cells exhibited a clear cell shrinkage morphology after treatment with drug at 50 nM for 48 h and even more cells were dead (80–90%) with a higher concentration of vincristine (100 nM).Then we tested ET, an inhibitor of DNA topoisomerase II in the same concentrations. ET can block DNA synthesis during S-phase of the cell cycle, and then cause apoptotic cell death (Fearnhead et al., 1994). While screening we found out that the effective concentration of ET for activating apoptosis is between 50 and 100 mM. Also, significant reductions in the Y/C emission ratio were seen 72 h after addition of the drug, suggesting that ET may need more time to induce apoptotic cell death by inducing activation of caspase-3 in these HeLa-C3 cells than vincristine.

This demonstrates that HeLa-C3 cells are very sensitive in detecting caspase-3 activation induced by anticancer drugs bringing apoptosis. Based on the primary screening results of the above studies, we selected three drug concentrations (10, 50 and 100 nM) for testing all four compounds. The results of drug screening using HeLa-C3 cells were similar with the morphological observations of HeLa cells. Cells treated with 50 nM of drug concentration of vincristine started to round up at 16 h, and reached a maximum state of mitotic arrest at 48 h. Cell death as indicated by cell shrinkage started at 48 h, and most cells were dead after 48 h of drug treatment. Almost no cell death and reduction in the Y/C ratio were detected from cells treated with 10 nM of the second drug concentration for up to 96 h of treatment. With respect to etoposide a higher concentration of 350 µM was required to produce relevant effects. Whereas the leaf extracts of *Cocculus hirsutus* produced similar results at a concentration of 500 µM. Finally the leaf extract of *Caesalpinia pulcherrima* produced cell death at a concentration of 800 µM at the end of 72 h, although most of the results were obtained at the end of 48h of study.

Among all four compounds tested in this study, the compound of vincristine showed the highest potency against HeLa-C3 cells. At 50 nM, it was the only compound capable of reducing the Y/C emission ratio significantly in 48 h. We also noticed that the cell morphology changes during the course of drug treatment where after addition of 20 nM of the compound to HeLa cells for 12-24 h, many cells appeared to be arrested in mitosis with a shrinking morphology. More such cells were observed at the end 48 h. Etoposide showed similar activity at a concentration of 350 µM reducing the Y/C emission ratio in 48 h. In this study a cell-based, high throughput screen method for determining the anticancer activity of a few a potential herbal compounds was done. A stable cell line derived from HeLa cells that produced a caspase sensor protein was one of the basics of this study.

These sensor proteins can be cleaved by caspase-3 when HeLa-C3 cells undergo apoptotic cell death. Owing to this, the fluorescence emission changes from a yellowish green color to a cyan/blue color which can be easily detected by a fluorescent plate reader. We have also demonstrated that HeLa-C3 cells are only sensitive in detecting apoptotic cell death which are caspase-dependent but not necrosis.

The first GFP-FRET based in vivo assay for drug screening was reported by (Jay Jones et al., 2000). Demonstrating a good correlation between FRET changes and caspase-3 activation. The Y/C emission ratio generated by this system is independent of the number of cells involved. So a single threshold can be used to determine the outcome of the screening results. Most of the anticancer drugs tested in this experiment did produce the effect within 48 h of drug treatment. Thus, any compound that can reduce the value of the Y/C ratio can be considered a 'hit' in this process of drug screening. Further we need to develop a single threshold of Y/C ratio providing a simple and consistent method for high throughput drug screening for potential drug candidates and that will allow faster data processing along with faster data analysis. For compounds which significantly reduces the Y/C emission ratio, we should further study the slope of Y/C emission ratio changes because a curve of Y/C emission ratio with a steep slope generally indicates a fast activation of caspase-3, whereas a slope of gradual decline represents slow caspase-3 activation. We thus confirmed that the Y/C emission ratio measured by our HeLa-C3 cells responds to apoptotic inducers only but not to necrotic stimuli.

4. Conclusion

This is a novel method of drug discovery; by this method a lot of time and cost can be saved for screening anti-neoplastic drugs from plant sources moreover we have a vast natural resource that can serve as the background for such studies. We should focus to develop a threshold value that would correlate the decrease in the Y/C emission ratio and apoptotic activity of a large number of medicinal and aromatic plants while screening them.

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