Materials and Methods

ESSENTIALS OF CELL CULTURE

Water

All solutions and Media used in cell culture was prepared using Nuclease free Milli-Q™ (Millipore) purified water.

Phosphate- buffered saline (1X)

Material

- 137mM NaCl
- 2.7mM KCl
- 10mM Na_2HPO_4
- 1.8mM KH_2PO_4

Procedure

850ml nuclease-free water was used for dissolving above mentioned reagent to prepare 1l of PBS. The pH was adjusted to 7.4 using HCl and NaOH, the volume was maintained up to 1l. The solution was dispensed to aliquot 100ml each and sterilized by autoclaving at 15psi for 20min.

Growth Media (1X, DMEM)

Material

- 1X Dulbecco's Modified Eagle's medium (Gibco, Cat No: 12800-017)
- 7.5% $NaHCO_3$ solution (Sigma Aldrich, USA)
- 1N HCl
- 1N NaOH
- Nuclease free autoclaved water

Procedure

One sachet of DMEM was poured into 900ml nuclease-free autoclaved water in a sterile bottle and stirred gently until completely dissolved. 49.3ml of 7.5% $NaHCO_3$ solution was added aseptically and allowed to mix properly. The pH was adjusted to 7.2-7.4 using 1N HCl and 1N NaOH. The media was then filtered through a sterile 0.22μM membrane filter and aliquoted into 500ml sterile bottles and stored at 4˚C. Working aliquots (50ml) were supplemented with 10% FBS and antibiotics as required and can be stored at 4˚C for a week. The media was brought to room temperature prior to usage.

CELL LINE PROCUREMENT AND MAINTAINANCE

Material

- 0.25% trypsin-EDTA (Gibco, Cat No: 25200056)
- FBS (Gibco, Cat No: 10270-106)
- 10X antibiotics solution (Gibco, Cat No: 15240-062)
- T25 flask (Corning, Cat No: CLS3289)

Procedure

The Cell line A549 (Human lung adenocarcinoma cell line) and NIH3T3 (Mouse fibroblast cell line) were purchased from the NCCS Pune, India. Immediately after receiving, cells were checked under Lawrence and Mayo (NIB 100) inverted microscope for any artifact as well as for any contamination, incubated overnight at 37˚C with 5% CO_2 in a humidified CO_2 incubator (Thermo Fisher Scientific, USA). The transport medium was discarded, leaving behind a sufficient layer to grow. Once cells reached a 70-80% confluency, the leftover media carefully discarded, and cells were washed with PBS followed by the addition of 0.25% trypsin-EDTA solution (1ml/25cm^2 flask) and incubated at 37˚C for 3-7 min until cell get detached. Flask was struck once to assure total cell detachment from the substratum of the flask. The trypsin was inactivated using an equal volume of the complete growth medium, i.e., DMEM supplemented with 10% FBS. The entire solution containing cells, trypsin, and medium were transferred to a 15ml sterile centrifuge tube (Tarsons, Cat No: 546021) and centrifuged at 1000rpm for 10min. The supernatant was carefully discarded, and the resulting cell pellet was re-suspended in the complete growth medium. The cells were re-seeded in a T25 flask with 4-6ml growth media, i.e., DMEM supplemented with 10% FBS and 1X antibiotics solution (10,000 units/mL of penicillin, 10,000µg/mL of streptomycin, and 25µg/mL of Amphotericin B). Cell lines were further maintained in 25cm^2 flask (T25cm^2) and fed every 2-3 days with a complete growth medium. All experiments in A549 cell line were performed between passage number 78 to 86.

The strict aseptic condition was followed at every step. All process of cell culture was performed in the Laminar flow cabinet, and floor of the hood was swabbed with 70% isopropyl alcohol before and after use. All thermostable solutions were sterilized by autoclaving at 121˚C for 20min at 15psi. Pressure. However, the thermolabile solutions were filtered using a 0.22µM sterile filter.

CELL COUNTING

For re-seeding, cells were first trypsinized from the stock flask and counted using a hemocytometer. For counting, trypsinized cells were diluted with media or PBS (1:4) and applied to the coverslip chamber of the hemocytometer. Cells in the sixteen squares of the four outer corner grids of the chamber were counted microscopically. An average per corner grid was calculated and multiplied using the dilution factor, i.e., 4. Final cell numbers were multiplied by 10^4 to determine the number of cells per ml (volume occupied by a sample in the chamber is 0.1cm x 0.1cm x 0.01cm, i.e., 0.0001cm^3; hence, cell number x 10^4 is equivalent to cells per ml).

Cell number per ml= Average number of cells in the outer 16 corner square x10^4 x dilution factor

IN-OVO STUDY

Material

- 5% w/v povidone-iodine solution

Procedure

A part of this study was carried out using freshly laid fertile eggs of domestic hen (*Gallus domesticus*) of Rhode Island Red (RIR) breed. Eggs were procured from the intensive poultry unit of government poultry farm, Vadodara, India. All the eggs were wiped with 5% w/v povidone-iodine solution to remove any external contamination and candled to ensure the viability of the embryo. Non-viable and defective eggs were scraped out. Eggs were incubated at the temperature 37.5±0.5˚C and relative humidity of 75±5%. Eggs were hand-rotated on an hourly basis and kept in an incubator such that the broad end would face an upward position. The *in-ovo* study was conducted in compliance with the protocol approved by an Institutional Animal Ethics Committee (MSU-Z/IAEC/13-2017).

DOSE PREPARATION AND TREATMENT

Material

- DMF
- DMSO
- PBS
- 96-well plate (Corning, Cat No: CLS3997)
- 0.25% Trypsin- EDTA solution
- Complete growth media

Procedure

The 20mM stock solution of coumarin derivatives was prepared in DMSO or DMF and stored at room temperature. The working solution was prepared in PBS or in DMEM using a stock solution. For initial screening, a working solution of 0, 0.5, 1, 10, 25, 50, 75, and 100µM was prepared in PBS, aseptically. The volume was adjusted in such a way that, in all the treatment groups, the concentration of DMSO/DMF was not exceeding 0.5%.

The screening of derivatives was performed in the A549 cell line. Cells were harvested through trypsinization from the stock flask, counted on a hemocytometer, and seeded in 96-well plate with 1000cell/well. Allowed to adhere overnight, the next day, cells were treated with derivatives and incubated again for 48h in CO_2 humidified incubator at 37˚C, After 48h incubation, old media was discarded and washed with PBS, and initial screening was performed using MTT assay (the procedure was explained in another subheading of this section), and IC_{50} concentration for each derivative was calculated. The time-point study at 6h, 12h, 24h, 48h, and 72h was also performed for the derivative, which showed the lowest IC_{50} concentration in the A549 cell line.

Similarly, a narrow dose range study was also performed for correct IC_{50} concentration prediction using MTT assay. To achieve this, the derivative was further diluted with PBS, to attain the concentration 0, 0.05, 0.1, 0.5, 1.5, 2, and 2.5nM. The cytotoxicity of the derivative with the lowest IC_{50} concentration toward non-cancerous cell lines was estimated using the NIH3T3 cell line at the dose range of 0, 0.5, 1, 10, 25, 50, 75, and 100µM. Similar to the A549 Cell line, the NIH3T3 cells were also trypsinized, seeded at the density of 1000cell/well on the 96 well plates. The cytotoxicity potential of derivative on NIH3T3 was estimated using MTT assay.

MTT ASSAY

Material

MTT (Sigma Aldrich, Cat No: M5655)

5mg Thiazolyl Blue Tetrazolium Bromide powder dissolve in 1ml of PBS and filtered using 0.22µm sterile filter.

- Acidified Isopropanol (0.04 N HCl)
- 96-well plate (Corning, Cat No: CLS3995)

Procedure

Cells were trypsinized from the stock flask, counted on a hemocytometer and diluted with complete growth media to achieve 1000 cells per 100µl of media. Cells were re-seeded on the 96-well plate at the density of 1000 cells/well. Complete growth media (DMEM, 10% FBS, 1X antibiotic) was added to support the growth of the cells and allowed to adhere overnight. The next day, cells were treated with the coumarin derivatives (specific concentrations of derivative was mentioned in the concerned chapter) and incubated for 48h in CO_2 humidified incubator. On completion of 48h incubation, 100µl of MTT solution was added in each well and incubated for 4 hours in CO_2 humidified incubator at 37˚C. Post incubation, blue-purple formazan crystal can be seen in each well. 100µl acidified isopropanol was added in each well and re-suspended until the visible formazan crystal has been dissolved (5-10min). Absorbance was measured using a microplate reader (Metertech Σ960), at 570nm.

TRYPAN-BLUE EXCLUSION ASSAY

Material

- 0.4% Trypan blue dye (Gibco, USA)

Procedure

Cells were seeded in 12 well plates at the density of 1X 10^5 cells/well, allowed to adhere overnight in the growth medium. The Next day, cells were treated with 4-fluorophenylacetamide-acetyl coumarin and incubated for 48h in CO_2 humidified incubator at 37˚C. After 48h incubation, cells were trypsinized. The cells were diluted with trypan blue (1:1 ratio; 100µl cell suspension: 100µl 0.4% trypan blue) and immediately transfer the 100µl of dye-cell suspension at hemocytometer and the number of blue-stained cells (non-viable) and unstained cells (viable) were counted (Elkady, 2013).

FLOW-CYTOMETRIC ANALYSIS OF CELL CYCLE

Material

- 70% Ethanol (ice-cold)
- PBS (ice-cold)
- RNAase (Sigma Aldrich, Cat No: 6513)
- Propidium Iodide (Sigma Aldrich, Cat No: P4170)

Procedure

Cells were seeded in complete media at a density of 6 x10^7 cells per 25cm^2 flask and allowed to adhere overnight. The next day, cell synchronization was performed by replacing the complete media (10% FBS) with serum-free media and kept for 24h in CO_2 humidified incubator at 37˚C. Post synchronization, cells were treated with 4-fluorophenylacetamide-acetyl coumarin for 48h. After 48h, floating as well as adherent, both cells were collected and pellet down. The pellet was washed twice with ice-cold PBS at 1000rpm for 5min and fixed in ice-cold 70% ethanol overnight at -20˚C. After that, cells were pellet down through centrifugation, ethanol was pipette out carefully without disturbing the pellet and washed twice with PBS at 1000rpm for 5min. The pellet was re-suspended in PBS containing RNAase (100µg/ml) and Propidium Iodide (50µg/ml) and incubated for 30min at 37˚C (Chikara et al., 2017). Stained cells were analyzed by flow-cytometry using 'BD FACSDiva' software (Becton Dickinson & CO., USA).

ETHIDIUM BROMIDE/ACRIDINE ORANGE STAINING

Material

- Ethidium Bromide (Sigma Aldrich, Cat No: 7637)
- Acridine Orange (Sigma Aldrich, Cat No: 6014)
- 12-well plate (Corning, CLS3513)

Procedure

Cells were seeded in 12-well plate at the density of 1x10^5 cell/well and allowed to adhere overnight in CO_2 humidified incubator at 37˚C, followed by treatment with 4-fluorophenylacetamide-acetyl coumarin for 48h. Post derivative treatment, cells were washed with PBS, trypsinized, and pellet down at 1000rpm for 10min. Cells were counted and re-suspended in PBS to attain 1000cell/ml density. The trypsinized cells were stained with the 1:1 ratio of EtBr/AO solution (100µg/ml EtBr and 100µg/ml AO). 1µl EtBr/AO solution was immediately added to the 25µl of the cell suspension and gently mixed with the help of the pipette to loosen the pellet. The image was captured using a DM2500 fluorescence microscope (Leica, Germany).

LACTATE DEHYDROGENASE ASSAY

Material

- DMEM without phenol red (Gibco, Cat No: 21063029)
- LDH cytotoxicity kit (Pierce LDH Cytotoxicity Assay Kit, Thermo Scientific, USA)

Procedure

Cells were seeded in 96-well plate at the density of $1x10^4$ cell/ well in complete growth media without phenol red, allowed to adhere overnight. Next day treatment was given with 4-fluorophenylacetamide-acetyl coumarin for 48h. 50µl of supernatant was collected and transferred to a new 96-well plate and mixed with a 50µl reaction mixture provided with the kit. The plate was incubated at room temperature for 30min, protected from light, 50µl stop solution (provided with the kit) was added to stop the reaction, and absorbance was measured at 490nm and 680nm using a microplate reader (Metertech Σ960). To determine the actual LDH release form the cells, 680nm absorbance value (background signal from the instrument) was subtracted from the 490nm absorbance value.

COMET ASSAY

Material

- LMA -Low melting agarose (1% and 0.75% in PBS).
- NMA -Normal melting agarose (1% in PBS).
- **Lysing solution**

  2.5M NaCl, 0.1M EDTA Na_2, 10mM TrisHCl, 1%Triton X and 10%DMSO.
- **Electrophoresis buffer**

  Tris Borate EDTA buffer (pH 8.3)
- **Neutralization buffer** (0.4M Tris base, pH 7.2)

Procedure

The comet assay was performed as per the procedure of Olive and Banáth, 2006.

Slide preparation

Slides were dipped in 90% ethanol and wiped properly. One-third area of the slides was dipped in melted 1% NMA and removed is such a way that only a thin coating of agarose present on the slides and allowed to dry in a clean and dust-free environment. Once it dried, it was marked properly.

Cell preparation

Cells were harvested from the 12-well plate, having a density of $1x10^6$ cell/ml. 10µl of cell suspension was mixed with 10µl of 1% LMP and incubated on 40˚C water bath.

Sample loading

The NMA coated slides were also kept over the water bath, 50µl of the sample containing (1:1 ratio of cell suspension and 1% LMA) was loaded onto the pre-coated slides (as per marking). It was ensured that cell suspension was uniformly spread on the coated part of the slide. Following this, slides were allowed to dry for 20min at 4˚C and followed by coating with 0.75% LMA and kept for another 20min at 4˚C for drying.

Cell Lysis

To lyse the cells, coated slides were entirely submerged in pre-chilled lysis buffer for 4h at 4˚C.

Unwinding and electrophoresis

Following cell lysis, slides were incubated in electrophoresis buffer for 30min at 4˚C. Slides were placed in the electrophoretic unit taking care that slides do not touch each other or the wall of the unit. Electrophoresis was performed at 200mA/22V for 20min at 4˚C.

Neutralization and staining

Slides were washed thrice with Neutralization buffer and allowed to dry for 5min in absolute ethanol. Following neutralization, cells were stained with a staining solution of 20µl/ml EtBr and kept for 2-3min. Images were captured using a DM2500 fluorescence microscope (Leica, Germany).

DAPI STAINING

Material

- **DAPI (4'- 6'diamidino-2-phenylindole) (Sigma Aldrich, Cat No: 9542)**
  The stock solution of 1mg/ml was prepared in water aseptically. A working solution of 10µg/ml was prepared in methanol and can be stored for six months at 4˚C.
- 70% Ethanol (ice-cold)
- Triton X-100 (0.2%)
- Coverslip (Wiped with methanol, and UV treated for 20min)

Procedure

A549 cells were trypsinized from the stock flask and seeded on the coverslip at the density of $1x10^4$ cell/ml. 100µl of cell-suspension was added per coverslip and kept in 60mm dish filled with media, allowed to adhere overnight in a CO_2 humidified incubator at 37˚C. Following day, old media was replaced with fresh media containing 0.16nM of 4-fluorophenylacetamide-acetyl coumarin derivative and incubated for another 48h. Media containing derivative was pipette out,

and cells were washed with PBS thrice, 70% ethanol (ice-cold) was added to fix the cell. Washing was done with PBS to remove traces of ethanol. Further, cells were permeabilized with 0.2% Triton X-100 for 10min followed with washing using PBS. For staining, cells were incubated with DAPI solution (10µg/ml) for 10min. Once incubation was over, images were captured using a DM2500 fluorescence microscope (Leica, Germany).

DCFH-DA STAINING

Material

- **DCFH-DA (2'-7'dichlorofluorescin diacetate) (Sigma Aldrich, Cat No: 6883)**
  2mg DCFH-DA powder was dissolved in 1ml of DMSO to prepare a 2mg/ml stock solution, which can be stored at -20˚C in a bottle wrapped with aluminum foil to avoid direct contact with light. The working solution of 25µM was prepared in PBS by diluting 50µl of stock in 2ml of PBS. For quantitative analysis, the working solution of 10µM was prepared through diluting 6µl stock in 1.5ml PBS.

Procedure

$5x10^5$ cells/well was seeded on six-well plate. After adherence, cells were treated with 4-fluorophenylacetamide-acetyl coumarin derivative for 48h. Following incubation, cells were trypsinized, pellet down at 1000rpm for 10min, and washed thrice with PBS. The pellet was dipped in the DCFH-DA staining solution of 25µM for 30min at 37˚C, in the dark, covered with aluminum foil. The staining solution was pipette out and washed with PBS thrice (Maurya et al., 2011). Images were captured using a DM2500 fluorescence microscope (Leica, Germany).

For quantitative analysis, cells were trypsinized, and the pellet was collected in an Eppendorf tube, covered with aluminum foil. 100µl of working solution (10µM) was added in the Eppendorf tube having a cell pellet and incubated at 37˚C for 40min. Following incubation, the pellet was washed with PBS to clear background fluorescence, and fluorescence intensity was recorded at 480nm in Qubit® 2.0 Fluorometer (Invitrogen, USA).

BIOCHEMICAL ESTIMATION OF THE ANTIOXIDANT ENZYME SYSTEM

Material

- **KPE buffer**

  Potassium phosphate EDTA (KPE) Buffer (0.1M, 5 mM EDTA, pH 7.5)

  - Solution A: 6.8g of KH_2PO_4 was added in 500ml of H_2O_2.
  - Solution B: 8.5g of K_2HPO_4 or 11.4g of K_2HPO_4. $3H_2O$ was added to 500 ml of H_2O_2
  - Solutions were prepared separately and stored at 4˚C.
  - 0.1M KPE buffer was prepared by adding 16ml of solution A to 84ml of solution B. The pH was adjusted to 7.5, thereafter 0.327g of EDTA was added to prepare KPE buffer solution.

- **Extraction Buffer**

  0.1%Triton X-100, 0.6% sulfosalicylic acid in KPE

Procedure

The T25 cm^2 flask with 80% confluency was treated with derivative and incubated in a CO_2 humidified incubator at 37˚C, for 48h. Following incubation, cells were harvested using the trypsin-EDTA solution, pellet down at 1000rpm for 10min and washed with pre-chilled PBS. The pellet was re-suspended in extraction buffer and homogenized with Teflon pestle. Sonicated in ice for 2-3min with sonication cycle of 15sec sonication and 10sec resting followed with centrifugation at 20000rpm for 4min at 4˚C. The supernatant was collected in a pre-chilled tube, and assay for the antioxidant enzyme was performed using the supernatant.

GLUTATHIONE PEROXIDASE ESTIMATION

Material

- Phosphate Buffer (0.4M, pH 7)
- Glutathione (2mM) freshly prepared
- NaN_3 (10mM)
- H_2O_2 (10mM) freshly prepared
- Na_2HPO_4 (0.4M)
- DTNB (40mg of DTNB in 100ml of 1% sodium citrate)
- Metaphosphoric acid (30mM)

Procedure

40µl of supernatant was added in the reaction mixture containing 200µl Phosphate Buffer, 100µl Glutathione (2mM), 200µl NaN_3 (10mM), 200µl H_2O_2 (10mM), and 100µl water as mentioned in previous report (Paglia and Valentine, 1974). Incubated for 5min at RT. Following incubation, 200µl of metaphosphoric acid was added and kept in ice for 10min then centrifuged at 2000rpm for 10min. The supernatant was collected, and 60µl of supernatant was transferred to a new Eppendorf tube containing 60µl of Na_2HPO_4 and 3µl of DTNB, immediately reading was taken at 412nM in a microplate reader (Metertech ∑960).

SUPEROXIDE DISMUTASE ESTIMATION

Material

- Potassium phosphate buffer (0.2M, pH 8)
- Pyrogallol (25mg Pyrogallol in 1ml of 0.5N HCl)

Procedure

50µl Potassium phosphate buffer and 5µl Pyrogallol were mixed in an Eppendorf tube. Thereafter, 3µl of supernatant was added in the above solution, and immediately reading was taken at 420nm in a microplate reader (Marklund and Marklund, 1974).

CATALASE ESTIMATION

Material

- PBS (0.01M, pH 7)
- H_2O_2 (0.2M)
- Dichromatic acetic acid

  5% $K_2Cr_2O_7$ was prepared by dissolving $K_2Cr_2O_7$ in Glacial acetic acid at the ration of 1:4

Procedure

The 8µl of supernatant was mixed with the 50µl of H_2O_2 followed with addition of 80µl of PBS and incubated at room temperature for 1min. 80µl of dichromatic acetic acid reagent was added in the above mixture, and the reaction mixture was boiled for 10min, allowed to cool down. Absorbance was taken at 570nM in a microplate reader (Sinha, 1972).

RHODAMINE 123 STAINING

Material

- Rhodamine 123 (Sigma Aldrich, Cat No: 83702)

  The 5mg/ml Stock solution of Rhodamine 123 was prepared in ethanol. The working solution of 5µg/ml was prepared by diluting the stock solution with the PBS
- 70% ethanol (Ice-cold)
- PBS
- 35mm culture dish (Corning, Cat No: 430165)
- Sterile coverslip (Wiped with methanol, dried aseptically and UV treated)

Procedure

Cells were trypsinized from the stock flask and seeded on the coverslip at the density of $5x10^5$ cell/ml. 100µl of cell suspension was spread on the coverslip and kept in a 35mm dish, once cells get settled at the base, 1ml of complete media was poured. Cells were allowed to adhere overnight, the next day, old media was replaced with a fresh one, containing 4-fluorophenylacetamide-acetyl coumarin derivative and incubated in CO_2 humidified incubator at the 37˚C, for 48h. Following incubation, washing was done with PBS, thrice. Cells were fixed with ice-cold 70% ethanol for 30min. Washing was done thrice, with PBS to remove traces of ethanol. The cells were stained with 5µg/ml Rhodamine 123 solution and incubated again for 30min. Followed with washing, the images were captured using a Leica DM2500 fluorescence microscope.

ACRIDINE ORANGE STAINING

Material

- Acridine Orange (5µg/ml in PBS)
- Sterile coverslip (wiped with methanol, dried aseptically and UV treated)
- PBS

Procedure

Cells were harvested from the stock flask and re-seeded on the coverslip at the density of $1x10^5$ cells/ml, kept in 35mm dish, once cells get settled at the base, the dish was filled with complete media (1ml) and allowed to adhere overnight. Next day old media was replaced with fresh one containing 0.16nM derivative (4-fluorophenylacetamide-acetyl coumarin) and incubated for 48h. Following incubation, cells adhered coverslips were treated with 5µg/ml of Acridine orange solution and incubated for 15min. Images were captured with a fluorescence microscope.

CLONOGENIC ASSAY

Material

- **Crystal violet (Sigma Aldrich, Cat No: 6158)**

  0.1% w/v crystal violet solution was prepared by dissolving 10mg of crystal violet in 10ml of 10% ethanol.
- 35mm dish (Corning, Cat No: CLS430165)
- 70% ethanol (ice-cold)

Procedure

Cells were re-seeded on 35mm dish at the density of 1000cell/ml, allowed to adhere overnight and followed with treatment, (4-fluorophenylacetamide-acetyl coumarin derivative) for 48h. Post incubation, dishes were washed twice with PBS and supplemented with complete growth media (without derivative) and allowed to grow for an additional ten days in a CO_2 humidified incubator at 37˚C. The culture media was changed every three days. At the end of ten days, colonies, thus, obtained were washed with PBS thrice and fixed with 70% ethanol for 30min and stained with 0.1% crystal violet solution for one hour. The stained colonies were washed with PBS, and the images were taken to count the colonies in the dishes (Franken et al., 2006).

SOFT AGAR ASSAY

Material

- **2X DMEM media**

  1g of powered DMEM and 0.2g of $NaHCO_3$ was added in sterile water, and volume was adjusted to 50ml followed with filter sterilization using 0.22µm filter.
- Antibiotics (Gibco, Cat No: 15240-062)
- FBS (Gibco, Cat No: 10270-106)
- Six-well plate (Corning, Cat No: CLS3516)
- **1.6% agarose**

  1.6% agarose was prepared by adding 1.6g of agarose in 100ml of sterile water and autoclaved. This mixture can be stored in 4˚C in a closed lid glass bottle to maintain sterility until used.
- **0.8% Agarose**

  0.8% agarose was prepared by adding 0.8g of agarose in 100ml of sterile water and autoclaved. This mixture can be stored in 4˚C in a closed lid glass bottle to maintain sterility until used.

- 0.01% crystal violet solution in 10% ethanol.

Procedure

The Soft Agar assay was performed as per the procedure of Lui et al. (2016).

Bottom Agarose

1.6% Agarose was microwaved until completely dissolved, and the solution became clear. It was allowed to cool down in the Laminar flow cabinet by putting a bottle of agarose with an open lid in an ice bucket having warm water with a temperature of 40-45˚C. Then the 2X DMEM media (with antibiotic and without serum) was also kept nearby in the bucket. When the temperature of both solutions equilibrated in warm water, an equal volume of agarose and 2X media was added to adjust the concentration of agarose 0.8% for the bottom layer. Mixed immediately.

Cell suspension

Cells were harvested from the confluent flask, and cell number was counted on the hemocytometer. The Finale density of cells was adjusted to $6.5x10^3$ cell/ml in 2X complete media (20% FBS and 2X antibiotic).

Top Agarose

0.8% agarose was prepared in sterile water, autoclaved, and allowed to cool down in warm water. The cell suspension was added in an equal amount of agarose (1:1 ratio of agarose and cell suspension). To avoid premature hardening, prepare multiple tubes having 1ml of cell suspension, and 1ml of agarose would be added just before the plating.

Plating

1.5ml of top agarose was added immediately in a six-well plate and checked for any bubble at the upper surface if present allowed to settle down at the bottom of the layer. Kept at the room temperature in the Laminar flow cabinet for 30min to solidify properly before adding the top layer. Once the bottom agarose gets solidified, 1.5ml of the top agarose was added slowly onto it, to avoid any bubble formation. The final cell density would be around 5000cells/well in the six-well plate. Allowed to solidify at Laminar flow cabinet for 30min. 100µl of complete media was added before transferring to the CO_2 humidified incubator at 37˚C. Incubated overnight, next day old media was replaced with complete media having derivative and again incubated for 48h. Following incubation, media with derivative was replaced with fresh complete growth media and allowed to form colonies for ten days in CO_2 humidified incubator at 37˚C. Twice in a week, cells were fed with 100µl media. After incubation, the media was removed, and the upper surface of soft agar was washed with PBS. For staining, 500µl of crystal violet solution was added to each well and incubated for one hour, and images were captured using a microscope.

WOUND-HEALING ASSAY

Material

- Growth media with 1% FBS (DMEM, 1% FBS, 1X antibiotics)

Procedure

Cells were re-seeded on the six-well plate at the density of $2x10^5$ cells/well in complete media with 10% FBS. Incubated for continuous monolayer formation, when cell attains a monolayer with 90% confluency, The monolayer was scratched using 20µl micropipette tip, media having detached cells was removed, and washed with PBS, thrice (Yang et al., 2016). The plate was supplemented with growth media having 1% FBS and 0.16nM of derivative and incubated for 48h. The wound closure was monitored using Lawrence and Mayo inverted microscope, and the image was captured at 0h and 48h.

HANGING DROP AGGREGATION ASSAY

- 60mm dish (Corning, Cat No: 430166)
- Serum-free DMEM media

Procedure

Cells were re-seeded on culture dishes at the density of $2x10^5$ cells/ml and exposed to the 0.16nM of derivative (4-flurophenylacetamide-acetyl coumarin), incubated for 48h in CO_2 humidified incubator at 37˚C. Following treatment, cells were harvested and re-suspended in complete growth media such that 30µl of cell suspension would have 5000cells and placed as droplet at the inner surface of the 60mm culture dish lid, 20 drop-let per lid was placed and inverted on the dish having 5ml of serum-free media to avoid evaporation and drying of the drop. Incubated for 48h, the image was documented using a microscope at 0h and 48h.

HET-CAM TEST (HEN'S EGG TEST – CHORIOALLANTOIC MEMBRANE)

Material

- Paraffin wax
- 5% (w/v) Povidone-iodine solution
- 70% Ethanol

Procedure

Freshly laid fertile eggs of domestic hen (*Gallus domesticus*) of Rhode Island Red (RIR) breed were procured and inspected for any damages. The healthy eggs of uniform weight were selected and wiped and cleaned with 5% (w/v) povidone-iodine solution to remove external contamination. Candling was done to ensure the viability of the embryo. Non-viable and defective eggs were scraped out. Eggs were incubated for two days at the temperature of 37.5±0.5˚C and relative humidity of 75±5% in an automated incubator (Scientific Equipment Works, New Delhi) and hand-rotated on an hourly basis. On day 3, eggs were swabbed with 70% ethanol, 50µl of derivative (4-fluorophenylacetamide-acetyl coumarin) having a concentration of 0.16nM was injected via air sac method, aseptically in laminar airflow. The point of injection was sealed with molten paraffin wax and incubated for 48h at the temperature of 37.5±0.5˚C and relative humidity of 75±5% in an automated incubator, keeping horizontally with the sealed area facing upward and rotated every 1 hour. At day 5 (at HH27), 48h post-incubation, the embryos were isolated in the sterile Petri dishes of identical sizes. Images were observed under a light microscope (Magnus EM-210, India) and photographed using catcam eyepiece camera and catymage software (Catalyst Biotech, India).

PROTEIN EXPRESSION STUDY

Bradford reagent (Bradford 1976)

0.01% w/v Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid

Material

- Coomassie Brilliant Blue G-250 (SRL)
- 95% Ethanol
- 85% (w/v) Ortho-phosphoric acid

Procedure

For preparing Bradford reagent, 100mg of Coomassie Brilliant Blue G-250 was dissolved in 50ml of 95% ethanol thereafter, 100ml of 85% (w/v) ortho-phosphoric acid was added. The resulting solution was adjusted to a final volume of 1l. Filtered using Whatman filter paper # 1 and stored in an amber color bottle to protect from direct light at 4˚C.

Lysis Buffer

Material

- 10mM Tris (pH 7.4)
- 150mM NaCl
- 10mM $CaCl_2$

- 1% Triton X-100

Procedure

0.121g of Tris, 0.876g of NaCl, and 0.1g of $CaCl_2$ were weighed and dissolved in 70ml of distilled water. The pH was adjusted to 7.4 and checked usinhg pH meter (Analytical Instruments, USA). Finally, 1ml of Triton X-100 was added to the lysis buffer, and volume was made up to 100ml stored in 4˚C.

PROTEIN ESTIMATION

Material

- Bradford reagent
- Lysis buffer
- BSA

Procedure

$1x10^6$ cells were seeded in a six-well plate, incubated overnight for attachment. The next day, treated with 0.16nM of 4-fluorophenylacetamide-acetyl coumarin derivative and incubated in CO_2 humidified incubator at 37˚C. Post incubation, the media was removed, and the plate was transferred to ice, further, procedures were carried out on it. Cells were washed twice, with ice-cold PBS, and immediately 1ml ice-cold lysis buffer was added to each well. Cells were removed by gentle pipetting and collected in a microcentrifuge tube and centrifuged at 8000rpm for 10min at 4˚C. The supernatant was collected and transferred to a new vial, and the protein was estimated by the Bradford method (Bradford, 1976). 10µl of supernatant was taken in a 96-well plate in triplicate wherein 200µL of Bradford reagent was added and mixed well. Blank contained 10µL of distilled water instead of the protein sample. After 5min incubation, the absorbance was taken at 600nm in a microplate reader. The standard curve was used to determine the protein in unknown samples. Bovine Serum Albumin (BSA) was used as standard.

SDS-PAGE

Material

- Sample buffer (5X)
- 0.25M Tris-Cl (pH 6.6)
- β- Mercaptoethanol
- 10% SDS
- 50% Glycerol
- 0.5% Bromophenol blue

30% Gel stock

- 29% Acrylamide
- 1% Bis-acrylamide
- Distilled water to maintain the volume

4% Stacking gel

- 30% Gel stock
- 1.0M Tris-HCl (pH 6.8)
- 10% SDS
- 10% APS
- TEMED
- H_2O to maintain the volume

12% Resolving gel

- 30% Gel stock
- 1.5 M Tris Cl (pH 8.8)
- 10% SDS
- 10% APS
- TEMED
- D/W to maintain the volume

5X Tank buffer

- 0.125M Tris base
- 1.25M Glycine
- 10% SDS

Procedure

The gel casting assembly was set, and the bottom of the PAGE plate was sealed with 1% agar and allowed to solidify for 15 min. Resolving gel buffer was poured between PAGE plates in the casting assembly, and immediately, distilled water was added. The gel was left undisturbed and allowed to polymerize completely for 45min at room temperature. Excess water was removed, the stacking gel buffer was poured onto it, and Comb was inserted immediately. Once the stacking gel was completely polymerized, the assembly was transferred to the electrophoresis unit filled with tank buffer. Samples and molecular weight markers were loaded in the sample wells. The gel was allowed to run at a constant voltage of 100V until the dye front reached the bottom of the gel. The glass plates were separated carefully, and the gel was transferred into a staining box for further procedure.

Coomassie Brilliant Blue staining

Material

- 0.1 % CBB-R250
- 40% Methanol
- 10% Acetic acid

Procedure

For fixing, the gel was kept in 40% methanol for 30 min, after that, it shifted to a staining box containing the Coomassie stain and transfer on the rocker for 2 hours. Once the gel gets stained, the destaining of the gel background was done. As bands clearly visible and the background was cleared, the image of the gel was documented.

GELATIN ZYMOGRAPHY

Materials

Lysis Buffer

- 10mM Tris (pH 8)
- 150mM NaCl
- 1% Triton X-100
- 10mM $CaCl_2$

5X Sample buffer

- 1M Tris (pH 6.8)
- 10% Glycerol
- 2% SDS
- 0.002% Bromophenol blue

7.5% resolving gel (5ml)

- 30% Gel stock
- 1.5M Tris Cl (pH 8.8)
- 2.5% Gelatin
- 10% SDS
- 10% APA
- TEMED
- Distilled water

4% stacking buffer

- 30% Gel stock
- 1.5M Tris Cl (pH 6.8)

- 10% SDS
- 10% APA
- TEMED
- Distilled water

Triton wash buffer

- 20mM Tris Cl (pH 8)
- 0.15M NaCl
- 5mM$CaCl_2$
- 2.5% Triton X-100

Incubation buffer

- 50mM Tris Cl (pH 7.5)
- 0.2M NaCl
- $CaCl_2$
- 0.02% NaN_3
- 0.02% Brij35

0.25% Coomassie Brilliant Blue R250 staining solution

- Coomassie Brilliant Blue R250
- Methanol: H_2O (1:1v/v)
- Glacial acetic acid

Destaining solution (100ml)

Methanol: Glacial acetic acid: Distilled H_2O (50:10:40)

Procedure

$1x10^6$ cells were seeded in a six-well plate, allowed to adhere overnight, treatment with derivative was given for 48h. The next day, cells were homogenized with the lysis buffer and centrifuged at 8000rpm for 10min, the supernatant was collected, and estimation was done using Bradford reagent as per standerd procedure of SDS-PAGE. 30µg of the total protein from each sample was loaded on 7.5% SDS-PAGE (7.5% Resolving gel), containing 2.5% gelatin and allowed to electrophoresed at 100V (constant voltage). Then after, the gel was washed with Triton wash buffer, twice, each wash last for 1 hour. Further, the gel was incubated in incubation buffer, overnight at 37˚C to re-activate the MMPs and stained with 0.25% Coomassie Brilliant Blue R250 for 2-4h, followed by destaining with the destaining solution. The gel was destained till clear bands are against the dark blue background were visualized.

WESTERN BLOT

Material

- Methanol
- Whatmann filter papers
- Nitrocellulose membrane/ Polyvinylidene difluoride (PVDF) membrane
- Distilled water

5X Semidry transfer buffer (pH 9.2)

- 240mM Tris
- 39mM Glycine

Washing buffer (TBS-T)

- 0.1% Triton X-100 in TBS

0.1% Ponceau S staining solution (100ml)

- Ponceau S
- Acetic acid

  Volume made up to 100mL (with 5% acetic acid in 100ml ddH_2O) with double distilled water (100ml)

Blocking buffer (TBS-MT)

- 5% skimmed milk powder in TBS-T

Antibody dilution buffer

- BSA and Sodium azide added to TBS-T to a final concentration of 2 % and 0.02 % respectively.

BCIP-NBT stock solution (50X)

- BCIP 9.4 mg/ml
- NBT 18.75 mg/ml

  Prepared in 67% DMSO solution. Diluted to working concentration in ALP substrate buffer fresh before use.

ALP substrate buffer (pH 9.5)

- 0.1M Tris base
- 0.1M NaCl
- 0.05M $MgCl_2$

Procedure

Band Transfer

Following SDS-PAGE, the gel was transferred to the distilled water for removal of the buffer; then, it was placed in the transfer buffer for 15min. After soaking the PVDF membrane first in absolute methanol. Following this, the PVDF membrane was washed with a transfer buffer to remove the excess of methanol, and then it was shifted to the transfer buffer. Eight Whatman filter paper of 5cm×8cm size was soaked in a 1X transfer buffer for 10min. Once this set was ready the sandwich of 4 filter paper, gel, PVDF membrane, and 4 filter paper were made, and the proteins were transferred from the gel to PVDF by applying 100mA current for 20min in semidry transfer unit (Cat. no. MX-129501, Medox Biotech, India). Following the transfer, the membrane was stained with ponceau stain for 20sec (reversible) to check whether the proteins were transferred or not. The membrane was washed with distilled water and incubated with a blocking buffer for 45min at room temperature on a rocking platform (MedoxBio 1290-02 Gel rocker, Medox Biotech, India). The blocking buffer was discarded after incubation, and the membrane was incubated overnight at 4˚C with the respective primary antibody. The next day, the membrane was washed with 1X TBS thrice each for 20min.

Antibody probing

The membrane was blocked with a blocking buffer at room temperature for 1hour. It was then incubated in the primary antibody at 4˚C overnight. The membrane was washed with washing buffer thrice, for 10min each time. This was followed by incubation with a biotinylated secondary antibody for 30min, followed by washing with washing buffer thrice, same as the previous step. Streptavidin-ALP conjugate solution was added and incubated for 15min, followed by washing. The membrane was then incubated with ALP conjugated streptavidin for 45min at room temperature. Finally, 1ml of BCIP-NBT substrate was added to the membrane and observed for color development. Once the specific bands were observed on the membrane, the excess of the substrate was discarded in order to avoid non-specific color development. After the development of the western blot, densitometric analysis was done by measuring the band intensity using ImageJ software, and the values obtained were normalized with the band intensity of β-actin.

GENE EXPRESSION STUDY

RNA ISOLATION

Material

- DEPC water

0.1% DEPC in distilled water was prepared with vigorous vortexing. Autoclaved. All the reagents were made in DEPC water to protect RNA from RNAse during the RNA isolation procedure.

- TRIzol reagent (Life technologies, Cat No:10296010)
- Chloroform
- Isopropanol
- 70% Ethanol prepared in DEPC water
- 1% Agarose prepared in DEPC water
- 25mM Ethidium bromide

10X TBE buffer

- Tris base
- Boric acid
- 0.5M EDTA
- DEPC water to maintain the volume

5X RNA loading dye (1ml)

- 500mM EDTA
- 40% Formaldehyde
- Glycerol
- Formamide
- 2.5% Bromophenol blue
- 10X TBE
- DEPC water required for volume make up.
- All glassware and disposables were sterile, and all surfaces involved were cleaned prior to the experiment.

Procedure

Total RNA Isolation

$1x10^6$ cells/ml were seeded in the six-well plate, allowed to adhere overnight, followed with treatment with the 0.16nM concentration of the derivative(4-fluorophenylacetamide- acetyl coumarin), and incubated for 48h. Post-incubation, the media was removed, cells were washed with PBS, and 1ml TRIzol reagent was added (the plate was kept on ice, and the further procedure was performed on the ice). Cells were homogenized through gentle pipetting until all cells were detached completely. The supernatant was collected in a microcentrifuge tube and centrifuged at 8000rpm for 20min at 4˚C. The supernatant was transferred to a new Eppendorf

tube having 200µl of chloroform and shake vigorously for 15sec. The mixture was allowed to stand for 15min on ice with occasional mixing and centrifuged at 12000rpm for 15min at 4˚C. Centrifugation separated the mixture into three phases; the upper aqueous phase having RNA was collected and transferred to a new vial containing 500µl of 70% isopropanol. The tube was gently mixed by inverting and allowed to pellet out through putting it in the refrigerator at least for 1 hour and centrifuged at 12000rpm for 15min at 4˚C. The supernatant was removed carefully without disturbing the pellet and washed twice with 75% ethanol (1ml) by gentle mixing and centrifugation again at the same. The supernatant was decanted, and the tube was kept open on the ice for 5-7min to allow drying of the pellet. Overdrying was avoided. As ethanol evaporated completely, the pellet was dissolved in the 30µl of DEPC water and allowed to dissolve completely by putting it at 8-10˚C, overnight.

RNA QUANTIFICATION

Material

- 1% Agarose in DEPC
- DEPC water
- Ethidium bromide

Procedure

1µl of RNA solution was taken from the above isolation step and diluted in 1ml DEPC water. The reading was done at 260nm and 280nm on a UV-Vis spectrophotometer (Toshniwal). The quality of RNA was checked by OD_{260}/OD_{280}. A ratio of 1.8-2 was considered a good quality of RNA. Further, the integrity of RNA was checked using the electrophoresis of RNA. A 3µl of RNA was electrophoresed on a 1% Agarose gel containing Ethidium bromide. Three distinct and sharp bands were reflected, which showed good integrity of the RNA.

cDNA SYNTHESIS

cDNA was synthesized after RNA quantification of RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). It uses random hexamers to prime the polymerase chain reaction using reverse transcriptase. 1µg of RNA was used from each sample for cDNA synthesis. Following thermal cycle program was used for the cDNA synthesis (reverse transcription);

Program for cDNA Reverse transcription reaction

	Temperature	Time
▪ Step 1	25˚C	10min
▪ Step 2	37˚C	120min
▪ Step 3	85˚C	5min
▪ Step 4	4˚C	∞

2X Master Mix preparation (per 20µl reaction)

The kit components were thawed on the ice, and the following volume was taken to prepare 20µl of 2X Mastermix.

• 10X RT buffer	2µl
• 25X dNTP mix	0.8µl
• 10X RT random primers	2µl
• RT enzyme	1µl
• Water	4.2µl
• Total volume	10µl

All components were added and gently mixed on ice to prepare 2X Mastermix for the cDNA synthesis.

Procedure

10µl of 2X Mastermix was added in each tube having 10µl of RNA sample of 1µg/µl concentration and mixed gently by micro-centrifugation and run on the programme cDNA synthesis programme in a thermal cycler.

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Once cDNA was prepared, a semi-quantitative RT-PCR was performed on the T100 thermal cycler (BioRad, USA). The Primer sequence was obtained from the NCBI and listed in Appendix I. Master mix was obtained from the Sisco Research Laboratories, Mumbai. The PCR was performed as per below-mentioned programme with the reaction mixture of:

PCR Reaction Mixture (10µl)

- 2X Master mix 5.0µl
- Forward primer (5pmoles/µL) 1.0µl
- Reverse primer (5pmoles/µL) 1.0µl
- cDNA Sample 1.0µl
- Water 2.0µl

PCR Programme

	Temperature	Time	
• Initial Denaturation	95˚C	120sec	
• Denaturation	95˚C	10sec	40 cycles
• Annealing	60˚C	30sec	
• Extension	72˚C	30sec	
• Final Extension	72˚C	180sec	

Tubes were left at 12˚C in the thermal cycler until the further procedure.

AGAROSE GEL ELECTROPHORESIS

- 2% Agarose in TBE
- 25mM Ethidium Bromide
- 100bp ladder (Thermo Scientific, USA)

1X TBE

- Tris base
- Boric acid
- 0.5M EDTA

Volume made up to 1litter D/W water

6X DNA loading dye

Glycerol	30%
Bromo Phenol Blue	0.25%

Procedure

PCR product was checked on 2% agarose gel electrophoresis. The gel was prepared in 1X TBE buffer. For loading into a gel, PCR products were mixed with 1X DNA loading dye. Along with PCR product, the 100bp ladder was also loaded as a marker for confirmation of the correct size of PCR products. The gel was allowed to run at 100V for 30min. After that, the image was taken by GeNei Imaging System (GeNei, Merck, USA) in ultraviolet light.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION PCR (REAL-TIME QRT-PCR)

Quantitative gene expression analyses were carried out in real-time PCR using SYBR Green-based master-mix (Takara Bio, Japan) on a LightCycler 96 machine (Roche Diagnostics, Switzerland) using the following programme and component volume

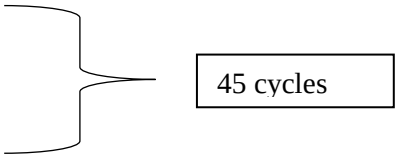
Component volume

- 2X SYBR Green Master mix 5µl
- Forward primer (5µM) 0.5µl
- Reverse primer (5µM) 0.5µl
- cDNA template 1µl
- Nuclease-free water 3µl

The reactions were run in 96-well plates (Genaxy, USA). Each gene was assayed in triplicates.

qReal-Time RT PCR programme

	Temperature	**Time**
•	95˚C	100 sec
•	95˚C	10 sec
•	60˚C	30 sec
•	72˚C	30 sec



After completion of the programme, the melt curve analysis was performed to assess the uniformity of the PCR product, primer-dimer formation and non-specific amplification. Following programme was used to analyse the melt-curve analysis:

Melt-curve analysis

	Temperature	**Time**
•	95˚C	10 sec
•	65˚C	60 sec
•	97˚C	1 sec

Data analysis

Data were analyzed using the LightCycler 96 software version. The comparative cycle threshold (Cq) method was used for relative quantification of gene expression where Cq was indicating the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR. Cq values were obtained for each well and normalized by subtracting the Cq values of the endogenous control gene (*18srRNA*) from those of the target gene. The mean of these normalized Cq values were plotted.

ΔCq = Cq of target – Cq of endogenous control

Moreover, fold change in expression was calculated by the ΔΔCq method of Livak and Schmittgen (2001).

ΔΔCq= ΔCq of treated gene - ΔCq of control gene

For fold change of gene expression in treated as compared to that in control

$$\textbf{Fold change} = 2^{-\Delta\Delta Cq}$$

ΔΔCq values were plotted on a graph with a vertical axis following a logarithmic scale. For biological significance, fold change values above 0.5 and below 2.0 were considered as significant up or downregulation.