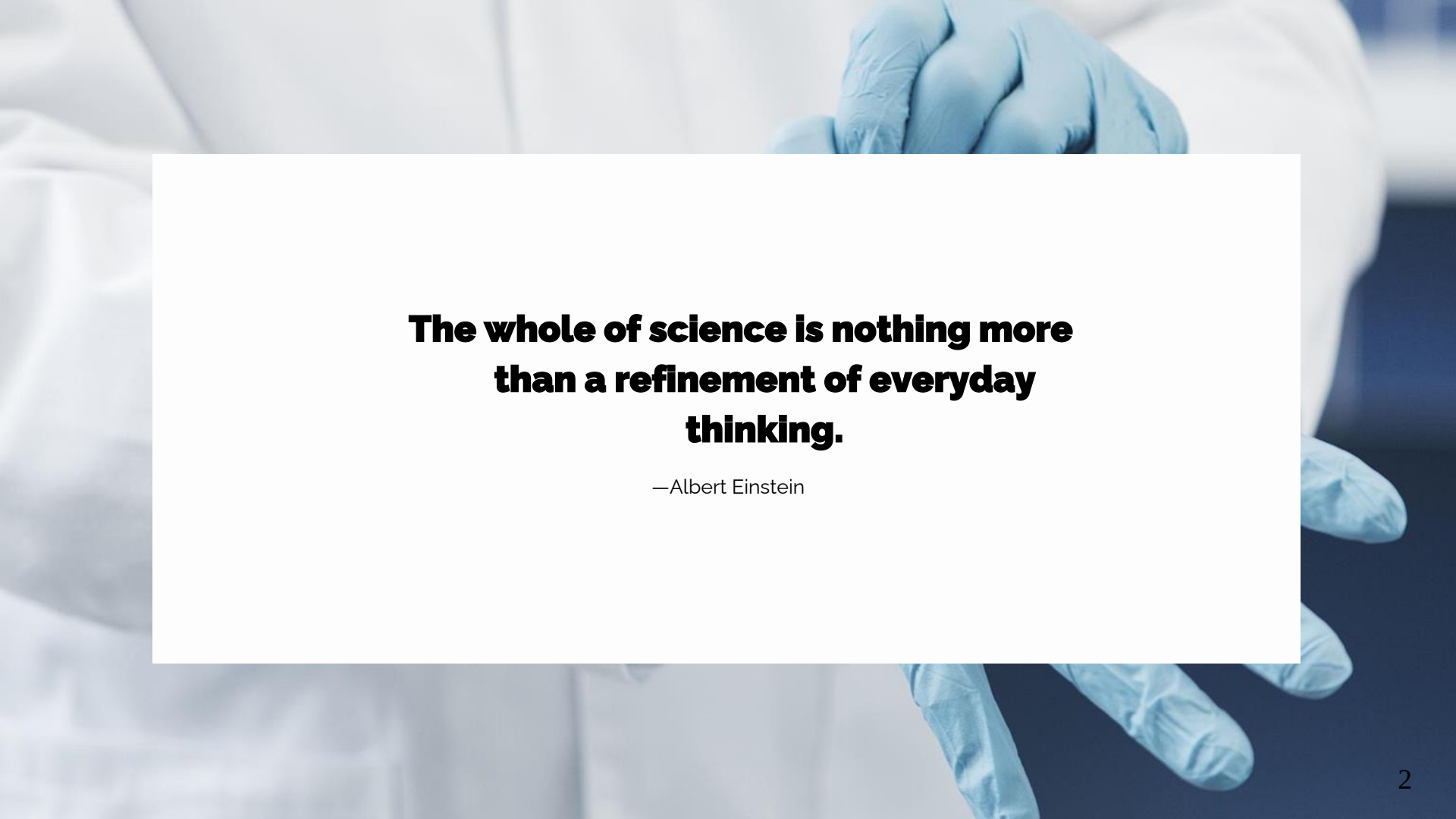
In the Name of God



The whole of science is nothing more than a refinement of everyday thinking.

—Albert Einstein

Investigation of autophagy as a mechanism of survival for Doxorubicin-resistant cell lines and it’s inhibition by Clarithromycin to improve anti-cancer effects In Vitro

Supervisors:

Prof. Mohammad Shokrzadeh, Dr. Fatemeh Shaki

Presenter:

Nahid Amani

(PhD candidate of Toxicology)

Date: 1399/4/8

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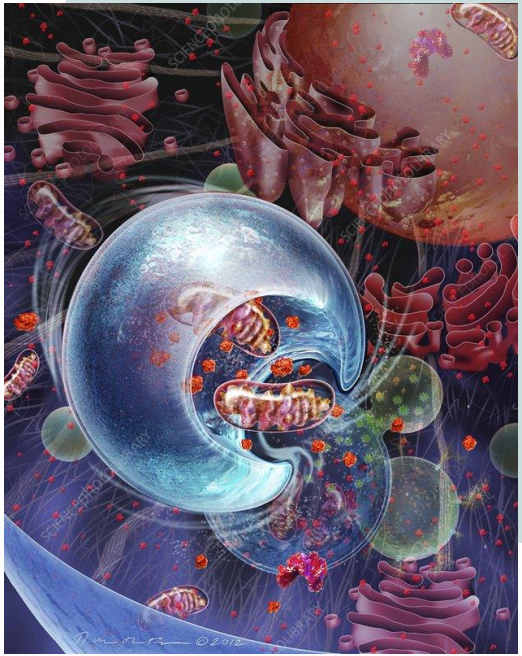


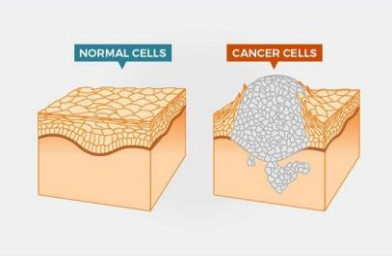
1

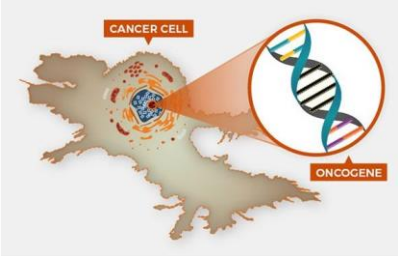
Overview

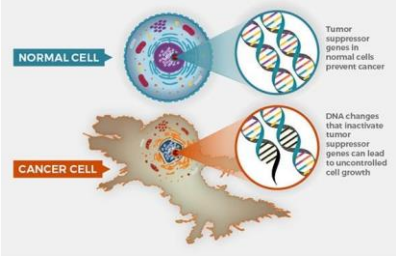
CANCER

- A terrifying illness
- A very heterogeneous group of diseases
- A complex problem in health care delivery and public policy

Cancer is a disease where cells grow out of control and invade, erode and destroy normal tissue








CANCER TREATMENT OPTIONS
HORMONE THERAPY
IMMUNOTHERAPY
SURGERY
RADIATION THERAPY
BONE MARROW TRANSPLANTATION
TARGETED THERAPY
CHEMOTHERAPY

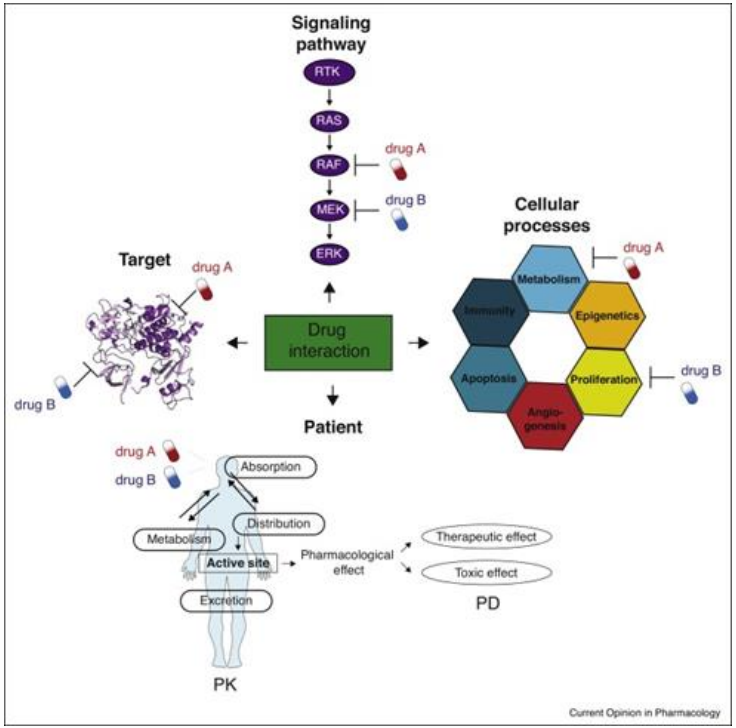
ANTI CANCER DRUGS

- Monotherapy
- **Combination therapy**

Efficacy

Toxicity

Drug resistance



COMBINATION THERAPY

- Combination therapy was first conceptualized in 1965, where *Emil Frei*, *James F. Holland* and *Emil J. Freireich* postulated the possibility of the first ever combination chemotherapy for acute leukemia.

- **Restrictive combinations of drugs**

- **Repurposing Drugs in Oncology (ReDO)**

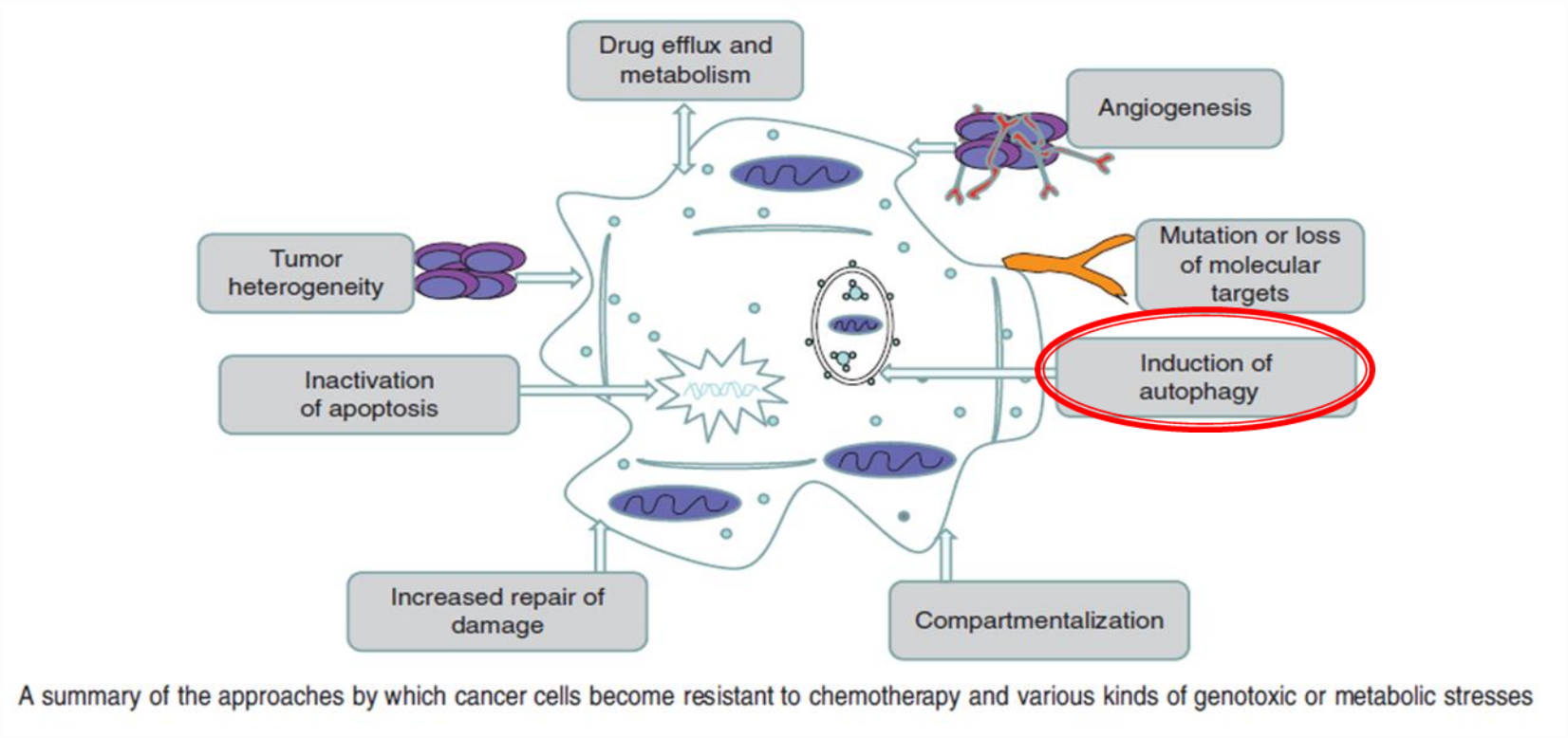
Mebendazole Nitroglycerin Cimetidine **Clarithromycin** Diclofenac Itraconazole

CANCER DRUG RESISTANCE

- A major problem facing current cancer research

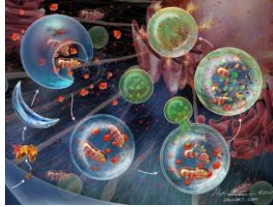
- Resistance to chemotherapeutics can be divided into two broad categories: intrinsic or **acquired**.

MECHANISMS OF DRUG RESISTANCE

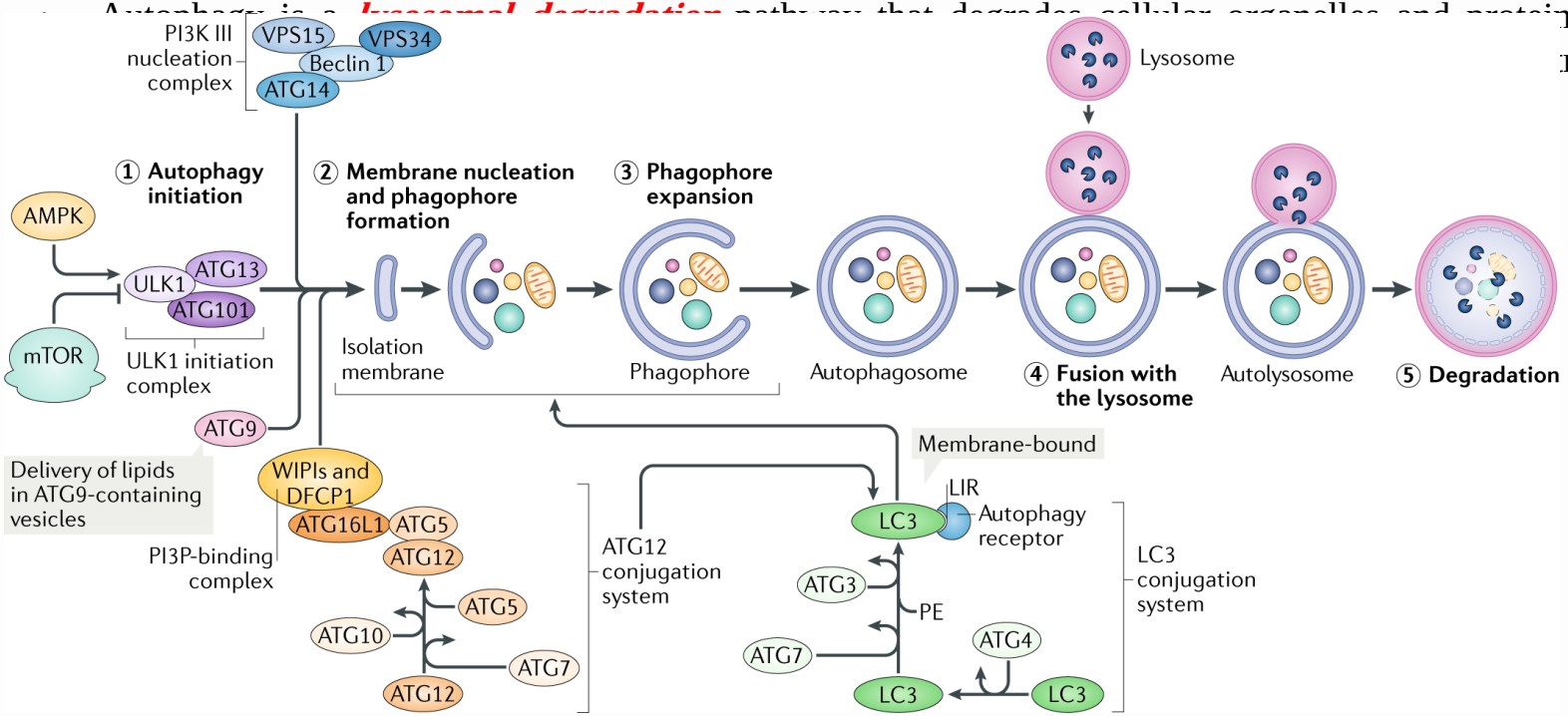


A summary of the approaches by which cancer cells become resistant to chemotherapy and various kinds of genotoxic or metabolic stresses

AUTOPHAGY



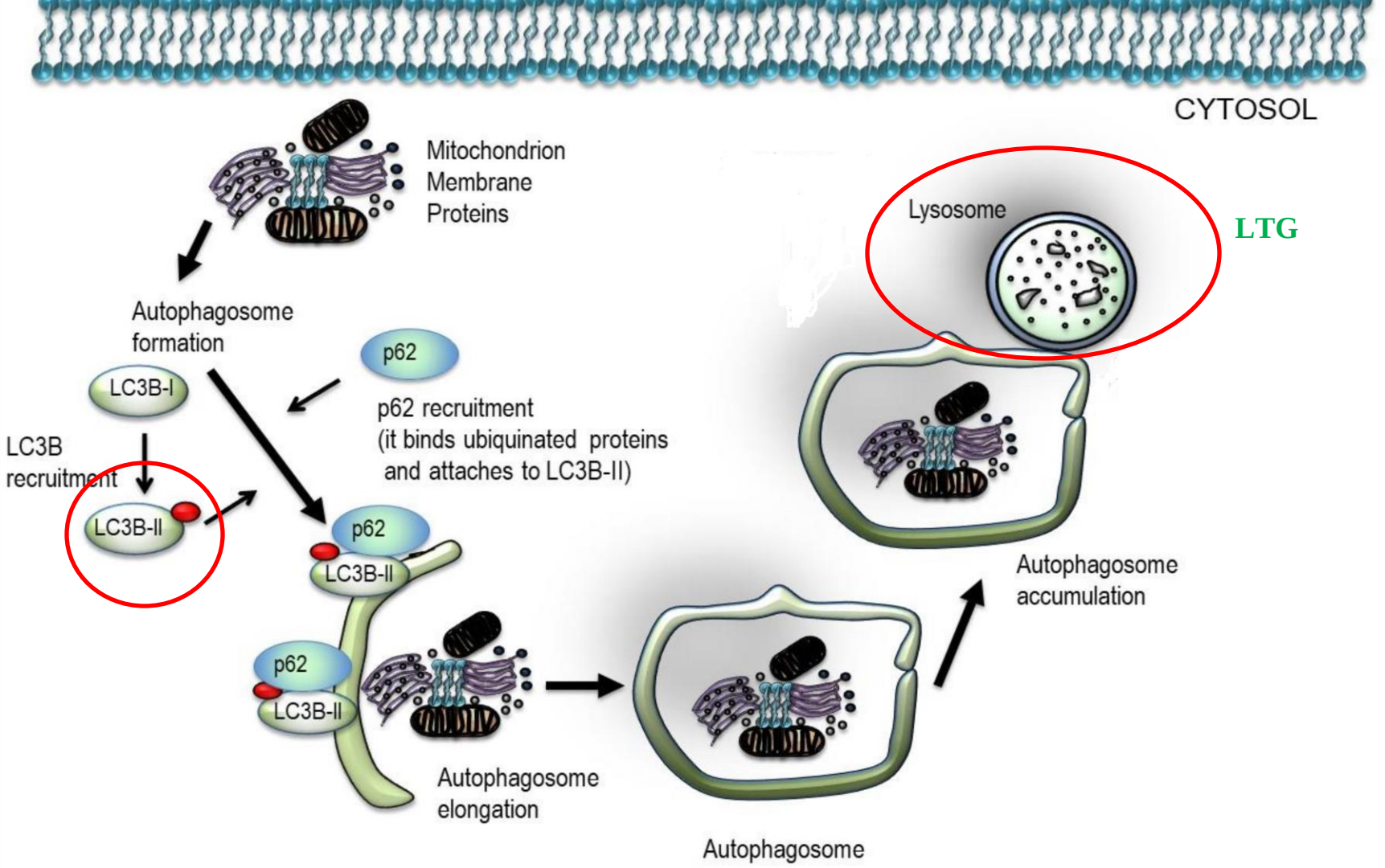
Autophagy is a ***lysosomal degradation*** pathway that degrades cellular organelles and proteins in ...rient



METHODS FOR MEASURING AUTOPHAGY

- **In vitro methods**

1. Transmission Electron Microscopy
2. Immunoblots for LC3 and SQSTM1/p62
3. LC3 and p62 Immunohistochemistry
4. LC3 Fluorescence Microscopy
5. Tandem GFP-RFP-LC3 to Assess Autophagic Flux
6. Nanoparticles
7. **Flow Cytometry–Based Methods**


CYTOSOL
Mitochondrion
Membrane
Proteins
Autophagosome
formation
LC3B-I
LC3B
recruitment
LC3B-II
p62
p62 recruitment
(it binds ubiquinated proteins
and attaches to LC3B-II)
p62
LC3B-II
p62
LC3B-II
Autophagosome
elongation
Autophagosome
Lysosome
LTG
Autophagosome
accumulation

AUTOPHAGY AND CANCER DRUG RESISTANCE

- **Autophagy** is prognostic of poor outcome in multiple tumor types, including cancers of the breast, lung, and colon.
- High levels of autophagy have been associated with ***resistance*** to systemic therapy in several preclinical and clinical models.
- Targeting of autophagy with pharmacological agents may be a mechanism to improve the effectiveness of anticancer drugs.
- Several autophagy inhibitors such as ***chloroquine*** and its derivative ***hydroxychloroquine*** have been studied in more than 30 phase I/II cancer clinical trials.

Requirement of high levels to obtain autophagy inhibition

CLARITHROMYCIN

- Repurposing Drugs in Oncology (ReDO)—**clarithromycin** as an anti-cancer agent
- Low toxicity
- Anticancer properties alone or in combination with conventional treatment
- **Clarithromycin is a potent and continuous inhibitor of autophagy**

DOXORUBICIN

- A broad-spectrum anthracycline
- The most widely used FDA approved anticancer drug
- **Autophagy**

2

Scope of work

AIMS OF STUDY

The aim of this thesis is to evaluate the effect of clarithromycin to improve the cytotoxicity of doxorubicin in two resistant-human cancer cell lines (lung adenocarcinoma (A549) and breast cancer (MCF7)) through inhibition of autophagy as the main underlying mechanism.

1. **At the first part of the project we aim to investigate whether CAM could enhance DOX toxicity in sensitive human cancer cell lines**
2. **Second part is to establish DOX-resistant cell lines**
3. **And whether exposure of cancer cell lines to DOX induces autophagic process as underlying mechanism of drug resistance**
4. **And the end part of the project will focused on the idea of autophagy inhibition as therapeutic strategy**

3

Methodology

PROJECT PHASES

1

Investigating the effect of CAM on DOX cytotoxicity in A549 and MCF7 cells

1.Time and dose-dependent growth inhibition of MCF7 cells by clarithromycin

2.Time and dose-dependent growth inhibition of A549 cells by clarithromycin

3.Combined treatment with doxorubicin and clarithromycin in MCF7 cells

4.Combined treatment with doxorubicin and clarithromycin in A549 cells

5.Drug interaction analysis between DOX and CAM in MCF7 cells

6.Drug interaction analysis between DOX and CAM in A549 cells

7.Evaluation of apoptosis induced by combined treatment with DOX and CAM in MCF7 cells

8.Evaluation of apoptosis induced by combined treatment with DOX and CAM in A549 cells

2

Establishment of DOX-resistant sub lines

1. Establishment of DOX-resistant MCF7 cells

2.Establishment of DOX-resistant A549 cells

3. Measurement of Drug resistance in MCF7.R cell lines

4. Measurement of Drug resistance in A549.R cell lines

3

Measurement of autophagy in resistant-sub lines

1. Measurement of autophagy in MCF7.Res cells by flowcytometry
2. Measurement of autophagy in A549.res cells by flowcytometry

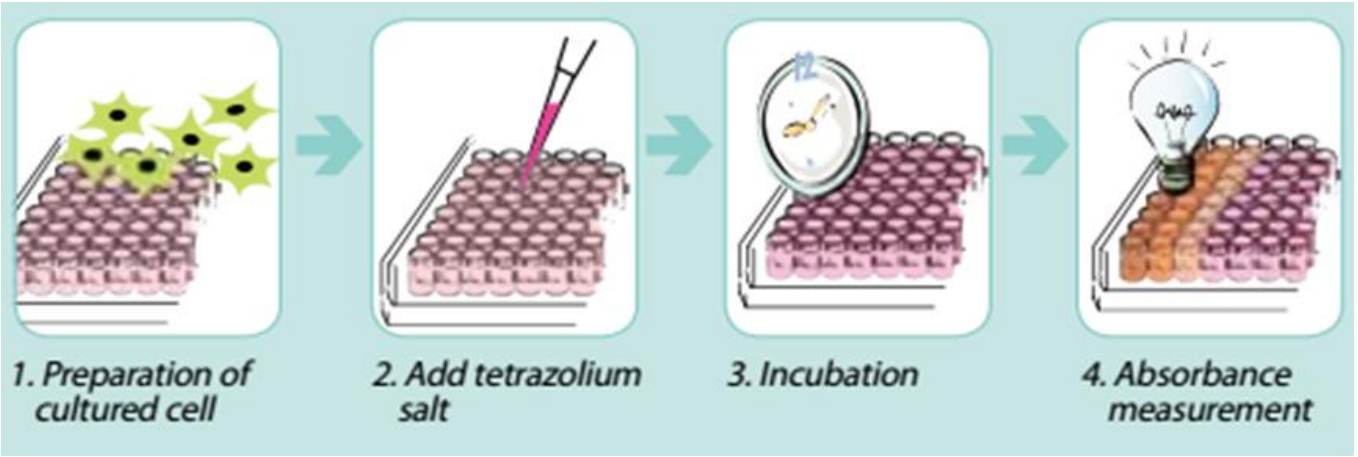
4

Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance

1. The effect of CAM on inhibition of autophagy in MCF7.Res cells
2. The effect of CAM on inhibition of autophagy in A549.Res cells
3. The effect of CAM on attenuating drug resistance in MCF7.Res cells
4. The effect of CAM on attenuating drug resistance in A549.Res cells
5. The effect of CAM on P-gp activity in MCF7.Res and A549.Res cells

1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 and A549 cells

- Time and dose-dependent growth inhibition of MCF7 and A549 cells by **Clarithromycin(CAM)**
- **MTT assay**



1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 and A549 cells

- Combination treatment with Doxorubicin(**DOX**) and **CAM** in MCF7 and A549 cells
- CAM(5, 100 and 500 μM)
- MTT assay
- **Comparison of DOX IC50**

1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 and A549 cells

- **Drug interaction analysis** between DOX and CAM in MCF7 and A549 cells
- **Median-drug effect analysis**
- **Chou and Talalay**
- **Compusyn software**

1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 and A549 cells

- **Drug interaction analysis** between DOX and CAM in MCF7 and A549 cells

- **Combination Index** $=[(D)_1/(D_{1\text{-}FA})_1]+[(D)_2/(D_{1\text{-}FA})_2]+[\alpha(D)_1(D)_2/(D_{1\text{-}FA})_1(D_{1\text{-}FA})_2]$

- ***The non-fixed ratio design***

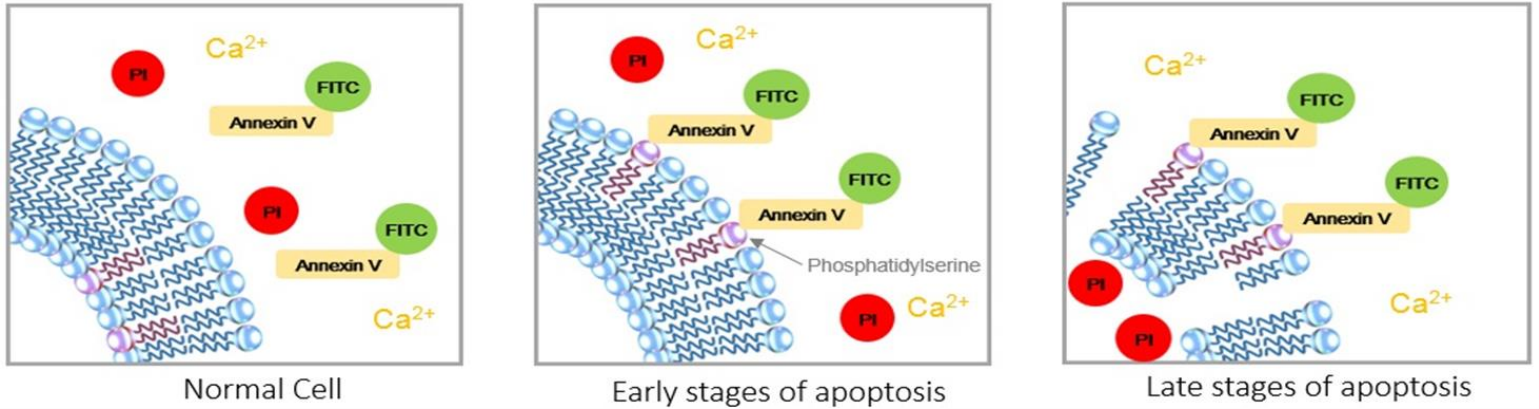
CI		Synergism/antagonism
<0.1	+++++	Very strong synergism
0.1–0.3	++++	Strong synergism
0.3–0.7	+++	Synergism
0.7–0.85	++	Moderate synergism
0.85–0.9	+	Slight synergism
0.9–1.1		Nearly additive
1.1–1.2	–	Slight antagonism
1.2–1.45	––	Moderate antagonism
1.45–3.3	–––	Antagonism
3.3–10	––––	Strong antagonism
>10	–––––	Very strong antagonism

Simplified CI values and their indication

<0.8	Synergism
0.8–1.2	Additive
>1.2	Antagonism

1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 and A549 cells

- Evaluation of **apoptosis** induced by combined treatment with DOX and CAM in MCF7 and A549 cells
- Annexin V apoptosis detection kit FITC



2 Establishment of DOX-resistant sub lines

- **Drug resistant cell models:**

1. Clinically relevant(pulsed treatment)
2. High-level laboratory(continues treatment)

- **Continues stepwise increasing**
- **Optimal initial dose**

⇩

Chronic inhibitory concentration 20(Chronic IC20)

⇩

IC20*1.5

⇩

IC20*1.5*1.25

2 Establishment of DOX-resistant sub lines

- Measurement of drug resistance in MCF7.Res and A549.Res cell lines

1. **Microscopic Images**

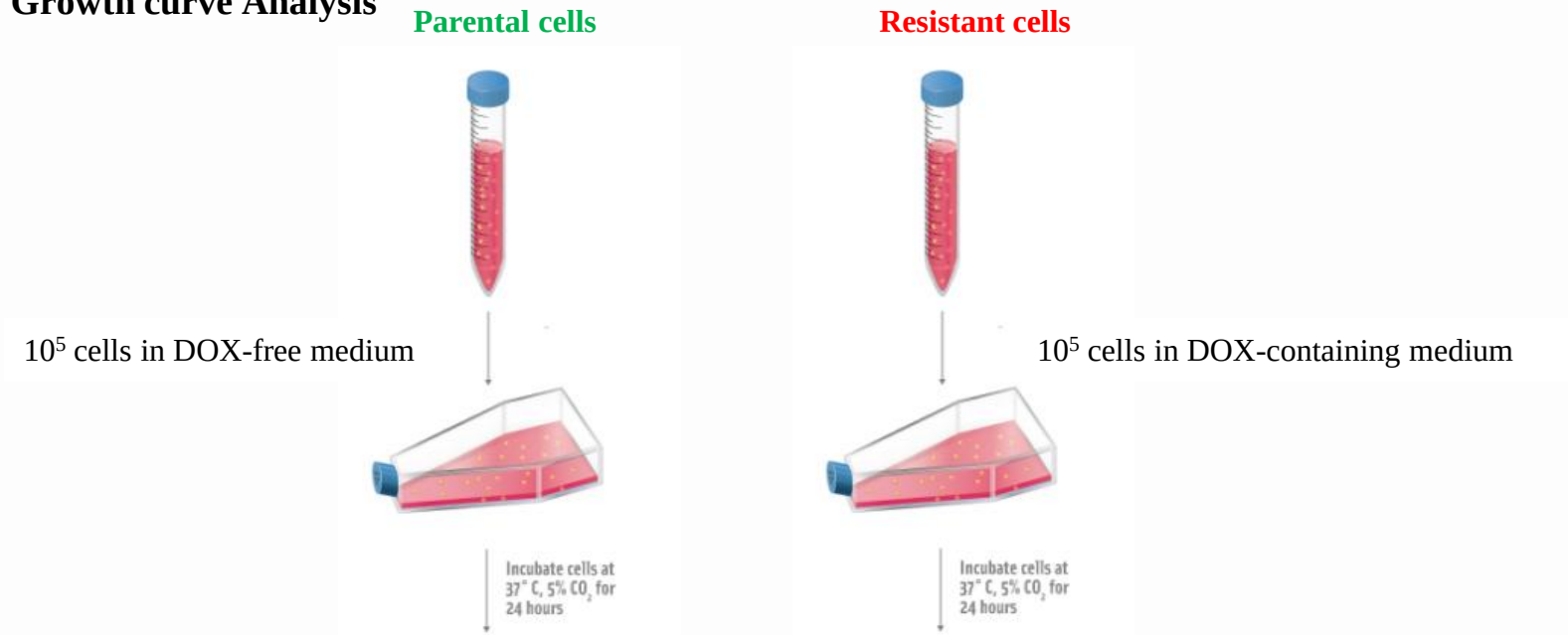
2. **Fold Resistance**

Fold Resistance=IC50 of resistant cell line/IC50 of parental cell line

3. **Growth curve Analysis**

2 Establishment of DOX-resistant sub lines

- **Growth curve Analysis**



Viable cells were counted using **trypan blue exclusion test** every 24 hours for 8 consecutive days

Doubling time=$t_{1/2}$=Ln2/K

3 Measurement of autophagy in resistant-sub lines

- **Indirect immunofluorescence LC3II labeling**

Trypsin-EDTA

Centrifuge for 5 min

Ice-cold methanol 100%
Place at -20°C for 5min

Rinse with PBS

Permeabilized with 0.25%
Triton X-100 for 15 min

Centrifuge for 5 min

LC3II polyclonal antibody(0.05 mg/ml) or rabbit immunoglobulin/30 min

Centrifuge for 5 min

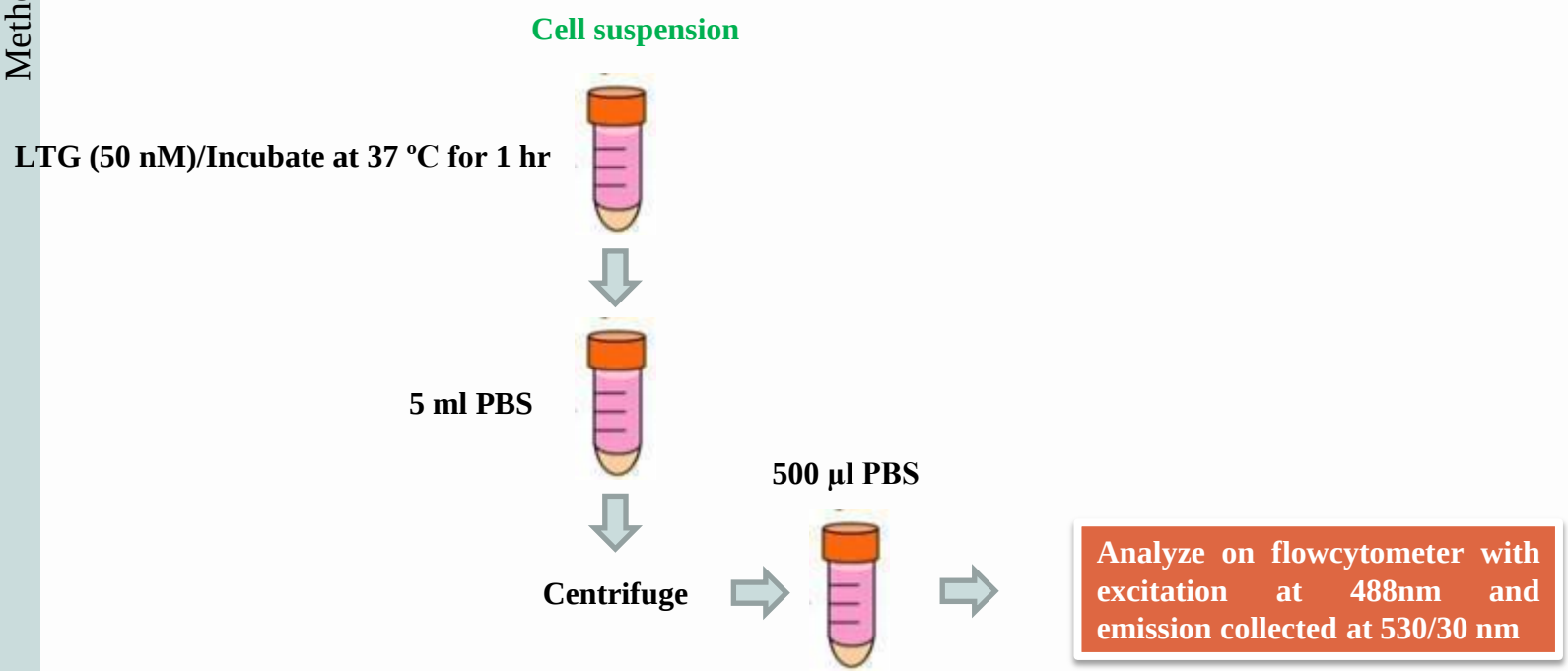
Goat anti-rabbit Alexa Fluor-647(0.025 mg/ml)/30 min

PBS(500 µl)

Analyze on flowcytometer with excitation at 633 nm and emission collected at 660/20 nm

3 Measurement of autophagy in resistant-sub lines

- **LysoTracker Green labeling**

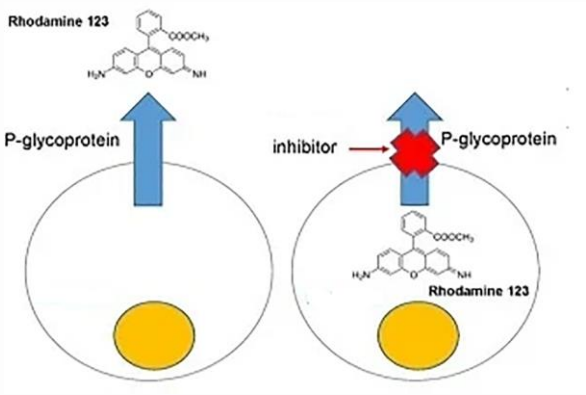


4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance

- **Cell growth inhibition after combined treatment of DOX+CAM in Resistant cells**
- **Determination of IC50 values of DOX in absence and presence of CAM**
  - ✓ Extra-sum of square F-test
- **LC3II expression in parental and resistant cells**
- **LTG expression in parental and resistant cells**
- **Apoptosis**

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance

- Determination of **P-glycoprotein activity** by **Rhodamin 123**



1. Cells were seeded in 6-well plate
2. After 24 h, 200 ng/ml Rh123 was added to the culture medium
3. After 1 h incubation, cells were washed with PBS
4. Then, cells fed with fresh Rh123- free media for another 1 h
5. Cells were then suspended in 1 ml PBS and cellular efflux of Rh123 was assessed by flow cytometry at 523 nm emission wavelength.

Statistical analysis

- **All experiments were performed at least in triplicate**
- **The results are presented as Mean ± SEM**
- **Data were analyzed using Student t-test, one-way and two-way ANOVA by GraphPad Prism 8**
- **Analysis of variance model was used to compare the differences among more than 2 groups and followed by Tukey post hoc test**
- **Statistical significance of differences was indicated as $p < 0.05$**
- **To compare IC50 values, the extra sum-of-squares F test approach was used.**
- **Compusyn software**

4

Results analysis

1 Time and dose-dependent growth inhibition of MCF7 cells by Clarithromycin

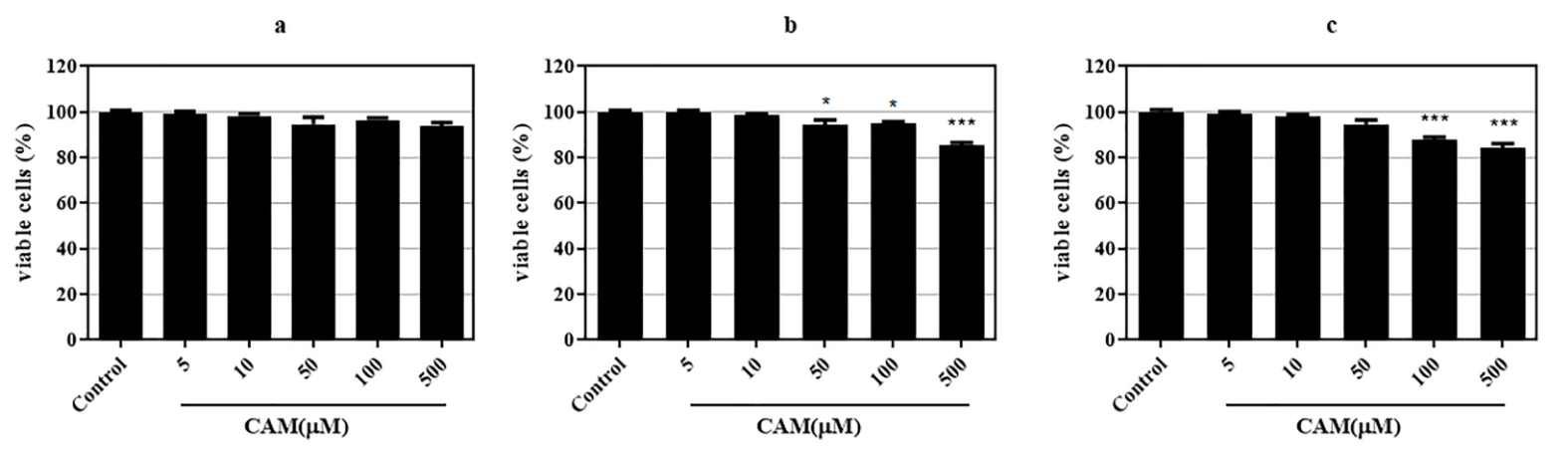


Figure 1. Cytotoxic effect of CAM on MCF7 cell lines. MCF7 cells were cultured with various concentrations of CAM for a) 24, b) 48 and c) 72 hours. The results are shown as Mean ± SEM. *$p < 0.05$ and ***$p < 0.001$ treated versus control

1 Time and dose-dependent growth inhibition of A549 cells by Clarithromycin

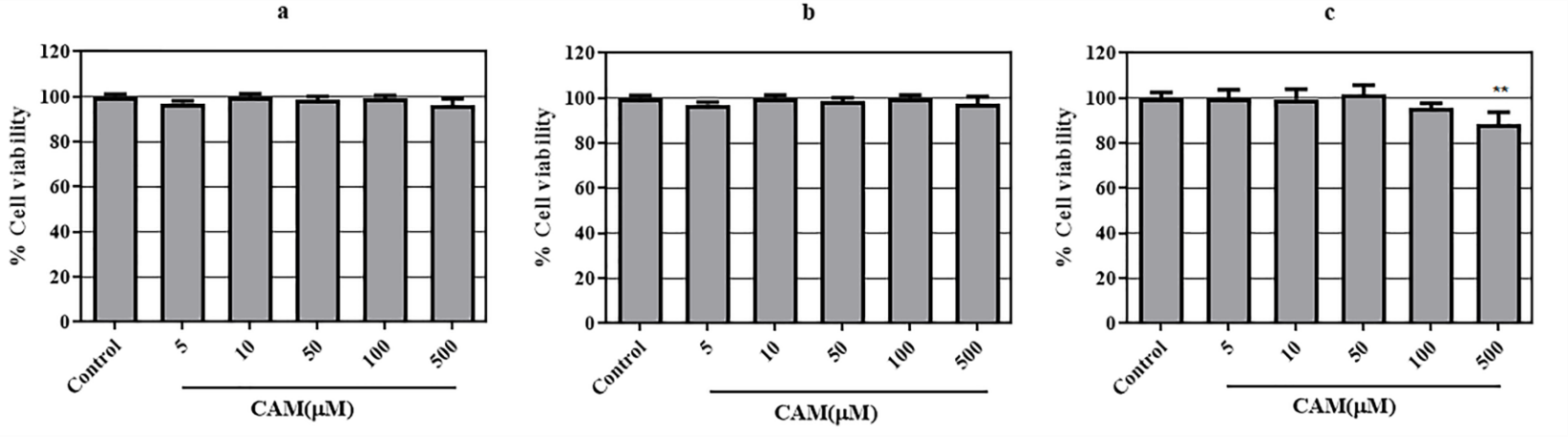


Figure 2. Cytotoxic effect of CAM on A549 cell lines. A549 cells were cultured with various concentrations of CAM for a) 24, b) 48 and c) 72 hours. The results are shown as Mean ± SEM. ***$p < 0.01$ treated versus control

- **CAM is not toxic in MCF7 and A549 cell lines**

Direct antineoplastic effects of CAM may depend on the tumor type

Authors	Year	Title	
Ohara T, et al.	2004	***Antibiotics directly induce apoptosis in B cell lymphoma cells derived from BALB/c mice***	
Qiao A-m, et al.	2006	***Involvement of mitochondria and caspase pathways in N-demethyl-clarithromycin-induced apoptosis in human cervical cancer HeLa cell***	
Hamada K, et al.	2000	***Adjuvant effect of clarithromycin on chemotherapy for murine lung cancer***	
Sassa K, et al.	1999	***Therapeutic effect of clarithromycin on a transplanted tumor in rats***	
Komatsu S, et al.	2012	***Clarithromycin enhances bortezomib-induced cytotoxicity via endoplasmic reticulum stress-mediated CHOP (GADD153) induction and autophagy in breast cancer cells***	

1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 cells

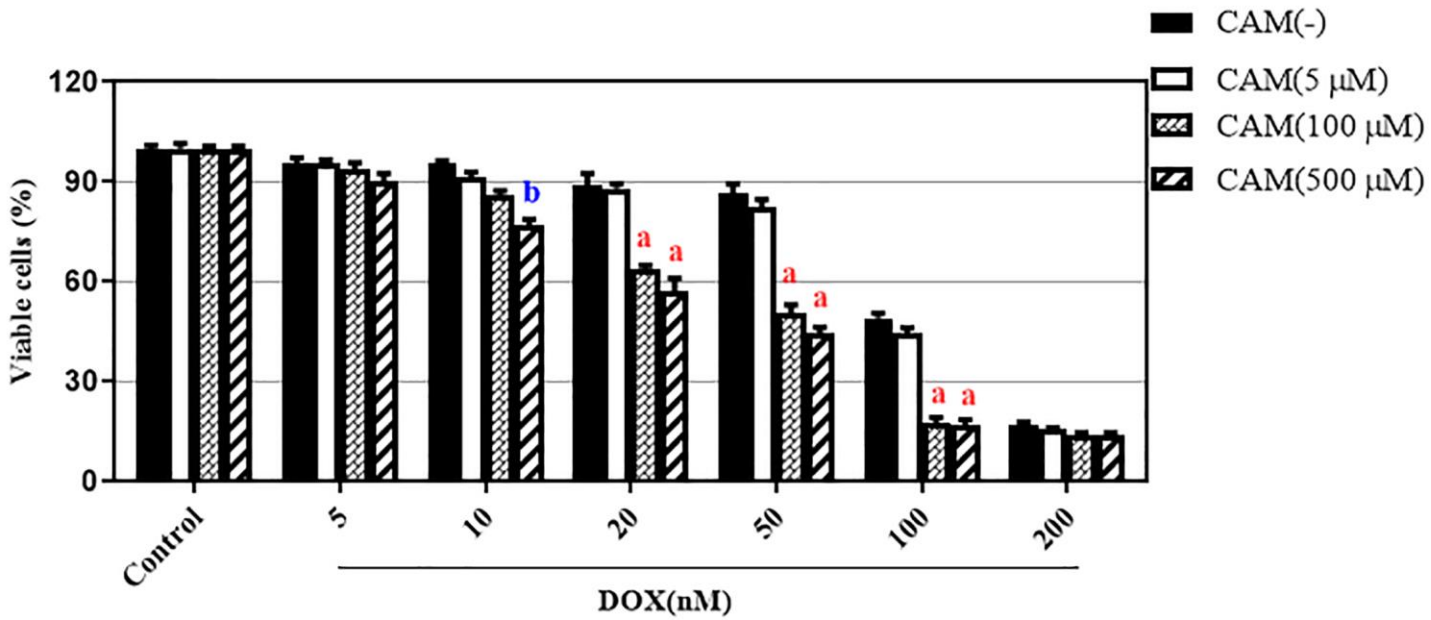


Figure 3. Cytotoxic effect on MCF7 cells after combined treatment with DOX plus CAM. MCF7 cells were cultured with DOX at various concentrations for 48 hours in the presence or absence of CAM at 5, 100 and 500μM. [^{a} $p < 0.001$ and ^{b} $p < 0.01$ CAM (-) vs CAM (+)]

1 Investigating the effect of CAM on DOX cytotoxicity in MCF7 cells

Table 1. IC50 value of DOX(nM) in MCF7 cells	
CAM(-)	95.29±5.35
CAM(5µM)	91.42±5.66
CAM(100µM)	39.84±6.54 *
CAM(500µM)	30.45±5.67 *

*significant values based on the extra sum-of-squares F test

1 Investigating the effect of CAM on DOX cytotoxicity in A549 cells

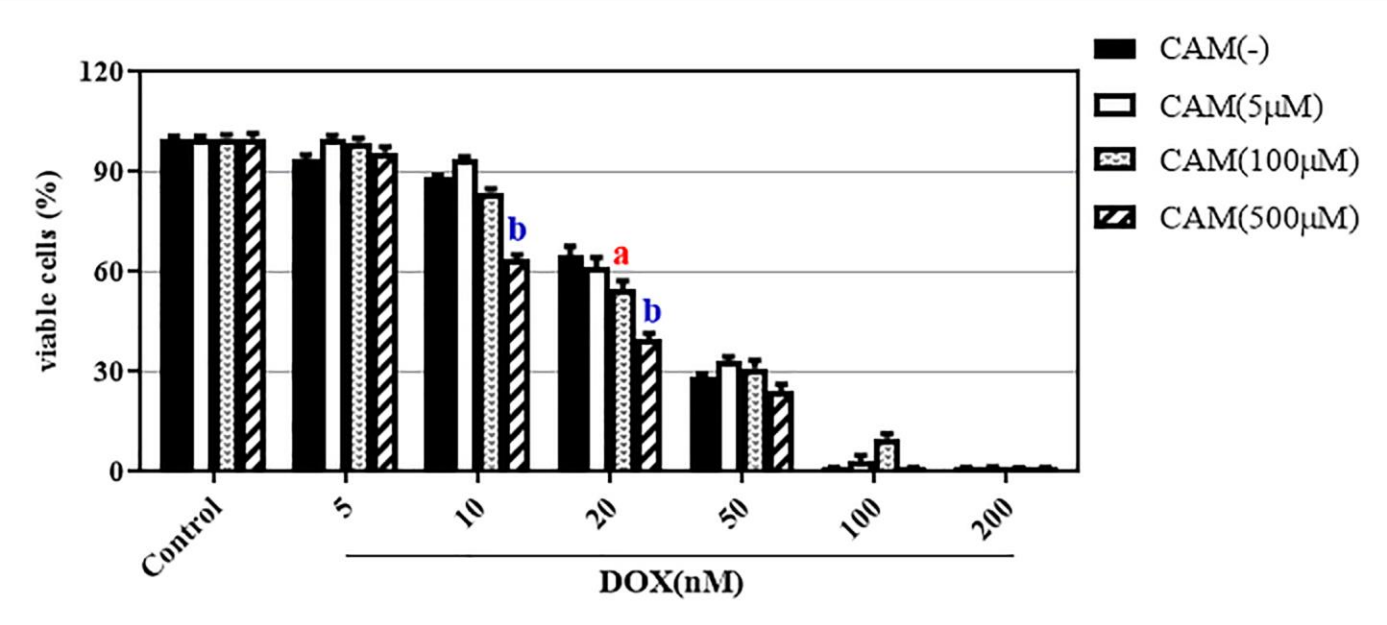


Figure 4. Cytotoxic effect on A549 cells after combined treatment with DOX plus CAM. A549 cells were cultured with DOX at various concentrations for 48 hours in the presence or absence of CAM at 5, 100 and 500μM. [^{a} $p < 0.01$ and ^{b} $p < 0.001$ CAM (-) vs CAM (+)]

1 Investigating the effect of CAM on DOX cytotoxicity in A549 cells

Table 2. IC50 value of DOX(nM) in A549 cells	
CAM(-)	30.86±1.38
CAM(5μM)	30.35±2.1
CAM(100μM)	25.28±1.87
CAM(500μM)	15.81±1.31*

*significant values based on the extra sum-of-squares F test

1 Drug interaction analysis between DOX and CAM in MCF7 cells

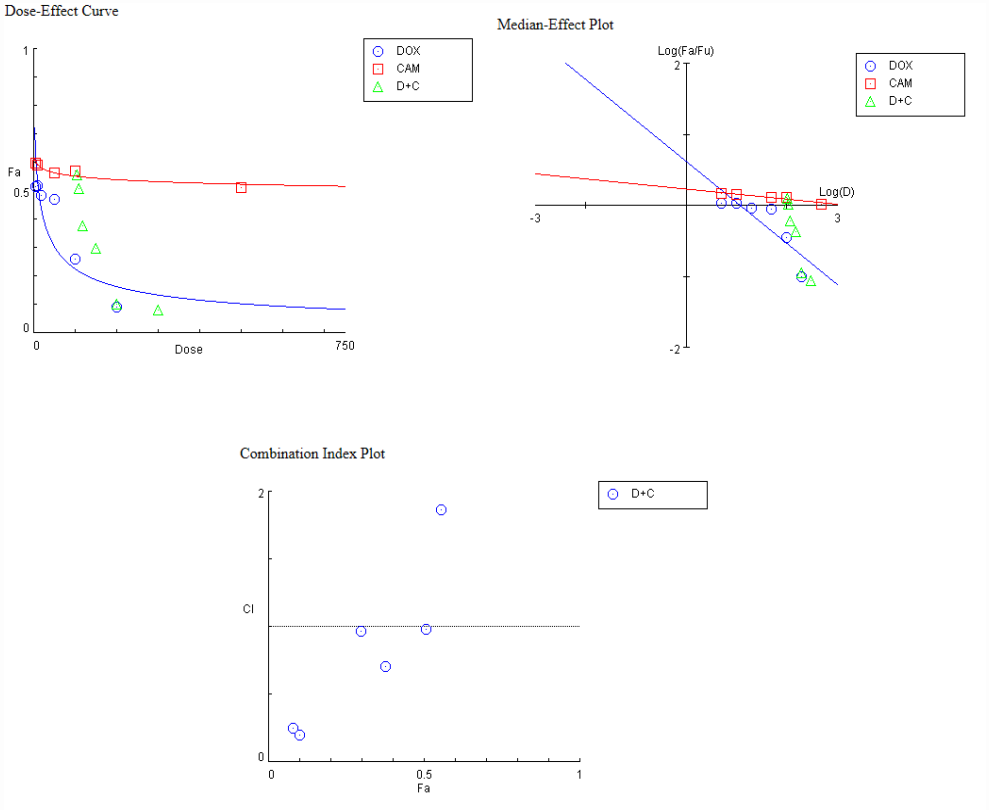


Figure 5. Dose-effect curve, median effect and interaction plots of DOX and CAM and their combination in MCF7 cells. Cells were treated with DOX or CAM alone or in combination at serial concentrations for 48 hrs. Cell viability was measured by MTT assay and median effect plot was generated using Chou and Talalay's method.
Synergy was defined as combination index plot lower than 1.
Abbreviations: **DOX – Doxorubicin; CAM – Clarithromycin; D + C – Doxorubicin + Clarithromycin.**

1 Drug interaction analysis between DOX and CAM in MCF7 cells

Table 3. Combination index values of DOX and CAM combination in MCF7 cells calculated by Compusyn software using Chou and Talalay's method. Synergy was defined as interaction index lower than 1.

DOX concentration(nM)	CAM concentration(μM)	Effect	Combination Index
5	100	0.556	**1.868**
10	100	0.509	**0.985**
20	100	0.377	**0.708**
50	100	0.299	**0.966**
100	100	0.103	**0.199**
200	100	0.08	**0.246**

1 Drug interaction analysis between DOX and CAM in A549 cells

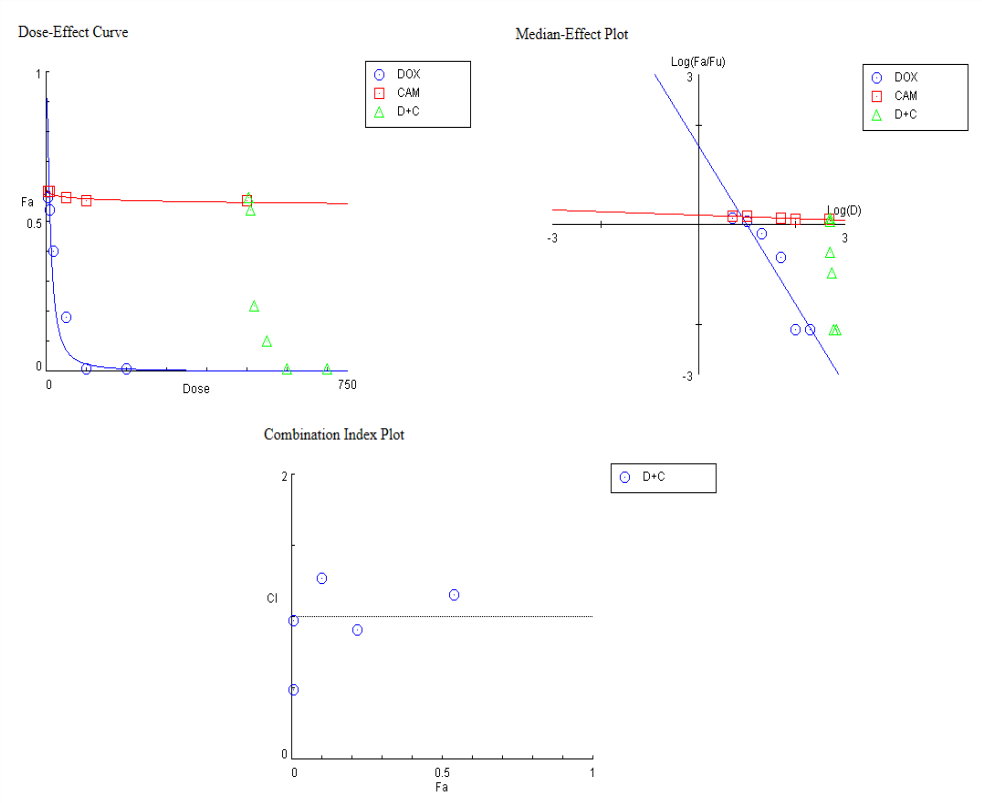


Figure 6. Dose-effect curve, median effect and interaction plots of DOX and CAM and their combination in A549 cells. Cells were treated with DOX or CAM alone or in combination at serial concentrations for 48 hrs. Cell viability was measured by MTT assay and median effect plot was generated using Chou and Talalay's method.
Synergy was defined as combination index plot lower than 1.
Abbreviations: **DOX – Doxorubicin; CAM – Clarithromycin; D + C – Doxorubicin + Clarithromycin.**

1 Drug interaction analysis between DOX and CAM in A549 cells

Table 4. Combination index values of DOX and CAM combination in A549 cells calculated by Compusyn software using Chou and Talalay's method. Synergy was defined as interaction index lower than 1.

DOX concentration(nM)	CAM concentration(μM)	Effect	Combination Index
5	500	0.58	**7.73**
10	500	0.54	**1.158**
20	500	0.22	**0.91**
50	500	0.17	**1.268**
100	500	0.008	**0.488**
200	500	0.008	**0.977**

1 Evaluation of apoptosis induced by combined treatment with DOX and CAM in MCF7 cells

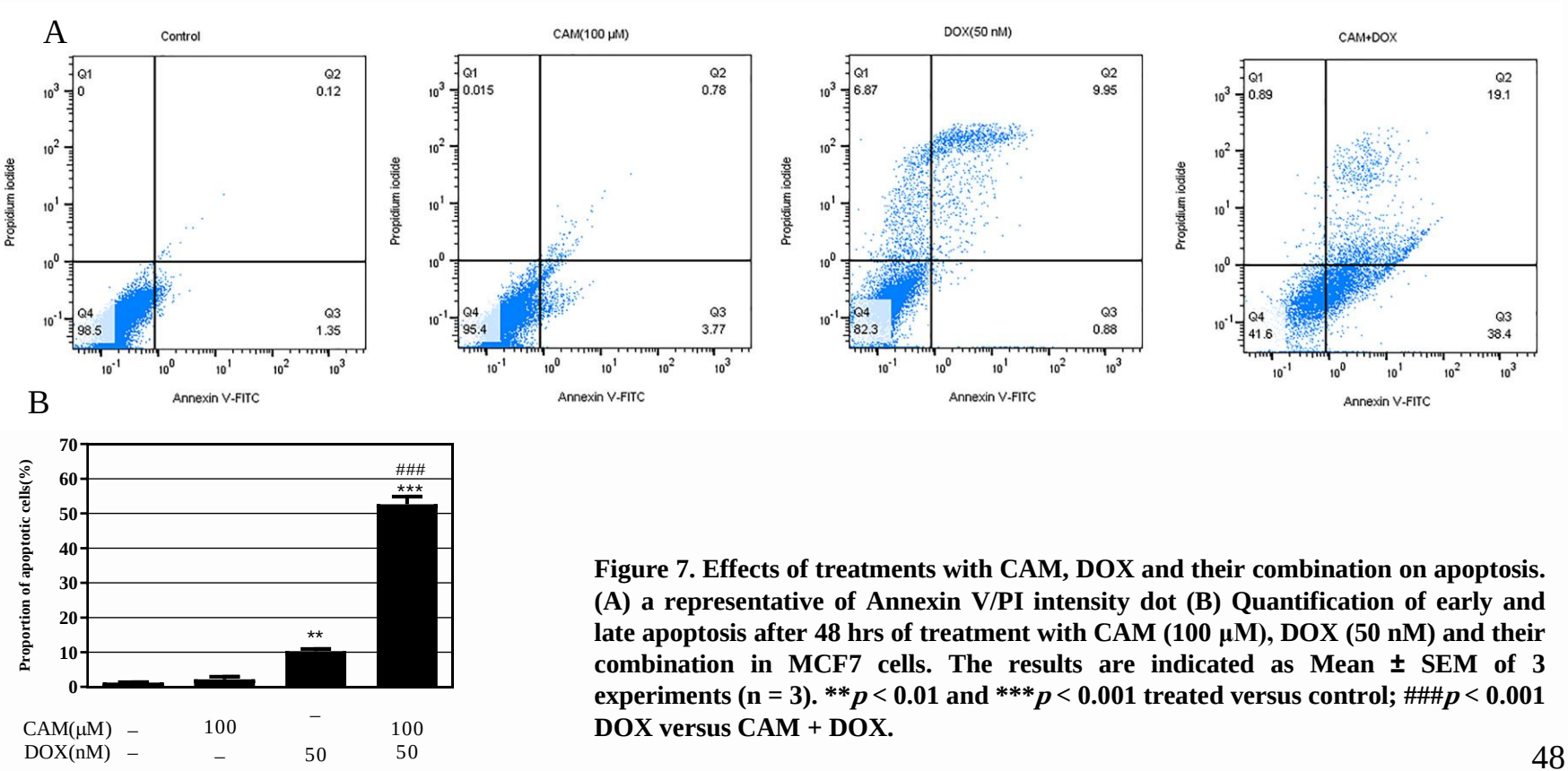
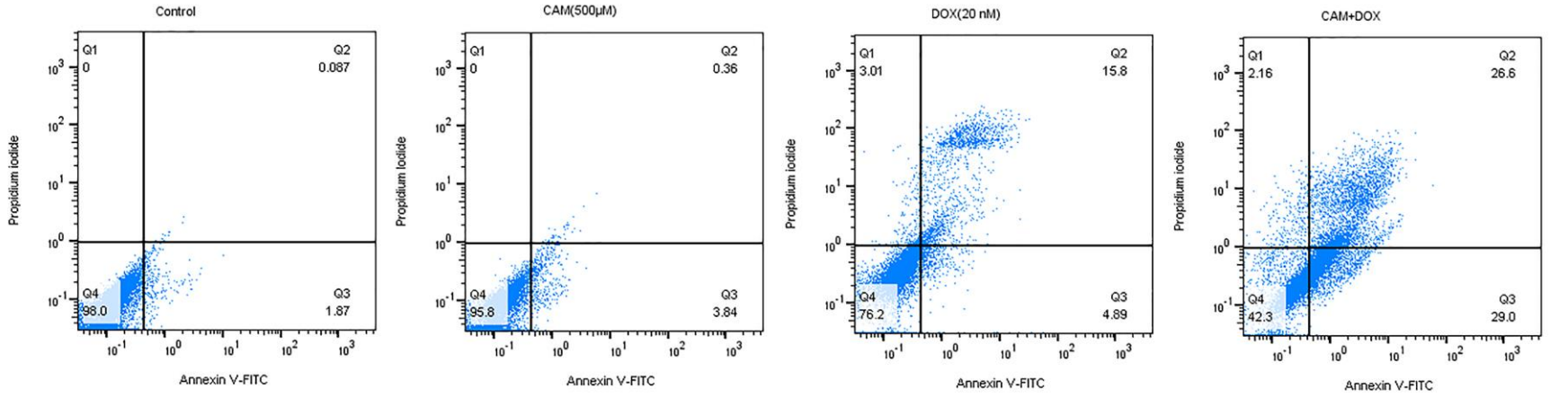


Figure 7. Effects of treatments with CAM, DOX and their combination on apoptosis. (A) a representative of Annexin V/PI intensity dot (B) Quantification of early and late apoptosis after 48 hrs of treatment with CAM (100 µM), DOX (50 nM) and their combination in MCF7 cells. The results are indicated as Mean ± SEM of 3 experiments (n = 3). **$p < 0.01$ and ***$p < 0.001$ treated versus control; ###$p < 0.001$ DOX versus CAM + DOX.

1 Evaluation of apoptosis induced by combined treatment with DOX and CAM in A549 cells



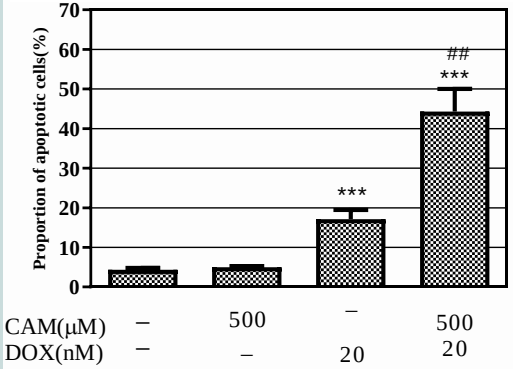


Figure 8. Effects of treatments with CAM, DOX and their combination on apoptosis. (A) a representative of Annexin V/PI intensity dot (B) Quantification of early and late apoptosis after 48 hrs of treatment with CAM (500 μM), DOX (20 nM) and their combination in A549 cells. The results are indicated as Mean ± SEM of 3 experiments (n = 3). ***$p < 0.001$ treated versus control; ##$p < 0.01$ DOX versus CAM + DOX.

- **CAM synergistically enhanced DOX-induced cytotoxicity through *elevating apoptotic cell death* in MCF7 and A549 cell lines**

1. **Clarithromycin synergizes with cisplatin to inhibit ovarian cancer growth in vitro and in vivo(2019)**
2. **Clarithromycin attenuates autophagy in myeloma cells. International journal of oncology(2010)**
3. **Adjuvant effect of clarithromycin on chemotherapy for murine lung cancer(2000)**
4. **Roxithromycin and clarithromycin, 14-membered ring macrolides, potentiate the antitumor activity of cytotoxic agents against mouse B16 melanoma cells(1999)**
5. **Therapeutic effect of clarithromycin on a transplanted tumor in rats(1999)**

2 Establishment of DOX-resistant sub lines

- High-level laboratory model: ***Continues stepwise increasing***
- Initial Dose and Dosing Interval Determination

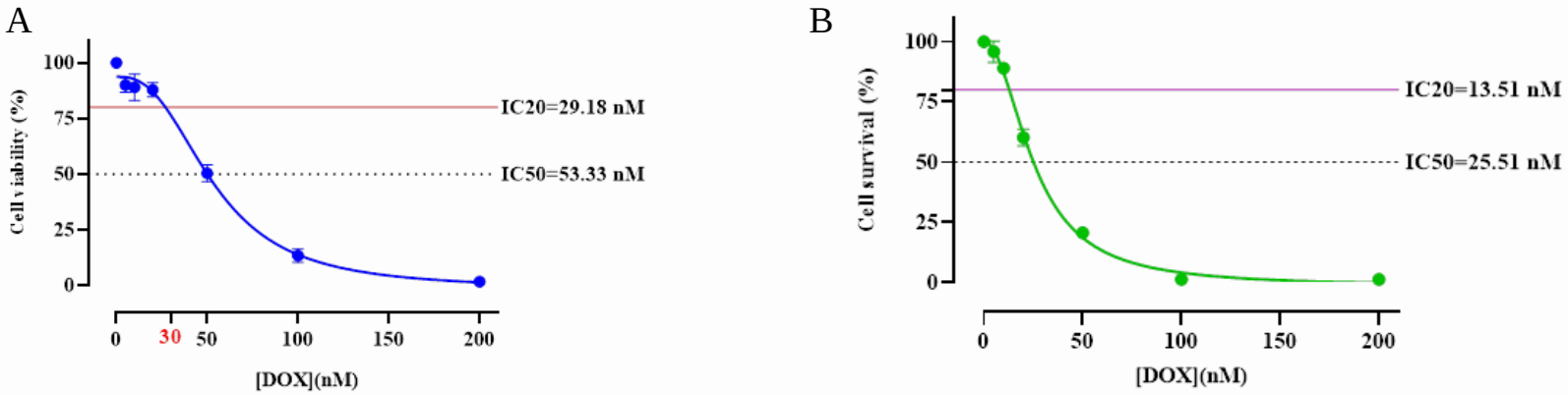


Figure 9. Dose–response curve for A) MCF7 and B) A549 cell viability after exposure to different concentrations of DOX for 72 hours. The IC20 value of DOX, measured by Fit spline/LOWESS method, was selected as the initial dose for resistance induction. DOX, doxorubicin.

2 Measurement of drug resistance in MCF7.Res and A549.Res cell lines

- **Microscopic Images**

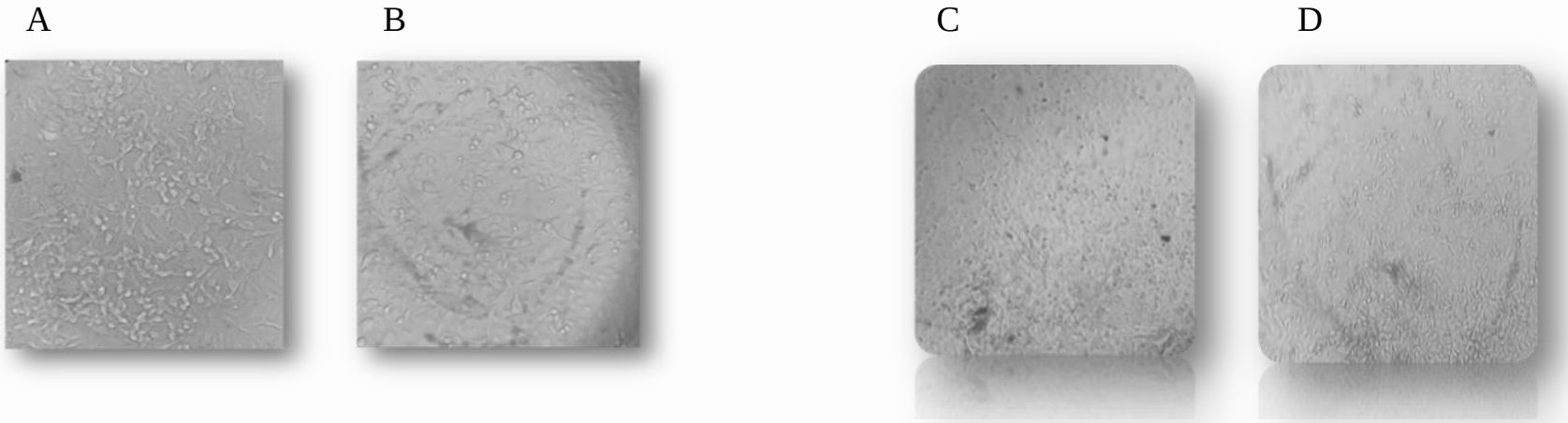


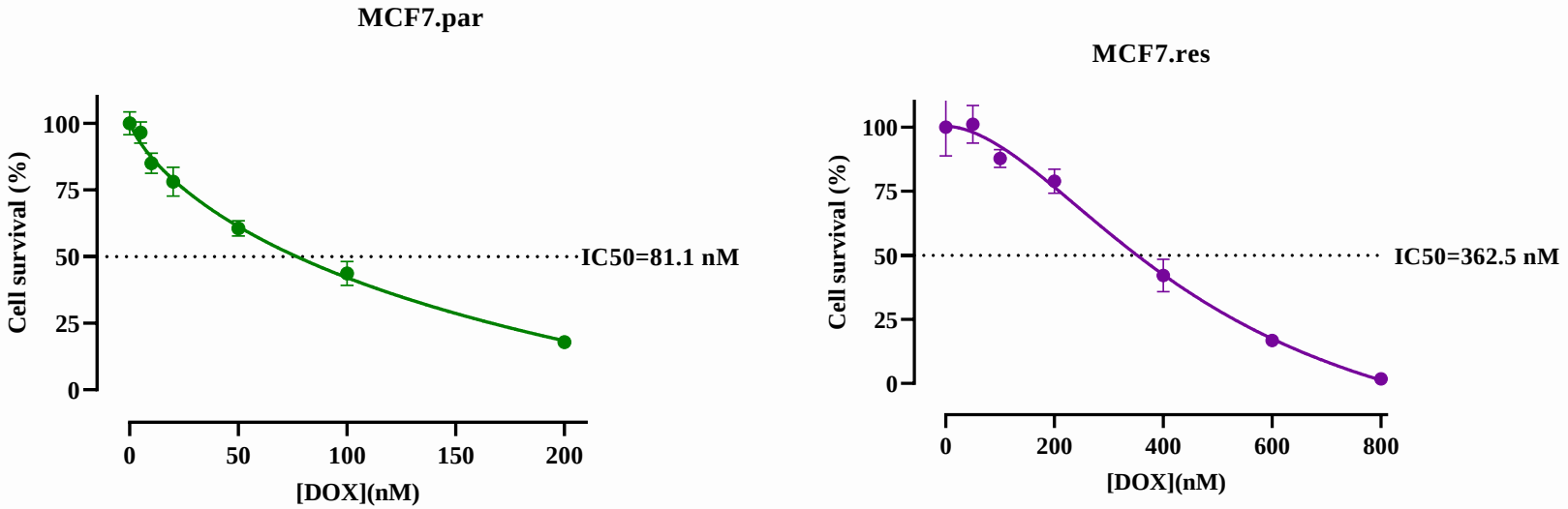
Figure 10. Cell morphology of A) parental MCF7, B) resistant MCF7 and C) parental A549, D) resistant A549 (× 10).

- **The morphological features of the resistant sublines were approximately similar to those of the parental cells**

1. **Gene expression alterations in doxorubicin resistant MCF7(2013)**
2. **Establishment and characterization of a cisplatin-resistant human osteosarcoma cell line(2014)**
3. **Increased sensitivity to platinum drugs of cancer cells with acquired resistance to trabectedin(2015)**
4. **Increased levels and defective glycosylation of MRPs in ovarian carcinoma cells resistant to oxaliplatin(2010)**
5. **Functional and transcriptomic characterization of carboplatin-resistant A2780 ovarian cancer cell lines(2019)**
6. **Generation of cisplatin-resistant ovarian cancer cell lines(2016)**
7. **Reversal of 5-fluorouracil resistance by EGCG is mediate by inactivation of TFAP2A/VEGF signaling pathway and down-regulation of MDR-1 and P-gp expression in gastric cancer(2017)**

2 Measurement of drug resistance in MCF7.Res cell lines

- **Fold Resistance**

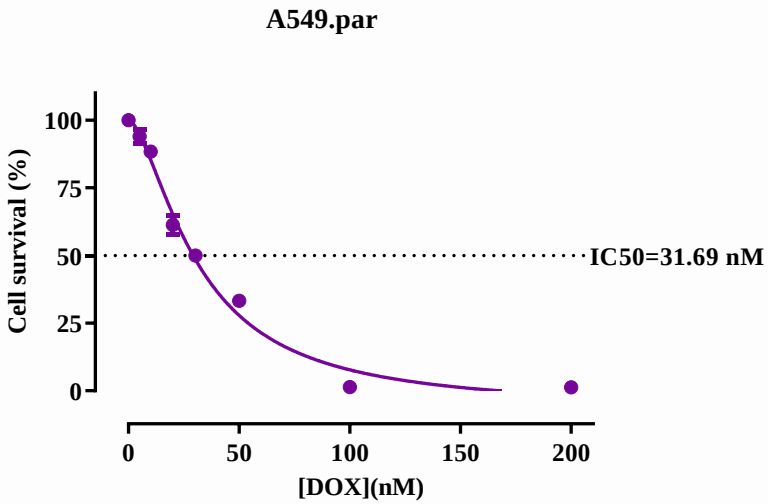


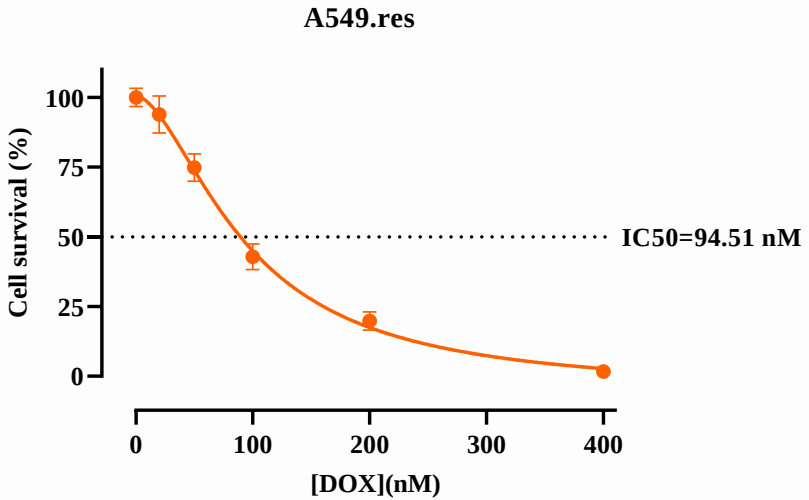
Fold Resistance=IC50 of resistant cell line/IC50 of parental cell line=362.5/81.1=4.46

Figure 11. Cytotoxicity of DOX in parental and resistant MCF7 cells after 48 hours drug exposure.

2 Measurement of drug resistance in A549.Res cell lines

- **Fold Resistance**





Fold Resistance=IC50 of resistant cell line/IC50 of parental cell line=94.51/31.69≈3

Figure 12. Cytotoxicity of DOX in parental and resistant A549 cells after 48 hours drug exposure.

2 Measurement of drug resistance in MCF7.Res cell lines

- **Growth curve Analysis**

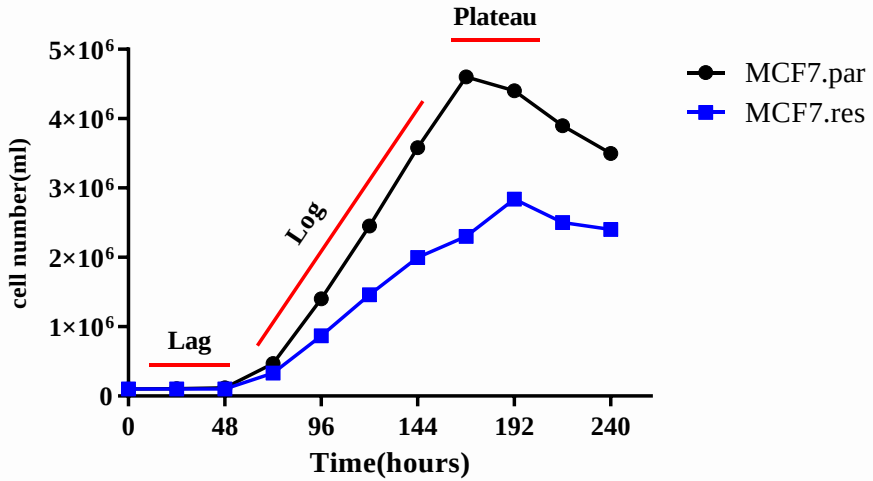
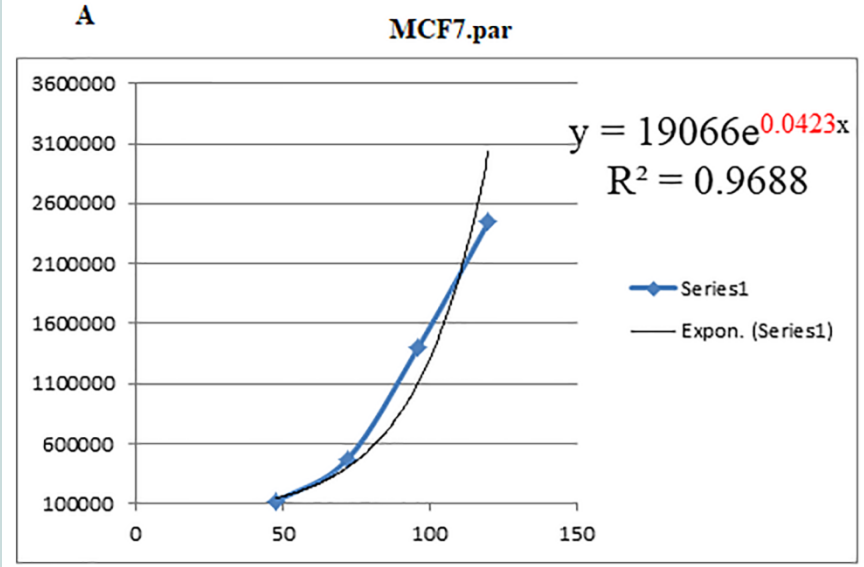


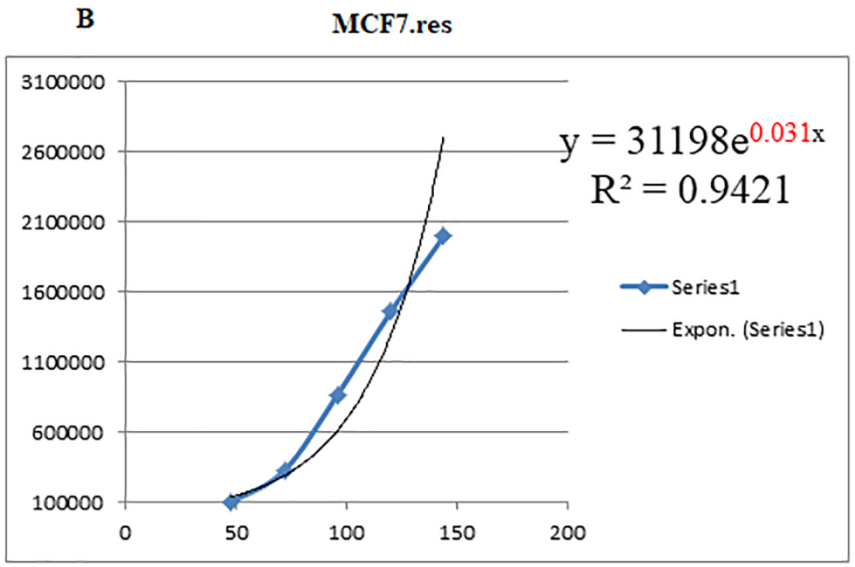
Figure 13. Growth curve of parental and resistant MCF7 cells. MCF7.par cells were cultured in DOX-free medium, whereas MCF7.res cells were plated in DOX-containing medium.

2 Measurement of drug resistance in MCF7.Res cell lines

- **Growth Analysis**



Td=Ln2/K=16.38 hrs



Td=Ln2/K=22.35 hrs

Figure 14. Population doubling time was calculated as follow: Td=Ln2/K (K, growth constant generated from exponential line equation).

2 Measurement of drug resistance in A549.Res cell lines

- **Growth Analysis**

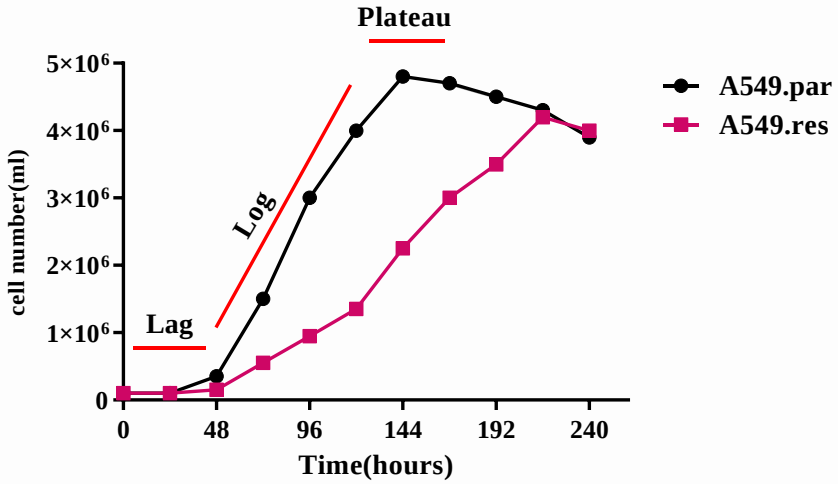
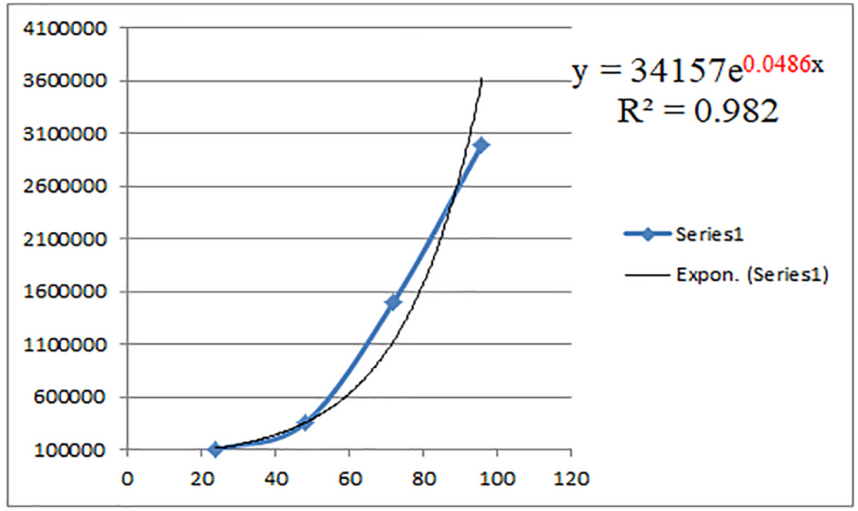


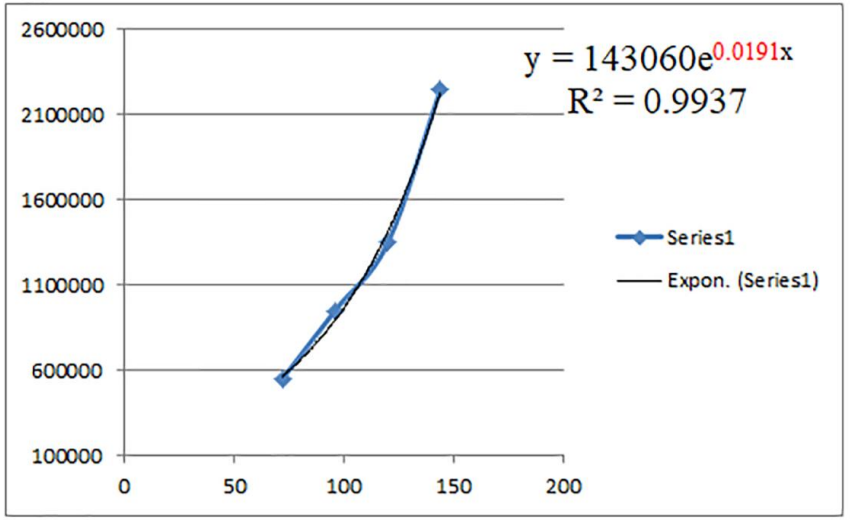
Figure 15. Growth curve of parental and resistant A549 cells. A549.par cells were cultured in DOX-free medium, whereas A549.res cells were plated in DOX-containing medium.

2 Measurement of drug resistance in A549.Res cell lines

- **Growth Analysis**



Td=Ln2/K=14 hrs



Td=Ln2/K=36 hrs

Figure 16. Population doubling time was calculated as follow: Td=Ln2/K (K, growth constant generated from exponential line equation).

- **The established resistant sublines had a usual growth curve; however, the growth rates were significantly decreased in resistant sublines compared with parental cells.**
- **There is a relationship between the growth rate and the drug resistance**

1. **Reversal of 5-fluorouracil resistance by EGCG is mediate by inactivation of TFAP2A/VEGF signaling pathway and down-regulation of MDR-1 and P-gp expression in gastric cancer(2017)**
2. **Reduced growth rate accompanied by aberrant epidermal growth factor signaling in drug resistant human breast cancer cells(2000)**
3. **Establishment of a human hepatoma multidrug resistant cell line in vitro(2010)**

3 Measurement of autophagy in MCF7.Res cell lines

- **Indirect immunofluorescence LC3II labeling**

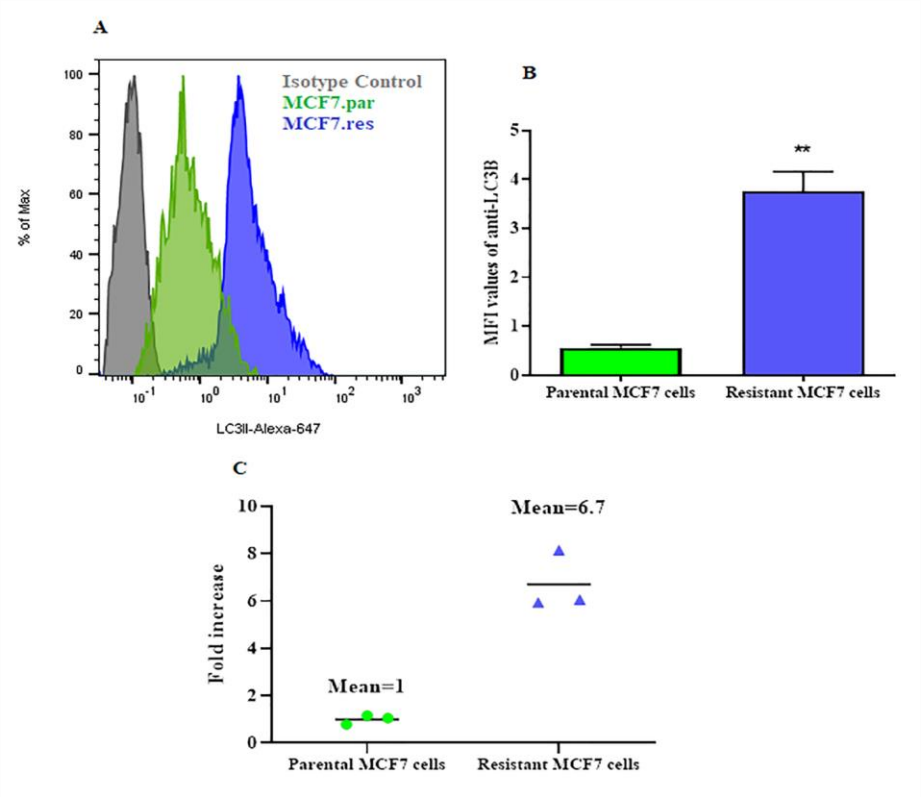


Figure 17. Histogram overlay presentation of LC3-II-Alexa-Flour647 in parental and resistant MCF7 cells (A). Median fluorescence intensity (MFI) values of LC3II-antibody for parental and resistant MCF7 cells are indicated in (B) and expressed as fold increase above parental cells in (C). T test, **p < 0.01 , parental vs resistant.

3 Measurement of autophagy in in MCF7.Res cell lines

- **LysoTracker Green labeling**

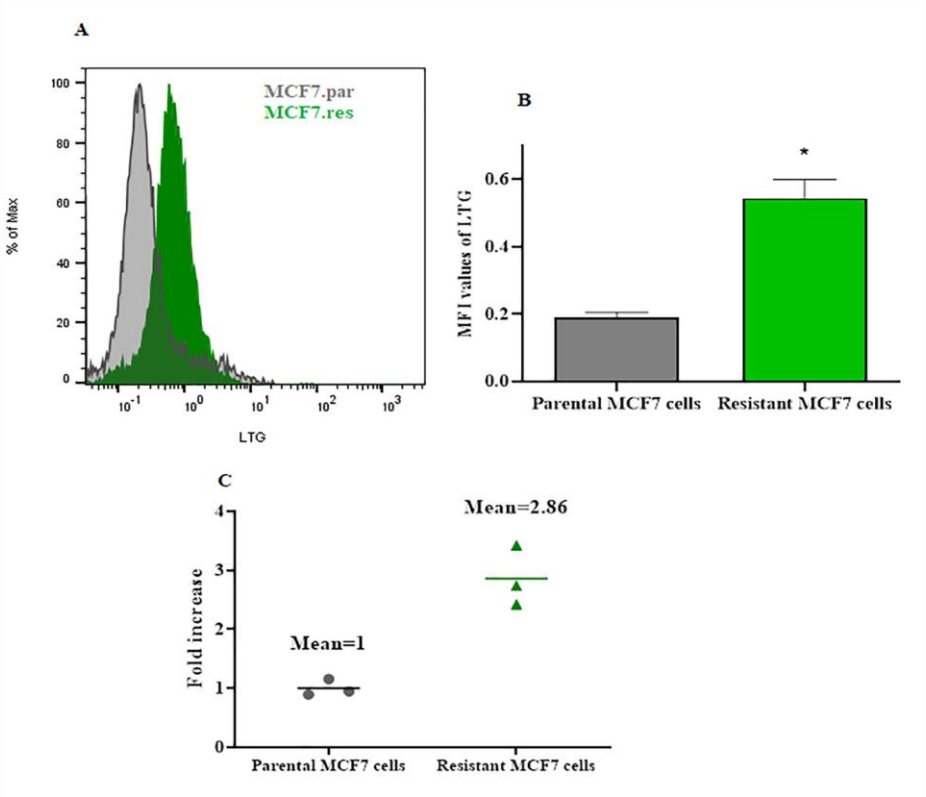


Figure 18. Histogram overlays of MCF7 LTG expression of parental and resistant cells (A). MFI values of LTG expression for parental and resistant MCF7 cells are indicated in (B) and expressed as fold increase above parental cells in (C). T test, *$p < 0.05$, parental vs resistant.

3 Measurement of autophagy in A549.Res cell lines

- **Indirect immunofluorescence LC3II labeling**

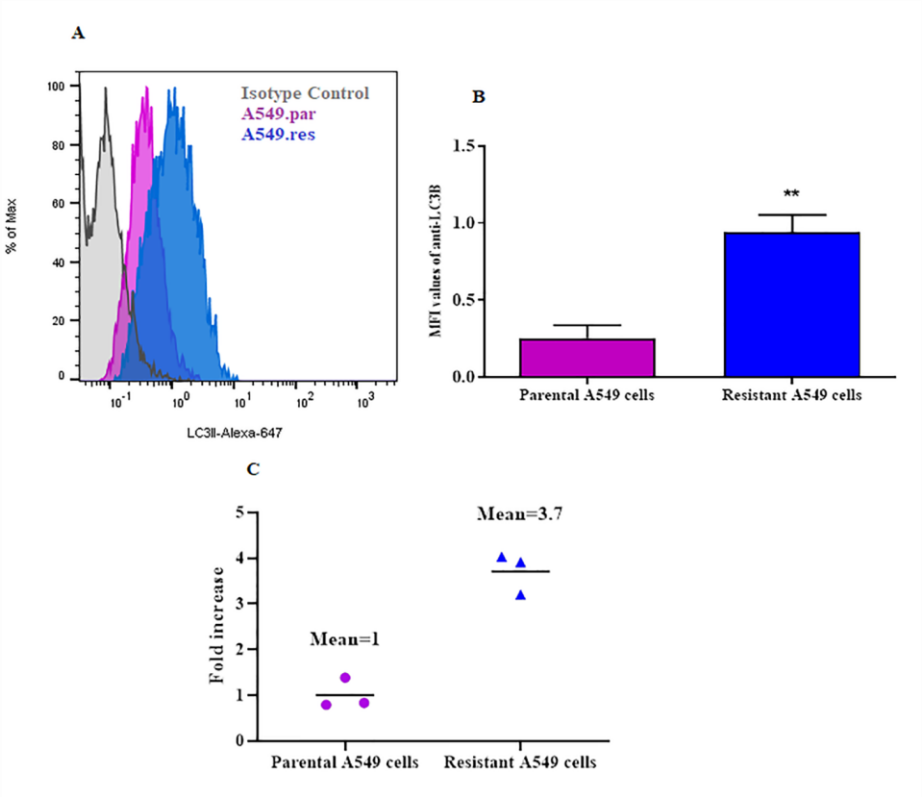


Figure 19. Histogram overlay presentation of LC3-II-Alexa-Flour647 in parental and resistant A549 cells (A). MFI values of LC3II-antibody for parental and resistant A549 cells are indicated in (B) and expressed as fold increase above parental cells in (C). T test, **$p < 0.01$, parental vs resistant.

Measurement of autophagy in in A549.Res cell lines

- **LysoTracker Green labeling**

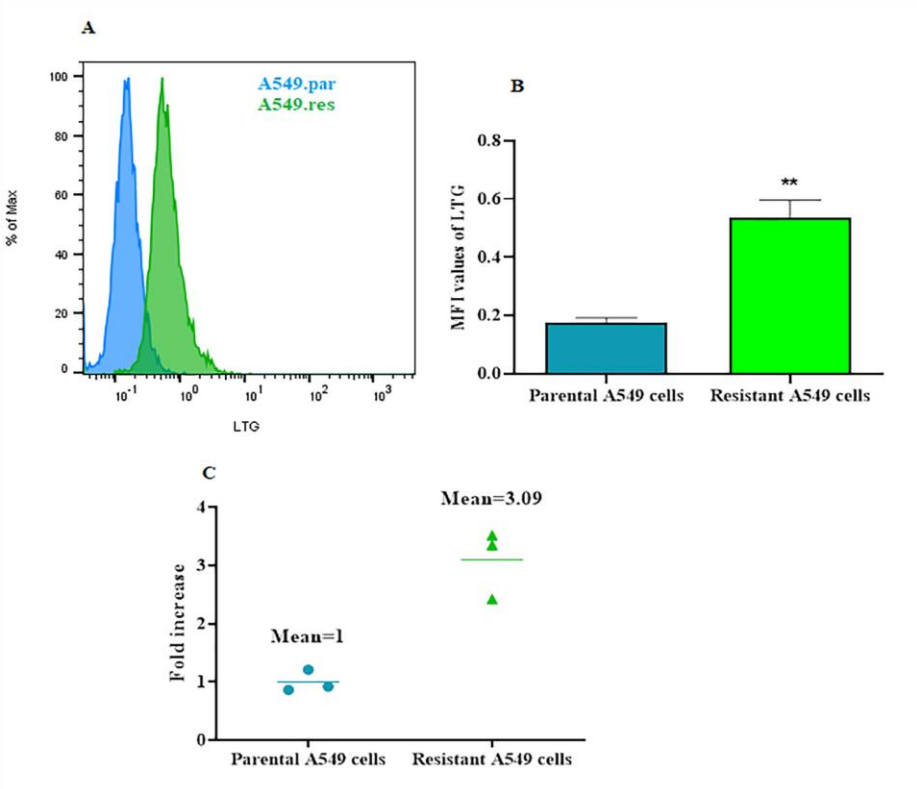


Figure 20. Histogram overlays of A549 LTG expression of parental and resistant cells (A). MFI values of LTG expression for parental and resistant A549 cells are indicated in (B) and expressed as fold increase above parental cells in (C). T test, **$p < 0.01$, parental vs resistant.

- **Contribution of autophagy in acquired drug resistance of DOX**

1. **Anthracyclines :molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity(2004)**
2. **Role of autophagy and lysosomal drug sequestration in acquired resistance to doxorubicin in MCF-7 cells(2016)**
3. **Enhanced cytotoxic activity of doxorubicin through the inhibition of autophagy in triple negative breast cancer cell line(2017)**
4. **MicroRNA-410 regulates autophagy-related gene ATG16L1 expression and enhances chemosensitivity via autophagy inhibition in osteosarcoma. Molecular medicine reports(2017)**
5. **Blocked autophagy by miR-101 enhances osteosarcoma cell chemosensitivity in vitro(2014)**
6. **Carvacrol nanoemulsion evokes cell cycle arrest, apoptosis induction and autophagy inhibition in doxorubicin resistant-A549 cell line(2018)**
7. **Autophagy in drug resistance of the multiple myeloma cell line RPMI8226 to doxorubicin(2015)**

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in MCF7.Res cell lines

- **Cell growth inhibition after combined treatment of DOX+CAM in Parental and Resistant cells**

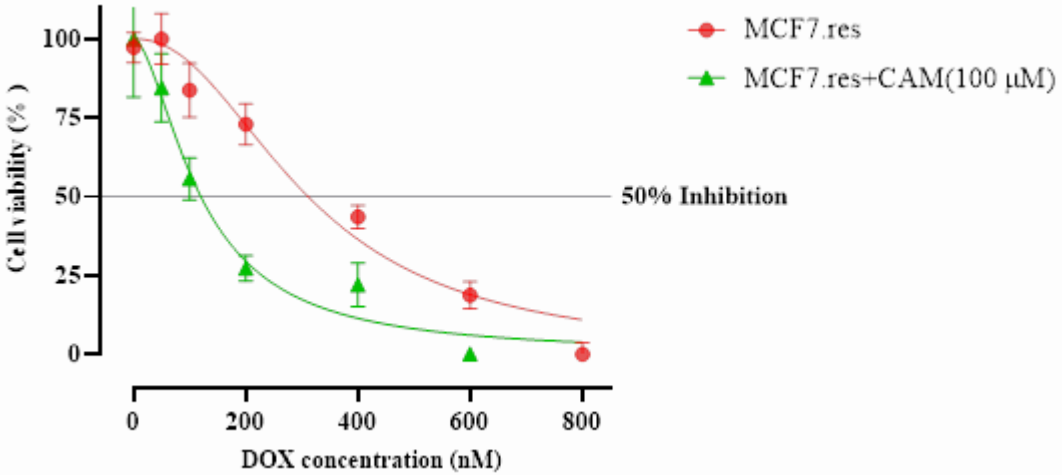
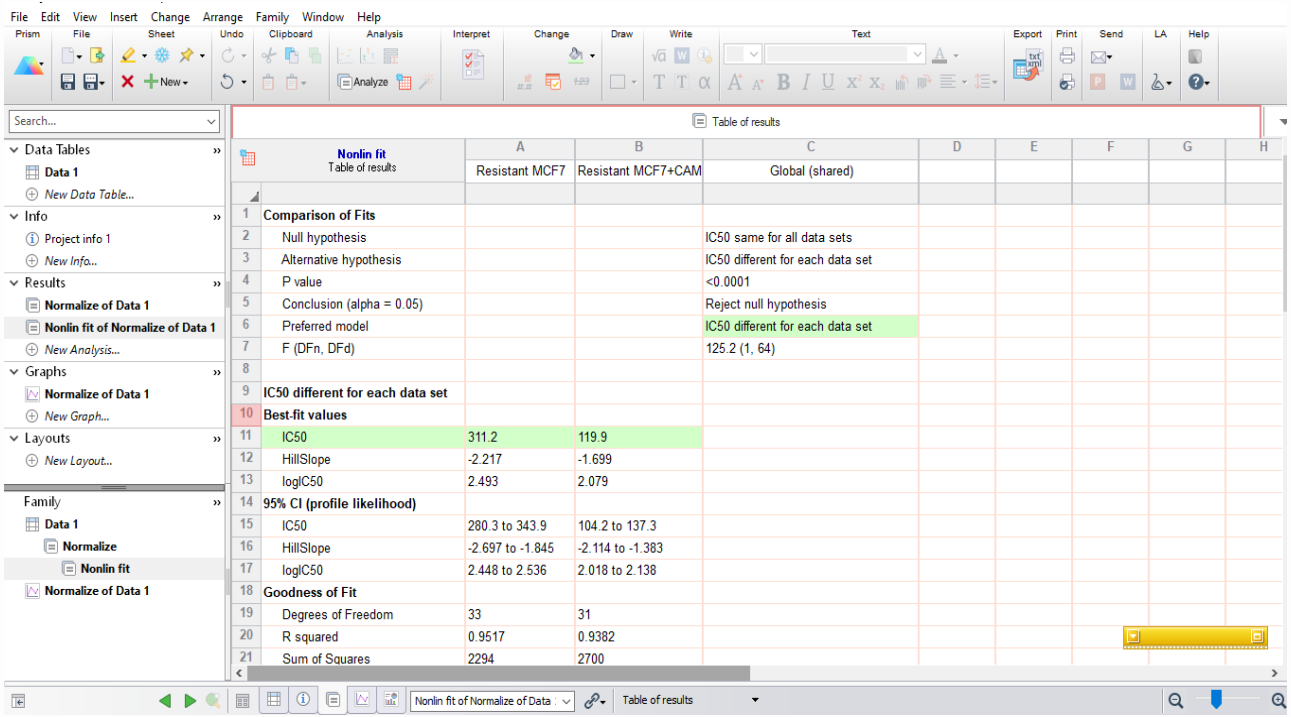


Figure 21. Cell growth inhibition of DOX in resistant MCF7 cells in absence and presence of CAM(100 μM)

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in MCF7.Res cell lines

- **Determination of IC50 values of DOX in absence and presence of CAM**

✓ Extra-sum of square F-test



	A	B	C	D	E	F	G	H
	Resistant MCF7	Resistant MCF7+CAM	Global (shared)					
1	**Comparison of Fits**							
2	Null hypothesis		IC50 same for all data sets					
3	Alternative hypothesis		IC50 different for each data set					
4	P value		<0.0001					
5	Conclusion (alpha = 0.05)		Reject null hypothesis					
6	Preferred model		IC50 different for each data set					
7	F (DFn, DFd)		125.2 (1, 64)					
8								
9	**IC50 different for each data set**							
10	**Best-fit values**							
11	IC50	311.2	119.9					
12	HillSlope	-2.217	-1.699					
13	logIC50	2.493	2.079					
14	**95% CI (profile likelihood)**							
15	IC50	280.3 to 343.9	104.2 to 137.3					
16	HillSlope	-2.697 to -1.845	-2.114 to -1.383					
17	logIC50	2.448 to 2.536	2.018 to 2.138					
18	**Goodness of Fit**							
19	Degrees of Freedom	33	31					
20	R squared	0.9517	0.9382					
21	Sum of Squares	2294	2700					

Figure 22. Comparison of IC50 values of DOX(nM) in resistant MCF7 in absence and presence of CAM(100 µM)

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in MCF7.Res cell lines

- **LC3II expression in parental and resistant cells**

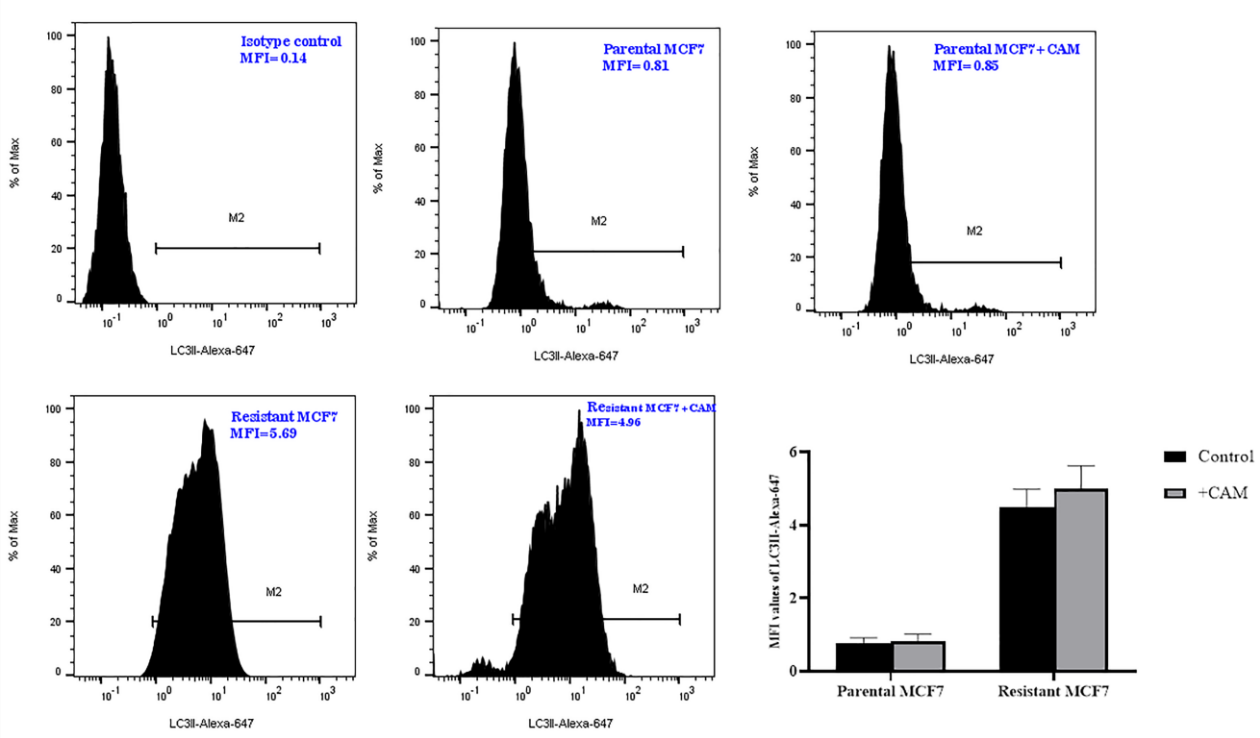


Figure 23. Histogram plots and MFI values of LC3II expression in parental and resistant MCF7 cells in absence and presence of CAM (100 µM). Data are presented as Mean ± SEM of three replicates.

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in MCF7.Res cell lines

- **LTG expression in parental and resistant cells**

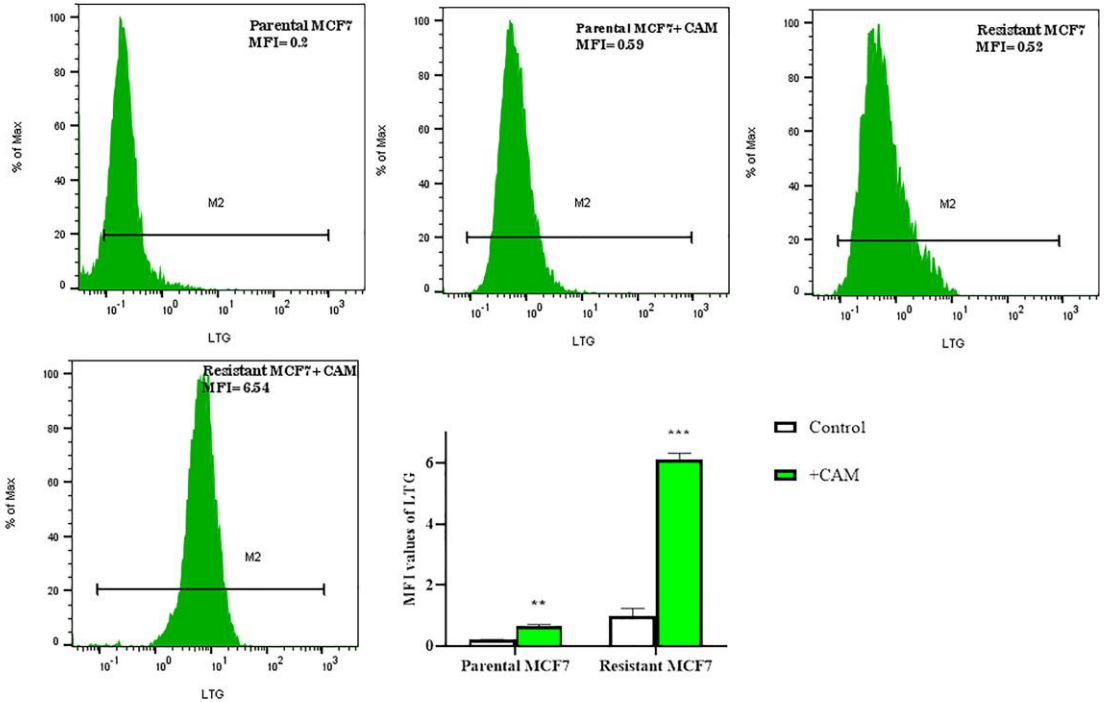


Figure 24. Histogram plots and MFI values of LTG expression in parental and resistant MCF7 cells in absence and presence of CAM (100 µM). Data are presented as Mean ± SEM of three replicates. ** p <0.01 and *** p <0.001, control vs (+CAM).

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in MCF7.Res cell lines

- **Apoptosis**

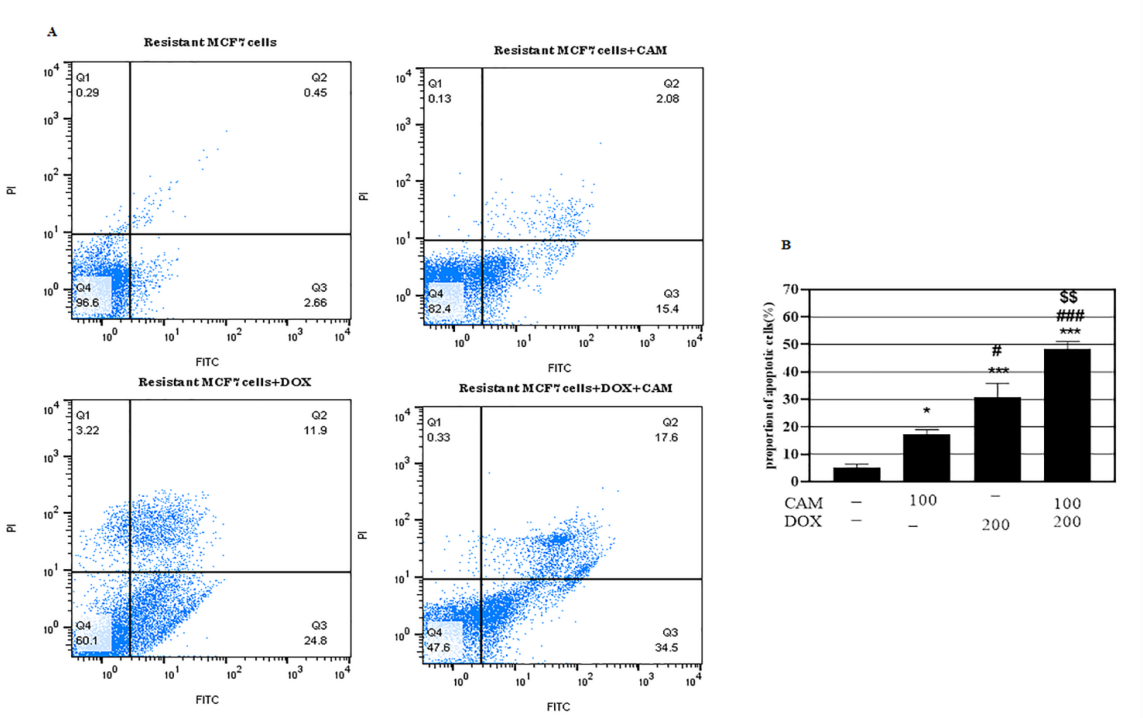


Figure 25. Quantification of apoptotic cell death after 48 hrs of treatment with CAM, DOX and their combination in resistant MCF7 cells. * $p<0.05$, *** $p<0.001$ treated vs control. # $p<0.05$, ### $p<0.001$ treated vs CAM and $$ $p<0.01$ treated vs DOX+CAM.

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in A549.Res cell lines

- **Cell growth inhibition after combined treatment of DOX+CAM in Parental and Resistant cells**

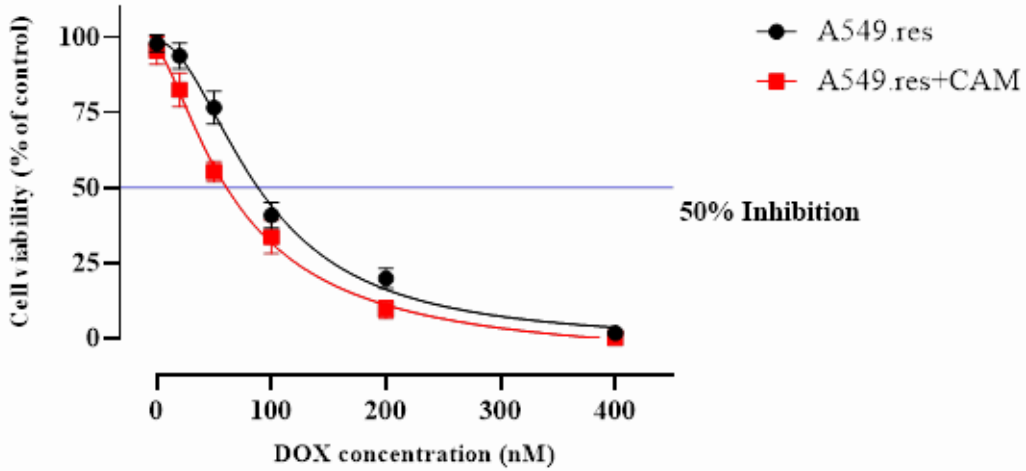


Figure 26. Cell growth inhibition of DOX in resistant A549 cells in absence and presence of CAM(500 μM)

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in A549.Res cell lines

- **Determination of IC50 values of DOX in absence and presence of CAM**

✓ Extra-sum of square F-test

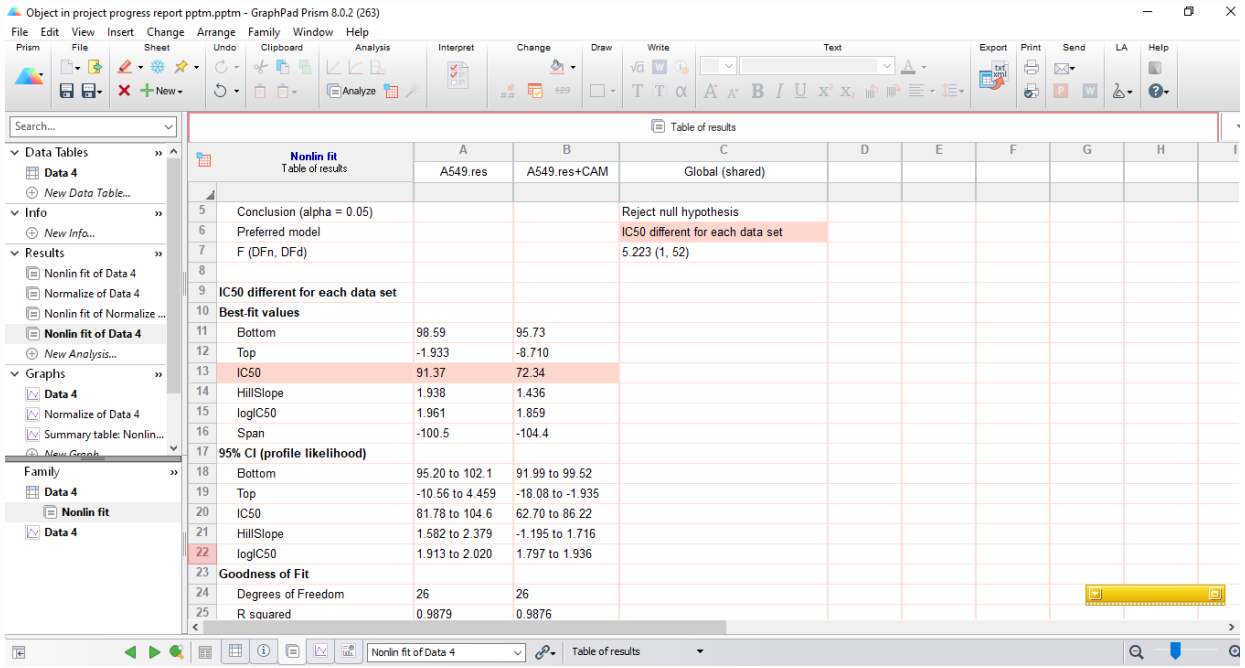


Figure 27. Comparison of IC50 values of DOX(nM) in resistant A549 in absence and presence of CAM(500 µM)

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in A549.Res cell lines

- **LC3II expression in parental and resistant cells**

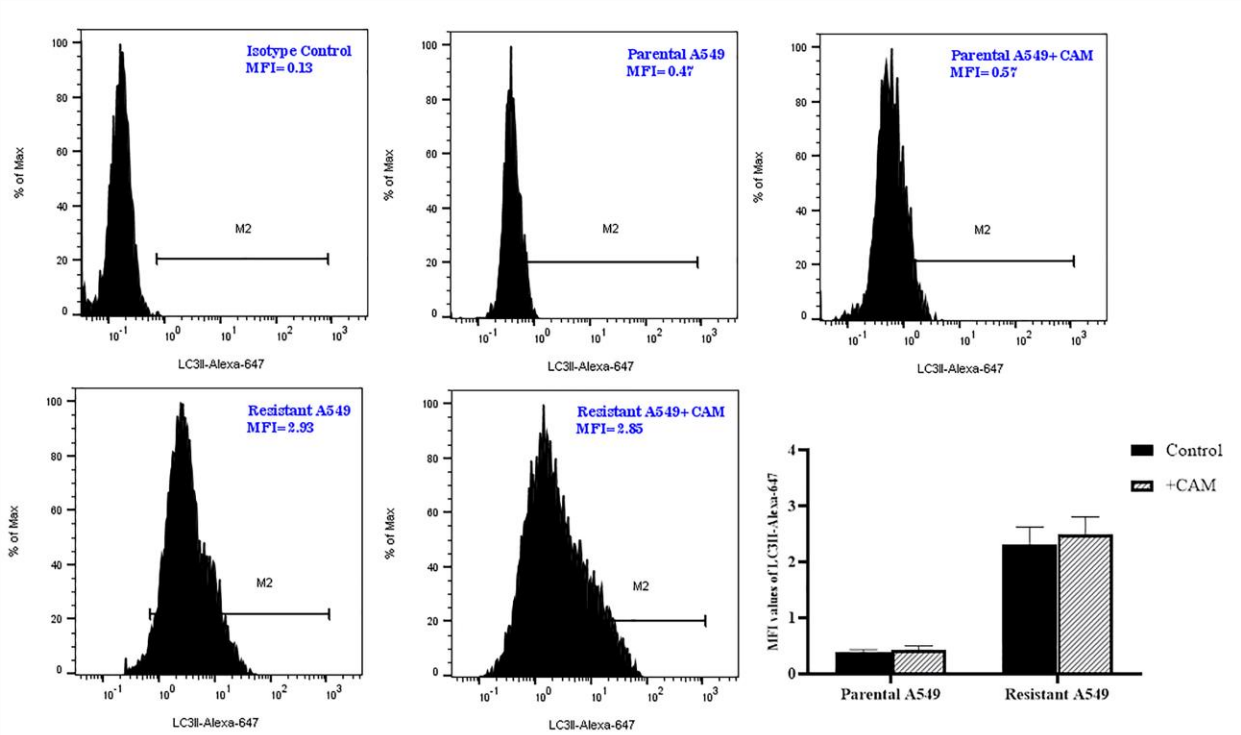


Figure 28. Histogram plots and MFI values of LC3II expression in parental and resistant A549 cells in absence and presence of CAM (500 µM). Data are presented as Mean ± SEM of three replicates.

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in A549.Res cell lines

- **LTG expression in parental and resistant cells**

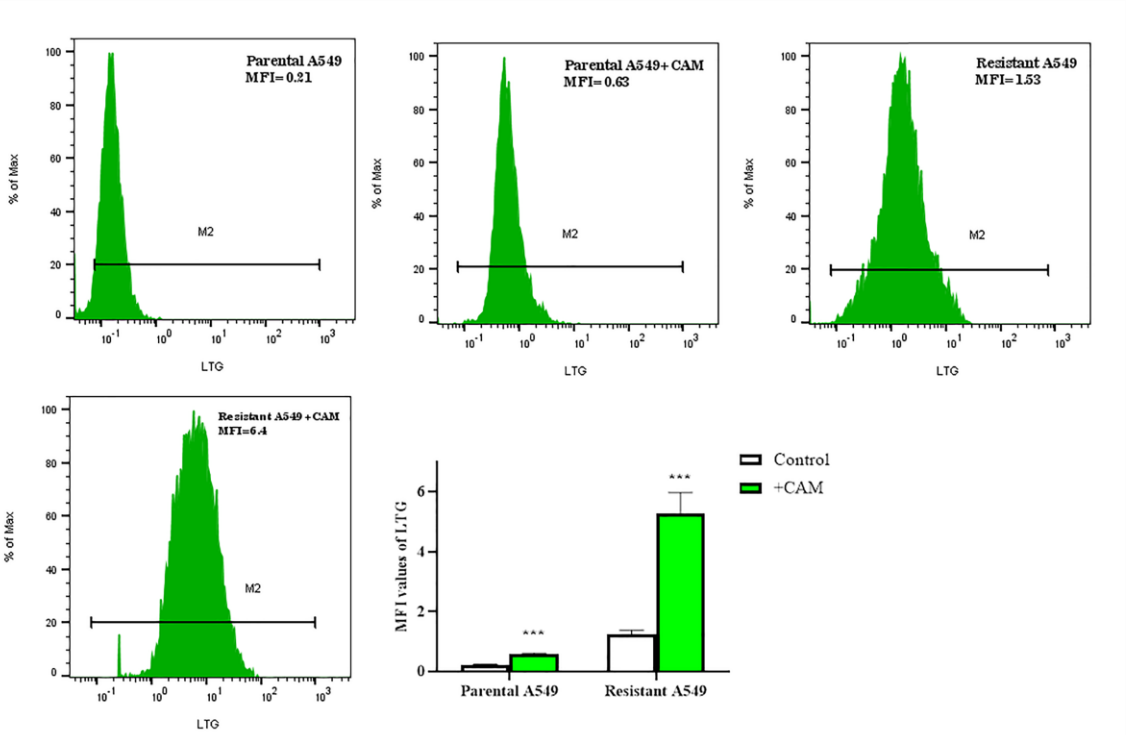


Figure 29. Histogram plots and MFI values of LTG expression in parental and resistant A549 cells in absence and presence of CAM (500 µM). Data are presented as Mean ± SEM of three replicates. *** p <0.001, control vs (+CAM).

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance in A549.Res cell lines

- **Apoptosis**

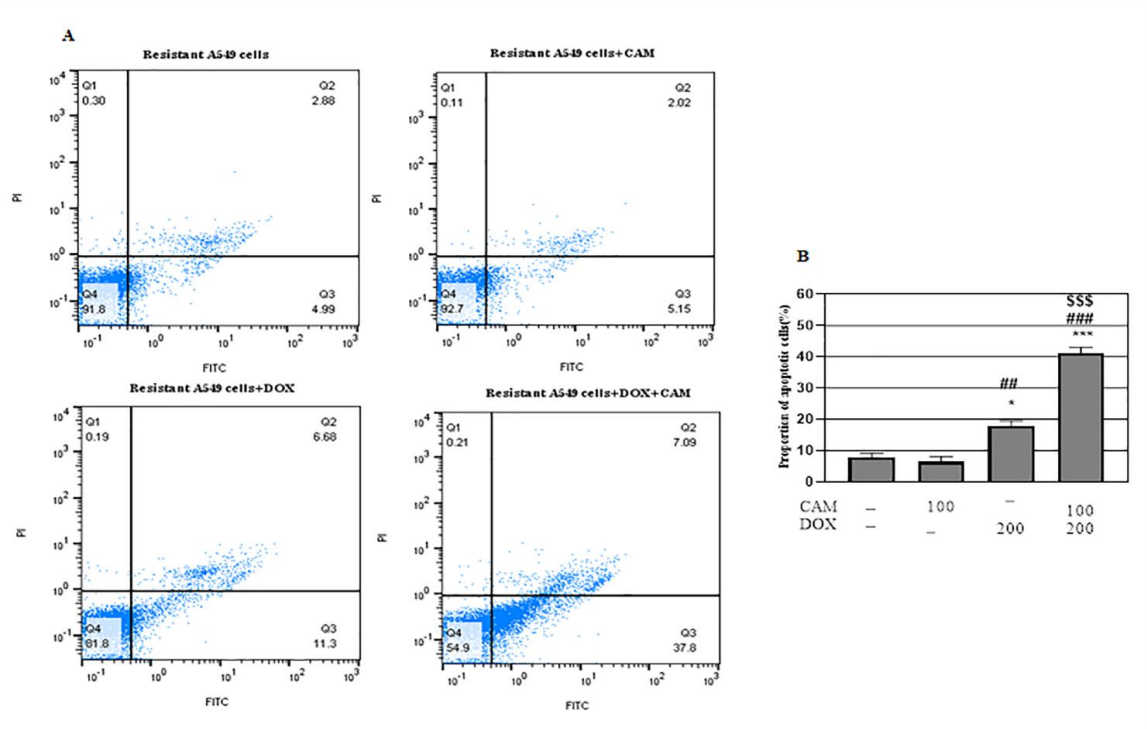


Figure 30. Quantification of apoptotic cell death after 48 hrs of treatment with CAM, DOX and their combination in resistant A549 cells. * $p<0.05$, *** $p<0.001$ treated vs control. ## $p<0.01$, ### $p<0.001$ treated vs CAM and $$$ $p<0.001$ treated vs DOX+CAM.

➢ Inhibition of autophagy is a therapeutic strategy

1. **Autophagy and doxorubicin resistance in cancer(2018)**
2. **Enhanced cytotoxic activity of doxorubicin through the inhibition of autophagy in triple negative breast cancer cell line(2017)**
3. **Role of autophagy and lysosomal drug sequestration in acquired resistance to doxorubicin in MCF-7 cells(2016)**
4. **PI3K/Akt/mTOR activation by suppression of ELK3 mediates chemosensitivity of MDA-MB-231 cells to doxorubicin by inhibiting autophagy(2016)**
5. **Berberine Reverses Doxorubicin Resistance by Inhibiting Autophagy Through the PTEN/Akt/mTOR Signaling Pathway in Breast Cancer(2020)**
6. **Carvacrol nanoemulsion evokes cell cycle arrest, apoptosis induction and autophagy inhibition in doxorubicin resistant-A549 cell line(2018)**
7. **HMGB1 promotes drug resistance in osteosarcoma(2012)**
8. **(S)-Ginsenoside Rg3 is a novel inhibitor of autophagy and sensitizes hepatocellular carcinoma to doxorubicin(2014)**

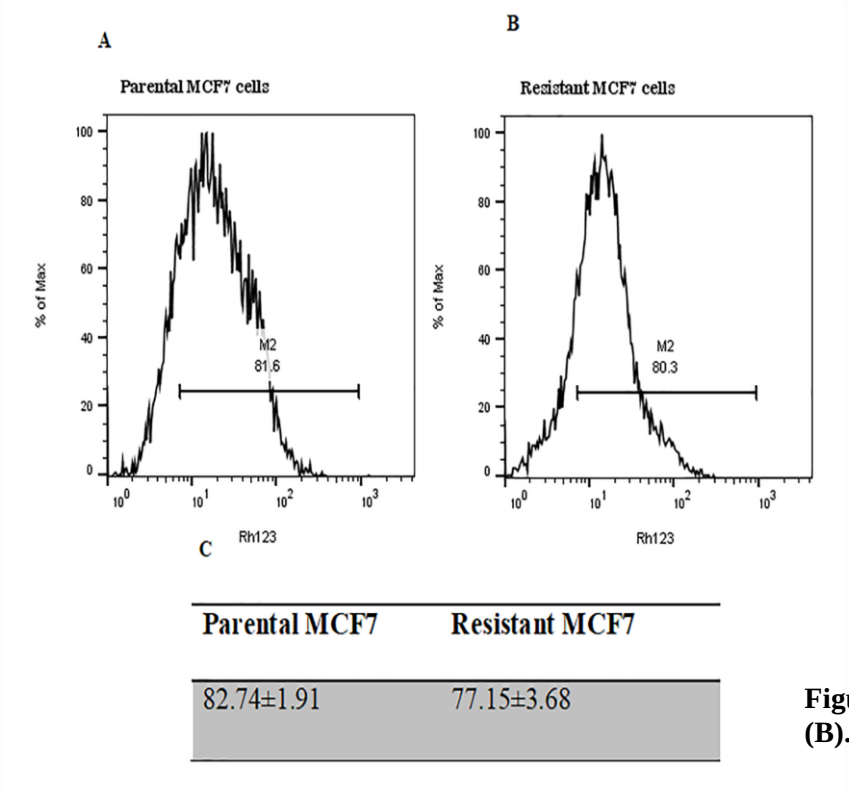
- **The relationship between autophagy and apoptosis is in an inhibitory manner**

- **CAM dysregulates autophagy in resistant sub lines and enhanced apoptosis**

1. **Self-consumption: the interplay of autophagy and apoptosis(2014)**
2. **Apoptosis and autophagy: the Yin–Yang of homeostasis in cell death in cancer(2015)**
3. **Role of the crosstalk between autophagy and apoptosis in cancer(2013)**
4. **Inhibition of autophagy increases proliferation inhibition and apoptosis induced by the PI3K/mTOR inhibitor NVP-BEZ235 in breast cancer cells(2015)**
5. **Autophagy prevents doxorubicin-induced apoptosis in osteosarcoma(2014)**
6. **Clarithromycin enhances dasatinib-induced cell death in chronic myeloid leukemia cells, by inhibition of late stage autophagy(2013)**

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance

- Determination of **P-glycoprotein activity** by **Rhodamin 123** in **MCF7.Res** cells

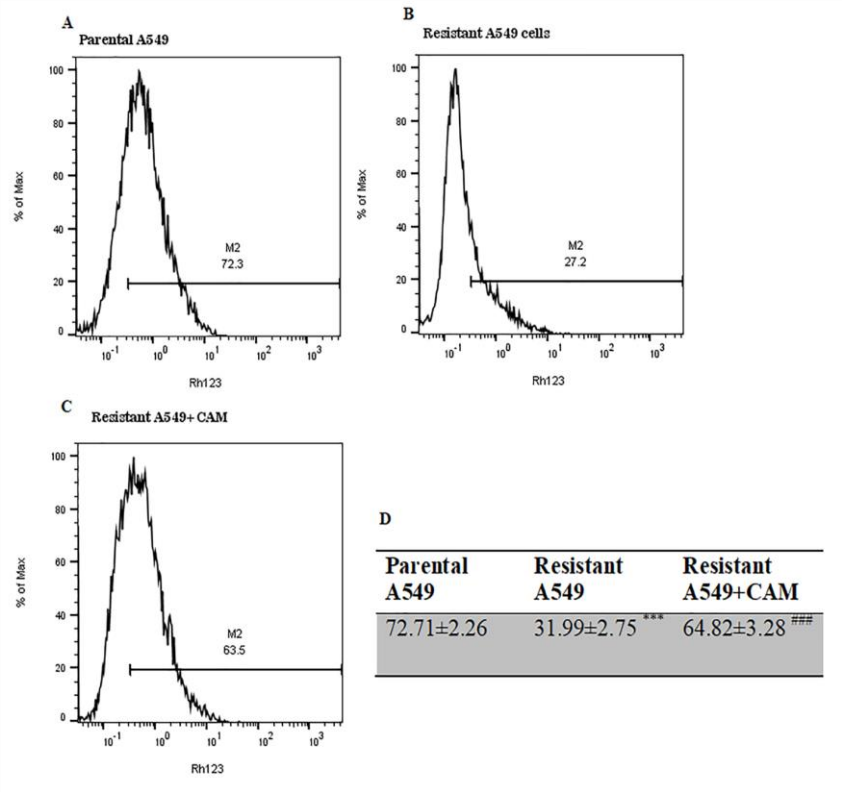


Parental MCF7	Resistant MCF7
82.74±1.91	77.15±3.68

Figure 31. Positive Rh123 parental (A) and resistant MCF7 cells (B). Data are presented as Mean ± SEM

4 Evaluating the effect of CAM on inhibition of autophagy and attenuating drug resistance

- Determination of **P-glycoprotein activity** by **Rhodamin 123** in **A549.Res** cells



D

Parental A549	Resistant A549	Resistant A549+CAM
72.71±2.26	31.99±2.75 ***	64.82±3.28 ###

Figure 32. Positive Rh123 parental (A) , resistant A549 cells (B) and resistant A549 cells with CAM. Data are presented as Mean ± SEM. * *p* <0.001 parental vs resistant cells, ### *p* <0.001 resistant vs resistant cells+CAM**

Conclusions

- **CAM synergistically enhanced DOX-induced cytotoxicity through *elevating apoptotic cell death* in MCF7 and A549 cell lines**
- **Contribution of autophagy in acquired drug resistance of DOX in MCF7.Res and A549.Res cell lines**
- **CAM dysregulates autophagy in resistant sub lines and enhanced apoptosis**
- **Taken together, these findings suggest that CAM is a suitable candidate for combination therapy. However, further investigations are needed to evaluate the application of CAM in adjuvant cancer therapy.**

Recommendations

1. **Evaluating the effect of combined treatment of DOX+CAM in normal breast and cardiac cell lines**

2. **Evaluating the effect of CAM on DOX-cardiotoxicity**

3. **Investigation the effect of CAM in combination with other chemotherapeutic drugs and in other caner cell lines**

4. **In vivo studies**

5. **Determination the relationship between autophagy and P-gp activity in MCF7.Res and A549.Res cell lines**

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Original research article

Clarithromycin effectively enhances doxorubicin-induced cytotoxicity and apoptosis in MCF7 cells through dysregulation of autophagy

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Department of Toxicology and Pharmacology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

ARTICLE INFO

Keywords:
Breast cancer
Doxorubicin
Clarithromycin
Autophagy
Apoptosis

ABSTRACT

Purpose: Use of autophagy inhibitors in combination with chemotherapy has become a novel chemotherapeutic strategy. In this study, we aimed to determine whether the effectiveness of doxorubicin (DOX) is augmented by clarithromycin (CAM) in MCF7 cells and the molecular mechanisms involved.

Materials and methods: Combined cytotoxicity of CAM and DOX was assessed by MTT assay and was analyzed using the Chou-Talalay's method. To clarify the underlying mechanisms, several factors, including apoptosis (Annexin V/propidium iodide staining), intracellular level of DOX (spectrofluorimetry) and P-glycoprotein activity (Rhodamin 123 efflux assay) were measured. In addition, autophagy was evaluated by intracellular la-

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Contribution of Autophagy in Acquired Drug Resistance of Human Breast Cancer Cells MCF7 to Doxorubicin

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Abstract

Introduction: At present, autophagy has attracted increased attention as a potential therapeutic target for breast cancer. Many investigators are trying to overcome drug resistance by inhibition of autophagy. The development of *in vitro* drug-resistant cell lines with well-characterized autophagy-related markers is required to discover new autophagy inhibitors. Therefore, in this study we established resistant human breast cancer cells MCF7 to doxorubicin (DOX) and used flow cytometric assays to study autophagic flux.

Materials and Methods: Resistant MCF7 subline (MCF7.res) was derived from parental MCF7 cells (MCF7.par) by continuous exposure to stepwise increasing concentrations of DOX (30–54 μM). Autophagic flux was assessed by the antibody labeling of the microtubule-associated protein LC3-II and analyzed by flow cytometry. In addition, lysosomal mass was measured flow cytometrically by LysoTracker Green (LTG) staining. The median fluorescence intensity of anti-LC3-II and LTG was compared between parental and resistant MCF7 cells. For detection of apoptotic cell death, Annexin V/propidium iodide staining was performed. After ~6 months, MCF7.res subline was obtained from MCF7.par cells.

Results: DOX resistance was confirmed by measurement of fold resistance and growth curve analysis. Our established MCF7.res subline exhibited 7.1-fold resistance to DOX compared with MCF7.par cells. Flow cytometric analysis revealed that LC3-II level and LTG signal were elevated in MCF7.res compared with MCF7.par, elu-

Clarithromycin synergistically enhances doxorubicin-induced apoptosis through inhibition of autophagy in MCF7 cells

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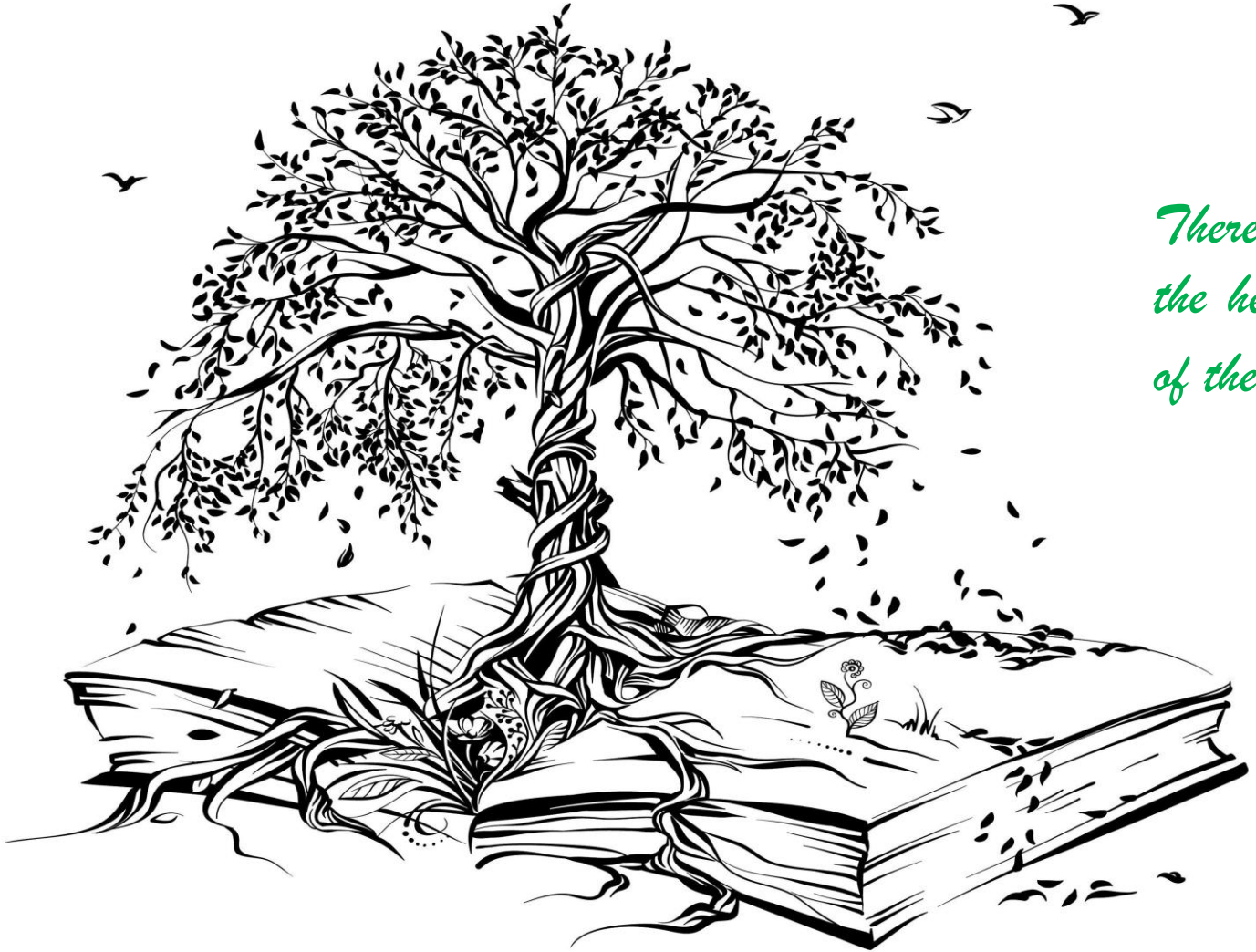
A growing number of studies have reported the correlation between autophagy and apoptosis, mostly, in an inhibitory manner, meaning autophagy blocks the induction of apoptosis. Due to this fact, using autophagy inhibitors in combination with chemotherapy has become a novel chemotherapeutic strategy. In the present study, we investigated if the effectiveness of doxorubicin (DOX) was augmented by using clarithromycin (CAM) as an autophagy inhibitor. The cytotoxic effects of DOX and CAM alone and in combination, on MCF7 cells were designed according to Chou-Talalay method and assessed by MTT assay. Also, the involvement of autophagy in the DOX-induced apoptotic death of MCF7 cells was investigated using flow cytometry. Simultaneous treatment with DOX (0.05, 0.1, 0.2, 0.5, 1, 2 µM) plus CAM (100, 500 µM) caused a significant reduction of DOX IC50 value. The median effect analysis generated by Compusyn software revealed that DOX and CAM combination in vitro exerted synergistic effect. Flowcytometry analysis indicated that CAM at 100 µM caused an accumulation of LC3II, and increased LysoTrackerGreen (LTG) signal, suggesting that CAM inhibits autophagic flux at late stage. On the other hand, DOX caused induction of autophagy flux that was confirmed with significant increasing in LC3II level but not LTG signal. Combination with CAM blocked autophagy flux induced by DOX that led to enhanced apoptosis. In summary, this study is in accordance with this theory that inhibition of autophagy would enhance apoptotic cell death of anti-cancer drugs.

Keywords: autophagy, apoptosis, breast cancer, clarithromycin



There is a wisdom of the head and a wisdom of the heart.

THANK YOU