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ANTITUMOR ACTIVITY OF MIXING GEMCITABINE WITH PRAVASTATIN IN A MICROEMULSION AGAINST A549 NON-SMALL CELL LUNG CANCER CELLS

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ABSTRACT

Combining several anticancer drugs in a nanoparticle is a new promising regimen in cancer therapy. In this study, the antimetabolite agent, gemcitabine (GEM), was combined with the cholesterol lowering drug, pravastatin (PRVA), in a microemulsion (ME) formula. The cytotoxicities and apoptotic effects of the combination formulas were *in vitro* evaluated in A549 non-small cell lung cancer cells. The cytotoxicity was assessed through using 3 (4,5-dimethylthiazole-2-yl)-2,5-diphyneltetrazolium bromide (MTT) assay, while the mechanism of cell death was evaluated by observing the morphological changes of the treated cells under light microscope, and detecting apoptosis by using ApopNexin apoptosis detection kit and 4′,6-diamidino-2-phenylindole (DAPI) dye. It has been found that a 5μM of mixed GEM and PRVA at a ratio of 2:1, respectively, in ME (2GEM/PRVA-ME) has the best cytotoxic and apoptotic effect on the A549 cells.This study suggests that combining GEM with PRVA in a ME formula has a great therapeutic effect on A549 cells.

KEYWORDS: *combination therapy, statin, antimetabolite, nanoparticles, apoptosis, cytotoxicity.*

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death in many countries. There are two kinds of this disease, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). About 80%–85% of the lung cancer cases are NSCLC, while about 15% of the cases are SCLC. Approximately half of the patients with NSCLC have advanced disease.[1]The newer approaches for cancer treatment is not only developing conventional chemotherapy and radiotherapy but also preventing damage to normal tissues and avoiding drug resistance. There has been a drive to find new routes for drug administration and novel drug compositions which allow efficient passage and high bioavailability of the drug. One form of formulating drugs is to deliver them in nanocarriers like microemulsions (MEs) which are optically isotropic and thermodynamically stable colloidal systems that consist of water, oil and amphiphile.[2]There are different structures of the MEs that can be produced depending on the constituents, oil-in-water (o/w), water-in-oil (w/o) and bicontinuous MEs. The o/w ME droplets are likely formed when the volume fraction of oil is low. In contrast, the w/o ME droplets are possibly formed when the volume fraction of water is low. The bicontinuous ME may result in systems where water and oil volumes are equivalent.[3]Gemcitabine (GEM), a chemotherapeutic agent, was recently formulated in a safe and effective ME delivery vehicle.[4]It has been found that the combination of the GEM and other drugs like statins improved the prognosis of cancer compared with GEM treatment alone.[5] This combination of GEM and statin resulted in an almost complete suppression and a marked delay in a relapse of tumor growth. A previous study demonstrated that statin inhibited proliferation, induced apoptosis in cancer cells and synergistically potentiate the cytotoxic effect of GEM.[6]The statin, Pravastatin (PRVA),a hydrophilic inhibitor of HMG-CoAreductase, haslimited penetration into the intracellular space of non-hepatic tissues and therefore got eliminated in the liver without being accumulated in plasma even with repeated administration.[7]Previous studies have demonstrated that statins may have antitumor activities because they impede the activity of HMG-CoA reductase which cause reduction in the farnesylation and geranylgeranylation of various vital proteins that are necessary for the cellular proliferation and survival of the cacner cells.[8,9]The objective of the present study was to *in vitro* evaluate the antitumor activity of the combination formula of the mixed GEM and PRVA loaded in a ME formula. In particular, the cytotoxic and apoptotic effect of the drug combination formulas was examined in the A549 non-small cell lung cancer cells.

MATERIALS AND METHODS

Materials and subjects

Polyoxyethylene Sorbitan monooleate (Tween 80), isopropyl Myristate (IPM), ethanol, sorbitan monolaurate (Span 20), penicillin-streptomycin antibiotic and gemcitabine hydrochloride (GEM) were purchased from Sigma (Missouri, US). Pravastatin (PRVA) sodium was obtained from the U.S. Pharmacopeia (Rockville, US). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS) 1X concentrated and pH 7.4, trypan blue (0.4%), Coomassie blue (10%) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) were purchased from Invitrogen life technologies (New York, US). Trypsin was purchased from HyClone (Utah, US). The (3-(4,5- dimethylthiazole-2-yl)-2,5-diphyneltetetrazolium bromide, MTT) cell proliferation assay kit was obtained from Cayman Chemical Company (Michigan, US). The ApopNexin FITC apoptosis kit was purchased from Millipore (MA, US). The A549 non-small cell lung cancer cell line was procured from the Tissue Culture unit at King Fahd Center for Medical Research, Jeddah, KSA.

Preparation of the ME formulations

A w/o ME formulation was prepared as described by Tsai *et al*..[4] In brief, it was produced by mixing 500 µl of IPM, 240 µl of Tween 80, 160 µl of Span 20 and 100 µl of 40% ethanol. The resulting ME was stored at room temperature (25°C). The tested ME formulas were blank ME, GEM-loaded-ME (GEM-ME), PRVA-loaded-ME (PRVA-ME), a 1:1 ratio of GEM:PRVA-loaded-ME (GEM/PRVA-ME), a 1:2 ratio of GEM:PRVA-loaded-ME (GEM/2PRVA-ME), and a 2:1 ratio of GEM:PRVA-loaded-ME (2GEM/PRVA-ME). Additionally, formulas, contained water instead of ME, were designated as GEM, PRVA, GEM/PRVA, GEM/2PRVA, and 2GEM/PRVA.

Cell culture

A549 cell lines were grown in a cell culture flask (25 cm^2)containing 10 ml of DMEM supplemented with 1% penicillin-streptomycin antibiotic and 10% FBS and were incubated for 24 h in a 95% air and 5 % humidified CO_2 incubator at 37 °C. Cells were fed until confluence and confluent cells were collected by trypsinization, washed and passaged.

Cytotoxicity screening using the MTT proliferationassay

The MTT assay provides a simple method for the determination of the viable cell number using standard plate absorbance readers. The cultured A549 cells were counted using hemocytometer and seeded at a density of 5 $x10^3$ cells per well into 96-well and incubated for 24 h at 37 °C in a humidified 5 % CO_2, flat-bottomed tissue culture plates containing 100µl of growth medium per well. Cells, treated with 100 µl of either 1 or 5 µM of the tested drug formula, were re-incubated for 48 h at 37 °C in a humidified 5 % CO_2 incubator. Untreated cells were used as control and triplicate wells were prepared for each individual formulation.Following the incubation time, a 5 µl of the yellow MTT reagent was added to each well in dark, mixed gently for one minute and incubated for 4 h at 37 °C in a humidified 5 % CO_2 incubator. After that, the MTT reagent was discarded, followed by the addition of a 100 µl of the crystal dissolving solution to each well and incubated for 10 min at 37 °C in a humidified 5 % CO_2 incubator. Finally, the absorbance of each well was measured at 540 nm using an ELISA plate reader (BioTek, US). The cytotoxicity effect of the drug formula on the cells was detected according to the percentages of cell viability calculated

by dividing the absorbance of the sample by the absorbance of the control and then multiplying by 100.

Characterization of cell morphology using light microscope

The cultured A549 cells were counted, plated into a 24-well flat-bottomed tissue culture plate at a density of 1 x10^4 cells per well containing 500 µl of growth medium per well and incubated for 24 h at 37°C in a humidified 5 % CO_2 incubator. Cells, treated with 500 µl of 5 µM of the tested drug formula, were re-incubated for 48 h at 37 °C in a humidified 5 % CO_2 incubator. After that, they were washed twice with PBS for 5 min and fixed by the addition of 4% formaldehyde. After 10 min, the fixation solution was discarded and the cells were stained with 10 % Coomassie blue dye for 15 min. Finally, the stain was washed with distilled water twice, and left to dry for 2h at room temperature, 25°C. The morphological changes were observed by phase contrast inverted microscope (1X2-SP Olympus, Japan).

Detection of apoptosis using the ApopNexin FITC apoptosis kit

The ApopNexin FITC apoptosis detection kit, utilized to investigate the stages of apoptosis, contains two stains. The former one is the green fluorescent which detects the cells at the early and middle stages of apoptosis. The latter one is the red fluorescent which stains the cells that are in the late stages of apoptosis or necrosis. The cultured A549 cells were counted, plated into a 24-well flat-bottomed tissue culture plates at a density of 5×10^4 cells per well containing 500 µl of growth medium, and incubated for 24 h at 37 °C in a humidified 5 % CO_2incubator. Cells were re-incubated with 500 µl of 5 µM of the tested drug formula for 48 h at 37 °C in a humidified 5 % CO_2 incubator. After that, cells were washed twice with 300 µl of PBS, detached with 200 µl of trypsin and incubated for 3 min followed by the addition of 500 µl of culture media. The supernatant containing detached cells was removed, put into a flow-cytometry tube and centrifuged at 1000 rpm for 5 min. Then, the supernatant was removed and the adherent cells were washed twice with 3 ml of cold PBS and centrifuged at 1000 rpm for 5 min. After that the buffer solution was replaced by a 200 µl of 1X binding buffer (10 mM HEPES /NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Finally, a 3 µl of FITC and a 2 µl of PI were added in the dark, incubated for 15 min at room temperature and then the tubes were transferred into ice-cold to be analyzed using the flow cytometry with FACSDivaTM lll software Version 6.1.3 (Tree Star Inc., Ashland, OR, USA).

Detection of nuclear alterations using DAPI kit

DAPI is a nuclear stain used in fluorescent techniques to detect the late apoptosis. The blue-fluorescent DAPI nucleic acid stains preferentially dsDNA.The cultured A549 cells were counted, plated into a 24-well flat-bottomed tissue culture plates at a density of 5×10^4 cells per well containing 500µl of growth medium and incubated for 24h at 37°C in a humidified 5% CO_2 incubator. Cells were re-incubated with 500µl of 5µM of the tested drug formula for 24h and 48h at 37°C in a humidified 5% CO_2 incubator. After the incubation time, they were washed twice with 300µl of PBS and fixed by the addition of 200µl formaldehyde (4%). After 10min, the fixation solution was discarded and the cells were stained by adding 300µl of DAPI staining solution and incubated for 5min at 25°C. All cells were viewed using afluorescence microscope(Leica DMI6000 B, Germany).

STATISTICAL ANALYSIS

Statistical analyses were implemented with one- way analysis of variance (ANOVA) test, and two- way ANOVA test using the MegaStat Excel (version 10.3, Butler University). The significant variations between the samples was considered when the p-value was less than 0.05.

RESULTS

Cytotoxicity screening using the MTT proliferation assay

As illustrated in Table 1, it has been found that all of the solution formulations were having insignificant cytotoxicity at either 1 or 5µM except GEM that have had slight cytotoxicity at 5µM. On the other hand, formulating the drugs in ME have improved their cytotoxicity with some discrepancies since the blank ME was having anti-proliferative effect as the % of cell viabilities were 62.38 ± 4 and 24.74 ± 1 at 1 and 5µM, respectively. Both of GEM-ME and PRVA-ME, as a single treatment, were having more inhibition effect than their combination formulas of GEM/PRVA-ME and GEM/2PRVA-ME. Conversely, the cytotoxicity of a 1µM of 2GEM/PRVA-ME was significantly greater than 1µM of GEM-ME and PRVA-ME. However, the cytotoxicity of 2GEM/PRVA-ME, GEM-ME and PRVA-ME were similar at 5µM.According to the results of MTT assays that screened the anti-proliferative effects of the entire formulations, the selection of the combination formulas for further studies was based on their inhibitory effect on the A549 cells.

Table 1

Percentages of cell viability of the drug formulations at 1 and 5 µM administered onto A549 cells.

Formulation	1µM	5µM
GEM	103.38±5	67.42±0
GEM-ME	72.61±1	34.59±1
PRVA	87.51±7	91.75±1
PRVA-ME	84.02±12	23.59±2
GEM/PRVA	119.75±6	106.64±6
GEM/PRVA-ME	117.82±8	95.25±1
GEM/2PRVA	107.66±5	112.98±9
GEM/2PRVA-ME	110.57±8	90.37±7
2GEM/PRVA	105.52±3	129.39±6
2GEM/PRVA-ME	48.15±11	37.46±1
ME	62.38±4	24.74±1

Characterization of cell morphology using light microscope

Light microscopy was employed to observe the mode of cell death, undergoing apoptosis when subjected into drug formulation. Signs of apoptosis are characterized by some alterations in the cellular morphology, such as shrinkage of the cell, chromatin fragmentation, rapid phagocytosis by neighboring cells and formation of apoptotic bodies, chromatid condensation and fragmentation. Cells were incubated with 5μM of the desired ME formulation for 24 and 48h.As exhibited in Figure 1, A549 cells treated with ME, GEM-ME, and 2GEM/PRVA-ME for 24h have endured membrane blebbing, formationof apoptotic bodies and digestive vacuoles indicating that the apoptosis was integrated with autophagocytosis.Following 48h incubation, late stages of apoptosis were observed in the treated cells as more chromatin fragmentation and increased intracellular spaces between the cells have occurred. Interestingly, ghost cells were observed when they were treated with PRVA-ME and ME within 24h and 48h, respectively. It should be noted that A549 cells, treated with solution formulations, were not observed under the light microscope because the water formulations did not have significant toxicity on the cells as detected by the MTT assay.

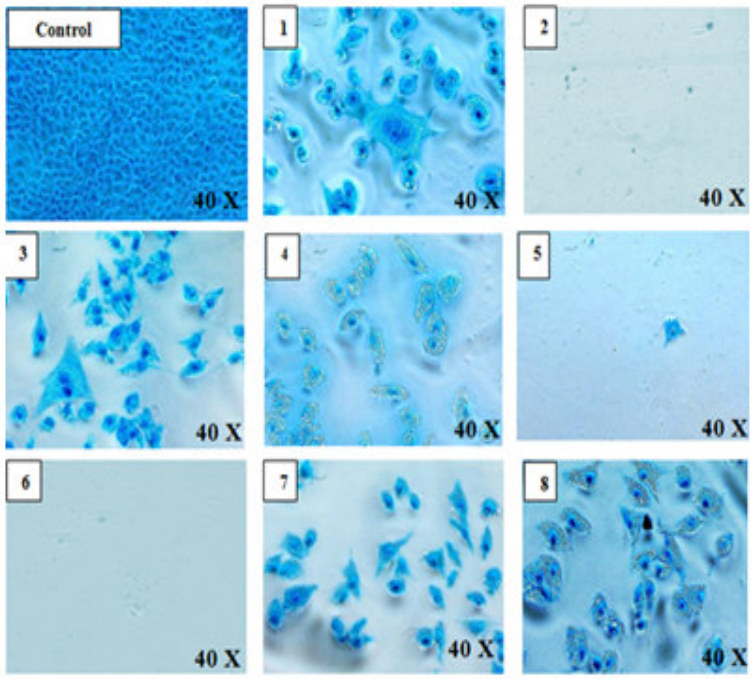

Figure 1

Light microscopy images of A549 cells treated with 5μM formulations at different periods of time.

(1) ME (24h), (2) ME (48h), (3) GEM-ME (24h),(4) GEM-ME (48h), (5) PRVA-ME (24h), (6) PRVA-ME(48h), (7) 2GEM/PRVA-ME (24h), and (8) 2GEM/PRVA-ME (48h).

Detection of apoptosis by ApopNexin FITC Apoptosis Kit

The apopNexin assay utilizes the flow cytometry in distinguishing between the cells according to the plot of the two fluorescents, green FITC and red PI, divided into four squares, designated as Q1, Q2, Q3 and Q4. Dead cells or necrotic cells locate in Q1 where they have low FITC but high PI while the viable cells are placed in Q3 where they have low FITC and low PI. On the other hand, late apoptotic cells have high FITC and high PI (Q2) whereas early apoptotic cells have high FITC but low PI (Q4).As illustrated in Table 2 and displayed in Figure 2, A549 cells were subjected into different ME formulations. The highest percentages of late apoptotic cells were recorded for the cells treated with the combination formula, 2GEM/PRVA-ME. The single treatment, GEM-ME, has induced apoptosis more than PRVA-ME. The viable cell percentages were the highest when the cells were treated with PRVA-ME.

Table 2

Detection of apoptosis using ApopNexin FITC apoptosis assay for 5μM of different drug formulas subjected onto A549 cells.

Type of cells (%)	ME	GEM-ME	PRVA-ME	2GEM/PRVA-ME
Necrotic	4.1	3.2	8.1	12.1
Late apoptotic	37.3	51.1	30.6	66.6
Viable	43.9	38.7	55.8	19.9
Early apoptotic	14.7	7	5.5	1.4

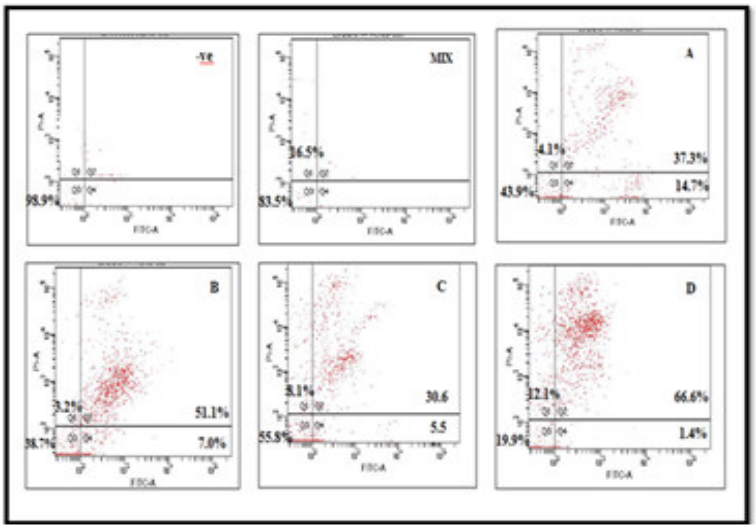

Figure 2

The fluorescein isothiocynate/propidium iodide (FITC/PI) flow cytometry plots of A549 cells treated for 24 hours with 5μM of drug formulations of (A) ME, (B)GEM-ME, (C) PRVA-ME, and (D) 2GEM/PRVA-ME.

DAPI assay for nuclear staining

DAPI is a useful tool in various cytological investigations because it forms blue fluorescent dye when it gets attached to the double-stranded DNA of the nuclei of the cells, undergoing apoptosis.As displayed in Figure 3, A549 cells were subjected into different ME formulations. All of the ME-formulations have changed the morphology of the nucleisuch as irregular edges around the nucleus, chromosome condensationand heavier coloring blue, which means that the cells were enduring apoptosis at late stages. Additionally, cells treated with GEM-ME and 2GEM/PRVA-MEhave had a massive effect on the nuclei of the cells as increased number of blue spots were seen.

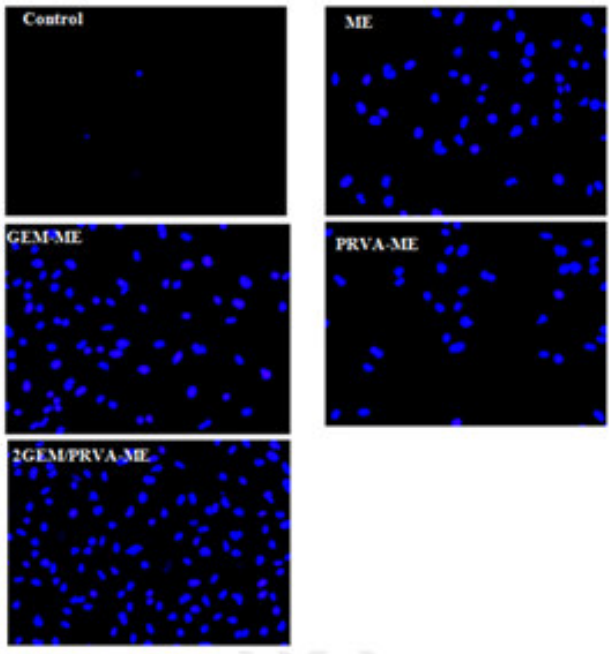

Figure 3

Fluorescence microscopy images of A549 cells, stained with 4',6-diamidino-2-phenylindole (DAPI), after treated with 5μM of drug formulations for 24h. Images were magnified at 20X.

DISCUSSION

According to the MTT assay that screened the anti-proliferative effects of the entire formulations,formulating the drugs in ME have improved their cytotoxicity against A549 cells more than water formulations, and these cytotoxicity more improved when the concentration of the formulas has increased from 1 to 5μM.The present study shows that PRVA-ME inhibits proliferation and induces apoptosis A549 cells. Therefore, combining PRVA with GEM at lower doses of both drugs has synergistically potentiate their cytotoxic effect on these cancer cells. Delivery by ME systems improved drug release, increased drug solubility, and enhanced bioavailability. In agreement with other previous studies performed by Paolino *et al*.[10]and Coimbra *et al*. [11] who

observed that the targeted delivery of GEM or PRVA can improve their anticancer activity by enhancing local drug concentration and direct regulation ofthe antitumor immune response. In the case of combination of GEM with PRVA, present results indicated that 2GEM/PRVA-ME was considered for more in-depth studies on their antitumor activity because 2GEM/PRVA-ME was having the best cytotoxicity effect on the A549 cells. It is noteworthy that all of the water combination formulas did not have a considerable cytotoxic effect on A549 cells. The combination of two hydrophilic drugs loaded into the hydrophobic core of ME could help the drugs to permeate the cells more efficiently. In general, MTT assay provides an indication of mitochondrial integrity and activity.[12] The drug formulations affected the total mitochondrial activity which is related to the number of viable cells. Previous studies on the specific combination of GEM with PRVA loaded on nanoparticles are very limited. However, there have been many studies on the combination formulas used for cancer treatment that incorporate either GEM or PRVA. Bocci *et al*. [6] have shown that fluvastatin deters cancer cell growth, stimulates apoptosis and bolsters the cytotoxic effect of GEM in human pancreatic cancer. Similarly, Mistafa and Stenius [13] found that combining atorvastatin and GEM have induced apoptosis in human pancreatic cancer whereas PRVA in combination with low doses of GEM did not increase inhibition of cell proliferation.Additionally, adding low-dose of simvastatin to GEM in advanced pancreatic cancer does not provide clinical benefit and does not result in increased toxicity.[14] In contrast, Cheng *et al*.[15] demonstrated that PRVA can be used as a cytoprotective agent prior to carboplatin chemotherapy as it can reduce oxidative stress and thus prevent cardiac apoptosis. In fact, there have been many researches on the single treatments, GEM and PRVA, encapsulated in nanoparticles. In agreement with the present study that demonstrated the improved effect of the lone drug-loaded-ME on the cancer cells relative to the free drug, Coimbra et al.,[11] elaborated that the delivery of PRVA using small long-circulating liposomes have affected the viability and proliferation of tumor cells, endothelial cells and macrophages. Furthermore, Arias*et al.*[16] found that chitosan nanoparticle loaded with GEM demonstrated improved antiproliferative effect over free GEM when it was administered into mice-bearing L1210 wt subcutaneous tumor. In addition, De Angel *et al*.[17] developed a novel stearoyl GEM solid-lipid nanoparticle formulation that are significantly more effective than GEM in controlling tumor growth in mouse models, and induce apoptosis without promoting the development of side effects. Viota *et al.*[18] illustrated that nanoparticles are able to release the antitumor drug GEM in liver, colon and breast tumor cells. They found that most of the drug is delivered inside the nuclei of the cancer cells after 2h.

CONCLUSION

In this study, the anticancer activity of the ME formulations loaded with different concentrations of GEM and PRVA was evaluated *in vitro* against A549. The ME formula, consisted of IPM as oil phase, a surfactant mixture of tween 80 and span 20, and aqueous phase of distilled water containing 40% ethanol.It has been found that the combination formula, 2GEM/PRVA-ME, has the best cytotoxicity effect on the A549 cells. Furthermore, the cytotoxicities of the mixed drugs-loaded-ME formulas against A549 cells were dose-dependent and significantly greater than the toxicities of the water formulations. The A549 cells treated with the ME formulations have endured apoptosis within 24h. Based on this study, it is recommended to establish further *in vivo* researches in order to give a complementary study on the effect of the combination therapy of GEM with PRVA, delivered in ME formula, on the body tissues. This ME formula is a promising formulation for delivery of other drug combinations.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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