Metformin-Loaded Nanostructured Plga: A New Strategy for Enhancing Efficacy of Metformin in Breast Cancer Treatment

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Abstract

Objective: Nano-formulation approaches would be more efficient in decreasing drug toxicity, preventing tumor development in relation to free form of drug.

Methods: Therefore, the purpose of this study is to construct poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs) loaded with Metformin (MET) to investigate their cytotoxicity as well as their impact on expression levels of apoptosis related genes in MDA-MB-231 breast cancer cells. Field Emission Scanning Electron Microscope (FE-SEM) and Fourier-transform infrared spectroscopy (FTIR) characterized the synthesized NPs. Then, MTT assay was used to evaluate and compare the cytotoxic effect of various concentrations of the chemotherapeutic molecules in pure and nano-formulated forms after 48 h exposure time. Moreover, the mRNA levels of apoptosis related genes expression were studied by quantitative PCR.

Results: By encapsulating MET into PLGA, the cytotoxic efficiency of the compounds considerably augmented for all concentrations. Furthermore, the results demonstrated that MET-loaded NPs could induce apoptosis in MDA-MB-231 breast cancer cell by upregulation of caspase-3, caspase-7 and BAX, along with Bcl-XL, hTERT and Cyclin D1 down regulation.

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Conclusion: Ultimately, this study revealed that the combination of MET–PLGA NPs with current cancer therapies holds promise for the potential of breast cancer treatment.

Keywords: *Metformin, PLGA, Nanoparticles, MDA-MB-231, Apoptosis*

Introduction

Nowadays, cancer is a major global health issue around the world (1)**.** Breast cancer is considered the most prevalent malignancy, with an estimated 2.3 million new cases (11.7%) in 2020 and a leading health challenge among females. According to some estimates in recent years, it is responsible for 6.9% of mortality cases in female breast cancers (2). Despite the significant progression in therapeutic strategies for breast cancer, many patients suffer from relapse and metastasis. The etiology of insufficient therapies can be related to ambiguous insight into molecular mechanisms involved in breast cancer initiation, progression, and metastasis. Multiple genomic and epigenetic events have long been considered hallmarks for the initiation of cancer formation and progression of the disease (3). Current approaches of cancer therapy including radiotherapy and chemotherapy are restricted because of owning various frustrating and unfavorable adverse effects such as unwanted toxicity on the normal cells. Therefore, it is essential that innovative strategies and approaches be developed to effective treatment of human breast cancer (4).

Currently, numerous non-hazardous natural compound have been derived, and their ability in moderating cancer relapse and improvement of treatment have been assessed (5). Some naturals compound like MET is promising products for therapeutic efficiency.

Metformin is a semi-synthetic oral hypoglycemic drug which mostly moderates blood glucose by activating of the vital AMPK/mTOR/p70S6K pathway, inhibiting hepatic glucose output and increasing glucose uptake (6). The anti-cancer ability of MET in various cancer cells including prostate, breast, ovary, cervix cancer, and leukemia incidents both *in vivo* and *in vitro* have been studied and recorded (7-9). In spite of the recognized efficacy of MET in treating cancer, the use of this drug in medication is related to some major challenges such as poor biological availability, short half-life, low solubility, low duration of stability in the bloodstream and degradation (10, 11). To overcome these limitations, use of nano-formulation system that allows controlled and sustained release of nano-formulated drugs can be a hopeful way to rise the activity and transfer of natural compounds to target cells.

Polymeric particles are a distinctive type of nanomaterials that have found many new applications in delivery systems for in situ controlled release

of therapeutic applications (12). Nanoparticle drug delivery supplies numerous advantages, due to high encapsulation efficiency, protected drug from degradation, improved biodistribution, delivered, and controlled drug release (5, 13)

In the current study, we aimed to encapsulate MET into PLGA NPs and investigate the improvement of its anti-cancer effects on MDA-MB-231 breast cancer cells through modulating the expression of apoptosis related gens, as the important molecules involved in tumorigenesis.

Materials and Methods

Synthesis of NPs

The improved w/o/w produced MET-encapsulated PLGA NPs with slight modifications (9) . Briefly, a premixed emulsion of PLGA (100 mg) and MET (6 mg) in DCM-Methanol (4:1) was added to the 2% PVA. The mixture was emulsified by sonication at 70,000 rpm for 1 min to produce w/o/w emulsion. Then, a rotary evaporator under a low vacuum was used to evaporate the DCM. The NPs formed by this method were gathered via three cycles of centrifugating at 12,500 g for 15 min.

Nanoparticle characterization

To characterize the surface charge (zeta potential), hydrodynamic particle size (nm) and polydispersity index (PDI) of designed NPs, dynamic light scattering (DLS) system (Zetasizer Nano ZS; Malvern Instrument) possessing a helium-neon laser beam at 633 nm wavelength. Furthermore, FE-SEM (Hitachi Ltd., Japan) was exploited to investigate the surface morphological features and Shape of prepared NPs. Also, to further evaluate MET's successful loading on prepared NPs, free MET and NPs, FTIR spectroscopy was utilized to investigate the chemical configuration of the samples in the range of 400–4000 cm^{-1}.

In vitro release study

The MET release pattern from PLGA NPs was obtained through immersing 80 mg of NPs into 10 mL of PBS (pH 7.4, 37 °C) under shaking at 200 rpm. At certain time intervals, 1 mL of incubation solution was transferred for UV-Vis spectroscopy absorbance measurement at 234 nm and replaced with 1 mL of fresh PBS. The cumulative drug release data were plotted as a function of time:

Cumulative amount of release (%) $= C_t / (C_\infty \times 100)$

where C_t is the quantity of MET discharged at time t and C_∞ refers to total quantity of MET loaded in 100 mg of NPs

Cell line maintenance

MDA-MB- 231 breast cancer cell line was gifted from researcher at our university. MDA-MB- 231 cell were grown in RMPI-640 supplemented with 5% FBS (v/v) and 1% pen/strep (v/v). The medium was replaced every other to remove floating debris and the flasks were fed with fresh medium

In vitro cytotoxicity

To evaluate the effect of synthesized NPs in increasing the cytotoxicity of MET on MDA-MB- 231, MTT assay was done. First, MDA-MB- 231, at a density of 8,000 cells per well, were seeded into 96-well plates and incubated for 24 h to become confluent. Then, the cells were treated with various concentrations of MET and MET-NPs and incubated for 24, 48, and 72 h. Then, the medium was aspirated and 50 µL MTT solution (2 mg/ml, Sigma Aldrich) was added to each well. Next, MTT solution was discarded and replaced with DMSO to dissolve the formazan crystals. The absorbance of solubilized formazan in 96-well plates was measured at a wavelength of 490 nm using an ELISA plate reader (Dynex MRX).

Real-time PCR assay

According to the manufacturer instructions, after exposure of A549 cells with different concentrations of the NPs for 48 h, the total RNA was isolated using TRIzol reagent (Invitrogen, USA). Then, a Nanodrop was applied to determine the purity and quantity of total RNA. RevertAid™ First Strand cDNA synthesis kit (Thermo Fisher Scientific, MA, USA) was used for synthesizing cDNA from 1 µg RNA. Next, the quantitative PCR (qPCR) was carried out in a Mic qPCR Cycler (BioMolecular Systems, Australia) by applying SYBR Green Premix PCR Master Mix (Roche, Mannheim, Germany), specific primers, and cDNA. The housekeeping GAPDH gene was applied as the internal control, and the quantification of the samples was analyzed using the $2^{-\Delta\Delta ct}$ method**.**

Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed using chi-square to determine the statistical significance between the two means evaluated at p<0.05. All experiments were replicated at least three times. Analyses were conducted in GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA).

Results

Characterization of drug-loaded NPs

The O/W single-emulsion solvent-evaporation process was successfully used for the formulation of the MET-PLGA NPs. The particle size distribution of NPs was assessed by a dynamic light scattering (DLS) method. According to the Table 1,

the typical particles size, polydispersity, and zeta potential of NPs was 150±1.17 nm, 0.175, and zeta potential of -7.3±0.27 mV, respectively. Drug encapsulated NPs presented larger diameter and a smaller polydispersity than blank NPs. This may include drugs that have amphiphilic copolymers that are completely bound.

Table 1. Mean (±SD) particle diameter, Polydispersity (±SD) and zeta potential of drug loaded PLGA-PEG NPs.

Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)
PLGA NPs	150±1.17	0.175	-7.33±0.27
MET-loaded PLGA/ PEG NPs	190±5.56	0.166	-6.2±3.1

The morphological characterization of MET NPs was studied by SEM. The images of the drug-loaded NPs revealed their regular spherical shape (Figure 1). The surface morphology of NPs was smooth with a regular spherical shape. The size distribution of NPs was narrow with a mean particle diameter ~ 80 nm. The ranges of drug encapsulation efficiencies (EE) were about 76.8 % with a loading capacity of 12.3 ± 2.2, respectively.
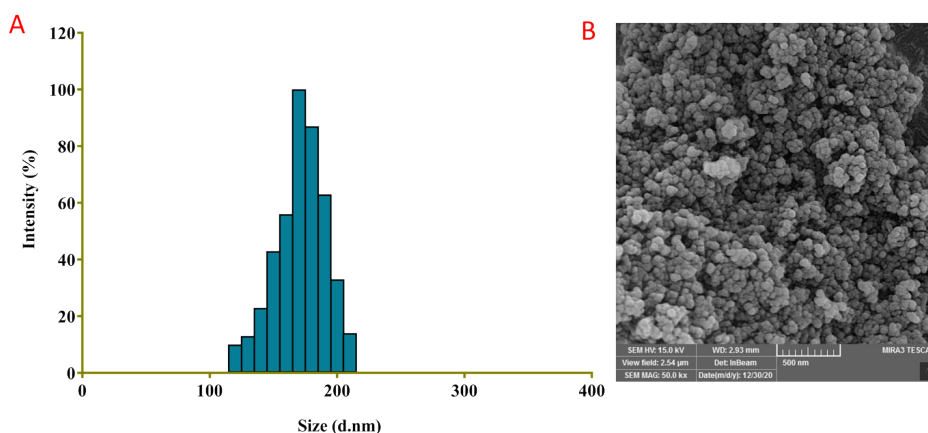

Figure 1. *A DLS histogram showing the size distribution of MET NPs. The average size ranged from 130–200 nm. B Field emission scanning electron microscopy (FE-SEM) image of surface morphology of MET-Cur NPs*

FTIR was applied to describe the functional groups present in pour and drugs loaded NPs (Fig. 2). The FTIR spectrum of pure MET showed two typical peaks at 3369 cm^{-1} and 3294 cm^{-1} relatives to the N–H primary stretching

vibrational mode and a peak at 3155 cm^{-1} because of the N–H secondary stretching, and characteristic peaks at 1626 cm-1 and 1567 cm^{-1}, attributed to C-N stretching. The physical mixture spectrum can be considered as the sum of pure MET and PLGA spectra. The spectrum of MET-loaded NPs shows the typical bands at 3300, 3200, 1625, 1583 cm^{-1} which are assigned to N-H asymmetric stretching, N-H symmetric stretching, C=N stretching and N-H bending groups. The characteristic spectral peaks corresponding to MET functional groups were present in nano-formulations. Also, all the characteristic absorption bands of MET functional groups in the drug loaded polymer, indicating the MET encapsulation into NPs
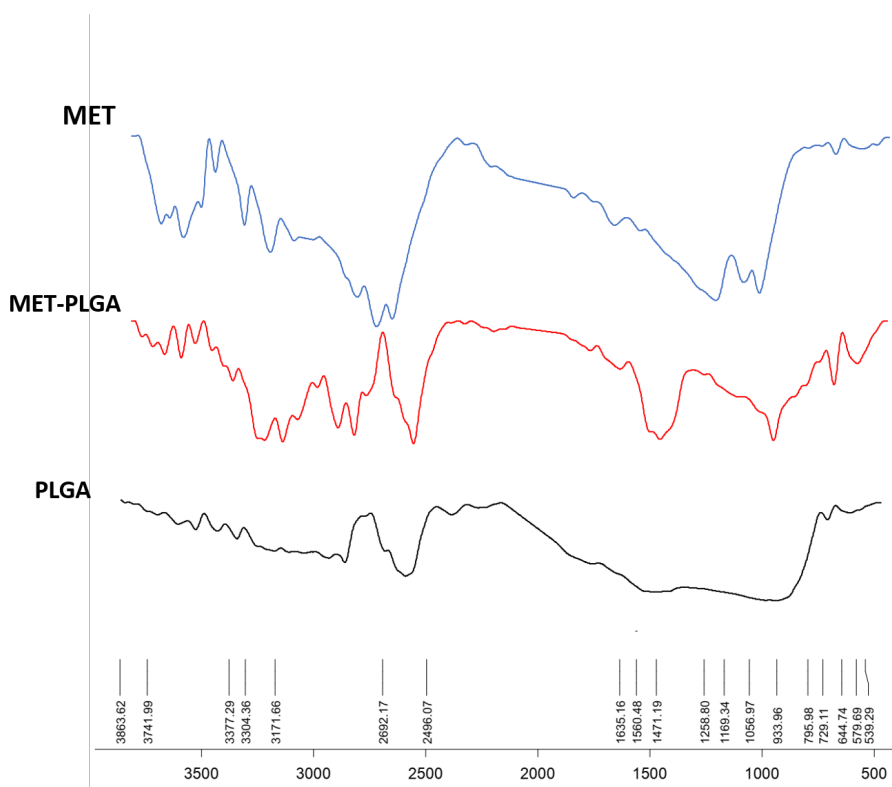

Figure 2. *Infrared spectra of MET, PLGA, MET NPs.*

Drug release

Cumulative MET release from MET-NPs was assessed for 140 h at 37 °C. As shown in Fig. 3, MET-NPs exhibited an initial rapid drug discharge pursued by slower constant release rates, which was consistent with the previously reported release kinetics of MET from PLGA NPs. In the first 48 and 72 h, roughly 55.3% and 72.4% of MET were released from the PLGA NPs, respectively.
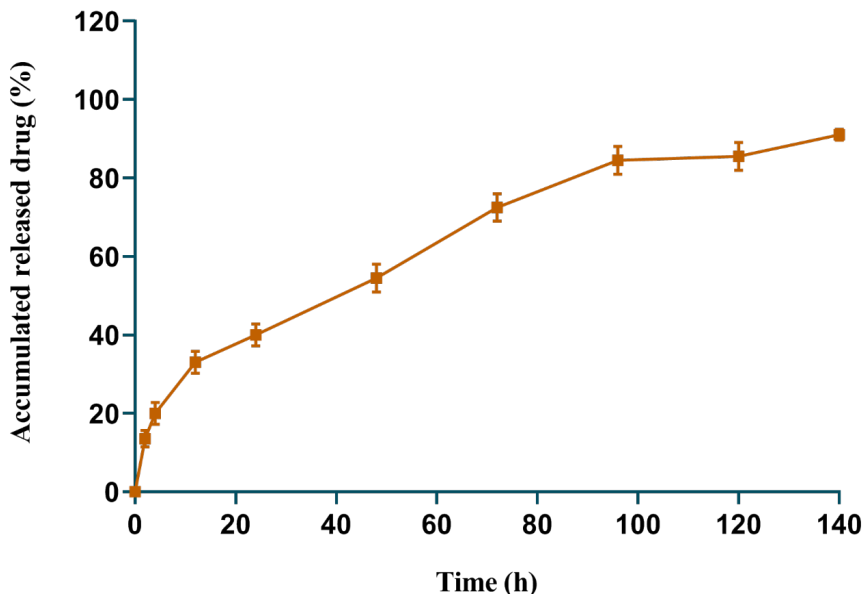
Figure 3. *Cumulative release (%) behavior of MET from NPs in phosphate buffered saline (pH 7.4).*

Cell cytotoxicity and synergistic analysis

To evaluate the growth inhibition effect of MET and MET-PLGA NPs, MTT assay was applied using different doses (0-30 mM) of free MET and MET-PLGANPs on MDA-MB-231 breast cancer cells during 24, 48, and 72 h (Fig. 4 and Fig. 5). Cells without treatment were used as control groups.
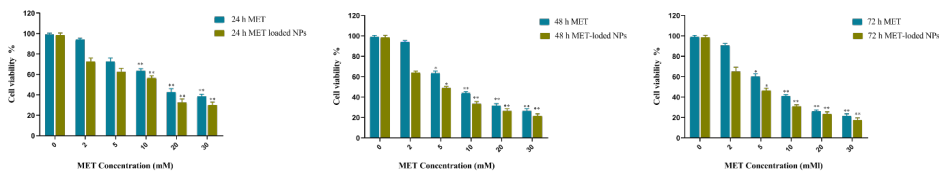

Figure 4. *The in vitro cytotoxicity by MTT assay. The viability of MDA-MB-231 cells receiving various treatments of free MET and MET–NPs, Cur–NPs. Error bars indicate standard deviations. (* p value < 0.05, ** p value < 0.001).*
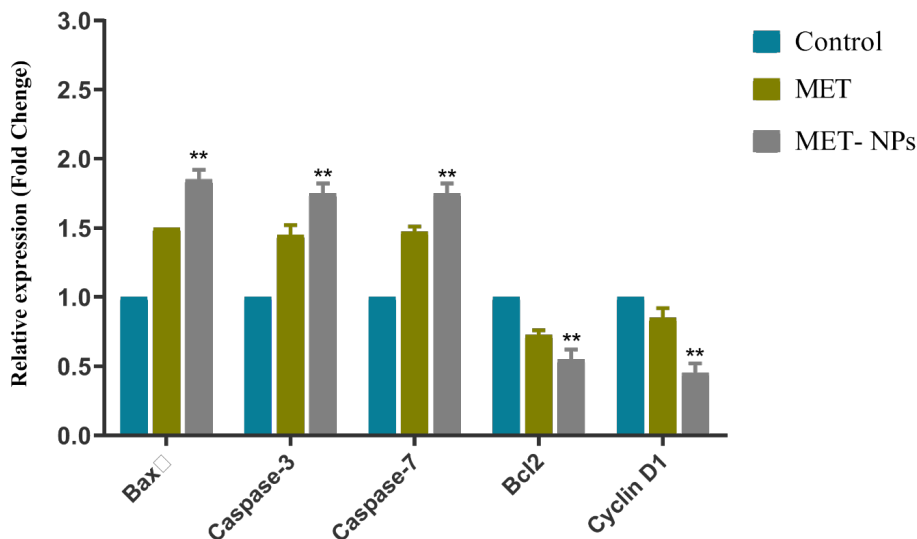
Figure 5. *Relative mRNA expression levels of CyclinD1, P53 and apoptosis related genes in MDA-MB-231 cancer cell line treated with by free and nano-formulated form of MET. *p<0.05, **p<0.01 and ***p<0.001 vs. control was considered significant.*

Gene expression findings

The expression of apoptosis related genes including caspase-3 caspase-7, bcl-2, Bax, Cyclin D1 in MDA-MB-231 cancer cell lines after exposure to MET and MET-PLGA NPs for 48 of incubation was determined by real-time PCR. GAPDH region normalized and calculated by the $2^{-\Delta\Delta ct}$ method to assess the alterations in expression levels of apoptosis related genes between the control and treated MDA-MB-231cancer. Our results showed that MET in free and nano-capsulated forms altered the expression levels of mentioned genes more than free MET and untreated cells. Moreover, our results displayed, caspase-7, caspase-3, and Bax (apoptosis markers) mRNA levels were meaningfully increased in the cells treated with nano-formulated forms of MET compared to control.

Discussion

As previously described, release of drug from nanoparticles indicates the combined effects of swelling, matrix erosion, degradation processes and in situ cross-linking (14). The sustained and sequential release pattern of chemotherapeutic agents could be valuable for inducing apoptosis and attaining an effective approach in cancer treatment (15).

As revealed in Fig. 4 in both free and nano forms of MET showed a concentration-dependent growth inhibitory effect on MDA-MB-231 cells. Remarkably, MET-loaded NPs exploited so much higher cytotoxicity relative to the free form of MET against the MDA-MB-231 cells. Improved growth inhibitory activity of drugs in nano-capsulated form might be distributed to the controlled release of drugs from the PLGA NPs and several procedures that included in the high cellular uptake of encapsulated drugs and as a result, leads to the effective discharge of drugs from the PLGA NPs into the cancer cells cytosol.

In one study, the effect of MET- NPs showed a greater inhibitory effect than pure MET by reducing the expression of the hTERT gene in T47D and MDA-MB-231 cell lines in *in vitro* [16]. Liou and co-colleges showed that MET and SIL in combination form prevent the survival of human cervical cancer cells (17).

Bcl-2 is a member of a substantial family of apoptosis regulatory proteins, which regulate apoptosis by either inducing proapoptotic proteins or inhibiting anti-apoptotic proteins(18). Caspases as a unique family of cysteine proteases are the executive proteins. Caspase-3 and caspase-9 are a family of protease enzymes that have a key role in both intrinsic and extrinsic apoptotic pathways (19). The p53 is a tumor suppressor encoded by the gene TP53, and has properties of a transcriptional activator, and it seems to act a role in the regulatory control of normal cellular proliferation. It is also noteworthy that, p53 contributes directly in the intrinsic path of apoptosis by interacting with the Bcl-2 family that prompt mitochondrial outer membrane permeabilization (20). These observations led to the suggestion that P53/BCL2/BAX apoptosis signaling pathway plays an essential role as a coordinator of apoptosis.

Various studies in consistent with our study have shown that   free MET and MET-NPs as well as in combined with other chemotherapeutic molecules can alter the expression levels of apoptosis genes (16, 21). In the preliminary study conducted in our group, it was found that MET in combination with SIL exhibits synergistic antiproliferative effects via down-regulating Cyclin D1 and hTERT (16). Moreover, in another study conducted by our group, it was shown that MET loaded into polymeric PLGA-PEG NPs may be a convenient drug delivery system to enhance its anticancer effects for ovarian cancer therapy(9).

In the present study, we proved that delivery of MET in nanoparticles, which are identified as appropriate delivery system for in situ drug delivery and formulation of anticancer drug with low solubility in water leads to strong anti-proliferative effect on lung carcinoma cells.

Conclusions

In this study, we had successfully fabricated a PLGA-PEG NPs as vehicle to nanocarrier structure to deliver of MET into breast cancer cells for natural based compound chemotherapy. According to the achieved results, MET loaded PLGA-PEG NPs where be able of killing MDA-MB-231 breast cancer cells much more than free drug, which is attributing to the sustained and controlled release. Furthermore, MET strongly prevent proliferation of cell and induces apoptosis via reducing of cyclin D1 and bcl-2 mRNA expression levels. In addition, MET increases the RNA transcription level of caspase-3, caspase-7 and Bax as well as p53, resulting in growth inhibition and apoptosis induction. This preliminary study showed that the loading of the chemotherapeutic molecules into PLGA might lead to developing novel and safe drug nano-delivery systems to treat breast cancer effectively.

Acknowledgements

Declaration of Competing Interest

The authors declare that they have no competing interests.

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