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Research Article

Metformin-Loaded Microsphere Promotes an Anti-tumour Response through the Caspase-Mediated Apoptotic Pathway Against MDA-MB-231 Cells

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ABSTRACT

Metformin has been reported to have antidiabetic effects. Metformin also has anti-inflammatory and antibacterial activity. However, the target specificity remains the major problem regarding this. Therefore, as it lacks target specificity, nanoformulation or microsphere formulation of this compound might have a good chance of overcoming this problem. Thus, to overcome this problem, we have made a metformin-loaded microsphere that shows target-specific activity. Furthermore, the microsphere-loaded metformin was administered to the EAC cell, MDA-MB-231. Results showed that the formulated drug was more potent against the MDA-MB-231 cell line. It shows dose-dependent inhibition in cell growth. For further evaluation, we investigated this matter and found that the drug-induced cytotoxicity is ROS-dependent and may occur through alterations in mitochondrial membrane potential. Additionally, this causes apoptotic marker proteins, such as cleaved PARP and cleaved caspase-3, to increase in expression. Overall, our data indicated that the formulated metformin was more effective than the free form in reducing cancer growth and enhancing survivability. Thus, the findings suggest that the formulated drug may be effective in reducing cancer growth and improving survivability.

Introduction

One of the most often given and advised drugs for the treatment and long-term control of type 2 diabetes (T2D) is metformin, as is well known. Due to its ability to effectively lower blood glucose levels, its good safety record, affordability, and additional advantages, including promoting modest weight loss and enhancing insulin sensitivity, it is frequently regarded as the first-line pharmaceutical therapy. It is thought to have an antidiabetic effect by inhibiting hepatic gluconeogenesis, which may be linked to an increase in skeletal muscle glucose absorption mediated by insulin.[1] Experimental research and epidemiological data have recently demonstrated the antitumor impact of metformin against several cancer forms, including melanoma.[2,3] Nevertheless, little is known about the precise mechanisms by which metformin slows the growth of tumours. It can be challenging to assess metformin's anti-proliferative effects *in-vitro* in patients, particularly when it only exhibits efficacy at supraphysiological concentrations *in-vivo*. Metformin's systemic effects on carcinogenesis are linked to a reduction in hyperinsulinemia, which is linked to a poor prognosis for several cancer types, including prostate, colon, and breast cancer. Further research has demonstrated that metformin can directly affect cancer cells, primarily through independent and AMP-activated kinase (AMPK)-dependent pathways.[4] Furthermore, metformin has

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been shown to impact the immune system in both healthy individuals and conditions, including cancer, TB, and autoimmune diseases. Additionally, some research has shown that metformin influences T-effector cell subsets and stimulates memory T-cell production through the AMPK pathway.[5] Metformin can alter the release of multiple cytokines, including interleukin (IL)-10, IL-17, IFN-γ, IL-22, and IL-6, and it has an impact on lymphocytes, neutrophils, macrophages, and other immune cells.[6] The inactivation of the mammalian target of rapamycin (mTOR), primarily due to the activation of adenosine monophosphate-activated protein kinase (AMPK), alterations in intracellular reactive oxygen species (ROS) levels, the inhibition of mitochondrial functions, or other mechanisms linked to the drug[7,8] may be the mechanism through which metformin exerts its inhibitory effect.

The unique energy metabolism of cancer cells distinguishes them from normal cells in both function and behaviour. It draws attention to the way cancer cells rewire their energy-producing systems to enable quick development, multiplication, and survival in frequently unfriendly microenvironments [9]. Consequently, tumour cells use more glucose than healthy cells. To compensate for mitochondrial respiratory failure in tumour cells, increased anaerobic glycolysis is used [10]. Since many tumour cells maintain both normal mitochondrial structure and normal respiratory activity, it remains debatable whether the failure of mitochondrial respiration is a characteristic shared by all tumour cells. Notwithstanding the disagreement, the failure of several sophisticated or conventional anticancer treatments has increased interest in the Warburg effect.

In this regard, recent research has focused more on how metformin's metabolic interference contributes to its anticancer effects [11-14]. Conversely, metformin generally has comparatively little action specific to tumour cells [15]. The drawback of metformin also lies in its poor solubility. Regarding this, the usage of microspheres may be a procedure that researchers are investigating to improve cellular availability in a target-specific manner. Certain dose forms, known as microspheres, can deliver medication to specific locations within the gastrointestinal system. Microspheres may play a significant role in providing a sustained influence on drug response at the target cells, which further sustains dose-dependent drug release. The addition of the metformin microsphere to the dose form can help prolong anticancer chemotherapy. [16-19] Formulations in microspheres offer a longer period of medication effect with less discomfort to the stomach. The drug is embedded in a matrix composed of sodium alginate and guar gum in microsphere preparations, allowing for continuous, gradual release over an extended period [12-14]. Together with sodium alginate, guar gum was used as a matrix-building substance to provide a controlled and prolonged release over an extended duration. In this investigation, an optimal concentration of each component was used to construct metformin microspheres. Microspheres are therapeutically adjusted dosage forms because they consistently distribute the drug to the target region over an extended period. The drug is progressively freed from its matrix embedding over time. In this work, we investigate the novel application of metformin microspheres and their distinct effects on cancer cell lines. In particular, we assessed its possible differential effect on Ehrlich Ascites Carcinoma (EAC) cells and MDA-MB-231, a highly aggressive triple-negative breast cancer cell line.

Two primary features of these findings are novel: (1) the creative use of metformin delivery via microspheres to enhance anticancer efficacy, and (2) the identification of the apoptotic mechanism as a crucial pathway of metformin-induced cell death in triple-negative breast cancer. These findings may lead to new approaches for the more effective and tumour-selective construction of targeted treatment plans with metformin. Overall, the pharmaceutical advantage of using metformin in a microsphere-loaded form lies in its target-specific anticancer potential against aggressive tumor cells. In a microsphere form, safety, affordability, and compatibility with existing cancer therapies make it a strong candidate for repurposing in oncology, particularly to enhance treatment outcomes.

Materials and Methods

Chemicals

We purchased JC-1, DCFDA, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (USA). We purchased primary antibodies containing fluorescence-tagged cleaved PARP and cleaved caspase-3 from Santa Cruz, CA, USA. Penicillin, streptomycin, neomycin (PSN), fetal bovine serum (FBS), trypsin, ethylene diamine tetra-acetic acid (EDTA), Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute Medium-1640 (RPMI-1640) were acquired from HIMEDIA (India). Analytical-grade compounds were also used.

Cell Lines and Cell Culture

The aggressive breast cancer cell line MDA-MB-231 and Ehrlich ascites carcinoma (EAC) cells were obtained from the NCCS in Pune. Peripheral blood mononuclear cells (PBMCs) were separated and used as a normal cell control. In a humid environment (5% CO2; 95% air), each cell was incubated separately at 37°C using DMEM or RPMI-1640 cell culture medium with 5% fetal bovine serum (FBS) and 100 µg/mL of streptomycin and penicillin. The cells were collected following a brief incubation in 0.02% (w/v) EDTA in PBS. The cells were routinely maintained as subcultures in tissue culture flasks.

Preparation of the Drug Solution

The solutions of free metformin and metformin-loaded microspheres were prepared by dissolving the test compounds in sterile phosphate-buffered saline (PBS, pH 6.8).

Characterisation of Microsphere

Morphological analysis using scanning electron microscopy (SEM)

The texture of microspheres was discovered using a SEM (CARL ZEISS EVO 18 special edition equipment). Additionally, the cross-sectioned surface of the treated microsphere was analyzed for morphology. The platinum coating was subjected to an SEM analysis using QUORUM Q150 TES.

XRD analysis provides information regarding whether the drug integrated into the final microspheres is crystalline or amorphous. With a copper target slide (10 mm) and a 5 mg sample on the holder, an X-ray diffractometer of model Ultima-111, manufactured by Rigaku (Japan), was used to perform an XRD analysis. The samples' diffractogram was read between 2θ = 1 and 100° at a scanning rate of 5°/min. To investigate any potential chemical interactions between the medication molecule and other components used in the microsphere formulation, FTIR analysis was performed.

Estimation of drug entrapment efficiency (EE)

Using spectrophotometry, the percentage of EE (%EE) in the microspheres was determined. To completely dissolve the polymer in DCM, 10 mg of drug-loaded core-shell microspheres were broken up, ground into powder, and dissolved in 5 mL of DCM. The mixture was then agitated for 10 minutes using a magnetic stirrer. Furthermore, 10 mL of methanol was added to the resulting solution, which was then magnetically stirred for two minutes at 40 to 45°C before being filtered. A double-beam UV-visible spectrophotometer (UV1, Thermo Scientific, Great Britain) was used to measure the absorbance of the final solution at 270 nm, with methanol as the blank.

The % EE was calculated by applying the following equations:

$$Percentage\ entrapment\ efficiency = \frac{Wt-Wf}{Wt} \times 100$$

Here, Wt is the total initial amount of 5-FU incorporated during formulation development, and Wf is the amount of free drug in the solution after formulating the drug-entrapped microspheres

In-vitro Cell cytotoxicity assay of metformin-loaded microsphere

The cytotoxicity of free metformin, metformin-loaded microspheres, and blank microspheres was assessed using the MTT test, which measures the activity of the mitochondrial succinic dehydrogenase enzyme that converts MTT, a tetrazolium salt, into formazan crystals. The studies were conducted using 96-well flat-bottomed culture plates (BD Biosciences, USA). MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/mL. The plate was then incubated for 24 hours after various doses of the test sample (0.5, 1.0, 5.0, 10, 20, 10 µg/mL) were added. After that, each well received 20 µL of the MTT solution, which was then incubated for four hours at 37 °C. After removing the culture medium, 200 µL of DMSO was used to dissolve the formazan crystals. A microplate reader was used to measure the absorbance of the formazan dye at 570 nm. The cells treated with a blank microsphere and the cells without treatment (MTT group) served as the negative controls. This work evaluated the cytotoxicity of metformin-loaded microspheres in comparison to free metformin, a common anticancer medication, without the use of a positive control. The quantity of live cells is comparable to the amount of formazan generated. Cell viability was calculated using the following equation:

$$\text{Cell viability\%} = \text{Absa/Absc} \times 100$$

Where Absa is the absorbance of the sample treated with metformin (free or microencapsulated), and Absc is the absorbance of the control group cell.

In-vitro detection of intracellular ROS

A fluorimetric study was used to perform the DCF-DA experiment according to the published procedure. On 12-well plates, 5 × 104 MDA-MB-231 cells were seeded in each well. At the same time, the cells were treated with the indicated concentrations of metformin-loaded microspheres, blank microspheres, and metformin medication. Cells were collected after 24 hours, centrifuged, washed in PBS, and loaded with 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) for 10 minutes at 37°C. A fluorimeter was then used to analyse fluorescence.

In-vitro flurimetric analysis of mitochondrial membrane potential (Ψm)

To detect the mitochondrial membrane potential (Ψm), JC-1 (5,5′-6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide) was employed as a fluorescence probe and examined using a fluorimeter. Briefly, MDA-MB-231 cells were seeded in 12-well plates at a density of 5 × 10^4 cells per well. After 24 hours of incubation, blank microspheres, metformin medication, and metformin-loaded microspheres were added to each well. The cells were then cultured for an additional 24 hours at 37°C in a 5% CO_2 environment. PBS was sprayed over the cells, following the manufacturer's instructions. Finally, fluorimetry was used to evaluate the material.

In-vitro cleaved Caspase 3 expression analysis

MDA-MB-231 cells were seeded at a density of 50,000 cells per well in a 12-well plate and allowed to adhere for

24 hours. Following adhesion, the cells were treated with either metformin or metformin-loaded microspheres. After treatment, the cells were incubated with a fluorescently labeled antibody against cleaved caspase-3, following the manufacturer's instructions. The fluorescence intensity was then measured using a fluorimeter to assess caspase-3 expression.

In-vitro cleaved PARP expression analysis

MDA-MB-231 cells were seeded at a density of 50,000 cells per well in a 12-well plate and allowed to adhere for 24 hours. Following adhesion, the cells were treated with either metformin or metformin-loaded microspheres. After treatment, the cells were incubated with a fluorescently labeled cleaved anti-cleaved PARP antibody, following the manufacturer's instructions. The fluorescence intensity was then measured using a fluorimeter to assess caspase-3 expression.

Results and Discussion

Formulation Development

Solvent extraction or evaporation processes, which create the desired formulation with a modified drug release profile, are the most widely accepted tradition for the manufacture of microspheres. However, the emulsification of a drug and a polymer-solubilised organic solution in a continuous aqueous phase is the only way that traditional solvent extraction or evaporation procedures can incorporate lipid-soluble substances. Water-miscible pharmaceuticals are therefore incorporated using modified procedures, such as creating several emulsions (W1/O/W2) (Fig. 1A) or including them in the aqueous phase as a saturated solution [20, 21, 22]. This contemporary W1/O/W2 solvent evaporation method for microsphere formation heavily relies on implied processing parameters. Therefore, the systemic investigation of the parameters that directly affect the properties of the microspheres (Fig. 1B) was the main emphasis of our current work.[20]

Preparing a polymeric microsphere with a high drug EE and predefined drug release kinetics was the aim of the formulation development project. Metformin-loaded microspheres were created during formulation development using varying concentrations of Guar (20, 30, 40, and 50 mg) to examine the percentage of entrapped medication (EE), surface shape, and release behavior of the entrapped medication at pH 1.2 and pH 6.8. Due to the varying guar concentrations, the microsphere's %EE varied considerably, ranging from 59.45 ± 3.18% to 79.25 ± 4.25%. Under scanning electron microscopy, however, the microsphere's surface appeared smoother and more spherical as the gum concentration increased (diameter 314.45 ± 7.80 with 50 mg Guar). However, up to a certain point (50 mg), the increase in %EE indicated the growth in Guar within the inner phase; after that, it decreased. In contrast to the low viscosity of the W1 phase, which inhibits the leaching of the drug from the inner phase to the external phase, the higher viscosity of the W1 phase causes the development of a homogeneous emulsion with many internal droplets in the W1/O emulsion, which encourages the leakage of inner core materials to the external aqueous phase [20].

The microspheres that were produced without surfactant in the oil phase became elongated, while those that were formed with surfactant at the intermediate concentration were spherical and individual (Fig. 1B). They lost their individuality and became aggregates as the Span 80 concentration increased. The microspheres exhibited a monomodal size distribution at a concentration of 1% (w/v) of Span 80. The microsphere had a mean diameter of 260.35 ± 6.50 µm using Span 80 (1%, w/v). The microspheres' %EE was higher at the middle emulsifier concentration than it was at other values. The tension-active properties of the Span 80 stabilized the remaining emulsion. They inhibited the rapid coalescence of the dispersed droplets, which explains why the %EE and particle size distribution of the formulated microparticles are strongly connected with the stability of the primary emulsion.

It was also examined how agitation affected the final emulsification process used to create drug-loaded microspheres. Various stirring rates of 700, 780, and 900 rpm were maintained while the microsphere formulations were being prepared. The average particle size, %EE, and shape of the microspheres all changed significantly when the stirring speed was increased from 700 to 900 rpm. As the emulsion's tension increases due to a mechanical stirrer's increased speed, the emulsion's droplets split apart, leading to the production of tiny particles. A smooth and spherical microsphere was created with a low stirrer speed (Figs 1A and B) [16, 20]. Microspheres with good drug release behavior, size, and form were created at an 800-rpm stirring speed (Fig. 1C) [20].

Here, the mean diameter of the microsite increased negligibly due to a rise in the volume of the aqueous phase. It was observed that the percentage of EE was higher in 5 mL of the aqueous phase than in 7 mL. It becomes evident that a larger concentration of gum, which eventually forms a viscous inner layer, is reflected in the inner phase's (W1) lower volume. As a result, it might create a rather thick layer on the organic phase (O), which could reduce drug release (pH-4.0) by acting as a barrier to the entrapped drug's diffusion to the exterior acidic aqueous phase. Additionally, compared to microspheres produced with a greater internal phase volume (7 mL), those generated with a lower internal phase volume (4 mL) were found to deliver the encapsulated medication in the dissolving media more slowly (Fig. 1C).[20]

DCM increases the organic phase's viscosity and tends to prevent the medication from migrating from the inner

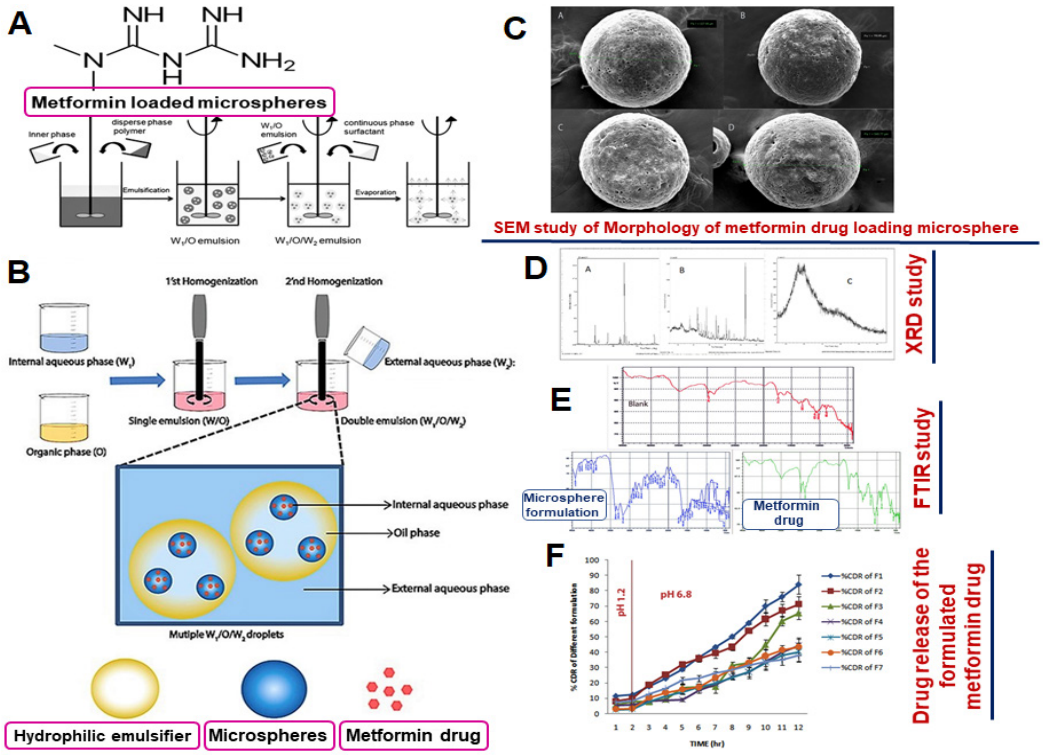

Fig. 1: Physicochemical characterisation of Metformin-loaded microsphere. (A) (B) W1/O/W2 emulsion technique of metformin loaded microsphere preparation, (C) SEM study of the morphology of metformin drug loaded microsphere, (D) XRD study of metformin loaded microsphere, (E) FTIR spectrum of drug-loaded microsphere preparation, (F) In-vitro release profile of the optimized formulated metformin loaded microsphere[16,20]

phase to the outer aqueous phase. When compared to microspheres made at 28 °C, the drug entrapment effectiveness of metformin-loaded microspheres at 38 °C was greater. One may argue that a higher rate of organic solvent evaporation at high temperatures caused the polymer to solidify quickly and resulted in a high percentage of EE. The rapid phase separation and evaporation of DCM during the shrinking and hardening step caused the microspheres to harden quickly and the surface to become rough at a high temperature (38°C) (Fig. 1A).[16,20] With the villi in the colon wall, the microsphere's rough surface helps to improve the dosage form's mucoadhesive properties, creating an ideal setting for prolonged drug release.

The diameter of the microsphere was found to be 258.37 ± 5.22 µm in the final formulation with the optimum parameters based on results from process variable patterns. Ultimately, within the parameters of our study, the optimal Guar microsphere formulation yielded a percentage of 91.73 ± 2.15%, a cumulative drug release of 82.63 ± 3.15% at 12 hours, and a drug entrapment efficiency of 76.53 ± 1.79% [16,20].

An interaction between the API and natural polymer was investigated by comparing the FTIR spectra of enhanced formulations with the spectra of pure medication and polymer. No such interaction was observed when the metformin-loaded and blank microspheres' FTIR spectra were displayed on -NH, -OH, and C=O (Fig. 1E).[16,20]

The crystalline nature of the pure medication is shown by its XRD pattern. According to powder crystalline theory, crystalline materials can have a distinct peak structure due to the way molecules are arranged in their inner framework. Atoms and ions form a stable network in three dimensions, which can produce a regular diffractogram from the crystalline structure with sharp peaks (Fig. 1D).[16,20] This is a perfect example of a microsphere process development method that uses the multiple emulsion solvent evaporation technique to describe drug release. One crucial factor in the creation of microspheres is the release of medications. One fundamental requirement for creating a formulation that adheres to controlled release medication delivery is the continuous release of the drug from the microsphere matrix. To trap the drug inside the microsphere, a matrix including the gum and the solvent can be prepared using multiple emulsion solvent evaporation (W1/O/W2) procedures. This is the main prerequisite for encapsulating the medication within the matrix. To meet the need for a sustained action dosage form, the matrix releases the medication gradually and slowly.

To formulate the W1/O/W2 emulsion, a primary emulsion must be prepared. This emulsion must meet the requirements for preparing a W1/O primary emulsion type because it must contain the water phase (W1), which is

the formulation's matrix, and the external oil phase (O), which is simply the organic phase that contains dichloro methane (DCM) as the organic solvent. The preparation of the microsphere requires the use of this specific solvent system. To stabilize the formulation with span-80 in the organic phase of the W1/O emulsion, the polymer ethyl cellulose was added to the W1/O main emulsion while it was in the organic phase. Since it will be mostly in charge of keeping the matrix in the microsphere, this primary emulsion serves as the foundation for the W1/O/W2 multiple emulsion. Different amounts of tween80 stabilized the water phase, from which the solvent was removed. Changes in these characteristics can alter how the microsphere is shown, changing its appearance significantly in scanning electron microscopy (SEM). As the factors alter to represent the visual changes in the microspheres (Fig. 1C), different images of the microspheres in the SEM studies are highly relevant [16,20]. Studies using FTIR and XRD further corroborate the microsphere's other characteristics, such as the absence of chemical interaction between its constituents or the polymorphic forms.

In the preparation of microspheres concentration of gum is an important criterion. In the present study, we have used guar gum to build up the matrix of the microsphere. We have seen the variation and texture of the microsphere with the increasing concentration of guar gum. As guar gum is a big bulk molecule produces a big viscous preparation while stirring in water. This produced a variation in drug entrapment. It has been observed that with the increasing concentration of guar gum, drug entrapment also increased to 66.78 ± 2.1 percent which established the fact that with the increase in guar gum in the internal phase the drug entrapment was also increased on the other hand particle size also increases gradually with the increase of guar gum concentration and it goes up to 540.42 ± 2.55 µm where the mean particle size can get for the optimized formulation f2 was 392.08 ± 1.96 µm the surface of the microsphere was smooth and clean with a fantastic round shape of the sphere. The release of the drug varies probably because, although initially, it exhibits higher release, the increasing concentration of the gum builds the viscosity in the internal aqueous phase, and leaching of the drug from the internal phase to the external phase. The mean release of the drug becomes 79.65 ± 2.16%, but ultimately, the big, viscous molecule in the internal aqueous phase hinders the release of the drug with increasing concentration of guar gum, establishing the peculiar nature of the guar gum.

Thereafter, tween 80 we used in the aqueous phase to establish the formulation. It was seen that tween 80 at 0.2 % produced maximum drug entrapment at 51.18 ± 2.52%. The solvent evaporated from the external phase helped the microsphere to become a consolidated shape, and the optimum size became 391.18 ± 1.52 (µm). The release of the drug also produced a protocol by releasing a mean of 72.76 ± 1.81 % as the evaporation of the solvent provided a stabilized formation.

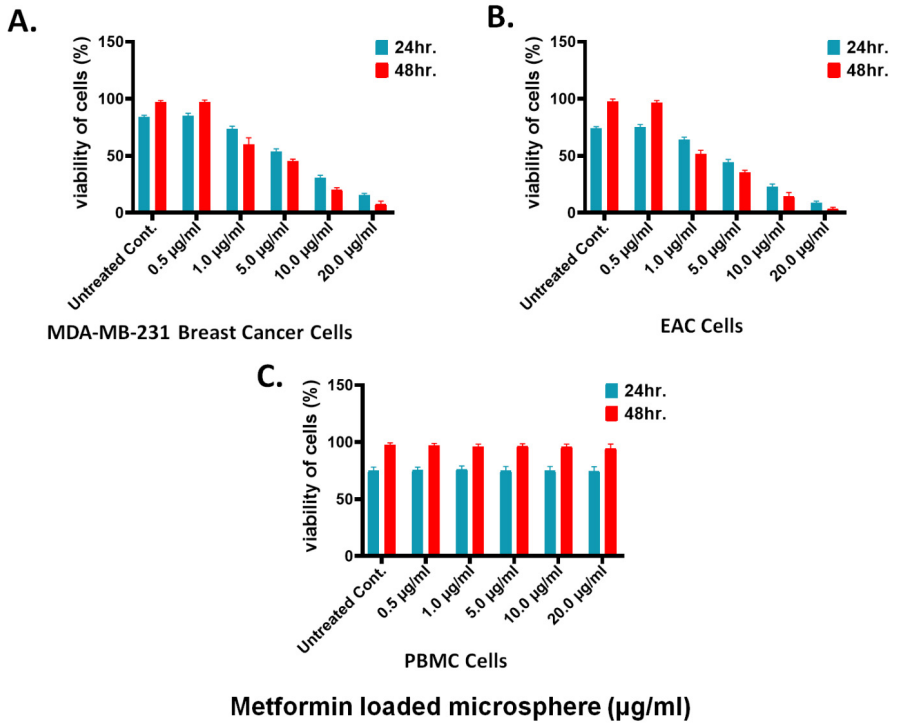

Fig. 2: MDA-MB-231 breast cancer cells, EAC tumour cells (A), and PBMC (B) cells were exposed to various concentrations of metformin-loaded microspheres at various doses, and cell proliferation assays were performed. Data are exposed as mean ± SEM, *n* 3. MDA-MB-231: epithelial human breast cancer cell; EAC: Ehrlich Ascites Carcinoma; PBMC: Peripheral Blood Mononuclear Cell

The processing temperature is an important parameter as it helps the formulation to be stabilized with the increase in temperature, showing a mean temperature to solidify from the external aqueous phase with stirring. Here we found an optimum temperature of 38ºc to provide drug entrapment of 50.58 ± 1.5% and a mean particle size of 390.18 ± 1.41 µm, showing a release of 73.45 ± 3.12%. It was found that with more increase in this temperature, the rate of solidification may be higher, but the particle size was also higher, 523.25 ± 1.8 µm. The surface of the microsphere becomes smooth and spherical with a fine shape at a mean temperature of 38ºC

The primary emulsion was made in the homogenizer at varying speeds. Primary emulsion (W1/O) is the basis for preparing the (W1/O/W2) emulsion microsphere system, as it stabilizes the emulsion. If the primary emulsion (W1/O) is not proper, then the final microemulsion (W1/O/W2) will not be stabilized. So, there is an optimum speed of rotation (4500 rpm/) of the homogenizer which makes the emulsion system (w1/o/w2) most stable to prepare finer microspheres with an optimum size range of 395.78 ± 2.89 µm with a smoother surface. The drug entrapment 52.93 ± 1.15 (%) and drug release 71.38 ± 1.95(%) also showed an optimum drug entrapment and release, confirming the stability of the final microemulsion (W1/O/W2) as the primary emulsion(W1/O) stabilized at 4500 rpm.

The aqueous phase volume is the primary indicator of the suspension produced in the matrix, as it contains (d.w) water as the vehicle and gum (guar gum) used as A.P.L. in the formulation. The concentration of the vehicle is optimum where it will reflect (5ml) the maximum stability of the microsphere.

The particle size is also optimum at 348.42 ± 1.85 (µm), and the drug entrapment is higher at 51.82 ± 1.8% as the matrix gets its maximum grip into the formulation with a good suspension of A.p.l. in the vehicle (d.w). So the release of the drug is also higher at 74.48 ± 2.08% as the stability of the microsphere was built up with the optimum concentration in the internal aqueous phase.

The FTIR study reports were done to determine if there was any interaction between the ingredients or not. The test provides a peak at 3172 cm^{-1}.

Other peaks were observed at 1065 and 1590 cm^{-1}, corresponding to C-N and N-H bonding in the XRD spectrum. Additionally, crystalline peaks were observed with the pure drug, whereas an amorphous peak was detected with the gum, and the microsphere was found to produce polymorphic forms.

In-vitro Study

In-vitro cell cytotoxicity assay of Metformin-loaded microspheres on various cancer cell lines

EAC cells were treated with varying concentrations of free metformin, metformin-loaded microsphere, and blank microsphere (0.5, 1.0, 5.0, 10.0, and 20.0 µg/mL) after 48 hours, and their proliferation was assessed to investigate the impact of metformin on MDA-MB231 cell proliferation. After 48 hours of exposure, microspheres loaded with metformin effectively inhibited cell proliferation; however, as compared to free metformin and blank microspheres, concentrations of 1.0–40.0 µg/mL significantly reduced cell proliferation by 10.2–84.7%, respectively. The IC_{50} values for MDA-MB-231 and EAC were found to be 11 and 10 µg/mL, respectively (Fig. 2). A microsphere loaded with metformin strongly suppressed the growth of MDA-MB-231 and EAC cells in a concentration-dependent manner (***p <0.001). Metformin-loaded microspheres, on the other hand, demonstrated no cytotoxicity or anti-proliferative effect when applied to PBMC, demonstrating their target specificity.

According to the *in-vitro* MTT data, the blank microspheres did not exhibit any discernible cytotoxicity after 48 hours, suggesting that the microspheres function as a drug carrier without causing any harmful side effects.

Detection of intracellular ROS in in-vitro MDA-MB-231 breast cancer cells:

Microspheres containing the medication metformin cause the production of reactive oxygen species. We used DCFH-DA staining, a cell-penetrating dye that fluoresces when it reacts with ROS, to investigate intracellular ROS generation in MBA-MB 231 breast cancer cells (Fig. 3A). The amount of ROS in cells was rather low under baseline circumstances. MDA-MB-231 breast cancer cells exhibited a concentration-dependent, statistically significant increase in ROS generation in response to treatment with both free metformin and encapsulated metformin (metformin-loaded microsphere). Additionally, we saw that the blank microsphere left no mark, demonstrating its inertness to cellular activity.

Fluorimetric analysis of mitochondrial membrane potential (ΔΨm) on the in-vitro MDA-MB-231 breast cancer cells

We used JC-1 staining to measure the mitochondrial membrane potential (ΔΨm) of the treated cells in order to determine whether the mitochondria were involved in the activation of the apoptotic pathway following the drug treatments. On the MDA-MB-231 cell line, there were notable differences in the relative intensity of JC-1 in untreated cells (Fig. 3B). JC-1 integration was significantly higher in MDAMB-231 cells than in metformin-loaded microspheres and free metformin (***p <0.001). In contrast, metformin-loaded microsphere treatments increased JC-1 uptake in both the free and metformin-loaded microsphere treatments. This suggests that the apoptotic pathways in this breast cancer cell line had a greater impact on the mitochondrial membrane potential.

Determination of cleaved Caspase 3 expression as apoptosis analysis in the in-vitro MDA-MB-231 breast cancer cells

One of the main mediators of apoptosis is caspase [**21**]. The

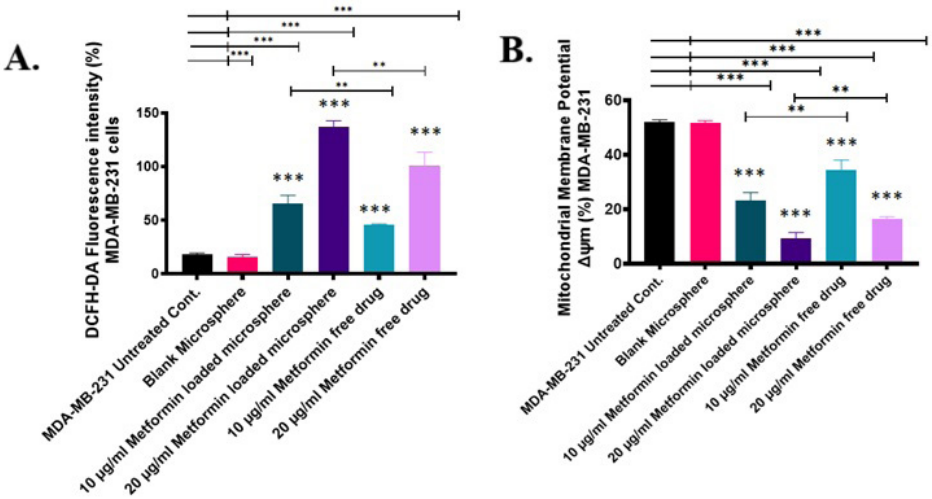

Fig 3: Metformin-loaded microspheres induced ROS generation and altered MMP. (A) *In-vitro* DCFH-DA ROS fluorescence intensity of MDA-MB-231 Breast Cancer cells on different concentrations of drug treatment. (B) *In-vitro* mitochondrial membrane potential (Δψm) assay of MDA-MB-231 breast cancer cells under different concentrations of drug. This metformin-loaded microsphere induced apoptosis through the loss of MMP potential

substrate proteins are broken down at aspartate residues by these cysteinyl aspartate proteinases. The precursor caspases undergo proteolytic processing to create an active component in response to an apoptotic signal. The primary downstream effector caspase that plays crucial functions in breaking down the bulk of important cellular components in apoptotic cells is caspase-3, one of the 11 caspases that have been identified in humans.[22] Flurimetric analysis was carried out utilizing antibodies to identify the cleaved forms of caspase 3 to determine if caspase is involved in free Metformin or Metformin-loaded microsphere-induced apoptosis. We found that the cleavage of caspase-3 increased gradually in a dose-dependent way when MBA-MB 231 breast cancer cells were treated with free metformin and encapsulated metformin (metformin-loaded microsphere) (Fig. 4A). These findings demonstrate that metformin-loaded microspheres triggered caspase-3 in a dose-dependent manner and that caspase-3 is involved in metformin-loaded microsphere-induced apoptosis. Additionally, we saw that the blank microsphere did not cause any caspase 3 cleavage, suggesting that it is a drug carrier with no apoptotic effect.

Determination of cleaved PARP expression as apoptosis analysis in the in-vitro MDA-MB-231 breast cancer cells

The type of cell death caused by metformin in MDA-MB-231 cells was identified by identifying cleaved PARP using fluorescence. It can be cleaved by caspases 3 and 7, and cleaved PARP is a hallmark of caspase-dependent apoptosis. The active form of the enzyme, cleaved caspase 3, was linked to increased PARP cleavage after treatment with metformin-loaded microspheres (Fig. 4B). Using fluorimetric analysis, we found that metformin-loaded microspheres significantly inhibited MDA-MB-231 cells more than metformin-free medicine and blank microspheres did. These results also show that PARP was cleaved in a dose-dependent manner by metformin-loaded microspheres. This suggests that a metformin-loaded microsphere triggers a pathway for apoptotic cell death.

Discussion

Metformin is a well-known antidiabetic drug. However, the incidence of diabetes, even after taking metformin, has been increasing day by day because metformin shows lesser target specificity [23]. To improve the target specificity, metformin was formulated in a microsphere. As we know, the microsphere encapsulation of several drugs increases the specificity of the drug [24]. Therefore, we aimed to design the formulation in such a way that metformin can achieve better target-specific delivery. Characterization of the formulation reveals that the microspheres are significantly loaded with metformin, providing sustained delivery.

Furthermore, this formulation drug was administered in the EAC and MDA-MB-231 breast cancer cell line. Data have shown that the formulated drug exhibits a more effective cytotoxic effect against these cell lines. The cytotoxic effect of the formulated drug was dose-dependent, with optimal activity observed at a suitable dosimetry. The cytotoxic effect was more potent in MDA-MB-231 cells than in EAC cells. Furthermore, we investigated the possible mechanism of such cytotoxicity using MDA-MB-231 cells. From experimental studies, we found that the cytotoxic effect was likely mediated by reactive oxygen species (ROS), as the administration of the formulated drug resulted in an upregulation of ROS levels.

Furthermore, the formulated drug was also found to downregulate the mitochondrial membrane potential. This suggests that the cytotoxic death was caused by

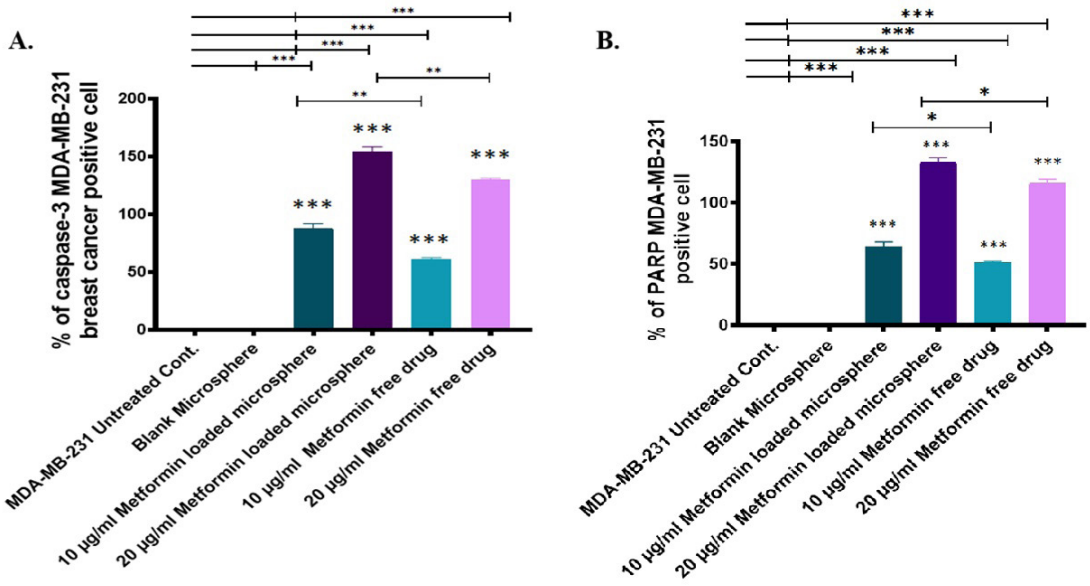

Fig. 4: (A) In-vitro caspase-3 assay of MDA-MB-231 breast cancer cells on different concentration drug drug-loaded microsphere treatment. (B) metformin-loaded microsphere-induced apoptosis. In-vitro PARP assay of MDA-MB-231 breast cancer cells on different concentrations drug drug-loaded microsphere treatment. Values are mean ± SD with n=3. $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05.

apoptosis. Regarding this, we have done ELISA studies of apoptotic markers. Studies have found that the metformin in a microsphere formulation upregulates cleaved caspase 3, cleaved PARP. In comparison with the free metformin, the formulated one was found to be more effective in inducing cell death and modulating the apoptotic marker proteins. This concludes that the formulation of metformin in a microsphere is more potent than the free form, as it bears more target specificity and sustained delivery into the target area. Further research on the formulated metformin-mediated cell death signalling mechanism is also required.

By using a specific and complex mechanism of action to cause cytotoxicity in aggressive and triple-negative MDA-MB-231 breast cancer cells, the metformin-loaded microsphere shows excellent promise as a novel therapeutic agent. The primary mechanism of the formulation is believed to be the increase in intracellular ROS levels, which disrupts the redox balance and causes cellular damage resulting from oxidative stress. The cancer cells may become more sensitive to apoptotic signalling pathways as a result of this pro-oxidative environment. This finding provides a strong foundation for further research into its molecular mechanisms and potential therapeutic options in oncology.

As a whole, our results show that metformin formulated in microspheres has a considerable more noticeable anticancer effect on MDA-MB-231 cells than on EAC cells. This suggests cell-type-specific responsiveness that may be influenced by the inherent molecular and metabolic variations of the two cancer models.

Notably, the study reveals that the activation of the mitochondria-dependent intrinsic apoptotic pathway is the mechanism by which metformin induces cytotoxicity in MDA-MB-231 cells. This mechanistic realization highlights the potential of metformin-loaded microspheres to precisely induce programmed cell death in aggressive breast cancer cells in addition to improving medication delivery. Improved cellular absorption, decreased systemic toxicity, and prolonged drug availability are potential benefits of using a controlled-release microsphere formulation.

Our results show that metformin formulated in microspheres has a considerably more noticeable anticancer effect on MDA-MB-231 cells than on EAC cells. This suggests cell-type-specific responsiveness that may be influenced by the inherent molecular and metabolic variations of the two cancer models.

Notably, the study demonstrates that metformin produces cytotoxicity in MDA-MB-231 cells through the activation of the mitochondria-dependent intrinsic apoptotic pathway. In addition to enhancing drug delivery, this mechanistic insight reflects the potential of metformin-loaded microspheres to cause programmed cell death in aggressive breast cancer cells accurately. Using a controlled-release microsphere formulation may extend medication availability, improve cellular absorption, and reduce systemic toxicity.

Conclusion

These findings promise significant advances in cancer treatments due to two primary and distinct features.

First, pharmacokinetics and targeted drug delivery were improved using the novel administration of metformin via biodegradable polymeric microspheres. Metformin can be encapsulated in microspheres for sustained release and enhanced absorption, which minimizes systemic toxicity and allows for higher local medication concentrations at the tumour site. One of the main drawbacks of traditional metformin therapy in oncology is that poor intratumoral concentrations frequently limit its efficacy; this approach also permits improved penetration into the tumour microenvironment.

The second finding of the study is that metformin-induced cytotoxicity is mainly caused by a mitochondria-mediated apoptotic pathway, especially in triple-negative breast cancer (TNBC), a subtype of the disease that is aggressive and resistant to HER2-targeted and hormone-based treatments. According to the results, metformin causes a decrease in mitochondrial membrane potential (ΔΨm) followed by the activation of caspase-3, which in turn induces apoptosis.

This leads to convergence on the intrinsic apoptotic cascade. When considered as a whole, these molecular discoveries not only enhance our understanding of metformin's anticancer effects when loaded into microspheres, but also justify incorporating the medication into precision medicine approaches.

The findings of the mitochondria-dependent death pathway open the door to combination regimens with other inhibitors. At the same time, the use of controlled-release microsphere formulations may provide temporal and geographical control of drug availability. These discoveries may open the door to the development of tumour-selective, metabolism-based treatment approaches, which are especially helpful in the treatment of refractory subtypes, such as TNBC, for which there are few traditional options.

Conflict of Interest

None to declare.

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