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FORMULATION AND CHARACTERIZATION OF METFORMIN-LOADED ETHOSOMES FOR TOPICAL APPLICATION TO EXPERIMENTALLY INDUCED SKIN CANCER IN MICE

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Abstract

We developed an ethosome formulation that acts as a metformin carrier and studied in vitro skin penetration. We also wanted to assess the anticancer efficacy of the best ethosomal formulation when given topically to animals with chemically generated skin cancer. We used a statistical Box-Behnken experimental design with three variables at three levels: lecithin, cholesterol, and a combination of ethanol and isopropyl alcohol concentrations. All formulations were created to calculate the entrapment efficiency, zeta potential, vesicle size, and drug release percentage after 1, 2, 4, 8, and 24 hours. The formulations' vesicles ranged in size from 124 ± 14.2 nm to 560 ± 127 nm, with entrapment effectiveness of 97.8 ± 0.23% to 99.4 ± 0.24% and drug release rates of 38 ± 0.82% to 66 ± 0.52% after 8 hours. All formulations were entered into the Box-Behnken software, which selected three formulations and assigned one as the ideal formula. Metformin-loaded ethosomal gel has a higher anticancer effect in vivo against skin cancer than the gel formulation containing free metformin. Lower lecithin, high ethanol and isopropyl alcohol, and moderate cholesterol levels increased penetration rate. Overall, we can conclude that metformin-loaded ethosomes are a promising remedy for treating skin cancers, and more studies are warranted to approve this activity in other animal models of skin cancers.

INTRODUCTION

One of the most common illnesses, skin cancer affects people on every continent in the world and has been steadily increasing in both prevalence and death. There are certain disadvantages to conventional therapy, such as chemotherapy and surgery. In this compilation, the ethosomal systems will be explored in detail and categorized according to their constituents: transethosomes, binary ethosomes, and classical ethosomes. Model lipid vesicular carriers with a significant amount of ethanol are called etherosomes.[1]

Metformin (1, 1-dimethylbiguanide hydrochloride) is an frequently used to treat type 2 diabetes. AMP-activated protein kinase (AMPK), which reduces hepatic gluconeogenesis and increases skeletal muscle glucose absorption, is the mechanism via which it has its anti-diabetic effect. Metformin's anticancer activity has garnered more interest, as multiple studies have demonstrated its anti-diabetic qualities. Metformin has been shown in fundamental investigations

to decrease the proliferation of cells in a number of human malignancies, including as pancreatic cancer, gastric cancer, endometrial cancer, and medullary thyroid cancer.[2] In xenograft mice models of melanoma, ovarian cancer, prostate cancer, and breast cancer, metformin also inhibited the formation of tumours. Furthermore, metformin reduced the incidence and extent of breast adenocarcinomas in Her2/c-Neu transgenic mice and prevented tobacco carcinogen-induced lung carcinogenesis in an animal model of cancer. Epidemiologic survey results verify that metformin significantly reduces the risk of tumour development. Patients with diabetes who take metformin have far lower odds of both cancer incidence and death. Patients with diabetes who were treated with metformin as part of their diabetic therapy for colorectal cancer seemed to have a better overall survival rate. Nevertheless, the intricacies of the processes responsible for metformin's inhibition of cancer growth are still mostly unclear.[3]

When metformin is taken orally, side effects include nausea, vomiting, and upset stomach. There have also been reports of pancreatitis and various forms of hepatotoxicity. Furthermore, lactic acidosis is also seen in patients with renal insufficiency, and metformin is known to cause vitamin B12 deficiency. Therefore, when it makes sense, it is best to choose the topical path.[4]

Ethosomes are the most studied vesicular system; they are lipid-based nanovesicles with enhanced deformability, softness, and elasticity. Ethosomes are phospholipid and ethanol-based multilamellar nanovesicles. Ethanol modifies the fluidity of the phospholipid bilayer, disrupts the stratum corneum's membrane barrier, and increases penetration power. Strong penetration enhancer ethanol offers vesicles unique properties such stability, size, negative electric potential, entrapment efficacy, and improved skin permeability. Ethamomes are able to penetrate the epidermis through the hair follicles and stratum corneum route. Once in the upper skin layer, the ethosomes release chemicals that cause the medication to progressively penetrate the epidermis while the phospholipids remain in the upper layer.[5]

The goal of the current work was to create and describe ethosomal preparations loaded with metformin and determine the ideal formula to test its topical anticancer efficacy against mice that were artificially induced to develop skin cancer. In order to treat skin cancer, this study also attempted to transport metformin to the skin's outer layers.

MATERIALS AND METHODS

Metformin hydrochloride (99.45% powder, BP 2012), 99.9% ethanol (v/v), isopropyl alcohol and carbopol 974p were obtained from JB Pharmaceuticals. Some 10× phosphate buffered saline was bought from Lonza Company. The 97% L-α-lecithin was granular, from soybean oil, CAS 8002-43-5, molecular weight = 750 g/mol and the method detection limit (MDL) number was MFCD00082428. The 97% cholesterol was bought from Acros Organics.

By using the Box-Behnken (BB) three-level three-factor design, we optimized and selected the formulation variables statistically for the preparation of ethosomes that carry metformin to

achieve the maximum EE%, small vesicle size, high ZP and the greatest DR%. The experimental design was generated and evaluated by the aid of the Design-Expert software.

Eight experiments were prepared, and the 3 independent variables were studied: L-α-lecithin concentration (2–4 w/w%) (X1), cholesterol concentration (0 to 1 w/w%) (X2) and ethanol and isopropyl alcohol concentrations (20-40 w/w%) (X3). On other hand, the EE% (Y1: EE%), vesicle size (Y2: VS), ZP (Y3) and DR% (Y4) were chosen as the dependent variables.

A concentration of 2-4 w/w% lecithin was used to prepare formulations of ethosomes. The concentration of ethosomes in most ethosomal formulations was 20-40 w/w%. A concentration of 0 to 1 w/w% cholesterol was used to prepare ethosomes in the most recent researches. The optimal formula was chosen based on its desirability, which was then subjected to further examination.

Formulation of Metformin-Loaded Ethosomes

Formulation of the metformin-carrying ethosomes was designed depending on the method reported previously. The aqueous and organic phases were prepared separately. Lecithin and cholesterol were dissolved in a mixture of ethanol and isopropyl alcohol to produce the organic phase, which was kept in a closed container. Metformin was insoluble in ethanol and isopropyl alcohol, so it was dissolved in distilled water to produce the aqueous phase. The aqueous phase was added to the organic phase drop by drop by a syringe pump. The mixture is stirred using a magnetic stirrer at a speed of 700 rpm for 5-30 min to obtain the required ethosomal formula at 30 °C. Finally, the ethosomal formulations were passed through a polytetrafluoroethylene (PTFE) filter with a pore size of 0.22 μm. Then, the filtrates were stored in closed containers at 4 °C.

Characterization of the Metformin-Loaded Ethosomes

Determination of entrapment efficiency %

EE% is the percent of the total amount of metformin encapsulated in vesicles in the formulations. The unentrapped metformin was separated using a cooling centrifuge rotating at 16,000 rpm at 4 °C. The supernatants were diluted in distilled water (10 mL, 3 min). The amount of entrapped metformin was estimated spectrophotometrically and the ƛmax of metformin was 234 nm; it was calculated using a standard calibration curve.

Vesicle Size Analysis

Vesicle size is evaluated by using the dynamic light scattering method that is performed in the Malvern Zetasizer. Distilled water was utilized to dilute all formulations and mixed by shaking before the measurements to improve the scattering intensity and remove the multiple scattering

phenomena. The particle size was measured after placing the samples in glass cuvettes. Three replicates were done for each formulation and presented as the mean ± SD.

Zeta Potential Analysis

We measured the ZP using a computerized Malvern Zetasizer (Instrument at Manipal University, Manipal, India) based on the electrophoretic mobility. The particle charge is an important parameter to ensure the ethosomal suspension stability.

In Vitro Release Study

Some 1-mL samples from each formula (1.7 w/w% of metformin) were added to a dialysis bag (Mw cut-off = 14,000 Da). Forty millilitres of Sorensen phosphate buffer (pH = 6.5) was used as a release medium. Then, the dialysis bag was immersed in the prepared release medium at 32 ± 0.5 °C in a dissolution apparatus at 100 rpm. New 1-mL samples were withdrawn from the medium and replaced with the same volume from the fresh medium at 1, 2, 4, 8, 12 and 24 h. Estimation of the sample concentrations was done spectrophotometrically at a 234 nm.

In Vitro Skin Permeation

The skin of rats was used fresh, as reported previously. Each diffusion membrane was mounted in a vertical diffusion cell (5 cm^2) as a donor compartment. Sorensen phosphate buffer (40 mL, pH = 6.5) was used as a receptor compartment. The diffusion membrane containing 1 mL of each formula (1.7 w/w% of metformin) was immersed in the receptor compartment, which was stirred in a water bath at 600 rpm, and the temperature equaled 37 ± 0.5 °C. After that, 1-mL samples were taken from the medium and substituted by equal volumes from the fresh medium at 1, 2, 4, 8, 12 and 24 h. Finally, the samples were measured by a spectrophotometer at a wavelength of 234 nm. The limit of quantitation (LOQ) was 0.84 µg, while the detection range was 1-20 µg/mL.

Gel Formulation

The optimum formula gel was prepared by adding 0.7 g from carbopol 974p to the optimal formula under vigorous stirring; then, trimethylamine solution (5%) was used to neutralize the mixture, which was added drop by drop until the gel was formed.

In Vivo Mouse Study for Screening of Antitumor Activity and Toxicity

Mice Preparation

Thirty male Swiss albino mice (weight range equaled 25-30 g, 6-8 weeks of age) were purchased from mahavir enterprises and placed in groups of six in plastic cages. A temperature range equal to 23 ± 5 °C, and the animals had free access to their normal diet and drinking water.

Induction of Skin Lesions

A 2 × 2 cm^2 dorsal skin area was shaved on all animals using a hair clipper 48 h prior to the experiment. To induce skin lesions in mice, one dose of DMBA, which acts as an initiator for skin tumors (100 μg in 200 μL acetone), was injected subcutaneously into each mice. After one week, there was an increase in the number of epidermal lesions (the lesions >1 mm in diameter for each mouse. The skin lesions were assessed first by skin morphology (lesion width and lesion length) and, also, by histological methods (thickness of the epidermis).

Regimen of Applying Metformin-Loaded Ethosomes

Each group contained 6 animals, and the selected optimal formula was applied topically on the dorsal region of the skin (10 mg/cm^2 of the affected area) per week for a total of 4 weeks.

Toxicological Screening

For testing any possibility of hepatic or renal toxicity due to the systemic absorption of the gel formula, a histopathological investigation was done for the liver and kidney specimens. The tissue samples were fixed in neutral-buffered formalin and processed for H&E staining and examination under light microscopy by an experienced blinded pathologist. In addition to the histopathological examination, the serum samples were directed for estimation of the liver enzymes (ALT and AST) and serum creatinine, urea and albumin.

Statistical Analysis

GraphPad prism was used to apply the statistical tests to the current data. Data were quantitative in nature and demonstrated in the form of the mean ± SD and analyzed using one-way ANOVA, as one factor (treatment regimen) was influencing the study groups. Bonferroni's test for multiple comparison analysis was at $p < 0.05$.

RESULTS

Selection of the Optimized Formula

We prepared ethosomes with a high percent of entrapment efficiency, small vesicle size, high ZP and high percent of DR% by using a three-level three-factor Box-Behnken design. The ANOVA

test analyzed and evaluated all the data collected from each response; then, an optimized formula was obtained using the desirability method. The formula that contained 2.083% w/w lecithin, 0.524% w/w cholesterol and 37.495% v/v ethanol was selected as the optimized formula, as it showed the best desirability index value (0.811).

The chosen optimal formula, #13, displayed an EE% of 98.40 ± 0.35%, a vesicle size equal to 124.01 ± 14.27 nm and a release % equal to 55.04 ± 0.98 %. The ZP of the optimized formula #13 was 60.08 ± 1.44 mV, which provided good stability.

In Vitro Studies to Evaluate Skin Permeation

In formula #9, the amount of permeated metformin was 1224.27 ± 18.1 µg/cm^2, and the steady-state flux was 2.93 µg/cm^2/h, while the percent of cumulative permeation was 72%. In the optimal formula, #13 showed an amount of permeated metformin equal to 1660 ± 32.4 µg/cm^2, while the steady-state flux was 3.61 µg/cm^2/h; however, the percent of cumulative permeation was 97.6%. In addition, formula #16 showed an amount of permeated metformin equal to 1547 ± 21.7 µg/cm^2, the steady-state flux was 3.26 µg/cm^2/h and the percent of cumulative permeation was 91%. Finally, the optimal formula #13 showed the best permeability at interval times with significance ($p < 0.05$), as this formulation had the highest ethanol and isopropyl alcohol concentration, lower lecithin concentration and moderate concentration of cholesterol. The TEER results of the measured electrostatic repulsion were above 30 ± 1.5 kΩ. That indicated a good state for the skin integrity.

	The Amount of Permeated Metformin (µg/cm^2)	**The Steady-State Flux (µg/cm^2/h)**	**The Percent of Cumulative Permeation (%)**
Formula #9	1224.27 ± 18.1	2.93	72
The optimum formula #13	1660 ± 32.4	3.61	97.6
Formula #16	1547 ± 21.7	3.26	91

Table 1: Skin penetration parameters after 24 hours

Morphological Characterization of the Ethosomes

The morphology of the ethosomes was characterized by using a transmission electron microscope. The optimal formula was freshly prepared, then used for the transmission electron microscopy (TEM) images. The ethosomes showed in the TEM images as black dots. The TEM images showed ethosomes in well-identified spherical shapes and homogenous and non-aggregated vesicles, which confirmed their nanovesicular characteristics for the ethosomes.

Thermal Analysis of Optimal Metformin-Loaded Ethosomes Formula

The pure metformin curve revealed a sharp endothermic peak at 242 °C, while the optimal metformin-loaded ethosome formula (#13) showed a peak appearing at 135 °C, but the thermogram of the empty formula (excipient) revealed two endothermic peaks at 103 °C and 148 °C. Metformin in the optimal metformin-loaded ethosome formula (formula #13) did not show a characteristic peak. These findings highlight that metformin was dissolved within the ethosomes during the formulation process

In Vivo Antitumoral Activity of the Optimized Metformin-Loaded Ethosomal Gel

The developed 7,12-dimethylbenz[α]-anthracene (DMBA)- induced lesions appeared at the back of each mouse and were monitored weekly. A specialized caliber was utilized to measure the width and length of each lesion.

The Body Weight and Lesion Length and Width

The weight of the mice and diameters of the lesions were measured to evaluate the skin cancer progression. The antitumor efficacy of the metformin-loaded ethosomes was evaluated in mice group #5. This metformin-loaded ethosome-containing gel produced a significant decrease in the lesion diameters compared with the other four gels over 28 days

The Thickness of the Hematoxylin and Eosin-Stained Skin Layers

We found that the vehicle + empty gel group displayed the normal morphological features of the mouse skin layers, with an apparent intact thin epidermal layer with intact keratinocytes and an intact dermal layer with well-organized collagen fibres and hair follicles without abnormal inflammatory cells infiltrates, as well as intact subcutaneous tissue.

Histopathological Examination of Kidney Specimens Stained with H&E

The kidney samples from the vehicle + empty gel group demonstrated intact morphological features of renal parenchyma, including renal corpuscles and different nephron tubular segments, including tubular epithelium, with intact interstitial tissue, as well as vasculatures. DMBA + empty gel or DMBA + ethosome gel showed a mild cystic dilatation of the renal tubular segments, accompanied by little interstitial mononuclear inflammatory cell infiltrates. The DMBA + free metformin gel group showed mild focal records of tubular degenerative changes with intact renal corpuscles, as well as interstitial tissues with few sporadic inflammatory cell infiltrates. The DMBA + metformin ethosome gel samples showed sporadic records of tubular degenerative changes with intact renal corpuscles, interstitial tissue and vasculatures.

Histopathological Examination of Liver Specimens Stained with H&E

Liver samples from the vehicle + empty gel group showed the normal morphological structure of hepatic parenchyma. The DMBA + empty gel samples showed mild hepatocellular degenerative changes in the pericentral, as well as periportal, regions with diffuse mononuclear inflammatory cells infiltrating in the hepatic parenchyma. Samples from the DMBA + free metformin gel group showed mild hepatocellular vacuolar degenerative changes with the dilatation of hepatic BVs and minimal inflammatory cell infiltrates. Samples from the DMBA + ethosome gel group showed mild hepatocellular degenerative changes with intact hepatocytes and mild focal pericentral and periportal mononuclear inflammatory cells infiltrates. Samples from the DMBA + metformin ethosome gel group showed almost apparent intact hepatocytes all over the hepatic parenchyma and moderate dilation of the portal BVs with minor focal perivascular inflammatory cell infiltrates.

Liver and Kidney Function Tests

We applied an ANOVA test on the data of the serum ALT, AST, albumin, urea and creatinine, but the data indicated nonsignificant differences among the study group

DISCUSSION

The goal of this study was to determine whether ethosomes could increase the quantity of metformin maintained on the skin, which would improve skin cancer treatment. Ethosomes can penetrate the stratum corneum to the deep layers due to their high alcohol concentration.[6]

The amounts of lecithin, ethanol, and cholesterol are crucial criteria while making ethosomes. Lecithin forms lipid bilayer membranes in the multilamellar vesicles of ethosomes. Cholesterol is responsible for metformin's stability and EE%.[7]

Ethanol gives the vesicles more freedom and stability by providing softness and a negative charge. Depending on the data collected from all formulations, a ethanol concentration of 40% was suitable to prepare the ethosomes that produced a high EE% and permeation. However, increasing the concentration of ethanol above 40% will dissolve the ethosome membranes, causing a decrease in the EE% and increase in the vesicle sizes. On the other hand, isopropyl alcohol is used with ethanol to prepare ethosomes as a skin penetration enhancer and to increase the EE%. The high entrapment efficiency of the formulations is due to adding isopropyl alcohol with ethanol; isopropyl alcohol decreases the vesicle size and increases the ZP and EE%. Isopropyl alcohol can release metformin in the long term, which achieves the goal of our study.[8]

A concentration of 2–4% lecithin was used to prepare formulations of the ethosomes. Increasing the lecithin ratio will increase the vesicle sizes. When the concentration of lecithin increases, this

will lead to increasing the ethosome vesicle sizes, as lecithin molecules tend to coalesce and aggregate.[9]

In our study, isopropyl alcohol and ethanol improved the metformin release from ethosomes, as they can increase the liquefaction and permeability that leads to an increased DR%. Cholesterol and lecithin decrease the metformin release from ethosomes, as increasing concentrations of cholesterol and lecithin are incompatible with metformin solubility. Lecithin has a negative effect on DR%, as increasing the lecithin level will cause an increase in the vesicle rigidity and will cause a decrease in the DR%.[10]

In our formulations, when the concentration of lecithin decreased, the permeation rate of metformin increased. Furthermore, decreasing the concentration of cholesterol caused an increase in the permeation rate of metformin. Similarly, one previous study found that, when the concentrations of lecithin and cholesterol increased, the rigidity of the ethosomal vesicle bilayer increased. Ethanol enhanced the permeation rate of the drug as it interacted with the polar head group of the SC lipid molecules, lowering the melting point of the SC lipids and thereby increasing the lipid bilayer fluidity and cell membrane permeability. The maximum permeability of the drug from the vesicles was due to a synergistic mechanism involving ethanol, vesicles and SC lipid molecules.[11]

The application of metformin-loaded ethosomes showed significant antitumor activity against the skin cancer compared to the application of free metformin. At the 14-days treatment point, the effect of the free metformin gel was better than the empty ethosome gel; this may be linked to the anticancer effect of the free metformin. However, at the following time points (day 21 and day 28), there was no significant differences among the two groups.[12]

CONCLUSION

When applied topically, metformin ethosomal gel significantly reduces chemically caused skin cancer in mice. The solubility of lecithin by ethanol was enhanced when isopropyl alcohol was added during the ethosome formation process. This resulted in an increase in the stability and efficacy of the ethosome vesicles. Additionally, isopropyl alcohol reduced the vesicles' particle size, which raised the EE% and prolonged the metformin's release. This is what our study aims to do. Ultimately, an increase in the penetration rate was attained with lesser lecithin, higher ethanol and isopropyl alcohol, and moderate cholesterol. As a result, the current research may pave the way for metformin to be formulated in the future and used as a therapeutic tool to treat skin cancer.

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