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ORIGINAL ARTICLE / ÖZGÜN MAKALE

EFFECT OF LAMOTRIGINE SOLID DISPERSIONS ON VIABILITY OF A549 AND RG-2 CELLS

LAMOTRİJİN KATI DİSPERSİYONLARININ A549 VE RG-2 HÜCRELERİNİN CANLILIĞI ÜZERİNE ETKİSİ

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ABSTRACT

Objective: *LMT is an antiepileptic drug that is among the drugs being repositioned/reprofiled beyond its original medical indications, thanks to its promising anticancer activity profiles on various cancer cells. In this study, the improvement of the poor aqueous solubility of LMT using solid dispersion technique with different polymeric/surfactant carriers in different ratios and the antiproliferative effect potentials of the obtained optimum LMT solid dispersion on A549 and RG-2 cells were determined.*
Material and Method: *LMT solid dispersions were prepared using four different carrier types (PEG 4000, PEG 6000, poloxamer 188 and poloxamer 407) and five different drug to carrier ratios (1:2 - 1:4 - 1:6 - 1:8 - 1:10) according to the melting method and the change in the solubility profile was determined by solubility studies performed according to the shake-flask method. The MTT assay protocol was used to determine the cytotoxicity profile of the selected optimum solid dispersion (F8) on A549 and RG-2 cell lines. The interaction of LMT with the formulation components and the solid-state characteristics within the formulation were evaluated by XRD, DSC, FTIR, and SEM analyses.*
Result and Discussion: *The solubility studies showed that LMT exhibits a pH-dependent solubility profile, and the highest solubility increase (2.3 fold) in solid dispersion formulations was obtained with the F8 formulation using poloxamer 407 at a 1:6 ratio. XRD, DSC, FTIR, and SEM analysis results explain the partial amorphization of LMT in the F8 formulation. In vitro cytotoxicity studies have shown that LMT has a dose-dependent antiproliferative effect on RG-2 cells, but no significant cytotoxic effect at low doses (10-80 µg/ml). For A549 cells, cell viability decreased with increasing doses, suggesting promising results regarding antiproliferative effects at higher doses.*
Keywords: *Antiproliferative effect, poloxamers, polyethylene glycols, lamotrigine, solid dispersion, solubility*

ÖZ

Amaç: *LMT, çeşitli kanser hücreleri üzerindeki ümit verici antikanser aktivite profilleri sayesinde orijinal tıbbi endikasyonlarının ötesinde yeniden konumlandırılan/yeniden profillenen ilaçlar arasında yer alan bir antiepileptik ilaçtır. Bu çalışmada LMT'nin zayıf sulu çözünürlüğünün farklı oranlarda farklı polimerik/yüzey aktif madde taşıyıcıları ile katı dispersiyon tekniği kullanılarak*

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iyileştirilmesi ve elde edilen optimum LMT katı dispersiyonunun A549 ve RG-2 hücreleri üzerindeki antiproliferatif etki potansiyelleri belirlenmiştir.

Gereç ve Yöntem: *LMT katı dispersiyonları eritme yöntemine göre dört farklı taşıyıcı türü (PEG 4000, PEG 6000, poloksamer 188 and poloksamer 407) ve beş farklı ilaç:taşıyıcı oranı (1:2 - 1:4 - 1:6 - 1:8 - 1:10) kullanılarak hazırlanmış ve çözünürlük profilindeki değişim şişe çalkalama yöntemine göre yapılan çözünürlük çalışmaları ile belirlenmiştir. Seçilen optimum katı dispersiyonun (F8) A549 ve RG-2 hücre hatları üzerindeki sitotoksisite profilinin belirlenmesinde MTT deney protokolü kullanılmıştır. LMT'nin formülasyon bileşenleri ile etkileşimi ve formülasyon içindeki katı hal karakteristiği XRD, DSC, FTIR ve SEM analizleri ile değerlendirilmiştir.*

Sonuç ve Tartışma: *Çözünürlük çalışmaları LMT'nin pH bağımlı bir çözünürlük profili sergilediğini göstermiş olup katı dispersiyon formülasyonlarında en yüksek çözünürlük artışı (2.3 kat) poloksamer 407'nin 1:6 oranında kullanıldığı F8 formülasyonu ile elde dilmiştir. XRD, DSC, FTIR ve SEM analiz sonuçları F8 formülasyonu içindeki LMT'nin kısmi amorfizasyonu açıklar niteliktedir. İn vitro sitotoksisite çalışmaları, RG-2 hücreleri için LMT'nin doza bağlı bir antiproliferatif etkisinin olmakla birlikte düşük dozlarda (10-80µg/ml) anlamlı bir sistotoksik etkiye sahip olmadığını göstermiştir. A549 hücreleri için, doz arttıkça hücre canlılığının azalması, daha yüksek dozlarda antiproliferatif etkiye ulaşılması ile ilgili umut verici sonuçlar alınabileceğini düşündürmektedir.*

Anahtar Kelimeler: *Antiproliferatif etki, çözünürlük, katı dispersiyon, lamotrigin, polietilen glikoller, poloksamerler*

INTRODUCTION

Epilepsy is a neurodegenerative disease in which spontaneous seizures occur repeatedly due to hyperexcitability and hypersynchronization in brain neurons [1]. Approximately 65 million people worldwide suffer from this disease, the causes of which remain partially unknown [2,3]. Nowadays, many drugs are used in the treatment of epilepsy, differing in their pharmacokinetics, efficacy, and side effect profiles, requiring treatment options to be tailored to individual needs [4]. The basic purpose of antiepileptic drugs is to improve neuronal mechanisms.

A phenyltriazine derivative, lamotrigine (LMT) is used to treat conditions like epilepsy and both simple and complicated seizures [5]. It is both a second generation anti-epileptic drug and a mood stabilizer [6]. LMT, which was first marketed in the UK (Lamictal®) in 1991, was approved for use in the United States (US) market in 1994 [7]. LMT is in Class II (low solubility and high permeability) according to the Biopharmaceutics Classification System (BCS). The solubility is defined as the maximum amount of substance that can be dissolved at constant temperature and pressure in a given solvent. Accordingly, the solubility of a drug refers to the maximum amount of solute substance of the drug at body temperature and in biological fluids. The therapeutic efficacy of a drug depends mainly on the solubility and permeability of the drug molecule. Therefore, solubility is one of the most important parameters that should be evaluated during drug research and development studies [8-10]. The poor aqueous solubility (~ 0.17 mg/ml) of LMT limits its oral absorption. LMT, with a pKa of 5.7, has been reported to undergo enzymatic first-pass metabolism in the liver and is eliminated mostly via renal excretion. It is also known to be a P-glycoprotein (P-gp) substrate, which accounts for LMT's limited access to the brain. Various biological and physiological barriers limit LMT from reaching the necessary therapeutic levels in the brain, necessitating higher concentration dosing in the clinic. Higher concentration dosing, in turn, is responsible for the undesirable side effects of LMT, such as skin rash, toxic epidermal necrosis, thrombocytopenia, hepatotoxicity, leukopenia, headache, nausea, and vomiting [11]. But, it is possible to overcome these problems with an appropriate formulation approach. One of these methods is the design of solid dispersions using polymeric and surfactant carriers.

Solid dispersions are defined as systems prepared by dispersing one or more active ingredients in a solid inert carrier using various methods (such as melting, kneading, hot-melt extrusion, solvent evaporation, spray-drying, and freeze-drying). The primary function of the carrier used in solid dispersions is to transform the drug from a crystalline to an amorphous structure and prevent the drug from crystallizing over time. In addition, the carrier is thought to increase the solubility of the drug by mechanisms such as increasing the wettability of the active ingredient, reducing particle size to the

molecular level, and preventing the active ingredient from settling upon contact with an aqueous medium due to the separation of active ingredient particles by the carrier particles [12,13].

A relatively recent method for finding alternate applications for well-known treatments that fall outside of their original medical indications is drug repurposing, commonly referred to as drug repositioning/reprofiling. When compared to traditional de novo drug development, this method may have several benefits, such as a shorter time to market and cheaper expenses. Central nervous system medications, particularly certain antidepressants and antipsychotic pharmaceuticals, are a class of drugs that may represent interesting candidates for repurposing in oncology [14]. For this reason, recent studies have focused on the possibility of drugs with different indications as repurposed oncological drug candidates. The literature has reported that antiepileptic drugs may be a good option for adjuvant cancer treatment. Some studies provide evidence of the anticonvulsant properties of some antiepileptic drugs as well as their potential inhibitory effects against the spread and metastasis of cancer cells. However, there are still insufficient studies to elucidate the clinical efficacy profiles of antiepileptic drugs in cancer treatment [15,16]. Also, many cancer patients have neuropathic pain after anticancer treatment. There are many studies investigating the efficacy of antiepileptic drugs in neuropathic pain caused by anticancer drugs. Some antiepileptic drugs, including LMT, are also effective in neuropathic pain and trigeminal neuralgia in cancer patients [17-19].

LMT has been reported to be used as an anticancer drug against colon cancer and chronic myeloid leukemia. Also, studies have confirmed that it exhibits strong antiproliferative effect on breast cancer cells. Furthermore, new research on a variety of cancer cell lines suggests that LMT-derived drug candidates have anticancer activities [20,21]. This study's objectives are to prepare LMT solid dispersions with various polymeric/surfactant carriers and carrier ratios to enhance their poor aqueous solubility and examine the antiproliferative effects of LMT solid dispersions on the rat glioblastoma cell line (RG-2) and human lung adenocarcinoma cell line (A549 cells).

MATERIAL AND METHOD

Materials

Lamotrigine (LMT) was received as a gift sample from Sanovel İstanbul, Turkey. The surfactant carriers (Poloxamer 188 (Kolliphor®P 188), poloxamer 407 (Kolliphor®P 407)) and polymeric carriers (polyethylene glycol 4000 (PEG 4000) and Polyethylene glycol 6000 (PEG 6000)) were purchased from Merck Millipore, Germany. Rat glioblastoma cell line (RG-2 cells) and human lung adenocarcinoma cell line (A549 cells) were provided by American Type Culture Collection (ATCC, Rockville, MD). Trypsin-ethylenediaminetetraacetic acid (EDTA) solution, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO, cell culture grade) were acquired from Sigma-Aldrich in the United States (US). Dulbecco's modified eagle's medium (DMEM), Dulbecco's Modified Eagle's Medium : Ham's F12 (1:1) (DMEM : Ham's F12), fetal bovine serum (FBS), and antibiotic solution (penicillin/streptomycin) were supplied by Biowest, US. The solvents that make up the mobile phase composition were HPLC grade, while all other chemicals were of reagent quality.

Determination of LMT by Chromatographic and Spectroscopic Methods

Development of UV Spectroscopic Method

Standard curve of LMT has been carried out in different pH medium such as distilled water, pH 1.2 (0.1 N HCl), pH 4.5 (Acetate buffer) and pH 6.8 (Phosphate buffer) as described in the World Health Organization (WHO) guideline [22]. Firstly, the stock solution of LMT (100 µg/ml) was prepared in each medium and maximum wavelengt (λ_{max}) in all solutions was recorded after scanning between 200 and 800 nm using a Ultraviolet (UV) Visible Spectrophotometer (Shimadzu UV-1700, Japan). The dilutions were made using the same medium to make solutions with different concentrations (5, 10, 15, 20, 25, and 30 µg/ml) for standard curve after the stock solution of LMT was prepared in each medium. The serial dilution technique was used to prepare standard solutions. Absorbance of prepared dilutions was measured at predetermined wavelengths with UV Visible Spectrophotometer.

Development of HPLC Method and Validation

The high performance liquid chromatography (HPLC) system (Agilent 1200, Germany) was used to perform the analyses. An Inert Sustain® C18 column (250 mm × 4.6 mm, 5µm) from GL Sciences, Tokyo, Japan, used as the stationary phase. Throughout the analysis, the mobile phase was a 70:30 (v/v) combination of acetonitrile and buffer (monobasic potassium phosphate solution, pH 4.5 modified with orthophosphoric acid). A flow rate of 1.0 ml/min was used to deliver it to the system. The detector signal was detected at a wavelength of 201 nm, and the injection volume was 20 µl. Throughout the analysis, the column temperature was maintained at 40 °C. The dilutions were done using the same medium for a standard curve, and the stock solution of LMT was prepared in the mobile phase (100 µg/ml). After preparing standard solutions using the serial dilution procedure (0.39, 0.78, 1.56, 3.125, 6.25, and 12.5 µg/ml), HPLC was used for analysis. The HPLC method was validated for linearity, accuracy, precision, and sensitivity (limit of detection (LOD) and limit of quantitation (LOQ)) in accordance with validation tests conducted in accordance with the International Conference on Harmonization (ICH) criteria [23].

Determination of Solubility Profiles of LMT

The protocol published by the WHO in 2018 formed the basis for the solubility studies [22]. The pH dependent solubility profile was studied in the pH range of 1.2 and 6.8 as described in the WHO guideline. Firstly, the 8 ml pH medium for each medium was taken in 10 ml amber coloured glass vials and closed after an excess amount of drug was added in each vials. These glass vials were placed in an orbital shaking water bath with the help of a suitable device in contact with the liquid. Temperature of the orbital shaking water bath was adjusted around 37 ± 1°C and the speed of it approximately 100 rpm. Syringe filters with a particle size of 0.22 µm were used to filter samples collected at 6, 24, 48, and 72 hours. UV Visible Spectrophotometer (Shimadzu UV-1700) was used to analyzed the drug's absorbance in the collected filtrate at the pre-scanned λ_{max} following appropriate dilutions using the same medium. The standard curve for the corresponding medium was then used to determine the concentrations in the collected samples. The guideline recommends at least three replicate solubility determinations at each pH condition. In this study, three samples were used for each time point.

Preparation of LMT Solid Dispersions

The melting method was used to prepare LMT solid dispersions. For this, a variety of carriers with surfactant (poloxamer 407) and polymeric (PEG 4000 and PEG 6000) characteristics were employed in varying ratios (drug to carrier ratios: 1:2 - 1:4 - 1:6 - 1:8 - 1:10). The drug was dispersed throughout the molten mass after each carrier was melted at melting point in an aluminum petri dish. The room temperature was used to cool the bulk. A screen (60 mesh) was used to sieve the hardened mixture after it had been ground into a powder in a mortar. LMT solid dispersions were stored in desiccators until entrapment efficiency and solubility studies were performed. The compositions of solid dispersions were given in Table 1.

Table 1. Composition of solid dispersions containing LMT

Formulation code	Ingredients (mg)				
	LMT	**Poloxamer 188**	**Poloxamer 407**	**PEG 4000**	**PEG 6000**
F1	100	200	-	-	-
F2	100	400	-	-	-
F3	100	600	-	-	-
F4	100	800	-	-	-
F5	100	1000	-	-	-
F6	100	-	200	-	-
F7	100	-	400	-	-
F8	100	-	600	-	-
F9	100	-	800	-	-
F10	100	-	1000	-	-
F11	100	-	-	200	-

Table 1 (*continue*). Composition of solid dispersions containing LMT

Formulation code	Ingredients (mg)				
	LMT	**Poloxamer 188**	**Poloxamer 407**	**PEG 4000**	**PEG 6000**
F12	100	-	-	400	-
F13	100	-	-	600	-
F14	100	-	-	800	-
F15	100	-	-	1000	-
F16	100	-	-	-	200
F17	100	-	-	-	400
F18	100	-	-	-	600
F19	100	-	-	-	800
F20	100	-	-	-	1000

Characterization of LMT Solid Dispersions

Entrapment Efficiency of LMT Solid Dispersions

To determine entrapment efficiency (EE%), equal amounts (5 mg) of each formulation were weighed and analyzed using a previously developed and validated HPLC method.

Solubility Studies

The shake-flask method, as outlined in the WHO's 2018 protocol, was used to conduct the solubility studies [22]. For this purpose, an excess amount of LMT and LMT solid dispersions were transferred into amber glass vials. Distilled water (2 ml) was added to each glass vial and the vials were tightly closed. The glass vials were placed in an orbital shaking water bath and shaken at 37 ± 1°C and 80 rpm for 24 hours under experimental conditions. At the end of the period, the collected samples were filtered through syringe filters (0.45 µm) and analyzed by the previously developed and validated HPLC method.

XRD Spectroscopy Analysis

The amorphous and/or crystalline structure properties of LMT, poloxamer 407, and optimum solid dispersion were determined using an Ultima X-ray diffractometer (XRD) instrument (Rigaku - D/Max 2200, Tokyo, Japan). Sample analyses were performed using Cu Kα radiation at a scanning rate of 6◦/min over a 2θ range of 5–50◦, and the resulting X-ray diffractograms were recorded.

DSC Analysis

The thermal characteristics and amorphous and/or crystalline structure qualities of LMT, poloxamer 407, and optimum solid dispersion were ascertained using a Differential Scanning Calorimetry (DSC) instrument (EXSTAR 7000, SII NanoTechnology Inc., USA). Heating curves were acquired at a scan rate of 10 ℃ /min in the range of 30 ℃ to 330 ℃ after 3-5 mg samples were packed into aluminum pans. This was carried out while nitrogen gas (20 ml/min) was flowing continuously. References were empty aluminum pans.

FTIR Spectroscopy Analysis

A Fourier Transform Infrared (FTIR) Spectroscopy system (NICOLET 6700, Thermo Fisher Scientific, USA) was used to clarify the molecular bond structures and interactions within the formulation of LMT, poloxamer 407, and optimum solid dispersion. The samples' FTIR spectra were captured at a spectral resolution of 1 cm^{-1} spanning the 400-4000 cm^{-1} range.

SEM Analysis

The surface morphology, shape, and particle size of LMT, poloxamer 407, and optimum solid dispersion were examined using a Scanning Electron Microscopy (SEM) instrument (LEO-EVO 40, Cambridge, England). For this purpose, samples were analyzed at 30 kV acceleration voltage, at 1000x and 2500x magnifications, and the resulting images were recorded.

In Vitro Cytotoxicity Studies of LMT Solid Dispersions in A549 and RG-2 Cells

MTT assay-based *in vitro* cytotoxicity studies were conducted to determined cytotoxicity properties of LMT and LMT solid dispersions [24]. The A549 cells were cultured in DMEM supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin, 10% FBS (complete medium-1) and RG-2 cells in DMEM : Ham's F12 supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin, 10% FBS (complete medium-2). After being seeded on a plate at a density of $5x10^4$ cells/ml (100 µl each well), the cells were taken out from the wells after an overnight incubation period. Sample dilutions (controls (complete mediums; negative controls), LMT, solid dispersions containing LMT; 10, 20, 40, and 80 µg/ml) prepared in complete mediums were applied to cells. Stock solutions of LMT and solid dispersions were prepared in DMSO and the complete mediums were used to make dilutions. To examine the potential toxic effects of DMSO ratios used in pharmaceutical standards on cells, DMSO controls (0.1, 0.2, 0.4, and 0.8%) were also applied to the cells. After the samples were administered, the cells were cultured for 24 hours. Each well was then filled with 25 µl of MTT solution (5 mg/ml), and the wells were incubated for an additional 4 hours. Following the addition of DMSO (200 µl) to each well, the Elisa Plate Reader was used to read on absorbance values.

Statistical Analysis

Mean ± standard error (SE) was used to present the data. Using the GraphPad Prism 8 statistical software, one-way ANOVA and post-hoc Tukey's multiple comparison tests were used to statistically analyze the *in vitro* cytotoxicity and solubility results. When the p-value was less than 0.05, the findings were deemed to differ significantly.

RESULT AND DISCUSSION

Determination of LMT by Chromatographic and Spectroscopic Methods

Development of UV Spectroscopic Method

The scanned λ_{max} values, linear equations and determination coefficient (R^2) values of standard curves of LMT in different pH media are given in the Table 2. The λ_{max} value of LMT in aqueous medium was found to be compatible with the literature [25,26]. According to the results, it seems that the pH of the environment has an effect on the λ_{max} of LMT. Also, there is a significant correlation (R^2 close to 1) between the concentration and absorbance. Therefore, it can be said that these methods are suitable methods for LMT analysis.

Table 2. The maximum wavelengths (λ_{max}), linear equations and determination coefficients in different media

Medium	λ_{max}(nm)	Linear equation (y = mx + c)	Determination coefficient (R^2)
Distilled water	305	y = 0.0253x – 0.0408	0.9997
0.1 N HCl (pH 1.2)	267	y = 0.0237x – 0.0736	0.9973
Acetate buffer (pH 4.5)	266	y = 0.0242x – 0.0012	0.9998
Phosphate buffer (pH 6.8)	305	y = 0.0255x – 0.0299	0.9998

Development of HPLC Method and Validation

The LMT solution that was made with the mobile phase had a λ_{max} value of 201 nm (Figure 1). The retention time was 4.9 min with the optimum HPLC conditions used and the HPLC chromatogram of LMT is given in Figure 1. The determination coefficient (R^2) for standard curve and the linear equation was $R^2 = 0.9997$, and y = 196.06x + 29.917, respectively. Our findings showed that, across the concentration range of 0.39–12.5 µg/ml, the association between concentration and peak area was linear. With respect to the LOD and LOQ, the values were calculated 0.18 µg/ml and 0.61 µg/ml. Examining the accuracy and precision results reveals that the recovery ranges from 93.8 to 102.7%, and the relative

standard deviation (RSD%) is less than 2%.

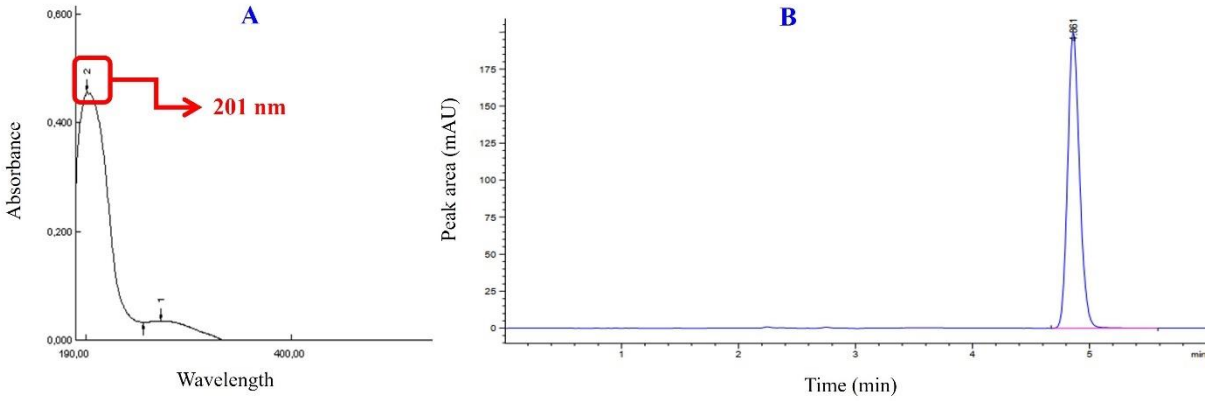

Figure 1. The UV spectrum (A) and the HPLC chromatogram (B) of LMT

Determination of Solubility Profiles of LMT

The equilibrium solubility values of LMT are summarized in Table 3. Also, the solubility profiles in different aqueous media of LMT are shown in Figure 2 separately. The results generally indicate that the solubility values are stabilized after a certain time. According to these results, the equilibrium solubility values of the drug in distilled water (0.29 ± 0.003 µg/ml) and pH 6.8 buffer (0.35 ± 0.083µg/ml) were close to each other but lower than other media. The lowest solubility was obtained in pH 6.8 buffer because LMT is nonionized in this basic medium. LMT contains amine groups with basic chemical properties [27]. The high solubility values in pH 1.2 (1.42 ± 0.060 µg/ml) and pH 4.5 (3.15 ± 0.146 µg/ml) buffers were attributed to the ionization of these amine groups in the acidic medium by taking protons. All these results show that LMT has a pH-dependent solubility, in agreement with the literature [28].

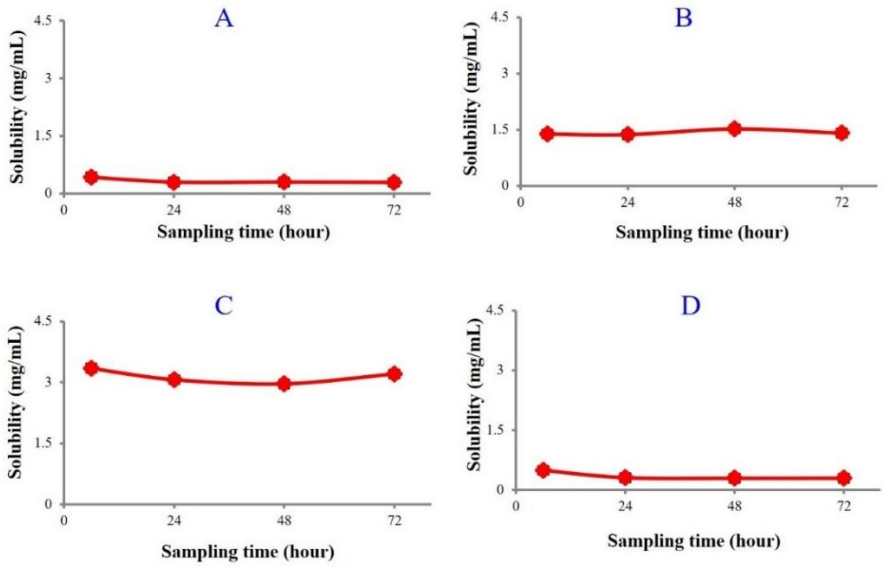

Figure 2. The solubility profiles in different aqueous media of LMT (A: distilled water; B: pH 1.2 buffer; C: pH 4.5 buffer; D: pH 6.8 buffer)

Table 3. The solubility values of LMT in different media (mean ± SE; n=3)

Medium	Solubility values
Distilled water	0.29 ± 0.003
0.1 N HCl (pH 1.2)	1.42 ± 0.060
Acetate buffer (pH 4.5)	3.15 ± 0.146
Phosphate buffer (pH 6.8)	0.35 ± 0.083

Characterization of Solid Dispersions

Entrapment Efficiency of LMT Solid Dispersions

For all prepared formulations, the entrapment efficiency (EE%) was calculated using a previously developed and validated HPLC method, with three replicates for each formulation. The entrapment efficiency values of LMT solid dispersions are given in the Table 4 and according to the results, the high entrapment efficiency values of LMT solid dispersions ranging from 86.1% to 105.3% was achieved. A study reported entrapment efficiencies between 97.76% and 98.62% for poloxamer 407 based LMT solid dispersions [29], while another study reported lentrapment efficiencies between 96.46% and 98.28% for polyviniyl prolydone (PVP) K30 and PEG 6000 based LMT solid dispersions [30].

Table 4. The entrapment efficiency and the solubility values of LMT solid dispersions (Mean± SE; n=3)

Formulation code	Entrapment efficiency (EE%)	Solubility (mg/ml)
F1	99.8 ± 1.825	0.550 ± 0.001
F2	91.7 ± 1.185	0.581 ± 0.001
F3	105.2 ± 1.355	0.554 ± 0.001
F4	89.3 ± 1.001	0.549 ± 0.001
F5	88.2 ± 2.012	0.555 ± 0.001
F6	96.7 ± 1.057	0.599 ± 0.003
F7	96.1 ± 0.971	0.596 ± 0.044
F8	98.2 ± 1.168	0.658 ± 0.002
F9	92.7 ± 2.815	0.615 ± 0.004
F10	88.8 ± 2.518	0.596 ± 0.002
F11	102.6 ± 1.461	0.504 ± 0.003
F12	102.1 ± 1.819	0.533 ± 0.004
F13	96.3 ± 0.624	0.477 ± 0.002
F14	94.5 ± 2.246	0.555 ± 0.002
F15	96.7 ± 1.179	0.555 ± 0.003
F16	100.3 ± 0.852	0.505 ± 0.001
F17	104.0 ± 2.459	0.490 ± 0.001
F18	103.6 ± 0.772	0.578 ± 0.002
F19	105.3 ± 1.953	0.462 ± 0.001
F20	86.1 ± 0.630	0.485 ± 0.002

Solubility Studies

According to the solubility studies, the solubility for LMT solid dispersions ranged between 0.46 mg / ml (1.6 fold increase) and 0.66 mg / ml (2.3 fold increase), and the highest increase was obtained in solid dispersions prepared with poloxamer 407 (1:6, drug: carrier). The results of the solubility study of LMT solid dispersions are presented in Table 4. The F8 formulation (prepared with poloxamer 407 at a ratio of 1:6), in which an approximately 2.3-fold increase in the aqueous solubility of LMT and a very high entrapment efficiency (98.2%) was achieved, was determined as the optimum solid dispersion. In general, higher solubility increase was obtained in solid dispersions prepared with surfactant carriers compared to those prepared with polymeric carriers. In addition, it was found that the solubility increases as the ratio of carrier in the solid dispersion increases, although it is not proportional. In a study, PEG

4000 and PEG 6000-based (1%, 2%, 5%, and 10% w/v) LMT solid dispersions were prepared using two different methods (melting and solvent evaporation method), and according to the solubility study data of the formulations, it was reported that PEG 6000 (10%) increased the solubility of LMT (0.17 mg/ml) more significantly (35-fold) compared to PEG 4000. These data were attributed to the greater solubility of PEG 6000 associated with the greater number of ether linkages [29]. In another study, a dissolution profile of approximately 1 mg/ml was obtained in the first 25 minutes with poloxamer 407-based LMT solid dispersions prepared by the melting method. The improvement in the solubility profile of LMT was attributed to the good surface activity properties of poloxamer 407. Furthermore, a linear relationship between the solubility of LMT and carrier concentration was reported [31]. According to the solubility study results, the obtained data are similar to the literature in that better solubility increases are achieved at higher carrier ratios.

XRD Spectroscopy Analysis

The crystalline structure of the formulation ingredients and the impact of formulation factors are ascertained using XRD patterns. The presence of sharp and repetitive peaks in the X-ray diffractograms of the formulation components indicates the crystalline state of the structure, while the absence of any sharp peaks is attributed to the amorphous state [10, 24]. The XRD diffractograms of LMT, poloxamer 407, and optimum solid dispersion are given in Figure 3. In this figure, the presence of a repeating peak pattern observed 13.96°, 17.78°, 25.34°, 26.14°, 26.56°, 27.69°, 28.24°, and 28.70° indicate the crystal structure of LMT (the percentage of crystallinity of LMT; 93.95 %) in line with the literature [25,32-34]. The XRD diffractogram of crystalline poloxamer 407 (the percentage of crystallinity of poloxamer 407; 95.87 %) was also observed to be similar to the literature, with sharp peaks in 9.04° and 23.20°, as well as peaks in 26.08° and 36.20° [35,36]. The decrease in the intensity of the sharp peaks of the LMT in the XRD diffractogram of the optimum solid dispersion and the decrease in the percentage of crystallinity (79.8%) calculated from the XRD analysis data, together with the DSC analysis data, supports partial amorphization of the LMT.

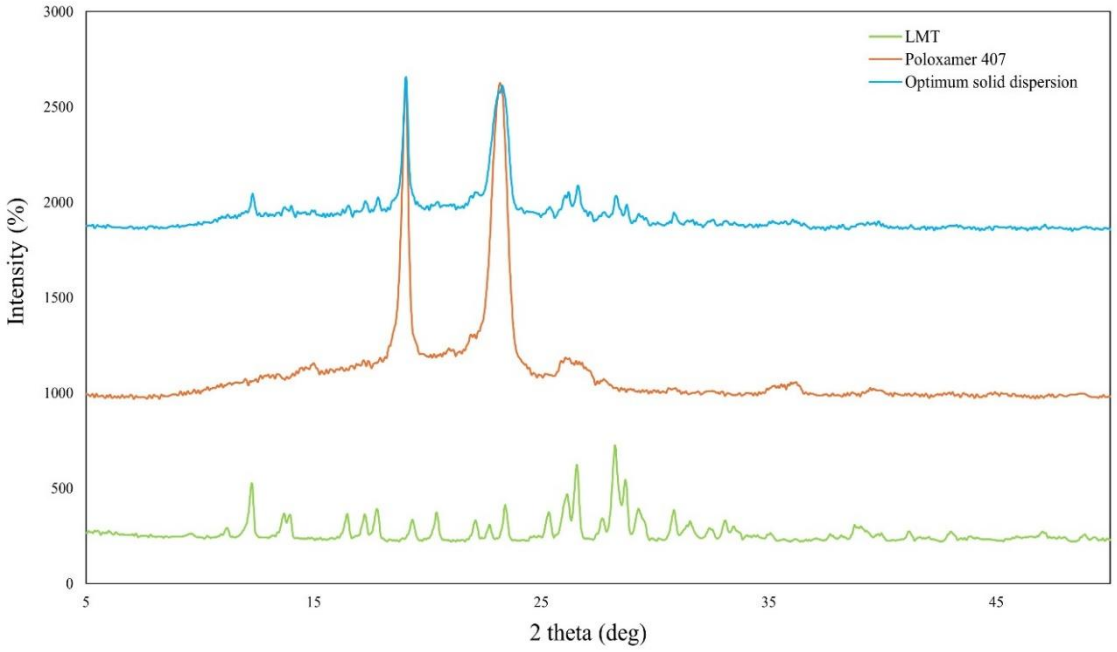

Figure 3. X-ray diffractograms of LMT, poloxamer 407, and optimum solid dispersion

DSC Analysis

The compatibility of excipients in a formulation and the potential effects of heat-treatment-based preparation techniques on the components can be elucidated using DSC analyses. Sharp peaks in DSC thermograms provide important information about the melting endotherms/exotherms of the ingredients [10,24]. The DSC thermograms of LMT, poloxamer 407 and optimum solid dispersion are shown in Figure 4. The DSC thermograms of poloxamer 407 and LMT showed a melting endothermic peak at 216.5°C and 55.8°C, respectively, and the enthalpy values (ΔH) were calculated 32.6 J/g and 44.4 J/g according to the data obtained from the DSC analyses. Our DSC results for poloxamer 407 and LMT are consistent with the literatüre [32,34,36-39]. However, the DSC thermogram of the optimum solid dispersion made using the melting method did not show the melting peak of LMT, suggesting that LMT was partially amorphized and covered with carrier during preparation of formulation.

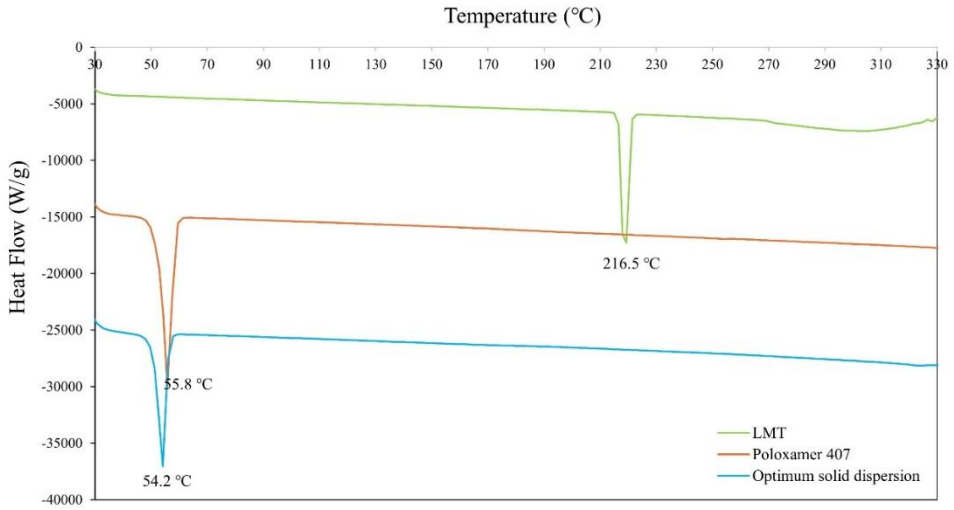

Figure 4. DSC thermograms of LMT, poloxamer 407, and optimum solid dispersion

FTIR Spectroscopy Analysis

The FTIR analysis allows the elucidation of functional groups and bonds in the structures of organic compounds, the presence of aromatic or aliphatic structures, and changes in the structure of two compounds after processing. The FTIR spectras of LMT, poloxamer 407, and optimum solid dispersion are given in Figure 5. The stretching bands of the functional groups in LMT supported the formation of this molecule: 3447.1 cm^{-1}, 3309.1 cm^{-1} and 3206.0 cm^{-1} (N–H), 1529.0 cm^{-1} and 1511.2 cm^{-1} (C=C), 1141.5 cm^{-1} (C–N), 789.2 cm^{-1} and 756.0 cm^{-1} (C–Cl), 716.4 cm^{-1} (C=H). The FTIR spectrum of poloxamer 407 showed distinctive peaks at 1097.4 cm^{-1}, 1341.3 cm^{-1}, and 2879.4 cm^{-1}, which have been found to correspond to the stretching of C-O (eter), O-H (in-plane O-H bend), C-H (aliphatic), respectively. FTIR spectra of LMT and poloxamer 407 showed characteristic peaks similar to those in the literature [32-34,36]. It has been determined that the FTIR spectrum of the optimum solid dispersion includes the stretching bands of LMT and poloxamer 407. However, only peaks attributed to poloxamer 407 were seen in the FTIR spectrum of the optimum solid dispersion. This was likely because the physical mixture's composition (LMT: poloxamer 407; 1:6) made it challenging to identify LMT signals.

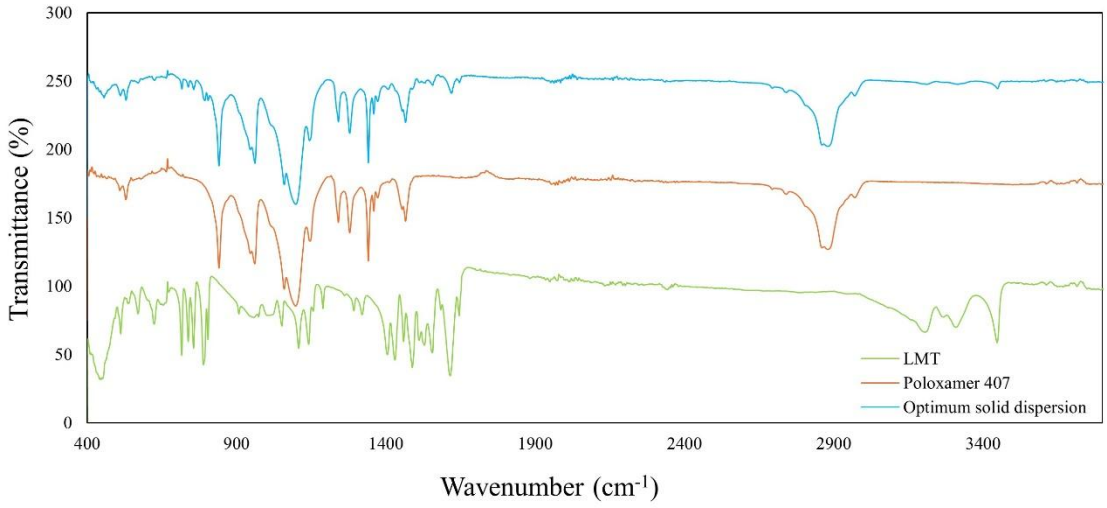

Figure 5. FTIR spectra of LMT, poloxamer 407, and optimum solid dispersion

SEM Analysis

The SEM images of LMT, polxomer 407 and optimum solid dispersion at different magnifications (1000x and 2500x) are illustrated in Figure 6. The SEM micrographs showed that LMT is in crystalline form with different particle sizes, while poloxamer 407 was observed as spheres of various sizes, smooth surfaces, pores, and irregular shapes. The SEM images of LMT and poloxamer 407 showed similar to those in the literature [38-40]. The SEM micrograph of the optimum solid dispersion reveals a smaller particle size, a more uniform, and homogeneous distribution. This indicates partial amorphization of the LMT within the solid dispersion, consistent with DSC and XRD analysis results.

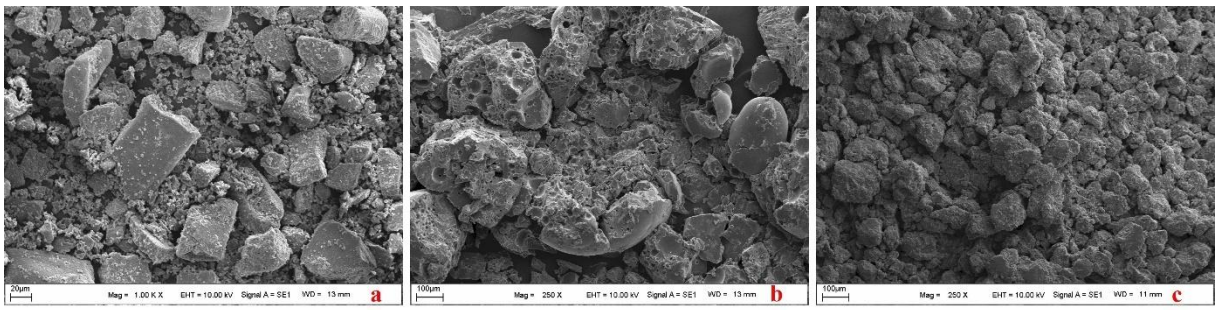

Figure 6. SEM images of LMT (a), poloxamer 407 (b), and optimum solid dispersion (c) (magnification of 1000x and 2500x)

In Vitro Cytotoxicity Studies of Solid Dispersions in A549 and RG-2 Cells

The cell viability results of RG-2 and A549 cells for LMT and LMT solid dispersions after 24 h incubation were summarized in Figure 7 and Figure 8. Dimethyl sulfoxide (DMSO) is an aprotic solvent used in the pharmaceutical field for drugs that are insoluble in water or have a low solubility profile and various *in vitro/in vivo* applications. It has been reported that DMSO concentrations greater than 10% (v/v) cause pore formation in the plasma membrane, leading to cell toxicity. It has also been emphasized that its use in low concentrations should be tested due to safety concerns [41]. Similar studies have reported that DMSO residuals do not cause significant cell toxicity if cell viability exceeds 80% at the DMSO concentrations used [10,24]. Therefore, the DMSO residue used in the preparation of stock

solutions of LMT and solid dispersions containing LMT in cell culture studies was tested by investigating the cell viability profile of DMSO controls. Even at the highest DMSO concentration (0.8%), cell viability was above 80% (92.8% for RG-2 cells and 80.6% for A549 cells), suggesting that DMSO did not have a significant toxic effect on the cells.

The cell viability on RG-2 cells decreased as the LMT dose increased. In a study conducted on colon cancer cells (Caco-2 cells) using the MTT assay protocol, the decrease in cell viability observed with increasing LMT doses (5, 10, 25, 50, 100, and 500 µM) is similar to our findings. [42]. However, for RG-2 cells, cell viability above 80% for all samples and all concentrations indicated that LMT solid dispersions did not have a significant antiproliferative effect on RG-2 cells in the selected concentration range. LMT is an anticonvulsant drug that inhibits voltage-gated sodium channels and reduces glutamate release. LMT has been reported to cause mild proliferation suppression in some cancer cells via voltage-gated sodium channel modulation and to suppress tumor growth by arresting the cell cycle through various signaling pathways, this is not observed in all tumor types due to differences in sensitivity among cells [43]. RG-2 cells are rapidly dividing, highly aggressive, and have strong resistance mechanisms (high P-glycoprotein (P-gp) expression, robust DNA repair mechanisms, and rich cancer stem cell-like subpopulation profiles) [44]. The lack of a significant antiproliferative effect on RG-2 cells may be attributable to their inherent resistance to weak antiproliferative agents such as LMT. Furthermore, these results related to the fact that LMT targets different pathways (voltage-gated sodium channels, glutamate release, N-methyl-D-aspartate (NMDA) receptor-associated pathways) in its antiproliferative effect profile, rather than the mechanisms that predominate in the proliferation of RG-2 cells (such as PI3K/AKT/mTOR, p53 mutations, and stimulating glomerular filtration rate (EGFR) overexpression). The cell viability for A549 cells was above 80% for nearly all samples and all concentrations except for the 40 µg/ml dose of solid dispersions prepared with PEG 6000 in a 1:2 ratio (76.2%), the 80 µg/ml dose of solid dispersions prepared with poloxamer 188 in a 1:2 ratio (73.9%) and the 80 µg/ml dose of solid dispersions prepared with poloxamer 407 in a 1:8 ratio (77.6%). For A549 cells, the decrease in cell viability as the dose increases suggests that an antiproliferative effect may be observed at higher doses. The relatively greater sensitivity of A549 cells compared to aggressive and resistant cells such as RG-2 cells may be related to the minimal effect observed at high doses. Therefore, further studies with increasing doses appear promising.

In vitro cytotoxicity studies have generally shown lower cell viabilities with polymeric carriers such as PEG 4000 and PEG 6000 compared to carriers with surfactant properties such as poloxamer 188 and poloxamer 407. This may be explained by some of the positive effects of poloxamers on cells. Studies have shown that poloxamer 188 temporarily integrates into damaged lipid regions in the cell membrane and stabilizes the cell membrane, reducing cell death [45,46]. Furthermore, poloxamers have been reported to support cell proliferation by reducing stress factors (such as oxidative stress, membrane tension, and protein denaturation) and increasing cell adhesion through surface modification. In contrast, it is known that PEG derivatives can affect cell volume by creating osmotic stress, leading to cell cycle arrest, and negatively affecting cell signaling pathways and cell-matrix interactions by altering the viscosity and macromolecular density of the medium [47,48]. These data suggest that poloxamer-based systems are superior in terms of biocompatibility in pharmaceutical designs. A549 cells are highly sensitive to osmotic imbalance and exhibit high dependence on glucose [49]. High-molecular-weight PEG derivatives, such as PEG 4000 and PEG 6000, affect lipids and integrins on the cell surface, leading to suppression of signaling pathways. Furthermore, they have been reported to disrupt osmotic imbalance within the cell, slowing the cell cycle, and impeding diffusion within cells due to their high viscosity, leading to reduced nutrient transport [50]. All this information explains the decrease in cell viability rates, especially in the high LMT dose and carrier ratio of PEG-based solid dispersions.

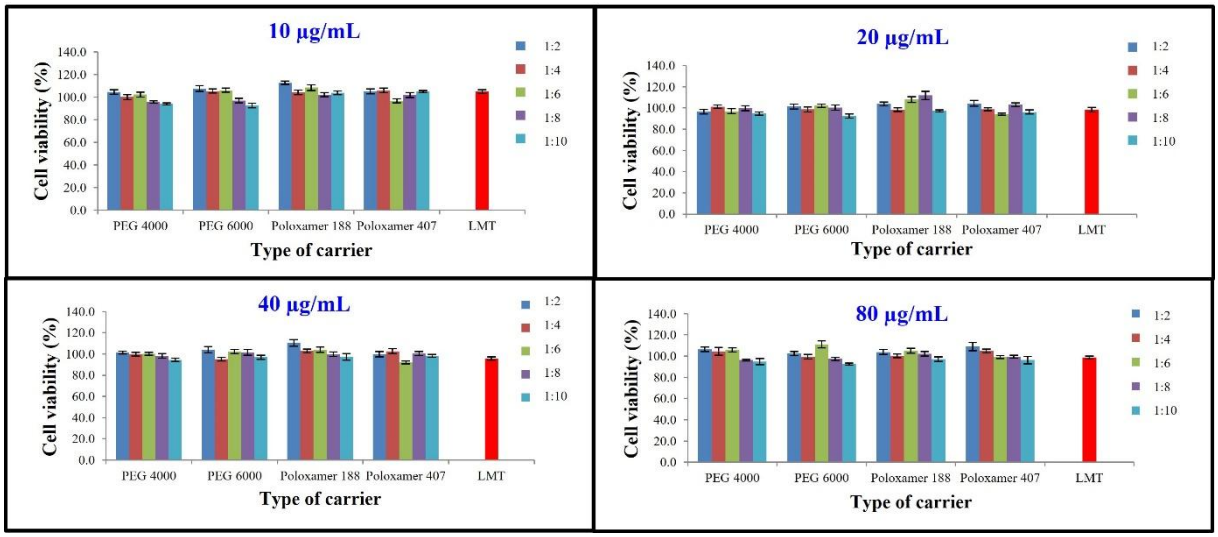

Figure 7. The cell viability results of RG-2 cells for LMT and LMT solid dispersions after 24 h incubation (Mean ± SE; n=3)

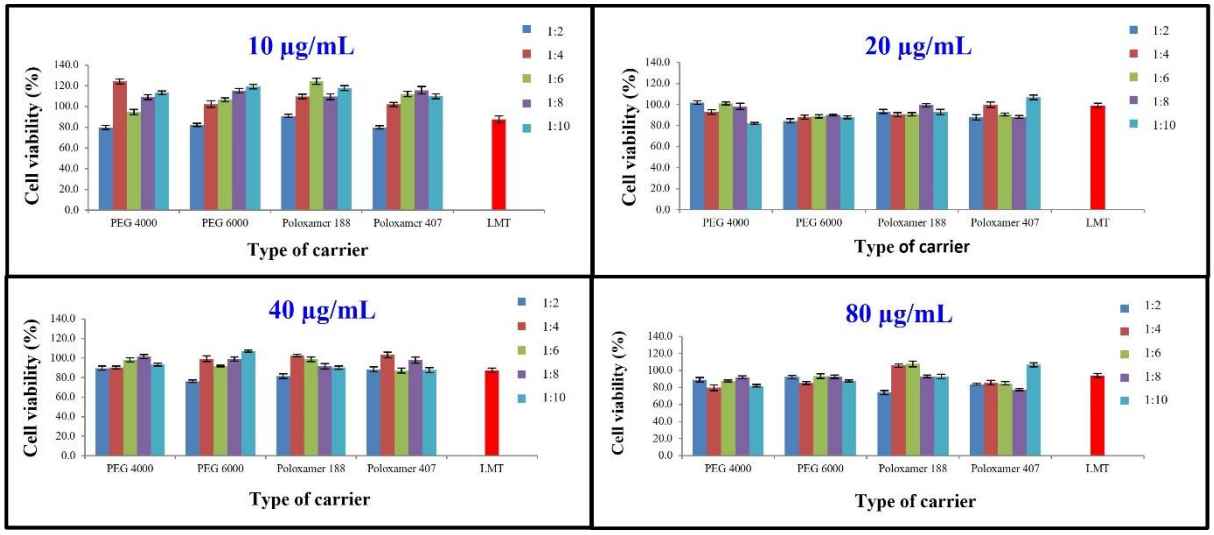

Figure 8. The cell viability results of A549 cells for LMT and LMT solid dispersions after 24 h incubation (Mean ± SE; n=3)

Conclusion

The advantages of lower cost and faster time to market compared to traditional de novo drug development have made drug repurposing popular in recent years. Current studies have focused on the possibility that some antiepileptic drugs, such as LMT, may be candidates for oncological drugs beyond their original indications, as they can improve neuropathic pain caused by oncological treatment and are effective on various cancer cells. However, low aqueous solubility seems to be a significant issue restricting the oral bioavailability of LMT. Therefore, in this study, we focused on the possibility of LMT being an oncological drug candidate for A549 and RG-2 cells by overcoming the problem of low aqueous solubility with the solid dispersion method. The studies have shown that the low aqueous solubility of LMT, which exhibits a pH-dependent solubility profile, can be increased statistically significantly with various polymeric and surfactant carriers (PEG 4000, PEG 6000, poloxamer 188 and poloxamer 407) by preparing solid dispersions according to the melting method, which is a very simple

and economical method. In general, higher solubility results were obtained with carriers with surfactant characteristics, and the highest solubility was obtained with the F8 formulation (solid dispersion using poloxamer 407 as carrier at a ratio of 1:6). Additionally, XRD, DSC, FTIR and SEM analyses were found to support the increase in solubility by correlating it with the partial amorphization of LMT. *In vitro* cytotoxicity studies have shown that polymeric carriers such as PEG 4000 and PEG 6000 generally lead to lower cell viability by creating osmotic stress in the cell, negatively affecting cell signaling pathways, and cell-matrix interactions. Carriers with surfactant properties, such as poloxamer 188 and poloxamer 407, have been observed to relatively support cell proliferation by stabilizing the cell membrane, reducing stress factors, and increasing cell adhesion. According to the data obtained, RG-2 cells maintained high cell viability at all concentrations. This demonstrated the strong resistance mechanisms of RG-2 cells and that the pathways affected by LMT do not coincide with the predominant proliferative mechanisms of this cell type. These results indicate the weakness of LMT as an effective antiproliferative agent for resistant cells such as RG-2 cells In contrast, the relatively higher sensitivity of A549 cells suggests that it may exhibit a dose-dependent antiproliferative effect, supporting the hope of repositioning the drug in the oncological field. In summary, these results demonstrate that a simple and scalable approach to improving the solubility of LMT has translational value that could support the drug's potential clinical use. Furthermore, this study lays the groundwork for more comprehensive future investigations of how the resulting solubility improvement will translate into *in vivo* pharmacokinetics and therapeutic effects, as well as the molecular mechanisms of antiproliferative activity and its effects in different tumor models.

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AUTHOR CONTRIBUTIONS

Concept: E.P.; Design: E.P.; Control: E.P.; Sources: E.P.; Materials: E.P.; Data Collection and/or Processing: E.P.; Analysis and/or Interpretation: E.P.; Literature Review: E.P.; Manuscript Writing: E.P.; Critical Review: E.P.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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