Novel Gamma linoleic acid encased in situ Lipogel for augmented anti-tumor efficacy against solid tumor: *In vitro* and *in vivo* evaluation

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Aim. The present investigation intended to develop novel thermo-reversible gamma-linolenic acid-liposome-hydrogel (GLG) as an in-situ depot system for intratumoralchemotherapy.**Methods.** Gamma-linolenic acid (GLA) entrappedliposome (GL) were prepared by ethanol injection method and homogenization. It wasoptimized using Box-Behnken design. To create the final GLG formulation, the GL was addedto a 22% w/v poloxamer hydrogel.The gel degradation tests,*in vitro* release studies and characterization were carried out simultaneously. In-depth *in vivo* biodistribution, anti-tumor efficacy,safety testing and histological investigation was done on DMBA-induced-solid tumor-bearing rats. **Results.** The optimized GL had a hydrodynamicparticle size of 104.2 ± 2.15 nm, PDI of 0.158 ± 0.0019, % EE 85.17 ± 2.05%, and %DL 12.65± 1.33 %. The TEM imaging revealed that GL had spherical shape, andwas uniformly dispersed. The final GLG formulation was adequately stable after 1 GLin poloxamer hydrogel because no noticeable changes in particle size, PDI, % EE,and gelation time were noticed. In addition, GLG demonstrated sustained in vitro drug releaseover 120 h. In vivo, intra-tumoral injection of GLG formed a depot that exhibited prolongedtumor retention, drug release lasting for 120 hours, and a special tumor inhibition ratecompared to free GLA and GL.

Keywords: Gamma Linolenic Acid, poloxamer, liposome, thermo-reversible hydrogel, intratumoraldepot.

1. INTRODUCTION

Oral or intravenous administration of, chemotherapeutic agents is limited by sub therapeutic concentrationat tumor site leading to systemic toxicity, non-specific drug distribution, and unwanted side effects [1-3]. Various factors associated with a tumor, like the type of tumor cells, tumor size, growth rate, location, and tumor microenvironment (pH, oxygen level, interstitial pressure), affect the percentage of drug reaching tumor mass [4]. Alternatively, direct tumoral administration of the chemotherapeuticagentis one of the methods for enhancing drug concentration at the tumor site [1,5]. Intra-tumoral delivery allows localized and augmented anti-tumor therapy [6]. Thetumor-specificitydeflects the cytotoxic drug from non-target organs, turn down systemic toxicity and improves drugefficiency [7]. One of the best possible methods to achieve direct into-tumoral delivery is through in situ gels fabricated through biodegradable andnon-biodegradable polymers [8].For the development of injectable in

situ gels and achieving sustained drug delivery at tumorsitethermosensitive polymerPoloxamer, hold great promise,[9]. Poloxamer 407 (P407), commonly known as Pluronic 127®, is a USFDA-approved temperature-sensitive polymer[10-13]. It possesses unique temperature-dependent phase transition properties. At room temperature (25°C) it behaves like a liquid with flowability, while at a temperature close to body temperature, it undergoes sol to gel transition with limited or no flowability [14-16]. In addition to phase transition properties, the biocompatibility, biosafety, and low immunogenic property of P407, make it an ideal polymer for drug delivery. Further, several reports exist stating the use of P407 in parenteral formulations [14-17]. Intra-tumoral injection of P407 sol will form a sustained release depot at the site of administration. However, a limitation of P407 gel is its low drug-loading capacity for hydrophobic drugs like Gamma-linolenic acid [10].

Gamma-linolenic acid (GLA), also chemically called cis-6,9, 12-octadecatrienoic acid) is a type of omega-6 polyunsaturated fatty acid (PUFA) [18-20]. Recently, omega-6 fatty acid has been implicated in the treatment ofcancer, arthritis, and inflammation.Also, its use in enhancing immunity, managing obesity, and regulating mood have been reported18-21].

GLA is most abundantly found in oil extracted from Oenothera (evening primrose) plant [19]. During the conversion pathway of linoleic acid into prostaglandins, GLA is formed as an intermediate by the action of the 4-6 desaturase enzyme on linoleic acid [22,23]. Reduced activity of this enzyme and GLA deficiency is found to be associated with many diseases like cancer, diabetes, skin disorder, and virus infection. Several reports showedthat the anti-tumor activityof GLA is mediated by the activation of macrophages**, free radical production, and lipid peroxidation leading to cell apoptosis**. Interestingly, GLA's mechanism of action is tumor-selective even at a large dose [18,24,25]. Unfortunately, poor biopharmaceutical properties of GLA restrict the delivery of GLA by intravenous route.

To facilitate the intravenous delivery of hydrophobic GLA, we selected liposomes ascarriers [26-27]. It is hypothesized that hydrophobic GLA is carried in the lipid bilayerof the liposome, which can coalesce with the cell membrane leading to the delivery of GLA. For sustained delivery, GLA-loaded liposomes were dispersed in thermosensitiveP407 gel.

Therefore, the present study is based on the hypothesis that localized intra-tumoral injection of GLA-liposome-loaded hydrogel will form an in-situ depot at the administration site; release the anti-cancer drug GLAin sustained manner at the tumor site. To the best of our

knowledge, this is the first report wherein , GLA-loaded-lipogel is prepared and; studied for in vivo anti-tumor activity, intra-tumoral release, and tissue distribution from in situ depot.

2. MATERIALS AND METHODS

2.1 Materials and animals

GLA (99.9%) was kindly gifted by Fermish Clinical Technology Private Limited (India). Lecithin was provided by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, Poloxamer 407 and 7,12-Dimethylbenz[a]anthracene (DMBA) were procured from Sigma Aldrich, USA. All reagents of analytical or chromatographic grade, were purchased from Qualigens Fine Chemicals (Mumbai, India), and used without further purification.

Albino Wistar rats, male (150g -200 g), were provided from the Central Animal House Facility, Jamia Hamdard, Delhi, India. The rats were housed at room temperature and humidity and had access to water and food *ad libitum*. All the experiments were performed as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA). The protocol isapproved by Institutional Ethical Committee of Jamia Hamdard, New Delhi (Approval No: 1777).

2.2 DEVELOPEMNT AND OPTIMIZATION OF GLA-LIPOSOMES (GL)

GLA-entrapped liposomes (GL) were prepared by ethanol injection method followed by homogenization. Briefly, lecithin (1%), cholesterol (0.1%), and GLA (0.1%) were dissolved in 3.0 ml of ethanol. Separately, 100 mg poloxamer (2%) was dissolved in 5 ml of distilled water with continuous stirring. The ethanolic phase was then rapidly injected into the aqueous phase under magnetic stirring using a 1 ml syringe attached to a 24-gauge needle. The liposomal suspension thus formed was stirred (1,000 rpm) at room temperature for 24 h. Finally, the suspension was homogenized with Heidolph Silent Crusher (Instruments S, Germany) at 15to 45 rpm for 1 to3 cycles. The formed liposomes were stored at 4°C until used.

Formulation parameters were optimized using 3^3 Box–Behnken design (BBD) (Design-Expert 11.1.0, State-Ease Inc., Minneapolis, USA). Fifteen experimental runs were suggested involving 12 factorial points and 3 replicates of the central point by defining the independent variables at three levels: low (−1), basal (0), and high (+1). The lecithin concentration used was 50 mg, 75 mg, and 100 mg; the homogenization cycles were 1, 2, and 3, and the homogenization speed was 15 rpm, 30 rpm, and 45 rpm. The responses were the size of

liposome vesicles R1 (nm), PDI (R2), and drug loading (%, R3). For optimized formulation, the response value was compared with the value predicted.

2.3 DEVELOPMENT AND OPTIMIZATION OF GLA-LIPOSOMAL -GEL (GLG)

The thermo-reversible, GL-loaded poloxamer hydrogel was preparedby modifying the "cold method" as described previously [10]. The poloxamer solution was prepared by dissolving 2.2 g of poloxamer in 10 mL PBS (pH=7.4) at 4°C for 12 h through continuously stirring. . For preparation of GLG,the GL suspension was dispersed in the poloxamer solution with continuous stirring at 4°C. The final formulation(GLG) was stored in a refrigerator (4°C), until used.

2.3.1 Optimization of GLG and Gelation Time Determination

. The concentration of poloxamer (18%w/v-24% w/v) was optimized by determination of gelation time at 25°C (room temperature), 37°C (body temperature), and 40°C (tumor temperature). Briefly, the aqueous poloxamer solution was stirred at 100 rpm at different temperatures in separate glass vialsand the time taken by a magnetic bead to stop revolving was observed as gelation time. Gelation time was also determined for GLG formulation. (Table 1).

2.4 IN VITRO CHARACTERIZATION OF GL AND GLG

The mean particle size and polydispersity index (PDI) of GL and GLG were determined by dynamic light scattering using zeta sizer (Malvern Zetasizer, Nano ZS, UK). The hydrodynamic diameter and PDI analysis were observed at a 90° scattering angle ata temperature of 25 ± 1 °C. The size and morphology were determined by transmission electron microscope (Tecnai, G20, FEI, Holland) and scanning electron microscope (SEM, JEOL, JSM6390LV, Tokyo, Japan) for GL and GLG, respectively. The GL suspension was mounted over formvar-coated copper grids, fixed with phosphotungstic acid (2% for 20 s), air dried, and then analysed by TEM. The lyophilized GLG sample was gold coated (ABG7340, Agar sputter coater, Agar scientific, UK) with sputter using argon gas at 0.5 bar pressure and analysed by SEM at 20 kV, resolution of 3 nm. The pH and viscosity of the gel were determined with the help of pH meter (Eutech pH Tutor, Effem Technologies, India) and a Brookfield viscometer (DV II + Pro, US). The gel was placed in the cup and maintained at a temperature of 40 ± 1 °C using a water bath. Viscosity was determined after applying shear at a rate of 60 rpm using 64-number spindles.

2.5 HPTLC ANALYSIS

The GLA in GL suspension and GLG was quantified using high-performance thin layer chromatography (HPTLC) method developed by our group [28]. Briefly, GLA concentration was determined using hexane: toluene: formic acid as a mobile phase in the ratio of 3:7:1.5 (v/v/v). Prior to analysis aluminium TLC plates (precoated with silica gel 60, Merck, Darmstadt, Germany) were activated in a hot air oven at 120 °C for 10 min. Then, samples were applied as 6 mm bands onto plates (20 cm × 10 cm) by using a 100-µL syringe , through a sample applicator (CAMAG Linomat V,Muttenz, Switzerland), with a 150 nL/s steady application rate, under a constant flow of N_2 gas. For GLA, a CAMAG TLC scanner III was used in the mode of reflectance absorption for scanning the developed plates at a wavelength of 200 nm using a tungsten (W) lamp. (CamagMuttenz, Switzerland). Rf value was 0.61 ± 0.05 for GLA. The linearity was found in the range of 1µg/spot to 3 µg/spot with correlation coefficients, intercepts, and slopes of 0.9138, 0.0547and 380.051, respectively. The LOD & LOQ were found to be 0.291µg/band and 0.725µg/band.

2.6 ENTRAPMENT EFFICIENCY AND DRUG LOADING OF GL AND GLG

For EE and DL the liposomes were separated by centrifuging the GL and GLG at 40,000 rpm for 60 min (Beckman Coulter, Optima TM LE-80K ultracentrifuge) to precipitate out liposome pellet. The supernatant was separated using micropipetteand the obtained liposome pellet was dissolved in ethanolandthe GLA concentration entrapped in the liposome was determined by the developed HPTLC method. The EE% and DL% were calculated using equation 1 and equation 2, respectively:

$$Entrapment\ Efficiency\ (\%) = \frac{Amount\ of\ GLA\ present\ in\ liposome}{Total\ Amount\ of\ GLA\ Added} * 100 \quad (1)$$

$$Drug\ Loading\ (\%) = \frac{Amount\ of\ GLA\ present\ in\ liposome}{Total\ weight\ of\ liposomes} * 100 \quad (2)$$

2.7 IN VITRO RELEASE STUDY OF GL AND GLG

In vitro drug release from GL and GLG, was determined using the dialysis membrane method. A pre-activated dialysis membrane with a 12kDa molecular weight cut-off was used to load an aliquot of GL and GLG samples, separately. The dialysis membrane containing the GLG sample was stored at 37°C for 1 min to induce gelation. Each GL and GLG sample containing dialysis bags were then suspended in flat bottom glass beakers containing 15 ml of receiving media pre-equilibrated at 37°C. The media was composed of PBS (pH 7.4) having

0.1% (v/v) tween 80 to maintain sink conditions. The glass vials were placed and shaken in a thermostatic shaker at 37°C at 40 ± 10 rpm. Samples (1 ml) were withdrawn at predetermined time points over 72 hours and replenished with the same amount of pre-warmed (37°C) fresh media every time. The concentration of GLA was estimated by the developed HPTLC methodas per the equation given below(3):

$$E(\%) = \frac{V_E \sum_{n}^{n-1} Ci + V_O C_n}{m_o} \quad (3)$$

Where E (%) stands for the percentage accumulated release, and V_E and V_O are the sampling volume (1ml) and the initial volume (15 ml), respectively. Ci and Cn are the drug concentrations at the ith and nth sampling time points. Finally, Mo is the total mass of the drug in the gel. In vitro drug release determination was done in triplicateby HPTLC and expressed as mean ± SD (n = 3).

2.8 IN VITRO GEL DEGRADATION STUDY OF GLG

The rate of hydrogel degradation was estimated by taking 2.5 ml of GLG solution in a test tube and storing it at 37℃ for 1 min until gel was formed. At first, the initial weight of the test tube before and after putting gel was recorded. The difference in weight was considered as 100% gel weight. Then, 5ml of PBS having 0.1% (v/v) Tween 80 was layered over the gel, and the entire liquified material was collected at regular intervals (12 h, 24 h, 48 h, 72 h, 120 h) corresponding to in vitro release time points. After collection, at each time point tube was replenished with fresh 5ml PBS. The change in weight of the test tube and the changes in the gel- PBS interface layer was observed at predetermined time points to estimate the hydrogel's degradation rate.

2.9 PHYSICAL STABILITY OF GLG

The stability of the GLG was determined by keeping the sample at 5±3°C in refrigerator for 90 days. The parameters like mean particle size, PDI, EE%, and gelation time were determined after stipulated time

2.10PRECLINICAL STUDIES

2.10.1 Induction of Tumor

The in vivo efficacy of the GLG was assessed in male albino Wistar rats (6 weeks old, 100-200 g). All the rats were kept at a temperature of 25±2°C and 55±5% relative humidity with 12 h light/dark cycles throughout the experiment. The rats were given a standard commercial

diet with tap water ad libitum and free access to movement, food, and water. Solid tumor in the rats was induced by subcutaneous injection of 7,12 Dimethyl Benz[a]anthracene (DMBA) in the right hind leg of the rat at a dose of 250 mg/kg. The DMBA solution was prepared by dissolving weighed quantity in a mixture of sunflower oil and acetone in equal proportion [29,30]. Rats were examined daily for tumor growth, and the treatment was initiated once tumor reached a volume of 150- 200 mm^3.

2.10.2 Intra-Tumoral Retention and Biodistribution

GLG depot residence time and Intra-tumoral GLA assay were performed in rats. The Wistar rats were weighed and randomly divided into three groups (n=3), and single intratumoral injections of free GLA suspension, GL, and GLG (having GLA equivalent to 10 mg/kg) were administered intra tumoral. The GLA suspension was prepared in 1:1 ethanol: PBS (pH 7.4) mixture. The tumor was excised at pre-designated time points viz. 4, 6, 12, 24, 48, 72, 96, and 120 h, selected based on the in-vitro release pattern of GLG. The tumor mass was weighed, minced, homogenized in PBS: ethanol mixture, and analysed by HPTLC method as mentioned above in section 2.4.

2.10.3 Biodistribution Studies

For biodistribution study, organs like heart, liver, spleen, kidney, and blood from cardia/c +puncture were collected from sacrificed animals at different times (4, 6, 12, 24, 48, 72, 96, and 120 h). Homogenized in PBS (pH 7.4) & ethanol mixture and analysed by HPTLC method; to determine the drug distribution pattern from GLA suspension, GL, and GLG.

2.10.4 Tumor Regression and Safety

The Wistar rats were weighed and randomly divided into four groups (n=3). Formulations intra-tumoral 200 µl of blank gel (control), free GLA suspension, GL and GLG (10 mg/kg GLA equivalent) were administered using a 1ml syringe. Post-treatment, the parameters like tumor volume and body weight were monitored for 14 days to assess treatment efficacy and safety. Tumor volume was calculated by equation 4:

$$Tumor\ volume\ (mm3)\ =\ [Length\ X\ Breadth2]\ /2 \qquad (4)$$

where the largest and smallest dimension of the tumor was considered as length and breadth (in mm), respectively. At the end of the study, tumor weight was determined by dissecting the tumor-bearing and standard legs, and the difference between the weights of the two legs was observed as tumor weight. During the experiment period (14 days), any changes in the pattern of each rat's food, water consumption, behavior, and body weight were monitored to evaluate

any possible toxic effects of the treatment [31]. Data obtained was interpreted by plotting tumor volume and body weight against time.

2.10.5 Histopathology

At the end of treatment, heart, liver, kidney, spleen, and tumor of the rats from all the groups were selected for histology observation. The organ/tumor was dissected, washed in PBS (pH 7.4), and fixed in 4% paraformaldehyde solution overnight, and then the sections were stained with haematoxylin and eosin (H&E) for histological analysis.

2.11 STATISTICAL ANALYSIS

The data were statistically analysed using IBM SPSS Statistic version 20 software. Values from the experiment were expressed as the mean and standard deviation in triplicate (SD). One-way analysis of variance was used to analyse the data, followed by the Turkey posthoc test. The statistical inference level was set at (* p 0.05), (** p 0.01), and (*** p 0.001) for significance

3. RESULTS

3.1 DEVELOPMENT AND OPTIMIZATION OF GL AND GLG

The GLA-Liposomes were synthesized by the combination of solvent injection and homogenization methods and optimized by Box- Behnken design. R1, R2 and R3, terms were analyzed and gave a "Predicted R2" value of 0.8279, 0.8965, and 0.8625 and "Adjusted R2 "values of 0.9591, 0.9727, and 0.9619. The following equations showed the quantitative effect and their interaction which affect the response of process variables.

Particle size = +136.067 -2.5625 * A + 2.45 * B + 10.4625 * C -8.2 * AB + 10.325 * AC -6.2 * BC -26.2458 * A2 + 1.92917 * B2 + 30.7042 * C2 (5)

PDI = +0.193333 -0.012 * A + 0.00175 * B -0.001 * C -0.02875 * AB -0.00625 * AC + 0.01025 * BC + 0.0147083 * A2 + 0.0252083 * B2 + 0.0287083 * C2 (6)

Drug loading = +11.3667 -2.1875 * A -1.4 * B + 1.6125 * C + 5.35 * AB + 2.075 * AC -2.9 * BC + 5.02917 * A2 + 1.90417 * B2 + 5.82917 * C2 (7)

where A, B and C represent the various independent variables. Lecithin conc. showed a negative outcome on the equations. In equation 1 and equation 2, the homogenization cycle had a positive effect, but equation 3 had adverse effects. The size of the particle and PDA considerably decreased with increasing the homogenization cycle, but the opposite response was in drug loading. The increase in homogenization speed caused an increase in the size of

particles. A significant decrease in PDI was detected on the rise in homogenization speed and the same in drug loading. With the help of these optimization studies, we observed that various independent factors on the response and a desirability function, as shown in Figure 1. For the optimized formulation, particle size, which is min in range, PDI, which is maximum in range, and maximum drug loading were carefully chosen. For proper validity, every formulation was made in triplicate. The optimized GL was formed using 75mg of lecithin, 2 homogenization cycles at a homogenization speed of 15rpm, and had a particle size of 104.2 ± 2.15 nm (predicted 140.51), PDI of 0.158 ± 0.0019 (predicted 0.220) and drug loading percentage of 12.65 % (predicted 12%); Table 2, Figure 3(A). The nanometer size of 104 nm of GL was also evident from the TEM image, Figure 3(B).

Gelation time was observed for different concentrations (18% w/v, 20% w/v, 22% w/v, and 24% w/v) of P407 blank hydrogel at three different temperature such as room temperature (25°C), body temperature (37 °C), and intra-tumoral temperature (40 °C), as shown in Table 2. At room temperature, 18% P407 gel failed to exhibit sol-gel transition, thus it was excluded. Gelation time at room temperaturewas 324 ± 6.5s, 269 ± 5.4 s, and 107±3.2 for 20%, 22%, and 24% P407 gel, respectively. While at body temperature, gelation time was found to be 32 ±1.0 s, 25 ±1.5, and 22 ±0.5 for 20%, 22%, and 24% P407 gel. The gel prepared from 22% P407 concentration showed optimum results. It remained fluid at RT long enough to allow ease in handling and administration; while undergoing quick sol-to-gel transition at body and tumor temperature (25 ± 1.5 s and 23 ± 0.5 s). On further loading, 22% P407 with GL did not show significant changes in gelation time at RT (252 ± 2.0 s), body temperature (23 ± 0.65 s), and 40°C (22 ± 0.82). Figure 2, showed sol to gel transition of optimized GLG.

3.2 IN VITRO CHARACTERIZATION OF GL AND GLG

The particle size measurements (DLS) of GL and GLG are shown in fig 3(A,C). The mean particle size of GL and GLG was 104.2 ± 2.15 nm and 186.7 ± 1.8 nm, respectively whereas PDI was 0.158 ± 0.0019 and 0.384 ± 0.0015 respectively.The slight increase in particle size as observed for GLG was due to the adsorption of water on the liposome micelle surface in the hydrogel. The TEM image, Figure 3 (B), showed that GL had a particle size of 118nm and was mono-dispersed and spherical. The viscosity of the gel formulation was found to be 2894 cps at 37°C temperature and 1112 cps at 25°C. The GLG showed a pH of 5.8 at room temperature as well as body temperature.

3.3 ENTRAPMENT EFFICIENCY AND DRUG LOADING OF GL AND GLG

The % EE forGL and GLG was found to be 85.17 ± 2.05% and 82.74 ± 1.62 %, respectively. Also, % DL was calculated and found to be 12.65 ± 1.33 % and 10.23 ± 0.95% for GL and GLG, respectively. The insignificant difference in EE and DL showed absence of drug leakage from liposomes.

3.4 IN VITRO DRUG RELEASE STUDIES OF GL AND GLG

The % cumulative release of GLA vs. time from GL and GLG is shown in fig 4.,Incomparision to GL, GLG exhibited more sustained release.Release of GLA was 77.4 ± 1.3% from GL and and 54.22 ± 1.8% from GLG at 24 h.

3.5 IN VITRO DEGRADATION OF GLG

Initially, clear phase interphase was observed between gel and PBS, but over time with gel degradation, the interphase became intermixed and turbid. Figure 5 showedat 48 h nearly 40% of the gel was degraded and about 86% gel degradation was observed by day 5 further resulting in disappearance of interphase.. This in vitro degradation behavior of thermosensitive poloxamer gel established gel could degrade in vivo.

3.6 PHYSICAL STABILITY OF GLG.

Following storage for 90 days at refrigerator no significant changes in gelation time was observed, (compared with original oe without storage).It showed sol-gel transition in 265 ± 4.039 s at 25 °C and 30 ± 2.5.s at 37 °C as gelation time. Further insignificant changes were observed in mean particle size (132.4 ± 2.3%), PDI (0.248), and EE% (80.6%). After storage for 90 days, no significant changes were observed in the assessed parameters.

3.7 PRECLINICAL STUDIES

3.7.1 Intra-Tumoral Retention and Biodistribution Studies

Following single intra-tumoral injection of GLA, GL, and GLG, the cumulative residual drug percentage at the tumor site was determined at pre-designated time points. The resident time of GLA suspension at the tumor site was upto 24h, after which none could be recovered from the tumor site. At 48 h, the total residual drug at the tumor site was observed to be 10.5% ± 1.9% for GL and 46.7% ± 1.2 % for GLG Figure 6.. At the same time, it was discovered that the GLG depot residence period was the longest of all 120 hours, with an estimated 12.6 ± 2.9% drug at the tumor.

The results of the biodistribution studies conducted in Wistar rats is shown in Figure 7. Data clearly showed that the blood clearance and RES uptake of GLA from suspension and

GLwere rapid compared to GLG. At 4h, following single intratumoral injectionof GLA suspension, the highest percentage was observed in the liver (29.87 ± 1.5%), followed by the kidney (4.8 ± 0.15 %) and spleen (5.1± 0.5%). Thus, free GLA suspension showcased hepatic metabolism and renal clearance as a major elimination route. In the case of GL, a significant uptake of GLA was observed in spleen (14.5 ± 0.2 %) and liver (10.6 ± 3.4%) at 4h and throughout tumoral residence time. However, in the case of GLG , the GLA percentage was small in liver (1.1 ± 1.02%) and kidney (0.5 ± 0.15%), inconspicuous compared to GLA suspension and GL. Also, insignificant amount of GLG was observed in the spleen (0.42 ± 0.04%, 4 h), indicating non-significant RES uptake after intratumoral administration of GLG. The GLA and GL showed slight uptake in heart tissue, but interestingly GLA was absent in heart following GLG administration. GLG showed 3-fold and 4-fold higher tumor to blood ratio of GLA compared at 24h (GLG:9.5 ± 0.5 %, GL: 3.3 ± 0.44% and GLA: 2.5 ± 1.1%). As observed in Figure 7f, the tumor-to-blood ratio of GLG was constantly higher compared to GL and GLA at all designated time points, which signifies prolonged drug release at the tumor site from the gel depot and lower off-target drug uptake.

3.7.2 Tumor Regression and Safety

The anti-tumor effect of the GLA, GL, and GLG asthe average tumor volume vs function of time, was showed in Figure 8a. All the 3 formulations exhibited a significant decrease in tumor volume compared to the control ($p < 0.05$, $p < 0.01$, $p < 0.001$). At day 14 , the tumor volume was found to be lowest ($p > 0.05$) for the group treated with GLG (698.198 mm^3), followed by GL (1808.45mm^3), and GLA (2498.56mm^3). The decrease in tumor volume for GLG was highest, 3.9 folds ($p > 0.01$) compared to control group... As shown in Figure 8b, at the end of 14 days, a 7.3% and 18.3% reduction in tumor weights were noted for GLA and GL, respectively, ($p > 0.001$) than the control. The GLA and GL showed a significant increase in tumor weights ($p > 0.05$) compared to GLG. Whereas GLG showed a marked 59.7% significant reduction ($p > 0.05$) in tumor weight compared to the control. The body weights of rats were measured throughout the experiment, Figure 8c. A relative increase of 4.4 %, 2.6%, 2.4%, and 0.6% in the body weights of rats were observed for blank gel (control), GLA, GL, and GLG, respectively. Comparing the three treatment groups, the relative change in body weight of GLG treated group was insignificant. The percent change in body weights for GLA and GL was also found to be significantly lower than the control group ($p > 0.001$), but the values against GLG were significantly higher ($p > 0.001$). From

the above results obtained for change in tumor volume, tumor weight, and body weight, it was concluded that GLG showed the highest anti-tumor activity in solid tumor-bearing rats.

3.7.3 Histopathology

To asses safety of treatment the histology of the spleen, liver, kidney, heart, and tumor was performed. As evident (Figure 9), following the administration of GLA, GL, and GLG, no morphological alterations were seen in either liver, kidney, or heart tissue, indicating absence of drug toxicity. Further, spleen histology indicated that GL conferred the highest RES uptake compared to all groups. The tumor cross section of control demonstrated (Figure 10) a vast population of proliferating nuclei and densely packed tumor cells. A decrease in the number of nuclei and a widening interstitial space between cells were observed in the tumor cross section of all the treatment group.Neverthless, a maximum decrease in tumor cell density and increase in interstitial space between cells were observed in GLG treated group.

4. DISCUSSION

Recently, the use of GLA in management of tumour has been widely published [18, 24, 25]. However, precise delivery of GLA to tumor is challenging and scarcely attempted and is the primary objective of the present research.

To achieve localized and sustained delivery of GLA, we formulated in situ gel using poloxamer. Since the addition of hydrophobic GLA to poloxamer gel was difficult, therefore GLA was loaded into liposomes and the formed liposomes were then incorporated into poloxamer gel. The GL was prepared by solvent injection and homogenization method following the addition of organic phase consisting of cholesterol, lecithin and GLA to 2% P407aqueous solution. The 2% P407 not onlyimproved the loading of GLAbut alsoimproved the stability of the liposomes by acting as a wetting and stabilizing agent. [36-38]. Utilizing the Box-Behnken design, the lecithin concentration, number of homogenization cycles, and homogenization speed was optimized for obtaining liposomes with smallest particle size, PDI, and maximum drug loading. The formulation developed using 75 mg lecithin processed for 2 homogenization cycles at 15 rpm yielded optimum GL with size, 104.0± 2.15 nm; PDI, 0.158 ± 0.0019; and DL,12.65%.The formation of small sized and uniform GL, was confirmed by TEM image. The optimized GL was then added to the P407 aqueous solution to form GLA-Liposome-Gel(GLG). The phase-transition property of poloxamer is concentration dependent [39-41] and thereforeThe ideal P407 concentration was determined through measurement of gelation time of different concentrations of P407 (18% w/v, 20% w/v, 22% w/v, and 24% w/v) at RT, body temperature, and intra-tumoral temperature. The

P407 concentration of 22% w/v was selected since it showed slow phase t.ransition (269± 2.5 s)at 25°C and rapid phase transition at body temperature(25±1.5 s) and tumor temperature (23±0.5 s). This allows easy handling and administration as intra-tumoral injectionfollowed by quick conversion into gel at tumor site allowing sustained release of GLA. ,Further, we found that addition of GL to 22% w/v P407 did not caused significant changes in gelation time.The results of the dynamic light scattering of GLG showed a slight increase in particle size of liposomes, which might be attributed to the hydration of outer PEO layers surrounding liposomes. Interestingly, incorporation of liposomes into gel did not alter the DE efficiency and ruled out the probability of drug leakage from entrapped liposomes.The same was apparent from the SEM image of GLG, which showed smooth surface, free fromdrug precipitation.Using a dialysis membrane technique the in vitro release of GL and GLG was studied. Within 48 hours of the investigation, the drug in GL released rapidly (97.2 ± 4.4.0% total cumulative release) from the lipoid membrane of the liposome. However, GLG demonstrated continuous drug release for up to120 hours with a 92.65 ± 2.5% total cumulative release. The suggested mechanism of drug release from GLG is gradual drug release from liposome micelles into the layers of poloxamer hydrogel surrounding them. Each poloxamer monomer consists of two outer hydrophilic units of polyoxymethylene (PEO) and one inner hydrophobic unit of PPO [41]. This hydrophobic PPOunit retains the liposome and aids in forming micelles within the poloxamer matrix. The surrounding hydrogel then serves as a second barrier to drug release and releases the drug gradually as the gel erodes or dissolves [42].

When physiological fluids dilute the gel, it breaks down and forms PEO-PPO-PEO free chains, isolated liposome micelles, or tiny micelle aggregates [43]. These broken fragments can further uphold the drug for longer and extend the drug release from the hydrogel matrix. This maintains the drug's sustained release at the tumor location [41,44]. In a test tube, gel degradation was carried out in the presence of PBS (pH 7.4) to anticipate the behavior of gel breakdown in vivo. It revealed that 14.4± 3.1% gel was still present after 120 hours. Thus, 15% of the gel maintains its integrity and supports the sustained release of the drug till 120h. Furthermore, thelyophilized GLG demonstrated stability after 90 days of storage at 25 ± 2 °C and 60± 5% RHas it showed non-significant changes in particle size, PDI, % EE, and gelation time. In vivo intra-tumoral retention studies, biodistribution, anti-tumor effect, and safety studies were carried out on DMBA-induced solid tumor-bearing Wistar rats [29]. The tumor retention result of GLG was in corroboration with its in vitro sustained release profile and demonstrated the presence of a total 16.6 ± 3.1 % drug in the tumor site after 120 hours. This

manifest in vivo prolonged retention and release of drug from GLG depot up to 120h. However, very lowtumor retention was observed for GL (up to 48 h) and GLA (up to 24 h. It was ascribed to their quicker systemic uptake and clearance from the body compared to GLG, as evidenced by biodistribution studies. At all the study's time points, the average GLG tumor retention was roughly 2.7 times higher than GL. The larger tumor-to-blood ratio of GLG than GL (~ 3.1-fold) at each indicated time point further demonstrated the substantial tumor retention of GLG,Figure 6. It is attributed to in situ depot formation and, thereby, sustained release of drugs fromit.

In biodistribution studies, after a single intra-tumoral injection of formulations, accumulation of GLA and GL was seen in the heart and lung. GLG, however, did not exhibit any such off-tumor accumulation. GLG showed renal clearance with limited hepatic metabolism as itsprimary elimination method. This is consistent with literature that identified renal clearance asthe primary method of elimination for poloxamer formulations [36]. For GLA, hepaticmetabolism and renal clearance was the main route of elimination. A comparatively highpercentage of GL was observed in the spleen. This explains the rapid clearance of GL within48 h only. While GLG showed noticeably reduced RES and systemic uptake. As a result, GLGshowed slower clearance and increased tumor retention and concentration compared to GL andfree GLA solution.

Anti-tumor efficacy among free GLA, GL, and GLG was compared by evaluating tumorvolume and weight in solid-tumor-bearing Wistar rats [3]. The rats treated with GLG showed nearly 4-fold reduced tumor growth compared to than control group. Similar efficacyresults were obtained in terms of mean tumor weight. This enhanced efficacy as observed for GLG, might be due to targeted and controlled delivery of GLA to tumor sites, which was certainly not seen in free GLA and GL group. Body weight loss was not observed in any of the study group , indicating high biosafety of Gamma-linolenic acid fornormal somatic cells. Following the administration of GLA, GL, and GLG, the histologicalstudy of the heart, kidney, and liver at 24 hours demonstrated that no degenerative changesoccurred in the respective organs. Compared to GLA and GL, the GLG-treated tumor histologyresult showed a significant reduction in tumor cell density and, thus, the highest anti-tumorefficiency. These outcomes can be linked to the in situ GLG depot's ability to maintain a highdrug concentration at the tumor for an extended period due to its longer tumor residency time.

5. CONCLUSION

Novel gamma-linolenic-acid-liposome-hydrogel was successfully developed andevaluated following intra-tumoral administration in DMBA-induced solid-tumor-bearing rats. Up on intravenous administration, the GLG formed in-situ drug depot with sustained release of GLA for about 120 hours resulting in enhanced antineoplastic activity as determined through preclinical studies. It showedencouraging outcomes as a therapeutically effective drug delivery system for localchemotherapy. However, further investigations are required to develop it for human use and to determine its safety and efficacy on long-term use .

Author Contributions:

Suma Saad- Conceptualization, Methodology, Validation, Writing - Original Draft, Data Curation.Lubna Siddiqui- Conceptualization, Nazeer Hasan- Resources, Sarwar Beg- Software, Resources, Asgar Ali- Supervision, Anshul Gupta- Writing - Review & Editing, Gaurav K Jain- Supervision, Farhan J Ahmad- Supervision, Conceptualization, Resources, Project administration. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Data will be made available on request

Conflicts of Interest

No conflict of interest

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Abbreviations

GLA- Gamma Linolenic Acid

GL-Gamma-Linolenic Acid (GLA) entrapped liposome

GLG-Gamma-Linolenic-Acid-liposome-hydrogel

TEM-Transmission Electron Microscopy

SEM-Scanning Electron Microscope

DMBA-7,12-Dimethylbenz[a]anthracene

EE-Entrapment Efficiency

DL- Drug Loading

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