RESEARCH ARTICLE

Multipronged, strategic delivery of paclitaxel-topotecan using engineered liposomes to ovarian cancer

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Abstract

Context: Synergistically active combinations have been used to enhance therapeutic efficacy for ovarian cancer chemotherapy.

Objective: The synergistically active combination of paclitaxel-topotecan (Pac-Top; 20:1, w/w) were loaded into folate-anchored PEGylated liposomes (FPL-Pac-Top) for safe and effective treatment of ovarian cancer.

Materials and methods: Coupling reactions were carried out using carbodiimide chemistry and confirmed by infrared spectral analysis. These liposomes were studied for shape and physical interaction (and integrity), in vitro drug release kinetics, hemolytic toxicity, ex vivo pharmacodynamics in OVCAR-3 cell lines, and pharmacokinetics in ovarian tumor-bearing mice.

Results: The differential scanning calorimeter studies exhibited melting of liposomes (~150 nm) at ~41 °C. The drug(s) release from liposomes followed Fickian diffusion model. The hematology studies revealed insignificant toxicity to blood cells. In vivo studies showed long circulatory behavior (increased AUC0–t and AUMC0–t) and selective accumulation of FPL-Pac-Top in the ovaries. FPL-Pac-Top showed less necrosis and more apoptosis in flow cytometry. Kaplan–Meier survival analysis revealed the doubling of the survival time with FPL-Pac-Top in comparison to Pac-Top solution.

Discussion and conclusion: Potentiated anti-cancer activity of FPL-Pac-Top was attributed to multiple features viz. thermosensitivity, long circulatory nature and targetability. Such approach could be a paradigm chemotherapeutic approach for safe and effective targeting of cancer.

Keywords

Ovarian cancer, ovcar-3, paclitaxel, thermo-sensitive liposomes, topotecan

Introduction

Cancer is paralyzing the world with increasing number of deaths projected to 21 million by 2030. In case of women, ovarian cancer is the primary cause of death. For the treatment of ovarian cancer, combination of drugs is found to be a better alternative to prevent toxicity, intolerance, and high relapse rate for long-term chemotherapy. It is reported that paclitaxel (Pac) and topotecan (Top) showed synergism and non-cross-resistance in case of ovarian cancer. Pac causes stabilization of microtubules thereby blocking cell division at the G2 and M phases of the cell cycle whereas Top showed cell cycle arrest in S phase. Liposomal formulations have been reported to stabilize Pac and Top. Hydrophilic stealthy shield of polyethylene glycol (PEG) renders long circulatory characteristics accounted to turning away RES (reticuloendothelial system) uptake. In addition to enhanced blood circulation, warranted therapeutic efficacy can be accomplished in liposomes using surface-oriented targeting ligand like folate and imparting stimuli responsiveness viz. temperature. Folate receptors (FR-α) being over expressed (>90% in ovarian cancer) were chosen to enhance target specificity. Above low critical solution temperature (LCST), the fabricated liposomal system gets disrupted causing immediate release of entrapped bioactive attributed to rapid gel-sol transition which can be attained by local hyperthermia at the site of interest. Promisingly, these smart liposomes maintain systemic stability at normothermia. For this reason, DPPC (Dipalmitoyl phosphatidylcholine) liposomes release entrapped bioactive in bulk at a region of local hyperthermia (41 ± 1 °C) generally reported in some tumors like ovarian carcinomas. Clinically tolerable Pac-Top (20:1, w/w) were co-encapsulated into the nanosteric liposomes (~150 nm) viz. Plain liposomes (L-Pac-Top), PEGylated liposomes (PL-Pac-Top), and FR-targeted liposomes (FPL-Pac-Top) at separate domains of residence using thin-film casting method as described in our previous report. It has been reported that ligand (like folate)-directed liposomes may fail to internalize because of PEG brush or development of resistance. If internalization of liposomes fails triggered release can enhance the localization of drug(s) at the target site. In addition, triggered release also facilitates abrupt
Preparation of liposomes
Liposomes were prepared using thin film casting method as reported previously by Jain et al.16. Following types of liposomes were developed: (a) Plain liposomes (L-Pac-Top; DPPC: DMPG = 85.5:9.5, molar ratio), (b) PEGylated liposomes (PL-Pac-Top; DPPC: DMPG: mPEG2000-DSPE = 85.5:9.5:5, molar ratio) and, (c) FR-targeted PEGylated liposomes (FPL-Pac-Top; DPPC: DMPG: mPEG2000-DSPE: DSPE-PEG-folate = 85.5:9.5:4.5:0.5, molar ratio). Briefly, lipid components and Pac (drug-to-lipid molar ratio = 1:33) were dissolved in chloroform: methanol mixture (80:20) in a round bottom flask and dried out using rotary evaporator (Rotavap; Steroglass Srl, Perugia, Italy) at 60±1°C to form thin lipid film. This lipid film was hydrated (PBS pH 5 containing Top; Pac-Top = 20:1, w/v) to get MLVs (multi-lamellar vesicles). These MLVs were then undergone sonication (Probe sonicator, Lark innovative technology, Chennai, India) for 5 min with 20 s ON and 20 s OFF mode in an ice-bath to get SUVs (small unilamellar vesicles, <200 nm)21.

Shape and physical interaction
Liposomes were visualized under Transmission Electron Microscope (Morgagni 268-D FEI, Holland) at suitable magnification using negative staining (1% phosphotungstic acid). The phase transition enthalpy of various excipient including liposomes was observed using a differential scanning calorimeter (DSC 8230, Rigaku Co., Tokyo, Japan) equipped with a thermal controller (F-25, Julabo Labortechnik GmbH, Seelbach, Germany) (n = 3).

In vitro drug release data modeling
The in vitro drug release study for liposomal formulations was carried out using dialysis tube (molecular cut-off point, 3500). Unentrapped drugs from formulations were removed using Sephadex G-50 mini column followed by centrifugation at 2000 rpm. This liposomal suspension (1 mL) was kept in a dialysis tube which was then suspended in a beaker containing PBS (20 mL, pH 7.4 at 37 ± 0.5°C) with constant magnetic agitation. Samples from release medium were taken out at defined time points and replenished immediately with the same volume of fresh medium. Drugs were estimated by HPLC method using acetonitrile–water (70:30, 0.1% TFA) on Phenomenex Luna C-18(2) column in isocratic mode at flow rate of 1.2 mL/min with UV detection at 227 nm22. The mechanisms and pattern of drugs release from different liposomal formulations were established by fitting in vitro release data to various kinetic models23: Baker and Lonsdale, Peppas, Hixon and Crowell, Higuchi Square Root Time, and First Order. The determination coefficient (R²) and sum of squares of residuals (SSR) were used as the indicators of the best fit to release data for each model. All model parameters of drugs release profiles were computed using Sigma Plot for Windows Version 11.0 (wpcubed GmbH, Germany).

Ex-vivo pharmacodynamics
Cell uptake and flow cytometry studies
The OVCAR-3 (Human ovarian cancer) cell lines were grown in RPMI 1640 medium (containing 10% fetal bovine serum + 2 mM L-glutamine). The cells (5 × 10⁶) were inoculated into 96-well microtiter plates and incubated at 37°C, 5% CO₂, 95% air and 100% relative humidity for 24 h prior to addition of any test formulations viz. Pac-Top, L-Pac-Top, PL-Pac-Top, and FPL-Pac-Top. The cells were sub-cultured every 48 h and harvested from sub-confluent cultures (~70%) using 0.05% trypsin-EDTA.

Methods
Synthesis of folate-PEG-DSPE
Folate-PEG-DSPE conjugate was synthesized using carbodiimide chemistry. Folate-PEG-NH₂ conjugate was synthesized using the reported method18 with FA:H₂N-PEG-NH₂:DCC:NHS:TEA in molar ratio 1:2:1:1.5:1.5:5. N-Succinyl DSPE conjugate was synthesized19 and activated with DCC (1 molar equivalent) to which an equimolar quantity of folate-PEG-NH₂ conjugate was added to obtain folate-PEG-DSPE. Linkages were examined by infrared spectroscopy. Folate-PEG-NH₂ was analyzed for folate content using spectrophotometric method at 363 nm and NH₂ content using Ninhydrin assay 19. Folate content of FA-PEG3400-DSPE conjugate was estimated using spectrophotometric method (0.05 mg/mL, methanol) at 285 nm20.

Materials
Distearoyl-phosphatidylethanolamine (DSPE), monomethoxy PEG 2000-distearoyl phosphatidylethanolamine (mPEG2000-DSPE), DPPC, Dimyristoyl phosphatidylglycerol (DMPG) and Distearoyl phosphatidyl-ethanolamine (DSPE) were obtained as gifts from Lipoid (Ludwigshafen Am Rhein, Germany). Gift samples of Pac and Top were obtained from Fresenius Kabi Oncology Ltd. (Gurgaon, Haryana, India). Folic acid (FA) was purchased from Himedia (Mumbai, India). PEG bisamine (PEG-3400 bisamine, H₂N-PEG-NH₂), Dicyclohexyl carbodiimide (DCC), Succinyl anhydride, Trifluoroacetic acid (TFA) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich. MITT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] was purchased from Sigma Aldrich (St Louis, MO). All other reagents and solvents of analytical or high-performance liquid chromatography (HPLC) grade were purchased from Merck India Ltd. (Mumbai, India).

Figure 1. Mechanistic scene: thermo-responsive behavior of smart liposomes.

dispersal of liposomal content in the cytosol during intracellular trafficking12,13–17. This debut study further explores smart liposomes-bearing Pac-Top for in vitro drug release kinetics modelling, stability studies, hemolytic toxicity studies, ex vivo pharmacodynamics in OVCAR-3 cell lines, and pharmacokinetics in ovarian cancer xenograft model in tumor-bearing mice. Figure 1 represents mechanistic scene of thermo-responsive behavior of smart liposomes.

DOI: 10.3109/03639045.2015.1036066
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To acquire hyperthermia, cells were exposed at 41 ± 1°C for 5 min. Cell viability was assessed by absorbance at 570 nm measured on a Biorad microplate reader. The fluorescence due to uptake of liposomal formulations was analyzed quantitatively with flow cytometer (FACS caliper, Becton Dickinson, San Jose, CA). The effect of coupled FA on cell uptake of liposomal formulations was assessed by pre-incubating the cells with excess of free FA (10 μM) for 2 h prior to addition of formulation. After the treatment with the formulation, the cells were detached (0.05% trypsin-EDTA) and washed (PBS, pH 7.4 at 4°C) for estimation of apoptotic potential. Then, 2.5 mL annexin-propidium iodide dye was added and the cells were incubated for 15 min followed by centrifugation (1500 rpm, 5 min) to obtain a cell pellet. The cells were rinsed thrice with PBS (pH 7.4) and subsequently analyzed using a flow cytometer (FACS caliper, Becton Dickinson, San Jose, CA).

Hemolytic toxicity

Hematological study was performed in Balb/c mice. The experimental protocol was duly approved by the Institutional Animal Ethical Committee (Protocol No: Animal Eths. Comm./10/5), Dr. H. S. Gour Central University, Sagar, MP, India. These mice were divided into five groups having five mice in each group, Pac-Top, L-Pac-Top, PL-Pac-Top and FPL-Pac-Top were intravenously administered to animals of Groups I, II, III & IV, respectively every day at an equivalent dose [4.5 mg Pac + 0.23 mg Top in PBS (pH 7.4) per kg mice] via tail vein for a week. Mice of Group V were kept as control. To get hematological parameters, the total white blood corpuscles (WBC), red blood corpuscles (RBC), platelets count and hemoglobin (Hb) content were estimated on 8th day using a semi-automated blood cell counter with digital display (Sysmex cc-130, Toa Medical Electronics Ltd., Japan).

Pharmacokinetic analysis

Tumor was induced in female Balb/c mice (average body weight, 21 ± 2 g; 4–6-weeks-old) by inoculating subcutaneously 5 × 10⁶ OVCAR-3 cells (in 100 μL PBS, pH 7.4) in the flank of each animal. When the tumor volume had reached approximately 50–100 mm³, the mice were randomly divided into four groups with 20 mice in each group (i.e. 20 × 4). They were fasted overnight before administration of dose. To first group, free Pac-Top solution [4.5 mg Pac + 0.23 mg Top in PBS (pH 7.4) per kg mice] was administered intravenously via tail vein. To the second, third, and fourth group of mice, L-Pac-Top, PL-Pac-Top and FPL-Pac-Top with equivalent i.v. dose were administered, respectively. Four mice from each group were sequentially sacrificed (performed in triplicate) after 0.5h, 1h, 4h and 24h post-administration. After cardiac puncture, blood was collected and centrifuged (Remi Elektrotech Ltd., Mumbai, India) at 3000 rpm for 10 min to separate RBCs and serum. Collected serum was thoroughly mixed with ethyl acetate (2 mL) followed by removal of organic layer using centrifugation. Different organs, i.e. liver, spleen, lungs, kidney, heart, and ovary were excised, isolated and dried with tissue paper and weighed. Percent drugs (Pac-Top) recovered in each case were then analyzed using reported HPLC method. Various pharmacokinetic parameters were computed using Thermo Kinetica Version 5.0 – Trail Version (Thermo Fisher Scientific Inc., Waltham, MA).

Anti-tumor activity and survival analysis

Tumor-bearing mice were regularly monitored every day for tumor growth after inoculation (method stated before). Mice were divided into five groups; first four groups were given intravenously (via tail vein) Pac-Top, L-Pac-Top, PL-Pac-Top, and FPL-Pac-Top, respectively with an equivalent dose [4.5 mg Pac + 0.23 mg Top] per kg mice after obtaining sufficient tumor growth (50–100 mm³) and animals of fifth group were kept as control. Electronic Vernier calliper was used to measure the major and minor axes of tumor at preset time points and tumor volume (V) was calculated using the following formula: 
\[ V = \frac{1}{2} \times \text{length} \times \text{width} ^ 2 \]
Acute toxicity was checked by monitoring the body weight of mice. Survival analysis was also carried out on another group of animals (n = 7) using Kaplan–Meier survival curves.

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical analysis was performed with the two-tailed unpaired test for two group comparison or one-way ANOVA, followed by Tukey’s multiple comparison test using NCSS 2007 Version 07.1.14 (UT). In vitro drugs release kinetics model fitting and Kaplan–Meier survival analysis were carried out using Sigma Plot for Windows Version 11.0 (wpcedb GmbH, Germany). All pharmacokinetic parameters were determined using Thermo Kinetica Version 5.0 – Trail Version (Thermo Fisher Scientific Inc., Waltham, MA). A difference with p ≤ 0.05 (i.e. 5%, level of significance) was considered to be statistically significant.

Results and discussion

Folate-PEG-DSPE was synthesized using carbodiimide chemistry. Infrared spectral analysis of folate-PEG-DSPE revealed characteristic peaks of FA (843.8 cm⁻¹, aromatic C–H bending; 1697.1 cm⁻¹, aromatic C = C bending and stretching), bisamine PEG (1106.9 cm⁻¹, C–O stretch, ether) and DSPE (2918.1 cm⁻¹, –CH₂– stretching of stearyl backbone). Peaks at 1637.2 cm⁻¹ (C = O stretch amide) and 3433.3 cm⁻¹ (N–H stretch of amide) confirmed introduction of amide bond in Folate-PEG-DSPE conjugate (Figure S1). The ratio of folate to free amine groups in Folate-PEG-NH₂ conjugate was ~1 (i.e. 50% yield). Similarly, folate content of folate-PEG-DSPE (UV spectrophotometry at 285 nm) was found to be 0.23 mMol/g (i.e. 96% yield). Transmission electron microscopy (TEM) showed spherical shape (size ~150 nm) without any aggregation or fusion confirming the physical integrity of vesicles (Figure 2). DSC analysis (Figure 3) showed loss of the tightly bound lattice water from Top at ~110°C and melting at ~210°C. Melting of Pac occurred at ~218°C followed by decomposition while DPPC melted at ~218°C.

Figure 2. TEM photomicrograph of FPL-Pac-Top.
at 41 °C\(^{29}\). The melting of liposomes (FPL) at 41 °C could be due to combined effect of DMPG (\(T_c \sim 25^\circ C\))\(^{30}\) and PEG (\(T_c \sim 61^\circ C\))\(^{31}\). DSC revealed no significant interaction in either of the components maintaining drugs integrity without leakage from liposomes\(^{32}\).

Developed liposomal formulations were further studied for in vitro drug(s) release at physiological pH 7.4 (37 ± 0.5 °C) and release data were fitted to different release kinetic models to predict mechanism and pattern of drug(s) release (Figure 4; Tables 1–3). The release data showed initial burst release of Pac at 1 h while it was suppressed for Top owing to high Pac-Top ratio (~17) and high entrapment of Pac (~90%) opposed to Top (~20%), and differential drug residences within liposomes that reinforced comparatively higher Pac gradient across lipid – release medium interface. As speculated, Pac-Top released for more than 72 h in all liposomal formulations at normothermia indicating sustained release pattern\(^{33}\).

Four kinetic models namely Baker and Lonsdale, Peppas, Hixon and Crowell, Higuchi, Square Root Time, and First Order were fitted to in vitro drug(s) release data. \(R^2\) close to 1 and less SSR indicated Peppas model as the best fit model. The ‘‘\(n\)’’ (diffusion release exponent) in case of Peppas model was used to predict the mechanism of drug(s) release from liposomes. As it is reported for nanoparticles of spherical shape, \(n \leq 0.43\) – Fickian diffusion, \(n = 0.43–0.85\) – Anomalous (non-Fickian) diffusion, \(n = 0.85\) – Case II transport, and \(n > 0.85\) – Super-case II transport\(^{34}\). In all cases, \(n\) was found to be lesser than 0.4 which indicated Fickian diffusion. The biphasic pattern of drug(s) release, i.e. initial burst release followed by sustained mode of release is characteristic of Peppas model. Statistical tests (at \(\alpha = 0.0500\)) such as normality test (Shapiro–Wilk), W Statistic and Constant Variance Test were applied to validate the kinetic models fitting to release data\(^{23}\).
Table 1. Release kinetics models curve fitting for L-Pac-Top.

| Controlled release kinetics model | Equation | Parameter | Coefficient | $R^2$ | SSR | Normality test (Shapiro–Wilk) | W Statistic | Constant variance test | Remarks |
|----------------------------------|----------|-----------|-------------|-------|-----|------------------------------|------------|------------------------|---------|
| **L-Pac-Top (Pac)**              |          |           |             |       |     |                              |            |                        |         |
| Baker and Lonsdale               | $F = \frac{a + c \cdot x^{0.5} + e \cdot x + \varepsilon \cdot x^{1.5} + k' \cdot x}{1 + b \cdot x^{0.5} + d \cdot x^{0.5} + h \cdot x^{0.5} + j \cdot x^{2.5}}$ | $k$ | 0.0025 | 0.8905 | 339.4144 | Failed | $p = 0.0242$ | 0.8183 | Passed | $p = 0.3270$ |
| Peppas                           | $F = k \cdot t^n$ | $k$ | 18.4149 | 0.9838 | 50.2773 | Passed | $p = 0.4384$ | 0.9290 | Passed | $p = 0.3842$ |
| Hixon and Crowell                | $F = 100^{\frac{1}{1 - (1 - k \cdot t)^3}}$ | $k$ | 0.0136 | 0.2091 | 2450.6962 | Failed | $p = 0.0119$ | 0.7931 | Passed | $p = 0.0977$ |
| Higuchi, square root time        | $F = k' \cdot \sqrt{t}$ | $k$ | 9.6957 | 0.7566 | 754.0804 | Failed | $p = 0.0061$ | 0.7693 | Failed | $p = 0.2583$ |
| First order                      | $F = 100^{\frac{1}{1 - \exp(-k \cdot t)}}$ | $k$ | 0.0423 | 0.3669 | 1961.7310 | Failed | $p = 0.0155$ | 0.8025 | Passed | $p = 0.3455$ |
| **L-Pac-Top (Top)**              |          |           |             |       |     |                              |            |                        |         |
| Baker and Lonsdale               | $F = \frac{a + c \cdot x^{0.5} + e \cdot x + \varepsilon \cdot x^{1.5} + k' \cdot x^{2.5}}{1 + b \cdot x^{0.5} + d \cdot x^{0.5} + h \cdot x^{0.5} + j \cdot x^{2.5}}$ | $k$ | 0.0025 | 0.8402 | 592.2012 | Passed | $p = 0.2546$ | 0.9060 | Passed | $p = 0.6814$ |
| Peppas                           | $F = k \cdot t^n$ | $k$ | 17.7425 | 0.9477 | 377.2132 | Passed | $p = 0.3152$ | 0.8278 | Passed | $p = 0.9186$ |
| Hixon and Crowell                | $F = 100^{\frac{1}{1 - (1 - k \cdot t)^3}}$ | $k$ | 0.0140 | 0.2874 | 2640.3096 | Passed | $p = 0.1261$ | 0.8787 | Failed | $p = 0.0186$ |
| Higuchi, square root time        | $F = k' \cdot \sqrt{t}$ | $k$ | 9.7252 | 0.7248 | 1019.5918 | Passed | $p = 0.0197$ | 0.8949 | Passed | $p = 0.2915$ |
| First order                      | $F = 100^{\frac{1}{1 - \exp(-k \cdot t)}}$ | $k$ | 0.0436 | 0.4200 | 2148.9375 | Passed | $p = 0.1678$ | 0.8896 | Passed | $p = 0.0736$ |

SSR, sum of squares of residuals; $\alpha$, level of significance; all other symbols are test statics.
Table 2. Release kinetics models curve fitting for PL-Pac-Top.

| Controlled release kinetics model | Equation | Parameter estimates ($p < 0.0001$) | Statistical tests ($\alpha = 0.0500$) | Remarks |
|----------------------------------|----------|------------------------------------|-------------------------------------|---------|
|                                  |          | Parameter                           | $R^2$ | SSR | Normality test (Shapiro–Wilk) | W Statistic | Constant variance test | Remarks |
| PL-Pac-Top (Pac)                 |          |                                    |       |     | Passed                          |            | Passed                |         |
| Baker and Lonsdale               | $F = \left( a + c x^{0.5} + e x + g x^{1.5} + i x^2 + k l x^{2.5} \right) / \left( 1 + b x^{0.5} + d x + f l x^{1.5} + h x^2 + j x^{2.5} \right)$ | k     | 0.0019 | 0.8643 | 346.1367 | Passed | $p = 0.0539$ | $0.8472$ | Passed | $p = 0.0599$ | Peppas model is best fitted. $R^2$ – Close to 1, SSR – Minimum. All statistical tests – passed |
| Peppas                           | $F = k^*t^n$ | k     | 16.8250 | 0.9796 | 52.1310 | Passed | $p = 0.2259$ | $0.8208$ | Passed | $p = 0.9728$ |
| Hixon and Crowell                | $F = 100^* (1 - (1 - k^*t)^n)$ | k     | 0.0072 | 0.0000 | 2644.5742 | Failed | $p = 0.0048$ | $0.7605$ | Passed | $p = 0.1988$ |
| Higuchi, square root time        | $F = k^* \sqrt{t}$ | k     | 8.8035 | 0.7481 | 642.4105 | Failed | $p = 0.0246$ | $0.8189$ | Passed | $p = 0.1988$ |
| First Order                      | $F = 100^* \left( 1 - \exp(-k^*t) \right)$ | k     | 0.0300 | 0.1795 | 2092.6790 | Failed | $p = 0.0120$ | $0.7933$ | Passed | $p = 0.2583$ |
| PL-Pac-Top (Top)                 |          |                                    |       |     | Passed                          |            | Passed                |         |
| Baker and Lonsdale               | $F = \left( a + c x^{0.5} + e x + g x^{1.5} + i x^2 + k l x^{2.5} \right) / \left( 1 + b x^{0.5} + d x + f l x^{1.5} + h x^2 + j x^{2.5} \right)$ | k     | 0.0019 | 0.8010 | 613.7687 | Passed | $p = 0.4402$ | $0.9292$ | Passed | $p = 0.3270$ | Peppas model is best fitted. $R^2$ – Close to 1, SSR – less. All statistical tests – passed |
| Peppas                           | $F = k^*t^n$ | k     | 16.2221 | 0.9371 | 375.9591 | Passed | $p = 0.2307$ | $0.9020$ | Passed | $p = 0.4044$ |
| Hixon and Crowell                | $F = 100^* (1 - (1 - k^*t)^n)$ | k     | 0.0073 | 0.0496 | 2931.7880 | Passed | $p = 0.0929$ | $0.8673$ | Passed | $p = 0.0977$ |
| Higuchi, square root time        | $F = k^* \sqrt{t}$ | k     | 8.8058 | 0.7015 | 920.7961 | Passed | $p = 0.5064$ | $0.9357$ | Passed | $p = 0.2746$ |
| First Order                      | $F = 100^* \left( 1 - \exp(-k^*t) \right)$ | k     | 0.0305 | 0.2392 | 2346.9415 | Passed | $p = 0.2412$ | $0.9038$ | Passed | $p = 0.0977$ |

SSR, sum of squares of residuals; $\alpha$, level of significance; all other symbols are test statics.
Table 3. Release kinetics models curve fitting for FPL-Pac-Top.

| Controlled release kinetics model | Equation | Parameter estimates ($p<0.0001$) | Statistical tests ($\alpha = 0.0500$) | Remarks |
|----------------------------------|----------|------------------------------------|--------------------------------------|---------|
|                                  |          | $R^2$ | SSR       | Normality test (Shapiro–Wilk) | W statistic | Constant variance test |                     |
| **FPL-Pac-Top (Pac)**            |          |       |           |                                  |             |                       |                     |
| Baker and Lonsdale               | F = $\left(\frac{a+c^*x^2+e^*x^5+g^*x^{1.5}+i^*x^7+k^*x^{2.5}}{1+b^*x^2+d^*x+f^*x^{1.5}+h^*x^2+j^*x^{2.5}}\right)$ | k | 0.0016 | 0.8824 | 270.8370 | Passed | $p = 0.1717$ | 0.8905 | Failed | $p = 0.0427$ | Peppas model is best fitted. $R^2 –$ Close to 1 SSR – Minimum All statistical tests – passed |
| Peppas                           | F = $k^*t^n$ | k | 14.7659 | 0.9742 | 59.4594 | Passed | $p = 0.1712$ | 0.8904 | Passed | $p = 0.9457$ |
| Hixon and Crowell                | F = $100^* \left(1-\frac{1}{(1-k^*t)^3}\right)$ | k | 0.0057 | 0.0332 | 2226.7952 | Failed | $p = 0.0191$ | 0.8099 | Passed | $p = 0.0977$ |
| Higuchi, square root time        | F = $k^*\sqrt{t}$ | k | 8.1509 | 0.7947 | 472.7904 | Passed | $p = 0.1343$ | 0.8811 | Passed | $p = 0.0665$ |
| First Order                      | F = $100^* \left(1-\exp(-k^*t)\right)$ | k | 0.0231 | 0.2025 | 1836.9144 | Failed | $p = 0.0197$ | 0.8110 | Passed | $p = 0.1853$ |
| **FPL-Pac-Top (Top)**            |          |       |           |                                  |             |                       |                     |
| Baker and Lonsdale               | F = $\left(\frac{a+c^*x^2+e^*x^5+g^*x^{1.5}+i^*x^7+k^*x^{2.5}}{1+b^*x^2+d^*x+f^*x^{1.5}+h^*x^2+j^*x^{2.5}}\right)$ | k | 0.0016 | 0.8181 | 500.1988 | Passed | $p = 0.1770$ | 0.8917 | Passed | $p = 0.6814$ | Peppas model is best fitted. $R^2 –$ Close to 1 SSR – least All statistical tests – passed |
| Peppas                           | F = $k^*t^n$ | k | 14.3553 | 0.9391 | 324.5845 | Passed | $p = 0.1232$ | 0.8778 | Passed | $p = 0.7326$ |
| Hixon and Crowell                | F = $100^* \left(1-\frac{1}{(1-k^*t)^3}\right)$ | k | 0.0058 | 0.0930 | 2494.6478 | Passed | $p = 0.0798$ | 0.8616 | Passed | $p = 0.1988$ |
| Higuchi, square root time        | F = $k^*\sqrt{t}$ | k | 8.1723 | 0.7408 | 712.8375 | Passed | $p = 0.2808$ | 0.9100 | Passed | $p = 0.2583$ |
| First Order                      | F = $100^* \left(1-\exp(-k^*t)\right)$ | k | 0.0234 | 0.2435 | 2080.7424 | Passed | $p = 0.1457$ | 0.8842 | Passed | $p = 0.0977$ |

SSR, Sum of squares of residuals; $\alpha$, level of significance; all other symbols are test statistics.
The cytotoxic activity of plain drugs and different liposomal formulations was determined using MTT assay at 37 ± 1°C after 72 h and 41 ± 0.1°C after 4 h incubation with OVCAR-3 cells. Cytotoxicity of Pac-Top, L-Pac-Top, PL-Pac-Top, and FPL-Pac-Top was investigated at different concentrations to expound the dose-dependent anti-cancer activity. All formulations showed significantly increased cytotoxicity with increasing concentrations. At normothermia, liposomes were found to be less cytotoxic due to relatively lesser availability of free Pac-Top to cancer cells as compared to Pac-Top solution. At the same time, PL-Pac-Top revealed the least cytotoxicity owing to steaing effect while folate ligand enhanced cytotoxicity potential of FPL-Pac-Top. This favored the fact that ovarian cancer cells possess over-expressed folate receptors, i.e. more than 90% opposed to normal cells. However, at the same concentration, all liposomal formulations as compared Pac-Top showed potentiated cytotoxicity at hyperthermia (41 ± 1°C) due to abrupt dispersal of Pac-Top from the liposomal formulations upon melting of lipids rendering accessible Pac-Top for killing ovarian cancer cells.

In cellular uptake studies, time-dependent increased cell uptake of all liposomal formulations was observed. FPL-Pac-Top showed enhanced uptake, i.e. ~4 fold and ~2 fold as compared to Pac-Top and non-targeted liposomes, respectively, in cancer cells at 37 ± 0.5°C attributed to receptor-mediated endocytosis (clathrin-independent pathway, i.e. caveolar pathways of uptake) by the folate receptors, which clearly indicated the targeting potential.

Figure 5. Cell cytotoxicity and cell uptake potential of various formulations.
of FPL-Pac-Top$^{38,39}$. In order to assure this phenomenal uptake, the receptor blockade or competitive binding assay was also carried out via pre-incubating cells with FA (1 mM). Interestingly, the cell uptake of FPL-Pac-Top in presence of FA was found to be reduced significantly similar to PL-Pac-Top (Figure 5). However, no competitive inhibition was observed with uncoupled liposomes. This again assured that enhanced uptake of FPL-Pac-Top was mediated by folate receptor-mediated endocytosis. At hyperthermia condition, cell uptake of FPL-Pac-Top as compared to Pac-Top solution and non-targeted liposomes was more pronounced because of combined effect of endocytosis and thermo-responsive drug release, however, there were also higher cell uptake of non-targeted liposomes attributed to enhanced availability of free Pac-Top after thermo-responsive burst release.

Flow cytometry studies were carried out to work out the cytotoxicity potential of different liposomal formulations in terms of apoptosis and necrosis. The outcomes of this investigation showed >95% cells in live stage in control group. Cells treated with Pac-Top showed ~48% cells in apoptotic pathway, and ~45% cell death because of necrosis. This outcome indicated that killing could be attributed to the combined influence of apoptosis and necrosis$^{40}$. It is also suggested that Pac-Top is enormously cytotoxic in nature, while L-Pac-Top, PL-Pac-Top, and FPL-Pac-Top have exhibited 12.6 ± 0.7%, 3.5 ± 0.2%, and 10.7 ± 0.8% cell death by necrosis, respectively, and cell death by combination of early apoptosis and apoptosis caused by FPL-Pac-Top was ~17-fold higher than control (Figure 6). Additionally, PL-Pac-Top showed lesser necrosis due to hydrophilic nature of PEG$^{41}$.

An evident decrease in blood cells count such as WBC count, RBC count, Hb content, platelet count was observed after injecting Pac-Top solution for a week as compared to liposomal formulations. This could be due to immediate contact of free drugs with the blood while encapsulation of drugs in liposomes reduced hematological toxicity accounted to protective and biocompatible nature of lipids used in liposomes leading to very less leaching of the drugs in blood. Moreover, hydrophilic PEG brush in PEGylated liposomes revealed insignificant hematological implications opposed to plain liposomes (Table 4)$^{42}$.

Following i.v. dose into tumor-bearing mice, the time course of Pac-Top in blood was determined for Pac-Top solution and different liposomal formulations (Figure 7). Free drugs appeared to have markedly shorter blood retentions than liposomal formulations. A greater fraction of liposomal Pac-Top was recovered from blood in initial hours$^{43}$. L-Pac-Top was found to have the shortest serum retention (~2% after 4 h) and faster clearance (i.e. rapid uptake by liver and spleen) as compared to PEGylated liposomal formulations. This revealed that liposomes which were not surface engineered were not effective to enhance the serum retention and thereby failed to avoid untoward exposure of drugs to vital organs albeit these performed comparatively better as compared to Pac-Top solution. PL-Pac-Top showed extended circulation lifetime compared to both L-Pac-Top and FPL-Pac-Top accounting to stealth effect of PEG brush which bestowed it long circulatory features$^{44,45}$. Interestingly, FPL-Pac-Top as compared to other two liposomal formulations not only extended the circulation lifetime (RES uptake evasion) but also targeted FR$^+$ sites (ovarian cancer cells). Results indicated that co-administration of Pac had no impact on the serum elimination profile of Top which supported the reported literature$^{46}$.

Various pharmacokinetic parameters computed from serum-drugs profile are summarized in the Table 5. The AUC$^{0–t}$, AUC$^{0–\infty}$, AUMC$^{0–t}$, AUMC$^{0–\infty}$ for FPL-Pac-Top as compared to Pac-Top solution were found to be higher, namely 4.44 (Pac) and 14.83 (Top) folds, 5.79 (Pac) and 20.24 (Top) folds, 32.62 (Pac) and 144.37 (Top) folds, 67.91 (Pac) and 360.11 (Top) folds, respectively. Moreover, PL-Pac-Top performed even better than Pac-Top solution in terms of enhanced circulation lifetime showing higher AUC$^{0–t}$, AUC$^{0–\infty}$, AUMC$^{0–t}$, AUMC$^{0–\infty}$ as ~2.5 (Pac-Top) fold, ~6 (Pac-Top) fold, ~3 (Pac-Top) folds, ~20 (Pac-Top) fold, respectively. The MRT of FPL-Pac-Top was found to be 11.72 (Pac) and 17.86 (Top) folds, and 3.38 (Pac) and 3.31 (Top) folds, as compared to Pac-Top solution and L-Pac-Top.
respectively. Further, PL-Pac-Top showed ~3.3 folds higher MRT than FPL-Pac-Top. The significant increase in the pharmacokinetic parameters such as AUC, AUMC, \( t_{1/2} \), and MRT undoubtably depict improved bioavailability of Pac-Top with PL-Pac-Top and FPL-Pac-Top, suggestive of long circulatory behavior, which designate them as smart liposomes for controlled delivery of Pac-Top.

The RES represents a major mechanism for clearance of circulating liposomes. Therefore, deposition of Pac-Top in liver, spleen, lung, kidney, heart and ovary (included with the intent to test toxicity of the formulations in off-targeted vital organs) was investigated (Table 6). In case of Pac-Top solution, serum concentration of Pac was almost negligible at 4 h, and it was rapidly taken up and cleared by the liver, spleen and lung. The concentrations were higher for liposomal Pac than that of free Pac in spleen and lung (consistent with their role in RES-mediated clearance) attributed to rapid sequestration of drug followed by a more rapid clearance than observed for drug administered as free Pac. Because neither spleen nor lung likely played a significant role in the elimination of Pac, it was probably cleared off by remobilization. Top was excreted by the kidneys (~40%) to a significant extent. It is transformed in liver to inactive metabolites, so it was found to concentrate more in the kidney rather than liver and other organs. A high percentage of Top usually got converted to its less active form, i.e. carboxylate form but liposomal formulations found to be effective in maintaining its active form, i.e. lactone form in an acidic aqueous core of liposomes for longer periods.

PEGylated liposomes (PL-Pac-Top) demonstrated increased bioavailability as they remain in the blood circulation for extended periods of time (i.e. \( t_{1/2} \approx 40 \) h), and only 10–15% of the dose was recovered from the liver. This revealed the significant improvement over conventional liposomes (L-Pac-Top), where typically 80–90% of the dose deposited in the liver. Evidently, long-circulating liposomes were also found to localize at tumor site (ovaries i.e. ~3% even after 24 h) which accounted to enhanced permeability and retention effect. FPL-Pac-Top had faster clearance (~50% at 1 h) than PL-Pac-Top (~15% at 1 h).

Table 5. Various pharmacokinetic parameters in serum of tumor-bearing Balb/c mice.

| Formulations | Parameter | Drugs solution | L-Pac-Top | PL-Pac-Top | FPL-Pac-Top |
|--------------|-----------|----------------|------------|------------|------------|
|              | C\(_{\text{max}}\) (\(\mu\)g/mL) | 82.3 | 31.9 | 87.2 | 86.1 | 94.6 | 93.5 | 88.1 | 87.2 |
|              | T\(_{\text{max}}\) (h) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
|              | AUC\(_{0-t}\) (\(\mu\)g/mL × h) | 156.66 | 45.73 | 389.96 | 372.00 | 1756.16 | 1739.68 | 696.18 | 678.24 |
|              | AUC\(_{0-\infty}\) (\(\mu\)g/mL × h) | 174.06 | 47.03 | 400.02 | 380.61 | 5938.46 | 5507.38 | 1008.46 | 951.90 |
|              | AUMC\(_{0-t}\) (\(\mu\)g/mL × h\(^2\)) | 198.74 | 43.44 | 2071.26 | 192.4 | 19818.1 | 19527.3 | 6829.72 | 6271.78 |
|              | AUMC\(_{0-\infty}\) (\(\mu\)g/mL × h\(^2\)) | 298.36 | 50.07 | 2375.92 | 2180.79 | 404373 | 347433 | 20263.6 | 18031.2 |
|              | \( t_{1/2}\) (h) | 1.19 | 0.76 | 4.35 | 4.23 | 47.09 | 43.68 | 13.95 | 13.12 |
|              | HVD (h) | 1.19 | 0.69 | 2.10 | 2.04 | NA* | NA | 2.32 | 2.38 |
|              | MRT (h) | 1.714 | 1.06 | 5.93 | 5.72 | 68.09 | 63.08 | 20.09 | 18.94 |
|              | Ch\(_{\text{last}}\) (\(\mu\)L/h) | 10.2 | 1.2 | 1.7 | 1.5 | 61.8 | 59.9 | 15.9 | 14.8 |

NA*, not applicable; Probability \( p \leq 0.05; \) SD <5%.

C\(_{\text{max}}\), peak serum concentration; T\(_{\text{max}}\), time to reach peak serum concentration; AUC, area under serum drug concentration over time curve; AUMC, area under the first moment curve; \( t_{1/2}\), elimination half-life; Ch\(_{\text{last}}\), Clearance at last time point; MRT, mean residence time; HVD, half-value duration.

Figure 7. Percent dose recovered in serum after i.v. administration of various formulations [values represent mean ± SD (\( n = 5 \))].
### Table 6. Organ-distribution studies of free drug(s) and liposomal formulations.

| Organ     | Pac-top solution | Liposomes (L-Pac-Top) | PEGylated liposomes (PL-Pac-Top) | FR-targeted liposomes (FPL-Pac-Top) |
|-----------|------------------|----------------------|----------------------------------|-------------------------------------|
|           | 0.5 h            | 1 h                  | 4 h                              | 24 h                                |
|           | Pac-top          | Pac-top              | Pac-top                          | Pac-top                            |
|           | Liver 4.7 ± 0.23 | 3.1 ± 0.15           | 11.2 ± 0.56                      | 4.8 ± 0.24                          |
|           | Spleen 2.4 ± 0.12| 2.4 ± 0.12           | 5.6 ± 0.28                       | 2.6 ± 0.13                          |
|           | Kidney 1.7 ± 0.08| 10.8 ± 0.54          | 2.5 ± 0.12                       | 12.5 ± 0.62                         |
|           | Heart 0.9 ± 0.04 | 0.9 ± 0.04           | 1.3 ± 0.06                       | 0.3 ± 0.02                          |
|           | Lung 2.1 ± 0.1   | 1.1 ± 0.05           | 4.7 ± 0.23                       | 0.9 ± 0.04                          |
|           | Ovary 0.4 ± 0.02 | 0.3 ± 0.02           | 0.8 ± 0.04                       | 0.4 ± 0.02                          |
|           | Liver 2.1 ± 0.11 | 2.2 ± 0.11           | 14.4 ± 0.72                      | 13.9 ± 0.69                         |
|           | Spleen 1.2 ± 0.06| 1.1 ± 0.05           | 7.8 ± 0.39                       | 7.6 ± 0.38                          |
|           | Kidney 0.6 ± 0.03| 0.5 ± 0.02           | 2.1 ± 0.1                        | 2.3 ± 0.12                          |
|           | Heart 0.4 ± 0.02 | 0.5 ± 0.02           | 1.2 ± 0.06                       | 1.3 ± 0.06                          |
|           | Lung 1.1 ± 0.05 | 1.2 ± 0.06           | 3.8 ± 0.19                       | 3.1 ± 0.12                          |
|           | Ovary 0.3 ± 0.02 | 0.2 ± 0.02           | 0.7 ± 0.03                       | 0.9 ± 0.03                          |
|           | Liver 0.3 ± 0.02 | 0.4 ± 0.02           | 2.1 ± 0.11                       | 2.0 ± 0.1                           |
|           | Spleen 0.2 ± 0.02| 0.2 ± 0.02           | 1.4 ± 0.07                       | 1.5 ± 0.07                          |
|           | Kidney ND        | ND                   | 0.3 ± 0.02                       | 0.4 ± 0.02                          |
|           | Heart ND         | ND                   | 0.2 ± 0.02                       | 0.2 ± 0.02                          |
|           | Lung 0.2 ± 0.02  | 0.2 ± 0.02           | 0.4 ± 0.02                       | 0.5 ± 0.02                          |
|           | Ovary 0.2 ± 0.02 | 0.2 ± 0.02           | 0.6 ± 0.03                       | 0.7 ± 0.03                          |
|           | Liver 1.1 ± 0.2  | 1.2 ± 0.21           | 5.2 ± 0.76                       | 4.9 ± 0.74                          |
|           | Spleen 0.8 ± 0.04| 0.7 ± 0.03           | 3.6 ± 0.23                       | 3.5 ± 0.22                          |
|           | Kidney 1.5 ± 0.12| 1.7 ± 0.13           | 1.9 ± 0.45                       | 1.4 ± 0.47                          |
|           | Heart 0.4 ± 0.02 | 0.4 ± 0.02           | 0.5 ± 0.03                       | 0.7 ± 0.03                          |
|           | Lung 0.6 ± 0.08  | 0.8 ± 0.09           | 1.7 ± 0.33                       | 1.5 ± 0.32                          |
|           | Ovary 0.3 ± 0.02 | 0.2 ± 0.02           | 2.2 ± 0.06                       | 3.3 ± 0.06                          |

Values represent mean ± SD, n = 5; ND, not detected.

Figure 8. Survival analysis of tumor-bearing mice (Kaplan–Meier survival curve).
followed by leukocytes, kidney, lung, bone marrow, intestine, brain and portions of the central nervous system whereas lowest observed in heart and skeletal muscle\textsuperscript{53}.

Overall, the extensive RES uptake and non-instantaneous release of drug from L-Pac-Top was avoided using circulating liposomes that limit the systemic exposure of Pac-Top to non-RES tissues. These effects were initially confined a greater fraction of Pac-Top to the central compartment (blood), thus reducing the peak concentrations to which critical normal tissues are exposed. The subsequent release of Pac-Top from PL-Pac-Top provided a slower tissue distribution rate and lower tissue volume of distribution. Additionally, FA anchoring to PL-Pac-Top potentiated targeted delivery of Pac-Top to the FR\textsuperscript{+} organs such as ovary (cancer site) and liver followed by kidney.

Further, the group treated with FPL-Pac-Top showed significant prolongation in median survival time (47 d) in comparison with the control group (25 d), Pac-Top (32 d), L-Pac-Top (35 d), and PL-Pac-Top (42 d)-treated group in Kaplan–Meier survival analysis ($p<0.001$) (Figure 8).

Concurrent measurement of body weight changes depicted that FPL-Pac-Top showed fewer body weight shifts compared to group treated with Pac-Top (Figure 9). Extended median survival time with noteworthy decrease in tumor volume (Figure 9) evidently suggested the biocompatibility, reduced toxicity with potential anti-cancer activity of FPL-Pac-Top in comparison with Pac-Top\textsuperscript{17}. The leaky vasculature of tumor tissue (EPR effect) and folate receptor-mediated endocytosis facilitated effective targeting to cancer cells\textsuperscript{54–56}.

**Conclusion**

In this study, FPL-Lac-Top was found to show controlled delivery of Pac-Top at normothermia. The \textit{in vitro} and \textit{in vivo} experiments depicted promising cancer-targeting potential of FPL-Pac-Top against tumor-bearing mice (OVCAr-3). Pharmacokinetic and biodistribution studies in tumor-induced mice, smart liposomes exhibited sustained release of Pac-Top with improved pharmacokinetics and preferential deposition into tumor tissue, respectively. Tumor inhibition studies showed the significant delay in tumor growth by FPL-Pac-Top in comparison with Pac-Top solution with considerably prolonged survival time. Thus, these smart liposomes attacked tumor with multimodalities and proved to a promising strategy to treat cancers.

**Acknowledgements**

We are highly thankful to Dr. Dhiraj Khattar (Director, Formulation Development & Delivery Systems), Fresenius Kabi Oncology Ltd.; Haryana (India) for providing the gift samples of Pac and Top. We are also gratified to Mr. Prakash Arora (GE Healthcare, India) for gifting Hi-Trap\textsuperscript{®} columns.

**Declaration of interest**

Financial support (A. J.) was obtained from University Grant Commission (UGC), Delhi as Junior Research Fellowship (JRF) to Mr. Ankit Jain. The authors declare no competing financial interest, direct or indirect, in the subject matter or materials discussed in the article.

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Supplementary material available online
Supplementary Figure S1