Original Article

Pazopanib Colon Targeted Liposomal Drug Delivery for Colorectal Cancer: High-pressure Homogenization Process Optimization and *in-vivo* Evaluation

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ABSTRACT

Background: Pazopanib is second-generation tyrosinekinase inhibitor used in Colorectal cancer (CRC) which is effective orally. Targeted liposomal drug delivery will reduce the unwanted side effects of the drug. The application of High-pressure homogenizers for the preparation of systems like liposomes and lipid dispersions is rising because of its ability of vesicle disruption. **Aim:** Major objective of present research work was to optimize high pressure homogenization process for formulation of colon targeted liposomal drug delivery system of Pazopanib and its *in-vivo* evaluation. To study the influence of homogenization Pressure and number of cycles on some parameters, such as vesicle size and polydispersity index (PDI). **Materials and Methods:** The liposomes were formulated with HSPC (Hydrogenated Phosphotidylcholin from Soybean) m-PEG DSPE-2000 (Phospolipid) and Cholesterol using Ethanol injection method followed by downsizing by EmilsiFlex High pressure Homogenizer. **Results and Conclusion:** The liposomes were evaluated for entrapment efficiency, *in-vitro* drug release, osmolality, particle size, size distribution, polydispersity index, FEG-SEM and stability studies. Optimization studies concluded that the optimized formulation with homogenization pressure of 1000, 1500, 2000 psi and number of cycle 9, 6, 6 respectivly gives particle size of 109 nm with PDI 0.998 and desirability 0.975. *In-vivo* studies in wrister rats in which carcino genesis was done using 1,2- dimethylhydrazine (DMH), indicated that Pazopanib liposomes caused significant tumors growth suppression in terms of tumor volume and weight as compared to control. Histo-pathological evaluation showed that the animals treated with pazopanib liposomes had moderate dysplasia where as untreated animals had severe dysplasia.

Key words: Liposome, Pazopanib, High-pressure homogenizer, Colon targeted, Colorectal.

INTRODUCTION

Colorectal cancer (CRC) is the third recurrently diagnosed tumor worldwide in society. Presently conventional approaches like targeted chemotherapy, surgery, and radiotherapy are being used for the treatment of CRC. The targeted delivery of chemotherapeutics to their site of action increases efficiency with reduced side effects.[1] The conventional non-targeted chemotherapy produces untoward effects like anemia, neutropenia, liver toxicity gastrointestinal (GI) toxicity, mucositis, fatigue, and hematologic disorders.[2] In CRC, there is need to develop targated drug delivery to the required site of the colon in a expected and reproducible manner.[3]

Pazopanib is an orally bio-available second generation tyrosine-kinase inhibitor. It acts on VEGFR1, VEGFR2, VEGFR3, PDGFR alpha, PDGFR beta, FGFR1, FGFR2, c-kit, and with moderate activity against c-FMS.[4] It significantly reduces progression and prolongs lifespan of the patient of complex cancer. In preclinical evaluation Pazopanib has shown excellent anti-angiogenic and anticancer activity in relapsed colorectal cancer.[5,6]

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Commonly the colon is targeted by using the techniques such as pro-drug, pH or microflora activated systems, and time dependent release systems, but all had limited success. Some of the modern techniques used includes pulsatile release, micro and nano-particulate system.

The use of liposome for the delivery of drug at targeted sites is gaining considerable attention now a days.[7] Liposomes are one of the most often studied nanostructures in novel drug delivery due to their adaptable structure; bio-compatibility. They are non-toxic, non-immunogenic, and bio-degradable in nature.[8]

High-pressure homogenizers are employed for the formulation of liposomes and lipid dispersions because of its vesicle disruption ability. The preliminary dispersion of multilamellar liposomes is downscaled in the homogenizer device with application of high energy. The dispersion of liposome is introduced in homogenization chamber at high and constant pressure. Then due to high shear the turbulence, cavitation, and re-structuring of liposome occurs.[9]

The objective of present research work was to optimize high pressure homogenization process for formulation of colon targeted liposomal drug delivery system of Pazopanib and its *in-vivo* evaluation. To study the influence of homogenization Pressure and number of cycles on the parameters, such as vesicle size and poly-dispersity index (PDI).

MATERIALS AND METHODS

Pazopanib was procured from GSK Ltd, Mumbai, Hydrogenated phosphotidylcholine from soaybean, Cholesterol, and m-PEG DSPE-2000 (Phospolipid) were obtained from Wockhardt Research Centre, Aurangabad where as other chemicals such as stearic acid, methanol were of analytical grade from Loba chemie.

Liposome Formulation Method

The crude liposome are first prepared using ethanol injection method as described,[10] followed by downsizing using high pressure homogenizer (EmilsiFlex High pressure Homogenizer C-55, AVESTIN, Inc).

Ethanol injection

For preparation of crude liposome without drug, the excipients mentioned in Table 1 were weighed and ethanolic solution of these lipids was directly injected rapidly to an excess of normal saline solution through a fine needle under magnetic stirring. The liposomal dispersions were incubated with moderate stirring at 50°C for 30 min.

Table 1: Formulation composition for blank Liposome.

Sr. No.	Ingredients	Quantity for 500 ml batch
1	HSPC (Hydrogenated Phosphotidylcholin from Soybean)	5.155 gm
2	m-PEG DSPE-2000 (Phospolipid)	1.970 gm
3	Cholesterol	1.910 gm
4	Ethanol (absolute)	60 ml

High Pressure Homogenization

Crude liposomes were down sized using EmilsiFlex High pressure Homogenizer, where the liposomal dispersion was passed through orifice under high pressure causing downsizing. The homogenization process was optimized using 3^3 full factorial and D- optimal design as per Table 2. The independent variables were Pressure and number of cycles whereas Polydispersity index (PDI) as dependent variables.

Cassette Dialysis

The drug free liposomes were concentrated by cassette dialysis. One portion of feed flow (permeate) was passed through the membrane whereas, the remaining portion (retentate) is circulated again to the reservoir. The aggregating molecules sweeps away that forms a membrane-clogging gel (gel polarization), by flow of sample solution across the membrane surface allowing molecules smaller than the membrane pores to move toward and through the membrane. The process involves taking away liquid from a solution at the same time keeping the solute molecules. As solution volume decreases the concentration of the solute increases. At Diafiltration is a fractionation process that washes smaller molecules through a membrane leaving larger molecules in the remaining portion without any change in concentration. It can be used to remove salts or replace the buffers. It can remove trace solvents like ethanol also other additives. So, the process of making the liposome to a more concentric by exchange of buffer i.e ammonium sulfates with sucrose solution at about 50%. This will be determined by checking the conductance in between process.

Remote Drug Loading

Drug was loaded by keeping drug (2mg/ml) with histidine (1.55 mg/ ml) in washed liposomes at temperature of 60°C for 30 min. The finally the pH was adjusetd to 6.5 using 0.1N HCl or 0.1N NaOH. The volume was made

Table 2: Selection of Variable for Design of Experiment.				
Sr. No	Factors	Experimental values (screenings)		
		Low level (-1)	Medium level(0)	High level (+1)
1	1000 psi	3	6	9
2	1500 psi	3	6	9
3	2000 psi	3	6	9

up with 9% sugar solution up to desired batch size after incubation.

The homogenization process was optimized using 3^3 full factorial and D- optimal design as per Table 2. Accordingly 27 batches were prepared as per Table 3 and 4. The independent variables were Pressure and number of cycles whereas Polydispersity index (PDI) as dependent variables.

Entrapment efficiency[11]

It was done by the rupturing of the liposomes with absolute ethanol followed by 10 min sonication. The amount of Pazopanib released in ethanol was determined at 214 nm using a UV-visible spectrophotometer. The entrapment efficiency was calculated by

$$\% EE = [TD - FD/ TD] \times 100$$

[TD = Total Drug, FD = Free Drug]

Drug Release Studies

A drug-release study was conducted in 7.9 pH phosphate buffer media by inserting liposome into a pre-hydrated 13,000 MW cutoff dialysis membrane (Bio-design) with 5mL of release media, and sealed. The membrane was then placed in a beaker containing 50 mL of the media placed on magnetic stirrer at 100RPM. The Sink condition was maintained by addition of fresh media. Aliquots of 0.5mL were removed at regular time intervals and fresh preheated media was replaced. The removed aliquot was then analyzed for Pazopanib by UV spectrophotometry 214 nm.

Osmolality: Liposome can either osmotically active or inactive. In osmotically active liposomes the osmosis will occur through the lipid membrane but not in osmotically active liposomes. The osmotic behavior varies with size, lamellarity and lipid composition of liposomes. It is measured by freezing point depression method using 250µl of liposomal formulation. The limit is 270-330 mOsmo.[12]

Particle size distribution: Malvern zetasizer was used for the particle size analysis of the liposome formulations. Each liposomal formulation was evaluated for average particle size, size distribution and polydispersity index.

Field Emission Gun-Scanning Electron Microscopes (FEG-SEM): The surface morphology of liposomal formulation was done at IIT Mumbai by FEG-SEM. (JEOL JSM/7600F/FEG/SEM).

Stability Studies: The stability of the optimized batch of liposomes, was performed at 2–8°C and 25 ± 2°C/ RH60% for a period of 3 months. The change in the parameters like % EE and particle size of the stored liposomal dispersion were to assess before and after storage.[13]

Preparation of colon targeted capsules

For the colon targeting, the lyophilized liposomes were filled hard gelatin capsule shells which were coated using Eudragit S100. The capsules were dip coated by using 10%w/v Eudragit-S100 solution in acetone.[14]

Release of Drug from lyophilized liposomal powder filled in Eudragit S 100 coated capsules

To simulate the *in-vivo* conditions the dissolution studies were carried out in pH 1.2 for two hrs then at pH 6.8 for 3hr finally at pH 7.2 for 3hr using USP- I apparatus. The 5 ml sample was withdrawn at definite time interval and analyzed at 214nm.

In-vivo Studies

Animals

In-vivo study Protocol was approved by IEC committee of Y.B.Chavan College of Pharmacy, Aurangabad (CPCSEA/IAEC/P'ceutics-49/2019-20/155). The male Wistar rats (300–400g) were used for the study. The animals exposed to light–darkcycle of 12hr with standard diet and *ad libitum* water.

Experimental groups and technique

The animals were divided in two groups randomly:

Group 1 (G1): Treated with Pazopanib Liposomes, Group 2: Control.

The colorectal carcinogenesis was done using 1, 2- dimethylhydrazine (DMH) according to method described by Mario Jorge Juca *et al.*[15] DMH is an indirect inducer drug and have ability to promote DNA hyper-methylation of colonic epithelial cells in the segment. DMH was dissolved 0.9% NaCl solution containing1.5% EDTA. The pH of the solution was adjusted to 6.5 with 0.1 N NaOH. It is administered by SC route once a week for five weeks at a dose of 65 mg/kg/week.

The liposomal suspension was administered equivalent to 30mg/kg of body weight orally daily for 30 days

where as the control group treated with distilled water as placebo. The animals were sacrificed after 30 days treatment. The tumor growth was evaluated in terms of tumor volume and weight for control and treated group. Tumor growth was monitored by calipers, and tumor volumes were calculated according to the formula 0.5 × length × width^2.

RESULTS AND DISCUSSION

Preliminary Formulation Batches

1-5 Trials were taken with number of cycles as planned on the basis of DoE criterion then we got particle size in between 320 to 215 and polydispersity 0.3 to 0.150. In respect to these trials next trials were taken by changing the number of cycles with the variables. The temprature were kept constant at 60°C. In this 6 to 10 trial were taken trial 09 was found near to the targeted size and polydispersity so again in next trials change were made with number of cycles with respect to variables. Trial 11 to 15 were taken by manipulating the number of cycles the results were not found optimum so again next trials were planned with changing the no. of cycles (Table 3).

In trial 16 to 21 the two trials were found i.e trial 18 and trial 20 having the size 95 nm and polydispersity 0.09 and 96 nm and 0.093 PDI respectivly. Now further trials were taken.

In trial 22 to 27 the two trials were in the required range i.e trial 24 and trial 26. The number of cycles was 9, 3, 9 and 9, 6, 6 respectively and further studies were made by optimizing with the Design expert software (Table 4).

Full Factorial Design 3^3 by using 2FI model

The 3^3 full factorial designs were selected and D- optimal design was also selected to identifying the most vital variable. To study the effect of independent variables 1000 psi, 1500 psi, 2000 psi on dependent variables i.e. Particle size and Polydispersity.

Table 3: Polydispersity and Partical size of Experimental Trials of batch 1-15.

Trial No	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
No. of Cycles of 1000 psi	3	3	3	3	3	3	3	3	3	6	6	6	6	6	6
No. of Cycles of 1500 psi	3	3	3	6	6	6	9	9	9	6	6	6	3	3	3
No. of Cycles of 2000 psi	3	6	9	3	6	9	3	6	9	6	3	9	3	6	9
Particle Size of liposome	320	280	240	270	216	154	201	136	108	174	222	117	263	239	167
Polydispersity	0.3	0.25	0.189	0.21	0.157	0.116	0.143	0.108	0.103	0.133	0.162	0.105	0.19	0.177	0.126

Table 4: Polydispersity and Partical size of Experimental Trials of batch 16-27.

Trial No	16	17	18	19	20	21	22	23	24	25	26	27
No. of Cycles of 1000 psi	6	6	6	9	9	9	9	9	9	9	9	9
No. of Cycles of 1500 psi	9	9	9	9	9	9	3	3	3	6	6	6
No. of Cycles of 2000 psi	3	6	9	9	3	6	3	6	9	3	6	9
Particle Size of liposome	135	115	95	80	96	85	170	140	97	156	102	89
Polydispersity	0.108	0.104	0.09	0.08	0.093	0.083	0.13	0.11	0.094	0.117	0.101	0.087

Table 5: ANOVA for Full Factorial design 3^3 of response R1 (100 nm).

Source	Sum of Squares	Degree of Freedom (df)	Mean Square	*F* Value	*p*-value Prob > F	Model Significant/ Non-Significant
Model	4.40	18	0.24	23.37	**< 0.0001**	**Significant**
A-1000	1.77	2	0.89	84.65	< 0.0001	
B-1500	1.52	2	0.76	72.70	< 0.0001	
C-2000	0.99	2	0.50	47.38	< 0.0001	
AB	0.056	4	0.014	1.35	0.3333	
AC	0.014	4	3.603E-003	0.34	0.8408	
BC	0.046	4	0.012	1.10	0.4191	
Residual	0.084	8	0.010			
Cor Total	**4.48**	**26**				

Table 6: ANOVA for Full Factorial design 3^3 of response R^2 (0.100 PDI).						
Source	**Sum of Squares**	**Degree of Freedom (df)**	**Mean Square**	***F* Value**	***p*-value Prob > F**	**Model Significant/Non-Significant**
Model	3.10	18	0.17	128.14	< 0.0001	Significant
A-1000	1.18	2	0.59	441.39	< 0.0001	
B-1500	1.07	2	0.54	400.41	< 0.0001	
C-2000	0.56	2	0.28	209.80	< 0.0001	
AB	0.18	4	0.045	33.76	< 0.0001	
AC	0.042	4	0.010	7.75	0.0074	
BC	0.050	4	0.012	9.31	0.0042	
Residual	0.011	8	1.342E-003			
Cor Total	**3.11**	**26**				

By using the design expert 9.0.2.0 software ANOVA and Multiple regression analysis were done. Table 5 and 6 Shows ANOVA for dependent variable (R1) 100nm and (R^2) 0.100 PDI. The variables 1000 psi, 1500 psi, and 2000 psi were found to be significant at $p<0.05$ substantiate that effect of all the variables was significant on the selected responses.

The Model *F*-value of 23.37 and 128.14 mean that the model was significant. There is only a 0.01% chance that an F-value this much large could occur due to noise. Similarly R-squared near to zero indicated towards a good model. In all cases "Predicated R-squared" values were in reasonable agreement with the "Adjusted R-squared" values. The Adeq-Precision was the measure of the signal to noise ratio. A ratio > 4 was desirable. In our case the Adeq-Precision values were 17.761 and 43.309 for R1 and R2 respectively which indicated an adequate signal.

Equation

100 nm (Y) =

+5.03+0.28* A[1] +0.052* A[2] +0.27* B[1] +0.030* B[2] +0.23* C[1] +9.096E-003* C[2] +0.043* A[1]B[1] +0.037* A[2]B[1] -3.109E-003* A[1]B[2] +1.075E-003* A[2]B[2] +0.016* A[1]C[1] -0.014* A[2]C[1] -0.012* A[1]C[2] +0.041* A[2]C[2] -0.038* B[1]C[1] +0.065* B[2]C[1] +0.042* B[1]C[2] -0.011* B[2]C[2]

Equation

0.100 PDI (Y)

-2.06+0.25* A[1] +9.028E-003* A[2] +0.24* B[1] -4.872E-004* B[2] +0.17* C[1] +7.637E-003* C[2] +0.14* A[1]B[1] -0.016* A[2]B[1] -0.048* A[1]B[2] * A[2]B[2] +0.065* A[1]C[1] -0.025* A[2]C[1] -0.021* A[1] C[2] +0.038* A[2]C[2] +6.768E-003* B[1]C[1] +0.045* B[2]C[1] +0.032* B[1]C[2] -1.738E-003* B[2]C[2]

Response Surface Plot

To study the effects of independent variables on the response such as 100 nm and 0.100 PDI the response surface plots were produced using Design Expert 9.0.2.0 software (Figure 1).

The model obtained from the regression analysis used to build a 3-D graphs in which the responses were represented by Sloped surface as a function of independent variables.

Half Normal Plot

In that half normal plots (Figure 2) of R1 and R2 were generated interaction of variables. In the R1 AB, AC, BC were not found in optimum limit and in R2 AC, BC were not found in optimum limit. Hence main model was found significant.

Model Validation (optimization)

The three formulation trails were selected as the model formulation as per Table 7. The Response R1 100 nm and R2 0.100 was found within the limit. When predicted and experimental values were campared the results were with in limits confirms the validation of medel (Table 8). From the Design Expert it can be concluded that F3 formulation is the optimized formulation which shows the number of cycles of 1000, 1500, 2000 psi was 9, 6, 6 respectivly.

Optimized Formulation Trail F 01, 02, 03. (using Design Expert software)

The final three formulations i.e F1, F2, F3 were optimized and it was correlated with the pridicted value

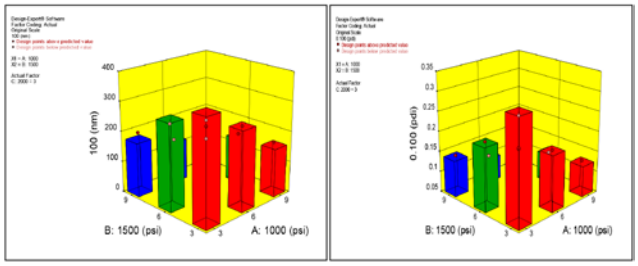

Figure 1: Response surface plots of Response R1 (100 nm) and R2 (0.100 PDI).

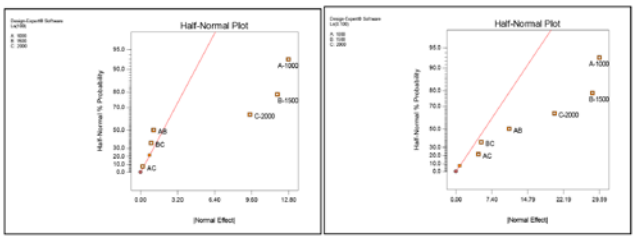

Figure 2: Half Normal Plot of R1 (100 nm) and R2 (0.100 PDI).

Table 7: Optimization (Model Validation).

Sr. No	1000 psi	1500 psi	2000 psi	R1 (100 nm)	R2 (0.100 PDI)	Desirability	Selected/ Not Selected
1	**9**	**6**	**6**	**109**	**0.0998**	**0.975**	**Selected**
2	9	3	9	107.57	0.094	0.818	
3	9	9	3	106.548	0.092	0.778	

Table 8: Comparison of Predicted and Experimental Values.

Sr. No	Responses	Optimized Formulation	
		Predicted	Experimental
1	100 nm	109.442	101.6
2	0.100 PDI	0.099875	0.096

which was obtained by Design Expert Software (Table 9). The Experimental values came witihn a range. Other Evaluation parameters were also done i.e osmolality, assay of lipid concentration, assay of drug concentration, entrapped drug, free drug, encapsulation efficiency were comes in optimum ranges.

Particle size determination: The particle size of the optimized batches F1, F2, F3 is 95.57, 106.8, 101.6 nm respectively (Figure 3).

In-vitro drug release studies

Cumulative amount of Pazopanib released from liposomes in PB- 7.9 pH media through a pre-hydrated

Table 9: Optimized Batch F 01, 02, 03.

Batch Code	F 1	F 2	F 3
No. of Cycles of 1000 psi	9	9	9
No. of Cycles of 1500 psi	9	3	6
No. of Cycles of 2000 psi	3	9	6
Particle Size of liposome	95.57	106.8	101.6
Polydispersity	0.089	0.098	0.096
Osmolality (mosmo)	290	295	305
Assay of Lipid conc.	99 %	97 %	100 %
Assay of Drug conc.	100 %	99 %	102 %
Entrapped Drug	98.4 %	99.3 %	98 %
Free Drug	1.6 %	0.7 %	2.0 %
Encapsulation efficiency	96.77 %	97.59 %	99.93 %

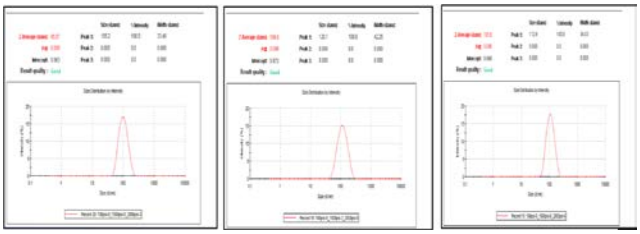

Figure 3: Partical size distibution of Trail F1,F2,F3.

13,000 MW cutoff dialysis membrane (Biodesign) is shown in Figure 4. All the optimized formulations showed good release more than 80% of drug released in 3hr. Amongst the three formulation F3 had showed good release profile so taken for further study (Figure 4).

Release of Drug from lyophilized liposomal powder filled in Eudragit S 100 coated capsules

The drug release studies were performed in pH 1.2 for two hrs then at pH 6.8 for 3hr finally at pH 7.2 for 3hr using USP- I apparatus. The results revealed that the coated capsule is able to prevent release the drug for 5hr and the drug will release in colonic area (Figure 5).

Field Emission Gun-Scanning Electron Microscopes (FEG-SEM)

The FEG SEM image analysis showed the porous surface morphology with spherical and uniform shape of liposomes. The study clearly showed the formulated liposomes are of sizes ranging 70 to 150 nm (Figure 6). This confirms that the desirable size range of liposome is achieved.

In-vivo activity study

Histopathology

After the tumor and Volume studies the tissues were fixed by 10% buffered formaldehyde for 24hr followed by dehydration using ethanol. Then tissue sections samples

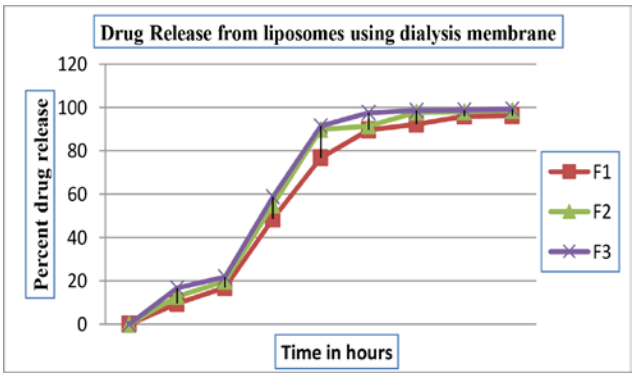

Figure 4: *In-vitro* drug release from liposomes using dialysis membrane.

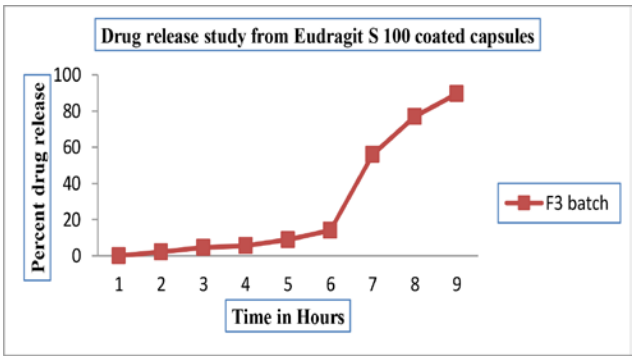

Figure 5: *In-vitro* drug release from liposomes filled in Eudragit S 100 coated capsules.

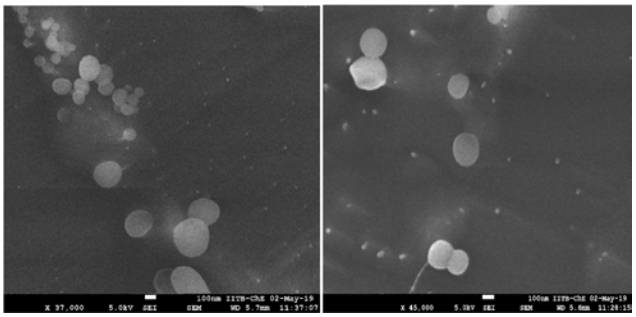

Figure 6: FEG SEM image of Pazopanib Liposome.

were collected by embedding the tissues in paraffin and then mounted on glass slides. The sections were then marked with Hematoxylin/eosin and observed under microscope.

Normal colorectal tissue were observed in the animals in the group (A). The pattern of staining in the nuclei was homogeneous. Mitotic tissue changes were not observed whereas the size or shape of the glands also uniform. In this animal tumor is not induced. Where as in the animals in group (B) treated with pazopanib liposomes reveals changes of reasonable dysplasia reflecting deformities nuclei of the cell with increased number of mitoses and

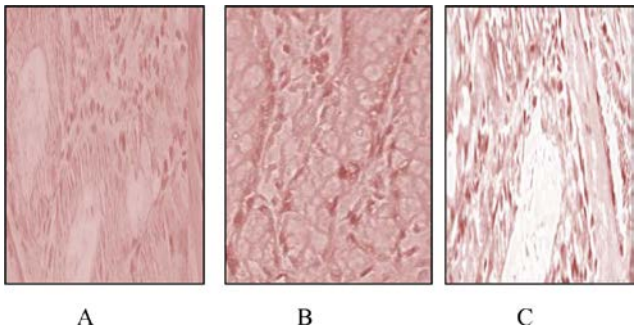

Figure 7: Histo-pathological evolution of Colon tissues.
(A): Normal Tissues (B): Pazopanib Treated group (C) Untreated Group (Control)

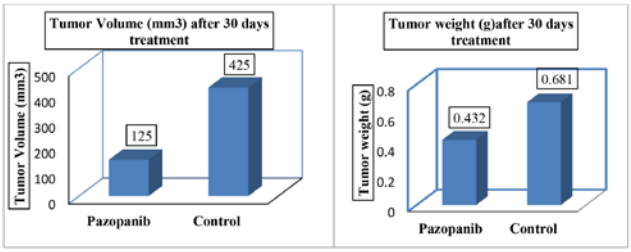

Figure 8: Tumor volume and tumor weight after 30 days of treatment (*n*=6).

thickening of the glandular epithelium. The animals in group (C), colon cancer induced control group showed severe dysplasia characterized by broad and round nuclei with higher nucleoli, and atypical mitotic Figures. (Figure 7)

The tumor growth was evaluated in terms of tumor volume and weight for control and treated group. Tumor growth was measued by calipers whereas the tumor volumes were calculated according to the formula ($0.5 \times \text{length} \times \text{width}^2$). Both tumor volume and weight of the group treated with pazopanib liposomes was significantly lesser as compared to control group (Figure 8). This indicates that Pazopanib liposomes caused significant tumors growth suppression in the treated group as compared to control.

CONCLUSION

On the basis of above study it can be concluded that Pazopanib colon targeted liposomes prepared by optimized high pressure homogenization process will serve as a better alternative to treat colon colorectal cancer. The process can be scalable for industrial production of liposomes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CRC: Colorectal cancer; **PDI:** Polydispersity index; **HSPC:** Hydrogenated Phosphotidylcholin; **MW:** Molecular weight; **DMH:** 1, 2- Dimethylhydrazine.

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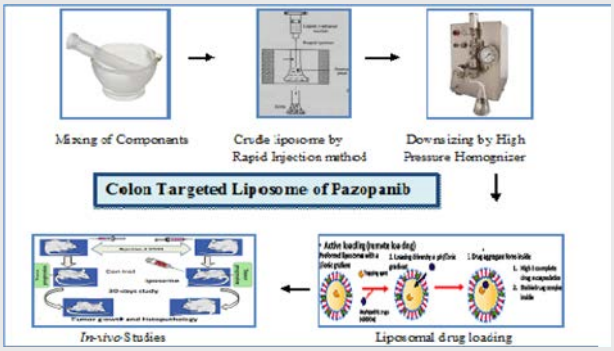
PICTORIAL ABSTRACT



SUMMARY

High pressure homogenization process produced the liposomes of required characteristics; when the process was optimized with respect the various process parameters. Pazopanib liposomes significantly suppressed the tumors growth in terms of tumor volume and weight as compared to control.

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