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ph-Responsive Alendronic Acid-Coated Liposomes for Improved Treatment Osteosarcoma

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Abstract

Objective

Current research work aimed to develop pH-responsive osteosarcoma-targeted liposomes composed of non-oncology drugs previously identified for repositioning purposes.

Material and Methods

Using thin film hydration method, the Ketoconazole (KCZ)-, Simvastatin (SVN)-, and Niclosamide (NSD)-loaded non-targeted liposomes (KLs, SLs, and NLs respectively) were developed individually. The Aledronic acid-Choleteryl hemiscuccinate conjugate (AA-CHSC) was synthesized and confirmed using FTIR and ^{1}H-NMR analysis. The AA-CHSC coated liposomes (pH sensitive and targeted liposome: PT-KLs, PT-SLs, and PT-NLs) were developed individually for the treatment of osteosarcoma with improved efficacy and reduced drug's primary effects. The developed non-targeted and targeted liposomes were characterized for drug contentt,vesicle size, surface morphology using transmission electron microscope (TEM), *in vitro* pH-sensitive drug release behaviour using dialysis bag technique, and *in vitro* cytotoxicity characteristics against osteosarcoma cells.

Results

All non-targeted liposomes showed %drug content and mean vesicle size in the range of 41-75% and 161-182 nm, respectively. The AA-CHSC insertion into the liposomal membrane (targeted liposomes) has substantially improved the drug solubility in the membrane as a result of its amphiphilic nature. Furthermore, the targeted liposomes

showed moderately increased mean vesicle size (203-210 nm) validating the surface modification. The TEM image revealed the formation of spherical, nearly spherical, and non-aggregated liposomes. The AA-CHSC insertion also caused liposomes sensitive to tumor pH (5.8); as a result, substantially higher drug release (58-80%) was observed as compared to non-targeted liposomes (34-52%). The cytotoxicity study results revealed substantially higher osteosarcoma cell (Saos-2) growth inhibition characteristics of targeted liposomes.

Conclusions

In conclusion, the developed targeted liposomes have potential application in the treatment of osteosarcoma. In addition, detailed anticancer activities should be revealed for the combination of targeted liposomes as the combination caused almost similar cytotoxicity as that of individual targeted liposomes.

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Keywords: Drug repurposing, Osteosarcoma, non-oncology drugs, pH-sensitive release.

1. INTRODUCTION

The most frequent primary paediatric bone cancer is osteosarcoma, which develops from prehistoric mesenchymal cells that produce osteoid. Owing to its highly variable expression, osteosarcoma can be classified into several subtypes based on the level of differentiation where it develops in the bone, and the histological variation.[1]It is commonly diagnosed in patients between the ages of 10 and 19, with an incidence rate of 1 to 5 cases per million people. Although there is a dose effect on treatment response, ample of studies have demonstrated that high-dose chemotherapy may not improve survival rates any more than less toxic moderate dosages.[2]The anti-osteosarcoma drugs now in use have a limited therapeutic index because of the tumour selectivity issues, the metastatic events, or the intricate aetiology of these bone tumours [3], and survival rates have not increased over the past three decades. The 5-year event-free survival rate for localised osteosarcoma is 60–70% when chemotherapy and surgery are combined.[4]Clinical research on osteosarcoma is tough to carry out because there aren't any new medications under development. Drug repurposing, an alternate research pathway that tries to use current pharmaceuticals as the foundation for novel therapeutic options with the ability to target many disease markers, offers an exciting answer to this issue.[5]

Our previous report has revealed the potential uses of ketoconazole (KCZ), simvastatin (SVN), and niclosamide (NSD) in the treatment of osteosarcoma in the direction of finding the non-oncology drugs that are both safe and efficient in place of harmful chemotherapy. In this early work, KCZ, SVN, and NSD individually and in combination (1:1:3) significantly increased the growth inhibition of Saos-2 and MG-63 cells (measured in picomoles), as well as the percentage of cells arrested at the S and G2/M phases.[6]Also, these three drugs have shown improved CDK1 interaction, suggesting that they may be able to fight cancer by inhibiting CDK1. In addition, the in vivo investigation using the combination (1:1:3) of these three medications did not find any notable differences in the rats' hematological and biochemical parameters, body weights, weights of their important organs, daily food and water intake, or overall behaviour.[7-8]

Following intravenous delivery, liposomal medications can exhibit better kinetics and dynamics, which can further broaden the therapeutic window and boost the efficacy and reduce the toxicities of encapsulated medications.[9-10] Additionally, the nanoparticulate drugs (including liposomal drugs) may decrease the primary effects of repurposed drugs by targeting them to selected area in the body. In comparison to liposomal membranes made simply of typical components like phospholipids and cholesterol, oils (such as vitamin E oil; VEO) in the membrane along with phospholipid and cholesterol have proven to have several advantages in our prior research.[11-12]The inclusion of VEO into the liposomal membrane has substantially improved the drug entrapment, reduced the drug leakage, and improved the chemical stability of the entrapped drug. Additionally, the presence of VEO in the membrane led to a regulated release of the medication in vitro, a reduction in hemolysis, and a reduction in in vivo toxicity.

The present research was aimed to individually develop liposomes containing identified non-oncology drugs KCZ, SVN, and NSD for the treatment of osteosarcoma with improved efficacy, and reduced primary effects. The Soyabean oil would be used as an alternative to VEO as one of the liposomal membrane components. The targeting of developed liposomes to osteosarcoma would be further improved by surface coting with

Aledronic acid (a bisphosphonate)-Choleteryl hemiscuccinate conjugate (AA-CHSC). The developed targeted liposomes would be evaluated primarily for drug loading, mean vesicle size, and in vitro drug release behaviour and would be compared with non-targeted liposomes. Finally, the *in vitro* cytotoxicity characteristics of targeted liposomes against osteosarcoma cells would be determined in comparison with non-targeted liposomal drugs.

2. MATERIALS AND METHODS

2.1. Materials

Ketoconazole (KCZ), Simvastatin (SVN), Niclosamide (NSD), Sodium Alendronate, Cholesteryl hemisuccinate, and Propidium iodide were obtained from Sigma Aldrich in Mumbai. HSPC was provided by Lipoid GmbH, Germany, while cholesterol was obtained from HiMedia Bangalore. Soybean oil was obtained from Molychem, India. MTT Powder, Foetal bovine serum (FBS), PenStrep, Trypsin, and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Invitrogen in Bangalore.

2.2. Cell Culture

Saos-2 and HEK-293 cells were obtained from ATCC in the United States. Saos-2 cells were cultivated in RPMI media, while HEK-293 cells were cultured in DMEM. The media was supplemented with 10% inactivated FBS, penicillin (100 IU/mL), and streptomycin (100 µg/mL). Cells were cultured in a humidified environment of 5% CO2 at 37oC until confluent. The cells were dissociated using cell dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS).

2.3. Methods

2.3.1. Preparation and optimization of Liposomes

The KCZ-loaded liposomes (KLs) were developed using the thin-film hydration process. Briefly, the liposomal components were dissolved in 15mL of a methanol: chloroform mixture (1:2 v/v) using bath sonication. The solvent was evaporated at 65±2ºC using a rotary evaporator (BUCHI Rotavapor R-200, BUCHI India Private Ltd, Mumbai, India) to create a thin film. The film was then held under vacuum overnight to eliminate the remaining solvent. The film was moistened with sterile water for injection (SWI, 40mL) at 70±2ºC for 15 minutes. The resultant multilamellar liposomes reduced in size by probe sonication to produce small unilamellar vesicles (5 cycles of 1 minute each). Each cycle consists of 12 seconds of on time at 240V and 0.6A current followed by 8 seconds of off time.[13-14] The obtained liposomes were then processed by using the above procedure SVN-loaded liposomes (SLs) and NSD-loaded liposomes (NLs) were also developed.

2.3.2. Formulation optimization

The KCZ-loaded liposomes were optimized by Box-Behnken design (BBD) using a using Design Expert® software.[15-16]A total of 17 runs (F1-F17) were created *via* software and investigated the effects of independent variables on response variables at three levels. Three independent variables selected were HSPC (X_1), Cholesterol (X_2), and, Soyabean oil (X_3) whereas, % EE (Y_1) and the vesicle size (PS; Y_2). All independent and response variables with their coded and actual levels are depicted in Table 1. The KCN-loaded liposomes were fabricated according to the software-generated experimental design matrix (Table 2) and the results obtained were evaluated. The statistical significance of obtained results was established by analysis of variance (ANOVA).

Table:1: Variables and responses along with their coded levels and constraints used in BBD.

Independent variables	**Low (-1)**	**Medium (0)**	**High (+1)**
X_1- HSPC (mg)	35.81	53.72	71.63
X_2- Cholesterol (mg)	9.08	40.885	72.69
X_3- Soyabean oil (mg)	5.59	25.18	44.78
Dependent variables (Factors)			
Y_1- Drug content (%) - Maximum			
Y_2- Vesicle size (nm) - Minimum			

(Data shown as mean ± standard deviation, n = 3)

Table 2: Design matrix of KCN-loaded liposomes as per BBD

Runs	HSPC (X_1)	Cholesterol (X_2)	Soyabean oil (X_3)	% EE (Y_1)	PS (nm) (Y_2)
1	53.72	9.08	5.59	14.8±1.23	147.5±1.23
2	53.72	72.69	5.59	21.81±2.56	156.5±2.14
3	53.72	40.885	25.185	29.93±1.47	283.5±1.34
4	71.63	40.885	5.59	26.43±2.61	208.7±2.55
5	71.63	9.08	25.185	19.1±2.58	224.9±2.34
6	35.81	40.885	5.59	14.8±2.35	176.7±1.33
7	53.72	40.885	25.185	29.93±2.47	283.5±1.54
8	35.81	40.885	44.78	22.13±1.74	201.6±2.54
9	35.81	72.69	25.185	35.82±1.89	100.5±1.11
10	53.72	40.885	25.185	29.93±1.48	283.5±1.97
11	71.63	40.885	44.78	36.78±1.94	203.9±2.34
12	53.72	9.08	44.78	21.17±2.56	177.4±2.66
13	53.72	72.69	44.78	56.36±2.94	171.8±2.57
14	71.63	72.69	25.185	46.81±2.22	196.8±2.47
15	53.72	40.885	25.185	29.93±2.37	283.5±1.64
16	53.72	40.885	25.185	29.93±1.52	283.5±2.37
17	35.81	9.08	25.185	7.64±2.17	140.3±1.23

(Data shown as mean ± standard deviation, n = 3)

2.3.3. Characterization of Liposomes

2.3.3.1. %Drug content and % Drug Loading Capacity

Following centrifugation at 10,000 rpm for 10 minutes at room temperature, the liposomal dispersion supernatant (0.2 mL) was diluted to 10 mL with methanol, bath sonicated for 5 minutes, and then examined using a UV-Visible spectrophotometer with a lambda maximum of 244 nm for KCZ, 235 nm for SVN, and 332 nm for (NSD) against plain methanol as a blank. The formula below is used to determine the percentage drug content (%EE).[17]

$$\% EE = \frac{Amount\ of\ drug\ recovered}{Amount\ of\ drug\ added} \times 100$$

2.3.3.2. Mean vesicle size and zeta potential analysis

Horiba Zetasizer (HORIBA SZ-100) was used to evaluate the liposomal dispersions' mean vesicle size and zeta potential. The samples were filtered, and the resulting filtrate then went to an analysis after being diluted 100 times with double distilled water (DDW). Every measurement was done three times, and the results were given as Mean ± SD.[18]

2.4. Synthesis of Alendronic Acid (AA)-Cholesteryl hemi succinate (CHS) Conjugate (AA-CHSC)

Alendronate sodium (**1**, 1 Equiv) and cholesteryl hemisuccinate (**2**, 1 Equiv.) was taken in round bottom flask containg dichloromethane (DCM) and Tetrahydrofuran (THF) (3:1) as solvent. After that, the reaction mixture was added to EDC hydrochloride (1.1 Equiv), Hydroxybenzotriazole (HOBT) (1 Equiv), and 4-Dimethylaminopyridine (DMAP) (1.1 Equiv), and it was agitated for 10 hours at room temperature. (Figure 1) The solvent diminished once the process was finished. under vacuum to obtain the crude product alendronic acid-Cholesteryl hemisuccinate conjugate (AA-CHSC) (**3**). The obtained crude product was recrystalized by using ethanol.[19]

2.5. Characterization of Alendronate-Cholesteryl hemi succinate Conjugate

2.5.1. FTIR

Fourier transform infrared spectroscopy (Vertex 70, Bruker, Billerica, USA) was used for recording changes within the sample's functional groups. The FTIR spectrum of AA-CHSC was produced using the attenuated total reflectance (ATR) method. The FTIR spectra were recorded spanning 4000-400 cm−1 at a resolution of 4 cm−1 for 50 scans.[20-21]

2.5.2. H^1-NMR

The ^{1}H NMR studies was carried out at room temperature using a Bruker AV400 or Bruker AVIII HD 400 spectrometer. The chemical shifts of the spectrums were determined in ppm relative to the residual solvent peak, and the NMR spectra were evaluated using Topspin 3.0 software. The following abbreviations are used to describe multiplicities: s = singlet; br = broad; d = doublet; t = triplet; and m = multiplet.[22-23]

2.6. Preparation of AA-CHSC (ligand)-coated liposomes (Targeted and pH-sensitive liposomes)

The AA-CHSC was dissolved in Chloroform and methanol mixture (1:1 v/v). The solvent was evaporated to obtain thin film. The liposomes (KLs, SLs, and NLs) were transferred to beaker containing AA-CHSC film separately, warmed to 70°C on a water bath, and kept in water bath for 30 min with continuous manual shaking. The resultant targeted and pH-sensitive liposomal dispersions (TP-KLs, TP-SLs, and TP-NLs) were filtered and subjected for characterization.

2.7. Characterization of pH-sensitive and targeted liposomes

The TP-KLs, TP-SLs, and TP-NLs were subjected for %EE, mean vesicle size, and zetapotential analysis as described for uncoated liposomes.[24]

2.7.1. Transmission electron microscope (TEM) analysis

To ascertain the morphology of TP-KLs, TEM (JEOL 1011, Tokyo, Japan) investigation was performed. The TEM was run at an accelerating voltage of 100 kV throughout the investigation. A 5µL sample drop was applied on a 400-mesh copper grid that had been covered with Formvar film. After contrast enhancement using osmium tetroxide vapour, the sample was allowed to air dry. Samples were stained with a 5µL of 0.75% w/w uranyl acetate solution. Grids were kept at room temperature until analysis. The liposomal images were captured using ImageJ software.[25]

2.7.2. In vitro drug release study

The drug release study was conducted on TP-KLs, TP-SLs, and TP-NLs at pH 7.4 (blood pH) and 5.8 (tumour pH). The in-vitro release research was performed utilising the dialysis bag technique at 37±2°C in a USP class II (paddle) device. In brief, liposomal dispersions equivalent to 5 mg drugs were inserted into dialysis tubes (MWCO 12000), hermetically sealed, linked to a paddle, and immersed separately in a vessel containing 100mL of phosphate buffer solution (PBS) of pH 7.4 and pH 5.8 containing 5% (v/v) methanol.[26] The dissolution media were maintained at 37°C with continuous stirring at 100 rpm. The samples (3 mL) were taken out and subjected to analysis using a UV-visible spectrophotometer at 244 nm for KCZ, 235 nm for SVN, and 332 nm for NSD at specified time intervals (0, 1, 2, 4, 6, 12, and 24h). A new buffer was added in equal volume substitution of the sample. The %KCZ, %SVN, and %NSD released was calculated and plotted against time.

2.7.3. In vitro cytotoxicity study

Human osteosarcoma cells (Saos-2 cells) were used to test the in vitro cytotoxicity of coated and untreated liposomes. The combination effect of coated liposomes was also determined against Saos-2 cells. In short, 96 well-plates were seeded with 50000 Saos-2 cells per well, and the cells were then allowed to adhere and develop overnight at 37°C with 5% CO_2. The cells were then incubated with RPMI medium (100µL) containing different concentrations (100nM-0.001nM) of uncoated (KLs, SLS, and NLs), and targeted and pH-sensitive (TP-KLs, TP-SLS, and TP-NLs) liposomal drugs. After 48 hours of incubation, the test solutions were replaced with 100µL of MTT solution (6 mg/10mL in PBS) and incubated for 4 hours in the same conditions. Finally, the MTT solution was replaced with same volume of DMSO to dissolve the formazan crystals present in viable Saos-2 cells. Using a microplate reader, the absorbance of the final solution was calculated at 590 nm. Dose-response curves were then used to get the IC_{50} values.[27] Using the above procedure, the cytotoxic nature of the different combinations of targeted and pH-sensitive (TP-KLs, TP-SLS, and TP-NLs) liposomal drugs was also determined. Furthermore, the toxicity of these combinations against healthy human cells was also determined using human embryonic kidney cells (HEK-293).

3. RESULTS AND DISCUSSION

3.1. Preparation and optimization of KLs

Box-Behnken design (BBD) (i.e. 3 factors, 3 levels) is commonly used in the formulation development and optimization.Three independent variables were evaluated: HSPC (X_1) Cholesterol (X_2) and, Soyabean oil

(X_3). The % EE (Y_1) and the PS (Y_2) were selected as dependent variables given in **Table 2**. The concentrations of variables of the prepared KCN-loaded liposomes are as per BBD.[28-29]

3.2. Fitting of data to the model:

A total 17 runs were generated from the software and the findings obtained are shown in Table 2.The quadratic model was the best fit for the responses Y_1 (R^2: 0.8954) and Y_2 (R^2: 0.8577), which confirms that the suggested model can accurately predict the 89.54% and 85.77% variances in responses Y_1 and Y_2, respectively. By using ANOVA, the models' significance and effectiveness were evaluated and presented in Table 3 and 4, respectively.

The model term was confirmed to be significant by the Prob (p) value being <0.05. The model's F-value, which indicates the model's significance, was determined to be 18.33 and 11.72 for the response variables Y_1 (Table 3) and Y_2 (Table 4), respectively. The response surface analysis plots displaying the significant consequence of independent variables on the response variables are shown in Fig.1 and 2.

The X_1, X_2 and X_3 variables positively impacted the % EE (Y_1) (Fig.2). Similarly, the positive effect was noticed on Y_2 with X_1 and X_3. In contrast, X_2 displayed negative influence on Y_2(Fig.3). Furthermore, X_1 X_3 and X_2 X_3 had positive effect and X_1 X_2 had negative effect on Y_1. Similarly, X_1 X_3 and X_2 X_3 demonstrated negative effect and X_1 X_2 had positive effect on Y_2..

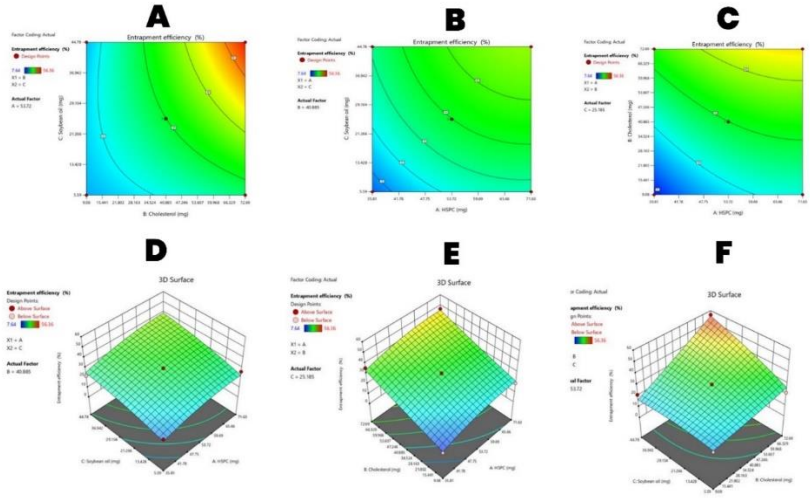

Figure. 1. 2D-Response surface (A, B, C) and 3D-Response surface plots (D, E, F) showing the effect of HSPC (X_1) Cholesterol (X_2) and, Soyabean oil (X_3) on % EE (Y_1) of KCN-loaded liposomesrespectively.

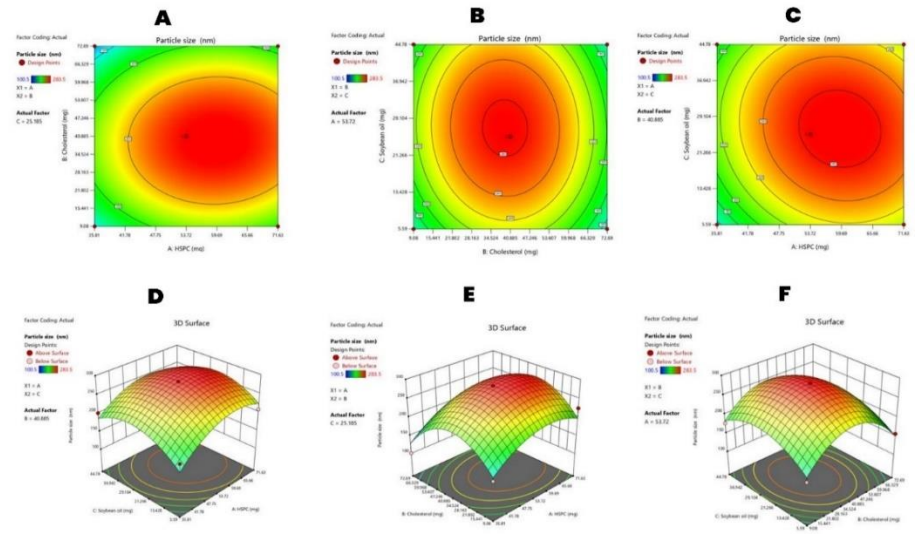

Figure.2. 2D-Response surface (A, B, C) and 3D-Response surface plots (D, E, F) showing the effect of HSPC (X_1) Cholesterol (X_2) and, Soyabean oil (X_3) on % Vesicle size (Y_2) of KCN-loaded liposomesrespectively.

These obtained findings signified that an increase in HSPC, cholesterol, and soyabean oil concentration resulted in an augment in the % EE of KCN in the liposomes. Further, an increase in the vesicle size of liposomes was noticed with an increase in the concentration of HSPC and soyabean oil. On the other hand, drop in vesicle size of liposomes was observed with an increase in the concentration of cholesterol.The polynomial equations derived for the responses Y1 and Y2 that demonstrate the relationship between independent and dependent variables are displayed below. The data of variables and, their levels of desirability constraints are tabulated in Table 1.

$$Y_1 = +29.93i+6.09\ X_1+12.26\ X_2+7.33\ X_3-0.1175\ X_1\ X_2+0.7550\ X_1\ X_3+7.05\ X_2\ X_3-3.04\ X_1^2+0.4562\ X_2^2-1.85\ X_3^2 \quad \text{Eq. 1}$$

$$Y_2 = +283.50i+26.90\ X_1-8.06\ X_2+8.16\ X_3+2.93\ X_1\ X_2-7.42\ X_1\ X_3-3.65\ X_2\ X_3-41.73\ X_1^2-76.15\ X_2^2-44.05\ X_3^2 \quad \text{Eq. 2}$$

Table 3: ANOVA for Quadratic model for % EE of KCN-loaded Liposomes

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2185.88	9	242.88	18.33	0.0005	**Significant**
A-HSPC	296.83	1	296.83	22.40	0.0021	
B-Cholesterol	1202.71	1	1202.71	90.75	< 0.0001	
C-Soybean oil	429.24	1	429.24	32.39	0.0007	
AB	0.0552	1	0.0552	0.0042	0.9503	
AC	2.28	1	2.28	0.1720	0.6907	
BC	198.53	1	198.53	14.98	0.0061	
A^2	39.01	1	39.01	2.94	0.1299	
B^2	0.8765	1	0.8765	0.0661	0.8044	
C^2	14.43	1	14.43	1.09	0.3314	
Residual	92.77	7	13.25			
Lack of Fit	92.77	3	30.92			
Pure Error	0.0000	4	0.0000			
Cor Total	2278.65	16				

Table 4: ANOVA for Quadratic model for PS of KCN-loaded Liposomes

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	51240.10	9	5693.34	11.72	0.0019	**Significant**
A-HSPC	5788.88	1	5788.88	11.92	0.0107	
B-Cholesterol	520.03	1	520.03	1.07	0.3353	
C-Soybean oil	533.01	1	533.01	1.10	0.3297	
AB	34.22	1	34.22	0.0704	0.7983	
AC	220.52	1	220.52	0.4539	0.5221	
BC	53.29	1	53.29	0.1097	0.7502	
A^2	7330.42	1	7330.42	15.09	0.0060	
B^2	24416.09	1	24416.09	50.26	0.0002	
C^2	8170.12	1	8170.12	16.82	0.0046	
Residual	3400.66	7	485.81			
Lack of Fit	3400.66	3	1133.55			
Pure Error	0.0000	4	0.0000			
Cor Total	54640.76	16				

3.3. Preparation and characterization of SLs and NLs

The SVN-loaded and NSD-loaded liposomes (SLs and NLs) are prepared using the procedure used for the preparation of KCZ-liposomes. The SLs and NLs are prepared using HSPC, Cholesterol, and Soybean oil at the molar ratios of optimized KLs. The %EE and mean vesicle size of KLs, SLs, and NLs are presented in

Table 5. The %EE of KLs and SLs is found almost same whereas, the %EE of KLs is found substantially higher than NLs. The Z-average of KLs, SLs, and NLs is found in the range of 161–182 nm.

Table 5. The %EE and mean vesicle size of optimized KLs, SLs, and NLs

Liposomes	HSPC (X_1)	Cholesterol (X_2)	Soyabean oil (X_3)	CHEMS	AA-CHSC	%EE (Y_1)	Vesicle size (Z-average) (nm) (Y_2)
KLs	53.72 mg (0.068mM)	72.69 mg (0.1879mM)	44.78 mg (0.04867mM)		--	75.6±2.9	172±11
SLs	53.72 (0.068mM)	72.69 (0.1879mM)	44.78 (0.04867mM)		--	78.8±4.4	161±12
NLs					--	41.8±4.7	182±9
	Targeted and pH-sensitive Liposomes						
TP-KLs	53.72 (0.068mM)	72.69 (0.1879mM)	44.78 (0.04867mM)	10 mol%	20 mol%	92.8±3.2	203±9
TP-SLs						97.7±2.5	210±15
TP-NLs						69.3±3.7	208±13

The amount of KCZ, SVN, and NSD used is 40mg. The millimoles of liposomal components are based of their molecular weights (HSPC: MW=790; Cholesterol: MW=386.65; Soybean Oil: MW: 920). CHEMS and AA-CHSC are used in mol% to that of total lipid content (HSPC, Cholesterol, Soyabean oil).

3.4. Synthesis and Characterization of AA-CHSC

As shown in the scheme (Fig.3), Alendronate sodium (1) and Cholesteryl hemisuccinate (2) were added together to synthesize the Alendronic acid-Cholesteryl hemisuccinate conjugate (AA-CHSC) (3) in DCM: THF (3:1). AA-CHSC (3) was identified by the use of IR and ^{1}HNMR spectra in addition to the presence of a newly formed amide group. The production of AA-CHSC (3) is confirmed by the IR spectra of the compound, which showed distinctive bands at 1638 cm-1 for the amide group, and the proton ^{1}HNMR spectrum, which showed the characteristic peak of singlet at 8.09 (s, 1H, -CONH-) for the amide.

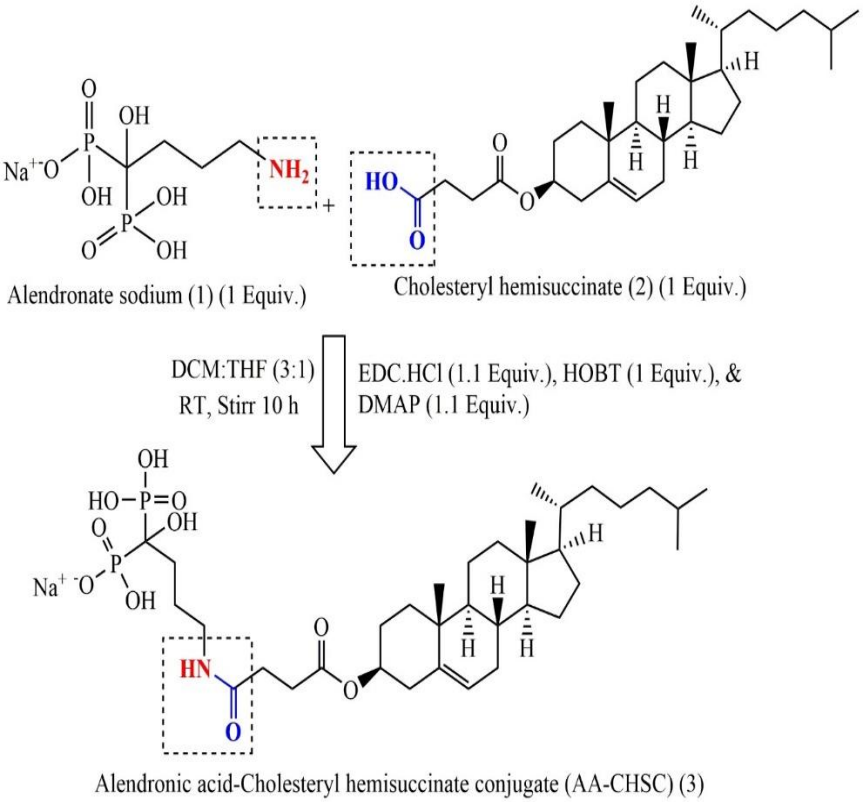

Figure. 3. Scheme for synthesis of Alendronic Acid (AA)-Cholesteryl hemisuccinate (CHS) conjugate (AA-CHSC)

3.5.1.IR cm^{-1}: 3332 (O-H), 2939 (C-H), 1727 (Ester-CO), 1638 (Amide-CO), 1559, 1449, 1388, 1210, 1162, 1058, 1001, 952, 821, 747, 638 (Fig.4A).

3.5.2.^{1}H NMR ($CDCl_3$, 400MHz): δ 11.98 (s, 3H, P(O-H), 8.09 (s, 1H, N-H), 6.42 (s, 1H, Vinyl C-H), 5.25 (s, 1H, O-H), 4.75 (s, 1H, OC-H), 3.39 (t, 2H, O-CH_2), 2.93 (t, 2H, N-CH_2), 2.21 (t, 4H, CH_2-CO), 1.78 (t, 2H, CH_2), 1.58 (t, 2H, CH_2), 1.05-1.48 (m, 18H, CH_2), 0.99-1.05 (m, 11H), 0.93-0.97 (m, 12H) (Fig.4B).

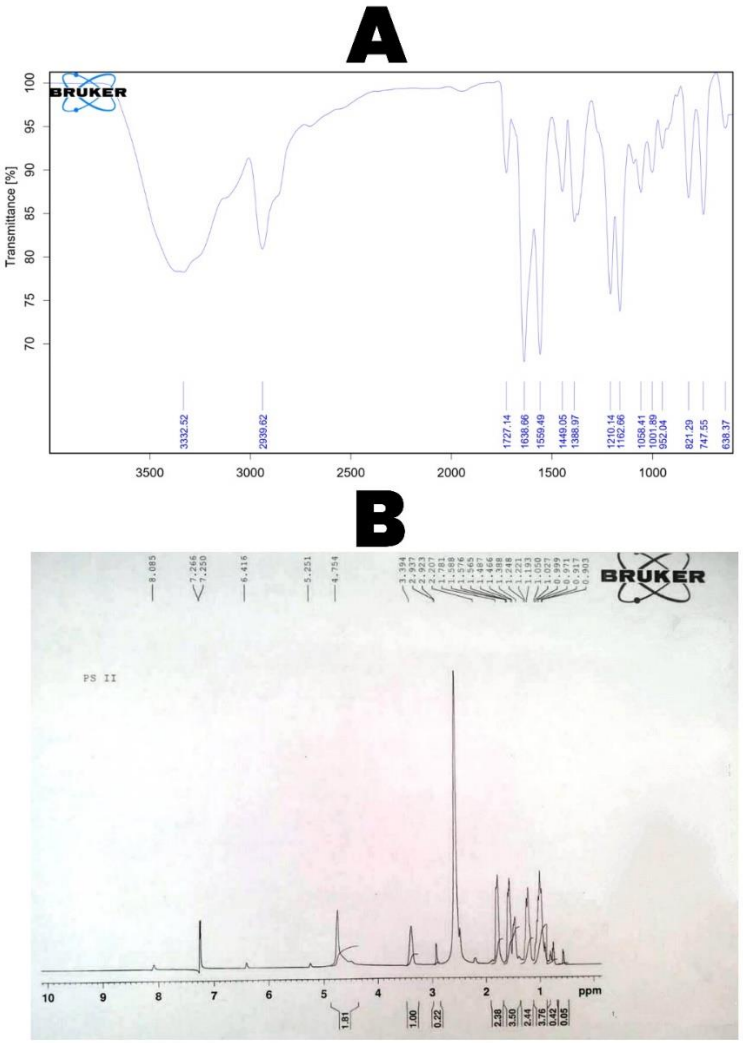

Figure. 4. A) FTIR Spectrum of Cholesteryl hemisuccinate-Alendronate conjugate B) NMR spectrum of Cholesteryl hemisuccinate-Alendronate conjugate.

3.6. Preparation and characterization Targeted liposomes

Despite making up more than 50% of body mass, bones only contribute 7% of cardiac output. Additionally, a significant portion of the bone is still isolated and less perfused. As a result, the desired concentration of chemotherapy drugs for tumour suppression is never reached. Targeted nanocarriers are anticipated to increase therapeutic efficacy, and multifunctional nanoparticles are crucial for medication delivery. Alendronate (ADN), risedronate, and etidronate are common examples of bisphosphonates that are employed as bone-targeted ligands for drug administration due to their strong affinity for the primary mineral component of bone (hydroxyapatite, HAp) [31].

Ryu and colleagues[32] described ADN-conjugated nano-diamonds (ADN-NDs) as bone-targeted drug carriers for possible osteoporosis treatment. Due to the inclusion of ADN, the ADN-NDs demonstrated significant Hap-affinity and demonstrated bone-targeting capabilities in vivo. Extracellular vesicles (EVs) generated from mouse mesenchymal stem cells were decorated with ADN to create ADN-EVs for osteoporosis treatment by Wang and colleagues. Extracellular vesicles (EVs) generated from mouse mesenchymal stem cells were decorated with ADN by Wang[33] and colleagues to create ADN-EVs for osteoporosis therapy. Because the ADN-EVs exhibited a strong affinity for HAp in vitro, they may be able to prevent osteoporosis in rats with osteoporotic ovaries by well-tolerated administration without causing any adverse effects.

Therefore, in the current study, AA-CHSC-decorated liposomes were developed to target osteosarcoma. The AA-CHSC was mixed with other components of the liposomes during preparation of thin film, the phosphate group of Alendronic acid may remain oriented on outer surface and towards internal aqueous compartment of liposomes. The developed TP-KLs, TP-SLs, and TP-NLs were subjected for %EE and mean vesicle size analysis and compared with non-targeted liposomes (Table 5). The inclusion of AA-CHSC into liposomal membrane substantially increased the entrapment of drugs in the membrane. The cholesteryl hemisuccinate is a hydrophobic component and alendronate sodium is completely water soluble in nature. Therefore, the conjugate of these two could be amphiphilic in nature and get inserted in the membrane. Furthermore, the substantially increased drug entrapment could be corroborated to its amphiphilic nature. In addition, it can dissolve the more drug in oil present in the membrane due to its amphiphilic nature. However, its amphiphilic nature could be evaluated in detail using suitable methods.

The mean vesicle sizes of targeted liposomes were determined and compared with the non-targeted liposomes. The mean vesicle size of all targeted liposomes is found moderately higher than non-targeted liposomes. The increased mean vesicle size indicates surface coating of liposomes with AA-CHSC.[30]

3.7. Transmission electron microscope (TEM) analysis

The TEM analysis of TP-KLs as representative of other targeted liposomes (TP-SLs and TP-NLs) is determined to validate liposome formation and to understand their structural features. The TEM images (Fig.5A) revealed the presence with spherical and nearly spherical unilamellar vesicles of moderately varied sizes.

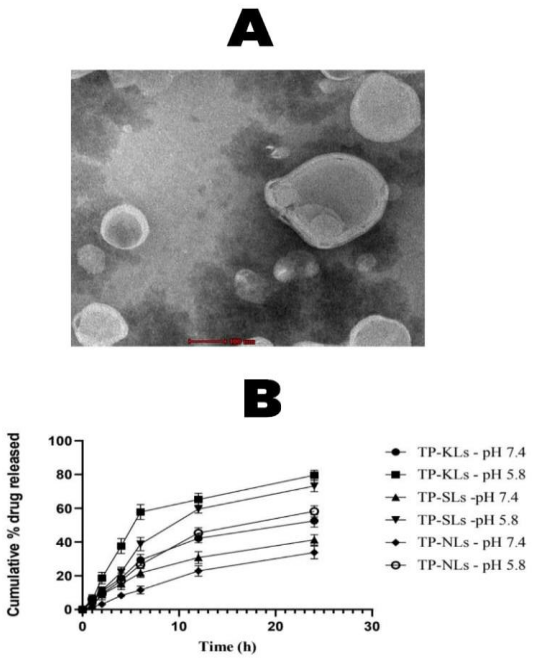

Figure. 5. A) Surface morphology of targeted and pH-sensitive ketoconazole-loaded liposomes (TP-KLs) using TEM B)pH-dependent release of KCZ, SVN, and NSD from their targeted liposomes (TP-KLs, TP-SLs, TP-NLs)

3.8. pH-dependent *in vitro* release behaviour of targeted liposomal drugs

Cholesteryl hemisuccinate (CHEMS) consists of succinic acid esterified to the beta hydroxyl group of cholesterol. The inclusion of CHEMS in the liposomal membrane has been reported to cause higher drug release at acidic pH (tumor microenvironment) than in systemic circulation. In acidic conditions, the CHEMS becomes partially protonated and destabilize the bilayer structure. The destabilizing effect as a result of protonation of CHEMS results in the destruction of the liposomal bilayer organization and payload release [34-35]

The cumulative percent drug released from different targeted liposomal drugs (TP-KLs, TP-SLs, and TP-NLs) at both blood pH (phosphate buffer pH 7.4) and tumour pH (phosphate buffer pH 5.8) is presented in Fig.5B. [36] During the study, 5% (v/v) methanol was included into the release medium to maintain the sink condition.[37]

The non-targeted (uncoated) liposomes showed moderately lower drug release (data not presented) when compared to targeted liposomes (AA-CHSC coated) at blood pH. The targeted liposomes showed substantially high drug release at tumour pH of 5.8 when compared to blood pH of 7.4 indicating pH-sensitiveness of the developed targeted liposomes which might be beneficial for the drug delivery in the tumour microenvironment. Further, the obtained results validate the hypothesis that a pH reduction encourages a destabilization of the liposomes and other nano systems culminating in larger drug release [38-39]

3.9. Substantially high *in vitro* cytotoxicity of targeted liposomal drugs

In the present study, the *in vitro* cytotoxic nature of liposomal formulations (KLs, SLS, NLs, TP-KLs, TP-SLS, and TP-NLs) were determined against Saos-2 cells (human osteosarcoma cell). All above formulations showed the concentration-dependent Saos-2 cell growth inhibition (Fig.6). The different combinations (taken in molar ratio) of targeted and pH-sensitive liposomal drugs (TP-KLs, TP-SLs, and TP-NLs) also showed the concentration-dependent Saos-2 cell growth inhibition (Fig.6). The IC_{50} values calculated for all above tested formulations and their combinations are recorded in Table 6.

The substantially higher cell growth inhibition (lower IC_{50} values) was noticed with targeted and pH-sensitive liposomal drugs when compared to non-targeted liposomal drugs; the IC_{50} values are not calculated for non-targeted liposomal drugs as they caused <50% cell growth inhibition. Amongst targeted and pH-sensitive liposomal drugs, the TP-SLs showed substantially lower IC_{50} (higher cell inhibition) when compared to TP-KLs and NLs.

In the current study, the combination of targeted liposomal drugs is also tested by varying their molar ratio. The combination effect is found almost same as individual liposomal drugs. The TP-KLs+TP-SLs+TP-NLs combination at 1:3:1 molar ratio exhibited moderately higher cytotoxicity (lower IC_{50} value) when compared to other molar ratios (1:1:3 & 3:1:1). This indicates that the increase in liposomal SVN (TP-SLs) concentration may further increase the *in vitro* combination cytotoxic effect.

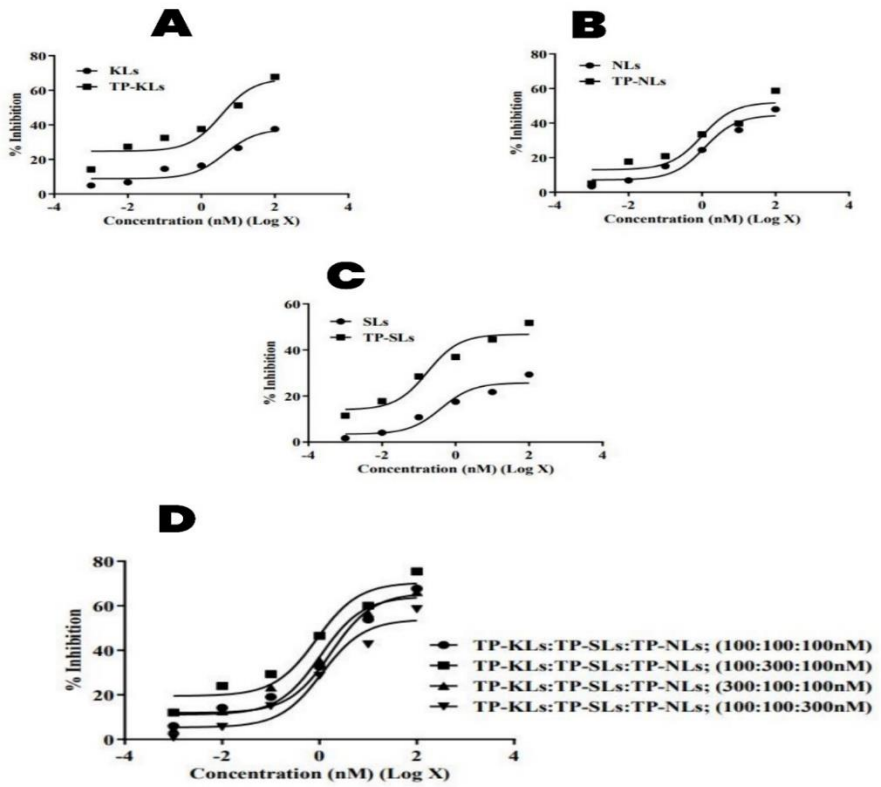

Figure. 6. Percent Saos-2 cell growth inhibition caused by different liposomal formulations.

Table 6. The IC_{50} values of different liposomal drugs and their combinations after 48h of Saos-2 cells treatment

Sample Name	IC_{50} Values (nM) (100-0.001nM)
KLs	ND
SLs	ND
NLs	ND
TP-KLs	3.543±0.24
TP-SLs	0.172±0.01
TP-NLs	1.01±0.13
TP-KLs:TP-SLs:TP-NLs; (100:100:100nM)	1.726±0.14
TP-KLs:TP-SLs:TP-NLs; (100:300:100nM)	0.875±0.16
TP-KLs:TP-SLs:TP-NLs; (300:100:100nM)	1.058±0.13
TP-KLs:TP-SLs:TP-NLs; (100:100:300nM)	1.125±0.21

Values presented are Mean±SD, ND: Not detected due to lesser cell inhibition (<50%)

Besides, the cytotoxic nature of TP-KLs+TP-SLs+TP-NLs combination (1:3:1 molar ratio) was also tested against human healthy kidney cells (HEK-293). This combination caused substantially lowest cell growth inhibition (18.9±4.2%) after 48h treatment indicating the lower toxicity and higher safety of liposomal combinations.

Additionally, MLs have demonstrated more cytotoxicity than other liposomal counterparts when combined with SLs. Consequently, these findings suggest that although drugs are present in liposomes, their slow-release nature, as demonstrated by in vitro release research [35], prevents them from having any discernible impact. Furthermore, in comparison to the widely utilised DSPE-PEG 2000 coating, which often results in increased cytotoxicity over non-coated liposomes, the long PEG chains of PF-108 prevalent over the liposomal surface may greatly prevent cell uptake. [40-41]

4.CONCLUSION

The present preliminary investigation has developed pH-responsive osteosarcoma-targeted liposomes for previously identified non-oncology drugs that are effective against osteosarcoma. The observations of targeted liposomes such as the mean vesicle size in the range required for intravenous administration, the lower drug-release behaviour in circulation and higher drug-release behaviours in tumour, and the substantially high osteosarcoma cell growth inhibition effect and lower cytotoxic effect against healthy cells clearly indicates their potential clinical applications in the treatment of osteosarcoma. The treatment of osteosarcoma with targeted liposomal combination requires further detailed studies as the combination in the ratio tested in the present study caused almost similar or moderately higher cytotoxicity when compared to individual targeted liposomes. Therefore, right combination of targeted liposomes and dosing frequency should be revealed for repurposing the liposomal non-oncology drugs in the effective intravenous treatment of osteosarcoma.

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ABBREVIATIONS
KCZ-Ketoconazole
SVN-Simvastatin
NSD- Niclosamide
AA-CHSC-Aledronic acid-Choleteryl hemiscuccinate conjugate
PT-KLs, PT-SLs, PT-NLs- AA-CHSC coated pH sensitive and targeted liposome
SLs -SVN-loaded liposomes
NLs-NSD-loaded liposomes
AA-Alendronic Acid
CHS-Cholesteryl hemisuccinate
BBD- Box-Behnken design
EE-Drug content
EVs- Extracellular vesicles
CHEMS- Cholesteryl hemisuccinate