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Oral solid self-emulsifying system containing non-oncology drug combination for repurposing in melanoma treatment: *In vitro* cytotoxicity, and *in vivo* hematotoxicity and pharmacokinetic study

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ABSTRACT: The goal of the current research was to identify a safe and effective non-oncology drug combination as a substitute to existing toxic chemotherapeutics for the treatment melanoma. Further intend was to develop an oral solid self-emulsifying drug delivery system (S-SEDDS) for concurrent delivery of identified combination. The drug containing S-SEDDS was prepared and characterized for flowability, compressibility, drug content, particle size and zeta potential, and *in vitro* cytotoxicity against melanoma cells. In silico molecular docking for drug with excipients interaction shows compatibility with each other. Moreover, the formulations were characterized *in vivo* for hepatotoxicity and pharmacokinetic study in rats. The S-SEDDS showed good flowability and compressibility. The particle size of S-SEDDS upon dilution was found in nanometer range. Furthermore, the *in vitro* cytotoxicity of S-SEDDS containing non-oncology drug (NOD) combination [ketoconazole (KCZ), tadalafil (TLF), disulfiram (DSR)] and docetaxel (DTX) was observed to be higher than S-SEDDS containing only NOD combination against mouse melanoma cells. No significant change in the hematological parameters, animal vital organs weights, and body weights were observed after oral administration of S-SEDDS containing non-oncology drug combination with DTX indicating their safety. In addition, significant ($p< 0.05$) improvement in the oral bioavailability of DTX was observed following its administration in the form of S-SEDDS when compared to the Taxotere in the rats. The developed S-SEDDS containing non-oncology drug combination alone and in combination with docetaxel could be a promising and safe approach in the effective treatment of melanoma.

KEYWORDS: Repurposing; melanoma; self-emulsifying drug delivery; cytotoxicity; acute toxicity; pharmacokinetics

1. INTRODUCTION

Skin cancer is one of the most common malignancies in the world, coming in at number 20 with respect to incidence. Melanomas and non-melanoma are the two main types of skin cancer, with melanomas being the most serious. Depending on the kind of skin cancer, chemotherapy is one of the main treatment modalities used to treat advanced skin cancer, either alone or in conjunction with other modalities.

Chemotherapy is one of the most widely used methods but deadly side effects, multi-drug resistance (MDR), non-selectivity, and extremely high drug costs [1] generally leads to the development of new chemical molecules. However, the search for and development of novel medicinal compounds is an expensive, time-consuming, and difficult process [2]. Additionally, due to their high cost and potential inability to supply the current demand for chemotherapeutics, this technique may result in high mortality in low- and middle-income nations [3].

Drug repurposing is a practice of using medications that have been licensed for one therapeutic application to treat a different ailment [4]. The main benefits proffered by this approach include recognized pharmacology, safety recital record, less investments, and a transient period. Moreover, the combined

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delivery of repurposed therapeutics can reduce the enzymatic metabolism of drugs thereby causing dose reduction and side effects. Furthermore, combined delivery of repurposed therapeutics can also result in synergistic effects thereby achieving better anticancer effectiveness.

Numerous malignancies have been successfully treated with medications like ritonavir (RTV), ketoconazole (KCZ), tadalafil (TLF), disulfiram (DSR), and valproic acid (VPA) [1, 5, 6]. The anticancer potential of the aforementioned medications promoted their screening against skin cancer. However, there are a number of obstacles to the conventional delivery of these repurposed therapies, including deprived biopharmaceutical and pharmacokinetic properties, oral bioavailability, stability, non-selectivity towards cancer cells, dose-related toxicities, etc [1, 5].

To get beyond the aforementioned obstacles in the way of repurposed medicines delivery, nanoparticulate drug delivery systems with polymeric and lipidic NPs may be helpful. It has been widely noted that lipid-based formulations are one of the prospective methods for increasing the oral bioavailability of hydrophobic medicines [6, 7]. It is generally known that lipid-based formulations, such as self-emulsifying drug delivery systems (SEDDS), can increase the oral bioavailability and absorption of weakly water soluble cargos [10-11]. The oral lipid-based self-emulsifying drug delivery systems (SEDDS) are generally isotropic mixtures of oils, hydrophobic surfactants, hydrophilic surfactants, both hydrophobic and hydrophilic surfactants, and cosurfactants; therefore, they could solubilise multiple drugs. SEDDS present drugs in nano or micro-sized droplet, enhance their dissolution and permeability [8-9], shield drugs against enzymatic hydrolysis, and reduce their hepatic first-pass metabolism by absorbing through the lymphatic system.[10] Therefore, to augment the recital of the elected drugs *via* oral route, the present study aimed to develop oral SEDDS composed of diverse category non-oncology drugs (NOD) and screen it for the *in vitro* anticancer activity against skin cancer cells, B16F10. Further, the study intended to develop a cocktail of NOD that shows the highest cytotoxic effect at the lowest concentration alone and in combination with the chemotherapeutic drug docetaxel (DTX).

2. RESULTS AND DISCUSSION

Furthermore, in the present study, we developed NOD combination-loaded solid self-emulsifying system as solid delivery systems (DS-SEDDS) are more convenient for oral administration of drug when compared to viscous liquid systems. Besides, to study the effect of inclusion of chemotherapeutic drug DTX to NOD combination on *in vitro* anticancer effect DS-SEDDS containing NOD combination and DTX were also developed in the present study.

In the current study, DS-SEDDS containing single drug was also developed and screened for drug content, drug retention upon dilution, and *in vivo* pharmacokinetic study as simultaneous estimation of four drugs present in the DS-SEDDS is challenging and not a validated method is available.

2.1. Preparation and optimization of blank L-SEDDS (BL-SEDDS)

The optimization procedure and parameters are discussed in detail in our previous publication [11]. Also, refer the optimization table provided as supplementary Table 1 and 2. In the optimization procedure: Leciva S-95 phospholipid was tested as emulsifier, stabilizer, and oil phase along with soyabean oil; The soyabean oil, tween-80, and span-80 were tested as oil phase, hydrophilic surfactant, and hydrophobic surfactant, respectively; Poly(ethylene glycol)- block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG–PPG–PEG; average Mn 14,600) was utilised as a cosurfactant along with tween-80; Distilled water was incorporated to reduce the viscosity of SEDDS and dispersion time.

The compositions of liquid self-emulsifying concentrate were optimized based on physical stabilities (phase separation) of the developed compositions at room temperature. Post stability study, the blank self-emulsifying concentrates such as BL-SEDDS-2 (without PF-108 and with 75 mg Leciva S-95), BL-SEDDS-8 (without PF-108 and with 150 mg Leciva S-95), and BL-SEDDS-9 (without PF-108 and Leciva S-95) are found highly stable and phase separation was not noticed for a period of 6 months and beyond. Therefore, these compositions were further selected for incormporation of drugs. In these compositions, the DTX (6 mg), TLF (1 mg), and DSR (15 mg) are found easily dissolved in a prepared mixture of span 80, soybean oil, and water (with and without Leciva S-95). As the KCZ was found insoluble in the same mixtures the tween-80 containing KCZ (15 mg) was prepared and added drop-wise into already prepared w/o emulsion with continuous stirring. The ratio of the amounts of drugs dissolved in preconcentrates is same as the ratio of the amounts of drugs tested in the *in vivo* acute toxicity study [11]. However, these self-emulsifying

preconcentrates containing high amounts of drugs (as per their solubility) could also be developed for other studies.

Based on dispersion time in 0.1 N HCl, viscosity, and mean particle size upon dilution (in 0.1 N HCl) the developed drug-loaded preconcentrates (DL-SEDDS-2, DL-SEDDS-8, and DL-SEDDS-9) were optimized [11]. The preconcentrate DL-SEDDS-9 (without Leciva S-95) showed lower dispersion time (1.2±0.2 min) owing to its moderately lower viscosity (52.3±2.4 cps) when compared to DL-SEDDS-2 (dispersion time:1.6±0.3 min; viscosity: 55.7±4.2 cps) and DL-SEDDS-8 (dispersion time: 3±0.8 min; viscosity: 64.7±7.7 cps) batches containing 75 mg and 150 mg Leciva S-95, respectively [11]. The mean particle sizes of DL-SEDDS-2, DL-SEDDS-8, and DL-SEDDS-9 after 1000-times dilution are found to be 291 ± 18 nm, 322 ± 31 nm and 217 ± 11 nm respectively [11].

2.2. Preparation of drug-loaded liquid SEDDS ((DL-SEDDS)

The physically stable BL-SEDDS (BL-SEDDS-2, BL-SEDDS-8, and BL-SEDDS-9) were converted into DL-SEDDS (DL-SEDDS-2, DL-SEDDS-8, and DL-SEDDS-9). To the optimized compositions of BL-SEDDS, the DTX (6 mg) [12], TLF (1 mg) [13], and DSR (15 mg) [14] were added sequentially up to 1h under stirring condition (800–1000 rpm). After probe sonication (2 min at V-200-240V and 560-590A), the tween-80 containing KCZ (15 mg) [15] was incorporated drop-wise under same stirring condition.

2.3. Preparation and characterization of drug-loaded solid SEDDS (DS-SEDDS)

In the present study, the DL-SEDDS-9 is considered for solidification owing to its moderately lower dispersion time and lower viscosity, and substantially ($p< 0.05$) lower mean particle size than the other two batches DL-SEDDS-2 and DL-SEDDS-8. The DL-SEDDS-9 is converted into DS-SEDDS-9 (DS-SEDDS) using neusilin as an adsorbent [16] to obtain the advantages of the solid oral solid dosage forms [8]. To solidify 1 mL of preconcentrate, 0.75 g of neusilin US2 was used (the total practical weight is found to be 1.75 ± 0.23 g).

A literature survey denotes the potential advantages of converting liquid nano emulsions to solid nano emulsions [17]. Ample techniques are available for conversion; however, adsorption on the micro-porous surface method is most commonly used to improve physical stability also it hass low cost, ease, easilty scalable and quick process [18].

2.4. Flowability and compressibility

The flowability of DS-SEDDS was assessed on the basis of angle of repose and Hausner's ratio. The DS-SEDDS showed angle of repose of 26.7±1.4^{o} and Hausner's ratio of 1.14±0.01 which reveals the good flowability of the prepared powder. The Carr's compressibility index is a measure of the compressibility of a powder. The DS-SEDDS displayed compressibility index of 13.6±1.8 which reveals good compressibility [19].

2.5.% Drug content retained, mean particle size, and zeta potential upon dilution

The DS-SEDDS containing only DTX in the oil phase was analyzed for %DTX retained upon dilution by using a 0.1N HCl. The percentage of DTX retained upon 100 and 1000-times dilution was 95±1% and 93±1%, respectively. The DS-SEDDS containing a single drug (DTX) are prepared separately to avoid issues associated with simultaneous analysis of multiple drugs (four drugs) [11].

Furthermore, the mean particle size and zeta potential of DS-SEDDS containing all four drugs upon 1000-times dilutions was observed to be 239±16nm and -16.5±2.4mV, respectively.

According to a previous publication, the increase in bioavailability is mostly caused by a reduction in globule size, which increases the amount of interfacial space open to absorption. The decreased globule size is mostly attributable to the correct S-mixture ratio and the SNEDDS with globule size in nanometer are suitable for improved oral delivery of drugs [20].

2.6. *In silico* drug-neusilin interaction study

To explore the binding mode and molecular interactions of neusilin with drug compounds such as disulfiram, docetaxel, ketoconazole, and tadalafil, molecular docking was employed using AutoDock4.2[9]. The minimum binding energy conformation of disulfiram, docetaxel, ketoconazole, and tadalafil with neusilin were found to be -3.51, -4.82, -6.47, -7.39 kcal/mol, respectively (Table 1, Figure 1A-1D). The docking analysis shows that the afore-mentioned drug compounds form a stable complex with the excipient neusilin as presented in Figure 1A-1D. The analysis of docked complex of neusilin-US2 with disulfiramshows the two hydrogen-bonding interactions between Sulphur (S7) and oxygen (O1) of the neusilin (3.73 Å) and Sulphur (S8) and oxygen (O31) of the neusilin (3.42 Å) shown in Table 1.

Table 1. Hydrogen bonding interaction of excipient, neusilin with different drug compounds. Here, energy is shown in kcal/mol.

Complex	Binding energy (kcal/mol)	Atoms Involved in bonding	Distance (°)	Angle (°)	Fig. Ref
Neusilin-Disulfiram	-3.51	UNK0:O1 - UNK0:S7	3.73	100.74	1A
		UNK0:O31 - UNK0:S8	3.42	103.31	
		UNK0:H2 - UNK0:O4	2.33	117.87	
		UNK0:H2 - UNK0:O6	2.59	124.49	
		UNK0:H2 - UNK0:O16	2.60	125.09	
		UNK0:H17 - UNK0:O31	2.66	147.94	
		UNK0:H17 - UNK0:O33	2.81	102.43	
		UNK0:H17 - UNK0:O34	2.28	129.76	
Neusilin-Docetaxel	-4.82	NUS0:O24 - TXL501:O12	2.99	107.31	1B
		TXL501:H29 - NUS0:O34	2.79	115.64	
		TXL501:H29 - TXL501:O8	1.66	158.28	
		TXL501:H29 - TXL501:O14	2.22	118.61	
		TXL501:H36 - NUS0:O24	1.81	161.75	
		TXL501:H25 - TXL501:O13	1.64	102.19	
		TXL501:H27 - TXL501:O6	2.37	100.10	
Neusilin-Ketoconazole	-6.47	KTN801:NH - NUS0:O1	2.27	111.50	1C
		KTN801:H3 - NUS0:O18	2.14	156.19	
		KTN801:H3 - NUS0:O27	3.06	117.96	
		KTN801:H3 - NUS0:O31	2.64	138.59	
		KTN801:H3 - NUS0:O34	2.63	105.64	
		KTN801:H25 - NUS0:O1	2.43	111.47	
		NUS0:Mg - KTN801	4.73	--	
		NUS0:MG35 - KTN801	4.36	--	
		NUS0:O4 - KTN801	3.48	--	
Neusilin-Tadalafil	-7.39	CIA501:H4 - NUS0:O18	2.77	113.04	1D
		CIA501:H6 - NUS0:O16	1.97	172.70	
		NUS0:O31 - CIA501	4.32	--	
		NUS0:O31 - CIA501	3.72	--	
		NUS0:O34 - CIA501	2.68	--	

Furthermore, the neusilin and disulfiram form carbon-hydrogen bonding interactions as shown in Table1. These hydrogen and carbon bonding interactions play important role in the stabilization of the complex. Next, the analysis of the neusilin-docetaxel complex reveals the only hydrogen bonding interactions between the hydrogen (H29) atom of docetaxel with O34 (2.79 Å), O8 (1.66 Å), O14 (2.22 Å), respectively, as shown in Figure 1B and Table 1. Similarly, the hydrogen atoms of docetaxel such as H36, H25, and H27 forms hydrogen bonding interactions with O24 (1.81 Å), O13 (1.64 Å), and O6 (2.37 Å) of neusilin as shown in Figure 1B and Table 1. The neusilin-docetaxel forms more number of interactions and this may help for the stabilization of the complex [21,22]. Next, the analysis of the neusilin and ketoconazole complex shows that hydrogen bonding interactions between the oxygen (O1) atom of neusilin and HN of the ketoconazole (2.27 Å) as shown in Figure 1C and Table 1. In addition, the complex also forms the carbon-hydrogen bonding interactions between the H3 atoms of ketoconazole with O18 (2.14 Å), O27 (3.06Å), O31 (2.64 Å), and O34 (2.63Å) (Table 1), and the H25 of ketoconazole with O1 atom of neusilin (2.43 Å) as shown in Figure 1C and Table 1. In addition, the complex forms the electrostatic interaction between the Mg atoms of neusilin with ketoconazole as shown in Figure 1C and Table 1.

Finally, the analysis of the neusilin and tadalafil complex makes the hydrogen bonding interactions between the hydrogen (H4) atoms of ketoconazole with O18 (2.77 Å) of the neusilin (Figure 1D and Table 1). Also, the complex forms the carbon-hydrogen interactions with the H6 and O16 (1.97 Å), and the electrostatic interactions include the O31 (4.32 and 3.72 Å), and O34 (2.68 Å) shown in Figure 1D and Table 1.

2.7. *In vitro* cytotoxicity study

The *in vitro* cytotoxicity of plain combination of DTX/KCZ/TLF/DSR, KCZ/TLF/DSR, blank SEDDS and DS-SEDDS containing all four drugs and DS-SEDDS containing only NOD combination [Ketoconazole (KCZ), Tadalafil (TLF), Disulfiram (DSR)] was executed against B16F10 cell lines *via* MTT assay. The %B16F10 cell growth inhibition was observed with the plain drugs cocktail and DS-SEDDS containing

all four drugs and DS-SEDDS containing only NOD in the concentration range of (1-0.00001nM) is presented in Figure 2.

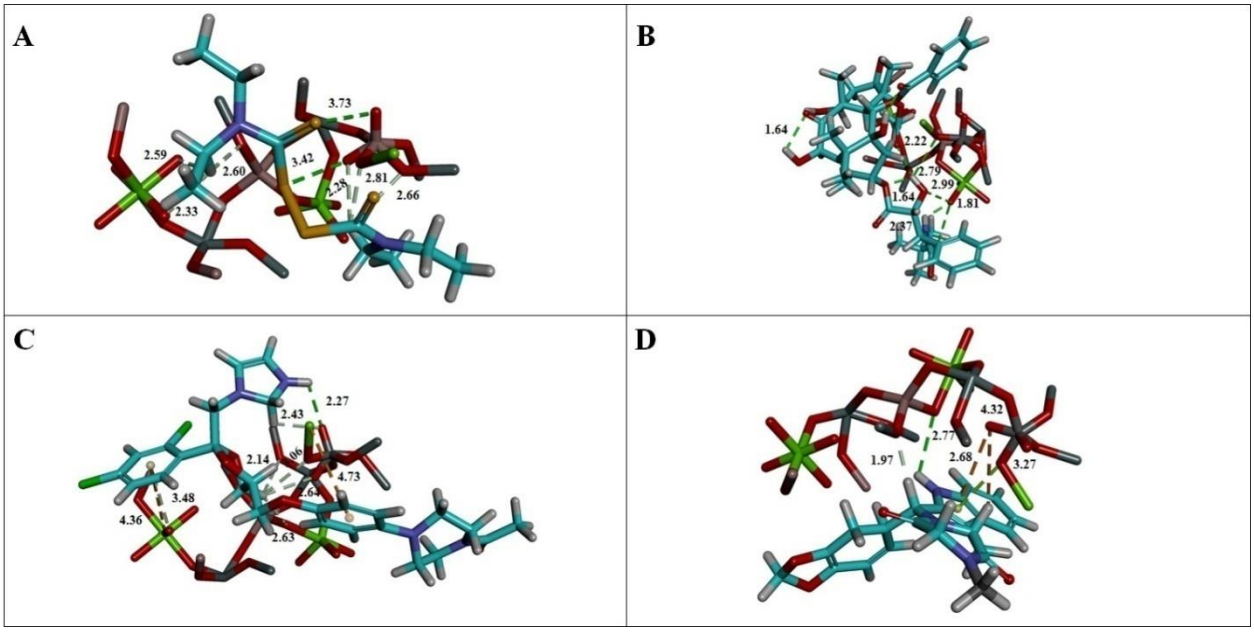

Figure 1. Binding mode of excipient Neusilin with drug A) Disulfiram, B) Docetaxel, C) Ketoconazole, D) Tadalafil. Here, drug compounds are shown in cyan color. The atoms of drugs such as C, N, O, and H atoms are shown in cyan, blue, red, and grey color, respectively. While, neusilin atoms such as Mg, Al, Si, O are shown in green, brown, grey, and red color, respectively. The hydrogen-bonding interactions, carbon-hydrogen bonding interactions are shown in green and light grey color, respectively.

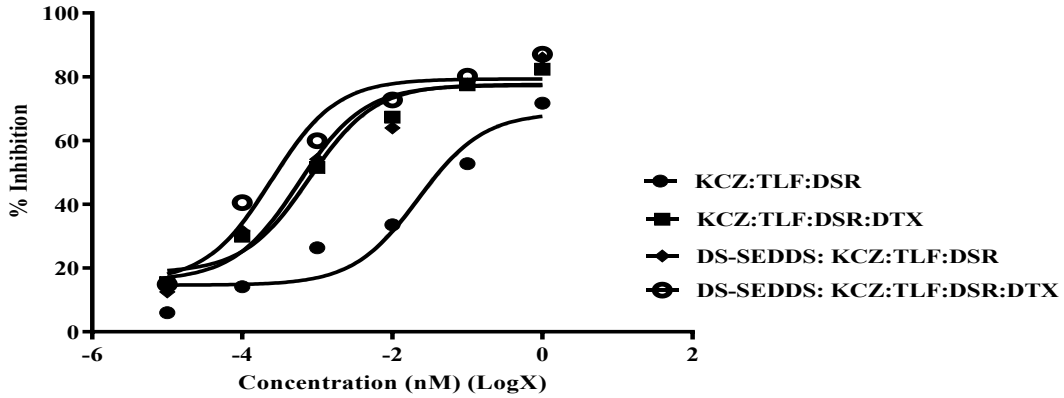

Figure2. The %B16F10 cell growth inhibition with the plain drugs cocktail and DS-SEDDS containing all four drugs and DS-SEDDS containing only NOD in the concentration range of (1-0.00001nM)

The plain drugs combination containing all four drugs (including DTX) showed substantially higher ($p< 0.0001$) *in vitro* cytotoxicity (IC_{50}: 0.00078±0.0001 nM) when compared to only NOD combination without DTX (IC_{50}: 0.022±0.003 nM). This indicates higher cytotoxicity in the presence of chemotherapeutic drug DTX. The same trend was noticed with the drug combinations in the form of solid SEDDS. The DS-SEDDS containing all four drugs showed higher cytotoxicity (IC_{50}: 0.024±0.001nm) than DS-SEDDS containing only NOD (IC_{50}: 0.056±0.005nm).The higher cytotoxic behavior of plain drugs combination containing all four drugs could be due to their synergistic effect along with drugs are molecularly dispersed in to lipid carriers and improving solubility,bioavalability by ultering permiabiltly which escilate anticancer potential of NOD [23]. The high IC_{50} value of both DS-SEDDS containing all four drugs and only NOD might be attributed to the sustained release of the drugs through the SEDDS. We observed negligible cytotoxicity with the blank

SEDDS against B16F10 cells. The obtained cytotoxicity results revealed potential of DS-ESDDS containing all four drugs and only non-oncology treatment of skin cancer.

2.8. In vivo toxicity

The *in vivo* toxicity of plain drugs (DTX/KCZ/TLF/DSR), and DS-SEDD is determined following oral administration at every 2nd day up to 14 days in rats. At the end of the study (on 14th day), the consequences of test solutions on hematological (Table 2) are determined.

Table 2. Summary of haematological parameters observed at the end of the study

Group	WBC (10^3/cu mm)	RBC (10^3/cu mm)	Hb (gm%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	PCV (%)	M.C.V (fl)	M.C.H (pg)	M.C.H.C (g/dl)
Group-A	13.73±2.18	7.47±0.38	13.73±2.18	24.33±9.85	47±7.66	1±0.01	2.5±2.12	39.9±2.4	54.05±0.49	17.4±0.14	32.2±0.13
Group-B	11.86±2.3	8.66±0.77	13.11±2.18	24.33±8.85	46±12.66	1.66±0.57	3.33±0.57	41.23±6.05	52.8±2.68	15.6±1.16	33.26±0.80
Group-C	14.66±8.98	6.91±0.37	12.96±0.81	28.66±11.93	45.66±10.8	3±1.0	4.33±2.52	37.83±1.16	51.4±0.95	16.6±0.60	34.23±1.10

Values presented are mean ± SD. N=6. **Group - A:** Untreated control; **Group - B:**Plain drugs cocktail (DTX/KCZ/TLF/DSR); **Group - C:**DS-SEDD containing DTX/KCZ/TLF/DSR

No significant changes were observed in the hematological parameters of drug combination (Group B) and DS-SEDD (Group C) treated groups when compared to the untreated control group (Group A) which is suggestive of the safety of the formulations following oral administration. However, moderate decrease in RBC count and % hemoglobin is observed with DS-SEDDS. This is correlated to improved absorption of DTX and other drugs when administered in the form of DS-SEDDS. Furthermore, the weight measurement of animal's body and their vital organs was carried out during the study (Table 3).

Table 3. % average group body weight and weights of vital organs measured during and after the study

Group	Mean body weight inclined (+)/ declined (-) (%)	Organ weight (g)						
		Liver	Heart	Lungs	Spleen	Brain	Kidney	
							Right	Left
Group – A	+4.8	9.05±0.14	1.27±0.06	1.96±0.11	0.69±0.06	1.47±0.03	1.19±0.04	1.18±0.04
Group – B	+3.5	8.97±0.19	1.23±0.07	1.91±0.31	0.64±0.08	1.40±0.07	1.10±0.03	1.06±0.06
Group – C	+2.8	8.93±0.16	1.18±0.04	1.86±0.25	0.60±0.05	1.35±0.09	1.04±0.04	1.02±0.05

Values presented are mean ± SD. N=6. **Group - A:** Untreated control; **Group - B:**Plain drugs cocktail (DTX/KCZ/TLF/DSR); **Group - C:**DS-SEDD containing DTX/KCZ/TLF/DSR

The % body weight of animals in the untreated group was noticed to be increased. However, not more than 2% loss in body weight was noticed in the group B and C animals treated with formulations in comparison to untreated control group. In addition, no substantial changes in the organs weights, food intake, and water intake is observed when compared to untreated control group. No death of animal is also observed from formulations treated groups (B and C). Thus, results obtained reveal the safety of test formulations.

2.9. Pharmacokinetic study

The blood concentration-time curves of DTX after p.o. administration of marketed Taxotere and DS-SEDDS containing DTX in rats is showed in Figure 3.

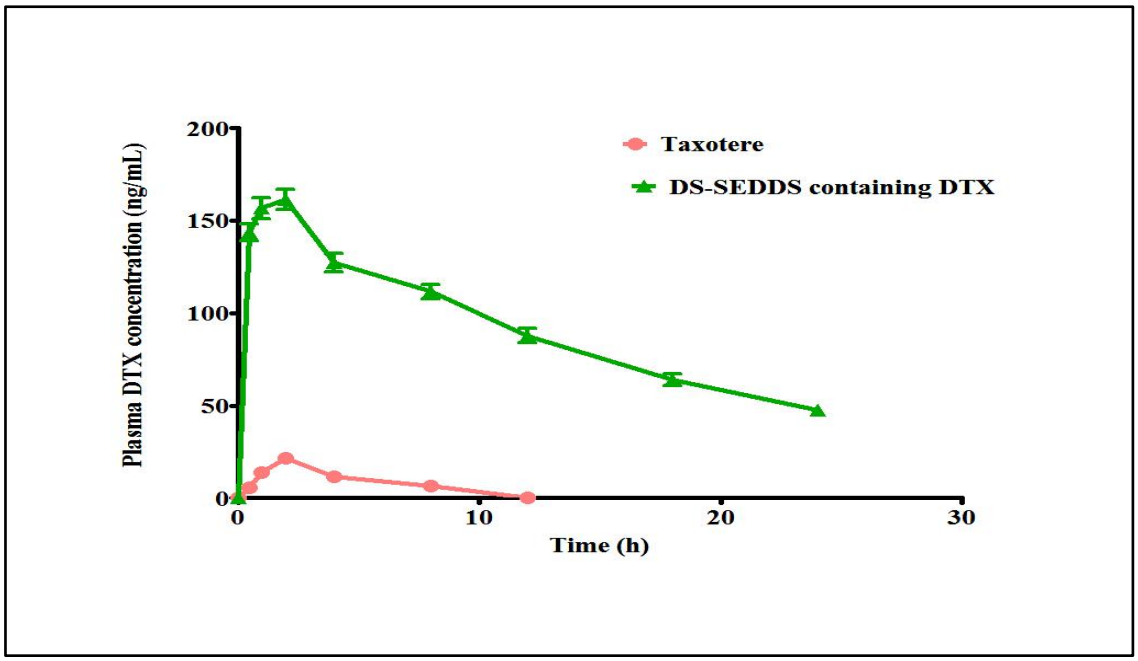

Figure 3. Plasma concentration-time profile of DTX following oral administration of DS-SEDDS and Taxotere

The pharmacokinetic parameters calculated using concentration-time curves are presented in Table 4.

Table 4. Pharmacokinetic parameters calculated from plasma concentration time profile

Formulations	Pharmacokinetic Parameters					
	C_{max} (ng/mL)	AUC_{0-t} (ng/mL*h)	$AUC_{0-\infty}$ (ng/mL*h)	$AUMC_{0-\infty}$ (ng/ml*h)	MRT (h)	$t_{1/2}$ (h)
Taxotere	31.4	116.75	119.61	338.47	2.829	1.528
DS-SEDDS	161.5****	2224.15****	3146.86****	61423.07****	19.518****	13.49****

Values presented are mean ± SD. N=6. ****P < 0.0001; P: Significant when compared to Taxotere group.

The DS-SEDDS showed significantly higher ($p< 0.0001$) area under the curve (AUC) and Cmax for DTX when compared to marketed Taxotere. This indicates higher oral bioavailability of DTX in the form of DS-SEDDS. Moreover, the DTX from DS-SEDDS absorbed at faster rate and showed prolonged mean residential time indicating when compared to DTX from Taxotere. The substantially improved pharmacokinetic profiles of DTX when administered in the form of SEDDS could be correlated to GI safety, improved oral absorption, and improved lymphatic absorption [24,25]. Globule size in nanometrs is mainly responsible for bioavailability enhancement, which is optimal for lymphatic uptake and mainly responsible for avoiding first-pass metabolism and decreasing P-GP-mediated gastrointestinal efflux [26]. Rapid and high absorption may be due to quick emulsification and a high dissolution rate of self-nano emulsions, which are attributed to an improvement in surface area for absorption. Lipids and surfactants are associated with bio-adhesive properties that contribute to the adhesion of self-nano emulsion to the apical membrane, which drastically improves residence time and thereby absorption and bioavailability [27].

3.CONCLUSIONS

The present study has identified cocktail of NOD (KCZ, DSR, and TLF) as an alternative to chemotherapeutics for the treatment of skin cancer. Its substantial *in vitro* anticancer effect against skin melanoma cells indicates its potential clinical application. The inclusion of docetaxel to cocktail of NOD has further increased its anticancer effect. In the current study, solid SEDDS were successfully developed and characterised for simultaneous oral delivery of identified NOD. The drug containing S-SEDDS showed excellent micromertic properties suitable for compression into tablet or filling into capsule. No incompatibilities between drugs and neusilin were releaved during in silico molecular docking study. The O/W nanoemulsions formed after the dispersion of solid SEDDS had narrow size distribution and all the

predetermined quality attributes. The substantial *in vitro* cytotoxicity, lower *in vivo* hematotoxicity, and higher oral pharmacokinetics profile of solid SEDDS indicate its potential application in oral treatment of skin cancer. However, further studies are needed to identify the correct proportion of these NOD and docetaxel, and right administration route for effective treatment of cancer. Besides, solid SEDDS composed of NOD alone and in combination with DTX could be used in melanoma treatment..However, further *in vivo* studies are needed to ascertain these facts.

4. MATERİALS AND METHODS

4.1. Materials

Docetaxel was gifted by Cipla Pvt. Ltd., Goa. Tadalafil and ketoconazole were provided as gift sample by Athena Drug Delivery Solutions Pvt. Ltd., Mumbai and Swapnaroop Drugs and Pharmaceuticals, Aurangabad, respectively. Disulfiram was obtained from Alfa Aesar Chemicals, Mumbai. Soyabean oil, Span 80, and Tween 80 were procured from Molychem, Mumbai. Popidium iodide was purchased from Sigma Aldrich, Mumbai. MTT Powder, Annexin V, FBS, PenStrep, Trypsin, and Dulbecco's Modified Eagle Medium (DMEM) were procured from Invitrogen, Bangalore. RNase A was procured from Bochringer Mannhein GmbH, Germany. Chloroform and Methanol were purchased from SD Fine Chemicals, Mumbai. All other reagents used were of analytical reagent grade and were used without further purification.

4.2. Cell culture

B16F10 cell lines were procured from ATCC, USA. The B16F10 cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS).

4.3. Preparation and optimization of blank L-SEDDS (BL-SEDDS)

The BL-SEDDS was prepared as per the composition shown in our previous publication (Supplementary Table 2) [11]. Briefly, in 10 mL beaker, span 80, soybean oil, Leciva S-95, and Poloxamer F108 (PF-108) were mixed through stirrering (800–1000 rpm) for 1h. Post mixing, under same stirring rate, distilled water was added drop-wise. Using probe sonication technique (LABMAN-PRO650), the w/o emulsion obtained was size reduced. Tween-80 was then added under same stirring conditions for 1h. All BL-SEDDS with different compositions (Supplementary Table 2) were stored in a sealed container at room temperature (25 ± 2 °C) for 6 months. At every day day up to one month and weekly after one month, the stored BL-SEDDS were carefully observed for any physical instability (phase separation) [28].

4.4. Preparation of drug-loaded liquid SEDDS ((DL-SEDDS)

The physically stable BL-SEDDS (BL-SEDDS-2, BL-SEDDS-8, and BL-SEDDS-9) were converted into DL-SEDDS (DL-SEDDS-2, DL-SEDDS-8, and DL-SEDDS-9). To the optimized compositions of BL-SEDDS, the DTX (6 mg) [26], TLF (1 mg) [27], and DSR (15 mg) [28] were added sequentially up to 1h under stirring condition (800–1000 rpm). After probe sonication (2 min at V-200-240V and 560-590A), the tween-80 containing KCZ (15 mg) was incorporated drop-wise under same stirring condition [29].

4.5. Preparation of drug-loaded solid self-nanoemulsifying drug delivery system (DS-SEDDS)

The optimized DL-SEDDS-9 containing all drugs in a mixture of mixture of span 80 (2mL) and soybean oil (2mL), water (1mL), and tween-80 (3mL) was converted into DS-SEDDS-9 (DS-SEDDS) using the adsorption technique wherein the DL-SEDDS-9 was added drop-wise into a porcelain dish containing Neusilin® US2 (a solid adsorbent carrier) with contineous mixing [12].

4.6. Characterization of DS-SEDDS

4.6.1. Flowability and compressibility

The flowability of DS-SEDDS-9 was determined using the angle of repose and Hausner's ratio. The compressibility of the S DS-SEDSS was checked using Carr's compressibility index. The experiment was performed using the procedure described in previous reports. Twenty grams of DS-SEDSS powder was used for analysis. The following equations were used to calculate the bulk density, tap density, Carr's index, Hausner's ratio, and angle of repose as described in previous reports[30].

4.6.2. % Drug content (Robustness to dilution)

The DS-SEDDS (500 mg) containing a single drug (DTX) was dispersed in 10mL and 100mL of 0.1N HCl maintained at 37±2°C for 2h, and then filtered. The 1mL of the filtrate was analyzed by RP-HPLC (Shimadzu Prominence series-I LC-2030c 3D Plus) following dilution with 10mL of methanol as described in the previous report.

4.6.3. Mean particle size analysis

The globule size of DS-SEDDS was measured and assessed using a particle size analyzer (HORIBA SZ-100). The diameter of the particle was calculated using the mean of the globule size distributions following the three-fold replication of each emulsion [31].

4.6.4. Zeta potential

Using the HORIBA SZ-100, the zeta potential of DS-SEDDS was determined. At 25°C, three separate samples were examined with a cell drive maintained at 150 mV [32].

4.7. *In silico* drug-neusilin interaction study

Molecular docking study

The molecular interactions of excipient Neusilin with drugs Disulfiram, docetaxel, ketoconazole, and tadalafil were studied using the molecular docking techniques through AutoDock4.2 [9]. The atomic coordinates of excipient and drug compounds were generated and further optimized using Discovery Studio Visualizer [33]. For molecular docking, a complete Neusilin molecule was enclosed in a grid box of 60×60×60 using a grid spacing of 0.375 Å. Here, we kept Neusilin as rigid and the drugs as flexible molecules. The Lamarckian Genetic Algorithm (LGA) was employed with the default parameters; g_eval was set to 2,50,000 (medium) [9]. The output docked conformation was further clustered using all-atom root means square deviations with a cut-off of 4 Å, and the clusters having maximum conformations were further selected and analyzed, similar to a previous study [34]. The out clusters were compared on their size, solvent accessible surface area and binding energy, van der Waals, and electrostatic energy calculated by the AutoDock4.2 scoring function. The least energy docked conformations were further used for interaction analysis through Discovery Studio visualize[22].

4.8. *In vitro* cytotoxicity study

A cytotoxicity study was carried out to determine the effect of plain mixtures of DTX:KCZ:TLF:DSR (1:1:1:1), KCZ:TLF:DSR (1:1:1), blank solid SEDDS, DS-SEDDS containing all four drugs (DTX/KCZ/TLF/DSR) at 1:1 molar ratios, and DS-SEDDS containing only NOD (KCZ/TLF/DSR) at 1:1 molar ratios separately on the viability of sensitive human prostate cancer cell lines (B16F10) in the MTT assay. 96 well plate was used to seed the cells and incubated at 37°C for 24h. Different concentrations of DS-SEDDS serial dilutions (1nM-0.00001nM) along with an extra 48 h of incubation were used to treat the cells. In addition, 6 mg/mL of MTT in PBS and 100 µL of MTT solution were added to each well, followed by 4 hours of incubation under the same conditions. Lastly, 100µL of DMSO was added to each well to solubilize the formed formazan crystals. A microplate reader has been used to measure the absorption at 570 nm [35].

4.9. *In vivo* toxicity study

Albino rats of either sex ranging from 200 to 250g were provided by Animal Care Facility, Biocyte Institute of Research and Development, Sangli (Protocol No.: IAEC/Sangli/2020/19). All *in vivo* experiments were approved by the Animal Care and Use Committee. All care and handling of animals were performed with the approval of the review board of animal experiments. The Albino rats were divided into 3 groups (6 rats per group): The group (A) is untreated control, group (B) received combination of plain drugs (DTX/KCZ/TLF/DSR), and group (C) received DS-SEDDS containing all four drugs. The cocktail of plain drugs was prepared by dissolving in 10mL of distilled water containing 1.0% v/v DMSO, while the DS-SEDDS containing all drugs was dispersed in 10mL of sterile water. The 0.1mL of above test solutions were diluted up to 1.0mL with distilled water and administered orally every second day up to 14 days. Each orally administered dose of these test formulations contains DSR (150µg), KCZ (150µg), TLF (10µg), and DTX (60µg). The body weights, food consumption, and water intake of animals were recorded during the study. Additionally, the rats were observed for the general toxicity signs such as impact on locomotion, behavior

(agitation, decreased activity, and somnolence), etc throughout the treatment. On 14th day, the blood samples were withdrawn and subjected for hematological and biochemical analysis [31, 32].

4.10. Pharmacokinetic study

The PK studies of DTX were carried out in Albino rats of either sex. For per-oral (p.o), the rats were divided in two groups (six rats per group). First group was treated orally with Taxotere and DS-SEDDS containing only DTX. The above test formulations were administered at dose equivalent to 20mg/kg. The blood samples were collected following predetermined time intervals (0, 0.5, 1, 2, 4, 8, 12, 18, and 24h) from the retro-orbital plexus and transferred into microcentrifuge tubes (containing 20µL of 1000 IU heparin/mL of blood). Blood samples were centrifuged (Remi, R8C) at 5000 rpm for 15 min to separate plasma and kept at -40±5°C until analysed [16,20].

*Extraction of drug from plasma and HPLC analysis:*Prior to HPLC analysis, the plasma samples were extracted with diethyl ether twice. The plasma (100µL) was mixed with 10µL paclitaxel (2µg/mL) as the internal standard. The samples were then vigorously mixed with diethyl ether (1mL) for 5 min and extracted. The above mixture was then centrifuged at 10000 rpm for 5 min; organic solvent was collected and dried at 40°C. The residue obtained was then dissolved in acetonitrile (200µL) and the solution was centrifuged for 5 min at 10000 rpm [11]. Then 25µL of the supernatant was injected into the HPLC system (Shimadzu Prominence series-I LC-2030c 3D Plus). The mobile phase used was Acetonitrile: Water (60:40 v/v) containing 0.1% v/v of orthophosphoric acid at a flow rate of 1.0mL/min at 239 nm [36].

4.11. Statistical analysis

Data are presented as the mean ± standard deviation of three independent experiments. GraphPad Prism software version 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. The obtained results were analyzed using one-way ANOVA and $P < 0.05$ was considered as statistically significant difference.

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