Research Article

Lipid based Nanocarriers of Tazarotene for the Treatment of Psoriasis: Cell Cytotoxicity & *In vivo* Studies

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ABSTRACT

The aim of the present work was to evaluate the safety and efficacy of tazarotene loaded lipid nanocarrieers for the treatment of psoriasis. Tazarotene loaded NLCs, SLNs with or without gel base were evaluated for cellular uptake study, *MTT assay, and cytotoxicity study using A431 skin cancer epithelial cell line and also dermal toxicity study, anti-psoriatic activity study using mice.* Cellular uptake and distribution were studied using coumarin – 6 fluorescent markers loaded NLS and SLN formulations by confocal microscopy. For the dermal toxicity study 18 mice were divided into equal three group (Normal control group, Formulation 1 treated group and formulation 2 treated group) study and 36 mice were divided into six equal groups for anti-psoriatic activity evaluation using Imiquimod induced psoriatic mode. Excise tissues were evaluated for its hydroxyproline content. The cell viability was in the range of 93-100% which indicates that the formulation was non-toxic to cells and safe for external use. The cellular uptake study indicated that the drug loaded SLNs and NLCs were taken up by the cells in peripheral region. The biochemical evaluation reveled no significant reduction in hydroxyproline content in treated group. Severity index was calculated by observing the psoriatic lesion by naked eye. %Relative epidermal thickness in test group for NLC gel and SLN gel were 56.38% and 51.28% respectively which was higher compare to drug gel-In house develop (36.82%) group, Marketed sample (40.24%) group but lesser that that of Normal control group (96.34%) which indicate good anti-psoriatic strength of the formulation. There was also less severity index observed in test compare to Drug Gel formulation and marketed formulation treated animal group. In-vivo animal study proved efficacy of formulations for the treatment of psoriasis.

Keywords: Tazarotene, NLC, SLN, Psoriasis, Melt emulsification, Topical drug delivery system.

INTRODUCTION

About 125 million people are affected from Psoriasis in all over world (1% to 3% of the population worldwide). Psoriasis is a chronic skin disorder marked by periodic flare-ups of sharply defined red patches covered by a silvery, flaky surface. the primary disease activity leading to psoriasis occurs in the epidermis (a hyper proliferative skin disease with a markedly increased 5-6 times normal rate of epidermal turnover). The exact cause remains unknown. There may a combination of factors, including genetic predisposition and environmental factors. The immune system is thought to play a major role. Despite research over the past 30 years looking at many triggers, the "master switch" that turns on psoriasis is still a mystery. For each patient, it causes a considerable, quantifiable reduction in quality of life. Given this great burden of illness, psoriasis warrants significant clinical and research attention. Over the past 20 years, there have been many developments in the understanding genetic, molecular and cellular mechanisms that underlie these inflammatory processes and many new and effective treatments have been developed. The negative impact of the disease on health-related quality of life is comparable to that of ischaemic heart disease, diabetes, depression and cancer[1].

Treatments for psoriasis can often control the disease for long periods. However, none of the available treatments is a cure. The disease can come back when treatment stops. Biologic agents are being introduced for the treatment of psoriasis and have substantial advantages over previously used systemic therapies because they have fewer risks and side effects. Two of the therapies currently being used, etanercept and remicade, are already available for the treatment of rheumatoid arthritis and cohn's disease. Both therapies are tumor necrosis factor (TNF) blocker, which work by interfering with specific immune responses that are responsible for psoriasis. Phototherapy, topical, systemic and Biologic treatment can induce reduction of psoriasis for months to years which depend on the severity of disease.[2] Mild psoriasis were treated by Topical therapies. Patients with severe psoriasis often use topical therapies. The main groups of topical therapies for psoriasis includes coal tar preparations, vitamin D and its analogues, topical corticosteroids, emollients, dithranol, and tazarotene (a topical retinoid)[3,4]. Different lipid nanocarriers such as solid lipid nanoparticles, nanostructured lipid carriers, liposomes, ethosomes, microemulsions and lipid nanocapules are studied as a topical drug delivery systems. This advanced novel formulations based on nanocarriers are promising

prospect to overcome the conventional formulations by reduction in dose, reduction in dosing frequency, deceases dose-dependent, less side effect with enhanced efficacy.[2,5,6]

Lipid nanoparticles such as SLNs and NLCs have many advantages compare to other nanocarriers as they provided close contact interaction with the stratum corneum leading in enhanced occlusion and skin hydration.[7] Topical preparations were used for the localized effects at the site of their application by virtue of drug penetration into the underlying layers of skin or mucous membranes.[8] By Topical drug delivery system bypass the first pass metabolism with the Avoidance of the risks and inconveniences of intravenous therapy and of the varied conditions of absorption, like pH changes, presence of enzymes, gastric emptying time are other advantage of topical preparations. [8]

Tazarotene is the first receptor-selective retinoid for the topical treatment of plaque psoriasis which regulates gene transcription and normalized keratinocyte differentiation, the pathogenic factor in psoriatic lesion.[3] Tazarotene hydrolyzed to its main metabolite, tazarotenic acid, which selectively binds to retinoic acid receptors (RARs) in the nucleus[9] and exhibits little affinity for retinoid X receptors[10]. The predominant type of RAR expressed in the human epidermis is RAR, indicating that it may be an important mediator of retinoid action in skin.[10-14] So that it was hypothesized that Tazarotene's SLNs and NLCs will enhanced anti psoriatic activity.

The aim of the present work was to evaluate the safety and efficacy of tazarotene loaded lipid nanocarrieers for the treatment of psoriasis by cellular uptake study, *MTT assay, and cytotoxicity study, dermal toxicity study and anti-psoriatic activity study*.

MATERIALS AND METHODS

List of materials

Tazarotene was provided as a gift sample by sun pharmaceutical industries ltd, Vadodara, India. Chremophor El, Cutina GMS, were procured from Gattefose, France. Triethanolamine was procured from LOBA Chemie, India. Carbopol 98 NF was provided by Lubrizol as a gift sample. Dulbecco's Modified Eagle Medium, fetal bovine serum, antibiotic solution, MTT dye and trypsin-EDTA solution were procure from HiMedia, India

Methods

Preparation of NLCs, SLN and Gel

Optimized formulation of Tazarotene loaded NLCs, SLN and gel were prepared using melt emulsification sonication method as per development and optimized by Mayur Parmar et al., (Mayur parmar et al., lipid based nanocarriers of tarazotene for the treatment of psoriasis: optimization and in vitro studies, manuscript no. WJPR/1566/8/2019, World journal of pharmaceutical research, 2015). In brief, solid lipid (Cutina GMS), and liquid lipid (Cremophor EL) and drug were taken in a beaker and heated up to 60° C. In another beaker, hydrophilic surfactant dissolved in dist. water was heated to same temperature as of lipid melt. The lipid melt was added to surfactant solution and was stirred by Ultra turrax T25 basic at 19000 rpm for 5 min with continued heating. The prepared emulsion was sonicated using probe sonicator for 5 min. The emulsion was allowed to cool naturally and the NLCs were evaluated. The drug loaded NLCs or SLNs gel were prepared using 0.8%w/w carbopol 980 NF by pH change method with the help of triethanolamine (0.23% w/v).

Cytotoxicity Study (MTT assay)

The cytotoxicity of formulation was carried out on A 431 (Skin cancer) Epithelial cell line procured from NCCS, Pune, India. Dulbecco's Modified Eagle Medium (HiMedia) with added 10% Fetal Bovine Serum and 0.5% antibiotic solution was used as media for culturing cells at 37°C. The cells of A 431 were maintained in 5% CO_2 incubator. After 70 – 80 % confluency of cells in culture flask, subculturing was carried out. The complete media from the flask was removed aseptically in laminar air flow hood. After gentle wash with PBS 7.4, the trypsin – EDTA treatment for NMT 2 min was carried out. The trypsin – EDTA treatment detaches the adherent cells from the flask and the roundup of the cells was visible under microscope. The cell suspension was then transferred to centrifuge tube. After centrifuge for 2 min at 1000 rpm, the supernatant was removed and the cells were re suspended in complete media. The cells were counted using neubauer's chamber under microscope after staining with tryphan blue. Accordingly, the cells were subcultured in another flask after appropriate cell density was obtained.

After counting the cells, 10^4 cells were seeded in each well of 96 well plate with 200 µl of complete media. After the cells get adhered at around 24 h, the formulation treatment was given for 6 h using 1000 µg/mL, 500 µg/mL, 100 µg/mL and 10 µg/mL concentration. After the treatment, the formulation was removed and 10 µL of MTT dye (5 mg/mL) was added to each well containing 100 µL of complete media. The cells were further incubated for 4 hr after MTT treatment. Then 100 µL of DMSO was added to each well for solubilization of Formazan crystal. Then the plate was incubated for 2 hr to allow the solubilization of formazan crystal and absorbance was read at 570 nm using ELISA plate reader. Based one comparative absorption of control cells, the % viability was calculated.

Cellular uptake using confocal microscopy

Qualitative cellular uptake was carried out after proliferating the cells on a coverslip in a 6 well plate. The cells were treated with formulations prepared by replacing the drug with Coumarin-6 dye for 4 hr. After treatment, the coverslips were washed with phosphate buffer saline pH 7.4 thrice. DAPI was used to stain nuclei. Following

staining, the coverslips were mounted using glycerin. The slides were observed using confocal microscopy (ZEISS LSM, Germany) and images were processed for color channeling using ZEN lite – Blue edition software

Animal Study

All the protocols of animals study were approved by the Institutional Animal Ethics Committee (IAEC) accordance to the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of social justice and empowerment, government of India, New Delhi, India.

Acute dermal toxicity study[13]

The acute dermal toxicity performed on mice (n=18) according to the Organization for Economic Co-operation and Development guidelines No. 402[13,14]. Approximately 24 hr before the test, 10% hairs of the body were removed from the dorsal area of the test animal by using sterile shaving blade. Animals were divided into three groups (Normal control group, Formulation 1 treated group and formulation 2 treated group) having 6 animals in each groups study. All animals were well observed during study

Anti-psoriatic activity evaluation (Imiquimod Induced psoriatic model)

Topical application of imiquimod (*MQ) can induce and exacerbate psoriasis.* [14,15] 8 – 11 weeks old mice (n=36) having weight 25g to 45 gm of either sex were kept under specific pathogen-free conditions and provided with food and water ad libitum. Animals divided into six group having six animals in each group (table 1). Animals except normal group's received a daily topical dose of 62.5 mg of commercially available IMQ cream (5%) (Aldara; 3M Pharmaceuticals) on the shaved back and the right ear for 5 or 6 consecutive days, translating in a daily dose of 3.125 mg of the active compound. Control mice were treated similarly with a control vehicle cream (Vaseline Lanette cream; Fagron). To score the severity of inflammation of the back skin, an objective scoring system was developed based on the clinical Psoriasis Area and Severity Index (PASI), except that for the mouse model the affected skin area is not taken into account in the overall score. Erythema, scaling, and thickening were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The level of erythema was scored using a scoring table with red taints. The cumulative score (erythema plus scaling plus thickening) served as a measure of the severity of inflammation (scale 0–12). At the days indicated, the ear thickness of the right ear was measured in duplicate using a micrometer (Mitutoyo). The increase in ear thickness was used to indicate the extent of inflammation[16]. At the end of the study, all animals were euthanized.

Table 1: Animal group

Groups (n=6)	Samples applied
Normal control	Placebo gel (In-house developed)
Disease control	Imiquimod (3.125 mg/mice)
Drug Gel (In-house developed)	Imiquimod (3.125 mg/mice) + 0.1%w/w gel
Zorotene® Gel (Marketed sample)	Imiquimod (3.125 mg/mice) + 0.1%w/w gel
NLC gel	Imiquimod (3.125 mg/mice) + 0.1%w/w gel
SLN gel	Imiquimod (3.125 mg/mice) + 0.1%w/w gel

Imiquimod applied topically for first 7 days. Later on treatment continued up to 21 days.

Biochemical estimation[17]

Excise tissues were store in formaline solution at minus 20° C until used for the estimation of hydroxyproline content.[17] The tissue samples were hydrolyzed with 6N HCL for 3 hr at 130° C then after neutralized to pH 7.0. After that, tissue was subjected to chloramines-T oxidation for 20 min. after 5 min 2.5 ml each to ehrlich reagent was added[18]. And sample tube immersed to in a water bath at 60° C for 25 min. tubes were transferred to ice bath for cooling. Samples were stirred after addition of 6.6 ml Isopropyl alcohol[18]. At last samples were analyzed in 1 cm cuvettes by UV spectrophotometer at a wavelength 557 nm against control in which the solution being analyzed was replaced by distilled water[17,18].

RESULTS AND DISCUSSION

Cyto-toxicity Assay

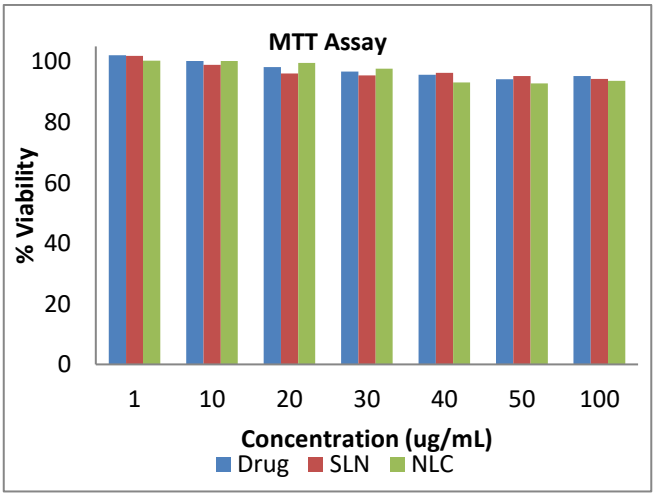

Figure 1: MTT assay

As shown in Fig. 1, the cell viability was in the range of 93-100 %. At higher concentration of SLN/NLC, the cell viability was less. This result when compared with pure drug, the only reason for decrease in % viability may be presence of surfactant. This surfactant molecules at higher concentration may have led to irreversible changes in cell that lead to apoptosis of cells. This in vitro cell toxicity study develops basis for in vivo or ex vivo study. From this %viability study, it was concluded that the formulation is safe for external use. On this basis, the formulation was carried forward for the ex-vivo and in-vivo study.

Cellular uptake using confocal microscopy

Cellular uptake and distribution were studied using coumarin – 6 fluorescent marker loaded NLS and SLN formulations[19]. As the drug tazarotene is hydrophobic in nature, coumarin-6 was chosen as hydrophobic model dye to mimic its nature[19]. Qualitative study of cellular internalization of formulation was studied by confocal LASER scanning microscopy. Figure 2. shows cellular image for cells incubated with NLC and SLN formulation. Dense nuclei area is stained in blue by DAPI flurophore, whereas green color is due to formulation loaded with coumarin – 6. Abundance of green color in formulation treated cell indicates internalization of formulation. Formulation do not appear in the nucleus area; however, its perinuclear location might be important for higher drug concentration around the nucleus. The punctual intracellular concentration of formulation within the cells indicates their location in certain compartments, such as endosomes or lysosomes.[20,21,24-44]

Animal studies

In Acute dermal toxicity study, sample topically applied at a dose of 1% w/w gel. After 14 days observation, all animals were normal and there were no changes in fur, eyes, and behavior of treated animals. There were no toxic reactions like inflammation redness, erthyma observed on skin. Hence, formulation was found to be safe for topical application.[14]

The result of the topical formulation on right ear thickness are given in Fig. 3. The maximum increase in ear thickness in the IMQ-treated group on day 7. After 7 day all group were daily treated as per group and further ear thickness was measured using micrometer regularly[14]. The maximum increase in ear thickness in the test groups was significant decrease on day 24 compare to other group II, III, IV showed no significant changes during the experiment.

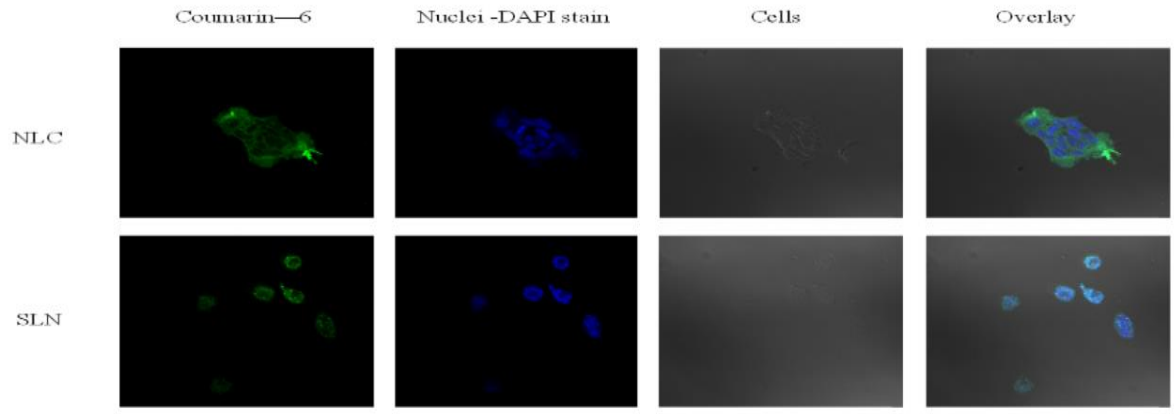

Figure 2: Assessment of cellular uptake

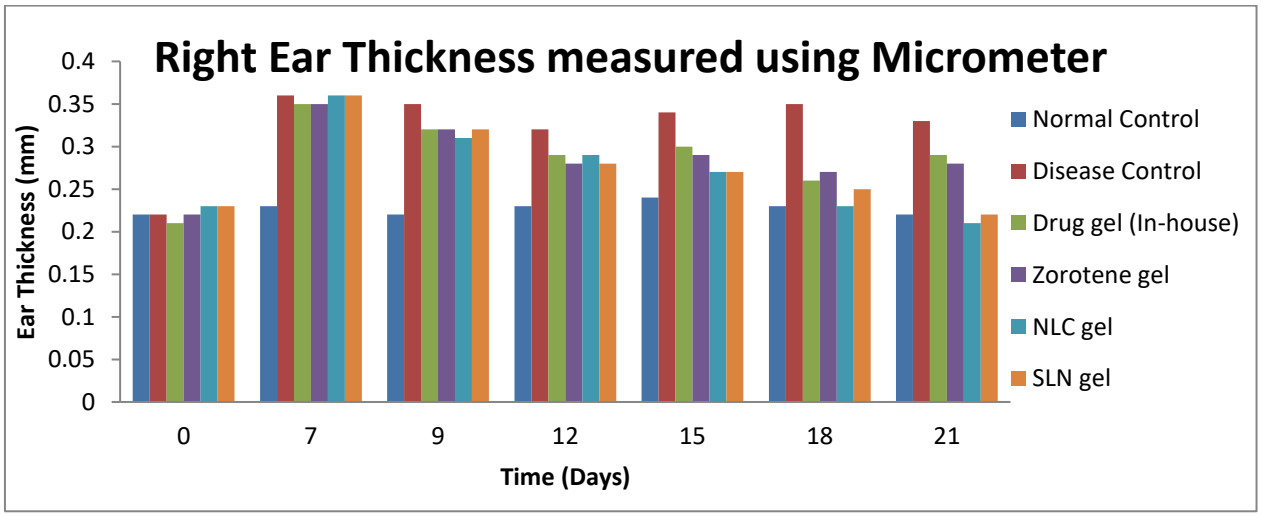

Figure 3: Effect of topical formulation on Right ear Thickness in psoriasis in rat

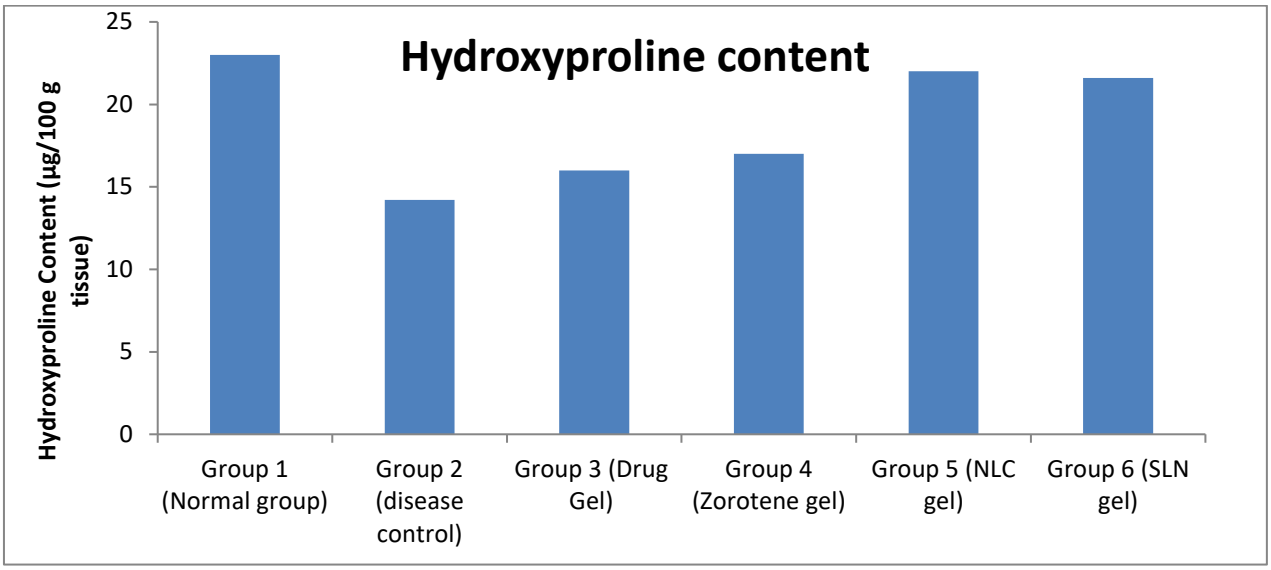

Figure 4: Effect of topical formulation on Hydroxyproline content in induced psoriasis in rat

Table 2: Macroscopic observation in animals

Group	% Relative Epidermal thickness (µm)#	Psoriasis Area and Severity Index (PASI)*		
		Erythema	Scales	Thickness
Group I (Normal control)	96.34	0	0	0
Group II (Disease control)	-	4	4	4
Group III (Drug Gel-In-house developed)	38.62	3	3	3
Group IV (Zorotene Gel)®	40.24	3	3	2
Group V (NLC gel)	56.38	2	2	1
Group VI (SLN gel)	51.28	2	3	1

#The % are calculated with reference to Disease control group.

*PASI: 0 – none, 1 – slight, 2 – moderate, 3 – marked, 4 – very marked

The result of the topical formulations on Hydroxyproline content are shown in figure 4. The biochemical evaluation reveled no significant reduction in hydroxyproline content in treated group. The hydroxyproline content of the test group V and VI was found to be almost equal which was higher than that of control group and standard groups tissues. An increase in collagen catabolism and regeneration is an important link in the pathogenesis of many diseases[21]. Twelve fourteen percent of all amino acid residues of collagen are hydroxyproline[22]. Collagen is major constituent of the dermis, and is therefore involved in many facets of skin disease including psoriasis as well as the recovery process[23].

The results of macroscopic observation in animals after topical application of formulations are shown in Table 2. Severity index was calculated by observing the psoriatic lesion by naked eye[24]. Result of %Relative epidermal thickness in both test group V (NLC gel) and Group VI (SLN gel) were 56.38% and 51.28% respectively which was higher compare to Group III (36.82%), group IV (40.24%) but lesser that that of Normal control group (96.34%) which indicate good anti-psoriatic strength of the formulation. There was also less severity index observed in test (Group V and Group VI) compare to Drug Gel formulation and marketed formulation treated animal group.

CONCLUSION

From the experimental data it can be concluded that the developed formulation of tarozotine is effective in treatment of psoriasis. The lipid nanocarriers incorporated in the gel show retention at the site of application due to enhanced interaction with the skin with sustained release at the site of application with negligible toxicity which is desirable characteristic of the formulation. The future aspect of the developed formulation should be should be commercial scale up once the safety and efficacy are confirmed from clinical trials.

Acknowledgements: The protocol for the study was duly approved by Institutional Animal Ethics Committee. The Protocol number is MPC/IAEC/24/2017. All experimental procedures were carried out as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines released by Ministry of Environment, Forests and Climate Change, Govt of India.

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