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Targeting tacrolimus to deeper layers of skin with improved safety for treatment of atopic dermatitis—Part II: *In vivo* assessment of dermatopharmacokinetics, biodistribution and efficacy

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ABSTRACT

The objective of present investigation was to study *in vivo* behavior of tacrolimus-loaded lipid-nanoparticles (T-LN) to understand its targeting potential for treatment of atopic-dermatitis-(AD). T-LN have shown significantly improved drug penetration to deeper epidermal and dermal skin-layers than commercial ointment-Protopic® and effectively reached target dendritic-immune-cells, responsible for immunopathogenesis of AD. Due to enhanced penetrability of T-LN, it became necessary to evaluate the toxicity of the nanocarrier and the drug at non-target tissues. This paper evaluates dermatopharmacokinetics (DPK), biodistribution, efficacy and safety of T-LN in comparison to Protopic® as reference. *In vivo* DPK in guinea pigs showed 3.02-fold higher bioavailability while γ-scintigraphy in albino-rats demonstrated 1.5-fold rapid penetration of radioactivity in skin for T-LN. Biodistribution in albino-rats revealed restricted localization at the target-skin-area with no general spreading to other body organs suggesting targeting potential of T-LN. *In vivo* efficacy studies in BALB/c mice showed highly efficient suppression of inflammatory AD-like skin-lesions with T-LN than reference and placebo. Dermal toxicity-studies revealed keratosis and collagenous mass-infiltration with repeated application of reference however interestingly, T-LN treated group showed no evident toxicity demonstrating significantly improved safety. Thus T-LN offered improved penetration to the target site without any toxic-effects and would represent an efficient and commercially viable alternative for AD treatment.

1. Introduction

Atopic dermatitis (AD) is a complex disease that manifests immunological abnormalities in the skin and represents one of the most common chronically relapsing inflammatory skin diseases accompanied by severe itching and eczematous skin lesions (Kaltoft et al., 1994; Lee et al., 2000; Zheng and Zhu, 2005). Incidence of AD has increased in recent years in industrialized countries, and it often relapses after the termination of therapeutic treatment period (Granlund et al., 1995; Pivarcsi, 2004). Even though considerable efforts are marshaled against AD, it continues to recur throughout the patient's lifespan and inflict an unacceptable burden on many developing countries. The treatment of local dermatological disease condition by directly applying a pharmaceutical formulation to the site is easy, convenient and well accepted by patients. Topical corticosteroids are routinely used to manage AD; however, its use is limited to shorter duration due to adverse side effects such as easy bruising of skin, telangiectasia, allergy, increased susceptibility to skin infection, skin thinning and atrophy (Furue et al., 2003; Yamamoto and Nishioka, 2003).

Tacrolimus, a macrolide immunosuppressant drug, has been found to be useful in the treatment of many immune mediated dermatoses including AD (Yoshida et al., 2004; Rubins et al., 2005). Unlike the conventional therapy which involves use of topical corticosteroids; use of topical tacrolimus does not produce significant side effects. Tacrolimus is therapeutically effective in patients having sensitive skin, infants, children and elderly where steroids are likely to cause many side effects (Ruzicka et al., 1999). Tacrolimus achieves immunosuppression mainly by inhibition of interleukin 2 (IL-2) transcription thereby preventing T lymphocyte activation (Denise and William, 2005; Sehgal et al., 2008). Although being effective, topical application of tacrolimus is associated with burning sensation, itching or stinging along with other potential local side effects (Zahir et al., 2001; Hultsch et al., 2005; Rubins et al., 2005; Spergel and Leung, 2006; Ständer et al., 2006).

At present tacrolimus is available commercially as an ointment formulation, Protopic® for topical application. However, ointments

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are difficult to wash off the skin and due to their greasy nature may create a sticky feeling and uneasiness to the patient (Kudla, 1979; Lin et al., 2005). In addition, several studies with tacrolimus ointment have reported notably variable absorption rates and high individual variation in mean disposition half-life values (Ruzicka et al., 1997; Cheer and Plosker, 2001; Kang et al., 2001; Paller et al., 2001). Patients have experienced sensation of warmth and stinging together with pain and redness during clinical trials of the ointment (Ständer et al., 2006; Svensson et al., 2011). Allergic reaction, fever, flu-like symptoms and skin infection have also been reported (Wijnen et al., 1992; Zahir et al., 2001; Spergel and Leung, 2006). Systemic exposure to tacrolimus can be even more catastrophic. It may cause hives, difficulty in breathing, swelling of face, lips, tongue, or throat and increased susceptibility to bacterial, viral, fungal, and protozoal infections including opportunistic infections. Side effects can be severe with increased long-term malignancy risk and serious drug interactions (Zahir et al., 2001; Spergel and Leung, 2006). It has also been reported to have a potential to increase the risk of development of lymphoma and other malignancies, particularly of the skin, due to immunosuppression. There are reports of lymphoproliferative changes in rodents and certain cynomolgus monkeys after tacrolimus use (Wijnen et al., 1992). This toxicology data suggest an increased risk with tacrolimus use in cases of extensive and/or prolonged use. In order to overcome these disadvantages, to enhance the bioavailability at the target site and reduce the related toxicity it is necessary to exploit delivery of tacrolimus using novel formulation approaches with significantly improved beneficial impact for treatment of AD.

A major problem associated with drug therapy is the inability to deliver the pharmaceutical to a specific site of the body without causing any side effects. The advantages of topical application are mellowed by protective barriers of skin, which limit drug penetration when formulations are applied topically to the skin. Therefore the attention of different research groups has focused on novel nanotechnology based delivery systems which hold great promise to improve the efficiency and enhance bioavailability of drugs (Couvreur et al., 1995; Mühlen et al., 1998; Barratt, 2000; Jenning et al., 2000; Maia et al., 2000; Porter and Charman, 2001). The smaller size and unique properties of the nanoparticles have substantially improved their application in drug delivery (Pople and Singh, 2006, 2011; Schäfer-Korting et al., 2007; Bhalekar et al., 2009; Nagi et al., 2008; Goebela et al., 2011). Studies have shown that nanoparticles can pass through the protective barriers of living organisms and accumulate at specific sites. The corollary is that nanoparticles could be distributed throughout the body, which may lead to undesirable toxic effects mainly because of their enormous surface area. The area of toxicology, known as nanotoxicology, reports deleterious effects due to substances which are ordinarily innocuous but can have adverse effects at the nanoscale (Service, 2004; Dechsakulthorn et al., 2007; Wani et al., 2011). Toxic effects due to nanoparticles have been documented at the pulmonary (Bartel et al., 2011), cardiac (McLeish et al., 2010), renal (Lei et al., 2008), cutaneous (Choksi et al., 2010) and cellular levels (Pakrashi et al., 2011). Effects on reproduction (Cañas et al., 2011) and genotoxic effects (Kumari et al., 2011) have also been identified so far. These documented toxic effects of nanoparticles on animals justify the urge for application of all useful measures to effectively ascertain the potential hazards of these delivery systems.

Although few novel approaches such as liposomal tacrolimus lotion (Erdogan et al., 2002), colloidal carrier system ME (Goebela et al., 2011) and modified nanolipid carrier (Pople and Singh, 2011) have been investigated for topical tacrolimus delivery, none of these have been subjected to thorough assessment of systemic biodistribution and safety evaluation.

In our previous report (Pople and Singh, 2010), tacrolimus loaded lipid nanoparticles (T-LN) were developed, optimized for process-product variables and characterized in detail for *in vitro* parameters including drug release and skin penetration. We found that T-LN significantly improved drug penetration to the deeper epidermal and dermal layers of the skin as compared to commercially available product Protopic® and effectively reached the target dendritic immune cells that are responsible for immunopathogenesis of AD. Due to enhanced penetrability of T-LN, it became necessary to evaluate the risk associated with application of the nanocarrier (*i.e.* nanotoxicity) and toxicity related to drug itself at non-target tissues. This paper evaluates the pharmacokinetics, pharmacodynamics and safety of T-LN in comparison to the commercially available ointment product Protopic® as reference. The bioavailability of T-LN was assessed employing dermatopharmacokinetic (DPK) approach in guinea pigs and bio-deposition was measured using gamma scintigraphy in albino rats. It is crucial that the drug reaches its specific site of action but it is equally important that it does not distribute to other organs of the body causing drug-related toxic effects. This becomes even more important when the drug is being applied topically and systemic toxicity needs to be avoided. Biodistribution studies were carried out using gamma scintigraphy in albino rats to confirm target specific delivery. Therapeutic efficacy was appraised using a hapten-induced murine model of AD in BALB/c mice. In addition, acute and repeated dose dermal toxicity studies (OECD 402 and 410) were performed in order to establish detailed safety profile. All experimental procedures involving animal use were reviewed and approved by Institutional Animal Ethics Committee.

Table 1
The composition of T-LN.

Tacrolimus	0.1%
Trimyristin	5 g
Sorbitan monooleate	3.5 g
Polyoxyethylene sorbitan monooleate	4.0 g
Double distilled water	q.s. 100 g

2. Materials

Tacrolimus was procured from Panacea Biotec Ltd., Punjab, India. Glyceryl trimyristate (Dynasan® 114) was a gift from Sasol (Germany). Stannous chloride dihydrate was purchased from Rankem RFCL Limited (New Delhi, India). Solvents used for TLC and HPLC analyses were of HPLC grade (Spectrochem, India). All other chemicals were of analytical reagent grade and were purchased from S. D. Fine Chemicals, Mumbai, India.

3. Methods

3.1. Characterization of tacrolimus-loaded lipid nanoparticles (T-LN)

T-LN dispersion was formulated using hot melt emulsification combined with high pressure homogenization technique and developed to a gel for topical application as described in our previous report (Pople and Singh, 2010). The composition of T-LN is provided in Table 1. Gaussian unimodal size distribution analysis was performed using Beckmen N5 Submicron Particle Size Analyzer (Beckmen, Inc., USA) based on the principles of dynamic light scattering (DLS). The scattered light intensity was detected at scattering angle of 90° at 20 °C. T-LN dispersion was diluted with double distilled water so as to adjust the light scattering intensity between $5e+004$ and $1e+006$. Size distribution processor (SDP) analysis was performed from the scattered light intensity fluctuations to provide the best evaluation of polydispersed distribution. Results represent an average of three different measurements. Unimodal fingerprint