Chapter - 7

Appendix
Nanomedicine

Event Proceedings

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110 - Solid Lipid Nanoparticles of Quercetin with Enhanced Brain Targeting Potential for the Treatment of Alzheimer

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The present study aims to formulate solid lipid nanoparticles (SLNs) of quercetin, a natural flavonoid with established antioxidant activity to improve its permeation across blood brain barrier and eventually to improve the therapeutic efficacy of this molecule in Alzheimer’s disease. Micro-emulsification technique was adopted for formulating SLNs using different ratios of lipid (Compritol 888 ATO) and surfactant (Polysorbate 80) as per the face-centered cubic design (FCCD). A total of 9 formulations were prepared as suggested by FCCD, evaluated & validated for various Critical formulation attributes (CFAs), viz. particle size, drug entrapment efficiency, amount of drug release in 20 h (Rel20) and zeta potential, which demonstrate the capability of SLNs for delivering quercetin across BBB into the CNS. The optimized formulation was selected & was found to have size below 200nm. Evaluation of in vivo cognitive performance of the rats using spatial navigation, maze acquisition and maze retention through Morris water maze test, and elevated plus maze paradigm showed better memory-retention in SLN-treated group compared to other animal groups. Nearly 5-fold augmentation in the brain levels of quercetin was observed in animals treated with the SLNs vis-a-vis those treated with pure quercetin. Biochemical estimations of rat brain homogenates indicated better regulation of glutathione, MDA and nitrite levels in highly significant manner. The studies successfully demonstrated the enhanced drug biodistribution potential of SLNs of quercetin for the treatment of AD.

111 - Lipid-based Nano-colloidal Carriers of Tretinoin with Enhanced Percutaneous Absorption, Photostability, Biocompatibility and Anti-psoriatic Activity

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Tretinoin (TRE) is a widely used retinoid for the topical treatment of acne, psoriasis, skin cancer and photaging. Despite unmatchable efficacy, it is associated with several unpleasant side effects like marked skin erythema, peeling and irritation which give rise to poor patient compliance. Its photo-instability and high lipophilicity also pose challenges in the development of a suitable topical product. The present study aims to develop biocompatible lipid-based nanocarriers of TRE to improve its skin delivery, photostability, biocompatibility and pharmacodynamic efficacy. TRE-loaded liposomes, ethosomes, solid lipid nanoparticles (SLNs) and nano lipid structured carriers (NLCs) were prepared and characterized for microemricics, surface charge, percent drug efficiency and morphology. Bioadhesive hydrogels of the developed systems were also evaluated for rheology, spreadability, photostability, ex vivo skin permeation and retention employing porcine skin, and anti-psoriatic activity in mouse tail model. Nanoparticulate carriers (SLNs, NLCs) offered enhanced photostability, skin transport and anti-psoriatic activity vis-a-vis the vesicular carriers (liposomes, ethosomes) and the marketed product. However, all the developed nanocarriers were found to be more biocompatible and effective than the marketed product. These findings are encouraging and can be employed for the proper selection of topical carrier systems to deliver problematic drugs like retinoids.

112 - In Situ Gelling Nanoparticles of Ornidazole for Treatment of Periodontitis using Bioresponsive Smart Polymers

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The present studies investigate the in situ gelling DDS of ornidazole using simple, effective and cost-effective intelligent polymers for the treatment of periodontal infections. Polymeric nanoparticles encapsulated in stimuli-responsive in situ gelling DDS were formulated for local drug delivery in the periodontal pocket. A face-centered cubic design was employed for rationally optimizing the blends of sodium alginate and chitosan for nanoparticles and gellan and Lutrol F68 for the in situ gelling system. Preformulation studies using FT-IR and DSC ratified lack of any significant drug-polymer interaction. The nanoparticles were optimized on the basis of maximizing the values of amount of drug release in 16h (Q16h), time required for 70% drug release (t70%), entrapment efficiency and zeta potential, while minimizing the particle size. Similarly, the optimized in situ gel (OPT GEL) was chosen by maximizing Q16h, t75%, biodegradability strength (Bb) and gel strength (Gs), while minimizing viscosity (e) and extrudability (Fs). The optimized nanoparticles were subsequently incorporated in the optimized in situ gels. Accelerated studies vouched high stability of the optimized nanoparticulate gel under refrigerated storage conditions. In vivo evaluation through histopathological studies in female Wistar rats by feature-induced periodontitis method, followed by microscopic and macroscopic evaluation corroborated significant improvement (p<0.001) in the periodontitis after once-a-day application of the optimized dual drug delivery formulation vis-a-vis twice-a-day application of the marketed conventional periodontal gel (METROHEX DGB®), as revealed by nonparametric statistical tests.

113 - Topical Delivery of Cyclosporin using Novel Self-Assembled Fibrillar Networks: A Promising Treatment Option

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Cyclosporin A (CyA), an immunomodulatory molecule, is one of the gold-standard choice in the treatment of T-cell mediated autoimmune skin disorders, such as atopic dermatitis, psoriasis, vitiligo, and rosacea. However, the current approaches of its administration and delivery is systemic, i.e., oral and parenteral. The later is, though very effective, marred because of the multiple problems associated with exposure of the molecule to entire bio-system. That may leads to skin cancer, cutaneous-extracutaneous infections, etc. For this reason, while skin (external surface) is the host of disease target, the topical delivery is considered to be more acceptable and meaningful. The present work aims to improve its topical potential by way of novel delivery carriers with safer and effective delivery into the skin. In this piece of study we developed a novel self-assembled fibrillar networks (NASPN) system that is a reverse micellar gel system by employing...
14 - Apolipoprotein C3 Polymorphism in First Degree Relatives of Metabolic Syndrome Subjects

udhakar Pemmineti

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Background of the study M. avium is the most common of the nontuberculous mycobacteria responsible for causing disease in human beings. The preferred treatment regimen includes a macrolide, together with rifabutin and ethambutol which have no limitations like lengthy treatment course and patient compliance. This emphasizes the need for an alternative strategy to overcome these drawbacks. In the present study, we have evaluated poly (D,L-lactide- caprolactone) (PLGA) nanoparticles for the pharmacokinetic and therapeutically potential of anti-M. avium drugs.

Pharmacokinetics: Pharmacokinetics of free and nanoparticles containing anti M. avium drugs were evaluated in mice after single oral dose administration at therapeutic concentrations. Therapeutic efficacy was assessed in M. avium infected mice after chemotherapy with free and nanoparticles drugs.

Observations: Prolonged release of various drugs was found to be up to 5-7 days compared to 24h for free drugs in plasma and other tissues. Nanoparticles contained drugs result in significant improvement in pharmacokinetic profile in comparison to free drugs. Four weeks of chemotherapy resulted in 10%, 35% and 0% clearance of bacilli from lungs, liver and spleen as compared to controls.

Conclusion: PLGA nanoparticles based drug delivery system hold great potential to produce sustained release of anti-M. avium drugs as observed that 4 doses of PLGA nanoparticle encapsulated M. avium drugs depicted an equivalent therapeutic effect as 28 doses of daily administered oral free drugs.

116 - Ayurveda and Nanomedicine

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This poster analyses the connectivity between the ancient Indian science of Ayurveda, with contemporary concepts in Nanomedicine based on a literature review on the subject. It establishes relationships between the two systems in terms of the principles of use of medicines in Ayurveda, in terms of methodology, principles in reducing particle size, targeting of drug to desired organ-systems, freedom from toxicity, and therapeutic dosing. It touches upon the use of metals as anti-cancer drugs and their administration in cancer treatment.

Use of drug delivery systems has shown promising results in past few decades especially with respect to cancer treatment. Drug resistance (MDR) in cancer, however poses a barrier to effective therapy & can be either inherent or acquired via exposure to chemotherapeutics. Drug delivery systems co-administering anti-cancer drug and anti-MDR compounds have been developed to facilitate drug uptake in resistant cancer cells. Several pharmaceutical excipients are known for their MDR-inhibitory properties. In this study, nanoparticles (NPs) in the size range of 100-300 nm were prepared using various excipients in the presence of surfactants. In vitro studies on drug sensitive and drug resistant cell lines were carried out to evaluate their cytotoxicity, cell adhesion and MDR inhibitory abilities. Maximal cell adhesion of Nile Red encapsulated NPs was observed with Solutol HS15 NPs (22.4±0.8%) as compared to PVA NPs (15.8±0.7%). In drug uptake studies, the drug concentration of Nile Red encapsulated NPs from the nanoparticle was found to be higher than from the free drug in drug resistant cell lines. The drug concentration in drug sensitive cell lines was found to be similar to that obtained from free drug and drug sensitive cell lines. This emphasizes the need for an alternative strategy to overcome these drawbacks. In the present study, we have evaluated poly (D,L-lactide-ε-caprolactone) (PLGA) nanoparticles for the pharmacokinetic and therapeutically potential of anti-M. avium drugs.

Pharmacokinetics: Pharmacokinetics of free and nanoparticles containing anti M. avium drugs were evaluated in mice after single oral dose administration at therapeutic concentrations. Therapeutic efficacy was assessed in M. avium infected mice after chemotherapy with free and nanoparticles drugs.

Observations: Prolonged release of various drugs was found to be up to 5-7 days compared to 24h for free drugs in plasma and other tissues. Nanoparticles contained drugs result in significant improvement in pharmacokinetic profile in comparison to free drugs. Four weeks of chemotherapy resulted in 10%, 35% and 0% clearance of bacilli from lungs, liver and spleen as compared to controls.

Conclusion: PLGA nanoparticles based drug delivery system hold great potential to produce sustained release of anti-M. avium drugs as observed that 4 doses of PLGA nanoparticle encapsulated M. avium drugs depicted an equivalent therapeutic effect as 28 doses of daily administered oral free drugs.
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OP-6: EFFECT OF ASHWAGANDHA (WITHANIA SOMNIFERA) AGAINST SCIATIC NERVE LIGATION INDUCED BEHAVIORAL AND BIOCHEMICAL ALTERATIONS: POSSIBLE INVOLVEMENT OF NITRIC OXIDE MECHANISM

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Background: The objective of the present study was to explore possible nitric oxide mechanism in the protective effect of ashwagandha (WS) against sciatic nerve ligation induced behavioral and biochemical alterations in rats. Methods: Chronic constriction injury (CCI) was performed in male wistar rats. Various behavioral parameters (thermal hyperalgesia, cold alldynia) as well as biochemical parameters (lipid peroxidation, reduced glutathione, catalase, and nitrite) were assessed in sciatic nerves. Drugs were administered for 21 consecutive days from the day of surgery. Results: CCI of sciatic nerve significantly caused thermal hyperalgesia, cold alldynia and oxidative damage. Chronic administration of WS (100 and 200mg/kg, po) significantly reversed hyperalgesia, cold allodynia and attenuated oxidative damage (as indicated by reduced lipid peroxidation, nitrite concentration, restoration of reduced glutathione and catalase activity). Further, L-NAME (5 mg/kg, ip) (nitric oxide synthase inhibitor) pretreatment with effective doses of WS (100 mg/kg, po) potentiated the protective effect of WS which was significant as compared to their effect per se. However, L-arginine (100 mg/kg, ip) (nitric oxide precursor) pretreatment with WS (100 mg/kg, po) significantly reversed the protective effects of WS in sciatic nerve. Conclusion: Result of present study suggests that nitric oxide mechanism might be involved in the protective effect of WS against CCI model of rats.

OP-7: SINGLE DOSE ORAL PHARMACOKINETIC AND TOXICITY STUDIES OF RIFAMPICIN LOADED SOLID LIPID NANOPARTICLES

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Background: Rifampicin (RIF) is a first line antitubercular drug, prescribed widely for the treatment of tuberculosis. Hypothesis: RIF loaded solid lipid nanoparticles (SLNs) were prepared to improve its bioavailability, reduce degradation and drug induced toxicity. Methods: Based on ternary phase diagram and interaction studies (FTIR and DSC), a suitable RIF-SLNs formulation was developed. A single oral dose (50 mg/kg) of free RIF suspension (1% CMC) or RIF-SLNs was administered to wistar rats and their plasma concentration-time profile was monitored over a period of 7 days using official microbioassay method. Acute and repeated 28 days oral toxicity study of RIF SLNs was performed in rats in accordance with OECD Guidelines. Results: Spherical SLNs with average particle size 141±3.5 nm, polydispersity index 0.320±0.032, zeta potential -3.5±0.8, drug loading 50%, total drug content 93±2.2 % and an encapsulation efficiency 76.71±4.84 % were developed. More than 70% release was obtained in phosphate buffer pH 6.8 within 9 days. A significant improvement in bioavailability in plasma (8.14 times) was observed with respect to free drug suspension. Nanoparticulate nature of RIF did not increase its toxicity. Conclusion: Developed RIF-SLNs hold significant potential in terms of reduced dose, dosage frequency and side effects.

OP-8: TOPICAL DELIVERY OF METHOTREXATE USING MODIFIED MEMBRANE VESICLES TO TREAT PSORIASIS

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Psoriasis is a dreadful skin disease affecting 2 – 3% population worldwide. It is an immune mediated, chronic and proliferative disorder mainly characterized by sharply demarcated, erythematic, silvery scales and papules preferentially localized to the extensor prominences and the scalp. Amongst all the available treatment options topical is the preferred one for localized cases. For this, methotrexate (MTX) is one of the potential drugs, but attempts hitherto made
could not succeed to bring any worthwhile results. This could be accounted to the failures of conventional formulation approach which remain deficient in addressing this delivery related issue. In the present work an attempt has been made to formulate MTX employing modified vesicular delivery system in order to exploit the potential of the drug, and compared with conventional vesicular system and a cream. Vesicles of the MTX were prepared using thin film hydration technique and were gelled in hydrocolloid base. The vesicles were optimized with respect to its various compositional parameters viz. drug-lipid ratio, type and percentage of charge inducer, and percentage of organic solvent. Formulation was characterized for morphology and micromeritics, drug entrapment and degree of deformability. Efficacy of the optimized formulation was assessed by performing \textit{in-vitro} and \textit{ex-vivo} studies using dialysis membrane and skin respectively. On comparing, the best performance was obtained with modified vesicles viz. release flux [21.392\,\mu g/cm^2/h], permeation flux [17.848\,\mu g/cm^2/h] and percent skin retention [14.284\%]. Further in the \textit{in-vivo} pharmacodynamic assessment using mouse tail model, the results have been encouraging with novel over the conventional formulations. This reveals the potential of the novel approach in transforming the character of the molecule like methotrexate. The developed formulation was also found to be stable in the six month accelerated stability study as per ICH guidelines.

**PP-1: MELATONIN POTENTIATES THE ANTIANXIETY AND ANTIOXIDANT LIKE EFFECT OF BUSPIRONE AGAINST IMMOBILIZATION STRESS: POSSIBLE INVOLVEMENT OF SEROTONERGIC MECHANISM**

Puneet Rinwa, Gurleen Kaur and Anil Kumar

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Stress is recognized to precipitate anxiety and other related psychological disturbances, characterized by wide range of biochemical and behavioral changes. The objective of the present study is to investigate the protective effects of melatonin, buspirone and their combination against 6 hours immobilization stress induced anxiety like behavioral and oxidative damage. Male Laca mice were pre-treated with melatonin, buspirone and their combination for consecutive five days followed by immobilisation stress on the day six. Immobilization stress significantly impaired body weight, locomotor activity and caused anxiety like behaviour along with oxidative damage as compared to naïve. Pretreatment with melatonin and buspirone significantly improved body weight, locomotor activity, and anxiety like behaviour (mirror chamber as well as elevated plus maze performance) along with anti-oxidant like effect as evidenced by reduced lipid peroxidation, nitrite concentration and restoration of reduced glutathione and catalase activity as compared to control. Further, combination of melatonin with buspirone significantly potentiated their protective effects as compared to their effects alone. Present study suggests that melatonin synergize the beneficial effect of buspirone against immobilisation stress induced anxiety like behavioral and oxidative damage in mice.

**PP-2: VERAPAMIL AUGMENTS THE NEURO-PROTECTIVE ACTION OF BERBERINE IN RAT MODEL OF TRANSIENT GLOBAL CEREBRAL ISCHEMIA**

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Objective- To evaluate the effect of a P-gp substrate, verapamil also acting as an inhibitor of this effluxer protein at blood brain barrier when concomitantly administered with a potential therapeutic molecule, berberine in animal model of acute ischemia reperfusion injury in brain. Hypothesis- We hypothesized that concomitant administration of berberine with P-gp inhibitor, verapamil may prevent the efflux of berberine ultimately increasing the brain bioavailability, thereby providing the beneficial neuro-protection. Method- Wistar rats were subjected to bilateral common carotid arteries occlusion to induce acute cerebral ischemia for 15 minutes followed by reperfusion resulted in transient global cerebral ischemia. The effect of berberine in doses of (5, 10, 20 \,mg/kg, p.o.) alone and concomitantly with verapamil (2 \,mg/kg, p.o.) for 18 days was evaluated by employing various neurobehavioural and biochemical parameters. The
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TOPICAL DELIVERY OF AN ANTIFOLATE ANALOG USING FLEXIBLE MEMBRANE VESICLES TO TREAT PSORIASIS

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KEYWORDS: Methotrexate, Psoriasis, Topical delivery, Vesicles, Dermal delivery

INTRODUCTION: Psoriasis is a debilitating skin disease affecting 2 – 3% population worldwide. It is an immune mediated, chronic and proliferative disorder. Due to the characteristic formation of skin lesions and eruptions, psoriasis gives its victims an unfavorable psychological outlook on life. Multifarious immunological activities are involved in the progression of psoriasis, however, the mechanism of its occurrence have not yet been clearly elucidated. In case of localized psoriasis, topical therapy is the preferred one amongst all the available treatment options. For this methotrexate (MTX) is one of the potential drug that found to be active against epidermal growth factor receptor and nuclear factor-κB apart from its antifolate activity, however, attempts hitherto made could not succeed to bring any worthwhile results. This could be accounted to the failures of conventional approach which remain deficient in addressing this delivery related issue.

OBJECTIVE: To assess topical delivery of MTX into the skin and its efficacy in psoriatic model.

EXPERIMENTAL METHODS:

Formulation development: Flexible membrane vesicles (FMVs) of MTX were prepared by thin film hydration. In brief, weighed quantities of drug, phospholipid (PL), and charge inducer (CI) were dissolved in chloroform-methanol (2:1 v/v) mixture in a round bottom flask. The solvent was evaporated at 50 °C ± 2°C under reduced pressure using rotary film evaporator and hydrated with aqueous phase. The suspension was kept for 2 h at room temperature for complete hydration process. Then, ethanol was added into the system, drop by drop, with continuous stirring on magnetic stirrer. The formulation was optimized for drug: PL ratio, for PL:drug:CI ratio and for percentage of ethanol. At every stage FMVs were characterized against MTX solution, FMVs gel, liposomal suspension, liposomal gel and conventional cream to check its efficacy.

Morphology and Micromeritics: Developed vesicles were examined for morphological characteristics using TEM and for micromeritics using DLS technique.

Drug Entrapment Efficiency: Mini-column centrifugation technique was used to determine the quantity entrapped within the vesicles. Sephadex G-50 (medium grade) was used to prepare mini-column.

Degree of Deformability: It is determined using mini filtration technique using 0.6 μm polycarbonate membrane. Vesicular size was monitored before and after filtration to determine degree of deformation.

Spreadability: Hydro-cellulose gel of the FMVs was evaluated using evaluated using Texture Analyzer™ equipment, equipped with a 5 kg load cell (Fig 1).

In-vitro Drug Release and Ex-vivo Permeation Studies: These studies were carried out using Franz diffusion cell.

Dialysis membrane and dorsal skin of Laca mice were used respectively for in-vitro drug release and ex-vivo drug permeation study (Fig 2).

In-vivo Pharmacodynamic Evaluation: Mice tail model of Bladon, et al., 1985 was used with slight modifications to evaluate the anti-psoriatic activity of the developed formulation (Fig 3).

RESULTS AND DISCUSSION: While optimization 10:1:1 ratio of PL, MTX and CI was found to be optimum with 20% v/v alcohol that showed abundant vesicular structures (165.5 x 103 vesicles/ mm²), lowest transmittance (55.7%) and maximum entrapment (69.962 ± 0.156%). On account of its in-vitro, ex-vivo and in-vivo study we found that the developed FMVs are suitable delivery option for the treatment of psoriasis.

REFERENCES:


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Tamoxifen-loaded novel liposomal formulations: evaluation of anticancer activity on DMBA-TPA induced mouse skin carcinogenesis

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Abstract

Purpose: Tamoxifen (TAM) is a non-steroidal estrogen receptor modulator known for its anticancer activity. Apart from marked breast cancer activity, this drug has also shown potential in treating other types of cancers including skin cancers. TAM is reported to be associated with serious side effects primarily due to its systemic distribution. The localized delivery of this drug in this regard would be highly beneficial with respect to safety as well as efficacy.

Methods: In the current studies, an endeavor has been made to investigate the efficacy of topically applied liposome-encapsulated TAM on skin cancer model. The drug was encapsulated in phospholipid-based vesicular systems viz. conventional liposomes and elastic liposomes. Incidence of papillomas and histopathological examination were employed to determine the efficacy of the tested formulations.

Results: The results demonstrated dose-dependent strong inhibition of skin carcinogenesis with encapsulated drug vis-à-vis drug in the solution form. The encouraging findings from the current work construe immense potential of the TAM-loaded liposomal systems in the management of skin cancer.

Keywords: Skin cancer, phospholipid, topical delivery, liposomes, chemoprevention

Introduction

Carcinogenesis, a multi-step process, involves initiation, promotion and progression of sequential changes. Cells gain a selective growth advantage as a result of accumulation of mutations and pass the phenotypic attributes of local invasiveness as well as distant metastasis (Boone et al., 1990). Accumulation of genetic lesions at the molecular levels results in progression. Use of one or multiple agents is the basis of chemoprevention of carcinogenesis (Boone et al., 1990; Einspahr et al., 2002; Smith et al., 2005). The mouse skin carcinogenesis model is a well-established model to study the genetic and biological changes leading to tumor promotion (Samaha et al., 1997; Holden et al., 1997). Some of the genetic changes like lesions and transition to squamous cell carcinoma are associated with the chemical initiation of benign papillomas and are characterized in this model (D’Agostini et al., 2005). Dimethylbenz[a]anthracene (DMBA), a potent polycyclic aromatic hydrocarbon, is repeatedly applied on mouse skin to induce skin lesions (Ishikawa et al., 2010). DMBA-diol-epoxide has been suggested as the ultimate carcinogen responsible for inducing chronic inflammation and causing oxidative damage to DNA by production of reactive oxygen species. This results in significant depletion of Langerhans cells and also local immunosuppression which results in the transformation of the erstwhile normal cells to tumor cells (Igawa et al., 2009; Ishikawa et al., 2010).

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Materials and methods

Materials

TAM citrate and saturated phospholipid (Phospholipon 90H) were the generous gift samples from M/s Biochem Pharmaceutical Industries, Mumbai, India and M/s Phospholipid GmbH, Naternannl, Germany, respectively. Triethanolamine (TEA), 7,12-dimethylen[a]anthracene (DMBA), 12-O-tetradecanoylphosphor-13-acetate (TPA), cholesterol and sorbitan monoooleate were procured from M/s Sigma Chemicals Co., St. Louis, MO, USA. Carbopol® 980 was obtained ex-gratis from M/s Lubrizol Co., Ohio, USA. All other chemicals and reagents used in the present study were of analytical grade. Distilled water was used in the study.

Preparation of topical TAM formulations

Drug-loaded liposomal formulations were prepared by thin-film hydration technique, as described previously (Bhatia et al., 2004). Briefly, a drug-loaded film, composed of phospholipid and sorbitan monooaleate (4:1 by weight ratio) in case of elastic liposomes, and phospholipid and cholesterol (2:1 by weight ratio) in case of conventional liposomes, was hydrated in water to obtain the corresponding liposomal suspensions. Carbopol® gel was prepared by dispersing it in warm de-mineralized water and stirring at 800 rpm (Remi Mechanical Stirrer, Mumbai, India) for 30 min. This Carbopol® dispersion was neutralized by the addition of TEA in the weight ratio of 1:1.5 (Carbopol®: TEA). Mixing was continued until a clear transparent gel was formed. Finally, the liposomal suspension was added to the clear gel to obtain a vesicular gel formulation for topical application. The drug solution was prepared by dissolving TAM in 5% ethanol (v/v).

The formulations employed in various studies were drug-loaded elastic liposomes in gel (TAM-EL), conventional liposomes in gel (TAM-CL) and hydro-ethanolic drug solution (TAM-SOL). All of the formulations contained 0.1% w/w of TAM.

Animals

Healthy female Swiss albino LACA mice with body weights ranging between 20 g and 25 g, and age between 4 and 6 weeks, were obtained from Central Animal House, Punjab University, Chandigarh, India. They were housed in polypropylene animal cages and acclimatized for a week with 12 h day-and-night cycle prior to the treatments at a relative humidity of 50–55% and temperature of 25–27°C. The mice were provided with a standard rodent diet. Drinking water was provided ad libitum. All the protocols were duly approved by the Punjab University Animal Ethics Committee and were performed with care.

Antitumor activity

Skin tumors were induced in mice according to the method already reported in the literature (Villasenor et al., 2002; Park et al., 2007). Depilatory was used to remove hair from the back of mice. The animals were divided into eight groups of 10 mice each (shown in Table 1) and administered respective treatments 2 days after removal of the hair.

The animals in Group 1 treated with acetone (100 μL) alone which served as the control. The frequency of topical acetone application was twice a week on the depilated back of each mouse for 20 weeks.
The animals of Group 2 were topically treated twice a week with DMBA (100 nmol/100 µL) on the depilated back of each mice for 2 weeks, followed by application by TPA (1.7 nmol/100 µL of acetone) per week for the next 18 weeks.

Pre-treatment groups (application of formulations immediately after the treatment, i.e. before the onset of tumors).

The animals of Groups 3 & 4 were treated with topical TAM-EL and TAM-CL formulations, respectively till 20 weeks. TAM topical formulations (500 mg; 2.69 mM conc. of TAM) were applied to the shaved area of mice skin with the help of index finger (hands were covered with surgical gloves). Following 1st week of exposure with TAM formulations, the animals were applied with DMBA and TPA similarly as in the case of Group 2.

The animals of Group 5 were treated with topical TAM-SOL (500 µL; 2.69 mM conc. of TAM) in the manner analogous to the case of Groups 3 & 4.

In post-treatment groups, the application of the formulations was initiated after 10th week, i.e. after the onset of tumors.

The above studies, as conducted for Groups 3-5, were repeated in case of post-treatment Groups 6-8, respectively. However, in this case, the formulations were applied after 10th week onwards, i.e. after the onset of tumors.

Evaluation

Animal bodyweight, incidence of papillomas on depilated skin and mortality were recorded at weekly intervals. Only those papillomas, which persisted for 2 weeks or more, were taken into consideration for the final evaluation. Comparative efficacy of the tested formulations was determined employing standard one-way ANOVA on Microsoft® Office Excel® version 2007 (Microsoft Corporation, USA). The value of \( p < 0.01 \) was considered to be statistically significant.

Histology

After completion of aforementioned study, the mice were sacrificed by cervical dislocation after administering inhalation anesthesia. Skin papillomas and skin tissues were removed immediately and were fixed in formalin, processed and embedded in paraffin. Sections (around 7 µm thick) were stained with hematoxylin and eosin (H&E), and observed under a high-power light microscope (Olympus) to carry out histological examination. The remaining animal carcasses were incinerated.

Results and discussion

Formulation details

The liposomal formulation, TAM-CL, possessed the mean vesicular size of 5.3 µm, drug entrapment of 57.5%, zeta-potential of 13.6 ± 1.2 mV and polydispersity index (PDI) of 0.441. On the other hand, the elastic liposomal formulation, TAM-EL, contained the vesicles with mean size of 0.125 µm, drug entrapment of 78.62%, zeta-potential of 15.4 ± 1.3 mV and PDI of 0.83. The values of drug permeation flux across LACA mice skin for TAM-CL and TAM-EL were found to be 59.87 pg cm\(^{-2}\)·h\(^{-1}\) and 46.05 pg cm\(^{-2}\)·hr\(^{-1}\) respectively. The formulation-specific details of TAM-CL have already been published by us elsewhere (Bhatia et al., 2004).

Body weight

The body weight of mice recorded during the entire 20-week duration of the study protocols is shown in Table 1. Significant reduction in the bodyweight of mice was observed in DMBA/TPA treated group (Group 2) as compared to the mice treated with TAM formulations at the end of study. However, there were no significant changes in the body weights of Groups 6-8 vis-a-vis Group 2 at the end of 10 weeks.

Animal mortality

Survival rate of the mice decreased significantly in DMBA/TPA treated mice as compared to the control group. Application of TAM vesicular formulations lead to higher survival rate (i.e. 80%), whereas TAM solution treated group showed lower survival rate (i.e. 50%). Similar results were obtained from the post-treatment groups too (Figure 1).

Incidence of lesions on mice skin

The mice skin lesions were counted in all the treatment groups and the percentage occurrence of lesions was
Figure 1. Effect of various treatments on the percent survival rate of mice.

Figure 2. Effect of different pre-treatments (a) and post-treatments (b) on the percent incidence of tumors in mice. Controls (i.e. Group 1) treated with acetone did not show any tumor formation. Data are expressed as means ± SD.

Calculated every week (Figure 2a and 2b). The onset of lesions was generally observed at the 8th week. A gradual rise in the incidence of tumors was noticeable from 6.8% at the 8th week to 100% at the 16th week in DMBA/TPA treated Group 2, as shown in Figure 2a. The incidence of lesions was delayed by 3 weeks in the TAM-EL treated Group 3 and by 2 weeks in TAM-CL treated Group 4. On the other hand, TAM-SOL treated Group 5 showed a delay of one week in the onset of papillomas in mice. Overall, significant decrease in the incidence of tumors was seen in Group 3, i.e. 51.35% and in Group 4, i.e. 28.41% on the completion of studies at the 20th week (p < 0.01). Reduction in the incidence of tumors for Group 5, however, was found to be 17.58% on the completion of study.

In post-treatment groups (Figure 2b), the application of different TAM formulations to the DMBA/TPA treated mice was started at 10th week onwards. At the end of 20th week, the pattern of decrease in the incidence of lesions for Groups 6, 7 and 8 (i.e. 41.68%, 22.35% and 10.16%, respectively) was comparable to that of the pre-treatment groups. There was no incidence of tumor found in the unexposed animals (i.e. acetone-treated) of Group 1 (Figure 3).

Histopathological studies

Histological changes in the mice skin and tumors were studied with H&E staining of the paraffin sections for all the groups. Skin sections of the control Group 1 showed normal histological features. The uniformly arranged epidermal and dermal layers with normal layer of keratin over the epidermis were observed (Figure 4A). In histopathological sections of DMBA/TPA treated mice tumors (Group 2), dyskeratosis of the epidermis and abnormally thickened epidermis, with deposition of numerous keratinocyte pearls (KP) was observed in dermis and epidermis. Islands of dysplastic squamous epithelial cells lying in the dermis distinctly indicated an invasive form of frank squamous cell carcinoma. Extensive hyperkeratosis, i.e. thickening of keratinized layer over the epidermis was also observed (Figure 4B and 4C). Furthermore, intraepithelial atypia of epidermis with the highest histological disorganization, necrotic keratinocytes and cytological atypia were observed in the skin sections of Group 2. Tumors of animals belonging to Group 3 (i.e. DMBA/TPA + TAM-EL-treated animals) displayed intact basement membrane with hyperplasia of the overlying epithelium (Figure 4D). The number as well as size of keratinocyte pearls was found to be the lowest as compared to other groups. The extent of developed lesions, keratinocyte pearls and abnormalities in skin cellular structures were found to observe the following descending order for different treatments:

Group 2 > Group 5 > Group 4 > Group 3 > Group 1

Analogous results were obtained for post-treatment groups (Groups 6-8), where TAM-EL showed significantly higher efficacy vis-à-vis other treatments. On application of one-way ANOVA for comparing the therapeutic efficacy of three tested formulations of TAM, the values of F-ratio were found to be 964.82, whereas the F-ratio for preventive efficacy of these formulations was found to be 977.04, which are markedly higher than the tabulated critical value of 5.53 (F0001 at d.f. 2,27). This indicates a highly statistically significant difference amongst the anticancer activity of the three formulations investigated during the studies (p < 0.001). The anticancer efficacy of the different treatments, as
determined from all the studied parameters, observed the following descending order:

TAM-EL > TAM-CL > TAM-SOL

Chemoprevention study shows a significant increase of 37.5% in tumor latency by application of TAM-EL in Swiss albino mice initiated by DMBA and promoted by TPA. This may be attributed to the delay in the promotion phase of carcinogenesis.

The enhanced efficacy of the vesicle-entrapped drug may be ascribed to its favorable interaction with the skin after topical application. The physicochemical characteristics of the vesicles, by virtue of the phospholipids may have allowed them to integrate intimately with the lipidic molecules of skin (Betz et al., 2001; El Maghraby et al., 2004). Also, the phospholipid molecules around the aqueous chambers containing drug allow it to behave as a water-bound lipoidal amphiphilic system. The latter facilitates drug penetration and permeation across the hard epidermal transportation barrier (Trotta et al., 2004; Giannantoni et al., 2006; Manconi et al., 2008).

However, as the skin milieu is lipid-enriched, it is also expected to retain the drug to a greater extent in vesicular form than in the un-entrapped system, e.g., drug in solution. The lower efficacy from TAM-SOL may also be ascribed to its faster elimination into microvasculature of the skin. On the other hand, the lipid-rich domains of vesicles could have helped to produce the depot effect for drug molecules within skin, thus leading to enhanced pharmacodynamic action vis-à-vis drug solution (Trotta et al., 2004; Sinico & Fadda, 2009; Benson, 2010).

The efficacy of TAM-EL was also found be better than that of the TAM-CL. This may be attributed to the elastic nature of vesicular bilayers facilitating penetration of the encapsulated drug in the skin and the enhanced efficacy of TAM. Whereas conventional
Figure 4. H&E stained sections of skin tumors of control and treated mice. (A) H&E stained section of control skin with normal cellular structures. (B) Characteristic squamous epithelial keratinocyte pearls and necrotic keratinocytes in tumors from DMBA/TPA-treated animals. (C) Squamous epithelial cells lying in the dermis clearly identifying the tumors as invasive in the DMBA/TPA-treated animals. (D) Intact basal layer of tumors and hyperplastic lesions indicating the hyperplasia of the overlying epidermis in tumors portraying relatively less damaged cellular structures in the skin of DMBA/TPA + TAM-EL treated animals. [Arrows show keratinocyte pearls].

liposomes cannot penetrate that much efficiently and tend to diffuse in upper skin layers leading to release of entrapped drug free in skin tissue (Cevc et al., 1998; Bouwstra et al., 2003; Choi & Maibach, 2005; 38-41; Mura et al., 2010). This may be responsible for lower efficacy of TAM-CL vis-à-vis TAM-EL.

Conclusion
In clinical practice, the systemic side effects of oral TAM largely preclude the use of this drug. TAM administered topically, if demonstrating effective, safe and acceptable profile, could be a promising agent for skin carcinoma. Accordingly, it can be concluded that the novel phospholipid-based vesicular systems hold appreciable potential in ameliorating efficacy and safety of TAM using topical route of administration.

Acknowledgements
The authors are thankful to Biochem Pharmaceutical Industries, (India), Lubrizol Co., (USA), and Phospholipid GmbH, (Germany), for generously providing the gift samples of TAM, Carbopol® and phospholipid, respectively.

Declaration of interest
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References
Synthesis, characterization and in vitro studies of pegylated melphalan conjugates

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¹Rungta College of Pharmaceutical Sciences and Research, Bhilai, (C.G), India, ²Drug Delivery Research Group, University Institute of Pharmaceutical Sciences, UGC-CAS, Panjab University, Chandigarh, India, and ³Institute of Chemical and Engineering Sciences, Singapore

Abstract
Melphalan, a drug used for the treatment of breast, ovaries and a certain type of cancer in the bone marrow, was conjugated to linear methoxy poly(ethylene glycol) (M-PEG) of 2000 and 5000 Da. An ester linkage between polymer and drug was used in the coupling to yield a polymeric prodrug. Purified esters were characterized by Maldi-Tof and IR spectroscopy methods. The modification allowed overcoming the known melphalan aqueous solubility problem (0.1 µg/ml) leading us to obtain a polymer-drug bioconjugate more suitable for oral and parental administration. It was found that molecular weight of M-PEG is critical for the conjugates stability, aqueous solubility (80 times and 123 times higher aqueous solubility for M-PEG 2000 and M-PEG 5000, respectively), and hemolytic activity. The melphalan caused 100% hemolysis above the concentration 5.5 µg/ml in 1 h, whereas conjugate of M-PEG 2000 and M-PEG 5000 shows 81.3 ± 0.5% and 48.8 ± 1.5% hemolysis respectively at 32 µg/ml after 1 h. Further in vitro anticancer activity of melphalan and its conjugates was performed with breast cancer MCF-7 cell lines. It shows that LD50 concentration was higher 1.14 and 2 µm for M-PEG 2000 and M-PEG 5000, respectively in comparison to pure melphalan (0.74 µm). Above studies revealed improved pharmacokinetics properties upon conjugation.

Keywords: Methoxy-polyethylene glycol, anti-cancer, MCF-7 cell line, aqueous solubility, hemolytic activity

Introduction
Melphalan (p-[bis(chloro-2-ethyl)amino]-L-phenylalanine), an alkylating agent, was introduced in the late 1950s and has since been established as an agent with a wide spectrum of antitumor activity. It is extensively used in the treatment of multiple myeloma, ovarian cancer, breast cancer, neuroblastoma, regionally advanced malignant melanoma and localized soft tissue carcinoma. The alkylating activity of melphalan is due to formation of highly reactive carbonium ion intermediates, which transfer alkyl groups to cellular macromolecules by forming covalent bonds. The position ‘7’ of guanine residues in DNA is especially susceptible, but other molecular sites are also involved. This results in cross-linking and abnormally high cell killing. Melphalan has issues with solubility as it is practically insoluble in water (0.1 µg/ml at 25°C).

A major problem with oral administration of melphalan is its variable bioavailability. The problem of melphalan bioavailability is further complicated by its rapid hydrolysis (degradation) at physiological pH and again rapid clearance from the systemic circulation due short circulatory half-life (90 min). In our research, a polymer conjugation approach based on PEG as carrier polymer was adopted to overcome the solubility problem that prevents an easy formulation of oral and parental administration and to modify the pharmacokinetics of the drug. The attachment of antitumor drugs to synthetic polymers is a promising strategy of modifying their biodistribution, of reducing drug toxicity and thus improving the therapeutic efficacy of anticancer agents. The phenomenon of modification of carrier system, herbal bioactive and drug conjugates with polyethylene glycol (PEG) is known as PEGylation.
Polyethylene glycols are non-ionic, water-soluble synthetic polymers, which are potential drug carriers due to their synthetic diversity and recognized biocompatibility. It has pronounced effects on biodistribution and pharmacokinetic by increasing blood circulation half-life, reducing the tissue distribution (RES and macrophageal uptake). PEG has unique ability to be soluble in both aqueous solutions and organic solvents (amphiphilic nature) make it suitable for end group derivatisation and chemical conjugation of bioactives under mild physiological conditions. PEG has the lowest level of protein and cellular absorption of any known polymer. It imparts increased aqueous solubility to hydrophobic drugs (e.g. Podophyllotoxin, Doxorubicin etc.). PEG offers unique advantage of being a telechelic or semitelichelic polymer and thus can be loaded quite predictably with drugs. PEG has extremely low level of immunogenicity and antigenicity. US FDA approved PEG for human intravenous, oral and dermal applications. The rationale of this approach is based on the assumption that properties of the polymeric carrier determine the above mentioned characteristics.

The chemistry of PEGylation is two step process where primary steps deals with derivatization and activation of PEG with suitable functional groups and the second step deals with the subsequent conjugation of these activated PEGs with Bioactives.

Based on these general properties we prepared PEG-melphalan conjugates using polymer with different molecular weight (MW 2000 and 5000). The obtained conjugates showed much increased solubility, slow hydrolysis in the serum, and decreased hemolytic activity of the conjugate in comparison to the pure melphalan. Furthermore, in vitro anticancer activity of melphalan and its conjugates was performed with breast cancer MCF-7 cell lines which show the significant cytotoxic activity. Stability studies were performed with both conjugates shows better stability characteristics.

### Experimental details

**Material**

Melphalan, N-hydroxysuccinimide, dimethylamino-pyridine (DMAP) Methoxy poly ethylene glycol (mPEG MW 2000) and methoxy poly ethylene glycol (mPEG MW 5000) were purchased from sigma aldrich USA. All the chemicals and reagents used in the study were of analytical grade.

**Methods**

**Synthesis of melphalan-mPEG (mw 5000) conjugate**

An excess quantity about 1.0 g (0.2 mmol) of mPEG-SPA (5000) and 0.06 g of DMAP (0.5 mmol) were dissolve in 10 ml volume of methanol. To the above solution 30 mg (0.1 mmol) of melphalan previously dissolve in 10 ml methanol was added drop wise. The reaction mixture was then transferred to glass stopper flat bottom flask and kept on the magnetic stirrer the flask covered by carbon paper and the reaction was carried out in the dark with continuous stirring for 24 h. The completion of reaction was monitored by TLC (Thin layer chromatography) in n-butanol: acetic acid: water (4:1:1). The same procedure was adapted for the mPEG MW 2000. The purification was carried out on preparative TLC plates coated with silica gel 60 F as stationary phase (600 μm thickness). A mixture of chloroform, methanol at ratio of 85:15 v/v, respectively was used as a mobile phase to separate MPPEG from the reaction mixture. Before separation the reaction medium was washed using 100 ml of distilled water. The MPPEG were extracted by 20 ml of dichloro methane (DCM). The organic solvent was dried using sodium sulfate, filtered and evaporated under vacuum. The residue was redissolved in 2 ml of chloroform and loaded on TLC plate. The major product was separated and extracted from silica by 50:50 (v/v) of methanol-DCM. The solvent was dried and the precipitated product was dissolved in a small volume of DCM and reprecipitated using DCM-Hexane.

**Chromatographic analysis**

The completion of reaction was assessed by TLC method. The TLC analysis of the conjugates was performed using silica-coated plates 60 F254 (Merck Darmstadt, FRG). The solvent system of composition n-butanol:acetic acid:water in 4:1:1 ratio was used to develop the plates. The solvent system was poured into the TLC chamber. A filter paper sheet was placed into it to provide rapid saturation so as to prevent edging effect. Chamber was sealed by placing a glass plate at the mouth of chamber with paraffin wax. The samples of the melphalan and reaction mixture were applied on the plate with the help of micropipette. The developed plates were visualized for spots under UV lamp. The calculated Rf values are given in Table 1. When there was no free melphalan left in the reaction mixture, the reaction was stopped and processed further. The spots off sample solutions were applied with the help of thin capillaries on the plates, at a distance of about 1.5 cm from the bottom and were allowed to dry in air. The distance between two spots was kept at least 10 mm.

**Characterization of melphalan-mPEG (mw 5000) conjugate**

Infrared spectrum of pure melphalan, PEG derivatives, melphalan-PEG conjugates were obtained using a Perkin Elmer (Japan) IR spectrophotometer (Perkin Elmer (Japan)) (Figures 1 and 2). Data interpretation for Mpeg-5000 and Mpeg-2000 conjugates shows better stability characteristics.

### Table 1. TLC analysis of conjugates (Rf values).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Melphalan and Conjugates</th>
<th>Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Melphalan</td>
<td>0.81</td>
</tr>
<tr>
<td>2.</td>
<td>Mpeg-5000</td>
<td>0.31</td>
</tr>
<tr>
<td>3.</td>
<td>Mpeg-2000</td>
<td>0.43</td>
</tr>
</tbody>
</table>
Figure 1. Schematic presentation of melphalan-mPEG conjugates.

Table 2. Important band frequencies in IR spectrum of MpPEG-5000.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Band frequency (cm$^{-1}$)</th>
<th>Named groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3432.9</td>
<td>N-H Stretching of 2° Amide</td>
</tr>
<tr>
<td>2</td>
<td>2888.9</td>
<td>C-H Stretching</td>
</tr>
<tr>
<td>3</td>
<td>1733.8</td>
<td>-C=O Stretching Vibration due to 2° Amide</td>
</tr>
<tr>
<td>4</td>
<td>1600.3</td>
<td>N-H Bending Vibration (II Amide)</td>
</tr>
<tr>
<td>5</td>
<td>1469.4</td>
<td>C-N Stretching</td>
</tr>
<tr>
<td>6</td>
<td>1242.0</td>
<td>Weak band due to interaction b/w N-H bending and C-N Stretching</td>
</tr>
<tr>
<td>7</td>
<td>1113.4</td>
<td>C-O-C Stretching</td>
</tr>
<tr>
<td>8</td>
<td>770.0</td>
<td>C-Cl Stretching</td>
</tr>
</tbody>
</table>

Table 3. Important band frequencies in IR spectrum of MpPEG-2000.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Band frequency obtained (cm$^{-1}$)</th>
<th>Named groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3432.9</td>
<td>N-H Stretching of 2° Amide</td>
</tr>
<tr>
<td>2</td>
<td>2818.9</td>
<td>C-H Stretching</td>
</tr>
<tr>
<td>3</td>
<td>1596.9</td>
<td>N-H Bending vibration (II Amide)</td>
</tr>
<tr>
<td>4</td>
<td>1464.6</td>
<td>C-N Stretching</td>
</tr>
<tr>
<td>5</td>
<td>1112.3</td>
<td>Symmetric C-O-C Stretching</td>
</tr>
<tr>
<td>6</td>
<td>768.3</td>
<td>C-Cl Stretching</td>
</tr>
</tbody>
</table>

Spec, 2E)31 using a nitrogen 337 nm laser. The MALDI-TOF spectra of PEG 2000 derivative, PEG 5000 derivative and their respective conjugates that are MpPEG5000 and MpPEG2000 were shown in Figure 3.

Drug content

The melphalan content of the conjugates was determined by using UV/Vis spectrophotometer (CINTRA GBC 10, Australia) at $\lambda_{max}$ 259 nm. Weighed about 5.0 mg of each conjugate were transferred to separate volumetric flasks. The volumes were made up to 10 ml in each case with methanol and above solutions were incubated for an hour. The absorbance of above solutions was recorded after appropriate dilutions using Cintra.
In vitro hydrolysis of conjugates in serum

The in vitro hydrolysis of the conjugates was determined by reverse phase HPLC in serum.34 HPLC (Shimadzu LC-10; Japan) with UV detector (259 nm), flow rate 1.0 ml/min and the syringe volume 20 µl was used, with column Bondapak C18 (250 × 4.6 mm, 10 µm), was equilibrated with an eluent mixture consisting of ethanol:water:acetic acid (4:4:1). A known quantity of the conjugates equivalent to 10 µg of melphalan content was incubated in 2 ml human serum in a micro centrifuge tube. Samples of 200 µl were withdrawn from the tube at the time intervals of 1, 2, 3, 4, 5 and 6 h and equal amount of acetonitrile was added to it than centrifuge at 10,000 rpm for 10 min using micro centrifuge. After centrifugation 20 µl supernatant was used for further HPLC analysis. The degradation was followed in HPLC (Shimadzu LC-10, Japan) by the decrease in the conjugate peak and the corresponding increase of melphalan. The observations of above analysis are presented in Table 6.

In vitro hemolysis of blood cells

The hemolysis was estimated by hemoglobin content in the supernatants of centrifuged RBC suspension spectrophotometrically at λmax 576 nm. The in vitro blood cell toxicity was used as a parameter to estimate the toxicity of melphalan-mPEG conjugates.32 The percentage hemolysis was calculated from the following equation:

\[
\% \text{ hemolysis} = \left( \frac{AB - AB_0}{AB_m - AB_0} \right) \times 100
\]

where AB is the absorbance of the test sample, AB0 is the absorbance of the blank, and ABm is the absorbance of the maximum absorbance of the test sample.

Table 5. Aqueous solubility of conjugates.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Preparation</th>
<th>Solubility of conjugate (mg/ml)</th>
<th>Corresponding melphalan solubility (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MpPEG-2000</td>
<td>0.0800 ± 0.06</td>
<td>7.2 ± 0.12</td>
</tr>
<tr>
<td>2.</td>
<td>MpPEG-5000</td>
<td>0.3100 ± 0.02</td>
<td>11.07 ± 0.6</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Time (h)</th>
<th>MpPEG-2000</th>
<th>MpPEG-5000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.98 ± 0.21</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>1.63 ± 1.2</td>
<td>0.63 ± 0.28</td>
</tr>
<tr>
<td>3</td>
<td>3.08 ± 0.52</td>
<td>1.21 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>5.26 ± 0.48</td>
<td>2.97 ± 0.62</td>
</tr>
<tr>
<td>5</td>
<td>7.83 ± 1.22</td>
<td>4.19 ± 0.86</td>
</tr>
<tr>
<td>6</td>
<td>10.11 ± 0.68</td>
<td>5.21 ± 1.2</td>
</tr>
</tbody>
</table>

Aqueous solubility of conjugates

Dissolution method was used to determine the aqueous solubility of purified conjugates.30,38 A known quantity of each conjugate was transferred in separate test tubes and 5 ml of distilled water was added to each tube. Contents of each tube were gently shaken and additional quantities of conjugates were added till the appearance of visible insoluble particles. The above mixtures were vortexed for 5 min and left aside for an hour. The contents of each tube were filtered through 0.45 µm filter paper and lyophilized to dryness using Heto Dry Winner and weighed. The observations of above experiment are presented in Table 5.

Figure 3. Comparative MALDI-TOF MS Spectrum of (a) PEG 5000 derivative (b) MpPEG 5000 conjugate (c) PEG 2000 derivative (d) MpPEG 2000 conjugate.

UV/Vis spectrophotometer at λmax 259 nm against methanol as blank. Results of above experiment are mentioned in Table 4.
Human venous blood was collected, using syringe pre-filled with acid citrate dextrose buffer. The RBCs were separated from the whole blood by centrifugation at 2000rpm for 5 min using refrigerated centrifuge. The supernatants and buffy coats were removed and discarded. The packed cell volume (PCV) was washed with phosphate buffer saline PBS (pH 7.4) and centrifuged again at 2000rpm for 2 min. The 3 ml of PCV was then diluted to 100 ml with PBS (pH 7.4) to get 3% RBC suspension. The above RBC suspension gave an absorbance of 0.56 at 576 nm on incubation with 5 µg of melphalan solution (100% hemolysis). This suspension was used to study the hemolytic activity of conjugates.

Stock solution of concentration, equivalent to 100 µg/ml of melphalan content were prepared for each conjugates in PBS (pH 7.4). While the stock solution of pure drug was prepared by dissolving 10 mg of the melphalan in minimum quantity of methanol and then diluting it with PBS (pH 7.4) to 100 ml.

Hemolytic activity of the above stock solutions was determined by following procedure: The 1.5 ml of 3% RBC suspension was placed in a series of micro centrifuge tubes and to this the concentration of 2 µg/ml to 32 µg/ml were prepared for each conjugate. The final volume in each case was adjusted to 2 ml with PBS (pH 7.4). The tubes were gently closed and occasionally shaken during incubation period of 1 h at room temperature. The RBC suspension incubated with PBS (pH 7.4) and 5 µg/ml of melphalan was considered as control of zero and 100% hemolysis, respectively. After 1 h of incubation, hemolysis was stopped by dipping the tubes in ice-cooled water. The non-lysed RBCs were separated out by centrifugation at 5000 rpm for 1 min using Tarson microcentrifuge.

The supernatants were analyzed for hemoglobin content, spectrophotometrically at 576 nm against PBS (pH 7.4) as blank using Cintra UV/Vis spectrophotometer. The percent hemolysis was calculated from the equation mentioned above. The observations for experiments carried out separately with pure melphalan, MpPEG-2000, and MPPPEG-5000 are summarized in Tables 7-9 and graphically represented in Figures 4-6, respectively. The purpose of this study was to assess the hemolytic activity of the conjugates over different time periods.

**In vitro anticancer activity**

*In vitro* anticancer activity of melphalan and its conjugates was performed with breast cancer MCF-7 cell lines. To distinguish between viable and non-viable cells on the hemocytometer (Sahli, India), trypan blue staining was used. Live cells can exclude the trypan blue stain, leaving them with a normal appearance under the microscope. Dead cells, however take up the stain making them appear blue. An equal volume of cells and stain (0.4%) were mixed and applied to the bright-line hemocytometer.

where, \( \text{ABS} = \text{Absorbance for the sample; AB}_0 = \text{Absorbance without melphalan-mPEG conjugates; AB}_{100} = \text{Absorbance in presence of 5 µg/ml of melphalan (100% hemolysis).} \)

<table>
<thead>
<tr>
<th>Table 7. Hemolytic activity of pure melphalan after 1 h.</th>
<th>Table 8. Hemolytic activity of MpPEG-2000 after 1 h.</th>
<th>Table 9. Hemolytic activity of MpPEG-5000 after 1 h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalent melphalan (µg/ml)</td>
<td>% Hemolysis (n = 3)</td>
<td>Equivalent melphalan (µg/ml)</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>1.0</td>
<td>7.2 ± 0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>1.5</td>
<td>24.0 ± 1.1</td>
<td>4.0</td>
</tr>
<tr>
<td>2.0</td>
<td>36.7 ± 2.8</td>
<td>4.5</td>
</tr>
<tr>
<td>2.5</td>
<td>58.9 ± 6.1</td>
<td>5.0</td>
</tr>
<tr>
<td>(-) Denotes no hemolysis found; C.H. = Complete hemolysis.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equivalent melphalan (µg/ml)</th>
<th>% Hemolysis (n = 3)</th>
<th>Equivalent melphalan (µg/ml)</th>
<th>% Hemolysis (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>18</td>
<td>18.4 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>20</td>
<td>23.9 ± 4.1</td>
</tr>
<tr>
<td>6</td>
<td>1.29 ± 0.9</td>
<td>22</td>
<td>29.4 ± 12.2</td>
</tr>
<tr>
<td>8</td>
<td>3.09 ± 0.2</td>
<td>24</td>
<td>39.4 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>4.94 ± 0.5</td>
<td>26</td>
<td>50.1 ± 10.5</td>
</tr>
<tr>
<td>12</td>
<td>7.14 ± 4.9</td>
<td>28</td>
<td>67.4 ± 11.4</td>
</tr>
<tr>
<td>14</td>
<td>10.43 ± 2.3</td>
<td>30</td>
<td>72.9 ± 1.2</td>
</tr>
<tr>
<td>16</td>
<td>14.85 ± 1.4</td>
<td>32</td>
<td>81.3 ± 0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equivalent melphalan (µg/ml)</th>
<th>% Hemolysis (n = 3)</th>
<th>Equivalent melphalan (µg/ml)</th>
<th>% Hemolysis (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>18</td>
<td>18.2 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>20</td>
<td>16.5 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>22</td>
<td>22.8 ± 1.7</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>24</td>
<td>28.6 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>26</td>
<td>34.8 ± 2.6</td>
</tr>
<tr>
<td>12</td>
<td>1.6 ± 0.4</td>
<td>28</td>
<td>38.2 ± 1.7</td>
</tr>
<tr>
<td>14</td>
<td>3.4 ± 1.2</td>
<td>30</td>
<td>43.2 ± 0.5</td>
</tr>
<tr>
<td>16</td>
<td>6.2 ± 1.8</td>
<td>32</td>
<td>49.8 ± 1.5</td>
</tr>
</tbody>
</table>

*(-) Denotes no hemolysis found. |

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Stability studies of conjugates

The stability of pure drug and its conjugates were determined by UV absorption at λmax 259 nm at different time intervals using samples freshly prepared at concentration of 2 mg/ml in methanol. The conjugates were kept in dry form at 4 ± 1°C and room temperature 25 ± 1°C in light protected amber coloured screw capped bottles for 4 months. During this period, samples were taken at time 0, 1, 2, 3 and 4 months.

The percent relative absorbance was calculated from the following formula:

\[
\text{% Relative absorbance} = \frac{\text{Absorbance at time } (t) - \text{Absorbance at time } 0}{\text{Absorbance at time } 0} \times 100
\]

Result and discussions

Synthesis of PEG conjugates of melphalan

The primary-NH2 group of melphalan provides an ideal site for attachment of mPEG-succinimidyl propionate (mPEG-SPA). The reaction of mPEG-SPA with primary amino group of melphalan is nucleophilic in nature catalysed by DMAP.35 During the course of reaction, the mPEG-SPA gets hydrolyzed to mPEG and N-hydroxysuccinimide. The reaction of melphalan and mPEG produces a relatively stable amide bond.

The mPEG conjugates of melphalan were synthesized by coupling the drug to mPEG-SPA (MW 5000 and 2000) in methanol in presence of DMAP. The reaction was carried out taking stoichiometric quantities of the reactants. The course of the reaction was monitored using TLC. 

The precipitation of the conjugates was carried out with chilled diethyl ether. 

The mPEG conjugate of melphalan with mPEG-SPA (MW 5000) gave a yield of 782 mg (~76%); while mPEG conjugate of melphalan with mPEG-SPA (MW 2000) gave a yield of 810 mg (~78.64%). The conjugates were obtained as light yellow colored powdered mass.

Cytotoxicity and LD50 with reference to drug concentration were recorded in Tables 10 and 11.

pellet. Percent cytotoxicity (Figure 7) was calculated by following formula:

\[
\text{Percent cytotoxicity} = \frac{\text{Blue cell count}}{\text{Total cell count}} \times 100
\]
TLC analysis
The TLC method was used to assess the completion of reaction. Both of the conjugates gave one spot each as visualized by UV light. This indicates that free drug is absent in the reaction mixture (Table 1). When there was no free melphalan left in the reaction mixture, the reaction was stopped and processed further. As an excess quantity of mPEG-SPA was used the product may contain some unreacted mPEG, which is harmless and it’s very difficult to separate the unreacted mPEG from the product because of the small difference in the molecular weight of the product and mPEG. After 1h the free melphalan spot in TLC plate was observed and hence reaction was extended till its completion (Figure 8).

FTIR spectrum
All the samples were crushed and then mixed with potassium bromide (1:100 ratios by weight) and pressed at 15,000 psig in a die to form pellets. Nitrogen gas was used carefully to purge the detector to increase the signal level and reduce moisture. The spectra were collected in the 400–4000 cm⁻¹ region with 8 cm⁻¹ resolution, 60 scans and beam spot size of 10–100 μm. The FTIR imaging in the present investigation was carried out using a Perkin Elmer (Japan) spectrophotometer (CDRI Lucknow, India). The FTIR spectrum of MpPEG5000 and MpPEG2000 (Figures 1 and 2) revealed the presence of characteristic peaks of both melphalan and mPEG. It is observed that doublet peak of primary-NH₂ stretching at 3400 cm⁻¹ replaced by singlet in the conjugates (N–H amide, stretching), while the new peaks at 1600.3 cm⁻¹ (N–H amide bending) and at 1733.8 cm⁻¹ (C=O stretching vibration) were appeared (Figures 1 and 2). All these peaks in FTIR spectrum of the conjugates provided a significant evidence of amide bond formation.

MALDI-TOF MS spectrum
Mass spectra were acquired using a Micromass Tof Spec 2E MALDI TOF MS JEOL Accutof-DARTMS mass spectrometer using a nitrogen laser. The matrix was a saturated solution of R-cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Sample and matrix were mixed 1:1, and 1 μL was spotted onto a 100-well sample plate. All spectra were acquired in positive mode over the range 600-2500Da under reflectron conditions (20 kV accelerating voltage, 350 ns extraction delay time) and 2-100kDa under linear conditions (25 kV accelerating voltage, 750 ns extraction delay time). Sodium adduct was extensively prepared for physical mixture and pure polymers. It shows high intensity at 5327 (Figure 3) that is the sum of the molecular weight of polymer mPEG (i.e. 5000) and drug melphalan (i.e. 304) it revealed the formation of conjugate and same case was reported with the MpPEG-2000 conjugate (Figure 3). It also confirms the mono substitution of the mPEG with drug.

Drug content
The melphalan content of MpPEG-5000 and MpPEG-2000 was found to be 0.2296 mg and 0.5288 mg per 5 mg of respective conjugate weight as determined by UV/Vis spectrophotometer (CINTRA GBC 10, Australia) at λmax 259 nm (Table 4).

Aqueous solubility of conjugates
Aqueous solubility of the conjugates was determined by dissolution method. The observed solubility of the MpPEG-2000 was found to be 0.0680 ± 0.06 mg/ml, which is equal to 7.2 ± 0.12 μg/ml in terms of melphalan content of the MpPEG-2000. The solubility of the MpPEG-5000 was found to be 0.2410 ± 0.02 mg/ml, which is equal to 11.07 ± 0.8 μg/ml in terms of melphalan content of MpPEG-5000 (Table 5). The reported aqueous solubility of melphalan is 0.1 μg/ml but we found it about 0.09 μg/ml. Thus based on this we can conclude that aqueous solubility of melphalan as MpPEG-2000 was increased approximately by 80 times, on the other hand in case of MpPEG-5000 it was increased approximately by 123 times. The reason behind enhanced solubility of the conjugated melphalan can be explained on the basis of PEG chain attached to the drug.36-39 The aqueous solubility of melphalan in MpPEG-5000 was more compared to that
in MpPEG-2000 this might be due the large PEG chain present in the conjugate.

In vitro hydrolysis of conjugates in serum
The percent cumulative hydrolysis in case of MpPEG-2000 was found to be 0.98 ± 0.21, 1.63 ± 1.2, 3.08 ± 0.52, 5.26 ± 0.48, 7.83 ± 1.22, 10.11 ± 0.68 after time period of 1, 2, 3, 4, 5, and 6 h, respectively, whereas with MpPEG-5000 only 5.21 ± 1.2% cumulative hydrolysis was observed after 6 h (Table 6). The hydrolysis rates of the conjugates were studied in vitro in serum. The degradation was followed in HPLC by the decrease in the conjugate peak and the corresponding increase of melphalan. The lower percent cumulative hydrolysis observed with MpPEG-5000 this may be interpreted as a protecting action of the PEG molecule on the ester linkage that may be due to the increased steric hindrance of the polymer. The amide bond of MpPEG-5000 might have less exposed to hydrolysis media as compare to MpPEG-2000 due to shielding effect of comparatively large PEG chain. Thus, MpPEG-5000 may provide the sustained drug release.46

Hemolytic activity of conjugates
The hemolytic activity of melphalan as well as conjugates was determined by estimating the hemoglobin content in the supernatants of centrifuged RBC suspensions. The hemolytic activity of the melphalan was observed at the concentration range of 0.5-5 μg/ml in 1 h. The melphalan caused 100% hemolysis above the concentration 3.5 μg/ml in 1 h (Table 7).

But with MpPEG-2000 the hemolytic activity was started at concentration of 6 μg/ml of melphalan content (Table 8). When the concentration of the MpPEG-2000 further increased, the % hemolysis was found to be increase in the same manner even at the concentration of 32 μg/ml 81.3 ± 0.5% hemolysis observed at 1 h. In case of MpPEG-5000 the hemolysis started at concentration of 12 μg/ml and at concentration of 32 μg/ml only 40.6 ± 1.5% hemolysis was observed at 1 h (Table 9). The lower hemolytic activity as observed with MpPEG-5000 and MpPEG-2000 might be based on the fact that properties of the macromolecular conjugate dominated by the properties of the polymeric carrier used which is PEG.41 Further, the length of the PEG chain has dramatic influence on the extent of melphalan induced hemolysis. The performance of MpPEG-5000 in retarding hemolysis found to be better than MpPEG-2000 even at higher concentrations.

Cytotoxic activity of conjugates

In the cytotoxic studies MCF-7 Cell Lines were used42 to compare the effectiveness of prepared conjugates the LD50 parameter was used that is the concentration required to kill the 50% of the cells in cell culture, the LD50 was estimated from the curve. Table 11 shows that LD50 concentration was higher in case of both of the Pegilated melphalan conjugates than the free melphalan because the drug content (Table 4) table shows the amount of melphalan present in conjugates (Mp-PEG 2000 and Mp-PEG 5000 are 0.5288/5 mg and 0.2296/5 mg, respectively). In the cytotoxic activity (Table 10) we have considered the amount of conjugates rather the equivalent concentration of melphalan and therefore the conjugates due to less amount of drug content showed low cytotoxic activity as compared to the pure drug.

Both melphalan and MpPEG-2000 showed about 100% activity at 30 and 50 μM concentration, respectively while in case of MpPEG-5000 it was about 83.42% cytotoxicity at concentration 150 μM. It might be due to the blocking of free amino group (-NH2-) present in melphalan which is used as a site for pegylation as reported by the Bergel and stock, 1960. In their studies they found that conjugation with free amino group of melphalan decreases its biological activity and at the same time retains its therapeutic index.

Thus, we can expect that hydrolytic cleavage of the amide bond of conjugates occur before the drug exerts a significant cytotoxic activity. Hydrolysis studies shows MpPEG-5000 are more stable than the MpPEG-2000 towards hydrolysis this might be the reason behind greater LD50 (Table 11) of MpPEG-5000 as it releases drug slowly and gives sustained effect.

Stability studies of conjugates
The chemical stability of the pure drug and conjugates were determined by measuring UV absorption at λmax 259 nm using samples freshly prepared at concentration of 2 mg/ml in methanol. On storage in dry form, no major change in the absorbance was observed for neither MpPEG-2000 nor MpPEG-5000 kept at 25°C or at 4°C throughout the period of 4 months. Both MpPEG-5000 and MpPEG-2000 lose only about 2% of their absorbance on storage in dry form. These data indicate that the conjugates remain stable, when stored.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Log % Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM</td>
<td>0</td>
</tr>
<tr>
<td>10 μM</td>
<td>1</td>
</tr>
<tr>
<td>20 μM</td>
<td>1.301</td>
</tr>
<tr>
<td>30 μM</td>
<td>1.477</td>
</tr>
<tr>
<td>40 μM</td>
<td>1.602</td>
</tr>
<tr>
<td>50 μM</td>
<td>1.698</td>
</tr>
<tr>
<td>60 μM</td>
<td>1.778</td>
</tr>
<tr>
<td>80 μM</td>
<td>1.903</td>
</tr>
<tr>
<td>100 μM</td>
<td>2</td>
</tr>
<tr>
<td>125 μM</td>
<td>2.096</td>
</tr>
<tr>
<td>150 μM</td>
<td>2.176</td>
</tr>
</tbody>
</table>

Table 10. Percent cytotoxicity with reference to concentration of formulations.

Stability studies of conjugates
The chemical stability of the pure drug and conjugates were determined by measuring UV absorption at λmax 259 nm using samples freshly prepared at concentration of 2 mg/ml in methanol. On storage in dry form, no major change in the absorbance was observed for neither MpPEG-2000 nor MpPEG-5000 kept at 25°C or at 4°C throughout the period of 4 months. Both MpPEG-5000 and MpPEG-2000 lose only about 2% of their absorbance on storage in dry form. These data indicate that the conjugates remain stable, when stored.

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</tr>
<tr>
<td>20 μM</td>
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</tr>
<tr>
<td>30 μM</td>
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</tr>
<tr>
<td>40 μM</td>
<td>1.602</td>
</tr>
<tr>
<td>50 μM</td>
<td>1.698</td>
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<tr>
<td>60 μM</td>
<td>1.778</td>
</tr>
<tr>
<td>80 μM</td>
<td>1.903</td>
</tr>
<tr>
<td>100 μM</td>
<td>2</td>
</tr>
<tr>
<td>125 μM</td>
<td>2.096</td>
</tr>
<tr>
<td>150 μM</td>
<td>2.176</td>
</tr>
</tbody>
</table>

Table 11. LD50 of different formulations.

S.No. | Formulation | LD50 (Log Conc. in μM) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Melphalan (Pure)</td>
<td>0.74</td>
</tr>
<tr>
<td>2</td>
<td>MpPEG-2000</td>
<td>1.14</td>
</tr>
<tr>
<td>3</td>
<td>MpPEG-5000</td>
<td>2.00</td>
</tr>
</tbody>
</table>
Table 12. Percent relative absorbance of conjugates on storage in dry form at 4 ± 1°C.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>99.87 ± 0.23</td>
<td>99.66 ± 0.11</td>
<td>99.45 ± 0.12</td>
<td>99.34 ± 0.65</td>
</tr>
<tr>
<td>MpPEG-2000</td>
<td>99.65 ± 0.73</td>
<td>99.48 ± 0.42</td>
<td>99.07 ± 1.2</td>
<td>98.64 ± 0.27</td>
</tr>
<tr>
<td>MpPEG-5000</td>
<td>99.87 ± 1.4</td>
<td>99.28 ± 0.68</td>
<td>99.04 ± 1.6</td>
<td>98.67 ± 0.62</td>
</tr>
</tbody>
</table>

Table 13. Percent relative absorbance of conjugates on storage in dry form at 25 ± 1°C.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>99.64 ± 0.19</td>
<td>99.12 ± 0.28</td>
<td>98.89 ± 0.12</td>
<td>98.29 ± 0.15</td>
</tr>
<tr>
<td>MpPEG-2000</td>
<td>99.48 ± 0.56</td>
<td>98.68 ± 0.44</td>
<td>98.12 ± 0.38</td>
<td>97.36 ± 0.42</td>
</tr>
<tr>
<td>MpPEG-5000</td>
<td>99.67 ± 0.24</td>
<td>99.12 ± 0.45</td>
<td>98.58 ± 0.62</td>
<td>97.70 ± 0.64</td>
</tr>
</tbody>
</table>

Table 14. Percent relative absorbance of conjugates on storage in dry form at 37 ± 1°C.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>99.56 ± 0.32</td>
<td>99.04 ± 0.48</td>
<td>98.52 ± 0.30</td>
<td>97.90 ± 0.42</td>
</tr>
<tr>
<td>MpPEG-2000</td>
<td>99.38 ± 0.64</td>
<td>98.68 ± 0.52</td>
<td>97.97 ± 0.38</td>
<td>97.21 ± 0.42</td>
</tr>
<tr>
<td>MpPEG-5000</td>
<td>99.67 ± 0.24</td>
<td>99.12 ± 0.45</td>
<td>98.58 ± 0.62</td>
<td>97.70 ± 0.64</td>
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</tbody>
</table>

The authors declared no conflict of interest.
Novel dithranol phospholipid microemulsion for topical application: development, characterization and percutaneous absorption studies

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Abstract

The objective of this study was to develop and characterize a novel dithranol-containing phospholipid microemulsion systems for enhanced skin permeation and retention. Based on the solubility of dithranol, the selected oils were isopropyl myristate (IPM) and tocopherol acetate (TA), and the surfactants were Tween 80 (T80) and Tween 20 (T20). The ratios of cosurfactants comprising of phospholipids and ethanol (1:10) and surfactant to co-surfactant (1:1 and 2.75:1) were fixed for the phase diagram construction. Selected microemulsions were evaluated for globule size, zeta potential, viscosity, refractive index, per cent transmittance, stability (freeze thaw and centrifugation), ex vivo skin permeation and retention. The microemulsion systems composed of IPM and T80 with mean particle diameter of 72.8 nm showed maximum skin permeation (82.23%), skin permeation flux (0.281 mg/cm²/h) along with skin retention (8.31%) vis-a-vis systems containing TA and T20. The results suggest that the developed novel lecithinized microemulsion systems have a promising potential for the improved topical delivery of dithranol.

Keywords: dermal delivery, colloidal delivery, phase diagrams, topical, anthralin, surfactant

Introduction

Dithranol (1,8-dihydroxy-9-anthrone; anthralin) has been used to treat various psoriatic disorders since many years. Despite being effective and safe, its application is difficult and troublesome owing to its irritating, burning, staining and necrotizing effects on the normal as well as the diseased skin. Further, the drug is highly lipophilic (log p = 2.3), poorly water soluble (< 2 μg/mL) and quiet unstable as it gets readily photo-oxidized (Mustakalio, 1981; Wang et al., 1987; Hiller et al., 1995).

Vesicular systems of dithranol, i.e. liposomes and niosomes, have been employed to circumvent these undesirable side effects (Agarwal et al., 2001). Microemulsion tends to possess several advantages over the vesicular carriers, namely, higher storage stability, lower preparation cost, good production feasibility, absence of organic solvents and oblation of intensive sonication (Paolini et al., 2002). Basic components of these colloidal formulations are surfactant, co-surfactant, oil and water combined in an appropriate ratio (Changez and Varshney, 2000). These systems have several specific physicochemical properties such as transparency, optical isotropy, low viscosity and thermodynamic stability (Hretschiol et al., 2008). Microemulsion systems can increase the dermal delivery of drug by different mechanisms. First, microemulsions can enhance the solubility of poorly water-soluble drugs via the finely dispersed oil droplet phase. Second, the increased thermodynamic activity of the drug favours its partitioning into the skin and third, the ingredients of microemulsion may increase the permeation rate of drug via skin by reducing the diffusional barrier of the skin (Delgado-Chauro et al., 1997; Gasco, 1997; Mohammed and Manoj, 2000).

Selection of various components for a microemulsion system should be conducted judiciously to prevent the skin irritation that might arise due to their specific physicochemical nature. It is well known that use of a blend of
Materials and methods

Materials

Free gift samples of dithranol and Phospholipon 90G were obtained from M/s Sujalam Chemicals (Mumbai, India) and M/s Phospholipid GmbH (Nattermanalle, Germany), respectively. Terpeneless oil, soybean oil and olive oil were obtained from M/s Protina (Kolkata, India) and isopropyl myristate (IPM), Tween 20 (T20) and Tween 80 (T80) were procured from M/s S.D. Fine Chemicals Ltd. (Mumbai, India). Tocopherol acetate (TA) was procured from M/s E-Merck Ltd. (Mumbai, India) while absolute ethanol from M/s Bengal Chemicals Ltd. (Kolkata, India). All other chemicals used for formulation development were of analytical grade.

Methods

Screening of oils, surfactants and cosurfactant

To find out the appropriate oil phase and surfactant in microemulsions, solubility of dithranol in various oils, such as IPM, soybean oil, olive oil, terpeneless oil and TA, and surfactants including T80, T20, Cremophor EL, Nikkol HCO 40, Nikkol HCO 50 and Nikkol HCO 60 were determined using shake flask method. In brief, an excess amount of dithranol was added to 5 mL of oil/surfactant and then the resulting mixture was shaken reciprocally at 37°C for 72 h followed by centrifugation at 12,000 rpm for 10 min. The supernatant was filtered through a membrane filter (0.45 μm) and the drug concentration in the filtrate was determined by UV-visible spectrophotometer after the appropriate dilution with chloroform-methanol mixture (2:1 v/v). Phospholipon 90G was selected as the cosurfactant along with ethanol for the formulation of microemulsions. The ratio of Phospholipon 90G and ethanol was selected on the basis of solubility of dithranol in the respective ratios starting from 1:2.5 to 1:30 (Phospholipon 90G: ethanol). The oil, surfactant and cosurfactant ratios that showed high solubility of dithranol were later used for the preparation of microemulsions containing 0.5% dithranol.

Phase diagram construction

On the basis of solubility studies of dithranol, IPM and TA were selected as the oil phase. T80 and T20 were used as surfactants and phospholipid and ethanol used as the corresponding cosurfactants. Acetate buffer (pH 3.3) was employed as the aqueous phase for the construction of phase diagrams. Oil, surfactants and cosurfactants were grouped in eight different combinations for phase studies; two S_m ratios (ratio of surfactant to cosurfactant) 1:1 and 2.75:1 for each surfactant and consequently four different combinations for each oil selected (Table 1). The ratio of aqueous phase and mixture of surfactant and cosurfactant (S_m) was varied (1:9-9:1), which were then titrated with oil till the appearance of turbidity. Similarly, ratio of oil and mixture of surfactant and cosurfactant was varied (1:9-9:1), which were then titrated with aqueous phase concerning of acetate buffer (pH 3.3), till the appearance of turbidity. The physical state of the lecithinized microemulsion was marked on a pseudo-three-component phase diagram with one axis representing aqueous phase, the other representing oil and the third representing a fixed S_m ratio. The microemulsion region was identified as the area in the phase diagram where clear and transparent formulations were obtained based on visual inspection (Kawakami et al., 2002).

Selection of formulations on the basis of phase diagrams

From each of the phase diagrams constructed, different formulations were chosen with minimum amount of emulsifier to a higher fixed value (approximately 60%) of emulsifier. This was assumed to represent the whole phase diagram.

Microemulsion formulation

According to the microemulsion areas in selected eight phase diagrams, various lecithinized microemulsions were selected at different component ratios. All microemulsion systems were prepared using analogous procedure. The oil phase was mixed with the phospholipid with the aid of heating (50-60°C) and the system was gently stirred on a magnetic stirrer (700 rpm for 10 min). The temperature of the above system was allowed to decrease and the surfactant was added to it. Then, the drug was dissolved in the above system to obtain a clear yellow solution. Required amount of aqueous phase (acetate buffer at pH 3.3) was added slowly under continuous stirring. Finally, ethanol was added to obtain a clear and transparent yellow coloured microemulsion.
Various studies were conducted to characterize and study the effect of different processes and formulation variables on the quality of microemulsions. The formulations were kept randomly for a stipulated period of time under the given experimental conditions to assess the sustenance of the characteristics of microemulsion.

**Drug content**

The amount of drug contained in the microemulsions was determined by extracting the drug in chloroform : methanol (2:1) mixture and analysed using UV spectrophotometer. The plain microemulsion (without drug) with same composition served as the blank. The absorbance of the drug in this solution was noted on the basis of which drug content was determined using the following equation

\[
\text{Concentration (mg/mL) = \frac{\text{absorbance}}{E_{1cm}}} \times \text{dilution factor} \times 10 \quad (1)
\]

**Globule size and zeta potential**

The mean globule size and polydispersity index were measured at 25°C by photon correlation spectroscopy (PCS) using Malvern Zetasizer, Nano ZS 90 (Malvern Instruments Co., Worcestershire, UK) (Kellmann et al., 2007). The same instrument was employed to determine the zeta potential of the formulations. The operating principle of this instrument is based on the Doppler shift caused by the movement of globules across interference fringes which are produced by the intersection of two laser beams (Skiba et al., 1996).

**Globule morphology**

Morphology and structure of lipid microspheres were determined using transmission electron microscopy (TEM) at Central Instrumentation Laboratory (CIL), Panjab University, Chandigarh, and photomicrographs were taken at suitable magnifications.

**Viscosity and refractive index**

Viscosity of the formulation was measured using Rheometer (Rheotab QC, Anton Paar, Germany) at 25°C. Apparent viscosity at shear rate (\(\gamma\)) 0.05-100 s\(^{-1}\) was obtained at 25 ± 1°C. Experiments were carried out in triplicate and the results were presented as mean ± SD (SD, standard deviation).

To quantify the clarity of prepared system, refractive index (RI) was determined using Abby’s refractometer at 37 ± 0.1°C. RI of the prepared formulations was compared vis-à-vis the RI of water.

**Per cent transmittance measurement**

The per cent transmittance of all the formulations was measured at 650 nm on a UV spectrophotometer (UV 1601, M/s Shimadza, Kyoto, Japan) keeping distilled water as a blank.

**Preparation of skin for ex vivo studies**

To obtain mice skin, animals were sacrificed after obtaining approval from the Institutional Animal Ethics Committee. Hair on the dorsal side of animals were removed with the help of 0.1-mm animal hair clipper, in the direction of tail to head, and the skin was separated from the animal body. Dermis part of the skin was wiped three to four times with a wet cotton swab soaked in isopropanol to remove any adhering fat material. A portion of the skin was further employed for permeation studies after washing with distilled water (Singh et al., 2005).

**Ex vivo skin permeation studies**

Ex vivo permeation studies were conducted using abdominal skin of Laca mice employing Franz diffusion cells (PermeGear, Inc., Hellertown, PA, USA) with an effective diffusion area of 3.14 cm\(^2\) and sink volume of 30.0 mL. The skin tissue was adhered to the upper surface of receptor compartment. The receptor compartment contained acetate buffer (pH 3.3) with ascorbic acid (1% w/v), sodium metabisulphite (0.5% w/v), ethylenediamine tetra acetate (EDTA; 0.5% w/v) and sodium chloride (0.9% w/v) as stabilizers along with Cremophor RH 40 (1% w/v) and methanol (20% v/v) as diffusion medium. Various formulations containing dithranol equivalent to 1 mg were applied onto the skin in the donor compartment. Plain dithranol drug suspension was also applied onto the skin of donor compartment for comparison. The formulations were uniformly spread and were maintained in intimate contact with the skin. At suitable time intervals, aliquots of 1 mL each were withdrawn through the sampling port and replaced with equal amounts of diffusion medium to maintain constant receptor volume. The samples were spectrophotometrically analysed after suitable dilution.

**Calculation of the ex vivo data**

The raw data obtained from permeation studies were analysed by applying factor for volume correction and drug losses during sampling using the following equation, which calculates the values of fraction of drug permeated

<table>
<thead>
<tr>
<th>Group</th>
<th>Oil</th>
<th>Surfactant</th>
<th>Ratio of surfactant: cosurfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>TA</td>
<td>T80</td>
<td>T80: cosurfactant (1:1)</td>
</tr>
<tr>
<td>II</td>
<td>IPM</td>
<td>T80</td>
<td>T80: cosurfactant (1:1)</td>
</tr>
<tr>
<td>III</td>
<td>TA</td>
<td>T80</td>
<td>T80: cosurfactant (2.75:1)</td>
</tr>
<tr>
<td>IV</td>
<td>IPM</td>
<td>T80</td>
<td>T80: cosurfactant (2.75:1)</td>
</tr>
<tr>
<td>V</td>
<td>TA</td>
<td>T20</td>
<td>T20: cosurfactant (1:1)</td>
</tr>
<tr>
<td>VI</td>
<td>IPM</td>
<td>T20</td>
<td>T20: cosurfactant (1:1)</td>
</tr>
<tr>
<td>VII</td>
<td>TA</td>
<td>T20</td>
<td>T20: cosurfactant (2.75:1)</td>
</tr>
<tr>
<td>VIII</td>
<td>IPM</td>
<td>T20</td>
<td>T20: cosurfactant (2.75:1)</td>
</tr>
</tbody>
</table>

Note: *Cosurfactant (Phospholipid 90 G: ethanol, ratio 1:10) has been kept constant in all the groups.

**Characterization**

The raw data obtained from permeation studies were analysed by applying factor for volume correction and drug losses during sampling using the following equation, which calculates the values of fraction of drug permeated.
and mean per cent drug permeated along with SD at varied times.

\[ \text{Corrected concentration} = C_t + \left( \frac{V_t}{V_s} \right) \sum C_i \]  

(2)

For the nth sample, \( V_s \) is the volume of sample withdrawn, \( V_t \) the total volume of receptor medium, \( C_t \) the corrected concentration, \( C_i \) the uncorrected concentration of the nth sample and \( C_i \) the uncorrected concentration.

Further, the corrected concentrations were used to calculate the values of the amount of drug released, and per cent drug released at each time of sampling. The flux values were calculated from the graph between the amounts of drug released per unit surface area versus time. The slope of the linear portion of the curve was taken as flux value.

Skin retention studies

Following permeation studies, the skin tissue mounted on the diffusion cell was removed and washed three to four times with distilled water. The treated skin area was weighed and dried using lint free cotton swab. Subsequently, the skin tissue was mashed with tissue homogenizer. The homogenate suspension thus obtained was mixed with 20 mL chloroform-methanol mixture (2:1, v/v) and shaken for 2 h at 37 ± 1°C for complete extraction of dithranol. Supernatant was filtered through a 0.45 μm membrane filter (M/s Millipore, Massachusetts, USA) and quantified for drug content spectrophotometrically. Fresh skin tissue, treated in a similar manner, served as blank for the above study. Each experiment was conducted in triplicate (Singh et al., 2005).

Statistical analysis

The permeation and skin retention data between different formulations were compared for statistical significance by the one-way analysis of variance (ANOVA) followed by Student’s t-test using Microsoft Excel.

Stability studies

All microemulsion formulations were subjected to drug loss studies at different temperatures. After measuring the initial drug content in all the formulations, these were stored in sealed glass ampoules (three each) at 5 ± 3°C, 30 ± 2°C and 45 ± 2°C for a period of at least 45 days. After every 7 days, the drug content was determined as per the method described in drug content studies.

Results and discussion

Screening of components for microemulsions

For poorly soluble drugs like dithranol, screening of microemulsion components (oil, surfactant and cosurfactant) on the basis of solubility is desirable to maintain the drug in solubilized state. The solubility of dithranol in various media was analysed. In five oils studied, the solubility of dithranol was the highest in TA (3.47 mg/mL) followed by IPM (3.15 mg/mL). The solubility in other three oils was relatively lower (olive oil, 2.59 mg/mL; soybean oil, 2.53 mg/mL; and terpeneless oil, 2.44 mg/mL). Both oils of ester nature, i.e. IPM and TA offered better sink to the drug, and both these oils were selected for further studies. Previous reports indicated that the superior dermal flux appeared mainly due to the large solubilizing capacity of the microemulsions, which led to larger concentration gradient towards the skin (Kreilgaard, 2002; Sisov and Shapiro, 2004). It has also been reported that IPM acts as penetration enhancer which helps in increasing the permeability of drug into the skin (Kogan and Garti, 2006), whereas tocopherol has antioxidant properties which will certainly enhance the photostability of the drug dithranol.

Proper selection of surfactants is necessary as larger amounts of surfactants may cause skin irritation on topical application. It is, therefore, important to determine the surfactant which, besides being used in minimum concentration, possesses the highest solubilization capacity for the drug. Non-ionic surfactants are relatively less toxic than their ionic counterparts and typically have lower critical micelle concentrations (CMCs: Kawakami et al., 2002). Out of the six non-ionic surfactants, the maximum solubility of dithranol was obtained in T80 (6.20 mg/mL) followed by T20 (6.00 mg/mL). Other non-ionic surfactants (Cremophore EL, 4.22 mg/mL; Nikkol HCO 40, 4.12 mg/mL; Nikkol HCO 50, 3.10 mg/mL; and Nikkol HCO 60, 1.99 mg/mL) not only offered less solubility to dithranol, but also resulted in quick degradation of dithranol (visual blackening of erstwhile yellow drug) as compared to T80 and T20.

Phospholipid 90G along with ethanol was used as cosurfactant. Phospholipid 90G helps in increasing the penetration of drug into the skin as phospholipids are the essential components of all biological membranes. When they are applied to skin as vehicles, due to their physicochemical properties and structures, they can interact with the stratum corneum lipids, perturb their structures and facilitate drug delivery (Yokomizo and Sagitani, 1996; Williams and Barry, 2004). The absolute alcohol used as the cosurfactant, was preferred since it is safe and non-toxic (Lawrence and Rees, 2000). Also, the presence of alcohol in the interfacial region causes a reduction in the rigidity of the otherwise-condensed lecithin film, allowing the curvature necessary for droplet formation (Ruth et al., 1995).

Ratio of the two cosurfactants, i.e. phospholipid and ethanol were selected on the basis of solubility of dithranol. The solubility values in all ratios were in the order 1:2.5 (2.48 mg/mL) > 1:0.5 (2.16 mg/mL) > 1:7.5 (2.08 mg/mL) > 1:2.5 (1.30 mg/mL) > 1:0.5 (1.28 mg/mL) > 1:10 (1.30 mg/mL) > 1:2.5 (1.28 mg/mL). Drug exhibited maximum solubility in the ratio 1:2.5, but a ratio of 1:10 was selected for further studies due to its comparable solubility to 1:2.5 ratio as well as being a cost-effective alternative. Further, S_{cas} ratio (ratio of surfactant to cosurfactant) was decided on the basis of solubility studies performed in five different ratios (1:1, 2.75:1, 5:1, 10:1 and 20:1). S_{cas} ratios containing T80 offered good solubility with the
maximum in ratio 2.75:1 (6.403 mg/mL) followed by 1:1 (8.148 mg/mL). The solubility values in other ratios were little less: 5:1 = 7.65 mg/mL, 10:1 = 6.32 mg/mL, and 20:1 = 5.86 mg/mL. Thus, two \( S_{\text{mix}} \) ratios were fixed to carry out the phase diagram studies.

**Construction of phase diagram**

The relationship between the phase behaviour of a mixture and its composition can be established with the aid of a phase diagram (Craig et al., 1995). Various pseudo-ternary phase diagrams of o/w microemulsions comprising of IPM/T80, T80/T20, lecithin, ethanol and acetate buffer, pH 3.3 are shown in Figures 1 and 2. The surfactant and cosurfactant mass ratio had been found to be a key factor influencing the phase properties, i.e., size and position of microemulsion region (Kreilgaard et al., 2000; Hua et al., 2004). The kind and concentration of oil employed also play an important role (Malcolmson et al., 1998; Yuan et al., 2000). With same surfactant T80 and \( S_{\text{mix}} \) ratio 1:1 (Figures 1(a) and 1(b)), it can be observed that for the given amount of emulsifier, a slightly bigger microemulsion region was obtained with IPM (Figure 1(b)) in comparison to that with TA (Figure 1(a)) as oil phase. The maximum amount of oil emulsified in case of TA was 15.10%, whereas in case of IPM it was 15.7%. This can be attributed to the fact that IPM is easily emulsified than TA, i.e., needs relatively lesser amount of the selected emulsifier for its emulsification. Effect of surfactant and cosurfactant mass ratio \( (S_{\text{mix}}) \) on microemulsion formation was prominent. When the concentration of cosurfactant increased \( (S_{\text{mix}} \text{ ratio 2.75:1}) \), in case of TA as oily phase and T80 as surfactant (Figure 1(c)), the microemulsion area increased and the oil solubilized increased up to 20% w/w, with the total surfactant concentration of 52% w/w. Similar observation was also noted when IPM (Figure 1(d)) was used as oily phase and T80 as surfactant. The maximum amount of oil that could be accommodated in the higher ratio (2.75:1) was 24% that too at a lower concentration of surfactant mixture, i.e. 44% w/w. This may be due to the substantial reduction of the o/w interfacial tension and increase in the interface fluidity with increasing amounts of cosurfactants (Azeem et al., 2009).

The microemulsion region was considerably smaller when T20 was used as surfactant instead of T80. In Figure 2(a) (TA as oily phase, \( S_{\text{mix}} \) 1:1), maximum amount of oil emulsified was only 4.74% and in Figure 2(b) (IPM as oily phase, \( S_{\text{mix}} \) 1:1), it was found to be 2.82%. On increasing the cosurfactant concentration \( (S_{\text{mix}} \text{ ratio 2.75:1}) \), in case of TA as oily phase, the maximum amount of oil that could be emulsified was 5.10% with the use of 66.39% emulsifier (Figure 2(c)) and in case of IPM, maximum amount of oil emulsified was 20% with the use...
of 58% emulsifier (Figure 2(d)). Pseudo-ternary phase diagrams with varied oil, surfactant and cosurfactant ratios suggested their influence on the formulation attributes. It can be generalized that T80 was found to be a better emulgent than T20 for given set of oil, and IPM was easily emulsified than TA.

Selection of formulations on the basis of phase diagrams

There are no reports of the proper basis of selecting different microemulsion formulations from a phase diagram, as hundreds of formulations can be prepared from microemulsion region of a single diagram. The variation in the number of formulations was because of the difference in the size of microemulsion region which could be due to the different surfactants employed; T80 for ternary diagrams (Figures 1(a)-1(d)) and T20 for ternary diagrams (Figures 2(a)-2(d)). From the first four pseudo-ternary phase diagrams (Figures 1(a)-1(d)), totally three formulations were selected from each phase diagram; one formulation comprised of minimum amount of emulsifier (irrespective of the amount of oil being emulsified), the other formulation consisted of 10% of oil, with a maximum amount of emulsifier being up to 65% and another formulation incorporated 70% emulsifier, irrespective of the amount of oil being emulsified. For the remaining ternary phase diagrams (Figures 2(a)-2(d)), two formulations were selected from each phase diagram - formulations using the minimum amount of emulsifier (irrespective of the amount of oil being emulsified) and formulation employing about 10% of oil. The compositions of all 20 microemulsion formulations are given in Table 2.

Table 2. Composition of all the formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Oil phase</th>
<th>S_mic (surfactant: cosurfactant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11</td>
<td>TA (4%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 65%</td>
</tr>
<tr>
<td>F12</td>
<td>TA (10%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 50%</td>
</tr>
<tr>
<td>F13</td>
<td>TA (13%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 45%</td>
</tr>
<tr>
<td>F21</td>
<td>IPM (5%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 50%</td>
</tr>
<tr>
<td>F22</td>
<td>IPM (11%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 63%</td>
</tr>
<tr>
<td>F23</td>
<td>IPM (16%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 56%</td>
</tr>
<tr>
<td>F31</td>
<td>TA (3%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 47%</td>
</tr>
<tr>
<td>F32</td>
<td>TA (10%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 54%</td>
</tr>
<tr>
<td>F33</td>
<td>TA (15%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 63%</td>
</tr>
<tr>
<td>F41</td>
<td>IPM (5%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 48%</td>
</tr>
<tr>
<td>F42</td>
<td>IPM (9%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 45%</td>
</tr>
<tr>
<td>F43</td>
<td>IPM (14%)</td>
<td>T80: (Phospholipid 90 G: ethanol) 50%</td>
</tr>
<tr>
<td>F51</td>
<td>TA (3%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 55%</td>
</tr>
<tr>
<td>F52</td>
<td>TA (7%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 65%</td>
</tr>
<tr>
<td>F53</td>
<td>TA (10%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 73%</td>
</tr>
<tr>
<td>F61</td>
<td>IPM (3%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 69%</td>
</tr>
<tr>
<td>F62</td>
<td>IPM (9%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 70%</td>
</tr>
<tr>
<td>F71</td>
<td>TA (3%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 60%</td>
</tr>
<tr>
<td>F72</td>
<td>TA (7%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 60%</td>
</tr>
<tr>
<td>F73</td>
<td>TA (10%)</td>
<td>T20: (Phospholipid 90 G: ethanol) 69%</td>
</tr>
</tbody>
</table>

Note: Formulations F11-F23 and F51-F62 comprise of fixed S_mic ratio i.e. 1:1 and formulations F31-F43 and F71-F73 contain S_mic ratio 2.75:1. Acetate buffer, pH 3.3, (q.s.) was used as the aqueous phase.
Table 3. Physicochemical characterization of formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Drug content (%)</th>
<th>Globule size (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
<th>Viscosity (cP)</th>
<th>RI</th>
<th>% Transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11</td>
<td>99.48</td>
<td>313.08</td>
<td>-2.11</td>
<td>0.331</td>
<td>160.67 ±0.15</td>
<td>1.38</td>
<td>96.1</td>
</tr>
<tr>
<td>F12</td>
<td>99.68</td>
<td>281</td>
<td>-0.122</td>
<td>0.402</td>
<td>136.67 ±0.58</td>
<td>1.40</td>
<td>97.9</td>
</tr>
<tr>
<td>F13</td>
<td>99.57</td>
<td>287.9</td>
<td>-0.134</td>
<td>0.435</td>
<td>113.00 ±0.60</td>
<td>1.41</td>
<td>98.1</td>
</tr>
<tr>
<td>F21</td>
<td>99.53</td>
<td>295.2</td>
<td>0.310</td>
<td>0.329</td>
<td>83.00 ±0.10</td>
<td>1.38</td>
<td>97.4</td>
</tr>
<tr>
<td>F22</td>
<td>99.47</td>
<td>356.6</td>
<td>-0.645</td>
<td>0.442</td>
<td>52.13 ±0.25</td>
<td>1.30</td>
<td>97.5</td>
</tr>
<tr>
<td>F23</td>
<td>99.65</td>
<td>285</td>
<td>0.354</td>
<td>0.356</td>
<td>52.67 ±0.21</td>
<td>1.30</td>
<td>97.8</td>
</tr>
<tr>
<td>F31</td>
<td>99.63</td>
<td>376</td>
<td>0.087</td>
<td>0.386</td>
<td>459.67 ±0.58</td>
<td>1.40</td>
<td>95.12</td>
</tr>
<tr>
<td>F32</td>
<td>99.67</td>
<td>327</td>
<td>-0.0350</td>
<td>0.376</td>
<td>370.67 ±0.58</td>
<td>1.42</td>
<td>96.75</td>
</tr>
<tr>
<td>F33</td>
<td>99.62</td>
<td>340.4</td>
<td>0.051</td>
<td>0.434</td>
<td>340.53 ±0.88</td>
<td>1.44</td>
<td>97.1</td>
</tr>
<tr>
<td>F41</td>
<td>99.81</td>
<td>172.2</td>
<td>-0.0027</td>
<td>0.372</td>
<td>221.33 ±0.58</td>
<td>1.30</td>
<td>97</td>
</tr>
<tr>
<td>F42</td>
<td>99.66</td>
<td>72.8</td>
<td>0.0731</td>
<td>0.331</td>
<td>226.67 ±0.58</td>
<td>1.40</td>
<td>97</td>
</tr>
<tr>
<td>F51</td>
<td>99.47</td>
<td>92.7</td>
<td>-0.0165</td>
<td>0.401</td>
<td>220.33 ±0.58</td>
<td>1.42</td>
<td>97</td>
</tr>
<tr>
<td>F52</td>
<td>99.70</td>
<td>400</td>
<td>0.0786</td>
<td>0.419</td>
<td>155.37 ±0.06</td>
<td>1.30</td>
<td>96.3</td>
</tr>
<tr>
<td>F61</td>
<td>99.67</td>
<td>474</td>
<td>0.255</td>
<td>0.409</td>
<td>162.17 ±0.06</td>
<td>1.40</td>
<td>96.4</td>
</tr>
<tr>
<td>F62</td>
<td>99.58</td>
<td>513.1</td>
<td>0.167</td>
<td>0.335</td>
<td>198.03 ±0.06</td>
<td>1.40</td>
<td>96.1</td>
</tr>
<tr>
<td>F71</td>
<td>99.54</td>
<td>269</td>
<td>0.0564</td>
<td>0.401</td>
<td>220.33 ±0.58</td>
<td>1.42</td>
<td>97.6</td>
</tr>
<tr>
<td>F72</td>
<td>99.49</td>
<td>432</td>
<td>-0.067</td>
<td>0.420</td>
<td>209.80 ±0.20</td>
<td>1.40</td>
<td>97</td>
</tr>
<tr>
<td>F81</td>
<td>99.51</td>
<td>397</td>
<td>-0.035</td>
<td>0.321</td>
<td>209.03 ±0.12</td>
<td>1.40</td>
<td>96.4</td>
</tr>
<tr>
<td>F82</td>
<td>99.62</td>
<td>771.5</td>
<td>-0.0523</td>
<td>0.386</td>
<td>263.27 ±0.29</td>
<td>1.42</td>
<td>96</td>
</tr>
</tbody>
</table>

Note: PDI, polydispersity index and RI, refractive index.

### Drug content

The drug content of various formulations ranged from 99.43% to 99.81% with a mean value of 99.55% (Table 3). These higher values of drug content ensured minimal drug loss during various stages of formulation development, hence, authenticated the method of preparation.

### Micromeritics and zeta potential

The zeta potential of all the prepared microemulsions was found to be approximately zero (Table 3). This may be attributed to non-ionic nature of both surfactants and cosurfactants used (i.e. T80 or T20 and lecithin). They do not impart any charge to the droplet surface and provide better stability to the microemulsion against any ionic interactions. Of all these formulations, the smallest globule size was of formulation F42 (72.8 nm) followed by formulation F32 (127 nm) and the largest globule size was of formulation F82 (771.5 nm). The differences in the globule size may be attributed to the use of different surfactants in these formulations which were T80 for F42 and F32 and T20 for formulation F82. Hence, these results point towards the better emulsifying capability of T80 in this study which resulted in smaller globule size of the oil droplets.

### Globule morphology

TEM revealed the spherical nature and size homogeneity of the microemulsion droplets (Figure 3).

### Viscosity and RI

The values for viscosity for the various formulations are given in Table 3. All microemulsions showed Newtonian flow behaviour, when shear stress was plotted against shear rate. These results were in accordance with the earlier reports (Cilek et al., 2006). The Newtonian flow behaviour indicated that the droplets were small and spherical in nature (Ktistis, 1990). The constancy in RI values (1.4 ± 2) of all the formulations is an indication of constant microemulsion structure (Cilek et al., 2006).

### Per cent transmittance measurement

The clarity of microemulsion was checked by transparencym, measured in terms of per cent transmission (%T).
Per cent transmittance values of all the formulations were around 97%, indicating high clarity of the formulations.

Skin permeation study
The *ex vivo* permeation of dithranol through abdominal skin of *Laca* mice from all microemulsions was determined in terms of per cent mean cumulative amount diffused at each sampling time point during time period of 24 h. The key factors that may have aided in enhancement of skin permeation are the choice of oil components, surfactant/cosurfactant and cosolvent in the formulation, particle diameter, mobility of the bioactive ingredient in the designed formulation and the concentration gradient (Lin et al., 2009). Statistically, the data obtained for skin permeation studies are highly significant (*p* < 0.001). In our study, the results indicated that the smaller globule diameters, as in two cases F42 and F32, resulted in enhanced skin permeation, in comparison to all other formulations (Figure 4). As shown in Figure 4 and Table 4, the per cent permeation and flux value of F42 were 82.23%, 0.281 mg/cm²/h, respectively, as compared to F32 which were 80.61% and 0.125 mg/cm²/h, respectively. Statistically, the difference in % permeation and flux values of F42 was highly significant (*p* < 0.005) vis-a-vis that of F32 (Table 4). The cumulative amount released, average flux values of all 20 microemulsions are given in Table 4. Microemulsions containing minimum amount of emulsifier showed a lesser permeation flux but as the concentration of emulsifier was increased to a certain extent keeping rest of the components constant, the permeation flux increased considerably; however, with further increase in emulsifier content up to 60%, the permeation flux did not increase appreciably. This could be attributed to the fact that high surfactant concentration decreases the thermodynamic activity of the drug in the vehicle, and the affinity of the drug to the vehicle becomes greater, consequently decreasing the flux (Shinoda and Kaneko, 1988). However, at lower concentration, due to the incorporation of phospholipoidal structures in the skin layers, surfactants can loosen or fluidize the lipidic matrix of the skin and can act as permeation promoters (Yokomizo and Sagitani, 1996; Williams and Barry, 2004). Therefore, it is conceivable that the permeation of dithranol is accompanied by the environment favourable to the partitioning of drug into the skin. Further, the formulations containing IPM as oily phase showed high permeation flux values in comparison to TA as oily phase because of the better solubilizing power of IPM, its penetration enhancement effect and higher emulsification by the surfactant.

Skin retention studies
In this study, percentage skin retention was estimated after 24 h for all 20 microemulsions (*p* < 0.001) (Table 4). Skin retention decreased with increase of emulsifier after a certain value. The findings are in consonance with the skin permeation studies. This may be ascribed to the fact that high concentration of alcohol (cosurfactant) had dissolved more of phospholipid as well as drug, resulting in the formation of pseudosolutions, therefore, increased permeation and retention. High levels of emulsifier might have formed their own vesicular systems consisting of various lamellae in which drug solution (because of increased ethanol content) is channelized and face few lamellar barriers for its release.

Maximum skin retention was achieved with the use of T80, while other variables were kept constant, i.e. maximum for formula F42 followed by F32. This high value of skin retention in the case of F42 (*p* < 0.005) when compared to F32 can be attributed to the nature of oil phase. These globules may have interacted with skin phospholipids and formed a depot resulting in increased skin retention. However, with TA microemulsions, the
Table 4. Permeation profiles and skin retention of dithranol through skin from various formulations selected at different temperatures for 45 days.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Cumulative amount released (cm² ± SD)</th>
<th>Permeation flux (µg/cm²/h)</th>
<th>Mean % skin retention¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11</td>
<td>0.626 ± 0.068</td>
<td>0.035 ± 0.010</td>
<td>5.42 ± 0.010</td>
</tr>
<tr>
<td>F12</td>
<td>0.662 ± 0.007</td>
<td>0.117 ± 0.012</td>
<td>5.97 ± 0.020</td>
</tr>
<tr>
<td>F13</td>
<td>0.632 ± 0.011</td>
<td>0.112 ± 0.007</td>
<td>5.63 ± 0.015</td>
</tr>
<tr>
<td>F21</td>
<td>0.608 ± 0.014</td>
<td>0.064 ± 0.009</td>
<td>5.77 ± 0.037</td>
</tr>
<tr>
<td>F22</td>
<td>0.679 ± 0.007</td>
<td>0.060 ± 0.015</td>
<td>6.24 ± 0.015</td>
</tr>
<tr>
<td>F31</td>
<td>1.000 ± 0.009</td>
<td>0.123 ± 0.016</td>
<td>6.73 ± 0.015</td>
</tr>
<tr>
<td>F33</td>
<td>0.683 ± 0.107</td>
<td>0.062 ± 0.004</td>
<td>5.84 ± 0.030</td>
</tr>
<tr>
<td>F33</td>
<td>1.384 ± 0.050*</td>
<td>0.125 ± 0.009</td>
<td>7.23 ± 0.0115</td>
</tr>
<tr>
<td>F35</td>
<td>0.718 ± 0.013</td>
<td>0.090 ± 0.011</td>
<td>6.13 ± 0.015</td>
</tr>
<tr>
<td>F41</td>
<td>0.719 ± 0.001</td>
<td>0.139 ± 0.015</td>
<td>6.09 ± 0.035</td>
</tr>
<tr>
<td>F41</td>
<td>1.299 ± 0.003</td>
<td>0.281 ± 0.009*</td>
<td>3.10 ± 0.017**</td>
</tr>
<tr>
<td>F43</td>
<td>0.752 ± 0.015</td>
<td>0.086 ± 0.001</td>
<td>7.32 ± 0.015</td>
</tr>
<tr>
<td>F51</td>
<td>0.718 ± 0.006</td>
<td>0.073 ± 0.016</td>
<td>5.10 ± 0.020</td>
</tr>
<tr>
<td>F52</td>
<td>0.629 ± 0.008</td>
<td>0.080 ± 0.007</td>
<td>5.77 ± 0.027</td>
</tr>
<tr>
<td>F61</td>
<td>0.680 ± 0.001</td>
<td>0.101 ± 0.009</td>
<td>5.27 ± 0.021</td>
</tr>
<tr>
<td>F61</td>
<td>0.656 ± 0.007</td>
<td>0.097 ± 0.013</td>
<td>5.15 ± 0.150</td>
</tr>
<tr>
<td>F71</td>
<td>0.637 ± 0.012</td>
<td>0.078 ± 0.010</td>
<td>4.87 ± 0.005</td>
</tr>
<tr>
<td>F72</td>
<td>0.710 ± 0.006</td>
<td>0.100 ± 0.014</td>
<td>4.51 ± 0.015</td>
</tr>
<tr>
<td>F81</td>
<td>0.610 ± 0.012</td>
<td>0.065 ± 0.014</td>
<td>5.12 ± 0.020</td>
</tr>
<tr>
<td>F81</td>
<td>0.869 ± 0.004</td>
<td>0.089 ± 0.019</td>
<td>4.99 ± 0.005</td>
</tr>
<tr>
<td>Plain drug</td>
<td>0.321 ± 0.014</td>
<td>0.021 ± 0.003</td>
<td>1.21 ± 0.012</td>
</tr>
</tbody>
</table>

Notes: Each value is the average of three different experiments ± SD. *p < 0.001 when compared to formulation F42. **p < 0.005 when compared to formulation F32 (for permeation flux). ***p < 0.005 when compared to formulation F31 (for mean % skin retention).

Table 5. Long-term stability of different formulations selected at different temperatures for 45 days.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Chemical stability (°C)</th>
<th>Physical stability (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5°C 30°C 45°C</td>
<td>4°C 30°C 45°C</td>
</tr>
<tr>
<td>F11</td>
<td>0.47 1.49 1.73</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F12</td>
<td>0.55 1.01 1.75</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F13</td>
<td>0.54 1.49 1.62</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F21</td>
<td>0.60 1.47 1.79</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F22</td>
<td>0.50 1.34 1.83</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F23</td>
<td>0.78 1.29 1.78</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F31</td>
<td>0.60 0.92 1.60</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F32</td>
<td>0.55 1.04 1.76</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F33</td>
<td>0.54 1.09 1.68</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F41</td>
<td>0.64 1.05 1.89</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F42</td>
<td>0.69 0.84 1.34</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F43</td>
<td>0.50 1.25 1.74</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F51</td>
<td>0.50 1.25 1.58</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F52</td>
<td>0.67 1.27 1.92</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F61</td>
<td>0.67 1.28 2.05</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F62</td>
<td>0.69 1.32 1.99</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F71</td>
<td>0.63 1.66 2.52</td>
<td>&gt; 45 &gt; 45 &gt; 45</td>
</tr>
<tr>
<td>F72</td>
<td>0.61 1.70 2.49</td>
<td>45 45 45</td>
</tr>
<tr>
<td>F81</td>
<td>0.61 1.75 2.09</td>
<td>45 45 34</td>
</tr>
<tr>
<td>F82</td>
<td>0.80 1.95 2.02</td>
<td>&gt; 45 45 34</td>
</tr>
</tbody>
</table>

Notes: Each value is the average of three different experiments ± SD. ¹ANOVA provided a p < 0.001 for various values of cumulative amount released, permeation flux and skin retention, respectively. *p < 0.05 when compared to formulation F42. **p < 0.005 when compared to formulation F21 (for permeation flux).

Conclusion

In conclusion, this study resulted in successful production of novel dithranol phospholipid-based o/w microemulsion employing various oils and surfactants/cosurfactants. Studies accomplished to establish the effect of various components on the design and development of emulsified microemulsion systems containing dithranol. Overall, our studies illustrated the enhanced dermal delivery of dithranol by lecithinized microemulsions. Therefore, novel dithranol phospholipid-based microemulsion has a promising potential for a new, cost-effective and commercial alternative for the improved topical application of dithranol.

Acknowledgements

The authors thank the University Grants Commission (UGC), New Delhi, India for their financial assistance. The authors also thank Phospholipid GmbH, Nattermannallee, Germany, for generously providing the gift sample of lecithin.

Declaration of interest

The authors report no conflicts of interest.

References

Novel dithranol phospholipid microemulsion 199


Report

Novel stain-free lecithinized coal tar formulation for psoriasis

Amit Bhatia, MPharm, Bhupinder Singh, PhD, Basant Amarji, MPharm, Poonam Negi, MPharm, Anshuman Shukla, MPharm, and Om Prakash Katare, PhD

Abstract

Background Coal tar has been a very popular traditional treatment for various types of psoriasis for over a century. It is the first-line treatment for scalp, hand and foot psoriasis. However, the application of coal tar on hair invariably causes staining, which results in a high degree of patient non-compliance, especially in patients with non-black hair. Thus, the treatment of scalp psoriasis with a topical coal tar formulation requires that special concern be paid to product esthetics.

Objective This study aimed to evaluate the hair-staining characteristics of a novel lecithinized coal tar (LCT) formulation on different types of mammalian hair.

Methods Samples of hair from different mammals, including human, sheep, rabbit and goat, were repeatedly exposed to the LCT formulation over 14 d. The color of hair samples treated with LCT was compared with that of untreated control hair samples.

Results The study revealed the distinct non-staining potential of the LCT formulation.

Conclusions This LCT formulation lacks the propensity to stain hair and thus has excellent potential to be exploited in the treatment of scalp psoriasis.

Introduction

The role of coal tar in the treatment of various dermatological conditions has been well known for several years. Over the past century, coal tar has been used in the treatment of scabies, sarcoidosis, neurodermatitis and pityriasis lichenoides chronicus. It has also been employed to treat chronic stable plaque psoriasis, scalp psoriasis, hand and foot psoriasis and seborrheic dermatitis. Coal tar preparations are more effective and cheaper than most other available treatments for scalp psoriasis, including corticosteroids and calcipotriol, and extend the length of remission without causing systemic side-effects. The exact mechanism of the action of coal tar has not yet been fully elucidated, as it contains a mixture of hydrocarbons. As 50-80% of psoriasis patients are known to have some involvement of the scalp, coal tar has immense promise in the treatment of this ailment. However, the greatest concerns associated with the application of coal tar refer to its obnoxious color and odor, and its tendency to stain hair as well as clothing, which combine to make it unappealing to patients. The unpleasant characteristics of coal tar formulations tend to affect rates of patient compliance and, ultimately, clinical response to therapy. Therefore, in order to overcome these obstacles, we developed a novel lecithin-based formulation in our laboratory by adopting an innovative formulation strategy.

The propensity of this lecithinized coal tar (LCT) formulation to stain clothes and hair must be evaluated before its potential application in scalp psoriasis can be assessed. We have already demonstrated the complete washability and non-staining properties of the formulation on different fabric types in our laboratory. This study, therefore, aims to evaluate its propensity to stain different mammalian hairs prior to its clinical application.

Materials and methods

Hair samples from human and three animal species, namely, rabbit, goat and sheep, were employed in this study. The human scalp and rabbit hairs were almost pure white in color and measured approximately 5.0 cm in length. The goat hairs were off-white and the sheep hairs were light brown in color; each of these measured approximately 7.5-10.0 cm in length. Each sample of hairs was tied together at one end to facilitate trouble-free handling and subsequently washed with shampoo before the study procedure began in order to remove any adherent material. The treatment encompassed a 14-d repeated application of the LCT formulation. Each hair sample was...
Figure 1 Human scalp hair in (a) an untreated control sample, (b) during incubation for 12 h in the lecithinized coal tar formulation, and (c) after 14 d of repeated treatment, washing and drying.

Figure 2 Sheep hair in (a) an untreated control sample, (b) during incubation for 12 h in the lecithinized coal tar formulation, and (c) after 14 d of repeated treatment, washing and drying.

Figure 3 Rabbit hair in (a) an untreated control sample, (b) during incubation for 12 h in the lecithinized coal tar formulation, and (c) after 14 d of repeated treatment, washing and drying.

Figure 4 Goat hair in (a) an untreated control sample, (b) during incubation for 12 h in the lecithinized coal tar formulation, and (c) after 14 d of repeated treatment, washing and drying.

The study subsamples were submerged in the LCT formulation for 12 h at room temperature in a glass Petri dish, after which they were gently washed with shampoo, rinsed in warm water, air-dried and kept aside for 12 h. Photographs were taken just before application and after 14 d of treatment. The brightness of the photographs was maintained at a uniform level to ensure consistency among images. All the hair samples were treated and photographed analogously.
Results

As the photographs demonstrate (Figs 1-4), none of the tested hair samples showed any observable difference in color between the hair treated with the LCT formulation and that in the untreated control subsample after a total of 14 repeated 12-h applications of LCT.

Figure 1a shows human scalp hair in the untreated control subsample. Figure 1b depicts the hair after the application of the LCT formulation, followed by a 12-h incubation at room temperature. Figure 1c shows that, after 14 d of repeated treatment, washing and drying, the study hairs were completely unstained. Figures 2-4 indicate analogous findings in hair samples from sheep, rabbit and goat, respectively. None of the hair samples tested showed any observable difference in color between hair treated with the LCT formulation and untreated control subsamples after 14 d of repeated application.

Discussion

The application of medication in scalp psoriasis differs from that in other forms of psoriasis that affect the skin tissue elsewhere because, when there is hair on the scalp, it is impossible to avoid applying the medication to the hair as well as to the skin. Coal tar is a commonly employed medication for the treatment of scalp psoriasis. It is available as liquids, lotions and shampoos. The main problem associated with its use concerns the staining of hair, which represents a significant reason for discontinuing coal tar therapy.1-13

The LCT formulation tested in this study was found to be completely devoid of any propensity to stain hair, even when used in a prolonged application protocol over 2 weeks. This may be attributed to the novel design of the formulation, which includes lecithin (i.e., a phospholipid) in combination with other excipients. Lecithin seems to play a major role in the action of this formulation and, in conjunction with moisture available from the emulsifying wax, leads to the formation of supra-structures.16,17 These supra-structures serve as hosts that entrap coal tar as guest molecules at the molecular level. This entrapment prevents the coal tar from directly interacting with the surface of the hair, thus accounting for its non-staining behavior.

Therefore, given the improved esthetics afforded by this formulation’s lack of tendency to stain hair, this LCT formulation has excellent potential for achieving good rates of patient compliance during the treatment of scalp psoriasis. However, further studies are required in patients with scalp psoriasis to corroborate these findings in clinical contexts.

References

ings will contribute towards rational design of immunologically safe Aβ-binding vesicles for the management of AD.

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[M.19]
Neuroprotection by Nerve Growth Factor (NGF) involves modulation of reactive gliosis and neuronal autophagy
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Keywords: Reactive gliosis; Neuroprotection; Synaptic homeostasis; Nerve Growth Factor

Nerve Growth Factor (NGF) plays a key role in development and function of specific neuronal populations of CNS and modulation of functional neuronal networks. NGF levels are regulated by a plasmin-dependent conversion of proNGF to mature NGF (mNGF) and its rapid degradation by metalloproteinases (MMP9). Decreased NGF availability is responsible for neuronal vulnerability during neurodegenerative diseases. We have used a rat model of peripheral nerve injury induced by spared nerve injury (SNI) to investigate mechanisms linking reactive gliosis, alteration of endogenous NGF levels and modifications of synaptic homeostasis. SNI-animals were characterized by extensive reactive gliosis (GFAP immunoreactivity), increased MMP9 activity and alteration of NGF/pro-NGF levels. Immunohistochemical studies also revealed modifications of glial (GLT1) and neuronal (EAAC1) glutamate transporters, glial glycine transporter (GlyT1), vesicular glutamate (vGLUT) and GABA (vGAT) transporters and glutamatic acid decarboxylase (GAD). These changes, paralleled by alterations of neurotransmitters and GSH levels, were partially or fully restored by i.t. administration of NGF or the MMP9 inhibitor (GM6001).

These data suggest a strong correlation between decreased NGF levels, mechanisms of reactive astrogliosis, alteration of synaptic homeostasis and astrocyte-mediated neuroprotection. Mechanisms of neuroprotection by NGF were also evaluated in NGF-differentiated PC12 cells, a well-established neuronal model for NGF activity. In neurally-differentiated PC12 cells, NGF deprivation induced a progressive decrease of survival that was preceded by mitochondrial dysfunction, increased ROS production, caspases activation and DNA fragmentation. In NGF-dependent PC12 cells, NGF was also important for stabilization of mitochondrial function following oxidative stress. Interestingly, we found that neuroprotection by NGF also involved modulation of neuronal autophagy, as indicated by several markers indicative of the autophagic flux.

Overall, our data strongly suggest that mechanisms of neuroprotection by NGF involve modulation of reactive gliosis, relevant for homeostasis of neuronal circuits and astrocyte-mediated neuroprotection, as well as modulation of neuronal autophagy.

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[M.20]
Vaccinology in the post-genomic Era
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Novartis Vaccines and Diagnostics, Italy

The scope of the presentation is to illustrate how the genomic era has remarkably revolutionized the field of vaccinology. The knowledge of the genome sequence and the capacity to rapidly express hundreds of recombinant proteins have allowed scientists to scan the entire proteome of a given pathogen using appropriate in vivo/in vitro assays so as to identify the protective antigens. The approach has proven to be successful in a number of new vaccine discovery processes. However, it requires screening a large number of proteins in time-consuming biological assays in order to select candidates amenable for vaccine development. To overcome this limitation, proteomic technologies have been set up that allow to narrow down the proteins to be screened for protective activity from few thousands (the average number of proteins in bacteria) to a few tens. Future efforts will focus on further reducing this pool of antigens to only those which ultimately are to be included in the vaccine formulation.

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[M.21]
Nano-bilosomes as potential vaccine delivery system for effective combined oral immunization against tetanus and hepatitis B
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1 University Institute of Pharmaceutical Sciences, UGC-CAS, Panjab University, India
2 Department of Pharmaceutical Sciences, Dr. H. S. Gour University, Sagar, M. P., India

Keywords: Mucosal; Vaccine; Oral; Immunization

Background: The currently available parenteral vaccines against hepatitis B and tetanus fail to induce mucosal antibody response. Thus, oral immunization appears to be an efficient alternative. However, owing to the degradation of antigen in the gut, periodic administration of its larger doses is required, leading eventually to oral tolerance. The purpose of the present study, therefore, was to surmount these problems using nano-bilosomal vaccine delivery system.

Experimental: Bilosomes containing HBsAg and Ttx were prepared by thin film hydration method. HBsAg- and Ttx-loaded bilosomes were characterized in vitro for shape, size and percent antigen entrapment. Stability was determined in SIF pH 1.2, SIF pH 7.5 and aqueous solutions of bile salt in different concentrations. The in vivo study comprised of estimation of anti-HBsAg-IgG, anti-Ttx-IgG, anti-HBsAg-sIgA and anti-Ttx-sIgA following oral immunization with different combinations of HBsAg- and Ttx-loaded bilosomes in BALB/c mice.

Results and discussion: The transmission electron microscopy indicated that the vesicles were unilamellar and spherical. The mean particle size was found to be 204 ± 20 nm. The percent entrapment of HBsAg and Ttx was found to be around 23–25% and 18–20%, respectively. The studies (Table 1) demonstrated significant antigen stability in various fluids.

The high dose of HBsAg-nano-bilosomes (50 μg) and Ttx-nano-bilosomes (5Lf) combination produced higher anti-HBsAg-IgG and anti-Ttx-IgG levels in serum vis-à-vis intramuscularly adminis-
Thus improved bioprocessing and formulation should improve oral delivery. After optimization, this novel formulation can release up to 4000-fold more live cells into a bile solution than unprotected dried LBV. Fold more live cells into a bile solution than unprotected dried LBV. Defined media. To improve delivery efficiency, we invented a novel formulation to block bile toxicity that utilises bile adsorbing Resins. After optimization, this novel formulation can release up to 4000-fold more live cells into a bile solution than unprotected dried LBV. Thus improved bioprocessing and formulation should improve oral vaccine function and reduce cost of goods. We are also exploring the possibility of improved delivery of probiotic bacteria using a similar formulations.

doi: 10.1016/j.jbiotec.2010.08.256

Table 1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Percent antigen retained in nano-bilosomes in various fluids</th>
</tr>
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<tbody>
<tr>
<td>HBsAg</td>
<td>5 mM 20 mM</td>
</tr>
<tr>
<td>TTx</td>
<td>83% 90% 90% 90% 79%</td>
</tr>
<tr>
<td></td>
<td>Bile salt solutions</td>
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</tbody>
</table>

Optimal integration of active immunization approaches into standard therapies suggests DNA vaccination as an effective personalized medicine to eradicate minimal residual diseases during clinical remission following standard chemotherapy in lymphoma patients.

References


Tamoxifen-loaded liposomal topical formulation arrests hair growth in mice

A. Bhatia, B. Singh, B. Amarji and O.P. Katare

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Summary

Background For several decades, androgens have dominated endocrine research in the domain of hair growth control. However, it has long been known that oestrogens also tend to alter hair follicle (HF) growth and cycling significantly by binding to locally expressed high-affinity oestrogen receptors (ORs). Tamoxifen (TAM) is a selective OR modulator.

Objectives The current work aims to investigate the effect of topically applied TAM on the hair growth of mice.

Methods Test formulations were applied once daily on the shaved back area of the mice for a period of 5 weeks. The effect of these formulations was studied by visual and histological examinations.

Results Animals treated with saline and placebo gel formulation showed significant hair growth on the 20th day. The number and length of follicles were also found to be normal. In contrast, no hair growth was observed in the animals treated with TAM gel, even after the termination of treatment. The HFs were found to be arrested in telogen phase with clear signs of follicle dystrophy.

Conclusion The hair growth-retarding effect of TAM observed in the current study clearly demonstrates its OR agonistic effect on hair growth. This work also provides a distinct lead towards the possible potential of TAM liposomal gel in the treatment of hirsutism.
a very effective means of delivery, especially for topical administration. The current studies have been undertaken to develop a topical liposomal formulation of TAM, and to investigate its efficacy in retarding hair growth on shaved mouse skin.

Materials and methods
TAM citrate and saturated phospholipid (soy phosphatidylcholine 90H) were generous gifts from Biochem Pharmaceutical Industries (Mumbai, India) and Lipoid GmbH (Ludwigsafen, Germany), respectively. Triethanolamine (TEA) and sorbitan monoleate were procured from Sigma Chemicals Co. (St Louis, MO, U.S.A.). Carbopol® 980 was obtained ex-pets from Lubrizol Co. (Wickliffe, OH, U.S.A.). All other chemicals used in the study were of analytical grade.

Preparation of lipid vesicular systems
Drug-loaded liposomes for topical application were prepared by the thin-film hydration technique, as described previously. Briefly, a dry film of lipid (phospholipid/sorbitan monoleate 4:1 w/w) and drug were hydrated in water to obtain the liposome suspension. Separately, Carbopol® gel was prepared by dispersing it in warm demineralized water and stirring at 800 r.p.m. (Remi Mechanical Stirrer, Mumbai, India) for 30 min. This Carbopol® dispersion was neutralized by the addition of TEA at 1:1.5 w/w Carbopol®/TEA. Mixing was continued until a clear transparent gel was formed. Finally, the liposomal suspension was added to the clear gel to obtain a vesicular gel formulation for topical application. Analogously, the placebo vesicular gel formulation was prepared employing the identical composition and method of preparation, but without addition of the drug.

Animals
Adult female laca mice, aged 4–5 weeks, with mean weight of approximately 25 g, were obtained from Central Animal House, Panjab University (Chandigarh, India). They were housed in polypropylene animal cages and acclimatized for a week with a 12-h day and night cycle prior to the treatments at a temperature of 25–27 °C and a relative humidity of 50–55%. The mice were provided with a standard rodent diet.

Fig 1. Effect of various topical formulations on hair growth of laca mice after 5 weeks of treatment. (a) Photographic images of dorsal side of mice exhibiting the effect of different treatments (group I–group III) on hair growth. (b) Photomicrographs showing hair follicles (HFs) mainly in the reconstructed anagen stage VI (marked by white arrows) with normal appearance and no sign of dystrophy in group I and group II mice. However, in group III mice, most HFs are seen in the telogen phase (marked by white arrows). Micrographs also portray abnormally dilated hair canals in group III without the hair shaft (marked by yellow arrows), confirming the signs of dystrophy (original magnification x 100). (c) Skin sections shown here (original magnification x 400) are the magnified images of the corresponding images in (b).
Drinking water was provided ad libitum. Taking cognizance that the research work adheres to the guidelines for the care and use of laboratory animals, all the animal investigations were performed as per the protocol approved by the Panjab University Animal Ethics Committee, duly approved for the purpose of control and supervision of experiments on animals by the Government of India.25

Treatment
Following 1 week of acclimatization, the mice were divided into three experimental groups, each composed of eight animals, as follows: animals treated with normal saline solution (group I, control); animals treated with placebo gel (group II, blank); animals treated with active gel, equivalent to 0.1% w/w of TAM (group III, active). The formulations were applied once daily on the shaved back area of the mice for a period of 5 weeks.

Laboratory analysis
Twenty-four hours after the last application of the formulations, treated skin areas were photographed employing a digital camera (Model 550; Nikon, Tokyo, Japan), the mice were decapitated by spinal dislocation, and their carcasses incinerated. Pieces of skin were punched out at the centre of the back of each mouse using a biopsy punch (Stiefel, 6 mm diameter), and were fixed in formalin. The fixed skin sections were appropriately processed and stained with haematoxylin and eosin.26,27 The processed skin sections were examined microscopically using a high-resolution microscope attached with camera (Model 90i; Nikon) at suitable magnification(s) to judge follicular behaviour in response to the administered treatments.18,29

Results
As depicted in Figure 1 (a), the animal groups treated with saline and placebo gel (I and II) started to exhibit hair growth on the 15th day. The animals showed significant hair growth on the 20th day onwards. In contrast, no hair growth was observed in animal group III treated with TAM gel, even after the termination of treatment. In this case, the arrowed HFs were found to be in telogen phase.

As illustrated in Figure 1 (b, c), animal groups I and II showed normal skin structures and HFs (i.e. anagen VI). The number and length of follicles were also found to be normal. However, animal group III exhibited no such normal growth patterns, with the HFs found to be arrested in telogen phase with signs of follicle dystrophy.

Discussion
These results are in close agreement with those of earlier studies10–14 showing that the naturally occurring 17β-estradiol arrests HFs in telogen phase, and produces profound and prolonged inhibition of hair growth, whereas treatment with the biologically inactive stereoisomer 17α-estradiol did not alter hair growth. Thus, the hair growth-retarding effect of TAM observed in the current studies clearly demonstrates its OR agonistic effect on hair growth. The present study also reveals appreciable pharmacodynamic activity of topically applied TAM, justifying the development of its topical gel formulation. The work provides a distinct lead towards the possible potential of TAM liposomal gel in the treatment of hirsutism.

What’s already known about this topic?
• Hirsutism, a condition of excessive growth of body hair in females, particularly on visible areas such as the face and chest, often causes great psychological distress.
• The existing treatments for excessive facial hair can lead to adverse reactions such as ingrown hair and infections.
• A convenient topical formulation that reduces production of facial hair would be of significant benefit to women with this condition.

What does this study add?
• This work provides a distinct lead towards the possible potential of tamoxifen liposomal gel in the treatment of hirsutism.

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MODELLING AND SIMULATION OF ERYTHROPOIETIN EXPRESSION FOLLOWING AAV-MEDIATED GENE TRANSFER

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SIMULATION OF VALPROIC ACID CONCENTRATIONS IN PEDIATRIC POPULATION WITH SPINAL MUSCULAR ATROPHY

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EVALUATION OF THE EFFICACY AND SAFETY IN PATIENTS WITH RIGIDS AND SYMPTOMS OF OSTEARTHRITIS OF THE KNEES.

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Purpose: Non-steroidal anti-inflammatory drugs (NSAIDs) are the "Gold Standard" in the treatment of various pain and inflammation related disorders like arthritis. However, their oral administration and use of newer selective COX-2 NSAIDs known to induce severe intolerance in patients. Thus, the present work aims to compare the efficacy and safety of a epicutaneously administered naproxen gel and placebo for relief of signs and symptoms in osteoarthritis (OA) of knee.

Methods: This was a randomized, double-blind, controlled trial on 56 patients with knee osteoarthritis. They were randomly assigned to naproxen gel, marketed gel and placebo, twice a day for 6 weeks. The patients were assessed by primary efficacy outcome measures included the changes from baseline to market gel and placebo, twice a day for 6 weeks. The patients were assessed by primary efficacy outcome measures included the changes from baseline to

Results: In naproxen gel treated group, the pain, stiffness and difficulty performing routine activities showed statistically significant improvements on <5 weeks of treatment compared to the other tested gels. While, all the treatments were found to be well tolerated with no observed adverse event. Conclusion: Diclofenac in liposomal gel is superior to other tested gels. The data was best performed for Kellgren-Lawrence criteria. Safety of the gel was also assessed by evaluating adverse events, vital signs, and irritation at the application site. Results: In liposomal gel treated group, the pain, stiffness and difficulty performing routine activities showed statistically significant improvements on <5 weeks of treatment compared to the other tested gels. While, all the treatments were found to be well tolerated with no observed adverse event.

Conclusion: Diclofenac in liposomal gel had statistically significant improvements over placebo in terms of pain, stiffness, and difficulty performing routine activities on <5 weeks of treatment. The safety of the gel was also assessed by evaluating adverse events, vital signs, and irritation at the application site. Results: In liposomal gel treated group, the pain, stiffness and difficulty performing routine activities showed statistically significant improvements on <5 weeks of treatment compared to the other tested gels. While, all the treatments were found to be well tolerated with no observed adverse event.

Conclusion: Diclofenac in liposomal gel had statistically significant improvements over placebo in terms of pain, stiffness, and difficulty performing routine activities on <5 weeks of treatment. The safety of the gel was also assessed by evaluating adverse events, vital signs, and irritation at the application site. Results: In liposomal gel treated group, the pain, stiffness and difficulty performing routine activities showed statistically significant improvements on <5 weeks of treatment compared to the other tested gels. While, all the treatments were found to be well tolerated with no observed adverse event.

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NOVEL LIPOSOMAL GEL OF AN ANTI-INFLAMMATORY AGENT: RANDOMIZED, DOUBLE-BLIND CLINICAL TRIAL FOR EVALUATION OF THE EFFICACY AND SAFETY IN PATIENTS WITH SIGNS AND SYMPTOMS OF OSTEOARTHRITIS OF THE KNEES.

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Purpose: Non-steroidal anti-inflammatory drugs (NSAIDs) are the “Gold Standards” in the treatments of various pain and inflammation related disorders like arthritis. However, their oral administration and use of newer selective COX-2 NSAIDs known to induce severe intolerance in patients. Thus, the present work aims to compare the efficacy and safety of a epicutaneously applied diclofenac within novel liposomal gel vis-à-vis popular marketed gel and placebo for relief of signs and symptoms in osteoarthritis (OA) of knee.

Methods: This was a randomized, double-blind, controlled trial on 36 patients with knee osteoarthritis. They were randomly assigned to liposomal gel, marketed gel and placebo, twice a day for 6 weeks. The patients were assessed by primary efficacy outcome measures included the changes from baseline to end of study on the WOMAC (Western Ontario McMaster Universities) Osteoarthritis Index. The radiographic imaging of OA in the knee was also performed for Kellgren–Lawrence criteria. Safety of the gel was also assessed by evaluating adverse events, vital signs, and irritation at the application site.

Results: In liposomal gel treated group, the pain, stiffness and difficulty performing routine activities showed statistically significantly improvements on 6 weeks of treatment compared to the other tested gels. While, all the treatments were found to be well tolerated with no observed adverse event.

Conclusion: Diclofenac in liposomal gel is superior to other tested formulations viz. marketed gel and placebo in the relieving the symptoms of OA of the knee. Hence, it can be concluded that diclofenac in liposomal gel can be a rational alternative to oral diclofenac formulations for management of various pain and inflammation related ailments including osteoarthritis.

Citation: