

Reduction in cisplatin genotoxicity (micronucleus formation) in non target cells of mice by protransfersome gel formulation used for management of cutaneous squamous cell carcinoma

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Cisplatin-loaded protransfersome system was prepared and characterized for *in vitro* drug permeation, drug deposition and antitumor effect. A histopathological study and a genotoxicity study were also done. The skin permeation data of cisplatin from protransfersome gel formulation revealed $494.33 \pm 11.87 \mu\text{g cm}^{-2}$, which was significantly higher than that from the control plain drug solution in 0.9 % NaCl ($p < 0.001$). Untreated group of animals showed invasive moderately differentiated keratinizing squamous cell carcinoma (malignant stage). However, with cisplatin loaded protransfersome gel system simple epithelial hyperplasia (pre-cancerous stage) with no cancerous growth was observed. Also, a significant induction in micronucleus formation was found in the group that was treated with injectable intraperitoneal cisplatin preparation in 0.9 % saline as compared to the group treated with topical protransfersome gel formulation. The findings of this research work appear to support improved, site-specific and localized drug action in the skin, thus providing a better option for dealing with skin related problems like squamous cell carcinoma.

Keywords: cisplatin, protransfersome, topical chemotherapy, genetic damage, bone marrow cells, micronucleated polychromatic erythrocytes

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Transdermal delivery of drugs offers many advantages compared to traditional drug delivery systems, including oral and injectable preparations. Due to their amphiphilic nature, lipid vesicles may serve as non-toxic penetration enhancers for drugs (1, 2). However, this approach is not very successful because of poor skin permeability, breaking of vesicles, drug leakage, aggregation and fusion of vesicles. To overcome problems of poor skin permeability, a novel vesicular system, transfersomes (ultradefensible lipid vesicles) were proposed for non-invasive delivery of drugs into deep skin strata or across

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the skin (3). Transfersomes incorporate edge activators (surfactants) which destabilize lipid bilayers and increase deformability of the vesicles (4). The problem of this carrier is self-stability. In the present study, liquid crystalline, pro-ultraflexible lipid vesicles (protransfersomes), would be converted into ultraflexible vesicles *in situ* by absorbing water from the skin. Protransfersomes are reported to have better stability and superior skin penetration ability than the traditional lipid vesicles, *e.g.* liposomes, miosomes, *etc.* (5, 6). Squamous cell carcinoma is a malignant tumor of squamous epithelium. Various topical chemotherapeutic approaches are reported in the literature, such as topical treatment of actinic keratosis, actinic cheilitis, Bowen's disease by 5-fluorouracil and tretinoin, imiquimod topical delivery for management of nonfacial, nodular and superficial basal cell carcinoma (7–9). Cisplatin is approved by the FDA to treat the various types of solid tumors or carcinoma and it is specially useful in the treatment of epithelial malignancies (10).

The aim of the present study was to investigate the feasibility of a topical drug delivery system (cisplatin-loaded protransfersome gel) in the treatment of cutaneous squamous cell carcinoma and to test the protective effect of the system in cisplatin genotoxicity on non-target cell (micronucleus study).

EXPERIMENTAL

Chemicals

Cisplatin was procured as a gift sample from Cipla Ltd, India. Soya lecithin (26 % phosphatidylcholine) was supplied by Acros Organics, USA. Sodium deoxycholate was obtained from Sigma Chemical Co, USA. May-Gruenwald and Giemsa stains were obtained from Central Drug House (P) Ltd, India. All other reagents and solvents used were of reagent grade.

Animals

Male Swiss albino mice, 6–8 weeks old, 20–25 g body mass, were obtained from the animal colony of the Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, India. Animals were housed in plastic cages and provided standard pellet diet and water *ad libitum*. All investigations were performed after approval from the Animals Ethical Committee of the Jawaharlal Nehru Cancer Hospital and Research Centre, and in accordance with the disciplinary principles and guidelines of the committee for the control and supervision of experiments on animals.

Preparation of cisplatin-loaded protransfersome

Protransfersome gel was prepared by the method reported by Perrett *et al.* (11), with minor modifications. Soya lecithin, surfactant (sodium deoxycholate) and isopropanol (100 %) were placed in a clean, dry wide-mouth round bottom flask warmed in a water bath at 60–70 °C until the ingredients were dissolved. Cisplatin in phosphate buffer saline (pH 7.4) was added dropwise into the flask and warmed on a water bath until a clear solution was formed. This mixture was converted into protransfersome gel after

cooling at room temperature for an hour. The final composition contained the ratio 2:1:2 (*m/m/m*) of lipid + surfactant/alcohol/aqueous phase. Drug concentration in the formulation was 1 % (*m/m*).

In vitro permeation and skin deposition

Preparation of mouse skin. – Abdominal skin of the mice was used in permeation experiments. Mice were slightly anesthetized by ether and hairs were removed from the abdominal skin. The animals were sacrificed and their abdominal skin was separated. The skin was stored at 20 °C until the permeation study. The skin was hydrated in normal saline and its adipose tissue layer was removed by rubbing it with a cotton swab.

Permeation of the drug under non-occlusive conditions. – A Franz diffusion cell (with effective diffusion area of 3.14 cm² and 15.5 mL cell volume) was used for drug permeation studies. The skin was clamped between the donor and receptor compartments of the diffusion cell. Protransfersome gel formulation (100 mg) was applied evenly onto the skin surface. The receptor compartment was filled with a freshly prepared phosphate buffer saline (pH 7.4) solution to solubilize the drug.

The receptor compartment was stirred with a magnetic stirrer and kept at room temperature. The samples (1-mL aliquots) were collected at suitable time intervals. Samples were analyzed for drug content with a UV/visible spectrophotometer (Shimadzu-1700, Japan) at 207.5 nm after making proper dilutions. Cisplatin in 0.9 % NaCl solution was used as control formulation. Cumulative corrections were made to obtain the total amount of drug permeated at each time interval. The cumulative amount of drug permeated across the skin was determined as a function of time.

Skin deposition. – At the end of the permeation experiment (after 24 hours), the skin surface was washed five times with ethanol/PBS pH 7.4 (1:1), then with water to remove excess drug from the surface. The skin was then cut into small pieces. The tissue was further homogenized with ethanol/buffer solution pH 7.4 (1:1) and left for 6 hours at room temperature. After shaking for 5 minutes and centrifuging for 5 minutes at 5000 rpm, the cisplatin content was analyzed after appropriate dilutions with PBS (pH 7.4) at 207.5 nm.

Evaluation of anti-tumor effect

Evaluation of the anti-tumor effect was done according to the reported method (12). In assessment of the anti-tumor effect, tumor growth inhibition rate (*IR*) was calculated using squamous cell carcinoma bearing mice. The mean estimated tumor volume in the treated group (*T*) and that in the control group (*C*) were measured.

Anti-tumor activity was also determined by comparing the mean survival time (*MST*) of the treated group with that of the control group. Anti-tumor activity was expressed as an increase in life span (*ILS*).

Histopathological studies

Protocol design. – Skin tumor was induced by topical treatment with the skin carcinogen DMBA (7,12-dimethylbenz[α]anthracene) + croton oil (13). The treatment was ap-

plied topically on a shaved area. On day 45 after tumor development, the tumor-bearing male Swiss albino mice were randomly divided into 2 groups. Each group comprised 3 animals. Group 1 served as a vehicle control group (untreated control, dimethylsulphoxide, DMSO), group 2 received local treatment with protransfersome gel containing 400 µg of cisplatin in each application (400 µg per 0.2 mL per animal) 3 times a week at tumor site.

Procedure. – For histopathological studies, tumors were retrieved whole and fixed into a fixative solution. Paraffin blocks were made by embedding the tissue in hard paraffin. Sections of 5 µm were cut using a microtome. The same procedure was followed after 45 days of treatment with cisplatin-loaded protransfersome (group 2). Animals from group 2 were sacrificed for histopathological assessment. Their dehydrated tissue was mounted in the paraffin block and processed for fixation, dehydration, paraffin infiltration, staining and blotting. Tissue was mounted on the slide with the help of DPX (Hi-media Laboratories, India) (a colorless synthetic resin, mixture of distyrene, plasticizer and xylene). Stained tumor tissue and treated tumor tissue were examined using a light microscope (Olympus BX 60 with camera, Olympus America Inc., USA).

Genotoxicity study

The genotoxic effects were evaluated in the mouse bone marrow after topical application of various cisplatin-loaded protransfersome gel formulations and intraperitoneally administered cisplatin solution. A bone marrow micronucleus test was carried out for genotoxicity evaluation. The presence of micronuclei in the cell is an indicator of chromosomal damage. Genotoxicity can be assessed by the frequency of micronucleated polychromatic erythrocytes (MNPCEs) (14).

Protocol design. – The animals were divided randomly into four groups (each group containing 3 animals). The first group was used as a negative control group. The second group was treated as a positive control group and was injected intraperitoneally with cisplatin at a dose of 8 mg kg⁻¹ body mass (250 µg per 0.2 mL per animal). The third and fourth groups were topically treated with doses of cisplatin-loaded protransfersome gel of 12.5 and 15 mg kg⁻¹ body mass, respectively (300 and 400 µg per 0.2 mL per animal).

Micronucleus test. – After 24 hours of treatment/drug application, animals were sacrificed by cervical dislocation and their bone marrow cells were harvested. The slides were prepared according to the modified method of Fenech *et al.* (15) for micronucleus evaluation.

Briefly, the animals were killed and their femurs were dissected out and cut open at the distal (knee) end, inserting a needle on a syringe (1 mL) containing the solution [composition: 0.15 % EDTA, 1 mL Hank's balanced salt solution (HBBS) and 1 % bovine albumin serum] for micronucleus assay, keeping the bone below the surface of the solution in a centrifuge tube and forcing the marrow out through the opening around the needle by flushing it with the contents of the syringe. After repeated aspiration and flushing, the bone should be visibly empty of marrow. The harvested cells were centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded. A drop of the cell suspension was applied to the surface of a clean, grease-free glass slide, allowing the fixative to evaporate quickly to stick the cells firmly to the glass surface. After drying, the slides were put

into methanol for 10 minutes for fixation followed by staining with May Gruenwald and 5 % Giemsa stain for 5 minutes each. After staining, a total of 1000 cells were scored for each animal using a light microscope. The data are expressed as the average number of micronucleated cells per thousand PCE (polychromatid erythrocytes) cells per animal (\pm SE).

The data are presented in MNPCE. The polychromatid erythrocytes/normochromatid erythrocytes (PCE/NCE) ratio was also calculated.

Statistical analysis

The results were compared to the vehicle control group using one tail Student's *t*-test.

RESULTS AND DISCUSSION

Different additives were employed in the protransfersome gel formulation. Lecithin was used as the vesicle forming agent, surfactant for providing flexibility, alcohol as the solvent and aqueous phase (buffering agent) as the hydrating medium. The method of preparation is based on the principle of coacervation phase separation in which concentrated protransfersomal gel was converted into stable vesicular transfersomal dispersion by dilution with excess aqueous phase.

The cumulative amount of drug permeated from the formulation across mouse skin was significantly higher ($p < 0.001$) than that from the plain drug solution (Fig. 1). The skin permeation data obtained from the present study revealed a transdermal flux of $20.59 \pm 2.05 \mu\text{g cm}^{-2} \text{h}^{-1}$. Table I shows the residual amount of cisplatin in the skin after 24 h of administration of cisplatin-loaded protransfersome formulation and the control plain drug solution. A significantly higher amount of drug was deposited in the skin through the full PTS-I₃ formulation than from the plain drug solution ($p < 0.001$).

Anti-tumor activity was checked by estimating the tumor volume in the treated groups and the control group after a 45-day treatment. The results are shown in Tables II and III. Tumor volumes in the group treated with protransfersome formulation were significantly smaller than those of the control group ($p < 0.001$), whereas there was no significant difference between the group treated with cisplatin solution in 0.9 % NaCl and the untreated control group. Results of this study showed effective activity of protransfersome formulation at doses of 300 and 400 μg per 0.2 mL per animal with inhibition rates of 90.1 and 99.1 %, respectively (Table II), a range in which cisplatin control solu-

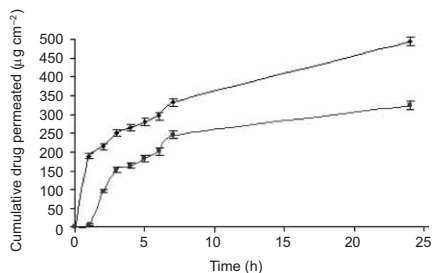


Fig. 1. *In vitro* drug permeation study: Permeation of drug across mouse skin for the protransfersome gel formulation (—■—) and control plain drug solution (—○—). Mean \pm SD, $n = 3$.

Table I. Permeability profile of cisplatin after 24 h and skin deposition from protransfersome gel formulation

Formulation	Cumulative drug permeated after 24 h (%) ^a	Cumulative drug permeated ($\mu\text{g cm}^{-2}$) ^a	Transdermal flux ($\mu\text{g cm}^{-2} \text{h}^{-1}$) ^a	Cisplatin deposited (%) ^a
Protransfersome	59.3 ± 3.8	494.33 ± 11.87 ^b	20.59 ± 2.05	43.4 ± 4.0 ^b
Saline control	38.9 ± 3.0	323.93 ± 10.95	13.49 ± 1.98	14.6 ± 3.0

^a Mean ± SD, $n = 3$.

^b Significant difference compared to control ($p < 0.001$).

Table II. Tumor growth inhibition rate after 45 days of treatment of tumor bearing mice

Group	Treatment/dose	Tumor volume (mm^3) ^a	IR (%) ^c
Control	Untreated	89.62 ± 5.78	–
Cisplatin solution in 0.9 % NaCl	400 μg per 0.2 mL per animal	64.79 ± 9.07	27.8
Cisplatin-loaded formulation	300 μg per 0.2 mL per animal	8.93 ± 2.75 ^b	90.1
Cisplatin-loaded formulation	400 μg per 0.2 mL per animal	0.78 ± 0.39 ^b	99.1

IR – Tumor growth inhibition rate.

^a Mean ± SD, $n = 3$.

^b Statistically significant difference from control group ($p < 0.001$).

tion was ineffective ($IR = 27.8\%$). Table IV summarizes the *MST* and *ILS* (%) of various groups of tumor bearing mice after topical administration of cisplatin solution in 0.9 % NaCl and cisplatin-loaded protransfersome formulation. Application of cisplatin-loaded protransfersome formulation with doses of 300 and 400 μg per 0.2 mL per animal signifi-

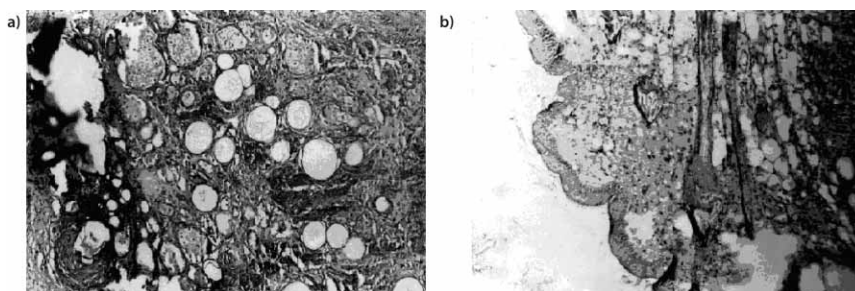


Fig. 2. Histopathological study: a) Moderately differentiated squamous cell carcinoma in which dermis is invaded by downward proliferating epidermal masses of cells which show atypical features and a few horn pearls with central laminated keratin are present in the skin of mice that received skin carcinogen, dimethyl benzanthracene (DMBA) + croton oil. b) Simple epithelial hyperplasia with no cancerous growth in the skin of mice which received local treatment of cisplatin-loaded protransfersome gel formulation.

cantly ($p < 0.001$) prolonged the MST (57.3 ± 2.8 and 63.3 ± 3.6 days, respectively) compared to the untreated control and cisplatin-solution in 0.9 % NaCl.

The animals treated with DMBA + croton oil (skin carcinogen) showed about 8-mm sized gross tumors at the site of application. The histopathological report of skin tumor suggests invasive moderately differentiated keratinizing squamous cell carcinoma in one animal of group 1 and in other animals early invasive cell carcinoma, which comprised vacuoles and bag-like structures containing malignant squamous cells (Figs. 2a and b). In group 2, when cisplatin-loaded protransfersome gel was applied, most of the tumors regressed and only skin scars remained, which was histopathologically observed. This observation indicates simple epithelial hyperplasia with no cancer growth.

Cisplatin delivery through the novel topical drug carrier system at doses of 300 and 400 μg per 0.2 mL per animal provided protection against micronucleus formation in bone marrow cells when compared with the positive control group (intraperitoneally administered cisplatin at a dose of 250 μg per 0.2 mL per animal). The main purpose of the micronucleus test is to determine the number of MNPCE. A significant difference ($p < 0.050$) was found in micronucleus formation between cells that were treated topically with cisplatin-loaded protransfersome formulation and the positive control group. Results obtained in this study are given in Table IV. It is noteworthy that differently treated groups were not cytotoxic (in terms of PCE/NCE ratio) compared to the solvent control group.

Table III. Increase in life span after 45 days treatment of tumor bearing mice

Group	Treatment/dose	MST (days) ^a	ILS (%)
Control	Untreated	40.8 ± 7.4	–
Cisplatin solution in 0.9 % NaCl	400 μg per 0.2 mL per animal	47.8 ± 4.0	17.1
Cisplatin-loaded formulation	300 μg per 0.2 mL per animal	57.3 ± 2.8	40.5
Cisplatin-loaded formulation	400 μg per 0.2 mL per animal	63.3 ± 3.6	55.2

MST – Mean survival time; ILS – Increase in life span.

^a Mean \pm SD, $n = 3$.

Table IV. Comparative study of micronucleus formation in mouse bone marrow cells

Group	Treatment/dose	MNPCE ^d	PCE/NCE ratio ^d
Group 1 ^a (vehicle control)	solvent treated	0.75 ± 0.50	0.95 ± 0.018
Group 2 ^b (positive control)	250 μg per 0.2 mL per animal	6.84 ± 2.28	1.34 ± 0.25
Group 3 ^c	300 μg per 0.2 mL per animal	1.50 ± 0.58^e	0.92 ± 0.15
Group 4 ^c	400 μg per 0.2 mL per animal	1.65 ± 0.77^e	1.34 ± 0.25

MNPCE – micronucleated polychromatid erythrocyte, PCE/NCE – polychromatid erythrocytes/normochromatid erythrocytes.

^a DMSO

^b Positive control group treated intraperitoneally with cisplatin in 0.9 % saline.

^c Topical treatment with protransfersome gel.

^d Mean \pm SE, $n = 3$.

^e Statistically significant difference from the respective positive control group ($p < 0.050$).

CONCLUSIONS

Protransfersome provides better non-invasive delivery of cisplatin in cutaneous squamous cell carcinoma. The findings of the research also appear to support improved, site-specific and localized drug action in the skin, thus providing a better option for dealing with skin related problems like squamous cell carcinoma. This potential drug delivery system has shown protection against cisplatin genotoxicity and cytotoxicity. It opens numerous challenges and research strategy for future development of novel improved therapies.

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S A Ž E T A K

Smanjenje genotoksičnosti cisplatina (stvaranje stanica s mikronukleusom) u neciljnim stanicama miša upotrebom gela s protransfersomima u terapiji karcinoma skvamoznih stanica kože

VANDANA GUPTA, RAMESH CHANDRA AGRAWAL i PIYUSH TRIVEDI

U radu je opisana priprema protransfersoma s cisplatinom te njihova evaluacija: permeacija lijeka *in vitro*, odlaganje lijekovite tvari, histopatološke studije, antitumorski učinak i genotoksično djelovanje. Permeacija kroz kožu iz protransfersoma bila je $494,33 \pm 11,87 \mu\text{g cm}^{-2}$, što je značajno više nego iz otopine lijeka u 0,9 % otopini NaCl ($p < 0,001$). Uz terapiju protransfersomima pojavila se obična hiperplazija epitela (prekancerozno stanje), dok su se u skupini netretiranih životinja pojavile invazivne, umjerenno diferencirane skvamozne stanice karcinoma (maligno stanje). Osim toga, stvaranje stanica s malim jezgrama u skupini životinja koje su tretirane intraperitonealnim injekcijama cisplatina u fiziološkoj otopini bilo je značajnije u odnosu na skupinu koja je tretirana s topičkom formulacijom protransfersoma. Rezultati rada pokazuju da je formulacijom s protransfersomima postignuto poboljšano, specifično i lokalizirano djelovanje u koži, što pruža mogućnost boljeg rješavanja problema kao što je karcinom skvamoznih stanica.

Ključne riječi: cisplatin, protransfersom, topička kemoterapija, oštećenje gena, stanice koštane srži, polikromatski eritrociti s mikronukleusom

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