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Effect of polyamidoamine dendrimer G3 and G4 on skin permeation of 8-methoxypsoralene—In vivo study

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ABSTRACT

In the present study we have assessed the ability of (PAMAM) dendrimers G3 and G4 to facilitate transdermal delivery of 8-methoxypsoralen (8-MOP) in vivo. In vitro study using Franz diffusion cell revealed an enhanced transdermal flux for 8-MOP in complex with G3 and G4 dendrimer in relation to standard 8-MOP solution. In present study in vivo skin permeation potential of 8-MOP complex with G3 and G4 PAMAM dendrimer was assessed using confocal laser scanning microscopy (CLSM), which revealed an enhanced permeation of the 8-MOP to the deeper layers of the skin and significantly higher concentration in comparison with standard 8-MOP solution. Skin tissue 8-MOP concentration, evaluated by HPLC indicates that G3 and G4 PAMAM application significantly increase 8-MOP skin deposition in comparison with standard 8-MOP solutions after 1 and 2 h. G4 appeared to be a more effective 8-MOP penetration enhancer than G3 PAMAM. Our results suggest the feasibility of G3 and G4 PAMAM dendrimers for transdermal delivery of 8-MOP resulting in better skin permeation and higher concentration of 8-MOP in epidermis and dermis of the drug that could help to improve effectiveness and safety of PUVA therapy.

1. Introduction

Photochemotherapy with psoralens and long-wavelength ultraviolet (UVA) radiation (PUVA) is an essential treatment for a variety of skin diseases. The psoralen mainly used in PUVA is 8-methoxypsoralen (8-MOP). 8-MOP is frequently administered orally because systemic PUVA therapy is a very effective way of treatment and easy to perform. It is now well established that oral administration of 8-MOP is associated with gastrointestinal side-effects and increases risk of serious complications such as carcinogenesis or glaucoma (Stern and Laird, 1994; Stern and Väkevä, 1997).

To reduce the side-effects of systemic PUVA, topical PUVA therapy has been developed. Currently available formulation of 8-MOP for topical use: solutions, emulsions and creams do not provide good transdermal permeability of 8-MOP and do not allow its penetration to deeper layers of the skin (Grundmann-Kollmann et al., 2001; Lüftl et al., 1997; De Rie et al., 1995).

Higher concentration of 8-MOP delivered to the skin allows to reduce UVA radiation dosage and side effects. Therefore the development of novel drug systems formulated with 8-MOP which improve their permeability and allow treatment of skin disorder with PUVA more safely and effectively is needed. Dendrimers seem to be good candidates for this purpose since they are known to possess capacity to improve solubility and bioavailability of several drugs and are able to release drug in a controlled manner (Yiyun and Tongwen, 2005; Chauhan et al., 2003; Gajbhiye et al., 2009). Recently polyamidoamine (PAMAM) dendrimers have been proven to effectively improve transdermal penetration of 5-fluorouracyl, ketoprofen, diflunisal, and 5-ALA (Venuganti and Perumal, 2008, 2009).

Our in vitro study showed that PAMAM G3 and G4 dendrimers enhanced 8-MOP permeation through prepared pig ear skin or polivinyldifluoride (PVDF) membrane (Borowska et al., 2010).

The aim of this study was to investigate the potential of PAMAM G3 and G4 dendrimers as transdermal drug carriers facilitating permeability of 8-MOP through the skin of experimental animal in vivo.

2. Materials and methods

2.1. Animals procedures

All investigations were performed as approved by the Institutional Ethical Committee of Medical University of Lublin. Male Rat Wistar 8- to 10-week-old, weight of 350–450 g were used

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in vivo study .The animals were kept in well-spaced ventilated cages and maintained on healthy and fixed diets prior to the studies for 16 h. The cages were placed in a quiet, temperature and humidity-controlled room (23 ± 3 C and 45 ± 5%) in which a 12/12 h light–dark cycle was maintained. While the animals were anesthetized with Urethane (5 ml/kg of 20% solution), the dorsal area (6 cm × 6 cm) of each rat was shaved prior to irradiation and/or dosing to remove as much fur as possible. The animals were divided into 6 groups with 5 rats in each group. Drug (1% 8-MOP) preparations in a volume of 1 ml were applied to the exposed skin and spread over the entire area using the side of a pipette tip.

Group I was administrated without any drugs and was used as a negative control; II. 8-MOP; III. 8-MOP + PAMAM G3; IV. PAMAM G-3; V. 8-MOP + PAMAM G4; VI. PAMAM G4. Tested formulations were applied for 1 h and then removed by wiping with dichloromethane. The same experimental protocol was repeated after 2 h application of tested 8-MOP formulation. Each animal was used only once.

2.2. Confocal laser scanning microscopy

Depth and mechanism of skin penetration of 8-MOP was investigated using confocal laser scanning microscopy (CLSM). The rat was sacrificed by decapitation under chloroform anesthesia; dorsal skin was excised and washed with distilled water. The full skin thickness was optically scanned at different increments through the *z*-axis of a CLSM microscope Axiovert 200 (Carl Zeiss, Germany) with an attached LSM 5 Pascal Zeiss epifluorescence microscope. Optical excitations were carried out with a 488 nm Argon laser beam and fluorescence emission was detected above 560 nm for RR. Depth of penetration of 8-MOP and the intensity of fluorescence (MFI) was noted. In order to evaluate the penetration of tested PAMAM dendrimers, both G3 and G4 dendrimers were conjugated with fluorescein as described previously (Filipowicz and Wołowiec, 2011), which allowed its detection in CLSM. By using special dyes of high affinity to nucleus-7-aminoactinomycin (7-AAD) and HCS CellMask™ Deep Red Cytoplasmic/Nuclear Stain confocal microscopy allowed to visualize cellular distribution of all 8-MOP formulation tested.

7-Aminoactinomycin D (7-ADD, Invitrogen USA) possesses strong affinity to double-stranded DNA and was used as a fluorescent marker for DNA for identification of cellular nuclei. HCS CellMask™ Deep Red Cytoplasmic/Nuclear Stain (Invitrogen USA) is designed for mammalian cells and produces labeling of the entire cell. Moreover, it allows for accurate identification of nucleus and cytoplasm. Skin samples were prepared according to manufacturer recommendation.

2.3. Analysis of 8-MOP concentrations in skin sections

HPLC – was employed for the determination of 8-MOP concentration in skin sections. Rat skin obtained punch via biopsy 6 mm diameter (6 pieces for each sample). 8-MOP was extracted with dichloromethane from samples of skin after its sonification.

Samples were cleaned with BakerBond octadecyl SPE microcolumns (500 mg, 3 mL; J.T. Baker, Phillipsburg, NJ, USA) previously activated with 10 mL methanol then 10 mL water.

Each extract was mixed with water to furnish 80% (v/v) aqueous solutions of the methanol extracts and these (10 mL) were then filtered through the columns under reduced pressure (SPE-12G chamber; J.T. Baker, Groß-Gerau, Germany). The eluates obtained were free from ballast compounds and contained free phenolic acids and aglycones.

HPLC was performed with an Agilent 1100 system coupled with an autosampler, a column thermostat, and a diode-array detector (DAD). Compounds were separated on a 250 mm × 4.6 mm stainless steel column packed with 5 μm Hypersil BDS C18 (Shandon, UK), with the use of a stepwise mobile phase gradient prepared from methanol (A) and water (B). The gradient was: 0 min, 50% A in B; 5 min, 60% A in B; 25 min, 80% A in B; 30–40 min, 100% A. The flow rate was 1 ml/min, the column temperature was 25 °C. The LC pumps, autosampler, column oven, and DAD were monitored and controlled by use of HP Chem Station rev. 10.0 software (Agilent). Compounds were identified by comparison of retention time and UV spectra with those of appropriate standards analyzed under the same conditions. Quantitative determination was performed at 254 nm (Skalicka-Woźniak et al., 2009).

2.4. Statistical analysis

Statistical analysis of differences between different treatments was performed using one-way analysis of variance Anova test with post-test Tukey–Kramer Multiple Comparisons Test. *P*-value is the probability that the null hypothesis that the data are sampled from populations with the same mean is false. 0.05 level of probability was taken as the level of statistical significance.

3. Results and discussion

3.1. HPLC analysis

Analysis by HPLC of 8-MOP content in the obtained skin samples of experimental animal revealed significantly higher 8-MOP concentration after its topical application in complex with G3 and G4 PAMAM in comparison with standard solution. After 2 h exposition to all the tested formulation, concentration of 8-MOP in rat skin was higher than after 1 h (Table 1). We did not detect the presence of 8-MOP in blood serum of animals after 1 and 2 h treatment of tested 8-MOP formulas (Table 2).

3.2. Confocal laser scanning microscopy

The visualization of 8-MOP penetration depth was carried out using CLSM. CLSM microscopic studies allowed also to measure the intensity of fluorescence, which reflects 8-MOP concentration revealed as increased fluorescence intensity and the depth of penetration after 1 h of application of 8-MOP loaded PAMAM G3 and G4 dendrimers as compared to standard 8-MOP solution. Both epidermal and dermal levels of 8-MOP increased for further 2 h after application of all tested 8-MOP formulation and achieved intensity of fluorescence was higher after its applications with PAMAM dendrimers in the following order: G4 > G3 > standard 8-MOP.

Both tested PAMAM dendrimers appear to enhance penetration of 8-MOP to epidermis and dermis in comparison with standard formulation. The G4 PAMAM seems to be more effective than G3. These results are in agreement with in vitro study, where the highest transdermal flux was achieved with G4 > G3 > 8-MOP. Using PAMAM G3 and G4 dendrimers conjugated with fluorescein confocal microscopy also revealed that both tested dendrimers possess the ability to penetrate to deep layer of the skin along with 8-MOP conjugated with dendrimers.

1 h after topical application of standard 8-MOP solutions fluorescence was detected at a border between *pars papillaris* and *reticularis cutis*. After 2 h the depth of penetration did not increase substantially, although intensity of fluorescence was higher in all affected skin layers. After the application of the 8-MOP complex with PAMAM G3 and G4 the 8-MOP fluorescence of the skin was observed in all skin layers and reach subcutaneous fatty tissue. The depth of penetration did not differ significantly after 1 and 2 h and was the same after application of complex with G4 and G3 although fluorescence intensity was higher in case of G4. No fluorescence from the skin was observed for G3 and G4 free of 8-MOP.

Table 1
Concentration of 8-MOP in ng/g in the rat skin samples obtained via biopsy 1 and 2 h after topical application of tested formulas. 8-MOP in the basis was used as a control. Results are presented as mean ± SD.

Time [h]	8-MOP	G3-8-MOP	G4-8-MOP	*P* value between columns	*P* value 8-MOP vs G3-8-MOP	*P* value 8-MOP vs G4-8-MOP	*P* value G3-8-MOP vs G4-8-MOP
1	1892 ± 195	4295 ± 534	5492 ± 592	0.001	0.0050	0.0070	0.0400
2	5745 ± 500	16899 ± 945	22532 ± 1234	0.001	0.0009	0.0001	0.0100

Table 2
Mean fluorescence intensity (MFI) of skin layers obtained by biopsy 1 and 2 h after topical application of tested formulas. Results are presented as mean ± SD.

Location; time [h]	8-MOP	G3-8-MOP	G4-8-MOP	*P* value between columns	*P* value 8-MOP vs G3-8-MOP	*P* value 8-MOP vs G4-8-MOP	*P* value G3-8-MOP vs G4-8-MOP
Epidermis; 1 h	38 ± 8	46 ± 9	52 ± 9	0.0001	0.0100	0.0010	0.5000
Epidermis; 2 h	40 ± 9	47 ± 9	54 ± 8	0.0001	0.0500	0.0010	0.0500
Dermis; 1 h	12 ± 5	20 ± 7	25 ± 7	0.0001	0.0100	0.0010	0.0500
Dermis; 2 h	18 ± 6	41 ± 9	47 ± 9	0.0001	0.0100	0.0010	0.0100

By using special dyes of high affinity to nucleus-aminoactinomycin (7-AAD) we found that 8-MOP accumulate mostly within nucleus (Fig. 1).

PUVA photochemotherapy is widely used to treat skin diseases, such as psoriasis, mycosis fungoides, and vitiligo. Although high dose of 8-MOP applied during PUVA orally might have deleterious side-effects, it provides a very good penetration to all skin layers (Stern and Väkevä, 1997). 8-MOP given topically poorly penetrates through the skin to its deeper layers which restricts its effectiveness in diseases such as lymphomas, sclerodermia and requires high dose of UVA radiation, witch essentially increases risk of side effects, such serious as carcinogenesis (Young, 1990). Consequently it seems to be essential for PUVA therapy to develop the carrier which enables more efficient 8-MOP penetration through skin and helps to achieve concentration of 8-MOP in the skin

In this study, we investigated in vivo the skin permeation of 8-MOP encapsulated in G3 and G4 PAMAM dendrimers. The tissue concentration of 8-MOP were analyzed in the whole skin tissue by HPLC after 1 and 2 h following skin application of tested 8-MOP formulation. Detailed distribution of 8-MOP in skin layers and cellular structures were analyzed using confocal microscopy with the application of appropriate dyes 7-AAD. Analytical data obtained by confocal microscopy and HPLC in the present study confirmed our previous in vitro observation that G3 and G4 PAMAM dendrimers significantly enhance transdermal delivery of 8-MOP in relation to its standard formula (Borowska et al., 2010). The depth of 8-MOP penetration and its attained concentration in epidermis and dermis was enhanced by both G3 and G4 dendrimers, the G4 being more effective than G3. Hairless rat skin was proved to be a good model for transdermal drug delivery studies due to similarity between the rat and human skin (Morimoto et al., 1992).

Confocal microscopy additionally visualized precisely the distribution of 8-MOP in the skin and proved that 8-MOP accumulated mostly in skin cell nuclei.

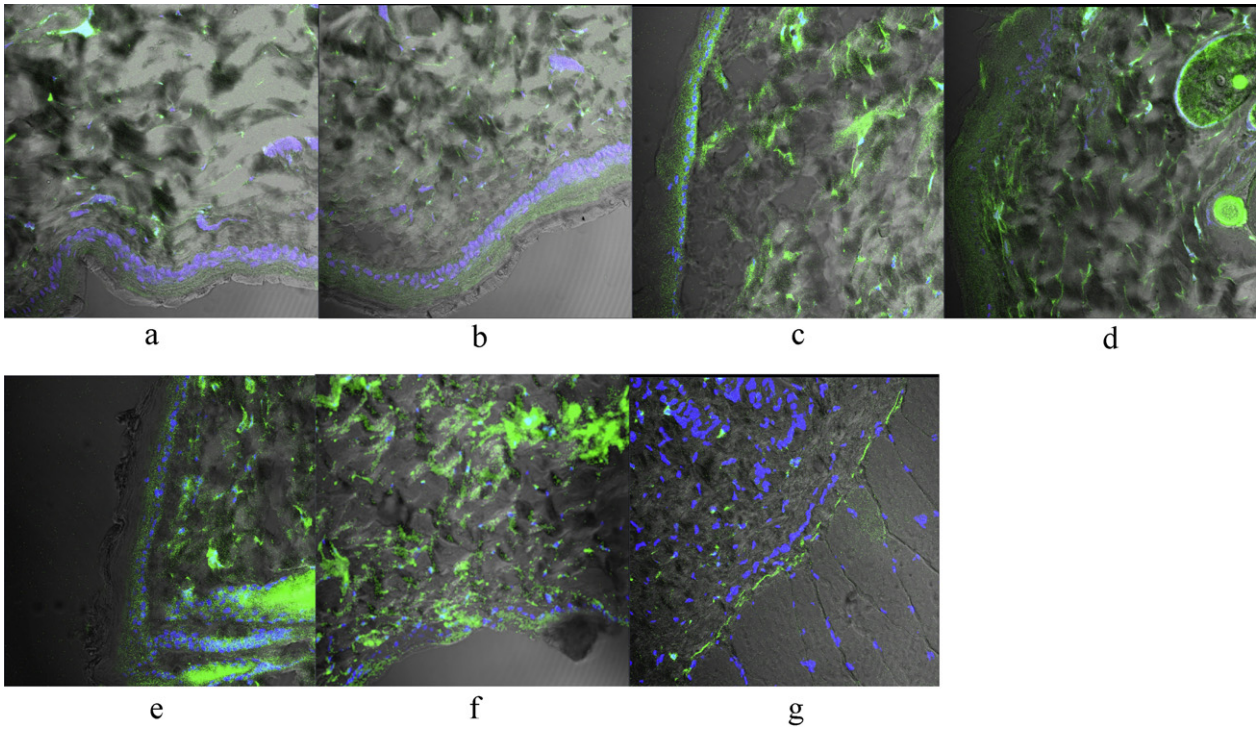

Fig. 1. Distribution of 8-MOP (green) in rat's skin samples obtained by confocal microscopy following skin application of tested 8-MOP formulation. Cellular nuclei were counterstained with 7 AAD (blue): (a) 8-MOP after 1 h; (b) 8-MOP after 2 h; (c) 8-MOP-G3 PAMAM after 1 h; (d) 8-MOP-G3 PAMAM after 2 h; (e) 8-MOP-G4 PAMAM after 1 h; (f) 8-MOP-G4 PAMAM after 2 h; (g) 8-MOP-G4 PAMAM after 2 h – all skin layers and subcutaneous fatty tissue.

It seems very important that 8-MOP exert its phototherapeutic activity mostly via inhibition of DNA replication. This is accomplished by intercalation of the psoralen between adjacent base pairs in the DNA duplex followed by photocycloaddition reactions generating a psoralen diadduct ND cross-links of DNA strands. Using PAMAM G3 and G4 dendrimers conjugated with fluorescein confocal microscopy also revealed that both dendrimers possess the ability to penetrate to deep layers of the skin along with 8-MOP encapsulated in dendrimers. According to our knowledge this is the first observation indicating the transdermal properties of PAMAM G3 and G4. Until now there are very few reports on the use of dendrimers for topical/transdermal drug delivery. Wang et al. (2003) found that G3 PAMAM dendrimers can be used to enhance penetration of hydrophilic tamsulosin. G4 (PAMAM) dendrimer effectively enhanced transdermall penetration and solubility of hydrophobic and lipophilic drug indomethacin (Chauhan et al., 2003). The exact mechanism by which PAMAM dendrimers improve transdermal delivery of drugs is still not fully clarified. It is possible that the large number of cationic charges of amine groups (Diallo et al., 2004) on the surface of G3 and G4 dendrimers could alter the skin barrier function and help 8-MOP–PAMAM dendrimer complexes to pass through (Chauhan et al., 2003). Cationic dendrimers like G3 and G4 might interact with negatively charged biological membranes and skin, which are negatively charged at physiological pH (Hong et al., 2004; Burnette and Ongpipattanakul, 1987) and might change the ionic composition of the skin and increase their permeability. G3 and G4 PAMAM dendrimers can increase the skin permeation of lipophilic drugs such as 8-MOP by increasing the drug's aqueous solubility (Chauhan et al., 2003; Cheng et al., 2007), which was achieved by facile preparation described previously (Borowska et al., 2010). There are several possible mechanisms of increased penetration of drugs across cellular and biological membranes by PAMAM dendrimer described previously (Cheng et al., 2008). Cationic dendrimers can interact with cellular lipids – hydrophilic head groups of ceramides and fatty acids (Venuganti and Perumal, 2008, 2009), change the architecture of the lipid bilayer (Shcharbin et al., 2006) and even induce hole formation in cell membranes (Mecke et al., 2004), which can contribute to increased transmembrane drug permeation. G4 PAMAM interacts with hydrophylic head groups of phospholipids and incorporates in the lipid bilayer, alters its structure and fluidizes the lipid bilayer (Gardikis et al., 2006) resulting in better membrane permeability.

Since the effectiveness of PUVA therapy and necessary dose of ultraviolet A irradiation depend on 8-MOP concentration in the skin, it seems that higher 8-MOP concentration achieved by its application with G4 or G3 PAMAM carriers will allow to reduce patients UVA exposure (Tanew et al., 2001) and thus could contribute to improved effectiveness and safety of PUVA therapy.

4. Conclusions

1. G3 and G4 PAMAM dendrimers are effective enhancers of transdermal delivery of 8-MOP resulting in higher concentration of 8-MOP in epidermis and dermis in relation to standard 8-MOP solution.
2. G4 PAMAM allows to achieve higher skin concentration of 8-MOP than G3 PAMAM dendrimer.

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