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Title: Combining Poly-Arginine with the Hydrophobic Counter-anion 4-(1-Pyrenyl)-Butyric Acid for Protein Transduction in Transdermal Delivery

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Corresponding Author: Dr. Hiroyuki Michiue, M.D. Ph.D

Corresponding Author's Institution: Okayama University

First Author: GERILE CANDAN, M.D.

Order of Authors: GERILE CANDAN, M.D.; Hiroyuki Michiue, M.D. Ph.D.; Sanae Ishikawa; Atsushi Fujimura, M.D.; Keiichiro Hayashi; Atsuhito Uneda, M.D.; Akiko Mori, Ph.D.; Iori Ohmori, M.D. Ph.D.; Tei-ichi Nishiki, Ph.D.; Hideki Matsui, M.D. Ph.D.; Kazuhito Tomizawa, M.D. Ph.D.

Abstract: Topical therapy is the most favored form of treatment for whitening against hyperpigmentation and sunburn because it lends itself to self-administration, patient compliance, and absence of systemic adverse effects. However, transdermal delivery of hydrophilic chemicals is difficult. The main purpose of this study is to develop a delivering system of hydrophilic drugs and proteins across the skin. Hydroquinone (HQ), a well-known tyrosinase inhibitor and antimelanogenesis compound, and enhanced green fluorescent protein (EGFP) were fused with eleven poly-arginine (11R). Both HQ-11R and EGFP-11R were efficiently delivered in B16 cells, a mouse melanoma cell line. HQ-11R was as effective as HQ alone at inhibiting melanin synthesis in B16 cells. EGFP-11R was efficiently delivered into cells of the epidermis with 4-(1-pyrenyl)-butyric acid (PB), a counteranion bearing an aromatic hydrophobic moiety, in vivo, but EGFP alone or EGFP-11R without PB was not. Finally, topical application of HQ-11R with PB significantly inhibited UV irradiation-induced pigmentation in guinea pigs compared with HQ alone. These results suggest that topical therapy using poly-arginine in combination with PB is useful for the delivery of hydrophilic drugs and proteins by the transdermal route.

Abstract

Topical therapy is the most favored form of treatment for whitening against hyperpigmentation and sunburn because it lends itself to self-administration, patient compliance, and absence of systemic adverse effects. However, transdermal delivery of hydrophilic chemicals is difficult. The main purpose of this study is to develop a delivering system of hydrophilic drugs and proteins across the skin. Hydroquinone (HQ), a well-known tyrosinase inhibitor and antimelanogenesis compound, and enhanced green fluorescent protein (EGFP) were fused with eleven poly-arginine (11R). Both HQ-11R and EGFP-11R were efficiently delivered in B16 cells, a mouse melanoma cell line. HQ-11R was as effective as HQ alone at inhibiting melanin synthesis in B16 cells. EGFP-11R was efficiently delivered into cells of the epidermis with 4-(1-pyrenyl)-butyric acid (PB), a counteranion bearing an aromatic hydrophobic moiety, *in vivo*, but EGFP alone or EGFP-11R without PB was not. Finally, topical application of HQ-11R with PB significantly inhibited UV irradiation-induced pigmentation in guinea pigs compared with HQ alone. These results suggest that topical therapy using poly-arginine in combination with PB is useful for the delivery of hydrophilic drugs and proteins by the transdermal route.

1. Introduction

Transdermal drug delivery has several advantages over other administration routes including convenience for the patient, avoidance of hepatic metabolism, and easy withdrawal of treatment if necessary [1]. Despite extensive studies of transdermal drug delivery, only a few drug formulations are commercially available [1,2]. Almost no hydrophilic chemicals and proteins can be delivered by transdermal systems. One reason for this is that the stratum corneum acts as a barrier to exogenous substances [1]. Another reason is that hydrophilic chemicals and proteins have no ability to penetrate cell membranes [3]. To develop transdermal drug delivery systems, these problems must be overcome using appropriate physical and chemical means. To enhance the ability of drug molecules to penetrate skin, several methods have been reported [4]. Occlusive dressing, a technique which hydrates the stratum corneum, is perhaps the simplest approach [4,5]. Chemical enhancers such as dimethylsulphoxide (DMSO), Azone, oleic acid, propylene glycol, terpenes and terpenoids that improve the diffusion coefficient of a substance in the stratum corneum by interacting with intercellular lipids have been studied [4,6]. As physical modifications, iontophoresis, which promotes skin permeability by using electropotential energy, electroporation, which produces small pores on the surface of the stratum corneum by adding electric pulses,

sonophoresis, which utilizes the cavitation of ultrasonic waves, and microneedles which produce new permeation routes, have been studied [4,6]. However, both chemical and physical enhancers to induce irritation, cause damage, and reduce the skin barrier function [4]. Recent studies showed that liposome formulations are effective at delivering chemicals and functional molecules through the stratum corneum. However, they probably increase penetration only through the transappendageal route [6,7]. It is desirable to deliver therapeutic agents to cells across the stratum corneum while main taining the normal skin barrier function.

Protein transduction system is a widely accepted method of delivering proteins, peptides, siRNA and biologically active compounds across the cell membrane by fusing with cell-penetrating peptides (CPPs) such as poly-arginine and the protein transduction domain of TAT [8-10]. This methodology attracted our attention not only as a means of cell biological study but also because of its potential for pharmaceutical vectors [9]. CPP-fused proteins are rapidly internalized by lipid raft-dependent macropinocytosis [11]. After internalization via the macropinocytotic pathway, the proteins are carried to macropinosomes, where most of them are then degraded [11]. Recent studies have shown that the ability to cross lipid bilayers and gain access to the cell interior of CPPs, especially poly-ariginine, is enhanced in the presence of the hydrophobic counter-anion

4-(1-pyrenyl)-butyric acid (pyrenebutyrate, PB) [12,13]. The negatively charged counteranions and high hydrophobicity of PB can exert a great influence on the translocation behavior of arginine peptides in artificial membranes [12,13]. These features of the combination of poly-arginine and pyrenebutyrate are thought to be useful for the transdermal delivery of hydrophilic chemicals.

Hydroquinone (HQ) is a tyrosinase inhibitor and a hydrophilic antimelanogenesis compound used as an active ingredient in cosmetics and pharmaceuticals [14-16]. In the present study, we investigated whether protein transduction using poly-arginine in combination with PB was capable of delivering functional hydrophilic molecules and proteins into skin.

2. Materials and methods

2.1. *Cell Culture*

Mouse B16 melanoma cells (B16-4A5) were provided by the European Collection of Cell Culture (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM, Life Technologies, Grand Island, NY) with 10% fetal calf serum (Life Technologies), 100 U/ml penicillin, 100 U/ml streptomycin and 0.2 % L-glutamine (Life Technologies). Cultures were maintained at 37 °C in 95% air and 5% CO_2 in a humidified incubator.

2.2. *Peptide and HQ-11synthesis*

1) Cys(Npys)-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg- Arg-Arg-Arg-NH_2

Peptide derivatives were assembled using an Applied Biosystems model 433 peptide synthesizer with Rink Amide MBHA Resin (0.34 mmol/g, 0.25 mmol) as the starting solid support. The protected peptide resin was treated with a deprotecting reagent [TFA-TIS-H_2O (95/2.5/2.5/, v/v)] at room temperature for 2 h. The crude S-Npys derivatives were isolated and purified by RP-HPLC. The purified Npys was used for the final disulfide formation procedure.

2) Cys[$(OH)_2C_6H_4S$-]-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH_2·11TFA

HQ was conjugated with poly-arginine peptides as shown in Fig. 1. To an aqueous solution (1.2 ml) of Cys(Npys)-11Arg-NH_2 (60 mg) was added $(OH)_2C_6H_4SH$(2-mercaptohydroquinone, 3.6 mg, 0.8 eq) with stirring at room temperature overnight under an Argon gas atmosphere. After the reaction was over, the reaction mixture was directly subjected RP-HPLC [YMC ODS column (30 x 250 mm, 0.1%TFA/H_2O)]. The desired peptide was obtained as a TFA salt (25 mg), as confirmed by RP-HPLC and Mass spectral analysis. Cys[$(OH)_2C_6H_4S$-]-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-NH_2·11TFA; Mol wt : 3232.6 as TFA salt, C75H144N46O14S2 MW:1978.4, MS analysis : m/z 660.4(M 3H)3, m/z 495.6([M 4H]4), Purity : 94.5%.

2.3. *Protein/peptide transduction into cells*

The transduction of protein and peptide into cells was carried out as described previously [12]. Briefly, cells were plated onto dishes (diameter 3 cm) and incubated in D-MEM containing 10% FBS (Life Technologies), 1% penicillin and streptomycin for 48 h in a humidified atmosphere containing 5% CO_2. After removal of the medium, the cells were washed twice with PBS and incubated with 50 μM 4-(1-pyrenyl)-butyric acid (PB, Sigma-Aldrich, St. Louis, MO) in PBS for 2 min at 37 ˚C. Cells were then

replaced with new PBS and protein or peptide dissolved in PBS was added. After 20 min, the cells were washed twice with PBS and incubated further in new medium for the period indicated.

2.4. *Cell Viability Assay*

Cell viability was determined using a CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI) following the manufacturer's instructions. After the application of HQ and HQ-11R, B16 cells (1×10^3 per well) seeded onto 96-well plates were cultured in D-MEM containing 10% fetal bovine serum, and 1 % penicillin and streptomycin for 24 h. After being washed with PBS, the cells were placed in fresh D-MEM and incubated further for 24, 48 and 72 h**.** Cell viability was measured using a CellTiter-Glo® Luminescent Cell Viability Assay kit with microplate luminometer device [MicroLumat Plus LB 96V, Berthold technologies, (Bad Wildbad, Germany]].

2.5. *Expression and purification of recombinant forms of EGFP and EGFP-11R*

The recombinant forms of enhanced green fluorescent protein (EGFP) and eleven poly-arginine-fused EGFP (EGFP-11R) were produced as described previously [17].

2.6. *Confocal laser microscopic analysis*

Cells (2 x 10^5) were plated onto 35-mm-diameter glass-bottomed dishes (Iwaki, Tokyo, Japan) coated with laminin and cultured for 48 h. Cells were preincubated with 50 μm PB or PBS for 2 min at 37 ˚C. 20 min after the transduction of 5 μM EGFP and EGFP-11R, the cells were washed twice with PBS and the medium was replaced with D-MEM containing 10% (v/v) calf serum, 1% penicillin and streptomycin. The EGFP signals in living cells were observed using a confocal laser microscope (FV300, Olympus, Tokyo, Japan) equipped with a 60 x objective lens at 0.5, 2, 4 and 8 h after protein transduction.

2.7. *Western blotting analysis*

Western blotting was carried out at high stringency, essentially as described previously [18]. The harvested cells were lysed by a sonicator in a boiled buffer containing 1% SDS. The cell lysate (50 μg) was subjected to SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). The blots were probed with primary antibodies against tyrosinase (1:100) (Abcam, Tokyo, Japan). Immunoreactive bands were visualized by enhanced chemiluminescence using

ECL plus (GE Healthcare UK Ltd, Buckinghamshire, England) and Bio-Rad Versadoc (Model 5000, Bio-Rad Laboratories, Inc., Hercules, CA).

2.8. *Measurement of melanin contents*

Melanin content was measured described previously [19]. Briefly, B16-4A5 cells dissolved in 1ml of 5% Trichloroacetic acid (TCA) were incubated on ice for 10 min. After centrifugation, the precipitate was dissolved in 2ml of EtOH-ether (3:1) and centrifuged. The precipitate was resuspended in 2ml of ether, and centrifuged for 10 min. Finally, the precipitate was air-dried, and resuspended in 0.5 ml of 2 N NaOH at 80 ˚C for 1 hr. The absorbance at 415 nm was measured.

2.9. *Topical application of EGFP-11R and EGFP*

The hair from the back of adult guinea pigs (Female Weiser-Maples, SHIMIZU Laboratory Supplies, Kyoto, Japan) weighing 450–500 g was removed carefully using depilatory cream 24 h before the study. All procedures of animal experiments were approved by the Animal Ethics Committee of Okayama University (OKU-2008019). Areas of 4.0 cm^2 were marked on the dorsal trunk of the animals using a template. For topical treatment of EGFP-11R, PB in propylene glycol [a mixture of 1 μL of PB (50

mM) and 99 µL of propylene glycol] was pre-applied on the skin. After 5 min, 11R-EGFP [a mixture of 25 µl of 11R-EGFP (50 µM) and 25 µl of propylene glycol] was applied to the same region. As a control, EGFP or EGFP-11R [a mixture of 25 µl of 11R-EGFP or EGFP (50 µM each) and 25 µl of propylene glycol] was applied on the skin of same guinea pigs. Skin sections were obtained at 0.5, 2, 4, and 8 h after the topical applications with a 3-mm dermapunch (Maruho, Osaka, Japan). Excised skin samples were immediately frozen in Optimal Cutting Temperature compound (Sakura Finetek, Japan) and sequentially sectioned at a thickness of 10 µm. The sections were fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 15 min. After being washed with PBS, the sections were incubated with Hoechst 33258 (1 µg/ml) for 5 min, and viewed using a confocal microscope (FluoView™ FV300, Olympus, Tokyo, Japan).

2.10. *UV-induced pigmentation and topical treatment in vivo*

Hyperpigmentation was induced on the backs of brown guinea pigs by the modification of a method as described previously [20]. Four separate areas (2 cm x 3 cm square) on the back were shaved and exposed to UV from Model UVM-57 lamps (Funakoshi, Tokyo, Japan), The total dose of UV was 15 mJ/cm^2 per day. Animals were exposed five days a week for two consecutive weeks (Fig. 2). Two days after the last UV

irradiation, HQ (a mixture of 50 µl of 1 mM HQ and 50 µl of propylene glycol) was applied to the tanning lesion. HQ-11R and HQ-GLHFPHIYVRD (a mixture of 50 µl of 1mM HQ or HQ-peptide and 50µL of propylene glycol) were applied to the tanning lesion 5 min after pre-treatment with PB（a mixture of 1 µl of 50 mM PB and 99 µl of propylene glycol）. Guinea pigs were treated with HQ and HQ-peptides once a day, 5 days a week, for a total of 10 days (Fig. 2). Three days after the last treatment, the animals were sacrificed and skin samples were taken with a 3-mm dermapunch (Maruho, Japan). As a control, the guinea pigs were received 100 µl of propylene glycol.

2.11. *Histology study*

Under general euthanasia, skin was removed and fixed in 4% paraformaldehyde in PBS. After paraffin processing, paraffin, embedded tissue sections 4.5 µm thick were processed for light microscopic examination. A hematoxylin and eosin (H&E) stain was used for studying the general histopathological changes in the skin. Melanin pigments were visualized with Fontana-Masson sliver staining followed by eosin background staining as described previously [21]. Melanin-positive cells were counted in a 500 x 400 µm area in 20 different fields.

2.12. *Statistical analysis*

Data are shown as the mean ± S.D. Data were analyzed using either Student's t-test to compare two conditions or ANOVA followed by planned comparisons of multiple conditions, and $P < 0.05$ was considered to be significant.

3. Results

3.1 Effect of poly-arginine-fused HQ (HQ-11R) with 1-pyrenebutyric acid (PB) on the viability of B16-4A5 melanoma cells.

To investigate the cell toxicity of HQ-11R with PB in B16-4A5, a cell line of mouse melanoma, the cells were incubated with each concentration of HQ and HQ-11R. When the cells were incubated with 0.5 and 5.0 μM of HQ alone or of HQ-11R with 50 μM PB, viability was the same as that of the control after 24, 48 and 72 h (Fig. 3A), suggesting that neither HQ and nor HQ-11R with PB had cell toxicity when used at concentrations of 0.5 and 5 μM. Viability was significantly inhibited when the cells were treated with 50 μM of HQ-11R. However, 50 μM of HQ also inhibited cell viability the same as HQ-11R (Fig. 3A), suggesting the inhibitory effect to be due to HQ.

3.2 Inhibitory effect of HQ-11R on melanin content.

To investigate whether HQ-11R inhibited melanogenesis, B16-4A5 cells were incubated with 10, 20 and 30 μM of HQ and HQ-11R and melanin content was measured. Both HQ and HQ-11R dose-dependently inhibited melanin synthesis (Fig. 3B). The inhibitory effect of HQ-11R was the same as that of HQ (Fig. 3B). HQ

fused with a control peptide (HQ-GLHFPHIVRD), which consisted of eleven amino acids and had no ability to penetrate cells, did not inhibit melanin synthesis when used at 10 and 20 μM (Fig. 3B). HQ –GLHFPHIVRD inhibited melanin synthesis at 30μM but its effect was weaker than HQ-11R and HQ alone (Fig. 3B). The 11R-peptide without HQ had no effect on melanin synthesis (Fig. 3B). HQ is a potent inhibitor of tyrosinase, a key enzyme in melanin synthesis, but did not affect the expression or degradation of the enzyme [22,23]. Some tyrosinase inhibitors display a hypopigmenting effect through post-transcriptional control of the tyrosinase. Linoleic acid decreases the amount of tyrosinase through increased tyrosinase ubiquitination and degradation by the proteasome [23]. *N*-Acetylglucosamine reduced melanin synthesis through the inhibition of tyrosinase glycosylation [24]. To investigate the possibility that the inhibitory effect of HQ-11R on melanin synthesis was due to the post-transcriptional control of tyrosinase, tyrosinase levels were examined in B16-4A5 cells treated with HQ and HQ-11R. Neither HQ nor HQ-11R affected the protein level (Fig. 3C), suggesting that HQ-11R may inhibit melanin synthesis through the inhibition of tyrosinase activity.

3.3 Transdermal delivery of EGFP-11R with PB.

Next, the efficiency of the delivery of EGFP fused with 11R (EGFP-11R) in B16-4A5 cells was examined. EGFP without 11R was not observed in the cells (Fig. 4A). The signals were observed in almost all of the cells 30 min after the addition of EGFP-11R regardless of PB (Fig. 4A). Strong signals were observed 2, 4 and 8 h after the transduction regardless of PB (Fig. 4A). Preincubation of PB did not affect the efficiency of EGFP-11R transduction in the cells (Fig. 4A).

We next examined the effectiveness of the topical application of EGFP-11R. When EGFP was topically applied, the signals were only observed on the surface of the skin (Fig. 4B). The topical application of EGFP-11R without PB delivered the protein into the dermis (Fig. 4B). However, signals were not observed in the epidermal layer (Fig. 4B). Strong signals in the epidermal layer were observed 0.5 and 2 h after the application but the signals were faint at 4 and 8 h. When EGFP-11R was applied with PB, strong signals were detected in both epidermis and dermis 0.5 and 2 h after the application (Fig. 4B). Moreover, the signals were spread out more and stronger in both layers after 4 and 8 h (Fig. 4B).

To investigate whether EGFP-11R was delivered in the cells of the epidermis and dermis, the skin sections were counter-stained with Hoechst (Fig. 4C). Most EGFP signals did not overlap with Hoechst staining when EGFP-11R was applied without PB,

suggesting that 11R-EGFP was delivered in the space of the epidermal layer (Fig. 4C). In contrast, when EGFP-11R was applied with PB, the signals overlapped with Hoechst staining in the cells of both epidermis and dermis 4 h after the application (Fig. 4C).

3.4 Inhibitory effect of HQ-11R on UV-induced pigmentation in guinea pig skin.

The whitening effect of HQ-11R was examined using UV-induced pigmentation of brown guinea pig skin. After UV irradiation for 10 days, guinea pigs were topically treated with HQ, and HQ-11R and HQ-GLHFPHIYVRD with PB for 10 days (Figs 2 & 5A). The treatments did not induce abnormal morphological changes in the epidermis and dermis (Fig. 5B). The HQ application tended to decrease levels of melanin but not significant (Fig. 5B & C). HQ-11R significantly reduced melanin levels in the basal cell layer of the epidermis whereas HQ-GLHFPHIYVRD lacking the ability to penetrate cells had no effect on melanin levels in the epidermis (Fig. 5C).

4. Discussion

Protein transduction method using CPPs such as 11R is widely accepted as useful for the delivery of proteins, peptides and cell-impermeable molecules into cells [25]. Previous studies have showed the effective transdermal skin delivery of proteins and peptides employing CPPs including Tat (Trans-activating transcriptional activator), YARA, WLR, and 9R peptides [26-29]. In the present study, we also showed that protein transduction method using 11R was valuable for the transdermal delivery of EGFP and HQ. However, EGFP-11R without PB was not delivered into cells of the epidermis although signals were observed in the layer. These results suggest that protein transduction using CPPs is capable of increasing the penetration of skin by proteins but most proteins delivered topically do not enter or function in cells of the epidermis and dermis. In contrast, EGFP-11R was delivered to cells of the epidermis and dermis and HQ-11R reduced melanin levels in the basal cell layer of epidermis when applied in combination with 4-(1-pyrenyl)-butyric acid (PB), suggesting that PB enhances the intracellular delivery of proteins and hydrophilic molecules delivered transdermally. The molecular mechanism by which CPP-fused proteins cross the cell membrane is different with that without PB. CPP fusion proteins are internalized rapidly by lipid raft-dependent macropinocytosis [11]. In contrast, the negatively

charged counteranions and high hydrophobicity of PB can exert a great influence on the translocation behavior of arginine peptides in artificial membranes [12,13]. Endogenous mechanism such as macropinocytosis and endocytosis are not necessary for the intracellular delivery of poly-arginine fusion proteins when applied with PB. The artificial membrane penetration may be convenient for the transdermal delivery of poly-arginine fusion proteins.

The backbone of PB is a pyrene, a polycyclic aromatic hydrocarbon consisting of four fused benzene rings and it contains a hydrophilic carboxylic acid. The structure is similar with benzopyrene, which consists of five benzene rings. Benzopyrene is found in coal tar and cigarette smoke, and shows an evidence of carcinogenicity in lung cells [30]. Therefore, PB is misunderstood to have toxicity. However, a previous study showed that PB had no significant cytotoxicity [12]. In the present study, moreover, PB did not affect the growth of B16-4A5 nor cause dermatitis in guinea pigs. These results suggest protein transduction using 11R and PB to be safe as a transdermal delivery method. However, further study is needed.

A previous study showed that bioactive proteins were transdermally delivered by non-covalently associated poly-arginine peptides [27]. We also examined whether HQ non-covalently associated with 11R was delivered in the dermis and epidermis of guinea

pigs when applied with 11R. However, HQ was not delivered in the skin (data not shown). These results suggest that transdermal delivery by non-covalently associated poly-arginine peptides may be suitable for proteins and peptides but not low molecules.

In conclusion, a topical approach using poly-arginine in combination with PB was proven to be beneficial for the delivery of proteins and hydrophilic drugs. HQ-11R is a candidate for a skin-whitening agent with the advantages of strong tyrosinase inhibition, effective penetration of the skin, and relatively little cytotoxicity.

4. Conclusion

The present results showed that a poly-arginine (11R) with pre-treatment of PB was effective at topical delivering system to enter or function in cells of the epidermis and dermis. It appears that our system can be used to deliver many kinds of proteins and whitening-agents into g transcription factors into various cells. *In vivo* topical protein transduction method with pyrenbutyrate may overcome the disadvantages of previous methods and become a promising modality in dermatological field and cosmetics industry.

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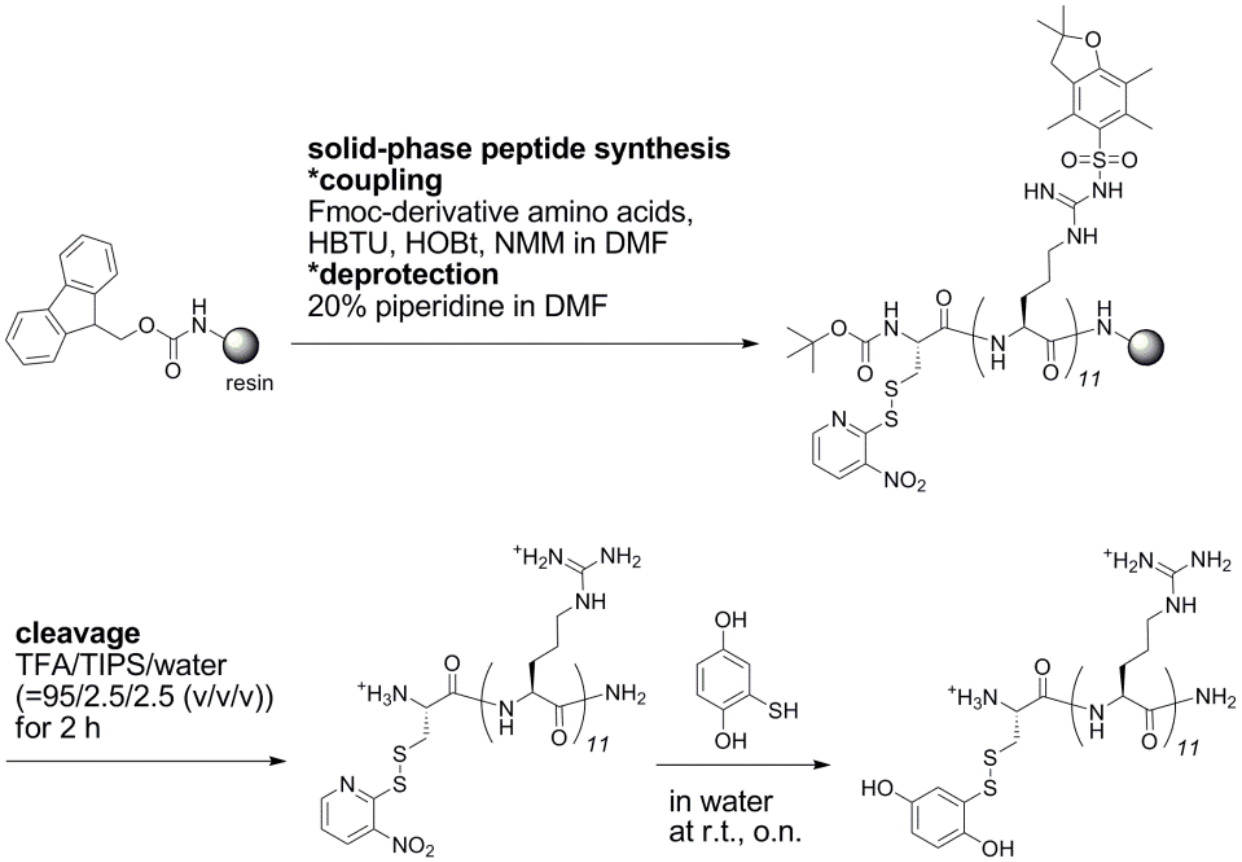
Figure

Fig. 1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Fmoc-derivative amino acids,
HBTU, HOBt, NMM in DMF
*deprotection
20% piperidine in DMF
resin
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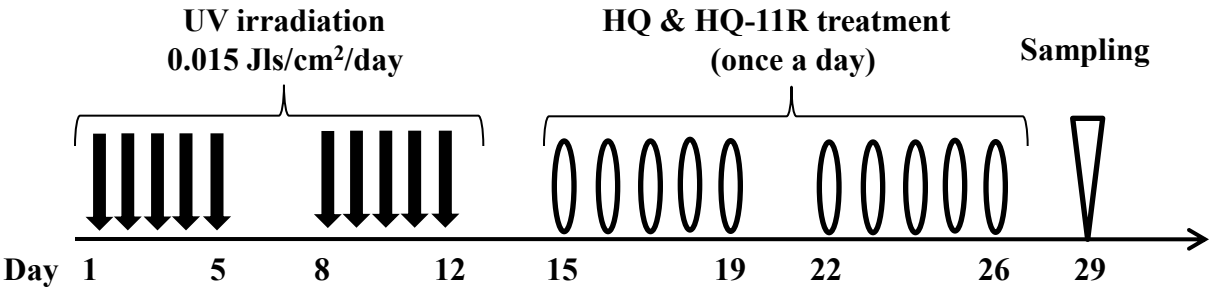
for 2 h
in water
at r.t., o.n.

Fig. 2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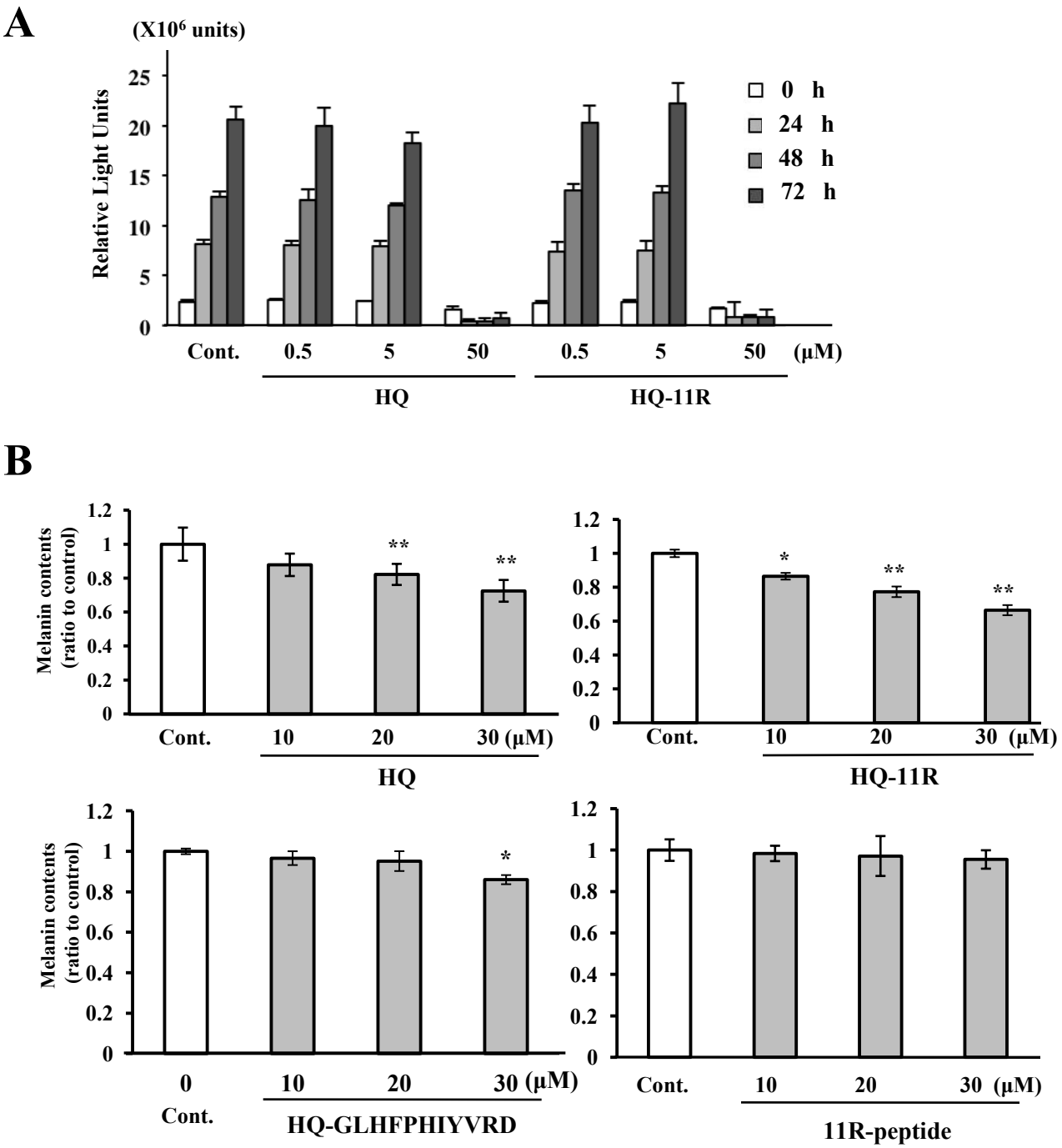
HQ & HQ-11R treatment
(once a day)
Sampling
Day 1 5 8 12 15 19 22 26 29

Fig. 3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(X10⁶ units)
Relative Light Units
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20
15
10
5
0
0 h
24 h
48 h
72 h
Cont.
0.5
5
50
0.5
5
50
(μM)
HQ
HQ-11R
B
Melanin contents (ratio to control)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Cont.
10
20
30 (μM)
HQ
*
**
**
Cont.
10
20
30 (μM)
HQ-11R
*
0
Cont.
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30 (μM)
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Cont.
10
20
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11R-peptide
C
Tyrosinase
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Cont. 0.5 5 0.5 5 0.5 5 0.5 5
HQ HQ-11R HQ HQ-11R
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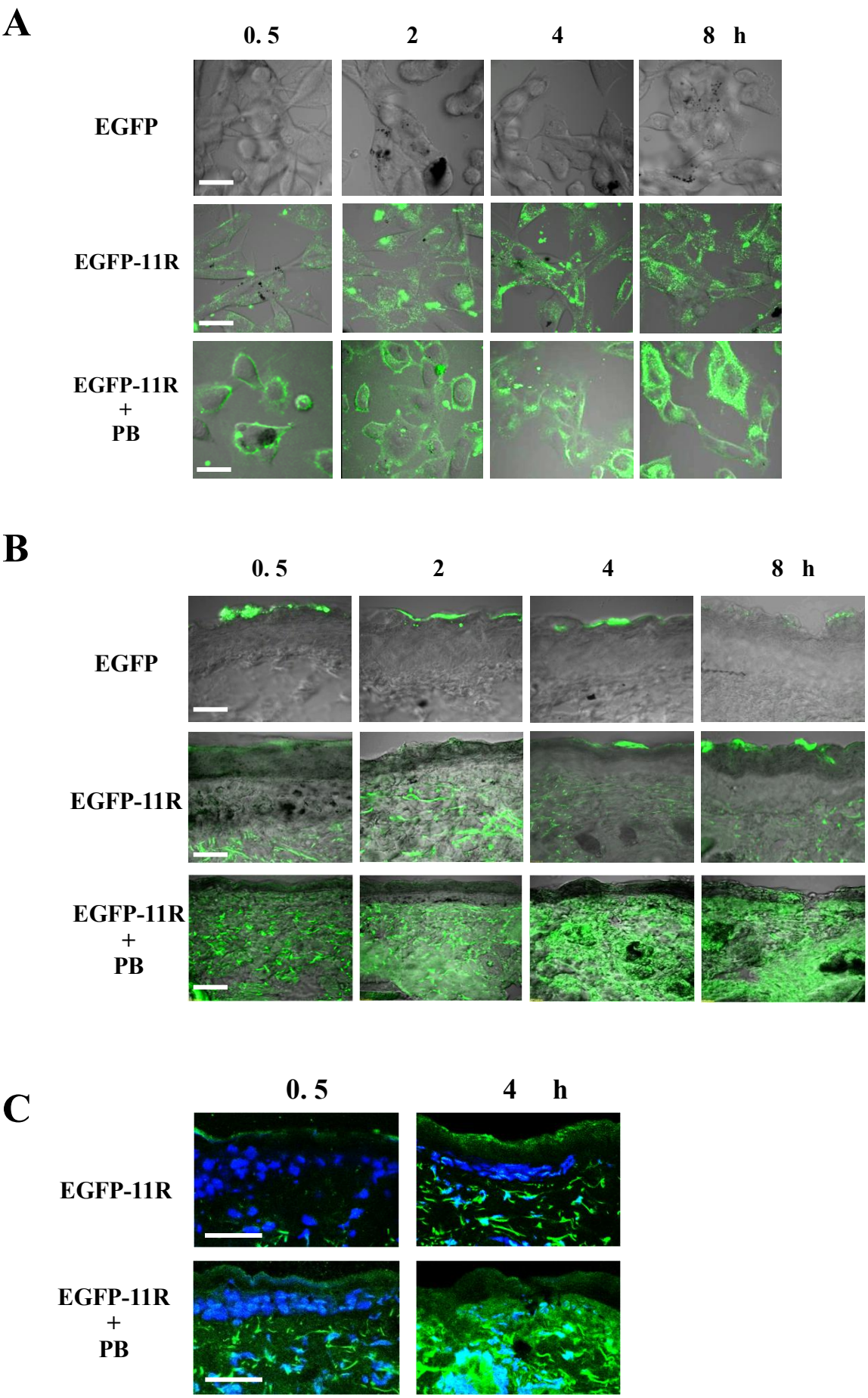
HQ HQ-11R HQ HQ-11R
12 h
24 h

Fig. 4


A
0. 5
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EGFP
EGFP-11R
EGFP-11R
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PB
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EGFP
EGFP-11R
EGFP-11R
+
PB
C
0. 5
4 h
EGFP-11R
EGFP-11R
+
PB

Fig. 5

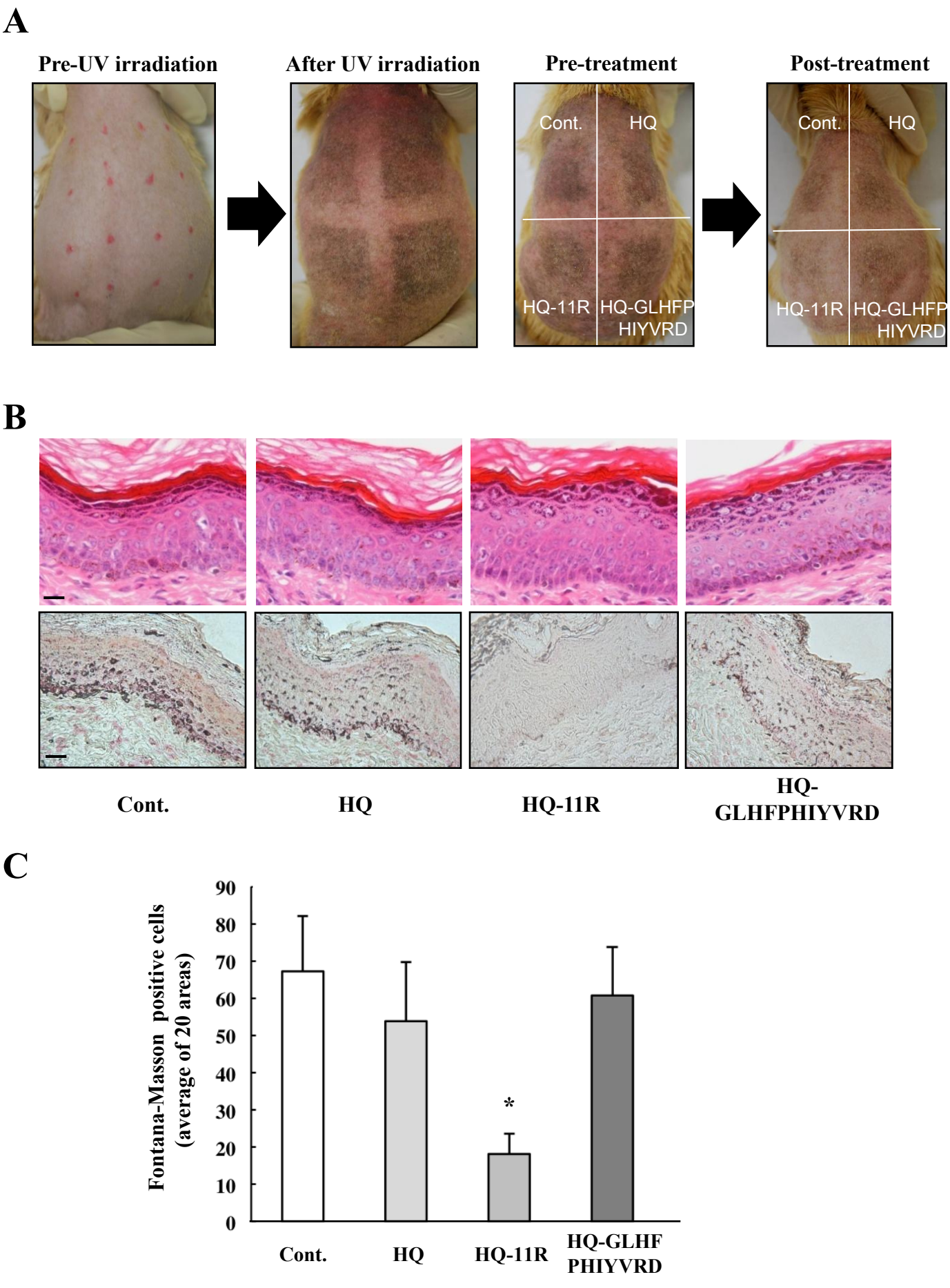
A
Pre-UV irradiation
After UV irradiation
Pre-treatment
Post-treatment
Cont.
HQ
HQ-11R
HQ-GLHFP
HIYVRD
Cont.
HQ
HQ-11R
HQ-GLHFP
HIYVRD
B
Cont.
HQ
HQ-11R
HQ-
GLHFPHIYVRD
C
Fontana-Masson positive cells
(average of 20 areas)
90
80
70
60
50
40
30
20
10
0
*
Cont.
HQ
HQ-11R
HQ-GLHF
PHIYVRD

Figure legends

Fig. 1. The schema of HQ-11R synthesis.

Fig. 2. Time schedule of UV-irradiation and applications of HQ and HQ-11R.

Fig. 3. Comparison of the inhibitory effect on melanin synthesis between hydroquinone (HQ) and eleven poly-arginine-fused HQ (HQ-11R) in B16-4A5 melanoma cells. (A) Cell viability was measured by the *Cell Titer-Glo Luminescent Cell Viability* assay. B16-4A5 cells were treated with 10, 20 and 30 μM of HQ alone or HQ-11R in the presence of PB for the periods indicated. Control cells (Cont.) were intact cells. Data are represented as the mean ± SD. n=6 each. (B) Measurement of melanin contents in the cells treated with 10, 20 and 30 μM of HQ, HQ-11R, HQ-GLHFPHIYBRD and 11R peptide for 24 h. Data are represented as the mean ± SD. n=6 each. $*P < 0.05$, $**P < 0.01$ (C) Effect of HQ and HQ-11R on tyrosinase levels in B16-4A5 cells. The cells were treated with HQ alone and HQ-11R with PB for the periods indicated. The cells were harvested at each time point and the lysates were used for Western blotting.

Fig. 4. Transdermal transduction of EGFR-11R with PB. (A) Time-dependent transduction of EGFR-11R with or without PB in B16-4A5 cells. The cells were treated with 1 μM EGFP-11R (EGFP-11R). After pre-treatment with PB for 5 min, 1 μM EGFP was added (EGFP-11R + PB). The signals were observed by confocal microscopy at each time point. Bars = 10 μm. (B) Time-dependent transdermal delivery of EGFP-11R. EGFP and EGFP-11R with/without PB were applied on the skin of guinea pigs. Skin sections were obtained at each time point and EGFP signals were observed with a confocal microscope. Bars = 200 μm. (C) The distribution of EGFP-11R in the epidermis and dermis of guinea pigs. EGFP-11R was applied with or without PB. The skin sections were counter-stained with Hoechst 33258 (blue). Bars = 50 μm.

Fig. 5. Inhibitory effect of HQ-11R on UV-induced pigmentation in guinea pig skin. (A) A representative image of the back of the Guinea pigs pre-radiation, post-radiation, and pre-treatment and post-treatment with HQ, HQ-non CPP peptide and HQ-11R. Each area is 2cm x 2cm square. (B) Biopsy specimens from HQ-, HQ-11R- or HQ-GLHFPHIYVRD-treated guinea pig skin after 10 days of topical application were examined by H.E. staining (upper panel) and Fontana-Masson silver

staining (lower panel). Bars = 50 μm. (C) Number of cells positive for Fontana-Masson stain. The positive cells were counted in a 500 x 400 μm area in 20 different fields. *P < 0.01.

AUTHOR DECLARATION

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We would like to draw the attention of the Editor to the following publications of one or more of us that refer to aspects of the manuscript presently being submitted. Where relevant copies of such publications are attached. We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. [OR] We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. **We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.**

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from biomaterials@online.be.

Signed by all authors as follows:

CANDANGERILE	CANDANGERILE II	date: 11/4/2012
Hiroyuki Michitue	Hiroyuki Michitue	date 4/10/2012
Sanae Ishikawa	Sanae Ishikawa	date 4/11/2012
Atsushi Fujimura	Atsushi Fujimura	date 4/11/2012.
Keiichiro Hayashi	Keiichiro Hayashi	date 4/11/2012
Atsuhito Uneda	Atsuhito Uneda	date 4/11/2012
Akiko Mori	Akiko Mori	date 4/11/2012
Tori Ohmori	Tori Ohmori	date 4/11/2012
Tei-ichi Nishiki	Tei-ichi Nishiki	date 4/11/2012.
Hideki Matsui	Hideki Matsui	date 4/11/2012
Kazuhito Tomizawa	Kazuhito Tomizawa	date 4/11/2012

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*Title Page

Combining Poly-Arginine with the Hydrophobic Counter-anion 4-(1-Pyrenyl)-Butyric Acid for Protein Transduction in Transdermal Delivery

CANDANGERILE [1*], Hiroyuki Michiue[1*#], Sanae Ishikawa[1], Atsushi Fujimura[1], Keiichiro Hayashi[1], Atsuhito Uneda[1], Akiko Mori[1], Iori Ohmori[1], Tei-ichi Nishiki[1], Hideki Matsui[1], and Kazuhito Tomizawa[2]

1. Departments of Physiology Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-Ku, Okayama 700-8558 Japan.
2. Department of Molecular Physiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjou, Kumamoto, 860-8556 Japan

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[*] These authors contributed equally to this work.
[#] Address Correspondence to:
Dr. Hiroyuki Michiue
Department of Physiology
Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,
Shikata-cho 2-5-1, Okayama 700-8558, Japan,
Tel.: +81-86-235-7105
Fax: +81-86-235-7111,
E-mail: hmichiue@md.okayama-u.ac.jp

Okayama University Graduate School of Medicine & Dentistry
2-5-1 Shikata-cho, Okayama 700-8558, Japan

Prof. D.F. Williams,
Editor-in-Chief
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Dear Prof. Williams:

Thank you for reviewing our paper and providing an opportunity for revision. We have carefully read the comments by the Editor and the reviewer, and changed the manuscript accordingly.
We hope that this revised version is considered worthy of publication in **Biomaterails**.
We appreciate your thoughtful review of this work, and look forward to hearing from you soon.

Answers to mandatory editor's requirements

1. We changed the title to " Combining Poly-Arginine with the Hydrophobic Counter-anion 4-(1-Pyrenyl)-Butyric Acid for Protein Transduction in Transdermal Delivery ".

Sincerely yours,

Hiroyuki Michiue M.D. Ph.D

Assistant Professor

Department of Physiology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Shikata-cho 2-5-1, Okayama 700-8558, JAPAN

Tel: 81-86-235-7105, Fax: 81-86-235-7111

E-mail: hmichiue@md.okayama-u.ac.jp