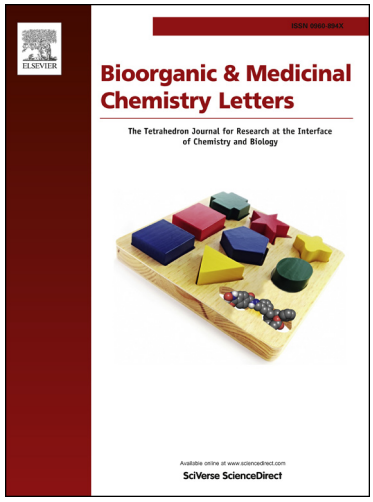

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Graphical Abstract

Regulatory effect of hydroquinone-tetraethylene glycol conjugates on zebrafish pigmentation

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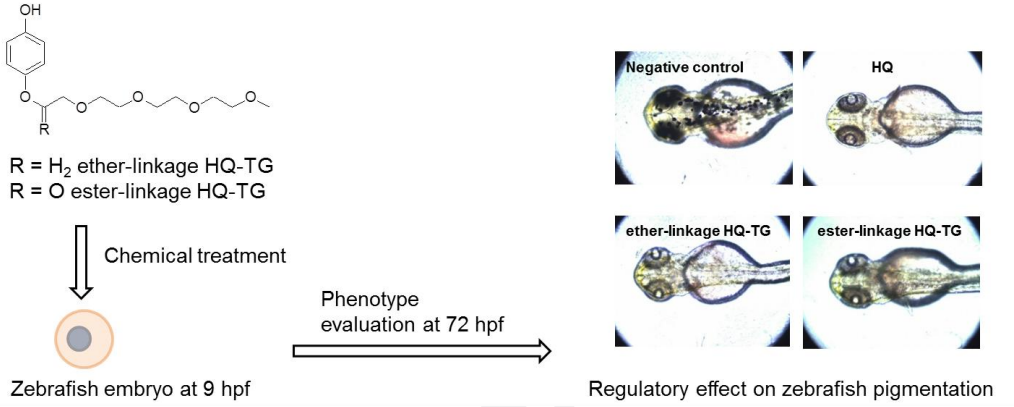

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Hoa Thi Le[a,†], Bin Na Hong[b,†], Young Ro Lee[b,c], Ji Hyun Cheon[a], Tong Ho Kang[b,c,*], Tae Woo Kim[a,*]

[a] *Graduate School of East-West Medical Science, Kyung Hee University, Gyeonggi-do 449-701, Republic of Korea*
[b] *Department of Oriental Medicinal Materials & Processing, College of Life Sciences, Kyung Hee University, Gyeonggi-do 449-701, Republic of Korea*
[c] *Graduate School of Biotechnology, Kyung Hee University, Gyeonggi, Gyeonggi-do 449-701, Republic of Korea*

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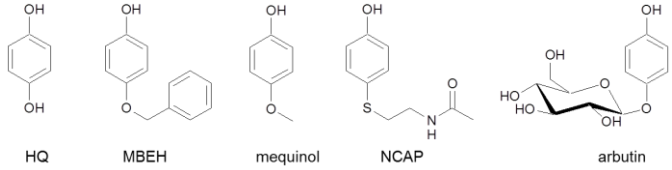
ABSTRACT

We synthesized two hydroquinone-tetraethylene glycol conjugates (HQ-TGs) and investigated their logP, photophysical stability, and redox chemical stability. HQ-TGs are a little more hydrophilic than hydroquinone (HQ) and show an enhanced photophysical and redox chemical stability compared with HQ. In addition we studied the effect of HQ-TGs on cell viability and on zebrafish pigmentation. MTT assay in HF-16 cells showed HQ-TGs are less cytotoxic than HQ. The phenotype-based image analysis of zebrafish larvae suggests that HQ-TGs suppress the pigmentation of zebrafish in a dose-dependent manner. The comparative experiments on stability, cytotoxicity, and zebrafish pigmentation between HQ and HQ-TGs suggest that mono tetraethylene glycol-functionalization of HQ is an alternative solution to overcome the adverse effect of HQ.

Hyperpigmentation is the darkening of an area of skin caused by inflammatory skin disorders, allergic/irritant contact dermatitis, sun damage, or other skin injuries.[1] Several compounds with bleaching properties have been used for the treatment of pigmentary disorders of the skin. The bleaching or depigmentation agents are divided into two groups, phenolic and nonphenolic compounds.[2] The phenolic group includes hydroquinone (HQ), monobenzyl ether of hydroquinone (MBEH), 4-methoxyphenol (mequinol), 4-isopropylcatechol, *N*-acetyl-4-*S*-cystaminylphenol (NCAP), arbutin, etc. Meanwhile, azelaic acid, tretinoin, L-ascorbic acid, kojic acid, and *N*-acetylcysteine, etc. are belong to the nonphenolic group.

In the above bleaching or depigmenting agents, HQ has been the gold standard for treatment of hyperpigmentation for over 50 years.[3] 2% HQ preparations are the only agents considered safe and effective for over-the-counter treatment of cutaneous hyperpigmentation.[4] However, it also has some adverse reactions. It may be irritating and unstable at high concentrations (5~10%). Even though not satisfying, other phenolic agents have been developed to overcome the conventional disadvantages of HQ. Chemically modified HQs are still attractive approach to develop a price-competitive skin lighter.

Zebrafish (*Danio rerio*) has emerged as an important vertebrate model organism in neurobiological and biomedical research during the last decades.[5] Its value for studying vertebrate development and human diseases has been recognized because of its ex utero, optically transparent embryogenesis and amenability to in vivo manipulation.[6] The embryos rapidly absorb low molecular weight compounds, diluted in the surrounding media, through skin and gills. In contrast, late stage zebrafish, from 7 days post fertilization (dpf) to the adult stage, absorb compounds orally.[7] Therefore, early stage zebrafish provide another advantage of testing percutaneous effects of medicinal and/or cosmetic compounds. In addition, melanin pigments of zebrafish are easy to observe without complicated experimental procedures. T. J. Yoon et al. validated zebrafish as a model for phenotype-based screening of melanogenic regulatory compounds.[8]



Scheme 1. Molecular structures of phenolic depigmenting agents. HQ = hydroquinone, MBEH = monobenzyl ether of hydroquinone, mequinol = 4-hydroxyanisole, NCAP = *N*-acetyl-4-*S*-cysteaminylphenol, arbutin = 4-hydroxyphenyl-β-glucopyranoside.

*Corresponding authors. Tel.: +82-31-201-3704; fax: +82-31-204-8119; e-mail: tw1275@khu.ac.kr (T. W. Kim), panjae@khu.ac.kr (T. H. Kang). †First two authors contributed equally to this work.

Our study commenced with the synthesis of HQ derivatives, which can suppress pigmentation but simultaneously overcome the disadvantages of HQ. Compared with HQ, the hydroquinone derivatives should reduce cytotoxicity and improve chemical stability against oxidation by light, air, or oxidant. In addition the compounds should be easy to synthesize and to be scalable. Scheme 1 represents the molecular structures of depigmenting agents on market. The depigmentation agents, except HQ itself, share a common structural motif, mono ether-functionalized HQ.

PEGylation (the covalent attachment of polyethylene glycol (PEG) to another molecule) of biologics improves their pharmacokinetic, pharmacodynamic, and immunological profiles.[9] In addition, the recent clinical development of PEG-naloxol (NKTR-118 from Nektar Therapeutics, San Carlos, CA) spotlighted the benefits and challenges of small molecular weight PEGylation of small molecules.[10,11] Small molecular weight PEGylation of HQ was studied in viewpoint of hydrolyzable prodrug concept,[12] but the depigmentation effect of HQ derivatives attaching small molecular weight PEG was not fully addressed.

In order to apply HQ-small molecular weight PEG conjugates as a depigmentation agent, should be taken into account the next issues; synthesis, stability, safety, and melanogenesis inhibitory effect. As a proof of concept, we synthesized two HQ-tetraethylene glycol conjugates (HQ-TGs) and investigated the logP, photophysical stability, redox chemical stability, and cytotoxicity of HQ-TGs. We also confirmed the depigmentation of HQ-TGs using zebrafish phenotype-based evaluation.

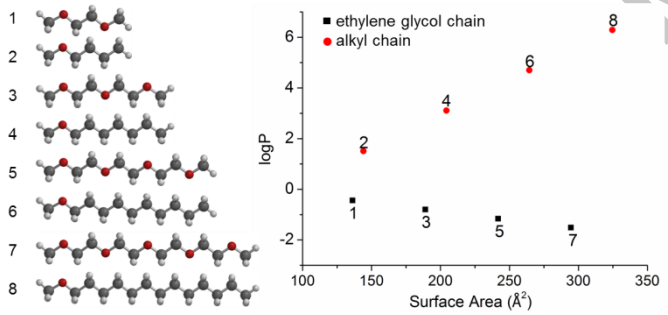
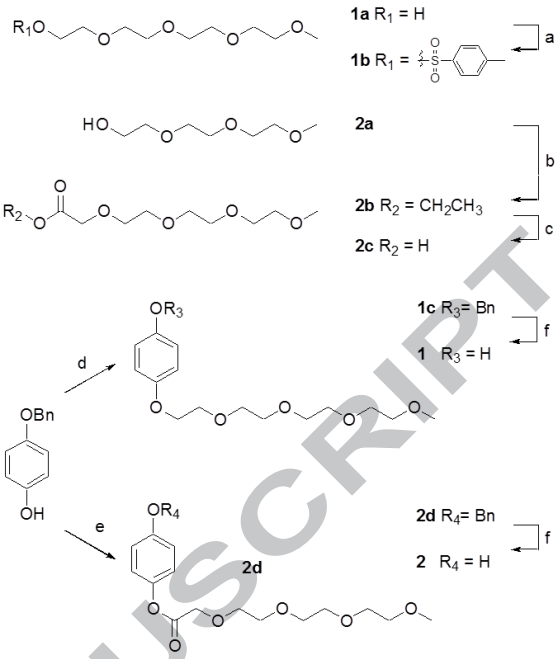


Figure 1. Surface area vs. logP correlation of ethylene glycol and hydrocarbon alkyl chain. The surface area (Å^2) was calculated using Spartan '08 (B3LYP calculation with 6-31G (d) basis set in vacuum, Wavefunction, Inc.). The logP values were calculated using Advanced Chemistry Development (ACD/Labs) Software V12.01.

Ethylene glycol chain (-(CH_2CH_2O)$_n$-) has unique physical properties compared with hydrocarbon alkyl chain (-(CH_2)$_n$-). The oxygen in ethylene glycol unit makes the chain more hydrophilic and the ethylene moiety provides hydrophobicity to the chain. The balance between hydrophilicity and hydrophobicity of ethylene glycol unit makes polyethylene glycols (PEGs) miscible in broad range of solvents; from water to many organic solvents. The surface area vs. logP correlation of ethylene glycol and hydrocarbon alkyl chains clearly shows the characteristic hydrophilicity of ethylene glycol chain compared with hydrocarbon alkyl chain. The longer hydrocarbon alkyl chains are the more hydrophobic, but the longer ethylene glycol chains are the more hydrophilic (Fig. 1). In general, the ethylene glycol modification of a molecule enhances both aqueous and organic solubility of the molecule at the same time. Usually ethylene glycol modification has been used for resolving the poor water-solubility problem of hydrophobic mother molecules.[13]



Scheme 2. Reagents and conditions. a) *p*-Toluenesulfonyl chloride, TEA, CH_2Cl_2; b) ethyl bromoacetate, NaH, THF; c) NaOH, CH_3CN, then HCl; d) **1b**, NaH, THF; e) **2c**, EDC·HCl, DMAP, CH_2Cl_2; f) H_2, Pd/C, THF. Bn = benzyl, EDC·HCl = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMAP = 4-(dimethylamino)pyridine.

1 and **2** were prepared by following Scheme 2. The tetraethylene glycol precursor for ether bond formation (**1b**) was prepared by tosylation of tetraethyleneglycol monomethyl ether. The other tetraethylene glycol precursor for ester bond formation (**2c**) was prepared by two step reactions: 1) Williamson ether synthesis of triethyleneglycol monomethyl ether and ethyl bromoacetate and 2) basic hydrolysis of ester and acidification. The tetraethylene glycol-hydroquinone conjugates having ether linkage (**1c**, **1**) were reported by N. N. Ekwuribe et al. but were not characterized.[12] The tetraethylene glycol-hydroquinone conjugates having ester linkage (**2d**) was prepared by esterification using EDC as a coupling reagent. **1** and **2** were prepared from debenzylation of **1c** and **2d** (H_2 balloon, 10 wt.% palladium on carbon). The synthesis and characterization of **1** and **2** was reported in Supplementary Data S1. The synthesis and NMR, MS characterization of **1c**, **1**, **2d**, and **2** were reported in SD S1.

It will be worth to mention the melting points of HQ and its derivatives in Scheme 1; HQ = 172.3, MBEH = 122, mequinol = 57, arbutin = 199.5 °C.[14] We failed to find out the literature melting point of NCAP. If considered structural similarity and molecular weight, it would be reasonable to suggest that NCAP has higher melting point than mequinol. The physical appearance of them is solid at ambient temperature. However, we observed that **1** and **2** are oily at ambient temperature. The liquid-like property of **1** and **2** would provide better opportunity for pharmaceutical and/or cosmetic formulation.

To test the effect of tetraethylene glycol moiety on HQ's hydrophobicity, logP values were measured by a UV spectrometer (Shake-flask method) (Supplementary Data S2). The tetraethylene glycol moiety made HQ more hydrophilic (logP (HQ) = 0.25 → logP (**1**) = –0.026, logP (**2**) = –0.036), but ΔlogPs are within 0.3 (Supplementary Data Table S2). The linkage type of **1** and **2** (ether or ester) did not effect on the logP

difference of them (ΔlogP (**1** and **2**) ~ 0.01). Though the measured and the calculated logPs are not coincident, the logP values show a same trend; HQ > **1** ≈ **2**.

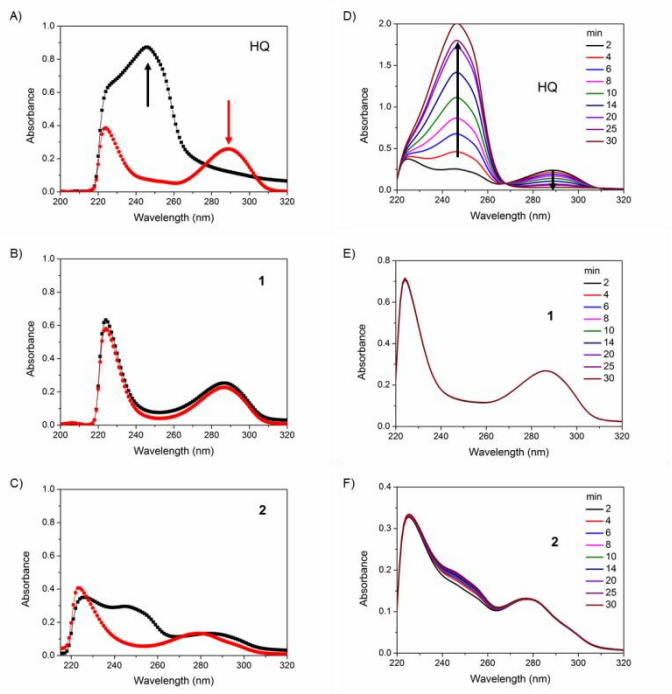


Figure 2. A, B, C) UV spectrum change before (red circle) and after (black square) UV exposure at ambient environment for 18 h. D, E, F) Time course UV measurement after treating Cu^{2+} (20 μM). [sample] = 0.1 mM in 1X PBS (pH 7.4).

The facile oxidation of HQ to benzoquinone (BQ) under ambient factors (light, oxygen, etc.) has been well-known.[15] HQ has UV absorption band at 289 nm (Fig. 2A red arrow). After UV exposure (maximum irradiation at 365 nm) at ambient environment, the characteristic UV absorption of HQ at 289 nm decreased and a new UV absorption at 244 nm increased (Fig. 2A black arrow). The new UV absorption after UV exposure agrees with the known UV absorption band (244 nm) of BQ.[16] Compared with HQ and **2**, **1** is more stable under UV exposure at ambient environment (Fig. 2B). We could not observe detectable UV changes of **1** after long UV duration (18 h). **2** was more labile than **1**, but still more stable than HQ (Fig. 2C). The results suggest that the HQ-TGs have better photophysical stability than HQ under UV exposure.

HQ itself is a reductant and can be easily oxidized by oxidizing chemicals. For example, the oxidation of hydroquinone was strongly accelerated by copper ions and afforded BQ, which is considered to be more toxic than HQ.[17] The Cu^{2+}-mediated oxidation of HQ also generates hydrogen peroxide (H_2O_2)[18] and the H_2O_2 may interact with copper and generate other reactive oxygen species (ROS) such as hydroxyl radical (•OH), single oxygen ($^1O_2$). The generation of ROS results in HQ-induced DNA damage.[19] HQ in the presence of Cu^{2+} ions was oxidized to BQ in a time-dependent manner (Fig. 2D). However, **1** did not show any detectable changes (Fig. 2E). **2** showed just small UV change, but the change will be ignorable when it is compared with HQ (Fig. 2F). The redox chemistry of HQ is a complicate chemical process. Thus we cannot generalize the effect of tetraethylene glycol conjugation on HQ redox chemical stability from the above experiment. However, it has to be considered that the tetraethylene glycol modification of HQ would be a way to enhance redox chemical stability of HQ.

pH is an important factor to determine the chemical transformation of a compound when it was treated in a living organism. We designed two HQ-TGs with different bonds to inquire into the relation between the pH stability and the melanogenic efficacy of HQ-TGs. Ether bonds are more robust than ester bonds in acidic or basic conditions. The HPLC monitoring of hydrolysis reaction at different pH condition shows that **1** (ether linkage) was not hydrolyzed at the pH 3 ~ 9, but **2** (ester linkage) was hydrolyzed at basic condition (Supplementary Data Fig. S3).

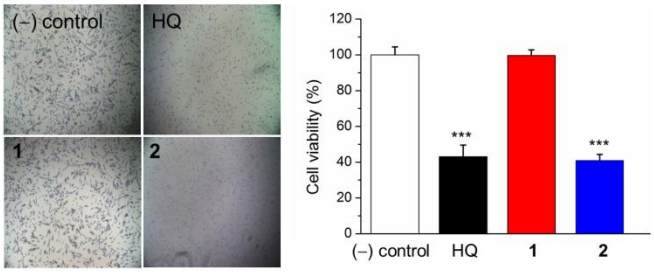


Figure 3. Cell viability of HQ, **1**, and **2**. Picture: 0.1% of DMSO ((–) control), 200 μM of HQ, **1** and **2** were treated in HF-16 cells and the pictures were taken after 24 hr. The MTT data are expressed as means ± SEM. *** $p < 0.001$ indicates a significant difference from (–) control group.

We investigated the cytotoxicity effect of **1** and **2** on the viability of HF (human fetal foreskin fibroblast)-16 cells. The viability of cultured HF-16 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cell viability of HF-16 cells was significantly decreased in HQ and **2** treatment (***, $p < 0.001$), but **1** did not show any cytotoxicity in the cells at 200 μM (Fig. 3). These data indicate that **1** is less cytotoxic than HQ or **2**.

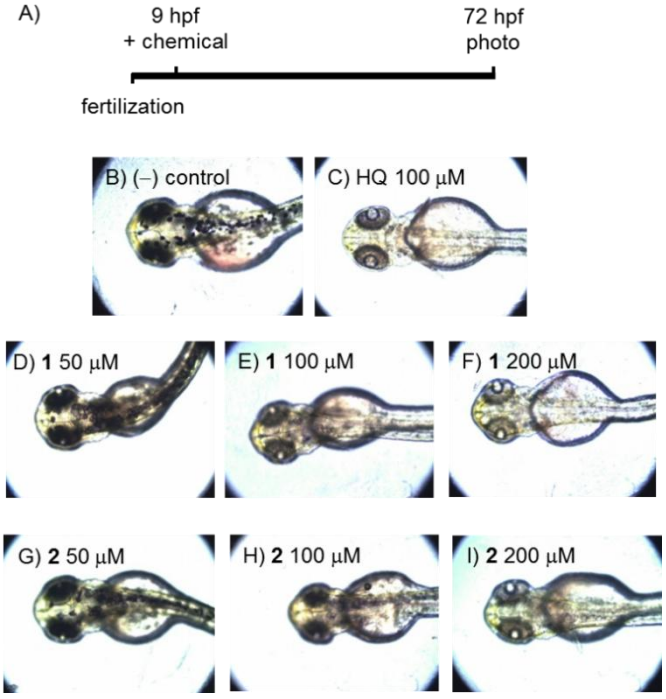


Figure 4. Phenotype evaluation of zebrafish laevae pigmentation. A) A schematic representation for the schedule of pigmentation study. Synchronized embryos were treated with HQ, **1**, and **2** at the indicated concentrations. Test compounds were dissolved in 0.1 % DMSO, and then added to the embryo medium, B) 0.1% of DMSO, C) 100 μM of HQ, D), E), F) treatment of **1** at 50, 100, and 200 μM, G), H), I) treatment of **2** at 50, 100, and 200 μM.

We evaluated the depigmentation effect of HQ-TGs in zebrafish larvae (Fig. 4). When HQ was treated as a positive control, the black spots of zebrafish larvae faded away and the eye colors of zebrafish larvae changed from black to gray (Fig. 4C). When **1** and **2** were treated, the black spots of zebrafish larvae also faded away and the eye colors of zebrafish larvae changed from black to gray (Fig. 4D-4F for **1** and 4G-4I for **2**). The degree of depigmentation of HQ at 100 μM was similar to that of **1** and **2** at 200 μM (Fig. 4C, 4F, 4I). The inhibitory effects of **1** and **2** on zebrafish pigmentation were dose-dependent. These phenotype-based observations suggest that **1** and **2** have the inhibitory effect on zebrafish pigmentation.

To quantitative comparison, the microscope images of zebrafish larvae were evaluated by image analysis software. The mock treatment (0.1% DMSO) was used as a negative control and the HQ treatment (100 μM) was used as a positive control. In dorsal view pictures the zebrafish pigment spots, except eyes and yolk, were manually selected and the binary threshold, the histogram, and the size of the black spots were analyzed.

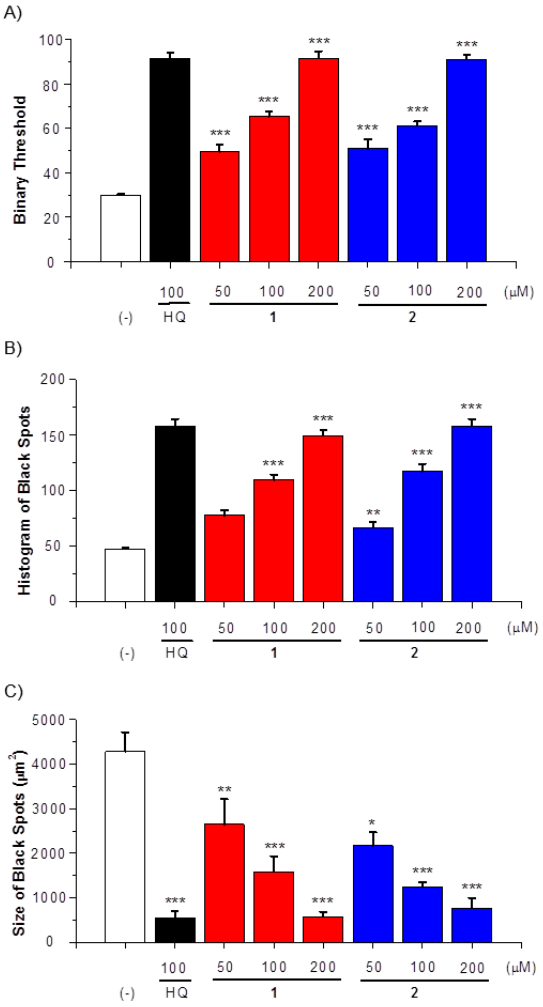


Figure 5. Pigmentation inhibitory effect on zebrafish. A negative control group was exposed to 0.1% DMSO. 100 μM of HQ used a positive control of whitening. **1** or **2** at 50, 100, and 200 μM were treated respectively. Data are expressed as means ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate a significant difference from (-) control group.

Binary threshold (BT) means a black and white threshold digit where the black spots disappear. The BT value is inversely proportional to black color intensity. The higher BT indicates the whiter image and the more efficient inhibition of pigmentation. The BT analysis showed that **1** and **2** inhibited the pigmentation of zebrafish in a dose-dependent manner (*** $p < 0.001$) (Fig. 5A). Histogram converts each RGB pixel in the image to gray scale and gives color tone information. As the color tone gradients move dark to light, the histogram of black spot (HB) value increases. The histogram analysis also showed that **1** and **2** inhibited the pigmentation of zebrafish (** $p < 0.01$, *** $p < 0.001$) (Fig. 5B). The area summation of the black spots of zebrafish larvae showed that the treatments of **1** and **2** downsized the size of black spot (SB) in a dose-dependent manner (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (Fig. 5C). These results suggest that **1** or **2** suppress the pigmentation of zebrafish depending on the concentration.

The bond type (ether or ester) between HQ and tetraethylene glycol did not have no detectable influence on the binary threshold, the black spot histogram, and the black spot size in zebrafish pigmentation. This observation may be related with the zebrafish early embryo's characteristics: the embryos directly absorb chemicals through skin and gills at early development stage instead of oral absorption. Skin and gills do not form a dynamic pH barrier compared with digestive organs. Meanwhile, **2** showed higher cytotoxicity than **1** in MTT assay (Fig. 3). If considered both depigmentation effect and cytotoxicity, **1** (ether linkage) would be a better option than **2** (ester linkage) as a potent depigmentation agent.

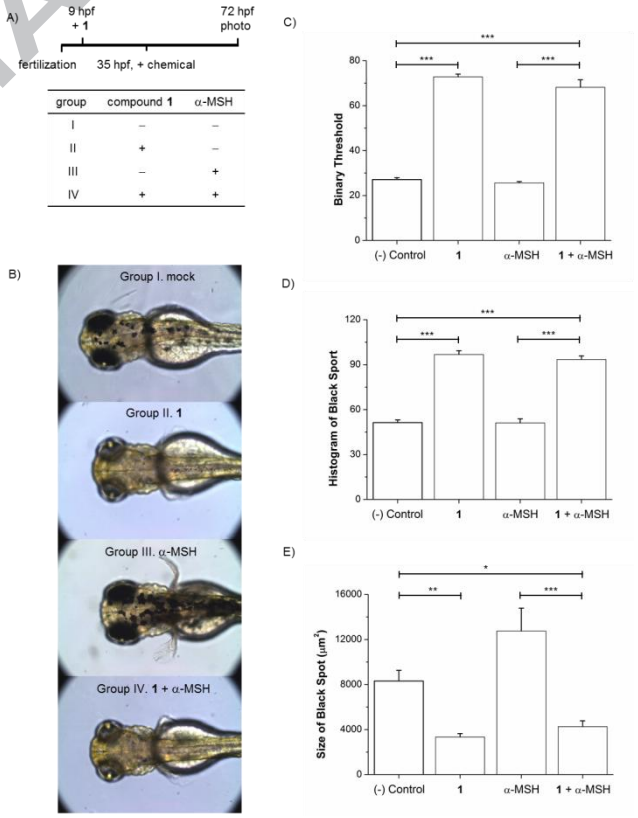


Figure 6. Inhibitory effect of **1** on α-MSH induced pigmentation. A) A schematic representation for the schedule of α-MSH pigmentation study. Synchronized embryos were pretreated with 0.2 mM **1** at 9 hpf, then washed intensively and bathed immediately in the fresh medium at 35 hpf. Chemicals were added and incubated for a further 37 h. B) Representative morphology of 72 hpf zebrafish larvae. C, D, E) Pigmentation image analysis of 72 hpf zebrafish larvae by binary threshold, black spot histogram, and black spot size. Data are expressed as means ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate a significant difference between groups.

Melanocyte stimulating hormones (MSHs) play a key role in producing coloured pigmentation found in the skin, hair and eyes. α-Melanocyte stimulating hormone (α-MSH), an endogenous tridecapeptide hormone produced by melanocortin is the most important one of the MSHs in stimulating melanogenesis. All groups were exposed with **1** during 9 ~ 35 hpf and then treated with mock (group I), **1** (group II), α-MSH (group III), and 1 + α-MSH co-treatment (group IV) (Fig. 6). The removal of **1** (group I) recovered the pigmentation (low BT, HB & moderate SB). As expected, the treatment of **1** (group II) suppressed the pigmentation (high BT, HB & low SB) and the treatment of α-MSH (group III) stimulated the pigmentation (low BT, HB & high SB). Interestingly, the co-treatment of **1** and α-MSH (group IV) showed the similar pattern with group II; a suppressed pigmentation (high BT, HB & low SB). These data indicate that **1** acts as a competitive inhibitor of α-MSH induced melanogenesis.

In summary, we synthesized two hydroquinone-tetraethylene glycol conjugates, **1** and **2**. The logP, photophysical stability, and redox chemical stability of them were compared with HQ. The mono tetraethylene glycol-functionalization of HQ improved the photophysical and Cu^{2+} redox chemical stability. MTT assay in HF-16 cells showed **1** is less cytotoxic than HQ or **2**. We observed the regulatory effect of **1** and **2** on zebrafish pigmentation. The quantitative image analysis of zebrafish larvae showed that **1** and **2** suppressed the pigmentation of zebrafish in a dose-dependent manner. The comparative experiments on stability, cytotoxicity, and zebrafish pigmentation between HQ, **1**, and **2** suggest that mono tetraethylene glycol-functionalization of HQ is an alternative solution to overcome the conventional disadvantages of HQ.

Acknowledgments

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Supplementary Material

Supplementary data associated with this article (synthesis and characterization of **1** and 2, experiment details of stability test, logP measurement, MTT assay, phenotype-based evaluation of zebrafish melanogenesis) can be found, in the online version, at http://dx.doi.org/xxxx/yyyyy.