Supplementary materials

Title

The surprising effect of phenformin on cutaneous darkening and characterization of its underlying mechanism by a forward chemical genetics approach

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Supplemental Figure 1. Phenformin darkens skin samples from a Hispanic donor and a Caucasian donor in tissue culture.

(a) Skin tissues obtained from a Hispanic subject (48y) were subjected to tissue culture with or without 300 μM phenformin for 8 days. The photographs shown are representative samples. Scale bars = 5 mm. (b) Treated skins were subjected to Fontana-Masson staining. Scale bars = 100 μm. The areas indicated by the squares are shown at higher magnification under each image. (c) Skin tissues obtained from a Caucasian subject (18y) were subjected to tissue culture with or without 300 μM phenformin for 6 days. The photographs shown are representative samples. Scale bars = 5 mm.

Supplemental Figure 2. Phenformin does not affect DOPA oxidase activity and the expression levels of melanogenesis-related proteins in melanocytes.

(a) After treatment of NHEMs with or without phenformin for 3 days (at the indicated concentrations), DOPA oxidase activity was measured. Values represent means ± SD of 6 independent samples (***P < 0.001 versus control (DMSO treatment) by Dunnett’s test). (b) Microscopic images of NHEMs treated with DMSO (control) or phenformin (300 μM or 1 mM). (c) After incubation with or without phenformin for 4 days (at the indicated concentrations), NHEMs were lysed and subjected to western blot analysis of Tyrosinase and TRP1 proteins. β-Actin = internal control.

Supplemental Figure 3. Evaluation of the potential effects of biguanide compounds on the regulation of skin color.

Skin tissues obtained from a Japanese subject (age unknown) were subjected to tissue culture with
or without (a) phenformin, (b) phenylbiguanide, (c) metformin, (d) buformin or (e) N-[amino(imino)methyl]piperidine-1-carboximidamide for 7 days. The photographs shown are representative samples. Scale bars = 5 mm.

**Supplemental Figure 4.** SAR (structure-activity relationship) analysis of biguanide compounds to evaluate their potential skin darkening effects.

Chemical structures of (a) a skin darkening compound (phenformin), and (b) non-skin darkening compounds (phenylbiguanide, metformin, buformin and N-[amino(imino)methyl]-piperidine-1-carboximidamide) are shown. We speculate that amino groups of phenformin are not involved in the darkening activity in contrast to the phenyl group.

**Supplemental Figure 5. Preparation of phenformin-immobilized FG-beads.**

The reaction scheme shows the immobilization of phenformin on magnetic FG-beads. The experimental procedure is described in “Materials and Methods”.

Supplemental Figure 1
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(a) control (DMSO) Phenformin 300 μM
(b) control (DMSO) Phenformin 300 μM
(c) control (DMSO) Phenformin 300 μM
Supplemental Figure 2
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(a) DOPA oxidase activity (% of control) for different concentrations of Phenformin.

(b) Images showing control (DMSO), Phenformin (300 μM), and Phenformin (1 mM).

(c) Western blot analysis showing Tyrosinase, TRP1, and β-Actin levels with different concentrations of Phenformin.
**Supplemental Figure 3**
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(a) | control (DMSO) | Phenformin  |
---|---|---|
| 30 μM | 100 μM | 300 μM | 1 mM |

(b) | control (DMSO) | Phenylbiguanide |
---|---|---|
| 30 μM | 100 μM | 300 μM |

(c) | control (DMSO) | Metformin |
---|---|---|
| 30 μM | 100 μM | 300 μM |

(d) | control (DMSO) | Buformin |
---|---|---|
| 30 μM | 100 μM | 300 μM |

(e) | control (DMSO) | N-[amino(imino)methyl]piperidine-1-carboximidamide |
---|---|---|
| 30 μM | 100 μM | 300 μM |

5 mm
Supplemental Figure 4
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(a) Skin darkening compound

Phenformin

(b) non-skin darkening compounds

Phenylbiguanide

Metformin

Buformin

N-[amino(imino)methyl]piperidine-1-carboximidamide
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\[
\text{NH}_2 \xrightarrow{\text{HCl}} \text{H}_2\text{N} - \text{CN} \xrightarrow{\text{NaCN} \cdot \text{HCl}} \text{H}_2\text{N} - \text{CN} \xrightarrow{\text{xylene reflux}} \text{H}_2\text{N} - \text{CH}_2\text{(CH}_2\text{)}_4\text{NH}_2\cdot\text{HCl}
\]