Thiamidol (isobutylamido thiazolyl resorcinol): A Highly Specific Human Tyrosinase Inhibitor for the Treatment of Hyperpigmentation

Tobias Mann[1], Wolfram Gerwat[1], Jan Batzer[1], Kerstin Eggers[1], Cathrin Scherner[1], Horst Wenck[1], Franz Stab[1], Vincent J. Hearing[2], Klaus-Heinrich Rohm[3], Ludger Kolbe[1]

[1]Front End Innovation, Beiersdorf AG, Hamburg, Germany; [2]DASS Manuscript, Haymarket, Virginia, USA; [3]Institute of Physiological Chemistry, Philipps University, Marburg, Germany

Abstract

Tyrosinase is the rate-limiting enzyme of melanin production, and accordingly, is the most prominent target for inhibiting hyperpigmentation. Numerous tyrosinase inhibitors have previously been identified; however, most lack clinical efficacy because they were identified using mushroom tyrosinase as the target substrate. Therefore, we used recombinant human tyrosinase to screen a library of 50,000 compounds and compared the active screening hits with well-known anti-pigmentation ingredients, including 4-butylresorcinol, kojic acid, rhododendrol, hydroquinone, and arbutin. Hydroquinone and its derivative arbutin only weakly inhibited human tyrosinase with a half-maximal inhibitory concentration (IC_{50}) in the millimolar range, and kojic acid showed a weak efficacy (IC_{50} > 500 µmol/L). The most potent inhibitors of human tyrosinase identified in this screening were resorcinol-thiazole derivatives, specifically the newly identified Thiamidol (Beiersdorf AG, Hamburg, Germany) (isobutylamido thiazolyl resorcinol), which had an IC_{50} of 1.1 µmol/L. In contrast, Thiamidol only weakly inhibited mushroom tyrosinase (IC_{50} = 108 µmol/L), demonstrating the specificity of inhibition for human tyrosinase. In melanocyte cultures, Thiamidol strongly, but reversibly, inhibited melanin production (IC_{50} = 0.9 µmol/L), whereas hydroquinone irreversibly inhibited melanogenesis (IC_{50} = 16.3 mmol/L), demonstrating Thiamidol's inhibition of melanin via tyrosinase inhibition as opposed to cytotoxicity. Clinically, Thiamidol visibly reduced the appearance of age spots within 4 weeks, and after 12 weeks, some age spots were indistinguishable from the normal adjacent skin. This data demonstrates the viability of Thiamidol as a suitable anti-melanogenic ingredient for use in topical cosmetic products.

Materials and Methods

Human Tyrosinase

A truncated, His-tagged form of hTyr (hTyr-DHis) comprising the catalytic domain of hTyr was expressed in HEK 293 cells and purified by metal affinity chromatography on Ni2þ-Sepharose (GE Healthcare, Munich, Germany) as described elsewhere (Cordes et al., 2013). The resulting preparation had the same catalytic properties as wild-type hTyr.

Sources of Inhibitors

From the Evotec compound library (Evotec, Hamburg, Germany), 50,000 compounds, covering a wide chemical space, were selected to conduct an HTS for hTyr inhibitors, assessed using the Tyr assay described in the next section. Derivatives of promising lead compounds were then synthesized for further optimization.

Tyr Assay and HTS Procedure

The L-Dopa oxidase activity of hTyr was assayed at 40ºC in 50 µM sodium phosphate buffer, pH 7.0, using a modification of the method of Winder and Harris (Winder and Harris, 1991). The reaction product, L-dopaquinone, spontaneously reacts with the assay ingredient MBTH (3-methyl-2-benzothiazolinone-hydrazone) to form a stable pink dye. The resulting increase of absorption at 490 nm was monitored over time using a microplate reader. In the primary screen, performed in cooperation with Evotec AG (Hamburg, Germany), the compounds were tested in singlicate at a concentration of 25 µM. The threshold was set to 3 σ, meaning that each compound result that was higher (meaning lighter) than 3 times the standard deviation of all results on the respective test-plate was considered a hit. These compounds were then retested to confirm the results and to get a dose-response curve. The final hit rate was about 1% of all tested compounds, and the hits were clustered according to their chemical similarity. These hit clusters were ranked according to their chemical accessibility to be modified and to explore structure-activity relationships. One of those hit clusters contained the resorcinol-aminothiazoles, and a MedChem program produced the final compound Thiamidol. For dose-response curves, various concentrations of each inhibitor were applied in triplicate at a fixed substrate concentration of 1 mM. Inhibitor concentrations at 50% inhibition (IC_{50} values) were estimated by fitting a four-parameter logistic equation to the profiles.

For a detailed *in vitro* kinetic analysis of inhibitors, reactions at 7 different substrate concentrations and 5 different inhibitor concentrations were measured. Inhibition constants Ki and their standard deviations were then estimated from global fits of appropriate model equations to the resulting data (Sun et al., 2014). The enzyme kinetics module of SigmaPlot 11 (Systat Software, San Jose, CA) was used for curve fitting.

Melanocyte Cultures

Melanocytes derived from African-American donors used in these studies were derived and cultured as previously described (Ebsen, 2011). Melanocytes were cultured for the times noted in the text with or without Thiamidol, after which the cells were photographed.

Clinical Studies

Two randomized *in vivo* studies (blinded for the test products, open for the untreated control) were conducted. One study enrolled 18 female subjects (56-71 years of age), with 17 subjects completing the study. The second study was performed with 19 subjects (18 females, 1 male; 58-70 years of age), with all 19 subjects completing the study. Each subject applied two different formulations twice daily to age spots on their volar forearms using a spot applicator. The formulations differed only in the active ingredient: 0.2% Thiamidol versus vehicle in the first study, 0.1% Thiamidol versus vehicle in the second study. One age spot per subject was treated with a formula containing the active ingredient, and a control spot was treated with the vehicle only. Pigmentation of the age spots was measured in both studies by spectrometry (Spectro-Pen™, L- values) and/or digital photography (EpiFlash™) before the first product application (baseline), and at certain points in time during the treatment. The SAS software package for Windows V9.1.3 was used for statistical analysis. Data were tested for normality using the Shapiro-Wilk test. Significance was tested using the Wilcoxon signed rank test. A value of $P \leq 0.05$ is considered statistically significant (two-sided hypothesis testing). The *in vivo* studies were conducted according to the recommendations of the current version of the Declaration of Helsinki and the guidelines of the International Conference on Harmonization Good Clinical Practice. All participants in these studies provided written informed consent. In addition, the studies were approved and cleared by the institutional review board of Beiersdorf AG (Hamburg, Germany).

Results

FIGURE 1. Chemical structure of inhibitory compounds evaluated in this study. Compound 1, Thiamidol (Beiersdorf AG, Hamburg, Germany) (isobutylamido thiazolyl resorcinol); compound 2, 4-butylresorcinol; compound 3, 4-hexylresorcinol; compound 4, 4-phenylethylresorcinol; compound 5, kojic acid; compound 6, hydroquinone; compound 7, arbutin; compound 8, dimethoxytolyl propylresorcinol; and compound 9, rhododendrol.

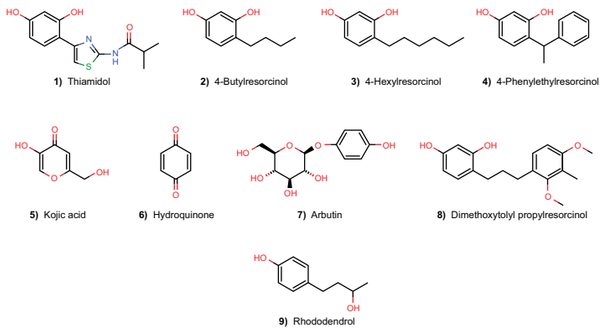

FIGURE 2. Inhibition of human tyrosinase, inhibitor concentration 50 (IC_{50}). *In vitro* assays using purified human tyrosinase in 50 mmol/L sodium phosphate buffer, pH 7.0, at a substrate (L-Dopa) concentration of 1 mmol/L and various concentrations of inhibitors as noted. Data represent mean ± standard deviation of three independent experiments.

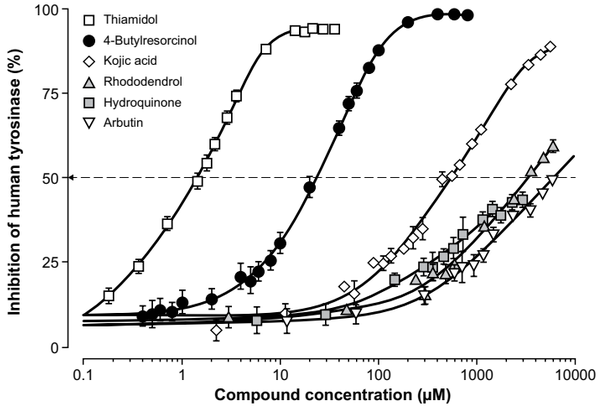

FIGURE 3. Thiamidol inhibition of melanin production in human melanocytes. Melanocytes from donors were cultivated for 2 weeks (left) with or (right) without 5 µmol/L Thiamidol (Beiersdorf AG, Hamburg, Germany) in microplate dishes; photographs were taken in bright field mode. Scale bars = 200 µm.

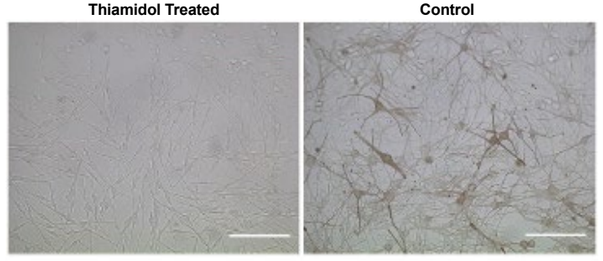

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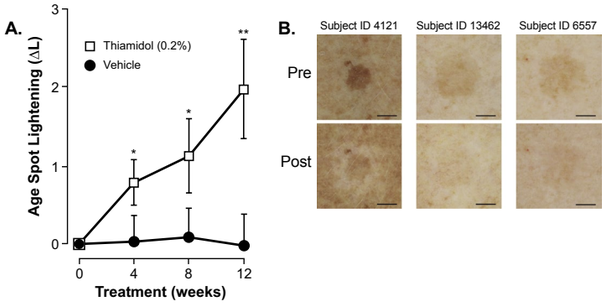
FIGURE 4. *In vivo* inhibition of hyperpigmentation (Age Spots). A. Age spots on the volar forearms of each subject were treated twice daily for 12 weeks with 0.2% Thiamidol (Beiersdorf AG, Hamburg, Germany) or with the vehicle only as a control using a spot applicator. Efficacy was evaluated after 4, 8, and 12 weeks. Data represent the mean ± standard error of the mean of subjects (n = 17). *P < 0.05, **P < 0.01; statistically significant versus the control. **B.** Visual monitoring of the lightening of age spots during treatment. Photographs were taken (top) before and (bottom) after 12 weeks of treatment. Images show three representative age spots. Scale bar = 5 mm. Pre, before treatment; Post, after 12 weeks of treatment.



Summary and Conclusions

- One of the safest and most effective ways to treat hyperpigmentation is to reduce melanin production via inhibiting tyrosinase activity
- Most tyrosinase inhibitors described in the literature lack clinical efficacy when incorporated into topical products, partly because of their specificity against mushroom tyrosinase vs human tyrosinase
- We have identified Thiamidol (isobutylamido thiazolyl resorcinol) out of 50,000 compounds identified
- Thiamidol is the most effective inhibitor (IC_{50} = 1.1 µmol/L) of human tyrosinase compared to commonly used anti-pigment ingredients including kojic acid, hydroquinone, and its derivative arbutin
- In human melanocytes, Thiamidol demonstrated inhibition of melanin production with no signs of cytotoxicity
- In two human clinical trials, Thiamidol significantly inhibited melanin production in age spots compared to vehicle control as early as Week 4 ($P < 0.05$), with maximum inhibition at end of study (Week 12, $P < 0.01$)
- This data demonstrates the viability of Thiamidol as a suitable anti-melanogenic ingredient for use in topical cosmetic products

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