ORIGINAL ARTICLE

4-n-butylresorcinol, a highly effective tyrosinase inhibitor for the topical treatment of hyperpigmentation

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Abstract

Background Hyperpigmentary disorders like melasma, actinic and senile lentigines are a major cosmetic concern. Therefore, many topical products are available, containing various active ingredients aiming to reduce melanin production and distribution. The most prominent target for inhibitors of hyperpigmentation is tyrosinase, the key regulator of melanin production. Many inhibitors of tyrosinase are described in the literature; however, most of them lack clinical efficacy.

Methods We were interested in evaluating the inhibition of skin pigmentation by well-known compounds with skin-whitening activity like hydroquinone, arbutin, kojic acid and 4-n-butylresorcinol. We compared the inhibition of human tyrosinase activity in a biochemical assay as well as inhibition of melanin production in MelanoDerm™ skin model culture. For some compounds, the *in vivo* efficacy was tested in clinical studies.

Results Arbutin and hydroquinone only weakly inhibit human tyrosinase with a half maximal inhibitory concentration (IC_{50}) in the millimolar range. Kojic acid is 10 times more potent with an IC_{50} of approximately 500 μmol/L. However, by far the most potent inhibitor of human tyrosinase is 4-n-butylresorcinol with an IC_{50} of 21 μmol/L. In artificial skin models, arbutin was least active with an IC_{50} for inhibition of melanin production > 5000 μmol/L. Kojic acid inhibited with an IC_{50} > 400 μmol/L. Interestingly, hydroquinone inhibited melanin production in MelanoDerms with an IC_{50} below 40 μmol/L, probably due to a mechanism different from tyrosinase inhibition. Again, 4-n-butylresorcinol was the most potent inhibitor with an IC_{50} of 13.5 μmol/L. *In vivo* efficacy of 4-n-butyl-resorcinol was confirmed in clinical studies. Subjects with age spots on the forearm treated twice daily two age spots with a formula containing 4-n-butylresorcinol and two control age spots with the corresponding vehicle. Within 8 weeks, 4-n-butylresorcinol reduced visibly the appearance of age spots, while the control spots showed no improvement. A second study showed that 4-butylresorcinol was more effective than 4-hexylresorcinol and 4-phenylethylresorcinol.

Conclusion The present *in vitro* and *in vivo* data prove the high inhibitory capacity of 4-n-butylresorcinol on human tyrosinase activity, exceeding by far the potency of hydroquinone, arbutin and kojic acid. The resulting clinical improvement of skin hyperpigmentations reveals 4-n-butylresorcinol as a very valuable active compound for the management of pigmentation disorders.

Conflict of Interest

The authors are employees of Beiersdorf AG. The sponsor has provided funding to support the work of this study.

Introduction

Hyperpigmentary disorders like melasma, actinic and senile lentigines and postinflammatory hyperpigmentation are a major cosmetic problem for which patients seek medical advice. These disorders affect populations with darker skin complexion, like Asians and Hispanics, with greater frequency and severity.[1] Numerous topical products are available, containing diverse active ingredients to reduce melanin production and distribution. In principle, skin depigmentation can be achieved by regulating the transcription and activity of tyrosinase and other melanosomal proteins, inhibition of melanocyte activation, interference with the uptake and distribution of melanosomes in keratinocytes and an accelerated turnover of pigmented keratinocytes.[2] Tyrosinase is the key regulator of melanin production and, consequently, the most prominent target for inhibitors of hyperpigmentary disorders. To date, many substances have been described in the literature as inhibitors of tyrosinase; however, most of them lack the efficacy and specificity necessary for practical applications and, thus, only

a few compounds are being used in topical products.[3–5] Among these, kojic acid and hydroquinone arbutin are most widely used.[6] Skin-whitening actives have to be applied as a long-term treatment and may cause various side-effects, like skin irritation and dyschromia; therefore, it is important to find an effective and well-tolerable alternative to reduce pigment irregularities and protect against the reappearance. We were interested in evaluating the inhibition of skin pigmentation by well-known compounds with skin-whitening activity like hydroquinone, arbutin kojic acid and 4-n-butylresorcinol, and therefore compared the efficacy in various *in vitro* and *in vivo* assay systems.

Material and methods

L-Dopa tyrosinase assay

The L-DOPA oxidase activity of human tyrosinase oxidizes L-DOPA into L-DOPA-quinone, which spontaneously reacts with the assay-ingredient 3-methyl-2-benzothiazolinhydrazone (MBTH) to a stable pink–red dye. The increase in absorption (at 490 nm) over time was determined for various inhibitor concentrations to calculate the concentration for a 50% inhibition of enzyme activity (IC_{50} values). The assay is a modified version of the method from Winder and Harris.[7] The measurements were performed using a truncated, His-tagged form of human tyrosinase that comprises the catalytic domain of the enzyme. The enzyme was expressed in HEK 293 cells essentially as described by Schweikardt[8] and purified by metal affinity chromatography on Ni2+-Sepharose.

Skin model assay

MelanoDerm™ from MatTek Corporation (Ashland, MA, USA) were incubated with various inhibitors for 13 days according to manufacturer's instructions to allow for melanin production. Afterwards, melanin of air-dried models was dissolved in 7.5 N NaOH (12 h at 99°C). The solutions were measured in a plate reader and the integral of absorption between 380 and 440 nm was calculated. The results are expressed as inhibition of melanin production compared with the untreated control (=0% inhibition). Some MelanoDerms were snap frozen at the start of the experiment and served as controls, indicating the initial melanin content that represent the level for maximum inhibition (=100% inhibition).

Clinical studies

Two single-centre, randomized *in vivo* studies (blinded for the test products, open for the untreated control) were conducted to verify the *in vitro* results. Study I enrolled 14 female subjects (55–69 years of age) with at least four measurable age spots on the inner forearm. Four different formulations were applied twice daily to age spots of the volar forearm. The formulas only differed in the active ingredient; one contained 0.3% 4-n-butylresorcinol, the second 0.3% hexylresorcinol and a third formula contained 0.5% phenylethylresorcinol. Vehicle-treated age spots served as control.

In study II, 15 female subjects, aged 53–70, treated age spots on the volar forearm with a spot applicator. Two age spots were treated with a formula containing 1% butylresorcinol, two age spots were treated with the respective vehicle.

In both studies, pigmentation of the age spots was measured by spectrometry (Spectropen, Hach Lange GmbH, Düsseldorf, Germany) and digital photography Epi-Flash, Beiersdorf AG, Hamburg, Germany before the first product application (baseline), and after various points in time of treatment.

The *in vivo* studies were conducted according to the recommendations of the current version of the Declaration of Helsinki and the guideline of the International Conference on Harmonization Good Clinical Practice (ICH GCP). In addition, this study was approved and cleared by the institutional ethics review board (Beiersdorf AG, Hamburg, Germany).

Statistical analysis

SAS Software Package for Windows V9.1.3 (SAS Institute GmbH, Heidelberg, Germany) was used for statistical analysis. Data were tested for normality using Shapiro–Wilk's test. Significance was tested with Wilcoxon's signed rank test. A value of $P \leq 0.05$ was considered statistically significant (two-sided hypothesis testing).

Results

Inhibition of human tyrosinase

All four compounds were tested over a wide range of concentrations, up to four orders of magnitude, for tyrosinase inhibition (Fig. 1). 4-butylresorcinol proved to be a highly effective inhibitor of human tyrosinase with an IC_{50} of 21 μmol/L and complete enzyme inhibition at concentrations above 100 μmol/L. The resorcinol derivatives 4-hexylresorcinol and 4-phenylethylresorcinol showed an IC_{50} of 94 and 131 μmol/L respectively (data not shown). Kojic acid was more than 20 times less potent with an IC_{50} at 500 μmol/L and maximum inhibition (89%) at 5.6 mmol/L concentration. Arbutin and hydroquinone are only poor inhibitors of human tyrosinase with IC_{50} values in the millimolar range, i.e. approximately 6500 μmol/L for arbutin and 4400 μmol/L for hydroquinone. Neither arbutin nor hydroquinone completely inhibited human tyrosinase.

Reduction of melanin production

Arbutin showed only marginal efficacy on melanin production in MelanoDerm skin models with an IC_{50} for inhibition of > 5000 μmol/L (Fig. 2). Kojic acid inhibited melanin synthesis with an IC_{50} > 400 μmol/L and showed a surprisingly steep dose–response curve. Concentrations below 200 μmol/L only marginally inhibited melanin production, i.e. 5% inhibition at 150 μmol/L. Interestingly, hydroquinone inhibited melanin production in MelanoDerms with an IC_{50} below 40 μmol/L, pointing towards a mechanism different from tyrosinase inhibition. 4-butylresorcinol was the most potent inhibitor with an IC_{50} of 13.5 μmol/L. A

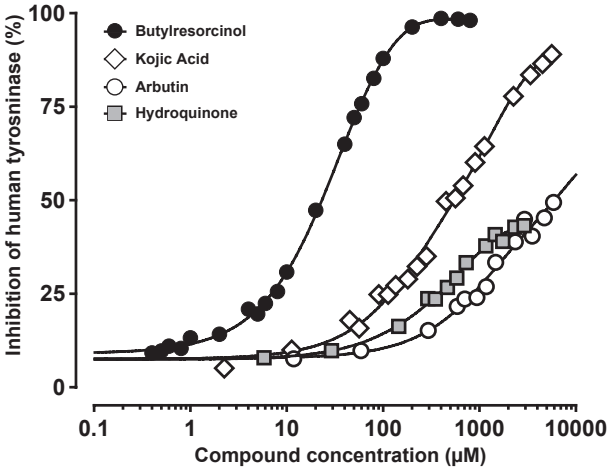

Figure 1. Inhibition of human tyrosinase by 4-butylresorcinol, kojic acid, arbutin and hydroquinone. The L-DOPA oxidase activity of tyrosinase was determined at various concentrations of the inhibitors to allow for the calculation of IC_{50} values. Data represent the mean of three independent experiments.

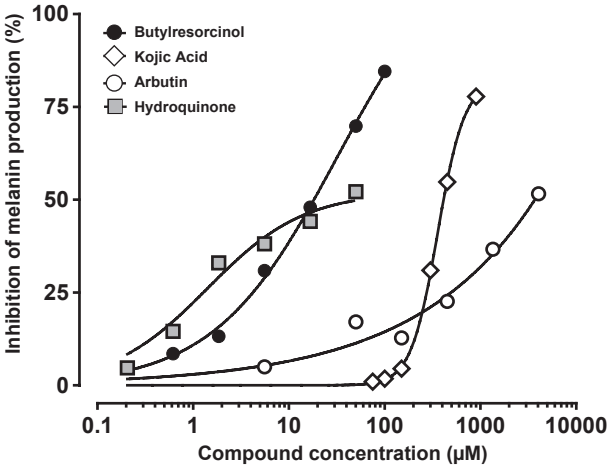

Figure 2. Inhibition of melanin production in MelanoDerm™ skin models by 4-butylresorcinol, kojic acid, arbutin and hydroquinone. Melanin content of skin models was determined after 13 days of cultivation in the presence of various inhibitor concentrations. Data represent the mean of five independent experiments.

comparison of the dose–response curves of hydroquinone and 4-butylresorcinol reveals that at concentrations above 20 μmol/L, 4-butylresorcinol is slightly more effective than hydroquinone, at concentrations below 20 μmol/L hydroquinone is slightly more effective than 4-butylresorcinol.

Clinical studies

Elderly subjects treated age spots twice daily either with a formula containing 4-butylresorcinol, 4-hexylresorcinol or 4-phenylethylresorcinol (Fig. 3). Within 8 weeks, 4-butylresorcinol significantly reduced the appearance of age spots while 4-hexylresorcinol or 4-phenylethylresorcinol showed significant effects after 12 weeks (all in comparison to vehicle).

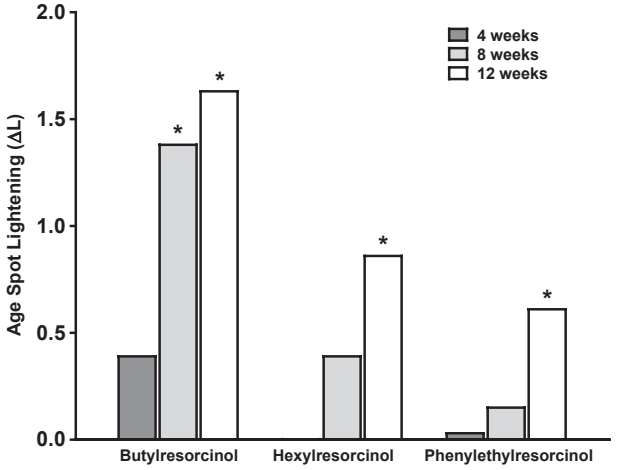

Figure 3. Age spot lightening by 4-butylresorcinol, 4-hexylresorcinol and 4-phenylethylresorcinol. The spots were treated twice daily for 12 weeks with a formula containing the respective inhibitor. Efficacy was evaluated after 4, 8 and 12 weeks. Data represent the mean of 14 subjects. *$P < 0.05$: statistically significant vs. the untreated control age spots.

Epi-Flash photographs revealed visible improvement in the appearance of age spots after 12 weeks of treatment with 4-butylresorcinol. Control age spots remained unchanged (not shown).

In a second study, subjects applied 1% 4-butylresorcinol to age spots of the volar forearm using a spot applicator. Control age spots were treated with a spot applicator containing the vehicle. Already after 4 weeks of treatment, the treated spots were lighter than the control spots. Improvement continued over the entire treatment period, and after 16 weeks some of the spots were undistinguishable from the surrounding skin (Fig. 4). After treatment, the age spots were monitored for several weeks. Even after 4 weeks without treatment, the age spots previously treated with 4-butylresorcinol were still significantly lighter than the vehicle-treated spots.

Discussion

Inhibition of tyrosinase activity is the most effective way to reduce hyperpigmentation. However, most topical products with tyrosinase inhibitors lack clinical efficacy. This is no surprise, as most screenings for inhibitors have been performed with the only commercially available enzyme, tyrosinase from the mushroom *Agaricus bisporus*.[9,10] This is mainly due to considerable difficulties to extract sufficient amounts of human tyrosinase from biological sources. In fact, many substances described as inhibitors of tyrosinase were only tested on mushroom tyrosinase and are, thus, very

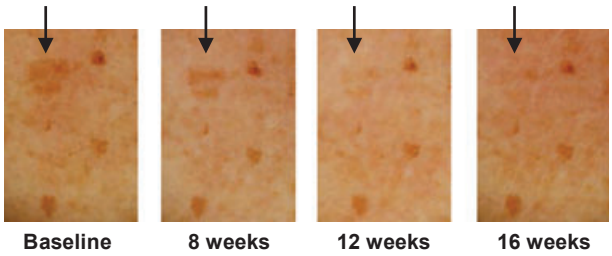

Figure 4. Clinical Study – monitoring of a treated age spot during treatment with a spot applicator. Photographs of age spots were taken at baseline and after 8, 12 and 16 weeks of treatment. The arrows mark the treated age spot. For comparison, untreated spots were included in the photographed area.

effective inhibitors of mushroom tyrosinase, but rather poor inhibitors of human tyrosinase. The objective of this study, therefore, was to compare the inhibitory capacity of arbutin, hydroquinone, kojic acid and 4-butylresorcinol on melanin production using human *in vitro* test systems and to select the best active ingredient for further *in vivo* studies. All four substances are known as tyrosinase inhibitors,[11] however, the published range of inhibitory activity is extremely broad and divergent.

In the medical literature, hydroquinone is considered the gold standard for the treatment of hyperpigmentation. However, there are severe concerns regarding the safety of hydroquinone. Banned in the EU from the use in cosmetics, it is still sold in the USA as over-the-counter drug in formulations with up to 2% hydroquinone. Recently, the FDA also expressed concerns[12]; however, a final ruling is still pending. The published IC_{50} values for hydroquinone in the mushroom tyrosinase inhibition cover a wide range from 1.1[13] to 680 μmol/L.[14] In our human assay, hydroquinone was remarkably ineffective and only marginally inhibited human tyrosinase, barely reaching 50% inhibition at 4400 μmol/L. Since Palumbo published his results in 1991[15] hydroquinone is considered a tyrosinase inhibitor; however, the cytotoxic effects seem to be more important for the efficacy of the molecule,[2] not only for the adverse effects. This view is substantiated by our results with the MelanoDerm skin models. Here, hydroquinone (IC_{50} < 40 μmol/L) is almost as effective as the potent tyrosinase inhibitor 4-butylresorcinol.

Although arbutin is considered a potent tyrosinase inhibitor, the published IC_{50} values for mushroom tyrosinase range from 40 μmol/L[16] to more than 30,000 μmol/L.[17] On human tyrosinase, we found an IC_{50} in the millimolar range (~6500 μmol/L), and also on MelanoDerm models we measured an IC_{50} in the same range (>5000 μmol/L). According to the literature, alpha-arbutin seems to be slightly more effective than beta-arbutin, but both are hydroquinone prodrugs and the activity depends on the release of hydroquinone from the molecule.[2] The European Union Scientific Committee on Consumer Products published a critical opinion on arbutin. The release of hydroquinone from the molecule raised their concern, and consequently the SCCP regards the use of arbutin in cosmetic products as unsafe.[18]

The published tyrosinase IC_{50} values for kojic acid vary between less than 20 μmol/L[19] up to more than 100 μmol/L.[20] On human tyrosinase, kojic acid is less effective with an IC_{50} of about 500 μmol/L. On MelanoDerms, kojic acid shows a surprisingly steep dose–response curve, from more than 75% inhibition at 900 μmol/L down to 5% at 150 μmol/L. This might explain, in part, the very limited efficacy of kojic acid *in vivo*. Concerning the safety of kojic acid, the European Scientific Committee on Consumer Safety[21] now considers kojic acid in concentrations up to 1.0% safe for cosmetic products when applied to healthy skin, this view is shared by the Cosmetic Ingredient Review Expert Panel.[22]

The 4-substituted resorcinol motif has been known for a long time as a very powerful chemical moiety for the inhibition of human tyrosinase.[23] Many natural compounds, mainly flavonoids, known as whitening ingredient contain this resorcinol motif.[19,24] Unfortunately, the bioavailability of flavonoids is generally very low, and we therefore searched for smaller resorcinols with high effectiveness and good bioavailability. Among these resorcinol derivatives, 4-butylresorcinol has been characterized as a strong tyrosinase[25] and TRP-1[26] inhibitor. We measured an IC_{50} in the human tyrosinase assay of 21 μmol/L for 4-butylresorcinol compared with 94 and 131 μmol/L for 4-hexylresorcinol and 4-phenylethylresorcinol respectively. Also on skin models, 4-butylresorcinol was most effective of all tested substances with an IC_{50} of 13.5 μmol/L. Therefore, 4-butylresorcinol was selected for several clinical studies to prove *in vivo* efficacy. In comparison with 4-hexylresorcinol and 4-phenylethylresorcinol, 4-butylresorcinol treated age spots showed a faster onset of improvement and also a higher degree of lightening after 12 weeks of treatment. A study with a spot applicator showed continuous improvement over the entire treatment period and even after ceasing treatment, the age spots remained for 4 weeks significantly lighter than control spots. Even 13 weeks later, some of the spots still showed some improvement. Based on these results, a skin care line was developed and tested under dermatological supervision on Asian women with pigmentary disorders like melasma, age spots or postinflammatory hyperpigmentation.[27] The results of the studies demonstrate a significant pigment-reducing efficacy of the test products and a clear clinical benefit of the tested product line in the management of facial hyperpigmentation.

In conclusion, the present data show that 4-butylresorcinol is a powerful human tyrosinase inhibitor with remarkable *in vivo* effectiveness. Topical products containing 4-butylresorcinol show strong efficacy on age spots, melasma[28] and other facial hyperpigmentation.

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