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Evaluation of anti-pigmentary effect of synthetic sulfonylamino chalcone

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ABSTRACT

The 4′-(*p*-toluenesulfonylamino)-4-hydroxychalcone (TSAHC), which bears inhibitory chemotypes for both α-glucosidase and tyrosinase, was evaluated for tyrosinase activity and depigmenting ability relative to compounds designed to only target tyrosianse activity. TSAHC emerged to be a competitive reversible inhibitor of mushroom tyrosinase. More importantly, it was also able to return the melanin content of α-melanocyte stimulated by α-MSH to base levels unlike other inhibitors that only targeted tyrosinase. The Western blot for expression levels of proteins involved in melanogenesis showed that TSAHC significantly decreased three main tyrosinase related protein in melanin biosynthesis, tyrosinase, TRP-1 and TRP-2.

1. Introduction

Excessive/uneven pigmentation is the root cause of numerous skin problems including age spots, melasma and chloasma. These maladies can be initiated by ultraviolet light, chronic inflammation as well as abnormal levels of α-melanocyte stimulating hormone (α-MSH) which results in overproduction of melanin [1,2]. It is thus not unsurprising that many efforts have been devoted to screening both recognized and putative depigmenting agents in a bid to develop drugs to attenuate hyperpigmentation. Most of these screens have in some way targeted tyrosinase, which has been considered to be an effective approach to treat a variety of hyperpigmentary disorders [3]. Tyrosinase in *Homo sapiens* is a membrane-bound glycoprotein with an active site containing two copper ions. This enzyme catalyzes two different processes involving the oxidation of tyrosine, first to L-DOPA (monophenolase) and then to dopaquinone (diphenolase). Tyrosinase is thus a perfect target in many ways because it is produced only by melanocytic cells and to reach its fully active form it requires processing (glycosylation) in the ER and Golgi, after which time it is trafficked to specialized organelles, melamosomes [3]. Thus numerous highly tyrosinase specific processes can be targeted to effect inhibition of melanin production. In principle, tyrosinase activity can be attenuated by regulating the transcription of tyrosinase mRNA, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2) [4] and its maturation via asparagine-liked oligosaccharide processing [5–7]. However, arguably the most direct route is to inhibit tryosinase (EC 1.14.18.1).

Numerous reports have focused on the inhibition of tyrosinase as the sole route to depigmentation of the epidermal layer [8,9]. Kojic acid is a representative tyrosinase inhibitor with an IC_{50} of 16 μM against mushroom tyrosinase. Its activity is ascribed to copper chelation. However, despite the wide knowledge garnered to date concerning depigmentation and the numerous ways to bring it about, we noticed that there has so far been no effort to target multiple proteins in the pathway to melanogenis in one inhibitor. For instance, one of the most widely employed strategies to effect a decrease in cellular tyrosinase activity which does not involve direct tyrosinase inhibition is the inhibition of enzymes involved in tyrosinase *N*-glycosylation [10]. This reduces tyrosinase

Abbreviations: IC_{50}, the inhibitor concentration leading to 50 % activity loss; K_i, inhibition constant; TRP-1, tyrosinase related protein-1; TRP-2, tyrosinase related protein-2; α-MSH, α-melanocyte stimulating hormone; TSAHC, 4′-(*p*-toluenesulfonylamino)-4-hydroxychalcone; NMR, nuclear magnetic resonance; TM4SF5, four-transmembrane L6 family member 5.

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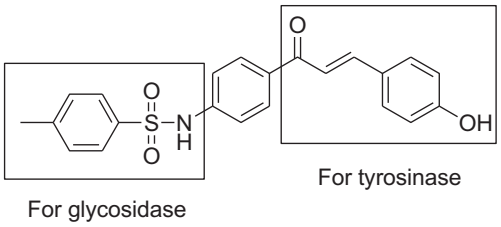


Fig. 1. Different chemotypes within TSAHC and their presumed targets.

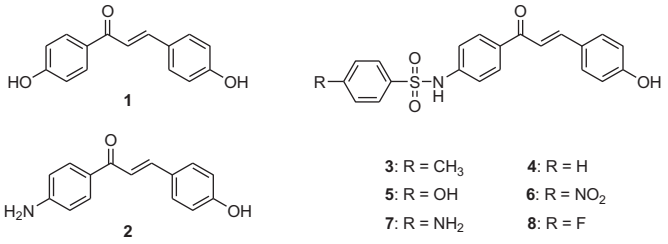


Fig. 2. Chemical structures of chalcone derivatives (**1–8**).

procesing and lowers tyrosinase activity because the tyrosinase can not reach its full maturity, and hence remains inactive. It thus occurred to us that a substance which can inhibit both tyrosinase and glycosidase may be a more promising depimenting agent.

Chalcones are natural products of widespread occurrence in plants. They have been reported to exhibit a wide range of pharmaceutical effects including antioncogenetic, anti-inflammatory, anti-ulcerative, antimalarial, antiviral, antifungal, and antibacterial activities [11]. Recently, they were highlighted as a new class of tyrosinase inhibitor in several publications. Nerya Ohad et al. reported that an OH group at the 4-position in B-ring of the chalcone is the major factor affecting inhibitory potency. This is presumably because these species have a phenol chemotype which the enzyme's native substrate, tyrosine, also contains [12]. In a previous communication, we showed that sulfonylamino chacones are potent α-glucosidase inhibitors with nanomolar IC_{50} values. Our structure activity relationship (SAR) studies unearthed some important requirements for α-glucosidase inhibitory activity (Fig. 1) [13,14]. In subsequent work, amino chalcones were able to reduce the tumorigenic proliferation induced by the transmembrane four L6 family member 5 (TM4SF5), the function of which is deeply related to *N*-glycosylation [15].

When all the above results were considered, we hypothesized that sulfonylamino hydroxy-chalcones (hybrids of tyrosinase inhibiting hydroxychalcones and glucosidase inhibiting sulfonamide chalcones) may have formidable antimelanogenic activity as they can inhibit both processing of tyrosinase as well as tyrosinase catalytic activity. Herein, we report that 4′-(*p*-toluenesulfonylamino)-4-hydroxychalcone (TSAHC) is an excellent depigmentary agent in a number of cell based assays through inhibition of tyrosinase's catalytic function and tyrosinase related proteins such as TRP-1 and TRP-2.

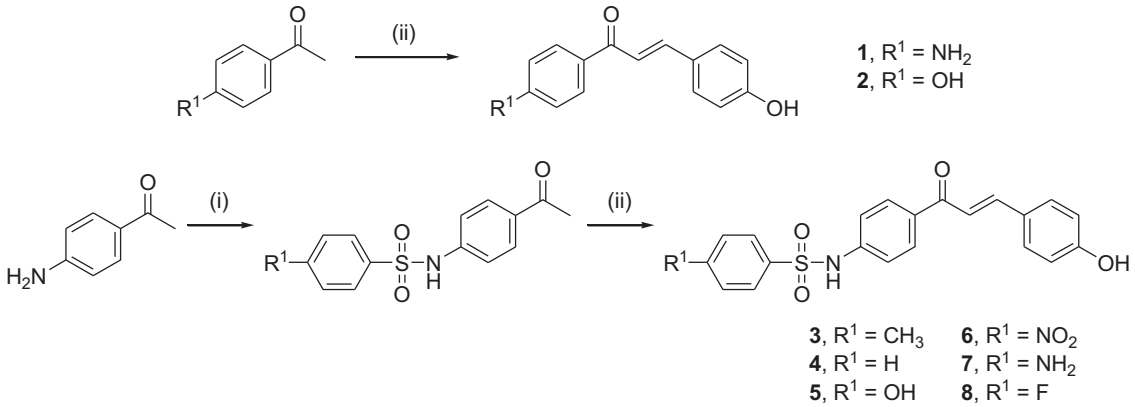
In these studies, we chose to focus our efforts on inhibitors bearing a *para*-hydroxy substituent in the B ring of the sulfonylamino chalcone. This is because it is well known that the corresponding catechol derived species are highly cytotoxic, being rapidly oxidized to *ortho*-quinones by tyrosinase [16]. As such they are not viable start points for *in vivo* screening of compounds which reduce pigmentation, which is the principal objective of this study. Furthermore, sulfonylamino chalcones bearing an unsubstituted phenyl unit on the ring B were not effective tyrosinase inhibitors *in vitro*, which is consistent with the general belief that a 4-hydroxy phenyl moiety on ring B of the chalcone is required for tyrosinase inhibitory activity.

2. Chemistry

Sulfonylamino chalcone derivatives were easily obtained through the Claisen–Schmidt condensation of hydroxybenzaldehyde and derivertized acetophenones using an acidic catalyst [13]. The 4′-(*p*-toluenesulfonylamino)-4-hydroxychalcone (TSAHC) was prepared from hydroxybenzaldehyde and *N*-sulfonylamino acetophenone using a catalytic amount of H_2SO_4 in MeOH (Scheme 1).

3. Results and discussion

The aim of the present work is to evaluate the anti-pigmentary effects of the bifunctional inhibitor TSAHC (**3**), which is adorned with functionalities for controlling both glycosidase and tyrosinase activities (Fig. 1). To allow us to draw meaningful comparisons we compare the efficacy of TSAHC with structurally similar inhibitors which only target tyrosinase. In our previous study, TSAHC inhibited α-glucosidase with 0.58 μM K_i value [13]. Molecular docking simulations revealed that sulfonylamino chalcones bind to the active site in a similar fashion to known inhibitors like acarbose and voglibose [14]. The sulfonylamino group plays the critical role in inhibitor/protein interaction. For example His111 and His348 within α-glucosidase can interact with the SO_2 function and indeed both these residues are crucial for an efficient enzyme/substrate binding interaction [14]. As shown in Fig. 2, the sulfonylamino chalcones (**3–8**) in this study also have a 4-hydroxychalcone moiety that was suggested to be instrumental in tyrosinase inhibiton by Nerya Ohad et al. [12].



Scheme 1. Synthesis of chalcone derivatives: (i) *p*-toluenesulfonyl chloride, pyridine, CH_2Cl_2, r.t, (ii) 4-Hydroxybenzaldhyde, H_2SO_4, MeOH, reflux.

In order to investigate and evaluate the activity of TSAHC against tyrosinase, we examined its tyrosinase inhibitory activity and compared it to all chalcone derivatives (**1–8**) as shown Fig 3A. Table 1 shows that all compounds exhibited a significant degree of monophenolase (L-tyrosine substrate to dopaquinone) inhibition, although sulfonylamino chalcones showed slightly lower inhibitory activities compared to the parent chalcones (**1**, **2**). The sulfonylamino chalcones inhibit tyrosinase dose-dependently with a similar pattern to the parent chalcones (**1**, **2**) (Fig. 3A). The inhibition of mushroom tyrosinase by TSAHC is illustrated in Fig. 3B representatively. Plots of the initial velocity versus enzyme concentration in the presence of different concentrations of TSAHC gave a family of straight lines, all of which passed through the origin. Increasing the inhibitor concentration resulted in a lowering of the line gradients, indicating that TSAHC was a reversible inhibitor. The mode of inhibition was analyzed by Lineweaver–Burk plots (Fig. 3C), which showed that TSAHC behaved as a competitive inhibitor because increasing concentration of substrate resulted in a family of lines which declined with a common intercept on the *y*-axis. The inhibition kinetics were illustrated by Dixon plots, which were obtained by plotting $1/V$ versus $[I]$ with varying concentrations of substrate. Dixon plots give a family of straight lines passing through the same point at the second quadrant, giving the inhibition constant ($-K_i$) (Fig. 3D). Most importantly, the plots of initial rate vs enzyme concentration (Fig. 3B) for TSAHC all consisted of straight lines passing through the origin, which strongly suggest that TSAHC is a competitive inhibitor for the monophenolase activity of tyrosinase. Through a similar analysis, all synthetic chalcones (**1–8**) have respective inhibition constants of 2.9, 3.9, 12.6, 13.3, 25.2, 11.4, 16.7, and 14.9 μM (Table 1).

Table 1
Tyrosinase inhibitory activity and melanin formation of chalcone derivatives (**1–8**).

Compound	Tyrosinase (enzyme)			Mealnin formation (cell)
	IC_{50} (μM)	Inhibition type	K_i (μM)	IC_{50} (μM)
1	4.8	Competitive	2.9	48.7
2	8.3	Competitive	3.9	28.1
3	23.3	Competitive	12.6	10.4
4	22.5	Competitive	13.3	24.2
5	58.8	Competitive	25.2	32.1
6	15.2	Competitive	11.4	–
7	30.7	Competitive	16.7	21.2
8	29.1	Competitive	14.9	19.8
Kojic acid	16.3	–	–	>50

The above data show that the chalcone skeleton exhibits optimal mushroom tyrosinase inhibition when sterically unencumbering groups are present on the A ring: only inhibitors **1** and **2** have IC_{50} and K_i values in the single digit micromolar range, whereas all inhibitors bearing the sulfonyl group had IC_{50} and K_i values around 3–8 fold higher. As all inhibitors studied were competitive, it is likely that there are unfavorable steric interactions between the bulky (tetrahedral) sulfonyl function and the active site. The crystal structure of inhibitor **3** shows that the aryl

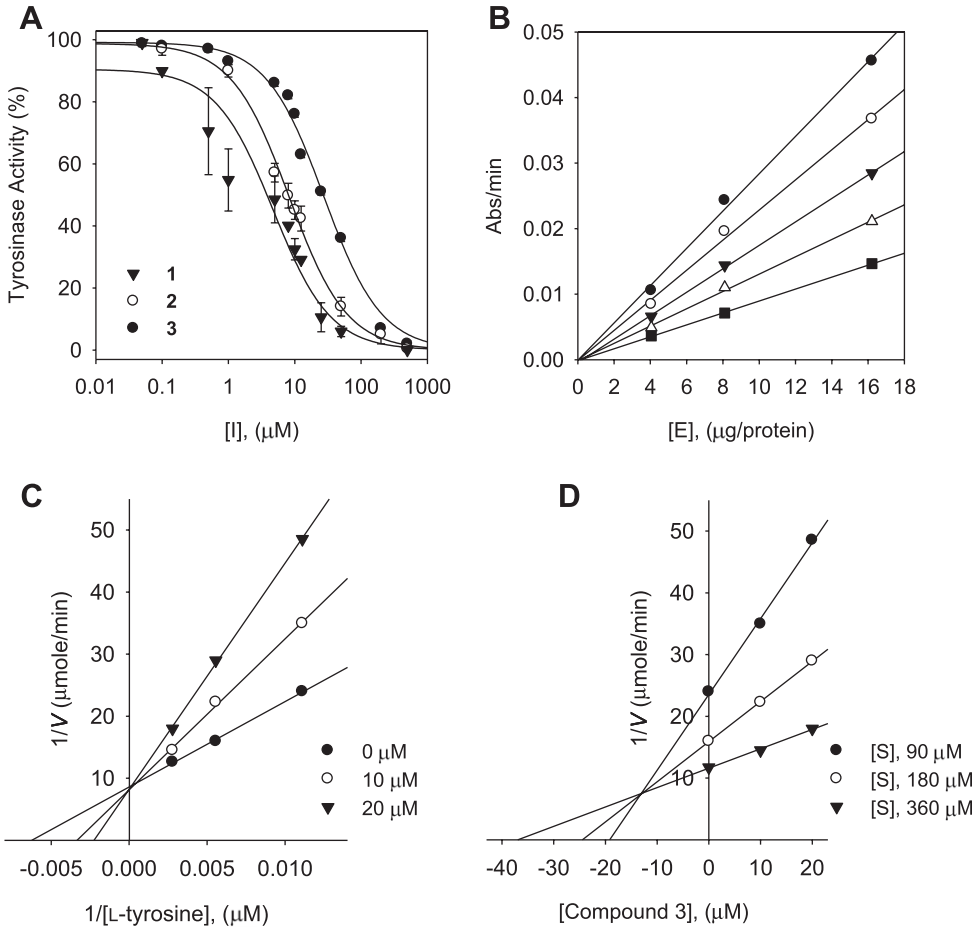


Fig. 3. (A) Effect of chalcone derivatives (**1–3**) on the oxidation of L-tyrosine by mushroom tyrosinase. (B) Relationship between the catalytic activity of tyrosinase and concentrations of compound **3**. Concentrations of compound **3** for lines from top to bottom were 0, 10, 15, 20, and 25 μM, respectively. (C) Lineweaver–Burk plots for the inhibition of the monophenoase activity of mushroom tyrosinase by compound **3**. (D) Dixon plots for the inhibition of the monophenoase activity of mushroom tyrosinase by compound **3** in the presence of different concentrations of L-tyrosine (90, 180, and 360 μM, respectively).

appendage within the sulfonamide moiety is orientated perpendicular to the ring system, which may further impede inhibitor binding (Fig. 4) [17]. The complex but relatively minor variation in IC_{50}s seen by varying the 4-substituent on the phenyl unit within the sulfonamide shows steric effects are much less important at this position.

Having characterized these inhibitors *in vitro*, we progressed to investigate their effects *in vivo* using cultured cells as well as in an animal model. It is well known that the hormone α-MSH upregulates tyrosinase and tyrosinase related protein production through the activation of a transcription factor, MITF. Therefore, we examined the effect of target compounds (**1**–**3**) on α-MSH-controlled tyrosinase expression levels in B16 melanoma cells. In this assay, B16 melanoma cells were incubated with 20 μM compounds (**1**–**3**) in presence of 10 ng/ml of α-MSH for 2 days. Cells under the same conditions but without added inhibitors were used as a control and the cells without any additives (α-MSH or inhibitor) were used as a baseline reference. As shown in Fig. 5, exposure of the cells to MSH more than doubled the melanin content of the cells. However, upon treatment with compounds **1**–**3** the pigmentation and melanin content of the MSH stimulated cells were both reduced. As may have been expected based upon its double inhibitor motif, TSAHC was the best inhibitor and was able to return the melanin pigment content to the levels seen in cells without α-MSH treatment. Most pleasingly, this compound also emerged to have very low cytotoxicity. An important point of note is that hydroxy-4-hydroxychalcone **1**, which showed the best inhibitory activity ($IC_{50} = 4.8$ μM) against mushroom tyrosinase *in vitro*, was not particularly effective at decreasing cell pigmentation. This result can be ascribed in part to the fact that the structure/regulation of mushroom tyrosinase differs significantly in several respects from mammalian tyrosinase: compounds that are active against mushroom tyrosinase do not show comparable results with mammalian tyrosinase [18]. In addition inhibitors **1** and **2** do not have any glucosidase ability and hence may be expected to be less potent than the hybrid inhibitors in the *in vivo* assays. For instance, 4-amino-4′-hydroxychalcone **2** showed a potent inhibitory effect on tyrosinase *in vitro*, but its efficacy against melanogenesis ($IC_{50} = 48.7$ μM) was significantly less than TSAHC ($IC_{50} = 10.4$ μM) *in vivo*. In a similar vein, the control inhibitor, kojic acid, was not able to reduce melanogenesis to the base level at 20 μM. The most important aspect of these assays is that the effects of the three chalcones studied are not due to their cytostatic activities because concentrations of inhibitors were chosen to be well below

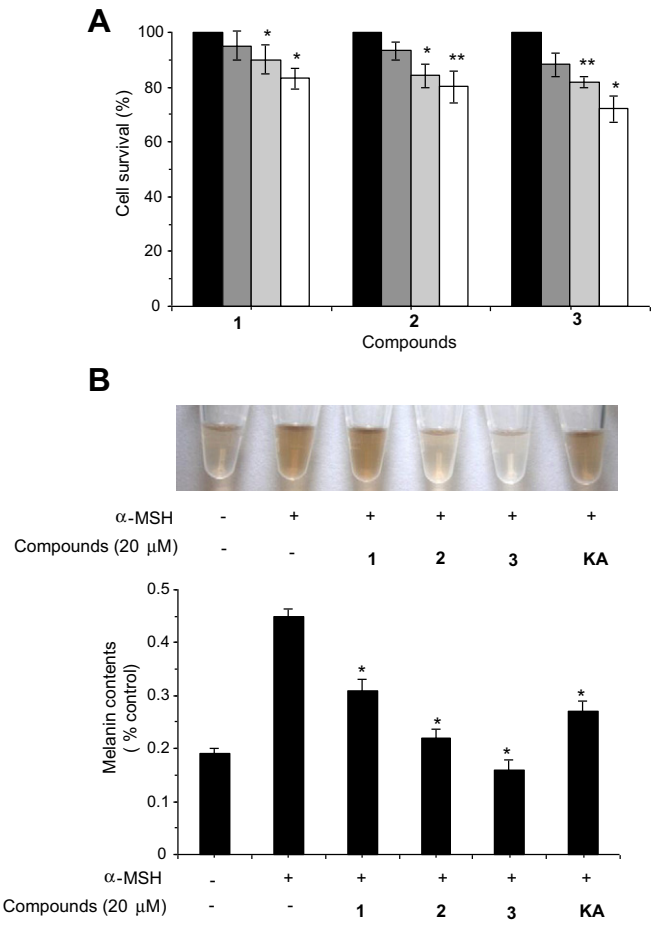


Fig. 5. (A) Effect of compounds **1–3** on the growth of B16 melanoma cells. (Black, 0 μM; dark gray, 10 μM; light gray, 20 μM; white, 30 μM). Cytotoxicity was measured by MTT assay of chalcone compounds (**1–3**) and control (DMSO) treated B16 melanoma cells after 48 h at the indicated concentrations. (B) Effect of chalcone compounds on melanin content in B16 melanoma cells. Cells (5×10^6 cells per well) were incubated separately with 20 μM of the indicated concentration of three chalcone compounds in the presence of 10 ng mL^{-1} α-MSH for 48 h; KA, Kojic acid. Results are showed as a percentage of α-MSH induced control and presented as mean ± SEM of three independent experiments. The data shown are representative of combined mean of three independent experiments. $^*P < 0.05$.

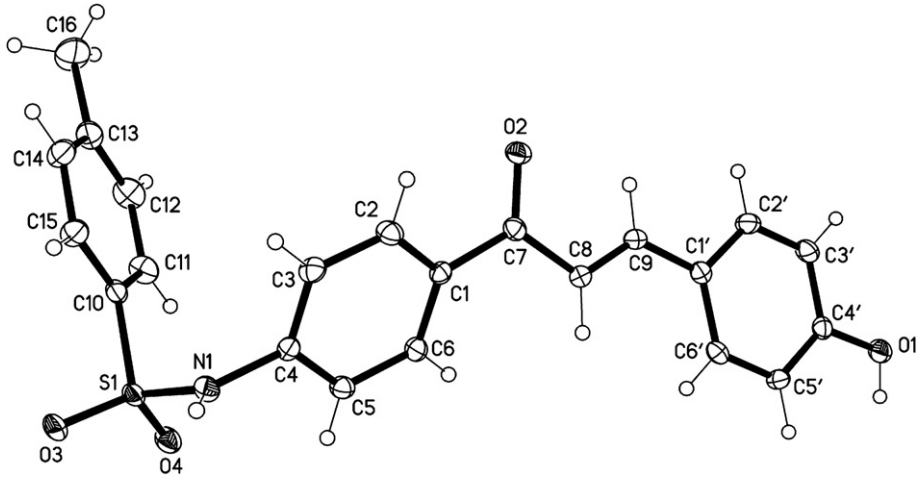


Fig. 4. ORTEP view of TSAHC (CCDC no. = 703762).

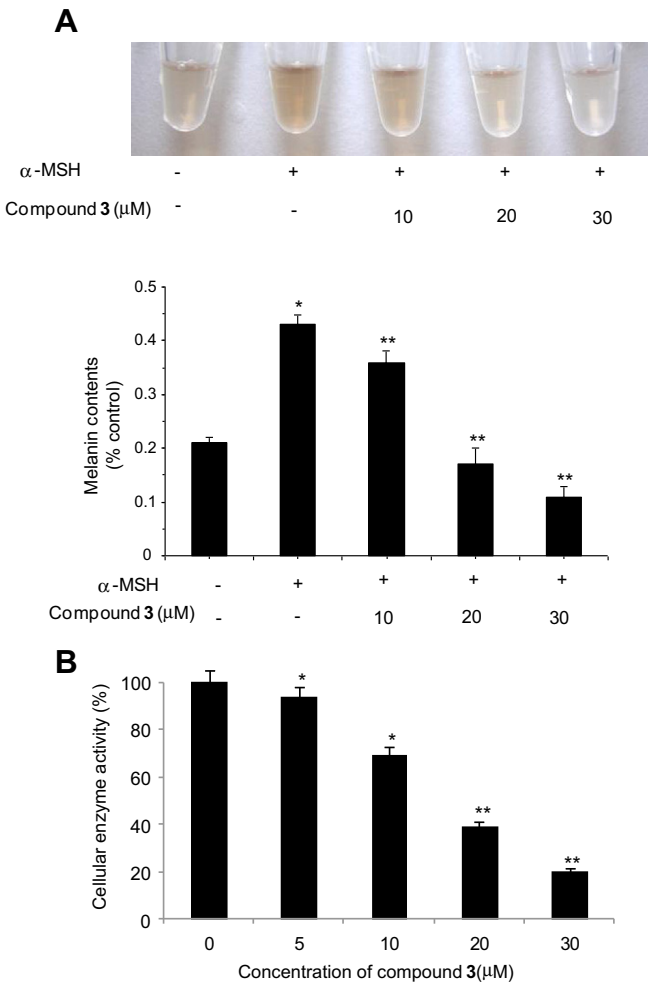


Fig. 6. Effect of various concentrations of compound **3** on cellular melanin content and celluar tyrosinase activity in B16 melanoma cells. (A) Effect of various concentrations of compound **3** on melanin content in B16 melanoma cells. (B) Cells (5×10^6 cell per well) were treated with the indicated concentration of compound **3** in the presence of 10 ng mL^{-1} α-MSH for 48 h. Cellular tyrosinase activity was determined as described in material and methods. The data shown are representative of combined mean of three independent experiments. $^*P < 0.05$; $^{**}P < 0.02$.

concentrations leading to significant cell growth inhibition. The effects of the inhibitors on growth were assessed using a calorimetric MTT-based assay as described in the experimental section. We progressed to analyze the action of TSAHC more thoroughly.

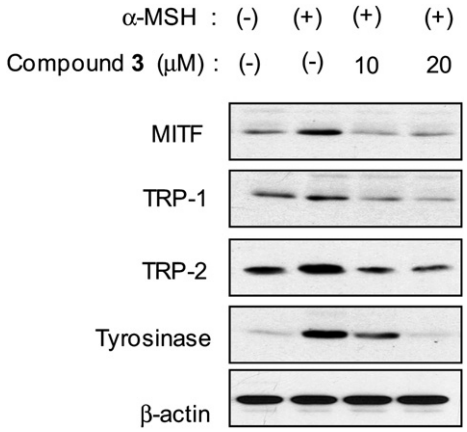


Fig. 7. Effect of compound **3** on α-MSH-induced increase of microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase related protein 1 (TRP-1) and TRP-2 expression analyzed by western blot (average of three results).

Repeating the experiments above using differing concentrations of the inhibitor showed that the effects of the latter were dose dependent (Fig. 6). Furthermore, the survival of cells grown in the presence of TSAHC varied as follows: 92% 10 μM, 84% at 20 μM, and 78% at 30 μM. These detailed results give quantitative backing to our claim that TSAHC inhibits melanin formation through a non-cytotoxic/cytostatic pathway. Thus at 20 μM concentration this inhibitor is able to regulate the melanin concentrations of hyper-stimulated cells to normal levels, while the vast majority of cells remain viable.

The above results spurred us on to investigate the effects of TSHAC at the biochemical level. Three enzymes are known to be involved in melanin biosynthesis in mammals, namely tyrosinase, tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) [4]. Therefore, we investigated the dose dependent effects that TSAHC exerted on the expression levels of tyrosinase and tyrosinase related proteins by western blot analysis in MSH-treated B16 melanoma cells. As anticipated, cells treated with MSH alone (in the absence of inhibitor) showed significant upregulation of MITF (transcription factor) which in turn led to increased TRP-1 and 2 and tyrosinase. However, the expression levels of MITF and tyrosinase related proteins were significantly reduced by a 2-day treatment of TSAHC at 20 μM concentration (Fig. 7). These results indicate that TSAHC exerts its strong depigmenting activity at least partially by downregulating expression levels of the various proteins required for tyrosinase processing/transcription. As we

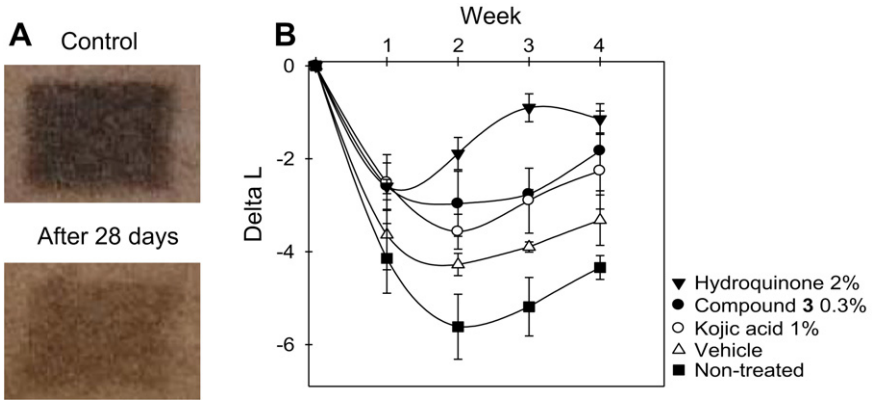


Fig. 8. (A) Photographs of the dorsal skin of guinea pig. Representative photographs of UV-irradiatted dorsal skin of guinea pig treated with 0.3% of TSAHC. (B) Degree of pigmentation. The degree of pigmentation (Δ*L* value) before and daily topical application of vehicle (△), non-treated (■), 2% of hydroquinone (▼), 1% of kojic acid (○), and 0.3% of TSAHC (●) was measured.

specifically designed TSAHC to exhibit dual inhibition characteristics this relatively complex behavior is not unexpected.

The skin whitening effect of TSAHC was next examined using a UV-induced hyperpigmentation model in brown guinea pigs. These animals are excellent models because they have functional melanocytes in their epidemis, which respond well to several stimuli including UV light. TSAHC was topically applied twice daily for 4 weeks to the dorsal skin of brown guinea pigs which had been tanned by irradiation once a week for three consecutive weeks. Hydroquinone (2%) and kojic acid (1%) were used as positive controls. A visible decrease in hyperpigmentation was observed 4 weeks after treatment with TSAHC, hydroquinone, kojic acid, and vehicle group. The change in pigmentation (ΔL) values were measured using a colorimeter to quantify the degree of pigmentation reduction induced by each compound. As shown in Fig. 8, the ΔL values of 0.3% TSAHC, 1% kojic acid, and 2% hydroquinone were obtained as 1.06, 1.49, and 2.18, respectively. Fig. 8 indicates that the skin returned to its original color after the TSAHC treatment without apparent side-effect.

4. Conclusion

In conclusion, we have rigorously investigated a range of inhibitors designed to be able to target both tyrosinase and glycosidase simultaneously. The 4′-(*p*-toluenesulfonylamino)-4-hydroxychalcone (TSAHC) bearing inhibitory chemotypes of both α-glucosidase and tyrosinase, showed potent inhibitory activities against mushroom tyrosinase *in vitro* as well strong depigmenting ability against B16 melanoma cells and UV-induced hyperpigmentation in brown guinea pigs skin. Our data suggest that TSAHC downregulated tyrosinase expression as well as a number of processing enzymes and transcription factors (TRP1, TRP2, and MITF). Our work has thus gone a long way to showing that the strategy of designing hybrid inhibitors targeting different aspects of tyrosinase maturation and activity can be used to treat skin pigmentation.

5. Experimental and methods

5.1. General

All reactions were monitored by thin layer chromatography (TLC) using commercially available glass-backed plates. Column chromatography was carried out using 230–400 mesh silica gel. The final solution before evaporation was washed with brine and dried over anhydrous Na_2SO_4. Melting points were measured on a Thomas Scientific Capillary Point Apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Bruker IFS 66. ^1H and ^{13}C NMR data were obtained on a Bruker AM 300 (^1H NMR at 300 MHz and ^{13}C NMR at 75 MHz) spectrometer in either MeOD or acetone-d_6; EIMS and HREIMS data were collected on a JEOL JMS-700 spectrometer.

5.2. General method for synthesis of chalcone and sulfonylamino chalcone derivatives

A solution of the required *para*-substituted acetophenone (0.1 mol) and 4-hydroxybenzaldehyde (0.12 mol) in MeOH with a catalytic amount of H_2SO_4 was refluxed for 1 day after which time it was neutralized with 15 % NaOH (50 mL). The organic layer was extracted with EtOAc, and dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by column chromatography eluted with hexane/acetone to give pure chalcone derivatives.

5.2.1. 4′,4-Dihydroxychalcone (**1**)

m.p. 205 − 206 °C; IR (KBr) 3440, 1687 cm^{-1}; ^1H NMR (300 MHz; MeOD) δ 6.85 (2H, dd, $J_1 = 8.64$, $J_2 = 2.0$ Hz), 6.90 (2HHH, dd, $J_1 = 6.83$, $J_2 = 2.0$ Hz), 7.52 (1H, d, $J = 15.6$ Hz), 7.53 (2H, d, $J = 8.9$ Hz), 7.70 (1H, d, $J = 15.5$ Hz), 7.98 (2H, dd, $J_1 = 6.8$, $J_2 = 2.0$ Hz); ^{13}C NMR (75 MHz; MeOD) δ 115.1, 115.6, 118.3, 126.5, 129.9, 130.3, 130.9, 144.4, 159.9, 162.3 and 189.8; EIMS *m/z* 240 [M^+]; HREIMS *m/z* 240.0788 [M^+] (calculated for $C_{15}H_{12}O_3$, 240.0786).

5.2.2. 4′-Amino-4-hydroxychalcone (**2**)

m.p. 79–80 °C; IR (KBr) : 3510, 1665 cm^{-1}; ^1H NMR (300 MHz; MeOD) δ 6.44 (2H, dd, $J_1 = 6.9$, $J_2 = 1.9$ Hz), 6.85 (2H, dd, $J_1 = 6.9$, $J_2 = 1.8$ Hz), 7.55 (3H, m), 7.68 (1H, d, $J = 15.5$ Hz), 7.91 (2H, dd, $J_1 = 7.9$, $J_2 = 2.0$ Hz); ^{13}C NMR (75 MHz; MeOD) δ 113.1, 115.5, 118.5, 126.5, 126.8, 130.1, 131.0, 143.4, 153.8, 159.7 and 189.1; EIMS *m/z* 239 [M^+]; HREIMS *m/z* 239.0948 [M^+] (calculated for $C_{15}H_{13}NO_2$, 239.0946).

5.2.3. 4′-(*p*-Toluenesulfonamide)-4-hydroxychalcone (**3**)

m.p. 105–107 °C; IR (KBr): 3520, 1675 cm^{-1}; ^1H NMR (300 MHz; MeOD) δ 2.25 (3H, s), 6.82 (2H, d, $J = 8.6$ Hz), 7.23 (4H, m), 7.42 (1H, d, $J = 15.5$ Hz), 7.51 (2H, d, $J = 8.6$ Hz), 7.66 (1H, d, $J = 15.5$ Hz), 7.73 (2H, d, $J = 8.3$ Hz), 7.90 (2H, dd, $J_1 = 8.7$, $J_2 = 2.0$ Hz); ^{13}C NMR (75 MHz; MeOD) δ 20.1, 115.6, 118.0, 118.5, 126.4, 126.9, 129.4, 129.8, 130.5, 133.5, 136.6, 142.4, 144.1, 145.2, 160.2 and 189.7; EIMS *m/z* 393 [M^+]; HREIMS *m/z* 393.1033 [M^+] (calculated for $C_{22}H_{19}NO_4S$, 393.1035).

5.2.4. 4′-(Benzensulfonamide)-4-hydroxychalcone (**4**)

m.p. 206–208 °C; IR (KBr): 3535, 1655 cm^{-1}; ^1H NMR (300 MHz; MeOD) δ 6.84 (2H, d, $J = 8.6$ Hz), 7.27 (2H, d, $J = 8.7$ Hz), 7.55 (6H, m), 7.71 (1H, d, $J = 15.5$ Hz), 7.88 (2H, d, $J = 8.6$ Hz), 7.96 (2H, d, $J = 8.7$ Hz); ^{13}C NMR (75 MHz; MeOD) δ 115.5, 117.9, 118.5, 126.3, 126.8, 128.4, 129.7, 130.5, 132.8, 133.6, 139.7, 142.3, 145.2, 160.3 and 189.6; EIMS *m/z* 379 [M^+]; HREIMS *m/z* 379.0873 [M^+] (calculated for $C_{21}H_{17}NO_4S$, 379.0878).

5.2.5. 4′-(4-Hydroxylbenzensulfonamide)-4-hydroxychalcone (**5**)

m.p. 102–103 °C; IR (KBr): 3525, 1670 cm^{-1}; ^1H NMR (300 MHz; Acetone-d_6) δ 6.94 (3H, m), 7.37 (1H, d, $J = 8.7$ Hz), 7.67 (3H, m), 7.77 (2H, d, $J = 8.8$ Hz), 8.04 (2H, m), 8.70 (3H, s); ^{13}C NMR (75 MHz; Acetone-d_6) δ 115.6, 115.8, 118.4, 118.5, 126.8, 129.6, 128.9, 130.3, 130.7, 133.7, 142.4, 144.0, 159.9, 161.5 and 187.6; EIMS *m/z* 395 [M^+]; HREIMS *m/z* 395.0830 [M^+] (calculated for $C_{21}H_{17}NO_5S$, 395.0827).

5.2.6. 4′-(4-Nitrobenzensulfonamide)-4-hydroxychalcone (**6**)

m.p. 136–138 °C; IR (KBr): 3513, 1656 cm^{-1}; ^1H NMR (300 MHz; Acetone-d_6) δ 6.92 (2H, d, $J = 8.6$ Hz), 7.42 (2H, d, $J = 8.7$ Hz), 7.68 (4H, m), 8.08 (2H, d, $J = 8.7$ Hz), 8.17 (2H, d, $J = 8.9$ Hz) and 8.41 (2H, d, $J = 8.9$ Hz); ^{13}C NMR (75 MHz; Acetone-d_6) δ 116.3, 118.7, 119.3, 125.3, 126.3, 128.8, 130.5, 131.4, 134.2, 141.7, 144.6, 145.1, 150.5, 160.6 and 187.9; EIMS *m/z* 424 [M^+]; HREIMS *m/z* 424.0728 [M^+] (calculated for $C_{21}H_{16}N_2O_6S$, 424.0729).

5.2.7. 4′-(4-Aminobenzensulfonamide)-4-hydroxychalcone (**7**)

m.p. 216–217 °C; IR (KBr): 3526, 1663 cm^{-1}; ^1H NMR (300 MHz; Acetone-d_6) δ 6.70 (2H, d, $J = 8.7$), 6.93 (2H, d, $J = 8.6$ Hz), 7.37 (2H, d, $J = 8.7$ Hz), 7.61 (2H, d, $J = 8.8$ Hz), 7.70 (4H, m) and 8.06 (2H, d, $J = 8.7$ Hz); ^{13}C NMR (75 MHz; Acetone-d_6) δ 113.1, 115.9, 118.2, 118.6, 125.7, 126.9, 129.2, 129.8, 130.6, 133.4, 142.9, 143.9, 153.1, 159.9 and 187.6; EIMS *m/z* 394 [M^+]; HREIMS *m/z* 394.0985 [M^+] (calculated for $C_{21}H_{18}N_2O_4S$, 394.0987).

5.2.8. 4′-(4-Fluorobenzensulfonamide)-4-hydroxychalcone (**8**)

m.p. 179–180 °C; IR (KBr): 3544, 1671 cm^{-1}; ^1H NMR (300 MHz; MeOD) δ 6.84 (2H, d, $J = 8.6$ Hz), 7.26 (4H, m), 7.55 (3H, m), 7.72 (1H, d, $J = 15.5$ Hz) and 7.94 (4H, m); ^{13}C NMR (75 MHz; MeOD) δ 115.5, 115.8, 116.1, 117.9, 118.7, 126.3, 129.7, 129.9, 130.5, 133.8, 135.8, 142.1,

145.2, 160.3 and 189.6; EIMS *m/z* 397 [M^+]; HREIMS *m/z* 397.0782 [M^+] (calculated for $C_{21}H_{16}FNO_4S$, 397.0784).

5.2.9. X-ray crystal structure analysis of **3**

$C_{22}H_{19}NO_4S$, $M = 393.44$, monoclinic, space group, $P2_1$, $a = 11.0821(7)$ Å, $b = 13.1497(8)$ Å, $c = 13.5886(8)$ Å, $\alpha = 90°$, $\beta = 102.2520(10)°$, $\gamma = 90°$, $V = 1935.1(2)$ Å^3, $Z = 4$, $T = 446(2)$ K, $D = 1.350$ mg/m^3, $\mu = 0.196$ mm^{-1}, $F(000) = 824$, $R_1 = 0.0561$, $R_w = 0.1280$ for 4195[R(int) = 0.0916] independent reflections [17].

5.3. Mushroom tyrosinase assay

The mushroom tyrosinase (EC 1.14.18.1) (Sigma Chemical Co.) was used for *in vitro* bioassays as described previously with some modifications [19]. In this experiment, L-tyrosine was used as a substrate. In a spectrophotometric experiment, enzyme activity was monitored by dopachrome formation at 475 nm with a UV-Vis spectrophotometer (Spectro UV-Vis Double beam; UVD-3500, Labomed, Inc.) at 30 °C. All samples were first dissolved in EtOH at 10 mM and used for the experiment with dilution. First, 200 μL of a 2.7 mM L-tyrosine ($K_m = 180$ μM) aqueous solution was mixed with 2687 μL of 0.25 M phosphate buffer (pH 6.8). Then, 100 μL of the sample solution and 13 μL of the same phosphate buffered solution of mushroom tyrosinase (144 units) were added in this order to the mixture. Each assay was conducted in triplicate. The inhibitor concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve by Eq. (1):

$$\text{Activity } (\%) = 100[1/(1 + ([I]/IC_{50}))] \quad (1)$$

5.4. Cell culture and chemicals

The B16 murine melanoma cells were grown in Dulbecco's modified minimum essential medium (DMEM) (Invitrogen, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum (Invitrogen, CA, USA), L-glutamine (2.5 mM), HEPES [40 mM (Biopure, CA, USA)], and antibiotics [penicillin (50 unit/mL) and streptomycin (50 μg/mL)] (Gibco, Invitrogen, CA, USA)] at 37 °C in a 5% CO_2 humidified atmosphere. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolidium bromide (MTT), α-MSH and kojic acid were obtained from Sigma. Anti-tyrosinase, anti-MITF, anti-TRP-1 and anti-TRP-2 were purchased from SantaCruz (USA, CA). TSAHC treatment began 24 h after plating, and cells were harvested after 2 days of incubation.

5.5. Cell viability assay

After treatment with kojic acid or one of the three compounds, cells were assayed for growth activity using a MTT 3-((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolidium bromide, Sigma, USA)-based colorimetric method, as previously described [20]. Briefly, Cells were seeded at densities of 5000 cells/well in 96-well culture plates. Cells were treated with chalcone compounds for 72 h. After treatment with these compounds, the attached cells were incubated with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 0.5 mg/mL, 1 h) and subsequently solubilized in DMSO. The absorbency at 550 nm was then measured using a microplate reader. The GI_{20} is the concentration of agent that reduced the cell viability by 20% under the experimental conditions.

5.6. Measurement of melanin content

The melanin content was measured using the method reported by Park et al. [21] with a slight modification. The cells, pretreated with the indicated concentrations of compound, were treated with α-MSH for 48 h. Then the media was aspirated and the cells were washed twice with PBS. Cells were harvested from each well in a 20 mM Tris – 0.2 % Triton X-100 buffer. Cells were pelleted by centrifugation (10,000 *g*) and the melanin was dissolved by treatment with 1 N NaOH for 15 min at 80 °C. Then, 200 μL crude cell extracts were transferred into 96-well plates. Relative melanin concentration was measured at 400 nm with microplate reader (Molecular Devices, Sunnyvale, CA, USA).

5.7. Measurement of cell-free tyrosinase activity

The tyrosinase activity was measured by the method of Akao et al. [22] with a slight modification. Briefly, B16 melanoma cells, pretreated with the indicated concentrations of chalcones for 3 h, were treated for 48 h with α-MSH (10 ng mL^{-1}). The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and extracted and then centrifuged at 10,000 *g* for 10 min. Samples of cell extract supernatant were incubated in duplicate for 1 h at 37 °C sodium phosphate buffer (pH 7.4) containing 0.1% L-DOPA. Dopachrome formation was monitored by measuring absorbance at 470 nm.

5.8. Western blotting

In order to measure cellular levels of MITF, tyrosinase, TRP-1 and TRP-2, B16 cells, pretreated with the indicated concentrations of chalcones for 3 h, were treated with α-MSH (10 ng mL^{-1}) for 48 h. Cells were washed with PBS and scraped in lysis buffer [40 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.1 % Nonidet P-40, and protease inhibitors]. They were then centrifuged at 14,000 *g* for 15 min at 4 °C and supernatants (total cell lysates) were collected and aliquoted. These lysates were either used on the day of preparation or were immediately stored at −78 °C until required. For western blot analysis, 30 μg of lysate was resolved on 12.5% and 16% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked with 5% fat-free milk in PBS (pH 7.4) for 30 min at room temperature and then incubated with appropriate mono- and polyclonal primary antibodies in blocking buffer for 2 h to overnight at 4 °C. After washing, membranes were incubated with anti-mouse or anti-rabbit horse radish peroxidase conjugated secondary antibodies for 1 h at room temperature, and then subjected to ECL solution and visualized using Hyperfilm (Amersham Biosciences). Densitometric analysis of Western blots was performed with the use of the Quantity one software system (Bio-Rad XRS).

5.9. UV-induced hyperpigmentation in brown guinea pigs

UV-induced hyperpigmentation was induced on the backs of brownish guinea pigs weighing approximately 500 g (Jungang Animal Co) using a modification of the methods reported by Hideya et al. and Imokawa et al. [23,24]. The guinea pigs were anesthetized with pentobarbital (30 mg/kg), and separate areas (1 cm^2) on the back of each animal were exposed to UV irradiation (Waldmann UV 800, Herbert Waldman GmbH, Philis TL/12 lamp emitting 280–305 nm). The total energy dose of UV was 500 mJ/cm^2 per exposure period. Each animal was exposed to UV radiation once a week for three consecutive weeks. The ΔL value was calculated using the L value (brightness index) measured with the colorimeter (CR-300; Minolta, Osaka, Japan) as follows:

$$\Delta L = L \text{ (at each day measure)} - L \text{ (at day 0)} \quad (2)$$

5.10. Statistical evaluation

Means ± SEM were calculated. Statistical analyses of results were performed using the *t*-test for independent samples. $P < 0.05$ was considered significant.

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References

[1] S. Im, J. Kim, W.Y. On, W.H. Kang, Br. J. Dermatol. 146 (2002) 165–167.
[2] W.H. Kang, K.H. Yoon, E.S. Lee, J. Kim, K.B. Lee, H. Yim, S. Sohn, S. Im, Br. J. Dermatol. 146 (2002) 228–237.
[3] S. Briganti, E. Camera, M. Picardo, Pigment Cell Res. 16 (2003) 101–110.
[4] T. Kobayashi, K. Urade, A.J. Winder, C. Jimènez-Cervantes, G. Imokawa, T. Brewington, EMBO J. 13 (1994) 5818–5822.
[5] B.S. Kwon, A.K. Haq, S.H. Pomerantz, R. Halaban, Proc. Natl. Acad. Sci. USA 84 (1987) 7473–7477.
[6] G. Műller, S. Ruppert, E. Schmid, G. Schűtz, EMBO J. 7 (1988) 2723–2730.
[7] A. Ujvari, R. Aron, T. Esenhaure, E. Cheng, H. Parag, Y. Smicun, J. Biol. Chem. 276 (2001) 5924–5931.
[8] M. Furumura, F. Solano, N. Matsunaga, C. Sakai, R.A. Spritz, V.J. Hearing, Biochem. Biophys. Res. Commun 242 (1998) 579–585.
[9] N. Branza-Nichita, A.J. Petrescu, R.A. Dwek, M.R. Wormald, F.M. Platt, S.M. Petrescu, Biochem. Biophys. Res. Commun. 261 (1999) 720–725.
[10] H. Ando, H. Kondoh, M. Ichihashi, V.J. Hearing, J. Invest. Dematol. 127 (2007) 751–761.
[11] (a) H.M. Yang, H.R. Shin, S.H. Cho, S.C. Bang, G.Y. Song, J.H. Ju, M.K. Kim, S.H. Lee, J.C. Ryu, Y. Kim, S.H. Jung, Bioorg. Med. Chem. 15 (2007) 104–111; (b) L. Svetaz, A. Tapia, S.N. Lopez, R.L.E. Furlan, E. Petenatti, R. Pioli, G. Schmeda-Hirschmann, S.A. Zacchino, J. Agric. Food Chem. 52 (2004) 3297–3300; (c) H.P. Avila, E.F.A. Smania, F.D. Monache, A.S. Junior, Bioorg. Med. Chem. 16 (2008) 9790–9794; (d) J.N. Dominguez, C. Leon, J. Rodrigues, N. Gamboa de Dominguez, J. Gut, P.J. Rosenthal., J. Med. Chem. 48 (2005) 3654–3658.(e) A. Alain Valla, B. Valla, D. Cartier, R.L. Guillou, R. Labia, L. Florent, S. Charneau, J. Schrevel, P. Potier, Eur. J. Med. Chem. 41 (2006) 142–146.
[12] O. Nerya, R. Musa, S. Khatib, S. Tamir, J. Vaya, Phytochemisty 65 (2004) 1389–1395.
[13] W.D. Seo, J.H. Kim, J.E. Kang, H.W. Ryu, M.J. Curtis-Long, H.S. Lee, M.S. Yang, K.H. Park, Bioorg. Med. Chem. Lett. 15 (2005) 5514–5516.
[14] K. Bharatham, N. Bharatham, K.H. Park, K.W. Lee, J. Mol. Graph. Model. 25 (2007) 813–823.
[15] S.A. Lee, H.W. Ryu, Y.M. Kim, S. Choi, M.J. Lee, T.K. Kwak, H.J. Kim, M. Cho, K.H. Park, J.W. Lee, Hepatology 49 (2009) 1316–1325.
[16] S. Khatib, O. Nerya, R. Musa, M. Shumuel, S. Tamir, J. Vaya, Bioorg. Med. Chem. 13 (2005) 433–441.
[17] Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as deposition No. CCDC-703762. Copies of data can be obtained, free of charge, on application to the Director CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk).
[18] G.M. Jacobsohn, M.K. Jacobsohn, Biochim. Biophys. Acta 1116 (1992) 173–182.
[19] S.H. Jeong, Y.B. Ryu, M.J. Curtis-Long, H.W. Ryu, Y.S. Baek, J.E. Kang, W.S. Lee, K.H. Park, J. Agric. Food Chem. 57 (2009) 1195–1203.
[20] Y.J. Lee, D.Y. Chung, S.J. Lee, G. Ja Jhon, Y.S. Lee, Int. J. Radiat. Oncol. Biol. Phys. 64 (2006) 1466–1474.
[21] J.Y. Yang, J.H. Koo, Y.G. Song, K.B. Kwon, J.H. Lee, H.S. Sohn, B.H. Park, E.C. Jhee, J.W. Park, Acta. Pharmacol. Sin. 27 (2006) 1467–1473.
[22] K. Ohguchi, M. Ito, K. Yokoyama, M. Iinuma, T. Itoh, Y. Nozawa, Y. Akao, Biol. Pharm. Bull. 32 (2009) 308–310.
[23] A. Hideya, R. Atsuko, H. Akira, O. Masahiro, I. Masamitsu, Arch. Dermatol. Res. 290 (1998) 375–381.
[24] G. Imokawa, M. Kawai, Y. Mishima, I. Motegi, Arch. Dermatol. Res. 278 (1986) 352–362.