MedChemComm

CONCISE ARTICLE

View Article Online
View Journal | View Issue

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

Cite this: *Med. Chem. Commun.*, 2014, **5**, 1410

Benzylidene-linked thiohydantoin derivatives as inhibitors of tyrosinase and melanogenesis: importance of the β-phenyl-α,β-unsaturated carbonyl functionality

Hye Rim Kim,[a] Hye Jin Lee,[a] Yeon Ja Choi,[a] Yun Jung Park,[a] Youngwoo Woo,[a] Seong Jin Kim,[a] Min Hi Park,[a] Hee Won Lee,[a] Pusoon Chun,[b] Hae Young Chung*[a] and Hyung Ryong Moon*[a]

Received 16th April 2014
Accepted 25th June 2014

DOI: 10.1039/c4md00171k

www.rsc.org/medchemcomm

Based on the structural characteristics of the heterocyclic scaffolds of substituted benzylidene-hydantoin, -pyrrolidinedione, and -thiazolidinedione derivatives with potent tyrosinase inhibitory activity, substituted benzylidene derivatives with a 2-thiohydantoin heterocyclic scaffold were synthesized by modified Knoevenagel condensation between benzaldehydes and 2-thiohydantoin with a view toward producing more potent, safer tyrosinase inhibitors capable of being utilized in the agricultural, food, cosmetics, and pharmaceutical industries. Of the twelve compounds synthesized, three compounds, **2c**, **2d** and **2i**, exhibited even more potent inhibitory activities against mushroom tyrosinase than kojic acid or resveratrol, which are well-known potent tyrosinase inhibitors. The inhibitory pattern of compounds with a thiohydantoin template differed from that of compounds with a hydantoin, pyrrolidinedione, or thiazolidine scaffold, probably because of the loss of the hydrogen bonding ability of the thiocarbonyl group of thiohydantoin. Considering the high tyrosinase inhibitory activities of 5-(substituted benzylidene)thiohydantoin derivatives, the thiohydantoin template is considered a near perfect surrogate for hydantoin, pyrrolidinedione, and thiazolidinedione scaffolds. (*Z*)-5-(2,4-Dihydroxybenzylidene)-2-thiohydantoin (**2d**, IC_{50} = 1.07 ± 2.30 μM) had 24 times the inhibitory effect of resveratrol (IC_{50} = 26.63 ± 0.55 μM) and 18 times that of kojic acid (IC_{50} = 19.69 ± 4.90 μM) against mushroom tyrosinase and showed anti-melanogenesis through the inhibition of tyrosinase activity in B16 cells with no appreciable cytotoxicity, which suggests that **2d** is a promising candidate for the development of safer and more potent fruit and food browning preventatives and skin-lightening medicines. This result and our previous data indicate that it is the "β-phenyl-α,β-unsaturated carbonyl" group that is essential for potent anti-tyrosinase activity.

Introduction

Over the past couple of decades, tyrosinase (EC 1.14.18.1), a polyphenol oxidase, has received considerable attention as an indispensable tool in the search for potent tyrosinase inhibitors from natural and synthetic sources. Tyrosinase is a copper-containing multifunctional, glycosylated oxidase that catalyzes both the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to L-dopaquinone, the latter of which is finally converted into melanin through a series of enzymatic and nonenzymatic reactions.[1]

Tyrosinase exists widely in bacteria, fungi, plants, insects, vertebrates, and invertebrates and is the rate-determining enzyme in the biosynthesis of melanin pigments that are responsible for the coloration of hair, skin, and eyes and the undesired enzymatic browning of vegetables and fruits.[2,3] Tyrosinase has become increasingly important in the medicinal and cosmetic industries. For example, tyrosinase inhibitors are used to treat hyperpigmentation, melasma, freckles, and age spots, and as skin whitening agents, whereas tyrosinase activators that increase melanogenesis can protect the skin from UV damage.[4] In the food industry, tyrosinase is an important enzyme in the context of maintaining the quality of fruit and vegetables under storage because of the damage-associated browning that occurs during postharvest handling. The development of undesirable flavors post-harvest also lowers the values of agricultural products. Quinone compounds are produced by successive oxidative reactions of tyrosinase during

[a]*Molecular Inflammation Research Center for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea. E-mail: mhr108@pusan.ac.kr; Fax: +82-51-513-6754; Tel: +82-51-510-2815*

[b]*College of Pharmacy, Inje University, Gimhae, Gyeongnam 621-749, Republic of Korea. E-mail: hyjung@pusan.ac.kr; Fax: +82-51-518-2821; Tel: +82-51-510-2814*

This journal is © The Royal Society of Chemistry 2014

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

'browning' and are highly reactive species, which are capable of triggering Diels–Alder and Michael addition reactions with the Lys-NH_2 (amino) and Cys-SH (sulfhydryl) groups of proteins.[5] Therefore, tyrosinase inhibitors could possibly prevent and control enzymatic browning reactions by inhibiting quinone formation and thus improve the quality and nutritional value of agricultural products.

Furthermore, recent studies have demonstrated that tyrosinase inhibitors can be used to control insect pests by inhibiting their developmental and defensive functions,[6–8] and thus, the development of suitable novel tyrosinase inhibitors could also improve the output and quality of agricultural products by reducing the impact of insect pests.

Over the last few decades, a large number of naturally occurring and synthetic tyrosinase inhibitors have been reported. Currently, arbutin, a hydroquinone glycoside extracted from the bearberry plant of the genus *Arctostaphylos* is used in the cosmetic industry as a whitening agent due to its tyrosinase inhibitory activity. However, it has also been reported that arbutin is decomposed by temperature (10% decomposition at 20 °C for 15 days), enzymes, such as β-glycosidase, and by intestinal bacteria to glucose and hydroquinone, the latter of which could cause immunotoxicity or possibly cancer.[9] Moreover, several reportedly active agents, such as arbutin, kojic acid, and tropolone, have not been demonstrated to be clinically efficacious when critically analyzed in carefully controlled studies. Therefore, there is a continuous need for tyrosinase inhibitors that are potent enough to be of practical use and yet safe enough to be used in pharmaceutical, cosmetic, food, and agriculture fields.

Results and discussion

Recently, we synthesized benzylidenehydantoin **I**, benzylidene-pyrrolidinedione **II**, and benzylidenethiazolidine-2,4-dione **III** derivatives as potential tyrosinase inhibitors (Fig. 1) and showed that these compounds are promising anti-browning agent candidates in food and agricultural products and promising active ingredients in skin-lighting cosmetics and in medicines for the treatment of diseases associated with hyperpigmentation.[10–13]

Compound **I** was designed by mimicking the chemical structures of the tyrosinase substrates, L-tyrosine and L-dopa.[10] Like the primary amines of L-tyrosine and L-dopa, the amide NH at position 1 of hydantoin **I** has the ability to form hydrogen bonds with amino acids at the active site of tyrosinase. Furthermore, the imido group of **I** could serve as an acid like the carboxylic acids of tyrosinase substrates. Compounds **I**, **II**, and **III** exhibited more potent tyrosinase inhibition than kojic acid the positive control (according to reports, kojic acid is three fold or more potent than arbutin), which prompted us to synthesize benzylidene-heterocyclic compounds with another surrogate of carboxylic acid. During our continued efforts to identify more potent, safer tyrosinase inhibitors, 5-(hydroxyl- or alkoxy-substituted benzylidene)thiohydantoin analogs **2a–2l** with a monothioimido group as a carboxylic acid surrogate were designed, synthesized, and evaluated with respect to their tyrosinase inhibitory and anti-melanogenic activities. Currently, hydantoin analogs, including phenytoin and fosphenytoin, are used for the treatment of epilepsy.[14] Therefore, we considered that replacement of the carbonyl group of hydantoin analogs **I** by a thiocarbonyl group might diminish the potential anticonvulsant effect and increase tyrosinase inhibitory activity and bioavailability by enhancing lipophilicity. Benzylidene-thiohydantoin derivatives have been reported to show potent fungicidal, aldose reductase inhibitory, anti-tuberculosis, and selective COX-2 inhibitory activities.[15–19] However, to the best of our knowledge, the anti-tyrosinase effects of benzylidene-thiohydantoin analogs have not been previously reported.

The target compounds **2a–2l** were prepared by modified Knoevenagel condensation between benzaldehydes with various substituents and 2-thioxoimidazolidin-4-one, also known as 2-thiohydantoin. Refluxing 2-thiohydantoin and benzaldehydes with hydroxyl, methoxyl, and/or ethoxyl substituents at different positions in the presence of sodium acetate and acetic acid afforded benzylidene-thiohydantoin derivatives **2a–2l**, as shown in Scheme 1 and Table 1. Twelve 5-(substituted benzylidene)-2-thiohydantoin analogs were obtained in moderate yields. In all reactions, (*Z*)-isomers were produced predominately. The products were characterized by ^{1}H and ^{13}C NMR and mass spectroscopy and all spectral data were identical to those of authentic compounds.

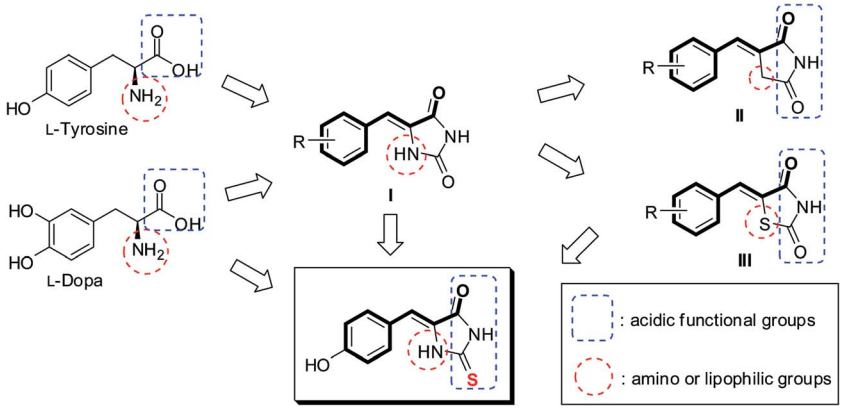
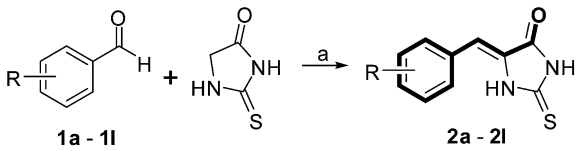


Fig. 1 The rationale for the design of benzylidene-linked thiohydantoin derivatives.

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.



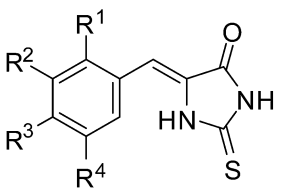
1a - 1l 2a - 2l

Scheme 1 Preparation of (*Z*)-5-(substituted benzylidene)-2-thiohydantoin derivatives. Reagents and conditions: (a) NaOAc, AcOH, reflux, 4–24 h, 15.1–84.6%.

Mushroom tyrosinase is commonly used for screening and characterizing potential tyrosinase inhibitors, because it is commercially available, relatively inexpensive, and provides reliable results. Accordingly, it was used in the present study to evaluate the tyrosinase inhibitory abilities of the synthesized thiohydantoin compounds. The mushroom tyrosinase inhibitory activities of the synthesized compounds were examined as described previously, with minor modifications[20] and are shown in Table 1. All tyrosinase inhibitions were measured at concentrations of 25 or 50 μM. 2,4-Dihydroxybenzylidene-2-thiohydantoin **2d** (96.54% inhibition at 25 μM) inhibited mushroom tyrosinase activity more than the other eleven thiohydantoins synthesized. The 2,4-dihydroxyl functional group on the benzylidene remarkably increased the activity. This tendency was also previously observed for benzylidene-pyrrolidinedione and benzylidene-thiazolidinedione derivatives reported by our laboratory.[11–13] Mono- and di-methoxybenzylidene analogs with a hydantoin, pyrrolidinedione, or thiazolidinedione scaffold showed only low inhibitory activities against mushroom tyrosinase.[10–13] Therefore, it was somewhat surprising that 4-methoxybenzylidene and 2,4-dimethoxybenzylidene analogs **2c** and **2i** with a thiohydantoin scaffold displayed impressive mushroom tyrosinase inhibitory activities (72.62 and 58.71%, respectively). Although most 3,5-dihydroxybenzylidene analogs synthesized previously by our laboratory exhibited only weak or no inhibition against mushroom tyrosinase, their congener **2f** with a thiohydantoin scaffold interestingly exhibited moderate inhibitory activities with 46.22% inhibition at 50 μM. It was also interesting to note that 4-hydroxybenzylidene derivatives constructed on templates, such as hydantoin, pyrrolidinedione, or thiazolidinediones, showed moderate to high inhibitory inhibition against mushroom tyrosinase[10,11] whereas the 4-hydroxybenzylidene analog **2a** constructed on a thiohydantoin template showed almost none. This dramatic change in heterocyclic scaffold-dependent tyrosinase inhibitory activity could be due to the hydrogen bonding abilities of scaffolds with tyrosinase. For 4-hydroxybenzylidene-hydantoin, -pyrrolidinedione, and -thiazolidinedione compounds, the carbonyl group of the heterocyclic templates could play an important role in interactions with tyrosinase, probably *via* hydrogen bonding. However, conversion of the heterocyclic template into thiohydantoin provides a thiocarbonyl group, instead of a carbonyl group, and this might prevent this hydrogen bonding and result in a loss of inhibitory activity.

We further investigated the IC_{50} values of **2c**, **2d**, and **2i** because these three compounds exhibited strong inhibitory activity. The IC_{50} values of **2c**, **2d**, **2i**, resveratrol, and kojic acid for the inhibition on mushroom tyrosinase are shown in Table 2. The inhibitory effects of **2c**, **2d**, **2i**, resveratrol, and kojic acid

Table 1 Reaction yields and tyrosinase inhibitions of the synthesized (*Z*)-5-(benzylidene)-2-thioxoimidazolidin-4-ones **2a–2l**[a]



Compound	R^1	R^2	R^3	R^4	Yield (%)	Tyrosinase inhibition[b] (%)
2a	H	H	OH	H	45.0	3.78 ± 3.62
2b	OH	H	H	H	61.4	42.60 ± 5.09
2c	H	H	OMe	H	64.0	72.62 ± 0.58
2d	OH	H	OH	H	58.1	96.54 ± 1.80[c]
2e	H	OH	OH	H	76.6	23.73 ± 4.76
2f	H	OH	H	OH	52.6	46.22 ± 4.95
2g	H	OMe	OH	H	59.4	10.43 ± 3.58
2h	H	OEt	OH	H	15.1	20.45 ± 1.09
2i	OMe	H	OMe	H	80.3	58.71 ± 0.35
2j	H	OMe	OMe	H	71.7	33.97 ± 3.48
2k	H	OMe	OH	OMe	84.6	5.47 ± 7.54
2l	H	OMe	OMe	OMe	52.8	19.27 ± 4.75
Kojic acid						78.26 ± 2.31

[a] Inhibition values represent means ± SEs of three independent experiments. [b] Tyrosinase inhibition was measured using L-tyrosinase as a substrate at 50 μM. [c] Tyrosinase inhibition was measured using L-tyrosinase at 25 μM.

 This journal is © The Royal Society of Chemistry 2014

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

Table 2 IC_{50} values for tyrosinase inhibition by **2c**, **2d**, **2i**, resveratrol, and kojic acid

Compound	Conc. (μM)	Tyrosinase inhibition[a] (%)	IC_{50}[b] (μM)
2c	1.0	31.12 ± 3.05	7.36 ± 1.79
	5.0	50.43 ± 3.05	
	10.0	61.67 ± 2.75	
	25.0	70.32 ± 1.04	
	50.0	72.62 ± 0.58	
2d	1.0	41.79 ± 4.07	1.07 ± 2.30
	5.0	62.25 ± 4.53	
	10.0	73.49 ± 2.92	
	25.0	96.54 ± 1.80	
2i	1.0	39.48 ± 2.59	14.12 ± 3.49
	5.0	44.96 ± 4.53	
	10.0	49.28 ± 5.38	
	25.0	53.89 ± 2.25	
	50.0	68.59 ± 5.29	
Resveratrol	0.5	13.67 ± 4.24	26.63 ± 0.55
	1.0	16.26 ± 0.75	
	5.0	27.09 ± 7.79	
	10.0	38.05 ± 1.84	
	20.0	47.29 ± 1.07	
	30.0	47.78 ± 1.30	
Kojic acid	2.0	11.70 ± 4.07	14.27 ± 4.07
	10.0	44.47 ± 4.07	
	20.0	63.30 ± 4.07	
	30.0	68.30 ± 4.07	
	40.0	74.47 ± 4.07	

[a] Values represent means ± SEs of three independent experiments. [b] 50% inhibitory concentration.

on mushroom tyrosinase were determined using L-tyrosine as a substrate; all compounds inhibited mushroom tyrosinase in a concentration-dependent manner. Compounds **2c** (IC_{50} = 7.36 ± 1.79 μM) and **2i** (IC_{50} = 14.12 ± 3.49 μM) were found to be 3.6- and 1.9-fold more potent, respectively, than resveratrol (IC_{50} = 26.63 ± 0.55 μM), a well-known tyrosinase inhibitor, and 2.7- and 1.4-fold, respectively, more potent than kojic acid (IC_{50} = 19.69 ± 4.90 μM), the compound most frequently used as a reference for the evaluation of tyrosinase inhibitors. More interestingly, (*Z*)-5-(2,4-dihydroxybenzylidene)thiohydantoin (**2d**, IC_{50} = 1.07 ± 2.30 μM) was found to be 24- and 18-fold more potent than resveratrol and kojic acid, respectively.

To evaluate the cytotoxic effects of compound **2d**, B16F10 melanoma cells (B16 cells, murine melanoma cells) were used. The effect of compound **2d** on B16 cell viability is presented in Fig. 2(a). No significant cytotoxic effect was found up to a concentration of 20 μM.

Next, effects of **2d** on cellular tyrosinase activity and melanogenesis were investigated using B16 cells within 20 μM concentration at which **2d** showed no appreciable cytotoxicity. To assess the inhibitory effect of compound **2d** on melanogenesis, we quantified the melanin contents of B16 cells treated with compound **2d** (Fig. 2(b)). The melanin content of B16 cells treated with compound **2d** in the presence of a 100 nM α-melanocyte-stimulating hormone (α-MSH) decreased as the concentration of **2d** was increased, that is, 160.50% at 5.0 μM and 138.25% at 20 μM, as compared with 100 nM α-MSH-only-treated cells (186.25%) and untreated controls (100%). In view of our cytotoxicity results (Fig. 2(a)), the inhibition of melanogenesis in B16 cells by compound **2d** did not appear to be due to the cytotoxic effect or cell growth inhibition, which suggested that it could have been caused by the inhibition of murine-derived tyrosinase by **2d**. To identify the mechanism involved, we examined the inhibitory effect of **2d** on cellular tyrosinase activity using murine-derived tyrosinase. After incubation for 24 h with compound **2d**, murine-derived tyrosinase activities decreased dose-dependently, showing 379.82% at 5.0 μM and 332.11% at 20 μM (Fig. 2(c)), compared with 100 nM α-MSH-only-treated cells (466.06%) and untreated controls (100%), indicating that the anti-melanogenic effect of **2d** was due to its inhibition of murine-derived tyrosinase.

These results suggest that the heterocyclic thiohydantoin serves as an effective scaffold for novel tyrosinase inhibitors, and that **2d** is a promising preventative of vegetable and fruit browning, a pesticide, and a promising skin-whitening agent. From this point of time, we strongly suggest that considering these results and our previous experimental data reported, the "β-phenyl-α,β-unsaturated carbonyl" group might serve as a key pharmacophore for high tyrosinase inhibition.

Materials and methods

General

Melting points are uncorrected. ^1H and ^{13}C NMR spectra were recorded on Varian Unity INOVA 400 and Varian Unity AS 500 instruments. Chemical shifts are reported with reference to the respective residual solvent or deuterated peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD, δ_H 7.27 and δ_C 77.0 for $CDCl_3$). Coupling constants are reported in hertz. The abbreviations used are as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), and brs (broad singlet). All the reactions described below were performed under argon or nitrogen atmosphere and monitored by TLC. All anhydrous solvents were distilled over CaH_2 or Na/benzophenone prior to use.

General procedure for the synthesis of (*Z*)-5-(substituted benzylidene)thiohydantoin analogues (2a–2l). A mixture of substituted benzaldehydes (1.53–2.46 mmol), 2-thiohydantoin (1.1 eq.) and sodium acetate (3.0 eq.) in acetic acid (4 mL/1.0 g of sodium acetate) was refluxed for 4–24 h. After cooling, water was added and the precipitates generated were filtered and washed with water and ethyl acetate and/or methylene chloride, depending on the physical properties of the remaining starting materials, to give (*Z*)-5-(substituted benzylidene)-2-thioxoimidazolidin-4-one derivatives as solids in 15.1–85.2% yields.

(*Z*)-5-(4-Hydroxybenzylidene)-2-thioxoimidazolidin-4-one (2a). Green solid; reaction time, 4 h; yield, 45.0%; melting point, >300 °C; ^1H NMR (500 MHz, DMSO-d_6) δ 12.23 (s, 1H, NH), 11.96 (s, 1H, NH), 10.02 (s, 1H, OH), 7.61 (d, 2H, J = 8.0 Hz, 2′-H, 6′-H), 6.79 (d, 2H, J = 8.5 Hz, 3′-H, 5′-H), 6.41 (s, 1H, vinylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.9 (C2), 166.5 (C4), 159.7 (C4′), 133.1 (C2′, C6′), 125.8 (C5), 124.0 (C1′), 116.5 (C3′, C5′), 113.5 (benzylic C); LRMS(ESI) m/z 219 $(M - H)^-$.

(*Z*)-5-(2-Hydroxybenzylidene)-2-thioxoimidazolidin-4-one (2b). Dark greenish yellow solid; reaction time, 4 h; yield, 61.4%;

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

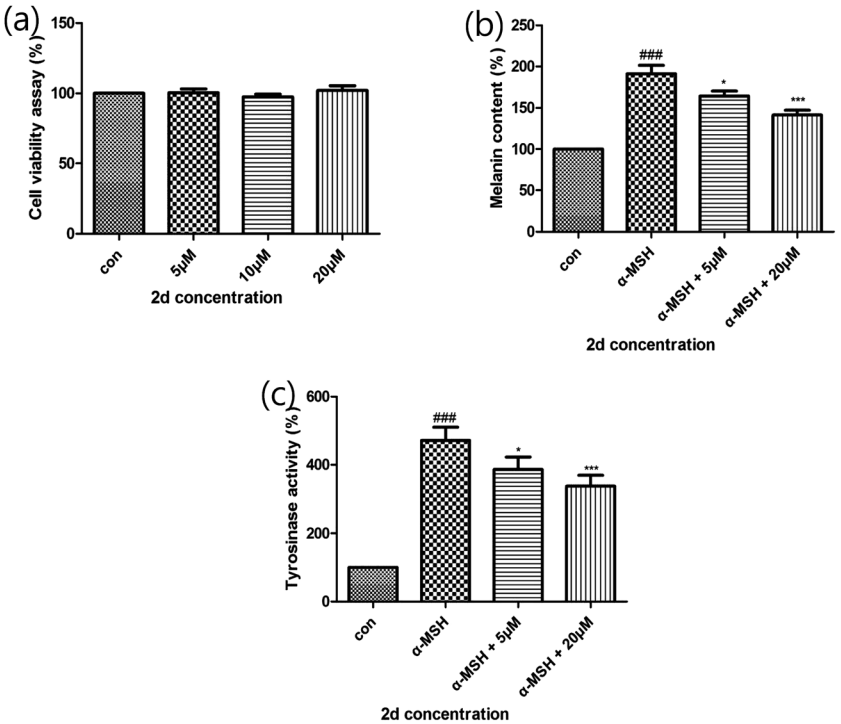


Fig. 2 Effects of **2d** on cytotoxicity, melanin production and tyrosinase activity in B16 cells. (a) The effect of **2d** on the viability of B16 cells. Cells were treated with various doses of **2d** (0–20 μM) and its effect on cell viability was examined by performing MTT assays. Data are expressed as percentages of non-treated controls. (b) The ability of **2d** to inhibit melanogenesis in the presence of 100 nM α-MSH in B16 cells. Melanin levels were measured at 405 nm. Values represent the means ± SEs of three independent experiments. Data are expressed as percentages of non-treated controls. ###$p < 0.001$ *vs.* untreated controls, *$p < 0.05$ and ***$p < 0.001$ *vs.* cells treated with 100 nM α-MSH. (c) Inhibition of tyrosinase in B16 cells by **2d**. B16 cells were co-treated with 5 or 20 μM of **2d** and 100 nM α-MSH for 24 h. Results are expressed as percentages of non-treated controls, and columns represent the means ± SEs of three independent experiments. ###$p < 0.001$ *vs.* non-treated control, *$p < 0.05$ and ***$p < 0.001$ *vs.* cells treated with 100 nM α-MSH.

melting point, 283.2–285.1 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.28 (s, 1H, NH), 11.84 (s, 1H, NH), 10.30 (s, 1H, OH), 7.66 (d, 1H, $J = 7.5$ Hz, 6′-H), 7.19 (t, 1H, $J = 8.0$ Hz, 4′-H), 6.87 (d, 1H, $J = 8.0$ Hz, 3′-H), 6.82 (t, 1H, $J = 7.5$ Hz, 5′-H), 6.70 (s, 1H, vinylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.0 (C2), 166.4 (C4), 157.0 (C2′), 131.7 (C4′), 131.1 (C6′), 127.6 (C5), 120.1 (C1′), 120.1 (C5′), 116.2 (benzylic C), 107.9 (C3′); LRMS(ESI) m/z 219 $(M - H)^-$.

(*Z*)-5-(4-Methoxybenzylidene)-2-thioxoimidazolidin-4-one (2c). Green solid; reaction time, 4 h; yield, 64.0%; melting point, 266.9–267.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.26 (s, 1H, NH), 12.03 (s, 1H, NH), 7.70 (d, 2H, $J = 8.8$ Hz, 2′-H, 6′-H), 6.94 (d, 2H, $J = 8.8$ Hz, 3′-H, 5′-H), 6.43 (s, 1H, vinylic H), 3.77 (s, 3H, 4′-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.2 (C2), 166.5 (C4), 160.9 (C4′), 132.8 (C2′, C6′), 126.5 (C5), 125.5 (C1′), 115.1 (C3′, C5′), 112.8 (benzylic C), 56.0 (4′-OCH_3); LRMS(ESI) m/z 233 $(M - H)^-$.

(*Z*)-5-(2,4-Dihydroxybenzylidene)-2-thioxoimidazolidin-4-one (2d). Yellowish green solid; reaction time, 5 h; yield, 58.1%; melting point, >300 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.13 (s, 1H, NH), 11.65 (s, 1H, NH), 10.30 (s, 1H, OH), 9.92 (s, 1H, OH), 7.56 (d, 1H, $J = 8.5$ Hz, 6′-H), 6.68 (s, 1H, vinylic H), 6.34 (d, 1H, $J = 2.0$ Hz, 3′-H), 6.27 (dd, 1H, $J = 2.0$, 8.5 Hz, 5′-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.7 (C2), 166.5 (C4), 161.4 (C4′), 158.9 (C2′), 132.6 (C6′), 124.7 (C5), 111.8 (C1′), 109.3 (benzylic C), 108.5 (C5′), 102.9 (C3′); LRMS(ESI) m/z 235 $(M - H)^-$.

(*Z*)-5-(3,4-Dihydroxybenzylidene)-2-thioxoimidazolidin-4-one (2e). Greenish yellow solid; reaction time, 5 h; yield, 76.6%; melting point, >300 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.20 (s, 1H, NH), 11.93 (s, 1H, NH), 9.63 (s, 1H, OH), 9.04 (s, 1H, OH), 7.09 (dd, 1H, $J = 2.0$, 8.0 Hz, 6′-H), 7.07 (d, 1H, $J = 1.5$ Hz, 2′-H), 6.75 (d, 1H, $J = 8.0$ Hz, 5′-H), 6.32 (s, 1H, vinylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.9 (C2), 166.5 (C4), 148.3 (C4′), 146.1 (C3′), 126.0 (C5), 124.4 (C1′), 123.6 (C6′), 118.5 (C5′), 116.5 (C2′), 114.1 (benzylic C); LRMS(ESI) m/z 235 $(M - H)^-$.

(*Z*)-5-(3,5-Dihydroxybenzylidene)-2-thioxoimidazolidin-4-one (2f). Dark brown solid; reaction time, 6 h; yield, 52.6%; melting point, >300 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.29 (s, 1H, NH), 12.03 (s, 1H, NH), 9.38 (s, 2H, OH), 6.52 (s, 2H, 2′-H, 6′-H), 6.27 (s, 2H, 4′-H, vinylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.8 (C2), 166.4 (C4), 159.1 (C3′, C5′), 134.5 (C1′), 128.5 (C5), 113.1 (benzylic C), 108.9 (C2′, C6′), 104.7 (C4′); LRMS(ESI) m/z 235 $(M - H)^-$.

(*Z*)-5-(4-Hydroxy-3-methoxybenzylidene)-2-thioxoimidazolidin-4-one (2g). Dark yellow solid; reaction time, 4 h; yield, 59.4%; melting point, °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.13 (br s, 2H, 2 × NH), 9.15 (br s, 1H, OH), 7.27 (s, 1H, 2′-H), 7.25 (d, 1H, $J = 8.5$ Hz, 6′-H), 6.79 (d, 1H, $J = 8.0$ Hz, 5′-H), 6.37 (s, 1H,

 This journal is © The Royal Society of Chemistry 2014

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

vinylic H), 3.83 (s, 3H, 3′-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.1 (C2), 167.2 (C4), 149.1 (C3′), 148.4 (C4′), 127.3 (C5), 125.4 (C6′), 124.8 (C1′), 116.4 (C5′), 114.7 (C2′), 113.6 (benzylic C), 56.6 (3′-OCH_3); LRMS(ESI) m/z 249 $(M - H)^-$.

(*Z*)-5-(3-Ethoxy-4-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (2h). Yellow solid; reaction time, 24 h; yield, 15.1%; melting point, 175.4–177.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.22 (br s, 1H, NH), 12.01 (br s, 1H, NH), 9.54 (s, 1H, OH), 7.20 (d, 1H, $J = 8.8$ Hz, 6′-H), 7.20 (s, 1H, 2′-H), 6.79 (d, 1H, $J = 8.8$ Hz, 5′-H), 6.39 (s, 1H, vinylic H), 4.08 (q, 2H, $J = 6.8$ Hz, 3′-OCH_2), 1.31 (t, 3H, $J = 6.8$ Hz, CH_2CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.0 (C2), 166.5 (C4), 149.6 (C3′), 147.6 (C4′), 125.8 (C5), 125.5 (C6′), 124.4 (C1′), 116.5 (C5′), 116.0 (C2′), 114.1 (benzylic C), 64.8 (3′-OCH_2), 15.3 (CH_2CH_3); LRMS(ESI) m/z 263 $(M - H)^-$.

(*Z*)-5-(2,4-Dimethoxybenzylidene)-2-thioxoimidazolidin-4-one (2i). Green solid; reaction time, 4 h; yield, 80.3%; melting point, 237.1–238.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.23 (br s, 1H, NH), 11.93 (br s, 1H, NH), 7.73 (d, 1H, $J = 8.5$ Hz, 6′-H), 6.68 (d, 1H, $J = 1.5$ Hz, 3′-H), 6.60 (s, 1H, vinylic H), 6.57 (dd, 1H, $J = 2.0$, 8.5 Hz, 5′-H), 3.85 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.8 (C2), 166.5 (C4), 162.8 (C4′), 159.9 (C2′), 132.0 (C6′), 126.4 (C5), 114.3 (C1′), 107.2 (benzylic C), 106.6 (C5′), 98.8 (C3′), 56.5 (2′-OCH_3), 56.2 (4′-OCH_3); LRMS(ESI) m/z 263 $(M - H)^-$.

(*Z*)-5-(3,4-Dimethoxybenzylidene)-2-thioxoimidazolidin-4-one (2j). Greenish yellow solid; reaction time, 4 h; yield, 71.7%; melting point, 236.2–238.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.30 (br s, 1H, NH), 12.14 (br s, 1H, NH), 7.35 (dd, 1H, $J = 2.0$, 9.0 Hz, 6′-H), 7.23 (d, 1H, $J = 2.0$ Hz, 2′-H), 6.98 (d, 1H, $J = 8.5$ Hz, 5′-H), 6.45 (s, 1H, vinylic H), 3.83 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.3 (C2), 166.5 (C4), 150.9 (C3′), 149.5 (C4′), 126.5 (C5), 125.7 (C1′), 125.0, (C6′) 114.0 (benzylic C), 113.4 (C5′), 112.4 (C2′), 56.5 (OCH_3), 56.2 (OCH_3); LRMS(ESI) m/z 263 $(M - H)^-$.

(*Z*)-5-(4-Hydroxy-3,5-dimethoxybenzylidene)-2-thioxoimidazolidin-4-one (2k). Yellowish green solid; reaction time, 4 h; yield, 84.6%; melting point, 240.1–242.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.15 (br s, 1H, NH), 11.98 (br s, 1H, NH), 9.10 (br s, 1H, OH), 7.57 (s, 2H, 2′-H, 6′-H), 6.50 (s, 1H, vinylic H), 3.76 (s, 6H, 3′-OCH_3, 5′-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.9 (C2), 164.2 (C4), 148.1 (C3′, C5′), 138.8 (C4′), 128.5 (C5), 123.7 (C1′), 121.9 (benzylic C), 109.5 (C2′, C6′), 56.6 (3′-OCH_3); LRMS(ESI) m/z 279 $(M - H)^-$.

(*Z*)-2-Thioxo-5-(3,4,5-trimethoxybenzylidene)imidazolidin-4-one (2l). Pale brown solid; reaction time, 4 h; yield, 52.8%; melting point, 264.0–266.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.25 (br s, 1H, NH), 12.08 (br s, 1H, NH), 7.54 (s, 2H, 2′-H, 6′-H), 6.52 (s, 1H, vinylic H), 3.79 (s, 6H, 3′-OCH_3, 5′-OCH_3), 3.69 (s, 3H, 4′-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.9 (C2), 164.2 (C4), 153.1 (C3′, C5′), 139.6 (C4′), 130.1 (C1′), 128.7 (C5), 120.3 (benzylic C), 109.0 (C2′, C6′), 60.8 (4′-OCH_3), 56.5 (3′-OCH_3, 5′-OCH_3); LRMS(ESI) m/z 293 $(M - H)^-$.

Materials

Mushroom tyrosinase, L-tyrosine [3-(4-hydroxyphenyl)]-L-alanine, (*S*)-2-amino-3-(4-hydroxyphenyl)propionic acid, kojic acid [5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one] and α-MSH (alpha-melanocyte stimulating hormone) were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). Solvents used for organic syntheses were redistilled. All other chemicals and solvents were of analytical grade and used without further purification.

Cell culture

B16 cells (obtained from the Korean Cell Line Bank) were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 IU/50 μg mL^{-1}) in a humidified atmosphere containing 5% CO_2 in air at 37 °C. B16 cells were cultured in 24-well plates for melanin quantification and enzyme activity assays.

Cell viability

Cell survival was quantified by a colorimetric MTT assay that measures mitochondrial activity in viable cells. This method is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to MTT-formazan crystal by mitochondrial enzymes as previously described.[21] Briefly, cells seeded at a density of 3×10^4 per cell in a Corning 48-well plate (Corning, NY, USA), were allowed to adhere overnight; the culture medium was then replaced with fresh serum free DMEM. MTT was freshly prepared at 5 mg mL^{-1} in phosphate-buffered saline (PBS). Aliquots of 500 μL of MTT stock solution were added to each well, and the plate was incubated at 37 °C for 4 hours in a humidified 5% CO_2 incubator. After 4 hours, the medium was removed. To each well, 500 μL of EtOH–DMSO (1 : 1 mixture solution) was added to dissolve formazan. After 10 min, the optical density of each well was measured spectrophotometrically with a 560 nm filter. Results from three experiments are shown.

Assay to measure inhibitory effects of compounds on mushroom tyrosinase activity

Mushroom-derived tyrosinase was used as the source of the enzyme for the entire study. Tyrosinase activity was determined as described previously with minor modification.[20] Briefly, 20 μL of an aqueous solution of mushroom tyrosinase (1000 units) was added to a 96-well microplate (Nunc, Denmark), in a total assay mixture volume of 200 μL containing 1 mM L-tyrosine solution, and 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 30 minutes. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm (OD_{492}) using a microplate reader (Hewlett Packard). IC_{50}, inhibitory concentration-50, is the concentration of a drug that inhibits a standard 50% response. IC_{50} is a value derived from the *X*-axis and is determined from the alignment of the dose–response curve on the dependent *Y*-axis. In the present study, dose-dependent inhibition experiments were performed in triplicate to determine the IC_{50} of a drug. According to the inhibition percentage of three doses in each experiment, log-linear curves and their equations were determined. Then, the individual IC_{50} was calculated as the concentration at which the

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

Y-axis value equaled 50% inhibition. Results are shown from three experiments.

Assay of murine tyrosinase activity

Murine tyrosinase activity was estimated by measuring the rate of oxidation of L-DOPA.[22] Cells were plated in 24-well dishes at a density of 5×10^4 cells per mL. B16 cells were incubated in the presence or absence of 100 nM α-MSH and then treated for 24 hours at various concentrations of (*Z*)-5-(2,4-dihydroxybenzylidene)-2-thioxoimidazolidin-4-one (**2d**) (0–20 μM). Cells were washed and lysed in 100 μL of 50 mM sodium phosphate buffer (pH 6.5) containing 1% Triton X-100 (sigma) and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and then frozen at −80 °C for 30 min. After thawing and mixing, cellular extracts were clarified by centrifugation at 12 000 rpm for 30 min at 4 °C. Eight μL of the supernatant and 20 μL of L-DOPA (2 mg mL^{-1}) were put in a 96-well plate, and the absorbance at 492 nm was read every 10 min for 1 hour at 37 °C using an ELISA plate reader. The final activity was expressed as ΔO.D. per min for each condition. Results from three experiments are shown.

Determination of melanogenesis in B16 cells

Determination of the melanin content was done using a modification of the method reported by Bilodeau *et al.*[23] The amount of melanin was used as an index of melanogenesis. B16 cells (5×10^4) were plated on 24-well dishes and incubated in the presence or absence of 100 nM α-MSH. Cells were then incubated for 24 hours with various concentrations of (*Z*)-5-(2,4-dihydroxybenzylidene)-2-thioxoimidazolidin-4-one (**2d**) (0–20 μM). After washing twice with PBS, samples were dissolved in 100 μL of 1 N NaOH. The samples were incubated at 60 °C for 1 hour and mixed to solubilize the melanin. The absorbance at 405 nm was compared with a standard curve of synthetic melanin. Results from three experiments are shown.

Statistical analysis

The inhibition of tyrosinase activity is expressed as a percentage of inhibition based on: $100 - [(A \times 100)/B]$, where $A = OD_{492}$ with a test sample and $B = OD_{492}$ without a test sample. Data collected are presented as means ± standard errors ($n = 3$). The statistical significance of differences between groups was determined by one-factor analysis of variance (ANOVA) followed by Fischer's protected least significant difference *post hoc* test. Values of $^*p < 0.05$ were considered statistically significant.

Conclusions

In conclusion, based on the structural characteristics of the heterocyclic scaffolds of substituted benzylidene-hydantoin, -pyrrolidinedione, and -thiazolidinedione derivatives showing potent tyrosinase inhibition, thiohydantoin was selected as a novel alternative heterocyclic scaffold for the design of potent, safe tyrosinase inhibitors for use in the agricultural, food, cosmetics, and pharmaceutical sectors. Twelve 5-(substituted benzylidene)-2-thiohydantoin analogs were synthesized by modified Knoevenagel condensation between benzaldehydes and 2-thiohydantoin. The inhibitory effects of the synthesized thiohydantoin analogs on mushroom tyrosinase were assessed using kojic acid and resveratrol as positive reference compounds. Compounds with a thiohydantoin template were found to have a tyrosinase inhibitory pattern that differed from that of compounds with a hydantoin, pyrrolidinedione, or thiazolidine scaffold, presumably due to the inability of the thiocarbonyl group of thiohydantoin to form a hydrogen bond. Of the twelve compounds synthesized, compounds **2c**, **2d**, and **2i** were found to have greater inhibitory effects than kojic acid or resveratrol. In particular, (*Z*)-5-(2,4-dihydroxybenzylidene)-2-thiohydantoin (**2d**) was found to be a 24- and 18-fold more potent tyrosinase inhibitor than resveratrol or kojic acid, respectively, and to inhibit melanin production through the inhibition of tyrosinase activity in B16 cells without exhibiting a cytotoxic effect. These results suggest that **2d** should be regarded a lead compound for the development of safer, more potent vegetable and food browning preventatives and skin-lightening medicines. Considering the tyrosinase inhibitory activities of 5-(substituted benzylidene)thiohydantoin derivatives, we consider the thiohydantoin template a near perfect surrogate for hydantoin, pyrrolidinedione, and thiazolidinedione scaffolds in the context of inhibiting tyrosinase activity. In addition, these results proved in part that the "β-phenyl-α,β-unsaturated carbonyl" group might be an essential template for great tyrosinase inhibition.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (Grant no. 2009-0083538) and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Grant no. NRF-2013R1A1A2009949).

Notes and references

1 V. J. Hearing and K. Tsukamoto, *FASEB J.*, 1991, **5**, 2902–2909.
2 A. M. Mayer and E. Harel, *Phytochemistry*, 1979, **18**, 193–215.
3 J. R. Whitaker, Polyphenol Oxidase, in *Food Enzyme: Structure and Mechanism*; ed. D. W. S. Wong, Chapman & Hall, New York, 1995, pp. 271–307.
4 M. Brenner and V. J. Hearing, *Photochem. Photobiol.*, 2008, **84**, 539–549.
5 G. H. Muller, A. Lang, D. R. Seithel and H. Waldmann, *Chem.–Eur. J.*, 1998, **4**, 2513–2522.
6 S. O. Andersen, Sclerotization of insect cuticle, in *Molting and Metamorphosis*, ed. E.Ohnishi and H. Ishizaki, Japan Sci. Soc. Press, Tokyo, 1990, pp. 135–155.
7 K. J. Kramer and T. L. Hopkins, *Arch. Insect Biochem. Physiol.*, 1987, **6**, 279–301.
8 M. T. H. Khan, *Curr. Med. Chem.*, 2012, **19**, 2262–2272.
9 C. Coiffard, L. J. M. Coiffard and Y. De Roeck-Holzhauer, *Pharm. Ind.*, 1999, **61**, 574–576.

 This journal is © The Royal Society of Chemistry 2014

Published on 02 July 2014. Downloaded by University of Windsor on 30/10/2014 09:12:27.

10 Y. M. Ha, J.-H. Kim, Y. J. Park, D. Park, J. M. Kim, K. W. Chung, E. K. Lee, J. Y. Park, J. Y. Lee, H. J.Lee, J. H. Yoon, H. R. Moon and H. Y. Chung, *Biochim. Biophys. Acta, Gen. Subj.*, 2011, **1810**, 612–619.
11 Y. M. Ha, J.-A. Kim, Y. J. Park, D. Park, Y. J. Choi, J. M. Kim, K. W. Chung, Y. K. Han, J. Y. Park, J. Y. Lee, H. R. Moon and H. Y. Chung, *MedChemComm*, 2011, **2**, 542–549.
12 S. H. Kim, Y. M. Ha, K. M. Moon, Y. J. Choi, Y. J. Park, H. O. Jeong, K. W. Chung, H. J. Lee, P. Chun, H. R. Moon and H. Y. Chung, *Arch. Pharm. Sci. Res.*, 2013, **36**, 1189–1197.
13 K. W. Chung, Y. J. Park, Y. J. Choi, M. H. Park, Y. M. Ha, Y. Uehara, J. H. Yoon, P. Chun, H. R. Moon and H. Y. Chung, *Biochim. Biophys. Acta, Gen. Subj.*, 2012, **1820**, 962–969.
14 R. E. Ramsay and F. M. Pryor, *Status Epilepticus*, 2006, 545–552.
15 J. Marton, J. Enisz, S. Hosztafi and T. Timar, *J. Agric. Food Chem.*, 1993, **41**, 148–152.
16 K. Inagaki, I. Miwa, T. Yashiro and J. Okuda, *Chem. Pharm. Bull.*, 1982, **30**, 3244–3254.
17 K. Kiec-Kononowicz and E. Szymanska, *Farmaco*, 2002, **57**, 909–916.
18 D. Lazewska, P. Maludzinski, E. Szymanska and K. Kiec-Kononowicz, *Biomed. Chromatogr.*, 2007, **21**, 291–298.
19 Y. Song, D. T. Connor, R. Doubleday, R. J. Sorenson, A. D. Sercel, P. C. Unangst, B. D. Roth, R. B. Gilbertsen, K. Chan, D. J. Schrier, A. Gulietta, D. A. Bornemeier and R. D. Dyer, *J. Med. Chem.*, 1999, **42**, 1151–1160.
20 J. K. No, D. Y. Soung, Y. J. Kim, K. H. Shim, Y. S. Jun, S. H. Rhee, T. Yokozawa and H. Y. Chung, *Life Sci.*, 1999, **65**, 241–246.
21 H. Tada, O. Shiho, K. Kuroshima, M. Koyama and K. Tsukamoto, *J. Immunol. Methods*, 1986, **93**, 157–165.
22 D. S. Kim, S. Y. Kim, J. H. Chung, K. H. Kim, H. C. Eun and K. C. Park, *Cell. Signalling*, 2002, **14**, 779–785.
23 M. L. Bilodeau, J. D. Greulich, R. L. Hullinger, C. Bertolotto, R. Ballotti and O. M. Andrisani, *Pigm. Cell Res.*, 2001, **14**, 328–336.