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Research Article

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Umbelliferone-Thiazolidinedione Hybrids as Potent Mushroom Tyrosinase Inhibitors

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ABSTRACT

In search for the potent mushroom tyrosinase inhibitors, six umbelliferone-thiazolidinedione hybrids, as well as their constitutive compounds, (5-substituted benzylidene)-thiazolidine-2,4-diones were examined on their inhibitory activity both, on monophenolase and diphenolase activity. The results showed that all tested compounds exhibited tyrosinase inhibition at 100 μM concentration, but diphenolase activities were found less inhibited than monophenolase. Umbelliferone-thiazolidinedione hybrids showed the greater monophenolase and diphenolase inhibitory activity than the constitutive thiazolidinediones. The most potent inhibitors of tyrosinase were umbelliferone-thiazolidinedione hybrids bearing 4- or 3-hydroxyphenyl moiety at position five of thiazolidinedione ring, showing IC_{50} of 0.17 and 0.35 μM for monophenolase, and 0.25 and 1.63 μM for diphenolase activity, respectively. Obtained data indicates importance of umbelliferone moiety for increase of inhibitory activity of thiazolidinediones bearing 3-hydroxyphenyl and 4-hydroxyphenyl moiety, and vice versa.

Key words: *mushroom tyrosinase, inhibition, umbelliferone, thiazolidinedione*

INTRODUCTION

Tyrosinases are enzymes with a binuclear copper centre able to insert oxygen in an *ortho*-position of an existing hydroxyl group in aromatic ring, followed by oxidation of diphenol to the corresponding quinone. These oxidoreductases are widely distributed among plants, animals, fungi and bacteria. Besides their positive developmental and defensive physiological role in various organisms, such as sclerotization and pigmentation of insect cuticles, wound healing, defence against herbivores and pathogens in plants [1, 2], tyrosinase has negative impact on hyperpigmentation's in humans, such as *senile lentigo*, melisma, freckles and pigmented ace scars [3, 4, 5], as well as in browning reaction in food industry [6]. Therefore, development of new potent tyrosinase inhibitors, that may be used for treatments of dermatological disorders related to melanin hyper accumulation in medicine and cosmetic industry, as well in the prevention of browning reaction in food industry, is of interest.

Recent reports on the ability of several coumarin derivatives to act as potent mushroom tyrosinase inhibitors [3, 4, 5, 7, 8], as well as reports on the significant inhibition of tyrosinase activity by 5-substituted benzylidene-thiazolidine-2,4-diones [9, 10], gave us idea to investigate tyrosinase inhibition by six thiazolidinediones and their corresponding coumarin derivatives (umbelliferone-thiazolidinedione hybrids) that have been previously synthesised and characterized by our research group [11]. Based on the report of Ashraf et al. [4] who found increased tyrosinase inhibitory activity of umbelliferone analogues substituted at position-7 of coumarin ring, as well as reports on increased inhibitory activity of coumarin derivatives substituted at position-3 of coumarin ring [3, 5, 7], we assumed that (5-substituted benzylidene)-thiazolidine-2,4-diones added at position-4 of umbelliferone ring might increase

inhibitory activity of both, umbelliferone and thiazolidinediones, thus leading to the new potent class of tyrosinase inhibitors.

Since inhibitory activity of thiazolidinediones against mushroom tyrosinase were determined with L-tyrosine as substrate (monophenolase activity) [9,10], and of coumarin derivatives with L-DOPA as substrate (diphenolase activity) [3, 4, 5, 7, 8], in our study we have examined inhibitory activity of six umbelliferone-thiazolidinedione hybrids and their corresponding 5-substituted benzylidene-thiazolidine-2,4-diones against mushroom tyrosinase with both, L-tyrosine and L-DOPA as substrates.

MATERIALS AND METHODS

Chemicals

Mushroom tyrosinase (5771 U/mg solid), L-3,4-dihydroxyphenylalanine (L-DOPA), and umbelliferone were purchased from Sigma-Aldrich (USA), sodium phosphate monobasic monohydrate, sodium phosphate dibasic dihydrate, and L-tyrosine from Merck (Germany), while dimethyl sulfoxide (DMSO) from Kemika (Croatia).

Thiazolidinediones **1a-f** ((5-substituted benzylidene)-thiazolidine-2,4-diones) and their umbelliferone hybrids **2a-f** (3-coumarinyl-5-aryliden-1,3-thiazolidine-2,4-diones) (Figure 1) were synthesized and characterized as previously reported [11].

Standard stock solutions (10 mM) of tested compounds were prepared with DMSO prior to inhibition studies.

Tyrosinase activity assay

Tyrosinase activity was determined with L-tyrosine (monophenolase) and L-DOPA (diphenolase) as substrates. Reaction mixture (1 mL) for monophenolase activity contained 1 mM L-tyrosine in 50 mM phosphate buffer (pH = 6.5) and 100 units of mushroom tyrosinase, or 0.5 mM L-DOPA in 50 mM phosphate buffer (pH = 6.5) and 100 units of mushroom tyrosinase for diphenolase activity. The reaction was carried out at 25 °C and increase in absorbance at 475 nm was measured using a double-beam spectrophotometer Specord 200 (AnalytikJena, Germany). Change in absorbance was recorded every minute during 20 min (L-tyrosine), or every 10 s for 100 s (L-DOPA), and enzyme activity was measured from the linear portion of curve. One unit of tyrosinase activity was defined as the change in absorbance of 0.001 per min and mL of enzyme. Activity measurements both, with L-tyrosine and L-DOPA were carried out in triplicate.

Inhibition studies

Mushroom tyrosinase activity (monophenolase and diphenolase) was measured in the presence of six thiazolidinediones (**1a-f**), six umbelliferone-thiazolidinedione hybrids (**2a-f**) (Figure 1), and umbelliferone as positive control. Compounds were dissolved in DMSO at 10 mM concentration and added (10 µL) to the reaction mixture containing 400 µL of 50 mM phosphate buffer (pH = 6.5) and 30 µL of aqueous solution of mushroom tyrosinase (1000 U/mL) for monophenolase, or 920 µL of 50 mM phosphate buffer (pH = 6.5) and 30 µL of aqueous solution of mushroom tyrosinase (1000 U/mL) and pre-incubated 5 min at 25 °C. Reaction was started by addition of 500 µL of 2 mM L-tyrosine in 50 mM phosphate buffer (pH = 6.5) (monophenolase), or 50 µL of 10 mM L-DOPA in 50 mM phosphate buffer (pH = 6.5) (diphenolase), and tyrosinase activity was measured as described in previous section. Final concentration of tested compounds in the reaction mixture was 100 µM, and of DMSO 1%. Controls without inhibitors, but containing 10 µL of DMSO were routinely carried out.

For compounds showing significant inhibition of tyrosinase activity at 100 µM concentration, IC_{50} value (a concentration giving 50% inhibition of tyrosinase activity) was determined by interpolation of the dose-response curves.

The percent of inhibition of tyrosinase catalysed reaction was calculated as following:

$$\text{Inhibition rate (\%)} = [1 - [(S - B)/(C - B)]] \times 100$$

where S, B, are the absorbance's for sample and blank, and C absorbance for control without inhibitor, but containing 10 µL DMSO.

RESULTS AND DISCUSSION

Synthesis of compounds

Synthesis of tested compounds was performed as described in our previous work [11]. Compounds **1a-f** (5-arylmethylidene-1,3-thiazolidine-2,4-diones) were prepared by conventional Knoevenagel reaction by reacting 1,3-thiazolidine-2,4-dione with appropriate aromatic aldehydes. When compounds **1a-f** were reacted with 7-hydroxy-4-bromomethyl-2-oxo-2*H*-chromene, 3-(7-hydroxy-2-oxo-2*H*-chromen-4-ylmethyl)-5-arylidene-1,3-thiazolidine-2,4-diones **2a-f** were synthesized (Figure 1).

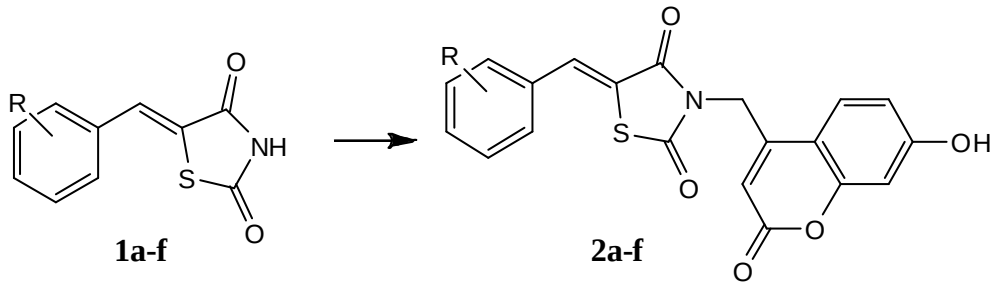

Figure 1. Synthesis of tested compounds (R = a: 3-OH; b: 4-OH; c: 4-OCH$_3$; d: 2-Cl; e: 3-phenoxy; f: 4-OH-3-OCH$_3$)

Inhibitory activity

Synthesized compounds were tested on inhibitory activity against mushroom tyrosinase at 100 μM concentration in the reaction mixture containing 1 mM L-tyrosine for monophenolase, and 0.5 mM L-DOPA for diphenolase activity. Results on monophenolase activity inhibition are shown in Figure 2, and diphenolase activity inhibition in Figure 3.

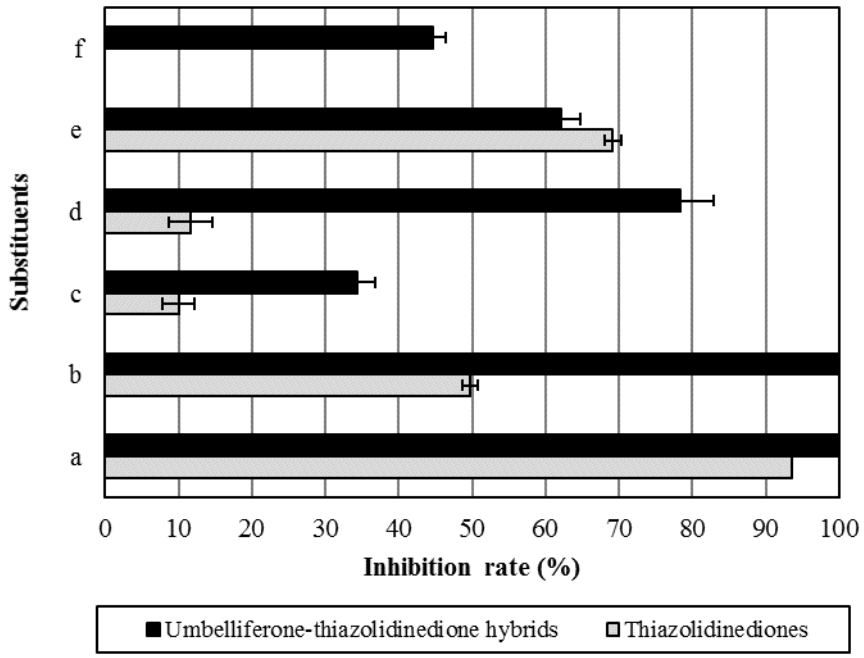

Figure 2. Inhibitory effect of thiazolidinediones and umbelliferone-thiazolidinedione hybrids on monophenolase activity of mushroom tyrosinase. Concentration of tested compounds in reaction mixture was 100 μM
Results present mean value ± standard deviation of triplicate measurements

All examined compounds, except thiazolidinedione bearing 4-hydroxy-3-methoxyphenyl moiety (**1e**), exhibited monophenolase inhibitory activity (Figure 2). Among tested thiazolidinediones, the greatest inhibition of monophenolase activity of 93.5% was observed in the presence of thiazolidinedione bearing 3-hydroxyphenyl moiety (**1a**), followed by those one bearing 3-phenoxyphenyl (**1e**) (69.2%) and those bearing 4-hydroxyphenyl moiety (**1b**) (49.8%). Similarly could be observed for umbelliferone-thiazolidinedione hybrids **2a-e** (Fig. 2), but in most cases inhibitory effect was greater than the constitutive thiazolidinediones. Umbelliferone-thiazolidinedione hybrids bearing 3- or 4-hydroxyphenyl moiety completely inhibited monophenolase activity at 100 μM concentration, while those one bearing 3-phenoxyphenyl moiety showed similar inhibitory effect as corresponding thiazolidinedione. Moreover, inhibitory effect of umbelliferone-thiazolidinedione hybrids bearing 2-chlorophenyl

and 4-hydroxy-3-methoxyphenyl moiety was found significantly augmented compared to the constitutive thiazolidinediones. This might be attributed to the presence of umbelliferone moiety, since umbelliferone alone was found to inhibit monophenolase activity for 62.7%.

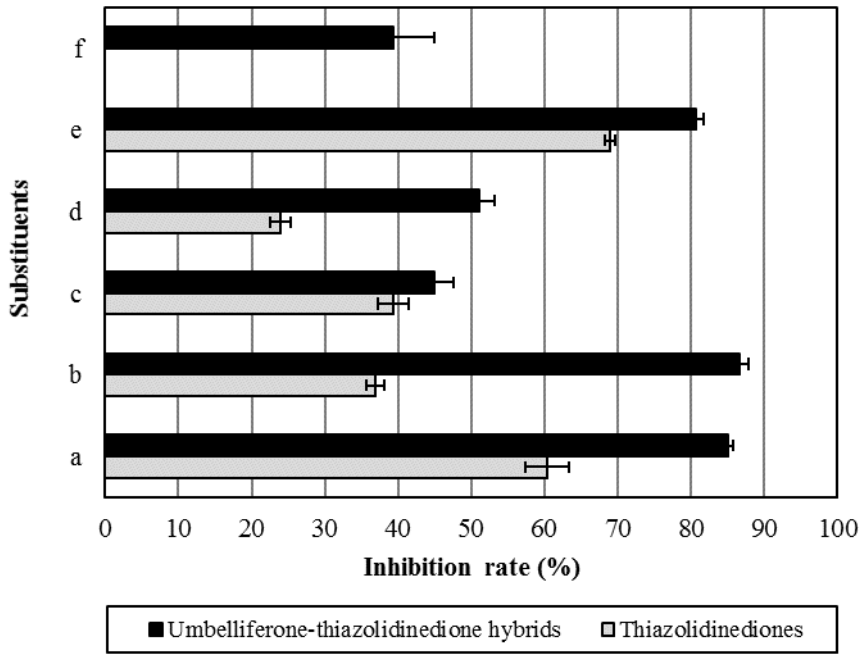

Figure 3. Inhibitory effect of thiazolidinediones and umbelliferone-thiazolidinedione hybrids on diphenolase activity of mushroom tyrosinase. Concentration of tested compounds in reaction mixture was 100 µM
Results present mean value ± standard deviation of triplicate measurements

Almost identical trends were found for mushroom tyrosinase diphenolase activity inhibition (Figure 3). Thiazolidinediones (**1a-f**) showed lower diphenolase inhibitory activity than the corresponding umbelliferone-thiazolidinedione hybrids (**2a-f**). The highest diphenolase inhibition was achieved with compounds **2b**, **2a and 2e** bearing 4-hydroxyphenyl, 3-hydroxyphenyl and 3-phenoxyphenyl moiety, as well as **2d** and **2f** hybrids bearing 2-chlorophenyl and 4-hydroxy-3-methoxyphenyl moiety which showed significantly increased inhibition than the constitutive thiazolidinediones. However, umbelliferone itself was found to only slightly inhibit diphenolase activity (13.4%), and thiazolidinedione **1b** bearing 4-hydroxyphenyl moiety showed lower diphenolase inhibiting action (36.9%).

Due to the great inhibition of mushroom tyrosinase activity by both thiazolidinediones and umbelliferone-thiazolidinedione hybrids bearing 3-hydroxyphenyl, 3-phenoxyphenyl and 4-hydroxyphenyl moiety, their IC_{50} values, both for monophenolase and diphenolase activity were determined (Table 1). In addition, IC_{50} value for umbelliferone as positive control, as well as constitutive compound of umbelliferone-thiazolidinedione hybrids was determined.

Table 1. Concentration of selected thiazolidinediones and umbelliferone-thiazolidinedione hybrids causing 50% inhibition of monophenolase and diphenolase activity of mushroom tyrosinase (IC_{50})

Compounds/ substituents	**Monophenolase**	**Diphenolase**
	IC50 (µM)*	
Thiazolidinediones		
1a	6.21 ± 0.40	56.50 ± 6.08
1b	98.99 ± 8.95	175.24 ± 19.09
1e	46.22 ± 5.91	45.97 ± 1.29
Umbelliferone-thiazolidinedione hybrids		
2a	0.35 ± 0.01	1.63 ± 0.15
2b	0.17 ± 0.01	0.25 ± 0.01
2e	33.78 ± 9.54	12.21 ± 0.90
Umbelliferone	71.88 ± 6.13	402.40 ± 10.78

** IC_{50} values were determined from logarithmic concentration–inhibition curves and are given as means ±SD of triplicate measurements.*

Results showed that the most potent inhibitors of mushroom tyrosinase are umbelliferone-thiazolidinedione hybrids **2a** and **2b** bearing 4- or 3-hydroxyphenyl moiety at position five of thiazolidinedione ring, showing IC_{50} of 0.17 and

0.35 μM for monophenolase, and 0.25 or 1.63 μM for diphenolase activity, respectively (Table 1). In comparison with their constitutive thiazolidinediones **1a** and **1b**, umbelliferone-thiazolidinedione hybrid **2a** showed 18 and 35-fold increase in monophenolase and diphenolase inhibitory activity, and even greater for hybrid **2b** where 580 and 690-fold increase in inhibitory activity was observed. This clearly indicates the importance of umbelliferone moiety for achieving significant mushroom tyrosinase inhibition. From the tested thiazolidinediones **1a-f**, only those one bearing 3-hydroxyphenyl moiety (**1a)** was found to significantly inhibits monophenolase activity (IC_{50} = 6.21 ± 0.40 μM; Table 1), what is similar to previous reports on monophenolase activity inhibition by thiazolidinediones bearing 2,4-dihydroxyphenyl (IC_{50} = 3.55 μM) and 3-hydroxy-4-methoxy (IC_{50} = 9.87 μM) moiety [9, 10]. However, its inhibitory activity toward L-DOPA oxidation was 9-fold reduced (IC_{50} = 56.50 ± 6.08 μM; Table 1). Reduced diphenolase activity inhibition in comparison with monophenolase was recorded for the most tested substances (Table 1). Significant differences, up to 12.6-fold, between IC_{50} values for monophenolase and diphenolase activity inhibition of mushroom tyrosinase was reported by Li et al. [12] when chlorobenzaldehyde thiosemicarbazones was tested as inhibitors. The most probable reason for observed differences in inhibition action of selected compounds against monophenolase and diphenolase activity are differences in catalytic cycle mechanism of monophenol (L-tyrosine) and diphenol (L-DOPA) oxidation by mushroom tyrosinase [13, 14]. However, significant inhibition of monophenolase steady-state activity without prolongation of lag time, as already reported by Li et al. [12], cannot be excluded. Tyrosinase activity toward L-tyrosine as monophenol substrate requires lag-phase for its transformation to diphenol, a substrate to diphenolase [13, 15].

Umbelliferone differently inhibited monophenolase and diphenolase activity. It's inhibiting effect on diphenolase activity observed in our study (IC_{50} = 402.40 ± 10.78 μM; Table 1) was similar to those (0.42 mM) reported by Masamoto et al. [16], but there are no data regarding umbelliferone inhibiting effect on monophenolase activity (IC_{50} = 71.88 ± 6.13 μM; Table 1) reported in available literature, to compare. Therefore, it seems that for the first time here we report inhibitory activity of umbelliferone against monophenolase activity of mushroom tyrosinase toward L-tyrosine, as substrate. When umbelliferone IC_{50} values were compared with those of umbelliferone-thiazolidinedione hybrids **2a** and **2b**, then at least 200-fold increase in monophenolase and diphenolase inhibitory activity could be observed for hybrid bearing 3-hydroxyphenyl moiety (**2a**), and 400- and 1500-fold increase in monophenolase and diphenolase inhibition by hybrid bearing 4-hydroxyphenyl moiety (**2b**). This indicates that attachment of various groups on position-4 of umbelliferone ring might significantly increase inhibitory activity. Increase in umbelliferone inhibiting activity of mushroom diphenolase by addition of various substituents at position-3 has been reported by Ashraf et al. [4].

Literature survey for IC_{50} values of various coumarin derivatives inhibiting mushroom tyrosinase has shown that umbelliferone-thiazolidinedione hybrids bearing 4- or 3-hydroxyphenyl moiety investigated in our research, belongs to the group of the most potent coumarin derivatives inhibiting tyrosinase. This includes 5,7-dihydroxy-3-(3-thiophenyl)coumarin with IC_{50} value of 0.19 μM [5], 8'-epicleomiscosin A, a natural coumarin derivative isolated from the aerial parts of *Rhododendron collettianum* with IC_{50} value of 1.33 μM [17], 2-(1-(coumarin-3-yl)ethylidene)hydrazinecarbothioamide causing 50% of diphenolase inhibition of mushroom tyrosinase at 3.44 μM concentration [3], and 2-oxo-2-[(2-oxo-2H-chromen-7-yl)oxy]ethyl-2,4-dihydroxybenzoate with IC_{50} of 8.96 μM [4]. Therefore, umbelliferone-thiazolidinedione hybrids bearing 4- or 3-hydroxyphenyl moiety has potential to be used as tyrosinase inhibitors in medical, pharmaceutical and/or agricultural and food fields. However, further elaboration on their potential cytotoxic effect, as well elucidation of inhibiting mechanism on both monophenolase and diphenolase activity is of interest.

CONCLUSION

This study showed that some of synthesised derivatives exhibited significant inhibitory effect against monophenolase and diphenolase activity of mushroom tyrosinase. The two most active compounds were umbelliferone-thiazolidinedione hybrids bearing 4- or 3-hydroxyphenyl moiety at position five of thiazolidinedione ring, showing IC_{50} of 0.17 and 0.35 μM for monophenolase, and 0.25 or 1.63 μM for diphenolase activity, respectively. The presence of umbelliferone moiety was found important for increase in inhibitory activity of thiazolidinediones bearing 3-hydroxyphenyl and 4-hydroxyphenyl moiety, and *vice versa*. Therefore, it seems that 3-(7-hydroxy-2-oxo-2*H*-chromen-4-ylmethyl)-5-arylidene-1,3-thiazolidine-2,4-diones modulates the inhibitory action of both, umbelliferone and thiazolidinediones, what may lead to their potential use in medical, pharmaceutical and/or agricultural and food fields dealing with tyrosinase inhibition.

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