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A Potent Activator of Melanogenesis Identified from Small Molecule Screening

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Abstract

Small molecules that increase the cellular level of melanin can be used to study melanogenesis, and have therapeutic potential for melanin-related diseases such as albinism. We describe the identification of a potent activator of melanogenesis from a targeted combinatorial library. Treating melanocytes with our most active molecule results in a 1.8-fold increase in melanin, and an increase in tyrosinase-catalyzed oxidation of L-tyrosine, a key step in melanin biosynthesis.

Keywords

melanogenesis; tyrosinase; combinatorial chemistry; drug discovery

Melanoma, which most often results from genome instability and malignant growth of melanocytes, is a worldwide epidemic. As reported by the World Health Organization, 160,000 new cases of melanoma, and 48,000 melanoma related deaths occurred worldwide in 2006.^[1] Genome instability in melanocytes, leading to melanoma, is most often caused by chronic exposure to UVA-and UVB-radiation, which results in the generation of highly reactive free radicals, as well as photoinduced dimerization of thymine nucleotides in genomic DNA. As a mechanism of combating these deleterious effects of UV-radiation on the cell, a large number of organisms, including humans, produce melanin, a complex photoprotectant polymer capable of absorbing 99.9% of UV-radiation.^[2] While produced in a number of areas including the brain, eyes, adrenal gland, and hair, production of melanin in melanocytes results in the pigmentation of skin. The production of melanin (melanogenesis) is a complex cellular process, involving over 100 genes, which regulate melanin biosynthesis, intracellular trafficking of melanogenic enzymes to melanosomes, and intercellular trafficking to keratinocytes.^[3] Although numerous reports have shed light on key steps in melanin biosynthesis, regulation and trafficking, our current understanding of melanogenesis remains incomplete.

Small molecule reagents that upregulate melanin biosynthesis in melanocytes hold the potential to reduce UV-radiation induced skin damage and the onset of melanoma. In

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addition, such reagents may find use in the treatment of hypopigmentation disorders such as albinism. Conversely, a significant number of skin diseases such as melasma, postinflammatory melanoderma, and solar lentigines are the result of increased melanin biosynthesis.^[4] Small molecule reagents that increase or decrease melanin biosynthesis in melanocytes can be used as tools to examine the cellular mechanisms underlying melanogenesis, and potentially reveal previously unknown therapeutic targets for melanin-related diseases.

Currently, only a small number of molecules are known to alter the cellular level of melanin in melanocytes. The naturally occurring molecule forskolin^[5] (Figure 1), and synthetic reagents such as isobutylmethylxanthine^[6] (IBMX, Figure 1) are known to regulate adenylyl cyclase and phosphodiesterases, respectively, resulting in an increase in melanin biosynthesis in melanocytes. When applied topically to mice, forskolin provides protection against UV-induced carcinogenesis of skin.^[7] Recently, a synthetic reagent dubbed “melanogenin” was identified from small molecule screening, that increases melanin production in melanocytes (Figure 1).^[8] Unlike forskolin and IBMX, which act on known members of the canonical melanogenesis pathway, melanogenin was found to bind prohibitin and mitochondrial F₁F₀-ATPase resulting in re-trafficking of tyrosinase and tyrosinase-related protein 1. This example,^[8] and others,^[9] highlight the utility of small molecules with unique biological function to examine complex cellular processes, such as melanogenesis, and identify potential therapeutics and novel therapeutic targets.

As part of a research program designed to identify additional synthetic molecules capable of altering the cellular level of melanin in melanocytes, we synthesized and screened a 75-member targeted combinatorial library of functionally diverse amides. Library members were synthesized from carboxylic acid (**A–E**) and amine (**1–15**) building blocks in a two-step process to generate functionally diverse amides from inexpensive starting materials (Figure 2). All library members were characterized by mass spectrometry, and purity was determined by high performance liquid chromatography (HPLC). All compounds were determined to be 90% pure, and stored as 25 mM solutions in DMSO.

Screening the library for molecules that alter melanin levels in melanocytes was performed in triplicate in an immortalized murine melanocyte cell line (melan-a). Briefly, 1×10^5 melan-a cells were plated in a 24-well tissue culture plate and allowed to stably adhere in 1 mL of RPMI 1640/10% FBS/0.2 μ M TPA (referred to herein as growth media) for 18 hours at 37 °C under 5% CO₂. After such time, media was removed and replaced with growth media containing either 2.5 μ M forskolin, which serves as a positive control for increased melanin levels, or 2.5 μ M library member. As a negative control, cells were treated with growth media containing 0.1% DMSO (referred to herein as vehicle control).

After 72 hours, cells were trypsinized from the tissue culture multi-well plate, individually placed in a 1.5 mL plastic tube, pelleted, resuspended, and washed twice with phosphate buffered saline (PBS, pH 7.2). Following resuspension in 1 mL of PBS, a small aliquot of each cell mixture was removed, and cells were counted by hemocytometry. Cell count was used to measure the cytotoxicity of each library member, as well as to normalize the cellular level of melanin per cell in different samples.

After counting, the cell samples were repelleted, and lysed in 800 μ L of 1 M NaOH/PBS for 2 hours on ice. Cell lysate was homogenized by pipetting, and melanin levels were measured by Abs₄₉₀. The cellular level of melanin in each sample was determined by reference to a A₄₉₀ standard curve using purified melanin (Sigma Aldrich) and normalized to cell count. From this screen, four compounds were identified that increase the cellular level of melanin by 1.5-fold in comparison to treatment with vehicle control. The most active compounds

identified in our screen share structural features. 2-ethyl-quinoline and furan moieties represent the carboxylic acid building block, and three of the four compounds have either *ortho*- or *para*-fluorinated aryl groups as the amine building block. Comparison of these four identified compound A7 as the most effective activator of melanogenesis. Treating melan-a melanocytes with 2.5 μ M A7 resulted in a 1.8 ± 0.3 -fold increase in the cellular level of melanin, in comparison to treatment with vehicle control (Figure 3).

Treatment of melan-a cells with 0.5, 1.0, 2.5, or 5.0 μ M A7 resulted in a dose-dependent increase in the cellular level of melanin, with a maximum effect observed at a dose 2.5 μ M. In contrast, compound A7 did not have a significant effect on melan-a proliferation under the concentrations tested (Figure 4). In addition to these data, an A7-dependent increase in the cellular level of melanin was confirmed by microscopy. Melan-a melanocytes were grown on glass slides and treated with growth media containing either vehicle control or 2.5 μ M A7 and incubated at 37 °C under 5% CO₂ for 72 hours. Cells were then washed three times with PBS and imaged. Unlike cells treated with vehicle control, which were absent of dark features, cells treated with 2.5 μ M A7 were observed to have significant pigmentation. Closer analysis of cells treated with 2.5 μ M A7 revealed a large number of dark punctate foci within each cell, consistent with high levels of melanin present in melanosomes (Figure 5).^[10] Taken together, these data are consistent with a model in which the cellular level of melanin is significantly increased by treatment with compound A7.

In addition to an increase in cell pigmentation, we observed significant changes in the morphology of melan-a cells following treatment with 2.5 μ M A7 (Figure 5). This observation further supports an A7-dependent increase in melanogenesis. Previous reports have shown a strong correlation between changes in melanocyte morphology, such as dendricity, and the cellular level of melanin.^[11] In order to measure the observed change in morphology, as well as obtain additional data on the response of melanocytes to compound A7, we measured the dendricity of melan-a cells 72 hours after treatment with vehicle control or 2.5 μ M A7. 1×10^4 melan-a cells were plated on a MatrigelTM-coated glass slide and allowed to stably adhere for 18 hours. Cells were then treated in triplicate with growth media containing either vehicle control or 2.5 μ M A7 and cultured for 72 hours at 37 °C under 5% CO₂. Cells were washed three times with PBS and fixed with 4% formaldehyde/PBS. The cell nucleus was stained with hematoxylin, and cells were imaged by microscopy. The number of dendrites per cell were counted manually. On average, $36\% \pm 3.1\%$ of cells treated with growth media containing vehicle control had greater than 2 dendrites per cell. In comparison, $69\% \pm 5.2\%$ of cells treated with 2.5 μ M A7 had greater than 2 dendrites per cell (Figure 6). These data confirm our initial observations, and support A7-dependent induction of melanogenesis in melan-a cells.

We next sought to identify cellular targets of A7. Tyrosinase is a well characterized enzyme that catalyzes the oxidation of phenols (such as tyrosine) and is known to play a critical role in the canonical melanin biosynthetic pathway.^[12] In this pathway, tyrosinase catalyzes the hydroxylation of tyrosine to generate L-DOPA. An increase in the activity or expression of tyrosinase therefore results in an increase of melanin in melanocytes.^[13]

The direct correlation between the catalytic activity of tyrosinase or tyrosinase overexpression, and melanin production, makes tyrosinase a good starting point to examine potential cellular targets of compound A7 that result in an increased cellular level of melanin. In addition, because the role of tyrosinase in the canonical melanogenesis pathway is well established, the effect compound A7 has on tyrosinase activity and expression provides a valuable starting point for future efforts to further elucidate the mechanism of action of compound A7.

Tyrosinase activity was measured using a previously reported technique.^[14] The cellular level of tritiated water, which is formed as a byproduct of tyrosinase catalyzed hydroxylation of L-tyrosine-3,5-^{[3]H} was measured by scintillation (Figure 7). An increase in ³H₂O levels would support an increase in tyrosinase activity, whereas a decrease in ³H₂O levels supports a decrease in tyrosinase activity.

Briefly, 1×10^5 melan-a cells were plated in a 24-well tissue culture plate and allowed to stably adhere for 18 hours. Cells were then treated in quadrupet with growth media containing either vehicle control or 2.5 μ M A7 and cultured for 72 hours. Melan-a cells were trypsinized from the tissue culture plate, pelleted, washed three times in PBS, and repelleted. Cells were then lysed in 75 μ L of 80 mM K₂PO₄, 1% CHAPS, 2 mM PMSF, containing PICO2 protease inhibitor cocktail (CalBioChem) for 2 hours on ice. Total protein levels in each cell lysate sample were determined by Bradford Assay. 5.0 μ g aliquots of total protein were incubated in 250 μ L of 80 mM K₂PO₄ containing 250 nM L-tyrosine, 25 μ M L-DOPA and 0.7 μ Ci of L-tyrosine-3,5-^{[3]H} for 60 minutes at 37 °C. Total protein was then precipitated with 375 μ L of 0.2% bovine serum albumin and 375 μ L of 10% trichloroacetic acid, and pelleted. The resulting supernatant was added to 500 μ L of a washed charcoal slurry to remove particulate. Charcoal was then pelleted, and the level of tritiated water in the supernatant was analyzed by a scintillation counter. Supernatant from melan-a cells incubated in growth media containing either 2.5 μ M forskolin or vehicle control was used as a positive and negative control, respectively. Supernatant from melan-a cells that were untreated, and boiled after lysis, to denature total cellular protein, was used to determine background count level. Background was subtracted from the value obtained for each sample to provide an absolute measurement of tyrosinase activity.

On average, cells treated with vehicle control had $73,422 \pm 781$ scintillation counts per minute. In comparison, cells treated with 2.5 μ M A7 or 2.5 μ M forskolin had $99,567 \pm 10,390$ scintillation counts per minute and $121,588 \pm 5,016$ scintillation counts per minute, respectively (Figure 8A). On average, this correlates to a 35% increase in tyrosinase activity in cells treated with compound A7 and a 65% increase in tyrosinase activity in cells treated with forskolin. These data suggest that treating melan-a cells with compound A7 results in a statistically significant increase in the activity of tyrosinase, a known participant in the canonical melanin biosynthetic pathway.

At least two models explain an A7-dependent increase in tyrosinase activity. In one model, treatment with compound A7 has no effect on the cellular level of tyrosinase, but does result in increased catalytic activity of tyrosinase. For example, a direct or allosteric interaction between compound A7 and tyrosinase, as well as an interaction between compound A7 and a member of the canonical melanin biosynthetic pathway upstream of tyrosinase, could influence the catalytic activity of tyrosinase. In another model, treatment with compound A7 does not influence the catalytic activity of tyrosinase, but does result in the overexpression of tyrosinase. Overexpression of tyrosinase would result in an increase of tyrosinase-dependent oxidation of tyrosine, resulting in higher cellular levels of melanin.

In an effort to provide support for one of these models, the cellular level of tyrosinase was measured in melan-a melanocytes treated with growth media containing either vehicle control or 2.5 μ M A7. 1×10^5 melan-a melanocytes were plated in a 12-well tissue culture plate and allowed to stably adhere for 18 hours at 37 °C under 5% CO₂. Cells were then treated with growth media containing either vehicle control or 2.5 μ M A7, and cultured for 72 hours. After such time, growth media was removed and cells were washed three times with PBS. Cells were then lysed in RIPA buffer containing protease inhibitor cocktail (CalBioChem) and tyrosinase levels were measured by Western blot and normalized to β -actin. Cells treated with vehicle control or A7 were found to have similar cellular levels of

tyrosinase (Figure 8 B and C). These data are consistent with a model in which treatment with compound A7 in melanocytes has no effect on tyrosinase expression, but does increase the activity of tyrosinase through mechanisms that are currently unknown.

The production of melanin in melanocytes is a critical defense mechanism to combat genome instability and disease, as a result of chronic exposure to UV-radiation.^[2] Small molecules that increase the cellular level of melanin in melanocytes therefore have significant therapeutic potential, and may be used to treat diseases such as albinism. In addition, a number of diseases are the result of hyper-melanogenesis.^[4] Small molecules that upregulate melanin production can be used to study melanogenesis, and have the potential to reveal new therapeutic targets for melanin-related diseases.

In this communication, we described the synthesis of a targeted combinatorial library of functionally diverse amides, and the screening of this library for molecules that alter the cellular level of melanin in melanocytes. From this screen, four compounds were identified that resulted in a 1.5-fold increase in melanin production in melan-a cells, in comparison to cells treated with vehicle control. Treating melan-a melanocytes with the most active molecule identified in our screen, A7, resulted in a 1.8 ± 0.3 -fold increase in the cellular level of melanin, in comparison to cells treated with the vehicle control. An A7 dose-dependent increase in the cellular level of melanin was observed in melan-a cells treated with 0.5 μ M – 5.0 μ M A7, further supporting A7-dependent melanogenesis. In addition to an increase in pigmentation, an increase in the dendricity of melanocytes has been shown to correlate well with melanogenesis.^[11] On average, 69% of melan-a cells treated with 2.5 μ M A7 had > 2 dendrites per cell, whereas 36% of melan-a cells treated with vehicle control had > 2 dendrites per cell. Tyrosinase-catalyzed oxidation of L-tyrosine to L-DOPA is a well studied, and critical step in melanin biosynthesis. As a starting point to examine the mechanism of A7-dependent melanogenesis, we examined the effect compound A7 has on the catalytic activity of tyrosinase, as well as cellular levels of tyrosinase. On average, treating melan-a cells with 2.5 μ M A7 increased tyrosinase-catalyzed oxidation of L-tyrosine-3,5-^{[3]H} by 35%, in comparison to vehicle control. Conversely, treating melan-a cells with 2.5 μ M A7 did not result in a significant change in the cellular level of tyrosinase.

Taken together, our data show that compound A7 is a novel and potent activator of melanogenesis in melan-a melanocytes, and supports a model in which compound A7 acts in a way that increases the tyrosinase-dependent oxidation of tyrosine, resulting in higher cellular levels of melanin. A7 is simply prepared in a two-step synthesis, and has significant potential as a tool to study melanogenesis, as well as mechanisms by which tyrosinase activity can be increased in a small molecule-dependent manner. Future studies examining the role A7 plays in melanogenesis and tyrosinase activity, as well as structure activity relationships centered on A7 are currently underway, and will be reported in due course.

Experimental

Library synthesis

Carboxylic acid **A** was prepared using a previously reported method.^[15] Carboxylic acids **A–E** (1.0 eq.) were individually placed into a 5 mL glass vial, and dissolved with 1 mL anhydrous DCM. To this solution was added oxalyl chloride (3.0 eq), and catalytic DMF (0.01 eq). Evolution of gas was observed following addition of DMF. The reaction vial was capped with a rubber septum and stirred at 25 °C under a nitrogen atmosphere for 18 hours. After such time, solvent was removed under reduced pressure. Acid chlorides were re-dissolved in 7.0 mL of anhydrous DCM, and 0.5 mL of each acid chloride solution was individually placed into a 5 mL glass vial. To each reaction was added triethethyl amine (1.05 eq), DMAP (0.05 eq), and one of the amine building blocks (**1–15**, 1.05 eq). Vigorous

evolution of gas was observed upon addition of the amine building block. The reaction vial was capped with a rubber septum and stirred at 25 °C under a nitrogen atmosphere for 36 hours. Crude reaction mixtures were passed through a plug of silica gel, eluted with 1:1 hexanes:ethyl acetate, and dried under a stream of nitrogen. Remaining solvent was removed under freeze drying. The purity of each compound was determined by HPLC on a Shimadzu LC-2010A chromatograph using a Shim-pack CLC-ODS-(M) C₁₈ column with a detector setting of 254 nm. Structures were confirmed by ESI-MS. The most effective compound identified in our screen, A7, was further characterized by ¹H NMR, ¹³C NMR, IR, and HRMS (supplemental information).

Compound A7

¹H NMR (400 MHz CDCl₃)—8.01 (1H, d, J = 10 Hz); 7.96 (2H, s); 7.72 (2H, t, J = 7.5 Hz); 7.51 (1H, t, J = 7.5 Hz); 7.30 (1H, q, J = 10.5 Hz); 7.05 (1H, d, J = 7.5 Hz); 6.99-6.94 (2H, m); 5.94 (1H, app t); 3.77 (2H, q, J = 10 Hz); 3.09 (2H, q, J = 10 Hz); 2.993 (2H, t, J = 6.5 Hz); 1.35 (3H, t, J = 7.5 Hz); **¹³C NMR (400 MHz CDCl₃)**: 8168.8, 164.0, 162.0, 161.1, 148.0, 141.1, 141.0, 134.5, 130.5, 130.3, 129.9, 128.8, 127.6, 126.5, 125.5, 124.4, 124.4, 115.7, 115.6, 113.7, 113.6, 40.8, 35.3, 29.9, 13.9; **FTIR (Neat)**: 3282, 3069, 2979, 2931, 1649, 1585, 1540, 1485, 1451, 1439, 1318, 1296, 1256, 1238, 1205, 1143, 1188, 1050, 922, 878 cm⁻¹; HRMS Calculated for (M⁺H) 323.1560, found 323.1554.

Cell culture—melan-a cells^[16] were obtained from the Wellcome Trust Functional Genomics Cell Bank, and were cultured in RPMI 1640 medium (Sigma) with 10% FBS, 2 mM glutamine, 5 I.U. penicillin, and 5 µg/mL streptomycin. All cells were cultured at 37 °C with 5% CO₂.

Tyrosinase activity

Tyrosinase activity was determined using a previously reported method.¹⁴ melan-a cell pellets were lysed in 75 µl of tyrosinase harvesting buffer (80 mM K₂PO₄, 10% CHAPS, 0.5 mM PMSF, 5 µg/ml aprotinin, pH 6.8) for 2 hours on ice and spun at 13,000 rpm for 15 minutes at 4 °C to pellet cellular debris. Total protein in the soluble lysate was quantified via Bradford assay (BioRad). In quadruplicate, 5 µg of total protein was transferred to a 2.0 ml tube containing 250 µl of 80 mM K₂PO₄ pH 6.8. To one of the tubes serving as the boiled control, 2 µl of 2-mercaptoethanol was added and this sample was boiled for 30 minutes. To each tube, 20 µl of tyrosinase assay solution (3.375 µM L-tyrosine, 337.5 µM 3,4-Dihydroxy-L-phenylalanine, 0.7 µCi [³H]-tyrosine (Perkin Elmer, Boston, MA, 5uL of stock from supplier), 40 mM K₂PO₄, pH 6.8) was added and let incubate at 37°C for 1 hour. Then, 375 µl of 0.2% BSA, followed by 375 µl trichloroacetic acid was added to each tube. After addition of trichloroacetic acid, proteins precipitated and were spun at 13,000 rpm for 10 minutes. 750 µl of the cleared supernatant was transferred to a 2.0 ml tube containing 500 µl of a prewashed 33 % charcoal slurry in 40 mM K₂PO₄, pH 6.8 and nutated for 1 hour at 23 °C. Charcoal was then pelleted by spinning at 13,000 rpm for 15 minutes. 1.0 ml of the resulting supernatant was transferred to scintillation vials filled with 5 ml of scintillation fluid, and [³H] levels were counted.

Western blot—Melan-a cells were washed once with 4° C PBS 72 hours after treatment. Cells were lysed with 200 µL RIPA buffer (Boston Bioproducts) containing a protease inhibitor cocktail (CalBioChem) for 15 minutes. The resulting cell lysate was resolved by SDS-PAGE on a 10% acrylamide gel (Invitrogen). The proteins on the gel were transferred by electroblotting onto a nitrocellulose membrane (BioRad) and membranes were blocked in 5% milk for 1 hour. Membranes were then incubated in primary antibody against tyrosinase protein (Zymed Co., San Francisco, CA, T311; mouse monoclonal) and b-actin (Abcam, Boston, MA; mouse polyclonal) in 5% milk overnight at 4° C. The membrane was

washed three times with PBS and treated with secondary antibody (HRP goat anti-mouse IgG (Sigma Co.) in blocking buffer (Li-COR Biosciences) for 30 minutes. The membrane was washed three times with 50 mM Tris, pH 7.4 containing 150 mM NaCl and 0.05% Tween-20. Visualization of immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL). Densitometry was performed using Image J. Representative data are shown in Figure 8.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Forskolin, IBMX, and melanogenin: Compounds known to increase the cellular level of melanin in melanocytes.

Figure 2.

Synthesis of a targeted 75 member combinatorial library from functionally diverse carboxylic acid and amine building blocks. Carboxylic acids (A-E) are converted to acid chloride by treatment with oxalyl chloride and catalytic DMF, followed by reaction with an amine building block (1–15) to generate mature library members. All compounds used in screening were 90% pure by HPLC, and stored as 25 mM solutions in DMSO

Figure 3.

Fold change in melanin and viable cell count in melan-a cells 72 hours after treatment with vehicle control (0.1% DMSO), 2.5 μ M forskolin, or 2.5 μ M of library member. Cellular level of melanin was determined by ($Abs_{490}/10^6$ cells) and is represented by dashed bars. Cytotoxicity was determined by hemocytometry and is shown by solid bars. Values are normalized to treatment with vehicle control (0.1% DMSO). In each sample, cells demonstrated very low levels of small molecule-dependent cytotoxicity; however, cells treated with the positive control (forskolin) and library members A5, A7, C1, and C5 resulted in a 1.5-fold increase in melanin. Values and error bars represent the mean and $\pm$ SD of three independent experiments. The structures of the most active compounds identified in the screen are shown (above).

Figure 4.

Concentration-dependent increase in A7-dependent melanin content. Fold change in melanin was determined by ($A_{490}/10^6$ cells) 72 hours after treatment with 0.5, 1.0, 2.5, or 5.0 μ M A7 and is represented by dashed bars. Fold change in cytotoxicity was measured by hemocytometry 72 hours after treatment with 0.5, 1.0, 2.5, or 5.0 μ M A7, and is represented as solid bars. Values are normalized to treatment with a vehicle control (0.1% DMSO). An A7 concentration-dependent change in melanin is observed. In contrast, cytotoxicity was uniformly low in the concentrations tested. Values and error bars represent the mean and SD of three independent experiments.

Figure 5.

Brightfield microscopy images of melan-a cells 72 hours after treatment with either vehicle control (0.1% DMSO) or 2.5 μM A7. Cells treated with vehicle control are non-pigmented whereas cells treated with A7 are pigmented, and contain a large number of dark punctate foci, consistent with high levels of melanin in melanosomes. Exposure time = 10 msec. Scale bar = 100 μm .

Figure 6.

A7-dependent changes in the dendricity of melan-a cells. (A) Melan-a melanocytes imaged 72 hours after treatment with either vehicle control (0.1% DMSO) or 2.5 μ M A7. The nucleus of each cell is stained with hematoxylin, a representative sample of cells are shown. Scale bar = 50 μ m. (B) Percentage of cells with > 2 dendrites 72 hours after treatment with vehicle control (0.1% DMSO) or 2.5 μ M A7. Treatment with 2.5 μ M A7 results in ~ 2-fold more cells with > 2 dendrites. Values and error bars represent the mean and $\pm$ SD of three independent experiments.

Figure 7. Tyrosinase catalyzed oxidation of L-tyrosine-3,5-[³H] to generate L-DOPA and ³H₂O.

Figure 8.

(A) Tyrosinase activity in melan-a cells treated with vehicle control (0.1% DMSO), 2.5 μM A7, or 2.5 μM forskolin for 72 hours. Absolute tyrosinase activity levels is plotted. In comparison to treatment with DMSO, treatment with A7 and forskolin result in a 35% and 65% increase in tyrosinase activity, respectively. (B) Densitometry analysis of cellular level of tyrosinase protein in melan-a cells treated with 2.5 μM A7 or vehicle control (0.1% DMSO). (C) Representative Western blot of melan-a cells treated with either 2.5 μM A7 or vehicle control (0.1% DMSO). In (A) and (B), values and error bars represent the mean and $\pm$ SD of four and three independent experiments, respectively.