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High Throughput, High Content Screening for Novel Pigmentation Regulators Using a Keratinocyte/Melanocyte Co-culture System

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Abstract

Skin pigmentation is a complex process including melanogenesis within melanocytes and melanin transfer to the keratinocytes. To develop a comprehensive screening method for novel pigmentation regulators, we used immortalized melanocytes and keratinocytes in co-culture to screen large numbers of compounds. High-throughput screening plates were subjected to digital automated microscopy to quantify the pigmentation via brightfield microscopy. Compounds with pigment suppression were secondarily tested for their effects on expression of MITF and several pigment regulatory genes, and further validated in terms of non-toxicity to keratinocytes/melanocytes and dose dependent activity. The results demonstrate a high-throughput, high-content screening approach, which is applicable to the analysis of large chemical libraries using a co-culture system. We identified candidate pigmentation inhibitors from 4,000 screened compounds including zoxazolamine, 3-methoxycatechol, and alpha-mangostin, which were also shown to modulate expression of MITF and several key pigmentation factors, and are worthy of further evaluation for potential translation to clinical use.

Keywords

pigmentation; high content screening; co-culture; microphthalmia transcription factor; melanocyte; keratinocyte

INTRODUCTION

Pigment synthesis occurs within the epidermal melanin unit, a system which relates to the density of melanocytes among keratinocytes, the number, type, size and transfer of melanosomes and their degradation rate (1-2). Pathologic hyperpigmentation is a common and undesirable cutaneous disorder. Various agents have been used for the treatment of hyperpigmentation or esthetic purposes (3). Currently utilized pigmentation inhibitors

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including corticosteroids and tyrosinase inhibitors may be clinically effective, but may also be associated with certain toxicities (4). The development of high-throughput, high-content screening approaches may aid in the identification of additional pigment-modulating agents. Several methods have been commonly utilized for screening libraries of natural extracts as well as new synthetic entities that typically target the melanogenesis pathway. These screens commonly utilize melanocyte or melanoma cell culture systems (5).

Keratinocytes modulate melanocyte function including pigment formation (6-8). Recently, a luciferase reporter assay was used for pigmentation screening based upon measurements of Tyrosinase promoter activity (9). Reconstructed pigmented skin equivalents also have been used (10) which have advantages in assessing an integrated pigmentation phenomenon, but are difficult to use in high-throughput or high-content screening due to cost, time, and effort-consuming procedures. Lei et al. have developed an assay comprised of a co-culture of immortalized mouse melanocytes and keratinocytes (11). This assay was performed in 6-well plates, and there were no obvious barriers to scaling the assays into 96-well format for high-throughput screening.

To develop high-content screening for pigmentation regulators, a co-culture system was optimized to provide a broad physiologic context in which to identify chemical modulators of pigmentation in a time and effort-efficient manner. We present a model system that uses immortalized melanocytes and keratinocytes in a co-culture assay that provides a read-out of pigmentation, which encompasses keratinocyte signals to melanocyte pigment machinery, and actual melanin content.

MATERIALS AND METHODS

Compounds for screening

A total of 4,000 compounds were screened. 2000 screened compounds were derived from the Spectrum (MicroSource, Gaylordsville, CT) library (drug components (60%), natural products and other bioactive components (40%) <http://www.msdiscovery.com/spectrum.html>). Another 2000 compounds from the ChemBridge CNS library (ChemBridge Corp., San Diego, CA) were selected for chemical diversity and biased toward increased lipophilic properties (http://www.chembridge.com/screening_libraries/targeted_libraries/index.php#CNS-Set).

All compounds were dissolved in dimethyl sulfoxide (DMSO) and were compared to DMSO (vehicle) control. Resveratrol was examined both in crude form (5%, Oriza Yuka Co. Ltd, Japan) and in purified form (All-trans, 99%, DSM Co. Ltd, Switzerland).

Cell culture and Compound treatment

Murine SP1 keratinocytes and melan-A melanocytes were grown in high glucose DMEM (HEPES, 10mM; fetal calf serum, 10%; penicillin, 100,000 U/L; streptomycin sulphate, 100 mg/L; Glutamax, 100×2) (Life Technologies, Grand Island, NY) and RPMI 1640 (Fetal calf serum, 10%; penicillin, 100 000 U/L; streptomycin sulphate, 100 mg/L; Glutamax, 100×; TPA, 200 nM (for melanocyte proliferation and differentiation) (Life Technologies, Grand Island, NY). SP1 cells and Melan-A cells were passaged at 2×10^4 cells/ml and 3×10^4 cells/ml every 3-5 days.

On day one, 20,000 cells/well of SP1 keratinocytes were plated into 100 ul/well in 96-well clear bottom plates (Corning, Tewksbury, MA) and then incubated at 37°C for 48 hours. On day 3, culture media was removed and Mitomycin C, 8 ug/ml solution in SP1 media was added in the amount of 40 ul/well using MatrixWellMate (ThermoScientific Inc, Waltham, MA) to arrest keratinocyte proliferation and diminish background noise in the high

throughput assay. After incubation for 3h, Melan-A cell suspension, 20,000 cells/ml in SP1 growth media was dispensed at 100 μ l/well and incubated for 24 hours. On day four, 200 μ l of each test compound was pin-transferred and incubated for 48 hours. On day 6, media was removed and the cells were fixed with cold 1:1 acetone and methanol 100 μ l/well. The plates were incubated at -20°C for 15 minutes to complete fixation. The co-culture plates were air-dried and stored at room temperature before imaging.

For other experiments, B16-F10 murine melanoma cells were cultured in RPMI-1640 medium (Fetal calf serum, 10%; penicillin, 100 000 U/L; streptomycin sulphate, 100 mg/L; Glutamax, 100x; TPA, 200 nM) and incubated in 5% humidified, 5% CO_2 , at 37°C .

High-throughput, high-content co-culture assay development

The analysis of 4,000 compounds was initiated with optimization of the co-culture system and its adaptation to a 96-well plate format. Several parameters including density, keratinocyte/melanocyte ratio, cell feeding frequency, and mitogen concentration were optimized in a series of initial experiments. To select the proper media for the co-culture, SP1 growth media, RPMI-based, and standard keratinocyte medium (SKM) low calcium media (Mediatech Inc, Manassas, VA), and Keratinocyte-serum free medium (SFM) (LifeTechnologies, Woburn, MA) were tested. Pigmentation was observed to increase in a dose-dependent manner proportional to keratinocyte density. A keratinocyte-to-melanocyte ratio of 10:1 cultured in the DMEM-based SP1 culture media and 20,000/well density of keratinocyte plating resulted in the optimal induction of pigmentation (data not shown). The co-cultures produced melanocytes with dendritic morphology and visible pigmentation (under brightfield microscopy) with melanosomes. The optimal cell-feeding interval was 48 hours. Further optimizations were also explored, including reduction of media volume from 200 μ l to 50 μ l for the final two days (day 5-6) of co-culture. Assay development steps resulted in the set-up of a robust co-culture screening system in 96-well plate format suitable for automated image-based analysis (Fig. 1a).

Image analysis

Images were obtained by automated microscopy (Olympus, automated stage, ZDC autofocus, Tokyo, Japan) and were analyzed by Metamorph Premier software (Molecular Device LLC, Sunnyvale, CA). First, the backgrounds (keratinocytes-only) were subtracted from each image and thresholds were determined manually, to detect pigmented areas using forskolin (FSK) (pro-pigmentation) and phenylthionuria (PTU) (anti-pigmentation) controls.

Toxicology assay

Cytotoxicity was assessed using the AlamarBlue (AbD Serotec, Raleigh, NC) viability assay per manufacturer's protocol as fluorescence at 530 nm (Ex590 nm), measured by a Victor X3 (Perkin Elmer) plate reader.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated using QIAGEN TurboCapture (Gaithersburg, MD) and reverse transcribed using the Applied Biosystems RT-PCR kits (Life Technologies, Grand Island, NY). qRT-PCR was carried out in 25 μ l reaction (12.5 μ l 2 $\times$ SYBR Green master mix (Bio-Rad), 0.25 μ l reverse transcriptase, 1 μ l of each primer (10 μ M stock) and 100 ng of total RNA. Reverse transcription was carried out at 48°C for 30 min followed by 40 cycles of PCR at 95°C for 15 seconds and 60°C for 30 seconds. Data were acquired and analyzed with 7500 Fast System SDS software (Life Technologies, Grand Island, NY). PCR primers are listed in Table S1.

NaOH lysis assay

Following in vitro treatments, B16 melanoma cells were lysed by treatment with 50 μ l/well of 1M NaOH, incubation for 3h at 70°C, cooling to room temperature and melanin absorbance at 405 nm was measured.

RESULTS

Assay system validation and primary hit selection

To validate the co-culture system, we used previously known chemical modulators of pigmentation: PTU 1 mM as a pigmentation inhibitor, and FSK 20 μ M as inducer of pigmentation. At 20 hours post-initiation of the co-culture, the media was replaced with fresh DMEM growth media containing either PTU or FSK or DMSO control. Image analysis was performed based on a pixel-cutoff method and showed robust differences between the representative compounds (Fig. 1b). The cutoff value was determined based on “keratinocyte-only” images that were taken under the same conditions. Any pixel that showed more than five standard deviations darker than the average in keratinocyte-only background was scored as “pigmented”. Z scores were calculated as the differences of populations and significant differences were between the treatments over 2σ (σ = standard deviation). To eliminate possible artifacts due to differences in numbers of plated cells or toxic effects of compounds, we calculated the ratio of pigmented area to total coverage. Wells with below 20% of the well’s surface area were regarded to be “dead” and excluded from further analysis. A total of 25% of all 4,000 compounds were excluded based on these criteria. Images with above 80 percent cell coverage were regarded to be out of focus and the images were retaken at different fields of the sample. Individual wells were normalized to DMSO control and average values of the single high throughput screening experiment with 2 duplicates were calculated. Table S2 represents the top hits with greater than 50% inhibition of pigmentation.

Secondary hit selection based on toxicity

Although visibly toxic compounds were excluded from the analysis, cell lines are capable of developing mechanisms to survive in tissue culture conditions. Therefore we included a secondary viability assay in primary human keratinocytes and melanocytes to ensure that general toxicity does not create a false positive whitening signal among the top hits. To this end primary normal human keratinocytes or melanocytes were treated with the top 34 hit compounds (< 30% pigmentation activity) at 10 μ M and 20 μ M concentrations for 48 hours. Twenty two of these compounds did not exhibit toxic effects even at the 20 μ M dose (Table S2).

Secondary hit selection based on a dose-dependent response to pigmentation inhibition

To characterize the positive hits, we tested dose responses for inhibition of pigmentation for the top 19 non-toxic compounds. “Cherry pick” plates (i.e., automated retrieval of specific compounds from the chemical libraries) with three different concentrations (2.5, 5, 10 μ M) of each compound were prepared. Out of 19 test compounds, 3-Methoxycatechol, Canrenone, Carapin, Tetracaine, 7925483, Trazodone, Carnosic acid, Oxydopamine, α -Mangostin, and Zoxazolamine showed dose-dependent inhibition of pigmentation in the keratinocyte-melanocyte co-culture system (Fig. S1a). To compare with single melanocyte cultures, B16 melanoma cells were treated with the same concentrations of compounds to test the ability to suppress forskolin-induced hyperpigmentation. Only 3-Methoxycatechol, Carnosic acid, and α -Mangostin suppressed pigmentation under basal conditions and to a higher extent upon FSK-induced pigmentation (Fig. S1b).

Expression of MITF, TYR, TRP1, and TRP2 in primary melanocytes treated with top-hit compounds

To investigate effects of the compounds on the pigment pathway, the expression patterns of MITF, TRP1, TRP2 and TYR mRNAs were studied using qRT-PCR. (Fig. 2a-d). Alpha-mangostin, 3-Methoxycatechol, and Zoxazolamine demonstrated dose-dependent and statistically significant down-regulation of MITF while Tetracaine, Trazodone, Canrenone, Oxydopamine showed effects at certain doses. The other pigmentation genes were affected by certain compounds as well.

Extended dose response analysis of primary hits

In the initial dose response experiments using the top 19 hits from the primary screen, 9 compounds failed to show dose dependent effects. Further analysis of the literature revealed that two of these compounds, Isoproterenol and resveratrol were identified as modulators of pigmentation. Isoproterenol-HCl (12) was used in doses similar to the ones used in our experiments. The former effects of resveratrol (13) were detected at 10 μ M and higher concentrations. Therefore we extended our initial experiments and explored the effects of resveratrol up to 150 μ g/ml with crude extract and a more purified preparation. In agreement with previous studies (13-14), we observed a suppression of pigmentation in the co-culture system at high dose of purified resveratrol (Fig. 3a). The suppression of pigment accumulation using the purified form of resveratrol was not related to cellular toxicity, while the crude extract was cytotoxic at higher concentrations (Fig 3b).

DISCUSSION

High-throughput screening approaches for the identification of pigmentation inhibitors have not been widely explored, with the exception of tyrosinase targeting assays (15-17). A melanocyte-keratinocyte co-culture system has been reported to offer advantages of mimicking a more physiologic induction of pigmentation due to the major role of keratinocytes in modulating melanocyte proliferation and differentiation (18). Keratinocyte-secreted soluble factors can determine the dendricity and cell-cell contacts of melanocytes as well as distribution patterns of melanosomes that can contribute to pigmentation (19-21). The process of melanosome transfer has been extensively studied (2, 22-25) and the importance of a co-culture system has been raised. *In vitro* co-culture systems were used to validate melanin transfer and to observe the effects on the pigmentation (26-29). Moreover, tyrosinase, TRP-1, and TRP-2 which are key eumelanogenesis enzymes, are induced under co-culture conditions containing keratinocytes and melanocytes (30). For the mechanism of retinoic acid's inhibition of melanogenesis, cellular retinoic acid binding protein-1 of melanocytes is thought to be influenced by keratinocytes (31). As one of the regulatory factors of pigmentation, activation or inhibition of protease-activated receptor 2 in keratinocytes also has been suggested to modulate a key keratinocyte-melanocyte interaction required for pigment transfer (32). Keratinocytes participate in additional crosstalk with melanocytes (33-35). UV induced DNA damage in keratinocytes results in p53-mediated upregulation of Melanocyte Stimulating Hormone, which stimulates melanocytic cyclic AMP and pigment synthesis (8).

A number of hit-compounds exhibited little or no effect on expression of melanogenesis related genes, suggesting that their primary targets might be post-transcriptional suppression of pigmentation machinery rather than affecting expression of genes involved in melanogenesis. We used immortalized murine cell lines for the co-culture instead of human primary keratinocytes and melanocytes due to greater ease and reproducibility of *in vitro* culturing, as compared to donor variability of human primary cultures (33) (although human primary melanocytes were utilized for followup validation studies). Importantly, the

reported assay integrated measurement of cell viability and excluded from further analysis compounds whose toxicity reduced cellular adherence to the tissue culture well. These efforts were further expanded by the measurement of toxicities in primary human melanocytes and keratinocytes at higher doses, for top hits from the primary assay. In previously published studies using a co-culture assay, melanin content was measured by NaOH lysis and absorbance (11) or spectrophotometric analysis (27, 28). These methods may be less suitable for high-throughput scale-up due to the fact that smaller well sizes will reflect lower amounts of melanin, which may fall below detection sensitivity. We therefore utilized this assay for reconfirmation of our primary hits. In this assay, we observed a higher sensitivity of the co-culture assay.

Among the hits from the screen reported here, Resveratrol and Miconazole, have been previously demonstrated to inhibit pigmentation and tyrosinase activity in B16 cells (14, 36). The identification of previously known agents as pigmentation inhibitors provided validation of the current screening approach. Alpha-mangostin is reported to have a role in inhibition of lipoprotein oxidation (37), and methoxycathechol inhibits carcinogenesis in the rat (38), however, their roles in suppression of pigmentation have not been previously elucidated. Carapin was isolated from the Brazilian Hard-Nut tree, *Carapa Guianensis* and has been used in Brazilian traditional medicine for anti-inflammatory, anti-allergic purposes although no evidence regarding regulation of pigmentation was found in the literature (39). Of course, it is always important to consider the possibility of unpredicted effects, such as the described role of forskolin in producing pigment-independent epidermal thickening (40).

Taken together, we executed a high-throughput, high-content image-based screen with 4,000 individual compounds and found several previously described agents (carnosic acid, resveratrol and miconazole) as well as novel modulators of melanin accumulation. The clinical relevance and utility of these agents will be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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JH Lee, IH Jung, V. Kolev, K.H. Aull, H. Chen, J. Wang, S. Miyamoto, J. Hosoi performed the research, JH Lee and A. Mandinova analyzed the data and wrote the paper, DE. Fisher conceived and designed the research study and wrote the paper.

Abbreviations

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FSK	forskolin
MITF	microphthalmia transcription factor
PTU	phenylthiourea
SKM	standard keratinocyte medium

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Figure 1.

(a) Schematic flowchart for high-throughput, high-content screening for pigmentation regulators. On day 1, SP-1 keratinocytes were plated in 96-well clear bottom plates and incubated for 48 hours. On day 3, after the treatment of 8 $\mu\text{g/ml}$ mitomycin for 3 hours, Melan-A cells were plated. On day 4, 4,000 individual compounds were pin transferred to each wells in duplicates. On day 6, growth media were removed and fixed with fixer solution. Auto-imaging and pixel cutoff analysis were performed to get the primary hits. (b) Representative images and histograms for DMSO (neutral control), forskolin (darkening control) and phenylthiourea (PTU, whitening control). Panel 'a' shows brightfield images. Red color indicates threshold pixels (b panel). Histograms and respective threshold pixel percentage are shown (c panel). In the co-culture system, cells were grown for 72 hours total, preserved by drying in 1:1 acetone:methanol, and imaged under brightfield at 25X magnification.

Figure 2.

Expression of melanin regulating genes MITF, TYR, TRP-1, and TRP-2 in primary human melanocytes. Primary human melanocytes were treated with different concentrations (0, 5, 10 uM) of the top hit compounds. The relative expression level of pigmentation related genes was determined by quantitative RT-PCR at 24 hours. LRP-11 was used as loading control and all values were normalized to LRP-11. 3-Methoxycatechol, α -Mangostin and Zoxazolamine showed dose-dependent suppression of MITF. (a) 3-Methoxycatechol and Zoxazolamine down-regulated MITF expression in a dose-dependent manner. Tetracaine, Oxydopamine, and α -Mangostin showed MITF gene down-regulation at certain concentrations. (b) 3-Methoxycatechol, Canrenone, Tetracaine, Trazodone, and α -Mangostin down-regulated TYR gene expression. (c) TRP1 gene was down-regulated by 3-Methoxycatechol, Carnosic acid, α -Mangostin and Zoxazolamine. (d) TRP2 mRNA was down-regulated by Canrenone, Tetracaine, Trazodone, Oxydopamine, and α -Mangostin. The values shown represent mean and standard errors from quadruple replicates. An asterisk

indicates statistically significant down-regulation with p value <0.05 . A double asterisk indicates statistically significant up-regulation with p value <0.05 . (ANOVA test)

Figure 3.

Dose-dependent inhibition of melanin production by resveratrol. (a) The assay was performed using higher doses of resveratrol in a co-culture system of mitomycin treated SP-1 keratinocytes and Melan-A melanocytes. 10 to 150 $\mu\text{g/ml}$ of crude vs. purified resveratrol were compared. PTU and forskolin were used as positive and negative controls respectively. (b) Toxicity test of crude and purified resveratrol in co-culture assays. The values represent the mean and standard errors from quadruple replicates. An asterisk indicates statistically significant down-regulation with p value < 0.05 . (Student T-test)