

Research Article

Tyrosinase Inhibitors with Potent Anti-Senescence Activity in Human Neonatal Keratinocyte Progenitors

Anan Abu Ubeid and Basil M Hantash*

Escape Therapeutics, Inc. 3800 Geer Road, Suite 200, Turlock, CA 95382, USA.

*Corresponding Author: Basil M Hantash, Escape Therapeutics, Inc. 3800 Geer Road, Suite 200, Turlock, CA 95382, USA. E-mail: basil@escapetherapeutics.com

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Abstract

Background: Sirtuins exhibit pleiotropic effects on premature aging, cellular senescence, longevity, and a wide range of aging disorders.

Aim: We assessed effects of decapeptide-12, a known tyrosinase inhibitor, on sirtuin gene expression levels in human neonatal keratinocyte progenitors.

Methods: We quantitated effects of decapeptide-12 on 7 sirtuin genes using RT-PCR and its effects on cellular viability and proliferation after 72-h incubation with various concentrations of decapeptide-12 and oxyresveratrol.

Results: 100 μ M decapeptide-12 increased transcription of *SIRT1* by $141 \pm 11\%$ relative to control cells, whereas levels of *SIRT3*, *SIRT6*, and *SIRT7* were increased by $121 \pm 13\%$, $147 \pm 8\%$ and $95 \pm 14\%$, respectively.

Conclusion: Decapeptide-12 upregulated sirtuin transcription to similar levels as oxyresveratrol but with reduced cytotoxicity.

Keywords

Anti-senescence, anti-aging proteins, decapeptide-12, hypopigmentation, oxyresveratrol, sirtuins, tyrosinase inhibitors, anti-inflammation

Introduction

Skin manifests the consequences of chronological and photoaging rendering us constantly aware of the aging process and seeking remedies to slow or reverse its impact. Skin aging has traditionally been categorized as extrinsic or intrinsic [1,2]. Recent evidence indicates that both types are characterized by connective tissue damage [3], reduced procollagen synthesis, and enhanced matrix metalloproteinase expression [4].

Aging of human skin results in diminished cellular proliferation and differentiation and increased senescent cells [5-7]. These alterations result from free radical damage by various native reactive oxygen species (ROS) [8]. *In vivo* data further supports this theory as linkage between skin aging and cellular senescence were shown to result from mitochondrial oxidative damage [9]. Furthermore, mutagenesis and photoaging resulted from ROS triggered by ultraviolet (UV) radiation [10,11]. Consistent with these data, UV exposure also altered sirtuin expression supporting the importance of sirtuins in skin aging [12,13].

Cellular senescence involves arrest of cell division with resultant specific phenotypic changes such as altered chromatin and secretome coupled with tumor-suppressor activation [14-16]. Numerous reports helped establish sirtuins as potent anti-aging proteins orchestrated via modulating pathways involving telomere length, DNA repair, and oxidative stress [17-23].

Seven sirtuin genes (*SIRT1-7*) are present in mammals, each with unique localization and activity [24,25]. Sirtuins maintain NAD^+ -dependent lysine deacetylase activity [18,26] and serve as important metabolic regulators, cellular energy and redox sensors, and oxidative stress modulators [27,28].

These findings have triggered interest in developing small molecule activators or pharmaceuticals to help slow the progression of aging and its wide range of age-associated disorders. The effects of *SIRT1* on aging and longevity have been thoroughly studied. For instance, resveratrol, a well-known anti-aging nutraceutical, derives its anti-aging effects via *SIRT1* activation [29,30] and up-regulation of AMP-activated protein kinase activity to diminish cellular senescence and proliferative dysfunction [31].

We have previously reported on decapeptide-12's potent hypopigmenting efficacy in human skin [32]. Further clinical studies revealed an overall improvement in facial skin appearance in patients with dyschromia who were treated twice daily with topical cream containing 0.01% decapeptide-12 for 8 weeks [33,34].

These findings led us to hypothesize that decapeptide-12 may modulate sirtuin activity to improve overall skin appearance. To clarify this possibility, we assessed the effects of decapeptide-12 on sirtuin transcription in human neonatal keratinocyte progenitors.

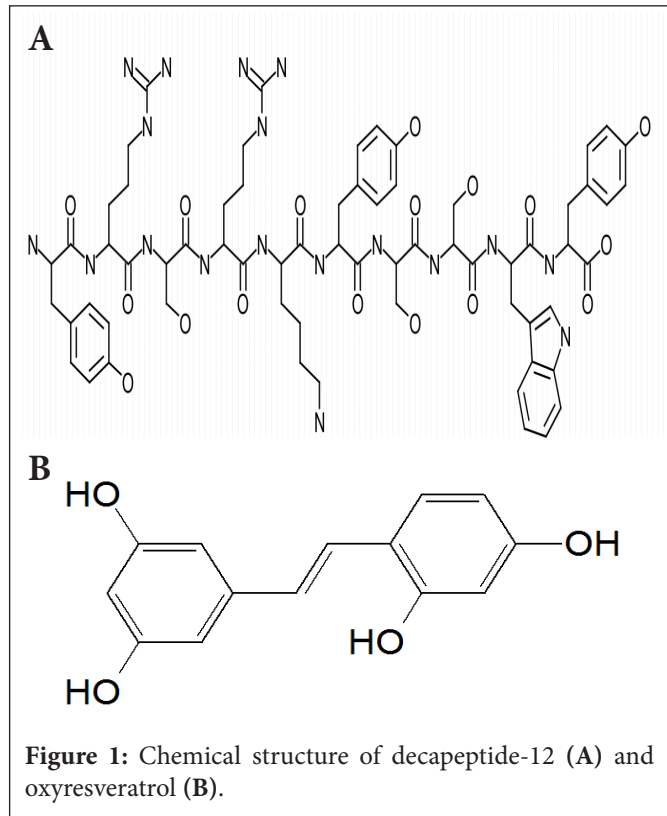


Figure 1: Chemical structure of decapeptide-12 (A) and oxyresveratrol (B).

Materials and methods

Chemicals

Solid-phase Fmoc chemistry was utilized to synthesize decapeptide-12 (YRSRKYSWY; Figure 1A) (Bio Basic, Inc., Ontario, Canada) [32]. Oxyresveratrol (Figure 1B) and all other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell culture

Human neonatal keratinocyte progenitors (Thermo Fisher Scientific, NY) were seeded in 6-well plates at a density of 2×10^5 cells/well. Each well received 2 ml of Epilife media containing 60 μ M calcium chloride (Thermo Fisher Scientific, NY). Plates were incubated in a humidified chamber at 37°C and 5% CO₂. Twenty-four hours later, cells were treated with various concentrations of oxyresveratrol or decapeptide-12 dissolved in PBS containing 5% DMSO. Control wells received vehicle only (5% DMSO and PBS). Final concentration of DMSO in each well was 0.05%.

Total RNA extraction, quantitation, and cDNA synthesis

Gene	Primer sequence (5'-3')
<i>SIRT1</i>	F GCCAATCATAAGATGTTGCTGAAC
	R TAGAGCCTCACATGCAAGCTCTA
<i>SIRT2</i>	F AACCTCCCTCATCTCTAACT
	R GTCTCCAATAAGCAATGTCT
<i>SIRT3</i>	F GTTGGTTACAAGATCCAGAC
	R AGATAGAAAGTGCTGGAATG
<i>SIRT4</i>	F AGAGCTGTGAGAGAATGAAG
	R TTTCTGACCTGTAGTCTGGT
<i>SIRT5</i>	F TCTTCCATACACTTTACTACCTT
	R TTTATATGATAGTGTCTTTGTTGC
<i>SIRT6</i>	F CAGCTTAAACAGGAGTGAAC
	R TTATTGCATTGAGGACTTTT
<i>SIRT7</i>	F GACATTTTTAGCCATTTGTC
	R CATCCAGTACAGAGAGGATT
<i>18S</i>	F CGGAGGTTCGAAGACGATCAGATA
	R TTGGTTTCCCGGAAGCTGCC

Table 1: Primer sequences for *SIRT 1-7* and *18S*.

After a 72-h incubation period, cells were trypsinized and total RNA extracted, using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration was determined using nanodrop (Thermo fisher scientific, NY). Two μ g of total RNA were used to synthesize cDNA using oligo dT primers and TaqMan reverse transcription reagents (Thermo fisher scientific, NY). A DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA) was used to carry out reactions as follows: annealing at 25°C for 10 min, first strand synthesis at 48°C for 1 h, and heat inactivation at 95°C for 5 min.

Semi-quantitative analysis

SIRT1-7 primers (Table 1) were designed using Primer 3 [35]. Semi-quantitative PCR reactions were performed on a DNA Engine Peltier Thermo Cycler (Bio-Rad, Hercules, CA). PCR was carried under the following conditions: denaturation at 94°C for 2 min and primer extension at 54°C for 30 s in 34 cycles for *SIRT 1-7* and the housekeeping gene *18S*.

Samples were run and resolved on a 1.5% agarose gel containing 0.5 μ g/ml of ethidium bromide and imaged using the FluorChem HD2 Imaging System (Protein simple, San Jose, CA). AlphaEase FC software (Protein simple, San Jose, CA) was used to carry out densitometry analysis. Intensity value for each gene divided by the intensity value of the internal control gene *18S* represented the intensity ratio.

Viability/proliferation and cytotoxicity assays

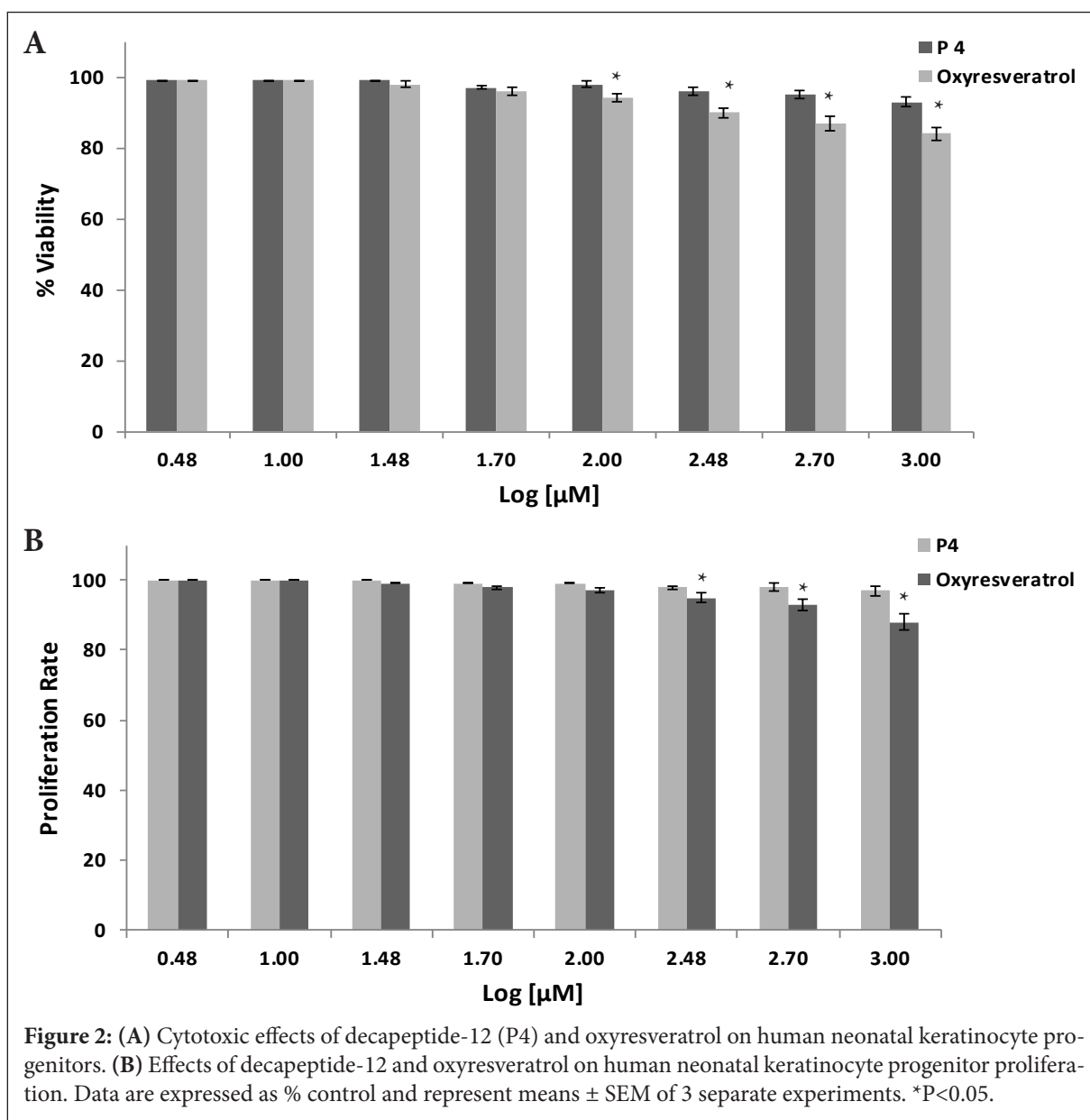
TACS® MTT Cell Proliferation Kit (R&D systems, Minneapolis, MN) was used to determine proliferation rates. Cells were seeded at 2.5×10^4 /well in 96-well plates in a humidified atmosphere with 5% CO₂ at 37°C. Twenty-four hours later, decapeptide-12 or oxyresveratrol was added to the corresponding wells at varying concentrations (0, 3, 10, 30, 100, 300, and 1000 μM), cultures were then incubated for 72 h, and manufacturer's protocol was followed for the remainder of the procedure.

Trypan blue exclusion assay was used to measure cellular toxicity. Cells were cultured in 6-well plates at a density of 4×10^5 cells/well and then treated with various concentrations of decapeptide-12 or oxyresveratrol (0, 3, 10, 30, 100, 300, and 1000 μM). Plates were incubated at 37°C in a humidified 5% CO₂ chamber.

After 72 h, an aliquot was taken, and cells counted using a hemacytometer. Cytotoxicity was measured according to the following formula: $[1 - (\# \text{ of cells in control} - \# \text{ of live cells in test sample})/\# \text{ of cells in control}] \times 100\%$.

NK cytotoxic killing and PBMC proliferation assays

Cryopreserved human peripheral blood mononuclear cells (PBMCs) were purchased from Astarte Biologics (Redmond, WA), activated with phytohemagglutinin (PHA), and proliferation assessed according to previously described method [36]. Interleukin (IL)-2 activated human natural killer (NK) cell cytotoxic killing of human K562 cells was assessed using a CytoTox96 non-radioactive cytotoxicity assay kit (Promega) as previously described [36]. Protease inhibitor was added to the media to prevent degradation of decapeptide-12.



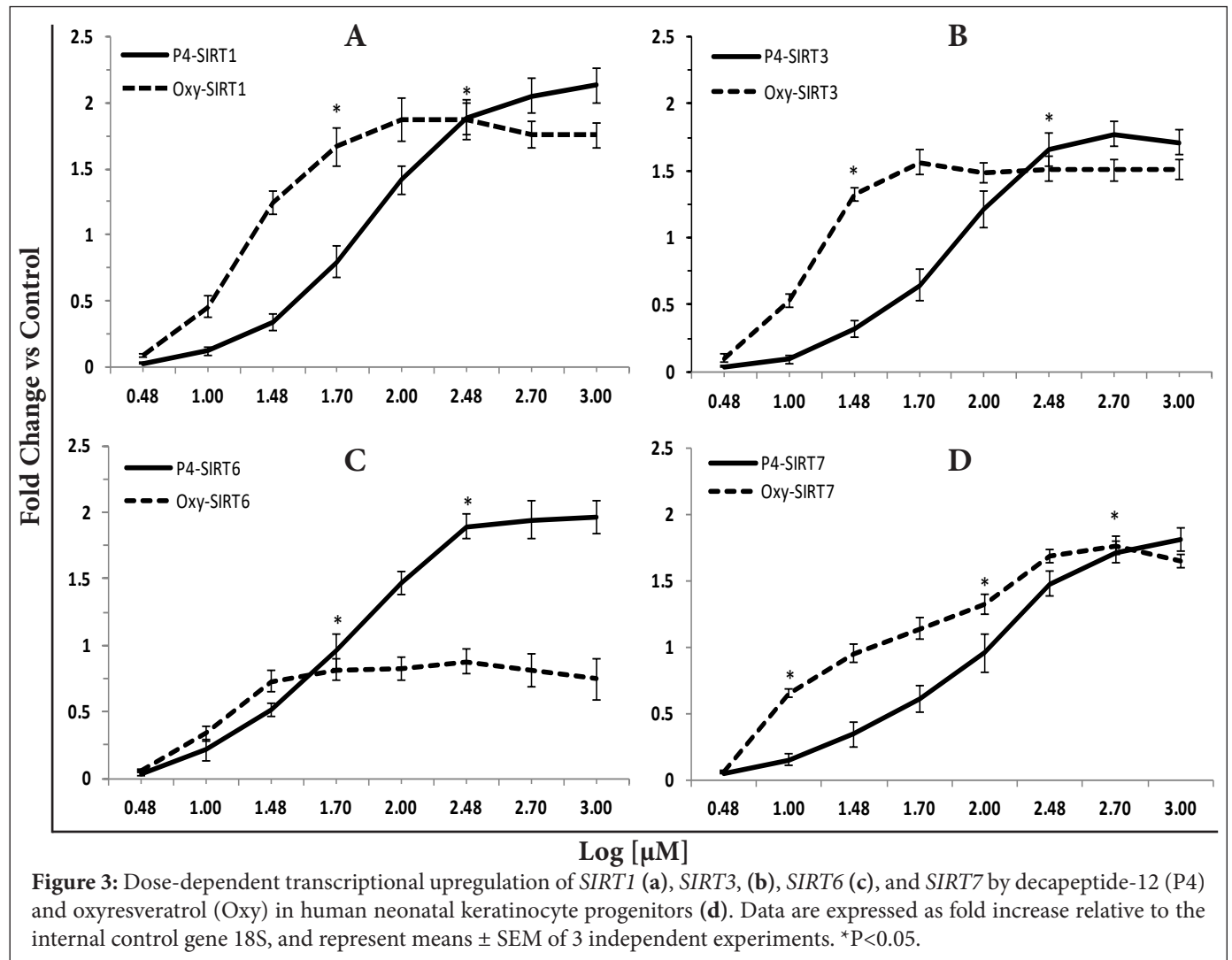


Figure 3: Dose-dependent transcriptional upregulation of *SIRT1* (a), *SIRT3* (b), *SIRT6* (c), and *SIRT7* by decapeptide-12 (P4) and oxyresveratrol (Oxy) in human neonatal keratinocyte progenitors (d). Data are expressed as fold increase relative to the internal control gene 18S, and represent means \pm SEM of 3 independent experiments. * $P < 0.05$.

Statistical analysis

Three independent trials were performed for each experiment. Microsoft Excel (Seattle, Washington) was used to calculate means and standard errors and statistical significance was determined using unpaired analysis of variance or two-tailed student T-test. P values < 0.05 were taken to be statistically significant.

Results

Effect of decapeptide-12 on keratinocyte progenitor proliferation and cytotoxicity

We first assessed the cytotoxic effect of decapeptide-12 and oxyresveratrol on human neonatal keratinocyte progenitors. Treatment with 100 μ M of decapeptide-12 and oxyresveratrol resulted in $3 \pm 1\%$ and $6 \pm 1\%$ cell deaths, respectively (Figure 2A). At 1 mM, decapeptide-12 and oxyresveratrol resulted in $7 \pm 2\%$ and $16 \pm 2\%$ of cell death, respectively.

We also evaluated the effects of decapeptide-12 and oxyresveratrol on the viability and proliferation of human neonatal kerati-

noyte progenitors. Figure 2B shows that treatment with 300 μ M of decapeptide-12 and oxyresveratrol resulted in $2 \pm 1\%$, and $5 \pm 1\%$, reduction in cell proliferation rates, respectively. However, unlike 1 mM decapeptide-12 which reduced proliferation $3 \pm 2\%$, 3-day incubation with oxyresveratrol reduced proliferation $12 \pm 2\%$.

Decapeptide-12 upregulated transcription of *SIRT1-7* in keratinocyte progenitors

We next assessed the effect of oxyresveratrol and decapeptide-12 on sirtuin expression in human neonatal keratinocyte progenitors. Figure 3 and Table 2 show that treatment with decapeptide-12 and oxyresveratrol modulated transcription of *SIRT1-7* in a dose-dependent fashion. At 30 μ M of oxyresveratrol, *SIRT1* transcription levels were upregulated by $125 \pm 9\%$ relative to control cells, whereas *SIRT3*, *SIRT6*, and *SIRT7* were upregulated by $133 \pm 5\%$, $73 \pm 8\%$ and $95 \pm 7\%$, respectively. One hundred μ M of decapeptide-12 increased transcription of *SIRT1* by $141 \pm 11\%$ relative to untreated cells, whereas *SIRT3*, *SIRT6* and *SIRT7* increased by $121 \pm 13\%$, $147 \pm 8\%$ and $95 \pm 14\%$, respectively (Figure 3).

Table 2a

Decapeptide-12 [μM]	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
3	3 ± 1%	1 ± 1%	4 ± 1%	3 ± 1%	3 ± 1%	3 ± 1%	5 ± 1%
10	12 ± 3%	4 ± 3%	9 ± 3%	8 ± 4%	5 ± 3%	21 ± 8%	15 ± 4%
30	34 ± 7%	11 ± 5%	32 ± 6%	12 ± 7%	21 ± 7%	52 ± 5%	34 ± 9%
50	79 ± 12%	22 ± 5%	65 ± 12%	41 ± 13%	33 ± 6%	95 ± 13%	61 ± 10%
100	141 ± 11%	35 ± 2%	121 ± 13%	71 ± 14%	46 ± 7%	147 ± 8%	95 ± 14%
300	188 ± 12%	61 ± 7%	165 ± 12%	115 ± 2%	67 ± 9%	189 ± 10%	148 ± 10%
500	205 ± 13%	76 ± 6%	177 ± 9%	145 ± 3%	87 ± 15%	194 ± 14%	171 ± 8%
1000	213 ± 13%	76 ± 7%	171 ± 9%	151 ± 13%	92 ± 17%	167 ± 12%	181 ± 8%

Table 2b

Oxyresveratrol [μM]	SIRT1	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
3	9 ± 1%	8 ± 2%	10 ± 3%	8 ± 1%	7 ± 1%	6 ± 1%	6 ± 1%
10	45 ± 8%	15 ± 2%	53 ± 5%	12 ± 2%	12 ± 3%	34 ± 6%	65 ± 3%
30	125 ± 9%	4 ± 2%	133 ± 5%	49 ± 7%	45 ± 4%	73 ± 8%	95 ± 7%
50	166 ± 15%	56 ± 8%	156 ± 9%	52 ± 7%	46 ± 4%	8 ± 8%	114 ± 8%
100	187 ± 17%	4 ± 8%	148 ± 7%	64 ± 7%	36 ± 7%	8 ± 8%	132 ± 8%
300	187 ± 15%	39 ± 9%	152 ± 9%	67 ± 9%	33 ± 7%	87 ± 9%	168 ± 5%
500	176 ± 10%	33 ± 12%	151 ± 8%	61 ± 9%	35 ± 8%	81 ± 12%	177 ± 7%
1000	175 ± 9%	31 ± 12%	151 ± 7%	71 ± 9%	37 ± 7%	75 ± 15%	165 ± 5%

Table 2: Gene expression profile of *SIRT 1-7* in response to treatment with decapeptide-12 (a) or oxyresveratrol (b). Results are averages ± SEMs of 3 independent runs.

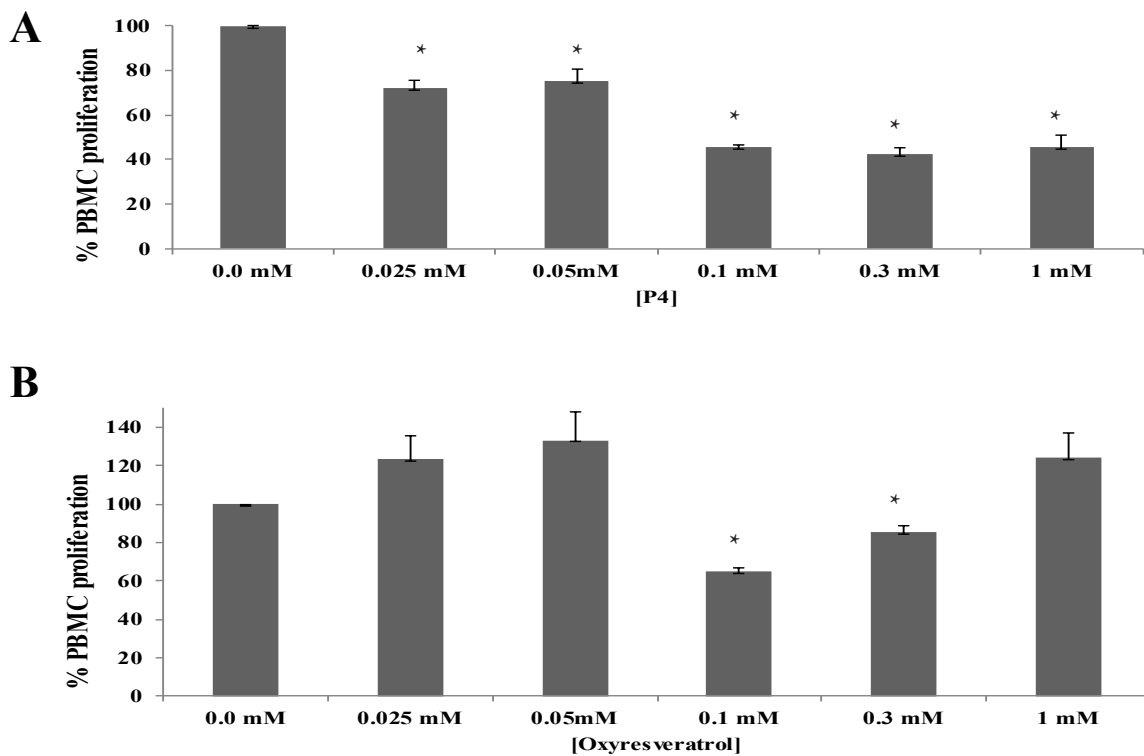
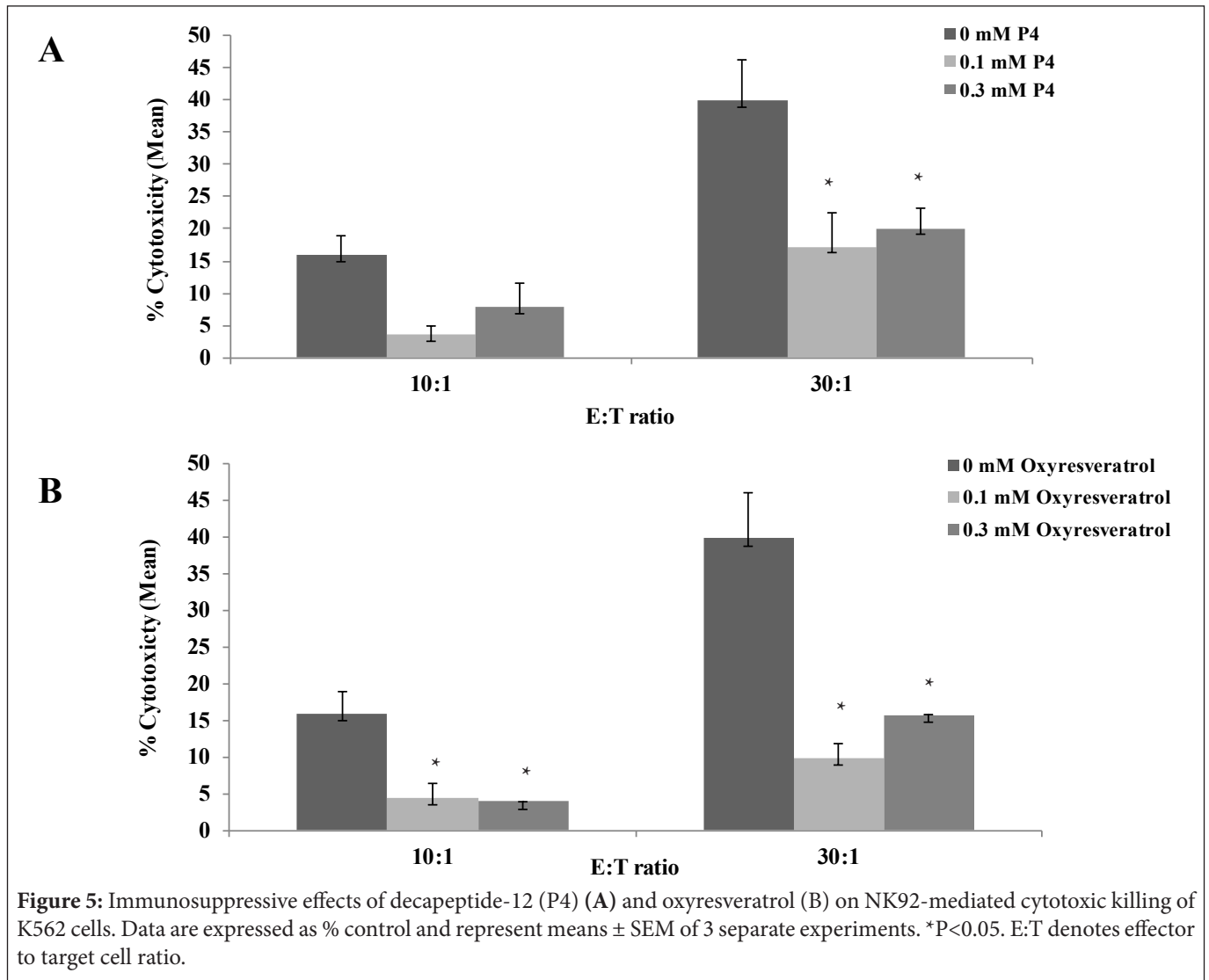


Figure 4: Immunosuppressive effects of decapeptide-12 (P4) (A) and oxyresveratrol (B) on PHA-stimulated PBMC proliferation. Data are expressed as % control and represent means ± SEM of 3 separate experiments. *P<0.05. E:T denotes effector to target cell ratio.



Decapeptide-12 is immunosuppressive

We also explored the effects of decapeptide-12 and oxyresveratrol on PBMC proliferation rates after exposure to PHA for 72 h. Figure 4A shows decapeptide-12 statistically significantly ($p < 0.02$) reduced proliferation by $28 \pm 4\%$ at 0.05 mM and $54 \pm 1\%$ at 0.1 mM. No significant additional reduction was achieved at 0.3 or 1 mM ($p > 0.05$). Oxyresveratrol also reduced proliferation by $35 \pm 2\%$ ($p < 0.02$) at 0.1 mM although only by $15 \pm 3\%$ at 3 mM ($p < 0.02$) (Figure 4B). Other concentrations tested did not show a statistically significant change ($p > 0.14$) (Figure 4B).

We concluded by assessing the impact of decapeptide-12 on IL-2 primed NK-mediated cytotoxic killing of K562 cells. Figure 5A illustrates that decapeptide-12 reduced NK killing by $81 \pm 1\%$ and $59 \pm 4\%$ at 0.1 and 0.3 mM, respectively ($p > 0.05$), at 10:1 ratio of effector to target (E:T) cells. At a 30:1 ratio, decapeptide-12 reduced NK killing by $65 \pm 5\%$ and $45 \pm 3\%$ at 0.1 and 0.3 mM, respectively ($p < 0.04$). Figure 5B shows that oxyresveratrol diminished NK killing by $89 \pm 2\%$ and $86 \pm 1\%$ at 0.1 and 0.3 mM, respectively ($p < 0.03$), at E:T ratio of 10:1. At a 30:1 ratio,

oxyresveratrol blocked NK killing by $73 \pm 2\%$ and $64 \pm 3\%$ at 0.1 and 0.3 mM, respectively ($p < 0.03$).

Discussion

Sirtuin pleiotropic effects are believed to underlie delayed senescence and aging. Therapeutic use of resveratrol as a *SIRT1* activator and potential anti-aging agent has been extensively researched and documented [37-39]. By activating *SIRT1*, a nuclear deacetylase [26], resveratrol conveys protection of human endothelium from H_2O_2 -induced oxidative stress and senescence [29]. Similarly, oxyresveratrol is also a potent antioxidant and free radical scavenger. However, unlike resveratrol, it exhibits less cytotoxicity and better water solubility. Consequently, we elected to use it as a positive control against which we compared decapeptide-12's performance and ability to modulate sirtuin transcription in human neonatal keratinocyte progenitors.

Even though all 7 sirtuins were upregulated after treatment with decapeptide-12, our discussion will focus on those sirtuins directly implicated in skin aging. At 100 μ M and 1 mM, decapeptide-12 in-

creased *SIRT1* transcription by an impressive 141% and 213%, respectively. *SIRT1* exerts effects on metabolism, stress response, genome stability, and cell survival, proliferation, and differentiation [40-42]. In keratinocytes, *SIRT1* was reported to protect against UVB- and H₂O₂-induced cell death by modulating p53 and c-Jun N-terminal kinases leading authors to speculate that *SIRT1* activators may serve anti-aging agents [12]. Furthermore, via suppression of NF-κB signaling, *SIRT1* may delay aging and extend lifespan [43]. This is achieved by deacetylating the p65 subunit of NF-κB complex leading to improved oxidative metabolism and earlier resolution of inflammation [44]. Thus, *SIRT1* has been firmly established as a crucial anti-aging protein [45].

SIRT3 transcription was increased by 121% following treatment with 100 μM decapeptide. *SIRT3* effects include regulation of mitochondrial β-oxidation, ATP generation, ROS [46], and hematopoietic stem cell self-renewal [47]. This latter discovery helps lay the path for future stem cell-based interventions for metabolic disorders resulting in premature aging.

SIRT6 is another sirtuin family member with pleiotropic effects on metabolism, inflammation, tumor suppression, and DNA repair [45]. In a landmark study, *SIRT6* knockout caused severe premature aging phenotypes and mouse lifespan reduction to 1 month [48]. Whole body *SIRT6* overexpression resulted in increased lifespan in mice [49]. *SIRT6* also exerts an anti-inflammatory effect by histone deacetylation of NF-κB target gene promoters thus reducing NF-κB activity [50].

Baohua *et al.* showed that *SIRT6* plays a key role in the process of skin aging via modulation of collagen metabolism and NF-κB signaling. They reported that blocking *SIRT6* significantly decreased hydroxyproline content by inhibiting transcription of type 1 collagen, prompting matrix metalloproteinase 1 secretion and increased NF-κB signaling [51]. Hence, decapeptide-12, which significantly enhanced *SIRT6* transcription, may hold great promise as a therapeutic anti-aging candidate to address the often-concurrent phenotypes of premature skin aging and photodamaged skin.

Our studies also showed that decapeptide-12 and oxyresveratrol exhibited anti-inflammatory effects as measured by two methods: 1) blockade of PHA-stimulated PBMC proliferation, and 2) inhibition of NK-mediated cytotoxic killing. The effect of decapeptide-12 appeared dose-dependent in contrast to oxyresveratrol which exhibited a very narrow inhibitory concentration range. In fact, the general trend, although not statistically significant, showed oxyresveratrol may have biphasic effects as concentrations of 0.3 and 1 mM showed progressively less inhibition than did 0.1 mM. This was not the case for decapeptide-12 which exhibited a plateau or maximum inhibition at 0.1 mM in the concentration range tested. This can be explained by dose-dependent differences in activation of downstream signaling pathways or feedback loops. Indeed, careful examination of dose-dependency curves of sirtuin expression patterns presented herein reveal biphasic effects with higher concentrations becoming inhibitory.

In contrast, abrogation of NK killing appeared dose-dependent for both decapeptide-12 and oxyresveratrol, with the latter showing more pronounced inhibition at all concentrations tested. The inhibitory effects were greater at an E:T ratio of 10:1 than 30:1 for both decapeptide-12 and oxyresveratrol. This may be due to blockade of NKG2D and perforin mediated cytotoxicity but remains to be determined. Our findings are consistent with those of Boscolo *et al.*, who reported that resveratrol strongly inhibits PHA-induced proliferation at 0.1 mM [52]. They suggested that this suppression effect may be due to inhibition of NF-κB, which as noted above, is also regulated by sirtuins and strongly linked to immune and inflammatory responses as well as regulation of cell proliferation and apoptosis, amongst other effects.

Taken together, the immunosuppressive effects observed in our study suggests that unique and specific regulatory pathways are engaged in different arms of the immune system. Further studies are needed to clarify these pleiotropic effects. For example, assessment of the impact of these two agents on pro-inflammatory mediators such as TNFα, IL-1β, IFNγ, and IL-6 would be insightful. In addition, determination of translational and other transcriptional effects of activated versus resting PBMCs would be insightful.

In summary, decapeptide-12 was shown in this report to significantly upregulate transcription levels of *SIRT1*, *SIRT3*, and *SIRT6*, all 3 of which play significant roles in counteracting skin aging and other age-associated pathologies. Finally, clinical studies with various topical formulations containing decapeptide-12 and/or oxyresveratrol are currently being designed to help validate the in vitro findings and test the efficacy of these potent sirtuin activators in *vivo*.

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