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In vitro and *in vivo* toxicity assessment of the senotherapeutic Peptide 14

Alessandra Zonari [a,*], Lear E. Brace [a], Thuany Alencar-Silva [b], William F. Porto [b,c], Daniel Foyt [a], Mylieneth Guiang [a], Edgar Andres Ochoa Cruz [a], Octavio L. Franco [b,d,e,f], Carolina R. Oliveira [a], Mariana Boroni [a,g], Juliana L. Carvalho [b,h]

[a] *OneSkin, Inc., San Francisco, CA 94107, USA*
[b] *Genomic Sciences and Biotechnology Program, Catholic University of Brasilia, Brasília 70790-160, DF, Brazil*
[c] *Porto Reports, Brasília 72236–011, DF, Brazil*
[d] *Center of Proteomic Analyses and Biochemistry, Genomic Sciences and Biotechnology Program, Catholic University of Brasilia, Brasilia, 70790–160, DF, Brazil*
[e] *S-Inova Biotech, Biotechnology Program, Catholic University Dom Bosco, Campo Grande 79117–010, MS, Brazil*
[f] *Molecular Pathology Program, University of Brasilia, Brasilia 70.910–900, DF, Brazil*
[g] *Bioinformatics and Computational Biology Lab, Brazilian National Cancer Institute (INCA), Rio de Janeiro 20231–050, RJ, Brazil*
[h] *Interdisciplinary Biosciences Laboratory, Faculty of Medicine, University of Brasília, Brasília 70.910–900, DF, Brazil*

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ABSTRACT

Senotherapeutic molecules decrease cellular senescence burden, constituting promising approaches to combat the accumulation of senescent cells observed in chronological aging and age-related diseases. Numerous molecules have displayed senotherapeutic potential, but toxicity has been frequently observed. Recently, a new senotherapeutic compound, Peptide 14, was developed to modulate cellular senescence in the skin. In order to assess the potential toxic and genotoxic effects of the peptide, we observed the viability of human primary dermal fibroblasts and epidermal keratinocytes with Peptide 14 treatment, and show that it is mostly non-toxic in concentrations up to 100 μM. Cancer lines were also used to investigate its potential of modulating proliferation. Different concentrations of the peptide promoted a discrete reduction in the proliferation of cancerous cells of the MeWo and HeLa lineages. In full-thickness human skin equivalents, topically formulated Peptide 14 also failed to exert any significant irritation, nor cellular toxicity when added to the culture media. Genotoxic assays including the Ames, micronucleus, and karyotyping tests also indicate the safety of the peptide. Finally, the irritative potential of the peptide was assessed in human subjects in a repeated insult patch test executed using 1 mM peptide. No visible skin reactions were observed in any of the 54 participants. Taken together, the present data support that Peptide 14 is a senotherapeutic molecule with a positive safety profile as tested with cruelty-free models, justifying further studies involving the peptide.

1. Introduction

Senotherapeutic compounds are defined as molecules that reduce cellular senescence either by suppressing such a cellular state or selectively eliminating senescent cells [1]. Given the increasing knowledge regarding the role of senescent cells in aging [2], as well as in diverse age-related diseases [3–6], interest in senotherapeutic molecules is continuously rising. Indeed, the elimination of senescent cells has been shown in experimental models to alleviate age-related phenotypes and disorders, such as hair density and renal function [7], sarcopenia and cataracts [8], hematopoietic system aging [9], atherosclerosis [3], and degenerative joint disease [10], ultimately extending life and healthspan [2,11]. Nevertheless, clinical evidence of the value of senotherapeutic use in humans is still scarce [12], and the toxicity of some senotherapeutic approaches is a concern that needs to be overcome prior to the clinical application.

Our group developed a screening platform to identify novel senotherapeutic compounds [13]. After screening the potential of a 164-peptide library to reduce cellular senescence levels of primary fibroblasts obtained from Hutchinson-Gilford progeria syndrome donors *in vitro*, we selected the 4 top performing peptides to be used as reference amino acid sequences to generate 764 leads. After confirming the lack of cellular toxicity and the senotherapeutic potential of the top performing peptides, we chose Peptide 14 for further characterization and

* Correspondence to: 953 Indiana Street, San Francisco 94107, CA, USA.
E-mail address: alessandra@oneskin.co (A. Zonari).

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technological development. While the systemic toxicity of novel compounds still requires animal-based models, compounds designed for topical applications, such as Peptide 14, can be tested using exclusively cruelty-free systems. In the present study, we assessed the safety of Peptide 14 as measured by *in vitro* toxicity and irritation tests, genome stability *via* karyotyping, mutagenicity by Ames and micronucleus tests, and the sensitization potential of the peptide in human subjects in a Repeated Insult Patch Test (RIPT). The ability of Peptide 14 to induce cancer cell proliferation was also tested. All evidence obtained from such assays demonstrated the safety of the peptide, justifying further studies with such a senotherapeutic molecule.

2. Methods

2.1. Peptide synthesis

Peptide 14 (ETAKHWLKGI) (Sup. Fig. 1) was purchased from CPC Scientific Inc. (USA), which synthesized the peptide by solid phase (Fmoc) on a Rink amide resin, with > 95% purity, in the form of acetate salt. The molecular mass and purity were confirmed by the manufacture using mass spectral analysis and RP-HPLC chromatogram. The peptide was ressuspended in water at a stock solution of 10 mM.

2.2. Cell culture

Healthy normal human dermal fibroblasts and human keratinocytes were either obtained from MatTek Life Science (Ashland, MA), or isolated from *ex vivo* human skin explants obtained from ZenBio (Research Triangle, NC). Cells purchased from MatTek Life Science included human dermal fibroblasts (HDF) 60 yr (F13400A, XX, African-American), keratinocytes 60 yr (K13400A, XX, African-American), neonatal HDFs (F90800, XY, foreskin, caucasian), neonatal keratinocytes (K90800A, XY, foreskin, Caucasian). The cancer cell lines SW1990, H358 were obtained from ATCC and were cultured in RPMI (Invitrogen) supplemented with 10% v.v. Fetal Bovine Serum (FBS; VWR) and 1% v.v. Penicillin-Streptomycin (Invitrogen). The cell lines HeLa and MeWo were obtained from Banco de Células do Rio de Janeiro and cultured in Dulbecco's Modified Eagle Medium (Invitrogen), supplemented with 10% v.v. Fetal Bovine Serum (FBS; VWR) and 1% v.v. Penicillin-Streptomycin (Invitrogen) CHO-WBL cells were cultured in McCoy's 5 A medium, supplemented with 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, and containing 5% fetal bovine serum or cultured serum-free. Cells isolated from *ex vivo* human skins were obtained from ZenBio (Research Triangle, NC). All skin samples were from XX donors, Caucasian, and from the abdominal area. The ages of the donors were 35 and 55 years. Cell isolation was performed as described by [14]. Briefly, the tissue samples were cut into small pieces and incubated in PBS containing dispase (2.5 U/mL, BD Biosciences) overnight at 4 °C. The epidermis was then mechanically separated from the dermis, using two forceps, and incubated in 0.5% trypsin-EDTA (Gibco, USA) for 7 min at 37 °C to isolate the keratinocytes. The cells were separated from the remaining tissue using a 100 μm pore size cell strainer (BD Biosciences, USA) and the cell suspension was centrifuged at 300 g for 5 min. Human keratinocytes were seeded at a density of 100, 000 cells/ cm^2 in Keratinocyte Serum Free Medium (KSFM) supplemented with Epidermal growth factor and Bovine pituitary extract (Gibco). For the isolation of HDF, the dermis was incubated in PBS containing collagenase IA (250 U/mL, Sigma) for 3 hr at 37 °C. The HDF were separated from the remaining tissue using a 100 μm pore size cell strainer, centrifuged at 300 g for 5 min and seeded at a density of 50,000 cells/cm^2 in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA), supplemented with 10% v.v. Fetal Bovine Serum (FBS; VWR) and 1% v.v. Penicillin-Streptomycin (Invitrogen).

2.3. Full-thickness 3D skin equivalents

Neonatal human dermal fibroblasts were embedded in collagen I gels, which were seeded with keratinocytes and cultured for 24 hrs. An air-liquid interface was then created and kept for additional 7 days, to allow epidermal cornification, as described [13].

2.4. Cell toxicity tests

The viability of various cell types treated with different concentrations of Peptide 14 (0.01–100 μM) was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]−2, 5-diphenyltetrazolium bromide) assay, which is based on the reduction of tetrazolium salt to formazan crystals by the dehydrogenase present in living cells mitochondria. Cell viability was assessed after 24 hr incubation with the peptide. Briefly, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well, followed by incubation for 4 h at 37 °C, 5% CO_2. The resulting formazan salts were solubilized with 100 μL DMSO and the optical density of the solution was evaluated with a microplate spectrophotometer at 570 nm. Cell viability was then calculated as the percent cellular viability of the negative control samples, which received vehicle only.

2.5. In vitro skin irritation and toxicity

The *in vitro* skin irritation assay was performed according to OECD 439 [15], using full thickness 3D skin equivalents. Briefly, the skin equivalents were maintained in an air-liquid interface and treated topically with 20 μL of 1, 10, or 100 μM of Peptide 14. PBS was used in negative irritation controls, and 5% aqueous SDS was used in positive irritation control samples. After 1 hr, the applied material was removed. After 42 hr incubation, the skin equivalent viability was assessed using the MTT method. Tissue viability was then calculated as the percent tissue viability of the negative control samples. *In vitro* toxicity was also evaluated by treating 3D skin equivalents with Peptide 14 or PBS vehicle in the culture media. Briefly, the skin equivalents were maintained in an air-liquid interface and Peptide 14/vehicle was added to the media reaching the final concentrations of 3.12, 6.25, and 12.5 μM. After a 24 hr incubation, the skin equivalent viability was assessed using the MTT method.

2.6. Histological analysis

Human skin equivalents were treated as described in the *in vitro* skin irritation and toxicity section. Then, samples were fixed with 10% Formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) following standard protocols.

2.7. Ames test

The Ames test was performed by Wuxi AppTech, Inc. Briefly, *S. typhimurium* was preincubated with various concentrations of Peptide 14 (100, 25, 5, 1, 0.5 μg/mL) with sufficient histidine or tryptophan, with or without S9 (S9 is obtained from the 9000 g supernatant of a liver homogenate and contains many enzymes. Therefore, the addition of S9 allows the assessment of both phase I and phase II metabolites). Then, the culture was diluted into medium without histidine or tryptophan. After 48 hrs, cells undergoing reversion to prototrophy would grow into colonies, hence the medium color changed from purple to yellow. One or less positive wells were considered as negative results. Two non-adjacent positive wells were considered equivocal.

2.8. Micronuclei test

The micronucleus test was performed by Wuxi AppTech, Inc. The study design was based on the OECD Guideline 487, adopted 29 July 2016, and ICH guidance on genotoxicity testing and data interpretation

for pharmaceuticals intended for human use S2 (R1), November 2011. Briefly, CHO-WBL cells were either cultured in the absence of activation systems for 3 and 24 hr, or in the presence of activation stimuli (Aroclor 1254 induced rat liver S9) for 3 hr. All cells were cultured for 24 hr after initiation of test article treatment. Sterile water for injection was the solvent of choice based on the solubility of Peptide 14 and compatibility with the CHO-WBL cells. Duplicate cultures of CHO-WBL cells were incubated with Peptide 14 at 500, 295.75, 177.25, 118.30, 100.00, 59.15, 29.57, 14.78, 7.39, 3.69, 1.84, 0.92, 0.46, 0.23, 0.11, and 0.05 μg/mL in all three treatment series. No precipitate was observed in the treatment medium at any concentration during treatment. Concentrations at 500, 295.75, and 177.25 μg/mL were scored for evaluation of cytotoxicity (cytostasis) and micronucleus formation in the all three treatment series. Cytokinesis-Block Proliferation Index was calculated using the frequency of CHO-WBL cells containing one, two, or more nuclei, in at least 500 total cells. Cytokinesis-Block Proliferation Index (CBPI) was calculated on the following formula: CBPI = (Number of mononucleated cells +2 × Number of binucleated cells +3 × Number of multinucleated cells)/ Total number of cells. The percentage of cells in cytostasis was calculated using the following formula: % Cytostasis = 100–100{(CBPIT-1)/ (CBPIC-1)}, in which T is test article treatment culture and C is vehicle control culture. Thus a CBPI of 1 (all cells were mononucleate) was equivalent to 100% cytostasis. As positive controls, cyclophosphamide monohydrate (CP), an agent that requires metabolic activation to its clastogenic form, was evaluated concurrently at 5.0 μg/mL in the S9-activated exposure series. Also as a positive control, Mitomycin C (MMC), a clastogen that does not require metabolic activation, was evaluated concurrently at 0.3 μg/mL in the non-activated 3 h exposure series, and 0.1 μg/mL in the non-activated 24 h exposure series.

2.9. Karyotyping analysis

The karyotyping analysis was performed by Cell Line Genetics, Inc, following internal standard operational procedures, based on [16]. Briefly, human primary fibroblasts obtained from different donors were treated with 1 μM Peptide 14 for 10 days. Cells were treated with colcemid for 20 min, then with hypotonic KCl solution. Cells were fixed with a methanol/acetic acid solution and used for slide preparation. Slides were then aged in a 90 ^{o}C oven for 1 hr and immediately processed for GTL banding, being immersed in trypsin-EDTA for 20 s, and rinsed with water. Then, slides were immersed in working Leishman's stain once, water twice, and blown dry before visualization. Twenty metaphases were analyzed. The same gain of a particular chromosome/structural alteration had to be present in at least two different metaphase cells to be considered a clone. In the case of chromosome loss, at least three cells had to present such karyotypic alteration.

2.10. RIPT

The RIPT study was approved by the Allendale Investigational Review Board and has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Accordingly, informed consent was obtained from participants before the initiation of the research. Briefly, 56 participants, male and female, 18–77 years of age were recruited to participate in the study. The upper back between the scapulae served as a treatment area. Approximately 2 mL of the Peptide 14 formulated in a standard solution containing glycerin, PIP-097 and PIP-093 was applied to 1"x1" absorbent pad of a clear adhesive dressing, which was applied to the skin to form a semi-occlusive patch. Patches were applied 3 times a week (*e.g.* Monday, Wednesday, Friday) for 3 weeks during the induction phase. After supervised removal, participants were instructed to remove the patches at home. After at least 10 days following the final induction patch application, a challenge patch was applied to a virgin site, following the same procedure as described for induction. The patch was removed and the site scored at the clinic day 1 and 3 post-application, according to the occurrence of no visible skin reaction (0), barely perceptive (0.5), mild (1), moderate (2), marked (3), or severe skin reaction (4). The occurrence of edema (E), dryness (D), staining (S), papules (P), vesicles (V), bullae (B), ulceration (U), and spreading (Sp) were also observed and registered.

2.11. Statistical analysis

Cell and human skin equivalent experiments were performed in triplicates. Data are presented as the mean and standard deviation. Statistical comparisons were performed using two-way analysis of variance (ANOVA) and Bonferroni's post-hoc test. For the micronucleus test, Fisher's exact test was used to identify significant differences between the treated and concurrent vehicle control groups. P-value < 0.05 were considered to be statistically significant.

3. Results

3.1. In vitro toxicity and skin irritation potential

Human dermal fibroblasts from three donors (35, 55 and 60 y) were cultured for 24 h in the presence of increasing doses of Peptide 14 (0.01–100 μM). After treatment, cell viability was determined by the MTT assay and revealed that Peptide 14 did not significantly alter cell viability in concentrations up to 100 μM (Fig. 1A.1).

The potential toxicity of the peptide was also assessed in keratinocytes derived from three donors (35, 55 and 60 y), and also corroborated that the peptide is not toxic to human skin cells (Fig. 1A.2). Considering that the peptide presents senotherapeutic potential in concentrations as low as 0.5–12.5 μM [13], these data suggest that the peptide is not toxic to cells even if present at 20 times its effective dose.

The hazard identification of irritant chemicals can be performed using different methodologies. Here, we assessed the irritation of Peptide 14 using full thickness 3D skin equivalents, adapting the recommendations from OECD 439 for reconstructed human epidermis test model. According to the guidelines, after topical treatment, a compound should be considered an irritant if it decreases the viability of human skin equivalents by more than 50%. Peptide 14 was tested in increasing concentrations and not only did it not decrease the viability of treated tissues even when used at 100 μM, but all tested doses improved the viability compared to untreated (Fig. 1B.1). The observation that Peptide 14 is not a skin irritating substance is also supported by qualitative histological analysis of skin equivalents processed similar to those used for the MTT assay. As shown, no significant differences could be observed between the overall histological structure of human skin equivalent samples of the negative control and Peptide 14 treatment groups (Fig. 1B.2). Similar to control samples, Peptide 14 treatment maintained the epidermal basal layer, as well as epidermal stratification, preserving epidermal thickness, which is an important parameter for skin toxicity assessment [17].

The effect of Peptide 14 on full human skin equivalents was also assessed after the peptide was added to the culture media and incubated for 24 h. Once again, the peptide did not promote toxicity in any of the tested concentrations (3.12–12.5 μM), as demonstrated by MTT and histological analysis (Fig. 1C).

Finally, the toxicity of the peptide was tested in several cancer cell lines including melanoma (MeWo), cervical cancer (HeLa), pancreatic cancer (SW1990) and lung cancer (H358). In Fig. 2A, a significant decrease of the cell viability compared to non-treated controls were observed only in MeWo cells. Increased viability indicates cell proliferation and was not observed in any cancer cell line tested with Peptide 14. Doxorubicin, a known chemotherapeutic was used as a positive control, and as expected induced cell death in a dose-dependent response in all cancer cell lines tested compared to non-treated controls (Fig. 2 B).

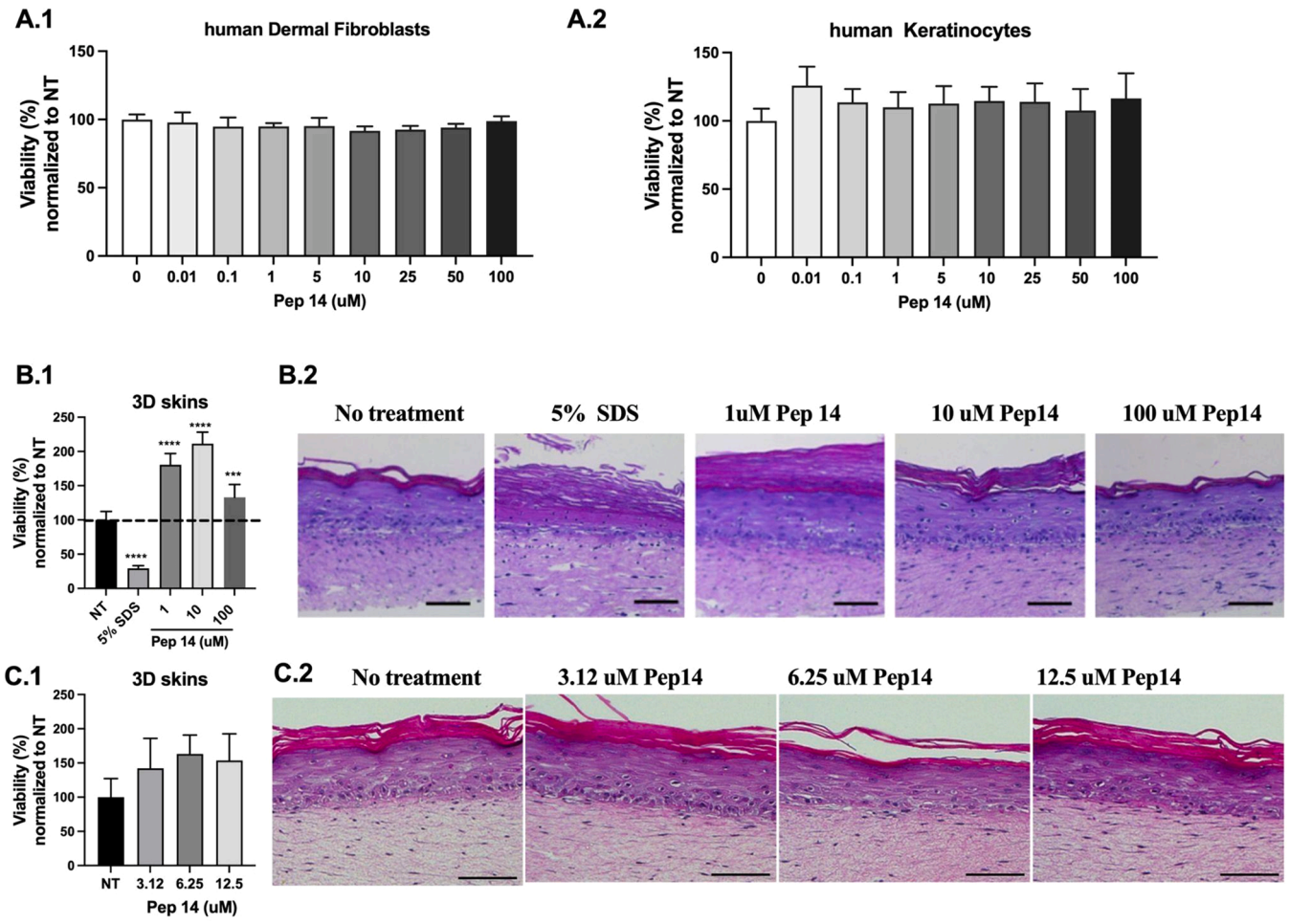

Fig. 1. Viability studies with Peptide 14 tested on human skin cells and 3D skins equivalents. Different cell lines and 3D skin equivalents were exposed to Peptide 14 at several concentrations and cell viability was assessed after 24–42 h using MTT assay. A.1) Cell viability of human dermal fibroblasts, graphs represent the average of 3 donors (35, 55 and 60 y) A.2) Cell viability of human keratinocytes, graphs represent the average of 3 donors (35, 55, and 60 y). B.1) Cell viability of 3D skin human equivalents treated topically with different concentrations of Peptide 14. B.2) Representative images of H&E staining of 3D skin equivalent. Scale bar: 200 μm. C.1) Cell viability of 3D skin human equivalents treated in the media with different concentrations of Peptide 14. C.2) Representative images of H&E staining of 3D skin equivalent. Scale bar: 200 μm. All experiments were performed in triplicate and were analyzed using ONE way anova and Bonferroni post-hoc test. * **$p < 0.001$; * ** *$p < 0.0001$, represents a significant difference compared to untreated controls.

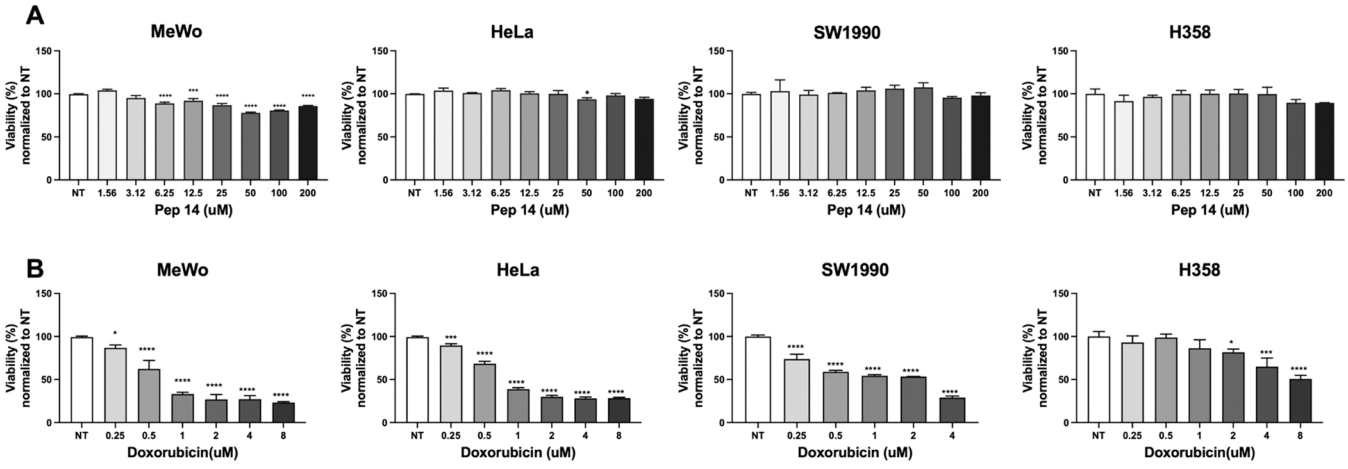

Fig. 2. Viability studies with Peptide 14 tested on different cancer cell lines. Different cancer cell lines were exposed to Peptide 14 (A) or Doxorubicin (B) at increasing concentrations and cell viability was assessed after 24 h using MTT assay. All experiments were performed in triplicate and were analyzed using ONE way anova and Bonferroni post-hoc test.*$p < 0.5$, * *$p < 0.01$, * **$p < 0.001$; * ** *$p < 0.0001$, represents a significant difference compared to untreated controls.

3.2. Mutagenicity and Genotoxicity testing

3.2.1. Ames Test

The reverse bacterial mutation test, also known as the Ames test, is a biological assay to assess the mutagenic potential of new compounds using bacteria. This assay was performed by WuXi AppTec, Inc, using standard industry procedures. Briefly, the bacteria *S. typhimurium* was preincubated with Peptide 14 with sufficient histidine or tryptophan, with or without S9. In the presence or absence of S9, which mimics compound metabolization in the liver, the number of revertant colony counts for each experimental condition (positive wells) did not exceed the values of vehicle control (DMSO). Therefore, Peptide 14 was considered not to be mutagenic up to 100 μg/mL (Table 1). As evidence that the assay was correctly executed, the positive controls of nitrofluorene and aminoanthracene did induce mutation reversal in a significant number of wells (47 and 48 out of 48 wells, respectively).

3.2.2. Micronuclei test

In the presence of genotoxic compounds, mammalian cells may accumulate an array of chromosomal aberration types, which include the formation of micronuclei. Micronuclei are formed in the nuclei of damaged cells as a consequence of chromosome breakage and encapsulation in membrane-like coatings. Therefore, the cytotoxicity of Peptide 14 was assessed according to the percentage of cells presenting cytostasis (Table 2). No obvious cytotoxicity (cytostasis, reduction of CBPI relative to the concurrent solvent control) was observed under any treatment condition. Mitomycin-C was used as positive control for the test. Since Peptide 14 was not cytostatic in the tested concentrations, the concentration of 500 μg/mL was selected as the highest dose for the micronucleus formation analysis (Table 3) and two lower doses 295.75 and 177.25 μg/mL were also analyzed. In all tested conditions, no statistically significant increase in the frequency of MN-BN (Bi-nucleated cells with micronuclei) cells was observed in any peptide-treated samples relative to the concurrent negative control groups ($p > 0.05$). The frequency of MN-BN for the concurrent positive control articles was significantly higher than the concurrent negative/vehicle controls ($p \leq 0.05$). Based on these results, the micronucleus assay was considered valid and Peptide 14 was considered non-cytotoxic, failing to halt the cell cycle and to induce the formation of micronucleated cells in CHO-WBL cell cultures.

3.2.3. Karyotyping

Karyotyping is another genotoxicity assay in which tested compounds undergo cytogenetic evaluation in the search of potential chromosomal damage, including structural and numerical aberrations. Differing from the Ames and micronucleus tests, karyotyping was performed using human cells (primary dermal fibroblasts), following a longer peptide treatment period of 10 days. As depicted below, human primary dermal fibroblasts (obtained from a 31 year old donor) treated with Peptide 14 at 1 μM for 10 days were considered "apparently normal", since, from 20 cell metaphases counted, 19 were normal and a single non-clonal aberration was detected, which is considered an artifact of the method (Fig. 3).

Table 2
Cytotoxicity Data for Micronucleus Assay.

Concentration (μg/mL)	Concentration (μM)	3 h treated with S9% Cytostasis	3 h treated without S9% Cytostasis	24 h treated without S9% Cytostasis
Sterile water	–	0	0	0
Peptide 14				
0.05	0.04	NS	NS	NS
0.11	0.09	NS	NS	NS
0.23	0.19	NS	NS	NS
0.46	0.39	NS	NS	NS
0.92	0.78	NS	NS	NS
1.84	1.56	NS	NS	NS
3.69	3.12	NS	NS	NS
7.39	6.25	NS	NS	NS
14.78	12.5	NS	NS	NS
29.57	25	NS	NS	NS
59.15	50	NS	NS	NS
100	84.6	NS	NS	NS
118.3	100	NS	NS	NS
177.25	150	-7	3	-11
295.75	250	-6	-1	-6
500	422	-5	4	-6
Mitomycin C 0.1		22	47	39

Table 3
Frequency of MNBN for micronucleus assay.

Frequency of MNBN cells/ Cells scored (%)				
Concentration (μg/mL)	Concentration (μM)	3 h treated with S9	3 h treated without S9	24 h treated without S9
Sterile water	–	0.95	0.95	0.70
Peptide 14				
177.25	150	1.20	1.10	0.85
295.75	250	1.00	1.00	0.85
500	422	1.10	1.15	0.65
Cyclophosphamide monohydrate 5.0	18	16.85*	22.55*	17.55*

* $P < 0.05$, represents a statistically significant difference compared to vehicle control (sterile water) according to Fisher's exact test.

3.3. Repeated Insult Patch Test (RIPT)

The RIPT was executed to determine the potential of Peptide 14 to induce primary or cumulative irritation and/or allergic sensitization in human subjects. To do so, 54 participants (15 males and 39 females) aged 18–77 years received semi-occlusive patches with 1 mM Peptide 14 on their backs for a total of 9 alternating applications (3 times a week) for 3 weeks of induction phase. Ten days following the induction phase, a challenge patch was applied in a virgin test site. No visible skin reaction was observed in any participant (Table 4).

Table 1
Ames Test.

without S9					with S9				
Strain	Compound	Dose	Concentration	Positive wells	Strain	Compound	Dose	Concentration	Positive wells
S. Typhimurium TA98	Peptide 14	100 μg/mL	84 μM	0	*S. Typhimurium TA98*	Peptide 14	100 μg/mL	84 μM	0
		25 μg/mL	21 μM	0			25 μg/mL	21 μM	0
		5 μg/mL	4.2 μM	0			5 μg/mL	4.2 μM	1
		1 μg/mL	0.84 μM	0			1 μg/mL	0.84 μM	2
		0.5 μg/mL	0.52 μM	0			0.5 μg/mL	0.52 μM	0
	2 Nitrofluorene	1 μg/mL		47		2 Aminoanthracene	1 μg/mL		48
	DMSO	0 μg/mL		1		DMSO	0 μg/mL		1

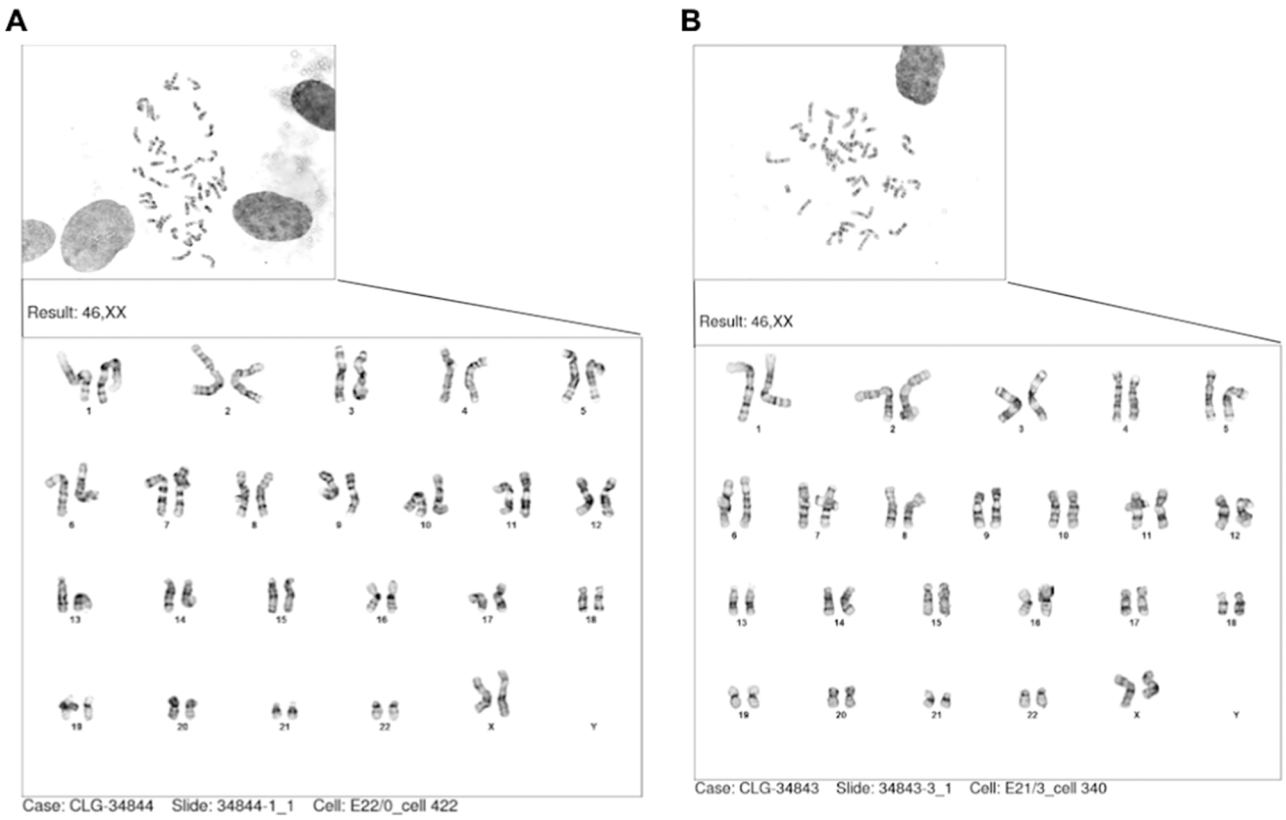

Fig. 3. Karyotyping analysis of primary fibroblasts treated with Peptide 14. Representative karyotype analysis of human dermal fibroblasts (31 y) control (A) and 10 days treated with 1 μM Peptide 14 (B). As depicted, 19 out of 20 metaphases analyzed showed normal karyotype. Peptide 14 was therefore considered safe.

Table 4
Demographics and reaction grade of the Repeated Insult Patch Test.

Number of participants		54
Female		39 (72.2%)
Male		15 (27.8%)
Range		18–77
Reaction grade	Reaction description	Participants with reactions n (%)
0	No visible reaction	54 (100%)
0.5	Barely perceptible	0
1	Mild	0
2	Moderate	0
3	Marked	0
4	Severe	0

4. Discussion

The possibility of treating several age-related diseases at once by decreasing the cellular senescence burden in aged individuals is an appealing concept, which has resulted in increased characterization of potential senotherapeutic compounds. So far, few senotherapeutic molecules have been described, including natural and synthetic compounds. Currently, natural senotherapeutics that exert low toxicity seem to be less effective than targeted senolytics which, on the other hand, seem to present more frequent toxic effects [11,18]. For instance, Navitoclax, an inhibitor of BCL-2 and initially described as an anti-tumor drug, was later described as "one of the most potent and broad-spectrum senolytic agents identified to date" [18,19] though it caused platelet toxicity and hemorrhagic events in cancer patients [20]. Other senotherapeutics that target the FOXO4-p53 pathway or the mammalian target of rapamycin (mTOR) also present toxicity [18,19].

Peptide 14 was discovered following a screening and optimizing approach, and was shown to reduce cellular senescence burden in skin cells [21]. Given that the clinical application is dependent on the establishment of a safety profile, we assessed the toxicity potential of the peptide. Also, given that the response to senotherapeutics appears to be tissue-dependent [22], and that Peptide 14 was shown to reduce senescence burden associated with aging in the human skin and would be applied topically, we evaluated the toxicity profile of the peptide specifically in skin cells and 3D skin equivalents, in addition to other tests on humans which are standard in topical product development.

Peptide 14 did not exert any relevant cytotoxic effects in human primary dermal fibroblasts and human primary keratinocytes, regardless of the donor age, as well as in CHO-MBK cells and cancer cell lines. The concentrations tested surpassed the concentration in which the peptide presents senotherapeutic effect by at least 10 times. In more realistic scenarios, using human skin equivalents, the peptide also failed to induce cell death, as measured by the MTT metabolization method. Since senescence is considered a mechanism to prevent further cancer development, we also evaluated if Peptide 14 would be able to induce cancer cell proliferation. The data showed that Peptide 14 does not increase cancer cell viability.

Considering the possibility of the peptide exerting mutagenic effects independently from cell toxicity, we assessed the genotoxicity of the peptide using industry's standard protocols of the Ames Test, Micronuclei Test, and Karyotyping. Once again, no genotoxicity was detected, supporting that the peptide is safe in the tested conditions. Finally, the RIPT assay tested on human subjects with formulated peptide reported no adverse events detected in any of the 54 participants, confirming Peptide 14 is a non-irritating compound.

In summary, under the conditions of this study, Peptide 14 was shown to be safe and non-irritating for topical application in human

skin. Further clinical studies are necessary to confirm the potential of Peptide 14 in promoting skin benefits.

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Competing interests

MB, AZ, CR, LB, EA, and JC are named as inventors of a patent directed at this invention, which is solely owned by OneSkin, Inc. MB, AZ, CR, EA and JC are co-founders of OneSkin Inc.

CRediT authorship contribution statement

Conception and design: AZ, MB, CR, LB, EC, and JC. Peptide library design and lead optimization: OF, WP. Wet lab experiments: AZ, LB, DF, MG. Drafting the article: JC, AZ, LB, MB. Final revision: All authors. The authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Zonari, Mariana Boroni, Carolina Reis de Oliveira, Lear Brace, Edgar Andres Ochoa Cruz, Mylieneth Guiang, Thuany Alencar-Silva, and Juliana L Carvalho are named as inventors of a patent directed at this invention, which is solely owned by OneSkin, Inc. Alessandra Zonari, Mariana Boroni Carolina Reis de Oliveira, Edgar Andres Ochoa Cruz, and Juliana L Carvalho are co-founders of OneSkin Inc.

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.07.018.

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