International Journal of Cosmetic Science

International Journal of Cosmetic Science, 2021, **43**, 102–106 doi: 10.1111/ics.12669

Enhanced retinoid response by a combination of the vitamin A ester retinyl propionate with niacinamide and a flavonoid containing *Ceratonia siliqua* extract in retinoid responsive *in vitro* models

E.C.S. Lam*, R. Li*, M.R. Rodrigues*, L. Vires†, R.L. Adams†, J.D. Sherrill† and J.E. Oblong†

*Procter and Gamble International Operations SA SG Branch, Singapore, Singapore and †The Procter and Gamble Company, Cincinnati, OH, USA

Received 2 September 2020, Revised 2 October 2020, Accepted 6 October 2020

Keywords: retinyl propionate, keratinocytes, niacinamide, *Ceratonia siliqua*

Abstract

OBJECTIVES: Retinoids have been used for decades as efficacious topical agents to treat photoaged skin. The purpose of our present research is to evaluate whether the activity of the vitamin A ester retinyl propionate (RP) can be enhanced by niacinamide (Nam) and a flavonoid containing *Ceratonia siliqua* (*CS*) fruit extract in retinoid responsive *in vitro* models.

METHODS: Retinyl propionate was tested alone and in combination with Nam and *CS* in an RARα reporter cell line for promoter activation and compared to *trans*-retinoic acid (tRA) activation. These treatments were also tested in keratinocytes for gene expression profiling by qPCR using a panel of 40 retinoid responsive genes.

RESULTS: tRA or RP elicited RARα reporter activation in a dose-dependent manner. The combination of 0.5 μM or 2 μM RP with 10 mM Nam had a 56% and 95% signal increase compared to RP, respectively. The addition of 1% *CS* to 0.5 μM or 2 μM RP with 10 mM Nam elicited a further increase of 114% and 156%, respectively, over RP and Nam combinations. All retinoids elicited an increase in expression of 40 retinoid sensitive genes over control levels. Of the 40 genes, 27 were enhanced by either 0.1 μM RP or 0.5 μM RP with 10 mM Nam and 1% *CS*. Nam or *CS* had very modest activity in both models.

CONCLUSION: The combination of RP with Nam and *CS* showed a higher retinoid response than RP in two separate retinoid responsive *in vitro* models. We hypothesize Nam and *CS* enhances RP activity by modulating metabolism to tRA via increasing NAD^+ pools and inhibiting reduction of retinal (RAL) back to retinol, respectively. The findings provide evidence that this combination may have enhanced efficacy for treating the appearance of photoaged skin.

Abstrait

OBJECTIFS: Les rétinoïdes sont utilisés depuis des décennies comme agents topiques efficaces pour traiter la peau photo-âgée. Le but de notre recherche actuelle est d'évaluer si l'activité du propionate rétinyl ester de vitamine A (RP) peut être augmentée par le niacinamide (Nam) et un flavonoïde contenant un extrait de fruit de *Ceratonia Siliqua* (*CS*) dans les modèles in *vitro* sensibles aux rétinoïdes.

MÉTHODES: RP a été testé seul et en combinaison avec Nam et *CS* dans une ligne de cellule rapporteur de RARα pour l'activation du promoteur et par rapport à l'activation de l'acide *transrétinoïque* (tRA). Ces traitements ont également été testés dans les kératinocytes pour le profilage d'expression génique par qPCR à l'aide d'un panel de 40 gènes rétinoïdes sensibles.

RÉSULTATS: tRA ou RP ont provoqué l'activation du promoteur RARα d'une manière dépendante de la dose. La combinaison de 0,5 μM ou 2 μM de RP avec 10 mM de Nam a permis une augmentation respectivement de 56% et 95% du signal par rapport à RP. L'ajout de 1 % de *CS* à 0,5 μM ou 2 μM de RP avec 10 mM de Nam a permis une nouvelle augmentation de 114 % et 156 %, respectivement, qu'avec la combinaison RP et Nam. Tous les rétinoïdes ont provoqué une augmentation de l'expression de 40 gènes sensibles aux rétinoïdes sur les niveaux de contrôle. Sur les 40 gènes, 27 ont été améliorés soit par 0,1 μM de RP ou 0.5 μM de RP avec 10 mM de Nam et 1% de *CS*. Nam ou *CS* avaient une activité très modeste dans les deux modèles.

CONCLUSION: La combinaison de RP avec Nam et *CS* a montré une réponse rétinoïde plus élevée que RP dans deux modèles *in vitro* séparés sensibles aux rétinoïdes. Nous émettons l'hypothèse que Nam et *CS* améliorent l'activité RP en modulant le métabolisme de tRA par l'augmentation des groupement NAD^+ et en inhibant la réduction du rétinal (RAL) en rétinol, respectivement. Les résultats fournissent la preuve que cette combinaison peut améliorer l'efficacité du traitement de l'aspect de la peau photo-âgée.

Correspondence: John E. Oblong, The Procter & Gamble Company, Cincinnati, OH, 45040, USA. Tel.: +1 513 646 5064; E-mail: oblong.je@pg.com

Introduction

The skin is the largest organ of the human body and one of its primary functions is to provide protection from damaging environmental stressors such as solar UV radiation, carbon emissions and pollution. Cumulative exposure to these damaging stressors leads to premature structural and functional changes that manifest as photoageing. Solar UV radiation is considered the most significant stressor and has been estimated to account for ~85% of the premature ageing cascade in skin [1,2].

Retinoids are lipophilic vitamin A derivatives that have been used for decades in the treatment of photoaged skin [3]. Mechanistically,

retinoids play an important role in epidermal homeostasis, particularly regulating proliferation and differentiation of keratinocytes and maintaining epidermal thickness [4–6]. The primary active retinoid form is *trans*-retinoic acid (tRA), which binds to members of the retinoic acid receptor (RAR) family of nuclear hormone receptors. Upon binding, the RAR complex translocates into the nucleus to activate selective gene expression [7,8]. Although systemic vitamin A is obtained via diet and oral absorption, retinoids can also become bioavailable when applied topically [9,10]. Epidermal keratinocytes and dermal fibroblasts are enzymatically capable of converting retinol (ROL) and retinyl esters to tRA via an NAD^+ dependent oxidative pathway [11–13].

RP is a vitamin A ester analogue and has been reported to clinically impact photoaged skin with minimal irritation [14–16]. In addition to its overall efficacy and skin tolerability, RP has also been reported to have a better chemical stability profile compared to other retinyl esters, thereby increasing its half-life on the skin's surface during topical delivery [17]. In the present work, we wished to further enhance efficacy potential and minimize any risk of irritation by identifying compounds which could boost RP activity in retinoid responsive models. Previous approaches to increase retinoid activity and overcome retinoid resistance have focused on inhibition of P_{450} hydroxylases, particularly members of the CYP26 family, which metabolize tRA to the inactive form of 4-oxo-retinoic acid [18]. This has led to the development of retinoic acid metabolism blocking agents (RAMBA) such as the azoles liarazole and rambazole, potent inhibitors that can reduce retinoid resistance [19]. We evaluated an alternate approach to retinoid enhancement by focusing on the metabolism pathway of RP to tRA via the ROL and RAL intermediates that require NAD^+ as a cofactor. We hypothesized that the NAD^+ precursor Nam would facilitate this conversion by incorporating into cellular NAD^+ pools and there by enhance RP activity. This is of particular relevance in photoaged skin since it has been established that cellular NAD^+ pools in skin decline significantly with age, which impacts the role it has in maintaining skin homeostasis [20,21]. Additionally, we evaluated the flavonoid content in a *Ceratonia siliqua* (carob) fruit extract (*CS*, Silab, France) since flavonoids have been reported to inhibit AKR1B10 and carbonyl reductases, enzymes that can reduce RAL back to ROL [22,23]. *CS* sourced raw materials are of keen interest for human health benefits, including topical skin care usage, based on their flavonoid and polysaccharide content [24,25]. A total polyphenolic Folin–Ciocalteu quantification assay was performed on the *CS* fruit extract used in this work, and it was calculated to have a total flavonoid content of 104 μg/mL (data not shown, Polyphenol Quantification Assay Kit, Bioquochem, Asturias, Spain).

A RARα reporter (Luc)-HEK293 cell line that contains a stably transfected luciferase gene under control of a retinoic acid receptor response element along with a full length human RARα gene (Promega, Madison, WI) was used to quantify the effect of retinoids on induction of luciferase activity. This reporter system requires the presence of tRA and thus is an indirect measure of enzymatic conversion of ROL and RP into tRA inside the cells. Briefly, cells were grown in assay media and seeded into a 96-well plate at a density of ~30 000 cells per well. Cells were exposed to respective treatments for 24 h after which viability was measured using CellTiter-Fluor as per manufacturer's instructions (Promega, Madison, WI). Subsequently, luciferase activity was measured by BioGlo reagent as per manufacturer's instructions (Promega, Madison, WI). Both fluorescence and luminescence measurements were performed using a Cytation 3 multi-mode plate reader (BioTek Instruments, VT, USA). For data normalization, ratio of the luminescence and fluorescence value for each well was obtained and the response of the materials in percentage fold change was then expressed relative to the control.

Cells were treated with tRA between 0.00001 and 0.1 μM to establish a luciferase signal range Figure 1a. All tRA doses significantly induced luciferase activity by 618% (at 0.00001 μM), 682% (at 0.0001 μM), 928% (at 0.001 μM), 1203% (at 0.01 μM) and 1895% (at 0.1 μM) as a percentage of vehicle control. Cells were

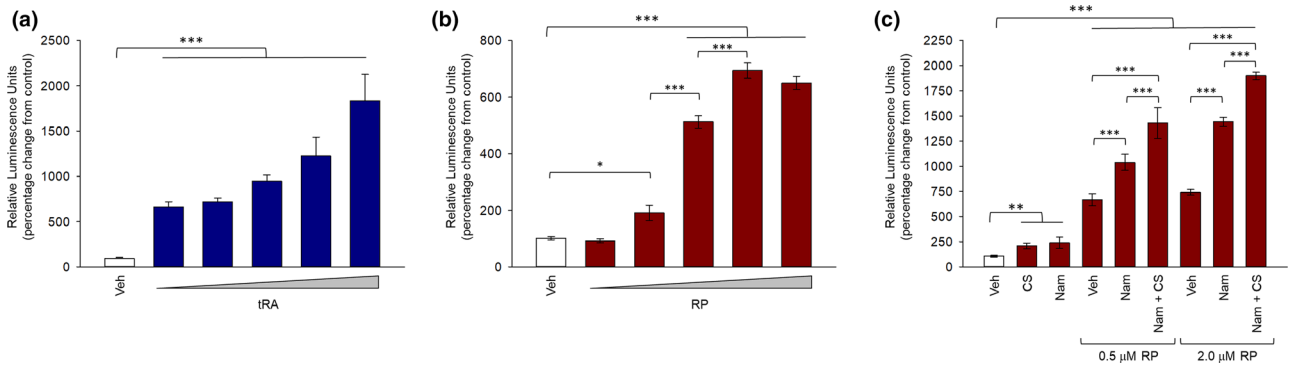

Figure 1 RARα activation by RP can be enhanced when combined with niacinamide and *Ceratonia siliqua*. HEK293 cells containing a stable RARα reporter construct were treated for 24 hours with tRA, ROL, RP, niacinamide (Nam), *Ceratonia siliqua* (*CS*) or a combination of RP with Nam and *CS*. Luciferase activity normalized to cell viability was measured, and the values were expressed as percentage change relative to vehicle (Veh) control treatment (Student's t-test, ***$p < 0.001$, ** $p < 0.005$, * $p < 0.01$, n = 3). (a) Normalized luciferase signal from cells treated with 0.00001, 0.0001, 0.0001, 0.01 or 0.1 μM tRA (blue bars, increasing dose represented by rising triangle) compared to vehicle control (white bar). (b) Normalized luciferase signal from cells treated with 0.02, 0.1, 0.5, 2 or 10 μM RP (red bars, increasing dose represented by rising triangle) compared to vehicle control (white bar). (c) Normalized luciferase signal from cells treated with 1% *CS*, 10 mM Nam, 0.5 μM RP or 2.0 μM RP and the combinations of 0.5 μM RP or 2.0 μM RP with 10 mM Nam or with 10 mM Nam and 1% *CS* (red bars) compared to vehicle control (white bar)

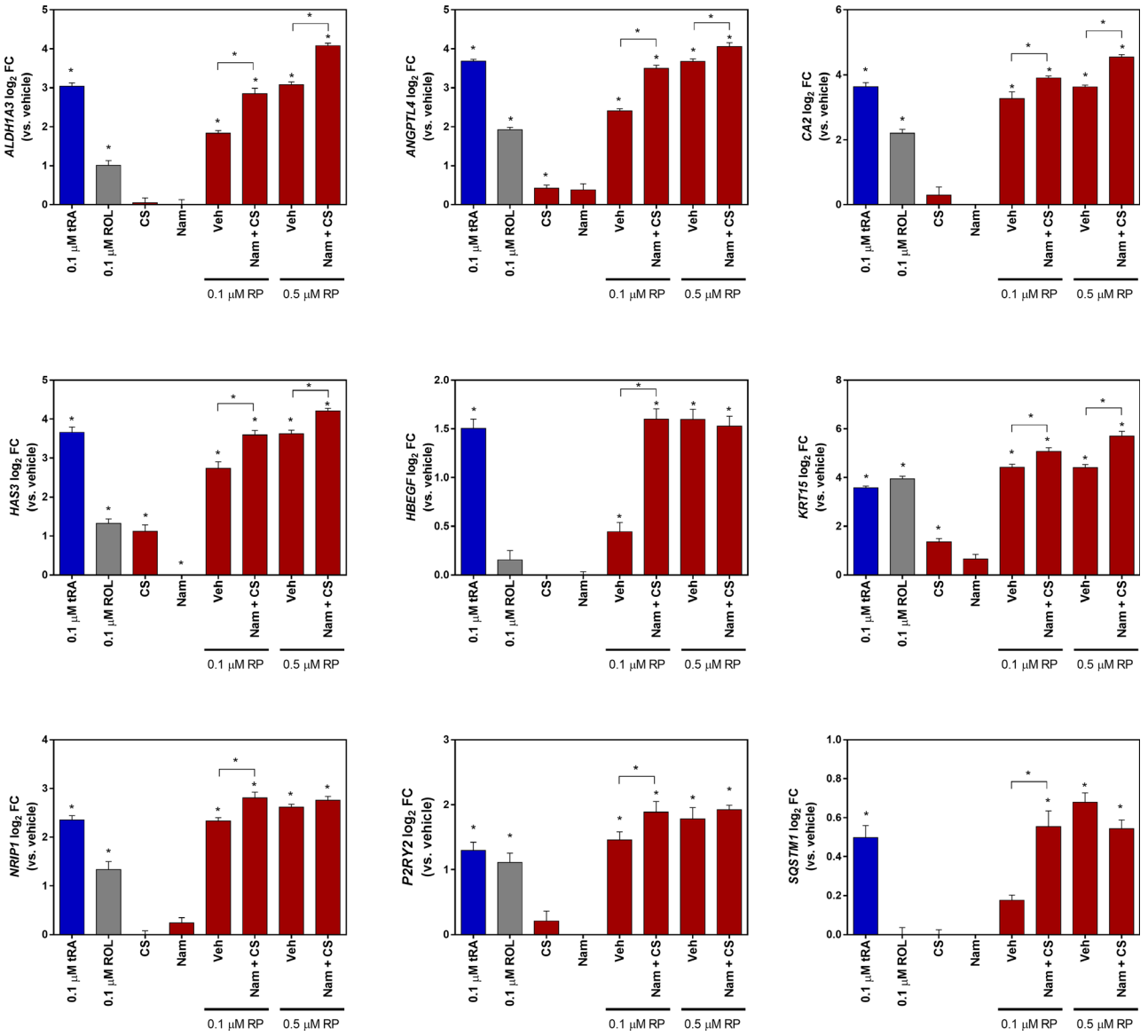

Figure 2 Retinoid responsive gene expression quantification shows increased response to combination of RP with niacinamide and *Ceratonia siliqua*. Quantitation of retinoid responsive target genes by qPCR in hTERT keratinocytes after treatment for 24 hours. Shown are the log2 fold changes (FC) of 9 select retinoid target genes (versus untreated). These include ALDH1A3, ANGPTL4, CA2, HAS3, HBEGF, KRT15, NRIP1, P2RY2 and SQSTM1. Treatments included single material of 0.1 μM tRA (blue bar), 0.1 μM ROL (grey bar), 1% *Ceratonia siliqua* (*CS*), 1 mM niacinamide (Nam), 0.1 μM RP or 0.5 μM RP (red bars). Additionally, a treatment combination of 0.1 μM RP or 0.5 μM RP with 1% *CS* and 1 mM Nam (red bars) was performed. Student's t-test, $*p < 0.05$. n = 12 per treatment group. Error bars represent log2 SEM

also treated with 0.02, 0.1, 0.5, 2 or 10 μM RP Figure 1b. RP significantly induced luciferase activity by 90% ($*P < 0.05$) at 0.1 μM and by 413%, 593% and 550% at 0.5, 2 or 10 μM, respectively ($***P < 0.001$) as a percentage of vehicle control. 0.5 and 2 μM RP were selected for combination testing Figure 1c since the basal signal increase by RP alone was in the lower range of the tRA dose–response curve Figure 1a. When 0.5 μM or 2 μM RP was tested in combination with 10 mM Nam, there was a 56% and 95% increase in signal over RP alone, respectively. Importantly, the combination of 1% *CS* and 10 mM Nam with 0.5 μM or 2 μM RP further increased the measured luciferase signal by an additional 115% and 157%, respectively, over RP alone.

We next evaluated the impact of RP alone and in combination with *CS* and Nam on the expression profiles of 40 retinoid responsive genes by qPCR. hTERT keratinocytes were cultured in EpiLife media with full supplements and treated for 24 h with

vehicle (DMSO) or retinoid treatments. Total RNA was isolated from cell lysates, quantitated, and cDNA generated. cDNA was plated onto a Wafergen MyDesign SmartChip (TakaraBio, p/n 640036) using the Wafergen Nanodispenser. qPCR was then performed on the chip, relative expression values of the 40 target genes were normalized to the geometric mean of 4 housekeeping genes (*ACTB*, *B2M*, *GAPDH* and *PPIA*), and fold changes over vehicle-treated cells were evaluated for significance using Student's *t*-test. A complete list of the target genes analysed can be found in Table S1.

Treatment with 0.1 μM tRA, 0.1 μM ROL, 0.1 μM RP or 0.5 μM RP all significantly increased expression of the target genes. 0.1 and 0.5 μM RP were selected based on these concentrations eliciting a similar expression induction level as measured in the lower range of a tRA dose–response curve (data not shown). All 40 target genes showed an increase in expression level in response to RP and 16 were RP dose-sensitive Table S1. Nine of the dose responsive genes (ALDH1A3, ANGPTL4, CA2, HAS3, HBEGF, KRT15, NRIP1, P2RY2 and SQSTM1) showed an increase in expression level by the combinations of 0.1 μM or 0.5 μM RP with Nam and *CS* Figure 2. In comparing to the other retinoids, 0.1 μM tRA shows an inconsistent pattern of higher expression when compared to 0.1 μM or 0.5 μM RP. Additionally, 0.1 μM ROL had overall lower induction levels compared with 0.1 μM RP. In contrast to RARα activation, RP with Nam did not show a consistent pattern of increased expression over RP alone on the 40 responsive genes (data not shown).

In conclusion, these collective data provide a body of evidence that RP in combination with Nam and *CS* can significantly increase RP activity in retinoid responsive *in vitro* models. Mechanistically, we believe this is via an increase in cellular NAD^+ pools by the precursor Nam which allows for optimized oxidation to tRA via ROL and RAL. Additionally, the total flavonoids present in *CS* may further boost metabolism efficiency by inhibiting the reduction of RAL back to ROL Figure 3. The *CS* carob fruit extract tested in this work also contains a high level of oligogalactomannans (data not shown, analysis by Silab, France). Thus, it is possible that these chemistries may also have a functional role in increasing the RAR response in the RP combinations. To better address these questions, future work is needed to confirm the retinoid metabolite profile from these retinoids and combinations in keratinocytes. We hypothesize that this combination has the potential to provide a stronger breadth and depth of efficacy response than RP alone and future work is needed to confirm this in clinical testing.

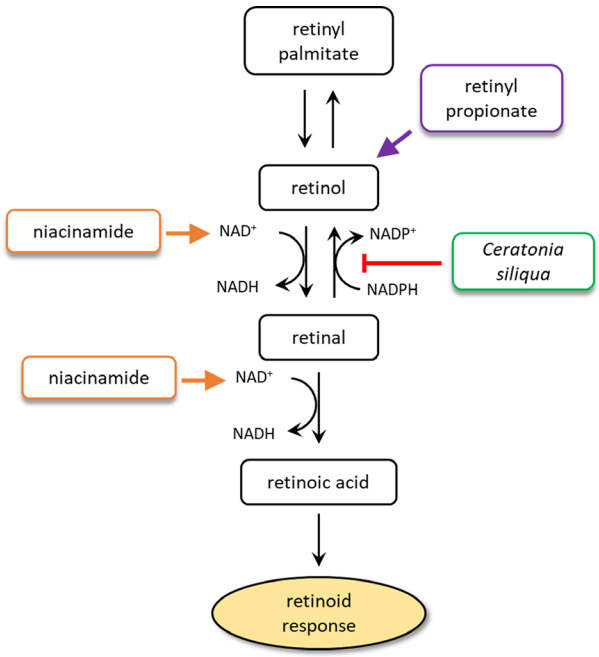

Figure 3 Schematic diagram of retinoid metabolism and hypothesized intervention points by niacinamide and *Ceratonia siliqua* for increased retinoid response when both are combined with RP. Enzymatic conversion of RP, RPalm and ROL to tRA is well known and utilizes NAD^+ as a key cofactor for the oxidation steps of ROL to RAL and RAL to tRA. Niacinamide, a known NAD + precursor, is proposed to further heighten conversion by increasing NAD^+ cellular levels. *Ceratonia siliqua (CS)* is proposed to inhibit the reverse reduction of RAL to ROL by functioning as a potent blend of flavonoids (104 μg/ml)

Acknowledgements

We thank Holly Rovito and John Bierman for assistance with keratinocyte qPCR experiments.

Conflict of interest

E.C.S.L., R.L., M.R., L.V., R.L.A., J.D.S. and J.E.O. are full-time employees of The Procter & Gamble Company, and all other authors declare no conflicts of interest.

References

1. Yaar M, Gilchrest BA. Photoageing: mechanism, prevention and therapy. *Br. J. Dermatol.* 2007;**157**:874–87.
2. D'Orazio J, Jarrett S, Amaro-Ortiz A, Scott T. UV radiation and the skin. *Int. J. Mol. Sci.* 2013;**14**:12222–48.
3. Zasada M, Budzisz E. Retinoids: active molecules influencing skin structure formation in cosmetic and dermatological treatments. *Postepy Dermatol. Alergol.* 2019;**36**:392–7.
4. Chopra DP, Flaxman BA. The effect of vitamin A on growth and differentiation of human keratinocytes in vitro. *J. Invest. Dermatol.* 1975;**64**:19–22.
5. Gilfix BM, Eckert RL. Coordinate control by vitamin A of keratin gene expression in human keratinocytes. *J. Biol. Chem.* 1985;**260**:14026–9.
6. Rosenthal DS, Roop DR, Huff CA, Weiss JS, Ellis CN, Hamilton T, *et al.* Changes in photo-aged human skin following topical application of all-trans retinoic acid. *J. Invest. Dermatol.* 1990;**95**:510–5.
7. Varani J, Fisher GJ, Kang S, Voorhees JJ. Molecular mechanisms of intrinsic skin aging and retinoid-induced repair and reversal. *J. Investig. Dermatol. Symp. Proc.* 1998;**3**:57–60.
8. Benbrook DM, Chambon P, Rochette-Egly C, Asson-Batres MA. History of retinoic acid receptors. *Subcell Biochem.* 2014;**70**:1–20.
9. Blomhoff R, Green MH, Norum KR. Vitamin A: physiological and biochemical processing. *Ann. Rev. Nutr.* 1992;**12**:37–57.
10. Yourick JJ, Jung CT, Bronaugh RL. In vitro and in vivo percutaneous absorption of

retinol from cosmetic formulations: significance of the skin reservoir and prediction of systemic absorption. *Toxicol. Appl. Pharmacol.* 2008;**231**:117–21.
11. Roos TC, Jugert FK, Merk HF, Bickers DR. Retinoid metabolism in the skin. *Pharmacol. Rev.* 1998;**50**:315–33.
12. Bailly J, Crettaz M, Schifflers MH, Marty JP. In vitro metabolism by human skin and fibroblasts of retinol, retinal and retinoic acid. *Exp. Dermatol.* 1998;**7**:27–34.
13. Kurlandsky SB, Xiao JH, Duell EA, Voorhees JJ, Fisher GJ. Biological activity of all-trans retinol requires metabolic conversion to all-trans retinoic acid and is mediated through activation of nuclear retinoid receptors in human keratinocytes. *J. Biol. Chem.* 1994;**269**:32821–7.
14. Oblong JE, Saud A, Bissett DL, Zhu C. Topical retinyl propionate achieves skin benefits with favourable irritation profile. In: *Cosmeceuticals and Active Cosmetics: Drugs Versus Cosmetics (Cosmetic Science and Technology Series)*. (Elsner P, Maibach HI, eds.), Chapter 25, pp. 441–63 (2005).
15. Kaczvinsky JR, Griffiths CE, Schnicker MS, Li J. Efficacy of anti-aging products for periorbital wrinkles as measured by 3-D imaging. *J. Cosmet. Dermatol.* 2009;**8**:228–33.
16. Fu JJ, Hillebrand GG, Raleigh P, Li J, Marmor MJ, Bertucci V, *et al*. A randomized, controlled comparative study of the wrinkle reduction benefits of a cosmetic niacinamide/peptide/retinyl propionate product regimen vs. a prescription 0.02% tretinoin product regimen. *Br. J. Dermatol.* 2010;**162**:647–54.
17. Semenzato A, Bovenga L, Faiferri L, Austria R, Bettero A. Stability of Vitamin A propionate in cosmetic formulations. *SÖFW-J.* 1997;**123**:151–4.
18. Isoherranen N, Zhong G. Biochemical and physiological importance of the CYP26 retinoic acid hydroxylases. *Pharmacol. Ther.* 2019;**204**:107400.
19. Njar VC, Gediya L, Purushottamachar P, Chopra P, Vasaitis TS, Khandelwal A, *et al*. Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases. *Bioorg. Med. Chem.* 2006;**14**:4323–40.
20. Massudi H, Grant R, Braidy N, Guest J, Farnsworth B, Guillemin GJ. Age-associated changes in oxidative stress and NAD+ metabolism in human tissue. *PLoS One*. 2012;**7**:e42357.
21. Oblong JE. The evolving role of the NAD+/nicotinamide metabolome in skin homeostasis, cellular bioenergetics, and aging. *DNA Repair (Amst)*. 2014;**23**:59–63.
22. Zemanova L, Hofman J, Novotna E, Kamil Musilek K, Lundova T, Havrankova J, *et al*. Flavones inhibit the activity of AKR1B10, a promising therapeutic target for cancer treatment. *J. Nat. Prod.* 2015;**78**:2666–74.
23. Shimada H, Hirashima T, Imamura Y. Effects of quinones and flavonoids on the reduction of all-trans retinal to all-trans retinol in pig heart. *Eur. J. Pharmacol.* 2006;**540**:46–52.
24. Stavrou IJ, Christou A, Kapnissi-Christodoulou CP. Polyphenols in carobs: A review on their composition, antioxidant capacity and cytotoxic effects, and health impact. *Food Chem.* 2018;**269**:355–74.
25. Zhu BJ, Zayed MZ, Zhu HX, Zhao J, Li SP. Functional polysaccharides of carob fruit: a review. *Chin. Med.* 2019;**14**:40.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Tabulation of the target genes analyzed for the effect of RP or the combination of RP with Nam and CS on gene expression response.