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Topical delivery of retinyl ascorbate co-drug: 6. Determination of toxic dose and antioxidant activity in cultured human epidermal keratinocytes

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Received March 2, 2005, accepted May 19, 2005

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Pharmazie 60: 794–795 (2005)

The maximum effective dose of retinyl ascorbate and its potential therapeutic benefits against induced oxidative damage were assessed *in vitro* using cultured human epidermal keratinocytes. RA-AsA exhibited toxic effects at concentrations >6 μM. The findings indicate to the potency of RA-AsA as free radical scavenger and cell proliferation regulator.

Retinyl ascorbate (RA-AsA), a co-drug of vitamins A (retinoic acid) and C (ascorbic acid), has been proposed as a topical agent for UV-induced skin damage (Abdulmajed and Heard 2004). In the current work, the maximum effective dose and potential therapeutic benefits against induced oxidative damage were assessed *in vitro* using cultured human epidermal keratinocytes.

Reactive oxygen species (ROS) are implicated in UV-induced damage to skin (Krutmann et al. 1992). This oxidative damage could be an initiator in the pathogenesis of skin cancer and photoaging (Peus et al. 1998). Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in the skin (Weber et al. 1997).

The therapeutic value of AsA (as antioxidant) and retinoids (mainly as cellular division regulators) and their stability in a physiological environment are well established (Boerman and Napoli 1996; May 2002; Reihl et al. 2004).

In our previous work the co-drug retinyl ascorbate (RA-AsA) was shown to penetrate and partially decompose within the skin, thereby regenerating both species *in situ* and having overcome the stratum corneum barrier (Abdulmajed and Heard 2004). The potent antioxidant activity of RA-AsA when reacted with the free radical 1,1-diphenylpicrylhydrazyl (DPPH) as a model for radicals encountered in oxidation process (Abdulmajed et al. 2005) has also been demonstrated, as has the chemical/enzymatic hydrolysis of the ester (Abdulmajed et al. in press).

In the current study, the maximum effective dose of RA-AsA and the potential therapeutic benefits against induced oxidative damage were assessed *in vitro* using cultured human epidermal keratinocytes (Parkinson and Yeudall 2002). The experimental parameter for the bioactivity was the number of living cells (using a haemocytometer) as counted after dying with trypan blue (Sigma 2001). Retinyl palmitate (Rol-Pal) and ascorbyl palmitate (AsA-Pal), often added to topical formulations, were evaluated in a similar manner for comparison.

The number of cells in the culture medium was determined as $2.8 \pm 0.7 \times 10^5$ per ml, with viability of 90 (±3)% over a period of 12 h. The effect of RA-AsA on cell growth was determined by treating the culture (1×10^5 cells per well) with increasing amounts of ester up to 100 μM. The effect was compared in parallel culture treated with similar concentrations of an equimolar mixture of Rol-Pal and AsA-Pal. An equimolar mixture of Rol-Pal and AsA-Pal was used to mimic the hypothesised dual antioxidant/cell division regulatory properties of RA-AsA.

The results (Fig. 1) suggest concentrations of ~6 μM of RA-AsA were beneficial in terms of maintaining the life of the culture at $\sim 9.8 \pm 0.2 \times 10^4$ cells per well, although concentrations above 6 μM caused rapid death of the cells, indicating that high doses of the ester would have a toxic effect. However, the initial concentrations of the equimolar mixture of Rol-Pal and AsA-Pal did not seem to have a significant effect on the number of cells in the culture until the concentration was above 50 μM, where the number of viable cells started declining. This was most notable at an equimolar concentration of 100 μM with $5.6 \pm 0.3 \times 10^4$ cells per well, whereas, at 100 μM of RA-AsA viable cells were counted at $1.2 \pm 0.4 \times 10^4$ cells per well at the end of 12 h incubation time. Excessive amounts of antioxidants are known to have adverse effects

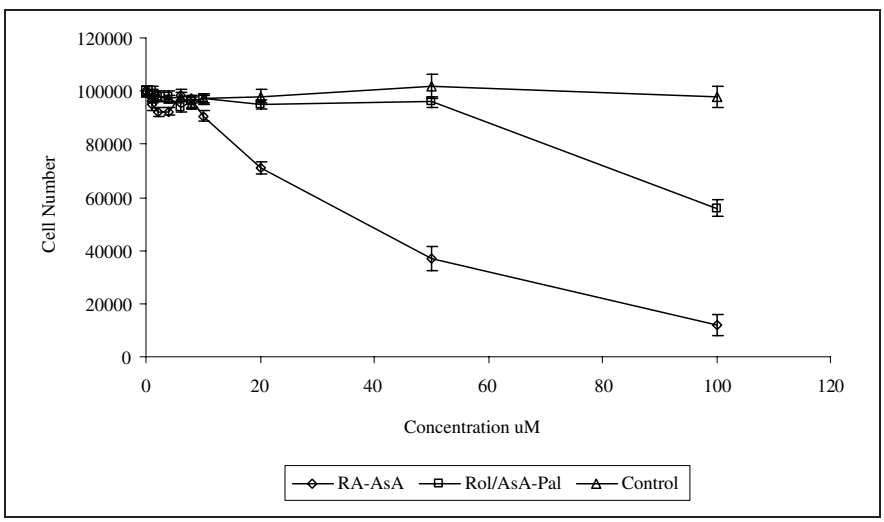

Fig. 1:
Toxicity profiles of RA-AsA and Rol/AsA-Pal on human epidermal keratinocytes, showing greater dose-dependant reduction of viable cells with increased concentrations of RA-AsA compared to AsA-Pal. Mean ± SD, n = 8

on cells. While AsA has a predominantly antioxidant property, it has been demonstrated that concentrations of AsA above 50 μM can cause lymphocyte apoptosis (Puskas et al. 2000). Pro-oxidant and pro-apoptotic effects of AsA may be related to hydroxylation and/or formation of ascorbyl radicals (Sakagami and Satoh 1997).

We previously demonstrated that RA-AsA exhibited potent antioxidant activity when reacted with the free radical DPPH (Abdulmajed et al. in press) as a model for hydroperoxyl (•OOH) and peroxyl (•OOR) radicals encountered in the oxidation process (Karki et al. 2000). RA-AsA was shown to undergo sequential degradation resulting in the generation of more antioxidants. In the same study, Rol-Pal was shown to have no antioxidant properties, whereas AsA-Pal exhibited such properties against DPPH.

While the constituent moieties, RA and AsA, were not isolated from the culture, it is assumed that the toxic effect of RA-AsA at >6 μM concentration was due to its potent antioxidant properties. This could be supported by the results obtained from the equimolar mixture of Rol-Pal and AsA-Pal. As cell death occurred at >50 μM, this is in agreement with those of the effect of excessive administration of AsA (Sakagami and Satoh 1997).

However, 6 μM of either compound seemed to have insignificant influence on cell proliferation within 12 h. But, when the pre-treated cells were exposed to 100 μM of H_2O_2, this concentration of H_2O_2 was shown to produce a significant toxic effect on keratinocytes *in vitro* (Hoogduijn et al. 2004), the number of cells incubated in medium containing 6 μM of RA-AsA ($\sim 8.4 \pm 1.4 \times 10^4$ cells per well) was significantly higher than those incubated in medium containing similar concentration of Rol-Pal and AsA-Pal ($\sim 3.5 \pm 0.8 \times 10^4$ cells per well), which is another indication of the antioxidant potency of RA-AsA (Fig. 2).

In cells incubated in media containing 100 μM of H_2O_2, and then placed in media containing 6 μM of either compound for 12 h, the number of those recovered in RA-AsA containing medium ($\sim 3.9 \pm 1 \times 10^4$ cells per well) was higher than the number of those in medium containing similar concentration of Rol-Pal and AsA-Pal ($\sim 1.2 \pm 0.6 \times 10^4$ cells per well) (Fig. 2). This was an indication of cell growth/regulatory enhancement properties of RA-AsA, which could be due to the release of the RA moiety of the compound. Rol-Pal could not deliver similar regulatory properties over 12 h. The Rol-Pal ester bond may require longer time for hydrolysis as was shown by other workers. Furthermore, the Rol moiety would have to be converted to RA by the cell. Workers previously demonstrated *in vivo* that 44% of topically applied Rol-Pal was converted to Rol in 72 h (Duell et al. 1997), thus the regeneration/repair of damaged cells is not likely to take place at the time of oxidative stress occurring.

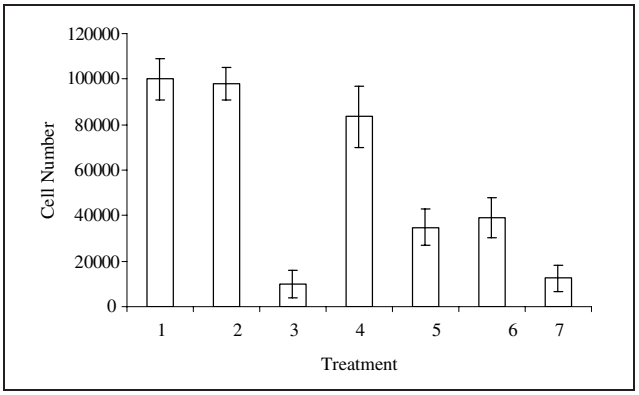

Fig. 2: Protective and regenerative activity of the test compounds on epidermal keratinocytes. 1. Starting population of viable cells, 2. Cell population after 12 h incubation in untreated medium, 3. Cells treated with 100 μM H_2O_2 for 1 h, 4. Cells pre-treated with 6 μM RA-AsA for 12 h and then exposed to 100 μM H_2O_2 for 1 h, 5. Cells pre-treated with an equimolar mixture of 6 μM Rol-Pal and AsA-Pal for 12 h and then exposed to 100 μM H_2O_2 for 1 h, 6. Cells treated with 100 μM H_2O_2 for 1 h and then 6 μM RA-AsA for 12 h, 7. Cells treated with 100 μM H_2O_2 for 1 h and then incubated in a medium containing an equimolar mixture of 6 μM Rol-Pal and AsA-Pal for 12 h, n = 8, ± SD in each case

In conclusion, RA-AsA exhibited toxic effects at concentration >6 μM. However, when subjected to an insult of 100 μM of H_2O_2 for 1 h at this concentration it exhibited >2 fold greater protection and >3 folds greater recovery in comparison with those exhibited by Rol-Pal and AsA-Pal at the same concentrations and experimental procedure. The prime potency of RA-AsA is as a protective agent. The findings indicate to the potency of RA-AsA as free radical scavenger and cell proliferation regulator.

Experimental

Cells were grown on 12 well flat-bottomed culture plates, placed in a humidified incubator with 5% CO_2 at 37 °C under light shield, with medium (SFEKM) renewal every 24 h. To avoid bias of senescence modification in cell metabolism, cells between third and fifth passage were used for the present study (Parkinson and Yeudall 2002). Cell viability was determined by the trypan blue dye-exclusion procedure. At the start of each experiment, the culture medium (SFEKM) was discarded and replaced with fresh medium containing RA-AsA or Rol-Pal/AsA-Pal, 50 : 50, at variable concentrations. The anti-oxidant activity towards DPPH was determined using standard techniques, described elsewhere in detail (Abdulmajed et al. 2005).

References

Abdulmajed K, Heard CM (2004) Topical delivery of retinyl ascorbate co-drug: 1. synthesis, penetration into and permeation across human skin. Int J Pharm 280: 113–124.

Abdulmajed K, McGuigan C, Heard CM (2005) Topical delivery of retinyl ascorbate co-drug: 4. Comparative anti-oxidant activity towards DPPH. Free Radical Res 39: 491–498.

Abdulmajed K, McGuigan C, Heard CM Topical delivery of retinyl ascorbate co-drug: 5. In vitro degradation studies. Skin Pharmacol Physiol, in press.

Boerman MH, Napoli JL (1996) Cellular retinol-binding protein-supported retinoid acid synthesis. J Biol Chem 271: 301–309.

Duell EA, Kang S, Voorhees JJ (1997) Unoccluded retinol penetrates human skin in vivo more effectively than unoccluded retinyl palmitate or retinoic acid. J Invest Dermatol 109: 301–305.

Hoogduijn MJ, Cemeli E, Ross K, Anderson D, Thody AJ, Wood JM (2004) Melainin protects melanocytes and keratinocytes against H2O2-induced DNA strand breaks through its ability to bind Ca2+. Exp. Cell Res. 294, 60–67.

Krutmann J, Czeeh W, Parlow F, Kapp A, Schopf A, Luger TA (1992) Ultraviolet radiation effects on human keratinocyte ICAM-1 expression: UV-induced inhibition of cytokine-induced ICAM-1 mRNA expression is transient, differentially restored for IFN versus TNF, and followed by ICAM-1 induction via a TNF-like pathway. J Invest Dermatol 98: 923–928.

May JM (2002) Recycling of vitamin C by mammalian thioredoxin reductase. In: Sies, H., Packer, L. (Eds.), Protein sensors and reactive oxygen species, part A. Methods in Enzymology, volume 347. Academic Press, San Diego, pp. 327–332.

Parkinson EK, Yeudall WA (2002) The epidermis. In: Freshney RI, Freshney MG (Eds.) Culture of specialised cells: culture of epithelial cells. 2nd Edition. Wiley and Sons, New York, pp. 65–94.

Peus D, Vasa RA, Meves A, Pott M, Beyerle A (1998) H_2O_2 is an important mediator of UVB-induced EGF-receptor phosphorylation in cultured keratinocytes. J Invest Dermatol 110: 966–971.

Puskas F, Gergely P, Banki K, Perl A (1996) Stimulation of the pentose phosphate pathway and glutathione levels by dehydroascorbate, the oxidised form of vitamin C. FASEB J 14: 1352–1361.

Reihl O, Lederer MO, Schwack W (2004) Characterization and detection of lysine–arginine cross-links derived from dehydroascorbic acid. Carbohydr Res. 339: 483–491.

Sakagami H, Satoh K (1997) Modulating factors of radical intensity and cytotoxic activity of ascorbate. Anticancer Res 17: 3513–3520.

Sigma Catalogue, 2000–2001, pp. 1848.

Weber C, Podda M, Rallis M, Thiele JJ (1997) Efficacy of topical applied tocopherols and tocotrienols in protection of murine skin from oxidative damage induced by UV-irradiation. Free Rad Biol Med 22: 761–769.