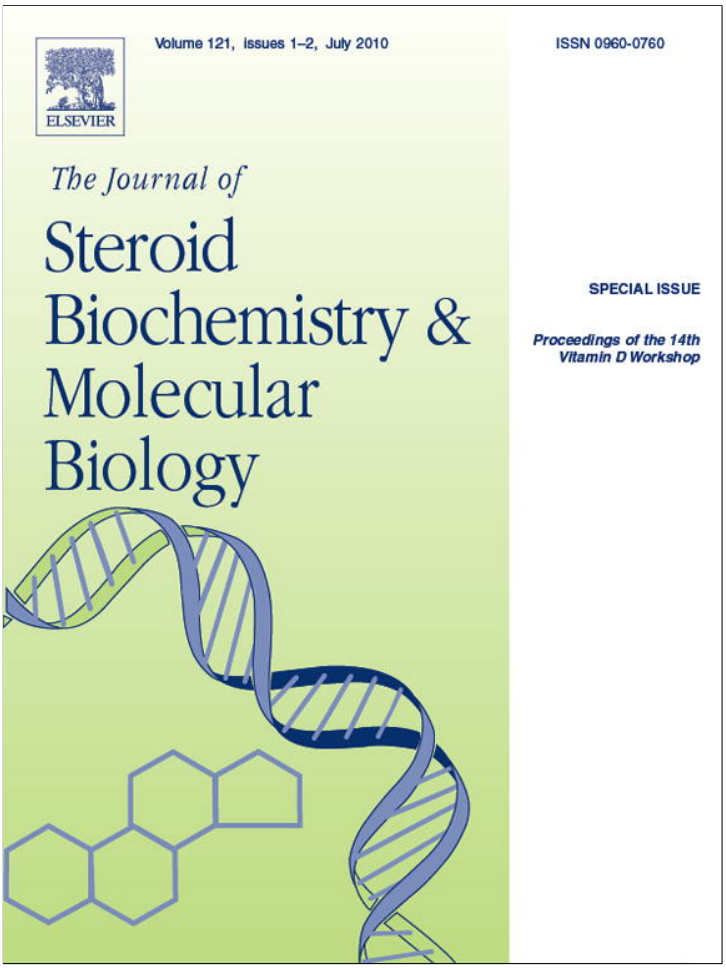

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Photoprotection by 1α,25-dihydroxyvitamin D and analogs: Further studies on mechanisms and implications for UV-damage☆

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ABSTRACT

Ultraviolet (UV) irradiation causes DNA damage in skin cells, immunosuppression and photocarcinogenesis. 1α,25-dihydroxyvitamin D3 (1,25D) reduces UV-induced DNA damage in the form of cyclobutane pyrimidine dimers (CPD) in human keratinocytes in culture and in mouse and human skin. UV-induced immunosuppression is also reduced in mice by 1,25D, in part due to the reduction in CPD and a reduction in interleukin (IL-6. The *cis*-locked analog, 1α,25-dihydroxylumisterol3 (JN), which has almost no trans-activating activity, reduces UV-induced DNA damage, apoptosis and immunosuppression with similar potency to 1,25D, consistent with a non-genomic signalling mechanism. The mechanism of the reduction in DNA damage in the form of CPD is unclear. 1,25D doubles nuclear expression of p53 compared to UV alone, which suggests that 1,25D facilitates DNA repair. Yet expression of a key DNA repair gene, *XPG* is not affected by 1,25D. Chemical production of CPD has been described. Incubation of keratinocytes with a nitric oxide donor, SNP, induces CPD in the dark. We previously reported that 1,25D reduced UV-induced nitrite in keratinocytes, similar to aminoguanidine, an inhibitor of nitric oxide synthase. A reduction in reactive nitrogen species has been shown to facilitate DNA repair, but in view of these findings may also reduce CPD formation via a novel mechanism.

1. Introduction

The epidermis or outer layer of skin consists primarily of keratinocytes which proliferate in the basal layer adjacent to the basement membrane and gradually differentiate into cornified envelopes as they move outwards. Melanocytes in the basal layer produce pigmented melanosomes that are transferred to adjacent keratinocytes. Fibroblasts are the main cell of the dermis, below the basement membrane and these cells produce collagen and elastin (Fig. 1). Dendritic antigen presenting cells, including Langerhans cells, are located in the epidermis [1].

The skin is exposed to solar irradiation, consisting of UVB (energetic wavelengths from 290 to 320 nm), UVA (320–400 nm), as well as infra-red and visible light [2]. UVC (wavelengths below 290 nm) are removed by atmospheric ozone. There are a number of consequences of this, including DNA damage, which, if unable to be repaired, lead to apoptosis, acute erythema and inflammation, immunosuppression and long term problems of photoageing and photocarcinogenesis [3]. There are three main types of DNA damage. The commonest are cyclobutane pyrimidine dimers (CPD). These occur when absorption of UVB by DNA opens up the 5–6 double bond of pyrimidines and a stable ring structure is formed to produce thymine dimers, thymine–cytosine or cytosine–cytosine dimers [4]. There is also evidence of chemical induction of pyrimidine dimers in the dark [5] and of indirect production through photosensitized triplet energy transfer [6]. Both CPD and another common form of DNA damage, 6–4 photoproducts [7], if not repaired properly, can result in UV "signature" mutations [3], but 6–4 photoproducts occur at a much lower rate and are repaired much faster than pyrimidine dimers [8]. Oxidative damage, which results in the mutagen-prone base product 8-hydroxy-2′-deoxyguanosine, may also play a role in sun-induced carcinogenesis [9]. Mutagenic DNA damage and systemic immunosuppression, which is also

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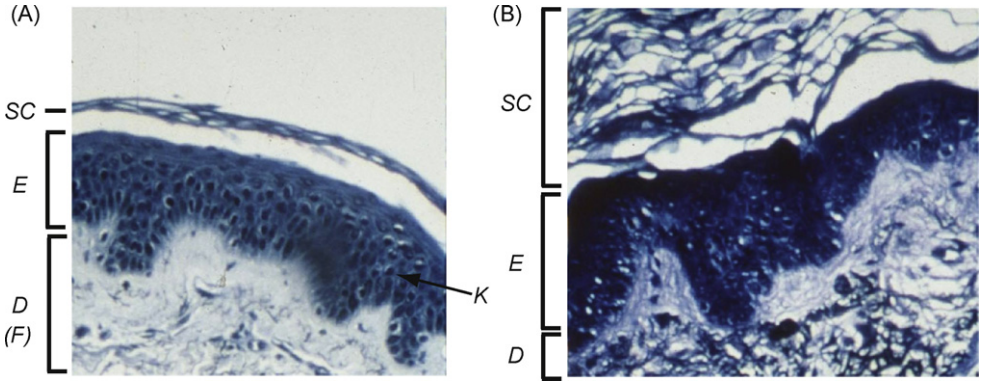

Fig. 1. Histological appearance of human epidermis from non-sun-exposed skin (A) and from sun-exposed skin (B). Note the increased thickness of the stratum corneum (SC) or cornified layer, and some hyperplasia of the epidermis (E), composed principally of keratinocytes (K), in sun-exposed skin. Dermis (D) contains fibroblasts (F).

caused by UV irradiation, are both needed to induce skin cancers [10,11].

2. Protection from UV-induced DNA damage

The two well known mechanisms of endogenous photoprotection are increased pigmentation and increased cornification [2,3,12]. The increased depth of the stratum corneum attenuates UV penetration (Fig. 1). Melanin, which absorbs UV and thus protects DNA, is produced in greater amounts by melanocytes after UV and is transferred to adjacent keratinocytes, where melanin caps are formed over the nuclei. These processes take hours to days, so that increased cornification and pigmentation protect from the next UV exposure, not the initial one. UV also produces previtamin D which thermally isomerizes into vitamin D [13]. Continued irradiation produces overirradiation products. There is evidence that vitamin D can be converted to 1α,25-dihydroxyvitamin D (1,25D) via 25hydroxyvitamin D (25OHD) in skin [14,15], though the process takes several hours. We previously reported that 1,25D could enhance pigmentation in melanocytes and increase cornification in keratinocytes [16,17]. We conducted studies in human primary skin cells to determine whether 1,25D might enhance the pigmentation response to UV in melanocytes or the cornification response to UV in keratinocytes. These assays required correction for the number of cells still viable several hours after irradiation. We found that the most reliable result we obtained was that more melanocytes or keratinocytes survived after UV in the presence of 1,25D than in the vehicle-treated wells [18,19]. Protection from UV-induced apoptosis has also been reported by others – including Lee and Youn [20], who showed evidence for metallothionein induction as a mechanism; by Manggau et al. [21], who provided evidence for a role of increases in sphingosine-1-phosphate; and De Haes et al. [22,23] who reported the involvement of PI3K/Akt, ERK and c-Jun kinase in this protective effect. For reasons that are not clear, the concentrations of 1,25D required for protection from apoptosis in the studies of Manggau et al. [21] and De Haes et al. [22,23] were rather high – 10^{-7} to 10^{-6} M, whereas the effect on cell survival was seen at 10^{-11} to 10^{-8} M in our studies.

Since apoptosis after UV mainly occurs as a result of skin cells acquiring so much DNA damage that this is unable to be repaired [3], the improved cell survival we noted seemed likely to be due to less DNA damage. We and others have reported that this is indeed the case, at least for CPD in human keratinocytes in culture [19,24–27], mouse skin [25–27] and human skin [28]. Along with the reduced DNA damage, we saw fewer sunburn cells (apoptotic keratinocytes) in mice and humans [25,26,28]. This effect of 1,25D to reduce CPD is concentration dependent, occurring at concentrations as low as 10^{-10} M, and occurs whether the agent is added 24 h before UV and again immediately after UV or just after UV [27].

3. Photoprotection by D compounds is via a non-genomic pathway

As previously reported, the *cis*-locked vitamin D analog, 1α,25-dihydroxylumisterol3 (JN), which has limited binding activity in the classical assay and almost no transactivating activity [29], mimics the photoprotective actions of 1,25D in reducing CPD and skin cell apoptosis, with much the same potency as 1,25D itself [25,26]. Further, the protective actions of 1,25D are unaffected by an antagonist of the genomic pathway, but completely reversed by an antagonist of the non-genomic pathway – 1β,25-dihydroxylumisterol3 [25]. Both these results support the proposal that photoprotection is mediated by a non-classical/non-genomic pathway.

4. Protection from UV-induced immune suppression

UV exposure results in immune suppression. Our studies in hairless mice showed that topical application of 1,25D reduced systemic immune suppression measured 2 weeks after the UV exposure [25], though 1,25D can be immunosuppressive under other conditions [30]. Since increased CPD and reactive nitrogen species both contribute to UV-induced immunosuppression [11,31], it is likely that both the reduced CPD and reduced reactive nitrogen species measured post-UV in the presence of 1,25D contribute to the reduced immunosuppression. An altered cytokine profile is probably a downstream effect [32]. Expression of the pro-inflammatory interleukin 6 (IL-6) protein in mouse skin 48 h after UV was markedly reduced by topical treatment with 1,25D (Fig. 2). This result *in vivo* complements a finding that 1,25D also reduced UV-induced IL-6 in human keratinocytes in culture [23]. The Skh:hr1 hairless mouse is a well established model for photodamage and photocarcinogenesis, but interpretation of results with vitamin D compounds may be complicated by observations that the *hairless* gene, which is mutated in these mice, regulates the genomic actions of the vitamin D receptor [33]. This might be less important if, as seems to be the case, the photoprotective mechanism is non-genomic. As might be expected for an agent which reduces UV-induced DNA damage and immunosuppression, preliminary analysis of hairless mice subjected to chronic low-dose solar simulated UV (50 exposures over 10 weeks) and treated immediately after exposure with 1,25D (22.8 pmol/cm^2) or vehicle, showed that the number of tumors per tumor-bearing mouse at 26 weeks was reduced from 2.0 ± 0.4 in vehicle-treated mice to 0.8 ± 0.2 in mice treated with 1,25D ($p < 0.01$). This result is consistent with studies showing that 1,25D and related analogs inhibit chemical carcino-

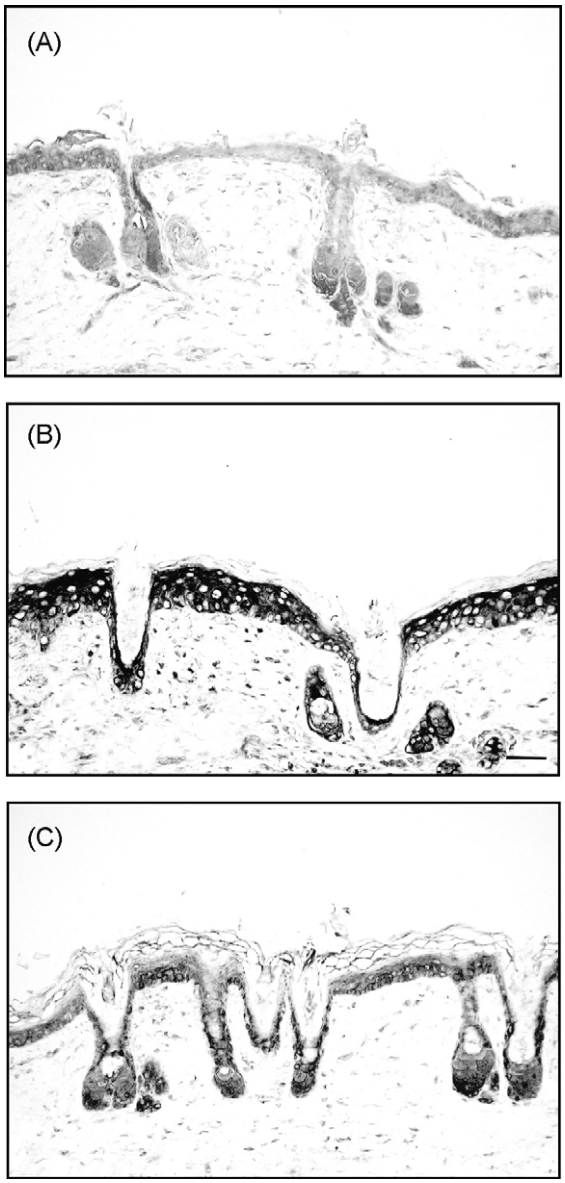

Fig. 2. Reduction of UV-induced IL-6 expression by topical application of 1,25D in mouse skin. Immunohistochemical detection of IL-6 in Skh:HR-1 hairless mice skin was with a monoclonal antibody to IL-6 (R&D Systems, Minneapolis, MN, USA) and a biotinylated secondary rabbit anti-goat IgG (Vector Laboratories, CA, USA). Figures are representative dorsal skin sections (A) non-irradiated skin or (B) after solar simulated radiation followed by 48 h treatment with vehicle, or (C) after solar simulated radiation followed by 48 h treatment with 1,25D (22.8 pmol/cm^2).

genesis in skin [34,35], while photocarcinogenesis is enhanced in vitamin D receptor knock-out mice [36].

5. Mechanisms of photoprotection

Incubation of UV-irradiated human keratinocytes with 1,25D results in a striking further enhancement of nuclear p53 expression above that produced by UV exposure alone [27]. Increased p53 expression is known to cause cell cycle arrest and to facilitate DNA repair [37]. We have also reported that 1,25D reduced nitric oxide products, measured as nitrite, after UV irradiation in keratinocytes, to an extent similar to that seen with the nitric oxide synthase inhibitor, aminoguanidine [27]. Reduction in nitric oxide and reactive nitrogen species also could be expected to enhance DNA repair [38]. CPD repair is by nucleotide excision repair (NER), a complex multistep process involving several different proteins. The XPG protein (xeroderma pigmentosum, complementation group G), is a key factor in this process [39]. It is responsible for the 3′ incision made during NER and works by stabilizing the NER pre-incision complex and is essential for the 5′ incision by another enzyme, XPF [40]. As shown in Fig. 3, there was no difference in XPG mRNA expression in keratinocytes treated with vehicle or 1,25D up to 5 h after UV. This does not exclude the possibility that there may be differences in activity of XPG or in the expression or activity of other proteins involved in NER. Yet the time-course of protection from UV-induced CPD [27] also is not entirely consistent with a mechanism solely of enhanced repair. CPD repair is relatively

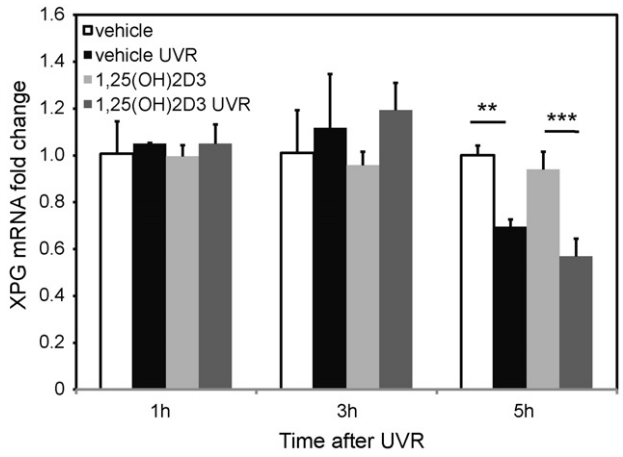

Fig. 3. Expression of XPG mRNA after UV in the presence of 1,25D or vehicle. XPG mRNA was quantified in extracts of keratinocytes treated pre- and post-UVR with vehicle or 1,25D (10^{-9} M) using TaqMan real-time RT-PCR fluorogenic probes and a ABI PRISM 7700 sequence detector system (Applied Biosciences, Foster City, CA, USA) at 1, 3 and 5 h post-UVR. Expression of XPG was measured as fold change in XPG mRNA ± SD using non-irradiated vehicle-treated keratinocyte mRNA as the calibrator sample; $^{**}p < 0.01$ significantly different from non-irradiated controls.

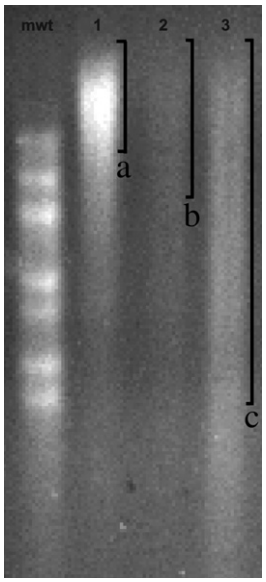

Fig. 4. Nitric oxide induction of CPDs in the absence of UV. DNA was extracted from unirradiated and vehicle-treated keratinocytes (lane 1), keratinocytes incubated for 3 h at 37 °C in the dark with SNP (2 mM) (lane 2) or keratinocytes subjected to UV radiation (lane 3) then subjected to digestion with T4 endonuclease V (T4NV; 10 units/10 μg DNA; Epicentre Biotechnologies, Madison, WI, USA) for 30 min at 37 °C. Digested DNA samples were electrophoresed on a 0.4% agarose gel in 50 mM NaCl, 4 mM EDTA buffer at 30 V for 18 h. Intact or less digested DNA shows as bright areas or smears in the lanes. a: largely intact DNA, b: virtually no intact DNA and c: smear showing little intact DNA. mwt: molecular weight markers.

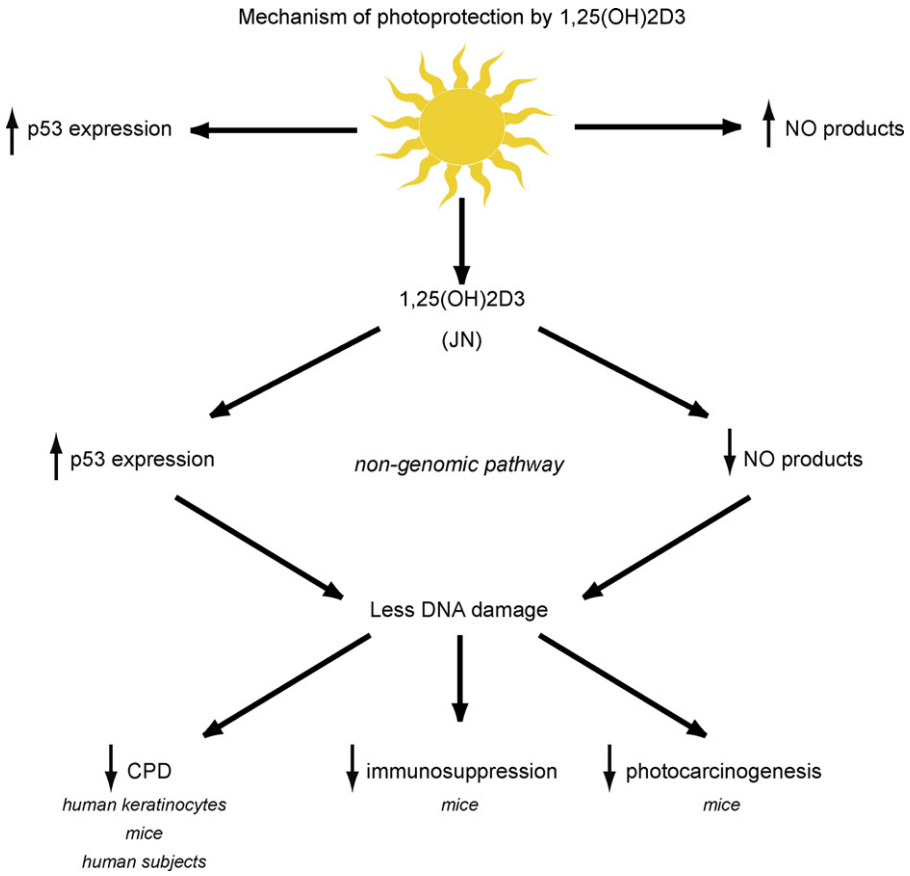

Fig. 5. Mechanism of photoprotection by 1,25(OH)2D3 and analogs. See main text.

slow with a half-life of 7 h or more [8], yet protection is seen with 1,25D as soon as 30 min after UV. Moreover, in that study, as in others, CPD appear to increase in keratinocytes up to 6 h after UV. Since CPD may be induced through photosensitized energy transfer [6] and by a purely chemical reaction [5], we tested whether an agent which increased reactive nitrogen species, a nitric oxide donor sodium nitroprusside (SNP), was able to induce CPD in the dark. The gold-standard technique for detection of CPD uses treatment of nuclear extracts with T4N5, an enzyme which specifically cuts DNA at the site of CPD followed by alkaline gel electrophoresis [41]. Fig. 4 shows the results of a typical gel. Intact DNA after enzyme digestion from unirradiated, vehicle-treated keratinocytes in lane 1 is shown as a bright area near the top of the image. In contrast, there is almost no intact DNA visible in lane 2, which shows enzyme digested DNA from cells treated with SNP (2 mM) in the dark, and only a white smear of partly digested DNA is visible in lane 3 from cells subjected to UV irradiation. Quantitation of remaining intact DNA [41], from this experiment using densitometry measurements by Image J software, showed that whereas more than 90% of DNA from unirradiated cells was intact after digestion with T4N5, intact DNA after digestion was less than 20% after incubation with SNP (2 mM) and less than 50% after UV irradiation. These results indicate the presence of substantial numbers of CPD in both SNP-treated and UV-treated keratinocytes. In light of this data, since 1,25D reduces reactive nitrogen species after UV [27], it may reduce CPD formation as well as enhancing CPD repair.

6. Conclusion

The mechanism we propose for photoprotection by 1,25D is shown in Fig. 5. Exposure of skin cells to UV causes an increase in nuclear p53 expression, which facilitates DNA repair and an increase in nitric oxide products which both reduce DNA repair and increase indirect CPD formation. Application of 1,25D immediately after UV exposure further enhances p53 expression and suppresses nitric oxide products. Both these actions would result in less DNA damage, with reduced CPD, reduced immunosuppression and reduced photocarcinogenesis and there is evidence for these effects in a number of systems. Observations that the non-genomic analog, JN mimics the actions of 1,25D and that a non-genomic antagonist abolishes the effects, support the proposal that this photoprotection is principally via the non-genomic pathway. UV increases 1,25D concentrations in skin, but this process takes several hours [14,15]. This endogenous delay makes it possible to demonstrate that exogenous 1,25D is photoprotective. It also means that like increased cornification and increased pigmentation, increased concentrations of D compounds in skin act to protect against the next, rather than the initial UV exposure. Vitamin D metabolites may be the diffusible factor predicted by work that showed early UV adaptation after UV, before cornification or pigmentation would be likely to develop [42]. These studies raise the possibility that analogs of 1,25D might be successfully incorporated into a sun-screen or after-sun lotion to reduce the damaging effects of UV exposure.

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