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Melanogenesis: a Realistic Target for Antimelanoma Therapy?

P.A. Riley

Melanin is a widely-distributed pigment in the biosphere. In the human adult, the enzymatically-catalysed process of melanin generation is the exclusive prerogative of melanocytes. Melanogenesis generates a number of reactive intermediates including orthoquinones and has been recognised as a potential hazard to melanocytes. Amplification of this cytotoxic hazard to selectively damage malignant melanogenic cells has been investigated as a rational therapeutic strategy for melanoma. A number of surrogate substrates for tyrosinase have been studied, including a range of phenols and catechols. Initial attempts to use these agents for the treatment of disseminated melanoma have foundered on problems due to unfavourable pharmacokinetics, primary toxicity or pharmacological actions of the analogue substrates, and the toxicity of hepatic metabolites. Successful exploitation of the undoubted potential of the metabolic targeting strategy presented by the subversion of melanogenesis depends on the development of prodrugs with minimal primary toxicity and improved pharmacokinetics. The range of possible novel approaches is being extended by the emergent understanding of the complexities of melanogenesis which are outlined.
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MELANINS

MELANINS ARE highly conjugated bathochromic aromatic polymers of uncertain structure. Although melanins are widely distributed in nature, it is not clear what evolutionary pathway has led to their generation. In animals, melanins generally comprise a high proportion of indoles which copolymerise with other residues to give a range of macromolecular pigments [1, 2]. Although the pronounced light and heat-absorbing properties render melanins a potentially important photoprotective, and possibly phonoprotective, pigment in vertebrates, the selective forces acting at earlier stages of evolution are likely to have been connected with the advantages conferred by the initial metabolic steps in its biosynthesis [3].

In mammals indolic melanin is metabolically derived from the aminoacid L-tyrosine by a complex process. The initial oxidation involves ring hydroxylation and subsequent dehydrogenation of tyrosine (see Fig. 1, structure **1**) to form the corresponding L-phenylalanine-3,4-orthoquinone [dihydroxyphenylalanine quinone, or dopaquinone (DQ, structure **3**)]. The enzyme catalysing this oxidation is tyrosinase which, whilst exhibiting stereospecificity with regard to the aminoacid side chain, is able to oxidise a range of analogous phenols and catechols. The corresponding orthoquinones are reactive molecules which readily undergo redox reactions and reductive addition reactions with nucleophiles (Michael addition [4] reactions). The capability of generating reactive quinones with irritant properties is important in defensive sprays of certain arthropods [5] and is probably the physiological basis of the action of the supposed obfuscating ink of cephalopods. Enzymatically-generated orthoquinones are involved in the process of cuticular hardening in insects [6]. Whether there are important functions of melanin precursors that have preserved this metabolic pathway in mammals is not known, but the relatively modest nature of the abnormalities present in albinos, in which mutations of the tyrosinase gene [7] result in greatly reduced levels of quinone generation, give little indication of such a role. Nevertheless, as a general rule, melanogenesis is ubiquitous among vertebrates and is a predominant function of a set of neural crest-derived dendritic cells, the melanocytes.

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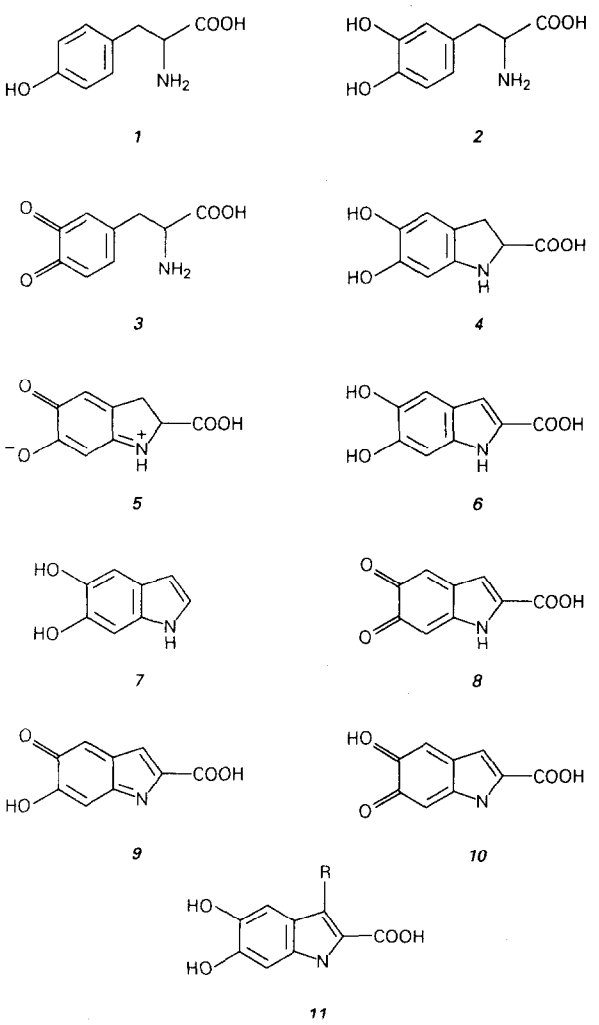


Fig. 1. Structural formulae of melanogenic precursors. With the exception of 5 the structures are shown in the un-ionised form. 1 = L-tyrosine; 2 = dihydroxyphenylalanine (dopa); 3 = dopaquinone (DQ); 4 = cyclodopa (CD) (leucodopachrome); 5 = dopachrome (DC); 6 = 5,6-dihydroxyindole-2-carboxylic acid (DHICA); 7 = 5,6-dihydroxyindole (DHI); 8 = indole-2-carboxylic acid-5,6-quinone (ICAQ); 9 = ICAQ-imine; 10 = ICAQ-methide; 11 = nucleophilic addition product at C3 of the indole ring, e.g. if R = OH the product illustrated would be 3,5,6 trihydroxyindole-2-carboxylic acid.

MELANOGENESIS

Melanogenesis may be viewed in two distinct stages (Figs 2 and 3): phase I in which the basic process is the tyrosinase-catalysed generation of the orthoquinone species; and phase II, the polymerisation process which can be regarded as a mechanism for reducing the steady-state concentration of intracellular orthoquinones and may involve further enzyme-catalysed steps. The basic step where tyrosinase is oxidised is the generation of dopaquinone which is able to undergo facile redox reactions with catecholic compounds, including those formed subsequently in the melanogenic pathway. Because tyrosinase is able rapidly to reoxidise 3,4-dihydroxyphenylalanine (dopa, 2), i.e. the reduced product of dopaquinone, a "dopa cycle" (Fig. 2) is established as the oxidative process which brings about vicarious oxidations at subsequent stages of the melanogenic pathway (Fig. 3). Despite the fact that tyrosinase has been shown to be capable of directly oxidising substrates such as 5,6 dihydroxyindole (DHI) [8, 9], their oxidation by dopaquinone and the reoxidation of dopa by tyrosinase is more efficient [10]. The processes involved in phase I melanogenesis were characterised in the classical studies of Raper [11] and confirmed and extended by H.S. Mason. The Raper–Mason pathway [12] describing the production from tyrosine of indolene-2-carboxylic acid-5,6-quinone (usually called dopachrome and written in the Zwitterionic isomeric form, 5) is illustrated in Fig. 2. In this eumelanogenic pathway dopaquinone undergoes endocylisation by molecular rearrangement of the amino group on the side chain to give the corresponding indolene compound, cyclodopa (CD, 4) and this is rapidly oxidised by redox exchange with dopaquinone to give rise to dopachrome whilst simultaneously reducing dopaquinone to dopa. Dopa is reoxidised by tyrosinase and this enzyme-catalysed oxidation gives rise to the oxidation cycle that drives the melanogenic process as mentioned above. Dopachrome is a relatively stable compound possessing limited reactivity. Thus, the disproportionation of dopaquinone illustrated in Fig. 2 is a mechanism which limits the dopaquinone concentration by generating a product with lower oxidation potential which presents less hazard to the cell.

There is now strong evidence that the further metabolism of dopachrome involves the enzyme dopachrome tautomerase [13–16] and this constitutes the first step of phase II melanogenesis [17], the details of which have yet to be elucidated but which leads eventually to the formation of the melanin polymer. Some progress in the understanding of phase II melanogenesis was discussed at a recent meeting in Manchester, in the Paterson Institute Symposium series, at which Prof. Hugo Wyler (Lausanne) showed that the mechanism of conversion of dopachrome to 5,6 dihydroxyindole-2-carboxylic acid (DHICA, 6) involves three successive deprotonations and that, therefore, an enzyme is necessary to catalyse the process at neutral pH. The preferential generation of DHICA may be important since the decarboxylated derivative [5,6 dihydroxyindole, DHI, 7] which can form spontaneously under certain conditions [18] possesses different reactivity and may be potentially more toxic. The tautomerisation of dopachrome involves desaturation of the indolene ring with concomitant reduction of the quinone function. DHICA formed from dopachrome is readily oxidised by a redox reaction with dopaquinone to yield indole-2-carboxylic-

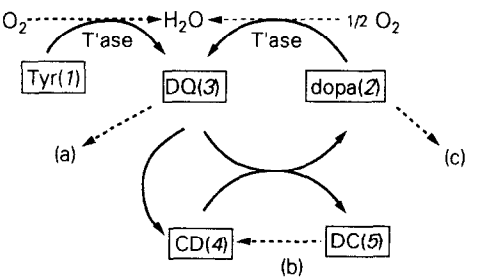


Fig. 2. Phase I melanogenesis. Schematic representation of the initial stage of melanogenesis. Tyrosine (1) is oxidised by tyrosinase to dopaquinone (DQ, 3) which is able to undergo three types of reaction: endocyclisation to yield cyclodopa (CD, 4), reductive addition reactions with nucleophiles [indicated by the pathway marked (a)], and redox reactions to give rise either to semiquinone radicals (not shown) or to dopa (2). This latter reaction accounts for the oxidation of cyclodopa to dopachrome (DC, 5) which is able in turn to be reduced under some conditions (e.g. in the presence of ascorbate) as indicated by the pathway (b). Dopa may act as a substrate for other enzyme-catalysed reactions (e.g. methylation by COMT) indicated by (c), but the major pathway is its oxidation by tyrosinase to form dopaquinone (DQ, 3). This tyrosinase-catalysed dopa/dopaquinone redox cycle acts as the oxidative drive of melanogenesis.

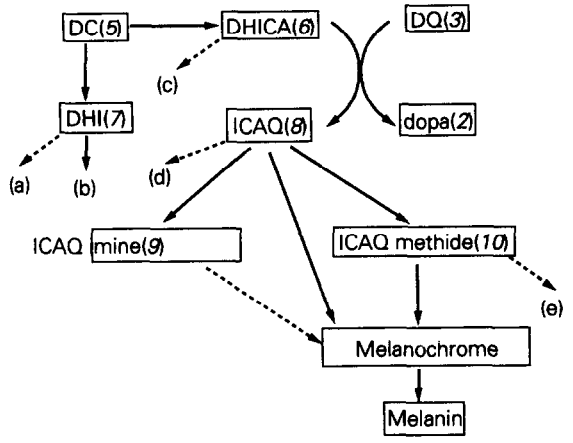
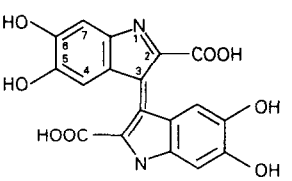


Fig. 3. Phase II melanogenesis. Schematic outline of events in melanogenesis subsequent to the formation of dopachrome. The major pathway catalysed by dopachrome tautomerase converts dopachrome (DC, 5) to dihydroxyindole-2-carboxylic acid (DHICA, 6). A minor pathway results in decarboxylation of dopachrome with the formation of dihydroxyindole (DHI, 7) which may be methylated (a) or undergo polymerisation (b) (see structures 13 and 14 in Fig. 4). DHICA may also be methylated (c) but in the presence of the "dopa cycle" is oxidised to indole-2-carboxylic acid-5,6-quinone (ICAQ, 8). ICAQ will take part in reductive addition (d) and polymerisation reactions possibly giving rise to "melanochrome". These may be direct or via the tautomeric imine (9) or methide (10) forms. The latter reaction is favoured because of the greater reactivity of the methide which may also take part in reductive addition reactions (e) to give products of the type indicated by structure 11 (Fig. 1).

acid 5,6-quinone (ICAQ, 8) and dopa (see Fig. 3). Kinetic studies by two groups [19, 20] using pulse radiolysis of hydroxylated and methoxylated indoles indicate that indole quinones can exist in three isomeric forms (Fig. 1) and evidence is accumulating to suggest that the formation of the methide isomer (**10**) may be crucial to subsequent stages in polymerisation and melanin formation [21].

Evidence from work on insect cuticular sclerotisation suggests that enzymatic control of the relative proportions of indole quinone isomers [22, 23] determines to what extent such compounds take part in the reactions leading to hardening of the cuticle (sclerotisation) or are involved in pigmentation of the exoskeleton [24]. It is possible that similar considerations apply to phase II melanogenesis in mammals and their enzymatic control may suggest functions for some of the melanocyte genes that have been recently cloned but for which functions are presently unknown [25–28]. ICAQ methide will react with nucleophiles to generate adducts at the C3 atom of the indole and the pulse-radiolysis data of Lambert *et al.* [20] are consistent with addition of water to form a tri-hydroxyindole species (**11**). Clearly other nucleophilic addition e.g. by thiols may occur at C3, and this may regulate the extent of melanin polymerisation by diversion of indole metabolism. If this is the case it strongly implicates the C3 carbon of ICAQ methide in the polymerisation process and a dimer generated by copolymerisation of ICAQ methide (Fig. 4) might form a structural unit of melanin. This hypothetical 3–3 dimeric melanin precursor (**12**) needs to be conjugated to be consistent with the characteristics of the absorption spectrum of "melanochrome". Further copolymerisation of such dimers is possible to enable extension of the conjugated syndiotactic polymer chain. A feature of this proposed reaction pathway is that it explains the inability of dopachrome to initiate polymerisation of melanin, since it is unable to form a methide isomer because the C3 carbon of the indolene ring is saturated. However, it must be emphasised that at the present time there is no clear agreement on the details of the polymerisation process. Polymerisation of DHICA by condensation at the 4 and 7 positions (Fig. 4, structure **15**) has been proposed [29] and NMR analysis of tyrosinase-generated oligomers of DHICA has confirmed the formation of 4–4′, 4–7′ and 7–7′ linkages [30]. Spontaneous (metal-catalysed) oxidation of DHI gives rise to a purple pigment which probably has the structure illustrated (**14**). In the case of DHI oxidised by tyrosinase the preferred site of condensation (structure **13**) is at the C2 carbon giving rise to 2–4′ dimers [30]. Further work is required to clarify phase II melanogenesis.

Nevertheless, what is abundantly clear is that melanogenesis involves as intermediates several highly reactive compounds which may possess important physiological actions but which constitute a potential hazard to melanocytes.



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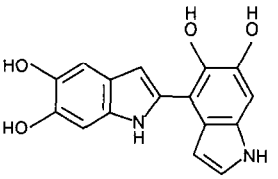

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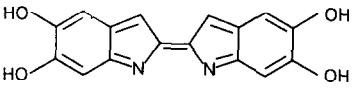

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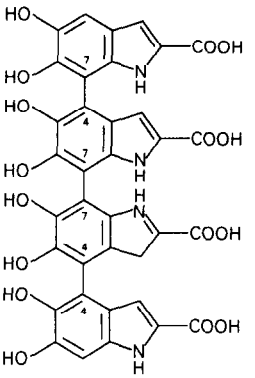

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Fig. 4. Proposed oligomeric indole condensation products. Hypothetical structure of melanochrome (12) arising from condensation of two molecules of the ICAQ-methide (10) with 3–3′ linkage. Subsequent polymerisation may involve 7–7′ condensations of this dimer. The structure (13) observed in the case of DHI dimerisation in the presence of tyrosinase favours 2–4′ linkage although 2–2′ linkage (14) occurs in metal-catalysed dimerisation. Since the C2 position is blocked by the carboxyl group in DHICA the alternative to C3 linkage is through the C4 and C7 carbons as illustrated in the tetramer (15). Natural melanins are known to be hypoindolic, i.e. to include other intermediates of the melanogenic pathway. This applies *a fortiori* to phaeomelanins [30].

PROTECTIVE MECHANISMS

Several protective mechanisms exist in melanocytes to diminish the hazard posed by the process of melanogenesis. The reactions are normally confined to specialised membrane-bound organelles so that the reactants are segregated from the remainder of the cytosol. These melanosomes contain a protein framework as well as the appropriate enzymes necessary for pigment generation. Electron microscopy of partially melanised granules [31] indicates that the melanin is deposited in a pattern imposed by the protein architecture. In view of the strong association of melanin with melanosomal structural proteins, it is likely that reductive addition reactions to nucleophilic groups in amino-acids are involved in a manner analogous to sclerotisation and tanning. The process also results in inactivation of the melanogenic enzymes; completely melanised melanin granules, which are transferred to surrounding structures, exhibit no residual tyrosinase activity. Thus, the potential cytotoxic hazard is diminished by polymerisation and/or macromolecular capture of the reactive intermediates such as the quinones and quinone methides.

Another important set of reactions involves low molecular weight thiols which readily undergo reductive addition reactions to quinone products of tyrosine oxidation. It appears that cysteine is the major reactant in this category present within melanosomes and nucleophilic addition of cysteine to dopaquinone gives predominantly 5-S-cysteinyldopa [32] which is regarded as an important branching point in the generation of phaeomelanins [33]. Whilst reactions involving nucleophilic addition of glutathione are of little significance in normal melanogenesis [34] this reaction may constitute a mechanism to protect the cell from quinones that have leaked out of the melanosome. Glutathione-dopa is formed in melanogenic cells [35] and is found in the plasma and urine.

An additional protective mechanism consists of the enzyme catechol O-methyl transferase (COMT) which has been identified in melanocytes [36, 37]. This enzyme, which appears to be located largely in the cytosol, is able to methylate one or more of the hydroxyl groups of a variety of dihydroxy compounds including those generated during melanogenesis, in particular, 5,6-dihydroxyindole (DHI, **7**) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA, **6**). The methylated derivatives are excreted in urine and constitute a useful indirect method of quantitating melanogenesis and their detection has been used in diagnosis of melanoma and monitoring of antimelanoma therapy [38]. The methylation of dihydroxy species exerts an important protective function since many catechols readily undergo auto-oxidation to generate reactive oxygen species (ROS), such as hydrogen peroxide, which are damaging to cells. Protective mechanisms including glutathione peroxidase and catalase exist for metabolising ROS generated by catechols autoxidation. In this regard it is interesting to note the co-existence of tyrosinase and catalase activity in melanosomes, and the suggestion that part of the sequence of tyrosinase may represent a haem attachment site accounting for catalase activity of tyrosinase [39]. However, recent oximetric data have adduced evidence that the catalase activity of tyrosinase is copper-dependent (Naish-Byfield, Brunel University).

THERAPY OF DISSEMINATED MELANOMA

The effectiveness of existing therapy against disseminated melanoma is inadequate and radiation therapy has been found generally to be ineffective. Most of the drugs currently used against melanoma exhibit less than 25% of responses [40, 41] and improvements in the chemotherapy for patients with disseminated melanoma is an important priority. Since many metastatic cells in disseminated melanoma are dormant or slowly proliferating, therapy based on selective damage to rapidly proliferating cells is inadequate and a need for novel targetting strategies is clear. Melanoma-targetting strategies based on specific cell surface markers [42, 43] and on melanin-targetted radiotherapy [44–46] are currently under investigation. However, the possibility of amplifying the potential toxicity of the melanocyte-specific process of melanogenesis itself by diversion of the intermediate oxidative products has been viewed for a long time as an attractive rational approach to melanoma targetting.

SUBVERSION OF MELANOGENESIS

This approach was suggested by the discovery that the mode of action of phenolic depigmenting agents is dependent on their ability to act as alternative substrates for tyrosinase catalysed oxidation [47–49]. Oxidation of phenols gives rise to reactive orthoquinones which become diverted from the normal melanogenic pathway. In principle, the toxicity of these quinones depends on their relative stability which results from the absence of a spontaneously endocyclysing side chain. Such products, provided they have appropriate solubility characteristics, are able to penetrate the melanosomal membrane and initiate toxic damage in melanocytes. Although it has been previously proposed [50] that the cytotoxic mechanism involves the initiation of lipid peroxidation by semiquinone radicals, which are known to be formed as a result of oxidation of analogue substrates [51], recent evidence [52, 53] suggests that the toxic mechanism predominantly involves the interaction of orthoquinones with crucial protein thiols.

A range of tyrosine analogues have been investigated as potential antimelanoma agents. Tyrosinase is able to catalyse both ortho-hydroxylation phenols and dehydrogenation of the corresponding dihydroxyphenols, and a number of phenols and dihydroxyphenols have been used as potential melanocytotoxic agents [54–60]. There is now considerable evidence that the cytotoxic effect of catecholic compounds is very largely the result of auto-oxidation of these compounds with the generation of reactive oxygen species [61, 62]. In addition, there are problems posed by the primary toxicity and the pharmacological activity of catechols.

Attempts to use phenolic substrates have also been impeded by primary toxic actions of phenols. For example, 4HA has been shown to have effects on ribonucleotide reductase activity [63], mitochondrial electron transport [64], and other aspects of cell physiology [65, 66], including the induction of micro-invasion [67]. Moreover, the cytotoxic effects of hepatic metabolites of these compounds pose a serious difficulty to their clinical use. The metabolic pathway of elimination of 4HA has been delineated by Pavel *et al.* [68] who have shown that in mice approximately 20% of the drug is metabolised through the intermediate generation of the 3,4-dihydroxy species. This material is probably generated by P450 metabolism and is potentially toxic through its auto-oxidation products. This limits the therapeutic concentration that can be achieved by parental administration, and serious hepatic and renal toxicity has been encountered in clinical practice (G. Rustin, Mt. Vernon Hospital). The human pharmacokinetics of 4HA infusion have been delineated [69] and the details of hepatic metabolism and *in vitro* cytotoxicity have recently been investigated [70]. 4HA has been investigated in clinical pilot studies [71–73]. Responses

have been observed mainly in cases treated for recurrent melanoma of the lower limb, the drug being given by intra-arterial infusion [71, 73]. An interesting observation relating to the responses is that they are usually delayed (B.D.G. Morgan, Mt. Vernon Hospital) suggesting the possibility that the mechanism of action involves the generation of neo-antigens which in some patients elicit an immune response. Jimbow and colleagues [60] have shown that 4-S-aminophenol derivatives have marked selective depigmenting action due to a specific cytotoxicity to melanocytes in hair follicles of black mice, and specific cytotoxic effects on implanted melanotic tumours in animals have been demonstrated. Although there is still no agreement on the precise cytotoxic mechanism, it seems likely that this is similar to the para-oxy-ethers such as 4HA.

If melanocyte-specific cytotoxicity is the result of tyrosinase-catalysed oxidation of the compounds to give rise to the corresponding reactive orthoquinone, it is possible that by inhibiting the melanocyte defences against the toxic action of diffusible and relatively stable quinones, would increase the specific cytotoxicity. Such procedures, including the reduction of the cellular glutathione levels and inhibition of COMT, remain to be investigated although some promising *in vitro* data have been published on the effects of BSO in lowering intracellular glutathione and increasing the toxicity of a catecholic substrate [74]. However, in view of the importance of glutathione peroxidase in diminishing damage due to catecholic auto-oxidation products these data may not be decisive.

Another approach could involve increasing the permeability of the melanosomal membrane so as to increase the likelihood of leakage of reactive melanogenic intermediates into the cytosol. An interesting aspect of this idea is that there is evidence of a considerable degree of abnormality of the melanosomal membrane in melanoma cells [75].

CONCLUSIONS

Subversion of melanogenesis to generate cytotoxic species from tyrosine analogues remains a promising rational approach to the chemotherapy of disseminated melanoma but there are many serious obstacles to its acheivement. It is possible that the emergent understanding of phase II melanogenesis will suggest novel variations on this strategy. Successful application will depend on the synthesis of structural analogues of melanogenic precursors that are devoid of significant tyrosinase-independent toxicity.

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