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Radicals and Melanomas [and Discussion]

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Radicals and melanomas

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The synthesis of melanin involves the oxidation of phenolic substrates by the enzyme tyrosinase. In vertebrates tyrosinase is present only in specialized cells (melanocytes), where it catalyses the oxidation of tyrosine and certain diphenolic intermediate products to quinones which polymerize to give rise to melanin. This specialized metabolic pathway provides a possible approach to the specific chemotherapy of malignant tumours of pigment cells (malignant melanoma). Certain analogues of tyrosine are oxidized by tyrosinase generating reactive orthoquinones with cytotoxic potential. One such analogue, 4-hydroxyanisole, has been investigated as a possible specific melanocytotoxic precursor. The parent compound inhibits DNA synthesis but exhibits little general toxicity, while the tyrosinase oxidation products are highly toxic to cells. The mechanism of this toxicity may involve semiquinone radicals. Encouraging initial results have been obtained from clinical pilot studies using intra-arterial infusion of hydroxyanisole in patients with localized recurrences of malignant melanoma.

Introduction

Melanins

One of the most widespread surface pigments in the animal kingdom is 'melanin', a term which is thought to have been first used by Berzelius to refer to dark pigments without specific chemical implications other than those of relative insolubility. Subsequent attempts to derive a more specific chemical definition have foundered because the detailed structures of most melanins are unknown, but they are formed by the copolymerization of quinones to give rise to structures in which there is sufficient conjugation between aromatic rings to lower the quantal energies required for light absorption: an effect sometimes termed 'bathochromicity'. The highly conjugated structure of melanins permits electron movement and electron exchanges with neighbouring molecules to take place relatively easily (Sealy *et al.* 1980). Commoner *et al.* (1954) were the first to demonstrate the presence of free radicals in melanins, and it was subsequently shown that the free-radical property of melanins is a result of semiquinones stabilized by resonance in the highly conjugated polymer and by steric restrictions on internal radical annihilation reactions (Mason *et al.* 1960).

Melanogenic pathway

Melanins are generally classified according to their predominant component, which in most cases is an oxidation product of the amino acid tyrosine. The metabolic pathway was first described by Raper (1928) and subsequent studies have established that the enzyme tyrosinase catalyses several oxidative steps leading to the formation of an indole-containing polymer (scheme 1). The evolutionary importance of this pathway is not clear (Riley 1977). Quinones

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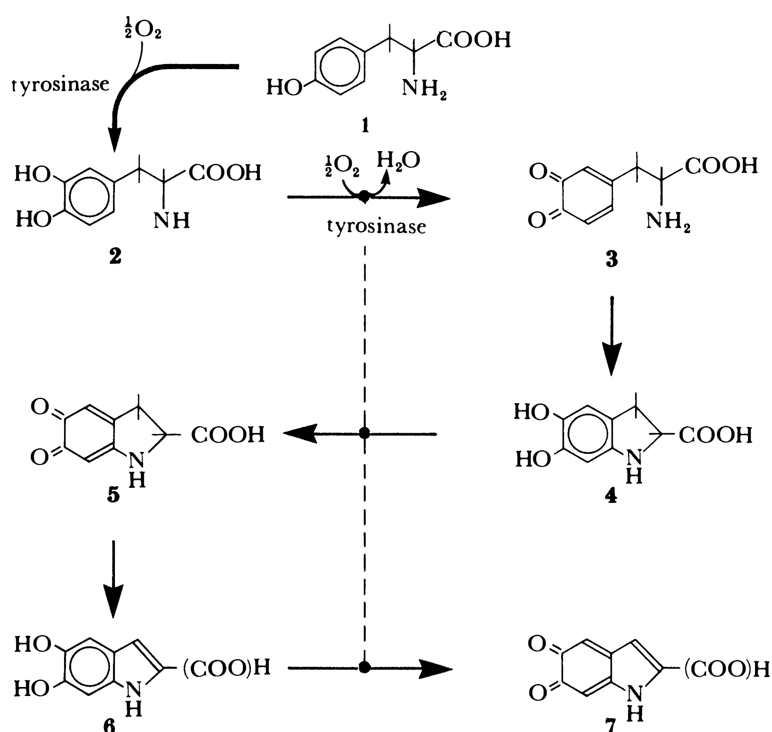

Scheme 1. Generalized scheme of the melanogenic pathway illustrating the formation of melanin precursors from tyrosine. The scheme shows that tyrosinase catalyses the hydroxylation of tyrosine (**1**) to 3,4-dihydroxyphenylalanine (**2**) and the dehydrogenation of dopa to dopaquinone (**3**). Spontaneous formation of the indolene ring **4** is followed by an oxidation step that is either a result of direct enzymatic action or to a redox exchange with dopaquinone being reduced to dopa and subsequently reoxidized by the enzyme. Dopachrome (**5**) undergoes spontaneous reduction with rearrangement to give 5,6-dihydroxyindole-2-carboxylic acid (**6**), which is reoxidized to indole-2-carboxylic acid 5,6-quinone (**7**) by either the direct or indirect mechanism described above. The possible linked reactions are indicated by the broken line. In some cases spontaneous decarboxylation leads to dihydroxyindole formation, and this is indicated by the brackets in structures **6** and **7**.

are found in the defensive secretions of several arthropods and millipedes, and are present in the surface secretions of many small insects. The protein-tanning effect of quinones seems to be of importance in the hardening of insect cuticles and in sclerotization reactions in other invertebrates. As pigments, the melanins have importance in the protective coloration of many species, and in man, melanins appear to be important in reducing radiation damage to exposed structures such as the skin and the retina.

Melanocytes

In adult mammals, melanogenesis takes place exclusively in specialized pigment cells (melanocytes) that are embryologically derived from the neural crest. These cells are located in the eye, the skin, the hair bulbs and other sites, and synthesize melanin which is donated to surrounding cells. Melanocytes generate melanin in specialized intracellular organelles known as melanosomes, which contain the active form of the enzyme tyrosinase. The melanin is deposited on a protein matrix within these organelles to give rise to pigment granules that are transferred to surrounding cells by a process in which portions of the melanocyte cytoplasm containing the melanin granules are phagocytosed.

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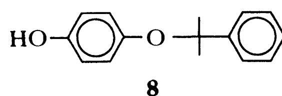
Melanomas

Tumours of melanocytes are known as melanomas and the malignant variety of melanoma is an extremely aggressive cancer. There is evidence that the incidence of malignant melanoma is increasing and affects a relatively young age group. The increased incidence of malignant melanoma is thought to be related to sun exposure on the basis of epidemiological evidence (Lee & Strickland 1980). Many malignant melanomas generate melanin, or melanin precursors, in large quantities and the tumour load may be estimated from the excretion of urinary melanogens. Therefore, it has for many years been the aim of rational chemotherapy for these tumours to use the melanogenic pathway as a means of targeting cytotoxic therapy.

Depigmentation

Phenolic antioxidants

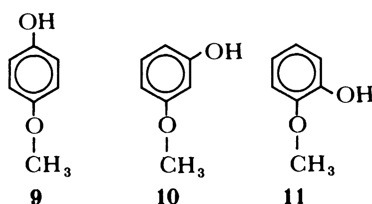
Our approach to this problem was prompted by investigations of the mechanisms of action of certain depigmenting agents. It was found by Oettel (1936) that dosing black cats with hydroquinone caused their hair to turn grey within six to eight weeks, and some time later it was shown that the monobenzyl ether of hydroquinone **8**, which was used as an antioxidant



in rubber gloves, caused severe and extensive depigmentation of the skin of black workmen in a tanning factory (Oliver *et al.* 1940). The depigmenting effects of phenolic compounds in man have been reviewed elsewhere (Searle & Riley 1979). Peck & Sabotka (1941) performed experiments on the depigmentation caused by the monobenzyl ether of hydroquinone when applied to the skin of guinea pigs and humans and showed that the microscopic depigmentation possessed the features of vitiligo. Extensive studies of depigmentation were made by Brun (1961), who showed that a number of antioxidants caused loss of pigmentation of the areolar skin of guinea pigs. Of the agents tested the most effective long-term depigmenting agent was 4-hydroxyanisole (**9**, 4-HA).

Structural requirements

The structural requirements for the depigmenting activity were investigated for a range of phenolic compounds applied topically to the ear skin of black guinea pigs. In these experiments it was found that hydroxylation in the *para* position relative to a non-polar side chain was an optimal requirement for the depigmenting activity (Riley 1969*a*). For the structural isomers of hydroxyanisole (**9**, **10**, **11**), this was shown to be independent of their rates of penetration



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into the skin. Depigmenting compounds with an ether link in the side chain were shown to be more effective than those with hydrocarbon chains.

4-Hydroxyanisole oxidation by tyrosinase

The structural resemblance to tyrosine initially suggested the possibility that loss of pigmentation was caused by competitive inhibition of tyrosinase, but it was shown that the depigmenting phenols were substrates for tyrosinase (Riley 1969*b*). Studies on cultured melanocytes showed that tritiated 4-HA is selectively incorporated into melanogenic cells in culture and transferred with pigment granules to keratocytes, suggesting that the substance is oxidized by tyrosinase and incorporated into melanin (Riley 1970). A stepwise reaction resembling the oxidation of tyrosine was suggested by spectrophotometric observations (figure 1) and the formation of a free-radical semiquinone intermediate was postulated (Riley 1969*b*),

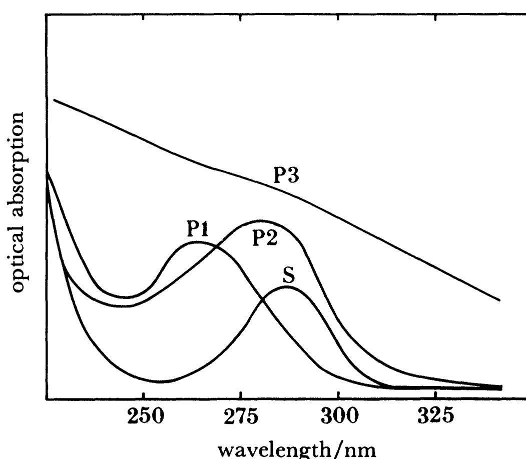

FIGURE 1. Ultraviolet absorption spectra of 4-hydroxyanisole and its tyrosinase oxidation products. The successive spectra are indicated as follows: S, substrate; P1, initial product; P2, secondary product; P3, spectral absorbance of final reaction mixture.

consistent with a new electron spin resonance signal generated in guinea pig skin depigmented by local application of 4-HA (Riley 1970). Erythrocyte suspensions exposed to a reaction mixture of mushroom tyrosinase and 4-HA showed evidence of membrane damage (Riley 1969*b*), and it was suggested that the cytotoxic action of 4-HA might be exerted as a result of the initiation of lipid peroxidation by a free-radical oxidation product (Riley *et al.* 1975). Melanocyte damage is visible in skin treated with depigmenting phenols, both at the light- and electron-microscopic levels (Bleehen *et al.* 1968; Riley 1969*a*) and this evidence makes it probable that the depigmenting action of 4-HA and related phenols is brought about by a reactive oxidation product that is able to initiate cellular damage in melanocytes.

TOXIC ACTIONS OF 4-HA

The cytotoxic actions of 4-HA have received much attention (Passi & Nazarro-Porro 1981; Breathnach *et al.* 1981; Breathnach *et al.* 1983; Nilges *et al.* 1984; Riley 1984). It appears that 4-HA has at least three main modes of action, two of which appear not to require prior

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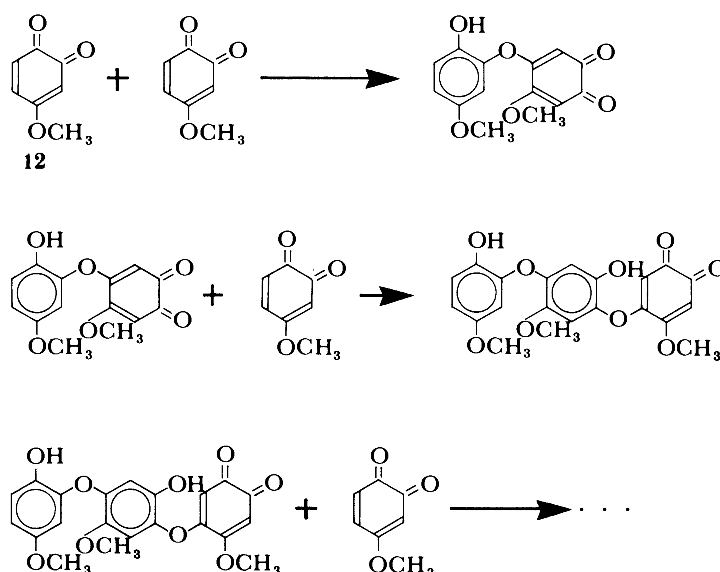
metabolism or activation of the compound. These direct actions include a phase-specific effect on the cell cycle (Galpine & Dewey 1984), probably by interference with the synthesis of DNA by inhibiting ribonucleotide reductase (Elford 1984), and an inhibition of mitochondrial oxidation (Passi *et al.* 1984). The third action depends on the oxidation of 4-HA by tyrosinase. There is now good evidence (Naish *et al.* 1985) that the initial product (P1) of tyrosinase oxidation of 4-HA is the corresponding anisyl-3,4-quinone (**12**). This may, under appropriate conditions, polymerize rapidly to melanoid products (scheme 2). Electron spin resonance



SCHEME 2. Schematic outline of proposed reactions in the linear polymerization of anisyl-3,4-quinone.

evidence (Nilges *et al.* 1984; Nilges & Swartz 1984; Tomasi 1984) shows that under certain conditions the anisyl semiquinone is formed. Whether the ultimate cytotoxic product of 4-HA is the quinone, as proposed for analogous compounds (Wick 1980), or the semiquinone species remains to be determined.

Formation of semiquinone

Nilges & Swartz (1984) have demonstrated that the semiquinone radical signal is inversely related to the oxygen concentration and interpret this finding in terms of a cycle of reduction of anisyl quinone to the corresponding hydroquinone, with simultaneous oxidation of a postulated trihydric phenol intermediate (Nilges *et al.* 1984), with enzymatic reoxidation of the 3,4-hydroquinone to the quinone. It is argued that, if the semiquinone is formed by reverse dismutation of the anisyl-3,4-quinone and the corresponding quinol, the steady-state concentration of the semiquinone will remain low while oxygen is available because the hydroquinone concentration will be small. The influence of tyrosinase on the rate of disappearance of anisyl-3,4-quinone has been examined. While the rate of removal of the quinone is significantly diminished in the presence of tyrosinase this effect cannot be the result of reoxidation of the corresponding 3,4-hydroquinone because it is also observed in a nitrogen atmosphere (figure 2). Also, recent studies of the stoichiometry of the oxidation of 4-HA suggest that the oxygen used is accounted for by the oxidation of 4-HA to anisyl quinone (figure 3). Thus, the role

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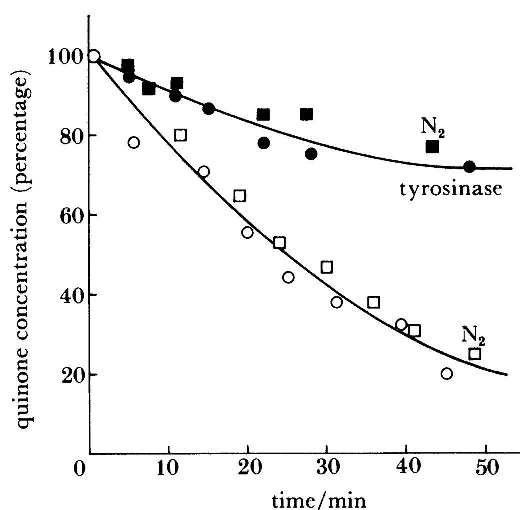

FIGURE 2. Rate of removal of anisyl-3,4-quinone in the presence and absence of tyrosinase (S. Naish, unpublished results). Anisyl-3,4-quinone (100 μM) was incubated at room temperature in 0.1 M phosphate-buffered saline (pH 7.4) in the presence (filled symbols) or absence (open symbols) of mushroom tyrosinase (15 $\mu g\ ml^{-1}$), and sampled at intervals. The quinone concentration was estimated by high-performance liquid chromatography, by a method essentially as described by Egan (1984). Incubations were performed in air (circles) and in nitrogen (squares).

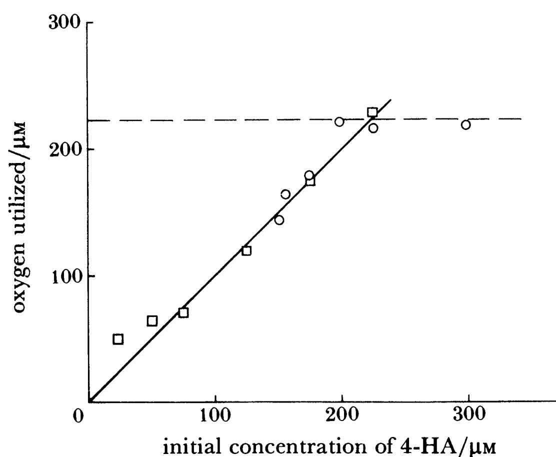

FIGURE 3. Stoichiometry of oxygen utilization as a function of 4-hydroxyanisole concentration in tyrosinase-catalysed oxidation (data of Dobrucki & Riley 1985). In two separate sets of experiments (○ and □), a range of 4-HA concentrations in 0.1 M phosphate buffer (pH 7.4) was incubated at 24 °C in the presence of mushroom tyrosinase (15 $\mu g\ ml^{-1}$), and the total oxygen utilized measured at the end of the reaction. The broken line indicates the saturating oxygen concentration in the incubation mixture (223 μM). With the possible exception of the lowest concentrations of 4-HA, the stoichiometry is close to a 1:1 molar ratio with oxygen.

of oxygen as a regulator of the semiquinone is obscure. The possibility that the concentration of semiquinone is diminished by the single-electron reduction of oxygen, with the implication that the generation of reactive oxygen species from the anisyl semiquinone might be a factor influencing the cytotoxicity of 4-hydroxyanisole, has not received support from recent e.s.r. studies (M. Davies, unpublished results), in which 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin trap for oxygen radicals.

If the anisyl semiquinone is not formed by reverse dismutation of the quinone with the

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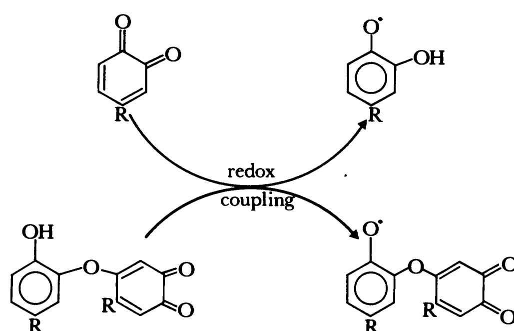
corresponding hydroquinone, an alternative pathway of generation needs to be postulated. A redox exchange between the anisyl-3,4-quinone and 4-hydroxyanisole in conditions where the substrate concentration exceeds the available oxygen concentration is one possibility, but to date no evidence has been obtained of the formation of the corresponding phenoxy radical. However, Tomasi (1984) has obtained e.s.r. spectra of radical species that may correspond to substituted phenoxyls, which could be derived by single-electron oxidation from oligomeric species of the class shown in scheme 2. Thus, redox-exchange reactions between the tyrosinase oxidation products of 4-HA may be the source of the free radicals detected, including anisyl semiquinone, as indicated in the speculative scheme (scheme 3). Whether this does, in fact, represent the processes occurring in the system, and to what extent the oxygen concentration influences the balance of reactions, remains to be determined.



SCHEME 3. Speculative scheme of redox coupling between anisyl-3,4-quinone and its polymerization products. The polymers are represented by a dimerization product ($R = OCH_3$), which may lose an electron to the quinone, generating the semiquinone radical and a substituted phenoxy radical.

Cytotoxic damage by melanin precursors

The possibility that toxic damage to cells could occur from the action of oxidation products of the melanogenic pathway has been previously recognized (Hochstein & Cohen 1969), and it may be for this reason that a specialized organelle is necessary for pigment production to protect the cell from potentially damaging species (Slater & Riley 1966). It is a matter of some interest as to why, when the oxidation products of 4-HA cause cell damage, the natural intermediates of melanogenesis, such as dopaquinone, are not overtly toxic to cells. This is the case even in model systems (see table 1). It may be that this is a result of the ability of natural

TABLE 1. EFFECT ON PLATING EFFICIENCY OF NATURAL AND ANALOGUE SUBSTRATES OF TYROSINASE

(from Holden 1984)

substrate	LD_{63}/μM
L-tyrosine	700
L-Dopa	> 1000
4-HA	11
p-ethoxyphenol	36
MBEH	31

Hamster lung fibroblast cultures (V79) were incubated in Ham's F12 medium in the presence of several concentrations of the agents, with mushroom tyrosinase (100 μg ml^{-1}), and the dose required to reduce survival to 37 % of controls was estimated from the plating efficiency data. Abbreviations used are as follows: Dopa = 3,4-dihydroxyphenylalanine; 4-HA = 4-hydroxyanisole; MBEH = monobenzyl ether of hydroquinone.

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substrates to form an indolene ring (see scheme 1), a reaction that takes place rapidly owing to the proximity of the amino group on the side chain of dopaquinone, so that the relative persistence of dopaquinone is very brief. An alternative explanation for differences in apparent toxicity may be the comparative lipid solubilities of the quinones, which may permit the oxidation products of analogue substrates more readily to enter and penetrate phospholipid membranes. For example, of the compounds listed in table 1, tyrosine and dihydroxyphenylalanine have aqueous solubilities at 25 °C of 0.45 and 1.65 gl^{-1} respectively, but are insoluble in most organic solvents; and at the other end of the scale the monobenzyl ether of hydroquinone dissolves readily in alcohol, ether or benzene, but is insoluble in water. 4-hydroxy-anisole is soluble in both, with a partition coefficient of about 15 between octanol and 0.1 M Tris–HCl buffer at pH 7.4 (Cheeseman 1984). It is probable that the corresponding *ortho* quinones exhibit similar solubilities.

Melanoma therapy

Although it is now known that 4-HA has several actions, some of which may not depend on metabolism, the relative lack of generalized toxicity of 4-HA (Hodge *et al.* 1949) suggested the possibility of its systemic use as a treatment for malignant melanoma (Riley 1970). Encouraging results from animal experiments (Dewey *et al.* 1977) led to pilot clinical studies in patients suffering from local recurrences and widespread metastatic melanoma. A number of routes of parenteral administration have been used; intralesional injection, intravenous infusion and intra-arterial infusion (Morgan *et al.* 1981; Webster *et al.* 1984). Therapy by the intra-arterial route has shown encouraging results for local recurrences and 4-HA has now been given intra-arterially to twenty patients, of which nine cases have shown a response (table 2). In two patients the recurrences disappeared completely after treatment (Morgan 1984) and in one of these cases there is no evidence of disease after three years. The usual pattern of

Table 2. Summary of cases treated by intra-arterial infusion of 4-hydroxyanisole

patient	age	sex	site treated	total dose/g	response[a]
LT	83	F	cheek	20	R
MP	75	F	leg	11	R
BN	53	F	foot	68	R
MG	31	F	arm	6	N
MH	42	F	arm	2	N
ED	76	F	leg	17	N
AM	40	F	leg	16	R
HH	50	M	oral	98	N
TG	46	M	arm	5	N
AD	73	F	leg	74	R
FT	70	F	leg	184	R
LY	70	F	leg	184	R
VG	53	F	leg	7	N
ML	65	F	leg	190	R
HB	53	M	liver	104	N
LP	41	M	leg	224	N
MN	63	F	foot	56	N
AG	44	M	leg	224	N
HS	60	M	leg	108	R
MA	64	F	leg	112	N

[a] Responses are indicated as R (positive) and N (negative).

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response has been for the intra-arterial infusion with 4-HA to halt the growth of tumour in the infused region, but for the disease to progress gradually elsewhere in the body. In general, it has been found that the larger metastases are more responsive to treatment (Riley *et al.* 1982). Although there are technical problems associated with the arterial route of administration of 4-HA, it is clear from the preliminary clinical results that a significant local response can be elicited in some patients.

Pharmacokinetics

Studies of the pharmacokinetics of 4-HA in mice (Holden & Dewey 1984) and some studies on patients receiving the drug by intra-arterial infusion (Morgan *et al.* 1984) have shown that the compound is rapidly cleared from the blood ($t_{\frac{1}{2}} = 9$ min in the human). Data from the human studies (Morgan *et al.* 1984) and investigations on rat-liver cells (Egan 1984) indicate that 4-HA is metabolized by the liver and that an inducible enzyme system is involved. The process of *o*-demethylation is apparently not implicated (Cheeseman 1984) and 4-HA is known to be a poor substrate for UDP-glucuronyl-transferase (Mulder & Van Doorn 1975). No recovery of 4-HA was observed after enzymatic deconjugation of the metabolic products formed from 4-HA in the rat-liver cell system (Egan 1984). It is possible that further ring hydroxylation is the preferred initial step in the metabolic pathway of 4-HA, with the subsequent possibilities of conjugation or oxidation to a quinone. Cummings & Prough (1983) have shown that the structurally related compound, BHA (E320) inhibits the mixed-function oxidase system through the formation of a quinone metabolite which diverts electrons from the microsomal electron-transport chain to the reduction of oxygen. In collaboration with other laboratories we are investigating modifications to 4-HA with the aim of improving its pharmacokinetic characteristics, by reducing hepatic metabolism without diminishing the tyrosinase affinity of the compound or its ease of oxidation by the enzyme. The key to systemic chemotherapy by using the tyrosinase analogue approach to the melanogenic pathway will be to solve the problem of the pharmacokinetics of 4-HA so that adequate doses can reach all the sites of metastasis of the tumour.

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Discussion

R. Marris (793 *Harrow Rd, London NW10 5PA*). Three or four years ago at least two substances were referred to in the correspondence columns of the *Lancet* with rather similar effects upon a few malignant melanomas. Has Professor Riley tried them alone or in combination with 4-hydroxyanisole?

P. A. Riley. The substances Mr Marris is referring to are straight-chain dicarboxylic acids with 9–12 carbon atoms in the chain. Azelaic acid has been used successfully to treat patients with lentigo maligna, a lesion that is regarded as an early form of malignant melanoma. We have no experience of the use of these compounds in the treatment of advanced malignant melanoma.

R. Marris. There is a variety of domesticated dog that is especially subject to malignant melanoma in the mouth. It may be useful in investigations; grey horses and Angora goats, which have been used elsewhere, are expensive (Cheville 1983).

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P. A. Riley. Thank you.