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Melanocytotoxicity and Antimelanoma Effects of Phenolic Amine Compounds in Mice *in Vivo*[1]

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ABSTRACT

A phenolic amine compound, 4-*S*-cysteaminylphenol (4-*S*-CAP), is a potent depigmenting agent. To develop more efficacious antimelanoma agents, we synthesized four homologues of 4-*S*-CAP: *N*-acetyl-4-*S*-CAP (*N*-Ac-4-*S*-CAP), α-methyl-4-*S*-CAP, 4-*S*-homo-CAP, and *N,N'*-dimethyl-4-*S*-CAP. We tested these five compounds in mice *in vivo*. After s.c. or i.p. injection of saline solution (in control groups) or one of the compounds, follicular melanocytes were examined by light and electron microscopy to assess the degree of melanocytotoxicity; *N*-Ac-4-*S*-CAP induced the most depigmentation (98%), whether given i.p. or s.c. After injection of 4-*S*-CAP or *N*-Ac-4-*S*-CAP, the number of murine B16F10 melanoma colonies formed in the lungs was determined; 4-*S*-CAP and *N*-Ac-4-*S*-CAP were almost equally effective, reducing the colonies to 32 and 25% of mean control, respectively. Metabolic studies of the urine showed 9% of 4-*S*-CAP and 20% of *N*-Ac-4-*S*-CAP injected i.p. were excreted unchanged in 24 h; 1.3% of the *N*-Ac-4-*S*-CAP was excreted as 4-*S*-CAP, indicating some conversion. We conclude that *N*-Ac-4-*S*-CAP is a suitable model for developing chemotherapy to treat melanoma characterized by high tyrosinase activity and melanin synthesis.

INTRODUCTION

Melanin pigment is synthesized solely within melanocytes and melanoma cells. The enzyme tyrosinase (EC 1.14.18.1) converts the amino acid tyrosine to L-DOPA and thence to dopaquinone, which gives rise to a melanin polymer. This process occurs in a specific secretory granule, the melanosome (1, 2). There have been several attempts to use this unique melanin pathway as a basis for developing chemotherapeutic agents selectively toxic to melanoma cells, in most cases based on L-DOPA and related catechols (3-7), but chemical instability and severe toxicity due to nonspecific oxidation have precluded success. Because phenolic compounds seem to possess fewer drawbacks and appear more promising for use in chemotherapy to combat melanoma, we developed a new subgroup of phenols, combining phenols with cysteine and cysteamine to yield cysteinylphenol and cysteaminylphenol, respectively (8).

Both 4-*S*-CP[4] and 4-*S*-CAP are substrates for mammalian tyrosinase, can depigment black hair, and possess an antimelanoma property *in vivo*, whereas their 2-*S*-isomers are not tyrosinase substrates and evidence no melanocytotoxic effects (8). Most importantly, 4-*S*-CAP has a significant antimelanoma effect (9). Padgette *et al.* (10), however, in developing hypotensive agents discovered that 4-*S*-CAP is a substrate of plasma MAO. Our earlier study confirmed this result *in vitro* and indicated that 4-*S*-CAP could produce a cytotoxic aldehyde through interaction with plasma MAO (11). To overcome these difficulties, we synthesized four homologues of 4-*S*-CAP; *N*-Ac-4-*S*-CAP, α-Me-4-*S*-CAP, 4-*S*-homo-CAP, and *N,N*-DiMe-4-*S*-CAP. We found that all four compounds are substrates for tyrosinase and that 4-*S*-HomoCAP is also a substrate for MAO (12).

The depigmentation potency in black follicles of test compounds given s.c. is a reliable screening method for evaluating melanocytotoxicity (13). In the present study, in mice *in vivo* we compared this property (visible change and chemical melanin content) of the four new phenolic amine compounds and the mother compound, 4-*S*-CAP, given both i.p. and s.c., and assessed the antimelanoma effect of 4-*S*-CAP and the most efficacious depigmenting compound by assaying the formation of melanoma colonies in the lungs.

MATERIALS AND METHODS

Animals. Breeding pairs of C57BL/6J black mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and their female progeny were used in the study when 8 weeks old and weighing 17.0 g. All procedures were approved by the University's Health Sciences Animal Welfare Committee.

Chemicals. The methods used to synthesize the phenolic amine compounds [4-*S*-CAP, *N*-Ac-4-*S*-CAP, α-Me-4-*S*-CAP, 4-*S*-Homo-CAP, and *N,N*-DiMe-4-*S*-CAP] were as reported previously (8, 9, 11). Briefly, the basic compound, 4-*S*-CAP, was prepared through refluxing a mixture of phenol and cysteamine with HBr (Fig. 1). In all experiments, the agent injected into controls was 1 N saline solution.

LD_{50}. The LD_{50} for each compound ($n = 6$ mice each) was established by a single i.p. injection of the test drug dissolved in normal saline solution, in a dose range of 100 to 1300 mg/kg body weight. The LD_{50} was 600 mg/kg for 4-*S*-CAP, 1200 mg/kg for *N*-Ac-4-*S*-CAP, 500 mg/kg for α-Me-4-*S*-CAP, 350 mg/kg for 4-*S*-HomoCAP, and 300 mg/kg for *N,N*-DiMe-4-*S*-CAP.

Melanoma Cell Line. The murine B16F10 melanoma cell line, with a strong metastatic property to form tumor colonies in lungs, was kindly supplied by Dr. B. M. Longenecker (Department of Immunology, University of Alberta). Cells were grown in T-75 flasks in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Melanocytotoxicity *in Vivo*. Black hairs were plucked manually from the back of the mice, to initiate new anagen growth and activate follicular melanocytes with increased tyrosinase activity. Thirty mice were randomized into six groups of five (one control and one for each compound). Starting on day 1, daily for 14 days the agent was injected i.p. or was infiltrated s.c. in an area where hair follicles had been plucked; the dose was 300 mg/kg body weight.

Melanin Content. In the same 30 mice, hair follicles were harvested on day 22 (early telogen phase) and their eumelanin content was analyzed. Details of the assay, including chemical degradation of melanin and HPLC, were as before (14). Briefly, a 10-mg hair sample is homogenized in water at a concentration of 10 mg/ml; the 200-μl homogenate is transferred to a screw-capped test tube, mixed with 1 M H_2SO_4 (800 μl), and oxidized with 3% $KMnO_4$. The product, PTCA, is analyzed by HPLC with a UVL detector; samples are measured in duplicate. The eumelanin content is expressed as PTCA (ng/mg) *versus*

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[4] The abbreviations used are: 4-*S*-CAP, 4-*S*-cysteaminylphenol; *N*-Ac-4-*S*-CAP, *N*-acetyl-4-*S*-cysteaminylphenol; α-Me-4-*S*-CAP, α-methyl-4-*S*-cysteaminylphenol; 4-*S*-HomoCAP, 4-*S*-homocysteaminylphenol; *N,N*-DiMe-4-*S*-CAP, *N,N'*-dimethyl-4-*S*-cysteaminylphenol; HPLC, high performance liquid chromatography; L-DOPA, L-dihydroxyphenylalanine; MAO, monoamine oxidase; PTCA, pyrrole-2,3,5-tricarboxylic acid.

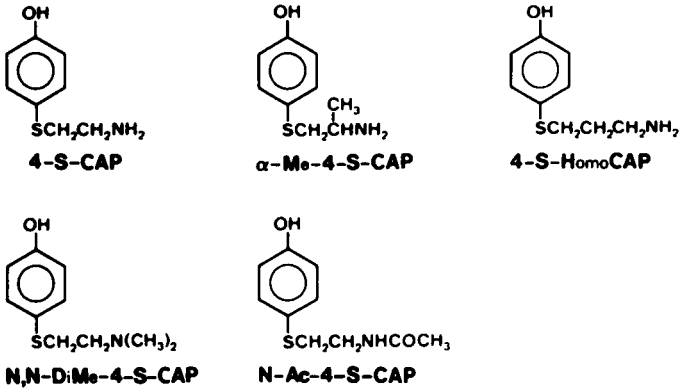

Fig. 1. Chemical structure of phenolic amine compounds, 4-*S*-CAP, α-Me-4-*S*-CAP, 4-*S*-HomoCAP, *N,N*-DiMe-4-*S*-CAP, and *N*-Ac-4-*S*-CAP.

the PTCA content of BALB/c albino mice as control (≤10 ng/mg, the background value with our method, in which 1 ng corresponds roughly to a eumelanin content of 50 ng), and the degree of depigmentation is expressed as a percentage: (PTCA content of control sample − PTCA content of experimental sample)/(PTCA content of control sample) × 100.

Light and Electron Microscopy. In six groups of three mice (1 control and 1 for each compound), samples of skin and regrowing hair follicles were excised under brief general anesthesia on day 10 (late anagen phase), fixed in Karnovsky's solution (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer), and refixed in 1% osmium tetroxide in cacodylate buffer. Tissues were stained *en bloc* with 1.5% uranyl acetate in 0.1 M veronal buffer for 30 min, dehydrated in graded ethanol solutions, embedded in epoxy resin, and sectioned with a Potter-Blum Ultramicrotome MT II. Sections 1.0-μm thick were stained with methylene blue for light microscopy; thin sections were stained with lead citrate for electron microscopy.

Assay of Melanoma Colony Formation in the Lungs. Murine B16F10 melanoma cells were cultured to subconfluence, harvested by applying a thin layer of 0.25% trypsin solution in EDTA, washed and resuspended in cold 1 N saline solution. Viable cells were identified by trypan blue dye exclusion and counted, the cell suspension was diluted to the desired concentration. Three groups of 10 mice were studied: control, 4-*S*-CAP, and *N*-Ac-4-*S*-CAP. On day 0, the mice were inoculated via the lateral tail vein with 5×10^4 cells in 0.2 ml of 1 N saline. The test drugs were dissolved in normal saline, sterilized by membrane filtration, and administered in a dose of 300 mg/kg (4-*S*-CAP) or 900 mg/kg (*N*-Ac-4-*S*-CAP); starting on day 5, the control solution or drug was injected i.p. daily for 14 days. On day 26, the mice were killed by cervical dislocation, their lungs were removed, and the number of melanoma colonies was counted under a dissecting microscope. The results were expressed as percentage of reduction in the number of colonies: $[a - b/a] \times 100$, where a = colonies in control group and b = colonies in experimental group.

Metabolic Assay. Fifteen mice randomly divided into three groups of five were given i.p. injections of a test agent (300 mg/kg) dissolved in 1 N saline solution. Group 1 were given injections of 4-*S*-CAP and group 2, *N*-Ac-4-*S*-CAP; group 3 were controls. The mice are kept in metabolic cages; their urine is collected 3, 8, and 24 h after injection, and the 4-*S*-CAP and *N*-Ac-4-*S*-CAP contents are measured by HPLC in a system consisting of a Waters 600 E liquid chromatograph, with a Bondapak C18 column (3.9 x 300 mm; particle size, 10 μm) and a Waters 460 electrochemical detector. Mobile phase is 0.1 M potassium phosphate buffer, pH 2.1, containing 0.1 mM Na_2EDTA:methanol, 80:20 (v/v), column temperature is 60°C, and flow rate is 1.0 ml/min. Urine samples are hydrolyzed with 0.1 M HCl before assay, and the phenols are detected at 850 mV against an Ag/AgCl reference electrode.

RESULTS

Macroscopically Visible Depigmentation of New Growth of Black Hair Follicles (Figs. 2 and 3). This was most marked after

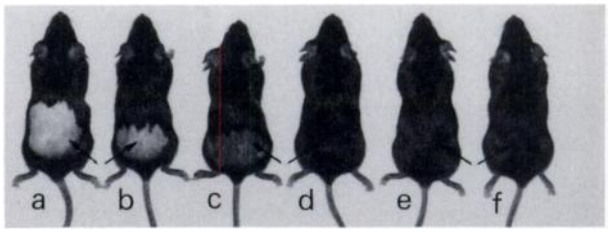

Fig. 2. Macroscopically visible changes in black hair follicles after i.p. injection of phenolic amine compounds, 300 mg/kg daily for 14 days: *a*, *N*-Ac-4-*S*-CAP; *b*, α-Me-4-*S*-CAP; *c*, 4-*S*-CAP; *d*, 4-*S*-HomoCAP; and *e*, *N,N*-DiMe-4-*S*-CAP. *f*, normal saline solution (control). *Arrows*, sites from which hairs had been plucked.

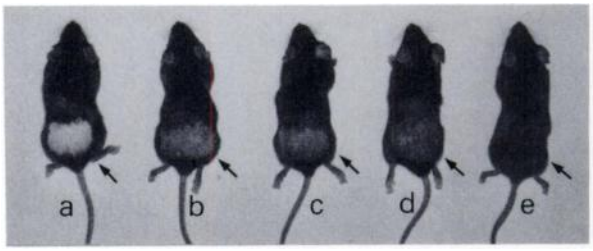

Fig. 3. Macroscopically visible changes in black hair follicles after s.c. injection of phenolic amine compounds, 300 mg/kg daily for 14 days. *a*, *N*-Ac-4-*S*-CAP; *b*, 4-*S*-HomoCAP; *c*, α-Me-4-*S*-CAP; *d*, 4-*S*-CAP; and *e*, *N,N*-DiMe-4-*S*-CAP. *Arrows*, plucked areas that were the site of s.c. infiltration.

injection of *N*-Ac-4-*S*-CAP, whether i.p. or s.c., with the new hair being almost pure white as in albino mice. The degree of depigmentation with the other phenolic amine compounds was α-Me-4-*S*-CAP > 4-*S*-CAP > 4-*S*-HomoCAP, > *N,N*-DiMe-4-*S*-CAP. Depigmentation after s.c. injection of *N*-Ac-4-*S*-CAP was similar with doses of 600 and 300 mg/kg. With 4-*S*-CAP and α-Me-4-*S*-CAP, i.p. administration induced more depigmentation than s.c. treatment, whereas 4-*S*-HomoCAP induced much more depigmentation when given s.c. than i.p. *N,N*-DiMe-4-*S*-CAP induced no visible changes in pigmentation in new follicles.

Melanin Content in Depigmented Follicles (Table 1). No side effects of the compounds were apparent. The most potent depigmenting agent was *N*-Ac-4-*S*-CAP, which induced 98% depigmentation in new growing follicles after either i.p. and s.c. administration, with little or no dose-related difference in degree. There was no statistically significant difference in melanin content between hair follicles treated with *N*-Ac-4-*S*-CAP and those of control albino mice. α-Me-4-*S*-CAP induced 89% depigmentation after i.p. injection and 58% after s.c. administration, and the latter caused some irritation of the skin in the plucked areas. The depigmentation potency of 4-*S*-CAP was similar to that of α-Me-4-*S*-CAP, being 87% after i.p. and 47% after s.c. administration. Interestingly, 4-*S*-HomoCAP evidenced greater potency when given s.c. (63.5%) than i.p. (12.1%); this was higher than with 4-*S*-CAP, α-Me-4-*S*-CAP, and *N,N*-DiMe-4-*S*-CAP given s.c. *N,N*-DiMe-4-*S*-CAP induced no significant depigmentation when given i.p. or s.c. (7.3 and 6.1%, respectively).

Light Microscopy of Depigmented Follicles (Fig. 4). In control samples (Fig. 4*a*) there were melanocytes in the bulb above the hair's papilla and both melanocytes and surrounding keratinocytes were filled with black pigment. On day 10, after *N*-Ac-4-*S*-CAP given i.p. (Fig. 4*b*) neither functioning melanocytes nor melanin pigments remained in the follicles but there were no vacuolar changes in cells of the hair bulb, and after α-Me-4-*S*-CAP i.p. (Fig. 4*c*) there was a similar loss of functioning melanocytes and melanin pigments. By contrast, after i.p. in-

Table 1 *PTCA content and depigmentation of new hair after treatment with phenolic amine compounds*

Compound injected (300 mg/kg)	PTCA content (ng/mg)	Depigmentation (%)	*t* test (*P*)
Intraperitoneal			
Normal saline (control)	1089.0 ± 48.9	0.0	
N-Ac-4-*S*-CAP	26.0 ± 10.3	97.6	<0.001
α-Me-4-*S*-CAP	121.3 ± 81.9	88.9	<0.001
4-*S*-CAP	146.1 ± 72.4	86.6	<0.001
4-*S*-HomoCAP	956.7 ± 77.9	12.1	>0.05
N,N-DiMe-4-*S*-CAP	1009.0 ± 193.5	7.3	>0.2
Subcutaneous			
Normal saline (control)	1089.0 ± 48.9	0.0	
N-AC-4-*S*-CAP	30.8 ± 9.1	97.2	<0.001
α-Me-4-*S*-CAP	458.3 ± 107.6	57.9	<0.001
4-*S*-CAP	577.9 ± 47.2	46.9	<0.001
4-*S*-HomoCAP	397.7 ± 279.3	63.5	<0.02
N,N-DiMe-4-*S*-CAP	1023.4 ± 110.1	6.1	>0.2

jection of 4-*S*-CAP (Fig. 4*d*) the hair follicles contained functioning melanocytes and melanin pigments transferred into keratinocytes, although they were much sparser than in the controls. After treatment with 4-*S*-HomoCAP (Fig. 4*e*) the follicles contained fairly numerous melanocytes and melanin pigments, almost the same as in controls. Few changes were evident after *N,N*-DiMe-4-*S*-CAP given i.p. (Fig. 4*f*).

Electron Microscopy of Depigmented Follicles after i.p. Injection for 10 Days (Controls, see Fig. 5*a*). No melanocytes or melanosomes were visible after treatment with *N*-Ac-4-*S*-CAP (Fig. 5*b*), whereas the follicles contained variable numbers of functioning melanocytes and melanosomes after injection of the other compounds. Melanocytes from mice treated with α-Me-4-*S*-CAP (Fig. 5*c*) and 4-*S*-CAP (Fig. 5*d*) contained immature melanosomes at stages II and III, whereas those from controls were full of mature melanosomes at stage IV (Fig. 5*a*). After 4-*S*-HomoCAP, also, the follicles contained stage III melanosomes (Fig. 5*e*), more numerous than with α-Me-4-*S*-CAP or 4-*S*-CAP. There was little or no change from control in numbers or melanization of melanocytes after *N,N*-DiMe-4-*S*-CAP (not shown).

Antimelanoma Effects of 4-*S*-CAP and *N*-Ac-4-*S*-CAP. The numbers of B16F10 melanoma colonies were significantly reduced after treatment with *N*-Ac-4-*S*-CAP and 4-*S*-CAP (Table 2). Furthermore, examination under a dissecting microscope revealed marked reduction in size of those remaining (diameter, 1.5–1.7 mm *versus* 2.5–3.1 mm in controls) and marked depigmentation or frank amelanosis of some. The only side effects noted were brief apathy and mild hypothermia immediately after i.p. injection; average body weight was the same on day 0 and at death.

Assay of Metabolites in Urine. Urinary excretion of the unchanged compounds, without conversion or degradation, was maximal 3 h after i.p. injection (5.2 mg/mouse); this was 7.7% (0.399 ± 0.018 mg) for 4-*S*-CAP and 13.9% (0.723 ± 0.027 mg) for *N*-Ac-4-*S*-CAP. Later excretion of these two compounds, respectively, was as follows: at 8 h, 8.5% (0.443 ± 0.023 mg) and 19.8% (1.034 ± 0.033 mg); and, at 24 h, 8.8% (0.458 ± 0.021 mg) and 20.4% (1.089 ± 0.028 mg). Most notably, 1.3% (0.066 ± 0.003 mg) of the *N*-Ac-4-*S*-CAP i.p. dose was excreted at 4-*S*-CAP, indicating conversion to the mother compound of part of this homologue administered by this route.

DISCUSSION

Our previous study (12) *in vitro* indicated that all of the five phenolic amine compounds tested were substrates of mushroom and melanoma tyrosinases but that only 4-*S*-HomoCAP and 4-*S*-CAP were substrates of tyrosinase and MAO. Interestingly, 4-*S*-CAP was the best substrate for mushroom tyrosinase, whereas 4-*S*-HomoCAP was the best for melanoma tyrosinase, a discrepancy attributed to the need for co-factors L-DOPA and L-ascorbic acid in the melanoma tyrosinase reaction mixture (15). The present study *in vivo* indicated *N*-Ac-4-*S*-CAP and α-Me-4-*S*-CAP as the compounds possessing greatest cytotoxicity for follicular depigmentation and 4-*S*-HomoCAP as having the least. This indicates that the selective melanocytotoxicity of phenolic amine compounds relates to melanin synthesis and tyrosinase activity, which are high in plucked hair, and not to

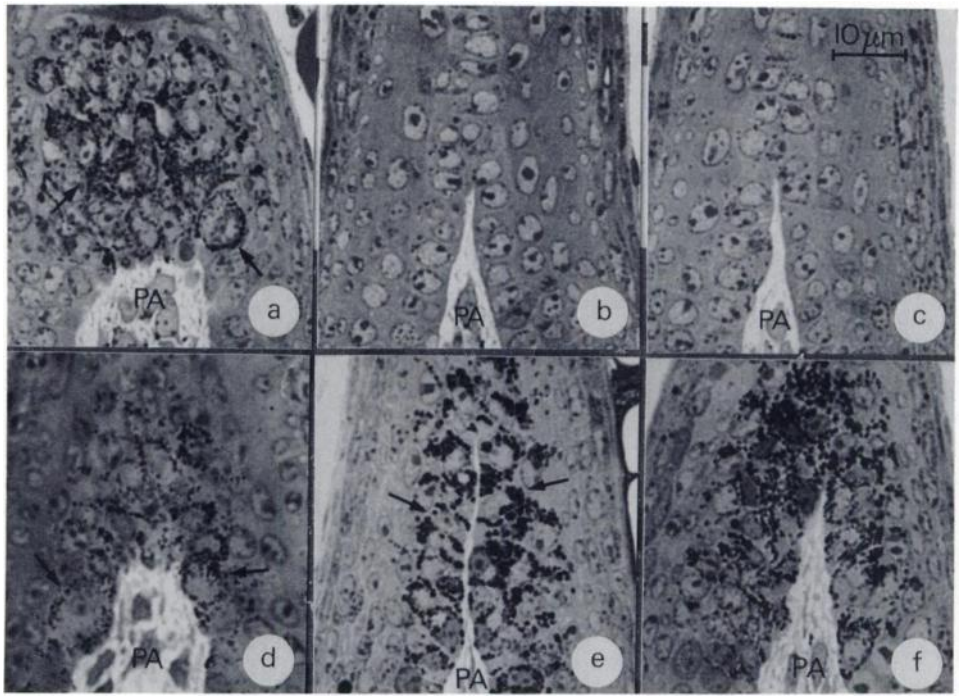

Fig. 4. Light microscopy of hair follicles after i.p. injection of phenolic amine compounds daily for 10 days. *Bar*, 10 μm. *PA*, papilla. *a*, control. *Arrows*, normal melanocytes of hair follicle of a C57BL/6J mouse. *b*, after treatment with *N*-Ac-4-*S*-CAP; no melanocytes or melanosomes are present. *c*, after α-Me-4-*S*-CAP; few melanocytes are seen in the melanocytic zone above the papilla. *d*, after 4-*S*-CAP; the number of melanosomes in the hair bulb is greatly reduced. *Arrows*, functioning melanocytes. *e*, after 4-*S*-HomoCAP; melanocytes and melanosomes are present in the melanocytic zone of the bulb (*arrows*). *f*, after *N,N*-DiMe-4-*S*-CAP; the numbers of melanocytes and melanosomes are similar to control (*a*). × 1100.

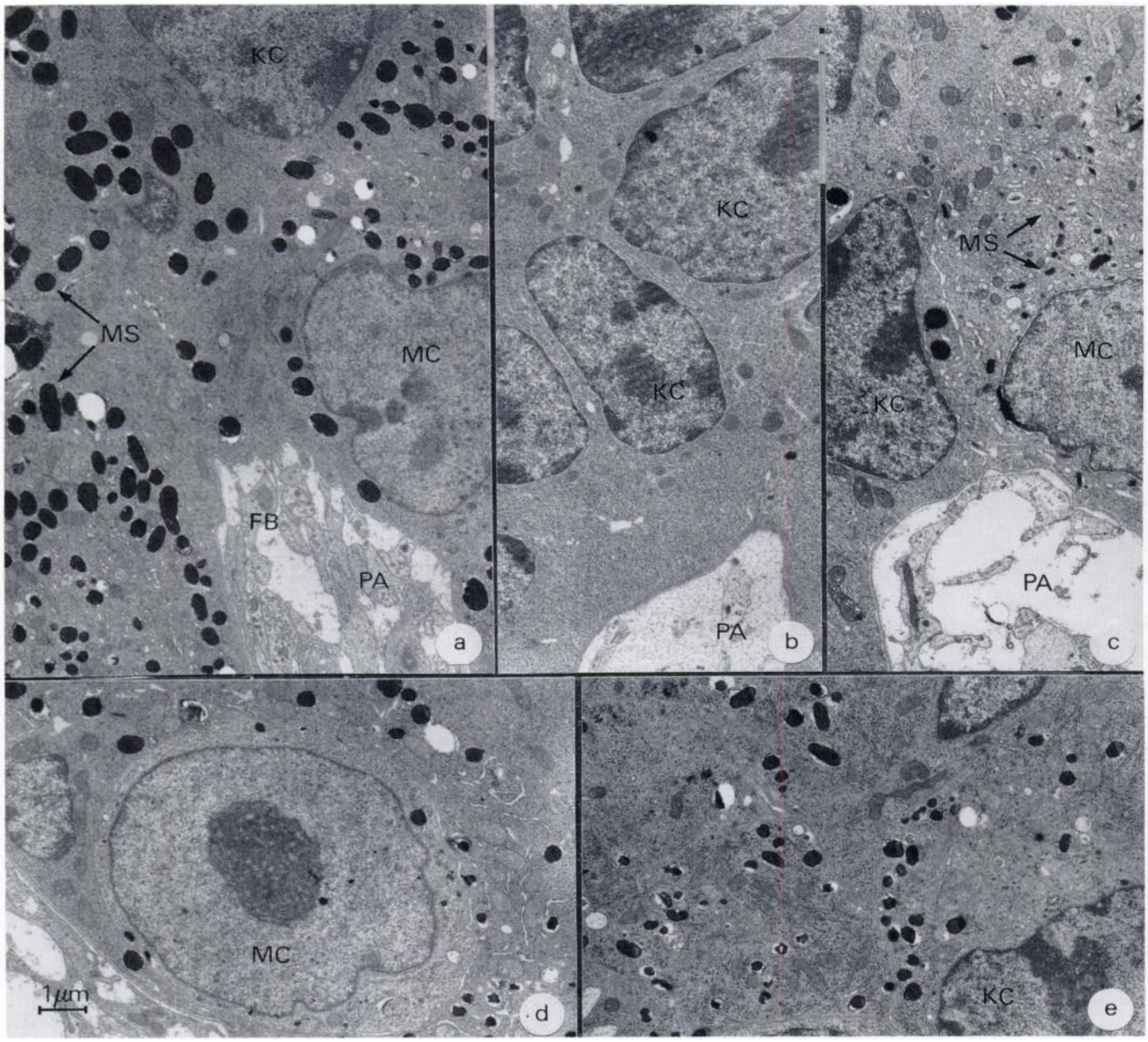

Fig. 5. Electron microscopy of hair follicles after i.p. injection with phenolic amine compounds (300 mg/kg) daily for 10 days. *Bar*, 1 μm. *MC*, melanocytes; *MS*, melanosomes; *KC*, keratinocytes; *FB*, papillary fibroblasts; *PA*, papilla. *a*, control; no degenerative changes are seen in melanocytes, melanosomes, keratinocytes, or papillary fibroblasts, and the melanocytes contain well developed Golgi complexes. *b*, after treatment with *N*-Ac-4-*S*-CAP; no melanocytes or melanosomes are present in the melanocytic zone of the hair bulb, but no degenerative changes are seen in the keratinocytes. *c*, after α-Me-4-*S*-CAP; individual melanosomes in the few remaining melanocytes appear sparser and more fragmented than in keratinocytes, and many are unmelanized (*arrows*). *d*, after 4-*S*-CAP; melanosomes are reduced in size and number. *e*, after 4-*S*-HomoCAP; cytotoxic effects are less than with the other compounds, consisting in some reduction in number and size of melanosomes. × 6400.

Table 2 *Antimelanoma effects of 4-S-cysteaminylphenol and N-acetyl-4-S-cysteaminylphenol on the formation of B16F10 melanoma colonies in mouse lungs excised 26 days after injection of the cell suspension*

Compound	No. of mice	No. of colonies per pair of lungs	No. of colonies % of control	*t* test (*P*)
Normal saline (control)	10	86.5 ± 32.9	100.0	
4-*S*-CAP, 300 mg/kg	10	28.0 ± 14.5	32.4	<0.001
N-Ac-4-*S*-CAP, 900 mg/kg	10	21.5 ± 10.8	24.9	<0.001

plasma MAO activity. The study of melanoma colonies in the lungs confirmed that *N*-Ac-4-*S*-CAP is a potent antimelanoma agent, but its effect was not absolute under the conditions and with the doses used in these experiments. The marked difference in depigmentation potency of 4-*S*-HomoCAP given i.p. and s.c. may relate to slower degradation of the compound given s.c. and, thereby, an enhanced ability to affect melanocytes directly and induce greater depigmentation.

Our study *in vitro* also showed that the rate of formation of *o*-quinone, a major intermediate of melanocytotoxicity that subsequently binds to SH-containing enzymes (*e.g.*, DNA polymerase), was very fast after the administration of 4-*S*-CAP and much slower after α-Me-4-*S*-CAP (11, 12). In the present study, however, the two compounds possessed similar ability for depigmentation *in vivo*, indicating that melanocytotoxicity related not only to the rate of *o*-quinone formation through tyrosinase but also to other mechanism(s); *e.g.*, DNA may be damaged by free radicals through the interaction of tyrosinase and phenolic amine compounds. Another factor responsible for selective melanocytotoxicity might be the binding of synthetic amine compounds to melanin, which could interact later with

tyrosinase. The absence of melanocytotoxicity *in vivo* in albino mice, in which tyrosinase and melanin are absent but unmelanized melanosomes are present, supports this possibility (12).

Further work is needed to establish whether phenolic amine compounds do in fact have selective affinity for melanin. Our findings indicate that *N*-Ac-4-*S*-CAP is an ideal model for developing rational chemotherapy to combat melanoma, but such development is hampered by the paucity of information about the compound *in vitro* as well as *in vivo*. Recently, the neurotoxicity of an amine (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was attributed to the binding of its active metabolite to neuromelanin (16). Many melanoma cells appear to be involved in pheomelanogenesis rather than eumelanogenesis (1), but it is not known whether the synthetic compounds we tested have high affinity for one of these pathways of melanin synthesis, how these compounds interact with γ-glutamyl transpeptidase, which is partly responsible for pheomelanogenesis (2, 17), or whether the active compound is 4-*S*-CAP or *N*-acetyl-4-*S*-CAP.

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