Glutathione Plays a Key Role in the Depigmenting and Melanocytotoxic Action of N-Acetyl-4-S-Cysteaminylphenol in Black and Yellow Hair Follicles

Frank Alena, Walter Dixon, Panakkezhum Thomas, and Kowichi Jimbow
Dermatology and Cutaneous Sciences, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada

This study examined the effect of glutathione on the *in vivo* depigmenting potency of N-acetyl-4-S-cysteaminylphenol (N-acetyl-4-S-CAP) in black and yellow mice after multiple intraperitoneal injections on 10 consecutive days. In black mice (C57BL/6J, a/a), N-acetyl-4-S-CAP showed dose-dependent depigmenting potency (0.5, 1.0, and 2.0 mmol/kg), which was in parallel to the tissue eumelanin content (98%, 28%, and 3% of controls, respectively) and to the tissue glutathione content (94%, 85%, and 76%, respectively). In lethal yellow mice (C57BL/6J, A^y/a), only a dose of 2.0 mmol/kg showed the color change of hair to dark, not to white as seen in black mice. This was reflected by the decrease of pheomelanin content (56%) and the increase of eumelanin content (28% of black mice). The simultaneous administration of N-acetyl-cysteine, which up-regulated glutathione content, completely abolished the depigmenting potency of N-acetyl-4-S-CAP, whereas administration of buthionine sulfoximine, which depleted the tissue glutathione content, enhanced the depigmenting potency of N-acetyl-4-S-CAP in black hair. In yellow mice, the darkening of hair follicles by 2.0 mmol/kg of N-acetyl-4-S-CAP was completely abolished by the combined administration of N-acetyl-cysteine, with the resulting hair color the same as in controls, whereas combined administration with buthionine sulfoximine caused some whitening of yellow hair follicles. Our data indicate that the tissue content of glutathione regulates melanocytotoxicity and depigmenting potency of N-acetyl-4-S-CAP and that this alteration of glutathione content may switch the melanogenesis type from pheomelanin to eumelanin. *J Invest Dermatol 104:792–797, 1995*

Phenols and catechols are known to be melanocytotoxic and to cause depigmentation of the skin and hair in humans and experimental animals [1]. The *p*-hydroxyphenyl derivatives, e.g., 4-hydroxyanisole, are the most extensively studied group of such depigmenting agents [2,3]. The common property of these depigmenting phenols is their capacity to act as substrates for the unique enzyme of pigment cells, tyrosinase (E.C. 1.14.18.1). It has been proposed that the observed selective melanocytotoxicity is mediated by the toxic products of tyrosinase, orthoquinones [4], and that pigment cells with active tyrosinase are susceptible to the cytotoxic action of *p*-hydroxyphenols.

In our earlier *in vivo* studies, we described the biologic effects of a new type of *p*-hydroxyphenol, N-acetyl-4-S-cysteaminylphenol (N-acetyl-4-S-CAP) [5]. N-Acetyl-4-S-CAP is a phenolic thioether and a substrate for tyrosinase [6]. It has been shown to possess a high depigmenting potency in the black hair follicles as well as a significant growth-inhibitory effect against murine melanoma [7]. The latter effect was found to be dose dependent [5].

Hair follicles of C57BL/6J mice have been recognized as a useful model for screening the *in vivo* melanocytotoxic effects of drugs [8,9]. These mice exist in several differently colored strains; the C57BL/6J (a/a) strain is black because of production of the black pigment, eumelanin, whereas the C57BL/6J (A^y/a) strain is yellow because of synthesis of the yellow pigment, pheomelanin, in hair follicles [8]. Plucking of hair follicles initiates the anagen phase of the hair cycle, followed by the activation of follicular melanocytes and high tyrosinase activity. Therefore, one might anticipate that during the anagen phase, a potent melanocytotoxic phenol that is a substrate for tyrosinase should cause depigmentation of newly grown hair in both black and yellow mice. Accordingly, depigmentation of hair follicles was shown to occur in black mice. However, attempts to depigment hair follicles in yellow mice using phenols have been unsuccessful [10].

In this study, we examined the difference in the *in vivo* susceptibilities of black and yellow follicular melanocytes to N-acetyl-4-S-CAP. The results suggest that the tripeptide glutathione (GSH) plays a key role in the depigmenting effect of N-acetyl-4-S-CAP, which enhances synthesis of pheomelanin by supplying cysteine. Using the GSH-modulating agents N-acetyl-L-cysteine (NAC) and D,L-buthionine-(S,R)-sulfoximine (BSO), which reduces GSH synthesis by inhibiting gamma-glutamyl cysteine synthetase, we show here that the depigmenting potency of N-acetyl-4-S-CAP in

Manuscript received June 6, 1994; final revision received January 10, 1995; accepted for publication January 13, 1995.

Reprint requests to: Dr. Kowichi Jimbow, 260G Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta T6G 2S2, Canada.

Abbreviations: AHP, aminohydroxyphenylalanine; BSO, D,L-buthionine-(S,R)-sulfoximine; DEM, diethyl maleate; NAC, N-acetyl-L-cysteine; N-acetyl-4-S-CAP, N-acetyl-4-S-cysteaminylphenol; PTCA, pyrrole-2,3,5-tricarboxylic acid.

0022-202X/95/$09.50 • SSDI0022-202X(95)00052-M •

follicular melanocytes is directly related to the GSH content in the target tissues of both black and yellow mice.

MATERIALS AND METHODS

Animals The black C57BL/6J (a/a) and yellow C57BL/6J (A^y/a) mice, 6-week-old females, were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were housed in the Animal House of the University of Alberta and fed *ad libitum* with water and rat/mouse chow.

Chemicals N-acetyl-4-S-CAP was synthesized in our laboratory using a method [6,7] based on that originally described by Wehrmeister [11]. BSO, diethyl maleate (DEM), and NAC were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytic grade.

Drug Treatment The mice were anesthetized briefly by inhalation of methoxyfluorane (Metophane), and their hair follicles were plucked manually from a small area of the back. N-Acetyl-4-S-CAP, BSO, and NAC were dissolved in 0.9% (w/v) NaCl (normal saline) solution, and these solutions were sterilized by membrane filtration. DEM was diluted in sesame oil for use. Seventy-two hours after plucking, the mice were given intraperitoneal injections of drug solutions daily for 10 consecutive days, as follows.

Dose-Dependent Effect of N-Acetyl-4-S-CAP: Black and yellow mice were given intraperitoneal injections of normal saline (control mice) or solutions of N-acetyl-4-S-CAP in normal saline at concentrations of 0.5, 1.0, and 2.0 mmol/kg, respectively.

GSH Depigmentation: Black mice were randomly divided into five groups and were treated as follows: group 1, intraperitoneal injection of normal saline; group 2, intraperitoneal injection of N-acetyl-4-S-CAP (1.0 mmol/kg); group 3, intraperitoneal injection of N-acetyl-4-S-CAP (1.0 mmol/kg) plus subcutaneous injection of NAC (3.0 mmol/kg) 1 h before and 8 h after the N-acetyl-4-S-CAP administration; group 4, intraperitoneal injection of N-acetyl-4-S-CAP (1.0 mmol/kg) plus intraperitoneal injection of BSO (2.0 mmol/kg) 1 h before the N-acetyl-4-S-CAP administration; and group 5, intraperitoneal injection of BSO (2.0 mmol/kg) alone.

The same protocol was used for lethal yellow mice, except that the dose of N-acetyl-4-S-CAP was increased to 2.0 mmol/kg and groups 4 and 5 mice received both an intraperitoneal injection of BSO (2.0 mmol/kg) and a subcutaneous injection of DEM (0.6 ml/kg) 1 h before N-acetyl-4-S-CAP injection. The end point of the assay was at day 21 after plucking (the beginning of telogen phase).

Melanin Content Newly grown hair from previously plucked areas on the backs of black and yellow mice was harvested, and eumelanin and pheomelanin contents were analyzed using a method described by Ito and Jimbow [12]. Briefly, the eumelanin content was measured by chemical degradation of eumelanin with $KMnO_4$. The product, pyrrole-2,3,5-tricarboxylic acid (PTCA), was analyzed using high performance liquid chromatography with an ultraviolet (UV) detector; samples were measured in triplicate. The results are expressed as ng of PTCA per mg of wet tissue; 1 ng of PTCA corresponds to about 50 ng of eumelanin. The pheomelanin content was measured by high performance liquid chromatography after chemical degradation of pheomelanin by hydriodic acid into aminohydroxyphenylalanine (AHP). The results are expressed as ng of AHP per mg of tissue; 1 ng of AHP is equal to 5 ng of pheomelanin.

GSH Assay

Dose-Dependent Depigmenting Effect of N-acetyl-4-S-CAP: Black and yellow mice, 12 from each strain, were plucked as described previously. Mice from each strain were then randomized into four groups: one control group and three experimental groups treated with different doses of N-acetyl-4-S-CAP. On day 5 after plucking, control mice were given a single intraperitoneal injection of normal saline solution, and mice of the first, second, and third experimental groups were given a single intraperitoneal injection of N-acetyl-4-S-CAP at concentrations of 0.5, 1.0, and 2.0 mmol/kg, respectively.

Effect of GSH-Modulating Agents: Hair follicles were plucked from black and lethal yellow mice, 12 from each strain, and the mice were randomized into four groups, consisting of one control group and three groups treated with the different drug regimens. On day 5 after plucking, control black and yellow mice were given a single intraperitoneal injection of normal saline solution, and black mice from groups 2, 3, and 4 received a single injection of N-acetyl-4-S-CAP (1.0 mmol/kg). In addition, black mice from group 3 were given a single subcutaneous injection of NAC (3.0 mmol/kg) and mice from group 4 received a single injection of BSO (2.0 mmol/kg) 1 h before N-acetyl-4-S-CAP. Yellow mice in groups 2, 3, and 4 were given a single intraperitoneal injection of N-acetyl-4-S-CAP (2.0 mmol/kg). In addition, the group 3 mice were given a single subcutaneous injection of NAC (3.0 mmol/kg), whereas the group 4 mice were given a single intraperitoneal injection of BSO (2.0 mmol/kg) and a subcutaneous injection of DEM (0.6 ml/kg) 1 h before N-acetyl-4-S-CAP administration.

At 2 h after injection of N-acetyl-4-S-CAP, the animals were sacrificed by cervical dislocation. Skin from the plucked areas was removed, immediately frozen in liquid nitrogen, and used later for measurement of total GSH content using a method described by Tietze [13] and Griffith [14]. Briefly, frozen tissues were weighed and then homogenized in 5.0 volumes (w/v) of 1% picric acid (w/v) on ice using a glass-glass homogenizer. The homogenates were centrifuged, and supernatants were removed and used to determine the total GSH content in tissue using a oxidized-GSH–reductase assay [14].

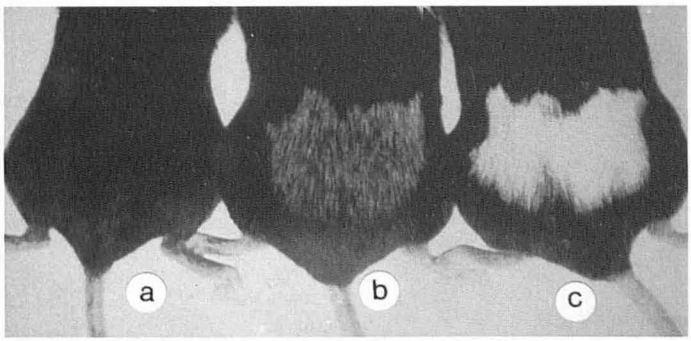

Figure 1. N-Acetyl-4-S-CAP shows a dose-dependent depigmenting effect in black hair follicles after intraperitoneal administration for 10 consecutive days. Drugs were dissolved in normal saline and given intraperitoneally in concentrations of 0.5 mmol/kg (*a*), 1.0 mmol/kg (*b*), and 2.0 mmol/kg (*c*).

RESULTS

N-Acetyl-4-S-CAP Can Induce Dose-Dependent Depigmentation

Black Hair Follicles: N-Acetyl-4-S-CAP administration during the anagen phase of hair regrowth resulted in a dose-dependent effect on follicular melanocytes, with depigmenting changes in their coat color. A dose of 0.5 mmol/kg of N-acetyl-4-S-CAP did not produce any visible depigmentation of the black follicles **(Fig 1*a*)**, whereas a dose of 1.0 mmol/kg was partially effective in that the newly growing hair follicles were gray **(Fig 1*b*)**. N-Acetyl-4-S-CAP at a dose of 2.0 mmol/kg was the most effective, resulting in pure white depigmented hair follicles **(Fig 1*c*)**.

Yellow Hair Follicles: The biologic effect of the N-acetyl-4-S-CAP treatment in lethal yellow mice was remarkably different from that seen in black mice. The doses of N-acetyl-4-S-CAP at 0.5 and 1.0 mmol/kg did not produce any visible change in pigmentation of the new growing hair follicles, and the hair color remained yellow-red, the same shade as in controls **(Fig 2*a*)**. However, in two of the five animals treated with N-acetyl-4-S-CAP at 2.0 mmol/kg, the newly growing follicles were dark, i.e., their color was incompletely black mixed with yellow-red **(Fig 2*b*)**. The hair color of the remaining three animals was the same as that of controls, with no dark (black) component.

GSH Depletion Affects the Depigmentation Potency of N-Acetyl-4-S-CAP

Black Hair Follicles: **Figure 3** shows the effects of three drug regimens on the pigmentation of newly growing hair follicles. A dose of 1.0 mmol/kg of N-acetyl-4-S-CAP caused a partial (gray) depigmentation of hair in black mice **(Fig 3*b*)**. The combination of N-acetyl-4-S-CAP (1.0 mmol/kg) and NAC (3.0 mmol/kg) (before and after administration of N-acetyl-4-S-CAP) almost completely abolished the depigmentation effect of N-acetyl-4-S-CAP **(Fig 3*a*)**, in that the newly growing hair follicles were as black as those of controls **(Fig 1*a*)**. The combination treatment with N-acetyl-4-S-CAP (1.0 mmol/kg) and BSO (2.0 mmol/kg) re-

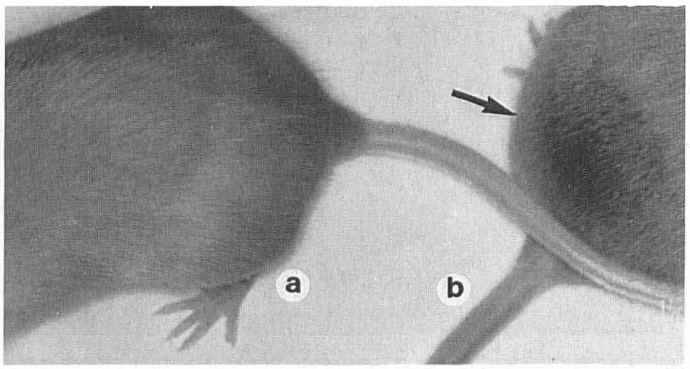

Figure 2. Yellow hair follicles show color changes after intraperitoneal administration of N-acetyl-4-S-CAP. Drugs were given for 10 consecutive days in doses of 1.0 mmol/kg (*a*) and 2.0 mmol/kg (*b*).

sulted in a significant enhancement of the N-acetyl-4-S-CAP depigmentation effect, turning the hairs almost completely white **(Fig 3*c*)**. This effect was equivalent to that produced by 2 mmol/kg of N-acetyl-4-S-CAP without BSO **(Fig 1*c*)**. Control treatment of mice using BSO alone did not produce any visible depigmentation of newly grown hair (data not shown).

Yellow Hair Follicles: The administration of N-acetyl-4-S-CAP (2.0 mmol/kg) to lethal yellow mice resulted in predominantly black pigmentation of the upper portion of newly growing hair follicles in one mouse and partially black (dark) pigmentation of newly growing hair follicles in another two of the five treated animals **(Fig 4*b*)**. In all animals treated with a combination of N-acetyl-4-S-CAP (2.0 mmol/kg) and NAC (3.0 mmol/kg), the color of newly growing hair was the same as that in the controls **(Fig 2*a*)**, i.e., yellowish-red **(Fig 4*a*)**. The combination drug treatment with N-acetyl-4-S-CAP (2.0 mmol/kg), BSO (2.0 mmol/kg) and DEM (0.6 ml/kg) had a different effect, i.e., the new hair follicles were mostly lightly yellow-red and some follicles were gray **(Fig 4*c*)**. The control treatment of yellow mice using BSO and DEM in combination, without N-acetyl-4-S-CAP, did not result in any visible change in normal hair pigmentation (not shown).

Melanin Content in the Hair Follicle Changes After Treatment With N-Acetyl-4-S-CAP and GSH-Modulating Agents

N-Acetyl-4-S-CAP: The eumelanin and pheomelanin contents in hair samples from black and yellow mice treated with different concentrations of N-acetyl-4-S-CAP are summarized in **Table I**. In the black mice, N-acetyl-4-S-CAP treatment with 0.5, 1.0, and 2.0 mmol/kg resulted in a dose-dependent reduction in eumelanin content of hair follicles to 98%, 28%, and 3% of that in the control group, respectively. Only nonsignificant amounts of pheomelanin were detectable in the hair follicles of black mice.

In lethal yellow mice, treatment with the 0.5- and 1.0-mmol/kg doses of N-acetyl-4-S-CAP did not significantly affect the pheomelanin content in newly grown hair follicles, and the AHP values were 99% and 90% of control, respectively. In these mice, practically no eumelanin was detectable (the eumelanin content was similar to that of albino mice) (data not shown). N-acetyl-4-S-CAP at 2.0 mmol/kg gave a dramatically different result. In this case, the pheomelanin content in newly grown hair follicles decreased to 56% of control levels and the eumelanin content became detectable, reaching levels as high as 28% of that seen in control black mice.

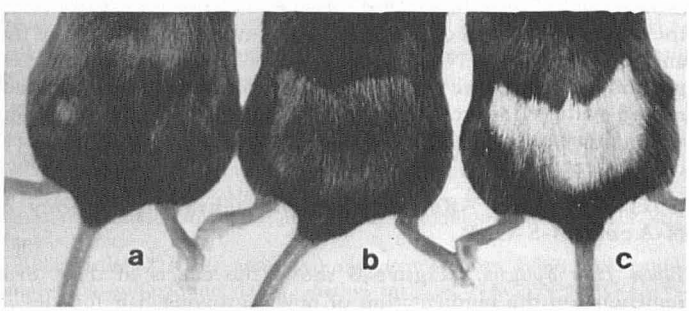

Figure 3. Black hair follicles show enhanced color changes after intraperitoneal administration of N-acetyl-4-S-CAP in combination of NAC and BSO. Drugs were given for 10 consecutive days in three different treatments: N-acetyl-4-S-CAP (1.0 mmol/kg) and NAC (3.0 mmol/kg), resulting in no hair color change (*a*); N-acetyl-4-S-CAP (1.0 mmol/kg) alone, resulting in gray hair color (*b*); and N-acetyl-4-S-CAP (1.0 mmol/kg) and BSO (2.0 mmol/kg), resulting in complete depigmentation (*c*).

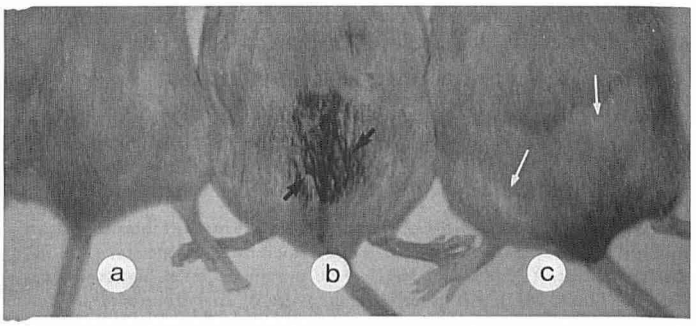

Figure 4. Yellow hair follicles show different color changes after intraperitoneal administration of N-acetyl-4-S-CAP in combination with NAC, BSO, and DEM. Drugs were given for 10 consecutive days in three different treatments: N-acetyl-4-S-CAP (2.0 mmol/kg) and NAC (3.0 mmol/kg), resulting in no change of hair color (yellow-red) (*a*); N-acetyl-4-S-CAP (2.0 mmol/kg) alone, resulting in dark hair (*arrows*) (*b*); and N-acetyl-4-S-CAP (2.0 mmol/kg), BSO (2.0 mmol/kg), and DEM (0.6 ml/kg), resulting in some gray hair follicles (*arrows*) (*c*).

GSH-Modulating Agents: **Table II**, presents the results of measurements of eumelanin and pheomelanin content in hair follicles of black and lethal yellow mice treated with three different drug regimens. In black mice, the gray hair follicles of mice treated with N-acetyl-4-S-CAP alone **(Fig 3*b*)** contained 29% of the eumelanin content of controls expressed as its PTCA derivative. Black hair contained 29% of the eumelanin content of controls expressed as its PTCA derivative. Black hair follicles from mice treated with NAC before and after the N-acetyl-4-S-CAP injection showed essentially no difference in eumelanin content compared with controls. In contrast, in the white-gray hair follicles from mice treated with N-acetyl-4-S-CAP and BSO **(Fig 3*c*)**, the eumelanin content was reduced to 14% of that in controls. Among lethal yellow mice, the follicles of control mice contained no significant amount of eumelanin (less than 1.4% of that of black controls). However, after treatment with N-acetyl-4-S-CAP alone, the eumelanin content became more obvious and increased to 20% of that in control black mice. At the same time, the pheomelanin content was reduced to 56% of that in control yellow mice. The hair follicles from mice treated with NAC before and after administration of N-acetyl-4-S-CAP revealed no significant difference in the total amounts of eumelanin and pheomelanin as compared with control yellow mice. Pretreatment of mice with BSO and DEM before administration of N-acetyl-4-S-CAP resulted in a significant reduction in pheomelanin content, up to 15% of control levels. Treatment with DEM resulted in a significant additive effect of BSO for the reduction of tissue GSH content (see **Table IV**).

Table I. Eumelanin and Pheomelanin Contents Vary in Black and Yellow Mouse Hair Follicles Treated with N-Acetyl-4-S-CAP[a]

Mouse Strain	Dose N-Acetyl-4-S-CAP (mmol/kg)	PTCA[b] (ng/mg)	AHP[c] (ng/mg)	PTCA/AHP Ratio
Black C57BL/6J (a/a)	None (control)	1079.0 ± 109.0	55.3 ± 4.1	19.51
	0.5	1058.0 ± 148.7	50.8 ± 10.2	20.83
	1.0	298.0 ± 20.6	58.1 ± 13.5	5.13
	2.0	33.3 ± 7.4	55.0 ± 9.8	6.61
Yellow C57BL/6J (A^y/a)	None (control)	16.1 ± 1.4	2882.1 ± 280.2	0.0056
	0.5	15.8 ± 0.6	2851.9 ± 330.4	0.0055
	1.0	15.9 ± 0.8	2590.9 ± 225.7	0.0061
	2.0	304.2 ± 41.2	1608.4 ± 324.9	0.1891

[a] N-acetyl-4-S-CAP was given intraperitoneally daily for 10 d. Data expressed as sample mean ± SD (n = 5).
[b] A eumelanin indicator.
[c] A pheomelanin indicator.

GSH Content in Skin Changes After Various Drug Treatments

N-acetyl-4-S-CAP: The N-acetyl-4-S-CAP treatments resulted in a dose-dependent depletion of GSH in the tissues of both black and lethal yellow mice. As shown in **Table III**, doses of N-acetyl-4-S-CAP at 0.5, 1.0, and 2.0 mmol/kg reduced the GSH content in the skin to 94%, 85%, and 76% of control levels in black mice, and to 99%, 95%, and 82% of control levels in yellow mice, respectively. The same doses of N-acetyl-4-S-CAP also resulted in a dose-dependent GSH depletion in the lung to 92%, 87%, and 81% of control levels in black mice and to 99%, 91%, and 86% in yellow mice, respectively; GSH was also depleted in the liver to 99%, 86%, and 64% of control levels in black mice and to 97%, 92%, and 77% of control levels in yellow mice, respectively.

GSH-Modulating Agents: The treatment with N-acetyl-4-S-CAP (1.0 mmol/kg in black and 2.0 mmol/kg in lethal yellow mice) resulted in depletion of GSH in the skin of both black and yellow mice to 85.6% and 80.0% of control levels, respectively **(Table IV)**. The pretreatment of animals with NAC significantly prevented GSH depletion in the two strains; the GSH content was 98.6% of control levels in black mice and 93.9% of control levels in lethal yellow mice. The combination treatment in black mice with N-acetyl-4-S-CAP and BSO, and in yellow mice with N-acetyl-4-S-CAP, BSO, and DEM, resulted in significant increases in GSH depletion, the GSH content being 47.8% and 33.4% of control levels in black and yellow mice, respectively.

DISCUSSION

The *in vivo* administration of N-acetyl-4-S-CAP to black C57BL/6J mice demonstrated remarkable depigmenting potential in activated follicular melanocytes. A clearly dose-dependent depigmentation was observed, ranging from no visible depigmentation through various shades of gray, to pure white depigmentation of newly grown hair. It is widely believed that the depigmenting action of phenolic agents against melanocytes derives from melanocytotoxicity mediated through the interaction of these agents with tyrosinase [1,3]. Tyrosinase is believed to oxidize phenols into highly reactive orthoquinones, which may give rise to semiquinone radicals and their derivatives. In the cytosol, these reactants are either detoxified by conjugation with GSH or, if not neutralized, are capable of inducing chemical damage to cells by the alkylation of macromolecules or by oxidative stress. Possible target organelles for this proposed pathway include the nucleus (e.g., inhibition of DNA polymerase), mitochondria (inhibition of oxidative phosphorylation), and the rough endoplasmic reticulum (inhibition of protein synthesis and glycosylation). The induced chemical damage could be reversible if it involved the perturbation of some nonessential function, such as melanogenesis, or could be irreversible with resulting cell death if it interfered with essential metabolic functions [15].

The effects of N-acetyl-4-S-CAP against black hair follicles may be explained by the concept of tyrosinase-mediated cytotoxicity of phenols. It may be assumed that N-acetyl-4-S-CAP molecules enter

Table II. Eumelanin and Pheomelanin Contents of Black and Yellow Mouse Hair Follicles Change After Treatment with N-Acetyl-4-S-CAP and Its Combination with NAC and BSO[a]

Mouse Strain	Treatment[b]	PTCA[c] (ng/mg)	AHP[d] (ng/mg)	PTCA/AHP Ratio
Black C57BL/6J (a/a)	None[e]	1090.0 ± 160.0	53.0 ± 3.2	
	Normal saline	1079.0 ± 109.0	55.3 ± 4.1	19.51
	N-acetyl-4-S-CAP	312.5 ± 56.8	52.1 ± 6.2	6.01
	N-acetyl-4-S-CAP + NAC	1037.5 ± 63.7	50.8 ± 7.0	20.41
	N-acetyl-4-S-CAP + BSO	154.2 ± 25.7	48.3 ± 3.8	3.19
Yellow C57BL/6J (A^y/a)	None[e]	4.4 ± 1.1	2930.0 ± 310.0	
	Normal saline	16.1 ± 1.4	2882.1 ± 280.2	0.006
	N-acetyl-4-S-CAP	212.5 ± 35.4	1608.4 ± 324.9	0.132
	N-acetyl-4-S-CAP + NAC	15.3 ± 0.8	2695.4 ± 301.1	0.006
	N-acetyl-4-S-CAP + NAC + DEM	4.3 ± 0.6	436.7 ± 111.3	0.010
White albino (KK/c/c)	None[e]	2.3 ± 1.2	15.0 ± 8.0	

[a] Data expressed as sample mean ± SD (n = 5).
[b] Treatment of black mice: N-acetyl-4-S-CAP (2.0 mmol/kg), NAC (3.0 mmol/kg), BSO (2.0 mmol/kg). Treatment of yellow mice: N-acetyl-4-S-CAP (2.0 mmol/kg), NAC (3.0 mmol/kg), BSO (2.0 mmol/kg), DEM (0.6 ml/kg).
[c] A eumelanin indicator.
[d] A pheomelanin indicator.
[e] Data previously reported by Jimbow et al [30].

Table III. GSH Content of the Skin, Lung, and Liver of Black and Yellow Mice Decreases 2 h After a Single Injection of N-Acetyl-4-S-CAP in Concentrations of 0.5, 1.0, and 2.0 mmol/kg[a]

Mouse Strain	Treatment (mmol/kg)	GSH Content (μmol/g of Tissue)		
		Skin	Lung	Liver
Black C57BL/6J (a/a)	Normal saline (control)	0.541 ± 0.037	2.014 ± 0.179	5.065 ± 0.234
	N-acetyl-4-S-CAP 0.5	0.512 ± 0.029	1.855 ± 0.106	5.014 ± 0.223
	N-acetyl-4-S-CAP 1.0	0.460 ± 0.033	1.750 ± 0.143	4.357 ± 0.284
	N-acetyl-4-S-CAP 2.0	0.372 ± 0.021	1.633 ± 0.138	3.205 ± 0.380
Yellow C57BL/6J (A^y/a)	Normal saline (control)	0.620 ± 0.041	2.095 ± 0.159	5.443 ± 0.359
	N-acetyl-4-S-CAP 0.5	0.617 ± 0.030	2.066 ± 0.155	5.282 ± 0.235
	N-acetyl-4-S-CAP 1.0	0.550 ± 0.058	1.903 ± 0.121	4.990 ± 0.195
	N-acetyl-4-S-CAP 2.0	0.499 ± 0.054	1.787 ± 0.112	4.166 ± 0.315

[a] Data expressed as mean ± SD (n = 3).

the melanocyte, and thence the melanosome, and there compete with L-tyrosine for binding to tyrosinase. Tyrosinase is the rate-limiting enzyme of melanogenesis, which implies that there is some maximum rate (limit) for the production of orthoquinone molecules. With an increased dose of N-acetyl-4-S-CAP, one may also expect increased production of orthoquinone molecules derived from N-acetyl-4-S-CAP, and thus an increased probability for the manifestation of its cytotoxic potential. From the present observation of different responses of follicular color in black and lethal yellow mice, it may be postulated that the bulk of reactive intermediates derived at the dose of 0.5 mmol/kg was insufficient to induce chemical injury and consequently failed to damage the follicular melanocytes. The net result was normal black pigmentation of newly grown hair. In contrast, a dose of 1.0 mmol/kg of N-acetyl-4-S-CAP was capable of inducing some chemical injury to the follicular melanocytes; however, this degree of cellular damage was compatible with survival in the majority of these follicular melanocytes. The result was partial inhibition of melanogenesis and gray depigmentation of new hair. The amount of reactive intermediates derived from a dose of 2.0 mmol/kg overcame the capacity of follicular melanocytes to survive the chemical insult and led to their total destruction. The result was complete depigmentation, and the newly grown hair was pure white. An alternative mechanism to explain the changes in hair color is to assume that N-acetyl-4-S-CAP is incorporated into melanin, resulting in different spectral characteristics. However, both *in vivo* and *in vitro* studies have shown that N-acetyl-4-S-CAP is cytotoxic to melanocytes, as revealed by morphologic (microscopy) studies of skin and biochemical assays on several melanoma cell lines [7].

We found that even a single intraperitoneal injection of N-acetyl-4-S-CAP (2.0 mmol/kg) was capable of producing complete depigmentation of new hair when administered at the beginning of the melanogenic phase of hair growth in black mice. However, the response effect of N-acetyl-4-S-CAP against activated follicular melanocytes in lethal yellow mice was different from that observed in black mice. The lower doses of N-acetyl-4-S-CAP (0.5 and 1.0 mmol/kg) did not affect the normal yellow pigmentation of new hair. However, the dose of 2.0 mmol/kg resulted in a dark, almost black color of new hair. The black color suggests the presence of eumelanin pigment, and this was confirmed through measurement of eumelanin and pheomelanin content of the hair follicles of lethal yellow mice after treatment with N-acetyl-4-S-CAP.

Previously, Geschwind *et al* [16] reported the conversion of pheomelanogenesis into eumelanogenesis in lethal yellow mice (A^y/a) after injections of high doses of α-melanocyte–stimulating hormone. Similarly, Ikejima and Takeuchi [17] and Jimbow and Takeuchi [18] reported the production of eumelanin in the hair-bulb melanocytes of ultraviolet-irradiated A^y/a mice. However, conversion of pheomelanogenesis into eumelanogenesis by synthetic drugs *in vivo* has not been described previously. In contrast, several studies had concluded that follicular melanocytes of yellow mice are not susceptible to depigmenting phenols [8,19]. A possible explanation for this phenomenon is based on the reported low level of tyrosinase activity (and resulting potentially cytotoxic intermediates of eumelanogenesis) in yellow follicular melanocytes [8]. Both eumelanin and pheomelanin have the same natural precursor, dopaquinone. Dopaquinone is a highly reactive electrophile with a very short life span [20,21]. In the presence of nucleophilic thiols such as cysteine or GSH, dopaquinone forms cysteinyldopa and/or glutathionedopa conjugates. Specifically, 5-S-cysteinyldopa is a major precursor for pheomelanin synthesis. In the absence of a significant concentration of thiols inside the melanosome, dopaquinone is cycled into dopachrome and further into indoles, which polymerize to eumelanin [22]. Thus, the critical factor that directs dopaquinone into the pheomelanic or eumelanic pathway is the presence or absence of thiols at the site of melanogenesis [23].

N-Acetyl-4-S-CAP is thought to be an inert lipophilic drug (phenolic thioether) that enters cells and exerts its cytotoxic effect by altering cytosolic GSH levels [24]. The formation of N-acetyl-4-S-CAP–GSH conjugates would lead to a relative depletion of GSH in the cytosol. Because GSH is a major reducing equivalent in cells, as well as a major physiologic reservoir for cysteine in many cell types, the GSH depletion may also lead to a shortage of reduced cysteine in the cytoplasm and consequently inside the melanosomes [25,26]. The reduced level of free cysteine in the melanosome could prevent pheomelanin synthesis. Under these circumstances, the tyrosinase product dopaquinone would enter the eumelanin-synthetic pathway by default. The outcome would be black pigmentation of newly grown hair in genetically yellow mice, as evidenced by the present study.

In this study, we used NAC, an effective agent for the stimulation

Table IV. GSH Content of the Skin of Black and Yellow Mice Decreases After Treatment with GSH-Modulating Agents *In Vivo*[a]

Mouse Strain	Normal Saline (Control)	N-Acetyl-4-S-CAP	N-Acetyl-4-S-CAP + NAC	N-Acetyl-4-S-CAP + BSO + DEM
Black[b] C57BL/6J (a/a)	0.533 ± 0.048	0.456 ± 0.020	0.526 ± 0.031	0.255 ± 0.041
Yellow[c] C57BL/6J (A^y/a)	0.608 ± 0.056	0.486 ± 0.043	0.526 ± 0.031	0.203 ± 0.036

[a] Data expressed as mean ± SD. GSH content in μmol/g of tissue (n = 5).
[b] Treatment of black mice: N-acetyl-4-S-CAP (1.0 mmol/kg), NAC (3.0 mmol/kg), BSO (2.0 mmol/kg).
[c] Treatment of yellow mice: N-acetyl-4-S-CAP (2.0 mmol/kg), NAC (3.0 mmol/kg), BSO (2.0 mmol/kg), DEM (0.6 ml/kg).

of GSH synthesis through nontoxic cysteine delivery [15], to increase GSH levels. To deplete GSH levels in tissues of both black and lethal yellow mice, we used a selective and irreversible inhibitor of gamma-glutamylcysteine synthetase, BSO. In addition, DEM, which could be combined with NAC, was used to deplete GSH in lethal yellow mice [27]. Recently, these drugs have been used for GSH modulation in pigment cells *in vitro* [28,29]. However, there have been no reports describing the use of NAC, BSO, or DEM in combination with a melanocytotoxic phenol for the purpose of hair depigmentation studies *in vivo*.

The modulation of the GSH concentration in tissues by the drugs described above strongly influenced the effects of N-acetyl-4-S-CAP. The repletion of GSH by supplying cysteine through NAC effectively inhibited the melanocytotoxicity in both black and lethal yellow mice; consequently, the newly growing hair had the same pigmentation as that of controls. The GSH depletion by BSO and/or DEM enhanced the intermediate depigmenting effect of N-acetyl-4-S-CAP into maximal depigmentation in black mice and visible depigmentation in lethal yellow mice. Our data thus indicate a direct relation between drug-induced changes in GSH levels in the skin and changes in the eumelanin/pheomelanin content of hair. Hence, the GSH content in the target tissue of black and yellow mice is one of the major regulatory factors for the depigmentation effect of N-acetyl-4-S-CAP.

This study was supported by grants-in-aid from National Cancer Institute of Canada (4145), Medical Research Council of Canada (PA1193), Alberta Cancer Board (R1-91), and Stiefel International Inc. KJ is an AHFMR medical scientist.

REFERENCES

1. Ortonne JP, Mosher DB, Fitzpatrick TB: Vitiligo and other hypomelanoses of hair and skin. In: *Dematology in General Medicine*. Parish JA, Fitzpatrick TB (eds.). Plenum Medical Book Company, New York, 1983, pp 479–507
2. Frank E, Pathak MA, Szabo G, Fitzpatrick TB: Selective action of mercaptoethylamines on melanocytes in mammalian skin. *Arch Dermatol* 97:465–477, 1968
3. Riley PA: Chemical leucoderma. In: Seiji M (ed.). *Phenotypic Expression in Pigment Cells*. University of Toyko Press, Toyko, 1981, pp 445–449
4. Hochstein P, Cohen G: The cytotoxicity of melanin precursors. *Ann N Y Acad Sci* 100:876–886, 1963
5. Miura T, Jimbow K, Ito S: The *in vivo* antimelanoma effect of 4-S-cysteaminylphenol and its N-acetyl derivative. *Int J Cancer* 46:931–934, 1990
6. Jimbow K, Iwashina T, Alena F, Yamada K, Pankovich J, Umemura T: Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma. *J Invest Dermatol* 100:231S–238S, 1993
7. Alena F, Jimbow K: Melanocytotoxicity and antimelanoma effects of phenolic amine compounds in mice *in vivo*. *Cancer Res* 50:3743–3747, 1990
8. Silvers WK: The coat color of mice. In: Silvers WK (ed.). Springer-Verlag, New York, 1979, pp 30–62
9. Ito Y, Jimbow K: Selective cytotoxicity of 4-S-cysteaminylphenol on follicular melanocytes of the black mouse—rational basis for its application to melanoma chemotherapy. *Cancer Res* 47:3278–3286, 1987
10. Quevedo WC Jr, Holstein TJ, Dyckman J, Nordlung JJ: Influence of depigmenting chemical agents on hair and skin color in yellow (pheomelanic) and black (eumelanic) mice. *Pigment Cell Res* 3:71–79, 1990
11. Wehrmeister HL: N-(arylmercaptoalkyl)amides by reaction of thiols with amino alcohols and carboxylic acids. *J Organ Chem* 28:2589–2591, 1963
12. Ito S, Jimbow K: Quantitative analysis of eumelanin and pheomelanin in hair and melanosomes. *J Invest Dermatol* 80:268–272, 1983
13. Tietze F: Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal Biochem* 27:507–521, 1969
14. Griffith OW: Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 106:207–212, 1980
15. Bridges JW, Benford DJ, Jubbard SA: Mechanism of toxic injury. *Ann N Y Acad Sci* 407:42–63, 1983
16. Geshwind II, Hyseby RA, Nishioka R: The effect of melanocyte-stimulating hormone on coat color in the mouse. *Recent Prog Horm Res* 28:91–130, 1972
17. Ikejima T, Takeuchi T: Environmental control on gene expression in the mouse hair pigmentation. *Jpn J Genet* 55:283–287, 1980
18. Jimbow K, Takeuchi T: Ultrastructural comparison of pheo- and eumelanogenesis in animals. *Pigment Cell* 4:308–317, 1979
19. Snell RS: Monobenzylether of hydroquinone. *Arch Dermatol* 90:63–70, 1964
20. Reszka K, Jimbow K: Electron-donor and acceptor properties of melanin pigments in the skin. In: Fuchs J, Packer L (eds.). *Oxidative Stress in Dermatology*. Marcel Dekker Inc, New York, 1993, pp 287–320
21. Prota G: Melanin and pigmentation. In: Dolphin D, Avramovic O, Poulson R (eds.). *Glutathione. Chemical, Biochemical, and Medical Aspects*. John Wiley & Sons, New York, 1988, Part B, pp 441–465
22. Wick MM: Melanin: structure and properties. In: Fitzpatrick TB, Wick MM, Kiyoshi T (eds.). *Brown Melanoderma. Biology and Disease of Epidermal Pigmentation*. University of Tokyo Press, Toyko, 1986, pp 37–44
23. Jimbow K, Fitzpatrick TB, Wick MM: Biochemistry and physiology of melanin pigmentation. In: Goldsmith LA (ed.). *Physiology, Biochemistry and Molecular Physiology of the Skin*. Oxford University Press, New York, 1991, pp 873–909
24. Chasseaud LF: Conjugation with glutathione and mercapturic acid excretion. In: Arias IM, Jakoby WB (eds.). *Glutathione, Metabolism and Function*. Raven Press, New York, 1976, pp 77–104
25. Reed DJ, Brodie AE, Meredith MJ: Cellular heterogeneity in the status and functions of cysteine and glutathione. In: Larsson A, *et al* (eds.). *Functions of Glutathione, Biochemical, Physiological, Toxicological, and Clinical Aspects*. Raven Press, New York, 1983, pp 39–49
26. Rorsman H, Rosengren E: Biosynthesis of melanin. In: Fitzpatrick TB, Wick MM, Toda E (eds.). *Brown Melanoderma. Biology and Disease of Epidermal Pigmentation*. University of Tokyo Press, Tokyo, 1986, pp 63–74
27. Plummer JL, Smith BR, Sies H, Bend JR: Chemical depletion of glutathione *in vivo*. In: Colowick SP, Kaplan ND (eds.). *Methods in Enzymology* 77. Academic Press, New York, 1981, pp 50–63
28. Karg E, Tunek A, Brotell H, Rosengren E, Rorsman H: Alteration of glutathione level in human melanoma cells: effect of N-acetyl-cysteine and its analogues. *Pigment Cell Res* 3:11–15, 1990
29. Prezioso JA, Fitzgerald GB, Wick MM: Effects of tyrosinase activity on the cytotoxicity of 3,4-dihydroxybenzylamine and buthionine sulfoximine in human melanoma cells. *Pigment Cell Res* 3:49–54, 1990
30. Jimbow K, Lee SK, King M, Hara H, Chen H, Dakour J, Marusyk H: Melanin pigments and melanosomal proteins as differentiation markers unique to normal and neoplastic melanocytes. *J Invest Dermatol* 100:259S–268S, 1993