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N-PROPIONYL-4-S-CYSTEAMINYLPHENOL EXERTS SELECTIVE MELANOCYTOTOXICITY THROUGH APOPTOTIC PROCESS

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ABSTRACT **Phenolic thioether amines, 4-S-cysteaminylphenol (4-S-CAP) and its N-acetyl derivative, N-acetyl-4-S-CAP, are tyrosinase substrates and potent depigmenting agents for skin and hair follicles. They also possess an antitumor effect on melanoma. However, it is not clear if their biological activity derives from a cytostatic or a cytocidal effect on melanocytes. We have recently synthesized a new derivative of 4-S-CAP, N-propionyl-4-S-CAP (NPr-CAP), in order to develop a better targeted chemotherapeutic or depigmenting agent. Structurally, NPr-CAP is more lipophilic than N-acetyl-4-S-CAP, hence it is expected to have better penetration into the cells and a more potent pharmacological effect. This study is aimed to identify the selectivity and potency of the biological effect of NPr-CAP on melanocytotoxicity. NPr-CAP was found to be a good tyrosinase substrate. The *i. p.* administration of NPr-CAP caused dose-dependent depigmentation of black hair follicles in C57 mice. However, the black hair follicles replaced the depigmented ones in the next hair cycle, suggesting the existence of dormant or drug-resistant melanocytes. Follicular melanocytes stained positively with fluorescein isothiocyanate by TUNEL (TdT-mediated dUTP-biotin nick end labeling) method, and by using the electron microscope, showed apoptotic changes as early as 12 h post-administration of NPr-CAP. However, surrounding keratinocytes and fibroblasts revealed no changes. NPr-CAP treatment induced irreversible inhibition of cell proliferation of melan-a2, an immortal melanocyte line of C57 black mice, but this did not occur in the control albino melanocyte line, melan-c. The agarose gel electrophoresis of DNA from drug-treated melan-a2 cells showed the nucleosomal DNA ladder pattern. The MTT assay and trypan blue test indicated that NPr-CAP can cause cell death of tyrosinase-positive melan-a2 cells, whereas, it transiently inhibits the proliferation of tyrosinase-negative melan-c cells. Thus, we propose that the melanocytotoxicity of NPr-CAP is selective and causes apoptosis in melanocytes through the interaction with active tyrosinase.**
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Key Words: Phenolic thioether amine, Apoptosis, Oxidative stress

Introduction

Melanocytes and melanoma cells have unique biological properties that can synthesize melanin pigments in the presence of tyrosinase. After converting tyrosine to dopa and subsequently from dopa to dopaquinone by tyrosinase, eumelanin or pheomelanin is formed[1]. Quinone and semiquinone derivatives

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produced during tyrosinase-mediated oxidation are potentially cytotoxic[2]. In our previous studies, sulfur analogues of tyrosine and their amine derivatives, phenolic thioether amines, have been found to be a better biological substrate of tyrosinase than tyrosine as a potent antitumor agent against malignant melanoma[2]. While the initial compound, 4-S-cysteaminylphenol (4-S-CAP), was a substrate of both tyrosinase and plasma monoamine oxidase, potentially causing the general toxicity by plasma monoamine oxidase[3-5], a newly developed N-acetyl derivative of 4-S-CAP, N-acetyl-4-S-CAP, was a substrate of tyrosinase but not of plasma monoamine oxidase[4] and possessed a significant melanocytotoxic and chemotherapeutic effect[6]. However, there was a possibility of having deacetylation of N-acetyl-4-S-CAP to produce 4-S-CAP after systemic administration[7]. In order to further develop a better targeted chemotherapeutic and/or depigmenting agent, we have recently synthesized an N-propionyl derivative of 4-S-CAP, N-propionyl-4-S-CAP (NPr-CAP), which is structurally more lipophilic and stable than 4-S-CAP and N-acetyl-4-S-CAP, hence it is expected to possess better penetrating properties into the cells and have a more potent melanocytotoxicity.

There are two major processes for cell death, apoptosis and necrosis. Apoptosis has morphological and biochemical characteristic properties, such as the condensation of nucleosomal chromatin and cytoplasm, cell shrinkage[8,9] and DNA fragments[10,11]. Therefore, it should be distinguished from necrosis. Apoptosis occurs during the process of forming and developing various normal tissues and cells, as well as during many kinds of environmental stresses, like antitumor agents[12,13] and oxidative stress[14,15]. Although previous studies have shown that phenolic thioether amines (*e. g.* N-acetyl-4-S-CAP) cause the selective degeneration of melanocytes, it is unknown if it occurs via the apoptotic or necrotic process. The following study is performed to examine the selectivity and biological potency of this newly developed compound, NPr-CAP. Specifically, we are interested in clarifying which one of the cell death processes is associated with this selective degeneration of melanocytes following the administration of NPr-CAP. Our results show that the melanocyte degeneration induced by NPr-CAP is mediated by apoptosis through interaction with tyrosinase.

Materials And Methods

Animals: C57BL/6J Jcl black adult mice (7~8-week-old females) and pregnant mice were purchased from CLEA Japan Inc. (Saitama, Japan) and maintained at our research animal farm. The newborn mice (3-days old) were obtained from the pregnant ones.

Chemical Amine: N-propionyl-4-S-cysteaminylphenol (NPr-CAP) was recently synthesized in our laboratory as a new derivative of 4-S-CAP[16]. It is a chemically stable compound even at boiling temperature of 100°C. For the *in vivo* depigmentation study, NPr-CAP was dissolved in warmed normal saline solution, supplemented with 7.3% propylene glycol at 30 mM and filtered to sterilize. For the *in vitro* culture study, it was dissolved in warmed RPMI 1640 (Bio Whittaker, Walkersville, MD) and filtered to sterilize.

Cell Lines and Culture: Several immortal murine melanocyte lines were used: melan-a2 and melan-c, which were kindly supplied by Dr. D. C. Bennett, London, UK[17,18]. The cells were grown as monolayers with a complete medium, *i. e.* RPMI 1640 supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies, Burlington, ON), penicillin (100 unit/m*l*, Bio Whittaker, Walkersville, MD), streptomycin (100 μg/m*l*, Bio Whittaker, Walkersville, MD) and phorbol-12-myristate-13-acetate (PMA, 200 nM, Calbio-Chem, San Diego, CA). These cells were incubated at 37°C humidified atmosphere of 5% CO_2 in air. One day prior to NPr-CAP exposure for any assay, exponentially grown cells were harvested and seeded at a specific density and cultured with a complete medium without PMA. The medium was then replaced by a medium containing fetal calf serum, penicillin, streptomycin and

specifically concentrated with NPr-CAP, but no PMA. The cells were incubated for 24 h under the same conditions for any assay.

In Vivo Drug Treatment: Twenty adult mice were anesthetized briefly by inhalation of diethyl ether, and then their hair follicles were plucked manually from the lumbar area following our previous method[19]. Seventy-two hours after plucking, each of the ten mice was given NPr-CAP (1.5 mmol/kg of body weight [BW]) by *i. p.* injection, or normal saline solution (50 m*l*/kg of BW) for 12 consecutive days. The day after completion of the repeated administrations of NPr-CAP, each of the five mice was biopsied. The other ten mice were kept alive to follow the development of white hair follicles. The newborn mice were given a single or repeated (for 3 days) *i. p.* injection of NPr-CAP solution with a quantity of 1.5 mmol/kg of BW or normal saline solution (50 m*l*/kg of BW). Each of the five newborn mice was sacrificed for electron microscopic and histochemical examinations at 4, 12 and 16 h following a single *i. p.* injection of the compound. Skin and hair follicles were biopsied from the lumbar area. Each of the five mice was kept alive to follow the development of white hair follicles.

Microscopic Preparation: Biopsied specimens were pre-fixed with Karnovsky solution (pH 7.2) and post-fixed with 1% osmium tetroxide in cacodylate buffer. After routine dehydration and embedding, they were processed to thin-sectioning, and thin sections were double-stained with uranyl acetate and lead citrate and observed under an electron microscope.

Tyrosinase Activity: The tyrosinase activity of melan-a2 and melan-c cells was examined by the incubation of 1% Triton X-100 phosphate-buffer-saline extract in 0.15% L-dopa in 0.05 M phosphate buffer, pH 6.8, 30 min at 37°C. The reaction product was assayed at OD 475 nm. Kinetics of tyrosinase for NPr-CAP, Km and Vmax were measured, following our previous reports[20,21].

TdT-Mediated dUTP-Biotin Nick End Labeling (TUNEL) Method: Biopsied samples were fixed in 4% paraformaldehyde solution and embedded in paraffin. After deparaffinization, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method was performed using the MEBSTAIN Apoptosis kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) to identify apoptotic cells. Sections were treated with proteinase K for 30 min at 37°C, followed by TdT buffer along with biotin-dUTP for 60 min at 37°C, blocking the solution for 10 min at room temperature and avidin-fluorescein isothiocyanate solution for 30 min at 37°C.

DNA Extraction and Gel Electrophoresis: The cells were seeded at a density of $5\text{-}10\times10^4$ cells/cm^2, exposed to NPr-CAP (0, 1, 2 and 4 mM) for 24 h and the adhered and detached cells were harvested for DNA extraction. Cell suspensions were lysed in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0% Triton X-100 (Boehringer Mannheim, Laval, Quebec) and 10 mM ethylenediaminetetraacetic acid for 20 min at 4°C, and were centrifuged at 13,000 g for 30 min. The removed supernatant was incubated sequentially with RNase A (400 μg/m*l*, Boehringer Mannheim, Laval, Quebec) for 70 min and proteinase K (400 μg/m*l*, Boehringer Mannheim, Laval, Quebec) for 70 min DNA was precipitated with 0.5 M sodium chloride in isopropyl alcohol, dried and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid. The samples were electrophoresed on 1.5% agarose gel at 90 V. Following electrophoresis, the gel was stained with 0.5 μg/m*l* ethidium bromide and photographed on an UV transilluminator. DNA molecular weight marker, 123 bp DNA Ladder (Gibco BRL, Life Technologies, ON), was used for calibration.

MTT Assay: Cell viability, activity and proliferation of drug-treated melan-a2 and melan-c cells were determined using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay as described previously[22]. MTT assay primarily measures the mitochondrial activity which reflects cell activity, viability and even proliferation. The cells were dispensed at a density of 1×10^4 cells/well within 96-well culture plates using a multichannel pipette. After 24 h, treatment with NPr-CAP was

performed by adding a specified concentration. At the end of the assay, 10 μl of MTT (Sigma, St. Louis, MO) solution (5 mg/ml in phosphate buffer saline) was added to each culture well and incubated for 4 h at 37°C. Following incubation, 100 μl of 0.04 N HCl in isopropyl alcohol was added to dissolve MTT-formazan product. After thorough dissolution, the absorbance at 550 nm was measured with a microplate reader (EAR 400 AT). Background samples (n=5) were prepared with cells in the concentrated medium with 0.04 N HCl in isopropyl alcohol but no addition of MTT solution. Cell activity/viability was expressed as the percentage of viable cells compared to two control groups, one derives from the cells treated without NPr-CAP for 24 h and the other from cells prior to drug treatment, following subtraction of background.

For the cell proliferation assay after exposure to NPr-CAP, the cells were dispensed at a density of 5×10^3 cells/well within 96-well culture plates. Following treatment with specified concentrations of NPr-CAP for 24 h, one plate of each cell line was removed from treatment. Other plates of each cell line were washed with RPMI 1640 and incubated with complete medium with and without PMA for 24 and 48 h. The subsequent procedures were the same as those described above. Cell proliferation was expressed as the ratio of viable cells at 24 and 48 h post-treatment compared to the NPr-CAP-exposed cells without subsequent incubation (0 h) following subtraction of background (n=5).

Trypan Blue Exclusion Test: The cell viability was also determined by using a trypan blue exclusion test. The cells were seeded at a density of 1×10^6 cells per 60 mm dishes, and exposed to NPr-CAP (0 mM, 1 mM and 3 mM) for 24 h. Detached and adhered cells were harvested and suspended in phosphate buffer saline. One volume of 0.4% trypan blue solution (Sigma, St. Louis, MO) was added to two volumes of the cell suspension, and the dye-excluding cells were quantified by using a light microscope in a hemocytometer at a magnification of ×100 (n=3).

Results

Chemical Structure and Tyrosinase Kinetics of Synthetic Compounds: The chemical structure of NPr-CAP and its kinetics for tyrosinase are compared with those of tyrosine, 4-S-CAP and N-acetyl-4-S-CAP (shown in Fig. 1). NPr-CAP possesses less affinity to, but reacts much faster to tyrosinase than tyrosine, 4-S-CAP and N-acetyl-4-S-CAP. This indicates that NPr-CAP will react more abundantly with tyrosinase than tyrosine, 4-S-CAP or N-acetyl-4-S-CAP, resulting in more chemical reaction.

Gross Appearance of Hair Follicles: Repeated administration of NPr-CAP for 12 consecutive days to adult C57 mice resulted in almost complete depigmentation of new hair follicles which possessed active tyrosinase and were grown at the site where hair plucking was carried out (Fig. 2A). The growth of these depigmenting hair follicles and the development of the animals were not apparently different from those of the control mice. There was no significant body weight loss. The findings suggest that the whitening of hair follicles by NPr-CAP treatment occurred through the selective interaction with pigment cells. One month later in the subsequent hair cycle, black hair follicles began to regrow and completely replaced depigmented ones in 6 months (Fig. 2B). After a single injection of NPr-CAP to newborn mice, the coat appeared to be silver-colored (Fig. 2C). Upon closer examination, the coat was found to consist of a mixture of white and black hair follicles. The coat of newborn mice injected with NPr-CAP for 3 days appeared much more depigmented than the coat of singly injected mice (Fig. 2D), indicating that this depigmentation was dose dependent. Depigmented hair follicles of the newborn mice were replaced by black ones at six weeks of the new hair cycle (Fig. 2E). The newly regrown hair follicles in the next hair cycle were not depigmented.

Morphological Findings of Follicular Melanocytes: Follicular melanocytes started to show some morphologic alterations, *e.g.* poor dendricities, but without any obvious degenerative changes, at 4 h

STRUCTURE	Km (m Mol)	Vmax μM/min/mg
HO — $CH_2CH(NH_2)COOH$ Tyrosine	0.021	0.164
HO — $S\text{-}CH_2CH(NH_2)COOH$ 4-S-cyteinylphenol	0.330	0.057
HO — $S\text{-}CH_2\text{-}CH_2\text{-}NH_2$ 4-S-cysteaminylphenol	0.117	0.039
HO — $S\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}COCH_3$ N-acetyl-4-S-cysteaminylphenol	0.375	1.050
HO — $S\text{-}CH_2\text{-}CH_2\text{-}NH\text{-}COCH_2CH_3$ N-propionyl-4-S-cysteaminylphenol	0.340	1.370

Fig. 1 Comparison of chemical structure and tyrosinase kinetics of NPr-CAP with other phenolic thioether amines. While the data shown is based upon the kinetics using mushroom tyrosinase, a mother compound, 4-S-CAP, was also shown to be a substrate for mammalian tyrosinase using mouse melanoma tissue[20,38].

after a single administration of NPr-CAP. There was, however, some electron dense material deposited selectively on the Golgi cisternae of the melanocytes. The distribution and electron density of the materials were similar to those described in our previous study of N-acetyl-4-S-CAP[21], which indicated that they derive from the oxidation product of NPr-CAP in the presence of tyrosinase (Fig. 3A). At 12 h post-administration, the poor development of melanocytic dendrites became much more pronounced. Their nuclear chromatin showed condensation in the periphery. Also, several endoplasmic reticula and melanosomal outer membranes were found to be dilated (Fig. 3B). At 16 h post-administration, numerous membrane-bound fragments associated with apoptosis were observed (Fig. 3C, Fig. 4A, Fig. 4B). Some of them showed vacuolation of endoplasmic reticula, but the fine structure of mitochondria and melanosomal membrane remained intact (Fig. 4A). Most of them were phagocytosed by adjacent keratinocytes and, to a great extent, were associated with a necrotic degeneration, secondary necrosis (Fig. 3C). These melanocytes showed selective degeneration and casting-off from the hair bulb (Fig. 4B). Importantly, there was no significant alteration in the fine structure of surrounding keratinocytes and fibroblasts (Figs. 4A, 4B). Osmiophilic changes in the cytoplasm of the melanocytes were observed in the entire degenerating process.

The Identification of Apoptosis Using TUNEL Method: As early as 4 h after a single administration of NPr-CAP, several melanocytes started to show positive staining for TUNEL method at the hair bulb (Fig. 5A). At 12 and 16 h after single NPr-CAP administration, TUNEL-positive melanocytes became much more obvious and numerous. The surrounding keratinocytes in the hair bulb were not stained positively and there were no positively stained cells in the hair bulbs of the control specimen (Fig. 5B). The identity of TUNEL-positive cells with melanocytes was confirmed by serial sections stained with toluidine blue and compared with control specimens (data not shown).

The Effect of NPr-CAP on In Vitro Melanocytes: Melan-a2 cells possessed a high tyrosinase activity (0.057 ± 0.007 at OD 475 nm/mg protein/min, n=6) whereas melan-c did not (0.000 ± 0.000 at OD 475 nm/mg protein/min, n=6). Melan-a2 and melan-c cells were treated with NPr-CAP to examine numerical and nuclear changes after drug administration. The discrete fragments of nucleosomal DNA

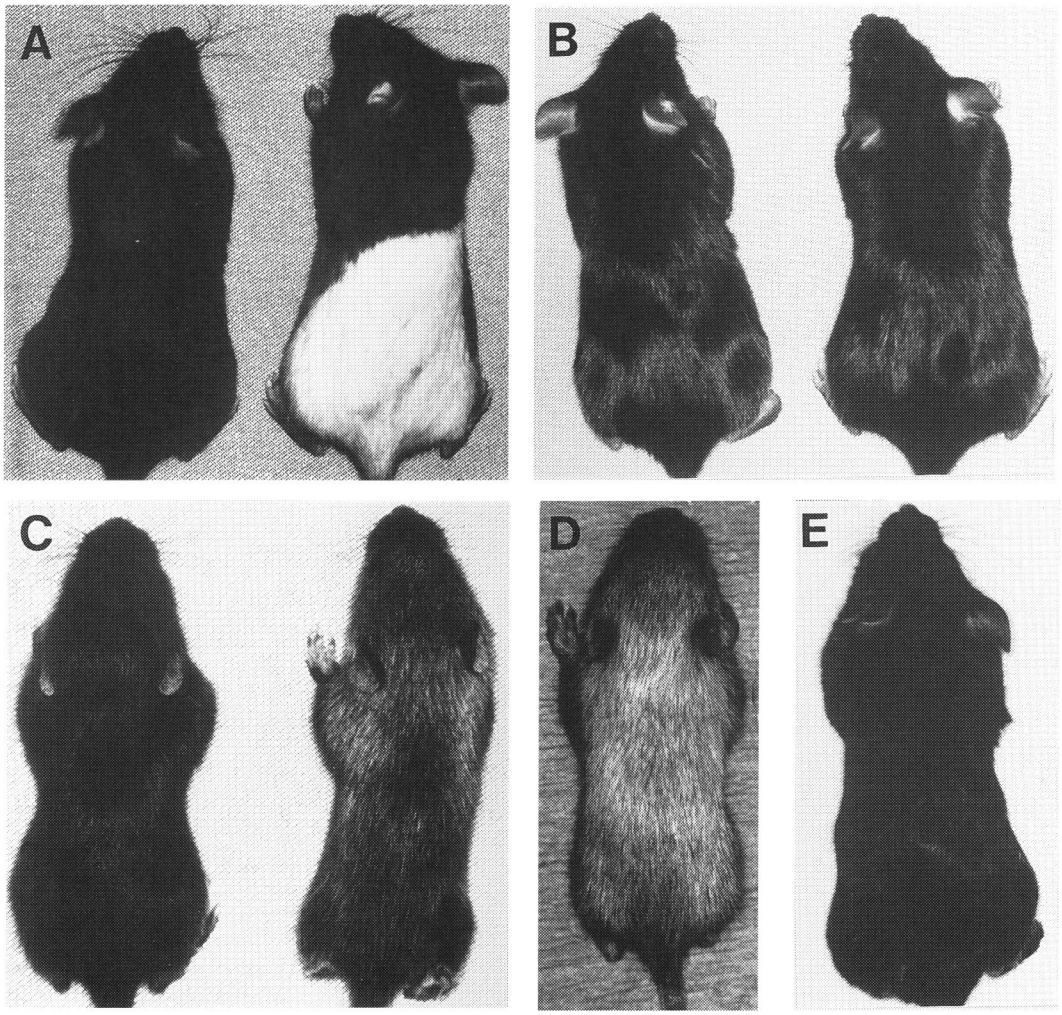

Fig. 2 Gross appearances of hair follicles after administration of NPr-CAP.
A: NPr-CAP-treated adult mouse (right) and control (left). Repeated administration of Npr-CAP for 12 consecutive days caused a marked depigmentation of black hair follicles at the site of hair-plucking in adult mice.
B: NPr-CAP-treated adult mouse (right) and control (left). The black hair follicles completely replaced depigmented ones by six months after treatment of NPr-CAP.
C: A single NPr-CAP-treated newborn mouse (right) and control (left). All mice treated with NPr-CAP showed mixed hair follicles consisting of white and black hair follicles. All hair follicles became black ones in the next hair cycle.
D: A newborn mouse injected with NPr-CAP once a day for 3 consecutive days. The population of depigmented hair follicles increased much more than that by a single injection.
E: A mouse treated with NPr-CAP injection for 3 consecutive days at new-born period (Fig. 2D) showing complete replacement of pigmented hair follicles after six weeks.

corresponding to multiples of about 180 bp, which is a characteristic pattern of apoptosis, were observed in melan-a2 cells treated with 1, 2 and 4 mM concentrations of NPr-CAP (Fig. 6), corresponding to the *in vivo* results of the involvement of apoptosis for melanocytotoxicity. Such a change was not observed in the melan-c cells.

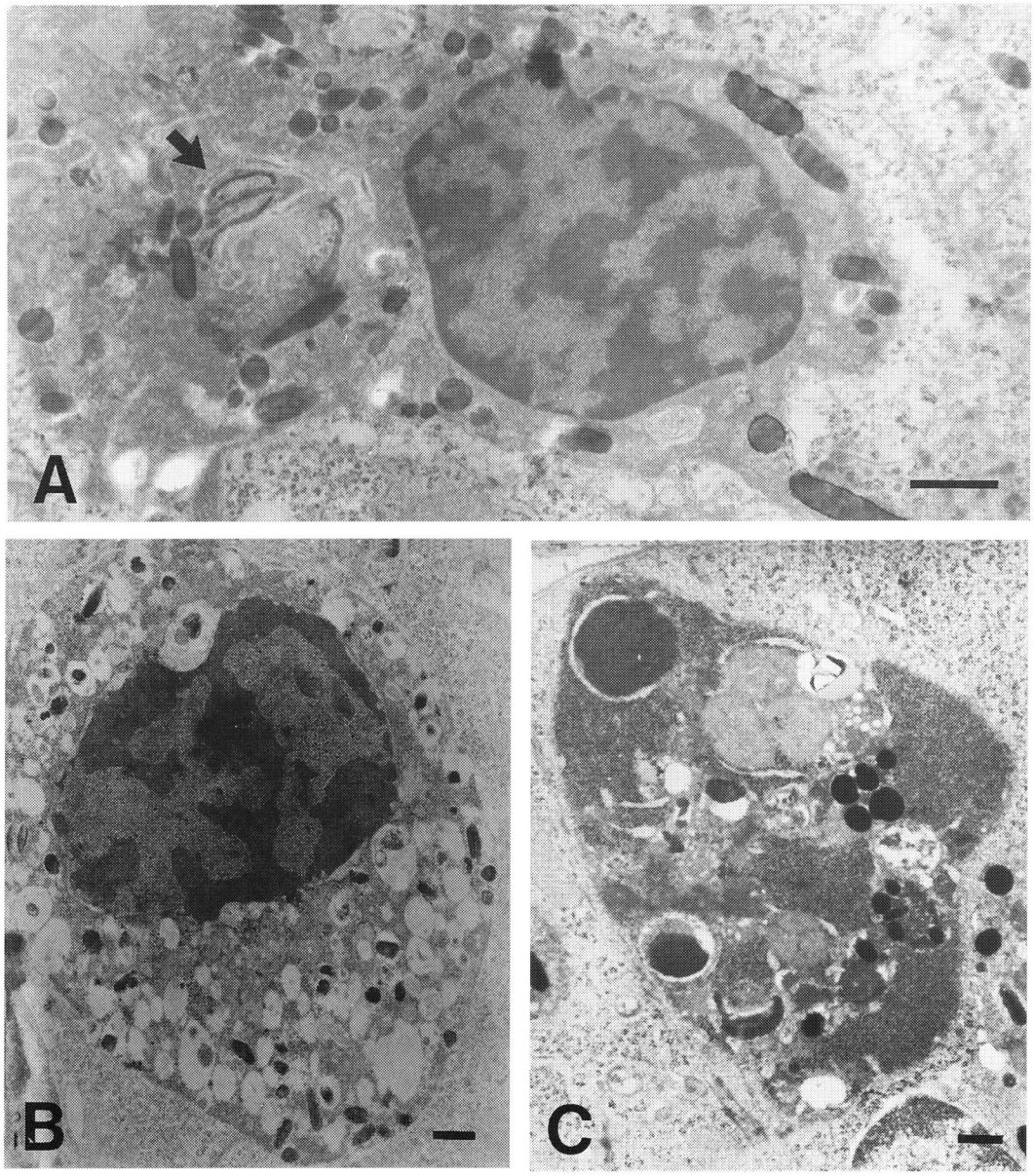

Fig. 3 Electron microscopic findings of sequential steps in melanocyte degeneration after a single *i. p.* injection of NPr-CAP to C57 black, newborn mice.
A: A melanocyte at 4 h post-injection, showing deposition of electron dense, melanin-like material (indicated by an arrow) in the Golgi cisternae and dark staining of entire cytoplasm. Such an increase of cytoplasmic electron density is not seen in surrounding keratinocytes.
B: A melanocyte at 12 h post-injection, showing condensation of nuclear chromatin, membrane swelling of melanosomes and endoplasmic reticula and increased electron density of cytoplasm.
C: A melanocyte at 16 h post-injection, showing an apoptotic body with secondary necrosis. *Scale bar*, 1 μm. in A, B and C.

The above *in vivo* study indicated that the biological effect of NPr-CAP was dose dependent and related to the presence of active tyrosinase. In order to confirm this finding, we performed an MTT assay. The results clearly showed that melan-a2 cells decreased their mitochondrial activity in the

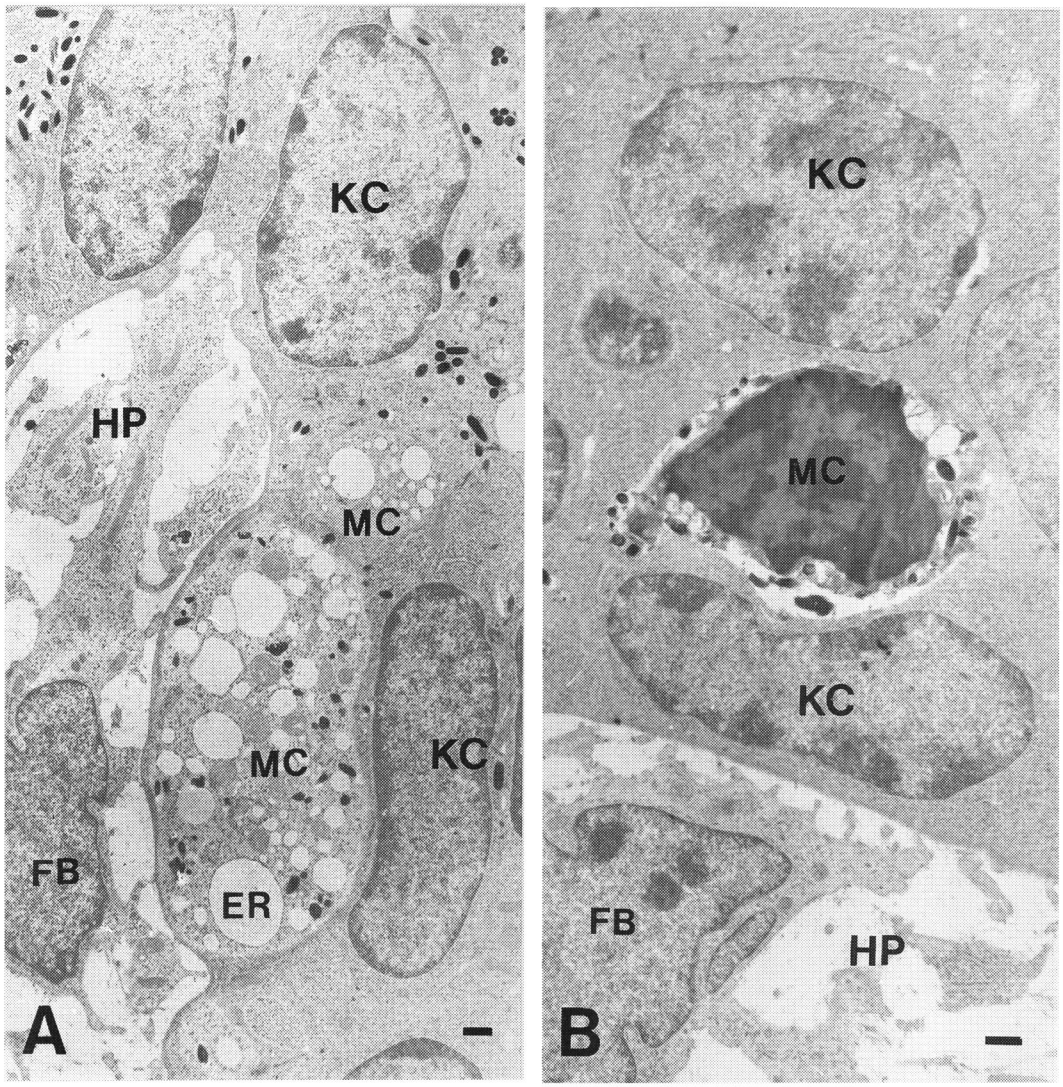

Fig. 4 Electron microscopic findings of late phases of melanocyte degeneration after a single *i. p.* injection of NPr-CAP to C57 black newborn mice.

A : At 16 h post-injection, showing marked vacuolation of endoplasmic reticula and cytoplasmic condensation, but without any obvious alteration in fine structure of mitochondria. Only melanocytes (MC) show such structural alterations and the fine structures of surrounding cells, *e. g.* keratinocytes (KC) in the hair bulb and fibroblasts (FB) in the hair papilla (HP) are well preserved.

B : At 16 h post-injection, showing selective degeneration and casting-off of a melanocyte (MC) within the hair bulb. Again, surrounding keratinocytes (KC) and fibroblasts (FB) do not show any structural alterations. *Scale bar*, 1 μm.

presence of NPr-CAP, in parallel to the drug concentrations administered (Table 1), confirming that the effect of NPr-CAP is a dose-dependent phenomenon. Similarly, melan-c cells reduced their cellular activity dose dependently to some extent. However, the extent of cytotoxicity was found to be far much less than that of melan-a2 (Table 1). We then examined the number of viable cells using trypan blue exclusion test to see if there was any difference between melan-a2 and melan-c cells after NPr-

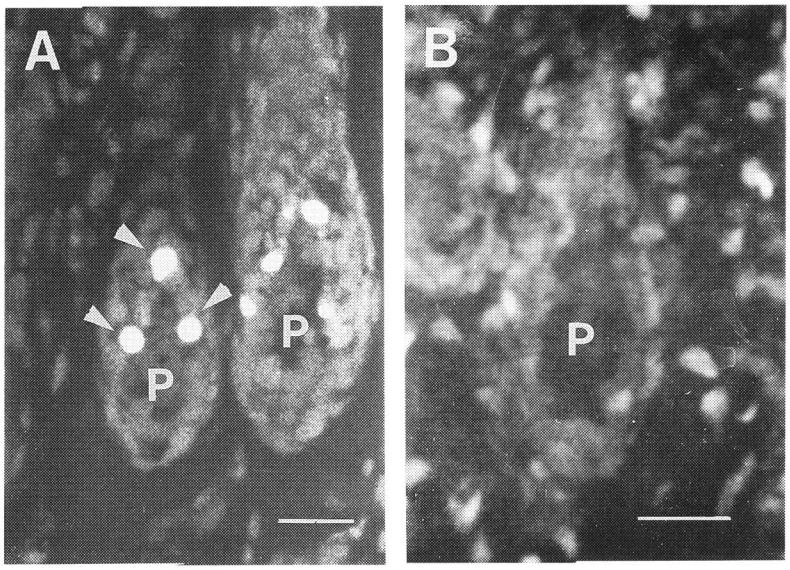

Fig. 5 The detection of apoptotic cells in hair bulbs using TUNEL method.
NPr-CAP-treated newborn mouse (A) and control (B). Melanocytes (arrow heads) of hair bulbs started to stain positively at 4 h after a single administration of NPr-CAP and became much more obvious at 12 h and 16 h after administration. These TUNEL-positive, fluorescent cells are located at the "melanocytic zone" above the basal lamina of the hair papilla. *Scale bar*, 30 μm.

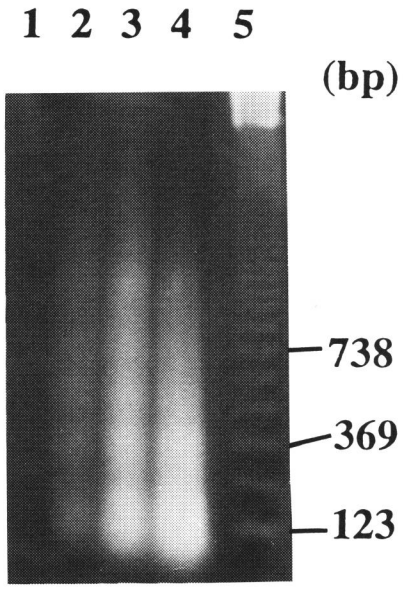

Fig. 6 Agarose gel electrophoresis of DNA from NPr-CAP-treated melan-a2 cells.
All cells were exposed to NPr-CAP for 24 h. Concentrations of the chemical used for treatment was 0 mM (lane 1), 1 mM (lane 2), 2 mM (lane 3) and 4 mM (lane 4). Treated cells were harvested, and then DNA was extracted from the cells and electrophoresed on 1.5% agarose gel at 90 V for 70 min. The DNA molecular weight marker was 123 bp DNA Ladder (lane 5). The ladder formations, consisting of multiples of about 180 bp nucleosome fragment, were seen in cells treated with more than 1 mM of NPr-CAP (lane 2-4).

Table 1 *Comparison of Cell Activity by MTT Assay and Cell Viability by Trypan Blue Test Between Melan-a2 and Melan-c Cells after Exposure to N-Propionyl-4-S- Cysteaminyl-phenol*

Drug Exposing Time and Concentration[a]	Melan-a2 Cells			Melan-c Cells		
	MTT Assay[d]		Trypan Blue	MTT Assay[d]		Trypan Blue
	Exp 1[b] (%)	Exp 2[c] (%)	Test[e] (%)	Exp 1[b] (%)	Exp 2[c] (%)	Test[e] (%)
0 h 0 mM		100.0±9.3			100.0±7.7	
(OD_{550})		(0.409±0.038)			(0.326±0.025)	
24 h 0 mM	100.0±4.6	111.5±5.1	94.6±1.5	100.0±5.5	145.4±8.0	96.9±0.2
(OD_{550})	(0.456±0.021)			(0.474±0.026)		
1 mM	96.7±4.1	107.8±5.9	87.8±4.1	86.5±2.7	125.8±4.0	90.8±2.0
2 mM	76.3±7.5	85.1±8.3	ND[f]	77.2±2.1	112.3±3.1	ND[f]
3 mM	37.1±2.0	41.3±2.2	10.6±1.2	66.7±4.4	96.9±6.4	80.2±7.7

[a] The assay conditions and procedures for MTT assay and trypan blue test are described in *Material and Methods*.
[b] The MTT value of the cells treated without drug is used as control.
[c] The MTT value of the cells prior to the treatment is used as control.
[d] Data represent mean ± SD (n=10). $p<0.0001$ *versus* control.
[e] Data represent mean ± SD (n=3).
[f] ND, not done

CAP treatment. The percentage of melan-a2 cells, excluding trypan blue, were altered significantly in parallel to concentrations of NPr-CAP. In contrast, the percentage of melan-c cells, excluding trypan blue, did not show such a significant reduction when treated with NPr-CAP. Importantly, melan-a2 cells showed only 10.6% cell viability, as judged by the trypan blue exclusion test at 3 mM drug concentration. In order to further examine the viability of cells after drug treatment, melan-a2 and melan-c cells were exposed to NPr-CAP for 24 h and then to the normal culture medium without NPr-CAP in

Table 2 *Cell Proliferation Rate of Melan-a2 and Melan-c Cells After Exposure to NPr- CAP with and without phorbol-12-myristate-13-acetate (PMA)*

Cell line[a]	NPr-CAP Exposed	Medium Replaced[b]	Cell Proliferation Rate[c]		
			0 h	24 h	48 h
melan-a2	0 mM	PMA (−)	1.00±0.06	1.24±0.01	2.46±0.10
		PMA (+)		2.34±0.05	3.12±0.21
	3 mM	PMA (−)	1.00±0.10	0.80±0.12	0.68±0.18
		PMA (+)		0.96±0.12	0.71±0.14
melan-c	0 mM	PMA (−)	1.00±0.05	1.56±0.04	1.82±0.04
		PMA (+)		1.64±0.01	1.94±0.13
	3 mM	PMA (−)	1.00±0.06	1.12±0.04	1.47±0.10
		PMA (+)		1.18±0.08	1.67±0.09

[a] Proliferation of melan-a2 and melan-c cells at 24 h and 48 h after complete "wash-out" of NPr-CAP exposed for 24 h.
[b] Abbreviations: + complete medium with PMA
− complete medium without PMA
[c] Data represent mean ± SD (n=5). $p<0.0001$ *versus* control (0 h).

the presence or absence of PMA (Table 2). Only melan-a2 cells showed a steady decline of cell proliferation rate (ratio of viable cells) at 24 h and 48 h after complete "wash-out" of NPr-CAP. In contrast, such a decline of viable cells was not seen in melan-c. Instead, they showed a steady increase of viable cells after NPr-CAP "wash-out" (Table 2). The decreased number of viable melan-a2 cells did not recover even when supplemented with PMA, whereas melan-c cells enhanced their cell proliferation after supplementation with PMA (Table 2). These results showed that NPr-CAP exerts a cytocidal effect caused by apoptosis on melan-a2 cells, but only a transient cytostatic effect on melan-c cells, and that the cytocidal effect of NPr-CAP depends on the presence or absence of active tyrosinase. Furthermore, our results show that the cytocidal and cytostatic effect of the drug on melan-a2 and melan-c cells, respectively, is not related to the different cell proliferation rate of the two cell lines.

Discussion

Our previous studies have clearly shown that phenolic thioether amines cause selective melanocytotoxicity and degenerative changes[7,19,23]. However, a detailed clarification of the exact mechanism has not been established. Specifically, it was unknown whether degeneration occurred via an apoptotic or a necrotic process. NPr-CAP was found to be a tyrosinase substrate. The *in vivo* follicular melanocytes showed the deposit of electron-dense, melanin-like materials in the Golgi cisternae where active tyrosinase is present. In our previous study using N-acetyl-4-S-CAP[21], such deposition of melanin-like materials was seen only in follicular melanocytes of C57 black mice with active tyrosinase activity while it was not seen in albino mice which do not have any active tyrosinase. Only follicular melanocytes with active tyrosinase and melanin synthesis showed morphological alteration after NPr-CAP administration. These *in vivo* findings suggest that the NPr-CAP-induced cytotoxicity is related to the presence of active tyrosinase. Wong and Jimbow[23] showed that the chromatin condensation occurred in follicular melanocytes of N-acetyl-4-S-CAP-treated mice, whereas the structure of mitochondria was unchanged in the early stage of this degenerative process. Another electron microscopic study also showed significant changes in the nucleus[19]. We also observed some necrotic changes in follicular melanocytes, however, they occurred during the phagocytosis process of apoptotic melanocytes. These findings were compatible with the so-called "secondary necrosis" of melanocytes. This apoptosis in melanocytes was further supported by using the TUNEL method and DNA ladder.

The chemical reaction within the cell and mechanism of melanocytotoxicity caused by NPr-CAP may be similar to that of other 4-S-CAP-derivatives. 4-S-CAP is thought to be oxidized by tyrosinase to an *o*-quinone form via 4-S-cysteaminylcatechol. Then the quinones conjugate with proteins through cysteinyl residues, thus exerting cytotoxic effects[24]. Yamada *et al*[25] showed that catalase prevented the melanocytotoxicity of 4-S-CAP, suggesting that the generation of free radicals in these processes probably play an important role. These free radicals may be the trigger to apoptosis in melanocytes after drug treatment. Apoptosis finally progresses to a common pathway which is associated with activation of DNase[11]. However, this activation has a variety of triggers, including many kinds of environmental stresses such as oxidative stress[14,15]. A decreased intracellular concentration of reduced glutathione results in cell death caused by oxidative injury[26-28]. Our previous studies showed that the melanocytotoxicity of N-acetyl-4-S-CAP was enhanced by a reduction in glutathione levels. This melanocytotoxicity was inhibited by the administration of N-acetylcysteine[29,30], which is not only a precursor and upregulator of glutathione but also an effective free radical scavenger[31].

Our previous *in vivo* study also showed that follicular melanocytes of albino mice were not affected by 4-S-CAP[19,21]. However, the present *in vitro* study showed that cultured murine albino melanocytes, melan-c cells, were transiently inhibited in their proliferation by NPr-CAP. These results may imply

that NPr-CAP affects, to some extent, any type of melanocytes, and that permanent, irreversible change which results from apoptosis occurs only in those cells with active tyrosinase activity and melanin synthesis. Melanocytes have been reported to possess lower activity of antioxidative enzymes than keratinocytes and fibroblasts[32]. Following the active or passive intracellular influx of NPr-CAP in melanocytes, NPr-CAP itself may cause transient, direct cell damage through a non-tyrosinase-mediated reaction. Thus NPr-CAP may exert, to some extent, the cytostatic effect on the albino melanocytes which do not have any active tyrosinase activity. However, the permanent cytocidal effects of NPr-CAP are associated with a tyrosinase-dependent mechanism, involving an apoptotic mechanism.

The biological potency of NPr-CAP for the depigmenting effect was restricted to newly grown hair follicles at the site of epilation, indicating that this NPr-CAP is effective only in activated, functioning melanocytes. The replacement of depigmented hair follicles with black hair follicles in the next hair cycle suggests the presence of unaffected, drug-resistant melanocytes or dormant melanocytes in hair follicles. Dormant melanocytes or melanocyte reservoirs are present in the resting hair follicles in adult black mice[33,34]. These melanocytes can be another source of functioning melanocytes activated during anagen hair growth. Similarly, Grichnik *et al*[35] showed that the precursor melanocyte reservoirs are present in the basal layer of the human epidermis. Okura *et al*[36] observed immature melanocytes without active tyrosinase in the murine epidermis, including the hair bulbs at birth. Slominski *et al*[37] suggested the existence of the chemoresistant precursor melanocytes which differentiate into functioning melanocytes in adult black mice. Our present study may also indicate that the replenishment of functioning melanocytes in the hair bulb in the next hair cycle after NPr-CAP treatment may derive from such precursor melanocyte reservoirs which are not affected by NPr-CAP.

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N-propionyl-4-S-cysteaminylphenol のアポトーシスを介した色素細胞障害効果

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これまで我々は，チロシナーゼの基質であるチロシンをイオウで化学的に修飾して phenolic thioether amine である 4-S-cysteaminylphenol (4-S-CAP) と，その N-アセチル配合体である N-acetyl-4-S-CAP を合成した．それらは皮膚と毛包の色素細胞に対して潜在的に脱色効果を有しており，悪性黒色腫に対して抗腫瘍効果も有している．しかし，それらの生物学的活性が色素細胞に対して cytostatic に作用するか，cytocidal に作用するかは明らかではない．我々はより効果的な化学療法剤として，また脱色剤として，4-S-CAP の N－プロピオン酸配合体である N-propionyl-4-S-CAP (NPr-CAP) を新たに合成した．NPr-CAP は N-acetyl-4-S-CAP より脂溶性であり，それゆえ細胞により浸透しやすく，より高い薬理学的効果を発揮することが期待される．今回の研究は melanocytotoxicity に対する NPr-CAP の生物学的効果の選択性と能力を調べることを狙いとしている．NPr-CAP はチロシナーゼの良い基質である．NPr-CAP の腹腔内投与は C57 黒マウスの黒毛を投与量依存性に脱色した．しかし，この脱色された毛は次の毛周期に黒毛に置換された．このことは休眠中の，または薬剤耐性の色素細胞の存在を示唆している．NPr-CAP 投与群において，毛包の色素細胞が TUNEL 法で陽性に染色されていることと，電子顕微鏡による形態学的所見から，投与 12 時間後にアポトーシスが起こっていることが示された．しかし，その周囲の角化細胞と線維芽細胞には何ら変化を認めなかった．NPr-CAP は C57 黒マウスから得た培養色素細胞である melan-a2 cell の細胞増殖を非可逆的に抑制したが，コントロールであるアルビノマウスから得た培養色素細胞である melan-c cell では抑制しなかった．NPr-CAP を投与された melan-a2 cell から得られた DNA のアガロースゲル電気泳動により DNA ladder が認められた．MTT アッセイとトリパンブルーテストは，NPr-CAP はチロシナーゼ陽性である melan-a2 cell に cell death を起こさせるが，チロシナーゼ陰性である melan-c cell には一時的に細胞増殖を抑制することを示した．以上より我々は，NPr-CAP はチロシナーゼとの相互作用により選択的に melanocytotoxicity を発揮し，色素細胞にアポトーシスを起こすと提唱する．