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Synthesis and Antitumour Effect of the Melanogenesis-based Antimelanoma Agent *N*-Propionyl-4-*S*-cysteaminylphenol

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ABSTRACT. Chemotherapy of malignant melanoma is still a great challenge, as no effective drugs are available. The development of melanogenesis-based drugs is a promising area of research because melanogenesis is a unique biochemical pathway operating only in melanoma cells (and their normal counterparts) so that the tumour can be targeted. We have been using cysteinylphenol, a sulphur-containing analogue of tyrosine, and derivatives for that purpose. *N*-Acetyl-4-*S*-cysteaminylphenol was found to have the best antimelanoma effect in cell culture systems and in mice bearing B16 melanoma tumours. It also caused depigmentation of the skin, suggesting the possibility of use as a hypopigmenting agent. To improve the efficiency of the drug, we thought of replacing the acetyl group in *N*-acetyl-4-*S*-cysteaminylphenol with a propionyl group in the hope that increased hydrophobicity would increase the cellular uptake of the drug. *N*-Propionyl-4-*S*-cysteaminylphenol was synthesized by condensing 4-hydroxythiophenol with 2-ethyl-2-oxazoline. The drug showed both cytostatic and cytocidal effects in a human melanotic melanoma cell line. The drug was found to be a good depigmenting agent for the black hair follicles of C57 black mice when given s.c. for 14 days. A 10-day treatment with *N*-propionyl-4-*S*-cysteaminylphenol at 300 mg/kg body weight reduced the growth rate of B16 melanoma s.c. tumours in mice by 36%. The propionyl derivative was found to increase the life span of mice bearing melanoma more effectively than did the acetyl derivative. BIOCHEM PHARMACOL **55**;12:2023–2029, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. tyrosinase; cysteaminylphenol; depigmenting potency; melanocytotoxicity; melanoma chemotherapy

Melanocytes and their neoplastic counterpart, malignant melanoma cells, possess a unique metabolic pathway, melanin synthesis, that is highly accelerated in the latter [1]. Currently, our research group has been involved in the development of depigmenting and antimelanoma agents by exploiting this unique metabolic pathway of melanocytes and melanoma cells. The key enzyme of this pathway is tyrosinase, which is always retained *in vivo* in melanoma cells, and, hence, melanin metabolites are always being synthesized, even though the whole tumour becomes nonpigmented and amelanotic. In our earlier studies, we demonstrated that melanin/melanosomes, *per se*, if overproduced, is/are toxic to melanocytes and may kill them [2]. This cytotoxicity due to melanin synthesis may be understood if one looks at 1) the role of tyrosinase in the melanin biosynthesis pathway, in which initial oxidation involves hydroxylation of tyrosine to form DOPA§ and 2) the oxidation of DOPA to form *o*-quinone (DOPA quinone), which is converted subsequently into dihydroxyindole derivatives through auto-oxidation/hydroxylation. The major melanin polymers and metabolites, therefore, can promote the oxidation of some compounds and the reduction of others. Products of tyrosinase action (melanin metabolites) can thus exhibit useful chemical cytotoxicity for melanoma cells, providing a rational basis for the development of targeted chemotherapy using tyrosine analogues [1, 3].

We recently synthesized the sulphur homologue of tyrosine, cysteinylphenol, and its amine derivative, cysteaminylphenol (4-*S*-CAP), for the purpose of melanoma chemotherapy. 4-*S*-CAP showed a potent depigmenting and antimelanoma effect, but it was not stable and caused non-specific cytotoxicity affecting even non-melanocytic

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§ *Abbreviations:* DOPA, 3,4-dihydroxyphenyl alanine; ILS, increased life span; 4-*S*-CAP, 4-*S*-cysteaminylphenol; *N*-acetyl-4-*S*-CAP, *N*-acetyl-4-*S*-cysteaminylphenol; and *N*-propionyl-4-*S*-CAP, *N*-propionyl-4-*S*-cysteaminylphenol.

cells rich in monoamine oxidase [4]. To overcome this difficulty, the *N*-acetyl derivative of 4-*S*-CAP, *N*-acetyl-4-*S*-CAP, was synthesized. Our *in vivo* melanocytotoxicity studies [5, 6] indicated that *N*-acetyl-4-*S*-CAP would be a potent melanocytotoxic agent *in vivo*. Furthermore, the i.p. injection of *N*-acetyl-4-*S*-CAP inhibited the lung colony formation by B16F10 melanoma cells in C57 black mice. It reduced the lung colony formation by 25% of the mean control [7]. This *in vivo* antimelanoma effect of *N*-acetyl-4-*S*-CAP was confirmed with human tumour xenograft in nude mice. We found that 400 mg/kg of *N*-acetyl-4-*S*-CAP induced significant inhibition of melanoma growth by 17 days when injected i.p. at 3-day intervals, but no effect on the ovarian tumour, JAM, was observed [8]. There was no *in vivo* cytotoxicity to albino melanocytes in which there was no production of active tyrosinase, indicating again the importance of the melanin biosynthesis pathway for selective melanocyte toxicity [6]. Importantly, our biodistribution study of *N*-acetyl-4-*S*-CAP, using the ^{14}C-labelled form, indicated that the label is selectively incorporated into and accumulated in melanoma tissues at 48 hr post-injection [9].

Although these studies have shown clearly that *N*-acetyl-4-*S*-CAP is a promising antimelanoma agent, our recent study indicates that it would require additional treatment (e.g. depleting of glutathione in tissue) to achieve a highly efficient melanoma therapy [9]. Therefore, we have synthesized a propionyl derivative of 4-*S*-CAP, *N*-propionyl-4-*S*-CAP, with the expectation that melanocytotoxicity and the antimelanoma effects of *N*-acetyl-4-*S*-CAP would be enhanced because of the more hydrophobic structure of the drug, leading to increased penetration into melanoma cells. Here, we present the chemical synthesis, chemical characterization, and the preliminary screening of the *in vitro* and *in vivo* melanocytotoxicity and antimelanoma effects of *N*-propionyl-4-*S*-CAP.

MATERIALS AND METHODS

Analytical Methods and Chemicals

Melting points were determined on a Buchi capillary apparatus and are uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were recorded on a Brucker AM-300 spectrometer. ^{1}H NMR assignments were confirmed by double irradiation experiments and the chemical shifts are provided in δ ppm downfield with respect to tetramethyl silane (TMS) as internal standard. ^{13}C NMR resonances were assigned by using the J-spin echo modulation (J_{mod}) technique to determine the number of attached hydrogen atoms. The purity of the compounds was checked on Whatman MK6F (250-μm thickness) silica gel microslides using dichloromethane:methanol (9:1, v/v; system A) as developing solvent. Silica gel column chromatography was carried out using Merck silica gel (100–200 mesh particle size). 4-Hydroxythiophenol, propionic anhydride, and 2-ethyl-2-oxazoline were purchased from the Aldrich Chemical Co. *N*-Acetyl-4-*S*-CAP was synthesized according to our previous report [9].

Synthesis of N*-propionyl-4-S-CAP*

A mixture of 4-hydroxythiophenol (25 g; 0.198 mol) and 2-ethyl-2-oxazoline (19.6 g; 0.198 mol) was heated at 160° under an inert atmosphere for 4 hr. A chromatographic examination of the reaction mixture showed no evidence of the starting reagents. Overnight cooling of the reaction mixture resulted in solidification of the crude product, which was purified on a silica gel column using CH_2Cl_2:MeOH (9.8:0.2, v/v) as eluent. This purified product, after recrystallization from dichloromethane, yielded 43.5 g (97%) of pure *N*-propionyl-4-*S*-CAP. R_f 0.34; m.p., 83°; ^{1}H NMR (CD_3COCD_3 + D_2O) − 1.04 (*t*, $J_{CH_3\text{-}CH_2}$ = 7.7 Hz, 3H, CH_3), 2.14 (*q*, $J_{CH_3\text{-}CH_2}$ = 7.7 Hz, 2H, propionyl CH_2), 2.88 (*t*, $J_{CH_2\text{-}CH_2}$ = 6.3 Hz, 2H, S-CH_2), 3.3 (*t*, $J_{CH_2\text{-}CH_2}$ = 6.3 Hz, 2H, CH_2 − NH), 6.79 (*d*, $J_{2,3} = J_{6,5}$ = 8.6 Hz, 2H, aromatic H-3 and H-5) and 7.28 (*d*, $J_{3,2} = J_{5,6}$ = 8.6 Hz, 2H, aromatic H-2 and H-6); ^{13}C NMR (CD_3COCD_3) − 10.14 (CH_3), 35.70 (S-CH_2), 39.68 (CH_2-NH), 116.94 (aromatic C-3 and C-5), 124.87 (C-4), 134.48 (C-2 and C-6 aromatic), 158.04 (C-1), and 174.45 (C═O); anal. for $C_{11}H_{15}NO_2S$; calcd., C, 58.63; H, 6.71; N, 6.21%; found, C, 58.88; H, 6.76; and N, 6.13%.

Activity as Tyrosinase Substrate

Murine B16F10 tumour cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Life Technologies). The cells were harvested with trypsin, washed with PBS, and homogenized by brief sonication in PBS. Equal volumes of the homogenate and 1 mM of *N*-propionyl-4-*S*-CAP in 50 mM of potassium phosphate buffer, pH 6.8, were mixed and incubated at 37°, and the absorbance at 360 nm was measured at different time intervals, as indicated.

Kinetic parameters for tyrosinase action on test compounds were determined following our previous reports [5, 10]. Mushroom tyrosinase (3670 units/mg; Sigma Chemical Co.) was used for enzyme tyrosinase kinetics with some modification. Briefly, the reaction mixture consisted of the substrate at different concentrations (10 μM to 1 mM), and mushroom tyrosinase (20 units) in 1.0 mL of 50 mM of sodium phosphate buffer, pH 6.8, at 37°. The enzyme reactions were stopped by cooling in ice. Product formation was measured spectrophotometrically at 360 nm.

In Vitro *Melanocytotoxicity Assays*

The cytotoxicity of *N*-propionyl-4-*S*-CAP was assessed *in vitro* in a melanoma cell line using two different methods: hexosaminidase assay and ATP assay. SK-MEL-23, a human melanotic melanoma cell line (gift from Dr. A. Houghton, Sloan–Kettering Cancer Center) was grown in

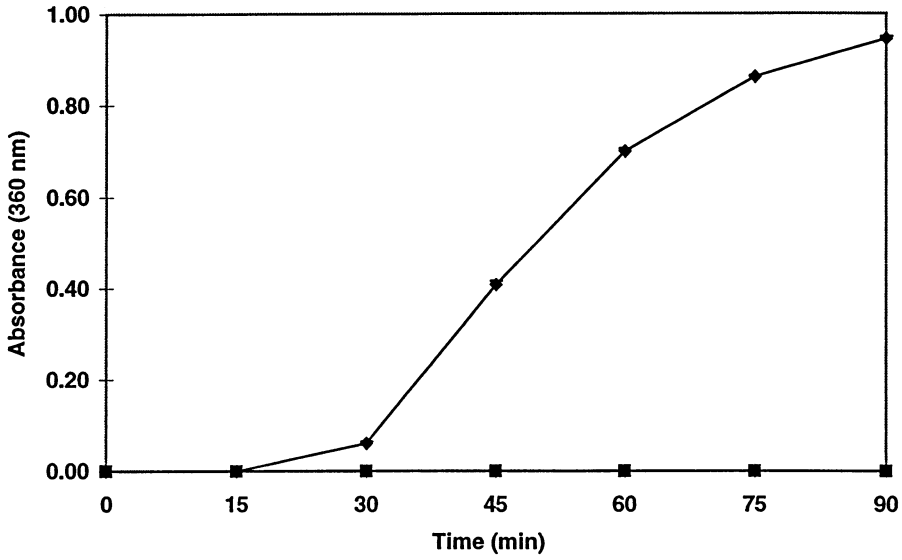

FIG. 1. Action of B16F10 homogenate on *N*-propionyl-4-*S*-CAP. The reaction conditions are given in Materials and Methods. The reaction mixtures consisted of 0.684 mg/mL of cellular protein, 0.5 mM of *N*-propionyl-4-*S*-CAP and 10 μM of DOPA without (◆) and with (■) 0.2 mM of phenyl thiourea. Values (means ± SD) from triplicate measurements are given.

RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) and passaged with trypsin. For the hexosaminidase assay, 2000 cells/well were seeded into 96-well plates. After 24 hr, the medium was replaced by medium containing the predissolved drugs at the indicated concentrations and incubated for 96 hr at 37° in a humidified atmosphere of 5% CO_2 in air. Then the plates were processed for hexosaminidase measurement [11]. Absorbance at 405 nm was measured using an ELISA plate reader (Easy Reader EAR 400AT, SLT-LAB Instruments).

For the ATP assay, $3–5 \times 10^5$ cells/well were seeded into 24-well plates. After 24 hr, the medium was replaced by medium containing the predissolved drugs at the indicated concentrations and incubated for 96 hr as above. After incubation, the medium was removed, the cells were washed twice with ice-cold PBS, and ATP was measured using the ATP Bioluminescence Assay Kit (Sigma Chemical Co.) according to the instructions given. Briefly, cells, after washing and being kept on ice, were suspended in 0.4 mL (per well) of ice-cold ATP Releasing Solution; aliquots (10–20 μL) were mixed with the diluted assay reagent, and light emission was measured immediately using a Lumat 9501 luminometer. Aliquots of 100–200 μL from each well were measured for protein [12].

Measurement of LD_{50}

Breeding pairs of C57BL/6J black mice were used in the study when they were 8 weeks old (weighing approximately 17.0 g). The LD_{50} for *N*-propionyl-4-*S*-CAP was established by a single i.p. injection of this drug dissolved in 1 mL of normal saline solution, in the dose range of 100–1400 mg/kg of body weight. The LD_{50} was found to be 1000 mg/kg of body weight for 4-propionyl-4-*S*-CAP. The LD_{50} of *N*-acetyl-4-*S*-CAP was reported to be 1300 mg/kg [10].

In Vivo *Melanocytotoxicity in Black Hair Follicles*

Black hair follicles of C57BL/6J black mice were plucked manually from the dorsal skin in order to initiate new anagen hair growth and induce tyrosinase activity as well as melanin synthesis in follicular melanocytes. *N*-Propionyl-4-*S*-CAP or normal saline (for control) was injected i.p. daily for 14 days, starting on day 2 after plucking the hair follicles on the dorsal side. The dose of injection (i.p.) followed that of our previous study with *N*-acetyl-4-*S*-CAP, 300 mg/kg of body weight, and the volume of injection was 1 mL.

In Vivo *Growth Inhibition of Melanoma Tumour and Increased Life Span of Melanoma Bearing Mice*

The murine B16F10 melanoma cell line was used for this study. B16F10 melanoma cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. When the cells reached the exponential growth phase, they were scraped and inoculated s.c. into C57BL/6J black mice (8-week-old females, 17.0 g). Seven mice were used in each experimental group. They were weighed and randomized at day 1 after s.c. inoculation of B16 melanoma cells (1×10^5 cells in 0.1 mL of normal saline). From day 5, *N*-propionyl-4-*S*-CAP (300 mg/kg of body weight), *N*-acetyl-4-*S*-CAP, and normal saline were administered i.p. in a volume of 1 mL daily for 10 days. The test compounds were dissolved in normal saline and sterilized by heating to 100° for 2 min. They were stable under this treatment, as judged by HPLC study [10]. The *in vivo* antimelanoma effects of the synthetic compounds, *N*-propionyl-4-*S*-CAP and *N*-acetyl-4-*S*-CAP, were evaluated by percent growth inhibition of the s.c. inoculated tumor (%

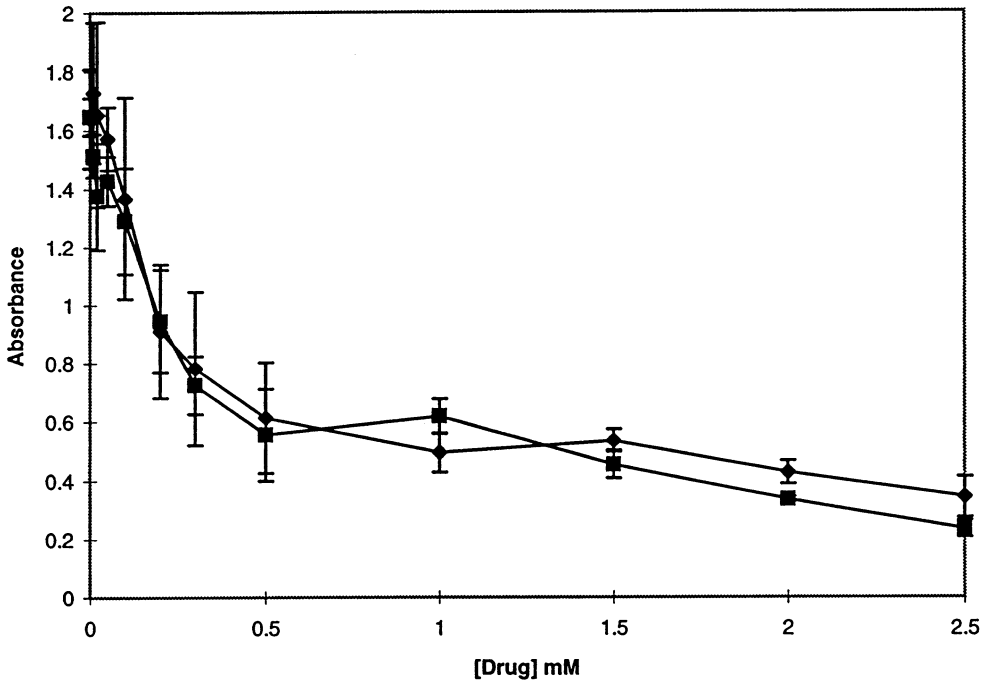

FIG. 2. Hexosaminidase assay for the melanocytotoxic action of cysteaminylphenol derivatives. The assay was carried out with SK-MEL-23 cells as described in Materials and Methods. Absorbance at 405 nm (mean ± SD from 7 readings) is plotted as a function of concentration of the drug for *N*-acetyl-4-*S*-CAP (◆) and *N*-propionyl-4-*S*-CAP (■).

GI) and percent increase of life span (% ILS). The percent GI was computed from the mean tumor volume at day 12 or 13 of the experiment, i.e. 100 × (1 − treated group/control group). Tumor volume was calculated using the formula: long axis × (short axis)^2 × ½. The size of the tumor, i.e. long and short axes (mm) of tumor, was measured every other day through the skin externally.

RESULTS

N-*Propionyl-4-S-CAP as Tyrosinase Substrate*

Figure 1 gives the time course for the action of B16F10 homogenate, a source of mammalian tyrosinase, on *N*-propionyl-4-*S*-CAP in the presence of 10 μM of DOPA as a cofactor. There was a time-dependent increase in the absorbance, suggesting that *N*-propionyl-4-*S*-CAP is utilized as a substrate by murine tyrosinase. That this reaction was due to tyrosinase was confirmed by the almost 100% inhibition of the reaction by 0.2 mM of phenyl thiourea, an inhibitor of tyrosinase.

Enzyme kinetics with mushroom and melanoma tyrosinase for 4-*S*-CAP and *N*-acetyl-4-*S*-CAP were reported previously [10, 13]. The K_m values for 4-*S*-CAP and *N*-acetyl-4-*S*-CAP were found to be 117 and 375 μM, respectively, whereas V_{max} values were 39.0 and 8.3 μmol/min/mg protein, respectively. Similarly, the kinetic parameters for our new synthetic compound, *N*-propionyl-4-*S*-CAP, were measured along with those for *N*-acetyl-4-*S*-CAP using mushroom tyrosinase. Both the compounds are oxidized by mushroom tyrosinase faster than is tyrosine, the natural substrate (data not given). A Lineweaver–Burk plot gave K_m and V_{max} values of 340 μM and 5.4 μmol/min/mg of protein, respectively, for *N*-propionyl-4-*S*-CAP.

In Vitro *Melanocytotoxicity of* N-*Propionyl-4-S-CAP*

The hexosaminidase assay results given in Fig. 2 indicate that *N*-propionyl-4-*S*-CAP was cytotoxic to melanotic melanoma cells, as expected. The effectiveness of the acetyl and propionyl derivatives was comparable in this assay. The results of ATP measurements in SK-MEL-23 cells treated with the drugs (Fig. 3) showed ATP depletion at high concentration of the drugs. The propionyl derivative seemed to be slightly more effective than the acetyl derivative in this assay.

In Vivo *Depigmenting Potency of* N-*Propionyl-4-S-CAP for Black Hair Follicles*

After the plucking of hair follicles, *N*-propionyl-4-*S*-CAP was injected s.c. for 10 consecutive days into the dorsal surface of C57 black mice where hair follicles were plucked manually. The regrowing new hair follicles at the site of hair plucking showed a complete depigmentation of hair follicles, starting (visible) at day 20 after initiation of the injection. The depigmenting effect is clearly seen from the pictures of control and treated mice given in Fig. 4. The extent of depigmentation was almost identical to that of *N*-acetyl-4-*S*-CAP, as reported previously [7].

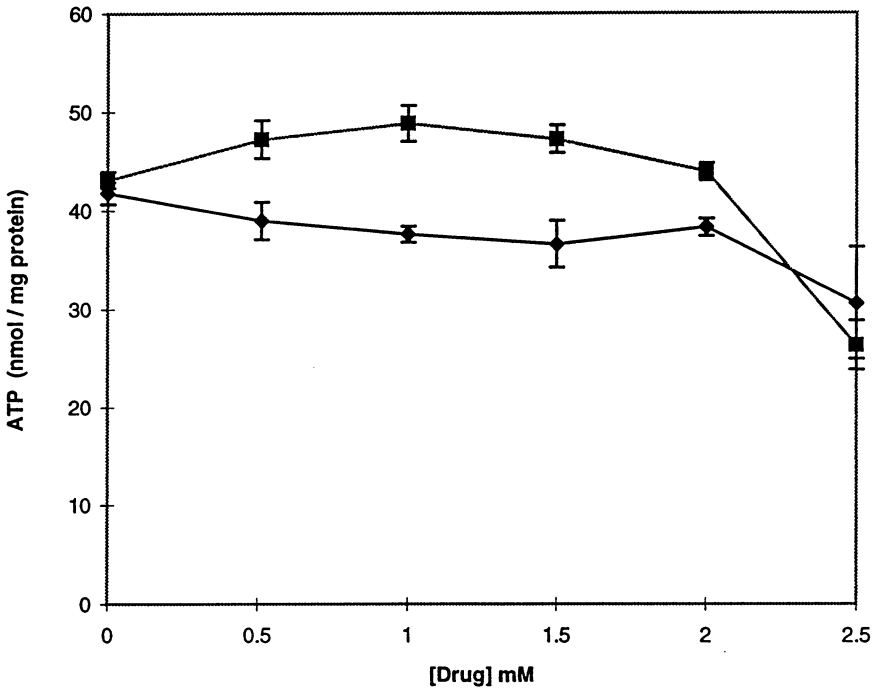

FIG. 3. ATP assay for the melanocytotoxic action of cysteaminylphenol derivatives. The assay was carried out with SK-MEL-23 cells as described in Materials and Methods. The ATP levels (means ± SD from triplicates) are plotted as a function of concentration of the drug for *N*-acetyl-4-*S*-CAP (◆) and *N*-propionyl-4-*S*-CAP (■).

In Vivo *Growth Inhibition of Melanoma Tumour and Increased Life Span of Melanoma Bearing Mice by N-Propionyl-4-S-CAP*

Both *N*-acetyl-4-*S*-CAP and *N*-propionyl-4-*S*-CAP caused a significant reduction in the tumour volume of melanoma tissues inoculated s.c. into C57 black mice, their percent GI being 42 and 36%, respectively (Table 1). The difference in tumour size between control and treatment groups was found to be statistically significant by *t*-test. There was also an increase in the life span of melanoma-bearing animals, their percent ILS being 27 and 39% in *N*-acetyl- and *N*-propionyl-4-*S*-CAP treated animals, respectively.

FIG. 4. Depigmenting effect of *N*-propionyl-4-*S*-CAP on C57BL/6J black mice hair follicles. The drug administration protocol is given in Materials and Methods. (A) a control mouse and (B) an *N*-propionyl-4-*S*-CAP-treated mouse.

DISCUSSION

As a part of our efforts to develop potent, yet safe antimelanoma agents, we have been focusing on various derivatives of cysteaminylphenol based on the hypothesis that these compounds will be taken up by the cell and will be converted into cytotoxic species by the action of the enzyme tyrosinase present only in melanoma cells (and normal melanocytes). Earlier studies have given promising results with *N*-acetyl-4-*S*-CAP, as this compound seemed to be better than 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (DTIC), the drug used presently for melanoma chemotherapy, in animal studies [1]. However, the potency was not spectacular. Thus, we are seeking more potent derivatives of cysteaminylphenol to develop an effective targeted chemotherapy of melanoma. One of our ideas was to improve upon *N*-acetyl-4-*S*-CAP by effecting structural changes that would enhance the cellular uptake of the drug. We thought that replacing the acetyl group by a propionyl group would increase the hydrophobicity and, hence, the lipophilicity of the drug molecule, which in turn was expected to increase the penetration of the drug into the cell through the cell membrane.

N-Propionyl-4-*S*-CAP was synthesized by condensing 4-hydroxythiophenol with 2-ethyl-2-oxazoline and was characterized by ^{1}H NMR, ^{13}C NMR, elemental analysis, and melting point. The compound was found to be very stable as a solid for several months at room temperature and as a solution in cell culture medium for several days. Targeted chemotherapy exploiting the melanin pathway requires that the compound be a tyrosinase substrate. This was tested using murine tyrosinase (as B16F10 cell homogenate) and mushroom tyrosinase (as a model), and the results confirmed that *N*-propionyl-4-*S*-CAP is a very good

TABLE 1. Antimelanoma effect of cysteaminylphenol derivatives in mice-bearing B16 melanoma

Treatment	Tumour volume (mm^3)	% GI	Median survival time (days)	% ILS
Saline	8977 ± 3778	0	16.5	0
N-Acetyl-4-*S*-CAP	5221 ± 2336	42	21.0	27
N-Propionyl-4-*S*-CAP	5744 ± 2155	36	23.0	39

% GI (growth inhibition of tumour) and % ILS (increased life span) of mice bearing B16 melanoma tumour were assessed as given in Materials and Methods. The differences in tumour volumes (given as means ± SD, N = 7) between saline and drug-treated groups were found to be significant at the level of $P < 0.05$ for both *N*-acetyl-4-*S*-CAP and *N*-propionyl-4-*S*-CAP.

substrate for tyrosinase. The enzyme kinetic parameters were found to be similar to those of the homologue, *N*-acetyl-4-*S*-CAP.

The melanocytotoxicity and antimelanoma effects were assessed *in vitro* and *in vivo* by different methods. The *in vitro* studies on the melanotic melanoma cell line SK-MEL-23 (Figs. 2 and 3) gave evidence for the melanocytotoxicity of *N*-propionyl-4-*S*-CAP. But the hexosaminidase and ATP assays gave widely different ranges for the toxic dose. This may be explained based on the fact that ATP depletion occurs only when there is cell killing (leaking), whereas hexosaminidase activity is determined by both cell killing and inhibition of cell proliferation. Thus, these results suggest that *N*-propionyl-4-*S*-CAP has both cytostatic and cytocidal activities, the latter requiring higher concentrations. The trend seen in Fig. 3 suggests that *N*-propionyl-4-*S*-CAP may have a better cytocidal effect than *N*-acetyl-4-*S*-CAP.

The *in vivo* effects of *N*-propionyl-4-*S*-CAP were assessed by two different tests: depigmenting effect on black hair follicles and the inhibition of melanoma tumour growth in mice. The depigmentation of hair follicles in black mice will be a direct assay for melanocytotoxicity and an indirect assay for antimelanoma effect, if depigmentation occurs as result of cytostatic or cytocidal effect. Our earlier studies with the parent compounds and *N*-acetyl-4-*S*-CAP showed that depigmentation results from a specific degeneration of follicular melanocytes [6, 7]. Hence, we used this simpler test as the first *in vivo* test for antimelanoma action. *N*-Propionyl-4-*S*-CAP was found to be a potent depigmenting agent in C57 black mice, as expected. Since 4-*S*-CAP- and *N*-acetyl-4-*S*-CAP-induced depigmentation occurs as a result of melanocyte degeneration, it is quite likely that *N*-propionyl-4-*S*-CAP also causes melanocyte killing. This was supported by the ATP-depleting effect of the drug. In the second test, as a preliminary assessment, we examined the antimelanoma effect directly by measuring the growth of tumour and life span of mice bearing B16 melanoma tumour. The results (Table 1) showed an antimelanoma effect of the drug, both in reducing the size of the tumour and in increasing the life span of the treatment group. The acetyl and propionyl derivatives were comparable in growth inhibitory effect, but the propionyl derivative was considerably better than the acetyl derivative in increasing the life span of mice bearing melanoma.

The *in vitro* cytotoxicity of substituted phenols has been shown to increase with the length of the side chain [14], in agreement with the present results. Thus, it is clear from these studies that *N*-propionyl-4-*S*-CAP will be a better choice than 4-acetyl-4-*S*-CAP for melanoma chemotherapy. Further studies to characterize the action in cell culture systems and the *in vivo* antimelanoma effect as well as biodistribution of the compound are in progress.

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