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MECHANISM OF GROWTH INHIBITION OF MELANOMA CELLS BY 4-*S*-CYSTEAMINYLPHENOL AND ITS ANALOGUES

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Abstract—Our previous studies have shown that 4-*S*-cysteaminylphenol (4-*S*-CAP) causes a significant inhibition of *in vivo* melanoma growth and a marked depigmentation of black skin and hair follicles. These studies have suggested a role of tyrosinase in the manifestation of these *in vivo* effects. In this study 4-*S*-CAP and its analogues were examined for their effects on the growth of human melanoma cells *in vitro*. 4-*S*-CAP and 4-*S*-HomoCAP exhibited strong cytotoxicity with effects much greater than those of α-methyl-4-*S*-CAP and *N*,*N*-dimethyl-4-*S*-CAP. The cytotoxicity of the former two amines was completely prevented by semicarbazide, an inhibitor of plasma monoamine oxidase, while that of the latter two was not prevented by semicarbazide, catalase, and phenylthiourea, a tyrosinase inhibitor. In culture medium 4-*S*-CAP was rapidly converted by the action of monoamine oxidase present in fetal bovine serum to the aldehyde which was then metabolized to the alcohol and the carboxylic acid when cells were present. α-Methyl-4-*S*-CAP was found to exert higher cytotoxicity to cells with higher tyrosinase activity and melanin content. These results suggest that the *in vitro* cytotoxicity of 4-*S*-CAP and 4-*S*-HomoCAP is mediated through conversion to the aldehydes while that of α-methyl-4-*S*-CAP appears to be dependent on tyrosinase activity to some extent.

Melanocytes possess a unique biochemical property, melanin synthesis [1]. The synthesis of melanin pigment from tyrosine is catalysed by tryrosinase (EC 1.14.18.1) present both in normal and malignant melanocytes. Previously, we synthesized 4-*S*-cysteinylphenol (4-S-CP‡), a sulphur homologue of tyrosine, and its amine derivative, 4-*S*-cysteaminylphenol (4-*S*-CAP), and showed that these phenols increased the life span of melanoma-bearing mice and inhibited the growth of melanoma cells both *in vitro* and *in vivo* [2, 3]. We also showed that topical application of 4-*S*-CP and 4-*S*-CAP caused depigmentation of black skin of guinea-pigs [4] and of black follicles of C57BL mice [5]. 4-*S*-CAP exhibited much more potent effects than did 4-*S*-CP. In a subsequent study, we found that 4-*S*-CP and 4-*S*-CAP were good substrates of mushroom and mammalian tyrosinases and suggested that in melanocytes these phenols are oxidized by tyrosinase to the corresponding *o*-quinone forms which conjugate with sulphydryl enzymes through cysteine residue, thus exerting cytotoxic effects [6].

In an attempt to develop antihypertensive drugs, Padgette *et al.* [7] prepared 4-*S*-CAP and its analogues and found that 4-*S*-CAP was a good substrate of plasma monoamine oxidase (MAO) while α-methyl derivative of 4-*S*-CAP was not. Furthermore, Kawase *et al.* [8] reported that various 3-substituted propylamines exert cytotoxicity through the corresponding aldehydes produced by enzymic oxidative deamination. Therefore, the possibility arose that 4-*S*-CAP might also exert its antimelanoma effect through a similar mechanism. In this study, we synthesized α-methyl, homo-, and *N*,*N*-dimethyl derivatives of 4-*S*-CAP (Fig. 1), compared the growth inhibition of melanoma cells by these phenols, and examined the mechanism of cytotoxicity. It was expected from the structures that 4-*S*-CAP and 4-*S*-HomoCAP may act as substrates of both tyrosinase and MAO while α-Me-4-*S*-CAP and *N*,*N*-DiMe-4-*S*-CAP may act as substrates of tyrosinase but not of MAO.

MATERIALS AND METHODS

Chemicals. Pargyline, catalase, plasma MAO (from bovine; 68 units/g protein), and yeast aldehyde dehydrogenase were purchased from the Sigma Chemical Co. (St Louis, MO). 4-Mercaptophenol was from Nakarai Chemicals (Kyoto, Japan) and the other chemicals were from Wako Pure Chemicals (Osaka, Japan). 4-*S*-CAP was synthesized as described by Padgette *et al.* [7]. 5-*S*-L-Cysteinyl-L-dopa and its diastereomer 5-*S*-L-cysteinyl-D-dopa were prepared chemically [9] and enzymically [10], respectively.

High-performance liquid chromatography (HPLC). 4-*S*-CAP and their metabolites were determined by HPLC under the following conditions. The HPLC system consisted of a Yanaco L-2000 liquid chromatograph, a Yanaco ODS-A reversed-phase column (4.6 × 250 mm, 7 μm particle size), and a

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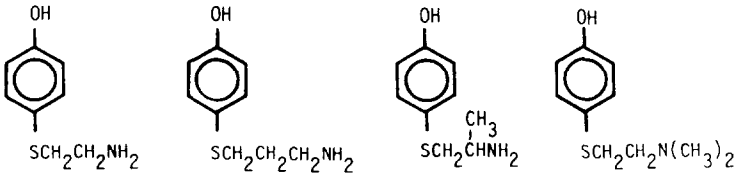
‡ Abbreviations used: CAP, cysteaminylphenol; CP, cysteinylphenol; HPLC, high-performance liquid chromatography; MAO, monoamine oxidase; PTU, phenylthiourea.



Abbreviation	Chemical name
4-S-CAP	4-(2-Aminoethylthio)phenol
4-S-HomoCAP	4-(3-Aminopropylthio)phenol
α-Me-4-S-CAP	4-(2-Aminopropylthio)phenol
N,N-DiMe-4-S-CAP	4-(2-N,N-Dimethylaminoethylthio)phenol

Fig. 1. Structures and names of 4-*S*-CAP and its analogues used in this study.

Yanaco VMD-101 electrochemical detector. The mobile phase was 0.1 M potassium phosphate buffer, pH 2.1, containing 1 mM Na_2EDTA : methanol, 88 : 12 (v/v). The column was maintained at 50° and the flow rate was 1.0 mL/min. The phenols were detected at +850 mV vs an Ag/AgCl reference electrode.

5-*S*-L-Cysteinyl-L-dopa and 5-*S*-L-cysteinyl-D-dopa were determined with the HPLC system consisting of a JASCO 880-PU liquid chromatograph, a JASCO catecholpak reversed-phase column (4.6 × 150 mm, 5 μm particle size), and a JASCO 840-EC electrochemical detector. The mobile phase was a mixture of 10 g of methanesulphonic acid, 12 g of 85% phosphoric acid, and 0.1 mmol of Na_2EDTA per litre, pH being adjusted to 3.3 with conc. NaOH. The temperature was at an ambient temperature and the flow rate 0.7 mL/min. The catechols were detected at +750 mV vs an Ag/AgCl reference electrode.

Synthesis of 4-S-*HomoCAP*. A general method for preparing 3-thiopropylamine derivatives [8] was adapted as follows. A mixture of 4-mercaptophenol (1.0 g, 7.9 mmol) and acrylonitrile (0.68 mL, 10.3 mmol) in ethanol (2 mL) was refluxed for 6 hr under an argon atmosphere in the presence of 25% trimethylphenylammonium hydroxide (0.2 mL). After cooling, the mixture was diluted with water and extracted with chloroform (2 × 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated to give a solid. Purification by column chromatography on silica gel (chloroform : ethyl acetate, 30 : 1) afforded colorless crystals of a nitrile (1.25 g, 88%): m.p. 72–73° (from benzene).

A solution of the nitrile (1.0 g, 5.6 mmol) in ether (5 mL) was added slowly to a vigorously stirred suspension of lithium aluminum hydride (0.48 g, 12.6 mmol) in ether (7 mL) at 0° under an argon atmosphere. The mixture was then refluxed for 3 hr under an argon atmosphere. After cooling in ice, ethyl acetate and then a saturated solution of potassium sodium tartrate were added. The mixture was filtered and the precipitate was washed with ethyl acetate and then with methanol. The combined filtrates were concentrated to give an oily residue which was dissolved in 6 M HCl (20 mL) and extracted with ether (30 mL). The aqueous phase was evaporated to dryness and the residue was chromatographed on Dowex 50W-X2 (2.0 × 16 cm) and eluted with 3 M HCl (15 mL/fraction). Evaporation of fractions 68–120 left the HCl salt of 4-*S*-HomoCAP which was dissolved in water and adjusted to pH 9 with conc. ammonia to give colorless crystals of 4-*S*-HomoCAP (0.31 g, 30%): m.p. 139–141°. Anal. Calcd for $C_9H_{13}NOS$: C, 58.98; H, 7.15; N, 7.64; S, 17.49%. Found: C, 58.87; H, 7.10; N, 7.66; S, 17.38%.

Synthesis of α-*Me*-4-S-*CAP*. The method of Padgette *et al.* [7] was modified as follows. To a mixture of ethyl orthoacetate (3.57 g, 47.5 mmol) and 1,2-dichloroethane (20 mL) was added 2-amino-1-propanol (7.7 g, 47.5 mmol). The resulting solution was refluxed for 9 hr under an argon atmosphere. After cooling, 4-mercaptophenol (5.0 g, 39.5 mmol) was added to the reaction mixture and was heated under reflux for 16 hr at *ca.* 130° under an argon atmosphere. The resulting mixture was evaporated in a rotary evaporator. The remaining solid was crystallized by adding benzene to a hot chloroform solution of the amide to give crystals of α-Me-*N*-acetyl-4-*S*-CAP (6.25 g, 70%). The amide (6.25 g, 27.8 mmol) was refluxed in 6 M HCl (110 mL) for 24 hr under an argon atmosphere. After cooling, the solution was extracted with ether (2 × 50 mL) and the aqueous phase was evaporated to dryness. The residue was dissolved in water (100 mL) and the solution was adjusted to pH 9 with conc. ammonia to give colorless crystals of α-Me-4-*S*-CAP (4.0 g, 78%): m.p. 105–106°. Anal. Calcd for $C_9H_{13}NOS$: C, 58.98; H, 7.15; N, 7.64; S, 17.49%. Found: C, 58.75; H, 7.16; N, 7.56, S, 17.04%.

Synthesis of N,N-*DiMe*-4-S-*CAP*. A common procedure of *N*,*N*-dimethylation of primary amines [11] was adapted. A mixture of 4-*S*-CAP (6.76 g, 40 mmol), 98% formic acid (15.2 mL, 400 mmol), and 37% formaldehyde (18.0 mL, 240 mmol) was heated under reflux for 1 hr. After cooling, 6 M

HCl (10 mL) was added and the mixture was evaporated in a rotary evaporator. The residue was dissolved in water (200 mL) and the solution was adjusted to pH 8 with conc. ammonia. The precipitated brown oil was decanted off and the supernatant was then adjusted to pH 9 to give pale yellow crystals of *N,N*-DiMe-4-*S*-CAP (4.3 g, 55%): m.p. 115–116°. Anal. Calcd for $C_{10}H_{15}NOS$: C, 60.88; H, 7.66; N, 7.10; S, 16.25%. Found: C, 60.71; H, 7.71; N, 7.05; S, 16.34%.

Cell culture. Human melanoma cell lines HMV-II (pigmented) and HMV-I (non-pigmented) were established by Kasuga *et al.* [12]. B16 mouse melanoma cell line was also used as a pigmented cell line. HeLa line was used as a non-melanotic, malignant cell line. These cell lines were cultured as monolayers at 37° in Ham's F-10 medium (with glutamine; from Gibco, Chargrin Falls, OH), supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) in a 5% CO_2 atmosphere.

Single cell suspension (2×10^5) in regular medium were inoculated into 60-mm Falcon Petri dishes, and cells were allowed to attach for 24 hr prior to exposure to the drug. The cells were incubated continuously with either regular medium or medium containing one of the drugs at various concentrations. After 24, 48, and 72 hr of incubation, cells were harvested by trypsinization and counted in a Model Z Coulter Counter.

Biochemical assays. Cells for biochemical assays were harvested 3 days after inoculation, suspended in phosphate-buffered saline and lysed with 1 mL of 0.2% Triton X-100 in phosphate-buffered saline. After centrifugation, the supernatant was used for tyrosinase assay and the precipitate was for analysing eu- and pheomelanin contents [13, 14].

Tyrosinase was assayed following the method of Carstam *et al.* [16]. Cell lysate (50 μL) was incubated at 37° with 450 μL of 0.1 M sodium phosphate buffer, pH 6.8, containing 1 mM L-dopa or D-dopa and 2 mM L-cysteine at final concentrations. After 5 min, a 100-μL aliquot was mixed with 100 μL of 0.8 M $HClO_4$. After centrifugation, a 10-μL aliquot was injected into HPLC. Tyrosinase activity was calculated as the stereospecific dopa oxidation [15] by subtracting the amount of non-enzymically formed 5-*S*-L-cysteinyl-D-dopa from that of enzymically formed 5-*S*-L-cysteinyl-*L*-dopa.

RESULTS

Figure 2 shows the effects of 4-*S*-CAP and its analogues on the growth of pigmented melanoma cells HMV-II and non-pigmented melanoma cells HMV-I. The four compounds examined may be grouped into two on the basis of degree of cytotoxicity. 4-*S*-CAP and 4-*S*-HomoCAP were highly cytotoxic to both cell lines, exerting toxicity at 50–100 μM concentrations. In contrast, α-Me-4-*S*-CAP and *N,N*-DiMe-4-*S*-CAP were approximately 40 times less toxic than the other two phenols. Among these four phenols, only α-Me-4-*S*-CAP exhibited some degree of selective toxicity to pigmented HMV-II cells.

The extreme difference in the cytotoxicity of these two groups might be ascribed to the difference in their biochemical properties: 4-*S*-CAP and 4-*S*-HomoCAP may act as substrates of MAO while α-Me-4-*S*-CAP and *N,N*-DiMe-4-*S*-CAP may not [7]. Therefore, effects of inhibitors of MAO and tyrosinase on the cytotoxicity were examined. Table 1 shows the effects of catalase, phenylthiourea (PTU), semicarbazide, and pargyline on the cytotoxicity of 4-*S*-CAP and its analogues. PTU is an inhibitor of tyrosinase [16], and semicarbazide and pargyline are inhibitors of plasma MAO and mitochondria MAO, respectively [17, 18]. Catalase and PTU did not protect the cells from the cytotoxicity of 4-*S*-CAP and its three analogues, indicating that hydrogen peroxide and tyrosinase were not involved in the cytotoxicity under these experimental conditions. Semicarbazide protected melanoma cells almost completely from the cytotoxicity of 4-*S*-CAP and 4-*S*-HomoCAP, whereas pargyline did not exert a significant protective effect. These results suggested that the strong cytotoxicity of 4-*S*-CAP and 4-*S*-HomoCAP was mediated mostly by MAO contained in fetal bovine serum. Oxidative deamination of amines by MAO produces hydrogen peroxide in addition to aldehydes. The inability of catalase to protect the cells indicated that the hydrogen peroxide produced did not play a major role in the cytotoxicity.

As the cytotoxicity of 4-*S*-CAP and 4-*S*-HomoCAP was suggested to be MAO-dependent, metabolism of 4-*S*-CAP in culture medium was next studied using HPLC analysis. As shown in Fig. 3, 4-*S*-CAP was rapidly metabolized with a half life of *ca.* 6 hr in medium irrespective of the presence or absence of cells; the time course of its decomposition coincided with each other. A new compound appeared in HPLC chromatograms when 4-*S*-CAP was incubated with medium alone. The compound was identified as the aldehyde on the basis that: (i) it was identical to the compound formed on incubation of 4-*S*-CAP with commercial preparation of plasma MAO, (ii) it gave a molecular ion peak (*m/e* 168) in a mass spectrum, and (iii) it exhibited an aldehyde signal (δ 9.46) in a nuclear magnetic resonance spectrum. When 4-*S*-CAP was incubated with medium in the presence of melanoma cells, the aldehyde did not appear as a discrete peak, but two peaks appeared instead. The compounds were identified as the alcohol and the carboxylic acid deriving from the aldehyde; reduction of the aldehyde with sodium borohydride gave a compound appearing at the position of the alcohol, while oxidation of the aldehyde with yeast aldehyde dehydrogenase and NAD^+ gave a compound appearing at the position of the carboxylic acid.

As it became apparent that MAO-dependent toxicity predominated when 4-*S*-CAP was exposed to cells in the medium containing fetal bovine serum, we then tried to overcome this unfavourable problem by using α-Me-4-*S*-CAP, a phenol that was not a substrate of MAO. Figure 4 compares the effect of α-Me-4-*S*-CAP on the growth of cell lines: pigmented B16 and HMV-II and non-pigmented HMV-I and HeLa. B16 line was the most susceptible among the four lines, followed by HMV-II line, and HMV-I and HeLa were less affected with similar degrees

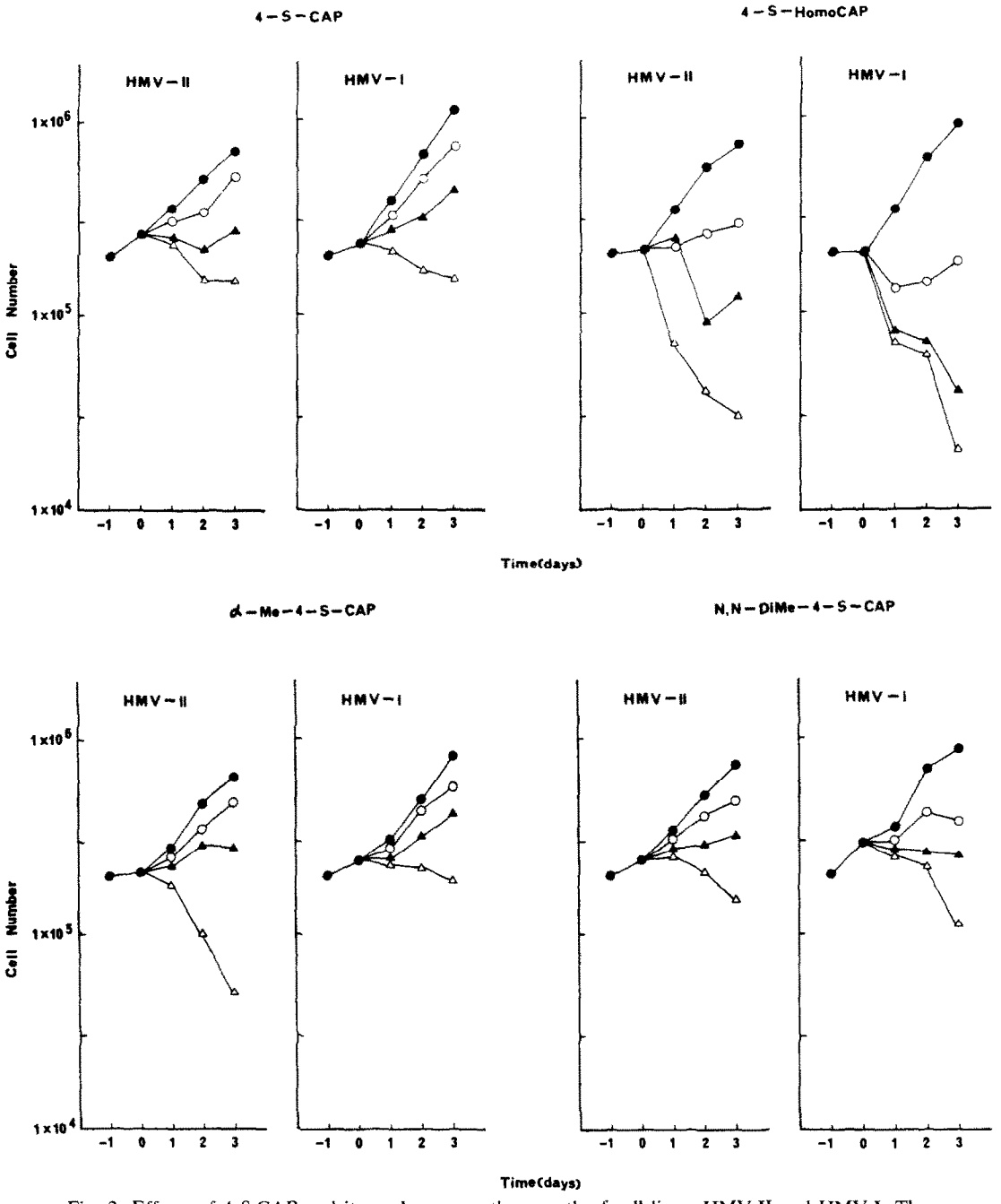


Fig. 2. Effects of 4-*S*-CAP and its analogues on the growth of cell lines, HMV-II and HMV-I. The experimental conditions were those described in Materials and Methods. (○) Control. For 4-*S*-CAP and 4-*S*-HomoCAP: (●), 25 μM; (△), 50 μM; (▲), 100 μM. For α-Me-4-*S*-CAP and *N*,*N*-DiMe-4-*S*-CAP: (●), 1 mM; (△), 2 mM; (▲), 4 mM. Results are means of three separate experiments.

of growth inhibition. The cytotoxicity of α-Me-4-*S*-CAP was compared with tyrosinase activity and content of eu- and pheomelanin in these cells. As shown in Table 2, these phenols exerted higher cytotoxicity to cells with higher tyrosinase activity and melanin content, i.e., B16 and HMV-II cells.

DISCUSSION

Melanin precursors and related catecholic compounds are highly toxic to melanoma cells *in vitro* and *in vivo* [19–22]. However, these catechols possess certain degrees of systemic toxicity that may result from auto-oxidation and concomitant production of active oxygen species [23]. In an attempt to overcome these difficulties, we synthesized and evaluated 4-*S*-CP and 4-*S*-CAP as antimelanoma agents [2–5]. These studies have shown that 4-*S*-CAP is selectively toxic to the *in vivo* melanocytes engaged in melanin synthesis [4, 5] but not to those melanocytes and keratinocytes of albino mice [5]. In

Table 1. Modification of toxicity of 4-*S*-CAP and its analogues by other agents

Cell line / Treatment	Growth inhibition (%)*				
	No phenol	4-*S*-CAP (50 μM)	4-*S*-HomoCAP (50 μM)	α-Me-4-*S*-CAP (2 mM)	*N*,*N*-DiMe-4-*S*-CAP (2 mM)
HMV-II					
Phenol alone	—	44 ± 4	39 ± 1	27 ± 3	29 ± 3
+ Catalase (100 μg/mL)	2 ± 1	50 ± 4	46 ± 6	42 ± 3	32 ± 2
+ PTU (100 μM)†	3 ± 2	46 ± 6	38 ± 2	24 ± 2	28 ± 3
+ Semicarbazide (1 mM)	0 ± 1	4 ± 2	4 ± 3	30 ± 4	31 ± 3
+ Pargyline (100 μM)	6 ± 3	39 ± 4	41 ± 3	28 ± 3	30 ± 3
HMV-I					
Phenol alone	—	40 ± 1	61 ± 5	23 ± 4	32 ± 7
+ Catalase (100 μg/mL)	0 ± 2	40 ± 2	72 ± 5	36 ± 4	35 ± 7
+ PTU (100 μM)†	1 ± 2	38 ± 2	62 ± 5	24 ± 1	33 ± 6
+ Semicarbazide (1 mM)	3 ± 1	5 ± 2	1 ± 1	18 ± 1	33 ± 7
+ Pargyline (100 μM)	3 ± 1	36 ± 4	69 ± 5	24 ± 1	34 ± 6

The experimental conditions were those described in Materials and Methods. The cells were incubated for 48 hr with either regular medium or medium containing a phenol and/or an agent.

* [(No. of control cells − no. of treated cells)/no. of control cells] × 100 by comparison with parallel control cultures. Mean ± SE of three separate experiments.

† Similar results were obtained with 1 mM PTU.

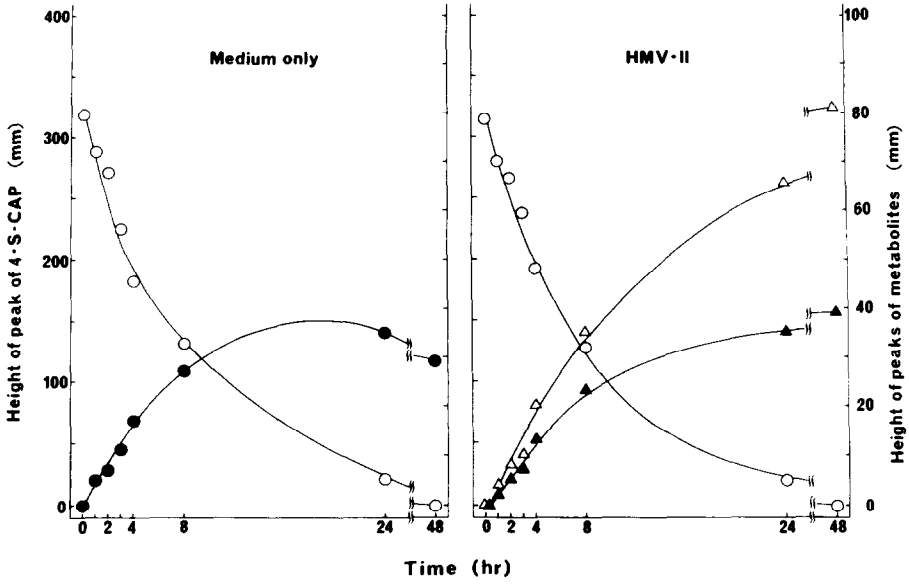


Fig. 3. Metabolism of 4-*S*-CAP in culture medium. 4-*S*-CAP at 50 μM was incubated with medium only or medium in the presence of cells. For the latter, single cell suspension (2×10^5) were allowed to attach for 24 hr prior to exposure to 4-*S*-CAP and medium containing 50 μM 4-*S*-CAP was then incubated with the cells. Aliquots (100 μL) of the medium were removed at intervals, diluted with 900 μL of 0.4 M $HClO_4$, and analysed directly by HPLC. (○), 4-*S*-CAP; (●), aldehyde; (△), alcohol; (▲), carboxylic acid. Results are means of two separate experiments. Experiments with HMV-I and HeLa cells gave results similar to those with HMV-I cells.

addition, [^{3}H]4-*S*-CP was shown to be selectively incorporated into HMV-II cells *in vitro* [24] and B16 melanoma tumour *in vivo* [25].

The present study as well as the study by Padgette *et al.* [7] has shown that 4-*S*-CAP is a good substrate of plasma MAO. The strong cytotoxicity of 4-*S*-CAP to cultured melanoma cells is mediated by the aldehyde formed by the oxidative deamination. On the other hand, α-Me-4-*S*-CAP was shown to exert higher cytotoxicity to cells with higher tyrosinase activity and melanin content, although the cytotoxicity was not inhibited by PTU, an inhibitor of tyrosinase. PTU has been shown to protect melanoma cells from cytotoxicity of tyrosinase substrates [16, 26, 27]. It is thus not clear at present why PTU failed to protect melanoma cells from the cytotoxicity of α-Me-4-*S*-CAP and *N*,*N*-DiMe-4-*S*-CAP, if it were tyrosinase-dependent. Furthermore, the cyto-

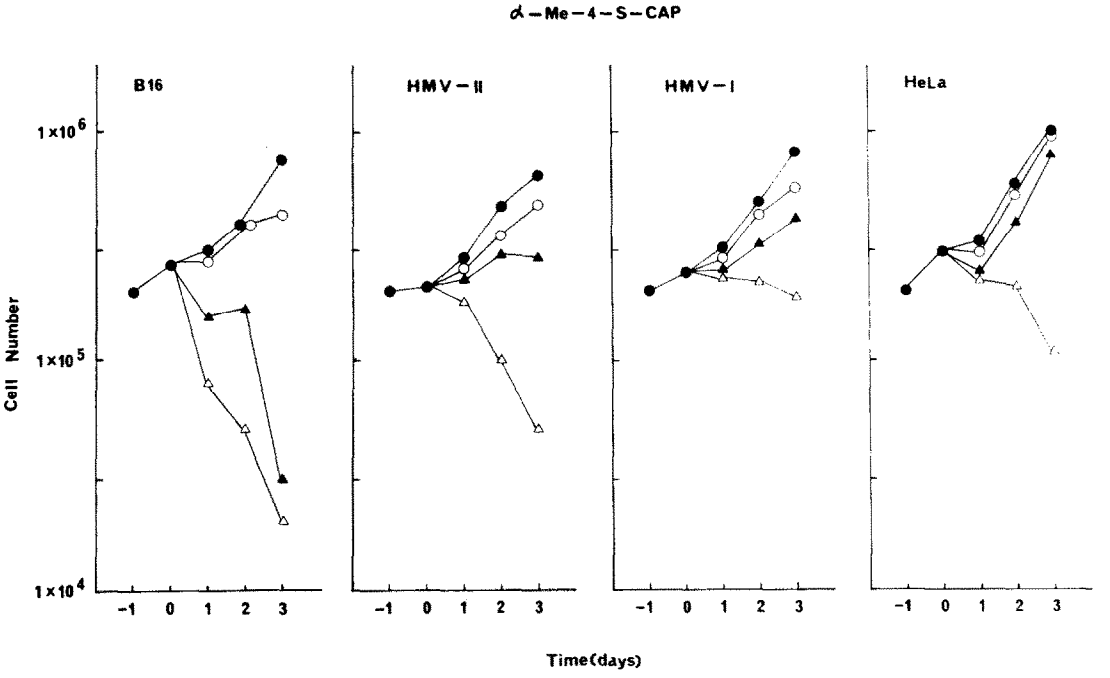


Fig. 4. Effects of α-Me-4-*S*-CAP on the growth of cell lines, B16, HMV-II, HMV-I, and HeLa. The data for HMV-II and -I lines are the same as those in Fig. 2, but included for comparison. (○), Control; (●), 1 mM; (△), 2 mM; (▲), 4 mM.

Table 2. Comparison of growth inhibition by α-Me-4-*S*-CAP, tyrosinase activity and melanin content

Cell line	Growth inhibition* (%)	Tyrosinase activity† (pmol/10^6 cells/min)	PTCA (ng/10^6 cells)	AHP‡ (ng/10^6 cells)
B16	98 ± 1	60 ± 5	2.9 ± 0.5	11 ± 0.2
HMV-II	92 ± 1	113 ± 27	1.7 ± 0.2	75 ± 3.8
HMV-I	78 ± 1	ND	<1.0	7.6 ± 1.3
HeLa	82 ± 2	ND	<1.0	15 ± 2.1

Results are mean ± SE of three separate experiments.
* Cells were incubated with 4 mM α-Me-4-*S*-CAP for 72 hr.
† Conversion of L-dopa to 5-*S*-L-cysteinyl-L-dopa in the presence of L-cysteine [15].
‡ PTCA and AHP are specific indicators of eu- and pheomelanin, respectively [13].
ND, not detected (below zero).

toxicity of these phenols to non-pigmented HMV-I and HeLa cells should be tyrosinase-independent. The mechanism of this tyrosinase-independent toxicity remains to be clarified.

A significant question emerged as a result of the present study: whether the selective toxicity of 4-*S*-CAP to normal and malignant melanocytes *in vivo* is mediated by tyrosinase or MAO. In order to answer this question, *in vivo* experiments were carried out in which the four phenols used in this study were compared for depigmenting effect on follicular melanocytes of C57BL mice [5]. Preliminary results show that α-Me-4-*S*-CAP is as potent a depigmenting agent as is 4-*S*-CAP.* This clearly indicates that MAO does not play a major role in selective toxicity of these phenols to melanocytes *in vivo*.

Finally, a role of melanin in the selective toxicity of 4-*S*-CAP and its analogues is discussed. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine was incidentally found to induce clinical features of Parkinson's disease in animals including man. Recently, as a possible mechanism of the neurotoxicity of the amine, it was postulated that the amine was first oxidized to an active metabolite by the action of MAO and the metabolite 1-methyl-4-phenylpyridine was selectively accumulated in dopaminergic neurons by binding to neuromelanin and was continuously released until it destroyed the cells [28]. A similar mechanism should also be considered for the selective toxicity of 4-*S*-CAP and its analogues: selective uptake to pigmented cells by virtue of the binding to melanin through the amino group [29] of

*Manuscript in preparation, F. Alena, K. Jimbow and S. Ito.

these phenols followed by tyrosinase-independent cytotoxicity.

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