In summary, using immunochemical techniques, we confirmed our previous report that the induction of cytochrome P-450 by MC in cultured chick hepatocytes is potentiated by EE_2. Immunological characterization suggests that the form(s) of cytochrome P-450 increased by the combined treatment with EE_2 and MC are identical to those induced by MC alone. The results may indicate the possible mechanism by which oral contraceptives are a risk factor in carcinogenesis and porphyria cutanea tarda, two disorders in which P-450 from the I family may have a role, as discussed previously [4].

Note added in proof: Hokama *et al.* (*J Biochem* **104**: 355–361, 1988) have recently found that 3,4,5,3′,4′-Pentachlorobiphenyl induces two forms of P-450 of 56K and 54K molecular weight, in chickens, which they conclude are the chicken equivalents of P-450IA, and P-$450IA_2$.

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Selective cytotoxicity of a phenolic melanin precursor, 4-*S*-cysteaminylphenol, on *in vitro* melanoma cells

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Our recent approach of using melanin precursors to design a rational chemotherapeutic agent against malignant melanoma may be of particular interest [1–6]. One would expect that, through the melanin synthesis pathway, compounds such as melanin precursors would selectively be incorporated into melanoma cells and would become toxic through conversion by tyrosinase, an enzyme unique to melanoma cells. A similar attempt to use a melanin precursor as an anti-tumor agent has been reported by a number of investigators [7–11]. They, however, utilized catecholic compounds.

Recently, we synthesized a sulfur homologue of phenol (tyrosine), cysteinylphenol (CP*), and its amine derivative, cysteaminylphenol (CAP), and tested for *in vivo* melanocytotoxicity and antimelanoma effects. We found that (a) 4-*S*-CP and 4-*S*-CAP are good substrates of mammalian tyrosinase to form melanin-like pigments, whereas their 2-*S*-isomers are not tyrosinase substrates [2, 3], (b) 4-*S*-CP and 4-*S*-CAP, in particular 4-*S*-CAP, possess *in vivo*

* Abbreviations: CP: cysteinylphenol; CAP: cysteaminylphenol; IMDM: Iscove's Modified Dulbecco's Medium; and DOPA: dihydroxyphenylalanine.

depigmenting potency on black skin and hair follicles whereas their 2-*S*-isomers do not show this effect [5, 6], and (c) 4-*S*-CAP possesses a significant *in vivo* antimelanoma effect on mouse melanoma growth and prolongs the life-span of melanoma-bearing mice [4]. It was indicated that these phenolic melanin precursors are oxidized by tyrosinase to the corresponding *o*-quinine forms which conjugate with sulfhydryl enzymes through cysteine residue, thus exerting melanocytotoxicity and an antimelanoma effect [2, 3]. To assess further the melanocytotoxicity of phenolic melanin precursors, we carried out, using 4-*S*-CAP as an experimental model, an *in vitro* thymidine incorporation test and a clonogenic assay on both human and murine melanoma cells.

Materials and methods

Tumors. B16 murine melanoma and K-562 leukemia cell lines were grown in RPM1-1640 medium supplemented with 10% fetal calf serum and antibiotics, and were passaged twice weekly. Human tumors, comprising malignant melanoma (MM 514), and ovarian (OXF 899) and lung (LXF 529) carcinomas were passaged in nude mice (NMR1, Lab. Dr. Fiebig, Freiburg, F.R.G.). Both B16 murine and human melanomas were highly melanotic and were expected to have high tyrosinase activity. The tumors were removed from the mice after the second to fourth passages. The animals were kept under laminar flow conditions and were fed mouse diet C14 (Altromin, Large, F.R.G.).

Chemicals. 4-*S*-CAP was prepared according to a method described previously [4, 12]. The compound was dissolved in sterile 0.9% NaCl solution, and further dilution was performed with Iscove's Modified Dulbecco's Medium (IMDM; Gibco Europe, Karlsruhe, F.R.G.) for the clonogenic assay and with Hanks' balanced salt solution for the thymidine incorporation test. Catalase was purchased from the Sigma Chemical Co. (St Louis, MO), and serially diluted in IMDM. Radiolabeled [6-^{3}H]thymidine (sp. act. 23 Ci/mmol) and [6-^{3}H]uridine (sp. act. 16 Ci/mmol) were obtained from Amersham & Bucheler (Braunschweig, F.R.G.); [4,5-^{3}H]leucine (sp. act. 60 Ci/mmol) was obtained from the New England Nuclear Corp. (Boston, MA).

Clonogenic assays. The tumor cells were cultured as described previously [13]. Briefly, human tumors passaged in nude mice were disaggregated mechanically into single-cell suspensions, and the viability of the tumor cells was assessed by trypan blue exclusion. Then, viable cells (1×10^5) were plated in the presence of 30% fetal calf serum in IMDM with 4-*S*-CAP. The plating efficiency was expressed by the number of visible cells obtained relative to the number of cellular units put into a plate. For tumor cell lines, B16 murine melanoma and K-562 leukemia cells (log phase 1×10^4) were also seeded. Methylcellulose at a final concentration of 0.9% (w/v) was used as a viscous support. The dishes were incubated at 37° in the presence of 7.5% CO_2 for 7–10 days. Aggregates of more than 30 cells with a diameter of 80–100 μm were considered to be colonies. Drug effects were expressed as percent survival of tumor cell colonies.

Precursor incorporation studies. A total of 5×10^5 tumor cells was exposed to 2.5 μCi/ml of [^{3}H]thymidine at 37° for 60 min for the cytotoxic study of 4-*S*-CAP on different tumor cells. In addition, to study the effect of 4-*S*-CAP on macromolecular synthesis. B16 murine melanoma cells (5×10^5) were incubated in triplicate flasks containing 4-*S*-CAP (2.5 μg/ml, 10 μg/ml) for 0–5 hr at 37° in the presence of 7.5% CO_2. Then, at a specified time, tumor cells were pulsed with either [^{3}H]thymidine (2.5 μCi/ml), [^{3}H]uridine (2.5 μCi/ml) or [^{3}H]leucine (2.5 μCi/ml) in a shaking water bath at 37° for 60 min. The cells were plated onto filter paper (2.3 cm; MN218, Macherey-Nagel, F.R.G.), and dried immediately. The filter discs were then washed twice with 5% trichloroacetic acid for 30 min and followed by ethanol/ether solution (1:1) for 20 min and ether solution for 10 min. The filter discs were placed in scintillation vials containing 5 ml of solution (Instant Scint. Gel, Packard, U.S.A.), and counted in a 2000CA Liquid Scintillation Analyzer (Packard, U.S.A.).

Results

Five different tumor cells (B16 murine melanoma, human melanoma, K-562 leukemia, and human ovarian and lung carcinoma) were tested for the cytotoxicity of 4-*S*-CAP on melanoma cells by two different assays, i.e. the precursor incorporation test and the clonogenic assay.

The precursor incorporation test by [^{3}H]thymidine supported the selectivity of 4-*S*-CAP-induced cytotoxicity on melanoma cells. As can be seen in Table 1, the inhibition of [^{3}H]thymidine incorporation into the two melanoma cell lines was particularly relevant even at the lowest concentration of 4-*S*-CAP, with no detectable inhibition of K-562 leukemia and ovarian and lung carcinoma cells.

The effect of 4-*S*-CAP on macromolecular synthesis was also studied. B16 murine melanoma cells were incubated with an appropriate concentration of 4-*S*-CAP (2.5 and 10 μg/ml) for 0–5 hr and, afterward, the tumor cells were pulsed with either [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine for 1 hr. The time–course data are shown in Fig. 1. At the low dose of 2.5 μg/ml, a time-dependent inhibition of thymidine incorporation was observed, with little effect on uridine. Furthermore, a rapid and selective inhibition of thymidine incorporation into DNA was found

Table 1. Inhibition of [^{3}H]thymidine incorporation into different tumor cells by various concentrations of 4-*S*-CAP*

Tumor	[^{3}H]Thymidine incorporation (% inhibition) 4-*S*-CAP (μg/ml)		
	5	10	25
B16 murine melanoma	0 ± 6	57 ± 4	78 ± 5
Human melanoma	52 ± 4	61 ± 4	82 ± 7
K-562 leukemia	9 ± 5	5 ± 2	8 ± 4
Human ovarian carcinoma	7 ± 4	8 ± 6	4 ± 4
Human lung carcinoma	2 ± 3	8 ± 5	11 ± 7

* Control cells incorporated 3,874 ± 826 dpm (B16 murine melanoma), 988 ± 266 (human melanoma), 13,839 ± 1,588 (K-562 leukemia), 923 ± 71 (ovarian carcinoma), and 1,146 ± 259 (lung carcinoma). Values, expressed as percent inhibition compared to the controls, are means ± SD from three separate experiments.

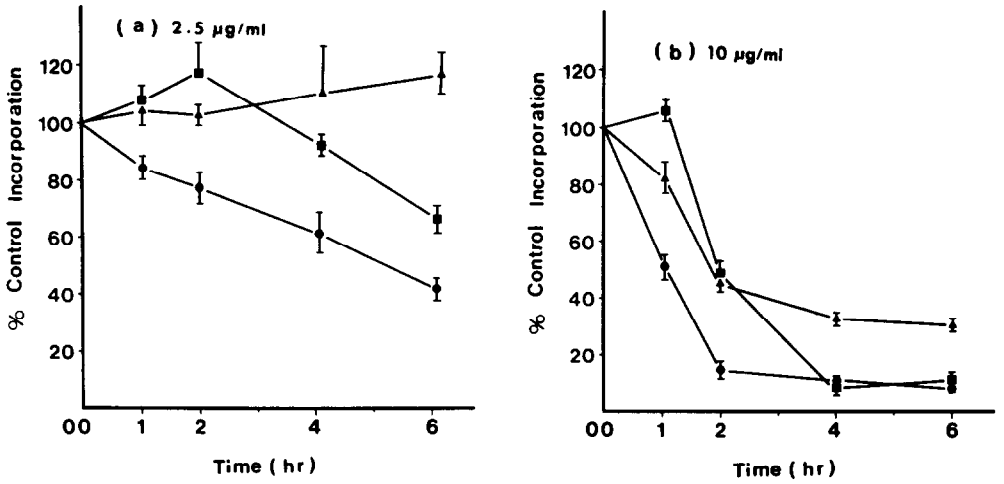

Fig. 1. Effect of 4-*S*-CAP on thymidine, uridine and leucine incorporation by B16 murine melanoma cells. Tumor cells incubated with 4-*S*-CAP, 2.5 μg/ml (a) and 10 μg/ml (b), were labeled (2.5 μCi/ml) with [^{3}H]thymidine (●—●), [^{3}H]uridine (■—■), or [^{3}H]leucine (▲—▲). Precursor incorporation into DNA, RNA and protein was determined by liquid scintillation counting as outlined in Materials and Methods. Experiments were done in triplicate. Values are means ± SD.

at a dose of 10 μg/ml, although incubation of more than 2 hr resulted in a decreased selectivity.

Figure 2a shows the dose–response curves for percent survival of colony for the different tumor cells. A marked colony reduction was observed in both human and murine melanoma cells compared to other control tumor cells. At a dose of 3 μg/ml drug, the values for percent survival of B16 murine and human melanoma cells were 18 and 43%, respectively, while no significant colony reduction was found for the other non-melanoma tumor cells. The numbers of colonies on the tumor cells at a drug concentration of 1 μg/ml were 49.3% ($P < 0.001$) in B16 murine melanoma (82 ± 15 for the 4-*S*-CAP-treated group vs 165 ± 9 for the control group), 41.4% ($P < 0.001$) in human melanoma

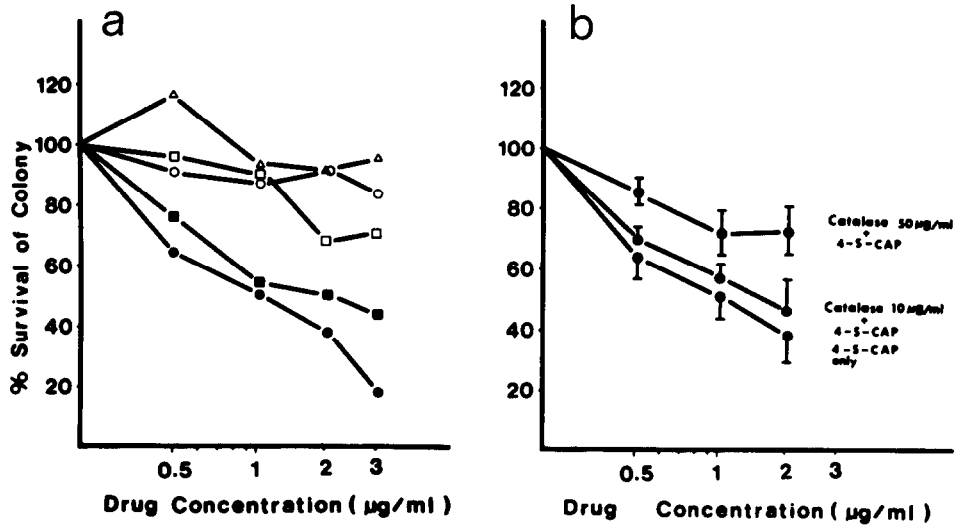

Fig. 2. Effect of 4-*S*-CAP on colony formation. (a) Survival of different tumor cells after continuous exposure to 4-*S*-CAP. Dose–response curves are shown for B16 murine melanoma (●—●), human malignant melanoma (■—■), K-562 leukemia (○—○), ovarian carcinoma (□—□) and lung carcinoma (△—△) cells. The effect is expressed as reduction of colony formation as a function of the concentration of 4-*S*-CAP. Each point represents the mean value of three to four dishes. Plating efficiency: B16 murine melanoma, 1.65; human melanoma, 0.08; K-562, 4.25; ovarian carcinoma, 0.08; and lung carcinoma, 0.04. Percent survival of colony = (control − 4-*S*-CAP-treated group)/control group × 100. (b) Influence of catalase on 4-*S*-CAP-induced cytotoxicity in B16 murine melanoma cells. Experimental conditions were similar to those of panel (a). Plating efficiencies: untreated cells, 1.65 ± 0.09; cells treated with 10 μg/ml catalase alone, 1.76 ± 0.03; and cells treated with 50 μg/ml catalase alone, 1.47 ± 0.02. Values are means ± SD.

(51 ± 2 vs 87 ± 5), 6.4% in K-562 leukemia (398 ± 26 vs 425 ± 21), 9.5% in ovarian carcinoma (76 ± 5 vs 88 ± 4) and 7.1% in lung carcinoma (39 ± 3 vs 42 ± 4). Compared to control groups, great colony reduction was observed for the two melanoma cells.

To examine whether the effect of 4-*S*-CAP on melanoma cells resulted from the formation of hydrogen peroxide, catalase was added to the clonogenic assay system. Figure 2b shows the effect of catalase on 4-*S*-CAP-induced cytotoxicity in B16 murine melanoma cells. The plating efficiency did not differ between the cells of the control and the treated groups. Treatment with 50 μg/ml catalase markedly prevented the effect of 4-*S*-CAP on B16 murine melanoma cells whereas little attenuation was found with 10 μg/ml catalase.

Discussion

This study demonstrated a rapid and marked inhibition of radiolabeled thymidine incorporation into both murine and human melanoma cell lines without any detectable inhibition in non-melanoma human cell lines, e.g. human leukemia, and ovarian and lung carcinoma cells. The comparison on macromolecular synthesis using thymidine, uridine and leucine suggested selective and rapid DNA damage in melanoma cells. In the clonogenic assay, 4-*S*-CAP also showed highly selective cytotoxicity in the two melanoma cell lines. Furthermore, catalase (50 μg/ml) attenuated markedly, but not completely, the 4-*S*-CAP cytotoxicity.

We and others have shown previously that phenolic and catecholic compounds cause depigmentation of the skin [14–16]. It was shown that these compounds selectively disintegrate the melanocytes [15]. In their first report, Wick *et al.* [7] found that a catecholic melanin precursor, L-DOPA, shows a selective growth inhibition of the melanotic S91A melanoma cell line compared to the nonpigmented control cells. The cytotoxicity corresponded to the rate of incorporation of [^{3}H]DOPA into the cells. They subsequently showed that L-DOPA methyl ester and dopamine treatment resulted in an increased life span of B16 melanoma bearing animals [8, 9, 17, 18]. Our previous *in vivo* studies with B16 murine melanoma indicated that, among the phenolic and catecholic compounds synthesized [4], the melanocytotoxicity is most prominent in the 4-*S*-CAP-treated group, the prolongation of life span and growth inhibition being 49 and 64%, respectively. Our subsequent studies [5, 6] ascertained that 4-*S*-CAP causes a selective destruction of melanocytes in skin and hair follicles. None of the degenerative changes were observed in non-melanin-forming cells, e.g. albine melanocytes, fibroblasts and keratinocytes. In the present study with five different human and mouse tumor cell lines, we also confirmed the selectivity of the 4-*S*-CAP cytotoxicity on melanoma cells by two different test systems.

We [2, 3] previously hypothesized that 4-*S*-CAP may be converted to an *o*-quinone via 4-*S*-cysteaminylcatechol and that the subsequent reaction with SH-containing enzymes may result in the covalent binding with sulfhydryl enzymes through cysteine residue, thus exerting the melanocytotoxicity. In the present study, we also examined the effect of catalase on 4-*S*-CAP cytotoxicity to B16 murine melanoma cells in the clonogenic assay. It was shown that catalase attenuated the effect of 4-*S*-CAP. This observation suggests that cytotoxicity to melanoma cells may result not only from DNA polymerase inhibition, as proposed previously [19–21], but also from hydrogen peroxide formed through mechanisms such as auto-oxidation of 4-*S*-CAP-derived melanin intermediate or oxidative determination of 4-*S*-CAP by monoamine oxidase [22].

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