[CANCER RESEARCH 35, 3126–3130, November 1975]

Tyrosinase-mediated Inhibition of *in Vitro* Leucine Incorporation into Mouse Melanoma by 4-Isopropylcatechol[1]

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SUMMARY

The effect of 4-isopropylcatechol (4-IPC), a potent, irreversible cutaneous depigmenting agent, on protein biosynthesis of malignant melanoma cells in mice was studied by examining the *in vitro* amino acid (leucine) incorporation into a microsome fraction in cell sap. The present study revealed that 4-IPC does not inhibit the protein biosynthesis of the cell-free system in mouse liver, but remarkably inhibits it in mouse melanoma cells, which contain a high level of tyrosinase. The enhanced inhibition was found also in the mouse liver cell-free system when tyrosinase was added. Air oxidation products of 4-IPC were not responsible for such inhibition. These results may indicate that 4-IPC directly inhibits protein biosynthesis, probably by some intermediates that occur in an early stage of enzymatic oxidation of 4-IPC.

INTRODUCTION

4-IPC[3] is a potent, irreversible depigmenting agent when applied topically to the skin of black guinea pigs (2, 9). Light and electron microscopy indicated that 4-IPC selectively affects the melanin-producing cell and results in a marked decrease in the population density of the functioning melanocytes (2, 9). Although a decrease in the population of functioning melanocytes can be derived from a number of processes, one wonders whether the selective action of 4-IPC on melanocytes is related to the presence of the melanin-forming enzyme, tyrosinase, and, if so, whether 4-IPC may be useful in the treatment of malignant melanoma, which contains a markedly high level of tyrosinase (7).

This study examines the effect of 4-IPC on *in vitro* protein biosynthesis of melanoma and liver cells with or without the presence of tyrosinase.

MATERIALS AND METHODS

Chemicals. Chemicals used in this study were obtained from the following sources: L-[1-^{14}C]leucine (28.7 mCi/mmole) from New England Nuclear, Boston, Mass.; L-tyrosine, DOPA, mushroom tyrosinase (grade III, 950 units/mg), ATP, GTP, phosphoenolpyruvate, and pyruvate kinase from Sigma Chemical Co., St. Louis, Mo.; 4-methylcatechol and 6-hydroxydopamine from Aldrich Chemical Co., Milwaukee, Wis.; 4-hydroxyanisole from Eastman Organic Chemicals, Rochester, N. Y.; hydroquinone from Fisher Scientific Co., Boston, Mass.; 4-IPC, courtesy of Fujisawa Pharmaceutical Corp., Tokyo, Japan; and puromycin dihydrochloride from the Nutritional Biochemicals Corp., Cleveland, Ohio.

Preparation of Cell Components. HP and B-16 mouse melanomas were serially transplanted s.c. into C57BL/6 and BALB/c mice (each strain, 5-week-old males). The melanomas were removed at the time of maximum growth, *i.e.*, HP at 8 weeks and B-16 at 3 weeks after transplantation. After removal, they were quickly chilled in an ice-cold PKMS solution. They were then weighed, minced, and homogenized in a loosely fitted glass-glass homogenizer using a PKMS solution with 3 times the volume of the tumor. All subsequent procedures were carried out in a cold environment. The homogenate was centrifuged at 15,000 × *g* for 15 min, and the postmitochondrial supernatant thus obtained was spun for 90 min at 105,000 × *g* to precipitate the microsomal fraction. After this ultracentrifugation, the cell sap was taken from the upper two-thirds of the supernatant.

Microsomal fraction and cell sap were also prepared from the liver of C57BL/6 mice in the same way as were the melanoma tissues, except that a glass-Teflon homogenizer was used.

The fraction of soluble, nonparticulate-bound tyrosinase was prepared from the HP mouse melanomas stocked in a frozen state at −20°. The melanomas were homogenized in distilled water with 3 times the volumes of tumors, using a glass-glass homogenizer. The homogenate was centrifuged at 15,000 × g for 20 min, and the supernatant was taken from the upper two-thirds of the centrifuge tube, which contained the soluble form of active tyrosinase.

Cell-free Incorporation System. *In vitro* incorporation of [^{14}C]leucine was studied using the microsome-and-cell-sap system of mouse melanoma cells, which contain the largest amount of active tyrosinase (18). Phosphate buffer at pH 7.4 was used instead of Tris, inasmuch as Tris buffer is known to inhibit DOPA oxidation mediated through tyrosinase (14), and the higher pH common for incorporating experiments stimulates air oxidation of 4-IPC. The sulfhydryl compound (*i.e.*, β-mercaptoethanol, dithiothreitol)

[1] This work was supported by USPHS Grants CA12108 and CA13651.

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[3] The abbreviations used are: 4-IPC, 4-isopropylcatechol; DOPA, L-3,4-dihydroxyphenylalanine; HP, Harding-Passey mouse melanoma; PKMS solution, solution of 20 mM potassium phosphate (pH 7.4), 50 mM KCl, 7 mM $MgCl_2$, and 0.25 M sucrose.

Received May 16, 1975; accepted August 1, 1975.

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was avoided in our incorporating system because it inhibits tyrosinase activity and binds to the intermediates of the tyrosine-to-melanin pathway (5, 12, 17).

The reaction medium for our cell-free incorporating system was prepared in a PKMS solution. One ml of the solution contained 4.0 mg protein of microsomal fraction of either liver or melanoma tissue, 6.0 mg protein of cell sap, 1 μmole of ATP, 0.25 μmole of GTP, 10 μmoles of phosphoenolpyruvate, 40 μg of pyruvate kinase, and 0.5 μCi of [^{14}C]leucine. Freshly prepared 4-IPC, DOPA, and tyrosine were incubated in the reaction medium with the concentration specified at 37° for 30 min.

Determination of Radioactivity. After incubation, the reactions were stopped by adding an equal volume of ice-cold 10% trichloroacetic acid. The precipitate was resuspended in 5% trichloroacetic acid, heated for 15 min at 90°, and washed 4 times with cold 5% trichloroacetic acid, twice with absolute ethanol, and finally with hot (60°) ethanol:ether (3:1, v/v). The precipitate was then dissolved in 2 ml of formic acid, transferred to stainless steel planchettes, dried, and weighed. Its radioactivity was measured in a low background automatic gas-flow counter with the correction of the self-absorption (Nuclear Chicago Model 181A-C115).

Preparation of the Air Oxidation Product of 4-IPC. 4-IPC, 300 mg, was dissolved in a 100-ml solution of 0.02 N KOH and air oxidized with a constant bubbling at room temperature. After 48 hr, the solution became brownish-dark red, resembling the color of the 4-IPC solution oxidized by the tyrosinase fraction of mouse melanoma. Chemical alteration of the air-oxidized 4-IPC was examined by spotting this solution on a silica gel thin-layer chromatogram, which was developed with chloroform:ethyl acetate (1:1, v/v). The separated spots were visualized by exposure to iodine vapors or to a saturated solution of ferric chloride in methanol. After air oxidation, 4-IPC changed its migration pattern on the chromatogram. There were 1major product and a few minor components that migrated differently from an intact 4-IPC.

RESULTS

Incorporation of ^{14}C-Labeled Leucine into the Cell-free System of Mouse Melanoma. A high incorporation of leucine into mouse melanomas was shown when [^{14}C]leucine was incubated in an *in vitro* cell-free system of B-16 mouse melanoma consisting of microsomes and cell sap. The incorporation of leucine was, however, remarkably inhibited when 0.5×10^{-3} M puromycin was added, indicating that the incorporation of ^{14}C-labeled leucine is mediated through the normal pathway of protein biosynthesis (11). The incorporating activity of the cell-free system of HP mouse melanoma, however, was always less than that of B-16 mouse melanoma. Seiji and Ogata (19) reported similar results for incorporation of ^{14}C-labeled leucine into ribosomes and rough membranes of HP mouse melanoma.

The microsomal fractions of both B-16 and HP melanoma were light brown. The reaction mixture, when it was immediately chilled and subjected to discontinuous sucrose-gradient (1.2 to 2.0 M) centrifugation (1) after incubation, revealed melanosomes in the pellet. The melanosome pellet,

Table 1

Incorporation activity of ^{14}C-labeled leucine into cell-free systems of B-16 and HP mouse melanoma

Two μCi of ^{14}C-labeled leucine were incubated for 30 min at 37° in a cell-free system containing microsome and cell-sap fractions of B-16 and HP melanomas.

	Incorporation (cpm/10 mg protein)	
	B-16	HP
Cell-free system without puromycin	707	124
Cell-free system with 0.5 mM puromycin	35	
Cell-free system with 1.0 mM puromycin	28	
Melanosomes[a]	6	11

[a] Melanosomes were isolated from the incubation mixture after chilling by discontinuous sucrose-gradient centrifugation.

Table 2

Effect of 4-IPC, DOPA, and tyrosine on incorporation of ^{14}C-labeled leucine into cell-free systems of B-16 and HP mouse melanoma

Type of mouse melanoma	Experiment	Incorporation (cpm/10 mg protein) into control, cell-free system	% incorporation after treatment with					
			4-IPC				DOPA, 2.0 mM	Tyrosine, 0.1 mM
			0.5 mM	1.0 mM	2.0 mM	4.0 mM		
B-16	1	160		53	31	23	105	97
	2	138	81	51				
	2a[a]	101	22	7				
	3	143			21	15		
	4	173		13	7			
	5	184		61	19			
	6	142		14	8			
HP	1	55			71	57	177	130
	2	38		71	55			

[a] In experiment 2a, tyrosinase fraction prepared from HP mouse melanoma (50 μg of protein) was added to both the control and the 4-IPC treated, cell-free system.

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Table 3

Effect of 4-IPC on the incorporation of ^{14}C-labeled leucine into a liver cell-free system with and/or without the addition of tyrosinase

Experiment	Type of tyrosinase added	Incorporation (cpm/10 mg protein) before treatment with chemicals	% incorporation after treatment with 4-IPC 1.0 mM	4-IPC 2.0 mM	4-IPC 4.0 mM	DOPA, 2.0 mM	Tyrosine, 0.25 mM
1	None	1112		82	75		
	HP tyrosinase[a]	740		23	11		
2	None	1398		83	76	103	98
	HP tyrosinase[a]	1340		62	49	83	98
3	None	1330	89	88		102	100
	HP tyrosinase[a]	1039	66	62		92	99
4	None	1395	94	86			
	25 μg of mushroom tyrosinase[b]	1650	15	5			
	50 μg of mushroom tyrosinase[b]	1473		4			

[a] Tyrosinase fraction, prepared from HP mouse melanoma, was added 50 μg protein per tube.

[b] Twenty-five μg of purified mushroom tyrosinase (Sigma Chemical Co.) correspond to 24 units of tyrosinase activity.

Table 4

Effect of the air oxidation product of 4-IPC on the ^{14}C-labeled leucine incorporation into cell-free systems of mouse melanoma and liver

System	Incorporation (cpm/10 mg protein) into control cell-free system	% incorporation after treatment with 4-IPC 1.0 mM	4-IPC 2.0 mM	Air-oxidation product[a] of 4-IPC 1.0 mM	Air-oxidation product[a] of 4-IPC 2.0 mM	Air-oxidation product[a] of 4-IPC 3.0 mM
B-16	184	61	19			
	192a[b]			99		
	204b[b]				82	
	200c[b]					72
HP	38	71	55			
	39a			81		
	40b				72	
Liver	1330	89	88			
	1180a			94		
	1182b				81	

[a] 4-IPC, 300 mg, dissolved in 100 ml of 0.02 N KOH was oxidized with air bubbles for 48 hr at room temperature (final pH, 7.8).

[b] One-hundred ml of 0.02 N KOH were oxidized in a way similar to that of 4-IPC, followed by adjustment of the pH to 7.8. To controls, a, b, and c, respectively, was added an equal amount of this solution to the solution containing the oxidation product of 4-IPC.

however, showed negligibly low radioactivity incorporation in both B-16 and HP mouse melanomas (Table 1).

Effect of 4-IPC on Incorporation of ^{14}C-Labeled Leucine into the Cell-free System of Mouse Melanoma. Incorporation of ^{14}C-labeled leucine in melanoma tissues *in vitro* was greatly decreased when 4-IPC was added to the cell-free system (Table 2). The decrease in the incorporation appears to correspond to the concentration of 4-IPC added to the system. DOPA and tyrosine did not show any inhibition of the incorporation by the cell-free system of the B-16 melanoma, whereas it rather enhanced the incorporation in the cell-free system of the HP melanoma.

Effect of 4-IPC on Incorporation of ^{14}C-Labeled Leucine into the Cell-free System of Mouse Liver. Table 3 shows that 4-IPC slightly inhibits incorporation of ^{14}C-labeled leucine by the liver cell-free system, which, however, exhibited weak DOPA and 4-IPC oxidation when measured in a Warburg apparatus. Interestingly enough, 4-IPC markedly inhibited leucine incorporation into the liver cell-free system when the tyrosinase fraction prepared from HP melanoma was added. For corroboration of this inhibitory effect of 4-IPC in the presence of tyrosinase, purified mushroom tyrosinase was added to the incubation mixture of the liver cell-free system; addition of mushroom tyrosinase alone did not inhibit incorporation, but marked decrease in incorporation was observed when both 4-IPC and mushroom tyrosinase were added. The air oxidation product of 4-IPC, however, showed only a slight inhibition of incorporation (Table 4). Therefore, even if the air-oxidized and the enzyme-oxidized 4-IPC are different, it is possible that some intermediate compound occurring at an early stage of enzymatic oxidation of 4-IPC is responsible for the inhibition of protein biosynthesis.

Effect of Other Depigmenting Agents on Incorporation of ^{14}C-Labeled Leucine into the B-16 Cell-free System. 4-IPC, and its effect on the incorporating activity of the B-16 cell-free system, was compared with other depigmenting agents that can be substrates of tyrosinase and inhibit the tyrosine-to-DOPA-to-melanin pathway: 4-methylcatechol, hydroquinone, and 4-hydroxyanisole (6, 8, 15, 16).

Among these agents, 4-IPC showed the highest inhibition of the incorporation of ^{14}C-labeled leucine (Chart 1). The inhibition was unexpectedly low in the system treated with 4-hydroxyanisole.

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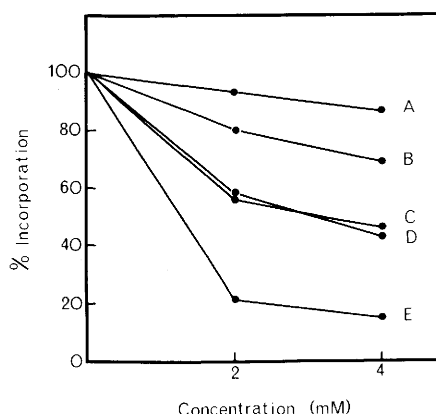

Chart 1. Effect of depigmenting agents 4-hydroxyanisole (*A*), hydroquinone (*B*), 4-methylcatechol (*C*), 6-hydroxydopamine (*D*), and 4-IPC (*E*), on the incorporating activity of leucine into the cell-free system of B-16 mouse melanoma.

DISCUSSION

Examination of the potency of chemical compounds for the induction of depigmentation is a reliable screening test for evaluation of their action on melanocytes and malignant melanoma cells. In 1968, our laboratory showed that 4-IPC, when applied topically, was the most potent depigmenting agent of 34 different compounds tested (2). It was shown that 4-IPC caused marked reduction of the epidermal melanocyte population and selective degeneration of remaining melanocytes without affecting other epithelial cells. Bleehen later demonstrated that 4-IPC, when injected i.p., caused an apparent retardation of the growth of HP melanoma in mice (3), but did not clarify the mechanism of the selective effects of 4-IPC on normal melanocytes and malignant melanoma cells. Our present study, however, indicates that the susceptibility of the malignant melanoma cells is due to the interaction of 4-IPC and tyrosinase. Furthermore, our study suggests that the inhibition of *in vitro* leucine incorporation caused by 4-IPC is related to the intermediate compounds generated in an early stage of the enzymatic oxidation of 4-IPC, inasmuch as 4-IPC incompletely oxidized by mushroom tyrosinase greatly inhibited the leucine incorporation into liver cells, whereas 4-IPC completely oxidized by air oxidation did not.

The significance of tyrosinase-mediated inhibition of protein biosynthesis by 4-IPC may be at least 2-fold with respect to the chemotherapeutic approach to malignant melanoma: (*a*) 4-IPC may selectively inactivate the melanoma cells, thus decreasing the functioning population (both melanotic and amelanotic); (*b*) it may also inhibit the biosynthesis of tyrosinase, thus decreasing the population of tyrosinase-positive melanotic melanoma cells with a concomitant increase in the population of amelanotic melanoma cells. A recent *in vitro* culture study by Bleehen (4) showed that 4-IPC caused the disruption of the subcellular architecture of human and mouse melanoma cells, whereas it did not affect the fibroblasts in the same culture condition. *In vivo* studies by Bleehen *et al.* (2) and Jimbow *et al.* (9, 10) showed that both 4-IPC and hydroquinone affect the normal epidermal and follicular melanocytes similarly, but the extent of the affect by each chemical is entirely different. The hydroquinone-treated sites still possessed a number of active functioning melanocytes, whereas 4-IPC-treated sites had only inactive melanocytes that contained a few unmelanized melanosomes after completion of depigmentation. In their studies it was also shown that 4-IPC and hydroquinone caused not only a decrease in synthesis and melanization of melanosomes but also abnormal disintegration of the inner structure of melanosomes and the membranous organelles, resulting eventually in necrosis or inactivation of whole cells.

Riley *et al.* (15, 16) indicated in their recent tissue culture studies that the toxic action of 4-hydroxyanisole, and possibly other similar substituted phenols, on pigment cells initiates the damage to cell membranes and involves a free radical mechanism initiating lipid peroxidation through tyrosinase. Unexpectedly, in our study, 4-hydroxyanisole did not inhibit the leucine incorporation into melanoma tissues to the same extent as 4-IPC did, although both compounds possess a similar depigmenting potency and appear to be affected by the same enzyme. Whether the inhibitory process of *in vitro* leucine incorporation by 4-IPC into melanoma tissues is similar to that of 4-hydroxyanisole, however, remains to be studied. Although the inhibition of protein synthesis in the *in vitro* system may account for the effect of 4-IPC and 4-hydroxyanisole on the intact melanoma cells, it could well be an unrelated toxic effect of the catechols that are peculiar to our incorporation system of leucine. Further studies are also needed to determine whether 4-IPC cytotoxicity for malignant melanoma can be initiated by the other intracellular enzymes, *e.g.*, peroxidase, or can be affected by a tyrosinase inhibitor, and whether this compound will provide a new chemotherapeutic approach to human malignant melanoma (13).

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. Y. Fujinuma for his preparation and analysis of air oxidation product of 4-IPC and to Dr. H. Obata for her preparation of melanoma tissues.

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