Table 1. Concentrations of several elements in the serum of rats after treatment with MCTP

Days after treatment*	Rx	Elements (ppm)						
		Iron	Magnesium	Phosphorus	Zinc	Potassium	Sodium	Calcium
3	DMF	21 ± 7	31 ± 4	219 ± 35	2.3 ± 0.4	499 ± 112	3382 ± 612	116 ± 19
	MCTP	9 ± 1	24 ± 1	162 ± 4	1.7 ± 0.1	235 ± 14†	2631 ± 642	93 ± 4
5	DMF	7 ± 1	24 ± 1	153 ± 9	1.7 ± 0.1	202 ± 19	2587 ± 238	88 ± 4
	MCTP	8 ± 1	22 ± 1	144 ± 4	1.6 ± 0.1	188 ± 16	2661 ± 160	89 ± 2
8	DMF	7 ± 1	24 ± 1	152 ± 5	1.7 ± 0.1	184 ± 11	2474 ± 12	96 ± 1
	MCTP	10 ± 2	24 ± 2	142 ± 7	1.6 ± 0.2	231 ± 27	2395 ± 56	86 ± 2†
14	DMF	5 ± 1	20 ± 3	157 ± 4	1.6 ± 0.1	200‡	2861 ± 102	88 ± 2
	MCTP	7 ± 1	23 ± 1	150 ± 4	1.6 ± 0.1	277 ± 46	2806 ± 100	87 ± 2

* Rats received MCTP (3.5 mg/kg) or DMF vehicle i.v. on day 0 and were killed on day 3, 5, 8 or 14. Values represent mean ± SEM, N = 3–10.

† Significantly different from DMF on the same day (Student's *t*-test, $P < 0.05$).

‡ N = 2.

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Growth inhibition of melanoma cells by N-protected dopa derivatives

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Melanocytes possess a unique biochemical property, melanin synthesis [1]. The synthesis of melanin pigment from tyrosine is catalysed by tyrosinase (EC. 1.14.18.1) present in both normal and malignant melanocytes: tyrosine is hydroxylated to dopa and then oxidised to dopaquinone, and the latter is converted to melanin pigment in a complex series of spontaneous reactions [2].

Wick *et al.* [3] showed that dopa is selectively toxic to pigmented melanoma cells *in vitro*. Subsequently, Wick [4–6] showed that catecholic compounds related to dopa, e.g. dopa methyl ester and 3,4-dihydroxybenzylamine, possess significant antitumour effect against mouse and human melanomas *in vitro* and *in vivo*. Several attempts have been made to enhance the antimelanoma effect of these catechols [7–9], and new types of dopa derivatives have also been evaluated [10–12].

In this study, we examined the effects of *N*-protected dopa derivatives, *N*-acetyldopa and γ-glutamyldopa, on the growth of melanoma cells *in vitro* and *in vivo*.

Materials and methods

Catalase, superoxide dismutase (SOD), phenylthiourea (PTU), reduced glutathione (GSH) and L-dopa were purchased from Sigma Chemical Co. (St Louis, MO), and the

other chemicals were from Wako Pure Chemicals, Ltd. (Osaka, Japan).

N-Acetyldopa was prepared as follows. To a suspension of 9.9 g of L-dopa in 100 ml of water was added at room temperature under stirring 50 ml of acetic anhydride in 5 ml portions every 5 min. The mixture was stirred for an additional hour, and the resulting solution was evaporated to dryness at 40° in a rotary evaporator. The oily residue was dissolved in 50 ml of water, and to this was added 100 ml of 4 M NaOH containing 2% $Na_2S_2O_5$. The mixture was stirred under an argon atmosphere for 1 hr and was acidified to pH 2 by adding 6 M HCl. The mixture was extracted with ethyl acetate, washed with water, and the ethyl acetate extract was dried over Na_2SO_4. Evaporation afforded 7.5–8.9 g of *N*-acetyldopa as an oily solid. This preparation contained 1 mole of acetic acid and 1 mole of water per mol.

γ-Glutamyldopa was prepared according to the chemical method [13] and was recrystallised from water–acetone.

Human melanoma cell lines HMV-II (pigmented) and HMV-I (non-pigmented) were established by one of the authors [14]. 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; GIBCO, Chagrin Falls, OH), supplemented with 10% fetal bovine serum, penicillin and streptomycine in a 5% CO_2 atmosphere.

Dopa and the dopa derivatives were determined by our HPLC method [15] with minor modifications, and GSH was determined by our HPLC method [16].

Results and discussion

Figure 1 shows the effects of the *N*-protected dopa derivatives on the growth of pigmented melanoma cells HMV-II, non-pigmented melanoma cells HMV-I and HeLa cells. *N*-Acetyldopa exhibited a dose-dependent cytotoxicity to melanoma cell lines. Non-pigmented HMV-I was slightly more susceptible than pigmented HMV-I cell line. HeLa cells were least affected; even at a 4 mM concentration, the number of cells did not decrease in 72-hr incubation. γ-Glutamyldopa also showed a dose-dependent cytotoxicity to melanoma cells. Contrary to *N*-acetyldopa, pigmented HMV-II line was more susceptible than non-pigmented HMV-I line; a significant difference was seen with 1 mM drug which caused a growth inhibition in HMV-II but not in HMV-I line. γ-Glutamyldopa also exhibited a much weaker toxicity to HeLa cells than to melanoma cells.

The mechanism of toxicity on melanoma cells by the *N*-protected dopa derivatives was next studied. The effects of catalase, SOD and PTU on the cytotoxicity of *N*-acetyldopa and γ-glutamyldopa are presented in Table 1. Catalase and SOD are scavengers of active oxygen species, hydrogen peroxide and superoxide radical, respectively. PTU is an inhibitor of tyrosinase [11]. When incubated alone, these agents were almost non-toxic to melanoma cells.

Catalase protected both HMV-II and HMV-I lines from cytotoxicity of the drugs to major extents, although not completely. This result indicates that the cytotoxicity results mainly from hydrogen peroxide produced outside of the cells by autoxidation of the drugs. On the contrary, SOD did not protect the cells, but augmented the cytotoxicity. This unexpected result might be related to the acceleration of hydrogen peroxide production from superoxide radical by SOD. PTU protected pigmented HMV-II cells from cytotoxicity of the drugs to a major extent, while it did not protect non-pigmented HMV-I cells. These results suggest that in HMV-II cells the cytotoxicity is mediated by both hydrogen peroxide and tyrosinase, while in HMV-I cells it is mediated mostly by hydrogen peroxide.

The effect of 24-hr incubation with 2 mM *N*-acetyldopa on the level of cellular GSH was studied. It was found that the treatment almost completely (99%) depleted cellular GSH in both HMV-II and I lines. This result suggests that *N*-acetyldopa exerts its cytotoxicity through the production of hydrogen peroxide (and the *o*-quinone derivative) followed by the depletion of cellular GSH.

The uptake of the drugs by melanoma cells was studied with an HPLC method. Dopa and the dopa derivatives were rapidly taken up by melanoma cells (data not shown). HMV-II line exhibited 2 times higher uptake than HMV-I line at 7.5 min, but after 30 min no difference in the uptake was observed. The uptake of the dopa derivatives by both cell lines were approximately 20 times lower than that of dopa.

Table 2 shows the effects of the drugs on the life span of B16 melanoma-bearing mice. *N*-Acetyldopa at a dose of 1000 mg/kg slightly prolonged the life span of B16 melanoma-bearing mice in two separate experiments. Although the median survival times of the control animals were longer than the reported values, an example of slow-growing B16 melanoma has been reported [7]. γ-Glutamyldopa did not show any increase in survival time. The dopa derivatives were not toxic to mice, as judged by weight and mobility of the treated animals.

Table 1. Modification of toxicity of *N*-acetyldopa and γ-glutamyldopa to melanoma cells by other agents

	Growth inhibition (%)*			
	HMV-II		HMV-I	
Treatment (concn)	Agent only	Drug + agent	Agent only	Drug + agent
N-Acetyldopa (2 mM)		22 ± 1		28 ± 1
+ Catalase (100 μg/ml)†	2 ± 1	6 ± 4	0 ± 2	5 ± 3
+ SOD (100 μg/ml)	1 ± 2	78 ± 2	2 ± 1	65 ± 3
+ PTU (50 μM)	3 ± 2	8 ± 1	1 ± 2	29 ± 2
γ-Glutamyldopa (2 mM)		44 ± 7		17 ± 6
+ Catalase (100 μg/ml)†	2 ± 3	11 ± 2	3 ± 3	8 ± 3
+ SOD (100 μg/ml)	3 ± 3	76 ± 3	4 ± 1	59 ± 6
+ PTU (50 μM)	9 ± 1	16 ± 4	2 ± 1	15 ± 4

The experimental conditions were similar to those described in legend to Fig. 1. The cells were incubated with either regular medium or medium containing one of the dopa derivatives and/or the agent for 24 hr.

* [(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.

† Heat-denatured catalase had little effect in preventing the growth inhibition.

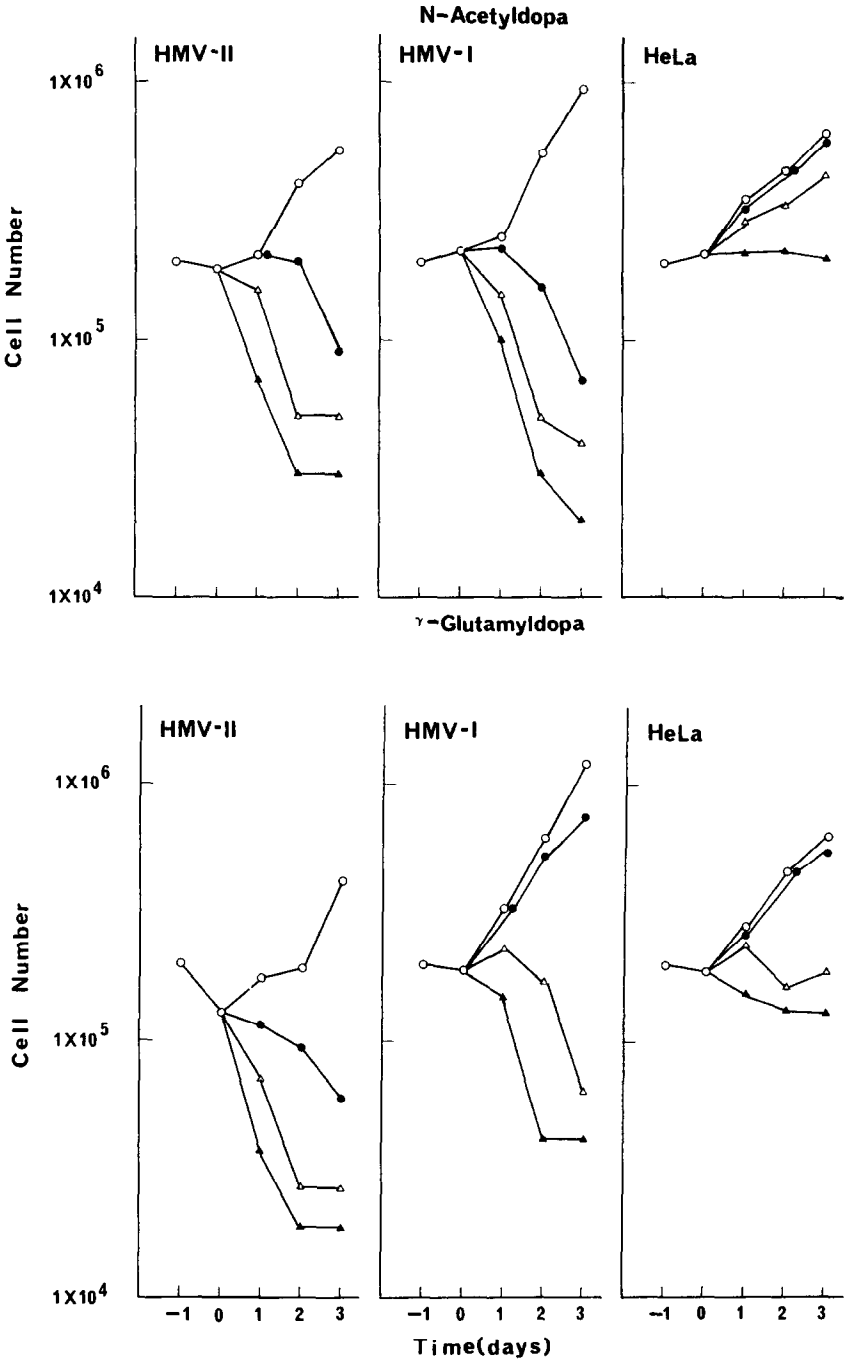

Fig. 1. Effects of *N*-acetyldopa and γ-glutamyldopa on the growth of cell lines, HMV-II, HMV-I, and HeLa. Single cell suspension (2×10^5) in regular medium were inoculated into 60-mm Falcon Petri dishes, and cells were allowed to attach for 24 hr prior to exposure to the drug. The cells were incubated continuously with either regular medium or medium containing *N*-acetyldopa or γ-glutamyldopa: ○, control; ●, 1 mM; △, 2 mM; ▲, 4 mM. After 24, 48, and 72 hr of incubation, cells were harvested by tripsinisation and counted in a Model Z Coulter Counter. Results are means of three separate experiments.

In this study, we hoped that the protection of the amino group in dopa might enhance the antitumour activity [17]. The results that the *N*-protected dopa derivatives showed barely significant effects *in vivo* may be ascribed to the low uptake by melanoma cells. It might also be possible that the dopa derivatives are rapidly metabolised and excreted before accumulated in melanoma tissues.

In summary, the *N*-protected dopa derivatives, *N*-acetyldopa and γ-glutamyldopa, exhibited dose-dependent cytotoxicity which is more pronounced in melanoma cells

Table 2. Effects of *N*-acetyldopa and γ-glutamyldopa on the life span of mice inoculated with B16 melanoma

Treatment (concn)	Survival time (days) Median	Range	%ILS*
Experiment 1			
Control	46	37–53	
N-Acetyldopa (500 mg/kg)	46	40–61	0
N-Acetyldopa (1000 mg/kg)	54	44–69	17 ($P < 0.01$)
γ-Glutamyldopa (500 mg/kg)	45	40–54	−2
γ-Glutamyldopa (1000 mg/kg)	47	35–54	2
Experiment 2			
Control	36	31–38	
N-Acetyldopa (1000 mg/kg)	40	32–45	11 ($P < 0.05$)

B16 melanoma cells were inoculated i.p. in young male C57BL/6 × DBA/2 F_1 mice, 10 animals per group, following the National Cancer Institute protocol [17]. Treatment of mice was started on day 1 and continued daily for 12 days. Drugs, dissolved in 1 ml of 0.9% NaCl, were adjusted to pH 7 with $NaHCO_3$ powder and were given i.p. once a day. Control animals received i.p. injection of 1 ml of 0.9% NaCl.

* Percentage of increase in the life span of treated versus control animals.

than in HeLa cells. The results obtained indicate that in pigmented melanoma cells the cytotoxicity is mediated by both hydrogen peroxide and tyrosinase, while in non-pigmented melanoma cells it is mediated mostly by hydrogen peroxide produced by autoxidation of the drugs.

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Drug effects on output of prostacyclin from isolated lungs

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Exogenous arachidonic acid (AA) is metabolized by lung tissue to a variety of biologically active cyclo-oxygenase products (COP) including prostaglandins (PG) and thromboxane (Tx). Synthesis of COP from endogenous AA esterified in lung lipids is stimulated by a variety of stimuli–chemical, physical or immunological [1]. In perfused isolated lungs, the active COP derived from either source of substrate which appear in effluent from lung are accompanied by inactive metabolites of these products, usually via the action of 15-hydroxy PG dehydrogenase (PGDH) [2–4]. Inactivation of PGs can also be demonstrated directly by perfusing PGE_2 or $PGF_{2\alpha}$ through the