0022-202X/ 79/0072-0002$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 72:67–69, 1979

Vol. 72, No. 2
Printed in U.S.A.

Selective Toxicity of 6-Hydroxydopa for Melanoma Cells

MICHAEL M. WICK, M.D., PH.D., LISKEN BYERS, B.A., AND JUDSON RATLIFF, B.A.

Divisions of Medical Oncology and Clinical Pharmacology, Sidney Farber Cancer Institute (LB, MMW and JR) and Department of Dermatology, Harvard Medical School, Boston, Massachusetts (MMW) U.S.A.

The growth inhibitory effect of 6-hydroxydopa, a cytotoxic analog of L-dopa, was studied in melanotic and amelanotic Cloudman melanoma, mouse fibroblast L929 and Chinese hamster ovary cells. A marked sensitivity of pigmented cells to 6-hydroxydopa was observed with a 10-fold increase in sensitivity of pigmented cells. Sensitivity correlated with the capacity of cells to incorporate radiolabeled exogenous L-dopa. The drug affected primarily DNA and RNA synthesis with greater inhibition observed in pigmented cells than the nonpigmented control cells. The mechanism of action may involve interaction with the melanocyte specific enzyme, tyrosinase, as a false substrate.

Melanocytic cells possess a unique biochemical apparatus for the conversion of L-dopa to the biopigment melanin [1]. Since this reaction is mediated by the enzyme tyrosinase which is restricted to normal and malignant melanocytes, a potential basis for a selective chemotherapeutic approach may exist. We have previously reported the efficient incorporation of exogenous L-dopa-^{3}H into pigmented S-91 Cloudman melanoma cells *in vitro* and *in vivo* [2,3]. L-dopa itself had been reported to be toxic to melanoma cells although no selective action [4,5] was noted until exposure times were reduced [6]. *In vivo* activity against the B-16 melanoma was observed when the more soluble ester of L-dopa was used [7].

Since L-dopa itself was cytotoxic at concentrations above 1000 mcg/ml, it was of interest to examine analogs with potentially increased potency. 6-Hydroxydopa was chosen as the prototype analog since it is known to be cytotoxic *in vivo* causing degeneration of adrenergic nerves [8,9]. Furthermore, it closely resembles L-dopa differing only by the addition of a hydroxyl group in the 6-position. Evidence of a stereochemical similarity is further supported by the results of Swan who has shown that 6-hydroxydopa is a substrate of the enzyme tyrosinase and is rapidly converted first to a deeply colored red intermediate and then to a melanin [10]. Recent results of Graham and Jeffs [11] have confirmed that 6-hydroxydopa is a substrate of the enzyme tyrosinase although it is probably not an intermediate in the conversion of L-dopa to melanin. Figure 1 summarizes the relationship of 6-hydroxydopa and L-dopa in the biosynthesis of melanin.

Evaluation of pharmacologic injury to cancer cells *in vitro* has been measured by a variety of techniques. In order to assess the relative sensitivity of melanoma cells to these agents, we examined the effects upon doubling time which in general are dose-dependent and correlate well with other methods [12,13]. We present the results of our study of the effect of 6-hydroxydopa upon the growth of melanotic clone of S-91 Cloudman melanoma as compared to the non-pigmented control cells, mouse fibroblast L929 and Chinese hamster ovary cells.

MATERIALS AND METHODS

Drugs

L-dopa and 6-hydroxydopa were obtained from the Sigma Chemical Co., St. Louis, Mo. All other materials were reagent grade and used without further purification.

Cell Lines

S-91A, a pigmented line, was obtained from the American Type Culture Collection (CCL 53.1), Rockville, Maryland. S-91B, a grossly amelanotic clone was a gift from Dr. Jewel Cobb, Conn. College, New London, Conn., and has been fully described [14]. Mouse fibroblast L929 and Chinese hamster ovary cells (CHO) were from Gibco, Grand Island, New York. All cell lines have been maintained in our laboratory in McCoy's 5A medium supplemented with 15% fetal calf serum and 100 units/ml streptomycin, and 100 μg/ml penicillin.

6-Hydroxydopa Toxicity

Single cell suspensions were inoculated into 60 mm Falcon Petri dishes and cells were allowed to attach for 24 hr prior to drug exposure. The 6-hydroxydopa was freshly prepared in cold 0.9% sodium chloride solution containing 2-3 drops of 0.1 N HCl and filtered (Millipore Corporation, Bedford, Mass.) before use. Plates were washed once with HBSS and 1 ml of HBSS containing 0.5, 1, 10, 25, and 50 μg of 6-hydroxydopa was added and cultures were incubated at 37°C. Cells were harvested by trypsinization with 0.25% trypsin-EDTA at 0, 24, 48 and 72 hr after exposure and counted in a Model Z Coulter Counter. Viability by trypan blue exclusion was about 90%. Results are expressed as % growth inhibition by comparison to parallel control cultures that were manipulated similarly except that they did not have drug added. Standard errors for triplicates were always less than 10%. Under the experimental conditions control cells had the following doubling times: S-91A, 22 hr, S-91B, 20 hr, L929, 24 hr and CHO, 16 hr.

L-dopa Incorporation

Experimental cultures were used 72 hr after plating, washed once with HBSS and 1 ml of HBSS containing 10^{-5}M L-dopa plus 2 μCi of tritiated L-dopa (s.a. 21 Ci/mmole, New England Nuclear, Boston, Mass.) added. Cells were incubated at 37°C in CO_2 humidified air for 30 or 60 min and medium removed. The monolayers were then washed 3 times with 0.9% sodium chloride solution and cells precipitated with 10% trichloroacetic acid for 30 min. The precipitate was washed twice with 0.9% sodium chloride solution, followed by addition of 1 ml of 1.0 N KOH and let stand at 4°C for 24 hr. An aliquot was added to scintillation fluid (Aquasol, New England Nuclear, Boston, Mass.) and counted in a Beckman LS 335 scintillation counter. Quenching was corrected by addition of an internal toluene standard. Values for incorporation represent mean ± SEM for 3 samples and are expressed as dpm/10^5 cells after 60 min incubation.

Macromolecule Biosynthesis

At the specified times following exposure to drug, 2 μCi/ml of either thymidine-methyl-^{3}H s.a. 2 Ci/mmole, uridine-5-^{3}H, s.a. 25 Ci/mmole or leucine-^{3}H, s.a. 41 Ci/mmole (New England Nuclear, Boston, Mass.) were added. After 60 min at 37°C medium was removed, cells were washed once with HBSS and 1 ml of 10% trichloroacetic acid added. The precipitate was washed 3 times with 0.9% sodium chloride solution

Manuscript received June 14, 1978; accepted for publication July 25, 1978.

Supported in part by National Cancer Institute Grant CA-06516. A portion of this work was presented at the Annual Meeting of the American Federation for Clinical Research, April, 1977.

Reprint requests to: Michael M. Wick, M.D., Department of Dermatology, Sidney Farber Cancer Institute, Harvard Medical School, Boston, Mass 02115.

Abbreviations:
CHO: Chinese hamster ovary cells
HBSS: Hanks Balanced Salt solution
6-hydroxydopa: L-2,4,5-trihydroxyphenylalanine
L-dopa: L-3,4-dihydroxyphenylalanine

and 0.5 ml of 1.0 N KOH added. Following digestion at 37°C for 4 hr, an aliquot was added to scintillation vials containing Aquasol (New England Nuclear, Boston, Mass.). Counting efficiency was about 30% and values are expressed as % of control for triplicate samples and represent the mean of 3 separate experiments. For examination of the immediate effects of 6-hydroxydopa upon precursor incorporation, drug and labeled material were added simultaneously and incubated for 30 min at 37°C and then processed as previously described.

In Vivo Antitumor Evaluation

6-Hydroxydopa was evaluated against the B-16 and S-91 melanomas *in vivo* according to standard National Cancer Institute protocols [15]. The method has been fully described and compares the survival of tumor-bearing mice as compared to nontreated controls [16].

RESULTS

6-Hydroxydopa Toxicity

Figure 2 represents the dose response curve for growth inhibition of melanoma cells 48 hr following treatment with 6-hydroxydopa. A selective inhibition of growth of the melanotic S-91A melanoma as compared to the nonpigmented S-91B and non-pigmented control cells is observed. Longer exposure times resulted in increased toxicity but decreased selectivity. It is apparent from these data that S-91A are at least 10-fold more sensitive to 6-hydroxydopa than nonpigmented control cells. Growth inhibition patterns were similar at 24 and 48 hr.

Table I compares the results of incorporation studies of radiolabeled L-dopa with sensitivity to 6-hydroxydopa. A correlation of the ability of the various cell lines to incorporate exogenous L-dopa with their respective sensitivity to 6-hydroxydopa is apparent. There is approximately a 10-fold increase in sensitivity of the pigmented S-91A as compared to the amelanotic S-91B and this corresponds to a 7-fold increase in radiolabeled L-dopa incorporation.

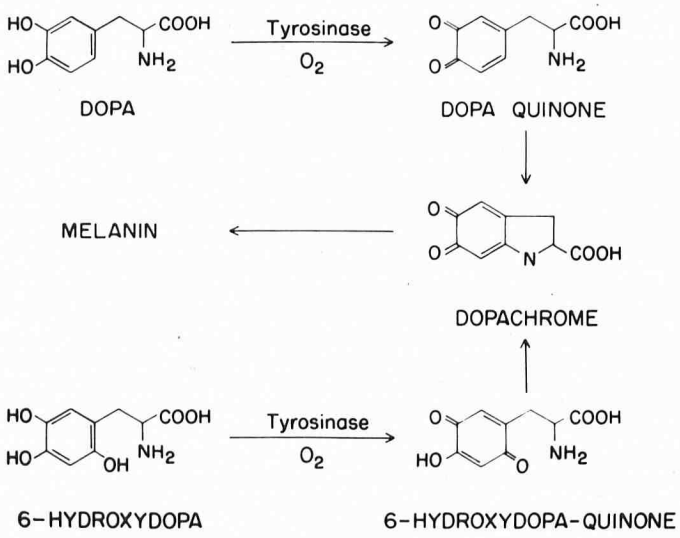

FIG 1. Biosynthetic scheme for the formation of dopachrome and melanin from L-dopa and 6-hydroxydopa by tyrosinase.

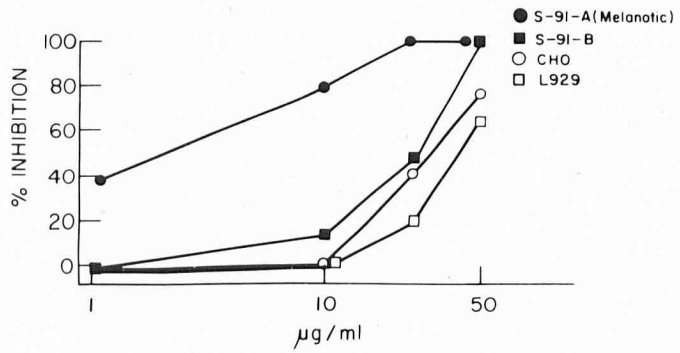

FIG 2. % Inhibition as a function of 6-hydroxydopa concentration. Values represent mean of triplicate samples 48 hr after drug exposure. Standard errors have been omitted for clarity but were less than ± 10%. Similar curves were obtained at 24 and 72 hr.

TABLE I. *Comparison of growth inhibition by 6-hydroxydopa with L-DOPA-^{3}H incorporation*

Cell type	L-dopa-^{3}H incorporation[a]	% Inhibition of growth[b]
S-91A	1531 ± 151	80%
S-91B	236 ± 21	18%
L929	80 ± 15	0%
CHO	92 ± 8	0%

[a] Values expressed as dpm/10^5 cells following a 90 min incubation. Represent mean ± SEM for triplicate samples.

[b] % Inhibition of growth of each cell line observed at 6-hydroxydopa, 10 μg/ml, 48 hr after exposure.

TABLE II. *Effect of 6-hydroxydopa upon thymidine, uridine and leucine incorporation by S-91A melanotic melanoma during and 4 hr following exposure*

	Concentration 6-hydroxydopa (μg/ml)	Immediate	4 hr
Thymidine[a]	10	13 ± 2	11 ± 5
	25	33 ± 1	80 ± 6
Uridine[a]	10	12 ± 2	22 ± 10
	25	29 ± 10	74 ± 10
Leucine[a]	10	5 ± 4	15 ± 13
	25	35 ± 3	33 ± 5

[a] Values represent mean ± SEM for 3 separate experiments and are expressed as a % inhibition as compared to control.

TABLE III. *Effect of 6-hydroxydopa upon thymidine incorporation 4 and 24 hr following exposure*

Cell line	Concentration	Thymidine	
	6-hydroxydopa (μg/ml)	4 hr	24 hr
S-91A	10	20 ± 5	17 ± 3
	25	80 ± 6	64 ± 11
S-91B	10	26 ± 10	3 ± 1
	25	61 ± 2	30 ± 5
L929	10	4 ± 5	5 ± 2
	25	13 ± 7	20 ± 4
CHO	10	22 ± 1	6 ± 2
	25	40 ± 7	19 ± 2

[a] Values represent mean ± SEM for 3 separate experiments and are expressed as % inhibition as compared to control.

Effects Upon Macromolecule Biosynthesis

Table II summarizes the effects of 6-hydroxydopa upon the pigmented melanoma cells during and 4 hr following exposure. It was apparent that at the concentrations examined, the degree of inhibition is a function of time following exposure to drug. There is almost no effect noted immediately but at 4 hr significant inhibition of thymidine and uridine incorporation is observed. The effect upon all 3 classes of macromolecule biosynthesis is consistent with a general disruption of cellular metabolism.

In order to confirm the selectivity observed in growth experiments, the effect of 6-hydroxydopa upon the non-pigmented control cells was also studied (Table III). Although inhibition of thymidine incorporation at 4 and 24 hr is observed it is less extensive in the nonmelanocytic control cells L929 and CHO with the amelanotic clone S-91B, which does have measurable tyrosinase activity (approximately 2% of that observed in the S-91A line) intermediate. These results confirm the observations made in the growth experiment regarding the relative sensitivity of each line to 6-hydroxydopa.

When tested against the B-16 and S-91 melanomas *in vivo*, no significant prolongation of survival of treated animals was

observed. The maximal tolerated dose for repeated injections was 75 mg/kg.

DISCUSSION

Melanocytic cells are unique in possessing the enzyme tyrosinase and a metabolic pathway for the conversion of L-dopa to melanin. Several agents have demonstrated selective toxicity against melanocytic cells *in vivo* and are in clinical use as depigmenting agents, e.g. hydroquinone and monobenzyl ether of hydroquinone [4,17]. Each has a quinone moiety as a common structural feature as does the prototype compound L-dopa itself.

In our studies, selectivity was apparent in both the effect upon the growth of pigmented as compared to nonpigmented control cells, and also in the corresponding greater inhibition of thymidine incorporation in pigmented cells. In contrast to L-dopa, a subsequent general cytotoxicity is supported by inhibitory effects observed upon incorporation precursors of each of the 3 types of macromolecules.

As compared to L-dopa, 6-hydroxydopa is approximately 100 times as potent, however, in addition to increased potency, there is also an increase in toxicity. It is this latter phenomenon that possibly accounts for the lack of *in vivo* antitumor acitivity. The toxicity is most likely neural, since 6-hydroxydopa is known to cause degeneration of adrenergic nerve endings, centrally and peripherally [18]. A possible method of limiting this toxicity might be to pretreat animals with inhibitors of catecholamine uptake such as imipramine since these agents are effective in limiting the neural effects of 6-hydroxydopa.

Although the mechanisms of action of L-dopa and 6-hydroxydopa are still undefined, toxicity appears to parallel the ability of the cells to incorporate L-dopa-^{3}H which in turn is dependent upon the presence of tyrosinase activity. Melanin exists as a complex polymer of 5,6-dihydroxyindole, which can undergo reversible oxidation reduction with formation of quinone-like free radicals [19,20]. 6-Hydroxydopa, a member of the class of compounds known as orthohydroquinones, can also form free radicals readily. It is possible that radicals are generated by the interaction of 6-hydroxydopa with melanin and/or tyrosinase, with consequent damage to cellular integrity.

Recently, Graham et al have discussed the mechanisms of cytotoxicity of melanin precursors [21]. They suggested that orthoquinones appear to operate as sulfhydryl group scavengers and thereby inhibit enzymes such as DNA polymerase. Our results with L-dopa and 6-hydroxydopa are in accord with their findings. 6-Hydroxydopa-quinone was shown to be a much less effective inhibitor of the polymerase than the catechol quinone which is consistent with our observations that L-dopa, its ester and dopamine, unlike 6-hydroxydopa cause a rapid inhibition of thymidine incorporation.

In summary, 6-hydroxydopa appears to be more potent than L-dopa as a melanocyte toxin, although unlike dihydroxy-derivatives of L-dopa, 6-hydroxydopa does not exhibit *in vivo* activity. Clinical evaluation of the therapeutic potential of this approach should focus, therefore, upon L-dopa and derivatives. We are presently evaluating the therapeutic potential of these compounds in humans.

We wish to thank M. Gorman for expert technical help and J. Walker for assistance in preparation of this manuscript.

REFERENCES

1. Luce JK, McBride CM, Frei III E: Cancer Medicine, Philadelphia, Pa, Lea and Febiger, 1974, pp. 1826–1828
2. Wick MM, Frei III E: Selective incorporation of L-3,4-dihydroxyphenylalanine by S-91 cloudman melanoma in vitro. Cancer Res 37:2123–2127, 1977
3. Wick MM, Kramer R, Gorman M: Effect of L-dopa decarboxylase inhibition upon uptake and distribution of L-dopa in melanoma in vivo. J Invest Dermatol 70:358–360, 1978
4. Pawelek J, Wong G, Sanson EM, Morowitz J: Molecular controls in mammalian pigmentation. Yale J Biol Med 46:430, 1973
5. Schatschabel D, Fisher R, Zilliken S: Untersuchenugen zur knotrolle der melanin synthesis in zellkulturen des harding passey melanomas. Hoppe Seyler's Physiol Chem 351:1402, 1970
6. Wick MM, Byers L, Frei III E: L-dopa: Selective toxicity for melanoma cells in vitro. Science 197:468–469, 1977
7. Wick MM: L-dopa methyl ester as a new antitumor agent. Nature 269:512–513, 1977
8. Berkowitz BA, Spector S, Brossi A, Focella A, Teital S: Preparation and biological properties of (−) and (+) 6-hydroxydopa. Experientia 26:982–983, 1970
9. Sachs C, Jonsson G: Degeneration of central and peripheral noradrenaline neurons produced by 6-hydroxydopa. J Neurochem 19:1561–1576, 1972
10. Chapman RF, Percival A, Swan GA: Studies related to the chemistry of melanin. XII. J Chem C:1664–1667, 1967
11. Graham DG, Jeffs PW: The role of 2,4,5-trihydroxyphenylalanine in melanoma biosynthesis. J Biol Chem 252:5729–5724, 1977
12. Foley GE, Lazarus H: The response in vitro of continuous cultures of human lymphoblasts (CCRF-CEM) cells to the chemotherapeutic agents. Biochem Pharmacol 16:659–664, 1967
13. Roper PR, Drewinko B: Comparison of in vitro methods to determine drug induced lethality. Cancer Res 36:2182–2188, 1976
14. Cobb JP, MacGrath A: S-91 mouse melanoma sublines following total in vitro versus in vivo passages. J Natl Cancer Institute 48: 885–871, 1977
15. Geran RI, Greenberg NH, MacDonald MM, Schumacker A, Abbott, BJ: Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep 25:1–66, 1962
16. Wick MM: Dopamine: A novel antitumor agent active against B-16 melanoma in vitro. J Invest Dermatol 71:163–164, 1978
17. Bleehan SS, Pathak MA, Hori Y, Fitzpatrick TB: Depigmentation of skin with 4-isopropyl catechol, mercaptoamines and other compounds. J Invest Dermatol 50:103–117, 1968
18. Sachs C, Jonsson G: Mechanism of action of 6-hydroxydopamine. Biochem Pharmacol 24:1–8, 1975
19. Blois MS: Observations of the semiquinone of benzoquinone. J Chem Phys 23:1351–1354, 1955
20. Commoner B, Townsend J, Pake G: Free radicals in biological materials. Nature 174:689–690, 1954
21. Graham DG, Tiffany SM, Vogel FS: Toxicity of melanin precursors. J Invest Dermatol 70:113–116, 1978