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Suppression of Tumor Cell Growth and Mitogen Response by Aporphine Alkaloids, Dicentrine, Glaucine, Corydine, and Apomorphine

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The aporphine alkaloids, dicentrine, glaucine, corydine, and apomorphine were shown to have inhibitory activity against several mouse tumor cell lines, leukemia P388 and L1210, melanoma B16, bladder cancer MBC2, and colon cancer Colon 26 in culture. These aporphine alkaloids also inhibited the mitogen-induced lymphocyte proliferation as well as the growth of IL-2 dependent CTLL2 line in a dose-dependent way. Of the four alkaloids apomorphine proved to be most potent in the inhibitory action. Apomorphine treatment resulted in some prolongation of survival time of the mice inoculated i.p. with P388, although its activity was not enough to meet the standard criterion for antitumor activity.

Keywords — inhibition; tumor cell growth; mitogen response; aporphine alkaloid; apomorphine; dicentrine; glaucine; corydine

Introduction

Aporphine alkaloids are found in many medical plants belonging to Annonaceae, Berberidaceae, Lauraceae, Magnoliaceae, Menispermaceae, Monimiaceae, Ranunculaceae, Papaveraceae, and Rhamnaceae.[1] Although a great number of aporphines have been isolated at the present time, their biological activities are rarely found in reports. Kupchan and Mollov *et al.* reported that thalicarpine (**1**), a dimeric benzylisoquinoline-aporphine alkaloid which was isolated from a root of *Stephania hernandifolia* (Menispermaceae), had significant inhibitory activity against Walker carcinosarcoma 256 in the rat[2,3] and KB cells in monolayer culture.[4] On the contrary, the aporphine alkaloids, glaucine (**2**), boldine (**3**), corydine (**5**), isocorydine (**6**), and bulbocapnine (**7**) appear to have no inhibitory activity against Walker 256 cells.[4] The fact suggested that the dimeric structure was needed for tumor-inhibitory activity.[5] However, the *in vitro* screening studies showed that there was no real correlation between the tumor-inhibitory activity and the molecular size.[6,7] We presented in this paper the inhibitory activity of apporphine alkaloids, dicentrine (**4**), glaucine (**2**), corydine (**5**), and apomorphine (**8**), against the various murine cell lines of P388 leukemia (P388), L1210 leukemia (L1210), B16 melanoma (B16), MBC bladder cancer (MBC2), colon adenocarcinoma C26 (Colon 26), and the suppressive activity to mitogen-induced proliferation of lymphocytes. In addition, antitumor activity of apomorphine was examined in mice.

Materials and Methods

Mice — Six week-old male BDF_1 mice were obtained from Japan SLC Co. (Hamamatsu, Shizuoka). Mice were maintained on water and routine mouse chow *ad libitum*.

Alkaloids — The aporphine alkaloids, dicentrine, glaucine and corydine were isolated from *Dicentra* and *Corydalis* species by the method of Asahina[8] and Kaneko *et al.*[9] The aporphine alkaloids were used after conversion to the hydrochlorides which were highly soluble in water. Apomorphine hydrochloride was prepared according to the literature.[10]

***In Vitro* Antiproliferative Activity** — a) [^{3}H]Thymidine([^{3}H]TdR) Uptake Method: P388, L1210, B16, or MBC2 cells were suspended at a concentration of 2.5×10^4 cells/ml in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-

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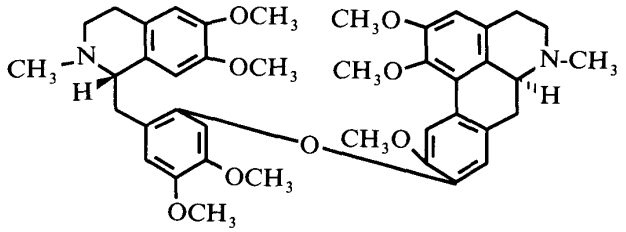

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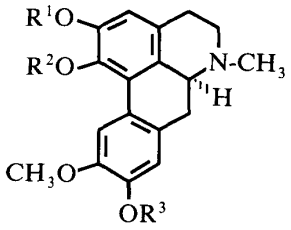

2: $R^1 = R^2 = R^3 = CH_3$
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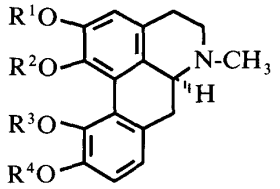
4: R^1, $R^2 = -CH_2-$, $R^3 = CH_3$



5: $R^1 = R^3 = CH_3$, $R^2 = H$
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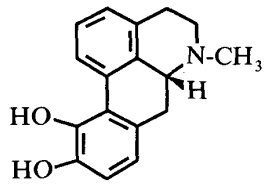
7: R^1, $R^2 = -CH_2-$, $R^3 = H$



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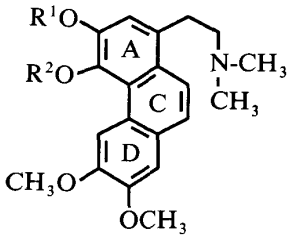

9: $R^1, R^2 = -CH_2-$
10: $R^1 = R^2 = CH_3$

inactivated fetal calf serum (General Scientific Laboratories, CA) 2 mM L-glutamine, 1 μM sodium pyruvate and 60 μg/ml kanamycin (culture medium). Aliquots (200 μl) of malignant cell preparations were delivered to each well in a 96-well flat bottom plate, followed by an addition of 10 μl of 0.01 M phosphate-buffered saline (pH 7.4, PBS) containing various concentrations of aporphine alkaloids. The plates were incubated for 24 h (P388 and L1210) or 72 h (B16 and MBC2) at 37 °C in a 5% CO_2 humidified incubator. Four hours prior to harvesting 0.5 μCi of [^{3}H]TdR (Amersham, 82 Ci/mmol) was added to each culture. Cells were harvested onto glass-fiber filters using a Skatron harvester (A. S., Lierbyen, Norway). The glass-fiber filters were then dried, placed in scintillation fluid and quantitated in a Beckman liquid scintillation spectrophotometer. The experiment was done in quadruplicate and the value was expressed as mean cpm ± S.E.

b) Cell Counting Method: P388, L1210, B16, MBC2, or Colon 26 cells (5×10^3 cells/ml) were suspended in culture medium and 2 ml each were delivered into a culture dish. After incubation for 5 h at 37 °C in a 5% CO_2 humidified incubator, various doses of the alkaloids in PBS or vehicle alone were added to culture dishes. Dishes were incubated for 72 h at 37 °C in a 5% CO_2 humidified incubator. The number of viable cells was counted using a hemacytometer.

Mitogen-Induced Proliferation — A single

cell suspension of spleens was prepared by gently mincing the splenic sacs with forceps and filtering through sterile nylon screens into cold PBS and then centrifuged at 200 × *g* for 5 min. Erythrocytes were lysed in Tris-buffered ammonium chloride solution (pH 7.0) during a 2 min incubation period at 37 °C. The leukocytes were washed three times and were brought to a concentration of 1.5×10^6 cells/ml in culture medium. Aliquots (200 μl) of spleen cell preparations were placed in 96-well microtiter plates and then were supplemented with 10 μl of concanavalin A (Con A type IV, 2 μg/ml, Sigma) or lipopolysaccharide (LPS, *E. coli* 055:B5, 40 μg/ml, Sigma), and with 10 μl of varing concentrations of aporphine alkaloids. The plates were allowed to incubate for 44 h at 37 °C in a 5% CO_2 humidified incubator. After incubation, 0.5 μCi of [^{3}H]TdR was added to each well and the cultures were allowed to incubate for an additional 4 h and the radioactivity incorporated was measured in a liquid scintillation spectrometer as described above.

Interleukin-2 (IL-2) Dependent CTLL2 Cell Proliferation — IL-2 dependent CTLL2 cells in exponential growth were treated with various doses of the alkaloids described above. After incubation with the alkaloids or vehicle for 20 h, CTLL2 cells were exposed to [^{3}H]TdR for 4 h and the radioactivity was counted in a liquid scintillation spectrometer.

Antitumor Activity of Apomorphine in Mice — P388 cells (10^6 cells) were inoculated intraperitoneally (i.p.) into BDF_1 mice and then apomorphine (1—100 mg/kg) was administered i.p. for 7 consecutive days from 1 d after the tumor transplantation. The mortality of mice was recorded for 2 weeks after the tumor inoculation. The improvement in survival was evaluated as follows.

$$T/C(\%) = \frac{\text{median survival days of treated mice}}{\text{median survival days of control mice}} \times 100$$

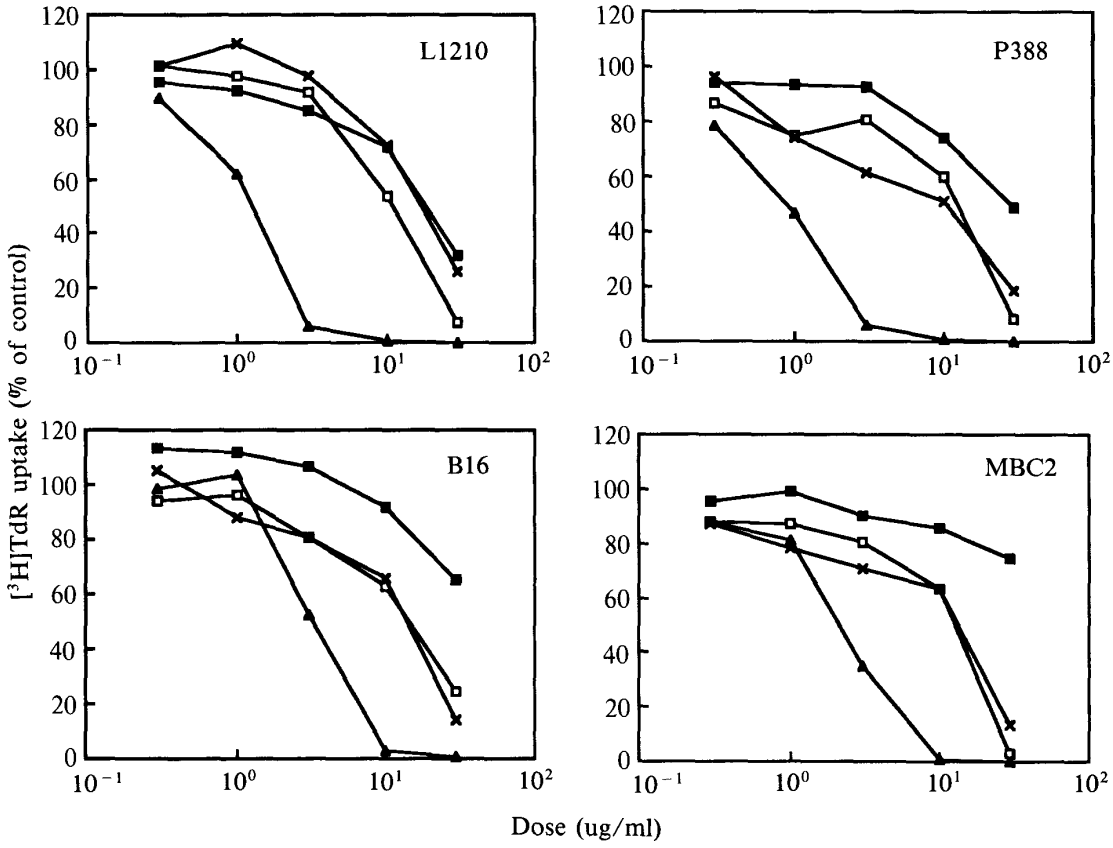

Fig. 1. Dose-Response Curve of Aporphine Alkaloids in the Inhibition of Tumor Cell Proliferation
Tumor cells were incubated with aporphine alkaloids, dicentrine (-□-), glaucine (-×-), corydine (-■-), or apomorphine (-▲-) for 24 or 72 h. Cell proliferation was assayed by the [^{3}H]TdR uptake method.

Results and Discussion

The present study evaluated the effects of four aporphine alkaloids on the growth of tumor cells and on the mitogen responses of mouse lymphocytes.

The antiproliferative effects of the aporphine alkaloids were examined using the different cell types of mouse tumor cell lines such as P388 and L1210, B16, MBC2, and Colon 26 and were assayed by the two methods, the [^{3}H]TdR-uptake method and the cell counting method. As shown in Fig. 1, dicentrine, glaucine, corydine, and apomorphine inhibited the [^{3}H]TdR incorporation into target tumor cells in a dose-dependent fashion. Table I shows the halfmaximally inhibitory concentrations (IC_{50}) of aporphine alkaloids obtained from Fig. 1 (Exp. 1) and that obtained from another experiment using the cell counting method (Exp. 2), in which Colon 26 was tested in addition to the four tumor cell lines used in Fig. 1. Both assay methods gave analogous IC_{50} values, indicating that the growth inhibition measured as the decrease of radioactivity incorporated into cells was not merely caused by the inhibition of [^{3}H]TdR transport. Apomorphine strongly inhibited the proliferation of all the five cell lines tested and its inhibitory activity was at least 4.3 times more potent than that of the other three aporphine alkaloids except for the growth inhibition of Colon 26 by glaucine.

Little is known concerning the chemical structure requirements in aporphine alkaloids for the antitumor activity. Wu *et al.* reported that a number of oxoaporphines showed significant activity against several malignant cell lines and removal of the oxo function markedly reduced their activity.[7] Furthermore, a Hofmann methine, dicentrine methine (**9**) exhibited a strong and wide range of inhibitory activity against A549, HCT8, KB, P388, and L1210 cells, while the activity of all other members including glaucine methine (**10**), were only marginal.[7] These facts suggest that the planarity of the A/C/D ring system may contribute to the activity. Regarding our results, each of four aporphine alkaloids revealed different efficacy against five cell lines tested in this study. The selectivity of action must depend upon the structural aspects of aporphines, but there is no evidence to draw a precise conclusion at the present time.

The effect of apomorphine on the i.p. P388 *in vivo* model was examined (Table II). Apomorphine treatment gave a dose-dependent prolongation of life time of mice, but it was not enough to meet the standard criterion ($T/C = 1.25$) for antitumor activity even at the highest dose ($T/C = 1.21$).

In vitro methods for generating and assaying proliferative responses of lymphocytes to mitogens are simple and are useful not only for primary screening of immunosuppressive agents, but also for analysis of toxic mechanisms at the cellular level. Effects of the aporphine alkaloids on the responses of spleen cells to T cell mito-

TABLE I. Antiproliferative Effects of Aporphine Alkaloids

Drug	IC_{50}(μg/ml)				
	P388	L1210	B16	MBC2	Colon 26
Exp. 1.					
Dicentrine	13.3	10.2	11.7	16.6	ND[a]
Glaucine	14.4	8.9	13.4	13.3	ND
Corydine	23.3	25.9	>30.0	>30.0	ND
Apomorphine	0.8	1.2	2.4	2.4	ND
Exp. 2.					
Dicentrine	22.5	13.8	14.3	18.0	14.0
Glaucine	11.5	11.7	>30.0	ND	0.7
Corydine	>30.0	>30.0	>30.0	>30.0	8.4
Apomorphine	2.7	1.2	2.7	1.9	1.3

Cell proliferation was assayed by the [^{3}H]TdR uptake method (Exp. 1) and by the cell counting method (Exp. 2). Antiproliferative activities are expressed as the drug concentration that resulted in 50% of the proliferation in control cultures. *a)* Not done.

TABLE II. Antitumor Activity of Apomorphine in Mice

Dose (mg/kg)	Survival time (d)	*T/C* (%)
None	7.6 ± 0.5	100.0
1	8.2 ± 0.4	107.9
10	8.6 ± 0.5	113.2
100	9.2 ± 1.5	121.1

P388 leukemia cells (10^6 cells) were inoculated i.p. with apomorphine for the 7 consecutive days from 1 d after the tumor inoculation.

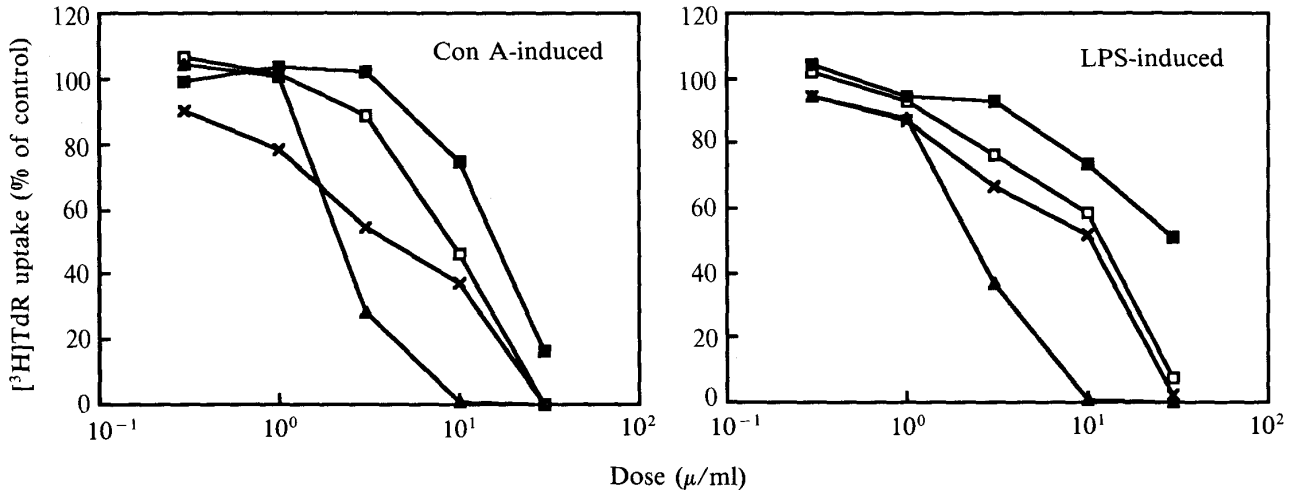

Fig. 2. Dose-Response Curve of Aporphine Alkaloids in the Inhibition of Lymphocyte Responses to T Cell and B Cell Mitogens

Spleen cells from CDF_1 mice were cultured with aporphine alkaloids in presence of Con A or LPS. Cell proliferation was assayed by the [^{3}H]TdR uptake method.

gen Con A and to B cell mitogen LPS were examined. The aporphine alkaloids suppressed the lymphocyte cell proliferations induced by Con A and LPS in a dose-dependent manner as shown in Fig. 2. Apomorphine was most potent in suppression of both the Con A and LPS responses in the same manner as presented in the antiproliferative activity to tumor cells. It is well known that T lymphocytes stimulated with mitogens such as Con A finally proliferate under IL-2 released by themselves. Aporphine alkaloids were tested for inhibitory effect on this step. The aporphine alkaloids were also found to inhibit the growth of CTLL2 cells in response to IL-2 (Table III). Apomorphine revealed a strong inhibitory action ($IC_{50}=0.5$ μg/ml). These results indicate that aporphine alkaloids inhibit the proliferation of normal cells as well as malignant cells, and the lymphocyte proliferation step in the course of Con A response is at least one site which is attacked by the alkaloids.

Many agents with antiproliferative activity have been reported to suppress immunofunction, and some of them such as azathiopurine and cyclophosphamide have been clinically used for immunosuppressive therapy. Therefore, it is interesting to know that apomorphine alkaloids modulate immunoresponse in the *in vivo* model system. It also remains to be elucidated at the molecular level how the alkaloids exert their action on proliferating cells.

TABLE III. Effects of Aporphine Alkaloids on the Mitogen Responses of Spleen Cells and on the Cell Proliferation of IL2-Dependent CTLL2 Line

Drug	Mitogen response (IC_{50}, μg/ml)		CTLL2 growth
	Con A-induced	LPS-induced	
Dicentrine	9.2	12.2	29.5
Glaucine	4.2	10.4	25.5
Corydine	15.7	>30.0	10.5
Apomorphine	2.2	2.0	0.5

Antiproliferative effects are expressed as the drug concentration that resulted in 50% of the [^{3}H]TdR uptake in control cultures.

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