In Vitro Cytotoxicity of the Protoberberine-Type Alkaloids

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In vitro cytotoxic activities of 24 quaternary protoberberine alkaloids related to berberine have been evaluated using a human cancer cell line panel coupled with a drug sensitivity database. Extending the alkyl chain at position 8 or 13 strongly influenced the cytotoxic activity, that is, relative lipophilicity as well as the size of the substituent affects cytotoxicity. The highest level of activity was observed in 8- or 13-hexyl-substituted derivatives of berberine. Structure−activity relationships are described.

The protoberberine alkaloids, such as berberine (**1**), are among the most widely distributed alkaloids of the isoquinoline series and are reported to exhibit several types of biological activities.[1,2] Berberine analogues modified at the 13 and/or 9 position, as well as 9-*O*-demethylated derivatives, have been prepared for investigation of their potential antitumor or antimicrobial activity, and some results on structure−activity relationships have been reported.[3,4] We have also reported antibacterial and antimalarial activities of berberine-related alkaloids, mainly C-8- or C-13-substituted derivatives.[5−10] We now report the cytotoxic activities of these alkaloids against various human cancer cell lines.

Three natural and 21 semisynthetic protoberberine alkaloids were selected to evaluate the impact of lipophilic substituents at various positions as well as variations in the alkoxyl groups naturally occurring on the A and D rings. The results of the studies on the structure−activity relationships of the tested alkaloids are discussed herein.

Results and Discussion

Cytotoxicity of the 24 protoberberines (**1**−**24**) was evaluated against a panel of 38 human cancer cell lines (seven lung, six colon, six CNS, six stomach, five ovarian, five breast, two renal, and one melanoma), and growth inhibition was represented by the mean graph produced by computer processing of the GI_{50} values, as described in the Experimental Section. The log GI_{50} for each cell line is indicated in the mean graph. The mean graph pattern of any compound with effective growth inhibition was compared with those of standard agents using COMPARE analysis,[11,12] which reveals the presence or absence of a correlation with standard agents based on similar patterns in the mean graphs of drugs with common modes of action. The growth inhibition of the protoberberines against a panel of 38 human cancer cell lines is presented in Table 1. Compounds **5**, **6**, **8**−**10**, and **20** were cytotoxic. Compounds **6** and **10** had the highest levels of cytotoxicity, with GI_{50} values of 0.83 and 0.41 μM, respectively, while compounds **5**, **8**, **9**, and **20** were less cytotoxic, with GI_{50} < 2.69 μM.

Table 1. Growth Inhibition of the Protoberberines against a Panel of 38 Human Cancer Cell Lines

compound	MG-MID[a] (μM)[e]	delta[b]	range[c]	γ[d]
1	−4.69 (20.4)	0.72	1.41	
2	−4.75 (17.8)	1.00	1.74	
3	−4.93 (11.7)	1.06	1.78	
4	−5.14 (7.24)	0.77	1.48	
5	−5.57 (2.69)	1.14	2.02	0.722−0.769
6	−6.08 (0.83)	0.53	1.35	
7	>−4.00 (100)	ND[f]	ND[f]	
8	−5.76 (1.74)	0.70	1.40	0.675−0.749
9	−5.88 (1.32)	0.76	1.42	0.653−0.755
10	−6.39 (0.41)	0.40	1.17	0.677−0.742
11	−4.51 (30.9)	0.43	0.68	
12	−4.23 (58.9)	0.97	1.20	
13	−4.94 (11.5)	0.69	1.63	
14	−5.17 (6.76)	1.16	1.90	
15	−4.09 (81.3)	0.49	0.57	
16	−4.14 (72.4)	0.75	0.89	
17	−4.25 (56.2)	0.52	0.77	
18	−4.46 (34.7)	0.47	0.94	
19	−4.88 (13.2)	0.77	1.33	
20	−5.62 (2.40)	0.81	1.43	0.594−0.611
21	−4.55 (28.2)	0.83	1.37	
22	−4.22 (60.3)	0.93	1.15	
23	−4.15 (70.8)	0.56	0.71	
24	−4.22 (60.3)	0.98	1.19	
mitomycin-C	−5.73 (1.86)			
adriamycin	−7.18 (0.066)			

[a] The mean of log GI_{50} values for 38 cell lines. [b] Difference between the MG-MID and the log GI_{50} of the most sensitive cell line. [c] Difference between the log GI_{50} of the most resistant cell line and the log GI_{50} of the most sensitive one. [d] Compare marginal ($0.5 \le \gamma_{MAX} < 0.75$), compare positive ($0.75 \le \gamma_{MAX}$). [e] GI_{50} in μM is presented in parentheses. [f] ND: not determined.

From a structure−activity point of view, some trends were observed.

For berberine (**1**) and palmatine (**15**) derivatives (**2**−**6** and **16**−**20**) bearing a 13-alkyl side chain, the cytotoxicity increased as the number of CH_2 units in the side chain lengthened, thus suggesting that extension of the alkyl function, and therefore relative lipophilicity of the C-13 substituent, may contribute to the activity.

12-Bromo-8-hexylberberine (**10**) was more cytotoxic than the corresponding 8-phenyl and 8-butyl derivatives (**8** and **9**), suggesting that length of the carbon unit at C-8 also

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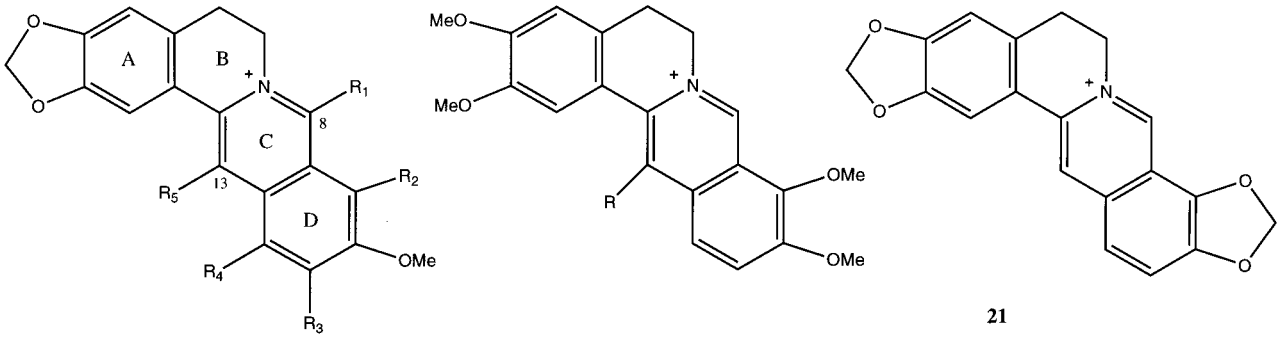

	R_1	R_2	R_3	R_4	R_5
1	H	OMe	H	H	H
2	H	OMe	H	H	Me
3	H	OMe	H	H	Et
4	H	OMe	H	H	n-Pr
5	H	OMe	H	H	n-Bu
6	H	OMe	H	H	n-He
7	H	OMe	H	H	OH
8	Ph	OMe	H	Br	H
9	n-Bu	OMe	H	Br	H
10	n-He	OMe	H	Br	H
11	H	OH	H	H	H
12	H	OH	H	H	Me
13	H	OEt	H	H	H
14	H	OEt	H	H	Et
22	H	H	OMe	H	H

	R
15	H
16	Me
17	Et
18	n-Pr
19	n-Bu
20	n-He

21

	R
23	H
24	Me

Figure 1. Structure of the protoberberines.

Table 2. Mean GI_{50} (μM) of 13-Substituted Protoberberines among the Several Strains in Some Cancer Cells

human cell (number of strains)	compounds/cytotoxicity (GI_{50} in μM)							
	5	**6**	**8**	**9**	**10**	**20**	mitomycin-C	adriamycin
BrBr[a] (5)	6.31	0.76	1.0	0.52	0.31	1.05	1.82	0.034
CNS[b] (6)	3.71	1.20	2.0	1.91	0.41	2.95	1.69	0.064
Co[c] (6)	2.51	0.72	1.55	1.11	0.44	1.82	2.55	0.18
Lu[d] (6 or 7)	1.78	0.49	2.19	1.54	0.41	2.14	0.40	0.023
Ov[e] (5)	5.25	1.17	2.45	1.38	0.48	2.88	4.35	0.12
St[f] (6)	3.9	0.76	1.91	1.29	0.34	3.47	5.82	0.10

[a] Breast cancer. [b] Brain cancer. [c] Colon cancer. [d] Lung cancer. [e] Ovarian cancer. [f] Stomach cancer.

influences the cytotoxicity. Bromination at the C-12 increased the cytotoxicity (compare **10** with **6**).

Replacing a 9-methoxy group with a 9-ethoxy group increased the cytotoxicity (compare **13** with **1** or **14** with **3**), while replacement of a methoxy group by a hydroxy group decreased cytotoxicity (compare **11** with **1** or **12** with **2**). These findings suggest that the bulk and/or relative lipophilicity of the C-9 substituent may contribute to the cytotoxicity.

Replacement of the dimethoxy groups of ring A with a methylenedioxy group increased the cytotoxicity (compare **1**−**6** with **15**−**20**). Replacement of both dimethoxy groups of the rings A and D with methylenedioxy groups also increased the cytotoxic activity (compare **21** with **15**). Shifting methoxy groups at C-9 and C-10 (e.g., **1**) to C-10 and C-11 (e.g., **22**) caused a decrease in cytotoxicity. These observations suggest that type and position of the oxygenated groups on rings A and D influence the activity.

Introduction of a hydroxy group at C-13 reduced the cytotoxicity (compare **7** with **1**), while compounds bearing a hydroxy group at C-9 (**11** and **12**) were less cytotoxic compared to those with a C-9 alkoxy group (see above). It should also be noted, however, that compounds **7**, **11**, and **12** adopt the phenolbetaine form (**7**, $R_4 = O^-$; **11** and **12**, $R_2 = O^-$), which could also account for reduced activity.

The protoberberinium salts were more cytotoxic than the quaternary dihydroprotoberberine derivatives (compare **1** with **23** or **2** with **24**). This result suggests that the presence of an iminium structure in the protoberberinium salts enhances activity, as shown by the observation that **23** and **24**, which do not have iminium ion structures, are less active. The correlation between iminium structure and biological activity has been noted by others as well.[13]

The mean GI_{50} for the individual cancer cell line is shown in Table 2. 13-Alkyl-substituted berberine derivatives (**5** and **6**) seem to be more effective against lung cancer, while the 8-substituted berberine analogues (**8** and **9**) and the corresponding derivative of palmatine (**20**) appear to be more selective against the breast cancer cell line. 12-Bromo-13-hexylberberine (**10**) showed potent (GI_{50} 0.41 μM) and wide spectrum activity against all cancer cells tested (Tables 1 and 2).

The known agents with the mean graphs resembling those of the active protoberberine analogues (**5**, **6**, **8**−**10**, and **20**) are shown in Table 3. COMPARE analyses of the mean graph revealed that the differential growth inhibition pattern of protoberberinium salts (**5**, **6**, and **8**−**10**) correlated with those of tetraphenylphosphonium chloride and rhodamine, which target mitochondria. In contrast, the palmatine-related alkaloid **20** correlated with the pattern for vinca alkaloids, which are tubulin inhibitors. Further investigations are needed to investigate the interaction of these compounds with tubulin and mitochondria.

On the basis of these results, it appears that several structural features of the protoberberines, such as the type of quaternary structures, the size of substituents at the C-8, C-9, and C-13 positions, and the type of the oxygenated substituents on rings A, C, and D, influence the cytotoxic

Table 3. COMPARE Analysis of the Protoberberinium Salts (**5**, **6**, **8**−**10**, and **20**)

compound	ranking order	agents	γ^a
5	1	tetraphenylphosphonium chloride	0.825
	2	tetraphenylphosphonium chloride	0.797
	3	rhodamine 123	0.783
6	1	tetraphenylphosphonium chloride	0.769
	2	tetraphenylphosphonium chloride	0.744
	3	rhodamine 123	0.722
8	1	tetraphenylphosphonium chloride	0.730
	2	tetraphenylphosphonium chloride	0.676
	3	rhodamine 123	0.674
9	1	tetraphenylphosphonium chloride	0.755
	2	tetraphenylphosphonium chloride	0.672
	3	dequalinium chloride	0.653
10	1	tetraphenylphosphonium chloride	0.742
	2	rhodamine 123	0.686
	3	rhodamine 123	0.677
20	1	vindesine	0.611
	2	vincristine	0.60
	3	vincristine	0.594

[a] Peason correlation coefficient.

activity. Extending the alkyl side chain at position 8 or 13 strongly increased the cytotoxic activity, suggesting that relative lipophilicity as well as the size of the substituent influences cytotoxicity. The combination of a hexyl substitution and bromination of the aromatic ring provided the most cytotoxic analogue (**10**). Among the potent protoberberinium salts, the activity decreased in the order **10** > **6** > **8**, **9** > **5**, **20**. Berberine-related alkaloids (**5**, **6**, and **8**−**10**) may target mitochondria. The mode of action will be probed in future studies.

Experimental Section

Test Compounds. Berberine **1** and palmatine **15** were obtained commercially (Alps-Pharmaceutical IND. CO., LTD). 13-Alkyl-substituted protoberberines **2**−**6** and **16**−**20**,[5] 13-hydroxyberberine **7**,[6] 8,12-substituted protoberberines (**8**−**10**),[7] 2,3,10,11-oxygenated protoberberine **22**,[6] and dihydroberberine *N*-metho salts (**23** and **24**)[14] were previously prepared. Coptisine **21** was obtained by oxidation of tetrahydrocoptisine isolated from *Chelidonium majus*.[15]

Cell Lines. Human breast cancer cell line MDA-MB-231 and leukemia cell line HL-60 were purchased from the American Type Culture Collection (Rockville, MD). Murine leukemia L 1210 and the following human cancer cell lines[16] were generously provided by the National Cancer Institute (Frederick, MD): lung cancer, NCI-H23, NCI-H226, NCI-H522, NCI-H460, DMS273, DMS114, and A549; colon cancer, HCC-2998, KM-12, HT-29, WiDr, HCT-15, and HCT-116; ovarian cancer, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; breast cancer, MCF-7; renal cancer, RXF-631 L and ACHN; melanoma, LOX-IMVI; and brain tumor, U251, SF-295, SF-539, SF-268, SNB-75, and SNB-78. Human stomach cancer, MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, and St-4, and human breast cancer, BSY-1, HBC-4, and HBC-5, were obtained as described elsewhere.[17,18] The cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum and penicillin (100 mg/mL) at 37 °C in humidified air containing 5% CO_2.

A Human Cancer Cell Line Panel and the Database. To evaluate drugs for the cell growth inhibition profile, a human cancer cell line panel combined with a database was established, modeled after the method of the National Cancer Institute.[11,12,19,20] Using this system, the antiproliferative effect of more than 200 standard compounds was examined and a new database was established.

Measurements of Cell Growth Inhibition and Data Analysis. The details of measuring cell growth inhibition are described elsewhere.[19,21] Briefly, the cells were plated at proper density in 96-well plates in RPMI 1640 with 5% fetal bovine serum and allowed to attach overnight. The cells were exposed to drugs for 48 h, then cell growth was determined according to the sulforhodamine B assay described by Skehan et al.[22]

Data calculations were made according to the method described previously.[19] Absorbance for the control well (C) and the test well (T) were measured at 525 nm. Moreover, at time 0 (addition of drugs), absorbance for the test well (T_0) was also measured. Using these measurements, cell growth inhibition (% of growth) by each concentration of drug was calculated as % growth $= 100 \times [(T - T_0)/(C - T_0)]$, when $T > T_0$, and % growth $= 100 \times [(T - T_0)/T]$, when $T < T_0$.

The GI_{50} was calculated as $100 \times [(T - T_0/C - T_0)] = 50$. The mean graph, which shows the differential growth inhibition of the drug in the cell line panel, was drawn based on a calculation using a set of GI_{50} values.[11,12] To analyze the correlation between the mean graphs of drug A and drug B, the COMPARE computer algorithm was developed according to the method described by Paull et al.[12] Peason correlation coefficients were calculated using the following formula: $r = (\sum(x_i - x_m)(y_i - y_m)/\sum(x_i - x_m)^2\sum(y_i - y_m)^2)^{1/2}$, where x_i and y_i are log GI_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

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References and Notes

(1) Preininger, V. In *The Alkaloids*; Brossi, A., Ed.; Academic Press: New York, 1986; Vol. 29, Chapter 1, pp 1−98.
(2) Bhukuni, D. S.; Jain, S. In *The Alkaloids*; Brossi, A., Ed.; Academic Press: New York, 1986; Vol. 28, Chapter 2, pp 95−181.
(3) Suffness, M.; Cordell, A. G. In *The Alkaloids*; Brossi, A. Ed.; Academic Press: New York, 1985; Vol. 25, Chapter 1, pp 188−198.
(4) Hong, S. W.; Kim, S. H.; J J,. A.; Lee, S. J.; Kim, S. U.; Kim, J. H. *Planta Med*. **2000**, *66*, 361−363.
(5) Iwasa, K.; Kamigauchi, M.; Sugiura, M.; Nanba, H. *Planta Med*. **1977**, *63*, 196−198.
(6) Iwasa, K.; Nanba, H.; Lee, D.-U.; Kang, S.-I. *Planta Med*. **1998**, *64*, 1−4.
(7) Iwasa, K.; Lee D.-U.; Kang, S.-I.; Wiegrebe, W. *J. Nat. Prod*. **1988**, *61*, 1150−1153.
(8) Iwasa, K.; Kamigauchi, M.; Ueki M.; Taniguchi, M. *Eur. J. Med. Chem*. **1996**, *31*, 469−478.
(9) Iwasa, K.; Kim, H.-S.; Wataya, Y.; Lee, D.-U. *Eur. J. Med. Chem*. **1988**, *33*, 65−69.
(10) Iwasa, K.; Nishiyama, Y.; Ichimaru, M.; Moriyasu, M.; Kim, H.-S.; Wataya, Y.; Yamori, T.; Turuo, T.; Lee, D.-U. *Eur. J. Med. Chem*. **1999**, *34*, 1077−1083.
(11) Boyd, M. R. In *Cancer: Principles and Practice of Oncology Update*; Devita, V. T., Hellman, S., Jr., Rosenberg, S. A., Eds.; Philadelphia: Lippincott, 1989; Vol. 3, pp 1−12.
(12) Paull, K. D.; Shoemaker, R. H.; Hodes, L.; Monks, A.; Scudiero, D. A.; Rubinstein, L.; Plowman, J.; Boyd, M. R. *J. Natl. Cancer Inst*. **1989**, *81*, 1088−1092.
(13) Caolo, M. A.; Stermitz, F. R. *Heterocycles* **1979**, *12*, 11−15.
(14) Onda, M.; Yuasa, K.; Okada, J.; Kataoka, K.; Abe, K. *Chem. Pharm. Bull*. **1973**, *21*, 1333−1337.
(15) Tani, T.; Takao, N.; Takao, S. *Yakugaku Zasshi* **1961**, *82*, 748−750.
(16) Stinson, S. F.; Alley, M. C.; Kop, p W. C.; Fiebig, H. H.; Mullendore, L. A.; Pittman, A. F.; Kenney, S.; Keller, J.; Boyd, M. R. *Anticancer Res*. **1992**, *12*, 1035−1053.
(17) Satoh, A.; Takayama, E.; Kojima, K.; Ogawa, H.; Yamori, T.; Sato, S.; Kawaguchi, T.; Tsuruo, T.; Yoshimoto, K.; Kine, T.; Matsumoto, I. *J. Biochem*. **1996**, *119*, 346−353.
(18) Motoyama, T.; Hojo, H.; Watanabe, H. *Acta Pathol. Jpn*. **1986**, *36*, 65−83.
(19) Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrichi, M.; Campbell, H.; Mayo, J. Boyd, M. *J. Natl. Cancer Inst*. **1991**, *83*, 757−766.
(20) Yamori, T.; Matsunaga, A.; Sato, S.; Yamazaki, K.; Komi, A.; Ishizu, K.; Mita, I.; Edatsugi, H.; Matsuba, Y.; Takezawa, K.; Nakanishi, O.; Kohno, H.; Nakajima, Y.; Komatsu, H.; Andoh, T.; Tsuruo, T. *Cancer Res*. **1999**, *59*, 4042−4049.
(21) Yamori T.; Sato S.; Chikazawa H.; Kadota T. *Jpn. J. Cancer Res*. **1997**, *88*, 1205−1210.
(22) Skehan P.; Storeng R.; Scudiero D.; Monks A.; McMahon J.; Vistica D.; Warren J. T.; Bokesch H.; Kenney S.; Boyd M. R. *J. Natl. Cancer Inst*. **1990**, *82*, 1107−1112.

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