Amaryllidaceae Alkaloids Belonging to Different Structural Subgroups Display Activity against Apoptosis-Resistant Cancer Cells†

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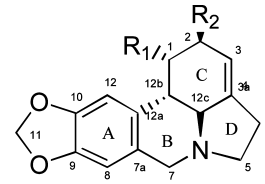
Received December 18, 2009

Fifteen Amaryllidaceae alkaloids (**1**−**15**) were evaluated for their antiproliferative activities against six distinct cancer cell lines. Several of these natural products were found to have low micromolar antiproliferative potencies. The log *P* values of these compounds did not influence their observed activity. When active, the compounds displayed cytostatic, not cytotoxic activity, with the exception of pseudolycorine (**3**), which exhibited cytotoxic profiles. The active compounds showed similar efficacies toward cancer cells irrespective of whether the cell lines were responsive or resistant to proapoptotic stimuli. Altogether, the data from the present study revealed that lycorine (**1**), amarbellisine (**6**), haemanthamine (**14**), and haemanthidine (**15**) are potentially useful chemical scaffolds to generate further compounds to combat cancers associated with poor prognoses, especially those naturally resistant to apoptosis, such as glioblastoma, melanoma, non-small-cell lung, and metastatic cancers.

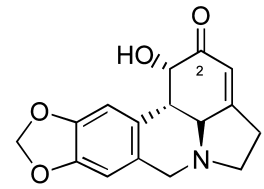
Among various organisms that have been investigated in the search for small-molecule constituents with potential use in cancer treatment, plants of the family Amaryllidaceae have been particularly fruitful.[1,2] The therapeutic property of these plants was known already in the fourth century B.C.E., when Hippocrates of Cos used the oil from the daffodil, *Narcissus poeticus* L., for the treatment of uterine tumors.[2]

In more recent times, more than 100 structurally diverse alkaloids, possessing a wide spectrum of biological activities, have been isolated from various Amaryllidaceae species.[3] Lycorine (**1**) was the first member of this family to be isolated in 1877.[2] Other examples of natural and synthetically derived compounds based on the pyrrolo[*de*]phenanthridine scaffold include amarbellisine (**6**) caranine (**2**), galanthine (**4**), pseudolycorine (**3**), norpluvine (**5**), and ungeremine (**7**). Another large alkaloid group from Amaryllidaceae is referred to as the lycorenine-type and is based on the [2]benzopyrano[3,4-*g*]indole skeleton, to which nobilisitine B (**8**) and clivonine (**9**, shown in hydrochloride form) belong, while tazettine (**10**) is the prototype representative of the pretazettine group. Alkaloids incorporating the 5,10β-ethanophenanthridine skeleton belong to the crinine-type of compounds and include, for example, ambelline (**11**), buphanamine (**12**), buphanisine (**13**), haemanthamine (**14**), and haemanthidine (**15**).

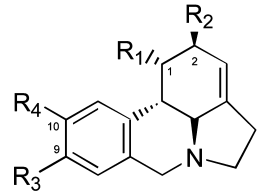
Many of the Amaryllidaceae alkaloids exhibit antiproliferative properties,[2] and it has been proposed that these compounds are active in this regard by disrupting eukaryotic protein biosynthesis.[4−6] Close analogues of these alkaloids, some nonbasic isocarbostyrils from Amaryllidaceae species, have been shown recently to exhibit cytostatic activity and impair cancer cell proliferation and migration by disorganizing the actin cytoskeleton.[7,8] The isocarbostyrils, narciclasine[9] and pancratistatin,[10,11] induce apoptosis in cancer cells of epithelial origin (carcinoma), but not in glioma cells,[8] and only at high concentrations, i.e., one log higher than their in vitro growth-inhibitory IC_{50} values.[9−11] This finding indicates clearly that induction of apoptosis is not the main route by which isocarbostyrils



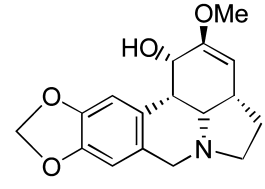
1 $R_1 = R_2 = OH$
1b $R_1 = R_2 = OAc$
1c $R_1 = OAc$, $R_2 = OH$
2 $R_1 = OH$, $R_2 = H$



1a



3 $R_1 = R_2 = R_4 = OH$, $R_3 = OMe$
4 $R_1 = OH$, $R_2 = R_3 = R_4 = OMe$
5 $R_1 = R_3 = OH$, $R_2 = H$, $R_4 = OMe$



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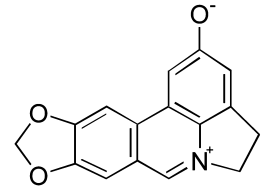

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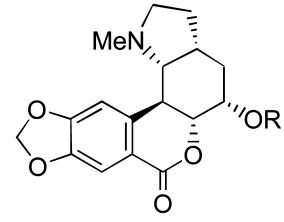

8 R = 3-Hydroxybutanoyl

† Dedicated to the memory of Prof. Carlo Rosini.

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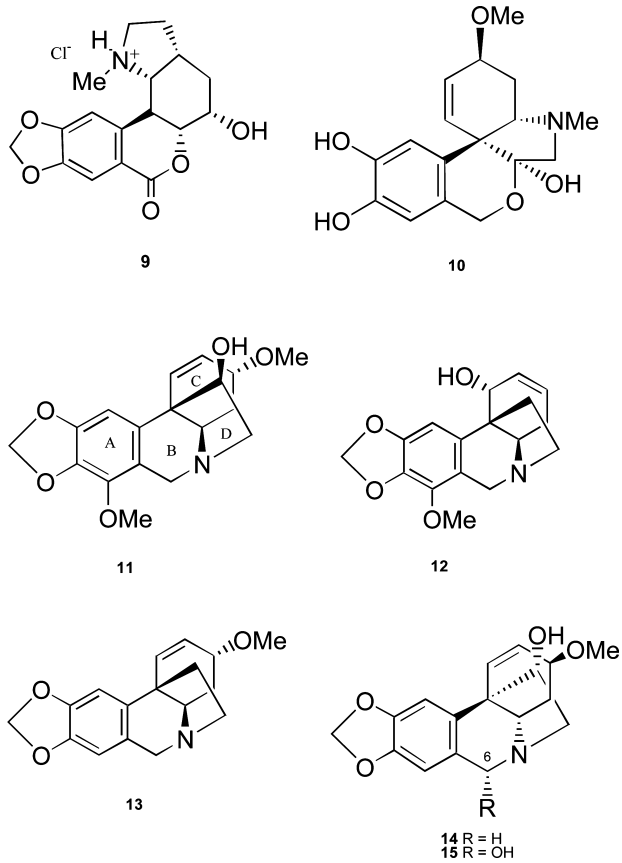
10.1021/np9008255
Published on Web 06/15/2010



from the Amaryllidaceae exert their activity, at least in vitro. Recently, our group analyzed the antiproliferative, apoptosis-inducing and anti-invasive activities in vitro of 29 Amaryllidaceae alkaloids and their derivatives and found significant in vitro activity for several compounds. Importantly, we observed that this activity did not occur systematically through activation of apoptosis.[12,13]

The present study is a continuation of the aforementioned investigation,[14] and it aims to determine whether Amaryllidaceae alkaloids, when active, display potential anticancer activity through cytostatic rather than cytotoxic in vitro effects. The growth-inhibitory IC_{50} values for each compound under study were determined in five human cancer cell lines and one murine mouse cancer cell line using a MTT colorimetric assay,[8,9,14] while the cytostatic versus cytotoxicity analysis was done with computer-assisted phase-contrast microscopy, i.e., quantitative videomicroscopy.[9,15,16] In addition, a review of the literature in this area[2,12] revealed that reduction of the amide functionalities to basic amines among the Amaryllidaceae isocarbostyrils leads to a significant drop in in vitro activities, whereas the opposite trend is observed in the lycorine series, where the activity disappears by rendering Amaryllidaceae alkaloids nonbasic by oxidation of the amine groups to the corresponding amides. Since activity in these series of compounds seems to be strongly influenced by their basicity, whether or not this effect is caused primarily by differences in compound bioavailabilities was considered. Therefore, log *P* values were calculated for each compound under study and correlated with the corresponding antiproliferative IC_{50} values. Last, alkaloids under study were evaluated for the ability to display differences in effects toward apoptosis-resistant compared to apoptosis-sensitive cancer cells.[17,18]

Results and Discussion

Six Amaryllidaceae alkaloids among the 15 assayed in the present study belong to the lycorine subgroup, with the pyrrolo[*de*]phenanthidine skeleton,[2] namely, amarbellisine (**6**), caranine (**2**), galanthine (**4**), norpluvine (**5**), pseudolycorine (**3**), and ungeremine (**7**). These alkaloids were isolated from different Amaryllidaceae species.[2,13] Our group recently analyzed 22 lycorine-related compounds and observed that seven displayed antiproliferative activities in the micromolar range for six distinct cancer cell lines, with four being resistant to apoptosis.[14] No compound was more potent in this regard than lycorine (**1**), which was at least 15 times more active against cancer cells than normal cells.[14] Structure−activity relationship (SAR) analysis revealed that the presence of an unmodified C-ring and C/D-ring junction are structural features important for biological activity, while a methylenedioxy group ring appeared to be nonessential.[14] The results of the present investigation are consistent with these previous data. In comparison with lycorine (**1**), the main representative alkaloid of this subgroup, amarbellisine (**6**), incorporates an altered stereochemistry of the C/D-ring junction and modified functionality of the C-ring, while preserving the nucleophilic nature of the C-1/C-2 fragment. It is likely that the latter structural feature is responsible for the activity of **6**, while caranine (**2**), which lacks the C-2 hydroxy group, is devoid of growth-inhibitory properties (Table 1). Galantine (**4**), norpluvine (**5**), and pseudolycorine (**3**) lack a methylenedioxy group at C-9 and C-10, with alternating hydroxy and methoxy groups in **5** and **3** and two methoxy groups in **4**. Since the opened ring in **3** results in complete retention of the antiproliferative activity of pseudolycorine, it is likely that the lack of the C-2 hydroxy group in **5** and

Table 1. Growth-Inhibitory Activity for Cancer Cell Lines and log *P* Values of Amaryllidaceae Alkaloids[a,b]

			cell line [in vitro IC_{50} (μM) growth-inhibitory values] ± SEM						
alkaloid	no.	log *P*	A549	OE21	Hs683	U373	SKMEL	B16F10	mean ± SEM
lycorine	**1**	0.16	4.2 ± 0.4	4.5 ± 0.7	6.9 ± 0.5	7.6 ± 0.4	8.4 ± 0.2	6.3 ± 0.2	6.3 ± 0.7
caranine	**2**	1.08	>10	>10	>10	>10	>10	>10	>10
pseudolycorine	**3**	0.16	7.4 ± 0.2	7.9 ± 0.3	7.9 ± 0.1	7.8 ± 0.1	>10[c]	7.5 ± 0.1	8.1 ± 0.4
galanthine	**4**	1.04	>10	>10	>10	>10	>10	>10	>10
norpluvine	**5**	0.99	>10	>10	>10	>10	>10	>10	>10
amarbellisine	**6**	0.64	7.2 ± 0.3	6.7 ± 0.2	8.2 ± 0.1	7.2 ± 0.4	8.3 ± 0.1	6.7 ± 0.1	7.4 ± 0.3
ungeremine	**7**	–1.82	>10	>10	>10	83 ± 1	>10	>10	>97
nobilisitine B	**8**	1.18	>10	>10	>10	>10	>10	>10	>10
clivonine hydrochloride	**9**	0.9	>10	>10	>10	>10	>10	>10	>10
tazettine	**10**	1.05	>10	78 ± 2	>10	>10	>10	>10	>96
ambelline	**11**	0.67	>10	86 ± 3	>10	>10	>10	>10	>98
buphanamine	**12**	1.17	>10	>10	>10	>10	>10	>10	>10
buphanisine	**13**	1.74	>10	97 ± 2	>10	>10	>10	>10	>99
haemanthamine	**14**	0.67	4.5 ± 0.6	6.8 ± 0.7	7.0 ± 0.3	7.7 ± 0.5	8.5 ± 0.2	6.8 ± 0.2	6.9 ± 0.5
haemanthidine	**15**	1.02	4.0 ± 0.4	3.7 ± 0.2	4.3 ± 0.2	3.8 ± 0.2	4.2 ± 0.2	3.1 ± 0.2	3.9 ± 0.2

[a] U373 (ECACC code 89081403), Hs683 (ATCC code HTB-138), SKMEL-28 (ATCC code HTB-72), A549 (DSMZ code ACC107), OE21 (ECACC code 96062201), and B16F10 (ATCC code CRL-6475) cell lines were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. [b] MEM and RPMI cell culture media were supplemented with 4 mM glutamine, 100 μg/mL gentamicin, and penicillin−streptomycin (200 U/mL and 200 μg/mL). [c] Insufficient quantity of compound was available to test at higher concentrations.

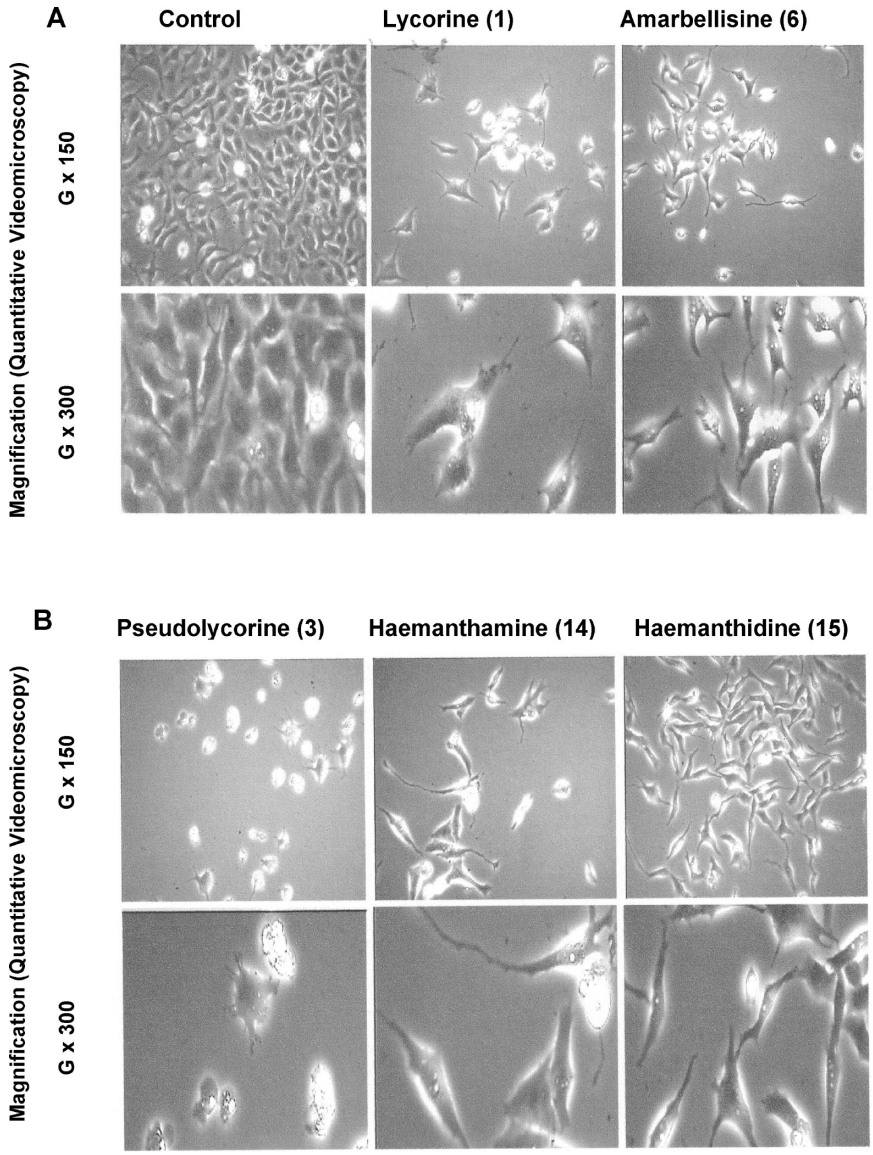


Figure 1. Computer-assisted phase-contrast microscopy (quantitative videomicroscopy) analyses of the antiproliferative effects displayed by lycorine (**1**), amarbellisine (**6**), pseudolycorine (**3**), haemanthamine (**14**), and haemanthidine (**15**) in human U373 glioblastoma cells. The cells were cultured for 72 h and left untreated (control; A) or treated with the IC_{50} concentrations specific for each compound in the U373 model, as detailed in Table 1. With the exception of pseudolycorine (**3**), which displayed cytotoxic effects in vitro (B), the remaining compounds displayed cytostatic effects in vitro (A and B).

the irreversible methylation of this hydroxy in **4** are responsible for the absence of activity of norpluvine and galanthine. Ungeremine (**7**), which is a betaine, incorporates an aromatized C-ring and quaternarized nitrogen as a consequence of the formation of an azomethine functionality with C-7 of the B-ring. This compound was also found to be inactive, possibly due to its highly polar nature.

The lycorenine subgroup of alkaloids, based on the [2]benzopyrano[3,4-*g*]indole ring system, includes nobilisitine B (**8**), isolated only from *Clivia nobilis*[22] thus far, and clivonine (**9**), from different Amaryllidaceae species.[2,19,20] Clivonine (**9**) and nobilisitine B (**8**) as well as tazettine (**10**), based on the pretazettine-ring type and obtained from different Amaryllidaceae species,[2,19] exhibit significant modifications of the carbon skeleton when compared with lycorine (**1**), but none of these compounds displayed notable in vitro activity.

The crinine subgroup of alkaloids, based on a 5,10β-ethanophenanthridine ring included ambelline (**11**), buphanamine (**12**), bupanisine (**13**), haemanthamine (**14**), and haemanthidine (**15**) in the present study, all obtained from different species of the Amaryllidaceae.[2,19,21,23] These compounds differ from lycorine (**1**) in the connectivity of both the B/D- and C/D-ring junctions and substitution of the C-ring. Within this subgroup only **14** and **15**, two alkaloids exhibiting an α-configuration at the ethano bridge, displayed significant in vitro activity against all the cancer cell lines under study. In contrast, **11**−**13**, alkaloids incorporating a β-ethano bridge, were weakly active or not active (Table 1). These data, therefore, emphasize the importance of having an unencumbered β-side of the molecule for Amaryllidaceae alkaloids to display antiproloferative activity. Additionally, the presence of the hydroxy group at C-6 of the B-ring in **15** seems to have no effect on the resultant activity (Table 1).

Lycorine (**1**) exerts its in vitro activity through cytostatic rather than cytotoxic effects.[14] Indeed, lycorine-induced apoptosis was not observed in human glioblastoma cells, even at a concentration 10-fold higher than its in vitro IC_{50} growth-inhibitory value.[14] In addition, in the same study, the cytostatic effects induced by lycorine in cancer cells could be suggested to occur by means of an increase in actin cytoskeleton rigidity.[14] The results illustrated in Figure 1 reveal that among the five active compounds against cancer cells, i.e., lycorine (**1**), amarbellisine (**6**), pseudolycorine (**3**), haeman-

thamine (**14**), and haemanthidine (**15**) (Table 1), compounds **1**, **6**, **14**, and **15** induced cytostatic effects (a delay in cell proliferation), while compound **3** induced cytotoxic effects (cell killing). Biochemical and molecular biology-related experiments are now warranted to investigate further the pathways by which these various compounds induced cytostatic versus cytotoxic effects for cancer cells.

The results reported in Table 1 also showed that the active Amaryllidaceae alkaloids, amarbellisine (**6**), haemanthamine (**14**), haemanthidine (**15**), and pseudolycorine (**3**), in addition to lycorine (**1**), exert similar antiproliferative activities toward cancer cell lines that are apoptosis-resistant (the human NSCLC A549,[22,23] U373 GBM,[24,25] OE21 esophageal cancer,[14] and SKMEL-28 melanoma[18] models) or apoptosis-sensitive (the human Hs683 oligodendroglioma[24,26] and mouse B16F10 melanoma[27] models). About 90% of cancer patients die from tumor metastases,[28] with metastatic cancer cells being intrinsically resistant to apoptosis and, therefore, unresponsive to a large majority of anticancer drugs available today, because the latter generally work through apoptosis induction.[17,29−31] As a barrier to metastasis, cells normally undergo apoptosis after they lose contact with the extracellular matrix or neighboring cells.[28] This cell death process has been termed "anoikis".[28] Tumor cells that acquire malignant potential have developed mechanisms to resist anoikis and thereby survive after detachment from their primary site when they travel through the lymphatic and circulatory systems.[28] Thus, drug resistance, either acquired or intrinsic, is often related to the inability of tumor cells to undergo apoptosis, thereby resulting in cancer cell survival and treatment failure.[17,29−31] One solution to apoptosis resistance entails the complementation of cytotoxic therapeutic regimens with cytostatic agents: the present data therefore argue in favor of lycorine (**1**) as a promising scaffold to develop novel cytostatic anticancer drugs that can overcome cancer cell resistance to apoptosis.

Last, we investigated whether partition measurements, which are presented as log *P* values in Table 1, can discriminate between active and inactive compounds. Experimentally determined log *P* values for lycorine (**1**) and three of its derivatives, i.e., lycorin-2-one (**1a**),[14] 1,2-*O,O′*-diacetyllycorine (**1b**),[14] and 1-*O*-acetyllycorine (**1c**), were generated.[14] The data in Figure 2 show that good fits between the experimental log *P* values and the corresponding values calculated by means of an appropriate algorithm were obtained[32] (see Experimental Section). The relatively good correlation observed between the experimentally determined and theoretically calculated log *P* values for lycorine (**1**) and its three derivatives (**1a**−**1c**) (Figure 2A) encouraged the calculation of log *P* values for the remaining alkaloids under study. Although the data in Figure 2B clearly indicate that log *P* value calculations cannot be used as a reliable predictive tool, all active compounds in this series were grouped within the 1−2 log *P* range. These findings might serve as a useful guideline for analogue design.

In conclusion, the results of the present study combined with our previous work in this area[8,12,14] demonstrate that lycorine (**1**), amarbellisine (**6**), haemanthamine (**14**), and haemanthidine (**15**) are potentially important anticancer chemical scaffolds to generate further compounds able to combat glioblastoma, melanoma, non-small-cell-lung cancers, and metastatic cancers, which are naturally resitant to apoptosis[2,17,18,31]

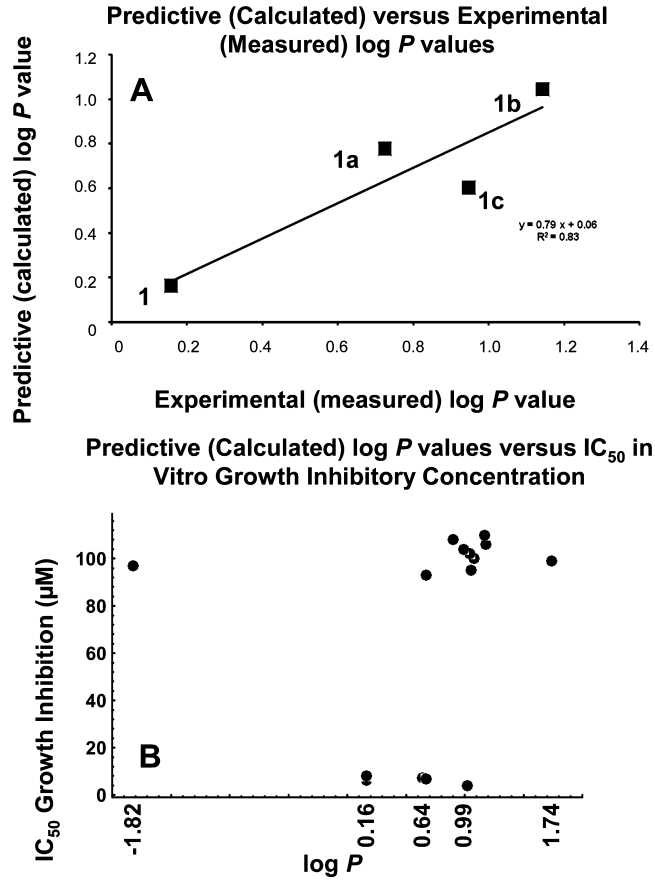


Figure 2. The distribution of a molecule between an aqueous phase and a hydrophobic phase partly conditions its biological properties such as the transport, access across membranes, bioavailability (distribution and accumulation), affinity for a receptor and fixing by a protein, pharmacologic activity, and toxicity. Therefore the theoretical (B) log *P* values of each compound under study were calculated in order to determine whether the IC_{50} in vitro growth-inhibitory concentrations (see Table 1) were related to log *P* values. The experimental log *P* values were calculated on four compounds (A) in order to validate the software calculations used to theoretically predict the log *P* value of each compound under study (B).

Experimental Section

Alkaloids. Lycorine (**1**) was obtained from dried bulbs of *S. lutea* using the procedures reported by Evidente and co-workers.[33] Amarbellisine (**6**),[2] nobilisitine B (**8**),[2] haemanthamine (**14**), ungeremine (**7**),[2] and tazettine (**10**)[2] were isolated from *Amaryllis belladonna* L., *Clivia nobilis* Regel, *Pancratium maritimum* L., and *Narcissus tazetta* L., respectively. Ambelline (**11**), buphanamine (**12**), buphanisine (**13**), galanthine (**4**), and pseudolycorine (**3**) were generously supplied by Dr. H. M. Fales, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, MD. Clivonine chlorohydrate (**9**) and norpluviine (**5**) were kindly supplied by Prof. C. Fuganti, Istituto di Chimica, Politecnico di Milano, Italy. Caranine (**2**) was prepared by lycorine using the procedures reported[34] and was identical to the natural alkaloids previously isolated.[35,36] Haemanthidine (**15**) was purified from the dried bulbs of *Lycoris aurea* Herb. as previously reported.[37] Lycorin-2-one (**1a**), 1,2-*O,O′*-diacetyllycorine (**1b**), and 7-*O*-acetyllycorine (**1c**) were obtained by chemical modification of lycorine as previously reported.[20]

Cell Lines. Human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), the European Collection of Cell Culture (ECACC, Salisbury, UK), and the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany). The code numbers and histological types of all the cell lines used in the current study are detailed in Table 1.

Biology-Related Experiments. The effects of alkaloids (**1**−**15**) on the overall growth level of human cancer cell lines were determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]diphenyltetrazolium bromide, Sigma, Bornem, Belgium) assay.[8,14] Briefly, the cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10 000 to 40 000 cells/mL culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. The assessment of cell population growth by means of the MTT colorimetric assay is based on the capability of living cells to reduce yellow MTT to a blue product, formazan, by a reaction occurring in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence: control) of the various compounds is directly

proportional to the intensity of the blue color produced, which was measured quantitatively by spectrophotometry, using a Biorad model 680XR (Biorad, Nazareth, Belgium) at a 570 nm wavelength (with a reference of 630 nm). Each experiment was carried out in sextuplicate. Use was made of quantitative videomicroscopy[8,14−16] to determine whether the best alkaloids identified as active (IC_{50} <10 μM for each of the six cancer cell lines under study) by means of the MTT colorimetric assay displayed cytotoxic versus cytostatic effects in the human U373 glioblastoma cell line.

Log *P* Calculations. 1-Octanol was purchased from Sigma-Aldrich (Germany). The aqueous phase used is Dulbecco's phosphate-buffered saline (DPBS, GibcoBRL/Life Technologies/Invitrogen, Merelbeke, Belgium). Briefly, 1.0 mg of each compound was dissolved into 10 mL of heterogenic phase solvents consisting of octanol and PBS buffer (1:1). The two-partitioned system was mixed for 30 min with a rotary mixer at 20 rpm. The experiment was performed at 25 °C and 1013 bar. After 24 h, 1 mL aliquots of each phase were transferred to spectrophotometric cells. The wave scan was monitored between 200 and 500 nm. The maximum of absorbance was measured, taking into account $K = A_{octanol}/A_{PBS}$. In addition, a predictive determination of the distribution coefficient was performed for each compound under study by means of an appropriate algorithm.[32]

Acknowledgment. The authors thank the Italian Ministry University and Research (MIUR), the Fonds National de la Recherche Scientifique (FNRS, Belgium), and the Fonds Yvonne Boël (Belgium). This is contribution DISSPAPA no. 217.

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NP9008255