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Bulbispermine: A Crinine-Type Amaryllidaceae Alkaloid Exhibiting Cytostatic Activity toward Apoptosis-Resistant Glioma Cells

Giovanni Luchetti,[a] Robert Johnston,[b] Véronique Mathieu,[c] Florence Lefranc,[c, d] Kathryn Hayden,[b] Anna Andolfi,[e] Delphine Lamoral-Theys,[f] Mary R. Reisenauer,[b] Cody Champion,[a] Stephen C. Pelly,[g] Willem A. L. van Otterlo,[g] Igor V. Magedov,[a] Robert Kiss,[c] Antonio Evidente,[e] Snezna Rogelj,[b] and Alexander Kornienko*[a]

The Amaryllidaceae alkaloid bulbispermine was derivatized to produce a small group of synthetic analogues. These, together with bulbispermine's natural crinine-type congeners, were evaluated in vitro against a panel of cancer cell lines with various levels of resistance to pro-apoptotic stimuli. Bulbispermine, haemanthamine, and haemanthidine showed the most potent antiproliferative activities as determined by the MTT colorimetric assay. Among the synthetic bulbispermine analogues, only the C1,C2-dicarbamate derivative exhibited notable growth inhibitory properties. All active compounds were found not to discriminate between the cancer cell lines based on the apoptosis sensitivity criterion; they displayed similar potencies in both cell types, indicating that the induction of apoptosis is not the primary mechanism responsible for antiproliferative activity in this series of compounds. It was also found that bulbispermine inhibits the proliferation of glioblastoma cells through cytostatic effects, possibly arising from rigidification of the actin cytoskeleton. These findings lead us to argue that crinine-type alkaloids are potentially useful drug leads for the treatment of apoptosis-resistant cancers and glioblastoma in particular.

Introduction

The anticancer properties of plants belonging to the Amaryllidaceae family were known as early as the fourth century B.C., when Hippocrates of Cos used oil from the daffodil *Narcissus poeticus* L. for the treatment of uterine tumors.[1] Using related biosynthetic pathways, these plants produce a large number of diverse alkaloids and related non-basic metabolites, the anticancer potential of which is being explored by many research groups worldwide.[2] Thus, various studies have identified promising anticancer effects exhibited by the isocarbostyrils narciclasine and pancratistatin, as well as the pyrrolophenanthridine alkaloid lycorine (Figure 1). These natural products have therefore been under intense scrutiny and are currently being pursued as clinical candidates for cancer treatment.[3]

Although the ethano-bridged phenanthridine alkaloids of the crinine type (Figure 2) have also been reported to display cytotoxic[4] and apoptosis-inducing[5] properties, their anticancer potential remains largely unexplored, and the mechanism(s) underlying their effects on cancer cells have not been investigated.

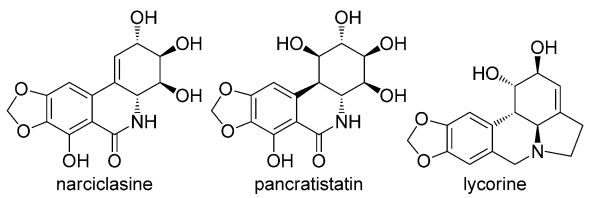


Figure 1. Structures of Amaryllidaceae anticancer constituents pursued as clinical candidates for cancer treatment.

We recently reported the results of biochemical experiments that provide mechanistic insight into the antiproliferative ef-

[a] G. Luchetti, C. Champion, Prof. I. V. Magedov, Prof. A. Kornienko
Department of Chemistry
New Mexico Institute of Mining and Technology, Socorro, NM 87801 (USA)
E-mail: akornien@nmt.edu

[b] R. Johnston, K. Hayden, M. R. Reisenauer, Prof. S. Rogelj
Department of Biology
New Mexico Institute of Mining and Technology, Socorro, NM 87801 (USA)

[c] Prof. V. Mathieu, Prof. F. Lefranc, Prof. R. Kiss
Laboratoire de Toxicologie, Faculté de Pharmacie
Université Libre de Bruxelles (ULB), 1050 Brussels (Belgium)

[d] Prof. F. Lefranc
Service de Neurochirurgie, Hôpital Erasme, Brussels (Belgium)

[e] Dr. A. Andolfi, Prof. A. Evidente
Dipartimento di Scienze del Suolo della Pianta dell'Ambiente e delle-Produzioni Animali
Università di Napoli Federico II, Via Università 100, 80055 Portici (Italy)

[f] D. Lamoral-Theys
Laboratoire de Chimie BioAnalytique, Toxicologie et Chimie Physique-Appliquée
Faculté de Pharmacie, Université Libre de Bruxelles (ULB), 1050 Brussels (Belgium)

[g] Dr. S. C. Pelly, Prof. W. A. L. van Otterlo
Department of Chemistry and Polymer Sciences, Stellenbosch University
Stellenbosch, Western Cape (South Africa)

fects induced by narciclasine and lycorine.[3a,b,d] We showed that at therapeutic concentrations, both narciclasine and lycorine do not induce apoptosis in cancer cells, but rather exhibit cytostatic effects by targeting the actin cytoskeleton. This mode of action explains their promising activities against cancer cells that are resistant to pro-apoptotic stimuli and therefore representative of tumors associated with poor prognoses such as melanoma, glioblastoma, and non-small-cell lung cancer. Herein we show that the crinine-type alkaloids exhibit useful cytostatic properties as well, and that these effects are most likely caused by targeting the actin cytoskeleton organization in cancer cells. These findings warrant further investigation of the crinine-type alkaloids as promising potential agents for the treatment of apoptosis-resistant cancers, such as glioblastoma.[6]

Results and Discussion

Isolation and structure confirmation

The alkaloid bulbispermine (Figure 2), previously isolated from various plants of the Amaryllidaceae family,[7] has been reported to have cytotoxic activity against HL-60 cells.[7c] We serendipitously isolated a significant quantity of this alkaloid from *Zephyranthes robustus* while investigating this plant as a potential source of pancratistatin.[8] Although this plant had not been previously reported as a source of bulbispermine, it appeared to contain significant quantities of this alkaloid (70 mg kg^{-1} of fresh bulbs). However, because of problems with plant availability, we subsequently switched to the known source of bulbispermine, *Crinum bulbispermum* (160 mg kg^{-1} of fresh bulbs).[7b] Although the NMR spectra of isolated bulbispermine were consistent with those reported previously,[9] we were concerned with their similarity to the NMR spectroscopic data of the stereoisomeric alkaloids, 11-hydroxyvittatine and hamayne (Figure 2). Therefore, we obtained unambiguous proof of stereochemistry by converting a portion of bulbispermine to its diacetate (compound **1**) and analyzing the structure by NOESY (Figure 3). Finally, in terms of natural product isolation, additional crinine-type alkaloids, namely haemanthamine, haemanthidine, buphanamine, buphanisine, and ambelline (Figure 2) were obtained with procedures used previously by our research group as detailed below in the Experimental Section.

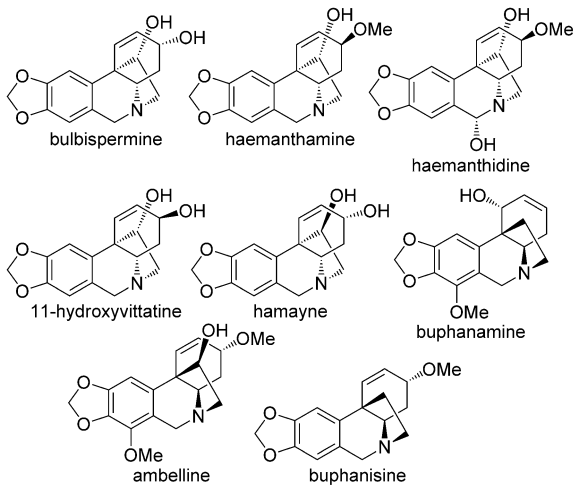


Figure 2. Structures of selected crinine-type alkaloids.

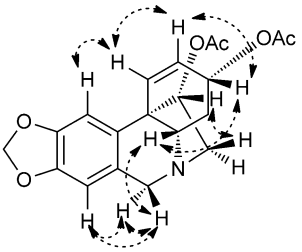


Figure 3. NOE correlations in compound **1**, confirming the stereochemical relationships.

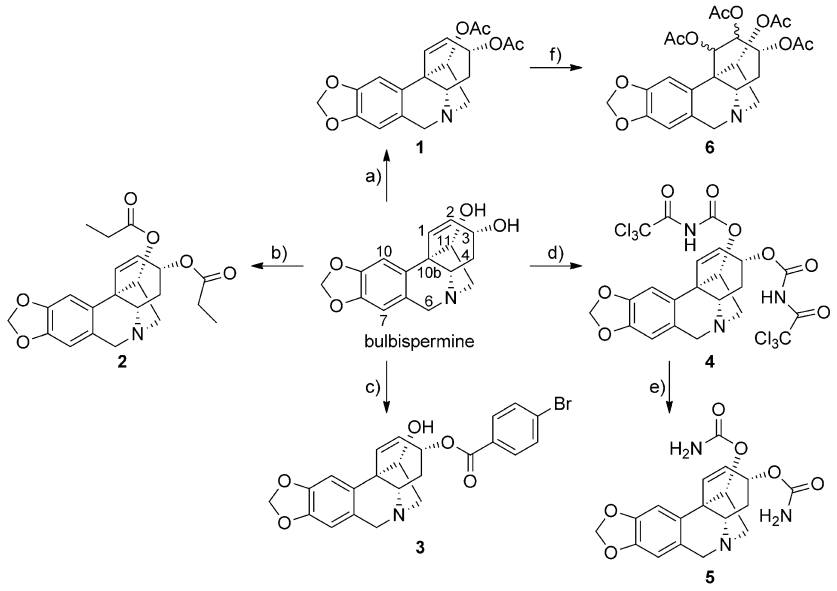
Synthesis

After isolating sufficient quantities of bulbispermine, we performed its chemical derivatization and subsequently obtained a small group of analogues. Thus, the hydroxy groups in bulbispermine were acetylated and propionylated to give esters **1** and **2** (Scheme 1). The reaction with one equivalent of *para*-bromobenzoyl chloride resulted in selective esterification of the more sterically accessible allylic hydroxy at C3. Notably, the other hydroxy group at C11 is located vicinal to the C10b quaternary carbon center, thus facilitating this outcome. As these derivatizations lead to the introduction of hydrophobic groups on the two hydroxy groups, we next sought to derivatize them as polar carbamates. This was done by first allowing bulbispermine to react with trichloroacetyl isocyanate to give intermediate **4**, which was then converted into dicarbamate **5** through basic hydrolysis. Our attempts to selectively dihydroxylate the double bond in diacetate **1** were unsuccessful, and we obtained tetraacetate **6** as a mixture of diastereomers at C1 and C2 (Scheme 1).

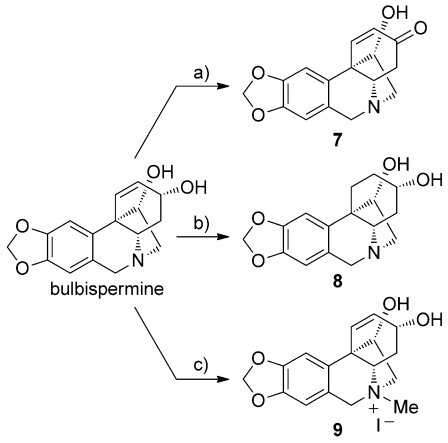
Additional bulbispermine derivatization (Scheme 2) involved selective oxidation of the allylic alcohol to give C3-ketone **7**, hydrogenation of the double bond to provide C1,C2-saturated analogue **8**, and lastly quaternization of the nitrogen atom to result in ammonium iodide salt **9**.

Biochemical experiments

The synthesized compounds were next evaluated for in vitro growth inhibition using the MTT colorimetric assay[3a,l] against a panel of five cancer cell lines. This included two that are resistant to pro-apoptotic stimuli: human U373 and T98G glioblastoma (GBM, from astroglial origin);[3a,10a,b] two apoptosis-sensitive tumor models: human Hs683 anaplastic oligodendroglioma[3a,10b] and HeLa cervical adenocarcinoma; and an additional human U87 glioblastoma cell line, the apoptosis-resistance properties of which have not yet been investigated.[10c] Analysis of the antiproliferative data reveals that active com-



Scheme 1. Derivatization of bulbispermine hydroxy groups. *Reagents and conditions:* a) Ac_2O, py, DMAP, RT, overnight, 78%; b) $(CH_3CH_2CO)_2O$, py, RT, overnight, 82%; c) *p*-Br-BzCl, py, DMAP, MeCN, RT, overnight, 48%; d) $Cl_3C(C{=}O)N{=}C{=}O$, CH_2Cl_2, 0 °C, 20 min, 75%; e) K_2CO_3, $MeOH/H_2O$, RT, 3 h, 98%; f) 1. OsO_4, NMO, $acetone/H_2O$, RT, 6 h, 2. Ac_2O, py, DMAP, RT, overnight, 59%.



Scheme 2. Additional bulbispermine derivatization. *Reagents and conditions:* a) Dess–Martin, $PhCH_3$, 65 °C, 2 h, 38%; b) H_2, Pd/C, MeOH, RT, overnight, 74%; c) MeI, MeCN, RT, 2 h, 97%.

pounds (bulbispermine, haemanthamine, haemanthidine, and synthetic derivatives **1** and **5**) do not discriminate between the cancer cell lines based on the apoptosis sensitivity criterion and display similar potencies in both cell types, indicating that apoptosis induction is not the primary mechanism responsible for antiproliferative activity in this series of compounds, at least in solid cancers (Table 1). Furthermore, three of the natural alkaloids containing the α-C11,C12-ethano bridge (bulbispermine, haemanthamine, and haemanthidine) are all active, in contrast to their counterparts incorporating this C11–C12 subunit at the β position (buphanamine, buphanisine, and ambelline). These observations are consistent with findings reported earlier,[4i,11] and they point to the criticality of this structural feature for antiproliferative activity in the crinine-type alkaloids. Of the synthetic bulbispermine derivatives, the only notable activity is observed for compound **5**, in which the C3,C11-hydroxy groups are converted into polar carbamates, demonstrating that derivatization of the hydroxy groups with hydrophobic moieties leads to an apparent loss of activity. Also of interest is the importance of the C1,C2-olefinic functionality, because analogue **8**, in which the double bond is hydrogenated, is inactive. Finally, the lack of anticancer activity for analogues **7**–**9** is consistent with previously published observations that similar modifications to the lycorine skeleton result in inactive compounds.[3d,11]

We also made use of computer-assisted phase-contrast microscopy (quantitative videomicroscopy) to analyze the principal mechanism of action associated with bulbispermine's in vitro growth inhibitory effects, as first revealed by the MTT colorimetric assay. Figure 4 shows that bulbispermine inhibits cancer cell proliferation without inducing cell death when assayed at its IC_{50} in vitro growth inhibitory value in U373 GBM cells or even at sevenfold its IC_{50} antiproliferative value in U87 GBM cells. Based on the phase-contrast images obtained by quantitative videomicroscopy, we calculated the global growth rate (GGR), which corresponds to the ratio of the mean number of cells present in the last image captured in the experiment (conducted at $t = 72$ h) to the number of cells present in the first image (at $t = 0$ h). We divided this ratio obtained in the bulbispermine-treated experiment by the ratio obtained in the control. Lycorine, which we established earlier to be a cytostatic compound in these cell lines,[3d] was used as a positive control. A GGR value of 0.6 in both cell lines means that 60% of cells grew in the bulbispermine-treated experiment relative to the control over an observation period of 72 h.

Our previous work with narciclasine[3a] and lycorine[3d] revealed that their cytostatic effects on cancer cells occur mainly during cytokinesis, through an increase in the rigidity of the actin cytoskeleton. Our experiments with bulbispermine and its active analogue **5** also revealed that at their in vitro growth inhibitory IC_{50} concentrations, these compounds markedly increase the levels of polymerized actin in Hs683 glioma cells (green fluorescence in Figure 5Cb,Db) relative to control (Figure 5Ab). These effects of increased rigidity of the actin cytoskeleton induced by bulbispermine and its synthetic analogue **5** thus closely resemble those of narciclasine (Figure 5Bb).

Correlations of the differential cellular sensitivities in the US National Cancer Institute (NCI) 60-cell-line screen confirmed

Table 1. Evaluation of natural and synthetic crinine-type compounds against cancer cells resistant or sensitive to pro-apoptotic stimuli.

Compd	GI_{50} [μM][a]				
	Resistant		Sensitive		Unknown
	T98G	U373	Hs683	HeLa	U87
bulbispermine	9	38	11	8	9
haemanthamine	8	6	3	3	6
haemanthidine	14	7	4	ND	6
buphanamine	>100	>100	>100	>100	>100
buphanisine	>100	>100	>100	>100	>100
ambelline	>100	>100	>100	>100	>100
1	98	ND	63	90	74
2	>100	ND	>100	>100	>100
3	>100	ND	>100	>100	>100
4	>100	ND	>100	>100	>100
5	91	>100	50	46	15
6	>100	ND	>100	>100	>100
7	>100	ND	>100	>100	>100
8	>100	ND	>100	>100	>100
9	>100	ND	>100	>100	>100

[a] Values represent the average of two independent experiments performed in vitro; ND: not determined.

this proposed mode of action for crinine-type alkaloids. Although bulbispermine has not been tested at NCI, data are available for its congener haemanthamine (Figure 2). Thus, the "Compare Correlation Coefficients" indicate that at its mean GI_{50} value (16 μM) characteristic of growth inhibition (Figure 6), the differential cellular sensitivities for haemanthamine are similar to those of known actin cytoskeleton modulators rapamycin[12] and isocarbostyril pancratistatin[3b] (the correlation coefficients are 0.5 and 0.35, respectively). However, the uniformity of the mechanism among these compounds disappears if the correlations are performed at their mean LC_{50} values, characteristic of cell death (no correlation obtained for 100 μM haemanthamine). Therefore, at cytotoxic doses, haemanthamine probably interacts with other lower-affinity targets, which are not necessarily the same for rapamycin and pancratistatin.

Computer modeling

The similarity of the effects on cancer cells exhibited by the previously investigated narciclasine and lycorine, as well as the currently described crinine-type alkaloids, gives rise to an important question of whether the structural skeletons associated with these three types of Amaryllidaceae constituents share the same chemical space and are capable of targeting the same intracellular macromolecules. For the purposes of investigating the structural relationship between narciclasine, lycorine, and bulbispermine (Figure 7), we undertook a conformational search to find the lowest-energy conformation for each structure, and these were superimposed. It is clear that the three structures have in common the hydrogen bond acceptor methylenedioxy moiety, the aromatic region, and the positioning of the nitrogen atom. Interestingly, in all three compounds this nitrogen would serve as a potential hydrogen bond donor group, as in narciclasine it is found as the amide –NH, and in lycorine and bulbispermine it will be in the protonated form at physiological pH. Therefore, it is highly probable that these three regions represent the common pharmacophore for the three structures in their mode of action. Diversification in the structures is tolerated in the hydroxy-containing portions of the three compounds, indicating that this region is possibly directed away from the putative binding site(s).

Conclusions

Malignant gliomas, which include anaplastic astrocytoma, oligodendroglioma, oligoastrocytoma, and glioblastoma, account for more than 50% of all primary brain tumors.[6] Of these, glioblastoma multiforme represents the highest grade of malig-

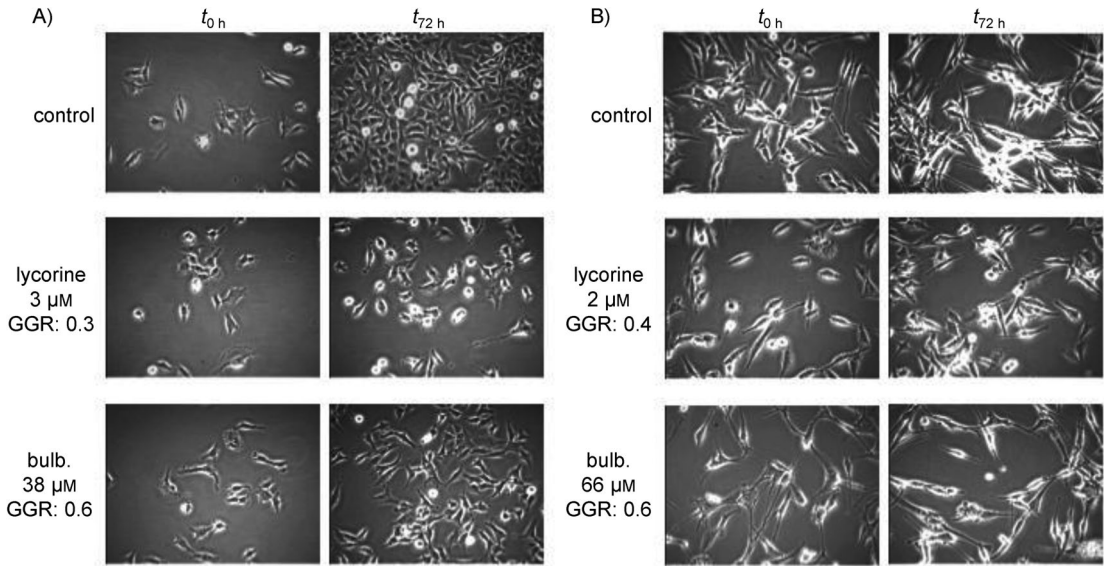


Figure 4. Cellular imaging of bulbispermine (bulb.) against A) U373 and B) U87 glioma cells (GGR = global growth rate), illustrating a non-cytotoxic yet cytostatic, antiproliferative mechanism at MTT colorimetric assay-related IC_{50} (U373) as well as $7 \times IC_{50}$ (U87) concentrations.

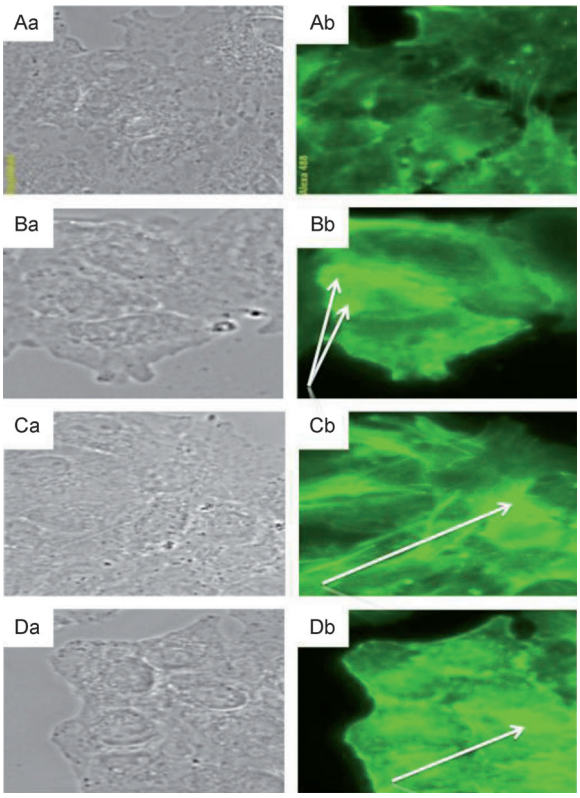


Figure 5. Effects on actin cytoskeleton organization by bulbispermine and analogue **5** at their in vitro growth inhibitory (IC_{50}) concentrations in Hs683 glioma cells. A) control (untreated) conditions (Aa: bright field; Ab: actin cytoskeleton). B) 100 nM positive control narciclasine-treated conditions (Ba: bright field; Bb: actin cytoskeleton). C) 10 μM bulbispermine-treated conditions (Ca: bright field; Cb: actin cytoskeleton). D) 50 μM analogue-**5**-treated conditions (Da: bright field; Db: actin cytoskeleton). Narciclasine (Bb), bulbispermine (Cb), and analogue **5** (Db) rigidify the actin cytoskeleton by increasing the amount of polymerized actin (white arrows).

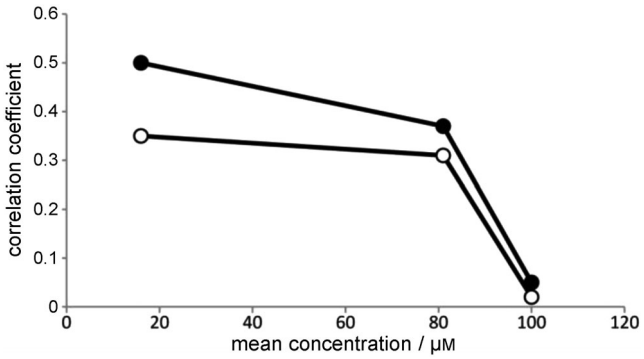


Figure 6. Correlations of the differential cellular sensitivities in the NCI 60-cell-line screen using the COMPARE algorithm. "Compare Correlation Coefficients" were generated by a computerized pattern-recognition algorithm and serve as an indication of similarities in differential cellular sensitivities or characteristic fingerprints for each compound. Haemanthamine was used as a seed to find significant correlations with the anticancer agents in the NCI Standard Compound Database, containing rapamycin and pancratistatin at the GI_{50}, TGI, and LC_{50} levels (for definitions of these parameters, see *DTP Human Tumor Cell Line Screen*: http://dtp.nci.nih.gov/branches/btb/ivclsp.html; accessed February 16, 2012). At the GI_{50} level (16 μM), the correlations identified rapamycin (●) and pancratistatin (○) as highly ranked agents among the compounds in the database with Compare Correlation Coefficients of 0.5 and 0.35, respectively. Whereas correlations at the TGI level (80 μM) were worse but still significant (Compare Correlation Coefficients of 0.37 and 0.34), the LC_{50} levels (100 μM) were not correlated at all.

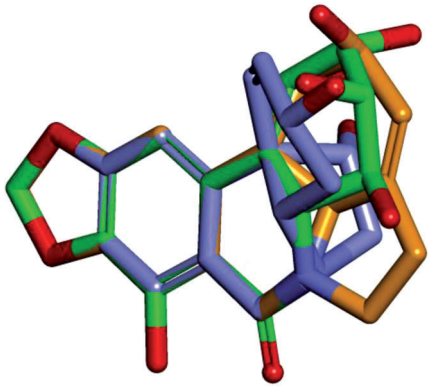


Figure 7. Superimposition of narciclasine (green), lycorine (orange), and bulbispermine (purple), showing high similarity in the methylenedioxy, aromatic, and nitrogen portions of the structures.

nancy and is associated with dismal prognoses. Glioblastoma cells display resistance to apoptosis, which contributes to their poor response to conventional chemotherapy with pro-apoptotic agents.[6] In our previous work we identified the Amaryllidaceae small-molecule constituents narciclasine and lycorine as promising anticancer agents that exhibit useful antiproliferative effects toward glioblastoma cells resistant to pro-apoptotic stimuli. Herein we demonstrate that another group of Amaryllidaceae constituents, namely crinine-type alkaloids, are also potentially useful drug leads for the treatment of apoptosis-resistant cancers and glioblastoma in particular. We show that a representative crinine-type alkaloid bulbispermine inhibits the proliferation of glioblastoma cells through cytostatic effects possibly arising from the rigidification of the actin cytoskeleton. Computer-assisted superimposition of the lowest-energy conformations of narciclasine, lycorine, and bulbispermine indicates that these three molecules, representative of three major Amaryllidaceae anticancer scaffolds, occupy similar regions in chemical space and they indeed could be targeting the same intracellular macromolecules to induce cytostatic effects in cancer cells.

Experimental Section

Chemistry

General: All commercially obtained reagents were used without purification unless otherwise noted. THF and toluene were distilled from sodium benzophenone-ketyl prior to use. CH_2Cl_2 was distilled from CaH_2 and kept under Ar. Reactions were monitored by TLC (EM Science, Silica Gel 60 F_{254}, 250 μm) and visualized with UV light or I_2. When appropriate, reactions were run under N_2 or Ar in oven-dried glassware using standard syringe, cannula, and septa techniques. Flash chromatography was performed on silica gel (Silica gel, 32–63 μm, 60 Å pore size). ^1H and ^{13}C NMR spectra were recorded on JEOL 300 MHz and Bruker 400 MHz spectrometers. HRMS was performed at the University of New Mexico mass spectrometry facility.

Alkaloids: Bulbispermine was isolated from dried bulbs of *Crinum bulbispermum* as previously reported.[7b] Haemanthamine was isolated from Egyptian *Pancratium maritinum*.[13] Ambelline, buphana-

mine, and buphanisine were generously supplied by Prof. Henry M. Fales, Department of Health, Education and Welfare, Bethesda, MD (USA). Haemanthidine was purified from dried bulbs of *Lycoris aurea*.[14] The purity of the samples was confirmed by TLC, mp, optical rotation, ^{1}H and ^{13}C NMR, NOESY, and ESIMS analyses.

Bulbispermine: ^{1}H NMR (CD_3OD, 300 MHz): $\delta = 6.85$ (s, 1H, H-10), 6.52 (s, 1H, H-7), 6.23 (dd, 1H, $J = 9.1$ Hz, 2.2 Hz, H-2), 6.04 (d, 1H, 9.1 Hz, H-1), 5.87 (s, 2H, -OCH_2O-), 4.36–4.29 (m, 1H, H-3), 4.27 (d, 1H, $J = 16.7$ Hz, H-6β), 3.95 (dd, 1H, $J = 8.3$ Hz, 6.0 Hz, H-11), 3.73 (d, 1H, $J = 16.7$ Hz, H-6α), 3.44 (1H, dd, $J = 13.6$ Hz, 7.1 Hz, H-4a), 3.25–3.17 (m, 2H, H-12), 2.15–2.03 (m, 1H, H-4), 1.98–1.91 ppm (m, 1H, H-4); ^{13}C NMR (CD_3OD, 400 MHz): $\delta = 148.5$ (C9), 148.0 (C8), 137.4 (C10a), 136.7 (C1), 125.2 (C6a), 124.2 (C2), 107.9 (C10), 104.4 (C7), 102.4 (-OCH_2O-), 80.4 (C11), 68.0 (C3), 67.7 (C4a), 63.3 (C12), 60.9 (C6), 51.6 (C10b), 33.8 ppm (C4).

Compound 1: To a solution of bulbispermine (10.0 mg, 0.0348 mmol) in pyridine (3.0 mL) was added Ac_2O (2.0 mL) and a catalytic amount of DMAP (0.1 mg, 0.9 μmol). The reaction was stirred at room temperature overnight, and the following day was co-evaporated several times with toluene to remove pyridine. The resulting residue was purified by preparative thin-layer chromatography (PTLC) using CH_2Cl_2/MeOH (98:2), yielding 5.1 mg of the diacetate (78%). ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 6.86$ (s, 1H, H-10), 6.47 (s, 1H, H-7), 6.22 (dd, 1H, $J = 10.4$ Hz, 2.2 Hz, H-1), 5.91 (s, 2H, -OCH_2O-), 5.86 (d, 1H, $J = 10.4$ Hz, H-2), 5.48–5.42 (m, H-3β), 4.99–4.31 (m, 1H, H-11*exo*), 3.34 (d, 1H, $J = 17.0$ Hz, H-6β), 3.71 (d, 1H, $J = 17.0$ Hz, H-6α), 3.40 (d, 2H, $J = 5.2$ Hz, H-12), 3.28 (dd, 1H, $J = 13.5$ Hz, H-4a), 2.12–2.17 (m, 2H, H-4), 2.09 (s, -OAc), 2.04 ppm (s, OAc); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 170.6$ (C=O), 170.3 (C=O), 146.9 (C9), 146.8 (C8), 134.0 (C10a), 131.4 (C1), 126.3 (C6a), 125.6 (C2), 106.8 (C10), 103.9 (C7), 101.1 (-OCH_2O-), 80.4 (C11), 70.0 (C3), 66.2 (C4a), 61.1 (C6), 49.4 (C10b), 29.8 (C4), 21.4 (CH_3), 21.3 ppm (CH_3); HRMS *m/z* (ESI$^+$) calcd for $C_{20}H_{22}NO_6$ $[M+H]^+$ 372.1447, found 372.1449.

Compound 2: To a solution of bulbispermine (5.0 mg, 0.0174 mmol) in pyridine (1.5 mL) was added propionic anhydride (1.0 mL). The reaction mixture was stirred at room temperature overnight, and the following day was co-evaporated with toluene several times to remove pyridine. The resulting residue was purified by PTLC using CH_2Cl_2/MeOH (98:2), yielding 5.6 mg of the dipropionate (82%). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 6.89$ (s, 1H, H-10), 5.50 (s, 1H, H-7), 6.23 (d, 1H, $J = 10.4$ Hz, H-1), 5.93 (s, 2H, -OCH_2O-), 5.87 (d, 1H, $J = 10.4$ Hz, H-2), 5.51–5.47 (m, 1H, H-3), 5.03–5.00 (m, 1H, H-11), 4.39 (d, 1H, $J = 17.2$ Hz, H-6β), 3.77 (d, 1H, $J = 17.2$ Hz, H-6α), 3.46–3.44 (m, 2H, H-12), 3.33 (dd, 1H, $J = 13.7$ Hz, 3.04 Hz, H-4a), 2.40–2.31 (m, 4H, -CH_2CO-), 2.23–2.19 (m, 1H), 1.19–1.14 ppm (m, 6H, -CH_3); ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 174.0$ (C=O), 173.6 (C=O), 147.0 (C9), 146.8 (C8), 131.5 (C10a), 125.3 (C2), 106.8 (C10), 104.0 (C7), 101.1 (-OCH_2O-), 79.8 (C11), 69.6 (C3), 66.1 (C4a), 60.9 (C12), 60.2 (C6), 49.4 (C10b), 29.6 (C4), 28.0, 27.9, 9.2, 9.1 ppm; HRMS *m/z* (ESI$^+$) calcd for $C_{22}H_{25}NO_6$ $[M+H]^+$ 400.1760, found 400.1761.

Compound 3: A solution of *para*-bromobenzoyl chloride in MeCN (0.5 mL, 0.0163 mmol) was added dropwise to a solution of bulbispermine (10.0 mg, 0.0384 mmol) in pyridine (0.5 mL). The reaction mixture was stirred at room temperature overnight, and the resulting solution was diluted with a 5% (*w/v*) solution of $NaHCO_3$ (1 mL). The aqueous solution was extracted from with EtOAc (3× 1 mL), and the organic layers were combined and dried with $MgSO_4$. After removal of the solvent, the residue was purified using PTLC and with the eluent system CH_2Cl_2/MeOH (95:5) to furnish 8.6 mg of mono *para*-bromobenzoate (48%). ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 8.94$ (d, 2H, $J = 5.1$ Hz, PhH), 8.59 (d, 2H, $J = 5.1$ Hz, PhH), 6.82 (s, 1H, H-10), 6.51 (s, 1H, H-7), 6.39 (d, 1H, $J = 12.0$ Hz, H-1), 6.24 (d, 1H, $J = 12.0$ Hz, H-2), 5.94 (s, 2H, -OCH_2O-), 5.75–5.66 (m, 1H, H-3), 4.36 (d, 1H, $J = 18.0$ Hz, H-6β), 4.09–4.05 (m, 1H, H-11), 3.71 (d, 1H, $J = 18.0$ Hz, H-6α), 3.49–3.34 (m, 2H, H-12), 2.46–2.32 (m, 2H, H-4), 2.25–2.16 ppm (m, H-4); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 165.6$, 146.7, 146.5, 134.1, 131.8, 131.4, 126.7, 125.4, 107.1, 103.3, 101.1, 80.3, 71.3, 66.1, 63.6, 61.4, 50.3, 31.1, 30.0 ppm; HRMS *m/z* (ESI$^+$) calcd for $C_{23}H_{20}BrNO_5$ $[M+H]^+$ 472.0583, found 472.0568.

Compound 4: To a solution of bulbispermine (5.0 mg, 0.0174 mmol) in CH_2Cl_2 (0.174 mL) cooled to 0 °C was added trichloroacetyl isocyanate (4.1 μL, 0.0348 mmol). The reaction was stirred at 0 °C for 20 min, and the CH_2Cl_2 was evaporated under reduced pressure. The crude material was purified by PTLC using 10% MeOH in CH_2Cl_2 resulting in 8.4 mg imidocarbamate (75%). ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 7.03$ (s, 1H, H-10), 6.67 (s, 1H, H-7), 6.46 (d, 1H, $J = 10.4$ Hz, H-1), 6.15 (d, 2H, $J = 10.4$ Hz, H-2), 5.97 (s, -OCH_2O-), 5.54–5.52 (m, 1H, H-3), 5.13–5.11 (m, 1H, H-11), 4.56 (d, 1H, $J = 15.9$ Hz, H-6β), 4.05 (d, 1H, $J = 15.9$ Hz, H-6α), 3.83–3.67 (m, 3H), 2.42–2.34 ppm (m, 2H, H-4); HRMS *m/z* (ESI$^+$) calcd for $C_{18}H_{20}N_3O_6$ (hydrolysis product $[M+H]^+$) 374.1352, found 374.1346.

Compound 5: To a solution of bulbispermine (5.0 mg, 0.0174 mmol) in CH_2Cl_2 (0.174 mL) cooled to 0 °C was added trichloroacetyl isocyanate (4.1 μL, 0.0348 mmol). After being stirred at 0 °C for 20 min, the CH_2Cl_2 was evaporated. The resulting residue was dissolved in MeOH (0.1 mL) and cooled to 0 °C. H_2O (0.5 mL) and K_2CO_3 (0.19 g, 1.38 mmol) was added, and the cooling bath was removed. After stirring at room temperature for 3 h, MeOH was evaporated, and the resulting aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were dried (Na_2SO_4) and concentrated to afford 4.7 mg of the dicarbamate (74%). ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 6.95$ (s, 1H, H-10), 6.59 (s, 1H, H-7), 6.35 (d, 1H, $J = 10.8$ Hz, H-1), 5.97 (d, 1H, $J = 10.8$ Hz, H-2), 5.96 (s, 2H, -OCH_2O-), 5.32–5.28 (m, 1H, H-3), 4.39 (d, 1H, $J = 16.5$ Hz, H-6β), 3.85 (d, 1H, $J = 16.5$, H-6α), 3.67 (s, 2H, H-12), 3.56 (*dd*,1H, $J = 14.2$ Hz, 7.7 Hz, H-4a), 3.45–3.40 (m, 1H, H-11), 2.15–2.07 ppm (m, 2H, H-4); HRMS *m/z* (ESI$^+$) calcd for $C_{18}H_{20}N_3O_6$ $[M+H]^+$ 374.1352, found 374.1352.

Compound 6: Diacetate **1** (5.0 mg, 0.0174 mmol) was dissolved in acetone (6 mL) and a solution of OsO_4 (0.25 mL, 2.5% wt solution in *t*BuOH) and NMO (0.038 g, 0.284 mmol) in H_2O (1.5 mL) were added over 5 min. The solution was stirred at room temperature for 6 h after which the solvents were evaporated under reduced pressure. To the obtained slurry were added EtOAc (2 mL) and saturated NH_4Cl (1 mL), followed by washing with saturated NaCl (1 mL). The extraction was repeated twice with EtOAc (2 mL), and the combined organic layers were dried with $MgSO_4$. The solvent was removed under reduced pressure and subjected to PTLC using CH_2Cl_2/MeOH (4:1), yielding an inseparable mixture of diastereomers. The mixture was subjected to standard acetylation conditions (2.2 equiv Ac_2O, pyridine, DMAP) in an attempt to resolve the mixture which yielded 4.9 mg (59%) of another inseparable mixture of the diastereomeric tetraacetates **6** that were biologically evaluated as a mixture; HRMS *m/z* (ESI$^+$) calcd for $C_{24}H_{28}NO_{10}$ $[M+H]^+$ 490.1713, found 490.1704.

Compound 7: An oven-dried flask was charged with bulbispermine (10.0 mg, 0.0348 mmol) and Dess–Martin periodinane (0.044 g, 0.103 mmol) and was subjected to several cycles of vacuum-argon purge. Dry toluene (2 mL) was added, and the reaction suspension

was heated at 65 °C for 2 h until the substrate was completely consumed. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by PTLC using CH_2Cl_2/MeOH (9:1), yielding 3.7 mg of the allylic ketone (38%). ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 7.69$ (d, 1H, $J = 10.2$ Hz, H-2), 7.11 (s, 1H, H-10), 6.69 (s, 1H, H-7), 6.24 (d, 1H, $J = 10.2$ Hz, H-1), 5.97 (d, 2H, $J = 5.4$ Hz, -OCH_2O-), 4.58 (d, 1H, $J = 17.1$ Hz, H-6β), 3.98 (d, 1H, $J = 17.1$ Hz, H-6α), 3.91 (dd, 1H, $J = 13.2$ Hz, 6.3 Hz, H-4a), 3.80 (d, 1H, $J = 18.6$ Hz), 3.51 (d, 1H, $J = 18.6$ Hz), 3.25 (d, 1H, $J = 11.6$ Hz), 2.76–2.61 ppm (m, 2H, H-4). ^{13}C NMR (CD_3OD, 400 MHz): $\delta = 206.5$ (C3), 197.0 (C9), 148.1 (C8), 143.7 (C10a), 130.7 (C1), 126.9 (C6a), 125.7 (C2), 107.0 (C10), 103.3 (-OCH_2O-), 101.5 (C11), 66.0 (C4a), 58.9 (C12), 58.3 (C6), 48.5 (C10b), 39.4 ppm (C4); HRMS *m/z* (ESI^+) calcd for $C_{16}H_{16}NO_4$ $[M+H]^+$ 286.1079, found 286.1046.

Compound 8: A solution of bulbispermine (10.0 mg, 0.0348 mmol) in MeOH (4.2 mL) was charged with Pd/C 5% (11.5 mg) and purged with an H_2 balloon. The stirred reaction mixture was kept under an H_2 atmosphere overnight at room temperature, after which the solution was filtered through Celite and the solvent evaporated under reduced pressure. The resulting white solid was purified by PTLC using CH_2Cl_2/MeOH (9:1) to yield 7.4 mg of reduced product (74%). ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 6.76$ (s, 1H, H-10), 6.51 (s, 1H, H-7), 5.89 (s, 2H, -OCH_2O-), 4.30 (d, 1H, $J =$ 16.7 Hz, H-6β), 4.04–4.03 (m, 1H, H-3), 3.75 (d, 1H, $J = 16.7$ Hz, H-6α), 3.67–3.61 (m, 1H, H-11), 3.40–3.39 (m, 1H, H-4a), 3.24 (dd, 1H, $J = 11.3$ Hz, 5.1 Hz, H-2), 3.09 (dd, 1H, $J = 12.8$ Hz, 4.84 Hz, H-2), 2.68 (dd, 1H, $J = 14.0$ Hz, 4.0 Hz, H-1), 2.12–2.03 (m, 2H, H-4), 1.99–1.78 ppm (m, 3H); ^{13}C NMR (CD_3OD, 400 MHz): $\delta = 146.9$ (C8), 146.2 (C9), 138.7 (C10a), 124.6 (C6a), 105.7 (C7), 103.2 (C10), 100.8 (-OCH_2O-), 81.2 (C11), 68.6 (C3), 66.9 (C4a), 62.0 (C12), 59.8 (C6), 45.8 (C10b), 35.4 (C4), 32.1 (C2), 25.6 ppm (C1); HRMS *m/z* (ESI^+) calcd for $C_{16}H_{20}NO_4$ $[M+H]^+$ 290.1392, found 290.1399.

Compound 9: To a solution of bulbispermine (5.0 mg, 0.0174 mmol) in dry MeCN (0.3 mL) was added 1.1 equiv MeI. After 5 min, the reaction turned milky, and was reacted at room temperature until starting material had been totally consumed after 2 h. The resulting precipitate was filtered and washed with cold MeCN, followed by $CHCl_3$, resulting in 7.3 mg of a white solid ammonium salt (97%). ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 7.02$ (s, 1H, H-10), 6.71 (s, H-7), 6.23–6.17 (m, 2H, H-1, H-2), 6.01 (s, 2H, -OCH_2O-), 4.69 (d, 1H, $J = 15.8$ Hz, H-6α), 4.62 (s, 1H), 4.44–4.39 (m, 1H, H-3), 3.79–3.75 (m, 2H), 3.35 (s, 3H, N-CH_3), 2.50–2.40 ppm (m, 2H, H-4); HRMS *m/z* (ESI^+) calcd for $C_{17}H_{20}NO_4$ $[M]^+$ 302.1392, found 302.1385.

Biochemical experiments

The overall growth level of human cancer cell lines was determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]diphenyltetrazolium bromide; Sigma, Belgium) assay.[3a,d] Briefly, the cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10000–40000 cells per 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 capacity of living cells to convert the yellow compound MTT into a blue product, formazan, by a reduction reaction occurring in the mitochondria. The number of living cells after 72 h culture in the presence (or absence: control) of the various compounds is directly proportional to the intensity of the blue, which is quantitatively measured by spectrophotometry—in our case with a Model 680XR instrument (Bio-Rad, Nazareth, Belgium)—at λ 570 nm (with reference at λ 630 nm). One set of experimental conditions included six replicates. Each experiment was carried out twice.

Cell proliferation was visualized by computer-assisted phase-contrast microscopy.[3a,d,15] The fibrillar actin cytoskeleton organization was determined by computer-assisted fluorescence microscopy.[3a,d] Phallacidin conjugated with the green-fluorescent Alexa Fluor488 (Molecular Probes Inc., Eugene, OR, USA) was used to label the fibrillar actin.

Computer modeling

Conformations of narciclasine, lycorine, and bulbispermine were generated using Accelrys Discovery Studio 3.1. All conformers were minimized, and structures with a RMSD > 0.2 were retained. For the purposes of the overlay, the lowest-energy conformation was selected for each structure, and superimposition using tethers was employed to bring into alignment the dioxolane and aromatic regions of the structures.

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