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3β-Hydroxy-6-aza-cholestane and Related Analogues as Phosphatidylinositol Specific Phospholipase C (PI-PLC) Inhibitors with Antitumor Activity

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Abstract—6-Aza steroid analogues were synthesized as PI-PLC inhibitors. The most active compound, 3β-hydroxy-6-aza-cholestane (**1**) showed potent PI-PLC inhibition ($IC_{50} = 1.8$ μM), similar to that of the commercially available steroid analogue U73122 ($IC_{50} = 1$–2.1 μM). Compound **1** exhibited significant growth inhibition effects ($IC_{50} = 1.3$ μM in each case) against MCF-7 and HT-29 cancer cells in in vitro cell culture. Compound **1** also inhibited the in vitro adhesion and transmigration of HT-1080 fibrosarcoma cells at 2.5 and 5.0 μM, respectively. In vivo, compound **1**, at 1 mg/kg/day, reduced the volume of MCF-7 tumors in xenograft models, without weight loss in mice. Structure–activity relationships of this series of compounds revealed that a hydrophobic cholesteryl side chain, 3β-hydroxy group and a C-6 nitrogen containing a hydrogen atom at position-6 are crucial for activity. *N*-Maleic amidoacid derivative **11** also exhibited weak inhibition ($IC_{50} = 16.2$ μM). © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Growth factors and certain oncogenes activate a range of phospholipid-mediated signal transduction pathways. Phosphatidylinositol specific phospholipase C (PI-PLC), an important enzyme for intracellular signaling, hydrolyses a minor membrane phospholipid, phosphotidylinositol (4,5)- biphosphate (PI $(4,5)P_2$) to give the second messengers inositol(1,4,5)- triphosphate ($I(1,4,5)P_3$), and diacylglycerol. The former releases Ca^{2+} from intracellular stores to increase intracellular free Ca^{2+} concentration while the latter activates the Ca^{2+} and phospholipid-dependent protein serine/threonine kinase, protein kinase C. Together, the increase in intracellular free Ca^{2+} and the activation of protein kinase C lead to a series of events that culminate in DNA synthesis and cell proliferation.

Several lines of evidence suggest that PI-PLC offers a good target for the development of drugs to inhibit the growth of cancer cells; some examples follow.[1] Most tyrosine kinase growth factor receptors lead to stimulation of PI-PLCγ1 in cells.[2] Microinjection of PI-PLCβ or PI-PLCγ into quiescent NIH 3T3 cells results in dose-dependent acute induction of DNA synthesis.[3] Microinjection of antibodies against PI-PLCγ but not against PI-PLCβ into NIH 3T3 cells blocks both serum stimulated and *ras*-stimulated DNA synthesis.[4] Specific mutation-restoration of PI-PLCγ binding to tyrosine-mutated PDGF (platelet derived growth factor) receptor is sufficient to confer a mitogenic response to PDGF.[5] A transforming Her2/*neu* oncogene shows constitutive tyrosine phosphorylation and activation of PI-PLCγ, while a non-transforming, kinase-defective mutant does not.[6]

PI-PLC activity in rat hepatomas is increased in the more aggressively growing tumors[7a] and PI-PLC activity is found to be increased in a number of human tumors. It has been reported that 76% of human breast cancers have detectable PI-PLCγ immunoreactive protein compared to only 6% of benign breast tissue.[7b] Cytosolic PI-PLC activity was found to be increased up to >4-fold in human non-small cell lung cancer and renal cell cancer compared to normal tissue, and was correlated directly with increased EGF (epidermal

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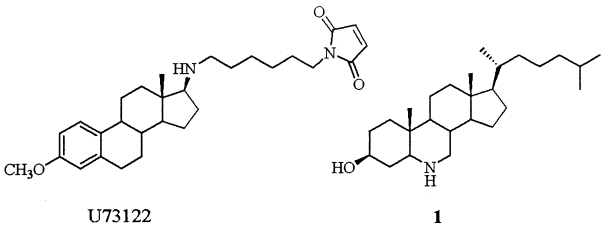
growth factor) receptor levels in the tumor.[8] An association between PI-PLCγ immunoreactivity and EGF receptor expression has been reported in human glial tumors.[9] Furthermore, PI-PLCγ activation was implicated to promote tumor invasion through EGF receptor mediated intracellular signaling. Finally, the growth inhibitory effect of tamoxifen on GH_4 C_1 cancer cells has been linked to inhibition of PI-PLC.[10c]

Screening of over 150 bacterial products as inhibitors of PI-PLCγ1 yielded five compounds in the micromolar range.[1] Two of these were the aminoglycoside antibiotics tobramycin and rhodomycin. Aminoglycoside antibiotics had been previously reported to inhibit PI-PLC.[11] Tobramycin and rhodomycin were also found to inhibit colony formation by HT-29 human colon adenocarcinoma cells. The three compounds found as inhibitors in the screen were the peptides myroridin K, streptothricin B and edeine. They were likewise found to inhibit colony formation by HT-29 human colon adenocarcinoma cells.[1] There is an urgent need for small-molecule inhibitors of PI-PLC. Two such compounds are commercially available. One of these is the surfactant-like molecule 1-*O*-octadecyl-2-*O*-methyl-*ras*-glycero-3-phosphorylcholine[12a](IC_{50} = 15 μM) and the other is the steroid analogue {1-[6-((17β-3-methoxyestra-1, 3, 5(10)-trien-17yl)aminohexyl]-1H-pyrrole-2, 5-dione}[12] U73122 (Fig. 1, IC_{50} = 1–2.1 μM). Since the dihydro analogue of U73122 is reported to be a very weak inhibitor of PI-PLC[12b] and is sold as a negative control, the mechanism of action of U73122 appears to be related to the double bond in the pyrrole-2, 5-dione ring. U73122, a specific PI-PLCγ inhibitor, or overexpressed dominant negative PI-PLCγ was found to greatly diminish invasiveness of DU-145 prostate tumor cells.[10]

During the course of a random screening, an impure sample of 3β-hydroxy-6-aza-cholestane (**1**) was found to inhibit PI-PLC (IC_{50} = 23 μM) and to show cytotoxicity to HT-29 human colon adenocarcinoma cells (IC_{50} = 2 μM). On the basis of this observation, we synthesized a pure sample of 3β-hydroxy-6-aza-cholestane (**1**) and related analogues for a structure–activity relationship study. Here we report the syntheses and biological activities of these compounds.

Chemistry

3β-Hydroxy-6-aza-cholestane (**1**) and related analogues were synthesized from cholesterol acetate. Ozonolysis of cholesterol acetate, as previously described,[13] gave 3β-acetoxy-5-oxo-5, 6-seco-cholestan-6-oic acid (**2**), which was converted into a separable mixture of 6-aza-cholest-3, 5-diene (**3**) and 3β-hydroxy-6-aza-cholest-5-ene (**4**) by classical Curtius rearrangement and hydrolysis,[14,15] or less efficiently by use of diphenyl-phosphoryl azide. Catalytic hydrogenation (Pd/C) of **4** gave the target compound 3β-hydroxy-6-aza-cholestane (**1**). In a similar manner, catalytic reduction of **3** gave the deoxy analogue 6-aza-cholestane (**5**). Acetylation of **4** and **1** gave the corresponding diacetyl derivatives **6** and **7**, respectively.



U73122 **1**

Figure 1. Chemical structures of U73122 and compound **1**.

Reaction of compound **1** with either dimethyl or diethyl acetylenedicarboxylate in methanol gave the fumarate esters **8** and **9**, respectively. The fumarate rather than the maleate structures are presumed based on prior literature[16] wherein the olefinic protons in fumarates appear at lower field in the ^{1}H NMR spectra than the corresponding maleate protons. In impure samples of **8** and **9**, it was possible to see signals for the minor components (ratios of minor to major ~1–7) upfield of the major components. By treatment of compound **1** with succinic, maleic, glutaric and phthalic anhydrides in DMF, the amidoacids **10**–**13**, respectively, were obtained.

To determine the effect of the lipophilic side chain in the D-ring on PI-PLC activity, 6-aza-pregnan-20-one (**14**) and 3β, 20-dihydroxy-6-aza-pregnane (**15**) were synthesized from pregnenolone acetate as previously described.[15]

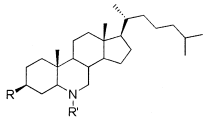
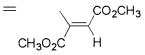
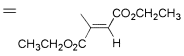
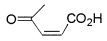
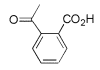
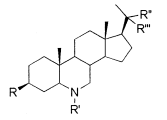
Results and Discussion

Structure–activity relationships

The PI-PLC inhibitory activities of compounds **1** and **3**–**15** are shown in Table 1. Compound **1** exhibited good inhibition (IC_{50} = 1.8 μM), similar to the commercially available steroid analogue U73122 (IC_{50} = 1–2.1 μM). As previously mentioned, the mechanism of action of U73122 appears to be related to the double bond in the pyrrole-2, 5-dione ring. Therefore, we synthesized some N-substituted derivatives of 3β-hydroxy-6-aza-cholestane (**1**) possessing double bonds conjugated to carbonyl groups, capable of acting as Michael acceptors, by analogy to the presumed mechanism of action of U73122. Michael addition of **1** to either dimethyl or diethyl acetylenedicarboxylate in methanol gave the fumarate esters **8** and **9**, respectively; both were found to be inactive. Reaction of **1** with succinic, maleic, glutaric and phthalic anhydrides in DMF gave the amidoacids **10**–**13**, respectively. Compounds **10**, **12** and **13** exhibited no activity, while maleic amidoacid **11**, with a double bond somewhat analogous to that in U73122, showed weak PI-PLC inhibition (IC_{50} = 16.2 μM).

The presence of a double bond at position C-5 (**4**) and conjugated double bonds at C-3 and C-5 (**3**) resulted in loss of inhibition. Acylation of the amino and 3-hydroxyl groups, as in **6** and **7**, resulted in loss of activity as observed for compounds **10**, **12** and **13**. The absence of the 3β-hydroxyl group as in **5** also resulted in loss of

Table 1. Effects of compounds **1** and **3**–**15** on PI-PLC,[a] growth inhibition for MCF-7 and HT-29 cells[b]

No.	Structures	PI-PLC IC_{50} (μM)	Growth inhibition	
			MCF-7 IC_{50} (μM)	HT-29 IC_{50} (μM)
				
1	R = OH, R′ = H	1.8 ± 0.0	1.3 ± 0.1	1.3 ± 0.2
3	R = H, $\Delta^{3,5}$	NA[c]		
4	R = OH, Δ^5	NA	1.3 ± 0.1	1.9 ± 0.1
5	R = R′ = H	NA		
6	R = OAc, R′ = Ac, Δ^4	NA		
7	R = OAc, R′ = Ac	NA		
8	R = OH, R′ = 	NA	2.3 ± 0.4	3.4 ± 0.3
9	R = OH, R′ = 	NA		
10	R = OH, R′ = -$CO(CH_2)_2CO_2H$	NA		
11	R = OH, R′ = 	16.2 ± 3.2		
12	R = OH, R′ = -$CO(CH_2)_3CO_2H$	NA		
13	R = OH, R′ = 	NA		
				
14	R = R′ = H, R″ = R‴ = O	NA		
15	R = OH, R′ = R″ = H, R‴ = OH	NA		

[a]Inhibition of PI-PLC activity was measured using bovine brain PI-PLCγ.[1,17]
[b]Cell growth inhibition was measured for MCF-7 cells by inhibition of monolayer growth over 5 days drug exposure and for HT-29 cells by inhibition of growth in soft agarose over 5 days.[1,17]
[c]NA is $IC_{50} > 100$ μg/mL.

inhibition. Replacement of the hydrophobic alkyl side chain in **1**, as in **14** and **15**, led to loss of inhibition, regardless of the presence of the C-3 OH group.

In summation, the PI-PLC activity of **1** is lost by removal of the C-3 OH group, acylation of the C-6 nitrogen or removal of the lipophilic C-17 side chain. Inhibition by an apparently different mechanism, involving the double bond system as in U73122, is observed for the maleic amidoacid **11**.

Colonogenic assays for HT-29 and MFC-7 tumor cell lines

Compound **1** was tested for its growth inhibition effects on HT-29 colon cancer and MCF-7 breast cancer cell lines in cell cultures[1,17] and exhibited an IC_{50} of 1.3 μM in each case (see Fig. 2). It is interesting that compounds **4** and **8**, while showing no PI-PLC inhibition, exhibited excellent growth inhibition of HT-29 and MCF-7 cancer cells; compound **4**, IC_{50} of 1.3 and 1.9 μM, and compound **8**, IC_{50} of 2.3 and 3.4 μM, respectively (Fig. 3). Compound **9** was not tested for growth inhibition, but presumably would be similar to **8**. The mechanism of growth inhibition for compounds **4** and **8** are not presently known.

NCI 60-human tumor cell line screening

The in vitro cytotoxicity of compounds **1** and **11** were evaluated at the National Cancer Institute against a panel of 60 human tumor cell lines representing nine different cancer types. The GI_{50}, TGI and GI_{50} values are shown in Table 2. Compound **1** showed a mean GI_{50} value (MG-MID) of 3.80 μM for 54 tumors. It was more selective against colon cancer lines HCC-2998 ($GI_{50} = 0.91$ μM) and HT-29 ($GI_{50} = 0.95$ μM), and melanoma cancer cell lines LOX IMVI ($GI_{50} = 1.00$ μM). TGI and LC_{50} values were in accord with the above GI_{50} values but of lower potency. It also showed some selective inhibition on BT-549 breast cancer cell with GI_{50} of 3.03 μM.

Compound **11**, which was a less potent PI-PLC inhibitor than compound **1**, was more potent than **1** against human cancer cell lines (MG-MID 3.09 μM), and more selectively inhibited NCI-H226 ($GI_{50} = 0.46$ μM, TGI = 0.91 μM, $LC_{50} = 1.79$ μM) and NCI-H460

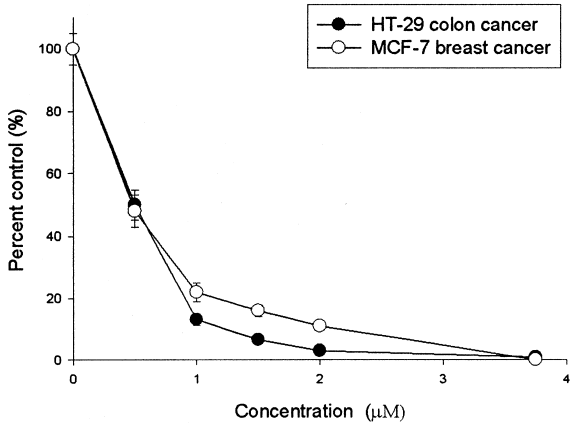


Figure 2. Inhibition of proliferation of HT-29 and MCF-7 cells by compound **1.** Cell growth inhibition was measured for MCF-7 cells by inhibition of monolayer growth over 5 days drug exposure and for HT-29 cells by inhibition of growth in soft agarose over 5 days.[1,17]

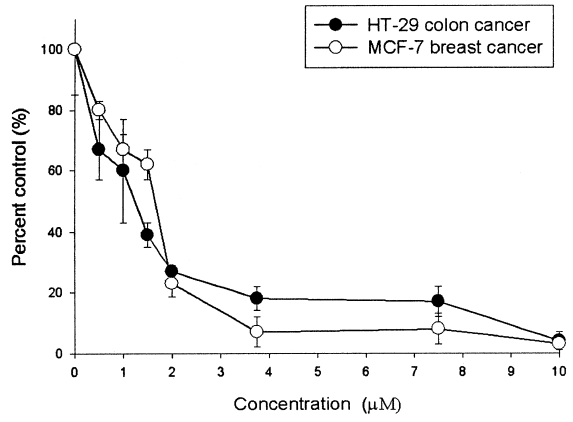


Figure 3. Inhibition of proliferation of HT-29 and MCF-7 cells by compound **8**. Cell growth inhibition was measured for MCF-7 cells by inhibition of monolayer growth over 5 days drug exposure and for HT-29 cells by inhibition of growth in soft agarose over 5 days.[1,17].

(GI_{50} = 0.56 μM, TGI = 1.37 μM, LC_{50} = 8.52 μM) non-small cell lung cancer cell lines, HCC-2998 (GI_{50} = 0.47 μM, TGI = 0.89 μM, LC_{50} = 1.67 μM) colon cancer cell line and LOX-IMVI (GI_{50} = 0.82 μM, TGI = 1.86 μM, LC_{50} = 6.13 μM) melanoma cancer cell line. It also showed inhibitions of HT-29, KM-12, SW-620 colon cancer cells with GI_{50} of 1.39, 1.43, 0.78 μM, and MDA-MB-435 breast cancer cell line with GI_{50} = 1.65 μM, respectively. Compounds **1** and **11** both showed selective inhibitions in the panels of colon cancer cell lines and breast cancer cell lines, and compound **11** exhibited good selectivity for non-small cell lung cancer cell lines. This is consistent with the observation that PI-PLC activity is found to be increased in a number of human tumors, like human breast cancers, human non-small cell lung cancer and colon cancer.[7,8]

Effects of compound 1 on the in vitro adhesion and invasion of HT-1080 cells

Accumulating evidence suggests that both the integrin and growth factor mediated signal transduction pathways may be involved in tumor cell adhesion, invasion and metastasis, although the detailed mechanisms have not been well characterized. PI-PLCγ activation was implicated to promote tumor invasion through EGFR mediated intracellular signaling. U73122, a specific PI-PLCγ inhibitor, or overexpressed dominant negative PI-PLCγ was found to greatly diminish invasiveness of DU-145 prostate tumor cells.[10] HT-1080 cells have shown a high invasive ability to penetrate reconstituted basement membrane and artificial blood vessel walls.[18] This cell line has been used as a target for studying new materials that inhibit the adhesion and migration of tumor cells through extracellular matrix (ECM) substrate.[19] The present data show that compound **1**, at a non-cytotoxic concentration (2.5 μM), significantly inhibited the adhesive abilities of HT-1080 cells to matrigel-coated or plastic surfaces (Fig. 4). In addition, 5 μM of compound **1** inhibited the ability of HT-1080 cells to transmigrate matrigel (Fig. 5). Thus, accumulating evidence suggests that PI-PLCγ may play an active role in the in vitro adhension and transmigration of HT-1080 tumor cells.

Table 2. NCI 60-human cell line screening of compounds **1** and **11**

Cell lines	1			11		
	GI_{50}[a]	TGI[b]	LC_{50}[c]	GI_{50}[a]	TGI[b]	LC_{50}[c]
Non-small cell lung cancer						
NCI-H226	5.95	14.1	>25.0	0.46	0.91	1.79
NCI-H460	ND[e]	ND	ND	0.56	1.37	8.52
Colon cancer						
HCC-2998	0.91	3.52	10.0	0.47	0.89	1.67
HT-29	0.95	3.66	9.56	1.39	4.46	11.0
KM-12	3.41	6.62	12.9	1.43	6.33	21.6
SW-620	2.19	>25.0	>25.0	0.78	2.60	9.63
Melanoma						
LOX IMVI	1.00	3.59	11.3	0.82	1.86	6.13
Breast cancer						
MDA-MB-435	3.89	7.24	13.5	1.65	6.06	17.5
BT-549	3.03	6.45	13.8	4.89	8.60	15.1
MG-MID[d]	3.80	8.62	16.9	3.09	7.77	15.6
Delta[f]	4.17	2.5	1.74	6.76	8.91	9.33

[a]GI_{50} represents the compound concentration (μM) required to achieve 50% inhibition of tumor cell growth.
[b]TGI represents the compound concentration (μM) required to achieve total growth inhibition of tumor cell.
[c]LC_{50} represents the compound concentration (μM) that is lethal to the survival of 50% tumor cell.
[d]MG-MID represents the calculated mean GI_{50}, TGI and LC_{50} for all panels.
[e]Not determined.
[f]Delta represents the greatest margin of the GI_{50}, TGI, and LC_{50} values for all panels, respectively.

In vivo antitumor activity of compound 1 against MCF-7 human breast cancer

To examine the antitumor activity of compound **1** in vivo, groups of eight mice were transplanted with MCF-7 xenografts and treated with compound **1** (Fig. 6). Tumor volume measurements and mice weights were conducted on the days indicated in Figure 6. The results demonstrated that the tumor volume in 1 mg/kg/day

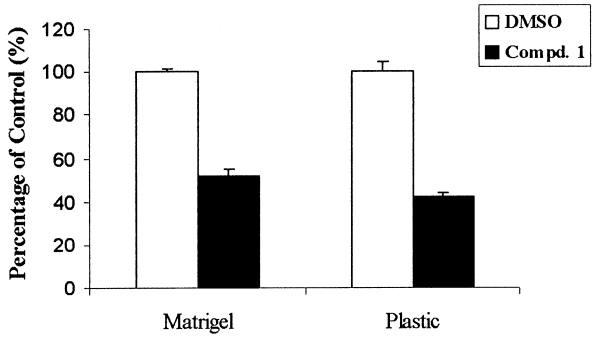


Figure 4. HT-1080 tumor cell adhesion by compound **1.** Inhibition of adhesion of HT-1080 cells to matrigel and plastic surfaces by compound **1**. Radioactive labeled HT-1080 cells were treated with 2.5 μM of **1** for 72 h before being placed on matrigel-coated plastic surfaces. After 90 min incubation, the cells binding to the matrigel plastic surface was quantified by radioactivity counting. Percentage cell adhesion was shown as the mean ± SD of three determinants, and values were calculated as a percentage of the DMSO control, taken as 100% cell adhesion.

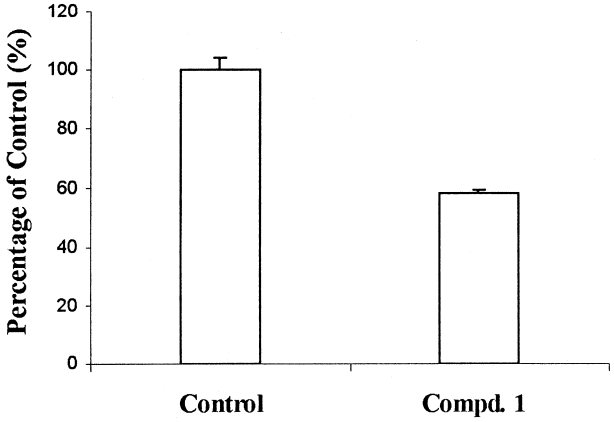


Figure 5. Inhibition of HT-1080 tumor cell invasion by compound **1.** Inhibition invasion of HT-1080 cells through matrigel-coated filter by compound **1**. ^{51}Cr labeled HT-1080 cells were placed on matrigel-coated filters (8 μm pores) in the presence of 5 μM compound **1** for 24 h. Invasion was measured as the percentage of HT-1080 cells traversed from the upper compartment of the transwell across the filter into the low compartment, by assessing the radioactivity associated with the cells below the filter (lower compartments) compared to the radioactivity of the total cells originally seeded on the upper compartments. The invasion was expressed as the mean ± SD of three determinants, and values were calculated as a percentage of the DMSO controls (100% cell invasion).

dose of compound **1** treated group significantly decreased as compared to controls (tumor volumes of 0.20 and 0.58 cm^3 at day 28, respectively). Treatment of mice with 1 mg/kg/day of compound **1** resulted in no weight loss compared to controls and at a later time point the weight gain was more than that of control mice (mice weight of 22 and 18 g, respectively). Treatment of mice with 2 mg/kg/day of compound **1** resulted in the death of the mice at day 12, revealing toxicity at higher doses.

Conclusion

A number of 6-aza steroid analogues were synthesized as PI-PLC inhibitors. The most active compound, 3β-hydroxy-6-aza-cholestane (**1**), showed potent PI-PLC inhibition (IC_{50} = 1.8 μM), similar to that of the commercially available steroid analogue U73122 (IC_{50} = 1–2.1 μM). Compound **1** also exhibited significant growth inhibition effects (IC_{50} = 1.3 μM in each case) against MCF-7 and HT-29 cancer cells in in vitro cell culture study. Compound **1** also inhibited the in vitro adhesion and transmigration of HT-1080 fibrosarcoma cells at 2.5 μM and 5.0 μM, respectively. Antitumor activity of compound **1** was demonstrated with the MCF-7 xenograft model where there was a significant decrease in tumor volume using 1 mg/kg/day. However, at 2 mg/kg/day compound **1** exhibited toxicity. Structure–activity relationships in this series of compounds revealed that a hydrophobic cholesteryl side chain, a 3β-hydroxy group and a free amino group at position-6 are crucial for activity. The *N*-maleic amidoacid derivative **11** showed weak inhibition (IC_{50} = 16.2 μM), presumably by a different mechanism. Two analogues, **4** and **8**, which showed no PI-PLC inhibition, exhibited excellent growth inhibition of HT-29 and MCF-7 cancer cells in the in vitro colonogenic assay.

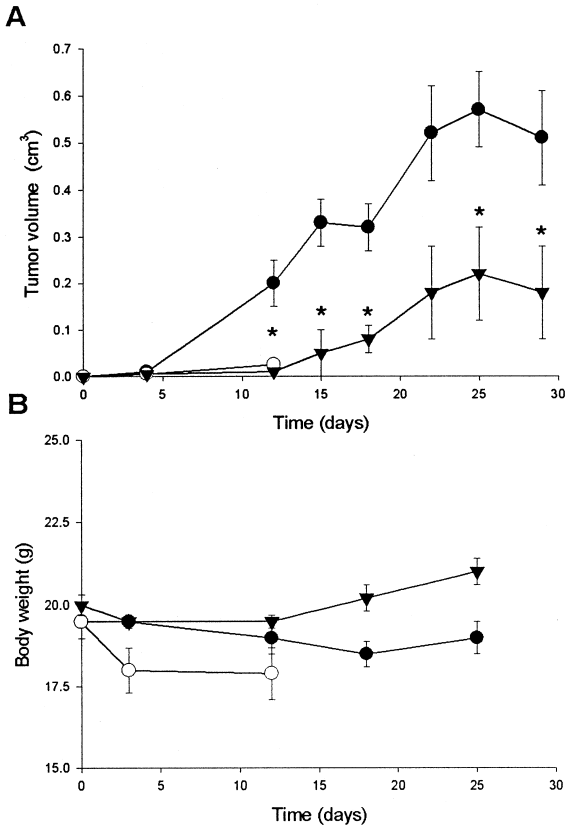


Figure 6. In vivo antitumor activity of compound **1** against MCF-7 breast cancer. Antitumor activity was tested against MCF-7 human breast cancer xenografts in the scid mouse. Groups of 8 mice were transplanted sc with 2×10^6 MCF-7 human breast cancer cells. Treatment with compound was begun ip on day 4 and continued daily for 14 days at 1 (▼) and 2 (○) mg/kg. Control mice received vehicle alone (●). All the mice receiving 2 mg/kg compound **1** died by day 12. The upper panel shows tumor volume and the lower panel body weight. Each point is the mean of 8 mice and the bars are S.E. *Indicates significantly different from the vehicle treated control. Details of the determination procedures have been reported previously.[21]

Experimental

Starting materials were purchased from Aldrich unless otherwise indicated. Thin layer chromatography analysis (TLC) was performed on aluminum sheets precoated with 0.2 mm of silica gel containing 60F254 indicator. Flash chromatography was run using 230–400 mesh silica gel. Reverse phase high performance liquid chromatography (HPLC) was run on a Phenomenex® LUNA 5μ C18 semi-preparative column. The homogeneity of all compounds was routinely checked by TLC on silica gel plates, and by HPLC. Fourier transformed infrared spectra were obtained on a Nicolet 520 FTIR spectrometer. ^{1}H (300 or 400 MHz), ^{13}C (75 or 100 MHz) NMR and DEPT spectra were recorded on either a Varian Gemini-300 or on a Varian XL-400 spectrometer. High-resolution mass spectra (EI or FAB) were recorded on a VG Analytical 70-SE mass spectrometer equipped with a 11-250J data system. Melting points were uncorrected. Elemental analyses were performed by Atlantic Microlab, Norcross, GA.

3β-Acetoxy-5-oxo-5,6-seco-cholestan-6-oic acid (2). Ozone was passed through a solution of 5 g (11.7 mmol) of cholesterol acetate in 300 mL petroleum ether at −60 °C until the solution turned a light blue color (40 min). Addition of piperidine (5 mL) and stirring at −60 °C yielded the piperidinium salt. Filtration gave 3.2 g of the piperidinium salt and concentration of the mother liquid gave an additional 1.6 g. The combined piperidium salt (4.8 g) was dissolved in 80 mL choloroform, the solution washed with 2M HCl (3×10 mL), then with water and dried over anhydrous Na_2SO_4. The residue of the evaporated solution was subjected to flash column chromatography on silica gel (petroleum ether:ethyl acetate 3:1) to yield 3-acetoxy-5-oxo-5, 6-seco-cholestan-6-oic acid (**2**), 3.3 g (6.9 mmol), 59% yield: mp 127–129 °C [lit[12] 129–130°] from petroleum ether; ^{1}H NMR ($CDCl_3$): δ 0.68 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.86 (d, J = 6.6 Hz, 3H), 1.04 (s, 3H), 2.01 (s, 3H), 2.24 (m, 2H), 2.43 (d, J = 14.3 Hz, 1H), 3.19 (dd, J = 14.3 Hz, J' = 4.4 Hz, 1H), 5.38 (br, 1H); FABMS m/z (relative intensity): 477 ($M^+ + 1$, 5%), 417, ($M^+ + 1$-AcOH, 88), 399 (100) 357 (40), 331 (45); EIMS m/z (relative intensity): 476 (M^+, 10), 416 (M^+-AcOH, 43), 398 (M^+-AcOH-H_2O, 21), 110 (100).

6-Aza-cholesta-3,5-diene (3) and 3β-hydroxy-6-aza-cholest-5-ene (4). 3β-Acetoxy-5-oxo-5, 6-seco-cholestan-6-oic acid (**2**) was converted into a mixture of 6-aza-cholesta-3, 5-diene (**3**) and 3β-hydroxy-6-aza-cholest-5-ene (**4**) as previously described.[13,14] The structures of the intermediate acid chloride and acyl azide were confirmed by their IR spectra (ν 1801 and 2134 cm^{-1}, respectively). Curtius rearrangement of the acyl azide occurred in benzene at reflux, yielding the isocyanate (ν 2275 cm^{-1}), which without further purification, was treated with 12 N HCl in acetone at reflux and after removal of the solvent 5% KOH in methanol at reflux. Upon extraction of the residue, obtained by evaporation of the latter, with chloroform:ethyl acetate (2:1), and drying over anhydrous Na_2SO_4, a crude mixture of **3** and **4** was obtained. Washing with ether afforded 3β-hydroxy-6-aza-cholest-5-ene (**4**): mp 170–172 °C [lit.[12] 172–175°] from acetone; ^{1}H NMR ($CDCl_3$): δ 0.66 (s, 3H), 0.83 (dd, J = 1.2 Hz, J' = 6.6 Hz, 6H), 0.88 (d, J = 6.6 Hz, 3H), 1.10 (s, 3H), 2.49 (m, 2H), 2.93 (m, 1H), 3.70 (m, 2H); EIMS m/z (relative intensity): 387 (M^+, 5), 369 (100, M^+-H_2O); HRMS calcd for $C_{26}H_{45}NO$ 387.3501, found 387.3506. Evaporation of the mother liquid and washing with petroleum ether gave 6-aza-cholesta-3, 5-diene (**3**)[13,14] as an amorphous solid; ^{1}H NMR ($CDCl_3$): δ 0.66 (s, 3H), 0.83 (dd, J = 1.2 Hz, J' = 6.6 Hz, 6H), 0.89 (d, J = 6.6 Hz, 3H), 1.41 (s, 3H), 2.38 (m, 2H), 5.69 (m, 1H), 5.84 (m, 1H); FABMS m/z (relative intensity): 370 ($M^+ + 1$, 30), 293 (100); HRMS calcd for $C_{26}H_{44}N$ ($M^+ + 1$) 370.3474, found 370.3476.

3β-Hydroxy-6-aza-cholestane (1). 3β-hydroxy-6-aza-cholest-5-ene (**4**) (100 mg), dissolved in 20 mL glacial acetic acid containing 15 mg 10% Pd/C, was hydrogenated at atm pressure and 40 °C for 6 h. Filtration, evaporation of solvent, adjustment to pH 8, extraction with chloroform and finally concentration of solvent gave 3β-hydroxy-6-aza-cholestane (**1**), 70 mg, 69%: mp 145–147 °C [lit[13] 147–148°] from petroleum ether/ether; ^{1}H NMR ($CDCl_3$): δ 0.65 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.89 (d, J = 6.6 Hz, 3H), 0.94 (s, 3H), 1.97 (m, 1H), 2.38 (m, 1H), 2.97 (dd, J = 2.2 Hz, J' = 6.9 Hz, 1H), 3.63 (m, 1H); EIMS m/z (relative intensity): 389 (M^+, 60), 374 (M^+-CH_3, 50), 316 (100); HRMS calcd for $C_{26}H_{47}NO$ 389.3658, found 389.3656; anal. ($C_{26}H_{47}NO$) C, H, N.

6-Aza-cholestane (5). Catalytic reduction of 6-aza-cholesta-3, 5-diene (**3**) as described above gave 6-aza-cholestane (**5**)[14] as an amorphous solid in 60% yield; ^{1}H NMR ($CDCl_3$): δ 0.66 (s, 3H), 0.85 (d, J = 6.6 Hz, 6H), 0.89 (d, J = 6.6 Hz, 3H), 0.94 (s, 3H), 1.98 (m, 1H), 2.38 (m, 1H), 2.97 (dd, J = 2.2 Hz, J' = 6.6 Hz, 1H); EIMS m/z (relative intensity): 373 (M^+, 100), 358 (M^+-CH_3, 45), 330 (20), 316 (92); HRMS calcd for $C_{26}H_{47}N$ 373.3709, found 373.3702.

3β-Acetoxy-6-acetyl-6-aza-cholest-4-ene (6). Compound **4** (20 mg, 0.05 mmol) on treatment with Ac_2O/pyridine/cat. DMAP, at room temperature for 4 h, gave, after extraction with CH_2Cl_2, a quantitative yield of **6**: mp 124–126 °C [lit[14] 122–123°] from acetonitrile; ^{1}H NMR ($CDCl_3$): δ 0.64 (s, 3H), 0.83 (d, J = 6.6 Hz, 6H), 0.87 (d, J = 6.6 Hz, 3H), 1.04 (s, 3H), 2.04 (s, 3H), 2.07 (s, 3H), 2.17 (m, 1H), 4.59 (m, 1H), 5.28 (m, 1H); FABMS m/z (relative intensity): 472 ($M^+ + 1$, 25), 412 ($M^+ + 1$-CH_3COOH, 100), 370 (70).

3β-Acetoxy-6-acetyl-6-aza-cholestane (7). Compound **1** (20 mg, 0.05 mmol) on treatment with Ac_2O/pyridine/cat. DMAP, at room temperature for 4 h gave, after extraction with CH_2Cl_2, a quantitative yield of **7**: mp 134–136 °C [lit[14] 132–133 °C] from methanol; ^{1}H NMR ($CDCl_3$): δ 0.65 (s, 3H), 0.84 (d, J = 6.6 Hz, 6H), 0.89 (d, J = 6.6 Hz, 3H), 0.94 (s, 3H), 1.97 (m, 1H), 2.01(s, 3H), 2.05 (s, 3H), 2.38 (m, 1H), 2.97 (dd, J = 2.2 Hz, J' = 6.9 Hz, 1H), 4.55 (m, 1H); FABMS m/z (relative intensity): 474 ($M^+ + 1$, 10), 414 ($M^+ + 1$-CH_3COOH, 100).

Reaction of 1 with dimethyl acetylenedicarboxylate to yield (8). To compound **1** (50 mg, 0.13 mmol) dissolved in 5 mL methanol, was added 1.5 equiv of dimethyl acetylenedicarboxylate with stirring at rt overnight. Concentration and washing the residue with petroleum ether:ethyl acetate (3:1) gave compound **8** as a light yellow gum (60 mg, 0.11 mmol, 85%). ^{1}H NMR ($CDCl_3$): δ 0.68 (s, 3H), 0.85 (d, J = 5.4 Hz, 6H), 0.89 (d, J = 5.4 Hz, 3H), 0.94 (s, 3H), 2.61 (m, 1H), 2.96 (m, 1H), 2.96 (m, 1H), 3.47 (m, 2H), 3.62 (s, 3H), 3.86 (s, 3H), 4.94 (s, 1H); EIMS m/z (relative intensity): 531 (M^+, 25), 516 (M^+-CH_3), 499 (95), 412 (100); anal. ($C_{32}H_{54}NO_5 \cdot 0.5H_2O$) C, H, N.

Reaction of 1 with diethyl acetylenedicarboxylate to yield (9). Reaction of **1** with diethyl acetylenedicarboxylate as described above gave **9** as a yellow gum (80%). ^{1}H NMR ($CDCl_3$): δ 0.65 (s, 3H), 0.83 (d, J = 5.4 Hz, 6H), 0.88 (d, J = 5.4 Hz, 3H), 0.93 (s, 3H), 1.21 (t, J = 7.2 Hz, 3H), 1.33 (t, J = 7.2 Hz, 3H), 2.54 (dd, J = 12.6 Hz, J = 10.8 Hz, 1H), 2.96 (m, 1H), 3.46 (m, 2H), 4.04 (q, 2H), 4.30 (q, 2H), 4.92 (s, 1H); FABMS m/z (relative intensity): 560 (M^+ + 1, 50), 390 (100); HRMS calcd for $C_{34}H_{58}NO_5$ (M^+ + 1) 560.4315, found 560.4253; anal. ($C_{34}H_{58}NO_5 \cdot 0.6H_2O$) C, H, N.

Reaction of 3β-hydroxy-6-aza-cholestane (1) with cyclic anhydrides to give amidoacids 10–13. General procedure: A solution of 3β-hydroxy-6-aza-cholestane, **1** (50 mg) and 1.0 molar equiv of the anhydride in 5 mL DMF was stirred at room temperature overnight. The amidoacid products were obtained by evaporation of the solvent followed by washing the residue with ether or chloroform and filtration. In all cases the products were obtained as amorphous solids. Compound **13** from phthalic anhydride: ^{1}H NMR ($CDCl_3$): δ 0.57 (s, 3H), 0.84 (d, J = 6.6 Hz, 9 H), 0.98 (s, 3H), 3.25 (m, 1H), 4.97 (m, 1H), 7.49 (m, 2H), 8.01 (m, 2H), 8.88 (br, 1H); FABMS m/z (relative intensity): 538 (M^+ + 1, 100), 390 (40); HRMS calcd for $C_{34}H_{52}NO_4$ 538.3896, found 538.3895; anal. ($C_{34}H_{52}NO_4 \cdot 0.6CHCl_3$) C, H, N. Compound **10** from succinic anhydride: ^{1}H NMR ($CDCl_3$): δ 0.65 (s, 3H), 0.83 (d, J = 6.6 Hz, 6H), 0.86 (d, J = 6.6 Hz, 3H), 0.89 (s, 3H), 2.96 (d, J = 15 Hz, 2H), 3.40 (m, 1H), 3.76 (m, 1H); FABMS m/z (relative intensity): 490 (M^+ + 1, 100), 472 (M^+-H_2O), 390 (25); HRMS calcd for $C_{30}H_{52}NO_4$ (M^+ + 1) 490.3856, found 490.3867. Compound **11** from maleic anhydride: ^{1}H NMR ($CDCl_3$): δ 0.65 (s, 3H), 0.83 (d, J = 6.6 Hz, 9 H), 0.87 (s, 3H), 2.70 (br, 2H), 3.30 (m, 1H), 3.76 (m, 1H), 6.28 (br, 2H), 8.16 (br, 1H), 8.70 (br, 1H); FABMS m/z (relative intensity): 488 (M^+ + 1, 5), 390 (100); HRMS calcd for $C_{30}H_{50}NO_4$ (M^+ + 1) 488.3740, found 488.3766. Compound **12** from glutaric anhydride: ^{1}H NMR ($CDCl_3$): δ 0.63 (s, 3H), 0.82 (d, J = 6.6 Hz, 6H), 0.86 (d, J = 6.6 Hz, 3H), 0.90 (s, 3H), 2.89 (d, J = 23.7 Hz, 2H), 3.38 (m, 1H), 3.69 (m, 1H). 8.00 (br, 1H); FABMS m/z (relative intensity): 504 (M^+ + 1, 15), 486 (M^+-H_2O), 390 (100).

6-Aza-pregnan-20-one (14) and 3β, 20-dihydroxy-6-aza-pregnane (15). Following the same procedures used in the preparations of 6-aza-cholestane (**3**), pregnenolone acetate was converted initially into 6-aza-pregnan-3, 5-dien-20-one:[14] IR (ν cm^{-1}) 3350 (NH), 1690 (C=O); ^{1}H NMR ($CDCl_3$): δ 0.78 (s, 3H), 0.98 (s, 3H), 0.98 (s, 3H), 2.02 (s, 3H), 2.44 (m, 2H), 5.96 (m, 1H), 6.18 (m, 1H); FABMS m/z (relative intensity): 300 (M^+ + 1,100), 285 (M^+ + 1-CH_3, 20), 243 (15). 6-Aza-pregnan-3, 5-dien-20-one upon reduction with 10% Pd/C in glacial acetic acid yielded 6-aza-pregnan-20-one (**14**)[14]: IR (ν cm^{-1}) 3350 (NH), 1701 (C=O); ^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H), 0.90 (s, 3H), 2.04 (s, 3H), 2.12 (t, J = 12.0 Hz, 1H), 2.44 (t, J = 10.2 Hz, 1H), 2.98 (dd, J = 2.2, 6.9 Hz, 1H); ^{13}C NMR: 12.30, 13.77, 20.68, 21.72. 23.24, 24.15, 25.84, 28.64, 31.83, 35.99, 36.67, 36.81, 39.22, 44.67, 52.41, 54.22, 54.29, 63.86, 65.81, 210.10; EIMS m/z (relative intensity): 303 (M^+, 90), 288 (M^+-CH_3, 40), 246 (100); HRMS calcd for $C_{20}H_{33}NO$ 303.2562, found 303.2541.

Similarly, as for the described preparation of 3β-hydroxy-6-aza-cholestane, **1** gave initially the acyl azide which upon Curtius rearrangement in benzene, then treatment with acid in acetone and finally hydrogenation (10% Pt/C) in glacial AcOH gave an epimeric mixture (5:1) of 3β, 20-dihydroxy-6-aza-pregnane (**15**): ^{1}H NMR ($CDCl_3$): δ 0.81 (s, 3H), 0.89 (s, 3H), 1.04 (d, J = 6.0 Hz, 3H), 2.18 (m, 1H), 2.80 (br, 1H), 2.88 (dd, J = 2.2, 6.9 Hz, 1H), 3.60 (m, 2H); CIMS m/z (relative intensity): 322 (M^+ + 1, 10), 304 (M^+ + 1-H_2O, 80), 288 (100); HRMS calcd for $C_{20}H_{35}NO_2$ 321.2668, found 321.2648.

Biological methods

The test cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All the cells were maintained in Dulbecco-modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). PI-PLCγ in vitro inhibition assay was measured using bovine brain PI-PLCγ as previously described.[1,17] Antiproliferative assay was measured over 7 days as previously described.[1,17]

The in vitro cytotoxicity assays were carried out at the National Cancer Institute. Details of the assay procedures have been reported previously.[20] In vitro antitumor activity was tested against MCF-7 human breast cancer xenografts in the scid mouse. Groups of 8 mice were transplanted sc with 2×10^6 MCF-7 human breast cancer cells. Treatment with compound **1** was begun ip on day 4 and continued daily for 14 days at 1 and 2 mg/kg. Control mice received vehicle alone. All the mice receiving 2 mg/kg compound **1** died by day 12. In Figure 6 the upper panel shows tumor volume and the lower panel body weight. Each point is the mean of 8 mice and the bars are S.E. *Indicates that it is significantly different from the vehicle treated control. Details of the determination procedures have been reported previously.[21]

In vitro binding assay

The effect of compound **1** on the adhesion of malignant HT-1080 cells to matrigel proteins was examined using

the binding assay. Briefly, 48-well polystyrene plates were coated with 50 μg matrigel per well at 4 °C overnight. The wells were rinsed three times with PBS and incubated with 1% bovine serum albumen (BSA) for 30 min. The tumor cells that had been grown to a subconfluent monolayer in T25 tissue culture flasks were treated with 2.5 μM compound **1** for 72 h and labeled with 200 μci of ^{51}Cr (ICN Biomedical, Irvine, CA, USA) for 24 h at 37 °C with 5% CO_2. DMSO (1:100) was used as a control for the binding assay. The labeled tumor cells were harvested with 5 mM EDTA, washed three times and resuspended in serum-free DMEM containing 1% BSA. Their viabilities were >95%. Tumor cells (5×10^5) in 0.5 mL 1% BSA were placed in triplicate into each matrigel-coated well and incubated for 90 min at 37 °C. The cells which did not adhere to the matrigel were gently washed off with warm PBS, and the remaining cells were collected with cotton swabs from each well followed by addition of 20 μL of 10% SDS into each well. Adherence was quantified by counting the radioactivity and values were calculated as a percentage of the control adherence.

In vitro invasion assay

The inhibitory effects of compound **1** on the invasive capability of HT-1080 cells were determined by using a modified Boyden chamber assay. Transwell chambers with 6.5 mm diameter polycarbonate membrane filters of 8 μm pore size (Costar, Pleasanta, CA, USA) were used to form dual chambers in a 24-well tissue culture plate. The filters were coated with 100 μg matrigel (Becton Dickinson, Bedford, MA, USA). Conditioned medium of Balb/3T3 cells (ATCC) was placed in the lower compartments (200 μL per chamber). HT-1080 cells were radiolabeled with ^{51}Cr (300 μci/T25 flask, ICN Biomedical) overnight. After being detached by trypsin-EDTA from the culture flasks and washed three times with plain DMEM containing 0.1% BSA, the radiolabeled HT-1080 cells were well mixed with 5 μM compound **1**, and 200 μL of the cell suspension (2×10^5) were added to each of the upper compartments in the dual chambers in triplicate. After 24 h of incubation, the fractions of the cells that had traversed the filters into the lower compartments were quantified by counting the radioactivity associated with the cells below the filter (lower compartments) compared to the radioactivity associated with all of the cells, i.e. percentage of cells transversing the filter = (cpm of the cells in lower chamber/cpm of total cells)×100.

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