

A β -lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line

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ABSTRACT Lactacystin, a microbial natural product, induces neurite outgrowth in Neuro 2A mouse neuroblastoma cells and inhibits progression of synchronized Neuro 2A cells and MG-63 human osteosarcoma cells beyond the G₁ phase of the cell cycle. A related β -lactone, *clasto*-lactacystin β -lactone, formally the product of elimination of *N*-acetylcysteine from lactacystin, is also active, whereas the corresponding *clasto*-lactacystin dihydroxy acid is completely inactive. Structural analogs of lactacystin altered only in the *N*-acetylcysteine moiety are active, while structural or stereochemical modifications of the γ -lactam ring or the hydroxyisobutyl group lead to partial or complete loss of activity. The inactive compounds do not antagonize the effects of lactacystin in either neurite outgrowth or cell cycle progression assays. The response to lactacystin involves induction of a predominantly bipolar morphology that is maximal 16–32 h after treatment and is distinct from the response to several other treatments that result in morphological differentiation. Neurite outgrowth in response to lactacystin appears to be dependent upon microtubule assembly, actin polymerization, and *de novo* protein synthesis. The observed structure–activity relationships suggest that lactacystin and its related β -lactone may act via acylation of one or more relevant target molecule(s) in the cell.

Lactacystin (structure 1 in Fig. 1) is a *Streptomyces* metabolite that inhibits cell proliferation and induces neurite outgrowth in the mouse neuroblastoma cell line Neuro 2A (1, 2). Cells induced to differentiate by treatment with lactacystin display a predominantly bipolar (two-neurite-bearing) morphology, particularly between 16 and 32 h after treatment, and become more multipolar (multiple-neurite-bearing) upon continued exposure, with increased branching of neurites. In contrast, serum deprivation or treatment of Neuro 2A cells with agents that increase intracellular cAMP levels tends to induce a predominantly multipolar type of neurite outgrowth (3, 4), whereas treatment with agents such as retinoic acid, natural gangliosides, and synthetic sialyl compounds tends to result in a predominantly unipolar (single-neurite-bearing) type of neurite outgrowth in the population of differentiated cells (3, 4).

Mature neurons exhibit a variety of phenotypes in terms of both morphology and physiology. In vertebrate development, neuroblasts from the neural tube and neural crest differentiate into specialized neurons with uni-, bi- or multipolar morphology depending on developmental history (5). The molecular mechanisms underlying these cell-fate determinations remain to be fully elucidated. Lactacystin may prove to be a useful reagent for studying the signal transduction pathways involved in neuronal differentiation and the induction of bipolar morphology.

MATERIALS AND METHODS

Materials. Lactacystin used in the biological assays was provided by S. Omura of the Kitasato Institute. Lactacystin analogs were synthesized by methods reported elsewhere (6–9). The structure of *clasto*-lactacystin β -lactone (structure 4 in Fig. 1) has been confirmed by single-crystal x-ray diffraction analysis (9). Compounds were dissolved in the minimal amount of methanol (MeOH) or dimethyl sulfoxide (DMSO) required for solubilization. No more than 0.1% solvent was present in any assay. When necessary, solutions were evaporated to dryness and resuspended in cell culture medium to their final concentrations before use.

Cell Culture. Neuro 2A, IMR-32, PC12, and MG-63 cells were obtained from the American Type Culture Collection. Neuro 2A and IMR-32 cells were cultured in Eagle's minimal essential medium (MEM) containing 10% (vol/vol) fetal bovine serum (FBS). PC12 cells were grown in RPMI 1640 medium containing 10% (vol/vol) horse serum and 5% FBS, and MG-63 cells were cultured in RPMI 1640 containing 10% FBS.

Neurite Outgrowth Assays. Neuro 2A cells were plated at a density of 1×10^4 cells per 1 ml per well in 12-well polystyrene dishes (22-mm-diameter flat-bottom wells) and grown for 24 h in MEM with 10% FBS prior to any treatment. In the relevant experiments, nocodazole, cytochalasin B, or cycloheximide was added 3 h before addition of lactacystin. In the serum deprivation experiments, cells were switched to serum-free MEM 24 h after plating and, when relevant, incubated another 24 h before addition of lactacystin and subsequently maintained in serum-free conditions.

Cell Cycle Analysis. MG-63 cells were plated at 7.5×10^4 cells per 3 ml per 25-cm² flask and grown for 24 h in RPMI containing 10% FBS. These subconfluent MG-63 cultures were synchronized in G₀/G₁ by changing the medium to RPMI containing 0.2% FBS and incubating for 64 h. This was followed by stimulation with 2 ml of RPMI containing 10% FBS and addition of compounds. Neuro 2A cells were grown to $\approx 2 \times 10^7$ in 175-cm² flasks in MEM with 10% FBS. Mitotic cells were harvested by shaking for 5 min at 100 rpm on a rotary shaker. The detached cells were replated at 1.5×10^5 cells per 2 ml per 25-cm² flask and incubated for 30 min to allow for reattachment prior to addition of lactacystin. Cells were harvested for cell cycle analysis 21 h after stimulation in the case of the MG-63 cells and 20 h after replating in the case of the Neuro 2A cells and then were processed for flow cytometry as reported (10). DNA histograms were obtained using a Becton Dickinson FACScan flow cytometer.

RESULTS

Neurite Outgrowth in Response to Lactacystin. A significant proportion of the lactacystin-treated cells exhibit a bipolar

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Abbreviations: FBS, fetal bovine serum; PKA and PKC, protein kinases A and C; DMSO, dimethyl sulfoxide; H7, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine.

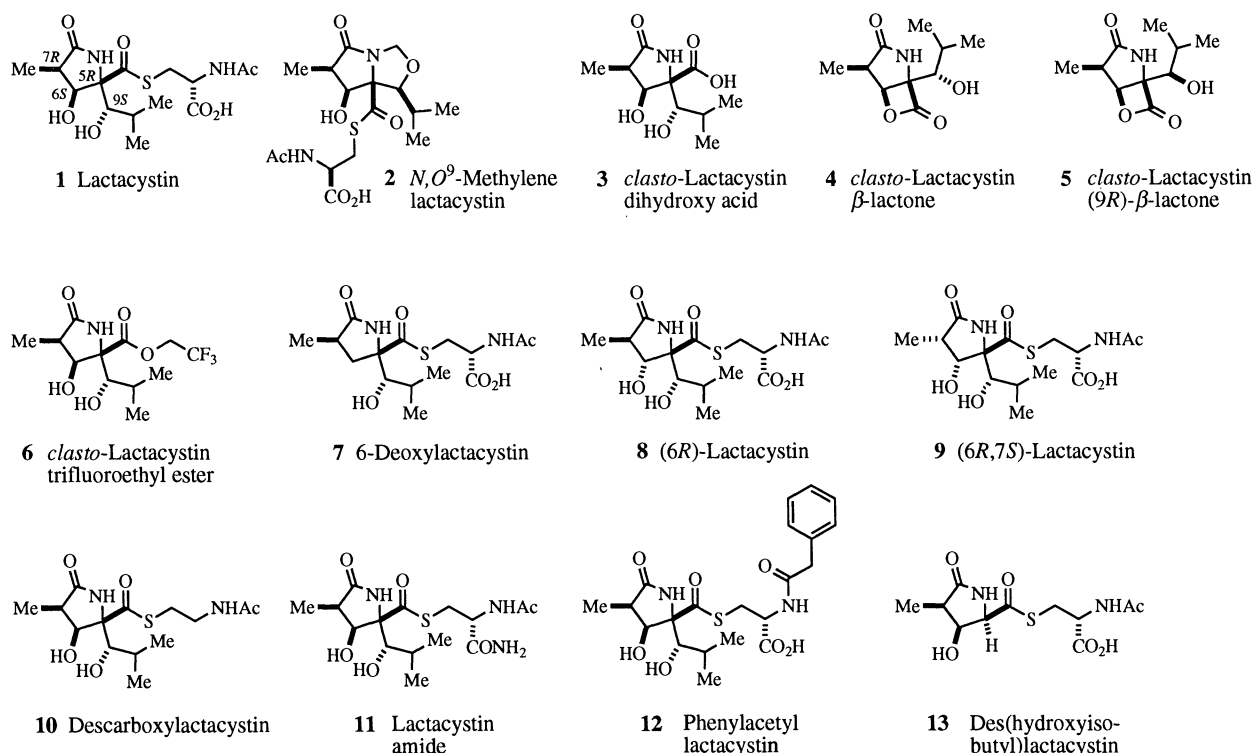


FIG. 1. Structure of lactacystin and several structural variants and stereoisomers used in this study.

morphology, in which two neurites extend from opposite sides of the cell body (see Fig. 3*B*) in the presence or absence of serum (see Table 1), with the highest proportion of bipolar cells between 16 and 32 h after treatment (Fig. 2*B*). With continued exposure, the cell population becomes more multipolar (Fig. 2*C*), and the neurites become more highly branched. Some dose-dependent toxicity is observable, particularly above 10 μ M, and is characterized by increased rounding-up and detachment of cells from the substratum. Neither PC12 rat pheochromocytoma cells nor IMR-32 human neuroblastoma cells elaborate neurites in response to lactacystin, although they are still sensitive to the dose-dependent toxicity of the compound.

The effects of inhibitors of cytoskeletal assembly and protein synthesis on lactacystin-induced neurite formation were examined. Nocodazole, an inhibitor of microtubule assembly (tubulin polymerization), inhibits morphological differentiation (Table 1). Treatment with cytochalasin B, an inhibitor of actin polymerization, blocks lactacystin-induced neurite outgrowth (Table 1), as does treatment with cycloheximide, an inhibitor of protein synthesis in eukaryotes (Table 1).

Neurite Outgrowth in Response to Other Treatments. In contrast to lactacystin treatment, serum deprivation results predominantly in differentiated cells of a multipolar morphology (Fig. 3*D*). Treatment of Neuro 2A cells with 0.1–1 mM 8-bromo-adenosine 3',5'-cyclic monophosphate (8-bromo-cAMP) also leads to predominantly multipolar-type neurite outgrowth as reported (4). Treatment with prostaglandin E₁ (3–150 μ M) or a combination of adenosine (0.1–1 mM) and isobutylmethylxanthine (0.1–1 mM) was found to result mainly in multipolar-type neurite outgrowth (data not shown). Sphingosylphosphorylcholine (50–100 μ M) was found to result in approximately equal proportions of uni-, bi- and multipolar cells by 24 h (data not shown). Unlike PC12 cells, Neuro 2A cells do not respond to 20–200 ng/ml of nerve growth factor (NGF) (2.5*S*) as reported (1).

Inhibition of Cell Cycle Progression by Lactacystin. Lactacystin inhibits progression of synchronized Neuro 2A cells and MG-63 osteosarcoma cells beyond the G₁ phase of the

cell cycle (Table 2). Synchronized MG-63 cells, harvested 21 h after serum stimulation, are inhibited from progressing beyond the G₁ phase of the cell cycle in the presence of lactacystin, while control cells are mostly in S phase at this time (Table 2). The cells exhibit diminishing sensitivity to lactacystin as a function of time following stimulation with serum (Table 2), with no inhibition at all observable when lactacystin is added at 12 h. In the absence of lactacystin, the majority of cells enter S phase between 16 and 19 h after stimulation (data not shown). These data suggest that lactacystin inhibits progression of the cell cycle at an earlier point in G₀/G₁.

Neurite Outgrowth and Inhibition of Cell Cycle Progression in the Presence of Lactacystin Analogs. Structural variants and stereoisomers of lactacystin were tested for their ability to induce neurite outgrowth in Neuro 2A cells (Table 1) and inhibit progression of MG-63 cells through the cell cycle (Table 2). The β -lactone 4, which completely lacks the *N*-acetylcysteine moiety, is highly effective (Tables 1 and 2 and Fig. 3*C*). The trifluoroethyl ester 6 is also active but is less potent than either the β -lactone 4 or lactacystin. Certain modifications to the *N*-acetylcysteine moiety appear to result in somewhat lowered activity relative to lactacystin in the neurite outgrowth assay at higher concentrations (Table 1; compounds 11 and 12).

In contrast to the β -lactone 4, the dihydroxy acid 3 is completely inactive even at 100 μ M (Tables 1 and 2). *N,O*⁹-Methylene lactacystin (structure 2 in Fig. 1) is completely inactive in the neurite outgrowth assay at 100 μ M (Table 1) but is weakly active in the cell cycle progression assay at this concentration (Table 2). The des(hydroxyisobutyl) analog 13 is completely inactive in both assays even at 100 μ M. The (9*R*)- β -lactone analog 5 displays markedly reduced activity in both assays (Tables 1 and 2). The 6-deoxylactacystin analog 7 is completely inactive in both assays even at 100 μ M, and the 6-*epi* (6*R*) variant 8 has reduced activity (Tables 1 and 2). The configuration of the methyl group on C7 is important since the di-*epi* (6*R*,7*S*) analog 9 is completely inactive in the neurite outgrowth

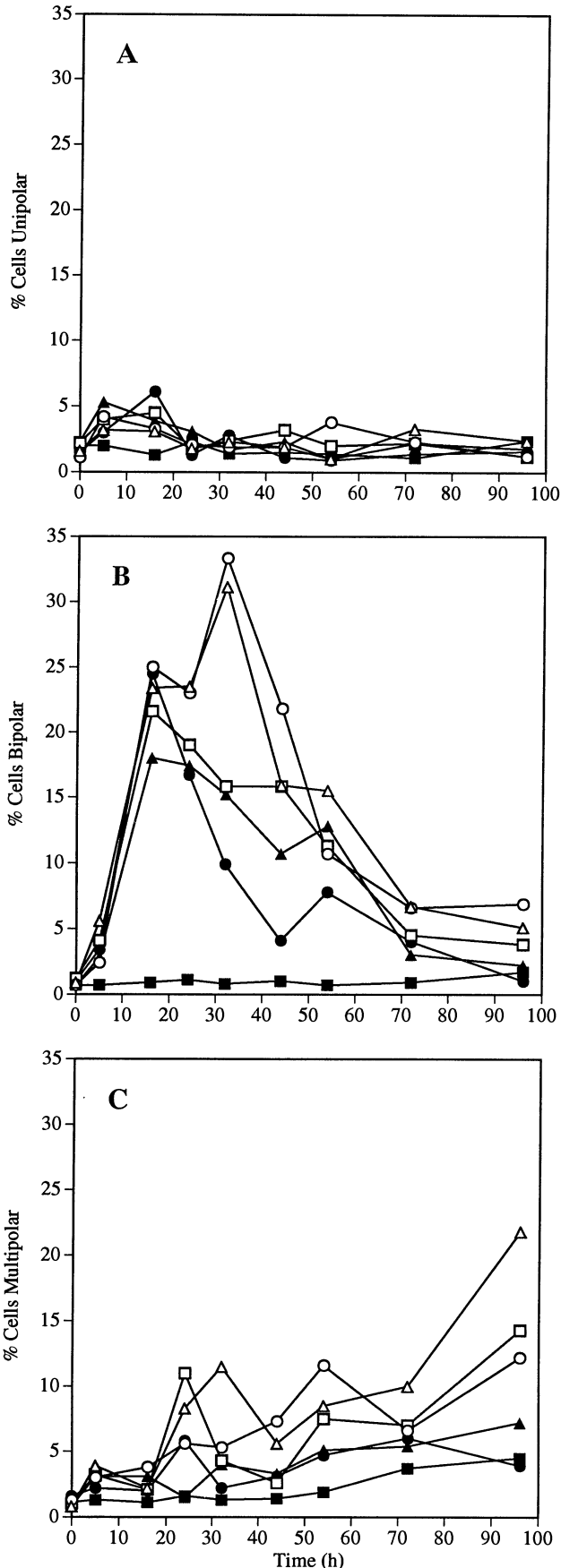


FIG. 2. Neurite outgrowth in Neuro 2A cells as a function of time after treatment with lactacystin. Cells were maintained in medium containing 10% serum (see *Materials and Methods*), and treatments were as follows: 0.1% MeOH (■); 2 μ M lactacystin (●); 4 μ M

Table 1. Neuritogenic activity of lactacystin and lactacystin analogs

Treatment	Activity				
	1 μ M	2 μ M	5 μ M	10 μ M	20 μ M
Compound					
1	-	+	++	+++	++++
2	-	-	-	-	-
3	-	-	-	-	-
4	-	+	++	+++	++++
5	-	-	-	-	+
6	-	-	+	++	+++
7	-	-	-	-	-
8	-	-	-	-	+
9	-	-	-	-	-
10	-	+	++	+++	++++
11	-	+	++	+++	+++
12	-	+	++	++	++
13	-	-	-	-	-
Nocodazole (3, 30 μ M) + 1	-	-	-	-	-
Cytochalasin B (5, 50 μ M) + 1	-	-	-	-	-
Cycloheximide (10, 50 μ M) + 1	-	-	-	-	-
Serum deprivation + 1	-	+	++	+++	++++
0.1% MeOH or DMSO				-*	
Nocodazole (3, 30 μ M)				-*	
Cytochalasin B (5, 50 μ M)				-*	
Cycloheximide (10, 50 μ M)				-*	
Serum deprivation (0.1% MEOH or DMSO)				-*†	
2, 3, 7, 9 or 13 (100 μ M)				-*	
2, 3, 7, 9 or 13 (100 μ M) and 1 (2 μ M)				+	

At least two independent experiments with triplicate samples were performed for each treatment. Plus signs indicate a significant difference in the percentage of cells exhibiting a bipolar morphology at 24 h relative to 0.1% MeOH- or 0.1% DMSO-treated control cells ($P < 0.05$ by Student's *t* test). A minus sign alone indicates no significant difference from control, while an additional dagger indicates that the differentiated cells exhibited a predominantly multipolar morphology.

*Activity at the concentrations noted in column 1.

assay even at 100 μ M (Table 1) and only weakly active in the cell cycle progression assay at this concentration (Table 2), whereas the 6-*epi* compound 8, in which the configuration of the 7-methyl group is not altered, is weakly active in the neurite outgrowth assay at ≥ 20 μ M and in the cell cycle progression assay at ≥ 10 μ M.

Potential antagonism of lactacystin effects by the analogs that do not exhibit activity in the neurite outgrowth assay was also examined. Treatment of cells with a 50-fold molar excess of each inactive compound over lactacystin does not result in antagonism by any of the inactive analogs (Tables 1 and 2).

DISCUSSION

Various agents that increase intracellular cAMP levels and lead to activation of cAMP-dependent protein kinase (PKA) induce neurite outgrowth of a predominantly multipolar type in Neuro 2A cells (3, 4). Intracellular delivery of the catalytic subunit of PKA induces neurite outgrowth in human neuro-

lactacystin (\blacktriangle); 6 μ M lactacystin (\square); 8 μ M lactacystin (\circ); 10 μ M lactacystin (\triangle). Values are the mean of triplicate samples for each treatment (SD $\leq 6\%$ in all cases). Data show the percentage of adherent cells exhibiting unipolar morphology (A), bipolar morphology (B), or multipolar morphology (C).

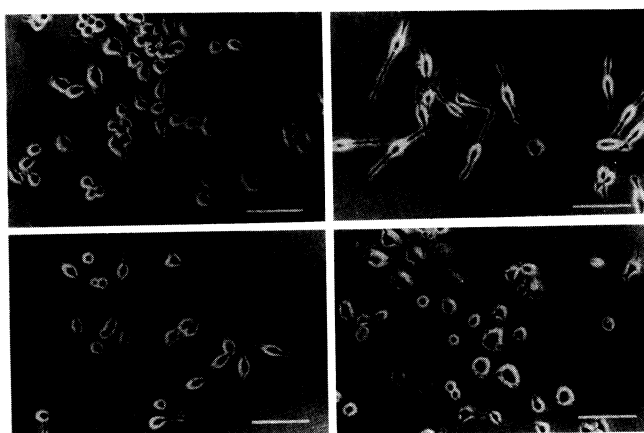


FIG. 3. Photomicrographs of Neuro 2A cells viewed 24 h after the following treatments: 0.1% MeOH (A); 5 μM lactacystin (B); 5 μM β-lactone (C); or serum deprivation (D). (Bar = 100 μm.)

blastoma cells (11), and simultaneous treatment with *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA 1004), which selectively inhibits cAMP- and cGMP-dependent protein kinases over protein kinase C (PKC) (12), blocks this response (11). HA 1004 alone does not induce any neurite outgrowth in Neuro 2A cells (13) or human neuroblastoma cells (11). Treatment of Neuro 2A cells with prostaglandin E₁ or a combination of adenosine and isobutylmethylxanthine, which inhibits cAMP phosphodiesterase, leads to a transient increase in intracellular cAMP levels within 15–30 min after treatment (1). Lactacystin treatment also results in transient accumulation of intracellular cAMP in Neuro 2A cells; however, peak cAMP levels are not reached until 24 h after treatment (1).

Staurosporine, a general inhibitor of protein kinase activity, induces morphological differentiation of mouse neuroblastoma cells (14) and human neuroblastoma cells (11). Treatment of Neuro 2A cells with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), another general protein kinase inhibitor, results mainly in unipolar-type neurite outgrowth at lower concentrations, with the proportion of bi- and multipolar cells increasing at higher concentrations (13). The neuritogenic effect of H7 is inhibited and reversed by the PKC activator phorbol 12-myristate 13-acetate in Neuro 2A cells (13), implying that the inhibition of PKC induces neurite outgrowth. Sphingomylophosphorylcholine also induces neurite outgrowth and has been shown to inhibit the activity of purified PKC in mixed micelle assays (15). However, calphostin C, a specific inhibitor of PKC that affects the diacylglycerol/phorbol ester-binding regulatory domain (16), does not induce any neurite outgrowth in Neuro 2A cells (17). Furthermore, analysis of the roles of PKC in the regulation of cellular processes is complicated by the existence of a number of PKC isoforms (18). Lactacystin does not inhibit PKC activity in rat brain preparations (1).

Cell-derived proteases and protease inhibitors are thought to play important roles in modulating neurite outgrowth and regeneration (19). An endogenous serine protease inhibitor, glia-derived nexin, possesses neurite-promoting activity (20). Thrombin inhibits neurite outgrowth induced by serum deprivation or treatment with protease nexin 1 (identical to glia-derived nexin) or the antithrombin hirudin in mouse neuroblastoma cells (21). Lactacystin does not inhibit thrombin or plasminogen activator (1). Furthermore, lactacystin-induced bipolar-type neurite outgrowth does not appear to be simply the result of inhibition of an activity found in serum, since it is observed even when cells are deprived of serum for 24 h prior to addition of lactacystin and subsequently kept in serum-free conditions.

Table 2. Effect of lactacystin and lactacystin analogs on cell cycle progression

Treatment	% cells in G ₀ or S phase			
	2 μM		10 μM	
	G ₀ /G ₁	S	G ₀ /G ₁	S
Compound				
1	74.5 ± 1.9*	11.5 ± 2.1	78.8 ± 1.8*	11.2 ± 0.6
2	12.6 ± 1.5	84.0 ± 1.7	14.6 ± 4.2	81.2 ± 5.9
3	11.3 ± 2.4	83.6 ± 2.8	13.3 ± 0.6	79.2 ± 2.5
4	67.3 ± 2.0*	22.1 ± 2.7	81.6 ± 2.3*	10.1 ± 1.0
5	14.3 ± 3.3	80.5 ± 2.9	38.7 ± 3.9*	55.6 ± 4.6
6	18.1 ± 1.8	77.6 ± 1.7	48.0 ± 1.3*	38.8 ± 2.5
7	13.1 ± 1.0	82.8 ± 1.5	13.0 ± 1.9	82.7 ± 3.5
8	14.5 ± 4.5	79.8 ± 6.4	49.8 ± 2.5*	37.9 ± 1.1
9	12.7 ± 1.6	80.9 ± 3.7	13.9 ± 1.7	83.7 ± 2.9
10	78.3 ± 1.9*	10.7 ± 1.0	78.7 ± 0.7*	12.5 ± 0.2
11	59.3 ± 2.1*	33.2 ± 3.2	76.7 ± 0.1*	12.6 ± 1.1
12	77.4 ± 2.6*	12.0 ± 4.7	78.0 ± 1.2*	11.0 ± 2.2
13	17.3 ± 1.8	77.0 ± 3.9	15.5 ± 2.4	76.4 ± 3.3
Neuro 2A cells treated with 1				
0.1% MeOH	72.8 ± 2.3 [†]	5.6 ± 3.6	75.7 ± 0.7 [†]	10.8 ± 1.7
0.1% DMSO	15.9 ± 3.0 [‡]		77.8 ± 5.6 [§]	
Stimulation with serum				
+ 1 (2 μM) 6 h later	65.1 ± 3.3* [‡]		13.0 ± 2.7 [§]	
+ 1 (2 μM) 9 h later	44.5 ± 1.1* [‡]		42.6 ± 1.4 [§]	
+ 1 (2 μM) 12 h later	13.5 ± 1.3 [‡]		75.1 ± 3.6 [§]	
0.1% MeOH; harvested 12 h after serum stimulation				
2 (100 μM)	89.4 ± 2.4* [‡]		5.5 ± 2.2 [§]	
2 (100 μM) + 1 (2 μM)	35.4 ± 2.3* [‡]		61.0 ± 2.6 [§]	
3 (100 μM)	16.4 ± 2.1 [‡]		73.6 ± 3.4 [§]	
7 (100 μM)	14.8 ± 1.4 [‡]		79.3 ± 2.5 [§]	
9 (100 μM)	49.7 ± 0.9* [‡]		40.2 ± 0.7 [§]	
13 (100 μM)	18.7 ± 1.6 [‡]		77.9 ± 1.5 [§]	
2 (100 μM) + 1 (2 μM)	76.4 ± 0.6* [‡]		13.0 ± 6.4 [§]	
3 (100 μM) + 1 (2 μM)	74.6 ± 3.0* [‡]		10.2 ± 2.9 [§]	
7 (100 μM) + 1 (2 μM)	76.1 ± 0.6* [‡]		10.4 ± 1.6 [§]	
9 (100 μM) + 1 (2 μM)	76.7 ± 0.7* [‡]		11.2 ± 2.8 [§]	
13 (100 μM) + 1 (2 μM)	75.2 ± 1.3* [‡]		10.9 ± 0.4 [§]	
Neuro 2A cells treated with 0.1% MeOH	36.4 ± 2.0 [‡]		26.1 ± 4.5 [§]	

The percentage of cells in the G₀/G₁ or S phases of the cell cycle (21 h after treatment with lactacystin or lactacystin analogs for MG-63 cells and 20 h for Neuro 2A cells) is displayed. Values are the mean ± SD of triplicate samples. Experiments were performed with MG-63 cells except as noted.

**P* < 0.002 relative to either 0.1% MeOH- or 0.1% DMSO-treated control MG-63 cells, as appropriate.

[†]*P* < 0.001 relative to 0.1% MeOH-treated control Neuro 2A cells.

[‡]% cells in G₀/G₁ after treatment at concentration shown in column 1.

[§]% cells in S after treatment at concentration shown in column 1.

Lactacystin does not appear to act at the level of the nerve growth factor signaling pathway, at least as it is manifested in PC12 pheochromocytoma cells, although it is nevertheless toxic to PC12 cells. The existence of a lactacystin target in other cell types is implied by the ability of lactacystin to inhibit progression of MG-63 osteosarcoma cells through the cell cycle. The induction of bipolar morphology in Neuro 2A cells, however, does not appear to be solely a default consequence of inhibition of cell cycle progression. Serum deprivation results in accumulation of cells in G₀/G₁ but does not lead to the induction of bipolar morphology, and treatment with calphostin C arrests Neuro 2A cells in G₀/G₁

without inducing any morphological differentiation (17). The general protein kinase inhibitor H7, on the other hand, inhibits cell proliferation and induces neurite outgrowth in Neuro 2A cells (13) but does not arrest cells in any specific phase of the cell cycle (17).

The morphological changes induced by lactacystin treatment appear to be dependent upon microtubule assembly and actin polymerization as well as *de novo* protein synthesis, which may reflect a requirement for new gene expression in mediating the response. The neurites of Neuro 2A cells treated with lactacystin for 4 days have been shown to contain parallel arrays of microtubules and intermediate filaments as well as large cored vesicles in the growth cones and thus to possess a structure resembling that of established neurites (1). The neurites of the lactacystin-induced bipolar cells form at opposite sides of the cell body. If the cytoskeletal lattice is mechanically integrated, tensile stress associated with increased cytoskeleton-linked adhesion to the substratum in one region may result in formation of the second neurite at the opposite pole of the cell body. Formation of the axis of neurite outgrowth may also involve the centrosome, the major microtubule-organizing center of the cell.

The lactacystin analog studies reveal the importance of several chemical groups for biological activity. The hydroxyisobutyl group and the configuration of the hydroxyl group on C9 are important, as are the configurations of the hydroxyl group on C6 and the methyl group on C7 of the γ -lactam ring. These groups may be involved in primary recognition of the target. In contrast, the *N*-acetylcysteine moiety is not required for activity. The compounds that are completely inactive in the neurite outgrowth assay do not compete with lactacystin in either assay. This implies (*i*) that the inactive compounds do not competitively bind to or modify the site on the target molecule(s) that mediates the effects of lactacystin or (*ii*) that they do not reach the target.

The mode of action of the lactacystin-related β -lactone 4 may be through acylation of a cellular target, involving attack of the β -lactone by a nucleophilic group on the target molecule and subsequent opening of the β -lactone ring. The natural product lipstatin and its derivative tetrahydrolipstatin are β -lactones that inhibit the activity of several lipases secreted by the gastrointestinal tract and may act via formation of a covalent acyl-enzyme complex (22, 23). A similar mode of action has been proposed for a β -lactone inhibitor of 3-hydroxy-3-methylglutaryl-CoA synthase (24, 25). Consistent with such a model for the mechanism of action of the β -lactone 4, the dihydroxy acid 3, corresponding to the hydrolysis product of 4, is completely inactive. The carboxyl of 3 is not as electrophilic as the carbonyl of the β -lactone ring of 4, and furthermore, the even less electrophilic carboxylate ion would predominate at physiological pH. Therefore, the dihydroxy acid 3 would not be expected to be a good acylating agent.

If lactacystin acts by covalent modification of a target molecule, its mode of action could involve acylation either by direct exchange of the thioester for a nucleophile on the target or by formation of the β -lactone 4 as an active intermediate. Both mechanisms would require a sufficiently reactive electrophilic carbonyl such as the thioester and result in release of the *N*-acetylcysteine. The trifluoroethyl ester 6 could likewise act by either mechanism if acylation were involved, and its somewhat reduced activity may be a function of the difference in electrophilicity of the carbonyl of the two types of ester. The 6-hydroxyl group is important for activity since the 6-deoxylactacystin analog 7 is completely inactive. The compounds that are the most active in both the neurite outgrowth assay and the cell cycle progression assay have an *S* stereocenter at C6 and an ester with a good leaving group and potentially could form the β -lactone 4. The 6-*epi* (6*R*) compound 8 is weakly active in both assays. If the electro-

philic carbonyl of the thioester were involved in a direct exchange, then the 6-hydroxyl group may be involved in primary recognition of the target, and the 6-*epi* compound may have reduced activity because of less efficient recognition. Alternatively, if the active compound must be a β -lactone, a spiro- β -lactone formed by attack of the 9-hydroxyl on the thioester could possibly account for the weak activity of 8.

The results of these analog studies suggest the possibility that covalent modification of a target molecule may be important in mediating the effects of lactacystin. Defining the direct molecular target of lactacystin and the downstream cellular events that are sensitive to this agent may shed light upon the regulatory pathways involved in neuronal differentiation and the induction of bipolar morphology.

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