

The Identification and Characterization of the Marine Natural Product Scytonemin as a Novel Antiproliferative Pharmacophore

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ABSTRACT

Marine natural products provide a rich source of chemical diversity that can be used to design and develop new, potentially useful therapeutic agents. We report here that scytonemin, a pigment isolated from cyanobacteria, is the first described small molecule inhibitor of human *polo*-like kinase, a serine/threonine kinase that plays an integral role in regulating the G₂/M transition in the cell cycle. Scytonemin inhibited *polo*-like kinase 1 activity in a concentration-dependent manner with an IC₅₀ of 2 μM against the recombinant enzyme. Biochemical analysis showed that scytonemin reduced GST-*polo*-like kinase 1 activity in a time-independent fashion, suggesting reversibility, and with a mixed-competition mechanism with respect to ATP. Although scytonemin was less potent against protein kinase A and Tie2, a tyrosine kinase, it did inhibit other cell cycle-regulatory kinases like Myt1, checkpoint

kinase 1, cyclin-dependent kinase 1/cyclin B, and protein kinase Cβ2 with IC₅₀ values similar to that seen for *polo*-like kinase 1. Consistent with these effects, scytonemin effectively attenuated, without chemical toxicity, the growth factor- or mitogen-induced proliferation of three cell types commonly implicated in inflammatory hyperproliferation. Similarly, scytonemin (up to 10 μM) was not cytotoxic to nonproliferating endotoxin-stimulated human monocytes. In addition, Jurkat T cells treated with scytonemin were induced to undergo apoptosis in a non-cell cycle-dependent manner consistent with its activities on multiple kinases. Here we propose that scytonemin's dimeric structure, unique among natural products, may be a valuable template for the development of more potent and selective kinase inhibitors used for the treatment of hyperproliferative disorders.

Tissue hyperplasia is a distinguishing characteristic of several chronic inflammatory disorders. Psoriasis, rheumatoid arthritis, and asthma all feature aberrant cell proliferation, angiogenesis, and inflammatory cell infiltrate as part of their pathology. Therapies to address these disorders are lacking and represent one of the great challenges for drug discovery. Historically, one abundant source of novel therapeutic agents has been natural products. For instance, 39% of the 520 new drugs approved between 1987 and 1994 were, or were derived from, natural products (Cragg et al., 1997). Many naturally derived therapies have come from terrestrial sources; how-

ever, over the last thirty years scientists have started exploring one of the greatest sources of biodiversity on the planet—the oceans. Between 1977 and 1987, approximately 2500 marine natural products were identified, many belonging to new chemical classes (Carte, 1996). Many of these have acted as pharmacophores or templates from which therapeutically useful agents have been designed. In fact, the first reported bioactive marine natural products, spongouridine and spongothymidine, served as templates for the development of cytosine arabinoside, an anticancer agent (McConnell et al., 1994). Others show potential as clinically effective agents themselves. Bryostatin, isolated from the brown algae, *Bugula neritina*, is currently in clinical trials as a chemotherapeutic due to its actions on protein kinase C (Varterasian et al., 1998). Hence, the exploration of natural products is still critical to the identification of novel chemical structures,

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ABBREVIATIONS. CDK1, cyclin-dependent kinase 1; GST, glutathione S-transferase; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; RSF, rheumatoid synovial fibroblast; PDGF, platelet-derived growth factor; *Sf*, *Spodoptera frugiperda*; PAGE, polyacrylamide gel electrophoresis; ECGF, endothelial cell growth factor; NHLF, normal human lung fibroblast; TUNEL, Terminal deoxynucleotidyl transferase dUTP fluorescein nick-end labeling.

which will lead to effective new treatments for inflammatory hyperproliferative diseases.

To date, microalgae have yielded a number of potentially useful antiproliferative agents, such as curacin D, the cryptophycin family of compounds, and nostodione A (Kobayashi et al., 1994; Marquez et al., 1998; Wagner et al., 1999). Another compound, closely related to nostodione A, is scytonemin (Fig. 1) (Proteau et al., 1993). This pigment, isolated from cyanobacteria, is believed to be the earliest developed mechanism of ultraviolet protection, more ancient than the flavonoids or melanins (Garcia-Pichel, 1998). Its ring structure, the "scytoneman skeleton", is unique among natural products and is thought to stem from the condensation of tryptophan- and phenylpropanoid-derived subunits (Proteau et al., 1993). Other attractive structural features include its lack of chirality, multiple dissection points, and phenolic groups that could be easily modified. These attributes and its relation to other antiproliferative agents make scytonemin a prime candidate for investigating its potential utility as a pharmacophore with which new therapies targeting hyperproliferative disorders can be developed.

Cellular proliferation is tightly regulated on multiple levels by a number of reversible phosphorylation events (Norbury and Nurse, 1992). Recent approaches used to identify and develop clinically useful antiproliferative agents have begun to focus on targeting the regulatory mechanisms at the later stages of the cell cycle; i.e., the G₂ to M transition. Cells appear less able to tolerate disruptions in the processes controlling normal entry into and through mitosis. Indeed, fewer transforming mutations have been identified in the enzymes controlling this stage of the cell cycle as compared with those at earlier checkpoints. To this end, our laboratory has focused on the kinases regulating the G₂/M transition and one in particular for which no small molecule inhibitor has been identified, the serine/threonine kinase, *polo*-like kinase 1. Originally identified in *Drosophila*, mutant *polo* phenotypes displayed abnormal mitotic divisions (Sunkel and Glover, 1988). It is believed that *polo*-like kinase 1, the mammalian homolog, functions at the G₂/M transition by phosphorylating and subsequently activating the cdc25C phosphatase, which in turn activates the CDK1/cyclin B complex, thus driving the cell's entry into mitosis (Kumagai and Dunphy, 1996; Roshak et al., 2000). As such, *polo*-like kinase 1 is a key enzyme at the G₂/M transition and provides a mechanism for

controlling cell proliferation not yet fully exploited (Lane and Nigg, 1996).

Here we describe scytonemin as the first characterized small molecule inhibitor of *polo*-like kinase 1. Preliminary biochemical mechanistic studies were conducted and selectivity studies revealed that scytonemin inhibited other cell cycle regulatory kinases, but was not totally "pan-active". Biological characterization showed it had the ability to inhibit proliferation without chemical toxicity and had no effect on a nonproliferating cell population. The inhibition of unchecked proliferation in Jurkat T cells, a tumor cell line, was accompanied by the induction of apoptosis, which unlike necrosis or chemical toxicity, implies that scytonemin affects specific biochemical processes in the cell. Together, these activities indicate that scytonemin is a nontoxic, antiproliferative agent, and its unique chemical structure offers a potential scaffold for further chemical modification that could be used to develop a new class of therapeutically useful drugs in treating hyperproliferative disorders.

Materials and Methods

Compounds. Scytonemin was extracted from *Stigonema* sp. collected from Walden Lake, Oregon, as described in Proteau et al. (1993) and purified to >95% purity as checked by nuclear magnetic resonance and thin layer chromatography. Hymenialdisine was obtained from Suntory Pharmaceuticals (Tokyo, Japan), and camptothecin was obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

GST-*polo*-Like Kinase 1 Activity and Kinetic Assays. The expression of GST-*polo*-like kinase 1 and GST-cdc25C was previously described in Roshak et al. (2000). Kinase reactions contained 350 nM GST-cdc25C, 0.5 μ Ci [γ -³²P]ATP (PerkinElmer Life Sciences, Meriden, CT), 10 μ M unlabeled ATP (Sigma-Aldrich, St. Louis, MO), and 20 nM GST-*polo*-like kinase 1 in kinase reaction buffer containing 20 mM HEPES (pH 7.4), 50 mM KCl, 10 mM MgCl₂, 1 mM EGTA, and 0.5 mM DTT. Enzyme and substrates were diluted individually in kinase buffer, and the *polo*-like kinase 1 solution was preactivated by incubating at 37°C for 1 h before its addition to the assay (Roshak et al., 2000). Reactions (50 μ l) were conducted in 96-well polypropylene plates. First, scytonemin (1 nM–10 μ M) dissolved in 100% DMSO was added, followed by the addition of enzyme and substrate (4% DMSO_(final)), and reactions were initiated by the addition of ATP. Reactions progressed for 60 min at 37°C, and then stopped with addition of EDTA to 25 mM and unlabeled ATP to 1 mM. Samples were transferred to 96-well 0.65 μ m Durapore filtration plates (Millipore Corporation, Bedford, MA), the proteins were precipitated with a 10% trichloroacetic acid, 5% sodium pyrophosphate solution, and the plates were filtered using a Millipore microplate filtration unit (Millipore). The plates were washed two times with the 5% sodium pyrophosphate solution, two times with 75 mM phosphoric acid, and two times with phosphate-buffered saline (PBS). After drying at room temperature, scintillation fluid was added to the wells, and [γ -³²P]ATP incorporation was measured on a Packard Top-Count scintillation counter (PerkinElmer Life Sciences). In certain experiments, *polo*-like kinase 1 activity was assessed by gel kinase assays and phosphorimaging analysis, previously described in Roshak et al. (2000). Briefly, GST-*polo*-like kinase 1 activity was assessed by measuring the phosphorylation of GST-cdc25C (1 μ Ci of [γ -³²P]ATP/assay). After termination, reactions were resolved by gel electrophoresis and subjected to phosphorimage analysis using Imagequest (Molecular Dynamics, Sunnyvale, CA). Hymenialdisine (10 μ M) served as a positive control for each assay described above due to its activity as a broad spectrum kinase inhibitor (Meijer et al., 2000). ATP competition assays were conducted as described above in the presence of increasing concentrations (10–25 μ M) of unlabeled

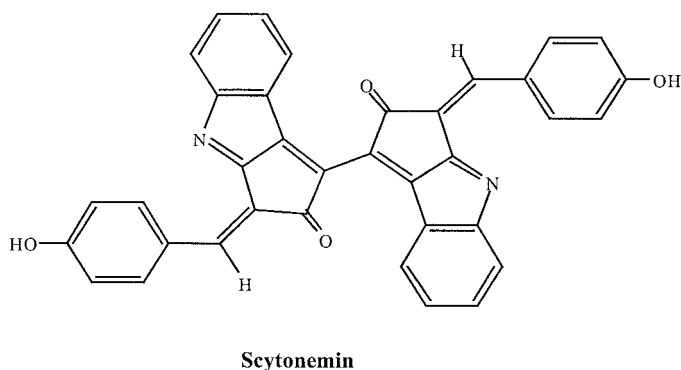


Fig. 1. The chemical structure of scytonemin. Scytonemin's symmetrical structure comprising phenolic and indolic subunits, termed the scytoneman skeleton, is believed to have been created from a condensation of tryptophan- and phenylpropanoid-derived subunits (molecular weight = 544).

ATP. For time dependence experiments, scytonemin was incubated with enzyme and substrate for up to 45 min before reactions were initiated with addition of the ATP.

Assessment of Cell Proliferation. Rheumatoid synovial fibroblasts (RSFs) (obtained through an agreement with Dr. Gene Mochan at Philadelphia College of Osteopathic Medicine, Philadelphia, PA), normal human lung fibroblasts (NHLFs) (Clonetics, San Diego, CA), and human umbilical vein endothelial cells (HUVECs) (Clonetics, San Diego, CA) were cultured in 96-well plates (Falcon, Franklin Lakes, NJ). RSFs (1.2×10^4 cells/well) and NHLFs (1×10^4 cells/well) were plated in Eagle's minimum essential medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 units penicillin-streptomycin (Pen-Strep) (Invitrogen, Carlsbad, CA). HUVECs (5×10^3 cells/well) were plated in CS-C media (Cell Systems, Seattle, WA) with 10% FBS. Cells were allowed to adhere for 24 h at 37°C, 5% CO₂. To quiesce the cells, FBS levels were reduced to 0.2% for 24 h prior to stimulation. Cells were incubated with compound or vehicle (0.5% DMSO_[final]) for 15 min at room temperature. RSFs were stimulated with 1 nM platelet-derived growth factor-BB (PDGF-BB) (R&D Systems, Minneapolis, MN), NHLFs with 5% FBS, and HUVECs with 0.5 μg/ml endothelial cell growth factor (ECGF) (Sigma, St. Louis, MO) for 24 h and then pulsed with 0.5 μCi/well [³H]thymidine (Amersham Biosciences, Inc., Piscataway, NJ) for an additional 24 h at 37°C, 5% CO₂. Toxicity was determined by visual morphological assessment of the cells, i.e., cells that are more spherical, shrunken, or floating would be considered necrotic. Jurkat T cells (American Type Culture Collection, Rockville, MD) (2×10^4 cells/well) were plated in RPMI 1640 (Invitrogen), containing 10% FBS and 10 units of Pen-Strep. Jurkat T cells were treated with compound at the time of plating, pulsed as above, and cultured at 37°C, 5% CO₂, for 24 h. All the cells were harvested onto 96-well GF/C filtration plates (PerkinElmer Life Sciences) using a Packard microplate cell harvester. [³H]Thymidine incorporation into the DNA was measured on a Packard Top-Count. Jurkat T cell proliferation was also analyzed by cell counts. Cells were grown in T25 flasks at 2×10^5 cells/ml in the presence of scytonemin from 1 to 30 μM and counted after incubating for 48 h at 37°C, 5% CO₂ using a hemocytometer. A parallel study was run in 96-well plates to compare cell number data to those using [³H]thymidine incorporation as a measure of cell proliferation. Cell viability was examined by adding a PBS solution containing 10% trypan blue to each well to approximate the level of necrotic or chemical toxicity mediated by scytonemin, as determined by the estimated percentage of cells that could not exclude trypan blue.

To determine effects on nonproliferating cells, the influence of scytonemin on human monocytes activated by lipopolysaccharide was studied. A monocyte-enriched peripheral blood leukocyte population was isolated from heparinized whole blood by double gradient centrifugation as previously described (Marshall and Roshak, 1993) and allowed to adhere in 24-well cell culture plates at 2×10^6 cells per well in 1 ml of RPMI 1640 containing 10% FBS. After 4 h, the medium was removed and washed once with 1 ml of RPMI 1640 containing 10% FBS. RPMI 1640 (1 ml) containing 10% FBS was then added to the cells followed by the addition of scytonemin (0.3–10 μM) with a final vehicle concentration of 1% DMSO. Cells were incubated with compound for 15 min before the addition of lipopolysaccharide to a final concentration of 200 ng/ml. Cells were incubated for 24 h at 37°C, 5% CO₂ after which the medium was removed. Cell viability was examined by adding a PBS solution containing 10% trypan blue to each well to approximate the level of necrosis or chemical toxicity mediated by scytonemin, as determined by the estimated percentage of cells that could not exclude trypan blue. Approximations of cell number were taken using standardized gridded areas for each sample before and after treatment with scytonemin to estimate effects on cell number.

Apoptosis Analysis. Jurkat T cells (2×10^5 cells/ml) were treated with vehicle (0.1% DMSO_[final]), 3 μM scytonemin, or 3 μM camptothecin (positive control) and incubated at 37°C, 5% CO₂ for

24 h. The cells were fixed in 1% paraformaldehyde, washed once with PBS, and stored in 70% ethanol at –20°C. Apoptosis was measured using the Promega Apoptosis Detection System (Promega, Madison, WI). Cells were counterstained with propidium iodide and analyzed for DNA content and fragmentation. Fluorescence incorporation was measured with a BD FACS Sort (BD Biosciences, San Jose, CA).

GST-Tie2 Kinase Activity Assay. To assess the effects of scytonemin on Tie2 kinase autophosphorylation, recombinant human, GST fusion constructs of the human Tie2 kinase domains were generated in a manner similar to those described in Huang et al. (1995). A partial Tie2 cDNA clone possessing an in-frame *Mun* I site was used to fuse the kinase domain to the *Eco*RI site of the pAcG1 expression vector (BD Pharmingen, San Diego, CA). This final construct was subcloned to baculovirus containing the entire *Schistosoma japonica* GST coding region and transfected into *Spodoptera frugiperda* (*Sf*) 9 cells. GST-Tie2 kinase was semipurified using a glutathione affinity column to greater than 90% purity, as determined via SDS-PAGE and Coomassie Blue staining. The kinase assay is carried out in 96-well flashplates and in the same kinase buffer used in the *polo*-like kinase 1 activity assays. Each reaction contained 5 μg of GST-Tie2 (intracellular kinase domain), 1 μCi of [^γ-³³P]ATP, and 30 μM unlabeled ATP in 60 μl. Compounds were solubilized in DMSO (1% DMSO_[final]). The reaction was incubated for 2 h at 30°C and terminated by washing the plate five times with 10 μM unlabeled ATP. Reactions were quantitated using a Packard Top-Count.

Protein Kinase A Activity Assay. Protein kinase A activity was measured by assessing its ability to phosphorylate Histone-IIA. Kinase reactions were conducted in 50 μl comprising 0.5 μg of protein kinase A (from bovine heart supplied by Sigma-Aldrich) and 0.1 mg/ml Histone-IIA (Sigma-Aldrich) in reaction buffer (50 mM MOPS, pH 6.5, 10 mM MgCl₂, 1 μM cAMP). Inhibitors were added at the indicated concentrations with a final DMSO concentration of 10%. An ATP solution was then added to the wells to a final concentration of 0.01 μM unlabeled ATP and 0.5 μCi of [^γ-³³P]ATP to start reactions. Reactions proceeded for 20 min at 37°C and were stopped with the addition of EDTA to 25 mM. The reactions were spotted onto filter paper, washed four times in a beaker of 75 mM phosphoric acid, and rinsed with acetone. Filters were air-dried and counted using a Beckman LS 6000LL liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Protein Kinase C Activity Assay. The effect of scytonemin on recombinant human protein kinase Cβ2 activity was measured by assessing the phosphorylation of a glycogen synthase peptide substrate. The cDNA encoding the protein kinase Cβ2 isoform was cloned from a human brain-derived cDNA library using PCR primers designed according to the sequence published by Coussens et al. (1986). The *Eco*RI fragment containing the coding region was subcloned into a baculovirus transfer vector to generate pVL-protein kinase Cβ2. *Sf* 21 cells cotransfected with AcNPV linear DNA (BD Pharmingen) and the baculovirus encoding protein kinase Cβ2 were incubated at room temperature for 4 days. The infected cells were resuspended in buffer containing 50 mM Tris, pH 7.5, 5 mM EGTA, 2 mM EDTA, 5 mM DTT, 1 mg/ml bacitracin, 50 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride at 2×10^6 cells/ml. The cells were sonicated on ice for six 10-s bursts at 60% output power. A Triton X-100 solution was added to the homogenate to a final concentration of 1% and extracted on ice for 1 h. Lysates were centrifuged at 100,000g for 20 min at 4°C. Supernatant was applied to a Whatman DE52 column (1 ml of supernatant/0.5-ml column) pre-equilibrated with buffer (20 mM Tris, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA). The columns were washed extensively with this same buffer, and fractions were eluted using 90 mM NaCl.

Kinase reactions were conducted in 50 μl consisting of 0.5 μg of recombinant human protein kinase Cβ2 and 0.01 mg/ml glycogen synthase peptide (Bachem Biosciences, King of Prussia, PA) in reaction buffer (10 mM Tris, pH 7.5, 40 μg/ml L-α-phosphatidyl-L-serine, 1 μg/ml 1,3-diolen, 0.9 mM EGTA, 1.1 mM CaCl₂, and 10 mM

Results

MgCl₂). Inhibitors were added at the indicated concentrations with a final DMSO concentration of 10%. An ATP solution was then added to the wells to a final concentration of 0.01 μM unlabeled ATP and 0.5 μCi of [γ-³³P]ATP to initiate the enzymatic reactions. Reactions were allowed to proceed for 20 min at 37°C and were stopped with the addition of EDTA to 25 mM. Two 25-μl aliquots of each reaction were spotted onto individual squares of filter paper, washed four times in a beaker containing 75 mM phosphoric acid, and rinsed with acetone. Filters were air-dried and counted using a Beckman LS 6000LL liquid scintillation counter.

GST-Myt1 Activity Assay. To assess the effects of scytonemin on GST-Myt1 kinase autophosphorylation, soluble, truncated, recombinant human GST-Myt1 was overexpressed in *Sf* 9 cells and purified. Briefly, a vector was constructed to include GST fused via a linker containing a thrombin cleavage site to the amino terminus of the human *Myt1* gene (truncated at amino acid 379 to remove the membrane anchoring domain). The construct was cloned into the pFast-Bac baculovirus expression system (Invitrogen). Purification occurred as described in Roshak et al. (2000) for GST-*polo*-like kinase 1 and GST-*cdc25C*. GST-Myt1 was determined to be 75% pure by SDS-PAGE analysis and Coomassie Blue staining. Dissociative enhanced lanthanide fluorescence immunoassays (DELFLIA) (PerkinElmer Wallac, Turku, Finland) were performed in 50 μl consisting of 0.25 μg of GST-Myt1 in reaction buffer containing 50 mM HEPES, pH 7.4, 2 mM Mn(OAc)₂, 1 μM ATP, and 1 mM DTT. Scytonemin or hymenialdisine was added in DMSO (1% DMSO_(final)). Reactions proceeded for 20 min at room temperature with shaking and were stopped with the addition of EDTA to 20 mM. After a 40-min incubation, to allow for protein binding, the reactions were developed as described by the manufacturer's protocol.

CDK1/Cyclin B Activity Assay. CDK1/cyclin B activity was assessed by phosphorylation of Histone H1. Baculovirus vectors expressing CDK1 and cyclin B1 were a gift from Dr. David Morgan at University of California, San Francisco (San Francisco, CA). Proteins were expressed and purified, following the method described in Desai et al. (1992), to 80% purity as determined by SDS-PAGE and Coomassie Blue staining. CDK1/cyclin B1 kinase activity was analyzed in a 96-well flashplate assay using bovine Histone H1 as a substrate (2.0 μg/well). Reactions were run in 50 μl with 0.25 μg of CDK1/cyclin B1 complex, in reaction buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.1 μM unlabeled ATP, 1 mM DTT, and 0.5 μCi of [γ-³³P]ATP). Scytonemin or hymenialdisine was added in DMSO (1% DMSO_(final)). Reactions, initiated with the addition of ATP, were allowed to proceed for 10 min at room temperature and were stopped by washing the plate five times with 300 μl/well of PBS. Incorporation of radioactivity was measured using a Packard Top-Count.

GST-Checkpoint Kinase 1 Activity Assay. GST-checkpoint kinase 1 activity was evaluated by assessing the phosphorylation of GST-*cdc25C*. Recombinant human GST-checkpoint kinase 1 and GST-*cdc25C* were expressed and purified as previously described in Jackson et al. (2000). The effects of scytonemin and hymenialdisine (positive control) on the activity of GST-checkpoint kinase 1 were measured using a flashplate kinase activity assay format previously described in Jackson et al. (2000).

Statistical Analysis. All data are presented as mean ± S.E.M.; *n* = 3 to 4 for each sample population in each study. One-way analysis of variance using the Student-Newman-Keuls method as a post test was used to determine statistical significance using the SigmaStat 2.0 statistical program (SPSS Science, Chicago, IL). Data calculated as percentage inhibition were done by using the formula: 100[1 - [(sample cpm - background cpm)/(control cpm - background cpm)]]. Percentage control data were calculated by using the formula: 100[1 - [(sample cpm - background cpm)/(control cpm - background cpm)]]. The data plotted for concentration-response curves and enzyme-inhibitor kinetic experiments were fit using the nonlinear least-squares method to standard equations using the GraFit statistical analysis program version 4.06 (Erithacus Software, Ltd., Horley, Surrey, UK).

Evaluation of Scytonemin's Effects on GST-*polo*-Like Kinase 1 Activity. As previously described (Roshak et al., 2000) GST-*polo*-like kinase 1 readily phosphorylates GST-*cdc25C*. As expected, *polo*-like kinase 1 activity was inhibited in the presence of the nonspecific kinase inhibitor, hymenialdisine (10 μM) (Fig. 2A). Scytonemin showed a concentration-dependent inhibition of *polo*-like kinase 1-mediated phosphorylation of *cdc25C* (Fig. 2A). An expanded concentration curve (Fig. 2B) shows the calculated IC₅₀ for scytonemin against *polo*-like kinase 1 activity in vitro to be 2.0 ± 0.1 μM.

Scytonemin-Mediated Inhibition of GST-*polo*-Like Kinase 1 Is Not Time Dependent. To ascertain whether there were any time-dependent changes in scytonemin's ability to inhibit GST-*polo*-like kinase 1 activity, the compound was preincubated with enzyme and substrate, as described under *Materials and Methods*. In this assay, GST-*polo*-like kinase 1 activity was reduced by ~58 and ~93%

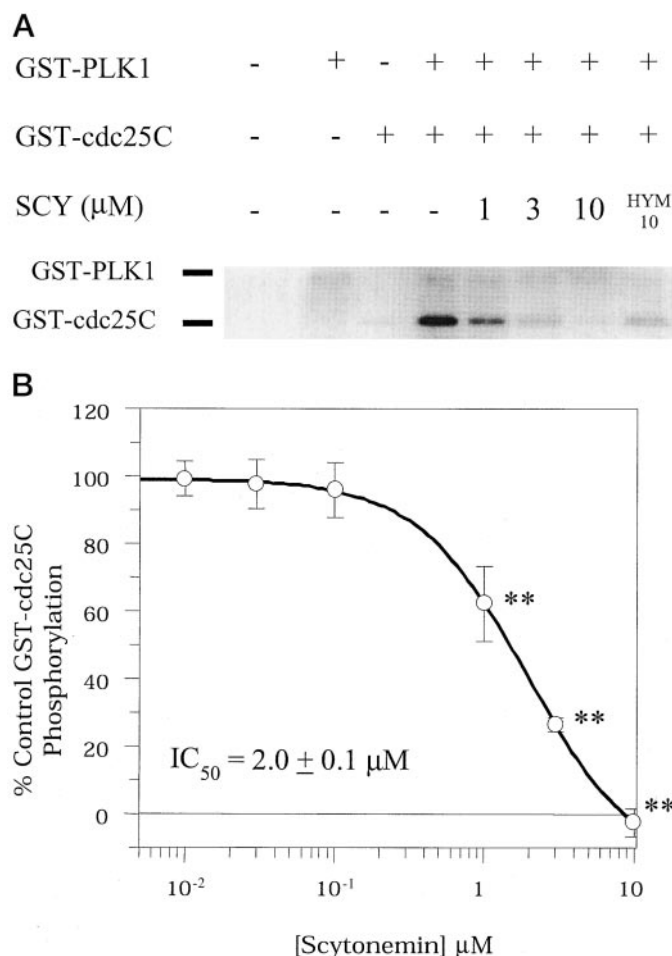


Fig. 2. Scytonemin inhibits GST-*polo*-like kinase 1 phosphorylation of GST-*cdc25C*. **A**, *polo*-like kinase 1-mediated phosphorylation of *cdc25C* was evaluated in the presence of scytonemin (SCY) or hymenialdisine. The reactions were carried out as described under *Materials and Methods*. ³²P incorporation was analyzed by SDS-PAGE and phosphorimage analysis (panel A). This figure is representative of two studies. **B**, to determine the IC₅₀ of scytonemin against GST-*polo*-like kinase 1, microtiter plate assays were performed as described under *Materials and Methods*. The amount of ³²P incorporation into GST-*cdc25C* was measured using a Packard microplate counter. This graph is representative of one of three studies. All error bars represent standard deviation; **, *p* < 0.001; IC₅₀ value is given as IC₅₀ ± S.E.M.

after preincubation with 3 μM and 10 μM scytonemin, respectively, consistent with the levels seen in Fig. 2. In addition, the ability of scytonemin to inhibit *polo*-like kinase 1 activity was unchanged regardless of preincubation time (Fig. 3).

Scytonemin Is a Mixed Inhibitor of GST-*polo*-Like Kinase 1 Activity. Studies were performed to determine whether scytonemin was acting as an ATP competitor. In this series of experiments, the effects of scytonemin on GST-*polo*-like kinase 1 activity were measured in the presence of increasing concentrations of unlabeled ATP. The double-reciprocal plot shown in Fig. 4 indicates that the mechanism for scytonemin may be one of mixed competition with respect to ATP. Comparable values for inhibition kinetics were calculated whether the data were fit as a pure ATP-competitive inhibitor ($K_i = 3.0 \pm 1.9 \mu\text{M}$) or as a noncompetitive inhibitor ($K_i = 2.3 \pm 1.9 \mu\text{M}$). The relatively equivalent K_i values for scytonemin signify that both competitive and noncompetitive components may be involved in the mechanism of scytonemin's inhibition of *polo*-like kinase 1.

Scytonemin Specifically Inhibits Actively Proliferating Cell Types. The ability of scytonemin to affect in vitro cell proliferation in response to growth factor or serum was measured by [^3H]thymidine uptake as described under *Materials and Methods*. PDGF-induced RSF and ECGF-induced HUVEC proliferation was inhibited by scytonemin with IC_{50} values of 1.5 ± 0.2 and $5.4 \pm 3.4 \mu\text{M}$, respectively (Fig. 5, A and B). Scytonemin was also able to inhibit the proliferation of NHLFs in response to serum with an IC_{50} of $2.5 \pm 0.6 \mu\text{M}$ (Fig. 5C). No toxicity (i.e., necrosis) was observed in any of the three cases as determined by visible morphological assessment. Jurkat T cells, a T cell lymphoma cell line, showed reductions in both thymidine incorporation ($\text{IC}_{50} = 7.8 \pm 0.2 \mu\text{M}$) (Fig. 5D) and cell number in the presence of increasing concentrations of scytonemin. As is evident from Fig. 6, A and B, the effect of scytonemin on Jurkat T cell proliferation was identical whether analyzed by counting cells (Fig. 6A) or by using [^3H]thymidine uptake (Fig. 6B). Cell counts also revealed that the concentration of scytonemin-treated Jurkats did not fall below their original values; i.e., the cells treated with 30 μM scytonemin for 48 h never fell below the original seeding density of 2×10^5 cells/ml. Additionally, trypan blue

exclusion tests showed that scytonemin was not toxic at concentrations up to 10 μM (data not shown). To determine whether scytonemin had any effect on nonproliferating cell populations, monocytes, from human whole blood, were exposed to the same concentrations of scytonemin as in the proliferation experiments and activated by lipopolysaccharide (this does not induce proliferation of monocytes). Scytonemin did not lower the original number of cells, nor did it affect the same cells' ability to exclude trypan blue (data not shown).

Scytonemin Induces Jurkat T Cell Apoptosis. To better understand the mode of scytonemin action with respect to inhibition of cell proliferation, Jurkat T cells were treated as above and examined for apoptosis, using an adapted TUNEL staining method. Also measured was the total DNA content of the cells, allowing us to track the ability of scytonemin to facilitate cell cycle arrest. The vehicle-treated group (Fig. 7, A and B) has populations of cells distributed throughout the phases of the cell cycle as measured by propidium iodide incorporation, with only a few cells showing evidence of apoptosis by possessing fragmented DNA. Cells treated with 3 μM camptothecin (Fig. 7, C and D), a DNA topoisomerase inhibitor used as a positive control for both cell cycle arrest and induction of apoptosis (Lee et al., 2000), are arrested at G_2/M phase and with 71% of the cells undergoing apoptosis after 24 h. Exposure to 3 μM scytonemin (Fig. 7, E and F) over the same period of time did not result in any cell cycle arrest, but did cause the cells to undergo apoptosis. After 24 h, 24% of scytonemin-treated Jurkat cells had TUNEL-positive staining, which is consistent with the level of inhibition of proliferation seen in the earlier experiments (Fig. 5D).

Evaluation of Scytonemin's Effects on the Activity of Other Protein Kinases. In light of scytonemin's inability to effect a G_2/M arrest, it was assayed against other kinases to assess its selectivity. These include mechanistically diverse kinases such as the tyrosine kinase, Tie2, and the tyrosine/threonine kinase Myt1, as well as additional serine/threonine kinases such as protein kinase A, protein kinase $\text{C}\beta 2$, CDK1, and checkpoint kinase 1. Scytonemin was first tested against the kinases not directly involved in CDK1 regulation, Tie2, protein kinase A, and protein kinase $\text{C}\beta 2$. Scytonemin was

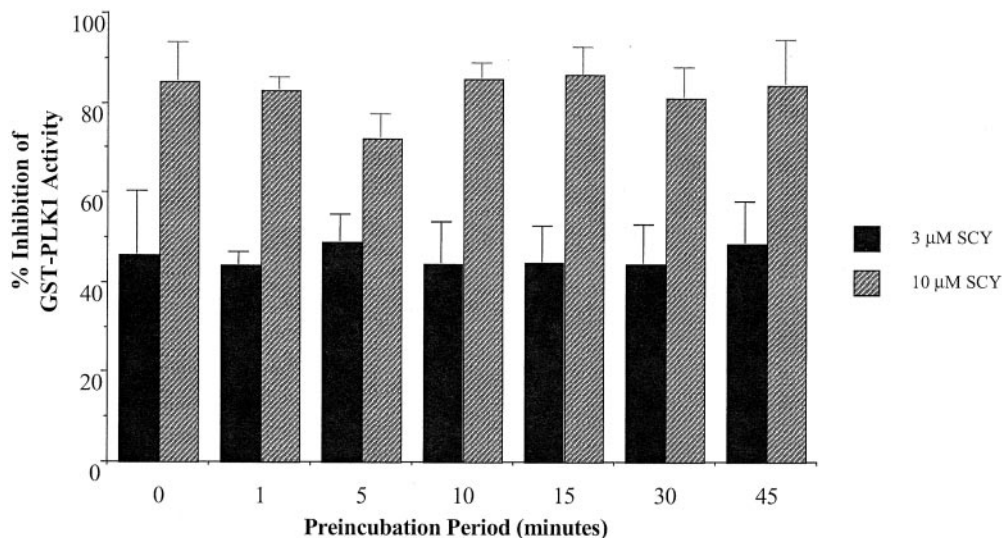


Fig. 3. Scytonemin-mediated inhibition of GST-*polo*-like kinase 1 is not time dependent. Scytonemin was preincubated with GST-*polo*-like kinase 1 and GST-*cdc25C* for 0, 1, 5, 10, 15, 30, and 45 min at room temperature and the reaction was initiated with the addition of ATP and run as described under *Materials and Methods*. Two concentrations (3 and 10 μM) of scytonemin (SCY) were used to test whether there were time-dependent changes in scytonemin's inhibition of GST-*polo*-like kinase 1 activity. Error bars represent standard deviation. This chart is representative of one of four studies.

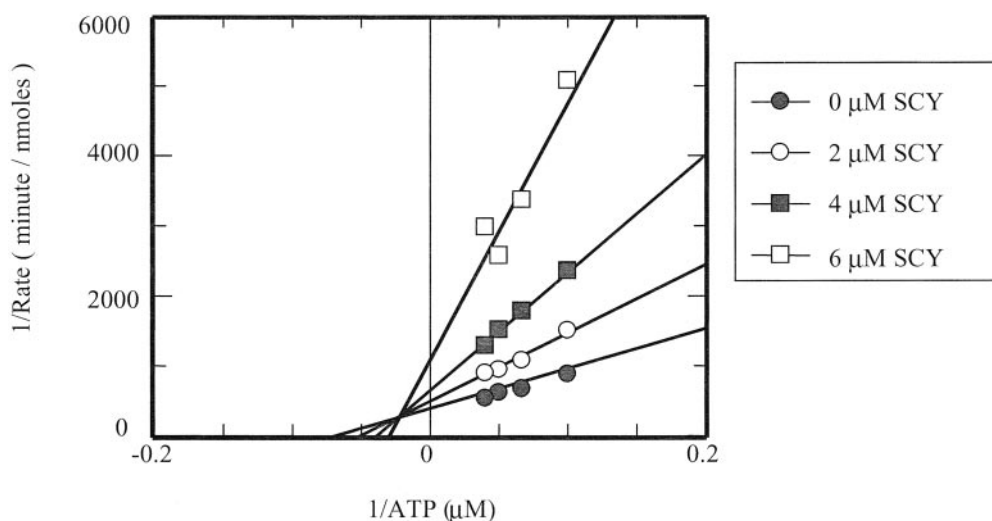


Fig. 4. Scytonemin is a mixed inhibitor of GST-*polo*-like kinase 1 activity. The levels of GST-*polo*-like kinase 1 inhibition were evaluated, as described under *Materials and Methods*, at three concentrations of scytonemin (SCY) (2, 4, and 6 μ M) in the presence of increasing concentrations of unlabeled ATP (10, 15, 20, and 25 μ M). The double-reciprocal plot was generated using the GraFit statistical analysis program. This analysis was done using data combined from three identical experiments.

unable to significantly block the activity of either GST-Tie2 or protein kinase A (Table 1) at concentrations equivalent to those that inhibited GST-*polo*-like kinase 1. Recombinant human protein kinase C β 2 activity was inhibited by scytonemin in a concentration-dependent manner with potency ($IC_{50} = 2.7 \pm 0.4 \mu$ M) similar to that for GST-*polo*-like kinase 1 (Table 1). In addition, scytonemin displayed activity on several other kinases involved in cell cycle regulation. CDK1/cyclinB, GST-Myt1, and GST-checkpoint kinase 1 were all inhibited by scytonemin in a concentration-dependent manner with IC_{50} values ranging from 1 to 3 μ M (Table 1).

Discussion

Screening natural products for potential therapeutic benefit and the identification of novel pharmacophores is still an integral aspect of drug discovery. We had preliminarily reported that scytonemin, isolated from a cyanobacterium, had the ability to inhibit *polo*-like kinase 1 in a flashplate screening assay (Stevenson et al., 2002). This assay was used to identify purified natural product compounds with the potential capacity of inhibiting kinases contributing to chronic hyperproliferative conditions. Here, we more fully describe scytonemin as the first characterized small molecule inhibi-

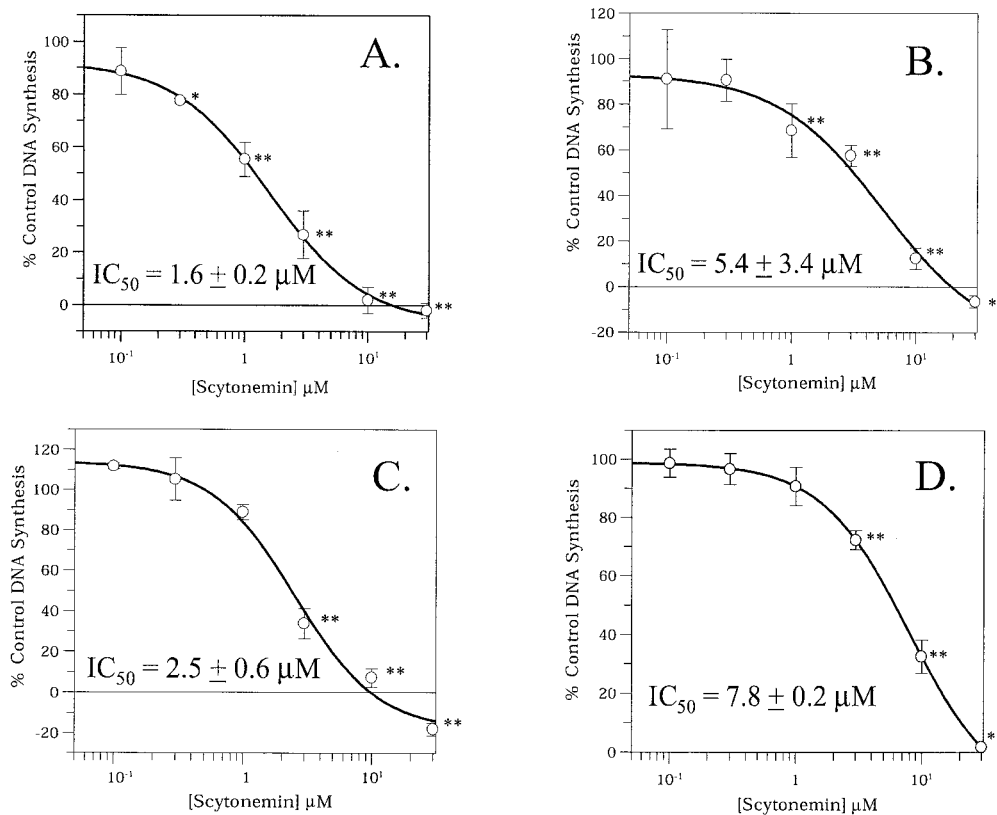


Fig. 5. Scytonemin inhibits growth factor-induced cell proliferation. Scytonemin was tested over a dose range of 100 nM to 30 μ M for its ability to inhibit [3 H]thymidine incorporation of PDGF-BB-stimulated rheumatoid synovial fibroblasts (A), ECGF-induced HUVECs (B), FBS-stimulated NHLFs (C), and FBS-stimulated Jurkat T cells (D), as described under *Materials and Methods*. Data are presented as the percentage of [3 H]thymidine incorporated as compared with the vehicle (0.5% DMSO) controls for each cell type. All plots shown are representative graphs. Error bars represent standard deviation; *, $p < 0.005$; **, $p < 0.001$; IC_{50} values are given as $IC_{50} \pm$ S.E.M.

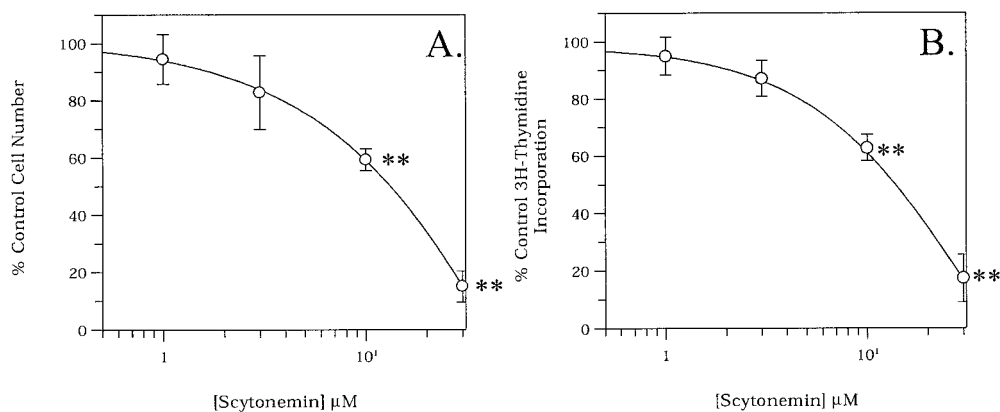


Fig. 6. Scytonemin inhibits Jurkat T cell proliferation. Scytonemin was tested over a dose range of 1 to 30 μM for its ability to inhibit cell proliferation in Jurkat T cells as assessed by cell counts (A) and [^3H]thymidine incorporation (B). Data are presented as the percentage of control cell number (A) or [^3H]thymidine (B) incorporated as compared with the vehicle (0.5% DMSO) controls. Both plots shown are representative graphs of two studies. Error bars represent standard deviation; **, $p < 0.001$.

tor of *polo*-like kinase 1 activity. Scytonemin inhibited the ability of GST-*polo*-like kinase 1 to phosphorylate GST-cdc25C in a concentration-dependent manner with an IC_{50} of $2.0 \pm 0.1 \mu\text{M}$, as characterized by both phosphorimage and filtration assays (Fig. 2, A and B). Investigations into the

possible kinetic mechanism underlying scytonemin's inhibition of GST-*polo*-like kinase 1, a serine/threonine kinase, revealed that there were no time-dependent aspects to its activities, suggesting that scytonemin's mechanism is reversible. This is an important discovery, in that several natural

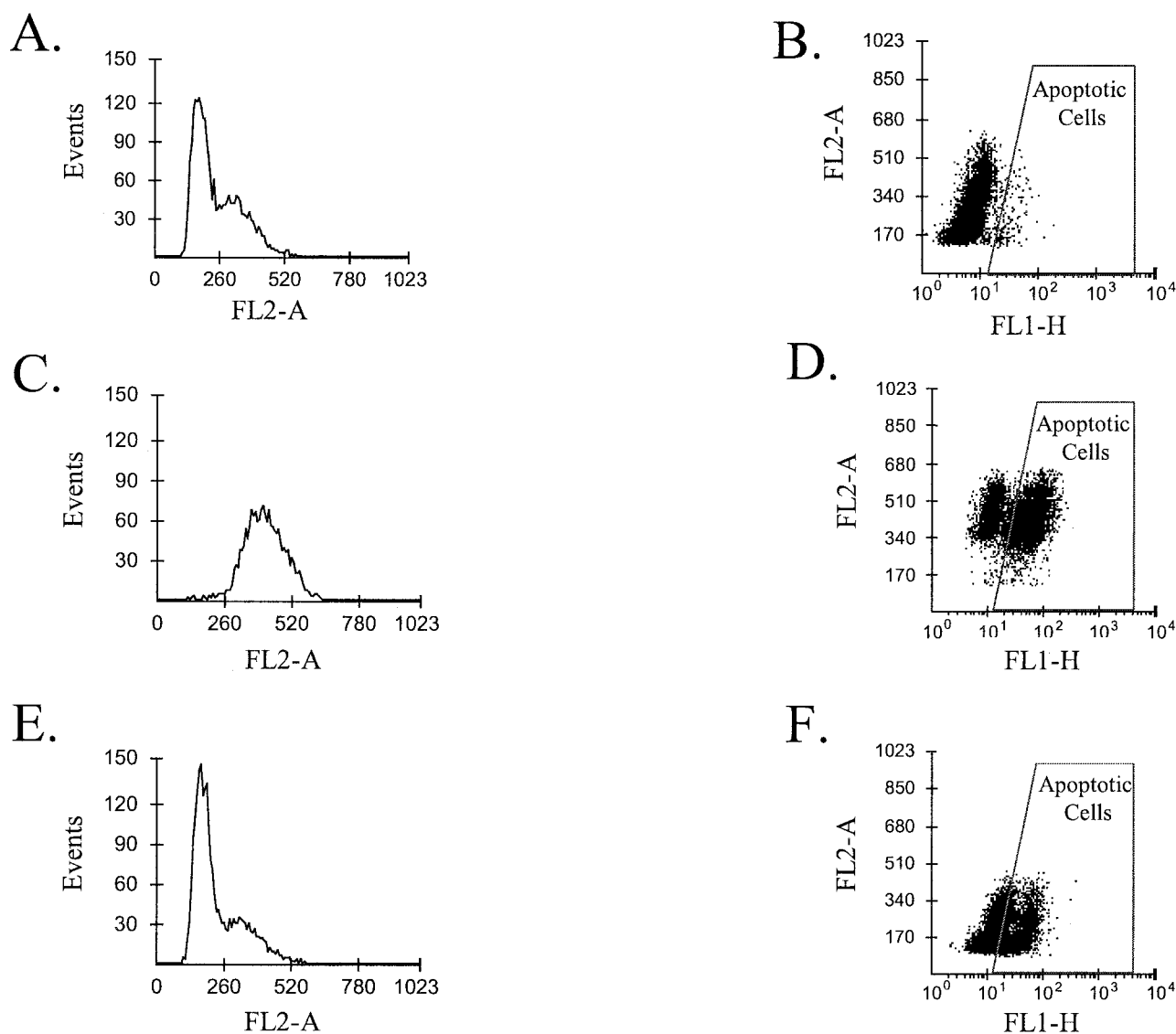


Fig. 7. Scytonemin induces apoptosis in Jurkat T cells. Jurkat cells were treated with 3 μM scytonemin (E and F), 3 μM camptothecin (positive control) (C and D) or 0.1% DMSO (vehicle control) (A and B). Samples were collected at 24 h and assayed for DNA content (FL2-A) (A, C, and E) and DNA fragmentation (FL1-H), i.e., apoptosis (B, D, and F), as described in the *Materials and Methods*. Shown are the results of one representative study.

TABLE 1

Effect of scytonemin on other kinase activities

The effects of scytonemin on the activity of various kinases were measured over a range of concentrations (10 nM– μ M). The values indicate the $IC_{50} \pm$ S.E.M. generated from one study, which was representative of at least two studies.

Protein Kinase	Kinase Type	IC_{50} of Scytonemin Inhibition
		μ M
GST-Tie2	Tyrosine	>10
Protein kinase A	Serine/Threonine	>10
Protein kinase C β 2	Serine/Threonine	2.7 \pm 0.4
CDK1/cyclin B	Serine/Threonine	3.0 \pm 0.3
GST-Myt1	Threonine/Tyrosine	1.2 \pm 0.2
GST-checkpoint kinase 1	Serine/Threonine	1.4 \pm 0.2
GST-polo-like kinase 1	Serine/Threonine	2.0 \pm 0.1

products have been found to work as irreversible inhibitors of certain enzymes, which prohibits their use as clinically effective agents and limits their potential utility as pharmacophores. Many kinase inhibitors are known to work via ATP competition (Garcia-Echeverria et al., 2000). Our results indicate, however, that scytonemin is a mixed inhibitor of GST-*polo*-like kinase 1 activity. Hence, using this structure as a template may prove useful in identifying structural elements that can lead to the development of either a specific, reversible allosteric inhibitor of *polo*-like kinase 1 or one that is ATP competitive. Nonetheless, definitive conclusions about scytonemin's kinetic mechanism against *polo*-like kinase 1 can not be drawn until additional studies using more purified forms of both enzyme and substrate are conducted, which would allow for more clarity in interpreting the kinetics of the enzyme and its inhibition. Evaluation of scytonemin's actions on other related and unrelated kinases revealed that it could inhibit other cell cycle kinases at similar concentrations but had little or no effect on either the tyrosine kinase, Tie2, or another serine/threonine kinase, protein kinase A. This suggests that scytonemin was not a "pan-active" kinase inhibitor and did display some selective activity. This is not an unusual finding in isolated marine natural products and does not prohibit the use of scytonemin's novel chemical structure as a scaffold or template for the design of new, more potent and selective compounds. Indeed, other natural-product compounds composed of indolic and phenolic subunits, such as staurosporine and balanol, have kinase-inhibitory activity (Garcia-Echeverria et al., 2000) and have served as structural templates to design more potent and selective inhibitors.

Consistent with its activity on cell cycle regulatory kinases, evaluation of scytonemin in several in vitro cell proliferation systems demonstrated its ability to inhibit proliferation. In rheumatoid arthritis patients, both PDGF-BB and its receptor are found at abnormally high concentrations in synovial tissue (Rubin et al., 1988; Remmers et al., 1991) and thought to contribute to RSF proliferation. This information led to the development of an in vitro system using RSFs, isolated from patients diagnosed with rheumatoid arthritis, and induced to proliferate with PDGF-BB (Butler et al., 1988). Similarly, the ECGF-induced HUVEC proliferation system used here was modeled after one developed to identify agents that may be effective in inhibiting angiogenesis, a process involved in the progression of many hyperproliferative disorders (Folkman and Haudenschild, 1980). Serum-stimulated NHLFs were used to represent the proliferative component of fibrotic pul-

monary disorders such as chronic obstructive pulmonary disorder. In all three cases, scytonemin effectively attenuated the proliferation of these cells in a concentration-dependent manner with IC_{50} values comparable to one another in the low micromolar range, as assessed by [3 H]thymidine incorporation, and with no significant chemical toxicity at concentrations up to 10 μ M, determined by visible morphological assessment.

Jurkat T cells, a tumor cell line, were used to assess scytonemin's ability to inhibit a cancer-derived cell type. Both [3 H]thymidine and cell counts demonstrated scytonemin's concentration-dependent reduction in Jurkat T cell proliferation, again with no chemical toxicity detected via trypan blue exclusion tests. Interestingly, more in depth investigation using flow-cytometric analysis revealed that scytonemin displayed no ability to arrest the cells at any one phase of the cycle, but was able to induce cells to undergo apoptosis independent of cell cycle phase. This induction of apoptosis is a significant finding in that, unlike necrosis or chemical toxicity, it indicates that scytonemin interrupts essential biochemical processes, which triggers the cell to undergo programmed cell death. Although *polo*-like kinase 1 inhibition could be one of the mechanistic reasons for this, scytonemin's actions on other kinases also likely contribute. Indeed, the lack of arrest at G₂/M suggests more than one site of action for scytonemin.

The effects of scytonemin were also evaluated in a model using cultured, nonproliferating, freshly isolated human monocytes activated by lipopolysaccharide. Monocytes exposed to the same concentrations of scytonemin as in the other cellular proliferation assays remained unaffected. Its lack of effect on cell viability and cell density, as determined by trypan blue exclusion and visual assessment, respectively, support the notion that scytonemin has a specific ability to target the cell cycle processes of actively proliferating cell populations, a process not invoked by nonproliferating cell populations. This is an attractive property for any prospective antiproliferative agent and provides a potential therapeutic window for specific antitumor or anti-hyperplasia activity.

These attributes advocate utilizing scytonemin as a novel pharmacophore for the development of new antiproliferative agents. The unique structural composition of these subunits in scytonemin provides a template that, along with tools like X-ray crystallography and molecular modeling, should yield another class of potent kinase inhibitor. Structural modifications through the development of a focused combinatorial library may be a good strategy toward the design of analogs. The compound's lack of chirality, obvious dissection points (1-1', 3--9, and 3'-9'), and phenolic groups are all attractive qualities making scytonemin amenable for this type of approach. In addition, related compounds, like nostodione A, that have similar cellular effects should be tested for comparable molecular effects. Such compounds could then serve as alternative chemical scaffolds to generate related combinatorial libraries. To that end, we feel that scytonemin, the first described inhibitor to *polo*-like kinase 1, shows promise as a novel chemical moiety, which can be further elaborated on using state of the art medicinal chemistry approaches, to develop more potent and selective antiproliferative agents.

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