The Cellular Target Specificity of Pateamine A

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The natural product pateamine A (pateamine) from the sponge *Mycale hentscheli* is active against a wide range of dividing cells and has been shown to inhibit the functions of the eukaryotic initiation factor 4A (eIF4A). We have identified that pateamine is additionally able to modulate the formation of actin filaments and microtubules *in vitro* but at higher concentrations than required for inhibition of eIF4A. Cell cycle analysis confirmed that actin and tubulin are not major mediators of the cellular activity of pateamine. The range of targets identified demonstrates the value of multiple approaches to determining the mode of action of biologically active compounds.

Key words: Pateamine, Actin, Tubulin

Introduction

Pateamine A (pateamine; Fig. 1) is a novel thiazole-containing natural product, first isolated in the early 1990's from samples of the marine sponge *Mycale hentscheli* collected off the southwest coast of the South Island of New Zealand (Northcote *et al.*, 1991). The biological activity of pateamine has been well characterized; it is highly toxic to a wide array of mammalian cell lines and has antifungal activity, but is essentially inactive against prokaryotes (Northcote *et al.*, 1991). A number of detailed studies have been undertaken with pateamine itself or with des-methyl des-amino pateamine A (DMDA-PatA), a simplified analogue that retains activity comparable to the natural product (Romo *et al.*, 2004). Like pateamine, DMDA-PatA shows a surprising lack of variability in activity towards a range of cell lines under normal growth conditions, with IC_{50} values typically within the 0.2–10 nM range for non-quiescent cells (Kuznetsov *et al.*, 2009). There is a significant difference in this activity between dividing and quiescent cells with quiescent cells being characterized as approximately 1000- (Kuznetsov *et al.*, 2009) to 2000-fold (Northcote *et al.*, 1991) less sensitive.

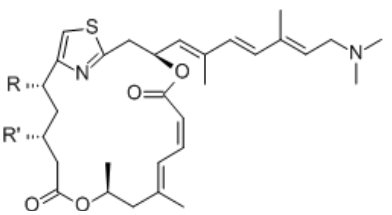

Fig. 1. The chemical structure of pateamine A (R = CH_3, R' = NH_2) and des-methyl des-amino pateamine A (R, R' = H).

This wide-spectrum activity and specificity for dividing cells suggest that pateamine interacts with one or more targets that are fundamental to eukaryotic cell survival and proliferation. Studies designed to determine the target of pateamine identified that it inhibits protein synthesis through inhibition of cap-dependent translation, and have revealed that members of the eukaryotic initiation factor 4A (eIF4A) family are mediators of this activity (Bordeleau *et al.*, 2005; Dang *et al.*, 2009). Members of the eIF4A family are ATP-dependant RNA helicases (Grifo *et al.*, 1983) and are the archetypal DEAD box helicases. There are three members of the eIF4A family in humans: eIF4AI and eIF4AII form a key component of the eIF4F complex that recruits the ribosome to a newly exported mRNA transcript (Conroy *et al.*, 1990; Grifo *et al.*, 1983; Yoderhill *et al.*, 1993) and melts secondary structure in the mRNA 5`-UTR immediately proximal to the cap allowing the ribosome to access the start codon (Pause *et al.*, 1994; Ray *et al.*, 1985; Svitkin *et al.*, 2001). The third member of the eIF4A family in humans, eIF4AIII, is required for non-sense-mediated mRNA decay (NMD) as

a part of the exon-junction complex and does not have a role in translation (Chan *et al*., 2004; Ferraiuolo *et al*., 2004; Li *et al*., 1999; Palacios *et al.*, 2004; Shibuya *et al*., 2004).

Although the eIF4A proteins are well-established as the most likely mediators of the activity of pateamine and its close analogues, there are indications that additional targets may contribute to this activity (Bordeleau *et al*., 2005) and potentially explain the complex structure-activity relationships exhibited by pateamine analogues (Romo *et al*., 2004). Recently Kuznetsov and coworkers (2009) have shown that DMDA-PatA causes some cell lines to arrest in the S-phase and have demonstrated direct inhibition of DNA polymerases α and γ, but at concentrations above those reported for *in vitro* or cellular activity against eIF4A.

As part of our study into its activity we generated a pateamine affinity resin to isolate cytosolic binding targets in an impartial fashion. In addition to the eIF4A family we identified cytokeratin and tubulin as proteins selectively isolated from HL-60 cells (Bordeleau *et al*., 2005). Whilst the interaction between pateamine and eIF4A was demonstrated to be functional and to occur at concentrations relevant to pateamine's cytotoxicity, the evaluation of the interaction with cytoskeletal elements remained unresolved. Herein we report the extension of the affinity chromatography study to include HeLa and SH-SY5Y cells, the consistent isolation of tubulin and actin but not cytokeratin as binding partners in addition to eIF4A, and the *in vitro* assessment of the impact of pateamine on the assembly of actin filaments and microtubules. We show that pateamine has the capacity to disrupt the cytoskeleton, but only at concentrations above those required for protein synthesis inhibition.

Results

Pateamine is cytotoxic to cultured cells

To ensure that the batches of pateamine supplied for this study were of comparable biological activity to samples tested in previous studies and to account for batch-to-batch variations in purity a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) was performed after 24 h exposure to pateamine with the three cell lines used for subsequent binding protein studies: leukemic HL-60 cells (Gallagher *et al*., 1979), cervical epithelial HeLa cells (Scherer and Hoogasian, 1954), and the neuroblastoma cell line SH-SY5Y (Biedler *et al*., 1978). Pateamine displayed similar cytotoxicity in the three cell lines with IC_{50} values of (1.43 ± 0.06) nM, (1.5 ± 0.04) nM, and (1.98 ± 0.08) nM for HL-60, HeLa, and SH-SY5Y cells, respectively. These values are comparable to those previously published (Hood *et al.*, 2001).

Identification of putative pateamine-binding proteins

Across each of the HL-60, HeLa, and SH-SY5Y cell lysates tested, only three proteins, 1–3, were consistently eluted from the pateamine resin that did not appear in the control resin (Fig. 2, lanes B, D, and F). The three putative pateamine-binding proteins were identified by peptide mass fingerprinting after trypsin digestion. In all cell lines except SH-SY5Y, binding protein 1 was identified as being a member of the β-tubulin family, however, the specific isotype(s) could not be determined due to a lack of discriminating peptides in the mass spectra. Whilst the band 1 from SH-SY5Y cells was of the same apparent molecular weight as that from the other cell lines, no significant hits were obtained from the mass fingerprinting. Binding protein 2 was identified as eukaryotic initiation factor 4A (eIF4A) as previously reported (Bordeleau *et al*., 2005). Analysis of the mass spectral information confirmed the presence of all three isoforms for each cell line tested consistent with our earlier identification of eIF4AI, -II, and -III from the HL-60 cell line (Bordeleau *et al*., 2005). Gel band 3 was identified as β/γ-actin from each of the three cell lines; no discriminating peptides were found to allow us to identify whether one or both isoforms were present. Low-abundance proteins and those not evident in all cell lines were not investigated. Two commonly seen higher-molecular weight bands at approximately 60 and 55 kDa were identified as originating from cytokeratin which was also identified in the control resin.

Pre-incubation of the HL-60 cell lysate with pateamine prevented the binding of any of the three previously recovered proteins to the pateamine resin and consequently there were no significant differences between the pateamine and control resins (Fig. 2, lane H). Washing the affinity resin with 1 M sodium chloride significantly

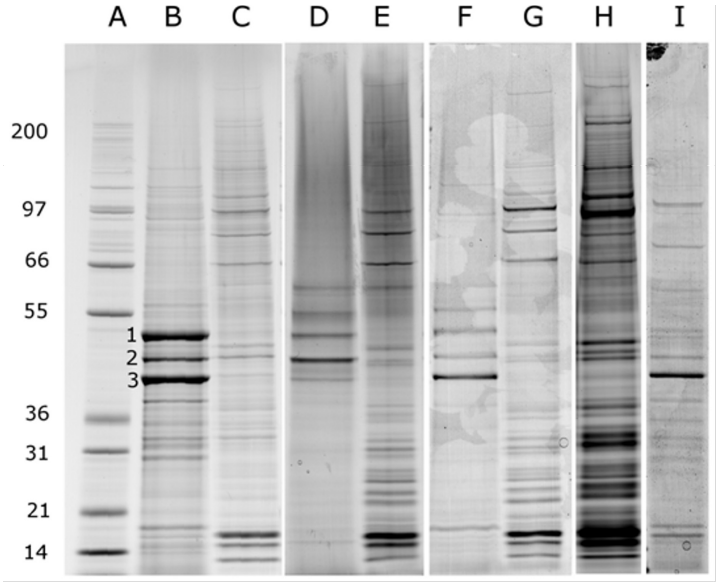

Fig. 2. Pateamine-binding protein isolation. Pateamine affinity resin was used to isolate putative targets from HeLa, HL-60, and SH-SY5Y cells (lanes B, D, and F, respectively). Bands 1–3 were not retained by the control resin (lanes C, E, and G) or after the addition of 500 μM pateamine to the HeLa cell lysate (lane H). Band 2 (confirmed as eIF4A by mass spectrometry) is retained by the pateamine resin after a high salt wash (lane I). Lane A, molecular size markers (kDa) as indicated.

decreased the amount of bands 1 and 3, but not of band 2 (Fig. 2, lane I), as confirmed by mass spectrometry highlighting a difference in the nature of the interaction of pateamine with actin and tubulin as compared to eIF4A.

Pateamine displays dual effects on in vitro microtubule polymerization

We used a turbidity assay to measure the effect of pateamine on microtubule polymerization which has been shown to be particularly valid for systems with low rates of nucleation (Hall and Minton, 2005). The effect of pateamine on the polymerization of bovine brain tubulin was compared to those of paclitaxel and colchicine which are known to enhance (Lopes *et al.*, 1997) and suppress (Borisy and Taylor, 1967) microtubule formation, respectively. Pateamine significantly enhanced the tubulin polymerization at 200 μM compared to the 2% ethanol control (Fig. 3A). In contrast 20 μM pateamine seemed to disrupt polymerization up to the 90 min end point to a level similar to that achieved by the same concentration of colchicine (Fig. 3B) with end point values not significantly distinguishable ($P > 0.03$, using a heteroscedastic Student's t-test based on absorbance values obtained between 88 and 93 min from three independent experiments performed in triplicate).

High concentrations of pateamine disrupt polymerization of rabbit α-actin in vitro

Pyrene-labelled actin was used to assess the *in vitro* effect of pateamine on microfilament formation (Kouyama and Mihashi, 1981). Fig. 4 shows that pateamine caused concentration-dependent suppression of both the initial rate of actin polymerization and the maximum fluorescence intensity attained indicating that a lower abundance of polymerized actin filament is formed, thus higher concentrations of non-polymerized actin remained in solution with higher concentrations of pateamine. Although evident at relatively low concentrations the IC_{50} values for both effects were well above 100 μM.

Pateamine does not specifically disrupt cell cycle progression

Given the well-established roles of both the actin microfilaments and microtubules in mitosis

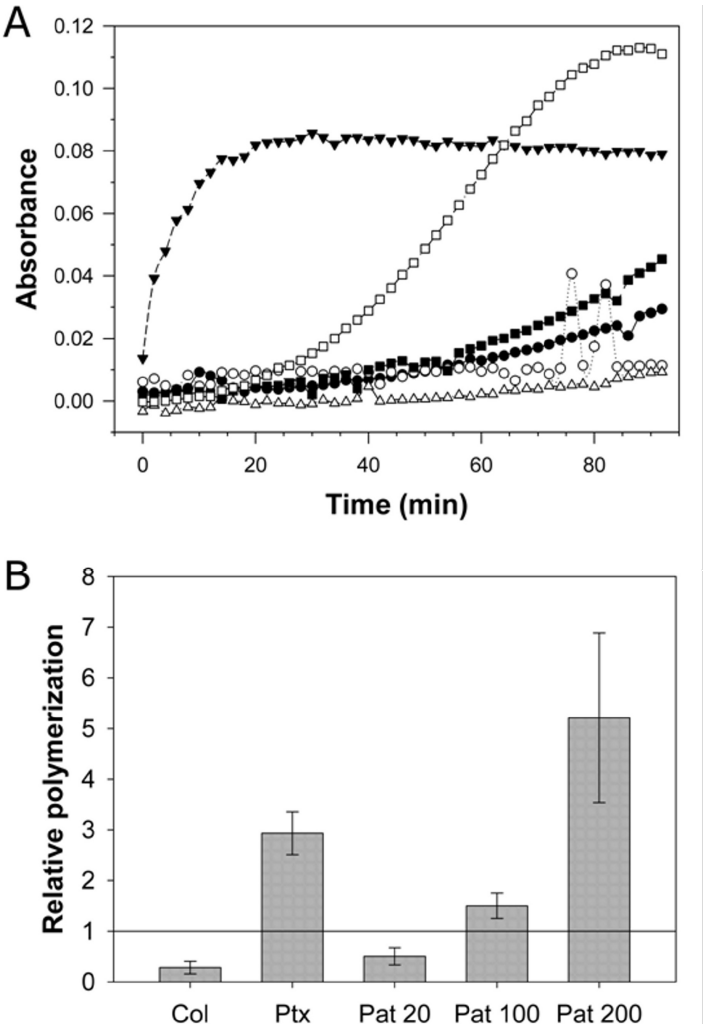

Fig. 3. Pateamine causes microtubule stabilization *in vitro*. (A) A typical tubulin polymerization time course experiment measured by absorbance at 365 nm. Fit lines were generated using a Loess biweight algorithm. Lines are 200 μM pateamine (open squares), 20 μM paclitaxel (closed triangle), 100 μM pateamine (closed squares), 2% ethanol control (closed circles), 20 μM colchicine (open circles), and 20 μM pateamine (open triangles). (B) End point polymerization relative to 2% ethanol carrier control derived from the 90 min time point. Col, 20 μM colchicine; Ptx, 20 μM paclitaxel; Pat 20, 20 μM pateamine; Pat 100, 100 μM pateamine; Pat 200, 200 μM pateamine.

and the observation that drugs that target these proteins tend to cause a change in the distribution of cells across the cell cycle (Lee and Keng, 2005; Lopes *et al*., 1997), the effects of pateamine on cell cycle distribution were studied using flow cytometry. At all concentrations tested pateamine caused a statistically significant increase in the amount of small debris in the sub-G_1 region (Fig. 5) (unpaired t-test, $P < 0.0003$) corresponding to an increase in apoptotic bodies, however, there was no evidence of cell cycle re-distribution as would be expected from compounds acting through actin or microtubule dynamics. This lack of cell-cycle effect was retained across a range of pateamine concentrations and potentially reflects rapid onset of apoptosis under the influence of pateamine. This was confirmed by identification of statistically significant caspase 3/7 activation and loss of phosphatidylserine asymmetry (data not shown).

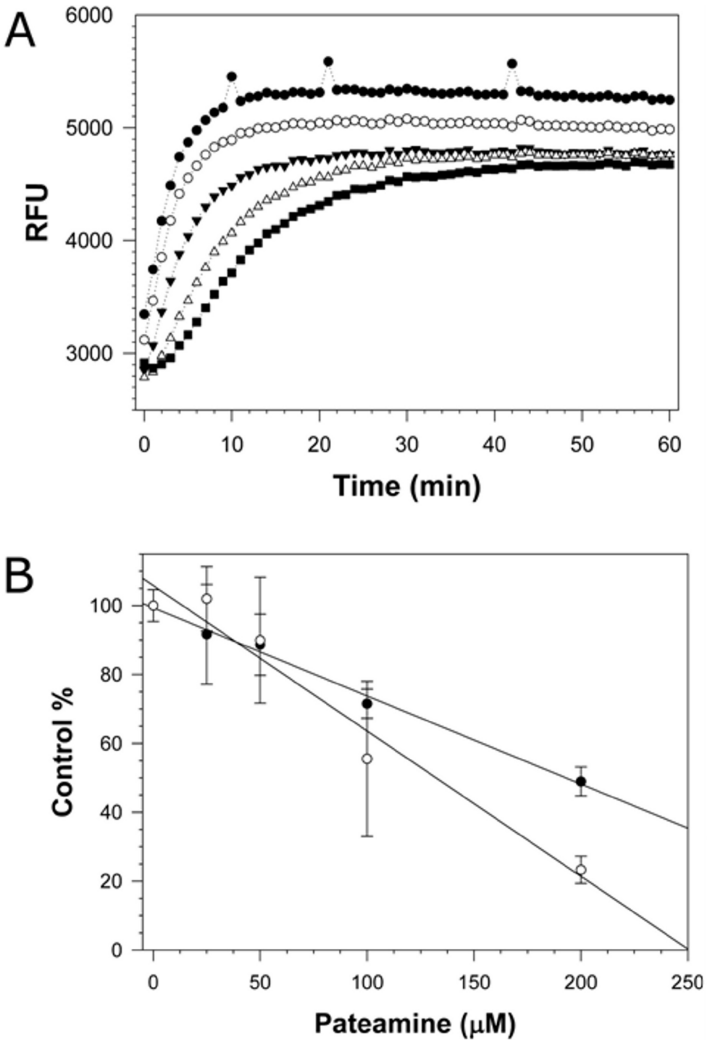

Fig. 4. Pateamine decreases both the amount of polymerized actin and the initial kinetics of polymerization in a concentration-dependent manner. (A) Typical pyrene-labelled actin polymerization experiment. Lines are 1% DMSO control (closed circles) and pateamine: 25 μM (open circles), 50 μM (closed triangles), 100 μM (open triangles), 200 μM (closed squares). (B) Changes in the maximum amount of polymerized actin (filled circles) are reported on the basis of differences in RFU from $t = 0$ to the steady-state average; initial rates (open circles) are drawn from the first 3 min of the time course. Error bars represent standard deviations of triplicate experiments.

Discussion

Although it has been shown previously that eIF4A is inhibited by pateamine (Bordeleau *et al*., 2005; Low *et al*., 2005), there were indications that pateamine may interact with multiple targets. In the current study we have used affinity chromatography to confirm our earlier observations of the interaction between pateamine and eIF4A and have now shown that eIF4A actin and tubulin are the only proteins significantly retained by pateamine from three representative mammalian cell lines. Whilst we did not isolate the DNA polymerases identified by Kuznetsov *et al.* (2009), this was expected as our cytosolic preparation would have excluded the nuclear fraction.

Table I summarizes the concentrations of pateamine required for functional effects against its full range of targets which has now been shown to include actin and tubulin. The impact on actin

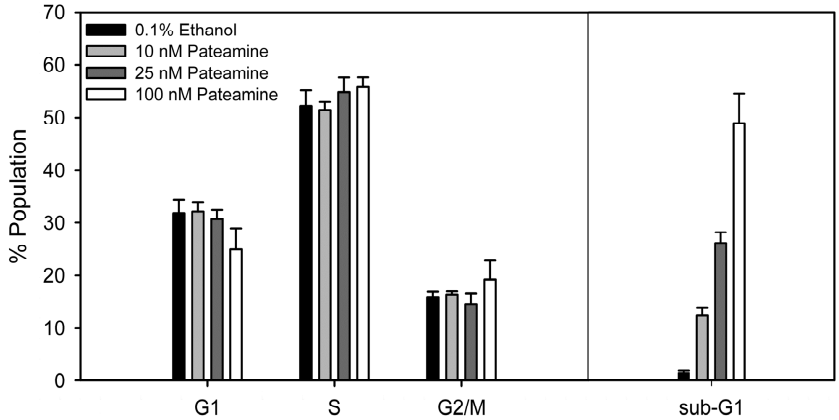

Fig. 5. Cell cycle distribution in the presence of pateamine. HeLa cells were treated for 18 h with pateamine at the concentrations shown in RPMI media or 0.1% ethanol carrier solvent control. Cells were washed, membranes permeabilized, and DNA stained with propidium iodide. DNA content was measured by flow cytometry, and the control cell samples were used to identify cell cycle stages. The left panel shows the relative proportion of cells in each cell cycle phase as a function of the total number of cycling (non-sub-G_1) cells. There are no statically significant differences between treatments. The right panel shows the proportion of sub-G_1 relative to the total gated population (cycling cells and sub-G_1 debris). The increase in sub-G_1 population is highly statistically significant ($P < 0.0003$, unpaired t-test).

Table I. A summary of *in vitro* target interactions with pateamine A and DMDA-PatA.

Concentration [μM]	Affect	Inhibitor (study)
0.2	IC_{50} cap-dependent translation	Pateamine A (Bordeleau *et al*., 2005)
0.5–1	eIF4AIII binding NMD inhibition through stabilization of the interaction between the EJC and mRNA	Pateamine A (Dang *et al*., 2009)
3.84	IC_{50} DNA polymerase α	DMDA-PatA (Kuznetsov *et al*., 2009)
10	10-fold increase in eIF4A ATPase activity	Pateamine A (Bordeleau *et al*., 2005)
10	Significantly increases binding of ATP to eIF4A in the presence of RNA	Pateamine A (Bordeleau *et al*., 2005)
10	Significantly increases RNA binding with and without ATP	Pateamine A (Bordeleau *et al*., 2005)
18.22	IC_{50} DNA polymerase γ	DMDA-PatA (Kuznetsov *et al*., 2009)
20	Equivalent suppression of microtubule formation to colchicine at 20 μM	Pateamine A (this study)
25	Mild increase in actin critical concentration	Pateamine A (this study)
130	IC_{50} initial rate of actin polymerization	Pateamine A (this study)
190	Increase in actin critical concentration to 50% of control	Pateamine A (this study)
200	Promotion of microtubule formation equivalent to or greater than paclitaxel at 20 μM	Pateamine (this study)

filament formation and microtubule stabilization occur only at concentrations much higher than those recorded for other targets, whilst the ability to destabilize microtubules occurs at a concentration similar to that required for effects on other targets although only noted at concentrations above those that lead to eIF4A inhibition. Microtubules are a common target for a number of cytotoxic compounds including the destabilizer colchicine (Borisy and Taylor, 1967) as well as the taxanes epothilones and peloruside A that stabilize microtubules (Hood *et al*., 2002; Jordan and Wilson, 2004; Singh *et al*., 2008).

To confirm that targeting microtubules and actin was not contributing significantly towards the cellular activity of pateamine cell cycle analysis was undertaken. Agents such as paclitaxel and colchicine that interfere with microtubule dynamics generally disrupt mitosis. In mammalian cells mitotic arrest leads to failure to pro-

gress through the G_2/M phase checkpoint of the cell cycle providing a characteristic marker on flow cytometric analysis (Honore *et al*., 2005). Pateamine treatment did not lead to G_2/M blockade nor did it result in failure to complete cytokinesis as is generally observed with compounds that inhibit actin function (Usui *et al*., 2004), but rather induced apoptosis in the absence of statistically significant changes in the ratios of G_1, S, and G_2/M cell populations. These results are consistent with those of Kuznetsov *et al*. (2009) according to which pateamine induced an S phase block in U-937 cells with no observable change in the G_2/M population. Interestingly the same study found no S phase block in COLO 205 or MDA-MB-435 cell lines despite the inhibition of DNA synthesis in the latter. On this basis an S phase effect cannot be ruled out for the HeLa cell line used here. However, the lack of an G_2/M block in HeLa cells suggests that effects on microtubules and actin filaments are unlikely to contribute significantly to the activity of pateamine.

Quiescent cells are more resistant to pateamine (Northcote *et al*., 1991; Kuznetsov *et al*., 2009). Cap-dependent translation initiation is non-essential in quiescent cells (Miskimins *et al*., 2001); since pateamine targets eIF4AI/II the resistance of these cells to pateamine is understandable, however, it would be expected that for these reasons pateamine should induce a G_1 cell cycle block (Huang *et al*., 2003). Furthermore knockdown of eIF4AIII in *Drosophila* cells causes a decrease in the G_2/M cell population (Boutros *et al*., 2004). Neither of these effects were observed by Kuznetsov *et al*. (2009) or in the current study using HeLa cells. Therefore a complete appreciation of the activity of pateamine must take into account the relative inhibition of eIF4AI/II, eIF4AIII, and DNA polymerase, the combined effect of which is to cause apoptosis at all stages of the cell cycle.

In the present study we have demonstrated an approach to the ascription of accurate biological ontologies to small molecules. The use of chemical proteomic approaches to identify targets in an impartial fashion coupled with *in vitro* assays against isolated targets and cell-based assays provides an important array of target-related information. Given the different strengths and weaknesses of the various techniques used, a full picture of activity is only revealed when such an array of approaches is used. The set of targets addressed by pateamine cover many of the pathways that would be desirable in a potent biologically active molecule, *i.e.* the synthesis of proteins and DNA along with perturbation of the cytoskeleton. Pateamine therefore provides an interesting starting point for programmes targeting any of these processes.

Experimental

Mammalian cell line maintenance and MTT assay

HL-60, HeLa, and SH-SY5Y cell lines were cultured according to standard tissue culture techniques (Freshney, 2005). Briefly all cell lines were grown in RPMI 1640 (GIBCO, Life Technologies New Zealand Limited, Auckland, New Zealand) medium supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (PenStrep) in a humidified incubator at 37 ºC and 5% CO_2. HL-60 cells were passaged by ten-fold dilution in fresh medium when the cell density reached $2 \cdot 10^5$ cells/ml. HeLa and SH-SY5Y cells were passaged after removal by trypsin treatment [2.5 mg/ml porcine trypsin in Hank's balanced salt solution (HBSS)] after reaching 70% confluence and were diluted ten-fold on re-seeding. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as previously reported by Hood *et al*. (2001).

Pateamine affinity chromatography

The pateamine-substituted affinity resin and a control resin were generated and used as previously reported (Bordeleau *et al*., 2005). Pateamine and control resin (30 μl each) were equilibrated in 0.5 ml of 0.1% Triton X-100 in 10 mM phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4 °C and washed with another 0.5 ml of the same buffer. The resins were incubated in 1 ml of cell lysate adjusted to a protein concentration of 1 mg/ml for 30 min with agitation for 30 min at 4 °C. Lysates were prepared from either $1 \cdot 10^7$ HL-60, SH-SY5Y, or HeLa cells that had been washed three times with 20 ml of ice-cold PBS and disrupted in 5 ml cold PBS with Complete Mini Protease Inhibitor tablets (Roche Dia-

gnostics, Mannheim, Germany) with a Dounce homogenizer. Triton X-100 was added to a final content of 0.1%, the homogenate rotated at 4 °C for 30 min and cleared by centrifugation at 100000 x *g* for 30 min (SS-34 rotor Evolution RC Sorvall; Thermo Fisher Scientific, Waltham, MA, USA). Beads were recovered by centrifugation and washed three times with 0.1% Triton X-100 in PBS. Bound proteins were eluted by addition of 30 μl of 2x LDS-PAGE (lithium dodecylsulfate polyacrylamide gel electrophoresis) sample loading buffer with reducing agent (Invitrogen, Carlsbad, CA, USA) and incubation at 70 °C for 10 min. Alternatively a high salt wash was performed with 1 M sodium chloride and 0.1% Triton X-100 in PBS, and the remaining proteins were eluted with LDS-PAGE loading buffer. Eluted proteins were separated using the NuPAGE LDS-PAGE system (Invitrogen) on 4–12% gradient gels under reducing condition in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer according to the manufacturer's instructions and visualized using standard colloidal Coomassie stain.

Identification of pateamine-binding proteins

Protein bands of interest were excised from the gel, de-stained, and subjected to in-gel trypsin digest following standard procedures (Young *et al*., 2009). The resulting peptides were purified using ZipTips-C18 (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Mass spectra were acquired using an Applied Biosystems DEPro MALDI-TOF mass spectrometer (Framingham, MA, USA) in positive ion reflector mode with accelerating voltage of 20000 V, grid voltage of 75%, and 180 ns delay time. Peptide masses were calibrated internally using trypsin autolysis peaks. The resulting mass peak list was used to search all eukaryotic species in the NCBI protein database via the ProFound search engine (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) (Zhang and Chait, 2000) allowing for methionine oxidation and acetamide adducts. A positive identification was considered achieved when more than five peptide masses were matched covering more than 25% of the peptide sequence with a mass error tolerance of 25 ppm and an expectation below 0.01.

Tubulin polymerization assay

Bovine microtubules were purified from the brain of an adult cow according to the method of Hamel and Lin (1981), and tubulin polymerization was followed by a variant of the turbidity assay of Gaskin *et al.* (1974) using tubulin in polymerization buffer (2 mg/ml, 95 μl), pateamine in 5 μl of 40% ethanol for final concentrations of 20, 100, and 200 μM ethanol (2%) as a carrier control, colchicine (20 μM) and paclitaxel (20 μM) as controls for microtubule destabilization and stabilization, respectively. Absorbance at 350 nm was recorded every 1.5 min for 93 min in a microtitre plate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

Pyrene-actin microfilament polymerization assay

In vitro actin polymerization was assessed using pyrene-labelled actin (Kouyama and Mihashi, 1981). The Actin Polymerization Biochem kit (Cytoskeleton Inc., Denver, CO, USA) was used according to the manufacturer's instructions. Pateamine was added in 5 μl DMSO for a final concentration range of 25–200 μM, 5 μl of DMSO were included as a carrier control. Polymerization was monitored on a fluorescent microtitre plate reader (SpectraMax Gemini; Molecular Devices), with excitation at 365 nm and monitoring emission at 407 nm, every 5 min for 1 h.

Cell cycle analysis

A 24-well plate was seeded with $2 \cdot 10^5$ HeLa cells and left overnight to attach in a humidified incubator at 37 °C with 5% CO_2. Cells were treated with 10 nM, 25 nM, or 100 nM pateamine in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 0.1% ethanol for 18 h in a humidified incubator at 37 °C and 5% CO_2, after which they were harvested with trypsin, washed twice with 1 ml of PBS, and their cell cycle distribution analysed by flow cytometry (Hood *et al*., 2001).

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