Stimulation of mammalian translation initiation factor eIF4A activity by a small molecule inhibitor of eukaryotic translation

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Communicated by David E. Housman, Massachusetts Institute of Technology, Cambridge, MA, May 23, 2005 (received for review April 12, 2005)

RNA helicases are the largest group of enzymes in eukaryotic RNA metabolism. The DEXD/H-box putative RNA helicases form the helicase superfamily II, whose members are defined by seven highly conserved amino acid motifs, making specific targeting of selected members a challenging pharmacological problem. The translation initiation factor eIF4A is the prototypical DEXD-box RNA helicase that works in conjunction with eIF4B and eIF4H and as a subunit of eIF4F to prepare the mRNA template for ribosome binding, possibly by unwinding the secondary structure proximal to the 5′ m7GpppN cap structure. We report the identification and characterization of a small molecule inhibitor of eukaryotic translation initiation that acts in an unusual manner by stimulating eIF4A-associated activities. Our results suggest that proper control of eIF4A helicase activity is necessary for efficient ribosome binding and demonstrate the feasibility of selectively targeting DEAD-box RNA helicases with small molecules.

The ribosome recruitment step of translation initiation is rate-limiting and an important regulatory point whereby cellular environmental cues (e.g., amino acid starvation, mitogenic stimulation, and hypoxia) are linked to the process of translation (1). Two distinct pathways exist for recruitment of the ribosome to the mRNA template. One mechanism is cap-dependent and is facilitated by the presence of the 5′ cap structure (m7GpppN, where N is any nucleotide) on the mRNA. It is catalyzed by the eIF4F class of translation initiation factors and involves the recruitment of ribosomes near the 5′ end of the mRNA template (1). The second mode involves ribosome recruitment in a cap-independent fashion to an internal ribosome entry site (IRES). Initiation factor requirement for internal ribosome binding varies among IRESes, with some not requiring any factors (2).

Preparation of the mRNA template for cap-dependent ribosome recruitment is achieved by eIF4F, eIF4A, eIF4B, eIF4H, and ATP hydrolysis (1). The eIF4F complex is comprised of three subunits: (i) eIF4E, which binds the mRNA cap structure in an ATP-independent fashion; (ii) eIF4A, an RNA helicase that exhibits RNA-dependent ATPase activity and ATP-stimulated RNA binding activity (3); and (iii) eIF4G, a modular scaffold that mediates mRNA binding of the 43S preinitiation complex through interactions with eIF3, eIF4B, and eIF4H to make its helicase activity more processive (4, 5). eIF4A exists as a free form (referred to herein as eIF4Aa) and as a subunit of eIF4F (eIF4Aa) and is thought to cycle through the eIF4F complex during initiation (6–8). When localized in the eIF4F complex, eIF4Aa is ~20-fold more efficient as an RNA helicase than when found alone (4, 9), leading to the proposal that eIF4Aa is the functional helicase for translation initiation (10). The helicase activity of eIF4F (via eIF4Aa) is thought to unwind local secondary structure in the 5′ UTR of mRNAs to facilitate cap-dependent ribosome recruit ment (6–8). In the crystal form, eIF4A has a distended “dumbbell” structure consisting of two domains (11–13), which undergo conformational changes in response to RNA and ATP binding (14).

There are three eIF4A family members: eIF4A1, eIF4AII, and eIF4AIII. eIF4A1 and eIF4AII show 90–95% similarity at the amino acid level, are involved in translation, and appear to have the same biological activity in vitro (15, 16). eIF4AIII is 65% similar to the other isoforms and is implicated in nonsense-mediated decay (17–20). The eIF4A isoforms are members of the DEAD-box putative RNA helicase protein family. These and related DEXD/H (where X is any amino acid) box proteins are characterized by seven highly conserved amino acid sequence motifs implicated in RNA remodeling. These proteins are involved in virtually all aspects of cellular RNA metabolism, including ribosome biogenesis, splicing, translation, and mRNA degradation (for examples, see www.helicase.net).

Small molecule targeting of DEXD/H family members would provide mechanistic insight into the properties of these proteins and help define their roles in normal and abnormal cellular and developmental processes. In this report, we identify and characterize a small molecule inhibitor of translation initiation that stimulates the RNA and ATP binding, ATPase, and helicase activities of eIF4Aa.

Materials and Methods

Pateamine Isolation and Generation of Affinity Matrix. Pateamine was isolated from Mycale sp., as described (21), and purity was established by NMR to be >95%. For the generation of a pateamine affinity matrix, epoxy-activated Sepharose 6B was prepared according to the manufacturer’s instructions and resuspended in 5 volumes of 60 mM pateamine A in methanol containing 66 mM triethylamine. Coupling was performed overnight at 45°C with agitation. After three washes with five volumes of methanol, the resin was lyophilized, resuspended in 1 M diethanolamine, and left overnight at room temperature with agitation to block any remaining epoxide groups. A control resin was generated by incubating the epoxy-activated Sepharose 6B with 1 M aqueous diethanolamine overnight at 4°C. Subsequent wash steps were performed according to the manufacturer’s instructions, concluding with three MilliQ water washes. Beads were lyophilized and stored at ~20°C until needed.

Protein Purification and Activity Assessment. Mouse eIF4A1 and the mutant (with an ^76AQSTGKT to ^76VOQTGTGKT alteration) cDNAs were subcloned into pET15b. Recombinant proteins were expressed in Escherichia coli BL21 codon + (DE3) and purified by...
using Ni-NTA agarose and Q Sepharose chromatography. ATPase assays, ATP and RNA crosslinking assays, and helicase assays were performed with recombinant eIF4AI, as described (4, 9, 22, 23). Recombinant Ded1p was expressed in *E. coli* as a histidine-tagged protein and purified as described (24).

**Results**

**Characterization of an Inhibitor of Eukaryotic Translation.** During the course of a high-throughput screening campaign to identify inhibitors of eukaryotic protein synthesis (25), we found a potent inhibitory activity associated with a marine natural product (Fig. 1). Pateamine is known to be cytotoxic to mammalian cells (21, 26), but its mechanism of action or biological targets are not defined.

A series of dose–response experiments in Krebs translation extracts were performed by using bicistronic mRNA reporters, two of which contain IRESes (Fig. 1B, FF/EMC/Ren and FF/HCV/Ren). Initiation at the EMC IRES utilizes eIF4G, eIF4A, and eIF4B, whereas these are dispensable for HCV IRES activity (27). For all three reporters, the IC50 for cap-dependent firefly luciferase activity in Krebs extracts was ∼0.2 μM (Fig. 1C). There was moderate renilla luciferase expression from FF/Ren mRNA, presumably due to ribosome reinitiation after termination at the upstream firefly cistron. Inhibition of renilla production from FF/Ren mRNA and from FF/EMC/Ren mRNA (due to internal ribosome recruitment) showed the same IC50 as for cap-dependent translation. However, translation of the renilla ORF from FF/HCV/Ren was ∼10-fold more resistant to inhibition by pateamine (Fig. 1C). No inhibition of prokaryotic translation was observed in *E. coli* S30 extracts with concentrations up to 10 μM (Fig. 6A, which is published as supporting information on the PNAS web site). These data suggest that at low concentrations (<0.8 μM), pateamine inhibits translation initiation, because expression of only the firefly cistron was decreased when extracts were programmed with FF/HCV/Ren mRNA (Fig. 1C). Pateamine exerted a similar inhibitory profile on translation of the mRNA reporters in rabbit reticulocyte lysates (Fig. 6B). We therefore used rabbit reticulocyte lysates to address whether pateamine inhibits 48S preinitiation complex formation (Fig. 2A and B). Complexes were trapped by using the nonhydrolyzable GTP analogue, GMP-PNP, and resolved by sedimentation velocity centrifugation. Pateamine prevented formation of the 48S preinitiation complexes when chloramphenicol acetyl transferase (CAT) mRNA was used as template (Fig. 2A) but not when the HCV IRES was used (Fig. 2B). These results indicate that pateamine acts before or at the ribosome recruitment step of translation initiation. In addition, they suggest that eIF4A,

![Chemical structure of pateamine](image)

**Fig. 1.** Inhibition of eukaryotic translation by pateamine. (A) Chemical structure of pateamine. (B) Schematic diagram of bicistronic constructs used to assess translation inhibition by pateamine. (C) Titration of pateamine in Krebs extracts programmed with bicistronic mRNAs. Translations were performed in the presence of the indicated amounts of pateamine and at a final mRNA and KCl concentration of 5 μg/ml and 100 mM, respectively. Firefly and renilla luciferase activity (RLU) were measured on a Berthold Technologies (Bad Wildbad, Germany) Lumat LB 9507 luminometer. Control translation reactions contained equivalent amounts of DMSO (data not shown). (Upper) Representative autoradiograph from an experiment performed with [35S]methionine. After separation of protein products on 10% polyacrylamide/SDS gels, the gels were treated with EN3Hance (PerkinElmer) dried, and exposed to X-Omat (Kodak) film. (Lower) Graphical representation of the effects of pateamine on translation of bicistronic mRNAs in Krebs extracts. The obtained luciferase activities were normalized to the activity obtained in the absence of compound (which was set at one). Each data point represents the average of three to seven translations, and the standard error of the mean is presented.

![Diagram of bicistronic constructs](image)
Pateamine inhibits ribosome recruitment. (A and B) 32P-labeled CAT and HCV/Ren mRNAs were incubated in rabbit reticulocyte lysate in the presence of 1 mM GMP-PNP or 1 mM GMP-PNP and 10 μM pateamine. After centrifugation (SW40; 39,000 rpm / 3.5 h), fractions from each sucrose gradient were collected using a Brandel (Bethesda, MD) Tube Piercer connected to an ISCO fraction collector and were individually counted. Total counts recovered from each gradient and the percent mRNA bound in 48S complexes were: (A) CAT mRNA/GMP-PNP (64,430 cpm, 21% binding) and CAT mRNA/GMP-PNP+pateamine (62,713 cpm, <0.7% binding), and (B) HCV/Ren mRNA/GMP-PNP (57,844 cpm, 7% binding) and HCV/Ren mRNA/GMP-PNP+pateamine (56,113 cpm, 6% binding). (C) eIF4A is specifically retained on a pateamine-Sepharose affinity matrix. Pateamine was coupled to epoxy-activated Sepharose, and total HL-60 cell extracts were loaded onto pateamine or control resin, washed with 0.1% Triton X-100 in 10 mM PBS, and eluted in SDS/PAGE sample buffer. Proteins were fractionated on a 4–12% polyacrylamide gel and stained with colloidal Coomassie blue. (D) Immunobots of HL-60 extract (LOAD) and eluents from control- and pateamine-affinity resins. The antibodies used in the immunobots are indicated. Recombinant eIF4AII, eIF4AI, and eIF4AIII proteins were used to assess antibody specificity. Recombinant eIF4AIII has a higher molecular mass than recombinant eIF4AI due to additional vector-derived sequences.

Fig. 2. Pateamine inhibits ribosome recruitment. (A and B) 32P-labeled CAT and HCV/Ren mRNAs were incubated in rabbit reticulocyte lysate in the presence of 1 mM GMP-PNP or 1 mM GMP-PNP and 10 μM pateamine. After centrifugation (SW40; 39,000 rpm / 3.5 h), fractions from each sucrose gradient were collected using a Brandel (Bethesda, MD) Tube Piercer connected to an ISCO fraction collector and were individually counted. Total counts recovered from each gradient and the percent mRNA bound in 48S complexes were: (A) CAT mRNA/GMP-PNP (64,430 cpm, 21% binding) and CAT mRNA/GMP-PNP+pateamine (62,713 cpm, <0.7% binding), and (B) HCV/Ren mRNA/GMP-PNP (57,844 cpm, 7% binding) and HCV/Ren mRNA/GMP-PNP+pateamine (56,113 cpm, 6% binding). (C) eIF4A is specifically retained on a pateamine-Sepharose affinity matrix. Pateamine was coupled to epoxy-activated Sepharose, and total HL-60 cell extracts were loaded onto pateamine or control resin, washed with 0.1% Triton X-100 in 10 mM PBS, and eluted in SDS/PAGE sample buffer. Proteins were fractionated on a 4–12% polyacrylamide/SDS gel and stained with colloidal Coomassie blue. (D) Immunobots of HL-60 extract (LOAD) and eluents from control- and pateamine-affinity resins. The antibodies used in the immunobots are indicated. Recombinant eIF4AII, eIF4AI, and eIF4AIII proteins were used to assess antibody specificity. Recombinant eIF4AIII has a higher molecular mass than recombinant eIF4AI due to additional vector-derived sequences.

eIF4B, or eIF4G may be the biological targets of pateamine, because ribosome recruitment to the HCV IRES is independent of these initiation factors (27) and is not affected by pateamine (Fig. 2B).

eIF4A Family Members Are Biological Targets of Pateamine. To identify the biological target(s) of pateamine, total cell extracts were prepared from HL-60 cells and loaded onto an affinity matrix containing immobilized pateamine. After extensive washing, polypeptides specifically retained on the matrix were separated and visualized by using SDS/PAGE. The bands denoted by asterisks were excised from the gel (Fig. 2C, lane 2), as well as the corresponding region of the gel from the control resin (lane 3), and were subjected to trypsin digestion followed by peptide mass fingerprinting (Fig. 2C, indicated with an asterisk and labeled a, b, and c). In silico comparison to peptide fragment masses generated from GenBank identified the proteins specifically retained by pateamine-Sepharose to be cytokeratin (band a), tubulin (band b), and eIF4A1/eIF4AII (band c) (Fig. 7, which is published as supporting information on the PNAS web site, and data not shown).

To assess whether other translation factors were retained on the pateamine affinity resin, we probed the HL-60 extracts (LOAD) and eluents from the control- and pateamine-resins for the presence of eIF4B, eIF2α, and eIF4E (Fig. 2D). None of these factors were recognized by pateamine, because they were not retained on the affinity matrix. In contrast, eIF4AI/eIF4AII and eIF4AI/eIF4AIII were specifically retained on the affinity matrix (Fig. 2D). (Note that in this experiment, we cannot distinguish between the eIF4AI and eIF4AII isoforms, due to lack of available isoform-specific antibodies.) These results indicate that members of the eIF4A family are selective targets of pateamine. Given the well documented role of eIF4AI and eIF4AII in translation initiation, and the relatedness of these two proteins, we focused the remainder of this study on characterizing the effects of pateamine on eIF4AI activity.

Pateamine Stimulates eIF4AI Activity. The effect of pateamine on the ATPase activity of eIF4AI was assessed and surprisingly showed a ~10-fold increase in initial rate of hydrolysis (Fig. 3A). This effect was RNA-dependent, because no stimulation was observed in the absence of exogenously supplied poly(U) (data not shown). We then assessed whether pateamine affected ATP binding to eIF4AI. In the absence of poly(U), there was no change in UV-induced crosslinking of ATP to eIF4AI (Fig. 3B, compare lane 1 with 2). However, in the presence of poly(U), binding of ATP to eIF4AI was significantly enhanced by pateamine (Fig. 3B, compare lane 3 with 4). The binding of eIF4AI to RNA has been previously characterized and is stimulated by ATP (Fig. 3C, compare lanes 1 and 3 with 2) (23). Pateamine-stimulated nonsequence-dependent RNA binding of eIF4AI (Fig. 3C, compare lane 4 with 3) and eliminated the stimulatory requirements of ATP for RNA binding (Fig. 3C, compare lane 6 with 5). We observed the same stimulatory effect on RNA binding by using UV light-induced crosslinking on body-labeled mRNA or using a filter-binding assay in which protein–RNA complexes were trapped on nitrocellulose filters (data not shown).

We also assessed whether pateamine affected the RNA- and ATP-binding properties of eIF4AII, eIF4AIII, and eIF4E (Fig. 3 D and E). Toward this end, eIF4F was chemically crosslinked to 32P-cap labeled oxidized mRNA in the absence or presence of pateamine (Fig. 3D). No observable effect on the RNA-binding properties of the eIF4E and eIF4A subunits was noted. eIF4E crosslinking was inhibited by m7GDP, and eIF4A crosslinking was ATP-dependent (data not shown). Crosslinking of α-[32P]ATP to eIF4A was not stimulated by pateamine (Fig. 3E). We conclude that pateamine affects primarily eIF4AI’s ATP- and RNA-binding properties.

The effect of pateamine on the RNA helicase activity of eIF4AI was assessed (Fig. 4A). As previously documented, eIF4AI is capable of unwinding an RNA duplex with a ΔG = −17.9 kcal/mol (Fig. 4A, compare lane 3 with 1) (4). This process requires ATP (compare lane 5 with 3) and is defective when an eIF4AI mutant [in which the ATPase A motif (7′QAGS.GTGKT)] is mutated to (7′QSGTGKT)] is used. This mutation abolishes the ability of eIF4AI to bind and hydrolyze ATP, as...
Pateamine did not inhibit eIF4AIf's helicase activity when using stimulated eIF4AIf helicase activity on this more stable duplex (Fig. 4) (9). Increasing the stability of the RNA duplex impairs Bordeleau Pateamine Inhibits Cap-Dependent Translation in Vivo.

If eIF4A is the relevant biological target of pateamine in mediating its inhibitory effect on translation initiation in vivo, then pateamine should not affect translation initiation mediated by the HCV IRES, under conditions where cap-dependent protein synthesis is inhibited. This was tested by transfecting HeLa cells with the bicistronic reporter, pcDNA/Ren/HCV/FF, and monitoring luciferase production in response to increasing pateamine concentration (Fig. 5A). Expression of renilla luciferase was inhibited in a dose-dependent fashion by pateamine, whereas firefly luciferase expression increased slightly in response to higher levels of pateamine (Fig. 5A). These results are unlikely the consequence of nonspecific mRNA degradation or activation of a cryptic promoter within the HCV IRES, because no evidence of truncated transcripts was observed by Northern blot analysis of the RNA from pcDNA/Ren/HCV/FF-transfected cells (Fig. 5B).

Discussion

Pateamine is a potent small molecule inhibitor of eukaryotic translation. Several lines of evidence indicate that eIF4A family members are the relevant biological targets of pateamine. (i) eIF4A/II and eIF4AIII are retained on a pateamine affinity resin, whereas eIF4E, eIF2α, and eIF4B are not (Fig. 2D). (ii) Initiation from the HCV IRES does not require eIF4A (27) and accordingly is not inhibited by concentrations of pateamine where cap-dependent translation is inhibited, both in vitro and in vivo (Figs. 1C, 2B, and 5A). (iii) Pateamine stimulates several eIF4A-associated activities, including RNA-dependent ATP hydrolysis, ATP and RNA binding, and RNA unwinding (Figs. 3 and 4). (iv) The compound appears selective for eIF4A because it failed to affect the helicase activity of the DEAD box family member, Ded1p (Fig. 4B) and of a DNA helicase from Pseudomonas aeruginosa, named dnaB (data not shown). In addition, no effect was observed on in vitro splicing reactions (Fig. 4C). Although it is difficult to assess whether the activity of other cellular helicases is affected by pateamine, none were well as to unwind RNA duplexes (Fig. 4A, compare lane 7 with 3) (9). Increasing the stability of the RNA duplex impairs eIF4AIf’s helicase activity (Fig. 4A, compare lane 10 with 3) (4). Pateamine did not inhibit eIF4AIf’s helicase activity when using the RNA-1/RNA-11 duplex as substrate (Fig. 4A, compare lane 4 with 3), nor did it eliminate the ATP requirement for helicase activity (Fig. 4A, compare lane 6 with 4). When we used an RNA duplex with a ΔG = -21.4 kcal/mol as substrate, pateamine stimulated eIF4AIf helicase activity on this more stable duplex (compare lane 11 with 10).

To gauge the selectivity of pateamine for eIF4A, we assessed its effects on another DEAD box family member, Ded1p, implicated in translation initiation in yeast (28, 29). No stimulatory (or inhibitory) effect on the helicase activity of Ded1p was observed when pateamine was present in helicase assays with Ded1p (Fig. 4B, compare lane 5 with 4). Splicing of eukaryotic mRNAs depends on the activity of 7–13 DEXD/H box helicases (30, 31). The addition of pateamine to an in vitro splicing reaction did not inhibit (Fig. 4C) or stimulate (data not shown) processing of the input mRNA template, indicating that pateamine is not a general inhibitor of splicing.

Pateamine Inhibits Cap-Dependent Translation in Vivo. Pateamine is a potent in vivo inhibitor of protein synthesis showing an IC50 of 5 nM in HeLa cells (Fig. 8A, which is published as supporting information on the PNAS web site). Metabolic labeling experiments revealed little effect on DNA or RNA synthesis when HeLa cells were exposed to 1 μM compound for 1 h (Fig. 5B). Whereas cells recovered from a translational block imposed by anisomycin within 1 h after removal from the media, no recovery is observed up to 6 h after removal of pateamine from the media, suggesting that binding of pateamine to its target is either extremely strong or irreversible (Fig. 8C). Analysis of polysomes from pateamine-treated cells revealed that very little translating ribosomes remain mRNA associated after a 20-min exposure to pateamine (Fig. 8D).
in the presence of 0.36 M ATP or 10 μM pateamine is indicated. Reaction products were resolved on 12% native agarose/formaldehyde gel, and transferred to Hybond N+. Probes were pro-

readily identified by direct binding to a pateamine affinity resin (Fig. 2C).

The mechanistic details of how eIF4A prepares the mRNA template for ribosome recruitment are not well understood. A current model postulates that eIF4F binds to the mRNA 5' cap structure to deliver eIF4A to the mRNA. This is suggested from experiments showing that a dominant-negative mutant of eIF4A inhibits eIF4F helicase and cap-binding activity by forming a more stable complex with eIF4G, presumably inhibiting eIF4A recycling through eIF4F (7, 8). In addition, the 20-fold higher helicase activity of eIF4AΔ relative to eIF4AΔ suggests that eIF4AΔ is the functional helicase for translation (6, 10). An eIF4A-binding domain within eIF4G (amino acid 737–774) alters the conformation of eIF4A to favor RNA binding (32). Once released on the mRNA, eIF4A (in conjunction with eIF4B and/or eIF4H) is thought to use ATP hydrolysis to melt the secondary structure (4, 6, 10). ATP hydrolysis may also be required for the initial release of eIF4A from the eIF4F complex (8), as well as for the release of eIF4F from the m7G cap structure (6, 23). Each initiation round may require multiple eIF4F-binding events to deposit eIF4A on the mRNA template (7), and eIF4F complexes devoid of the eIF4A subunit no longer recycle (6).

We propose that binding of pateamine to eIF4AΔ presets the protein in the high-affinity RNA-binding state, suggested by the ability of pateamine to stimulate eIF4AΔ RNA binding in the absence of ATP (Fig. 3C). This in turn stimulates ATP binding, hydrolysis, and RNA unwinding (Figs. 3 A and B and 4A). There are several models that could explain how pateamine inhibits translation. (i) Pateamine may inhibit channeling of eIF4A through the eIF4F complex. This would occur if a different conformation than pateamine-bound eIF4AΔ is required for eIF4G binding or the pateamine-binding site on eIF4A overlaps with or interferes in an allosteric fashion with eIF4G binding. If this model is correct, pateamine could be a useful tool for distinguishing between the...
Pateamine does not seem to alter the ATP- or RNA-binding properties of eIF4A. A dominant-negative mutant of eIF4A has been described that associates more strongly with eIF4G than wild-type eIF4A and subsequently inhibits eIF4E crosslinking to the cap structure (8). Translation inhibition by this dominant-negative mutant is thought to be a consequence of inhibiting eIF4E recycling (8). RNA aptamers targeting eIF4A have also been described that impair its ATPase activity and inhibit cap-dependent translation (37). Third, the tumor suppressor Pdcd4 is an inhibitor of translation that interacts with eIF4A and independently with eIF4G (38) and is thought to prevent translation by competing with eIF4G for binding to eIF4A and/or inhibiting eIF4A’s helicase activity. Fourth, DAP5/p97 functions as a general repressor of translation by forming translationally inactive complexes that include eIF4A and eIF3 but not eIF4E (39). Pateamine is the only small molecule inhibitor of translation that increases eIF4A’s helicase activity, and that can readily be used in vivo. The characterization of pateamine demonstrates the feasibility of selectively targeting DEXD/H box RNA helicases with small molecule inhibitors for pharmacological intervention.

We are grateful to Isabelle Harvey and Anne-Sophie Guenier for their experimental expertise during the course of this work and to Dr. Yuri Svitkin for helpful comments on the manuscript. We are grateful to Dr. Melissa Moore for help with the in vitro splicing reactions. We thank Ann Bracey and Maria Ferriauolo for advice regarding purification of recombinant eIF4A and anti-eIF4A immunoblotting. We thank the Jean-Louis Lèvesque Foundation and Valorisation-Recherche Québec for their contribution and support of the McGill Biochemistry and Cancer Center High Throughput Screening Facility. M.-E.B. was supported by a Canadian Institutes of Health Research (CIHR) Cancer Consortium Training Grant Award and a Fonds de Recherche en Santé du Québec studentship award. This work was supported by National Cancer Institute of Canada Grant (104313 to J.P.). J.P. is a CIHR Senior Investigator.