Published in final edited form as:

Leuk Res. 2010 April ; 34(4): 535–541. doi:10.1016/j.leukres.2009.07.043.

Synergistic effect of inhibiting translation initiation in combination with cytotoxic agents in acute myelogenous leukemia cells

Regina Cencic^a, Marilyn Carrier^a, Amanda Trnkus^a, John A. Porco Jr.^b, Mark Minden^c, and Jerry Pelletier^{a,d,*}

^a Department of Biochemistry, McGill University, Montreal, Quebec, Canada

^b Department of Chemistry, Center for Chemical Methodology and Library Development, Boston University, Boston, MA 02215, USA

^c Department of Medical Oncology and Hematology, University of Toronto, Princess Margaret Hospital, Toronto, Canada

^d Goodman Cancer Center, McGill University, Montreal, Quebec, Canada

Abstract

We have previously shown that inhibition of translation initiation, using the small molecule inhibitor silvestrol, induces apoptosis in a pre-clinical murine lymphoma model when combined with daunorubicin. Silvestrol blocks ribosome recruitment by targeting the RNA helicase, eIF4A, which is required for this process. Here we investigate the sensitivity of acute myelogenous leukemia (AML) cell lines to protein synthesis inhibition in combination with the standard cytotoxic agents daunorubicin, etoposide, and cytarabine. Silvestrol shows synergy with standard-of-care agents in AML cell lines and synergizes with ABT-737, a small molecule inhibitor of Bcl-X_L and Bcl-2. The *in vitro* synergy between silvestrol and the cytotoxic drugs used in AML therapy provides a basis for *in vivo* evaluation of these combinations.

Keywords

Silvestrol; AML; Translation initiation; Synergy; Chemotherapy

© 2009 Elsevier Ltd. All rights reserved.

* Corresponding author at: McIntyre Medical Sciences Building, Rm 810, 3655 Promenade Sir William Osler, McGill University, Montreal, Quebec, Canada H3G 1Y6. Tel.: +1 514 398 2323; fax: +1 514 398 7384. jerry.pelletier@mcgill.ca (J. Pelletier)..

Conflict of interest

The authors declare no potential conflicts of interest.

Contribution: Conceived and designed the experiments: RC, JAPJ, MM, JP. Performed the experiments: RC, MC, AT, JAPJ, MM, JP. Analyzed the data: RC, MC, AT, JAPJ, MM, JP. Wrote the paper: RC, JP. Final approval of the version to be submitted: RC, MC, AT, JAPJ, MM, JP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2009.07.043.

1. Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disease, thus increasing the challenge of developing a single effective therapy. While being sensitive to initial treatment, AMLs appear to become chemoresistant upon relapse. Current interests in developing treatment options for this disease lie in inhibitors of histone deacetylase, Flt-3 and farnesyltransferase, which, although having limited utility as single agents, do show synergy with conventional cytotoxic agents suggesting they may be more useful in combination chemotherapy [1,2]. Identification of other AML-specific biological modifiers has the potential to improve the treatment of this disease.

The PI3K/Akt/mTOR signaling pathway is elicited by a number of extracellular and intracellular stimuli and plays a critical role in many cellular functions, including being a major determinant for cell growth. Recent studies have indicated that the PI3K/Akt/mTOR axis is frequently activated in AML patient blasts and this strongly contributes to proliferation, survival and drug-resistance in these cells [3–5]. Rapamycin, a specific inhibitor of mTOR curtails down stream signaling from this pathway in primary AML cells to protein synthesis regulatory networks [6]. Studies performed in AML cell lines to test the synergistic effects of mTOR inhibitors *in vitro* revealed a time-dependent global enhancement of cytotoxic agents [7].

One of the primary functions of mTOR is to maintain protein synthesis in homeostasis with the cellular environment. It achieves this through modulating availability of the translation factor eukaryotic initiation factor (eIF) 4F, which is required for loading ribosomes onto mRNA templates. eIF4F is composed of three subunits: eIF4E, which binds to the cap structure present at the 5' -end of mRNAs; eIF4A, a DEAD-box RNA helicase implicated in preparing a ribosome landing pad for 43S pre-initiation complexes (40S ribosomal subunit and associated factors) by unwinding 5' mRNA structure; and eIF4G, a large scaffolding protein involved in recruiting the 43S preinitiation complex via its interaction with 40S-associated eIF3 [8]. Consequently, there is much interest in targeting eIF4F with small molecules to assess its contribution to tumor maintenance [9]. Indeed, targeting the eIF4E:eIF4G interaction with a small molecule inhibitor is cytotoxic *in vitro* [10] and down-regulation of eIF4E levels with anti-sense oligonucleotides significantly suppresses tumor growth in xenograft models [11]. Importantly, in the latter study although eIF4E levels were reduced by 80%, no significant cytotoxicity on normal tissue was observed [11]. We have previously shown that modulation of eIF4A activity with the small molecule, silvestrol, can resensitize lymphomas to the cytotoxic actions of doxorubicin in a pre-clinical mouse lymphoma model [12]. We also recently demonstrated that silvestrol shows activity as a single agent against solid tumors in mouse xenograft models, has anti-angiogenic activity, and has no cytotoxicity on normal tissues *in vivo* [13]. Additionally, silvestrol was demonstrated to exert anti-proliferative activity in the *Em-Tcl-1* mouse model (which develop a B-cell Lymphocytosis) and in SCID mice engrafted with 697 B-ALL cells [14].

These results prompted us to investigate the effects of silvestrol on AML cell lines either as a single agent, in combination with the cytotoxic agents daunorubicin, cytarabine-C (Ara-C) and etoposide, and in combination with the Bcl-X_L and Bcl-2 inhibitor, ABT-737. We

find that silvestrol proves to be a potent inhibitor of AML cell proliferation as a single agent and synergizes with ABT-737 and standard-of-care agents.

2. Materials and methods

2.1. General methods and cell maintenance

Silvestrol was resuspended in DMSO and stored at -70°C . Daunorubicin (Sigma) was dissolved in water and stored at 4°C . Etoposide and Ara-C (Calbiochem) were resuspended in DMSO and stored at -70°C .

U937 (a human histiocytic lymphoma cell line), NB4 (a human acute promyelotic leukemia cell line), and HL-60 (a human promyelocytic leukemia cell line) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. Human AML cell lines OCI/AML2 (human acute myeloid leukemia; AML FAB M4) and OCI/AML3 (human acute myeloid leukemia; AML FAB M4; NPM1 mutation A) were maintained in α -minimal essential medium (α -MEM) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin.

2.2. MTS assay

Cell proliferation was assessed using the MTS assay. U937 cells (35 000 cells/well) or HL-60, NB4, OCI/AML2 and OCI/AML3 cells (50 000 cells/well) were seeded in 96-well plates in the presence of increasing concentrations of silvestrol. Forty eight hours later CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was added to the plates and the plates further incubated for up to 3 h, followed by reading the OD₄₉₀ on a SpectramaxPlus³⁸⁴ (Molecular Devices) and using Softmax Pro 4.8.2 software. Values obtained were standardized against vehicle (DMSO) control, which was set at 1.

2.3. [³⁵S]-methionine metabolic labeling and Western blotting

U937, HL-60, NB4, OCI/AML2 and OCI/AML3 cells, 60 000 cells/well, were seeded in 24-well plates in the presence of 50 nM silvestrol for the indicated times. For the last hour of treatment, medium was changed to Met-free DMEM with 10% dialyzed FBS, during which the last 15 min [³⁵S]-Met was added. Cells were spun for 10 min at 2500 rpm at 4°C in a Sorval LegendRT table centrifuge, cell pellets washed with cold PBS, lysed in RIPA buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, 0.5% sodium desoxycholate, 0.1% SDS, 20 mM β -glycerophosphate, 10 mM NaF, 1 mM PMSF, 4 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ pepstatin) and the amount of TCA-insoluble [³⁵S]-Met determined. Protein content in the cell lysates was measured using the Bio-Rad DC ProteinAssay (Bio-Rad Laboratories) and used to standardize the counts obtained by TCA precipitation.

A parallel set of dishes (200 000 cells/well in a 6-well plate) was incubated with 50 nM silvestrol for the indicated times and the cells collected, washed in PBS and lysed in RIPA buffer. Equal amounts of protein (30 μg) were fractionated on a 10% SDS-polyacrylamide gel, followed by transfer to a PVDF membrane (Millipore). Primary antibodies used were

anti-Mcl-1 (Rockland, Gilbertsville, PA) and anti- β -actin (Sigma). Secondary antibodies conjugated to HRP were from Jackson Immuno Research (Burlington, ON).

To analyze PARP cleavage, NB4 cells (200 000 cells/well in a 6-well plate) were incubated with increasing concentrations of silvestrol for 48 h and the cells collected, washed in PBS and lysed in RIPA buffer. Equal amounts of protein (50 μ g) were fractionated on a 10% SDS-polyacrylamide gel and processed for Western blotting as described above. Primary antibodies used were anti-cleaved human PARP (Cell Signaling, Danvers, MA) and anti-GAPDH (Abcam, Cambridge, MA). Secondary antibodies conjugated to HRP were from Jackson Immuno Research (Burlington, ON).

2.4. Polysome profiling, Isolation of RNA and semi-quantitative RT-PCR analysis

For polysome profiling analysis 10 million NB4 cells were treated with either 1% DMSO or 10 nM silvestrol for 1 h. Cells were harvested by addition of 100 μ g/ml cycloheximide and centrifugation for 10 min at $2000 \times g$ at 4°C. The cell pellet was washed in cold PBS containing 100 μ g/ml cycloheximide followed by an additional spin as before. Pellets were resuspended in 425 μ l hypotonic lysis buffer (5 mM Tris_{7.5}, 2.5 mM MgCl₂, 1.5 mM KCl) followed by addition of 0.1 μ g/ml cycloheximide, 1 μ M DTT, 0.5% Triton X-100 and 0.5% sodium deoxycholate. Samples were loaded on 10–50% sucrose gradients and centrifuged in an SW40 rotor at 35 000 rpm for 2 h at 4°C. Gradients were analyzed by piercing the bottom of the tube with a Brandel tube piercer and passing a 60% sucrose solution through the bottom. Fractions were collected and recording of the data was performed using InstaCal Version 5.70 and Tracer-Daq Version 1.9.0.0 (Measurement Computing Corporation, Norton, MA).

To prepare RNA, fractions were separated into unbound and polysome (poly)-bound regions and RNA isolated using Trizol according to the manufacturer's instructions (Invitrogen). One twentieth of pooled RNA fractions was reverse transcribed using SuperscriptTMII (Invitrogen) primed with oligo(dT)_{12–18}. PCRs were performed with undiluted, 1:10, or 1:100 diluted cDNA samples with primers for Mcl-1. For PCRs with primers for β -actin, cDNA samples were additionally diluted 1:1000. Primers used were hMcl-1 [5' - TTCAGCGACGGCGTAACAAACT-3' and 5' -CCCATCCCAGCCTCTTTGTTTA-3'], and h β -actin [5' -TTCCTTCCTGGGCATGGAGT-3' and 5' -ACAGCACTGTGTTGGCGTAC-3']. Annealing temperature was 55°C and PCRs were optimized to detect the exponential phase of amplification. Samples were analyzed on 1.5% agarose gels.

2.5. Median effect analysis

To characterize the interaction between silvestrol and cytotoxic drugs, data were analyzed using the media effect method [15] and CompuSyn 1.0 software (ComboSyn Inc., NJ). U937 cells (35 000 cells/well) or HL-60, NB4, OCI/AML2 and OCI/AML3 cells (50 000 cells/well) were seeded in 96-well plates in the presence of increasing concentrations of silvestrol and a constant ratio of 1:1 daunorubicin, 1:10 Ara-C or 1:10 etoposide. Forty eight hours later, an MTS assay was performed as described above. Synergy analyses were performed using CompuSyn Version 1.0 software.

3. Results

We have previously reported that silvestrol has no significant haematological toxicity *in vivo* [13]. To determine if silvestrol's ability to chemosensitize tumor cells extended beyond *Eμ-myc* driven B-cell lymphomas, we examined the anti-proliferative potential of this compound on AML cell lines. When cells were cultured in the presence of silvestrol for 48 h and growth monitored by the MTS assay, we found that all cells showed a dose-dependent sensitivity (Fig. 1A). The IC₅₀ of silvestrol on the AML lines tested ranged from ~4 to 10 nM (Fig. 1A, Table 1), indicating that this compound is very potent *in vitro*. Treatment of NB4 cells with 7.5 nM silvestrol for 48 h inhibited protein synthesis >90% (Fig. 1A, inset middle panel). To determine if exposure to silvestrol resulted in cell death, we treated NB4 cells for 48 h with silvestrol and analyzed cell extracts for cleavage of poly(ADP-ribose) polymerase (PARP), a surrogate marker of apoptosis (Fig. 1B). At a concentration of 10 nM silvestrol, PARP appeared completely cleaved, with little consequence on total GAPDH levels (with the exception of slight inhibition at the highest concentration of 25 nM). These results indicate that silvestrol curtails proliferation of AML cells by triggering apoptosis.

We next assessed the consequence of combining silvestrol with three cytotoxic agents that are currently used in the treatment of AML. Combinations of silvestrol with daunorubicin, etoposide, and Ara-C were evaluated. Following exposure to compounds for 48 h, we then measured the median effect to calculate a combination index (CI) for each set of combination (Fig. 1C and Suppl. Fig. 1). The results indicate strong evidence for synergy between silvestrol and daunorubicin, etoposide, or Ara-C in all AML lines tested (Fig. 1C and Suppl. Fig. 1). Pre-exposure of the cell lines to silvestrol, before the addition of the cytotoxic compounds, did not improve the observed synergistic effect (R.C. and A.T., data not shown).

To investigate if the synergistic effect seen in these cell lines was related to silvestrol's potency as an inhibitor of eukaryotic translation initiation, we compared silvestrol to Flavagline 61, a less potent family member of the cyclopenta[b]benzofurans [13] (Fig. 2A). We found that the IC₅₀ of Flavagline 61 affecting cell growth was approximately 15-fold higher (100 nM compared to 7 nM) relative to silvestrol (Fig. 2B). The potency of Flavagline 61 compared to silvestrol on endogenous protein synthesis was also monitored. Whereas protein synthesis in NB4 cells was reduced by about 80% after 6 h exposure to 50 nM silvestrol, Flavagline 61 caused a decrease of ~45% when tested under similar conditions (Fig. 2C). Flavagline 61 displayed weak synergy on NB4 cells when tested with daunorubicin, etoposide, or Ara-C (Fig. 2D), compared to the more pronounced synergistic effects observed with silvestrol, as indicated by higher 1-CI values (compare Figs. 1C and 2D).

Myeloid Cell Leukemia sequence 1 (Mcl-1) is a highly unstable protein and requires active translation to maintain its expression levels [16]. It is necessary for early embryonic development as well as for maintenance of hematopoietic cell lineages [17]. In the *Eμ-Myc* mouse model, eIF4E translationally regulates Mcl-1 expression [18] and Mcl-1 expression is inhibited by silvestrol in the human breast cancer cell line MDA-MB-231 [13], as well as in CLL patient and the 697 ALL cell lines [14]. We therefore assessed the consequence on

Mcl-1 expression of exposing AML cell lines to silvestrol (Fig. 3). Exposure to 50 nM silvestrol caused a reduction in protein synthesis ranging from 40 to 80% in all cell lines tested (Fig. 3A). Under these conditions, levels of Mcl-1 protein were dramatically affected, decreasing by over 80% in all five-cell lines within 6 h of exposure to drug. In contrast, levels of β -actin remained unchanged (Fig. 3B).

To test, if the effect on Mcl-1 protein levels was due to silvestrol acting as an inhibitor of translation initiation, NB4 cells were treated with 10 nM silvestrol for 1 h followed by polysome profiling (Fig. 3C). Consistent with silvestrol inhibiting protein synthesis, we observed a reduction in polysomes and accumulation of free ribosomes. RT-PCR analysis of RNA isolated from unbound and polysome-bound fractions of DMSO and silvestrol treated NB4 cells showed a redistribution of Mcl-1 mRNA from polysome-bound into unbound fractions, whereas the distribution of β -actin mRNA remained unchanged (Fig. 3D).

The Bcl-2 and Bcl-X_L family members of the Bcl-2 subfamily have been associated with poor outcome in AML [19] and potential combination therapies for AML therefore include targeting Bcl-2 [20]. Sensitivity of the AML cell lines HL-60, KG1, NB4, U937 and OCI/AML3 to the small molecule inhibitor of Bcl-X_L and Bcl-2, ABT-737, has been shown to depend on Mcl-1 [21]. ABT-737 appears not to affect Mcl-1 activity, nor does it interact with A1, another member of the Bcl-2 subfamily [22]. We wanted to investigate, if silvestrol could synergize with inhibition of Bcl-2/Bcl-X_L. To this end, we titrated ABT-737 onto the NB4 cell line and tested for cell growth. ABT-737 showed an IC₅₀ of approximately 300 nM in this cell line (Fig. 4A). In experiments aimed to gauge synergy, ABT-737 synergized with silvestrol and daunorubicin, as single combinations, or with all three agents together (Fig. 4B).

4. Discussion

Acute myeloid leukemia remains a disease with poor outcome. Even though sensitive to initial treatments, the majority of patients will ultimately relapse and become resistant. A main problem lies in the heterogeneity of this disease. Research and treatment strategies focus on establishing prognostic factors and genotype based therapies as well as allogeneic stem cell transplantations (reviewed in [23–25]).

We have recently identified a small molecule inhibitor of the ribosome recruitment step of eukaryotic translation initiation, silvestrol, that resensitizes in *Pten*^{+/-} *E μ -Myc* lymphomas *in vivo* to the cytotoxic effect of doxorubicin [12]. It targets cap-dependent translation by altering the levels of eIF4F, a protein complex regulated by mTOR [26,27]. This is expected to alter translation of the oncoproteome, with preferential inhibition of “weak”, malignancy-related mRNAs that appear more dependent on eIF4F for ribosome recruitment [13]. Translation of mRNAs affected by silvestrol include cyclin D1 and Mcl-1, proteins known to drive malignant progression [28,29]. Silvestrol is also effective as a single agent in xenograft mouse models, shows anti-angiogenic effects [13], and is active in the *E μ -Tcl-1* mouse model (which develop a B-cell Lymphocytosis) and in SCID mice engrafted with 697 B-ALL cells [14].

These results led us to assess silvestrol's effect on AML cell lines as a single agent as well as in combination with standard-of-care agents. It appeared to be very potent as single agent, mediating increased apoptosis as assessed by increased PARP cleavage (Fig. 1B). When tested for its ability to synergize, silvestrol showed promising activity in all AML cell lines tested (Fig. 1C and Suppl. Fig. 1). A less potent analogue of silvestrol, Flavagline 61, showed weaker activity and decreased synergy, as would be expected if the resulting synergy was a consequence of blocking translation initiation (Fig. 2). Consistent with our results, the mTOR inhibitor rapamycin, which affects eIF4F assembly, has also been shown to synergize with daunorubicin in AML cell lines [7].

Activation of the PI3K/Akt/mTOR pathway in AML patient blasts has been shown to play a major role in chemoresistance. As well, Bcl-2 family members are known to additionally contribute to chemoresistance in AML, and have the potential to serve as prognostic markers (reviewed in [30]). Recent studies provided insight into the impact of the anti-apoptotic Bcl-2 subfamily member Mcl-1 by demonstrating that the Bcl-2/Bcl-X_L inhibitor, ABT-737, shows reduced activity in AML cell lines expressing high levels of Mcl-1 [21]. The production of Mcl-1 at the translation level is reduced by silvestrol in xenograft models [13], as well as in CLL patient cell lines and the 697 ALL cell line [14]. Silvestrol's preferential inhibition of translation of “weak”, malignancy-related mRNAs, such as Mcl-1 (Fig. 3D), is consistent with silvestrol targeting the eIF4F complex and inhibiting ribosome recruitment. These results do not exclude additional effects of silvestrol on Mcl-1 expression (e.g. protein stability) but do strengthen the tenet that silvestrol's main mechanism of action is through inhibition of protein synthesis. Silvestrol's ability to inhibit Mcl-1 production in AML cell lines (Fig. 3B) likely sensitizes these to ABT-737 (Fig. 4). This supports the idea that inhibition of synthesis of pro-survival targets by silvestrol can be an attractive therapeutic complement to current therapeutic modalities [21,31–35]. The demand on targeted therapy in the treatment of AML has put focus on developing combination treatments for this heterogeneous disease and stratifying genotype–drug response relationships. Our results highlight the ability of silvestrol to resensitize chemoresistant AML to standard-of-care therapeutics *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABT-737 was a kind gift from Dr. Gordon Shore (Dept. Biochemistry, McGill University). Flavagline 61 was a kind gift of Dr. H. Greger (University of Vienna, Vienna, Austria). We are grateful to Dr. Murray Tait (Cerylid Biosciences, Ltd.) for the generous supply of silvestrol. This work was supported by a Cole Foundation Fellowship Award (RC) and by funds from a CIHR grant (MOP-79385) to J.P. The study sponsors had no role in the study design, collection, analysis or interpretation of data, writing of the manuscript or decision to submit the manuscript for publication.

References

1. Yee KW, Schittenhelm M, O'Farrell AM, Town AR, McGreevey L, Bainbridge T, et al. Synergistic effect of SU11248 with cytarabine or daunorubicin on FLT3 ITD-positive leukemic cells. *Blood*. 2004; 104:4202–9. [PubMed: 15304385]

2. Adjei AA, Davis JN, Bruzek LM, Erlichman C, Kaufmann SH. Synergy of the protein farnesyltransferase inhibitor SCH66336 and cisplatin in human cancer cell lines. *Clin Cancer Res*. 2001; 7:1438–45. [PubMed: 11350915]
3. Kojima K, Shimanuki M, Shikami M, Samudio IJ, Ruvolo V, Corn P, et al. The dual PI3 kinase/mTOR inhibitor PI-103 prevents p53 induction by Mdm2 inhibition but enhances p53-mediated mitochondrial apoptosis in p53 wild-type AML. *Leukemia*. 2008; 22:1728–36. [PubMed: 18548093]
4. Kornblau SM, Womble M, Qiu YH, Jackson CE, Chen W, Konopleva M, et al. Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood*. 2006; 108:2358–65. [PubMed: 16763210]
5. Tamburini J, Elie C, Bardet V, Chapuis N, Park S, Broet P, et al. Constitutive phosphoinositide 3-kinase/Akt activation represents a favorable prognostic factor in de novo acute myelogenous leukemia patients. *Blood*. 2007; 110:1025–8. [PubMed: 17426258]
6. Zeng Z, Sarbassov dos D, Samudio IJ, Yee KW, Munsell MF, Ellen Jackson C, et al. Rapamycin derivatives reduce mTORC2 signaling and inhibit AKT activation in AML. *Blood*. 2007; 109:3509–12. [PubMed: 17179228]
7. Calabro A, Tai J, Allen SL, Budman DR. In vitro synergism of m-TOR inhibitors, statins, and classical chemotherapy: potential implications in acute leukemia. *Anticancer Drugs*. 2008; 19:705–12. [PubMed: 18594212]
8. Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem*. 1999; 68:913–63. [PubMed: 10872469]
9. Pelletier, J.; Peltz, S. Therapeutic opportunities in translation.. In: Mathews, MB.; Sonenberg, N.; Hershey, JWB., editors. *Translational control in biology and medicine*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor Laboratory: 2007. p. 855-95.
10. Moerke NJ, Aktas H, Chen H, Cantel S, Reibarkh MY, Fahmy A, et al. Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G. *Cell*. 2007; 128:257–67. [PubMed: 17254965]
11. Graff JR, Konicek BW, Vincent TM, Lynch RL, Monteith D, Weir SN, et al. Therapeutic suppression of translation initiation factor eIF4E expression reduces tumor growth without toxicity. *J Clin Invest*. 2007; 117:2638–48. [PubMed: 17786246]
12. Bordeleau ME, Robert F, Gerard B, Lindqvist L, Chen SM, Wendel HG, et al. Therapeutic suppression of translation initiation modulates chemosensitivity in a mouse lymphoma model. *J Clin Invest*. 2008; 118:2651–60. [PubMed: 18551192]
13. Cencic R, Carrier M, Galicia-Vázquez G, Bordeleau ME, Sukarieh R, Bourdeau A, et al. Antitumor activity and mechanism of action of the cyclopenta[b]benzofuran silvestrol. *PLoS One*. 2009; 4:e5223. [PubMed: 19401772]
14. Lucas DM, Edwards RB, Lozanski G, West DA, Shin JD, Vargo MA, et al. The novel plant-derived agent silvestrol has B-cell selective activity in chronic lymphocytic leukemia and acute lymphoblastic leukemia in vitro and in vivo. *Blood*. 2009; 113(19):4656–66. [PubMed: 19190247]
15. Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1984; 22:27–55. [PubMed: 6382953]
16. Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, et al. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev*. 2003; 17:1475–86. [PubMed: 12783855]
17. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003; 426:671–6. [PubMed: 14668867]
18. Wendel HG, Silva RL, Malina A, Mills JR, Zhu H, Ueda T, et al. Dissecting eIF4E action in tumorigenesis. *Genes Dev*. 2007; 21:3232–7. [PubMed: 18055695]
19. Del Poeta G, Bruno A, Del Principe MI, Venditti A, Maurillo L, Buccisano F, et al. Dereglulation of the mitochondrial apoptotic machinery and development of molecular targeted drugs in acute myeloid leukemia. *Curr Cancer Drug Targets*. 2008; 8:207–22. [PubMed: 18473734]

20. Marcucci G, Byrd JC, Dai G, Klisovic MI, Kourlas PJ, Young DC, et al. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. *Blood*. 2003; 101:425–32. [PubMed: 12393493]
21. Konopleva M, Contractor R, Tsao T, Samudio I, Ruvolo PP, Kitada S, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell*. 2006; 10:375–88. [PubMed: 17097560]
22. van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell*. 2006; 10:389–99. [PubMed: 17097561]
23. Kohrt HE, Coutre SE. Optimizing therapy for acute myeloid leukemia. *J Natl Compr Canc Netw*. 2008; 6:1003–16. [PubMed: 19176198]
24. Lancet JE, Giralt S. Therapy for older AML patients: the role of novel agents and allogeneic stem cell transplant. *J Natl Compr Canc Netw*. 2008; 6:1017–25. [PubMed: 19176199]
25. King ME, Rowe JM. Recent developments in acute myelogenous leukemia therapy. *Oncologist*. 2007; 12(2):14–21. [PubMed: 18039635]
26. Raught, B.; Gingras, A-C.; Sonenberg, N. Regulation of ribosome recruitment in eukaryotes. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 2000.
27. Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M. S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science*. 2006; 314:467–71. [PubMed: 17053147]
28. Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X, Holland EC. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. *Mol Cell*. 2003; 12:889–901. [PubMed: 14580340]
29. Mamane Y, Petroulakis E, Martineau Y, Sato TA, Larsson O, Rajasekhar VK, et al. Epigenetic activation of a subset of mRNAs by eIF4E explains its effects on cell proliferation. *PLoS One*. 2007; 2:e242. [PubMed: 17311107]
30. Irvine AE, McMullin MF, Ong YL. Bcl-2 family members as prognostic indicators in AML. *Hematology*. 2002; 7:21–31. [PubMed: 12171774]
31. Liu XS, Jiang J, Jiao XY, Wu YE, Lin JH, Cai YM. Lycorine induces apoptosis and down-regulation of Mcl-1 in human leukemia cells. *Cancer Lett*. 2009; 274:16–24. [PubMed: 18829157]
32. Gu C, Ye T, Wells RA. Synergistic effects of troglitazone in combination with cytotoxic agents in acute myelogenous leukaemia cells. *Leuk Res*. 2006; 30:1447–51. [PubMed: 16704876]
33. Tang R, Faussat AM, Majdak P, Marzac C, Dubrulle S, Marjanovic Z, et al. Semisynthetic homoharringtonine induces apoptosis via inhibition of protein synthesis and triggers rapid myeloid cell leukemia-1 down-regulation in myeloid leukemia cells. *Mol Cancer Ther*. 2006; 5:723–31. [PubMed: 16546987]
34. Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. *J Biol Chem*. 2005; 280:35217–27. [PubMed: 16109713]
35. Chen Y, Hu Y, Michaels S, Segal D, Brown D, Li S. Inhibitory effects of omacetaxine on leukemic stem cells and BCR-ABL-induced chronic myeloid leukemia and acute lymphoblastic leukemia in mice. *Leukemia*. 2009; 23(8):1446–54. [PubMed: 19322212]

Fig. 1.

Sensitivity of AML cells to silvestrol. (A) U937 cells (35 000 cells/well) or HL-60, NB4, OCI/AML2 and OCI/AML3 cells (50 000 cells/well) were seeded in 96-well plates in the presence of increasing concentrations of silvestrol. Forty eight hours later, cell growth was measured using the MTS assay and plotted relative to vehicle (DMSO) controls. Experiments were performed in triplicates and the error of the mean is shown. The inset of the middle panel shows NB4 cells treated for 48 h with 7.5 nM Sil and labeled for the last 15 min. Rel. Incorp. denotes the Relative [³⁵S]-Met Incorporation compared to DMSO control. The experiment was performed in triplicate (B) Silvestrol induces apoptosis in AML cells. NB4 cells, 200 000 cells/well, were seeded in 6-well plates in the presence of increasing concentrations of silvestrol. Forty eight hours later, cells were harvested in RIPA buffer and Western blot analysis performed on equal amounts of proteins fractionated on 10% polyacrylamide gels. (C) Plots showing combination index (CI) values for NB4 cells receiving combinations of silvestrol with etoposide, Ara-C, or daunorubicin at the indicated ratios. 1-CI values are plotted on the X-axis with values greater than 0.2 being synergistic, lower than -0.2 antagonistic and ranging between -0.2 and +0.2 being additive. The average of 2 experiments, each performed in triplicates, is presented. The error of the mean is shown.

Fig. 2.

The synergistic effect of silvestrol correlates with its potency as an inhibitor of translation initiation. (A) Chemical structures of silvestrol and Flavagline 61. (B) NB4 cells, 50 000 cells/well, were seeded in 96-well plates in the presence of increasing concentrations of silvestrol or Flavagline 61. Forty eight hours later, cell growth was measured using the MTS assay and plotted relative to vehicle (DMSO) controls. Experiments were performed in triplicates and the error of the mean is shown. (C) Inhibition of protein synthesis in NB4 cells following exposure to 50 nM silvestrol or Flavagline 61. The results are the average of triplicates with the error of the mean shown. Values are standardized against total protein content and plotted relative to DMSO controls, which were ~100 000 cpm/ μ g. (D) NB4 cells were treated with combinations of Flavagline 61 with etoposide, Ara-C or daunorubicin for 48 h at the indicated ratios. 1-CI values are plotted on the X-axis with values greater than 0.2 being synergistic, lower than -0.2 antagonistic and values between -0.2 and +0.2 being additive. The average of 3 experiments, each performed in triplicates, is presented. The error of the mean is shown.

Fig. 3.

Effect of silvestrol on protein synthesis in AML cells. (A) Inhibition of protein synthesis in AML cells following exposure to 50 nM silvestrol. The results are the average of duplicates with the error of the mean shown. Values are standardized against total protein content and plotted relative to DMSO controls, which were ~100 000 cpm/μg for all cell lines. (B) Western blot analysis probing levels of Mcl-1 in AML lines following exposure to silvestrol. Extracts prepared from cells exposed to 50 nM silvestrol for the indicated periods of time were resolved on 10% SDS-polyacrylamide gels. Western blotting was performed to investigate protein levels after treatment with silvestrol. (C) Polysome profiling analysis on NB4 cells following treatment with silvestrol. NB4 cells were harvested after 1 h treatment with either 1% DMSO or 10 nM silvestrol. Extracts were resolved on 10–50% sucrose gradients by centrifugation in an SW40 rotor at 35 000 rpm for 2 h. (D) Semi-quantitative RT-PCR analysis on fractions collected from extracts of DMSO or silvestrol treated cells. Fractions were pooled into unbound and polysome (poly)-bound fractions and monitored for distribution of Mcl-1 and β-actin mRNA.

Fig. 4.

Inhibition of protein synthesis synergizes with ABT-737 on AML cells. (A) NB4 cells (50 000 cells/well) were seeded in 96-well plates in the presence of increasing concentrations of ABT-737. Forty eight hours later, cell growth was measured using the MTS assay and plotted relative to vehicle (DMSO) controls. Experiments were performed in triplicates and the error of the mean is shown. (B) Plots showing combination index (CI) values against NB4 cells for combinations of silvestrol + ABT-737, silvestrol + daunorubicin, ABT-737 + daunorubicin or silvestrol + ABT-737 + daunorubicin at the indicated ratios. 1-CI values are plotted on the X-axis with values greater than 0.2 being synergistic, lower than -0.2 antagonistic and values between -0.2 and +0.2 being additive. The average of 3 experiments, each performed in triplicates, is presented. The error of the mean is shown.

Table 1

Average IC₅₀ of silvestrol on cell lines used in this study.

Cell line	IC ₅₀ [nM] ± SD
U937	7.08 ± 2.56
HL-60	7.70 ± 1.78
NB4	10.07 ± 4.82
OCI/AML2	3.82 ± 0.95
OCI/AML3	4.46 ± 1.74