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

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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.


## 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 chemoresistent 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 downstream 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 pre-initiation 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

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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 acitivity, and has no cyotoxicity on normal tissues *in vivo* [13]. Additionally, silvestrol was demonstrated to exert anti-proliferative activity in the *Eμ-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, cytarabinose-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 $A_{queous}$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 $OD_{490}$ on a SpectramaxPlus$^{384}$ (Molecular Devices) and using Softmax Pro 4.8.2 software. Values obtained were standardized against vehicle (DMSO) control, which was set at 1.

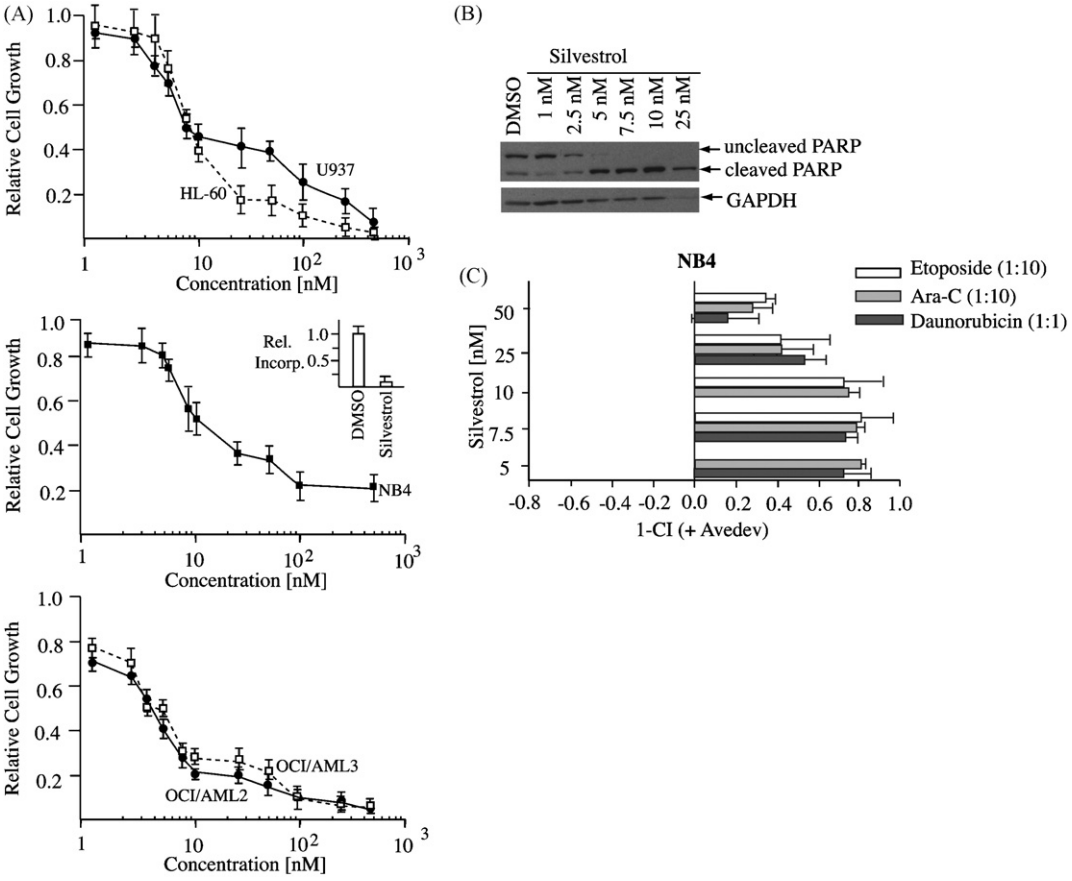


**Fig. 1.** Sensitivity of AML cells to silvestrol. (A) U937 cells (35 000 cells/well) or HL-60, NB4, OCI/AML2 and OCI/AML3 cells (50 000 cells/well) were seeded in 96-well plates in the presence of increasing concentrations of silvestrol. Forty eight hours later, cell growth was measured using the MTS 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 [$^{35}$S]-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.