

# Translation Inhibition by Rocaglates Is Independent of eIF4E Phosphorylation Status

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## Abstract

Rocaglates are natural products that inhibit protein synthesis in eukaryotes and exhibit antineoplastic activity. *In vitro* biochemical assays, affinity chromatography experiments coupled with mass spectrometry analysis, and *in vivo* genetic screens have identified eukaryotic initiation factor (eIF) 4A as a direct molecular target of rocaglates. eIF4A is the RNA helicase subunit of eIF4F, a complex that mediates cap-dependent ribosome recruitment to mRNA templates. The eIF4F complex has been implicated in tumor initiation and maintenance through elevated levels or increased phosphorylation status of its cap-binding subunit, eIF4E, thus furthering the interest toward developing rocaglates as antineo-

plastic agents. Recent experiments have indicated that rocaglates also interact with prohibitins 1 and 2, proteins implicated in c-Raf-MEK-ERK signaling. Because increased ERK signaling stimulates eIF4E phosphorylation status, rocaglates are also expected to inhibit eIF4E phosphorylation status, a point that has not been thoroughly investigated. It is currently unknown whether the effects on translation observed with rocaglates are solely through eIF4A inhibition or also a feature of blocking eIF4E phosphorylation. Here, we show that rocaglates inhibit translation through an eIF4E phosphorylation-independent mechanism. *Mol Cancer Ther*; 15(1); 136–41. ©2015 AACR.

## Introduction

Rocaglates are a family of natural products characterized by a common cyclopenta[b]furan skeleton exclusively found in the *Aglaia* genus of the Meliaceae family of angiosperms (1). Many members of this family are potent inhibitors of translation initiation and exhibit single-agent antineoplastic activity in pre-clinical cell and mouse models (2–4) as well as the ability to modulate chemoresponsiveness (5–9). A significant body of evidence indicates that these compounds inhibit translation initiation by disabling eIF4F assembly and interfering with ribosome recruitment to mRNA templates (2, 5, 9).

eIF4F is a heterotrimeric complex consisting of eIF4E, a cap-binding protein; eIF4A, the RNA helicase target of rocaglates; and eIF4G, a large scaffolding protein. eIF4F is required to unwind cap-proximal secondary structure within the mRNA 5' untranslated region (UTR) as a prelude to 43S preinitiation complex recruitment. Of all the initiation factors, eIF4E is the least abundant (10, 11), and mRNAs must compete for the limiting amounts of eIF4F during the initiation process. One determinant of competitive efficiency is the presence of structural barriers (e.g., stem-loop structures, protein–RNA interactions) within the mRNA 5' UTR, with elevated levels associated with poorly initiating mRNAs. Consequently, translation of these weaker mRNAs is

most affected upon eIF4F inhibition, and hence by rocaglates (2, 12–14).

eIF4A is an abundant factor that exists as a free form (eIF4A<sub>f</sub>) or as part of the eIF4F complex (eIF4A<sub>c</sub>). Biochemical assays using recombinant eIF4A (2, 5, 9), affinity chromatography experiments using immobilized epi-silvestrol (15), and chemogenomic profiling in yeast (16) have identified eIF4A as a predominant target of rocaglates. Mechanistically, rocaglates are thought to restrict efficient recycling of eIF4A through the eIF4F complex by increasing nonspecific RNA binding of eIF4A (Fig. 1A; ref. 8).

An alternative mechanism of rocaglate activity on translation initiation has been proposed based on their interactions with prohibitins (PHB) 1 and 2 (17). PHB1/2 are involved in a wide variety of cellular processes, including activation of the MAPK signaling cascade through direct interaction with c-RAF (18). In the presence of rocaglates, the PHB1/2:c-RAF interaction is inhibited, leading to dampened signaling through MEK and ERK 1/2 (17). Because MNK1 and MNK2 are activated by ERK signaling and phosphorylate eIF4E on S209, rocaglates are expected to inhibit eIF4E S209 phosphorylation. However, the effect of rocaglates on eIF4E phosphorylation status has yet to be assessed. If correct, this mechanism of action would have profound consequences on our understanding of the antineoplastic effects of these compounds because eIF4E phosphorylation is essential to its oncogenic activity (19, 20). As well, transcriptome-wide studies attributing alterations in mRNA translational efficiency to eIF4A inhibition by rocaglates would have to be reinterpreted if inhibition of eIF4E phosphorylation was a significant biologic property of rocaglates (12, 13).

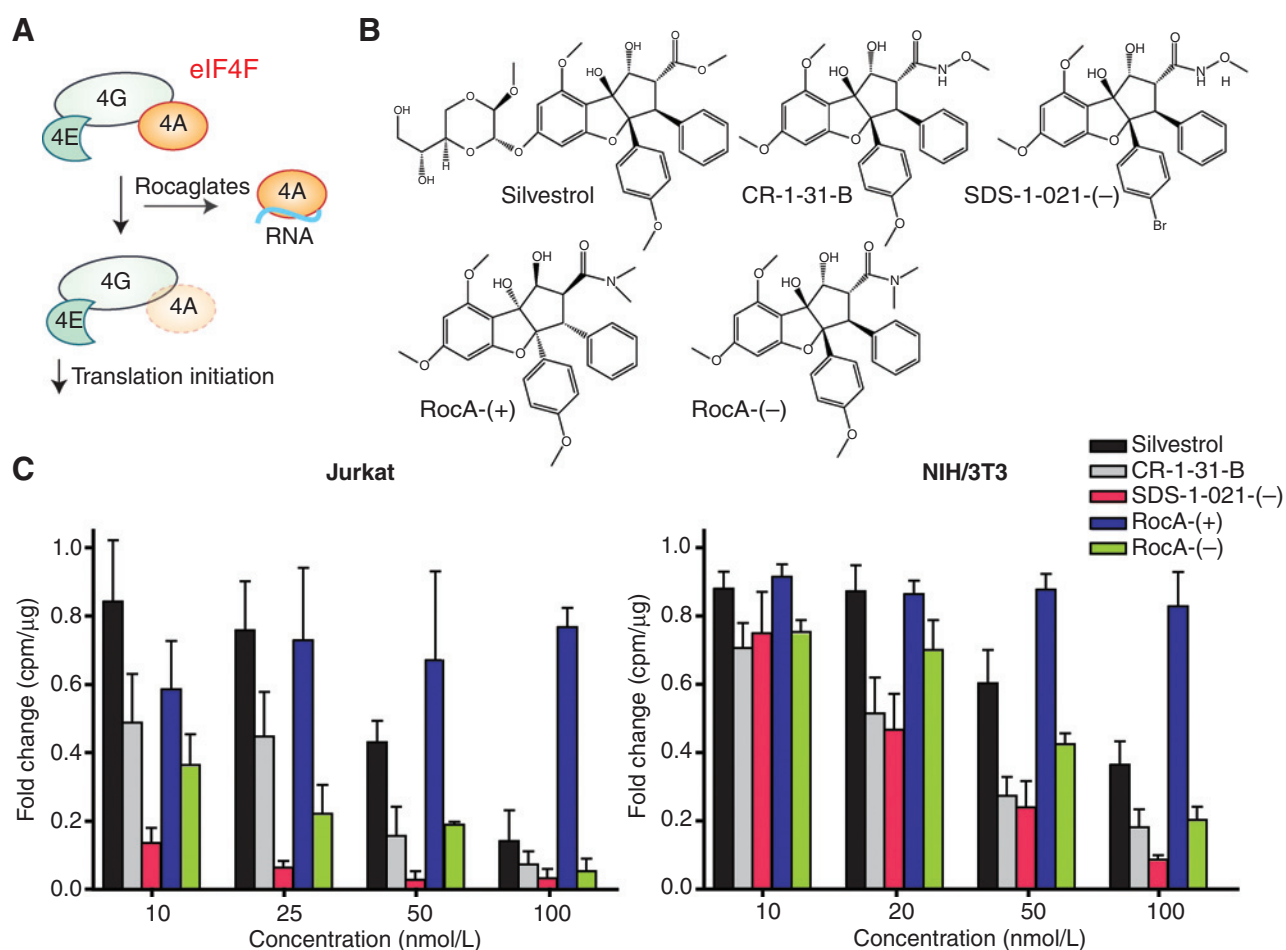
However, there are several lines of evidence inconsistent with inhibition of eIF4E phosphorylation contributing to the biologic activity of rocaglates. Firstly, rocaglates are potent inhibitors of eIF4A-dependent translation in *in vitro* extracts where RAS/MEK/ERK signaling is not maintained (5). Secondly, rocaglates have been shown to inhibit encephalomyocarditis internal ribosomal entry site (IRES)-driven translation (5), an event that is eIF4A, but

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**Figure 1.**

A, proposed mechanism of action of rocaglates on eIF4A recycling through the eIF4F complex. In this model, rocaglates stimulate eIF4A RNA binding, rendering it unavailable to enter into the eIF4F complex. B, structures of rocaglates used in this study. C, dose-dependent inhibition of translation by rocaglates in Jurkat and NIH/3T3 cells. Cells were incubated in the presence of compound for a total of 2 hours, and protein synthesis rates were determined as described in Materials and Methods. The relative rates of translation are calculated by normalizing to DMSO.  $n = 4$ ; error bars, error of the mean.

not eIF4E, dependent (21). Thirdly, rocaglates are potent inhibitors of global cap-dependent translation (2, 5), whereas loss of eIF4E S209 phosphorylation leads to more selective inhibition of mRNA translation (20, 22–24). It therefore remains an open question as to whether the reported suppression of RAS/MEK/ERK signaling by rocaglates represents an essential feature of these compounds. Herein, we report that the biologic activity of rocaglates cannot be explained by modulation of eIF4E phosphorylation.

## Materials and Methods

### General methods and reagents

Jurkat (in 2002) and NIH/3T3 (in 2005) cells were obtained from the American Type Culture Collection.  $MNK1^{+/+}MNK2^{+/+}$  and  $MNK1^{-/-}MNK2^{-/-}$  mouse embryonic fibroblasts (MEF) were obtained from Dr. Nahum Sonenberg in 2010 (McGill University, Montreal, Quebec, Canada). RAS-transformed NIH/3T3 cells were generated by infection with pBABE-Puro.H-Ras (V12) in 2015. Cells lines were not further authenticated. Jurkat, NIH/3T3, RAS-transformed NIH/3T3, and  $MNK1^{+/+}MNK2^{+/+}$  and  $MNK1^{-/-}MNK2^{-/-}$  MEFs were grown in RPMI 1640 and

DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37°C and 5% CO<sub>2</sub>. Cell extracts were prepared in lysis buffer (20 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% Triton-X100, 10% glycerol, 1 mmol/L EDTA, 10 mmol/L tetrasodium pyrophosphate, 100 mmol/L NaF, 17.5 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L PMSF, 4  $\mu$ g/mL aprotinin, 2  $\mu$ g/mL leupeptin, and 2  $\mu$ g/mL pepstatin). Extracts from Jurkat cells were prepared by lysing cells in 1 $\times$  NuPAGE LDS sample buffer (26.5 mmol/L Tris HCl, pH 8.5, 35.25 mmol/L Tris Base, 0.5% LDS, 2.5% glycerol, and 0.1275 mmol/L EDTA). Protein samples were resolved on 10% polyacrylamide gels and transferred to PVDF membranes (Bio-Rad). Antibodies used in this study were directed against: p-eIF4E (Ser209; #9741; Cell Signaling Technology), eIF4E (#9742; Cell Signaling Technology), p-ERK 1/2 (#9106; Cell Signaling Technology), ERK 1/2 (#9102; Cell Signaling Technology), eEF2 (#2332; Cell Signaling Technology), and GAPDH (ab8245; Abcam). SDS-1-021(-), Roc A(-), and Roc A(+) were synthesized using biomimetic kinetic resolution of chiral, racemic aglain ketone precursors as recently published (25) followed by amide formation (26). Silvestrol and CR-1-31-B were synthesized as previously reported (9, 27). The concentrations of

compound used were based on our experience with this class of small molecules, as well as previously published studies (2, 5, 6, 9, 12).

### Cell labeling and TCA precipitations

<sup>35</sup>S-methionine/cysteine protein labeling was performed as previously described (11). Essentially, the day prior to metabolic labeling, Jurkat cells were seeded at 500,000 cells/mL, and NIH 3T3, *MNKK1<sup>+/+</sup>MNKK2<sup>+/+</sup>*, and *MNKK1<sup>-/-</sup>MNKK2<sup>-/-</sup>* MEFs were seeded at 250 cells/mm<sup>2</sup>. On the day of labeling, cells were exposed to the indicated concentrations of rocaglates for 2 hours. During the last 30 minutes of incubation, [<sup>35</sup>S]-methionine/cysteine was added (150–200 μCi/mL; 1175 Ci/mmol; Perkin Elmer), and the labeling reactions were terminated with the addition of RIPA lysis buffer (20 mmol/L Tris, pH 7.6, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, 4 μg/mL aprotinin, 2 μg/mL leupeptin, and 2 μg/mL pepstatin). Lysates were then spotted onto 3 MM Whatman paper (2 cm<sup>2</sup> squares) that had been preblocked with MEM non-essential amino acid mixture (Gibco). After drying, filters were submerged in ice-cold 10% trichloroacetic acid (TCA)/0.1% L-methionine for 20 minutes, followed by boiling in 5% TCA for 15 minutes. The Whatman squares were washed twice with cold 5% TCA and then twice more with 95% ethanol, with each wash lasting 1 minute. Filter squares were dried, and the amount of radiolabeled precipitated protein was quantitated by scintillation counting (Beckman Coulter). Counts were standardized to total protein content that had been previously determined using the DC protein assay (Bio-Rad).

### RNA-binding assay

Body-labeled [<sup>32</sup>P]-labeled RNA was produced by *in-vitro* transcription of pSP/CAT (linearized with *PvuII*) using SP6 RNA polymerase. Recombinant eIF4AI was purified as previously reported (28). Binding assays were performed by incubating [<sup>32</sup>P]-labeled RNA (35,000 cpm) with recombinant eIF4AI in binding buffer (25 mmol/L Tris, pH 7.5, 1 mmol/L DTT, 100 mmol/L KCl, 1 mmol/L ATP, 5 mmol/L MgCl<sub>2</sub>) in the presence of 5 μmol/L of the indicated compound or 0.1% DMSO for 10 minutes at 37 °C, as previously noted (9). No preincubations were performed, and when reactions were assembled, rocaglates were added penultimately before the addition of [<sup>32</sup>P]-labeled RNA. Reactions were terminated by the addition of 1 mL stop buffer (25 mmol/L Tris, pH 7.5, 100 mmol/L KCl, and 3 mmol/L MgCl<sub>2</sub>) and then filtered through nitrocellulose (45 μmol/L HA; Millipore; preblocked with 0.1% sodium pyrophosphate). Filters were washed 3 times with 1 mL stop buffer, dried, and the amount of retained [<sup>32</sup>P]-labeled RNA was quantitated by liquid scintillation counting.

## Results

Our previous work investigating the consequences of silvestrol (2, 5), CR-1-31-B (a.k.a. hydroxamate (-)-9; ref. 9), and SDS-1-021-(-) (Regina Cencic, unpublished data; Fig. 1B) on translation indicate that these compounds target eIF4A and prevent its entry/recycling into the eIF4F complex (Fig. 1A). Polier and colleagues (17) have reported that rocaglates, such as RocA (Fig. 1B), can also inhibit PHB1/2 to downregulate ERK activation, although the downstream effects on eIF4E S209 phosphorylation were never

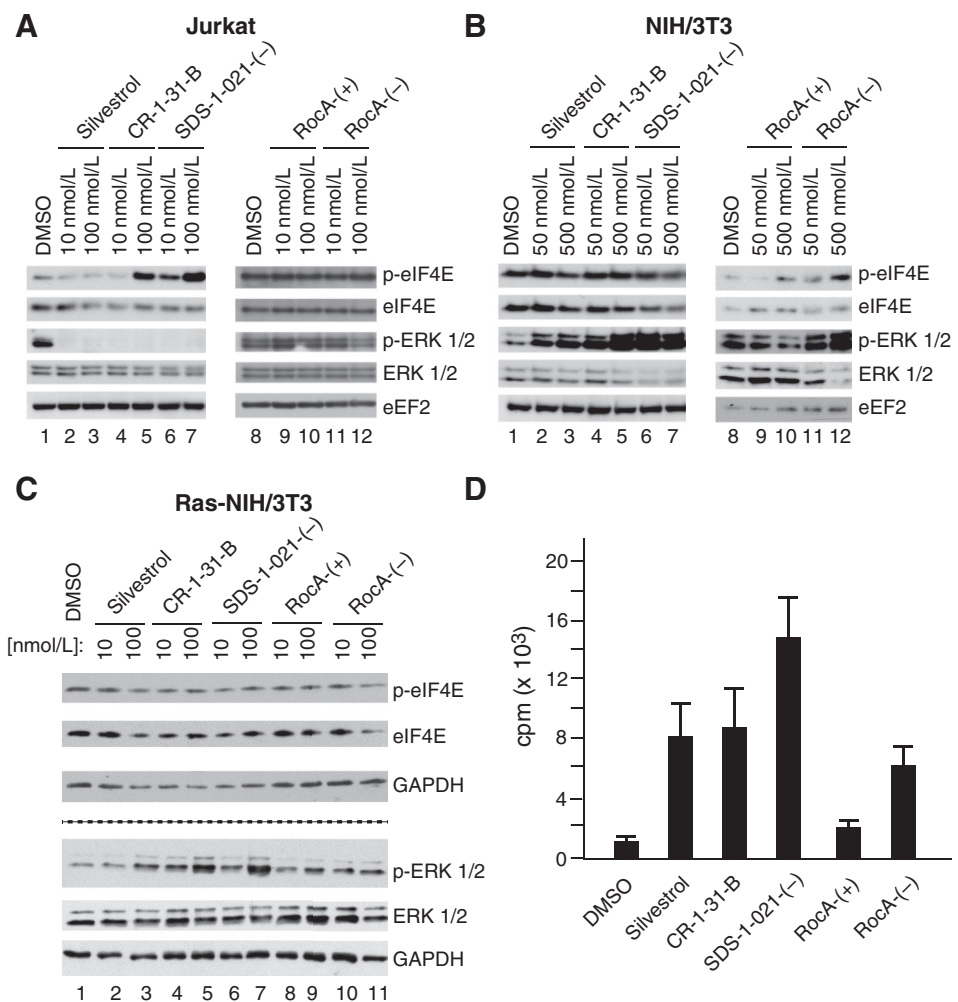
reported. We therefore decided to investigate this potential relationship and also took the opportunity to synthesize enantio-enriched preparations of RocA to tease out possible biologic differences between the stereoisomers (Fig. 1B).

To assess the relative potencies of the rocaglates on translation, we performed a series of titrations in Jurkat and NIH/3T3 cells and measured relative levels of protein synthesis using [<sup>35</sup>S]-methionine/cysteine metabolic labeling. To ensure that the measured values reflected the immediate effects of the compounds on translation, cells were exposed to the indicated rocaglate for only 2 hours, which was also the shortest incubation period used by Polier and colleagues to assess translation inhibition (17). Concentrations tested ranged from 10 nmol/L to 100 nmol/L, as rocaglates have been previously reported to be effective inhibitors of translation at nanomolar concentrations (2). Based on the relative IC<sub>50</sub>s, as defined by the concentration of compound required to inhibit translation by 50%, SDS-1-021-(-) was found to be the most potent rocaglate (IC<sub>50</sub> < 10 nmol/L) in Jurkat cells, followed by RocA(-) and CR-1-31-B (Fig. 1C). In NIH/3T3 cells, SDS-1-021-(-) and CR-1-31-B showed similar potencies, with IC<sub>50</sub>s ~20 nmol/L for translation inhibition under our test conditions (Fig. 1C). Silvestrol and RocA(-) were slightly less potent with IC<sub>50</sub>s of ~50 nmol/L. RocA(+) did not inhibit translation in either cell line highlighting the importance of compound stereochemistry for biologic activity, as previously noted (9). In sum, these results indicate that individual rocaglates exert differences in their ability to inhibit translation across cell lines, as previously reported (5, 9), and that within this small series, SDS-1-021-(-) is the most potent compound tested (Fig. 1C).

RocA has been shown to inhibit ERK 1/2 phosphorylation in Jurkat cells (17), although its effects on eIF4E phosphorylation have never been reported. We found that, with the exception of RocA(+), all other rocaglates suppressed ERK 1/2 phosphorylation in Jurkat cells (Fig. 2A). Silvestrol, CR-1-31-B, and SDS-1-021-(-) completely blocked phosphorylation, and modest inhibition was observed at 100 nmol/L RocA(-) (Fig. 2A). Surprisingly, eIF4E phosphorylation was not inhibited and, contrary to expectations, was stimulated by CR-1-31-B and SDS-1-021-(-). In contrast, in NIH/3T3 cells, we observed stimulation, not inhibition, of ERK 1/2 phosphorylation by all rocaglates tested, with the exception of the inactive RocA(+) enantiomer, compared with vehicle-treated cells (Fig. 2B). None of the rocaglates affected phospho-eIF4E status in NIH/3T3 cells under the tested conditions. To exclude the possibility that the absence of diminished eIF4E phosphorylation is a consequence of low levels of MEK/ERK activity in NIH/3T3 cells, we transformed the cells with RAS in order to elevate ERK signaling and treated with the rocaglate series (Fig. 2C). All rocaglates tested stimulated p-ERK levels in RAS-transformed NIH/3T3 cells with little effect on p-eIF4E status (Fig. 2C). This is consistent with what was noted in nontransformed NIH/3T3 cells, and we have not further investigated the underlying molecular mechanism. Nonetheless, there is little correlation between ERK and eIF4E phosphorylation status with the inhibition of translation documented above (Fig. 1C). One well-characterized activity of rocaglates is stimulation of the RNA-binding activity of eIF4A (2, 5, 9). To assess whether the rocaglate series under evaluation retained this activity, we performed RNA filter binding assays *in vitro* using <sup>32</sup>P-labeled RNA in the presence of 5 μmol/L of each compound, which is within the concentration range of silvestrol previously shown to stimulate RNA binding of eIF4A (ref. 2; Fig. 2D). With the exception of the inactive RocA(+)

**Figure 2.**

**A**, effects of rocaglates on eIF4E phosphorylation in Jurkat cells. Cells were incubated in the presence of the indicated compounds for 2 hours, lysed, resolved on a 10% NuPAGE Bis-Tris gel, and transferred to PVDF membranes for Western blotting. Blots were probed using antibodies directed to the proteins indicated to the right of the panel. **B**, effects of rocaglates on eIF4E phosphorylation status in NIH/3T3 cells. Drug incubations and Western blots were performed as described for **A**. **C**, effects of rocaglates on eIF4E phosphorylation status in RAS-transformed NIH/3T3 cells. Drug incubations and Western blots were performed as described for **A**. The dashed line indicates that probing for eIF4E and ERK was performed on different membranes. GAPDH levels were used as an internal standard to account for variations in extract levels between lanes. **D**, stimulation of eIF4A:RNA binding by rocaglates. Recombinant eIF4A (1.3  $\mu\text{mol/L}$ ) was incubated with 35,000 cpm of  $^{32}\text{P}$ -labeled RNA in the presence of 5  $\mu\text{mol/L}$  rocaglate and processed as described in Materials and Methods. eIF4A:RNA complexes retained on nitrocellulose filters were quantitated by scintillation counting.  $n = 3$ ; error bars, error of the mean.



enantiomer, all rocaglates stimulated binding of eIF4A to RNA, with SDS-1-021(-) being the most potent compound (Fig. 2D).

To further support the notion that eIF4E phosphorylation status is inconsequential to the inhibition of translation observed with rocaglates, we quantitated the effects of rocaglates on protein synthesis in  $MNK1^{-/-}MNK2^{-/-}$  MEFs, where eIF4E cannot be phosphorylated (Fig. 3A; ref. 29). Whereas we observed a slight difference in the sensitivities between  $MNK1^{-/-}MNK2^{-/-}$  and  $MNK1^{+/+}MNK2^{+/+}$  MEFs (~40%) toward silvestrol, there was no significant difference on translation exerted by the other tested rocaglates in these two cell types (Fig. 3A). Consistent with our results in Jurkat and NIH/3T3 cells, SDS-1-021(-) was the most potent inhibitor among the series tested (Fig. 3A). Western blots of extracts confirmed the complete absence of eIF4E phosphorylation in the  $MNK1^{-/-}MNK2^{-/-}$  MEFs as well as the absence of diminished eIF4E phosphorylation in  $MNK1^{+/+}MNK2^{+/+}$  MEFs (Fig. 3B). As a positive control, we included the Mnk1 inhibitor, CGP57380, and observed reduced phospho-eIF4E levels in  $MNK1^{+/+}MNK2^{+/+}$  MEFs (Fig. 3B; ref. 30).

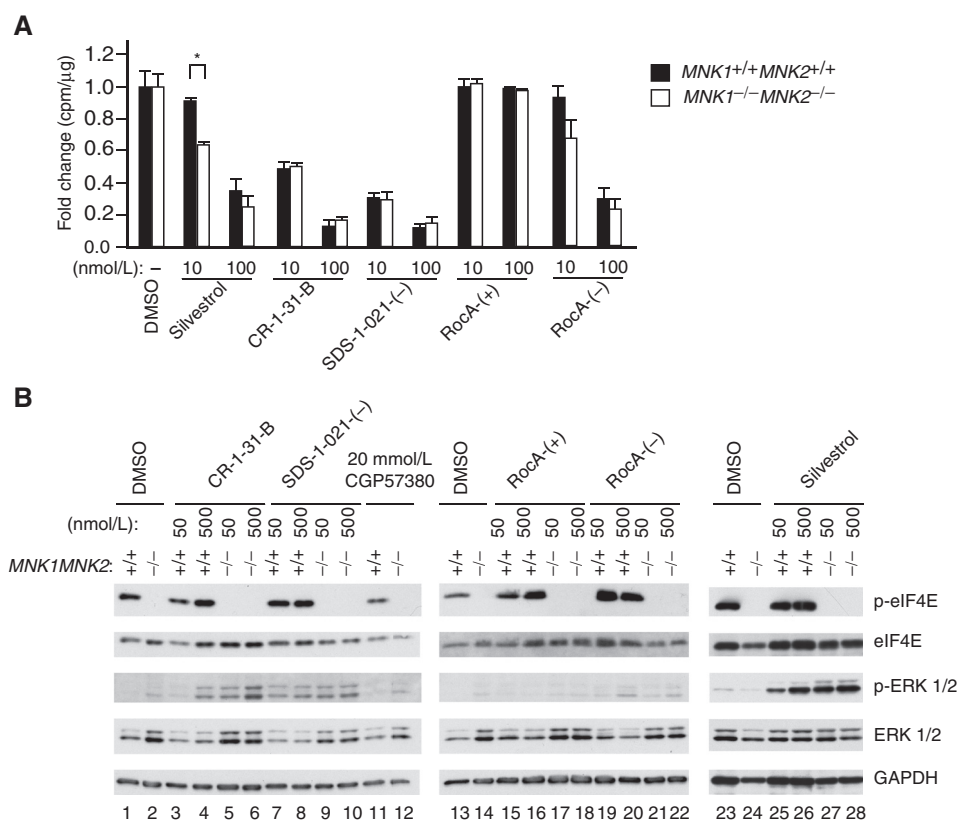
## Discussion

In this study, we report that translation inhibition by rocaglates is independent of eIF4E phosphorylation status. Although we

have not directly tested the ability of our rocaglate series to inhibit PHB1/2:c-RAF association, RocA has been previously shown to block this interaction (17). We find that the effects of rocaglates on p-ERK 1/2 and p-eIF4E status appear cell-type dependent overall do not correlate with rocaglate-induced translation inhibition (Fig. 2). It is clear that in the complete absence of eIF4E phosphorylation, the ability of CR-1-31-B, SDS-1-021(-), and RocA(-) to inhibit protein synthesis is unperturbed (Fig. 3). Silvestrol, but not CR-1-31-B, is a known Pgp-1 multidrug transporter substrate (ref. 31; Regina Cencic and Jerry Pelletier; unpublished data). Whether the increased sensitivity of  $MNK1^{-/-}MNK2^{-/-}$  cells to silvestrol is due to reduced expression of Pgp-1 and/or other drug response modifiers remains to be evaluated (Fig. 3A).

As reported by Polier and colleagues (17), we also find that rocaglates inhibit ERK 1/2 phosphorylation in Jurkat cells (Fig. 2A). However, in NIH/3T3 cells, we observed stimulation, not inhibition, of this posttranslational modification (Fig. 2B and C). The increase in p-eIF4E that we observed with the more potent SDS-1-021(-) and CR-1-31-B compounds in Jurkat cells is unlinked to p-ERK 1/2 status and may reflect activation of a stress kinase response—an effect that has been previously documented with other translation inhibitors, including anisomycin, onnamide A and theopederin B (32, 33). This increase in p-eIF4E levels is contrary to what would be expected upon ERK 1/2 inhibition,

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**Figure 3.**

A, rocaglates inhibit translation in *MNK1<sup>+/+</sup>MNK2<sup>+/+</sup>* and *MNK1<sup>-/-</sup>MNK2<sup>-/-</sup>* MEFs. Protein synthesis rates were determined as described in Materials and Methods. The relative rates of <sup>35</sup>S-Met incorporation are normalized to DMSO. *n* = 4; error bars, error of the mean. \*, *P* < 0.001 (Student *t* test). B, effects of rocaglates on eIF4E phosphorylation in *MNK1<sup>+/+</sup>MNK2<sup>+/+</sup>* and *MNK1<sup>-/-</sup>MNK2<sup>-/-</sup>* cells. Cells were incubated in the presence of the indicated compounds for 2 hours, lysed, resolved on a 10% polyacrylamide gel, and transferred to PVDF membranes for Western blotting. Blots were probed with antibodies directed to the proteins indicated to the right of the panel. GAPDH levels were used as an internal standard to account for variations in extract levels between lanes. Note that lane 24 is slightly underloaded based on the GAPDH internal standard.

which one would expect to stimulate, not inhibit, selective mRNA translation (20, 22–24).

Our results do not rule out the possibility that some rocaglate family members not tested here can block eIF4E phosphorylation given the appropriate context. Indeed, the compound RocAR has been reported to exhibit this activity in Human T-Lymphotropic Virus (HTLV)-infected ATL (adult T-cell leukemia/lymphoma) cells (34). However, our data indicate that this is not a general feature of this class of compounds and that rocaglate-induced translation inhibition is independent of eIF4E phosphorylation status. Taken together with previous data indicating that these compounds do not inhibit translation by increasing eIF2 $\alpha$  phosphorylation (5), the consequences of rocaglates on translation appear best explained by their effects on eIF4A activity.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

Conception and design: J. Chu, R. Cencic, J. Pelletier  
Development of methodology: J. Chu, J. Pelletier

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Chu, R. Cencic, J.A. Porco  
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Chu, R. Cencic, J. Pelletier  
Writing, review, and/or revision of the manuscript: J. Chu, R. Cencic, J. Pelletier  
Study supervision: J. Pelletier  
Other (providing all the molecules for this study): W. Wang

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