Abstract # 3797

Hyperphosphorylation and Desumoylation of RanGAP1•SUMO1

Irina Oussenko, James F. Holland, E. Premkumar Reddy, Takao Ohnuma
Mount Sinai School of Medicine, New York, NY

Introduction

Rigosertib Sodium (ON 01910.Na)

- ❑ Rigosertib (Figure 1A) is a potent anticancer antimitotic agent currently undergoing clinical studies
- ❑ Biological action is characterized by spindle abnormalities, selective G2/M-arrest and apoptosis
- ❑ In mantle cell lymphoma cells, the compound caused rapid decrease in cyclin D by blocking cyclin D1 mRNA translation through inhibition of the PI-3K/Akt/mTOR/eIF4E-BP pathway and triggering a cytochrome c-dependent apoptosis [Ref 1]
- ❑ Rigosertib induced hyperphosphorylation of RanGAP1•SUMO1 in all tumor cell lines tested [Ref 2].
- ❑ Okadaic acid, a known phosphatase inhibitor (PP1/PP2A), caused mitotic arrest, expression of hyperphosphorylated RanGAP1•SUMO1 and apoptosis similar to rigosertib [Ref 3]

RanGAP1 (Ran GTPase-activating protein 1)

- ❑ Key regulator of GTP/GDP cycle of Ran. Ran (RAs-related Nuclear protein) is essential for the translocation of RNA and proteins through nuclear pore complex, involved in control of DNA synthesis and cell cycle progression.
- ❑ SUMOylated RanGAP1 associates with nuclear pore complexes.
- ❑ During mitosis SUMOylated RanGAP1 associates with mitotic spindles.
- ❑ Phosphorylated by the M-phase kinase cyclin B/Cdk1
- ❑ Phosphorylation occurs before nuclear envelope breakdown and continues throughout mitosis.

SUMO-specific proteases

- ❑ Class C48 of cysteine peptidases
- ❑ N-ethylmaleimide (NEM) is an irreversible inhibitor of all cysteine peptidases, with alkylation occurring at the active site thiol group.

Materials and Methods

Cell lines and Drug Treatment
DU-145 prostate carcinoma cell line, maintained in RPMI1640 medium, was used. Medium was supplemented with 10% FBS and antibiotics (penicillin/streptomycin). Cells were seeded 24 h prior addition of rigosertib (to 1 μM final concentration) or drug vehicle control. Following 24h drug exposure cells were collected, washed, and total cell lysates prepared.

Preparation of Cell Lysates
Basic buffer (BB) Method (=no protease inhibitors, non-denaturing): cell lysates were prepared under non-denaturing conditions in the **BB** lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40), sample tubes were kept on ice for 30-40 min with periodical gentle vortexing and subsequent debris removal. In some experiments BB buffer was supplemented with rigosertib (at 100 μM) or okadaic acid (at 100 nM) as prospective phosphatase inhibitors.
LysA Method (=BB buffer + inhibitors, non-denaturing): cell lysates were prepared under non-denaturing conditions using BB lysis buffer supplemented with inhibitors including complete protease inhibitors (Roche), 0.5 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4 in some experiments LysA buffer was supplemented with N-ethylmaleimide at 2.5-40 mM.
1xSB Method (denaturing): cell lysates were prepared under reducing/denaturing conditions using gel sample buffer [SDS-PAGE sample buffer Laemmli (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) supplemented with inhibitors as in LysA method above and DNase I]. Chromosomal DNA was sheared by 25 G needle.

Western blot (WB) analysis
Samples from lysates prepared by "BB" and "LysA" methods as well as sample lysates after *in vitro* dephosphorylation reaction (see below) were diluted 2:1 with 3x gel sample buffer (Laemmli), then boiled prior loading on the gel. Samples prepared by "1xSB" method were just boiled prior loading on the gel. Samples were typically resolved on 7% SDS-PAGE alongside pre-stained protein standards. Proteins were transferred onto PVDF membrane, blocked in 1xTBS/T/5 % dry milk for 1 h at room temperature, then probed overnight at +4°C with primary antibody, then coupled to a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were visualized using ECL Plus (GE Healthcare) and imaged on Blue X-ray film CL-XPosure (Pierce). Data in Figure 5 was obtained using IRDye Secondary antibodies according to manufacturer protocol (*LI-COR*). The signal was then detected and quantified by using *Odyssey* Infrared Imaging System (*LI-COR*). Probing for β-actin was used for normalization of bands fluorescence intensity.

In vitro Dephosphorylation with Lambda Protein Phosphatase (λ PPase)
The enzyme and buffer components were from New England BioLabs. Two Reaction mixes (Rx) containing the enzyme buffer, $MnCl_2$ and cell lysate made either with (Rx1) or without (Rx2) N-ethylmaleimide, were prepared and kept on ice, then aliquoted into Eppendorf microtubes and supplemented, where appropriate, with the λ PPase to final volume 50 μl. Tubes were incubated in 30°C water bath for 30 min, transferred to ice and immediately subjected to Western Blot analysis.

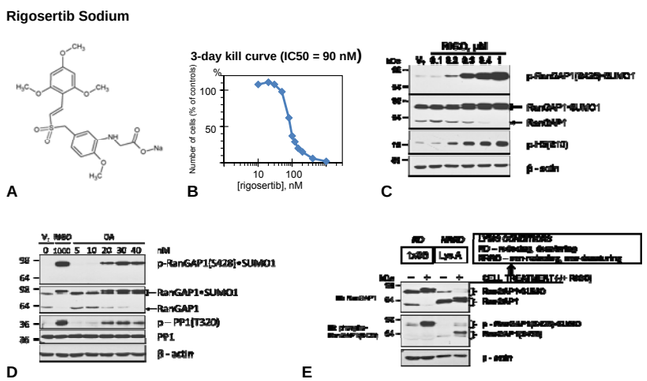


Figure 1. Introductory information on rigosertib and hyperphosphorylation of RanGAP1•SUMO1
A. Chemical structure. **B.** Rigosertib cytotoxicity assay in DU-145. **C.** Western Blot (WB) analysis of DU-145 cells exposed to rigosertib (RIGO) for 24h. RanGAP1•SUMO1 becomes hyperphosphorylated in a dose-dependent manner. Phospho-H3(S10) is a mitotic marker. **D.** Hyperphosphorylated RanGAP1•SUMO1 (HRGS) is also detected in cells treated with okadaic acid (OA), known PP1/PP2A phosphatase inhibitor. **E.** RanGAP1•SUMO1 is easily de-SUMOylated (SUMO-group is de-conjugated) under non-denaturing lysis conditions [Ref. 2].

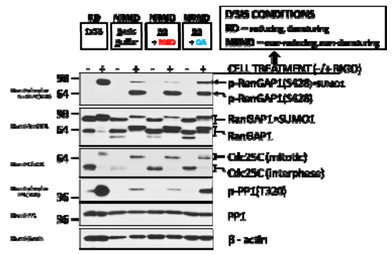


Figure 2. When the non-denaturing lysis buffer is utilized neither added rigosertib nor added okadaic acid protect hyperphosphorylated RanGAP1•SUMO1 against deSUMOylation
WB analysis. Cells were mock-treated or treated with 1 μM rigosertib for 24 h and processed, in pairs, for lysates under either reducing/denaturing conditions (method "1xSB") or under native conditions using Basic lysis Buffer alone or with additions of rigosertib (at 100 μM) or okadaic acid (at 100 nM) as prospective inhibitors (see Materials and Methods for details).
Mitotic (hyperphosphorylated) form of Cdc25C phosphatase was not affected by buffer conditions. In opposite, PP1, another important mitotic phosphatase, becomes dephosphorylated at T320 under non-denaturing lysis conditions with only slight opposing effect from okadaic acid. Cdk1/cyclin B kinase is known to inhibit PP1 by phosphorylating T320 before entry into mitosis.

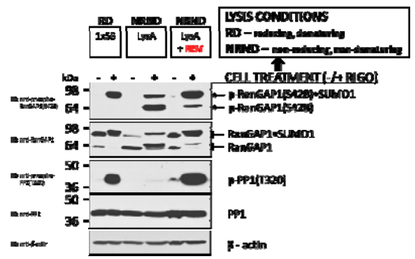


Figure 3. N-ethylmaleimide (NEM), added to non-denaturing lysis buffer, inhibits deSUMOylation of HRGS and keeps PP1 in T320-phosphorylated (inhibited) form
WB analysis. Cells were mock-treated or treated with 1 μM rigosertib for 24 h and processed, in pairs, for lysates under either reducing/denaturing conditions (method "1xSB") or under native conditions using LysA buffer alone or with added N-ethylmaleimide (at 20 mM) .

NOTA BENE: Dephosphorylation of PP1 phosphatase at the inhibitory Thr320 (and thus potential reactivation of PP1) did not affect phosphorylation status of RanGAP1 in the lysate.

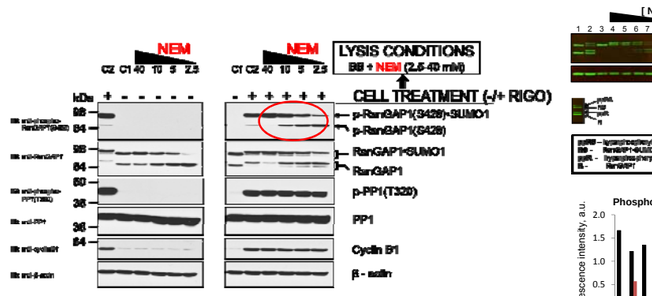
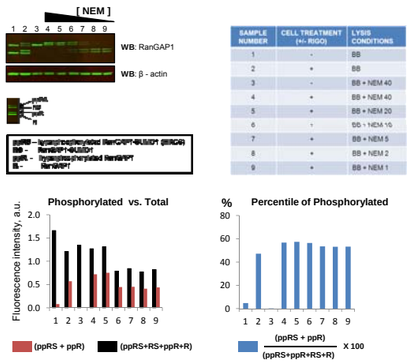


Figure 4. NEM–dependent dose response of HRGS deSUMOylation in cell lysates under non-denaturing conditions
WB analysis. Cells were mock-treated or treated with 1 μM rigosertib for 24 h, collected, washed, divided into equal portions within the corresponding cell pool, and processed for lysates using Basic lysis Buffer (BB) supplemented with 2.5 - 40 mM N-ethylmaleimide (NEM) as a sole inhibitor.
Circled in red: 40 mM NEM completely prevented deSUMOylation of HRGS in the lysate under the non-denaturing conditions. Gradual loss of SUMO from HRGS happens upon decreasing NEM concentration. Yet, there is no visible loss of phosphate.
In lysates derived from RIGO-treated cells, PP1 phosphatase remained in its inhibited, Thr320–phospho-rylated, form under all conditions tested.
C1, C2 – control lysates to provide baselines, made in LysA + 20 mM NEM from mock-treated cells and RIGO-treated cells respectively.



SAMPLE NUMBER	CELL TREATMENT (+/- RIGO)	LYSIS CONDITIONS
1	-	BB
2	+	BB
3	-	BB + NEM 40
4	+	BB + NEM 40
5	-	BB + NEM 20
6	+	BB + NEM 20
7	-	BB + NEM 5
8	+	BB + NEM 2
9	+	BB + NEM 1

Figure 5. Quantification of RanGAP1 protein forms under various lysis conditions reveals stability of its phosphorylation status
N-ethylmaleimide (NEM) was the only inhibitor added to the Basic lysis Buffer (BB, numbers in table are NEM concentration in mM). Samples were obtained in a replica experiment as in Figure 4. Quantitative WB analysis was performed as detailed in Materials and Methods. Fluorescence intensity of all RanGAP1 forms was normalized to that of corresponding β-actin.

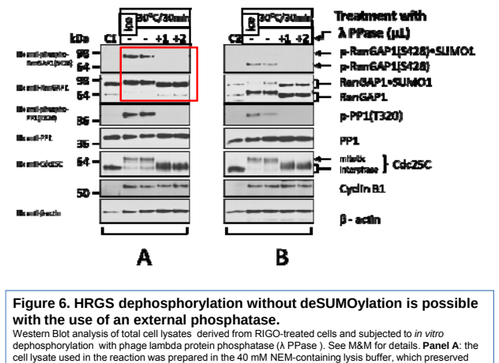


Figure 6. HRGS dephosphorylation without deSUMOylation is possible with the use of an external phosphatase.
Western Blot analysis of total cell lysates derived from RIGO-treated cells and subjected to *in vitro* dephosphorylation with phage lambda protein phosphatase (λ PPase). See M&M for details. **Panel A**: the cell lysate used in the reaction was prepared in the 40 mM NEM-containing lysis buffer, which preserved HRGS. **Panel B:** the cell lysate used in the reaction was prepared in the absence of NEM, therefore besides HRGS there was also present deSUMOylated, yet hyperphosphorylated, RanGAP1 (HRG).
Both hyperphosphorylated RanGAP1 forms , HRGS and HRG, were dephosphorylated by λ PPase.
Successful dephosphorylation (last two lanes in each panel) is manifested as an increase in protein mobility and down-shift of the protein band to the level of non-phosphorylated counterpart in lanes C1 or C2.
PP1 lost its inhibitory phosphate at Thr320 upon addition of λ PPase.
Mitotic form of Cdc25C was also dephosphorylated by λ PPase and converted into faster moving protein species.
C1, C2 – control lysates, prepared from mock-treated cells , to provide reference, i.e. non-phosphorylated version of corresponding protein. 1 μl of λ PPase = 400 activity units.

Summary

1) **Exposure of tumor cells to anticancer inhibitor rigosertib results in cell cycle arrest at mitosis, which is correlated with expression of Hyperphosphorylated RanGAP1•SUMO1 (HRGS). Previously, we suggested inhibition of a putative HRGS phosphatase as the mechanism of action.**
2) **HRGS becomes easily deSUMOylated under non-denaturing lysis conditions [making it difficult to study this protein *in vitro*].**
3) **N-ethylmaleimide (NEM), added to non-denaturing lysis buffer, inhibits deSUMOylation of HRGS in a dose – dependent manner.**
4) **RanGAP's acquired hyperphosphorylation is stable under various lysis conditions, regardless of its SUMOylation status.**
5) **HRGS dephosphorylation without deSUMOylation is possible with the use of an external phosphatase. This suggests an *in vitro* test system to identify the responsible enzyme that might be the target of inhibition by rigosertib.**

References:

1. Prasad, A., Park, I. W., Allen, H., Zhang, X., Reddy, M. V. R., Boominathan, R., Reddy, E. P., and Groopman, J. E. (2009). Styryl sulfonyl compounds inhibit translation of cyclin D1 in mantle cell lymphoma cells. Oncogene *28*, 1518-1528.
2. Oussenko, I. A., Holland, J. F., Reddy, E. P., and Ohnuma, T. (2011). Effect of ON 01910.Na, an Anticancer Mitotic Inhibitor, on Cell-Cycle Progression Correlates with RanGAP1 Hyperphosphorylation. Cancer Res *71*, 4968-4976.
3. Oussenko, I. A., Holland, J. F., Reddy, E. P., and Ohnuma, T. RanGAP1•SUMO1 hyperphosphorylation (RGSH) and mitotic arrest by ON 01910.Na, okadaic acid or tubulin agents [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 2639

Acknowledgements:

This work was supported in part by T.J. Martell Foundation for Cancer, Leukemia and AIDS Research, New York, NY; the Myra Shaw Cancer Research Fund, Brooklyn, NY and by Onconova Therapeutics, Inc., Newtown, PA.