Abstract # 2639

RanGAP1•SUMO1 hyperphosphorylation (RGSH) and mitotic arrest by ON 01910.Na, okadaic acid or tubulin agents

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Introduction

ON 01910.Na (Estybon™)

❑ ON 01910.Na (Figure 1) is a potent anticancer antimitotic agent currently undergoing Phase I and II clinical studies

❑ Biological action is characterized by spindle abnormalities, selective G2/M-arrest and apoptosis [1]

❑ 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 [2]

❑ Previous work from our laboratory showed that ON 01910.Na (abbreviated 1910) induced hyperphosphorylation of RanGAP1•SUMO1 (abbreviated RGSH) in all tumor cell lines tested. RGSH was not observed in the presence of 1911, inactive analog of 1910. However, RGSH was observed in the presence of tubulin depolymerizing agent nocodazole. Tubulin polymerization assay revealed that, unlike nocodazole, neither 1910 nor 1911 had effects on tubulin polymerization *in vitro* [submitted for publication]

❑ In this study, we examined whether other tubulin agents induce RGSH and whether known mitotic phosphatases are involved

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.

❑ RanGAP1•SUMO1 is transiently phosphorylated during mitosis. Upon treatment with Nocodazole it is converted quantitatively into the phosphorylated form [3].

Materials and Methods

Cell lines, Drug Treatment and Sample preparation

DU-145 prostate carcinoma and MOLT-3 ALL, maintained in RPMI1640 medium, were used. Medium was supplemented with 10% FBS and antibiotics (penicillin/streptomycin). Cells were seeded 24 h prior addition of drugs. Following drug exposure all cells were harvested, washed, and total cell lysates prepared by resuspending cells in reducing/denaturing 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 including complete protease inhibitors (Roche), 0.5 mM PMSF, 1 mM NaF, 1 mM Na3VO4 and DNase I]. Samples were passed 10 times through 25 G needle to shear chromosomal DNA.

Western Blot (WB) analysis

Samples of total cell lysates were 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: anti-RanGAP1 antibody, anti-Lamin B, anti-β-actin (Santa Cruz Biotechnology); anti-phospho-Cdc25C(T48), anti-Cdc25C (Cell Signaling Technology); anti-phospho-RanGAP1(S428) antibody (Sigma-Aldrich), 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).

Cytotoxicity Assay

Cell growth inhibition effect of the drugs ($2x10^3$ cells/well in quintuplicates in 96-well plate) was evaluated by the sulforhodamine B (SRB) assay. 24 h after cells were seeded graded concentrations of drugs were added and cells were incubated further for 3 days. After drug exposure, cells were fixed with 50% TCA at 4°C for 1 h, washed and stained with staining solution (0.4% sulforhodamine B dissolved in 1% acetic acid). Subsequently unbound dye was removed by washing with 1% acetic acid. The colorimetric endpoint, which correlates with cell density, was determined by measuring the absorbance at 540 nm on a microplate reader.

Cell cycle analysis

Following drug exposure cells were collected, fixed in 70% ethanol, stained with propidium iodide and analyzed by FACScan. Data for 20,000 events per sample were collected and analyzed using "CellQuest" software (Becton-Dickenson). To calculate percentage of cells in each cell phase from fluorescence histograms, we selected data from one sample in each treatment series (where picks corresponding to 2N and 4N DNA content, were clearly defined), and then positioned the gates over subG1, G1, S and G2/M areas of the histogram. Thus chosen, statistical gates were applied to the rest of samples in the corresponding series. SubG1 fraction was assumed to represent apoptotic cells.

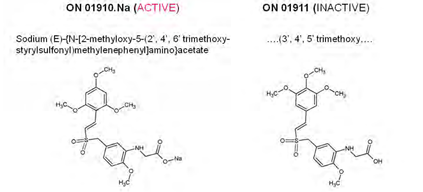


Figure 1. Chemical structures

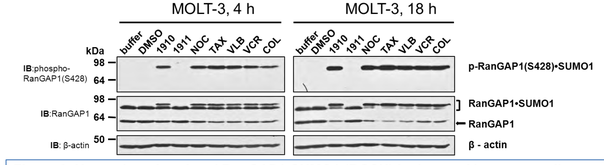
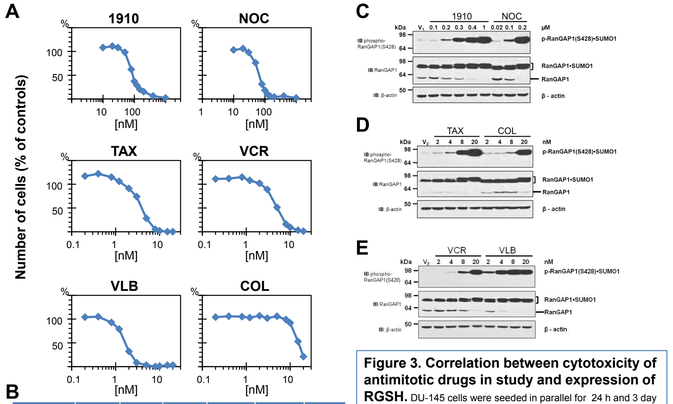


Figure 2. Hyperphosphorylated RanGAP1•SUMO1 (RGSH) is detected in cancer cells treated with 1910 or selected tubulin agents.
WB analysis of MOLT-3 cells exposed to 1910, 1911, nocodazole (NOC), paclitaxel (TAX), vinblastine (VLB), vincristine (VCR), colchicine (COL), each at 1 μM, or drug vehicle controls, for 4 h or 18 h. Expression of RGSH was confirmed in two ways: by probing with anti-phospho-RanGAP1(S428) antibody to detect specific phospho-protein signal and by probing with anti-RanGAP1 antibody (for total RanGAP1) to demonstrate RanGAP1•SUMO1 protein band up-shift that signifies presence of hyperphosphorylated RanGAP1•SUMO1, as we found previously. Drug vehicles and 1911, inactive analog of 1910, as expected, did not cause RGSH. All selected tubulin agents, known for their ability to bind tubulin, disrupt microtubule network [either by stabilization (paclitaxel) or depolymerization (other four)] and arrest cells in mitosis, caused expression of RGSH. Doxorubicin and camptothecin , both non-antimitotic agents, gave negative results (not shown).



	1910	NOC	TAX	VCR	VLB	COL
IC50, nM	90	60	4	5	1.5	15
IC90, nM	300	120	7	10	3	30*

*number taken from a different experiment

Figure 3. Correlation between cytotoxicity of antimitotic drugs in study and expression of RGSH. DU-145 cells were seeded in parallel for 24 h and 3 day drug exposure; then, in former, cells were collected and analyzed by WB (panels **C**,**D**,**E**) and, in latter, cell growth was evaluated in cytotoxicity assay (panel **A**,**B**, see M&M for details on the assay). IC90 of a drug correlated with minimal concentration of that drug sufficient to trigger expression of RGSH in 24 h. Abbreviations used: V_1 and V_2 ,buffer and DMSO correspondingly.

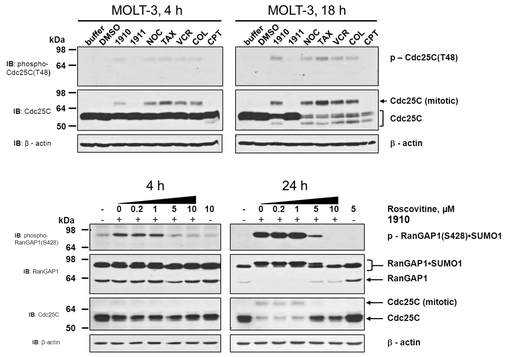
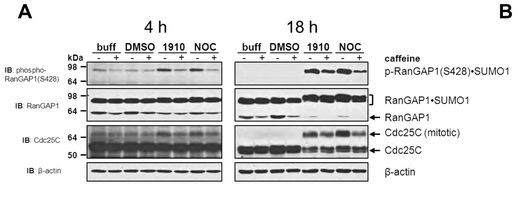


Figure 4. Extensively phosphorylated, mitotic form of Cdc25C is detected in cells treated with 1910 or selected tubulin agents.
WB analysis of MOLT-3 cells treated with 1910, inactive analog 1911, selected tubulin agents (NOC, TAX, VCR and COL) or camptothecin (used as negative control) for 4 h and 18 h. All drugs were used at 1 μM.
Cdc25C is a protein phosphatase responsible for dephosphorylating and activating cdc2, a crucial step in regulating the entry of all eukaryotic cells into mitosis. Full activation of cdc25C involves phosphorylation at more than 12 different sites including Thr48.

Figure 5. Inhibition of RGSH and mitotic Cdc25C in the presence of roscovitine
WB analysis of DU-145 cells treated with 1910 at 1 μM and increasing doses of roscovitine for 4 h and 24 h. Roscovitine is a purine analog that is a potent and selective inhibitor of cyclin-dependent kinases (cdk). In particular, it is a competitive inhibitor of Cdk1 (cdc2/cyclin B), which controls the entry of eukaryotic cells into mitosis. CDK1 is one of phosphorylating kinases for Cdc25C and RanGAP1.



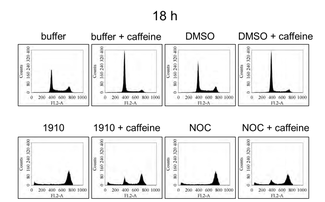


Figure 6. Inhibition of RGSH and mitotic Cdc25C in the presence of caffeine
WB analysis (**A**) and cell cycle distribution analysis (**B**) of DU-145 cells treated with 2.5 mM caffeine for two hours prior to addition of 1910 or nocodazole (both at 1 μM) and further incubation for 4 h or 18 h, Caffeine is known to non-specifically affect multiple cell targets, for example ATM and ATR kinases. In this experiment inhibition of RGSH and mitotic Cdc25C by caffeine is indirect due to arrest in G1 and inability of cells to enter mitosis. Abbreviations used: **B**, FL2-A (all X-axes) = fluorescence, arbitrary units, linear scale; COUNTS (all Y-axes) = cell count, linear scale

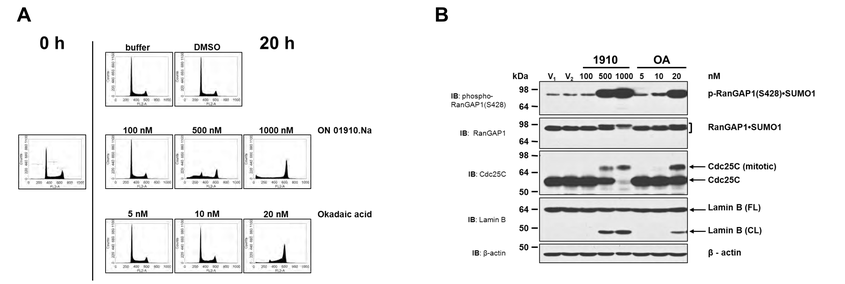


Figure 7. Okadaic acid, known phosphatase PP1 inhibitor, causes mitotic arrest, expression of RGSH and apoptosis similar to ON 01910.Na. Cell cycle distribution analysis (**A**) and WB analysis (**B**) of MOLT-3 cells treated with various concentrations of 1910 or okadaic acid (OA) for 20 h. Both 1910 and OA induced G2/M arrest, RanGAP1•SUMO1 hyperphosphorylation, induction of mitotic Cdc25C and cleavage of Lamin B (indication of apoptosis). Similar results were obtained with DU-145 (not shown). Abbreviations used: **B**, (FL) full length, (CL) cleaved

Results and Discussion

1) **Exposure of tumor cells to anticancer mitotic inhibitor, ON 01910.Na, resulted in cell cycle arrest at mitosis, that is correlated with RGSH**
2) **All tubulin agents tested, both tubulin polymerization enhancers and depolymerizing agents were able to produce RGSH**
3) **Cytotoxic drug concentrations of antimitotic drugs were correlated with those of RGSH expression**
4) **Extensively phosphorylated, mitotic form of Cdc25C is detected in cells treated with ON 01910.Na or all tubulin agents studied.**
5) **Roscovitine and caffeine inhibited RGSH and mitotic Cdc25C**
6) **Okadaic acid, known PP1/PP2A inhibitor, caused mitotic arrest, expression of RGSH and apoptosis similar to ON 01910.Na.**

These findings suggest that ON 01910.Na is a RGSH phosphatase inhibitor.

References

[1] Gumireddy K, Reddy MV, Cosenza SC, Boominathan R, Baker SJ, Papathi N, Jiang J, Holland J, Reddy EP (2005) ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. Cancer Cell 7(3):275-86.

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