Effect of ON 01910.Na, an anticancer mitotic inhibitor, on cell cycle progression correlates with RanGAP1 hyperphosphorylation

Irina A Oussenko¹, James F. Holland¹, E. Premkumar Reddy², and Takao Ohnuma¹.

Running Title: ON 01910.Na and M-phase arrest

Key Words: ON 01910.Na, G2/M arrest, RanGAP1, mechanism of action

Authors’ Affiliation: ¹Division of Hematology and Medical Oncology, The Samuel Bronfman Department of Medicine and Tisch Cancer Institute, The Mount Sinai School of Medicine, New York, NY; ²Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA.

Current address for E. Premkumar Reddy: Department of Structural and Chemical Biology, The Mount Sinai School of Medicine, New York, NY.

Corresponding author: Takao Ohnuma, M.D., Ph.D., Division of Hematology and Medical Oncology, Department of Medicine, and Tisch Cancer Institute, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029: takao.ohnuma@mssm.edu.
Abstract

The benzyl styryl sulfone, ON 01910.Na, is a novel anticancer agent that inhibits mitotic progression and induces apoptosis in most cancer cell lines. We examined ON 01910.Na effect on DNA damage signaling molecules upstream of Cdc25C, (Chk1, Chk2, and H2AX), as well as on RanGAP1·SUMO1, a mitosis coordinator. Prostate cancer, lymphoma and leukemic cells were incubated with drug for 4, 16 or 24 h. Cell lysates were resolved on SDS-PAGE and analyzed by Western blot. Camptothecin and doxorubicin treatment caused activation/phosphorylation of DNA damage-responsive molecules by 4 h, whereas ON 01910.Na did not. ON 01910.Na caused hyperphosphorylation of RanGAP1·SUMO1 within 4 h, which was sustained for more than 24 h. Mild phosphorylation of Chk2 was observed only after 24 h exposure, indicating that DNA damage response was not an initial effect of ON 01910.Na. MOLT-3 cells, synchronized by double thymidine block, when released into medium containing ON 01910.Na, accumulated mitotic cells at the peak of 10-14 h and remained near plateau for 20 h, which corresponded with the time of RanGAP phosphorylation. ON 01910.Na had minimal effects on tubulin polymerization. These findings imply that ON 01910.Na does not induce DNA damage directly nor act as a tubulin toxin. Its biological activity appears to rely on prolonged phosphorylation/hyperphosphorylation of RanGAP1·SUMO1. M-phase arrest and consequent induction of apoptosis follows possibly because of it. ON 01910.Na may act as an inhibitor of a RanGAP1·SUMO1 phosphatase or a stimulant of a new kinase. RanGAP1·SUMO1 appears to be a new target pathway for cancer chemotherapy.

Precis: Incubation of human tumor cell lines with ON 01910.Na, an anticancer mitotic inhibitor, resulted in prolonged phosphorylation and hyperphosphorylation of RanGAP1•SUMO1. It is possible that M phase arrest and apoptosis observed by the compound is related to this phenomenon.
Introduction:

ON 01910.Na (Figure 1) is a novel benzyl styryl sulfone, which showed cytotoxic activity against a variety of human tumor cell lines in vitro and growth inhibitory activity in xenografts in nude mice (1). The compound is currently in Phase 1 and II clinical trials (2-5). While the compound was originally considered to be a novel Plk1 inhibitor (1), a direct effect on Plk1 could not be confirmed in subsequent studies. Exact mechanism of action of ON 01910.Na, thus, remains unknown. Available data show that the drug produces 3 major abnormalities in tumor cells: (a) abnormal cell division including irregular chromosomal segregation and cytokinesis; (b) G2/M arrest and apoptosis in many tumor cell lines (in contrast, normal fibroblasts were arrested in G1 phase); and (c) decreased expression of Cdc25C (1).

In normal cells Cdc25C is phosphorylated by Plk1. Phosphorylated Cdc25C is a phosphatase, which catalyzes Cdc2 (=Cdk1) into its unphosphorylated state or active form, resulting in cell cycle progression from G2 to mitosis. ON 01910.Na decreases Cdc25C, which results in Cdc2 remaining in a phosphorylated (inactive) state. Consequently, cells fail to traverse mitosis and become blocked in the G2/M phase of the cell cycle. Since cells cannot be arrested in mitosis indefinitely, they are forced to activate apoptotic pathways.

During the initial characterization study we found that ON 01910.Na was not cytotoxic or inhibitory to yeast cells (Oussenko and Ohnuma, unpublished observation). In cross-resistance studies we found that many drug resistant cells were not cross-resistant to ON 01910.Na. Minor cross-resistance to ON 01910.Na was seen in cisplatin- and flavopiridol- resistant cells only. We attempted to develop ON 01910.Na-resistant cells by incubating tumor cells with increasing concentrations of ON 01910.Na in culture medium, but failed after 2 yr of attempts (6). These observations suggested that ON 01910.Na did not have direct cytocidal effects. Cell death might have occurred simply because cells cannot stay in G2/M phase indefinitely. Thus, G2/M arrest could be the primary lethal effects of ON 01910.Na.

In search of a mechanism of action we started with a working hypothesis that ON 01910.Na acts, as do many cytotoxic anticancer agents, on DNA damage response signal pathways (with emphasis on events upstream of Cdc25C). Niida et al (7)
provided a schematic molecular organization of DNA damage checkpoints throughout the cell cycle. This scheme identified target molecules for ON 01910.Na upstream of Cdc25C. One known upstream effector molecule is Plk1. Alternatively, ON 01910.Na might have activated upstream inhibitor molecules Plk3, Chk1 or Chk2. Plk3 is inhibited by ATM. Chk1 is activated by ATR. Chk2 is activated by ATM or DNAPK (8).

To our list of targets, DNA damage response signals (Chk1, Chk2 and H2A.X), we also added RanGAP1 (Ran GTPase-activating protein 1)(9-12). Ran is a Ras related GTPase that is required for nuclear transport, cell cycle control, mitotic spindle formation and post-mitotic nuclear re-assembly. In many instances RanGAP1 accomplishes its functions after covalently binding with SUMO1 (small ubiquitin related modifier 1) or SUMOylation (9-12). We termed RanGAP1•SUMO1 as mitosis coordinator. Because many tubulin toxins are effective anticancer agents, we also examined the effects of ON 01910.Na on tubulin polymerization in vitro.

**Materials and Methods**

**Anticancer and other agents** ON 01910.Na and its inactive analog, ON 01911, were provided by Onconova Therapeutics, Inc. (Newtown, PA). ON 01910.Na is a water soluble white solid. The material was dissolved at 10 mM in 500 mM NaHCO₃ buffer made in PBS (pH 7.4) and further diluted in distilled water to prepare a stock solution of 1 mM and was kept at -20°C. Its inactive isomer, ON 01911, was dissolved in DMSO at 1 mM concentration and was kept at -20°C. Doxorubicin, camptothecin and nocodazole were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell lines** MOLT-3 ALL (13) was maintained in our laboratory. DU-145 prostate cancer and U937 lymphoma cells were purchased from American Type Culture Collection (Rockville, MD). All cell lines were maintained in RPMI-1640 medium (Gibco-Invitrogen) supplemented with 10% FBS and antibiotics (penicillin and streptomycin). These cells were tested at Johns Hopkins Genetic Resources Core Facility, Baltimore, Md, by obtaining short tandem repeat (STR) profiles and authenticated by comparing them to the ATCC human cell line collection (in case of DU-145 and U937) or our own reference stocks (in case of MOLT-3).
Drug treatment, sample collection and preparation of total cell lysates

Exponentially growing cells were seeded at 200,000 cells/ml (U937, MOLT-3) or 20,000 cells/cm² (DU145) cell density and cultured for 24 h. Then, drugs and control vehicles were added appropriately, and cells were further cultured for designated time periods. Afterwards, suspension cells were collected by centrifugation, washed with ice-cold 1xPBS and spun again to obtain cell pellet. In case of cells in monolayer culture medium was aspirated and cells were carefully rinsed with ice-cold 1xPBS. Cells were then scraped in 1 ml of 1xPBS into Eppendorf tube and cell pellets were made by centrifugation at 600 g for four minutes at +4°C. Total cell lysates were prepared either by resuspending cells directly in reducing/denaturing SDS-PAGE sample buffer ("1xSB" method) or by lysing cells under non-denaturing conditions ("Lys A" method). Further details for "1xSB" method were as follows: cell pellets were resuspended proportionately to their wet weight in 0.6-0.9 ml of gel sample buffer (1xSB according to Laemmli (14): 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) supplemented with inhibitors (complete protease inhibitors (Roche), 1 mM NaF, 1 mM Na₃VO₄, 0.5 mM PMSF) and RNase-free DNase I (Roche) (1.5 unit per ml of the sample buffer); then chromosomal DNA was sheared by passing samples through 25 G needle 10 times up and down, and samples were stored at -80°C. Further details for "Lys A" method were as follows: cells were resuspended in 200-400 ul of lysis buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, supplemented with complete protease inhibitors), adapted from Agarwal et al (15), kept on ice for 30-40 min, during which time samples were vortexed gently every 10 min, then centrifuged to remove cell debris at 13,500 g for 30 min at +4°C, and the supernatants ("lysates") were transferred to fresh tubes and stored at -80°C. Protein content was determined by BCA method using kit from Pierce (Thermo Fisher).

Western Blot analysis and antibodies used

Western Blot analyses were performed in a standard manner (16). Shortly before loading on a gel, cell lysates of type “1xSB” were simply defrosted, while protein samples from cell lysates type “Lys A” were prepared by mixing 20 µl of cell lysate containing 20 µg of protein with 10 µl of 3xSB buffer by Laemmli; then both sample types were boiled for 5 min in water bath,
cooled on ice and microcentrifuged for 5 min. Samples were resolved on 7% (unless otherwise stated) SDS-PAGE alongside pre-stained protein standards (SeaBluePlus, Invitrogen, Carlsberg, CA). Using mini-Genie electro-blotting device (Idea Scientific, Minneapolis, MN) proteins were transferred from the gel onto PVDF membrane (Bio-Rad, Hercules, CA) for 1.5 - 2 h at 12 V in Towbin buffer supplemented with 0.2% SDS. PVDF membrane was pre-wetted in methanol, soaked in water for 5 min and equilibrated in the transfer buffer for 20 min prior to the electro-blotting procedure. After the transfer, membranes were blocked in 1xTTBS/5 % dry milk for 1 h at room temperature, then probed overnight at + 4ºC with chosen primary antibody: anti-RanGAP1, anti-Lamin B, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-SUMO-1, anti-phospho-Chk1 (S296), anti-phospho-Chk2 (T68), anti-Chk1, anti-Chk2, anti-phospho-Histone H2A.X (S139), anti-α-tubulin (Cell Signaling Technology); or anti-phospho-RanGAP1 (S428) antibody (Sigma-Aldrich, St. Louis, MO); followed by routine washing and then coupling to a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse and anti-goat antibodies were from Santa Cruz Biotechnology, and anti-rabbit antibody was from Alpha Diagnostic International (San Antonio, TX). Probed proteins were visualized using ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ) and the images were developed on Blue X-ray film CL-XPosure (Pierce, Rockford, IL).

**Cell synchronization** Double thymidine exposure technique was used (17, 18). Exponentially growing MOLT-3 cells were washed and seeded at 500,000 cells/mL density in RPMI-1640 medium containing 10% FBS and 2 mM thymidine (Sigma-Aldrich) and incubated 12 h at 37ºC. After the incubation, thymidine containing medium was removed, and cells and the flasks were rinsed with pre-warmed RPMI-1640 medium. Cells were re-plated in RPMI-1640 containing 10% FBS and incubated 16 h, after which cells were washed with RPMI-1640 and re-plated in RPMI-1640 containing 10% FBS and 2 mM thymidine for 12 h at 37ºC. After double thymidine exposure, cells were washed free of thymidine, divided into 3 portions and seeded in 100 mm plates for continued incubation in either regular medium (a.k.a. “release medium”), release medium supplemented with 1 µM of ON 01910.Na (final concentration) or supplemented with 1 µM of nocodazole (final concentration). At timed intervals plates of each type
were withdrawn, and cells were subjected to: (a) cell cycle distribution analysis, and (b) Western Blot analysis for expression of RanGAP1, phospho-RanGAP1 and Lamin B.

**Cell cycle distribution analysis by Flow Cytometry.** MOLT-3 cells from the synchronization experiment (see above) were collected by centrifugation, fixed in 70% ethanol, washed, resuspended in 1xPBS, treated with RNase A (DNase free) for 30 min at 37°C, mixed 1:5 with 25 µg/ml propidium iodide solution in 1xPBS, filtered through Falcon mesh caps to remove cell aggregates 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 on obtained 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.

In replicate synchronization experiment percentile of mitotic cells was determined simultaneously with cell cycle distribution analysis by double-staining fixed cells for phospho(Ser10)-histone H3 (marker of mitosis) as well as for DNA content with propidium iodide using protocol adapted from Xu et al (19). In short, after fixation the cells were resuspended in 1 ml of 1xPBS containing 0.25% Triton X-100 and incubated on ice for 15 min. After centrifuge the cell pellet was resuspended in 100 µl 1xPBS/1%BSA containing anti-pS^{10}H3 antibody at 1:30 dilution followed by incubation for 2 h at room temperature. Then cells were centrifuged, unbound antibody was washed off with 1 ml of 1xPBS/1%BSA, and cells were incubated in 100 µl 1xPBS/1%BSA containing FITC-conjugated secondary antibody at 1:30 dilution for 30 min in the dark. Subsequently cells were washed with 1 ml 1xPBS/1%BSA to remove the unbound antibody, resuspended in 1 ml of 1xPBS containing 25 µg/ml propidium iodide and 250 µg/ml RNaseA and incubated for at least 30 min at room temperature in the dark. Samples were filtered through Falcon mesh caps just before running them on FACScan. Proper gating and fluorescence compensation were performed on samples stained singly with either antibodies or propidium iodide. Anti-pS^{10}H3 antibody (primary antibody) was from Millipore, goat anti-rabbit IgG FITC conjugate (secondary antibody) was from Jackson ImmunoResearch Laboratories.
**Tubulin polymerization assay** We used fluorescence-based Tubulin Polymerization Assay Kit from Cytoskeleton (catalog # BK011P, Denver, CO) and followed manufacturer's protocol. The kit-based assay is an adaptation of an assay originally described by Bonne et al. (20), where polymerization is followed by fluorescence enhancement due to the incorporation of a fluorescent reporter into microtubules as polymerization occurs. Briefly, test compounds and their corresponding vehicles were pipetted into a 96-well microtiter plate, then tubulin reaction mix was quickly added to the wells, and the tubulin polymerization was initiated by transferring the plate to +37°C chamber of a plate reader. Fluorescence emission was measured at 425-445 nm (excitation wavelength was 340-360 nm) using Safire 2 Plate Reader (Tecan, Switzerland). Kinetics of changes in fluorescence was measured every minute for 60 min. Tubulin reaction mix was composed of 80 mM PIPES pH 6.9, 2.0 mM MgCl₂, 0.5 mM EGTA, 1.0 mM GTP, zero to 20 % glycerol and 2 mg/ml of highly purified porcine brain tubulin heterodimer. Three glycerol concentrations (10, 15 and 20%) were tested.

**Results**

**ON 01910.Na induced upshifting of RanGAP1•SUMO1** Western blot analyses of all 3 cell lines after various exposure times and drug concentrations are shown in Figure 2. In Panel 2A, DU-145 or U937 cell protein samples were resolved on 7% SDS-PAGE, transferred onto PVDF membrane and probed with anti-SUMO1 and anti-RanGAP1 antibodies. We found upshift of a protein band detected with anti-SUMO1 antibody. The reason for upshift was not determined at this point and new bands were tentatively named X•SUMO1, Y•SUMO1 and Z•SUMO1. SUMO-modified protein “Y” (Y•SUMO-1) appeared to be a dose-dependent upshift of SUMO-modified protein Z. Protein Z being RanGAP1•SUMO1 was confirmed by stripping the same blot and re-probing with anti-RanGAP1 antibody. Similar dose-dependent upshift of RanGAP1•SUMO1 was observed after treatment with ON 01910.Na (middle blot). In Panel 2B RanGAP1•SUMO1 was probed with anti-phospho-RanGAP1, showing that the upshift was due to phosphorylation of RanGAP1•SUMO1. In Panel 2C samples were
Ohnuma 9

blotted with antibody for shorter or longer exposure periods. At 2 h, dose response relationship was not clear, but a small upshifted band was already seen in RanGAP1•SUMO1. By 4 h clear upshift of RanGAP1•SUMO1 was evident even after short exposure. All 3 cell lines gave essentially identical results. Free RanGAP1 (62 kDa) was detected by this antibody as expected, but it appeared as a doublet in our early experiments (panel A as an example). It was later found to be largely due to loss of SUMO protein group from phospho-RanGAP1•SUMO1 when total cell lysates were prepared by “Lys A” method (see next section and Figure 3). After we changed our lysis method (panels B and C) free RanGAP1 was detected as a single band, even though phospho-RanGAP1•SUMO1 was also present.

Phosphorylation and hyperphosphorylation of RanGAP1•SUMO1 and not of free RanGAP1 is revealed when adequate lysis method is used. We compared two procedures for the preparation of total cell lysates (Figure 3). In Panel 3A DU-145 cells were treated with ON 01910.Na for 16 h and processed for total cell lysates in two ways, “1xSB” and “Lys A”. Method “1xSB” achieved better preservation of SUMOylated RanGAP1 (both, phosphorylated and non-phosphorylated), while “Lys A” resulted in substantial deSUMOylation yielding larger amounts of free RanGAP1 and otherwise undetectable phospho-RanGAP1. Observed deSUMOylation could be attributed to the presence of inadequate protease inhibitors in the non-denaturing lysis buffer in “Lys A” method. We routinely included standard protease inhibitor cocktail, which, according to the manufacturer (Roche) protects against serine-, cysteine- and metalloproteases; however, SUMO proteases represent a distinct class of proteases and require specific inhibitors like iodoacetamide or N-ethyl maleimide (21). In method “1xSB” cell lysis takes place under denaturing conditions thus preventing action from any type of proteases. We utilized “1xSB” method of preparing cell lysates throughout the rest of the experiments. However, aforementioned deSUMOylation of samples allowed us to distinguish at least two phosphorylation states of RanGAP1•SUMO1. Thus, samples in 3A prepared by “Lys A” method, resolved on 6% SDS-PAGE gel and probed identically (Panel 3B the inset oval) showed separation of three bands:
RanGAP1•SUMO1, phosphorylated RanGAP1•SUMO1 and hyperphosphorylated RanGAP1•SUMO1.

Phosphorylation of RanGAP1•SUMO1 was the initial event in the presence of ON 01910.Na, whereas phosphorylation of DNA damage response signaling molecules was the initial event of doxorubicin and camptothecin. The different kinetics of phosphorylation of DNA damage response molecules and the RanGAP1•SUMO1 mitosis coordinator are illustrated in Figure 4. By 4 h camptothecin and doxorubicin have already activated Chk1, Chk2 and Histone H2A.X, whereas ON 01910.Na and nocodazole did not. In contrast, ON 01910.Na and nocodazole produced phosphorylation of RanGAP1•SUMO1 by 4 h. By 24 h RanGAP1•SUMO1 continued being activated. Camptothecin or doxorubicin-induced activation of Chk1 and Chk2 has subsided by then with a concomitant reduction of Histone H2A.X phosphorylation. ON 01910.Na induced delayed and mild activation of Chk2, suppression of Chk1 and Chk1 phosphate, and strong activation of Histone H2A.X. Phosphorylation of Ser-139 residue of the histone variant H2A.X, forming gammaH2A.X, is an early cellular response to the induction of DNA double-strand breaks. Detection of this phosphorylation event has emerged as a highly specific and sensitive molecular marker for monitoring DNA damage initiation and resolution (22).

Phosphorylated histone H3 at serine 10, specific for mitotic cells, was detected in the presence of ON 01910.Na or nocodazole at 4 h and at 24 h indicative of mitotic cell arrest. In contrast, doxorubicin and camptothecin reduced mitotic cell fraction, consistent with known S phase cell kill.

These data show that early and prolonged phosphorylation of RanGAP1•SUMO1 has occurred after treatment with ON 01910.Na or nocodazole as an initial event, whereas, Chk2 and Histone H2A.X phosphorylation was a secondary phenomenon.

Camptothecin and doxorubicin produced no phosphorylation of RanGAP•SUMO1.

Synchronization of cell cycle confirmed ON 01910.Na– induced phosphorylation of RanGAP1•SUMO1 and mitotic cell arrest. Synchronization studies are illustrated in
Figure 5. MOLT-3 cells released into drug-free medium reached mitotic cell peak at 9-10 h and continued to cycle with synchronized transition into G1 at 15-16 h (Figure 5B). During this period, RanGAP1•SUMO1 was not phosphorylated, and no cleaved Lamin B was detected (Figure 5C). MOLT-3 cells, which were synchronized by double thymidine block and released into medium supplemented with ON01910.Na, resulted in peak accumulation of mitotic cells at 10-14 h. The G2/M cell fraction remained in near plateau for more than 20 h (Figure 5B). This time period correlated with the hyperphosphorylation of RanGAP1•SUMO1 (Figure 5D). Cleaved Lamin B detected by 16 h and thereafter in ON 01910.Na-containing release medium, confirmed an active apoptotic process during this period.

ON 01910.Na had no effects on tubulin polymerization Tubulin polymerization assay (Figure 6) revealed that both ON 01910.Na and control ON 1911 had only a minor inhibition or no effects, whereas nocodazole inhibited the process. Lack of effects of ON 01910.Na on tubulin polymerization is confirmatory of an earlier report (1).

Discussion

ON 01910.Na is a novel anticancer agent that inhibits mitotic progression and induces apoptosis in most cancer cell-lines. We examined whether ON 01910.Na activated DNA damage response proteins and found that ON 01910.Na induced no consistent activation or only delayed activation of proteins traditionally associated with this response. In contrast, both doxorubicin and camptothecin, used as controls, provided quick activation of Chk1, Chk2 and Histone H2A.X. We interpreted the delayed phosphorylation of Chk2 and Histone H2A.X, and suppression of Chk1 by ON 01910.Na and nocodazole as a secondary response. In contrast, we found that ON 01910.Na-induced hyperphosphorylation of RanGAP1•SUMO1 was correlated with accumulation of mitotic cells, prolonged M phase arrest and apoptotic cell death. Inactive analogue, ON 01911 was unable to induce phosphorylation of RanGAP1•SUMO1.

Based on these results, we conclude that ON 01910.Na is neither a DNA damage response inducer, nor a tubulin toxin, which are common mechanisms
associated with the action of many anticancer agents. ON 01910.Na's biological activity may at least in part rely on prolonged hyperphosphorylation of RanGAP1•SUMO1, which correlates with M phase arrest, leading physiologically to induction of apoptosis. Our observation is consistent with a report by Swaminathan et al (11), who showed that RanGAP1•SUMO1 was phosphorylated at the onset of mitosis and remained associated with RanBP2 (Ran-GTP-binding protein2) upon nuclear pore complex disassembly. Nocodazole-induced cell cycle arrest led to quantitative conversion of phosphorylated RanGAP1•SUMO1. We find these characteristics are shared with ON 01910.Na, although, we also found a striking difference between ON 01910.Na and nocodazole: nocodazole produced inhibition of tubulin polymerization, whereas, ON 01910.Na did not. Although ON 01910.Na did not have direct effect on microtubule polymerization in vitro, this does not exclude the possibility that it might indirectly affect polymerization in vivo.

It may be concluded that RanGAP1 hyperphosphorylation is one effect of ON 01910.Na that correlates with its effect on cell cycle progression. Whether there are any direct threads underlying the correlation of the effects of ON 01910.Na on cell cycle progression and hyperphosphorylation of RanGAP1•SUMO1 is unknown. It is possible that either a kinase or a phosphatase may be the target of the drug; however, further studies will be required to determine this. If the drug does target a kinase or a phosphatase, it is likely that RanGAP1 represents just one affected target. Further studies will also be required to determine whether RanGAP1 and/or other targets are functionally relevant to the observed cell cycle arrest.

Whether other agents which correlate with (hyper)phosphorylation of RanGAP1•SUMO1, per se, can be useful anticancer agents remains to be established. The impressive activity of ON 01910.Na in human cancer cell lines in vitro (1) and human tumor xenografts (23,24) and some clinical cancers (2,4,5) suggests that inhibition of dephosphorylation of RanGAP1•SUMO1-phosphate is a new molecular target for cancer chemotherapy.

Disclosure of Potential Conflicts of Interest
James F. Holland is a consultant to Onconova Therapeutics, Inc.; E. Premkumar Reddy is a consultant and stock-holder of Onconova Therapeutics, Inc.

Grant Support

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.

References


4  Sloand, EM, Olnes MJ, Galili M, et el. ON 01910.Na suppresses Cyclin D1 accumulation in trisomy 8 myelodysplastic syndromes patients while decreasing bone marrow CD34+ blast counts and aneuploid clone size. Blood (ASH Annual Meeting Abst 120), 2009; 114.


10 Dasso M. Emerging roles of the SUMO pathway in mitosis. Cell Division 2008;3:5-15


16 http://www.cellsignal.com/support/protocols/Western_BSA.html


18 Jachman J, O'Connor PM: Synchronizing cells at the onset of S-Phase by double


Figures legends

Figure 1. Chemical structures and IUPAC names of compounds used in the study. ON 01910.Na (active), Sodium (E)-2-(2-methoxy-5-((2,4,6-trimethoxy styrylsulfonyl)methyl)phenylamino)acetic acid. ON 01911 (inactive analog), (E)-2-(2-methoxy-5-((3,4,5-trimethoxystyrylsulfonyl)methyl)phenylamino)acetic acid.

Figure 2. Detection of RanGAP1•SUMO1 phosphorylation in cancer cells treated with ON 01910.Na. Panel A: Sample was prepared by Lys A method. Western blot analysis of U937 and DU-145 cells exposed to ON 01910.Na for 16 h probed with anti-SUMO1 antibody. SUMO-modified protein Y (Y•SUMO1, top panel) appeared to be a dose-dependent up-shift of SUMO-modified protein Z, which we assumed was RanGAP1•SUMO1. The same blot re-probed with anti-RanGAP1 antibody (middle panel) confirms dose-dependent up-shift of RanGAP1•SUMO1 in the presence of ON 01910.Na (abbreviated as 1910). Panel B-C: samples were prepared by 1xSB method.
Panel B: Western blot analysis of RanGAP1/RanGAP1•SUMO1 expression/phosphorylation in MOLT-3 treated with ON 01910.Na at various concentrations for 16 h. Panel C: Western blot analysis of RanGAP1•SUMO1 phosphorylation in DU-145 treated with ON 01910.Na at 1, 5 and 25 µM for 2 and 4 hours. Shown are shorter exposure (20 sec) and longer exposure (60 sec) blots.

Figure 3. Western blot analysis of RanGAP1•SUMO1 phosphorylation: phosphorylated versus hyperphosphorylated forms. Panel A: DU-145 cells were treated with ON 01910.Na for 16 h and processed for total cell lysates in two ways. “1xSB” samples were prepared by direct resuspension of cell pellet in reducing/denaturing SDS-PAGE sample buffer. In “Lys A” method cell pellets were lysed by incubation in a non-denaturing lysis buffer (see Methods) followed by centrifugation to clear the cell debris. Method “1xSB” achieves better preservation of SUMOylated RanGAP1 while “Lys A” results in substantial deSUMOylation. 7% SDS-PAGE was used. Panel B: Samples indicated in (A) by oval were also resolved on 6% SDS-PAGE gel and probed identically. RS, pRS and ppRS refer respectively to RanGAP1•SUMO1, phosphorylated RanGAP1•SUMO1 and hyperphosphorylated RanGAP1•SUMO1.

Figure 4. Western blot analysis of MOLT-3 treated with various drugs for 4 and 24 h. The second from the top blot was probed with anti-RanGAP1 antibody, the rest of blots were probed as indicated. In Panel A, cell lysates were resolved with 7% SDS-PAGE. In Panels B and C, cell lysates were resolved with 12% SDS-PAGE.

Figure 5. Synchronization of MOLT-3 cells by double thymidine block followed by release in drug-free medium or medium supplemented with either ON 01910.Na or nocodazole (both at 1 µM). Samples were collected at designated time points after release from the block and analyzed by Flow Cytometry for the cell cycle distribution (A, B) and by Western Blot analysis (C, D). Percentage of mitotic cells were separately obtained by fixing cells for phospho(Ser10)-histone H3 (Panel 5B). Abbreviations used (in C and D): FL, full length; CL, cleaved; pRS, phospho-RanGAP1•SUMO1; RS, RanGAP1•SUMO1; R, RanGAP1; β-A, β - actin.
Figure 6. Tubulin polymerization assay. A – 10% glycerol, B – 15% glycerol, C – 20% glycerol, (△) paclitaxel, (■) ON 01910.Na, (□) ON 01911, (O) DMSO, (▲) nocodazole.
Figure 2
Effect of ON 01910.Na, an anticancer mitotic inhibitor, on cell cycle progression correlates with RanGAP1 hyperphosphorylation


Cancer Res Published OnlineFirst June 6, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1603

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.