NOV-002 suppresses tumor cell growth by modulating redox-sensitive cell signaling

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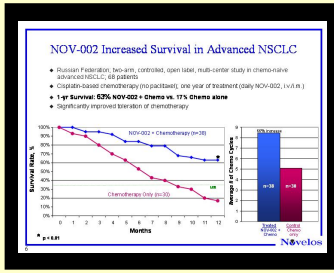
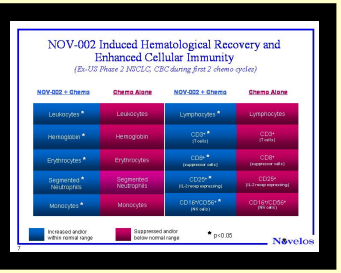
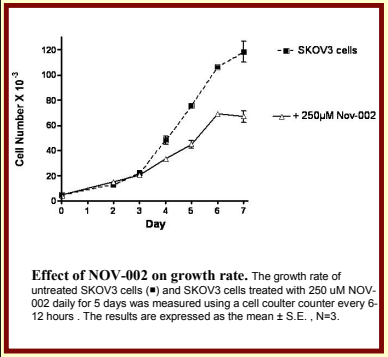
Abstract

Regulation of cell proliferation/survival is complex, impacted by multiple endogenous and exogenous stimuli capable of triggering overlapping and competing signaling pathways whose net effects can be quite cell dependent. Changes in redox status is one regulator of cell life/death decisions under both physiologic and pathologic conditions. Here we report on the anti-proliferative effects of a pharmacologically generated oxidative signal in human tumor cells using NOV-002, a glutathione disulfide-mimetic in advanced clinical trials for oncological indications. NOV-002 has demonstrated efficacy (increased survival and/or decreased tumor growth) in non-small cell lung, breast and ovarian cancers when combined with standard chemotherapeutic agents. In addition, treatment with NOV-002 mitigates chemotherapy-associated hematological toxicity. Pre-clinical data has linked this effect to proliferation of bone marrow progenitor cells subsequent to generation of an oxidative signal, intracellularly and at the cell surface of myeloid lineage cells, leading to activation of multiple kinases known to regulate cell proliferation (e.g. MAP kinases, JAK/STAT kinases). The data presented here extend redox modulation studies with NOV-002 to tumor cells. SKOV3 cells (a human ovarian tumor cell line) were exposed to NOV-002 (250 uM). Within 5 min after a single treatment, cellular levels of reactive oxygen species (ROS) were significantly elevated indicating the generation of an oxidative signal by NOV-002. This was not, however, sufficient to influence redox-sensitive cell signaling or tumor cell proliferation rate. In contrast, more prolonged exposure to NOV-002 (daily treatment for 5 days) resulted in a sustained elevation in ROS and dose-dependent activation of the proliferation-regulating MAP kinase, JNK, as evidenced by an increase in its active, phosphorylated form. Most importantly, this redox-activated cell signaling effect was associated with a significantly decreased proliferation rate of the SKOV3 cells that persisted even after stopping treatment with NOV-002. Thus, oxidative signal generation and MAP kinase pathway activation by NOV-002 appears to result in cell-type dependent effects on proliferation/survival. In myeloid lineage/bone marrow cells, chronic exposure to NOV-002 leads to increased proliferation while in tumor cells the result is a decrease in proliferation. These dichotomous effects may contribute to the unique clinical profile that NOV-002 has demonstrated to date -- increased anti-tumor efficacy and survival combined with enhanced recovery from chemotherapy-induced hematological toxicity.

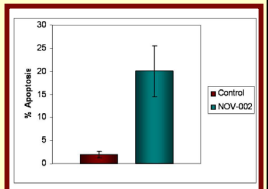




Chronic NOV-002 treatment induces apoptosis and inhibits SKOV3 cellular growth

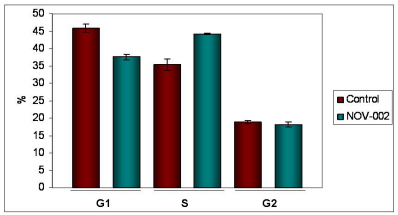


Effect of NOV-002 on growth rate. The growth rate of untreated SKOV3 cells (■) and SKOV3 cells treated with 250 uM NOV-002 daily for 5 days was measured using a cell coulter counter every 6-12 hours . The results are expressed as the mean ± S.E. , N=3.



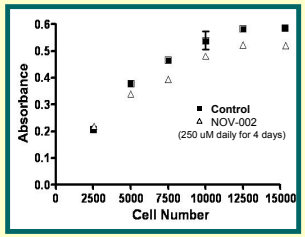
Effect of NOV-002 on apoptosis. The percent of apoptotic SKOV3 cells (■) and SKOV3 cells treated with 250 uM NOV-002 (■) daily for 5 days was measured in the flow cytometry facility at MUSC. Cells were pelleted and incubated for 15 minutes in the dark with Annexin-FITC antibody. Following incubation with Annexin V, cells were transferred to flow tubes and immediately analyzed using fluorescence-activated cell sorting analysis. The results are expressed as the mean ± S.E. , N=3.

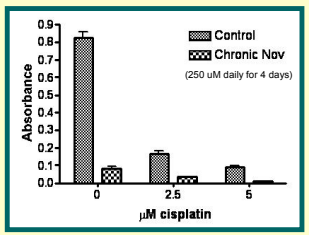
NOV-002 treatment leads to cell cycle alterations and enhanced apoptosis in SKOV3 cells



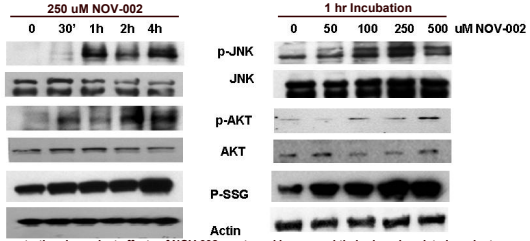
Effect of NOV-002 on cell cycle arrest. SKOV3 cells were treated with vehicle (■) or 250 uM NOV-002 (■) daily for 4 days. Cell cycle analysis was performed in the flow cytometry facility at MUSC. The results are expressed as the mean ± S.E. , N=3.

NOV-002 increases cytotoxicity towards SKOV3 cells in the presence of cisplatin (MTT method)



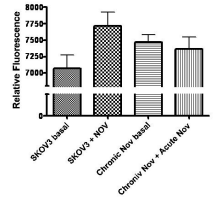


NOV-002 Activates Kinase Signaling and Increases Protein S-Glutathionylation in SKOV3 Cells



Time and concentration dependent effects of NOV-002 on stress kinases and their phosphorylated products were examined in SKOV3 cells. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred overnight onto nitrocellulose membranes (Bio-Rad). Non-specific binding was reduced by incubating the membrane in 10% blocking buffer for 1h containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% bovine serum albumin, 0.1% Tween 20, 1x protease inhibitors. Protein expression was determined by incubating membranes with specific primary in 5% blocking buffer according to manufacturer's recommendation. Briefly, the membranes were washed three times and incubated with the appropriate secondary antibodies, conjugated with horseradish peroxidase, in 5% blocking buffer for 1 h. The membranes were washed three times and developed with enhanced chemiluminescence detection reagents (Amersham Biosciences).

NOV-002 treatment leads to increased ROS levels in SKOV3 cells



Acute and chronic NOV-002 treatment leads to enhanced ROS production in SKOV3 cells. To examine the effects of acute NOV-002 treatment, SKOV3 cells were treated with 250 μM NOV-002 or control for 15 minutes. To examine the chronic effects of NOV-002, cells were treated with vehicle or 250 uM NOV-002 daily for 4 days prior to acute treatment. The Amplex® Red fluorescent dye (1 μM / L) and horseradish peroxidase (5 U/ 2 mL) were added and the release of H2O2 in the medium was detected at 37C in a fluorescence spectrophotometer. The excitation wavelength was 550 nm and the fluorescence emission was measured at 585 nm. The signal was generated from a calibration using known amounts of H2O2.

NOV-002 treatment leads to gene expression changes in SKOV3 cells

NADPH oxidase genes are up-regulated by chronic NOV-002 treatment in SKOV3 cells

NOX5 - 13.3-fold - NADPH oxidase, EF-hand calcium binding domain 5
- produces superoxide
- activated by calcium

CYBA - 2.1-fold - Cytochrome b-245 alpha polypeptide; p22 PHOX
- 22 KD subunit of NADPH oxidase
- produces superoxide anion

NCF1 - 2.3-fold - Neutrophil cytosolic factor 1; p47 PHOX; NOXO2
- 47 KD subunit of NADPH oxidase
- produces superoxide anion

NCF2 - 2.1-fold - Neutrophil cytosolic factor 2; p67 PHOX; NOXA2
- 67 KD subunit of NADPH oxidase
- produces superoxide anion

Other genes are up-regulated by chronic NOV-002 treatment in SKOV3 cells

IL8 - 39-fold - Interleukin 8
- CXC chemokine; mediator of inflammatory response
- Chemoattractant and angiogenic factor
- Induced by oxidative stress

VEGFA - 3.5-fold - Vascular endothelial growth factor A
- Angiogenic factor
- Induced by oxidative stress
- Activates NADPH oxidase resulting in superoxide production

JUN - 1.8-fold - Jun oncogene
- Activation can lead to apoptosis

Conclusions

- **Repeated treatment of SKOV3 tumor cells with NOV-002 leads to:**
  - Oxidative signaling as evidenced by formation of ROS, increased protein S-glutathionylation and up-regulation of ROS-induced genes
  - Concurrent activation of MAP kinase signaling pathways
  - Apoptosis and diminished tumor cell growth rate
  - Enhancement of cisplatin-induced tumor cell cytotoxicity
- **These data support the hypothesis that the improved efficacy seen in cancer patients treated with combinations of NOV-002 and standard chemotherapy may be due at least in part to direct anti-tumor effects of NOV-002**