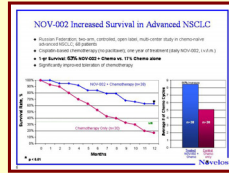
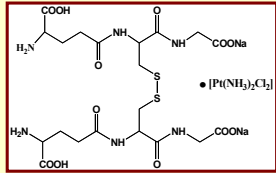


NOV-002, a redox modulating glutathione disulfide mimetic, leads to redox-mediated calcium influx and nitric oxide generation through eNOS activation in myeloid cells

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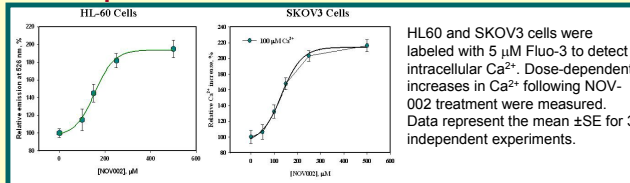
Abstract

NOV-002, a glutathione disulfide mimetic, is currently in a pivotal Phase 3 clinical trial in advanced non-small cell lung cancer. In clinical trials conducted to date, NOV-002 administered in combination with standard chemotherapeutic regimens has resulted in increased efficacy (survival, tumor response) and improved tolerance of standard chemotherapy (e.g. enhanced hematological recovery, immune stimulation). Recently, we showed that the myeloproliferative effect of NOV-002 in the pre-myeloid HL-60 cell line occurs in parallel with stress-induced S-glutathionylation and activation of kinase pathways (AKT, JAK2 and STAT5) that are known to regulate hematopoiesis. NOV-002 treatment produces oxidant signals intracellularly and at the cell surface. HL60 cells express the cell surface enzyme gamma-glutamyl transpeptidase (GGT). NOV-002 is a substrate for GGT and its cleavage alters the intracellular redox potential. Here we show that NOV-002 treatment in HL60 cells mediates changes of the plasma membrane potential as detected by the fluorescent probe bisOxonal. These changes were concurrent with time- and dose-dependent increases in the accumulation of intracellular Ca²⁺ as detected by Fura-2-AM and Fluo-3-AM fluorescent dyes. These effects were abolished by the addition of an extracellular Ca²⁺ chelator (EGTA) and decreased by the intracellular chelator, BAPTA-AM. These data suggest that NOV-002 induced calcium signaling involves entry of extracellular Ca²⁺ across the plasma membrane. Microarray analyses highlighted that NOV-002 treatment impacts expression of proteins involved in calcium signaling and nitric oxide (NO) metabolism pathways. NO generation following NOV-002 treatment was decreased by ~80% in HL60 cells transfected with siRNA to deplete eNOS levels. Overall, NOV-002 mediated alteration of cell surface redox status and transmembrane potential resulted in an induction of calcium influx, followed by NO generation through eNOS activation. These events may be pertinent to myeloproliferation that has been observed in cancer patients treated with NOV-002 plus standard chemotherapy.



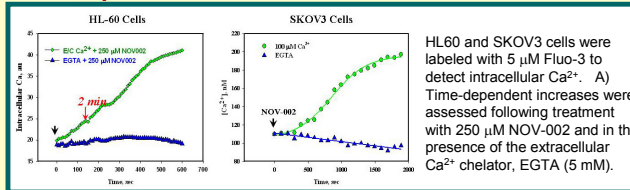
Intracellular Calcium Mobilization Following NOV-002

Dose-dependent



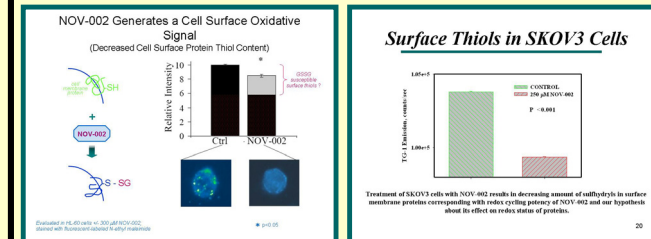
HL60 and SKOV3 cells were labeled with 5 μM Fluo-3 to detect intracellular Ca²⁺. Dose-dependent increases in Ca²⁺ following NOV-002 treatment were measured. Data represent the mean ±SE for 3 independent experiments.

Time-dependent

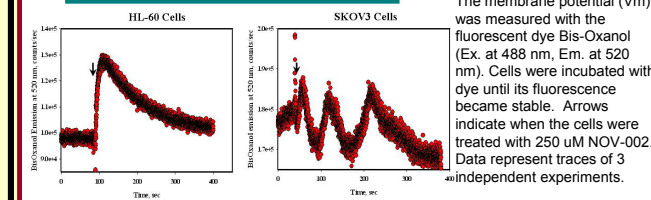


HL60 and SKOV3 cells were labeled with 5 μM Fluo-3 to detect intracellular Ca²⁺. A) Time-dependent increases were assessed following treatment with 250 μM NOV-002 and in the presence of the extracellular Ca²⁺ chelator, EGTA (5 mM).

Cell Surface Thiols Are Modulated By NOV-002

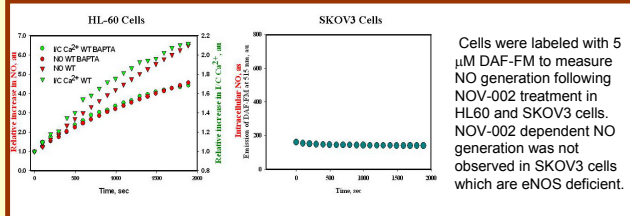


Effect of NOV-002 on Plasma Membrane Potential



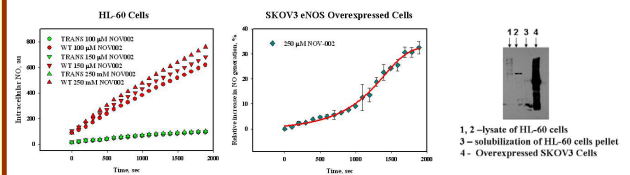
The membrane potential (Vm) was measured with the fluorescent dye Bis-Oxanol (Ex. at 488 nm, Em. at 520 nm). Cells were incubated with dye until its fluorescence became stable. Arrows indicate when the cells were treated with 250 μM NOV-002. Data represent traces of 3 independent experiments.

eNOS-Mediated NO Generation Following NOV-002



Cells were labeled with 5 μM DAF-FM to measure NO generation following NOV-002 treatment in HL60 and SKOV3 cells. NOV-002 dependent NO generation was not observed in SKOV3 cells which are eNOS deficient.

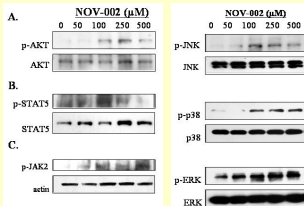
Effect of eNOS KD (HL-60) or Overexpression (SKOV3)



1, 2 – lysate of HL-60 cells
 3 – solubilization of HL-60 cells pellet
 4 – Overexpressed SKOV3 cells

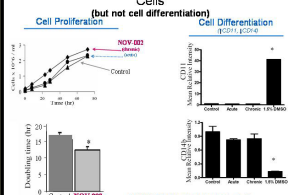
Cellular Kinase Signaling

JAK-STAT Pathway MAPK Pathway



Concentration dependent effects of NOV-002 on stress kinases and their phosphorylated products are consistent with actin-S-glutathionylation patterns. HL60 cells were treated for 1h with 0-500 μM NOV-002 in complete media.

NOV-002 Stimulates Proliferation of Pre-Myleoid Cells (but not cell differentiation)



Effects of NOV-002 on growth and differentiation in HL-60 cells. The growth rate of untreated HL60 cells (▲), HL60 cells + 300 μM NOV-002, "acute" (●) or HL60 cells + 300 μM NOV-002 every 24h, "chronic" (○) was measured using a cell counter every 6-12 hours. Flow cytometry was used to measure cell surface markers for differentiation, cd11 and cd14b. The results are expressed as the mean ± S.E., N=3.

Summary

- NOV-002 treatment of HL60 and SKOV3 cells decreased the levels of cell surface reduced thiols and alters the membrane potential.
- NOV-002 treatment of HL60 and SKOV3 cells results in plasma membrane hyperpolarization and influx of extracellular Ca²⁺ into the cytosol.
- NOV-002-mediated increases in intracellular Ca²⁺ are followed by NO generation through activation of eNOS.
- These effects may be pertinent to myeloproliferation observed after NOV-002 treatment and provide a rational for its combination with chemotherapy.