Niclosamide Stearate Prodrug Therapeutic (NSPT) Enhances Mitochondrial Proton Leak and Induces Potent Cytotoxicity in Osteosarcoma

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Background

- Osteosarcoma (OS) is the most common primary bone cancer in humans, accounting for 15% of all primary bone tumors confirmed through biopsy.[1]
- Typical treatment includes neoadjuvant chemotherapy (methotrexate, doxorubicin, cisplatin based therapies), surgical resection, and adjuvant chemotherapy.[2]
- Doxorubicin is known to cause cardiac toxicity, is associated with increased morbidity and is known to decrease the lifespan of patients, especially children.[2,3,4]
- Thus, there is a pressing need to identify safer, non-toxic, and effective chemotherapeutic alternatives to treat OS.

Purpose

- This study evaluates the mechanism of action of a novel chemotherapeutic, Niclosamide Stearate Prodrug Therapeutic (NSPT), a prodrug that increases the bioavailability of niclosamide, on canine and human OS cells.
- It has been previously proposed that niclosamide acts as an ionophore carrying protons from the matrix to the transmembrane space and thus disrupting the electron transport chain.[5]
- This study evaluated the bioenergetic effect of NSPT in OS mitochondria and its ability to induce apoptosis.

METHODS

DEVELOPMENT OF NSPT

- Rapid solvent exchange packages niclosamide into particles surrounded by lipid.
- Particle size was measured to ensure pharmaceutical quality utilizing dynamic light scattering (Figure 1).

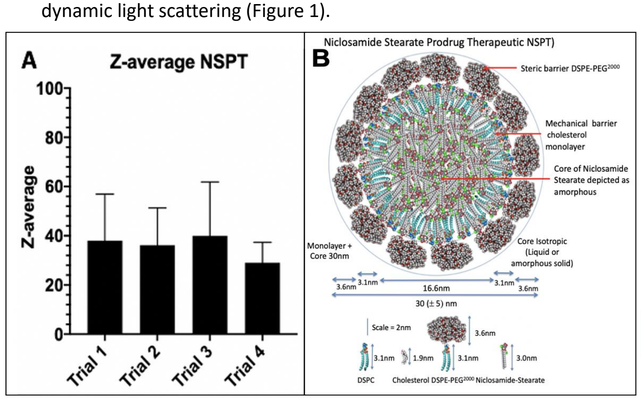

Figure 1: A) The Z-average of the NSPT and control across all 4 trials determined by dynamic light scattering. The average diameter of these molecules is well below the 100 nm threshold desired to potentially impact even the least vascularized tumors. B) Representative photo of NSPT taken from Reddy and Colleagues (2020)[6].

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METHODS (Continued)

CELL CULTURE

- 4 different OS cell lines:
  - 2 human (143B and MG-63)
  - 2 canine (D418 and D17)

BIOENERGETIC IMPACT of NSPT on OS MITOCHONDRIA

- Measured utilizing the the Seahorse XFe96 Analyzer (Bioscience, North Billerica, MA).
- The mitochondrial stress test measures the oxygen consumption rate (OCR) and is representative of the energy usage attributed to the electron transport chain in the cell.
  - From the OCR, approximations of the proton leak, ATP production, and reserve capacity of the cell were made.
- The extracellular acidification rate (ECAR) is representative of the amount of aerobic glycolysis utilized for energy in the cell.
- 3-hour and 3-day conditions were calculated with 143B, MG-63, D17, and D418 cells treated at 1/6 , 1/2, and their half maximal inhibitory concentration (IC50).

CYTOTOXICITY OF NSPT in MONO and SPHEROID CULTURES

- Cells were grown in monoculture and spheroid cultures.
- Treated with the cells Ic80:
  - Monoculture- on the day of plating and two days after plating.
  - Spheroids- 2 days after plating
- IncuCyte® S3 imaging allowed for a quantitative measure of growth inhibition (% confluence) and apoptosis (caspase 3/7 activity and the quantified fluorescent read out).
- The CellTiter-Glo® Luminescent Cell Viability Assay was utilized at the end-point of the assay to measure viability.

Ex Vivo Established Metastatic Disease Model

- Mice were injected by tail-vein with ~2,500,000 cells/ 100 uL.
- Their lungs were removed and the cells were allowed to grow for 5 days before treatment, which was meant to represent established disease in the lung parenchyma
- Cells were treated with either NSPT control, DMSO, 10 uM Niclosamide, 10 uM NSPT, or 10 uM Doxorubicin
- Tumor burden was measured at 0, 5, and 10 days post treatment

Results

NSPT SIZE

- NSPT had an average particle size of 25.39 +/- 10.23 nm and polydispersity index < 0.3:
  - Their size also indicates that, *in vivo*, they should be able to permeate even the least vascularized areas of the body.

REFERENCES (CONTINUED)

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Results (Continued)

BIOENERGETICS IMPACT OF THE MITOCHONDRIA

- The three hour condition showed an increase in proton leak, which is consistent with the proposed MOA of niclosamide (Figure 2).

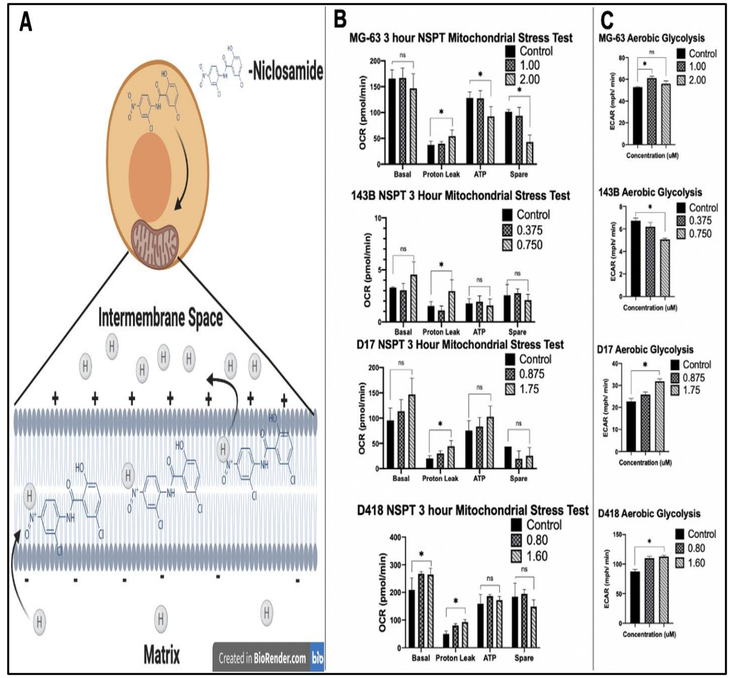

Figure 2: A) Diagram of the proposed mechanism of action of NSPT and, thus, niclosamide, on the inner mitochondrial membrane. It is thought that niclosamide acts as a shuttle, carrying protons from the matrix to the intermembrane space. B) Mitochondrial stress test after 3 hours of treatment showing that all cell lines tested demonstrate a significant increase in proton leak. This supports the uncoupling process diagrammed in panel A. C) ECAR, a measure of aerobic glycolysis, for each of the cell lines. The canine cell lines appeared to have an increase in aerobic glycolysis. This pattern was not observed in the human cell lines.

CYTOTOXICITY IN SPHEROIDS

- Increased levels of apoptosis in 143B, MG-63, and D17.
- Decreased number of viable cells at the end of the assay in all 4 cell lines (Figure 3).

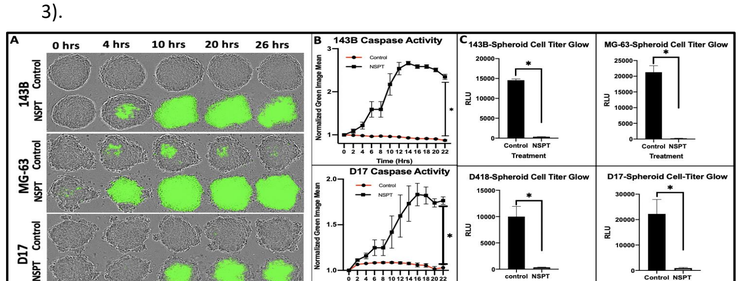

Figure 3: A) OS cell lines treated with either control or NSPT and a caspase 3/7 fluorescent marker. In all cell lines, the level of caspase 3/7 activity was markedly increased in the treatment group compared to the control. B) Significant differences were observed in the green image mean, indicating level of apoptosis, in the cell lines over a 24-hour period. C) CellTiter-Glo® end point assays show that the number of viable cells in the treatment group was significantly lower than that of the control group.

Ex Vivo Established Metastatic Disease Model

- NSPT with reduced tumor burden compared to its negative control and similar reduction to its positive control (Figure 4A).
- NSPT is as effective as the standard of care (Figure 4B).

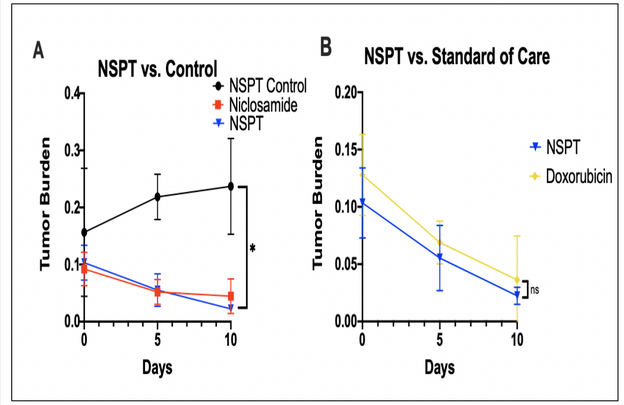

Figure 4:. A) NSPT's tumor burden is significantly reduced when compared to NSPT control B) NSPT can reduce tumor burden similar to that of the standard of care therapy Doxorubicin.

DISCUSSION

- OSs rely on a larger proportion of aerobic glycolysis for their energy needs than normal cells.[6]
- Aerobic glycolysis may be advantageous by allowing OS cells to shunt glucose into many precursors important for growth.[7]
- However, a reliance on aerobic glycolysis may leave OS cells energetically vulnerable in that they have very little reserve if their basal energy production is disrupted.[8]
- Therefore, a chemotherapeutic that targets the mitochondria may be efficacious in OS and other cancer cells.
- Further, the results of our ex vivo experiment may show that NSPT could be efficacious in established metastatic OS.

CONCLUSIONS

- Examination of the MOA of niclosamide established the presence of increased proton leak, which is consistent with its proposed MOA as a shuttle of protons across the membrane.
- NSPT is able to induce apoptosis across two human and two canine cell lines showing the ability to induce apoptosis is conserved and may be efficacious in most types of OS.
- An ex vivo model representing established metastatic OS was created and showed that NSPT is as efficacious as the current standard of care therapy, Doxorubicin, and that it may be efficacious in treating established metastatic disease.
- This study, in conjunction with the Eward Lab's canine study, supports further in vivo testing with NSPT and supports future human trials.
- Finally, NSPT could replace or reduce the amount of doxorubicin needed for treatment of OS, which might reduce cardiotoxicity and extend life.