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Glutamine metabolic inhibition synergizes with L-asparaginase in MYCN-amplified neuroblastoma

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Abstract

Neuroblastoma is the most common extracranial solid tumor in children. Though it accounts for about 10% of pediatric cancers, it is disproportionately responsible for 15% of pediatric cancer deaths. MYCN is amplified in 20% of neuroblastomas and correlates with adverse outcome. MYCN is known to drive tumor cell reliance on glutamine for cellular metabolism. 6-diazo-5-oxo-L-norleucine (DON) is a well-characterized glutamine analogue that inhibits glutamine metabolism by irreversibly inactivating multiple glutamine-utilizing enzymes. DON was well tolerated in a previous phase I clinical trial in pediatric patients, but it has never been systematically tested in neuroblastoma patients. We show that MYCN amplification confers sensitivity to DON therapy in *in vitro* models of neuroblastoma, and that DON administered by intraperitoneal injection twice weekly significantly reduces flank tumor volume in mouse models of MYCN-amplified neuroblastoma (mean tumor volume 1715 mm^3 vs. 207 mm^3 in control animals, p-value = 0.00017 by t-test). We have also developed an orally bioavailable DON prodrug, JHU083, and we found that this drug administered orally three times weekly was similarly able to suppress neuroblastoma tumor growth in mice (mean tumor volume 1115 mm^3 vs. 217 mm^3 in control animals, p-value = 0.0000088 by t-test). In stable isotope resolved metabolomics (SIRM) experiments, tracing glutamine and glucose donation of ^{13}C and ^{15}N via liquid chromatography/mass spectrometry, DON prevents asparagine synthesis, depleting intracellular asparagine levels by 40% in MYCN-amplified neuroblastoma cells compared to control cells (p-value = 0.006). We hypothesized that treatment with L-asparaginase would enhance DON efficacy. Indeed, DON combined with L-asparaginase synergistically inhibits growth of MYCN-amplified neuroblastoma cell lines (CI = 0.25 by the Chou-Talalay method, indicating strong synergy; p-value = 0.00011). We conclude that DON depletes cellular pools of asparagine and combination therapy with DON and L-asparaginase synergistically inhibits the growth of MYCN-amplified neuroblastoma. These studies provide the preclinical justification for potential clinical trials for the use DON or DON prodrugs in combination with L-asparaginase as new therapeutic options for patients with MYCN-amplified neuroblastoma.

Results

MYCN is highly expressed in a subset of human neuroblastoma cell lines and is involved in regulating glutamine metabolism

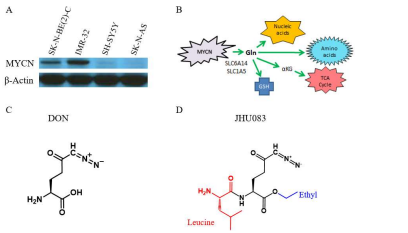
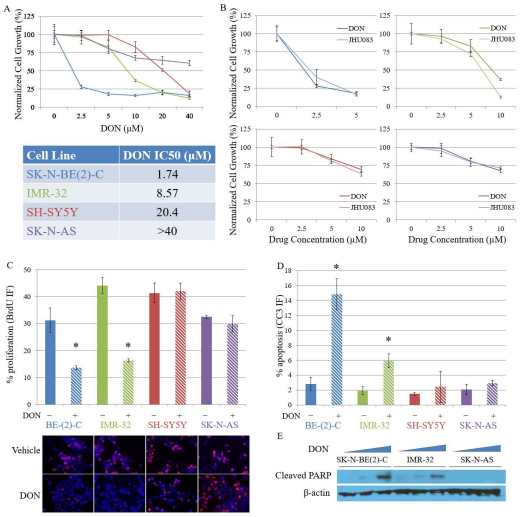


Figure 1. A) Western blot showing that SK-N-BE(2)-C and IMR-32 express high levels of MYCN, whereas SH-SY5Y and SK-N-AS do not. B) MYCN amplification may lead to increased reliance on glutamine by increasing glutamine transporters such as SLC6A14, as well as glutamine-utilizing enzymes such as glutaminase, which converts glutamine to glutamate. Glutamine contributes to nucleic acid synthesis, amino acid production, energy production via the TCA cycle and glutathione production – these diverse uses suggest that targeting glutamine metabolism with a broad inhibitor could be a novel therapeutic strategy for MYCN-amplified neuroblastoma, and for other MYC-dependent malignancies. C) Chemical structure of the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON). D) Chemical structure of the orally bioavailable DON prodrug JHU083.

6-diazo-5-oxo-L-norleucine (DON) and the DON prodrug, JHU083, inhibit growth and proliferation and induce apoptosis in MYCN-amplified neuroblastoma cell lines



Cell Line	DON IC50 (μM)
SK-N-BE(2)-C	1.74
IMR-32	8.57
SH-SY5Y	20.4
SK-N-AS	>40

Figure 2. A) MTS assay showing that MYCN-amplified neuroblastoma cell lines, SK-N-BE(2)-C and IMR-32, are more sensitive to DON therapy (72 hour treatment) than MYCN-non-amplified neuroblastoma cell lines (SH-SY5Y, SK-N-AS). B) MTS assay showing similar *in vitro* cell growth inhibition by DON and JHU083. C) Treatment with DON (10 μM) reduces proliferation in MYCN-amplified, but not MYCN-non-amplified, neuroblastoma cell lines, as measured by bromodeoxyuridine (BrdU) immunofluorescence; cells were treated for 72 hours before being fixed for staining (p <0.02 by Student t-test). D) 72 hour treatment with DON significantly induced apoptosis in MYCN-amplified but not MYCN-non-amplified neuroblastoma cell lines, as measured by cleaved caspase 3 (CC3) immunofluorescence (p <0.02 by Student t-test) and by F) cleaved PARP western blot. Similar effects on proliferation and apoptosis were seen with *in vitro* JHU083 treatment (data not shown).

DON and JHU083 treatment both significantly reduce MYCN-amplified neuroblastoma flank xenograft growth

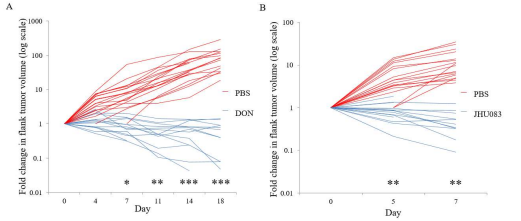


Figure 3. Flank injections of SK-N-BE(2)-C cells were performed in nude mice. Treatment was started when flank tumors achieved 5 mm in size. Spaghetti plots showing decreased neuroblastoma xenograft growth with A) DON treatment (20 mg/kg IP twice weekly; 40% of flank tumors entirely regressed for a period of time) and B) JHU083 treatment (30 mg/kg gavage 3 times weekly). * p < 0.03, ** p < 0.001, *** p < 0.0001 by Student t-test.

DON treatment primarily prevents asparagine synthesis in MYCN-amplified neuroblastoma cell lines

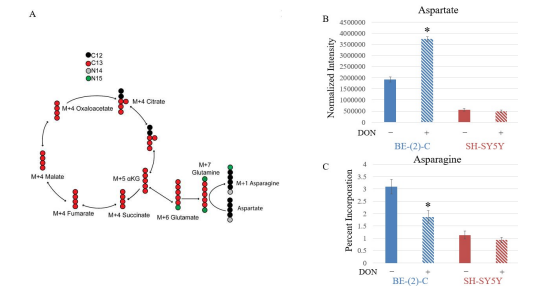


Figure 4. MYCN-amplified SK-N-BE(2)-C cells and MYCN-non-amplified SH-SY5Y cells were grown in culture until 80-90% confluent. The cells were treated with vehicle or DON 10 μM for a total of 3 hours. At hour 1, cells were switched to media containing uniformly labeled $^{15}N_2$,$^{13}C_5$-glutamine at a concentration of 4 mM. Metabolites were extracted from cells with 80% methanol and analyzed by LC/MS. A) Diagram of the TCA cycle showing how uniformly-labeled $^{15}N_2$,$^{13}C_5$-glutamine is metabolized. Asparagine is synthesized from aspartate by asparagine synthetase, using uniformly-labeled $^{15}N_2$,$^{13}C_5$-glutamine as a nitrogen donor. B) MYCN-amplified SK-N-BE(2)-C cells show a statistically significant increase (p = 0.004 by Student t-test) in levels of aspartate in response to DON treatment, whereas MYCN-non-amplified SH-SY5Y cells do not show any change in aspartate levels in response to DON treatment. C) MYCN-amplified SK-N-BE(2)-C cells show a statistically significant 40% decrease (p = 0.006 by Student t-test) in asparagine levels in response to DON treatment, an effect not seen in the MYCN-non-amplified SH-SY5Y cell line. Taken together, these data suggest that ASNS is being inhibited by DON treatment in MYCN-amplified neuroblastoma cells, causing a decrease in asparagine levels and a concomitant buildup of aspartate.

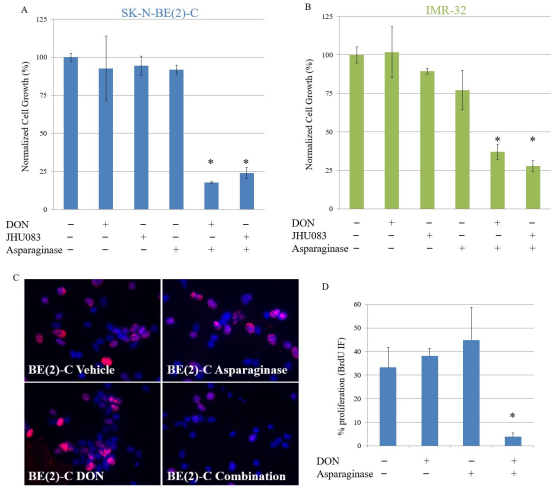


Figure 5. 72 hour treatment of MYCN-amplified neuroblastoma cell lines *in vitro* with DON or JHU083 +/- L-asparaginase shows synergistic growth inhibition with combination treatment. A) SK-N-BE(2)-C treated with DON or JHU083 0.625 μM +/- L-asparaginase 0.25 U/mL (p < 0.01 by one-way ANOVA). B) IMR-32 treated with DON or JHU083 1.25 μM +/- L-asparaginase 0.25 U/mL (p < 0.01 by one-way ANOVA). C-D) Bromodeoxyuridine (BrdU) immunofluorescence (Red = BrdU, Blue = DAPI) of SK-N-BE(2)-C cells treated for 72 hours with DON 0.625 μM and L-asparaginase 0.25 U/mL show synergistic inhibition of proliferation. CI < 0.3 for A-C by the Chou-Talalay method, indicating strong synergy.

JHU083 and asparaginase combination therapy *in vivo* more effectively reduces MYCN-amplified flank xenograft growth than treatment with either agent alone

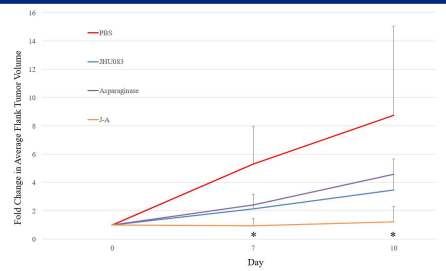


Figure 6. Flank injections of IMR-32 cells were performed in nude mice. Treatment was started when flank tumors achieved 5 mm in size. Graph of fold change in average flank tumor volume (normalized to day 0, first day of drug treatment) shows a statistically significant decrease in flank xenograft growth with combination therapy with JHU083 (20 mg/kg gavage twice weekly) and asparaginase (50 units IP twice weekly) compared to either medication alone (p < 0.0001 by one-way ANOVA). Interestingly, in this experiment, asparaginase alone reduced flank tumor growth compared to vehicle to a similar degree as DON alone (p < 0.02 by Student t-test).

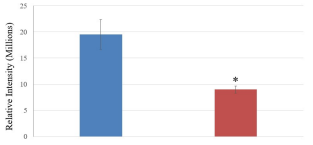


Figure 7. Quantification of N-acetyl-L-aspartate (NAA) peaks after unlabeled metabolomic analysis of neuroblastoma cell lines shows significantly higher levels in MYCN-amplified vs. MYCN-non-amplified neuroblastoma cells (p-value < 0.01 by Student t-test), suggesting NAA as a candidate metabolic biomarker to predict response to glutamine metabolic inhibition.

Conclusions

In MYCN-amplified neuroblastoma:

- Treatment with 6-diazo-5-oxo-L-norleucine (DON) or the DON prodrug, JHU083, reduces growth and proliferation and increases apoptosis *in vitro*
- Treatment with DON or JHU083 reduces flank tumor growth in murine xenograft models
- DON decreases production of asparagine
- The combination of DON and asparaginase decreases growth and proliferation *in vitro*
- The combination of JHU083 and asparaginase *in vivo* reduces flank tumor growth more effectively than either medication alone

Future Directions

- We predict that combination therapy will reduce growth of flank tumors generated from other MYCN-amplified neuroblastoma cell lines
- We plan to perform *in vivo* stable isotope resolved metabolomics to confirm that treatment with DON and DON prodrugs primarily prevents asparagine synthesis in MYCN-amplified neuroblastoma xenografts
- Based on related work in group C Myc-driven medulloblastoma in our laboratory, we hypothesize that the mechanism of increased cancer killing of the combination of DON and asparaginase is induction of the uncharged tRNA response/integrated stress response/GCN2-ATF4 pathway (see poster #3494)
- DON (or DON prodrugs) combined with asparaginase could be a potent metabolic therapy in certain carefully selected neuroblastoma patients
- We anticipate that our metabolomics approach will identify other potential metabolic combination therapies with DON and its prodrugs and thus help us to expand the treatment repertoire for MYCN-amplified neuroblastoma and other Myc-driven pediatric solid tumors.