Polyamine antagonist therapies inhibit neuroblastoma initiation and progression


Affiliations and addresses:
1Division of Oncology, The Children's Hospital of Philadelphia, Philadelphia, PA, USA.
2Children's Cancer Institute Australia, Sydney, Australia.
3UT Southwestern Medical Center, Dallas, TX, USA.
4Kids Cancer Centre, Sydney Children's Hospital, Sydney, Australia.
5School of Women's and Children's Health, Faculty of Medicine, University of New South Wales, Kensington, Australia.
6Lankenau Institute for Medical Research, Wynnewood, PA, USA.
7Department of Pediatrics and 8Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA.
9Department of Biostatistics, Temple University School of Medicine, Philadelphia, PA, USA.

*Authorship note: NFE and MH contributed equally to this work.
**Authorship note: MDN and MDH contributed equally to this work.

Running Title: Polyamine antagonism in neuroblastoma

Keywords: MYC, experimental therapeutics, preclinical models, difluoromethylornithine (DFMO), pediatric cancer

Funding to support this work came from the US Department of Defense W81XWH-10-1-0145 (to M.D.H and S.G.), and the Richard and Sheila Sanford Chair in Pediatric Oncology (to M.D.H.); the Children's Neuroblastoma Cancer Foundation (to N.F.E); The National Health and Medical Research Council (Australia) and Cancer Institute New South Wales (to M.H., G.M.M and M.D.N). Children's Cancer Institute Australia for Medical Research is affiliated with the University of New South Wales and Sydney Children’s Hospital Randwick, Sydney, Australia and is a member of the Kid’s Cancer Alliance.

Corresponding Author:
Michael D. Hogarty
Division of Oncology, The Children’s Hospital of Philadelphia
CTRB, Room 3020
3501 Civic Center Boulevard
Philadelphia, PA 19104-4318, USA
Phone: 215-590-3931
FAX: 215-590-3770
Email: hogartym@email.chop.edu

The authors disclose no potential conflicts of interest.

STATEMENT OF TRANSLATIONAL RELEVANCE

Hyperactive MYC signaling is an oncogenic driver for a large proportion of human tumors, yet to date no MYC-directed therapeutic has been approved for clinical use. MYC drives myriad signaling pathways that link cell cycle kinetics with the creation of cell biomass. Polyamines are cationic chaperones that support such MYC activities through ionic and covalent mechanisms, and their homeostasis is critical to both initiating and maintaining the cancer phenotype. Here we demonstrate in complementary models of the highly lethal childhood tumor neuroblastoma that therapeutics that deplete tumoral polyamines (such as DFMO, celecoxib and SAM486) synergize to block tumor initiation and regress established tumors. DFMO and celecoxib are FDA-approved drugs with activity in regressing diverse high risk neuroblastoma subtypes in vivo, alone and in combination with diverse chemotherapy regimens. These data strongly support the testing of such approaches in the clinic.
Polyamine antagonism in Neuroblastoma

ABSTRACT

Purpose: Deregulated MYC drives oncogenesis in many tissues yet direct pharmacologic inhibition has proven difficult. MYC coordinately regulates polyamine homeostasis as these essential cations support MYC functions, and drugs that antagonize polyamine sufficiency have synthetic-lethal interactions with MYC. Neuroblastoma is a lethal tumor in which the MYC homologue MYCN, and ODC1, the rate-limiting enzyme in polyamine synthesis, are frequently deregulated so we tested optimized polyamine depletion regimens for activity against neuroblastoma.

Experimental Design: We used complementary transgenic and xenograft-bearing neuroblastoma models to assess polyamine antagonists. We investigated difluoromethylornithine (DFMO; an inhibitor of Odc, the rate-limiting enzyme in polyamine synthesis), SAM486 (an inhibitor of Amd1, the second rate-limiting enzyme), and celecoxib (an inducer of Sat1 and polyamine catabolism) in both the pre-emptive setting and in the treatment of established tumors. In vitro assays were performed to identify mechanisms of activity.

Results: An optimized polyamine antagonist regimen using DFMO and SAM486 to inhibit both rate-limiting enzymes in polyamine synthesis potently blocked neuroblastoma initiation in transgenic mice, underscoring the requirement for polyamines in MYC-driven oncogenesis. Further, the combination of DFMO with celecoxib was found to be highly active, alone and combined with numerous chemotherapy regimens, in regressing established tumors in both models, including tumors harboring highest risk genetic lesions such as MYCN amplification, ALK mutation and TP53 mutation with multidrug resistance.

Conclusions: Given the broad preclinical activity demonstrated by polyamine antagonist regimens across diverse in vivo models, clinical investigation of such approaches in neuroblastoma and potentially other MYC-driven tumors is warranted.
INTRODUCTION

MYC genes coordinate transcriptional programs to promote cell proliferation, biomass production, self-renewal, and numerous other oncogenic attributes (1). Not surprisingly, MYC activity is tightly regulated in normal cells while deregulated expression is frequent in human cancers (2). Interest in pharmacologically antagonizing Myc is high as it acts at a network node governing growth signals and such antagonism might have broad clinical utility (3). However, Myc functions through protein:protein interactions generating a family of heterodimeric transcription factors with competing activities across thousands of sites in the genome (4). While direct antagonism of these complexes remains intractable, an alternative approach to antagonize Myc is to inhibit downstream pathways necessary for tumorigenesis. Polyamines represent a family of essential polycations that support Myc functions through ionic and covalent activities. Reduced levels of intracellular polyamines activate check-points that constrain proliferation, as seen in senescent and post-mitotic cells, while enhanced polyamine synthesis accompanies oncogenic proliferation (5). Since Myc regulates numerous polyamine enzymes this pathway has attracted attention as a therapeutic target in cancer (6), although specific testing for synthetic-lethal activity in tumors with deregulated MYC has been lacking.

The ornithine decarboxylase gene, ODC1, is a direct Myc target and bona fide oncogene that can substitute for MYC to transform cells in vitro (7) and in vivo (8). ODC1 encodes the rate-limiting enzyme in polyamine synthesis that decarboxylates ornithine to putrescine (Supplementary Figure S1). Step-wise conversion to the higher-order polyamines spermidine and spermine occurs via the aminopropyltransferases, SRM and SMS, respectively. The aminopropyl donor for these conversions is derived from the activity of the second rate-limiting enzyme in polyamine synthesis, S-adenosylmethionine decarboxylase (encoded by AMD1). Both Odc1 and Amd1 have the shortest half-lives of any mammalian enzymes (10-30 minutes). Odc activity is further regulated post-translationally by Odc antizymes that mediate its degradation (9), which are themselves regulated by antizyme inhibitors (10). Catabolism is regulated through polyamine acetylation by the spermidine/spermine-N-
acetyltransferase, SAT1, which controls flux through the pathway (11), while spermine can also be oxidized via SMOX activity to spermidine. Acetylated polyamines can be exported from the cell or back-converted by polyamine oxidase (PAOX) to spermidine or putrescine, respectively. Finally, an energy-dependent polyamine transport system can import polyamines from the microenvironment.

That homeostatic control over the repertoire of polyamines is so highly regulated at the transcriptional, translational and post-translational level (9, 12-14) underscores their importance to cell function and provides numerous opportunities for therapeutic intervention (Supplementary Figure S1).

Neuroblastoma is a lethal childhood tumor in which MYCN is the principal oncogenic driver (15). Its deregulation by genomic amplification correlates with aggressive disease and poor outcome (16). Gene expression profiles also identify strong MYC signatures in poor outcome neuroblastomas without MYCN amplification suggesting this may be a requisite pathway for the high-risk phenotype (17). Since MYC extensively regulates the polyamine pathway we and others have sought a role for polyamines in supporting neuroblastoma initiation and progression (18, 19). We previously used a transgenic mouse model of neuroblastoma to show that difluoromethylornithine (DFMO, Efornithine), an irreversible Odc1 inhibitor and FDA-approved drug for the treatment of Trypanosomiasis, delayed tumor initiation in a pre-emptive therapy model and synergized with chemotherapy to extend survival of mice with established tumors (18). Indeed, DFMO at doses up to 3 gm/m²/day were recently shown to be tolerable in children with neuroblastoma, and correlative studies supported depletion of systemic polyamine levels at this exposure (20). Here we sought to augment the efficacy of DFMO through synergistic targeting of multiple steps in polyamine homeostasis, and to extend these findings to complementary neuroblastoma xenograft models. We show that combined inhibition of Odc1 and Amd1 using DFMO and SAM486 prior to tumor initiation profoundly reduces tumor penetrance in TH-MYCN neuroblastoma-prone mice, validating this pathway as downstream of MYC. In the treatment of established tumors we show that the combination of DFMO and celecoxib, which induces Sat1, provides synergistic anti-tumor activity across models harboring highest risk genomic lesions such as
Polyamine antagonism in Neuroblastoma

MYCN amplification, ALK mutation and TP53 mutation with multidrug resistance. Further, we show augmented anti-tumor activity with multiple chemotherapy backbones including combinations widely used in neuroblastoma clinical protocols. Such broad pre-clinical data are required to prioritize agents for clinical investigation, as those with more limited pre-clinical testing often fail to demonstrate activity in human trials (21). Our findings support clinical testing of polyamine antagonist regimens in children with neuroblastoma, and potentially additional MYC-driven malignancies in which polyamine homeostasis is pivotal.
MATERIALS AND METHODS

Cell lines. Human neuroblastoma cell lines with \textit{MYCN} amplification (IMR5, NLF, SMS-KAN, BE2C) and without (SK-N-SH and SK-N-AS) were obtained from Garrett Brodeur (Children’s Hospital of Philadelphia). All are identity-confirmed by our group every 6 months using short tandem repeat (STR)-based genotyping (AmpFISTR, Applied Biosciences) and matched to the COG cell line database (www.cogcell.org). Murine neuroblastoma cell lines 844+/+ and 282+-/- were established from tumors arising in a homozygous and hemizygous \textit{TH-MYCN} mouse, respectively. All cells were grown in RPMI-1640 Media (Life Technologies) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml of penicillin and 100 mcg/ml gentamicin. Tissue culture was at 37°C in a humidified atmosphere of 5% CO$_2$. Polyamines were added to the culture media where indicated at 1 \textmu M concentration each for putrescine, spermidine and spermine (Sigma-Aldrich).

Colony Formation Assay. Neuroblastoma cells \textbf{were} exposed to DFMO at 5 mM for 72 hours, with or without supplemental polyamines (1 \textmu M each of putrescine, spermidine and spermine), in routine tissue culture media. Thereafter, 1,000 cells were plated in triplicate wells and stained by crystal violet after 14 days. For assessing drug interactions, BE2C cells were plated at 500 cells/well and SK-N-SH cells at 1,000 cells/well, and 5 hours later cells were exposed to DFMO, celecoxib or mafosfamide for 72 hours. Starting concentrations for BE2C cells were: 110 \textmu M DFMO, 14.8 \textmu M celecoxib and 0.51 \textmu M mafosfamide; and for SK-N-SH cells were: 40 \textmu M DFMO, 12 \textmu M celecoxib and 0.14 \textmu M mafosfamide. Drugs were kept at a constant ratio for three additional concentrations increasing by 1.5-fold. Colonies were fixed and stained after 10 days for BE2C and 14 days for SK-N-SH using 0.5% crystal violet/50% methanol, and counted using Quantity One 1-D Analysis software (Bio-Rad). Each experiment was performed in triplicate. Drug combination effects were determined using the CalcuSyn software (Biosoft, Cambridge, UK) which generates a combination index (CI) as a measure of the combined drug interaction. CI values were generated over a range of fractional cell kill.
(Fa) levels from 0.05–0.90 (5%–90% growth inhibition), and a Fa level of 0.75 was chosen to reflect the higher levels of growth inhibition needed when studying anti-cancer agents (22).

**TH-MYCN mouse model.** 129X1/SvJ mice transgenic for the *TH-MYCN* construct (23) were originally provided by Bill Weiss (Department of Neurology, UCSF). All murine studies were approved by the Institutional Animal Care and Utilization Committee at The Children’s Hospital of Philadelphia (Philadelphia) and the Animal Care and Ethics Committee of the University of New South Wales (Sydney). *TH-MYCN* hemizygous mice were bred and litters randomized to therapy as previously (18). Mice were genotyped from 1 cm tail-snip isolated DNA using Q-PCR (18). Mice were screened by experienced animal personnel and sacrificed for pathological signs of tumor burden. All mouse experiments were performed with a contemporaneous control arm. Transcriptome analyses were performed using available microarray datasets [(24); GEO GSE17740]] from the same *TH-MYCN* colony used herein.

**Pre-emptive therapy trials:** Pre-emptive DFMO therapy consisted of 1% DFMO added to water ad libitum from birth through day 70 [added to maternal drinking water days 0-21; after day 21 wean, added to pup drinking water; (25)]. SAM486 (Sardomozide, Novartis) therapy was given as 5 mg/kg IP three days per week from day 21 through day 70. Celecoxib was given daily by gavage at 100 mg/kg beginning day 21. In all studies vehicle was provided by similar route (IP or gavage) to control animals. Palpation for tumors was performed thrice weekly. Animals with tumors underwent serial abdominal ultrasonography under isoflurane sedation to determine in situ tumor volume (Vevo660; VisualSonics; Toronto, Canada). Mice without evidence of tumor were sacrificed at day >120 and a necropsy was performed to assess for the occult presence of tumor. No chemotherapy was used in pre-emptive trials and outcomes were analyzed by genotype (*TH-MYCN* +/-) with the time to tumor detection (Tumor Free Survival) and time to sacrifice due to signs of tumor progression (Survival) as end-points.
Established tumor trials: TH-MYCN mice were randomized at the time palpable tumors (~75-175 mm$^3$) were identified, to regimens including chemotherapy with or without polyamine antagonists: DFMO 1% added to water ad libitum; SAM486 at 5 mg/kg IP three days per week for up to 6 weeks; celecoxib at 100 mg/kg daily by gavage; started at enrollment. For the SAM486 + DFMO trial, both TH-MYCN hemizygous and homozygous mice were included. For all other trials, only TH-MYCN +/+ mice were included as they have less variable tumor progression kinetics (26). Chemotherapy regimens were temozolomide IP at 50 mg/kg (+/+ mice) or 10 mg/kg (+/- mice) for 5 days, irinotecan IP at 20 mg/kg for 5 days, topotecan IP at 2 mg/kg for 5 days, or topotecan IP at 0.5 mg/kg and cyclophosphamide IP at 10 mg/kg, or cyclophosphamide alone at 20 mg/kg for 5 days, as described.

Murine xenograft models. Cells used for xenograft studies were pathogen-free by IMPACT I PCR screening (RADIL, University of Missouri). Xenografts were established in the flank of NCR or BALB/c nu/nu athymic mice [Jackson Laboratories; (27)]. When tumor volume exceeded 200 mm$^3$, mice were randomly assigned to therapy arms. DFMO was given as 1% DFMO in drinking water ad libitum, SAM486 at 5 mg/kg IP 3 times per week for two weeks, and celecoxib was 100 mg/kg daily gavage for 5 days each week. Chemotherapy regimens included cyclophosphamide (30 mg/kg IP day 1 and 3) with topotecan (0.25 mg/kg IP day 1 and 3), and cyclophosphamide alone (30 mg/kg IP day 1 to 3; 90 mg/kg total). Animals were sacrificed when tumor volumes exceeded 2,000 mm$^3$ or if symptoms of tumor progression were apparent and this end-point used for Survival analyses.

Tumor histopathology. Tissues were harvested at sacrifice and fixed in 10% neutral buffered formalin and paraffin embedded for histologic studies, and flash frozen in liquid nitrogen for metabolic assays. Sections were H&E stained and assessed for differentiation, necrosis and...
mitotic/karyorrhectic cells. For immunohistochemistry, 5um sections were deparaffinized, hydrated and treated with appropriate antibodies. Caspase-3 antibody (R&D Systems AF835, Minneapolis MN) and Ki67 (Santa Cruz Biotechnology SC-7846, Santa Cruz, CA) staining were performed on an Autostainer Plus (DAKOCytomation, Carpinteria, CA). For Caspase-3, slides were incubated with Caspase-3 antibody at a 1:1,000 dilution for 30 minutes at RT, rinsed, then incubated with biotinylated anti-Rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 minutes. For Ki67, slides were incubated with Ki67 antibody at a 1:1,000 dilution overnight at 4°C, rinsed, then incubated with biotinylated anti-Goat IgG (Vector Laboratories) for 30 minutes. After rinsing, slides were incubated with avidin-biotin complex (Vector Laboratories) for 30 minutes, followed by rinsing and incubation with DAB (DAKO Cytomation) for 10 minutes at RT, then rinsed and counterstained with hematoxylin. Both Caspase-3 and Ki-67 staining was scored as the percentage of stained tumor nuclei.

**Polyamine content Assays.** Tumor tissues harvested at necropsy were flash-frozen in liquid nitrogen, ground to a fine powder, and stored at -80°C. For cell line assays, subconfluent cells were cultured with or without additional polyamines added to the culture media (1 µM each putrescine, spermidine, spermine; Sigma) as indicated, spun and rinsed three times and pelleted and frozen. For polyamine analyses, ground tissues were homogenized in 0.2 N perchloric acid and incubated at 4°C overnight. Dansylated polyamines were separated on a reversed-phase C18 HPLC column (28). Polyamine values were normalized to the amount of DNA in the tissue extracts.

**Radiolabeled Spermidine Transport Assays.** Radioactive spermidine (Net-522 Spermidine Trihydrochloride, [Terminal Methylene-3H(n)] from Perkin Elmer was used (specific activity 16.6 Ci/mmol). Cells were plated and grown to approximately 70% confluence. Three plates were used per condition: control, DFMO (5 mM), SAM486 (5 µM), celecoxib (10 µM), or combinations; treated for 48 hours. After repeated washing with PBS, 3H-SPD was added at 1 µM and incubated for 60 minutes at 37°C. Specimens were washed with cold PBS containing 5 µM spermidine and incubated in 500 µl
of 0.1 N NaOH at 37° C for 30 minutes to allow cells to dissolve. Contents were sonicated for 10 seconds to ensure uniform dissolution of cell debris. Then, an equal volume of 0.1N HCL was added to neutralize pH. Specimens were then aliquoted for scintillation counting. 50 µL of sample was then aliquoted for protein using mini-Biorad procedure. Results were expressed as CPM/400µL, then µg protein/400 µL was calculated and finally CPM/µg was calculated.

**Statistical analyses.** RNA expression levels between tumor and normal tissues were compared using the Wilcoxon Exact test. Tumor Free Survival and Survival analyses (defined above) were performed according to the method of Kaplan and Meier (29) with standard errors according to Peto (30). Comparisons of outcome between subgroups were performed by a 2-sided log-rank test. Neuroblastoma differentiation status was compared using the Fisher’s Exact test, and synergy studies were assessed as defined above. All other statistical comparisons were performed using the student’s t-test for independent sample sets and two-tailed design.
RESULTS

Polyamine homeostasis is faithfully recapitulated in the TH-MYCN neuroblastoma model. Human neuroblastomas with MYC activation coordinately deregulate polyamine enzymes to promote polyamine sufficiency and support Myc functions (18). We therefore tested whether similar polyamine deregulation accompanies tumor progression in the TH-MYCN mouse model. TH-MYCN mice have neural crest-targeted Mycn and develop tumors comparable to human neuroblastoma at the genetic (31), histologic (32) and ultrastructural level (23). The model is highly aggressive with 100% tumor penetrance in transgene homozygous (TH-MYCN +/+) mice with lethality by 7 weeks of age. Penetration is ~40% with extended latency in hemizygous (TH-MYCN +/-) mice (26).

Gene expression profiles were derived from nascent tumors through large invasive tumors (24) and compared to non-tumor cervical ganglia from both wild-type and TH-MYCN +/- mice (Figure 1). Like human neuroblastomas, the polyamine pathway was coordinately deregulated to expand polyamine capacity. ODC1 was upregulated while its antizyme, OAZ2, was downregulated. Pro-synthetic spermidine and spermine synthases (SRM and SMS) were also upregulated. Conversely, catabolic SAT1 and spermine oxidase, SMOX, were downregulated, while polyamine oxidase, PAOX, which cleaves acetylated polyamines to restore spermidine and/or putrescine was elevated. This pattern closely recapitulates the transcriptional changes found in human neuroblastomas and credentials the TH-MYCN model for pre-clinical polyamine antagonist studies (Supplementary Table S1).

Polyamine antagonists collaborate to block neuroblastoma initiation. We previously used the TH-MYCN model to show that pre-emptive DFMO therapy delayed tumor onset in homozygous mice, however, tumor penetrance remained complete (18). Tumors harvested from DFMO-treated mice had reduced putrescine levels reflecting Odc inhibition (on-target DFMO activity), however, spermidine and spermine were not reduced. We hypothesized that augmented Amd1 activity induced
Following DFMO exposure in vitro (33) and in vivo (19) might contribute to the rescue of Odc-inhibited cells. Since transcriptional changes were seen at the earliest stage of tumorigenesis, and since polyamine antagonists have chemopreventive activity in individuals at-risk for colon cancer (34), we reasoned that polyamines were required for tumor initiation. We tested the effect of pre-emptively inhibiting both Odc1 and Amd1 on tumor penetrance and latency in this model.

SAM486 (Sardomozide, Novartis) is an Amd1 inhibitor that demonstrates variable cytotoxicity against human neuroblastoma cells and lesser activity against murine TH-MYCN tumor cells (Supplementary Figure S2). We randomized TH-MYCN mice to receive SAM486 alone or with DFMO. SAM486 was initiated at day 21 when mice were of sufficient size for serial IP injections, relatively late with respect to tumor onset around day 28; (26, 35). Still, treatment with SAM486 reduced tumor penetrance in homozygous mice (p=0.02; Figure 2 and Supplementary Table S2). Notably, the combination of DFMO and SAM486 extended tumor latency (p<0.001) and reduced penetrance to ~60% in homozygous mice (n=67; p<0.01; Figure 2A-B) and from 66% to 17% in hemizygous mice (n=213; p<0.001; data not shown). Although therapy was stopped at day 70, tumors were rarely detected after this time-point. Tumors that arose under SAM486 (p=0.06), or SAM486+DFMO (p<0.05), showed neural differentiation, an effect typical of cytotoxic chemotherapy (36) that was absent from control tumors (Figure 2C). Tumors did not differ in hemorrhage, necrosis, mitosis/karrhyorhexis, or proliferative and apoptotic indices at the time of terminal tumor progression (Figure 2D). On-target activity was supported by reduced putrescine in DFMO and SAM486+DFMO treated tumors, while the addition of SAM486 to DFMO led further to reduced spermidine and spermine, biomarkers of Amd1 inhibition (Figure 2E).

Therapy with SAM486 or SAM486+DFMO was well tolerated. SAM486 treated mice grew at the same rate as control mice (day 42 weight 23.9±2.6 gms versus 25.2±2.3 gms; p=0.35). Mice treated with SAM486+DFMO were smaller at day 21, 17.1±2.9 gms versus 21.3±1.8 gms (p=0.01),
reflecting DFMO effects since SAM486 therapy started at day 21, but through day 42 mice treated with SAM486+DFMO, like DFMO-only treated mice, trended toward catch-up growth (Supplementary Figure S3). Thus, dual inhibition of the rate-limiting enzymes in polyamine biosynthesis reduced neuroblastoma initiation and this was correlated with augmented putrescine and spermidine deprivation, supporting a potent role for polyamines in MYCN oncogenesis. Tumors that did arise under polyamine deprivation stress had increased neural differentiation, a favorable histologic finding, compared with polyamine sufficient tumors.

**DFMO, but not SAM486, synergizes with chemotherapy in treating established tumors.**

We previously showed that DFMO treatment of TH-MYCN mice with palpable neuroblastomas extended overall survival (p<0.01) and enhanced chemotherapy activity, increasing survival when combined with vincristine, cyclophosphamide, or cisplatinum; and increasing durable complete regressions when combined with cisplatinum (18). We extended this testing additional regimens used for neuroblastoma therapy at diagnosis or following relapse and show that the addition of DFMO also improves survival when given with topotecan (p=0.02), topotecan and cyclophosphamide (p<0.02), temozolomide (p=0.03) or irinotecan (p<0.01); and induces durable complete regressions when given with irinotecan (p<0.05; Table 1 and Supplementary Figure S4). Therefore, we confirmed that the addition of DFMO improved outcomes across diverse chemotherapy classes (camptothecins, alkylators, platinators and microtubule poisons) without evident antagonism.

We next tested the addition of SAM486 to DFMO in the treatment of established tumors. TH-MYCN mice with palpable tumors were randomized to DFMO+SAM486, or vehicle, with or without cyclophosphamide (Figure 3A). Mice treated with DFMO+SAM486 (without chemotherapy) had survival increased >40% compared with vehicle treated mice (median 22 days versus 15 days; p=0.006) similar to effects of DFMO alone (18). The addition of SAM486 and DFMO to cyclophosphamide therapy did not improve outcome over cyclophosphamide alone but there was
instead a trend toward reduced survival (p=0.11). SAM486+DFMO was similarly tested in neuroblastoma xenograft models. Mice harboring IMR5 xenografts (MYCN amplified) had extended survival when DFMO (p<0.05) or DFMO+SAM486 (p<0.03) was added to cyclophosphamide. However, the addition of SAM486 to DFMO and cyclophosphamide did not further improve survival over DFMO and cyclophosphamide alone (p=0.40; Supplementary Table S2). Mice harboring BE2C tumor xenografts (MYCN amplified, TP53 mutant, multidrug resistant) were treated with cyclophosphamide and topotecan, a regimen often used after relapse, and again DFMO+SAM486 failed to improve survival (p=0.62; Figure 3B). Thus, SAM486 does not potentiate the activity of DFMO with chemotherapy in treating established murine or human neuroblastomas despite showing synergy in the pre-emptive therapy setting.

**Disabling polyamine synthesis leads to compensatory increases in polyamine import.**

Augmented uptake of polyamines from the microenvironment may rescue polyamine-depleted tumor cells (37). The mammalian polyamine transporter remains poorly characterized but spermidine uptake is a biomarker of this activity. Following Odc1 and/or Amd1 inhibition both murine and human neuroblastoma cells increased spermidine import up to 8-fold in response to polyamine depletion (Supplementary Figure S5). Adding polyamines to the culture media rescued DFMO-mediated inhibition in colony formation supporting polyamine uptake as functionally relevant (Figure 3C). Spermidine/spermine-N\(^1\)-acetyltransferase (SAT1) activity acetylates polyamines to promote their export from the cell and regulates flux through the pathway (13). We reasoned that SAT1 induction might further deplete non-acetylated polyamine pools. Cyclooxygenase inhibitors induce SAT1 and synergize with DFMO in reducing colorectal tumor recurrence in humans and in mouse models (38, 39). Since cox-2 is expressed in neuroblastoma and its inhibition by celecoxib has anti-tumor effects (40) we assessed celecoxib for synergy with DFMO.
Neuroblastoma cells modestly upregulated SAT1 mRNA and protein following celecoxib exposure (Supplementary Figure S6A-B). Although this led to reduced net polyamine import under basal conditions, it could not attenuate the increase in uptake following DFMO-mediated polyamine depletion (Figure 3D). However, polyamine acetylation and export is not reflected by spermidine uptake so we measured non-acetylated polyamine levels to show DFMO alone or with celecoxib markedly reduced putrescine and spermidine, while spermine levels were preserved (Figure 3E). In contrast, the addition of SAM486 to DFMO prevented putrescine and spermidine depletion by inhibiting Amd1. Neither the addition of supplementary polyamines nor extended exposure times altered these changes (Supplementary Figure S7). Celecoxib itself had no cytotoxicity against murine neuroblastoma cells, and had activity for two of five human neuroblastoma cell lines but only at exposures ≥100 μM not achieved with therapeutic dosing (Supplementary Figure S6C). At these higher concentrations we showed that supplementation with additional polyamines could partially rescue survival, supporting polyamine depletion as contributing to celecoxib effects (Supplementary Figure S6D). We next tested for synergy between DFMO and celecoxib in BE2C cells across concentrations achievable in vivo. DFMO and celecoxib showed synergy in reducing colony formation (Combination Index, CI, of 0.73) with virtual elimination of colonies at higher concentrations (Figure 3F). Synergy was not observed in SK-N-SH cells (absent MYCN amplification) when a CI was calculated from all 4 drug concentrations studied, however, synergy was observed (CI=0.56) when the highest concentration was excluded. We next assessed celecoxib with mafosfamide (the activated metabolite of cyclophosphamide) and demonstrated colony formation inhibition for both BE2C (CI=0.87) and SK-N-SH (CI=0.81) cells (Figure 3F).

Celecoxib synergizes with DFMO in vivo. Pre-emptive therapy of homozygous TH-MYCN mice with celecoxib alone from day 21 onward extended tumor free survival similar to DFMO (p=0.01; and Supplementary Table S2). We next tested celecoxib in the treatment of established tumors, combined with DFMO and/or chemotherapy. Unlike DFMO, celecoxib did not provide a survival
Polyamine antagonism in Neuroblastoma

Haber, et al., 17

benefit in the absence of cytotoxic agents, however, the combination of DFMO and celecoxib did (p=0.03; Table 1). With cyclophosphamide, the addition of either celecoxib (p<0.10), DFMO (p<0.10), or both celecoxib and DFMO (p<0.08) led to a trend toward improved survival, while mice treated with any polyamine antagonist (combined) had improved survival (p<0.02; Figure 4A-B). We next studied DFMO and celecoxib with two additional chemotherapy combinations, showing markedly extended survival when added to cyclophosphamide and topotecan (p<0.0005; Figure 4C) or irinotecan and temozolomide (p<0.0001; Figure 4D), compared with chemotherapy alone.

To evaluate the impact of DFMO and celecoxib in treating established human neuroblastomas we used xenografted tumors with high-risk genetic alterations, using IMR5 cells (MYCN amplified) and SK-N-SH cells (MYCN non-amplified, ALK F1174L mutant) established from tumors at diagnosis, and multidrug resistant BE2C cells (MYCN amplified and TP53 C135F mutant) established at the time of relapse (Table 1). The addition of DFMO to cyclophosphamide extended survival in mice harboring IMR5 tumors (p<0.05), including a durable complete response (culled at day 65) while all cyclophosphamide alone-treated mice were culled for tumor progression by day 26. The addition of celecoxib to DFMO and cyclophosphamide did not further extend survival (p=0.33; data not shown). Treatment of mice harboring SK-N-SH xenografts showed that even in the absence of chemotherapy the combination of DFMO and celecoxib extended survival (p<0.02; Figure 5A). One DFMO and celecoxib treated mouse was sacrificed for poor weight gain following complete tumor regression (no tumor at necropsy). Another had delayed tumor growth with sacrifice at >275 days. When combined with cyclophosphamide, the addition of DFMO and celecoxib increased survival (p<0.04). No mouse treated only with cyclophosphamide had a durable regression. In contrast, one mouse treated with cyclophosphamide and DFMO had slow tumor growth through day ~160 followed by regression until sacrifice at day 293, and 3 of 8 mice (37%) treated with cyclophosphamide, DFMO and celecoxib had complete and durable tumor regressions despite receiving no additional chemotherapy retreatment (Figure 5B).
The post-relapse BE2C xenograft was tested with two separate chemotherapy backbones, to define whether relatively therapy resistant tumors might have similar vulnerabilities. Remarkably, DFMO alone, celecoxib alone, or DFMO + celecoxib all extended survival of xenograft bearing mice >50% compared with control treated mice (p<0.01, p=0.02 and p<0.01, respectively), a survival advantage similar to that achieved by cyclophosphamide (Supplementary Figure S8). When added to cyclophosphamide, the combination of DFMO and celecoxib extended median survival ~40% though all mice were eventually culled for tumor progression (p<0.01; data not shown). Using a similarly non-curative cyclophosphamide and topotecan regimen, the addition of DFMO alone trended toward extended survival (p<0.10), while the combination of DFMO and celecoxib together extended survival (p<0.02) and led to durable complete regressions in 2 of 12 mice (Figure 5C-D). At the time of sacrifice no residual tumor could be identified nor could a cell line be propagated from the tumor residua.
DISCUSSION

Myc proteins are attractive drug targets as they are among the most frequent somatically activated genes in cancer (2) and drive diverse neoplasia-enabling transcriptional programs (41). Systemic genetic inhibition of MYC has been shown to provide anti-tumor efficacy with tolerable toxicity (42) supporting that a therapeutic index exists for MYC-directed therapies. Unfortunately, MYC operates through a network of broad, flat protein-protein interactions and pharmacologic inhibition has remained challenging. An alternative to directly antagonizing MYC is to target the downstream pathways necessary for its oncogenic activity. MYC drives resource utilization toward biomass production, including a dramatic increase in protein synthetic capacity (43). This evolutionarily conserved role in protein synthesis is critical to Myc oncogenesis and its inhibition has synthetic-lethal consequences (44). Among the genes highly regulated by MYC are polyamine homeostatic enzymes, constituting a core expression program downstream of MYC in transformation (45).

That polyamine sufficiency is required to support Myc oncogenesis was initially revealed in the Eu-MYC lymphoma model where genetic (ODC +/-) or biochemical (DFMO) polyamine antagonism was shown to extend tumor latency (46). We therefore explored its requirement in neuroblastomas, in which hyperactivation of MYCN through genomic amplification is present in 40% of high-risk tumors (47) and indirect deregulation of MYCN or MYC occurs in a large fraction of the remainder (17). Further, up to 20% of MYCN amplified tumors have co-amplification of ODC1, demonstrating targeted deregulation of an oncogenic transcription factor and its oncogenic target gene (18, 48, 49). Indeed, high-risk neuroblastomas showed coordinate deregulation of polyamine enzymes in a direction to support polyamine sufficiency (18) while high ODC1 expression was independently prognostic for poor outcome, supporting its potential value of this oncogene as a drug target.

We previously showed that pre-emptive DFMO therapy extended tumor latency in the TH-MYCN model, and that DFMO improved tumor free and overall survival when added to chemotherapy
Polyamine antagonism in Neuroblastoma

Haber, et al., 20

treatment of established tumors. Here we provide extended pre-clinical testing using robust complementary models that support transitioning optimized polyamine antagonist therapies to the clinic. In strong support that polyamine sufficiency is required for MYC-mediated oncogenesis, we show that inhibiting Odc1 and Amd1 with DFMO and SAM486 reduces tumor penetrance by ~75% in hemizygous mice and ~40% in homozygous mice (where lethal tumor penetrance is 100% without treatment). Tumors rarely arose following withdrawal of therapy suggesting a sustained chemopreventive effect. It is not clear whether this pre-emptive therapy eradicates nascent tumors or has specific effects that inhibit initiation or tumor stem cell viability. The latter is indirectly supported by the lack of SAM486 activity when added to DFMO in the treatment of established tumors, the finding that ODC1 expression is enriched in neuroblastoma tumor-initiating cell populations (50), and that AMD1 is both essential for stem cell self-renewal and downregulated concurrent with differentiation to the neural lineage (51).

Treating established tumors failed to show an additive benefit for combining Amd1 inhibition with Odc1 inhibition, in contrast to the potent activity seen in the pre-emptive setting. A metabolomic approach applied to colorectal cancer models led to the hypothesis that reduced cellular thymidine pools contributes to DFMO-induced cytostatic activity (52). DFMO-mediated polyamine depletion leads to compensatory hyperactivation of Amd1 with futile S-adenosylmethionine consumption, followed by depletion of folate-dependent metabolites and thymidine. Thymidine replacement rescued the cytostatic effect of DFMO without restoring polyamine levels. This is notable since the addition of SAM486 to DFMO is predicted by this model to antagonist the activity of DFMO by attenuating thymidine depletion. This is consistent with our findings but will require direct evaluation, including assessing these metabolites in both pre-emptive and established tumor models.

In seeking additional opportunities to antagonize polyamine metabolism, the cox-2 inhibitor celecoxib seemed particularly promising. Celecoxib induces SAT1, has intrinsic Odc inhibiting activity
Polyamine antagonism in Neuroblastoma

Haber, et al., 21

(53), and has been studied extensively in cancer trials together with chemotherapy, including in pediatric trials (54). Moreover, strong proof of concept for combining cyclo-oxygenase inhibition with DFMO was demonstrated by the striking reduction in recurrent colon adenoma in at-risk individuals through the preventive use of DFMO and sulindac (34). Consistent with this, the addition of celecoxib to DFMO extended survival when treating both murine (TH-MYCN) and human (xenograft) neuroblastomas, in combination with numerous clinically relevant chemotherapy regimens, and using genomically distinct high-risk tumor types (MYCN amplification, ALK mutation, TP53 mutation). It is plausible that celecoxib provides additional benefit to DFMO through the effects on spermidine depletion (an effect antagonized when SAM486 was added to DFMO), as this may be a biomarker for protein translation inhibition induced by polyamine deprivation via effects on eIF5A activities. However, pleiotropic cyclo-oxygenase activities have been postulated to impact tumor phenotype, including in neuroblastoma (55, 56), and warrant further consideration. Indeed, celecoxib has anti-tumor activity alone and combined with chemotherapy in neuroblastoma xenograft studies in which polyamine depletion stress is absent (40).

Although our pre-clinical studies support a cancer cell intrinsic activity for polyamine depleting agents, it does not exclude the contribution of cancer cell extrinsic activities. Indeed, polyamines (via the arginine-ornithine-polyamine axis) contribute to a tumor permissive microenvironment through myriad effects on immunosurveillance mechanisms (57-59), so efforts to study polyamine depletion in this context are warranted. Indeed, cyclo-oxygenase activity in tumors contributes to immune evasion and biochemical inhibition of these pathways restores anti-tumor immunity (60), providing an additional potential mechanism for the enhanced anti-tumor activities seen with DFMO and celecoxib in our models. Overall, demonstrating activity across a spectrum of pre-clinical models and in multiagent combinations, as done here, has been recommended in efforts to improve the rate of successful translation into human cancer trials (21). Our data support the integration of DFMO and
cyclo-oxygenase inhibitors such as celecoxib into chemotherapeutic regimens for neuroblastoma and potentially other MYC-driven embryonal cancers (61).
ACKNOWLEDGEMENTS

The authors gratefully acknowledge Pat Woster (Medical University of South Carolina) for DFMO; Robert Cozens (Novartis, Basel, Switzerland) for SAM486; William Weiss (UCSF) for TH-MYCN mice; Naomi Balamuth and John Maris (University of Pennsylvania) for TH-MYCN model transcriptome data-sets; Ashleigh Clark and Michelle Ruhle for expert technical assistance; and Andre Bachmann (Michigan State University) for helpful discussions.
REFERENCES

Polyamine antagonism in Neuroblastoma

Haber, et al., 26


52. Witherspoon M, Chen Q, Kopelovich L, Gross SS, Lipkin SM. Unbiased metabolite profiling indicates that a diminished thymidine pools is the underlying mechanism of colon cancer chemoprevention by alpha-difluoromethylornithine (DFMO). Cancer Discov 2013.


FIGURE LEGENDS

Figure 1. Coordinate deregulation of polyamine enzymes accompanies tumor progression in the TH-MYCN model. Polyamine gene expression levels for non-tumorous cervical ganglia (CG) from wild-type mice [white bars, n=3] and TH-MYCN +/+ mice [grey bars, n=9]; compared with expression from neuroblastomas (NB) arising in TH-MYCN +/+ mice [black bars, n=6 per group] at four stages of progression. NB-S, NB-M, NB-L and NB-UL represent small, medium, large, and ultra-large neuroblastomas, respectively, as defined in (24); *, Wilcoxon Exact p-values <0.05 in comparison with wild-type CG. Standard deviation error bars are shown. WT, wild-type.

Figure 2. Dual polyamine antagonism blocks neuroblastoma initiation. (A), Tumor free survival for TH-MYCN +/+ (n=68) treated with vehicle (n=19), SAM486 (n=25) or SAM486+DFMO (n=23); p-values by the method of Kaplan and Meier; all pair-wise comparisons are p<0.05 by log-rank test. Two mice were censored (depicted by a black-circle) at the time of procedure-related death in the absence of tumor. (B), Relative change in tumor penetrance in TH-MYCN +/+ and +/- mice treated with DFMO, SAM486 or both DFMO and SAM486 compared with genotype-matched contemporaneous vehicle treated mice (numbers of mice/group are shown). (C), Representative H&E stains of tumors demonstrating neural differentiation (arrowheads) induced by polyamine antagonist therapy; (D), Tumors arising under these conditions did not differ in necrosis, hemorrhage or proliferative (Ki67 IHC) or apoptotic (activated Caspase 3 IHC) indices. (E), Evidence for polyamine depletion in TH-MYCN tumors by dual antagonism with DFMO and SAM486. Ctrl, control; D, DFMO treated; S, SAM486 treated; D+S, DFMO and SAM486 treated. *, p<0.05 by two-tailed t-test; **, p<0.005 by two-tailed t-test; NS, not significant. Note: for panels B-E, DFMO-treated tumors were obtained from a prior DFMO trial in a manner identical to the other groups herein (18).

Figure 3. Effects of combined polyamine antagonist therapy. (A), TH-MYCN mice (hemizygous and homozygous; total n=126) with palpable tumors were randomized to receive DFMO+SAM486
Polyamine antagonism in Neuroblastoma

Haber, et al., 28

(n=33), DFMO+SAM486 with cyclophosphamide (n=35), cyclophosphamide alone (n=35), or vehicle (n=23). DFMO+SAM486 extended survival over control mice (p=0.006), as did cyclophosphamide (p<0.001). DFMO+SAM486 did not improve survival on a backbone of cyclophosphamide, however, but instead trended toward antagonism of efficacy (p=0.11). The same findings were seen when analyzed after stratifying by TH-MYCN genotype (data not shown). (B), BE2C xenografts established in NCR nu/nu mice (n=12 per arm) were treated with DFMO + SAM486 on a backbone of cyclophosphamide and topotecan, and compared with cyclophosphamide/topotecan alone or vehicle control. The addition of DFMO + SAM486 did not improve survival when added to chemotherapy when compared with chemotherapy alone (p=0.62). Treatment annotation is provided above the Kaplan-Meier curve and detailed in Methods. (C), Polyamine uptake is functionally relevant in neuroblasts as the effects of DFMO on colony formation activity are completely (844) or partially (IMR5) rescued through the provision of supplemental polyamines to the culture media. (D), Spermidine uptake for murine (TH-MYCN 282 and 844) and human (IMR5, BE2C, SK-N-AS) neuroblastoma cell lines, with or without pre-exposure to DFMO and/or celecoxib, as indicated. DFMO-mediated polyamine depletion induces increased spermidine uptake. Although celecoxib by itself reduces basal uptake it does not attenuate the increased uptake following DFMO exposure. (E), Polyamine antagonists alone or in combination (48 hour exposure) deplete neuroblastoma cells of polyamines, with profound reductions in putrescine and spermidine in DFMO and DFMO + celecoxib treated cells, while SAM486 rescues the spermidine depletion. For panels C-E: DFMO, 5 mM; SAM (SAM486), 5 µM; CEL (celecoxib), 10 µM; PAs, all polyamines added at 1 µM each. *, p<0.05 by two-tailed t-test compared with control, error bars show SEM. (F), DFMO and celecoxib (top), and celecoxib and mafosfamide (bottom) synergize to inhibit colony formation in BE2C cells. A combination index (CI) of <0.9 indicates synergy: for DFMO and celecoxib, CI = 0.73; for celecoxib and mafosfamide, CI = 0.87. Wells shown are representative of those generated by exposure of cells to the highest concentrations of each drug used to generate the median effect plot, as described in Methods.
Figure 4. Impact of DFMO and Celecoxib on established neuroblastomas in *TH-MYCN* mice. (A), Homozygous *TH-MYCN* mice (n=9-12 per arm) with palpable tumors were randomized to receive cyclophosphamide alone, or with the addition of DFMO, Celecoxib, or both DFMO and Celecoxib. Each arm that included a polyamine antagonist trended toward improved survival compared with cyclophosphamide alone (p<0.10 for all comparisons, as indicated). (B), Analysis of the same experimental data in A with the three polyamine antagonist arms combined (“any polyamine antagonist”) illustrates the survival benefit provided by a DFMO and/or Celecoxib based polyamine antagonist regimen when combined with cyclophosphamide chemotherapy (p<0.02). (C), Homozygous *TH-MYCN* mice (n=10 per arm) with palpable tumors were randomized to the combination of DFMO + Celecoxib on a backbone of cyclophosphamide and topotecan, a regimen commonly used for relapsed or refractory neuroblastoma, or chemotherapy alone. The addition of DFMO and Celecoxib improved survival (p=0.0005). (D), Similar results were obtained using an alternative salvage chemotherapy backbone, irinotecan and temozolomide, as the addition of DFMO and celecoxib improved survival compared with chemotherapy alone (p<0.001); p-values by the method of Kaplan and Meier. Treatment annotation is provided above the Kaplan-Meier curve and detailed in Methods.

Figure 5. Impact of DFMO and Celecoxib on neuroblastomas xenografts, alone and with chemotherapy. (A), BALB/c nu/nu mice (n=7-8 per arm) harboring SK-N-SH xenografts were randomized to receive DFMO, Celecoxib, or both (in the absence of chemotherapy). Neither DFMO nor Celecoxib alone significantly extended survival, however, DFMO + Celecoxib did (p<0.02) with a >2-fold extension in median survival. (B), The same model was used to test these combinations with cyclophosphamide: again, neither DFMO nor Celecoxib extended survival when added to cyclophosphamide, however, the combination of DFMO + Celecoxib did (p<0.04). All
cyclophosphamide-only treated animals were culled for tumor progression, one DFMO and
cyclophosphamide treated mouse had a late tumor regression with no recurrence, and three mice (of
8) treated with both DFMO + Celecoxib and cyclophosphamide had durable complete regressions
with no tumor at necropsy. (C); NCR nu/nu mice harboring therapy resistant BE2C xenografts were
randomized to receive cyclophosphamide and topotecan with or without DFMO or DFMO + Celecoxib,
or control (n=12 per arm). Mice receiving DFMO had a trend toward extended survival (p<0.10), while
mice receiving both DFMO + Celecoxib had markedly extended survival (p<0.02). (D), Tumor volume
over time is shown for mice in (C) receiving either cyclophosphamide/topotecan alone or combined
with DFMO + Celecoxib. As with SK-N-SH tumors, some mice receiving both DFMO + Celecoxib had
late and complete tumor regressions, despite receiving only a single chemotherapy course (days 1-3);
p-values by the method of Kaplan and Meier are shown. Treatment annotation is provided above the
Kaplan-Meier curve and detailed in Methods.
Table 1. Summary of pre-clinical efficacy data treating established neuroblastomas in complementary *TH-MYCN* and human xenograft models with polyamine antagonists.

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment Arms*</th>
<th>Outcomes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TH-MYCN +/- Tumors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle DFMO</td>
<td>DFMO alone improves survival</td>
<td>p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Celecoxib</td>
<td>DFMO + Celecoxib improves survival (NS compared with DFMO alone)</td>
<td>p&lt;0.03</td>
<td></td>
</tr>
<tr>
<td><strong>Vehicle</strong></td>
<td>DFMO + SAM486</td>
<td>DFMO + SAM486 improves survival (NS compared with DFMO alone)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>VCR</td>
<td>VCR + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.02 (Ref. 18)</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + DFMO</td>
<td>Addition of DFMO improves survival; more mice cured</td>
<td>p=0.03 (Ref. 18)</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.05</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.05</td>
</tr>
<tr>
<td>CISPLAT</td>
<td>CISPLAT + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.01 (Ref. 18)</td>
</tr>
<tr>
<td>IRN</td>
<td>IRN + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>TOPO</td>
<td>TOPO + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.02</td>
</tr>
<tr>
<td>TEM</td>
<td>TEM + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.08</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.10</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + Celecoxib**</td>
<td>Addition of Celecoxib trends toward improved survival</td>
<td>p&lt;0.08</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + DFMO</td>
<td>Addition of DFMO + Celecoxib trends toward improved survival</td>
<td>p=0.02</td>
</tr>
<tr>
<td><em><strong>Any polyamine depleting regimen (combined) improves survival</strong></em></td>
<td>CPM + DFMO + Celecoxib**</td>
<td>Addition of DFMO + Celecoxib improves survival</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>IRN/TEM</td>
<td>IRN/TEM + DFMO + Celecoxib</td>
<td>Addition of DFMO + Celecoxib improves survival</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CPM/TOPO</td>
<td>CPM/TOPO + DFMO + Celecoxib</td>
<td>Addition of DFMO + Celecoxib improves survival</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CPM/TOPO</td>
<td>CPM/TOPO + DFMO</td>
<td>Addition of DFMO improves survival</td>
<td>p=0.01</td>
</tr>
<tr>
<td>CPM/TOPO</td>
<td>CPM/TOPO + SAM486</td>
<td>Addition of DFMO + SAM486 improves survival (NS compared with DFMO alone)</td>
<td>p&lt;0.06</td>
</tr>
<tr>
<td>CPM</td>
<td>CPM + DFMO + SAM486</td>
<td>Addition of DFMO + SAM486 improves survival (NS compared with DFMO alone)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

| **TH-MYCN +/- Tumors**     |                 |                                                                          |               |
| Vehicle DFMO + SAM486      | NS              |                                                                          |               |
| TEM                        | TEM + DFMO      | Addition of DFMO improves survival                                        | p=0.03        |
| CPM                        | CPM + DFMO + SAM486 | Addition of DFMO + SAM486 improves survival (NS compared with DFMO alone) | p=0.03        |

| **BE2C Xenografts (MYCN amplified; TP53 mutation)** |                 |                                                                          |               |
| Vehicle DFMO               | DFMO alone improves survival | p<0.01                                                                 |               |
| Celecoxib                  | Celecoxib alone improves survival | p<0.03                                                                 |               |
| CPM                        | CPM + Celecoxib  | CPM + Celecoxib improves survival                                        | p<0.01        |
| CPM                        | CPM + DFMO      | NS                                                                        |               |
| CPM + Celecoxib            | NS              |                                                                          |               |
| CPM + DFMO + Celecoxib     | Addition of DFMO + Celecoxib improves survival                          | p=0.01        |
| CPM/TOPO                   | CPM/TOPO + DFMO | Addition of DFMO improves survival                                        | p=0.02        |
| CPM/TOPO + SAM486          | NS              |                                                                          |               |

| **IMR5 Xenografts (MYCN amplified)** |                 |                                                                          |               |
| CPM + DFMO                  | Addition of DFMO improves survival                                     | p<0.05        |
| CPM + DFMO + Celecoxib      | NS                                                                        |               |
| CPM + DFMO + SAM486         | Addition of DFMO + SAM486 improves survival (NS compared with DFMO alone) | p=0.03        |

| **SK-N-SH Xenografts (ALK F1174L mutation)** |                 |                                                                          |               |
| Vehicle DFMO                | NS              |                                                                          |               |
| Celecoxib                  | NS              |                                                                          |               |
| CPM + DFMO + Celecoxib***   | DFMO + Celecoxib improves survival (compared to DFMO, Celecoxib or Vehicle alone) | p=0.02        |
| CPM + Celecoxib***          | DFMO + Celecoxib improves survival compared with CPM                   | p=0.08        |
| CPM + DFMO + Celecoxib      | Addition of DFMO + Celecoxib improves survival                          | p<0.04        |

* CPM, cyclophosphamide; CISPLAT, cisplatinum; IRN, irinotecan; TOPO, topotecan; VCR, vincristine; TEM, temozolomide; NS, not significant.
**Figure 2.**

(A) *TH-MYCN +/+* 

![Graph showing Tumor Free Survival (TFS) over time.](#)

- **Control**
- **SAM486 (d21-70)**
- **SAM486 (d21-70) + DFMO (d1-70)**

(B) [% change in tumor penetrance](#)

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Change in Tumor Penetrance</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFMO</td>
<td>0%</td>
</tr>
<tr>
<td>SAM486</td>
<td>-24%</td>
</tr>
<tr>
<td>DFMO + SAM486</td>
<td>-39%</td>
</tr>
<tr>
<td>DFMO</td>
<td>-64%</td>
</tr>
<tr>
<td>SAM486 + DFMO</td>
<td>-74%</td>
</tr>
</tbody>
</table>

(C) ![Images of histological sections](#)

(D) [% Necrosis, % Hemorrhage, Ki67, Casp3](#)

(E) ![Box plots for Putrescine, Spermidine, and Spermine](#)
**Figure 3.**

**A.** TH-MYCN

* Cyclophosphamide (d1-5)  
* SAM486 (3x/wk d1-42)  
DFMO Rx

Survival vs. Time (days)

- Control  
- SAM486 + DFMO  
- Cyclophosphamide  
- SAM486 + DFMO + Cyclophosphamide

**B.** BE2C

* Cyclophosphamide (d1-3)  
* SAM486 + DFMO + Cyclophosphamide

Survival vs. Time (days)

- Control  
- Cyclophosphamide/Topotecan  
- SAM486 + DFMO + Cyclophosphamide/Topotecan

**C.** 844 cells

- Control  
- DFMO  
- DFMO + PAs

IMR5 cells

- Control  
- DFMO  
- DFMO + PAs

**D.** Spd Uptake

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TH-MYCN</th>
<th>BE2C</th>
<th>IMR5</th>
<th>BE2C</th>
<th>SK-N-AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFMO:</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Celecoxib:</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**E.**

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>IMR5</th>
<th>BE2C</th>
<th>844+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>[Graph]</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Spermine</td>
<td>[Graph]</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Spermidine</td>
<td>[Graph]</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
</tbody>
</table>

**F.**

- Control  
- DFMO  
- Celecoxib  
- DFMO + Celecoxib

- Mafosfamide  
- Celecoxib  
- Celecoxib + Mafosfamide
A. TH-MYCN +/+ 

Survival

* CPM (d1-5) 

Cyclophosphamide (CPM) 
CPM + DFMO  p<0.10 
CPM + celecoxib  p<0.10 
CPM + DFMO + celecoxib  p<0.08 

B. TH-MYCN +/+ 

Survival

* CPM (d1-5) 

Cyclophosphamide (CPM) 

CPM + any polyamine antagonist 

C. TH-MYCN +/+ 

Survival

* CPM+Topo (d1-5) 

Control 
Cyclophosphamide/Topotecan (CPM/Topo)  p=0.0005 
CPM/Topo + DFMO/Celecoxib 

D. TH-MYCN +/+ 

Survival

* IRN+TEM (d1-5) 

Control 
Irinotecan/Temozolomide (IRN/TEM)  p<0.0001 
IRN/TEM + DFMO/Celecoxib 

Figure 4.
Figure 5.

A. SK-N-SH xenografts

B. SK-N-SH xenografts

C. BE2C xenografts

D. BE2C xenografts
Polyamine antagonist therapies inhibit neuroblastoma initiation and progression

Nicholas F. Evageliou, Michelle Haber, Annette Vu, et al.

Clin Cancer Res Published OnlineFirst March 24, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-15-2539

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/03/24/1078-0432.CCR-15-2539.DC1

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, use this link http://clincancerres.aacrjournals.org/content/early/2016/03/24/1078-0432.CCR-15-2539. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.