**ODC1 Is a Critical Determinant of MYCN Oncogenesis and a Therapeutic Target in Neuroblastoma**

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**Abstract**

Neuroblastoma is a frequently lethal childhood tumor in which MYC gene deregulation, commonly as MYCN amplification, portends poor outcome. Identifying the requisite biopathways downstream of MYC may provide therapeutic opportunities. We used transcriptional analyses to show that MYCN-amplified neuroblastomas have coordinately deregulated myriad polyamine enzymes (including ODC1, SRM, SMS, AMD1, OAZ2, and SMOX) to enhance polyamine biosynthesis. High-risk tumors without MYCN amplification also overexpress ODC1, the rate-limiting enzyme in polyamine biosynthesis, when compared with lower-risk tumors, suggesting that this pathway may be pivotal. Indeed, elevated ODC1 (independent of MYCN amplification) was associated with reduced survival in a large independent neuroblastoma cohort. As polyamines are essential for cell survival and linked to cancer progression, we studied polyamine antagonism to test for metabolic dependence on this pathway in neuroblastoma. The Odc inhibitor α-difluoromethylornithine (DFMO) inhibited neuroblast proliferation in vitro and suppressed oncogenesis in vivo. DFMO treatment of neuroblastoma-prone genetically engineered mice (TH-MYCN) extended tumor latency and survival in homozygous mice and prevented oncogenesis in hemizygous mice. In the latter, transient Odc ablation permanently prevented tumor onset consistent with a time-limited window for embryonal tumor initiation. Importantly, we show that DFMO augments antitumor efficacy of conventional cytotoxics in vivo. This work implicates polyamine biosynthesis as an arbiter of MYCN oncogenesis and shows initial efficacy for polyamine depletion strategies in neuroblastoma, a strategy that may have utility for this and other MYC-driven embryonal tumors. [Cancer Res 2008;68(23):9735–45]

**Introduction**

Neuroblastoma is a common childhood tumor arising within the peripheral nervous system. The genetic feature most consistently associated with treatment failure is amplification of the MYCN proto-oncogene that strongly correlates with advanced disease (1, 2). Even in otherwise favorable localized disease, MYCN amplification portends poor outcome, underscoring its biological importance (3). In high-risk neuroblastomas that lack MYCN amplification, MYC itself may be deregulated (4, 5). Myc genes (including MYCN, MYC, and MYCL1) represent a family of basic helix-loop-helix leucine zipper transcription factors that are among the most frequently deregulated genes in cancer. Myc proteins form heterodimers with Max and are recruited to CACGTG (E-box) recognition sequences to transactivate target genes, or enter additional complexes to form repressors (reviewed in refs. 6, 7). It has been estimated that nearly one tenth of all genes may be directly or indirectly regulated by the Myc:Max axis (8), yet the determination of those necessary or sufficient to confer oncogenic properties remains empiric.

One compelling target that may be decisive in mediating MYC effects is ODC1 (9), a bona fide oncogene that encodes the rate-limiting enzyme in polyamine synthesis (10). Polyamines are organic cations that enhance transcription, translation, and replication (11) and support many cellular processes governed by MYC genes. Their maintenance is essential for cell survival as depletion activates growth arrest or apoptotic checkpoints (12). Thus, intracellular polyamines are kept under tight control through posttranscriptional as well as transcriptional regulation, with the rate-limiting enzymes ODC1 and AMD1 having among the shortest half-lives of any mammalian enzyme as a result (13). Odc activity is frequently elevated in cancer through deregulation of MYC, resulting in higher polyamine content to support rapid tumor cell proliferation (11). Considerable evidence links elevated polyamines to colon, breast, prostate, and skin carcinoma progression (14) but not embryonal tumors to date. Recently, the contribution of Odc activity to MYC-induced lymphomagenesis was investigated using the Eμ-MYC murine model in which transient biochemical ablation inhibited lymphomagenesis, whereas restoration of Odc activity allowed for delayed tumor onset (15).

We therefore sought to define whether Odc-mediated polyamine biosynthesis was a requisite metabolic biopathway supporting embryonal tumor initiation or progression. Most children with high-risk neuroblastoma have tumors that manifest a lethal course despite intensive multimodal therapy (16, 17). Thus, elucidating novel therapeutic pathways is paramount. We show that polyamine expansion through broad deregulation of regulatory enzymes, including ODC1, is a hallmark of neuroblastomas with MYCN amplification and have demonstrated an ability to reversibly inhibit neuroblastoma growth through inhibition of polyamine biosynthesis.
amplification. High ODC1 correlates with poor clinical outcome in a large cohort of patients, including those lacking MYCN amplification. Further, we show that biochemically disabling Odc inhibits neuroblastoma proliferation in vitro and has marked antitumor efficacy in a neuroblastoma-prone transgenic mouse model. Together, these data support that the adequate provision of polyamines is critical for MYC-driven proliferation and that targeted disruption of this pathway has therapeutic utility.

Materials and Methods

Patient Samples, Expression Profiling, Array Comparative Genomic Hybridization, and Real-time Quantitative PCR

Transcriptome profiles from 101 primary neuroblastomas from Children’s Oncology Group (COG) and the Children's Hospital of Philadelphia were obtained by our groups previously. Clinical and genetic features have been reported (18). Briefly, risk class was defined using COG criteria: 28 were localized biologically favorable neuroblastoma (low risk), 21 were intermediate-risk tumors, and 52 were high-risk tumors (of which 20 had MYCN amplification). cRNA was hybridized to Affymetrix U95Av2 oligonucleotide arrays containing 12,625 probe sets (Affymetrix) and statistical modeling of probe set behavior was conducted using Probe Profiler (Corimbia). A quantitative expression score (e-score) was calculated for each probe set. The data from this experiment are available online.9

Differential gene expression was measured with the Patterns from Gene Expression (PaGE; ref. 19) algorithm using binary comparisons of e-scores. All runs were done with 2001 permutations on unlogged data. A confidence level of 0.95 (1-FDR) was used to define differential expression for these analyses.

A subset of tumors (n = 80) had DNA available for detection of copy number alterations (CNA) and have been applied to a bacterial artificial chromosome (BAC) array comparative genomic hybridization (CGH) platform. The platform is described in detail by Greshock and colleagues (20) and its application and methodology for neuroblastoma CNA detection by Mosse and colleagues (21). Briefly, normalized log2 intensity ratios were averaged within slide for each BAC clone using the DNAcopy package within Bioconductor.10 A mean log2 ratio of ≥1.0 was considered a high-level amplification. Amplification of both the MYCN-containing BAC and the CTG-260DS17 clone that contains ODC1 and was used to define copy amplification.

A second independent cohort of 265 neuroblastomas from COG with available RNA was studied. Outcome data were available for 209 (79%). Informed consent was obtained from all subjects. Clinical characteristics and RNA/cDNA isolation procedures were previously described (22). ODC1 and MYCN expression was determined by real-time quantitative PCR (Q-PCR). The β2-microglobulin gene served as an internal control and the primers and probe sequences for β2-microglobulin and MYCN have been reported (22). ODC1 primer and probe sequences were 5’-GATGACTTCTGGATGAGTTGATGTA-3’ (forward primer), 5’-GGACCCGGATTTACAGAAGCT-3’ (reverse primer), and 5’-CGGATTCACGTCTAGGTCCT-3’ (probe). Q-PCR data were collected using a Prism 7700 Sequence Detection System (Applied Biosystems) and the level of target gene expression was determined using the ΔΔCt method (22). For all tumors in these studies, MYCN status was defined as >1-fold copies of MYCN compared with a 2p reference probe using fluorescence in situ hybridization (FISH).

Cell Lines and Tissue Culture

Neuroblastoma cell lines were obtained from the Children’s Hospital of Philadelphia Cell Line Bank courtesy of Garrett M. Brodeur and have been previously reported (23). Cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, 2 mmol/L l-glutamine, 100 units/mL penicillin, and 100 μg/mL gentamicin. Tissue culture was at 37°C in a humidified atmosphere of 5% CO2 as previously described (24). Cell line transcriptome profiles were obtained using the Affymetrix U133+2 oligonucleotide array and analyzed as above.

Cell Line ODC1 Expression

Real-time Q-PCR (Taqman) was used to quantify ODC1 mRNA. Total RNA (2 μg) was reverse transcribed in a 20 μL reaction using the SuperScript III First-Strand Synthesis System (Invitrogen). The probe and primer set used for ODC1 detection was Assay-on-Demand Hs0199739_m1 (Applied Biosystems). The genes SDHA and HPRT served as housekeeping genes for normalization. Triplicate reactions were performed and the mean expression values were used for calculating relative expression.

Cytotoxicity Assays

Cells were seeded into multiwell sensor microplates at 3 × 104 per well and allowed to adhere overnight. Cell index was obtained for each test condition in duplicate over 96 h using the real-time cell electronic sensor system (RT-CES, ACEA Biosciences). This label-free dynamic monitoring system uses electrical impedance to quantitate viable adherent cell number in real time (25). D-2Hfluoromethyl-α-ornithine (DFMO; Effornithine) was added to culture medium at a final concentration of 2.5, 5, or 10 μmol/L. DFMO for all studies was generously provided by Patrick Woster (Wayne State University) courtesy of ILEX Pharmaceuticals. All experiments were replicated three.

TH-MYCN Mouse Trials

129X1/Nj mice transgenic for the TH-MYCN construct (26–28) were graciously provided by William Weiss (Department of Neurology, University of California at San Francisco, San Francisco, CA) for establishment of TH-MYCN breeding colonies in both Philadelphia and Sydney. All 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).

Preemptive therapy trial (Philadephia). TH-MYCN hemizygous mice were bred and litters were randomized to water or water with 1% DFMO from birth onward. DFMO washes in breast milk so treated pups received DFMO from birth. After weaning at about day 28, mice were randomized to ad libitum water or water with 1% DFMO as initially randomized through day 70. Mice were genotyped from 1-cm tail snip isolated DNA. Primers to detect the human MYCN transgene were 5’-CACAAGGGCTCTACTTACC-3’ (forward) and 5’-AGGCAATCTTGGTTAGGATC-3’ (reverse). Hemizygous or homozygous transgene status was determined using real-time Q-PCR (Taqman) with the following: TH-MYCNf1 primer, 5’-CGACCAAAGGCTCATGCT-3’ (within exon 2); TH-MYCNr1 primer, 5’-AGGAGGAACGGCCGTCTC-3’ (exon 3); MYCN probe, FAM-CTCGAGGTCCTGCTTCCGGA-TAMRA; murine B-actinf1 primer, 5’-TTCGCTGTCCTGGATGCC-3’; murine B-actinr1, 5’-TACCGACTGTCGCGTC-3’; and murine B-actin probe, VIC-CGGGACCTGACTACTCTCATGA-TAMRA. For each analysis, 10 ng template DNA was used. All unknowns, FISH confirmed homozygous and hemizygous TH-MYCN controls, and nontemplate controls were loaded in triplicate. The average transgene dose was calculated using ΔΔCt, and calls were made based on comparison with control values within each run. Palpation for intra-abdominal tumors was performed thrice weekly. Animals with palpable tumors underwent serial abdominal ultrasoundography under isoflurane sedation to determine in situ tumor volume using a Vevo660 imaging system with 3D Acquisition and Visualization software (VisualSonics). Mice were screened by experienced animal personnel and sacrificed for pathologic signs of tumor burden (predominantly hunching and poor mobility).

Delayed treatment trial (Sydney). The specific characteristics of the Sydney breeding colony of TH-MYCN transgenic mice, including their maintenance, genotyping, and tumor incidence and latency, have been described previously (26). Cohorts of mice received plain water or 1% DFMO in their water ad libitum from day 25 (postweaning) onward. Animals were abdominally palpated twice weekly by experienced staff. Time to onset of tumor development and time to sacrifice according to well-defined humane end points were determined for all animals (26).

10 http://www.bioconductor.org

Cancer Res 2008; 68: (23). December 1, 2008 9736 www.aacrjournals.org
Combination chemotherapy treatment in TH-MYCN mice. Homozygous TH-MYCN mice with palpable tumors of 5 to 7 mm in diameter ($n \geq 10$ per group) were treated with cisplatin ($2$ mg/kg, i.p. daily $\times 5$ d; Pharmacia) or cyclophosphamide ($20$ mg/kg, i.p. daily $\times 5$ d). The control groups were treated with chemotherapy alone, whereas DMF0-treated groups received either continuous $1\%$ DMF0 in drinking water after completion of the cisplatin course or $1\%$ DMF0 in drinking water simultaneously with cisplatin or cyclophosphamide and continuously thereafter. Animals were sacrificed when tumors recurred, according to well-defined humane end points (26), or otherwise at 140 d of age.

Tumor Histopathology and Polyamine Content

Animal tumors and tissues were harvested at sacrifice and fixed in $10\%$ neutral buffered formalin and paraffin embedded for histologic studies as well as flash frozen in liquid nitrogen for metabolic assays. Tissue sections were stained with H&E and assessed histologically by a pathologist (B. Pawel) for confirmation of tumor type, and percentage of necrosis (average of five $40\times$ microscopic fields) and mitotic/karyorrhectic cells (average of five $600\times$ microscopic fields).

For immunohistochemistry, $5\mu$m sections were stained with antibodies to caspase-3 at a $1:1,000$ dilution (R&D Systems) and Ki67 at a $1:1,000$ dilution (Santa Cruz Biotechnology) on an Autostainer Plus staining system (DakoCytomation) using standard methods, including microwave antigen retrieval for $5$ min in $0.01\%$ mol/L citrate buffer at pH 7.6. Both caspase-3 and Ki67 staining were scored as the percentage of stained tumor nuclei using the average of five $600\times$ microscopic fields.

Tumor tissues were frozen in liquid nitrogen, ground to a fine powder, and stored at $-80^\circ$C. For polyamine analyses, ground tissues were homogenized in $0.2\%$ perchloric acid and incubated at $4^\circ$C overnight. Dansylated polyamines were separated on a reversed-phase C18 high-performance liquid chromatography column (29). Polyamine values were normalized to the amount of DNA in the tissue extracts.

Murine Paravertebral Ganglia Studies

To study the in vivo effects of perinatal Odc1 inhibition, litterers from TH-MYCN mice were randomized and treated with $1\%$ DMF0 in maternal drinking water as above. Pups were euthanized at postnatal days $0$ ($n = 26$), $7$ ($n = 40$), and $14$ ($n = 26$). Additionally, a cohort of pregnant females received DMF0 prenatally from embryonic days $14$ to $21$ and ganglia from pups were obtained at day $0$. Tissues were formalin fixed and paraffin embedded. A histologic audit was performed with each section scored for the presence of $>30$-cell neuroblast hyperplasia. Animals were TH-MYCN genotyped as previously described (30).

Paravertebral sympathetic ganglia were dissected from normal and TH-MYCN mice at postnatal day 14 and cultured for $3$ to $5$ d as described previously (27). DMF0 (1 mmol/L) was added to half the wells for $7$ d. On day $5$, cells were washed twice and $10\mu$g/mL anti-nerve growth factor (NGF) antibody (Chemicon) or isotype control (Cedarlane Laboratories) was added to alternate wells for $48$ h. The number of neurons surviving withdrawal of NGF was quantitated using immunofluorescent staining for the neuron-specific marker$jH$-tubulin, expressed as a percentage of neurons positive for$jH$-tubulin before and after NGF withdrawal.

For Odc1 immunohistochemistry, cells from murine ganglia were cultured as described (27) and then treated with $1\%$ DMF0 for $7$ d. Medium was replaced every $2$ to $3$ d with complete medium containing anti-NGF and DMF0. Cells were fixed with $4\%$ formaldehyde ($20$ min at room temperature) followed by methanol permeabilization ($20$ min at room temperature). Immunofluorescent staining was performed using a mouse Odc antibody (clone MP16-2; $1:25$ dilution; Sapphire Biosciences) or isotype control in conjunction with the Vector Labs M.O.M kit (Australian Laboratory Services). DMF0 competitively inhibits binding of this antibody to Odc1 (31). Cells were imaged using an Axiosplan 2 microscope (Zeiss) using a Sensicam charge-coupled device camera (PCO Imaging).

Statistical Analyses

Pearson's correlation coefficients were calculated to assess gene expression correlations. Two-tailed Student's t test was used to test significance unless otherwise stated. Survival analyses were performed according to the method of Kaplan and Meier (32) with SEs according to Peto and Peto (33). Comparisons of outcome between subgroups were performed by a two-sided log-rank test. Event-free survival (EFS) time was calculated as described previously (22) and death was the only event considered for the calculation of overall survival (OS) time. A continuous range of ODC1 Q-PCR values was used in outcome analyses. To categorize expression as either high or low, the following cut-points were tested: the mean, median, upper quartile, and upper decile values (34). The cut-point that maximized the difference in EFS between the two groups was selected, and that cut-point was applied to analyses of the overall cohort as well as the subgroups.

Results

ODC1 expression correlates with survival in neuroblastoma.

Odc1 is rate limiting for polyamine biosynthesis, a bona fide onconeural, and direct MYCN target. We therefore compared ODC1 mRNA expression with MYCN gene status, MYCN expression, and outcome in a large cohort of neuroblastoma patients. ODC1 expression was significantly higher in MYCN-amplified tumors (Fig. 1A) and strongly correlated with MYCN expression ($r = 0.80$; $P < 0.0001$). EFS for patients with high ODC1 expression (defined as the upper decile and determined by an optimal cut-point analysis) was significantly poorer than that of patients with low ODC1, with $5$-year rates of $38 \pm 11\%$ and $76 \pm 3\%$ (Fig. 1B). Similarly, worse OS was associated with high ODC1 ($P < 0.001$). High ODC1 was associated with worse EFS and OS when the groups were dichotomized around the mean, median, or upper quartile as well ($P < 0.05$ for each). In patients with stage IV metastatic disease, high ODC1 expression was again associated with reduced EFS or OS (Fig. 1C).

Because MYCN amplification has a negative influence on survival through regulation of numerous target genes (in addition to ODC1), we assessed tumors without MYCN amplification. Again, high ODC1 was associated with a worse EFS and OS, with $5$-year EFS rates of $43 \pm 19\%$ compared with $80 \pm 3\%$ (Fig. 1D), suggesting a role for ODC1 in promoting an aggressive phenotype (for dichotomization at the mean, median, or upper quartile, the $P$ value was again <0.05). In nonamplified neuroblastomas, there is no correlation between MYCN expression and outcome, so it is unlikely that this is a surrogate for MYCN activity (30). To assess the independent prognostic significance of ODC1 while controlling for known powerful prognostic factors such as tumor stage (stages I, II, and IV versus III and IV), age at diagnosis (<1 versus $\geq$1 year), and MYCN status, these factors were tested in a Cox proportional hazards model with dichotomized ODC1 expression. The addition of ODC1 expression by itself did not add independent significance to this highly prognostic model.

Neuroblastomas with MYCN amplification show coordinate pathway alterations that enhance polyamine biosynthesis. To further explore polyamine metabolism (see pathway, Fig. 2A), we mined transcriptome profiles from neuroblastomas of diverse risk classes (17). As predicted, MYCN and ODC1 mRNAs were significantly higher in HR-A tumors in comparison with all other groups (Fig. 2B). Odc1 is additionally regulated by antizymes (OAZs) that direct its degradation, whereas OAZIN inhibits this activity. OAZ2 was significantly reduced in HR-A neuroblastomas, further promoting Odc activity. Notable were alterations in numerous polyamine regulators in HR-A tumors, all in a direction promoting biosynthesis. Each prosynthetic enzyme was up-regulated (confidence level, $>0.95$), whereas there was a reduction in SMOX that catalyzes polyamines (note, no PAOX probe sets
were on the array). Together, these data show systematic alterations in polyamine metabolism correlated with MYCN amplification. High-risk tumors without MYCN amplification (HR-NA) also had higher ODC1 and reduced OAZ2 compared with low- and intermediate-risk tumors, suggesting pathway enhancement in these tumors as well.

ODC1, SRM, and AMD1 have been posited as Myc targets (Myc Cancer Gene Database\textsuperscript{11}) and our data support this. ODC1 was strongly correlated with MYCN across the entire cohort ($r = 0.53; P < 0.0001$; Fig. 2C). SRM and AMD1 yielded similar correlations ($r = 0.30; P = 0.001$ and $r = 0.39; P = 0.001$, respectively). SMS, despite no prior evidence as a MYC target, had the strongest correlation ($r = 0.69; P < 0.0001$), whereas OAZ2 was inversely correlated ($r = -0.42, P < 0.0001$; see Supplementary Fig. S1), although not a previously identified repressed target. Thus, MYCN-amplified neuroblastomas directly or indirectly promote polyamine pool expansion through coordinate alteration of multiple polyamine regulators through mechanisms that may include de novo transcriptional initiation or mRNA stability.

The correlation between MYCN and ODC1 expression was less evident in tumors with MYCN amplification (compared with other bona fide MYCN targets) due to the presence of outliers with exceptionally high ODC1 (Fig. 2C). ODC1 maps ~5.5 Mb telomeric to MYCN (2p24) using the University of California at Santa Cruz Genome Browser coordinates. We sought whether ODC1 was coamplified with MYCN as has been reported (35). Eighty of the 101 tumors had DNA available for determination of MYCN and ODC1 genomic copy number using a BAC array CGH platform (21). No tumors without MYCN amplification ($n = 64$) had ODC1 amplification. Sixteen MYCN-amplified tumors (by FISH) were confirmed to have high-level MYCN amplification using array CGH. Three of these (19%) had high-level ODC1 coamplification and each was an outlier with extremely high ODC1 expression (Fig. 2C, arrowheads; the fourth outlier did not have DNA for CNA determination). Thus, a subset of neuroblastomas coamplifies both the transcriptional regulator (MYCN) and target gene (ODC1) to augment effects on polyamine biosynthesis, a putative “amplification loop” that has not been previously postulated.

**Disabling ODC1 in neuroblastoma cell lines inhibits proliferation.** Across 26 neuroblastoma cell lines, there was a trend for higher ODC1 with MYCN amplification [$P = 0.10; P = 0.06$ if NBL-S with prolonged MucN half-life (36) is included in the “amplified” cohort; Supplementary Fig. S2]. We assessed Odc1 inhibition in vitro using DFM0, an irreversible Odc inhibitor. DFM0-mediated growth inhibition correlated with ODC1 mRNA expression and proliferative rates (Fig. 3A), was apparent by early

\textsuperscript{11}http://www.myccancergene.org
Figure 2. Polyamine regulation in neuroblastoma. A, polyamine metabolism: polyamines (putrescine, spermidine, and spermine) are synthesized from ornithine through decarboxylation and condensative processes. Synthetic (green) and catabolic (red) enzymes are shown. Underlined enzymes are highly regulated with the shortest half-lives of any mammalian enzymes. ODC1, ornithine decarboxylase; AMD1, S-adenosylmethionine decarboxylase; SRM, spermidine synthetase; SMS, spermine synthetase; SAT, spermine/spermidine-N-acetyltransferase; SMOX, spermine oxidase; OAZ1, ODC antizyme 1; OAZ2, ODC antizyme 2; OAZIN, ODC antizyme inhibitor.

B, polyamine regulator expression in primary neuroblastomas: LR, 28 low-risk tumors; IR, 21 intermediate-risk tumors; HR-NA, 32 high-risk tumors without MYCN amplification; HR-A, 20 high-risk tumors with MYCN amplification.* differential expression between HR-A and HR-NA; ** differential expression between HR-A and all others; *** differential expression between HR-NA and LR and IR (all with a confidence level >0.95 using PaGE analyses). Filled circle on Y axis, fetal brain (control) expression.

C, correlation between ODC1 and PTMA with MYCN expression using representative probe sets. Left, all 101 tumors; right, segregate HR-A tumors from the other groups. Pearson’s correlation coefficient and P values are given. Arrowheads, the three tumors that have both high-level MYCN and ODC1 coamplification.
time points (48 hours), and was seen in cells both with and without MYCN amplification. Indeed, Affymetrix expression data for neuroblastoma cell lines and fetal brain cDNA show up-regulation of polyamine synthetic enzymes and down-regulation of catabolic enzymes in nonamplified cells (Fig. 3B). This growth inhibition is not surprising as most cell types show cytostasis when Odc is inhibited in vitro, including neuroblasts (37). Tissue culture conditions do not provide the same rescue opportunities present to cells depleted of polyamines in vivo, where many markedly increase polyamine uptake, an option not present in polyamine-poor culture medium. We therefore focused on polyamine depletion effects in vivo.

Disabling Odc1 prevents MYCN-mediated oncogenesis. We determined the effect of disabling Odc (to impede polyamine

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**Figure 3.** Polyamine dependence in neuroblastoma cell lines. A, DFMO-mediated growth inhibition in vitro. Cell index, proportional to the viable cell number, was obtained with the RT-CES platform. Viable cell number following short-term exposure to DFMO is shown normalized to no treatment at 72 h. White columns, control; light gray columns, 2.5 mmol/L DFMO; dark gray columns, 5 mmol/L DFMO; black columns, 10 mmol/L DFMO. Triplicate wells are assessed for each experiment and multiple experiments are done for each condition. Bars, SD. B, Affymetrix expression (e-score) for polyamine regulatory enzymes in neuroblastoma cell lines, segregated by MYCN status and compared with fetal brain cDNA as a normal reference. Both amplified and nonamplified cells have expression alterations in a direction promoting polyamine expansion (more pronounced in the amplified cells).
Disabling Odc1 in Neuroblastoma

Figure 4. Extended tumor-free survival in neuroblasta-prone mice treated with DFMO. A, tumor-free survival curves for homozygous (TH-MYCN +/+) or hemizygous (TH-MYCN +/−) mice stratified by DFMO therapy. DFMO-treated mice (dashed lines) received DFMO from birth onward (preemptive treatment trial). DFMO therapy was stopped at day 70 in tumor-free mice, Control (solid line). DFMO therapy was continued thereafter (delayed treatment trial). TH-MYCN homozygous and hemizygous mice were randomized to DFMO (dashed lines) or control (solid lines) following weaning at day 25. Tumor-free survival for TH-MYCN homozygous mice with advanced tumor from the time of treatment with cisplatin alone (black line), cisplatin followed by DFMO (gray line), or cisplatin administered simultaneously with DFMO (dashed line) or cyclophosphamide alone (solid line) or combined with DFMO (dashed line). P values using the method of Kaplan-Meier are shown.

We evaluated whether Odc activity was required for MYCN-initiated oncogenesis by treating mice with DFMO from birth. All mice homozygous for the transgene (highest MYCN) developed tumors with complete penetrance; whereas hemizygous TH-MYCN mice have reduced (~30%) tumor penetrance (27, 28). Tumors develop within hyperplastic rests that are transiently present even in wild-type animals (27). Their number and persistence correlate with tumor penetrance and MYCN dosage. Tumors share biochemical features and orthologous genomic alterations with human neuroblastomas, suggesting preferred secondary pathways are recapitulated (28, 38, 39). Thus, the model provides a platform for evaluating biopathway targeted therapies (40).

DFMO enhances the therapeutic effect of chemotherapy. DFMO has been shown to induce cell cycle arrest in neuroblasts (37) and therefore may interfere with chemotherapy effects. We assessed Odc inhibition in combination with cisplatin, vincristine, or cyclophosphamide, first-line agents with high single-agent activity in neuroblastoma. TH-MYCN homozygous mice with palpable intra-abdominal tumors (75–150 mm3) were treated with the chemotherapeutic alone or in combination with DFMO. Cisplatin induced transient tumor regression with a mean latency to recurrence of 32 days. DFMO started concurrently or following cisplatin and continued thereafter did not interfere with cisplatin-induced regression and led to an extended relapse-free survival (P < 0.01; Fig. 4C). Similar findings were obtained using vincristine (data not shown), and a more marked augmentation of chemotherapeutic efficacy was seen with cyclophosphamide, where

Delaying Odc inhibition until after tumor onset also had a measurable effect. In a second trial, DFMO therapy was initiated after weaning when small tumors are invariably present in homozygous animals and in the majority of hemizygous animals (27). DFMO treatment of homozygous mice again inhibited progression (time to palpable tumor burden: mean, 47.5 ± 1.3 versus 38.6 ± 1.5 days) and time to death (mean, 49.2 ± 1.3 versus 42.6 ± 1.2 days; P = 0.001; Fig. 4B). Delayed DFMO treatment in hemizygous mice did not reduce penetrance (as the majority of tumors were present before therapy), yet there was a modest trend toward tumor inhibition based on a reduction in penetrance and extended tumor-free survival and OS.

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We evaluated whether Odc activity was required for MYCN-initiated oncogenesis by treating mice with DFMO from birth. All mice homozygous for the transgene (highest MYCN) developed tumors; however, tumor latency (mean, 31 ± 2 versus 43 ± 7 days; P < 0.001) and OS (mean, 43 ± 4 versus 59 ± 9 days; P < 0.001) were markedly extended by DFMO (Fig. 4A). Moreover, hemizygous mice (high MYCN) were protected from tumor initiation. Seven of 16 untreated hemizygous mice (44%) developed tumors, consistent with the penetrance observed historically, whereas only 6 of 38 DFMO-treated mice (16%) developed tumors (P = 0.035). Importantly, DFMO was removed at day 70, yet no tumors arose beyond this time point. This is consistent with a finite vulnerable period for embryonal oncogenesis and suggests that transiently inhibiting Odc1 provides long-lasting tumor protection.

Delaying Odc inhibition until after tumor onset also had a measurable effect. In a second trial, DFMO therapy was initiated after weaning when small tumors are invariably present in homozygous animals and in the majority of hemizygous animals (27). DFMO treatment of homozygous mice again inhibited progression (time to palpable tumor burden: mean, 47.5 ± 1.3 versus 38.6 ± 1.5 days) and time to death (mean, 49.2 ± 1.3 versus 42.6 ± 1.2 days; P = 0.001; Fig. 4B). Delayed DFMO treatment in hemizygous mice did not reduce penetrance (as the majority of tumors were present before therapy), yet there was a modest trend toward tumor inhibition based on a reduction in penetrance and extended tumor-free survival and OS.

DFMO enhances the therapeutic effect of chemotherapy. DFMO has been shown to induce cell cycle arrest in neuroblasts (37) and therefore may interfere with chemotherapy effects. We assessed Odc inhibition in combination with cisplatin, vincristine, or cyclophosphamide, first-line agents with high single-agent activity in neuroblastoma. TH-MYCN homozygous mice with palpable intra-abdominal tumors (75–150 mm3) were treated with the chemotherapeutic alone or in combination with DFMO. Cisplatin induced transient tumor regression with a mean latency to recurrence of 32 days. DFMO started concurrently or following cisplatin and continued thereafter did not interfere with cisplatin-induced regression and led to an extended relapse-free survival (P < 0.01; Fig. 4C). Similar findings were obtained using vincristine (data not shown), and a more marked augmentation of chemotherapeutic efficacy was seen with cyclophosphamide, where
cyclophosphamide alone resulted in long-term cure of ~20% of neuroblastoma-bearing mice. Concurrent administration of DFMO with cyclophosphamide increased OS to 80% ($P = 0.03$; Fig. 4D).

We noted no overt toxicity attributable to DFMO therapy in these trials. Wild-type mice receiving DFMO from birth weighed less than untreated littersmates following weaning (~day 28), yet they gained weight at the same rate or better thereafter, despite ongoing DFMO exposure (average weight gain of 0.45 g/wk in DFMO-treated mice versus 0.28 g/wk for control mice between weeks 14 and 20; $P = 0.12$; Supplementary Fig. S3). Mice receiving DFMO delayed until after weaning showed modest growth inhibition through 5 months of life compared with untreated animals (weight gain of 0.92 ± 0.04 versus 1.05 ± 0.04 g/wk; $P = 0.034$).

**Odc activity is not required for MYCN-mediated death resistance.** Tumors in TH-MYCN mice arise within hyperplastic rests in sympathetic ganglia. These are transiently present in wild-type mice but are increased in number and persist longer in a MYCN dose-dependent manner (27). We assessed the effect of DFMO on this process by performing a histologic audit. Prenatal DFMO treatment of pregnant mothers from embryonic days 14 to 21 did not affect the incidence of hyperplasia noted at day 0 (data not shown). Postnatal DFMO treatment of newborn pups did not have a demonstrable effect on neuroblast hyperplasia by day 7; however, by day 14, homozygote mice (highest MYCN) treated with DFMO showed a significant reduction (Fig. 5A). Together with in vivo data, this suggests that Odc activity is not required for basal neuroblast hyperplasia; however, MYCN supported maintenance and progression to tumor is at least partially Odc dependent. MYCN not only drives cell cycle entry but also protects against deprivation-induced apoptosis in TH-MYCN neural cells. Cultured perinatal ganglia from TH-MYCN mice show resistance to NGF withdrawal (27) analogous to that seen in postmitotic sympathetic neurons (41). Paravertebral ganglia from untreated normal and TH-MYCN homozygote mice at day 14 were cultured in the presence of NGF with or without DFMO for 7 days, after which NGF was withdrawn. Although DFMO-mediated Odc inhibition was supported using a conformation-specific antibody (Fig. 5B), there was...
no effect on the death resistance of ganglia cells, showing that Odc-mediated polyamine synthesis was not a critical component of MYCN-mediated apoptosis resistance (Fig. 5C).

**Neuroblastomas arising under DFMO may circumvent the polyamine depletion barrier.** We assessed whether tumors arising under Odc inhibition overcame this blockade or took an alternative route to transformation less dependent on polyamines. DFMO-treated and untreated mice developed cellular tumors that were undifferentiated (with the exception of one lymph node that had fibrillar neuropil) similar to poorly differentiated human neuroblastoma. DFMO-treated tumors had larger cells with reduced hemorrhage and necrosis but no differences in mitosis/karyorrhexis index (Fig. 6A). Tumors were notable for the large percentage of cycling cells (Ki67+) and caspase activation, although neither differed between groups. Serial ultrasonography in homozygous TH-MYCN mice confirmed similar tumor volume at the time of ascertainment (mean, 227 ± 61 mm³ versus 232 ± 64 mm³; P = 0.83), although tumors arose later in DFMO-treated mice. Tumors grew at similar rates (Δvolume/week of 166 ± 68 mm³ versus 156 ± 79 mm³ with DFMO; P = 0.75) and lethality. Thus, aside from being delayed in onset, DFMO-treated tumors manifested a similar aggressive phenotype in homozygous mice.

Polyamine assays from tumors harvested at culling showed reduced putrescine in DFMO-treated tumors, a trend toward reduced spermidine and no effect on spermine (Fig. 6B). These effects are consistent with experimental models of DFMO-induced Odc inactivation (42), including effects on neuroblastoma cell lines (37), and support that polyamine depletion is at least partially maintained. However, maintenance of spermidine and spermine through enhanced polyamine uptake, compensatory Amd1 induction, or altered metabolism cannot be formally excluded as a mechanism for circumventing the polyamine depletion barrier. Further studies are ongoing to determine whether neuroblastomas circumvent Odc inactivation to support polyamine homeostasis or take an alternate route to oncogenesis.

**Discussion**

Most children with high-risk neuroblastoma die from tumor progression and innovative treatment approaches are needed (16, 43). We show that neuroblastomas, particularly those with MYCN amplification, have altered polyamine metabolism and may be vulnerable to therapeutic polyamine depletion. Although MYCN amplification has long been associated with poor outcome (1, 2), the transcriptional targets governing this remain elusive as MYCN regulates thousands of genes (7). We show that MYCN amplification is correlated with deregulation of numerous enzymes that drive polyamine expansion, and such concerted alterations may be a hallmark of MYC oncogenesis (15). Odc1 is rate limiting in this pathway, and importantly, we show that its biochemical inhibition alone has measurable consequences on tumor progression in a transgenic model.
Whereas MYCN-amplified tumors deregulate diverse polyamine enzymes, high-risk tumors without MYCN amplification also have elevated ODC1 and reduced Odc antizyme (OAZ2). Deregulation of this rate-limiting step in polyamine biosynthesis may provide a therapeutic vulnerability here as well. This is supported by demonstration that polyamine regulator expression in MYCN-nonamplified cells parallels those for amplified cells, and by in vitro data showing DFMO-mediated growth inhibition in nonamplified cells. Of interest, we defined MYCN and ODC1 high-level coamplification in a subset of MYCN-amplified tumors associated with markedly elevated ODC1 expression. As lesser copy number gain of the ODC1 locus (2p25) is reported in half of high-risk neuroblastomas without MYCN amplification (21), we speculate that ODC1 gene dosage gain is a potential mechanism for pathway up-regulation in this subset. Alternative MYC deregulation in high-risk neuroblastomas may also transcriptionally drive ODC1 expression (4, 5). Importantly, we show that ODC1 expression is inversely correlated with survival in a large validation cohort even in tumors lacking MYCN amplification. Together, these data suggest that polyamine depletion strategies may be more broadly effective against high-risk tumors rather than selectively synthetic lethal for MYCN-amplified tumors.

Myo genes induce Csk1, a component of the SCF\(^3\)Skp2 E3 ubiquitin ligase complex that degrades p27\(^{kip1}\) (44). Because Odc is required both for Myo induction of Csk1 (44) and for polyamine biosynthesis, its activity licenses cell cycle entry at multiple steps. In vitro, Odc causes polyamine depletion and increased p27\(^{kip1}\) and Rb hypophosphorylation with G1 arrest in neuroblast (37). In vivo, we show that Odc activity is not required for basal hyperplastic rest formation (preneoplastic) nor neuroblast resistance to deprivation-induced apoptosis. However, Odc supports hyperplastic rests and promotes their oncogenic conversion. It is likely that DFMO-enforced growth arrest alters the stoichiometry of cycling cells within the peripheral sympathetic compartment to impede tumor initiation.

This selective pressure may prevent tumor onset in TH-MYC hemizygous mice, in which neuroblastoma initiation requires MYCN transgene amplification, indicating a higher threshold of MYCN permissive for oncogenesis (27). Of greater interest is that transient Odc inhibition through day 70 is capable of providing durable tumor protection. This contrasts with Eqi-MYC mice where DFMO withdrawal leads to lymphomagenesis at the expected interval from treatment cessation (15). This is consistent with a finite window for embryonal tumorigenesis beyond which the specific tissue milieu may be incapable of supporting transformation and suggests that chronic Odc inhibition may not be required for sustained therapeutic benefit.

In homozygous TH-MYC mice, disabling Odc from birth delays but does not prevent tumors. The polyamine depletion barrier imposed by Odc inactivation may be bypassed as only putrescine remains depressed and the rate of end-stage tumor progression is reminiscent of untreated animals. DFMO-mediated Odc inhibition is often accompanied by compensatory Amd1 up-regulation that may partly compensate. Alternatively, Odc inhibition may force premalignant neuroblasts to adopt an alternate pathway to transformation, as has been shown for lymphomas arising in Egf-MYC;Odc+/- mice (15). Still, Odc inhibition after tumor onset delays progression and augments sensitivity to cytotoxic stressors, providing clinical relevance to these studies.

The utility of therapeutic polyamine depletion has been limited to date (14). However, DFMO doses sufficient to inhibit Odc are well tolerated chronically and polyamine depletion as an anticancer strategy is in the midst of a reevaluation. Newer targeted agents are under development, including those that inhibit polyamine uptake from extracellular sources or target additional regulatory enzymes (45–47). That DFMO augments chemotherapy efficacy in our model allays concerns that enforced growth arrest via polyamine depletion will subvert traditional chemotherapeutics. This is reassuring as the entry of polyamine depletion agents into the clinic would likely be in concert with conventional cytotoxics. Interestingly, cisplatinum has been shown to alter Amd1, ODC1, Srm, and SAT in directions that antagonize polyamine synthesis (48). Yet, the dramatic responses with vincristine or cyclophosphamide and DFMO, including improved OS rates, suggest that this effect does not result from a unique synergistic opportunity provided by platinitators alone. Taken together, our data strongly support that polyamine depletion may provide an important addition to the neuroblastoma armamentarium and perhaps other embryonal malignancies governed by MYC. Potentiation of these effects with complimentary polyamine-targeted agents may further improve efficacy and deserve further evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ODC1 Is a Critical Determinant of MYCN Oncogenesis and a Therapeutic Target in Neuroblastoma

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