Disrupting Polyamine Homeostasis as a Therapeutic Strategy for Neuroblastoma
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Abstract  MYC genes are deregulated in a plurality of human cancers. Through direct and indirect mechanisms, the MYC network regulates the expression of > 15% of the human genome, including both protein-coding and noncoding RNAs. This complexity has complicated efforts to define the principal pathways mediating MYC's oncogenic activity. MYC plays a central role in providing for the bioenergetic and biomass needs of proliferating cells, and polyamines are essential cell constituents supporting many of these functions. The rate-limiting enzyme in polyamine biosynthesis, ODC, is a bona fide MYC target, as are other regulatory enzymes in this pathway. A wealth of data link enhanced polyamine biosynthesis to cancer progression, and polyamine depletion may limit the malignant transformation of preneoplastic lesions. Studies with transgenic cancer models also support the finding that the effect of MYC on tumor initiation and progression can be attenuated through the repression of polyamine production. High-risk neuroblastomas (an often lethal embryonal tumor in which MYC activation is paramount) deregulate numerous polyamine enzymes to promote the expansion of intracellular polyamine pools. Selective inhibition of key enzymes in this pathway, e.g., using DFMO and/or SAM486, reduces tumorigenesis and synergizes with chemotherapy to regress tumors in preclinical models. Here, we review the potential clinical application of these and additional polyamine depletion agents to neuroblastoma and other advanced cancers in which MYC is operative. (Clin Cancer Res 2009;15(19):5956-61)

Background

The MYC proto-oncogenes, which include MYC, MYCN, and MYCL, are among the most frequently deregulated genes in cancer. The MYC proto-oncogenes encode highly homologous basic-helix-loop-helix leucine zipper transcription factors that are biologically redundant, but differentially expressed spatiotemporally. MYC genes function through heterodimerization with Max and operate within a network of related proteins to regulate transcription through interactions at E-box sequences within promoters of diverse target genes (1). Many estimates have the MYC network governing the expression of > 15% of all human genes (2, 3) and a growing roster of noncoding RNAs (4). A simplified gene-specific model of transcriptional regulation has been expanded with the appreciation that MYC genes also contribute to global chromatin regulation. Loss of MYCN in neural stem cells, for example, leads to an aberrant nuclear structure mimicking a heterochromatin state accompanied by widespread histone modifications (5). Such higher-order regulatory activities may explain MYC's profound influence on transcription and the diversity in putative target genes identified across different model systems.

MYC activity is tightly regulated through transcriptional and posttranslational mechanisms, with rapid degradation of Myc protein in concert with cell cycle exit. In many cancers, MYC genes are deregulated through genomic translocation or amplification events that lead to supraphysiologic Myc expression. Although mutations in Myc have been identified in Burkitt's lymphoma cells (accompanying rather than replacing activating translocations; ref. 6), Myc oncogenesis typically results from deregulated overexpression of wild-type protein. Such large-scale biological reprogramming of cells through enforced expression of this promiscuous transactivator and chromatin regulator is highly oncogenic, and the ubiquity of MYC activation across tumor types makes it an attractive cancer target.

Inhibiting grossly deregulated transcription factors remains a daunting therapeutic challenge, yet pharmaceutical successes continue to whittle away at the list of domains considered “undruggable”; thus, direct Myc antagonism may be an achievable goal. However, a secondary concern for such an approach is that systemic interference with Myc might be quite toxic, as it is indispensable for cell cycle entry in response to mitogenic signals. This fear has been partially allayed by evidence that a profound dominant-negative Myc construct can be activated globally in mice without undue toxicity (7). An alternative approach to interfering directly with Myc is to define the critical
downstream pathways necessary for its oncogenic activity. Among these may be more immediately tractable drug targets that exploit cancer-specific aspects of Myc activity with a greater therapeutic index.

An improved understanding of Myc biology has emerged from high-dimensional assays that provide global transcriptome and/or Myc-chromatin binding site data. These platforms have generated daunting lists of genes and chromatin binding sites that underscore the widespread involvement of Myc in diverse biological processes. Still, patterns are discernible within this complexity. The most conserved set of MYC target genes functions in ribosomal biogenesis and protein metabolism and processing, and this is true for both MYC (3) and MYCN (8). Additionally, programs that direct carbon assimilation, anabolic pathways, and bioenergetics are all targeted by Myc (3). Thus, Myc orchestrates a program redirecting metabolism to provide for the energetic needs of the cell through augmented aerobic glycolysis (9) and glutaminolysis (10), as well as the biomass needs through enhanced synthesis and processing of RNA, DNA, protein, lipid, and polynamine precursors.

**Polyamines are multifunctional polycations found in nearly all living organisms.** Polyamines support biological processes through the stabilization of anionic macromolecules and modulate DNA:protein and protein:protein interactions. A detailed understanding of polynamine activities is hampered by the fact that they participate in mainly transient ionic interactions that are difficult to study. Still, polynamine homeostasis is essential for cell survival, and depletion activates cellular checkpoints that constrain proliferation or induce apoptosis (11). Reduced polynamines are seen in postmitotic and senescent cells, whereas enhanced polynamine biosynthesis accompanies normal as well as oncogenic proliferation (12, 13). That polynamine biosynthesis may be instructive in the cancer process, rather than simply a consequence of increased proliferation, emerged as molecular studies linked numerous cancer genes directly to polynamine metabolism (14, 15). For example, ornithine decarboxylase (ODC1), the rate-limiting enzyme for polynamine biosynthesis, is a MYC target gene (16) and a bona fide oncogene. Odc can substitute for Myc and cooperate with Ras to transform cells both in vitro (17) and in vivo (18). Thus, enhanced polynamine synthesis is essential to oncogenic signaling and may be specifically required to support Myc-governed functions.

Intracellular polynamine levels are modulated through tightly regulated synthetic, catabolic, uptake and export pathways (Fig. 1; reviewed in refs. 14, 15, 19). The rate-limiting biosynthetic enzymes are ODC1 and S-adenosylmethionine decarboxylase (AMD1). Odc homodimers decarboxylate ornithine, a urea cycle product, to the diamine putrescine, whereas Amd decarboxylates S-adenosylmethionine to provide the amino-ethyl donor for the conversion of putrescine to spermidine and spermine. These latter conversions are mediated by the aminopropyltransferases spermidine synthase (SRM) and spermine synthase (SMS), respectively. Multiple levels of control are exercised over Odc and Amd activities. They are highly transcriptionally regulated, with ODC1 being a direct MYC target (16); are posttranscriptionally regulated; and have the shortest half-lives (10-30 min) of any mammalian enzyme. Odc is rapidly degraded through a process initiated by Odc antizymes that bind monomeric Odc and present the protein to the 26S proteasome for degradation independent of ubiquitylation, while also inhibiting polynamine uptake. These antizymes (OAZ1, OAZ2, OAZ3) are themselves responsive to intracellular polynamines to provide a negative feedback loop (20). Furthermore, two mammalian antizyme inhibitors have been identified [AZIN1 (ref. 21) and, more recently, ADC (ref. 22)] that encode enzymatically inactive Odc homologs that compete to neutralize the antizymes and therefore constitute a positive regulator of Odc activity (23). This level of control underscores the importance of modulating Odc activity to ensure cellular fitness.

Polynamine catabolism occurs via the acetylation of spermidine or spermine by the readily inducible spermidine/spermine-N-acetyltransferase, SAT1 (24). Acetylated polynamines may be exported from the cell through specific transmembrane solute carriers to reduce intracellular levels (25). Alternatively, acetylated spermine and spermidine may be converted through PAOX oxidase activity to spermidine and putrescine, respectively, whereas SMOX oxidase activity can convert spermine to spermidine directly. These conversions allow for homeostatic control over the repertoire of natural polynamines, which may be important for maintaining functions unique to select polynamines [such as protein translation, in which spermidine acts as a gate-keeper by regulating the activity of eIF5A (ref. 26); Fig. 2]. Finally, an as yet uncharacterized energy-dependent polynamine transporter function to selectively import polynamines present in the microenvironment through dietary intake, export from neighboring cells, or synthesis by intestinal flora. This pathway can restore polynamine levels under conditions of biosynthetic blockade and may potentially undermine therapeutic efforts to diminish intracellular polynamines.

This multitude of regulatory controls highlights the critical need for polynamine homeostasis, and as polynamines operate downstream of Myc to support proliferation, they provide an intriguing cancer target. Indeed, substantial effort has been spent over 30 y to leverage polynamine disruption as an anticancer strategy with limited initial success. Inhibitors acting at nearly every step in this pathway have been developed and investigated, and despite encouraging preclinical results with diverse agents and cancer models, translation to clinical utility has been slow (comprehensively reviewed in ref. 14). Initial studies testing single polynamine-targeting drugs were disappointing, although not completely without successes, as activity has been seen in hematolymphoid (27, 28) and CNS neoplasms (29). To date, however, little attention has been paid to the potential for cancer genotype-specific responses, analogous to synthetic lethal interactions in yeast. Studies with transgenic cancer models suggest that Myc deregulation may provide an Achilles’ heel for cancer cells through a requirement for polynamine sufficiency that can be targeted therapeutically, and embryonal cancers may be particularly vulnerable.

**Clinical-Translational Advances**

Neuroblastoma is a childhood embryonal tumor that frequently presents with high-risk clinical and genetic features. Despite maximally intensive therapy, survival remains dismal and innovative treatment approaches are needed (reviewed in ref. 30). Several recurrent genomic alterations correlate with outcome, and of these, MYCN amplification is most strongly correlated with advanced disease and treatment failure (31, 32). In high-risk neuroblastomas that lack MYCN amplification, MYC is frequently deregulated instead (33–35), suggesting that MYC signaling may be essential for the high-risk phenotype. Still, despite
over 20 y of recognition that MYC deregulation is a seminal event in neuroblastoma, no molecularly targeted therapy has emerged to leverage this discovery. Polyamine homeostasis, deregulated downstream of MYC genes, may provide such a target.

First, polyamine regulators are aberrantly expressed in high-risk neuroblastomas to coordinately augment biosynthesis and reduce catabolism. ODC1 mRNA is significantly higher in high-risk tumors, whereas the antizyme OAZ2 is reduced, consistent with polyamine enhancement (note: unlike OAZ1, OAZ2 may not deliver Odc to the proteasome for degradation, yet it is equipotent at inhibiting Odc activity and polyamine uptake; ref. 36). Moreover, every prosynthetic enzyme (including AMD1, SRM, and SMS) is markedly upregulated in the highest-risk subset with MYCN amplification, whereas, conversely, there is reduced SMOX (37). A similar pattern is seen when evaluating neuroblastoma cell lines in comparison with fetal adrenal or neural tissues (37, 38). Second, ODC1 expression correlates with outcome in neuroblastoma, independent of MYCN amplification, supporting the finding that this upregulation has functional consequences (37). In addition to direct transactivation by MYC genes, ODC1 expression is influenced by a functional promoter polymorphism at the A317G SNP (39, 40). In neuroblastoma, the higher-expressing genotypes have an inferior survival, particularly when analysis is restricted to the MYCN nonamplified tumors, again supporting functional validity for this pathway (41).

Studies with a transgenic model of neuroblastoma support a requirement for polyamines in tumor initiation, progression, and therapy response. Mice carrying a neural crest-targeted MYCN transgene (TH-MYCN model) develop lethal neuroblastoma with complete penetrance in the homozygous state, and ~40% penetrance in the hemizygous state (42, 43). Tumors arise stochastically within peripheral sympathetic ganglia and recapitulate human neuroblastoma features, with cooperative genetic alterations at chromosome regions orthologous to those in the human disease (42, 43). Importantly, tumors arise in an appropriate microenvironment to recapitulate the heterotypic cell interactions important to cancer propagation and provide a relevant therapeutics-testing platform (44). As with human neuroblastomas, TH-MYCN tumors demonstrate altered polyamine regulator expression compared with sympathetic

![Diagram of polyamine metabolism](image-url)

Fig. 1. Schematic of polyamine metabolism required to support cell proliferation and therapeutic opportunities in this pathway. Putrescine (diamine), spermidine (triamine), and spermine (tetramine) are the major polyamines. Ornithine derived from the urea cycle provides the initial substrate for Odc-mediated decarboxylation to putrescine. Amd1 provides the aminopropyl donor to support SRM- and SMS-mediated conversion to higher-order polyamines. Pro-synthetic polyamine enzymes are shown in green; catabolic enzymes are shown in red (underlined enzymes are highly regulated and have among the shortest half-lives of any mammalian enzyme). Polyamine therapeutics and their sites of action are in blue (described in the text). Those shown are in preclinical or early phase clinical development as cancer therapeutics. ODC1, ornithine decarboxylase; SRM, spermidine synthase; SMS, spermidine synthase; AMD1, S-adenosylmethionine decarboxylase; AZIN1, Odc Antizyme inhibitor; SMOX, spermidine oxidase; PAOX, polyamine oxidase; SAT1, spermine/spermidine N-acetyltransferase; OAZ1,2,3, Odc Antizymes.
ganglia, with upregulated ODC1, AZIN, AMD1, SRM, and SMS and downregulated OAZ2, SMOX, and SAT1. Thus, the model likely reflects the polyamine pools, pathway flux, and compensatory mechanisms present in human neuroblastoma.

Treating TH-MYCN mice with α-difluoromethylornithine (DFMO), a suicide inhibitor of Odc, increases tumor-free survival. Moreover, tumor penetrance is reduced in hemizygous mice pre-emptively treated, supporting a requirement for Odc in tumor initiation downstream of MycN (37, 38). Of note, no tumors arose after DFMO withdrawal, consistent with a finite vulnerable period for embryonal oncogenesis. This differs from the Eμ-Myc lymphoma model in which protection from lymphomagenesis required persistent Odc inactivation (45). Neuroblastomas that arise under DFMO exposure may activate compensatory mechanisms to circumvent polyamine depletion. Since upregulated Amd1 accompanies Odc inhibition in mammalian cells, as confirmed in neuroblastoma (46), we tested the ability of DFMO and SAM486 (4-amidinoindan-1-one-2′-amidinhydrazone, a competitive Amd1 inhibitor from Novartis) to synergize in this model. Neuroblastoma penetrance was further reduced, including in homozygous mice; thus, optimized polyamine depletion contributes to markedly improved efficacy (47). A more practical test, however, requires treatment of established tumors. DFMO treatment of TH-MYCN mice harboring clinically detected neuroblastomas extends the time to tumor progression and augments the efficacy of numerous chemotherapeutics, supporting the idea that this strategy may have clinical relevance (37). Select DFMO and chemotherapy combinations improved survival as well, implying that these synergistic effects went beyond cytostasis.

Initial data with human neuroblastoma cell lines grown as xenografts in immunodeficient mice are similarly supportive of the finding that polyamine disruption interferes with tumor progression1, although these studies will need to be extended to assess the role MYCN amplification plays in modulating sensitivity to these inhibitors. It is encouraging that both DFMO and SAM486 inhibit neuroblastoma cell line growth in vitro independent of MYCN amplification (data not shown; ref. 37), likely reflecting a commonality of Myc deregulation in the high-risk phenotype (34). Mechanistically, Odc inhibition reduces Rb phosphorylation at Ser795 and Ser 807/811 through loss of MycN-mediated repression of p27 Kip1, leading to G1 growth arrest. Coincident with this, Akt phosphorylation at Ser 473 and GSK3B at Ser9 are induced, promoting survival (46, 48). Although DFMO similarly abolished p27 Kip1 induction in the Eμ-Myc lymphoma model in vivo, the effects in the TH-MYC neuroblastoma model may instead be mediated through the transcriptional upregulation of p21 Cip1 (38).

Strategies to integrate polyamine depletion therapeutics into neuroblastoma treatment are warranted. It is unlikely that traditional phase 1 and 2 studies for patients with relapsed or refractory high-risk neuroblastoma with only polyamine-targeted agents would generate enthusiasm. These pathway-targeted compounds might best be tested on a backbone of cytotoxic drugs to improve or restore chemoresponsiveness, as
shown with the mouse model, and such clinical trials are in development. However, maximizing the impact of such strategies requires a more complete understanding of tumor-specific polyamine flux and the compensatory mechanisms used to escape blockade in these pathways. For example, many tumor cells respond to Odc inhibition with enhanced polyamine uptake from the microenvironment (49). Radiolabeled spermidine uptake from neuroblastoma cell lines is not induced during DFMO or SAM486 exposure, although it remains possible that this represents accommodation to prolonged tissue culture, as polyamine content is nominal in culture media. Neuroblastoma cell lines established from the TH-MYCN model, however, do induce uptake from two- to six-fold under such conditions. Despite this, they remain sensitive to polyamine depletion in vivo.

There are potential opportunities for improving polyamine depletion responses for tumors with inducible polyamine uptake. First, compounds that antagonize polyamine uptake, such as D-lysine spermine (MQT-1426) and N1-spermyl-L-lysinamide (ORI202), are under preclinical development (50) and may cooperate with DFMO and other biosynthesis inhibitors to more profoundly reduce polyamine levels and improve therapeutic responses in vivo (51). Alternatively, "Trojan horse" approaches with polyamine-chemotherapy conjugates have been used and could cooperate with polyamine biosynthesis inhibitors. In this approach, DFMO or similar agents induce polyamine depletion in cancer cells that subsequently upregulate polyamine transport. This targets the delivery of polyamine-chemotherapy conjugates preferentially to tumor cells. Numerous such conjugates have been developed, but a subsidiary benefit of delivering a DNA-interacting cytotoxic in this manner is that the polyamine moiety not only enhances tumor-specific uptake, but also DNA binding through cationic interactions. The spermidine-podophyllotoxin conjugate F14512 (Pierre Fabre) has superior cytotoxicity in cells with enhanced polyamine uptake in vitro (IC50 in the nanomolar range) and regressed breast carcinoma xenografts in vivo (52), providing strong proof of principal for this approach.

Beyond inhibiting biosynthesis and impeding import, another therapeutic option is to enhance catabolism and/or export of polyamines. For example, NSAIDs influence polyamine acetylation and export through the upregulation of SAT1 via PPARγ (53). Although anticancer effects attributable to this drug class are pleiotropic, cox-inhibitor-mediated apoptosis in cancer cells can be rescued by polyamine supplementation, implicating this pathway (53). This may have therapeutic relevance, because sulindac and DFMO together have documented efficacy in reducing adenoma recurrence in an at-risk population, as demonstrated in a large chemoprevention trial (54). SAT1 induction also occurs downstream of platinator and other chemotheputics, in association with additional polyamine regulator changes to repress polyamine content, and this may provide synergy for select polyamine depletion-chemotherapeutic combinations (55). SAT1 acetylates polyamines and colocalizes with the putative export solute transporter SLC3A2 to facilitate export (25), leading to increased polyamine metabolic flux ~five-fold through upregulated biosynthetic enzyme activity in efforts to restore homeostasis (56). This provides another opportunity for synergy between SAT1 inducers (e.g., NSAIDs, disulfiram) and biosynthesis inhibitors (e.g., DFMO, SAM486) that prevent this response.

Finally, there is great interest in analogs that mimic native polyamines in homeostatic regulation. Such compounds can utilize the polyamine transporter to concentrate in cancer cells, leading to compensatory downregulation of polyamine biosynthesis and upregulation of catabolism, while not substituting functionally for natural polyamines. Agents of this class include assorted symmetrically and nonsymmetrically substituted analogs, conformationally restricted analogs, oligoaamines, and macrocyclic polyamine analogs. These agents are reviewed in (14) and are in preclinical and clinical studies. Of these, PG-11047 (a second-generation conformationally restricted analog) has been shown to have activity in vivo in preclinical non-small cell lung carcinoma models (57), although recent testing against pediatric tumors through the Pediatric Preclinical Testing Program (58) showed minimal activity as a single agent against neuroblastoma (59).

Optimism remains that polyamine depletion can be exploited therapeutically, and the agents discussed herein represent only a partial list of those under active drug development. DFMO (Eflornithine) has gained FDA approval for trypanosomiasis and has been the most rigorously tested agent in this class. Although it has demonstrated potent activity in colorectal adenoma chemoprevention, it has yet to demonstrate potency against more advanced cancers. Whether combination therapies that deprive cancer cells of major compensatory pathways will synergize is not yet known, but many rational combinations to do just that exist. Transitioning this class of agents from scientific discovery to preclinical anticancer activity appears to have been achieved, but bridging the chasm to demonstrate clinical utility remains a significant challenge.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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