Polyamine synthesis as a target of *MYC* oncogenes

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This paper is in recognition of the 100th birthday of Dr. Herbert Tabor, a true pioneer in the polyamine field for over 70 years, who served as the editor-in-chief of the *Journal of Biological Chemistry* from 1971 to 2010. We review current knowledge of MYC proteins (c-MYC, MYCN, and MYCL) and focus on ornithine decarboxylase 1 (*ODC1*), an important *bona fide* gene target of MYC, which encodes the sentinel, rate-limiting enzyme in polyamine biosynthesis. Although notable advances have been made in designing inhibitors against the "undruggable" MYCs, their downstream targets and pathways are currently the main avenue for therapeutic anticancer interventions. To this end, the MYC–ODC axis presents an attractive target for managing cancers such as neuroblastoma, a pediatric malignancy in which *MYCN* gene amplification correlates with poor prognosis and high-risk disease. ODC and polyamine levels are often up-regulated and contribute to tumor hyperproliferation, especially of MYC-driven cancers. We therefore had proposed to repurpose α-difluoromethylornithine (DFMO), an FDA-approved, orally available ODC inhibitor, for management of neuroblastoma, and this intervention is now being pursued in several clinical trials. We discuss the regulation of ODC and polyamines, which besides their well-known interactions with DNA and tRNA/rRNA, are involved in regulating RNA transcription and translation, ribosome function, proteasomal degradation, the circadian clock, and immunity, events that are also controlled by MYC proteins.

This article is dedicated to the 100th birthday of Dr. Herbert Tabor, a scientist with unparalleled knowledge and enthusiasm for the scientific exploration of polyamines. Although Dr. André Bachmann only had the privilege of meeting Dr. Tabor once in his career, at the 1996 Tokyo International Symposium on Polyamines at Shonan Village in Japan, it was an encounter that remains vivid in his memory to this day. More than a decade later, while chatting with one of Dr. Tabor's postdoctoral fellows on his way to the Gordon Research Conference on Polyamines, Bachmann learned that Dr. Tabor – then in his 90s – still arrives early every morning at his office and works in the lab until later in the afternoon, when he transitions to reviewing manuscripts for JBC. His relentless dedication to science and his untethered vision to unravel the mysteries around polyamines have greatly influenced all of us and inspired Bachmann's career. The polyamine group (also fervently referred to as the "Polyamigos") would have never evolved to where it stands today had it not been for Dr. Tabor's hard work and dedication and for his countless contributions to the scientific literature. We are blessed and grateful to know Dr. Tabor, an extraordinary scientist and human being. Happy 100th birthday!

The Bachmann laboratory has a longstanding interest in polyamines and, in particular, in the MYC–ODC axis, which forms the center point of this Minireview. Bachmann's work on polyamines began in 1992 when he studied the role of polyamines during foliar senescence of plant leaves (1). Because of the enthusiasm and encouragements by Dr. Alan Slusarenko and the late Dr. Philippe Matile (University of Zürich, Switzerland), he further delved into the world of polyamines in a quest to identify novel plant ornithine decarboxylase (ODC)[2] inhibitors, using α-difluoromethylornithine (DFMO) as a positive control (2–4). DFMO, also known as eflornithine and Ornidyl, is as a catalytic irreversible (suicide) inhibitor of ODC synthesized in 1978 by researchers at the Merrell-Dow Research Institute (5). DFMO reached FDA approval for the treatment of West African sleeping sickness (trypanosomiasis; intravenous formulation) (6) and for the treatment of excessive facial hair growth (hirsutism; topical formulation). More recently, DFMO, in an oral formulation (powder or tablets), has been under investigation in multiple clinical trials, for example, the chemoprevention of colorectal cancer and pediatric neuroblastoma (7–9).

In the late 1990s, Bachmann slowly drifted away from plant research toward the ODC/cancer field, and in 2002 began investigations toward repurposing DFMO for pediatric neuroblastoma (10, 11). Although the worlds of plant and cancer research are seemingly unrelated, polyamines exist in nearly all living cells. It was those early experiences in plant science that

This article is part of a series on "Polyamines," written in honor of Dr. Herbert Tabor's 100th birthday. A. S. B. is the sole inventor of U. S. patent 9,072,778 issued on July 7, 2015, entitled "Treatment Regimen for N-Myc, C-Myc, and L-Myc amplified and overexpressed tumors". A. S. B. is the co-founder, board member, and President of Hibiskus Biopharma, Inc.

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[2] The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; SPR, sepiapterin reductase; FDA, Food and Drug Administration; SSZ, sulfasalazine; SNP, single nucleotide polymorphism; BR, basic region; HLH–LZ, helix-loop-helix–leucine zipper; eIF, eukaryotic initiation factor; PI3K, phosphatidylinositol 3-kinase; mTOR, mechanistic target of rapamycin; DHPS, deoxyhypusine synthase; NSAID, nonsteroidal anti-inflammatory drug.

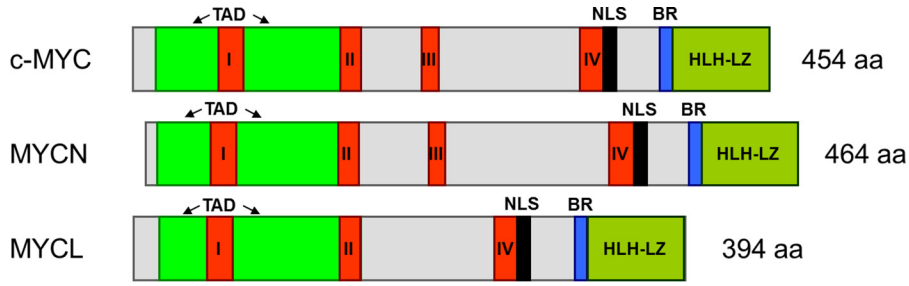

Figure 1. Human MYC proteins. Shown is a schematic alignment of human c-MYC, MYCN, and MYCL. *I–IV*, MYC homology boxes I–IV; *TAD,* transactivation domain; *NLS,* nuclear localization signal; *BR,* basic region; *HLH–LZ,* helix-loop-helix–leucine zipper domain; *aa,* amino acid. Depicted are the longest RefSeq isoforms at NCBI_gene (https://www.ncbi.nlm.nih.gov/gene): NP_002458.2 (c-MYC IF1), NP_005369.2 (MYCN IF1), and NP_001028254.2 (MYCL IF3). Domains are assigned based on Ref. 32 for c-MYC, and combined NCBI_gene annotation and BlastP alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for the other MYC proteins.

gave him the scientific knowledge on polyamines, which ultimately led to the idea of repurposing DFMO for the treatment of children with (*MYCN*-amplified) neuroblastoma.

Bachmann met the co-author of this Minireview, Dr. Dirk Geerts, in 2002 at the "Advances in Neuroblastoma Research" conference in Paris, France. It was at this conference that Bachmann "connected the dots" and concluded that DFMO should be beneficial to neuroblastoma patients, due to fact that MYCN activates the *bona fide* gene *ODC1* (10, 11). Although ODC as a drug target had been well-established by that time (12–14), the specific use of DFMO for the treatment of neuroblastoma–*in the clinical setting*-had not been seriously considered in the literature (10, 11). Over the following years, the authors continued their collaboration to investigate *MYCN*-driven ODC expression and polyamine regulation in neuroblastoma (8, 10, 11, 15–25). These preclinical efforts ultimately led to the first phase I neuroblastoma clinical trial with DFMO in 2010 (8). Today, multiple independent phase I and II DFMO neuroblastoma clinical trials are ongoing across the United States of America and in Australia (9).

MYC oncogenes

The *MYC* family of transcription factors is one of the most central–and most studied– gene groups in development and cancer. Three different *MYC* genes have been described: c-*MYC* (*MYCC*), *MYCN*, and *MYCL* (when all three genes are described in this Minireview, they will be named "*MYC*"). Initially discovered in the late 1970s (26), these three genes are aberrant in the majority of cancer types, performing oncogenic functions that correlate with aggressive tumor growth and poor patient prognosis. The human MYC proteins are around 400–450 amino acids in length (see Fig. 1) and are very homologous. This homology is highest in the four short "MYC homology boxes" I–IV that are important for MYC protein activity and oncogenic function. Two other longer and conserved regions exist in MYC: the N-terminal transactivation domain that can transfer transcription activity to the DNA-binding domain of another protein, and the C-terminal basic region (BR), necessary for binding to MYC target sites (the -CACGTG- "E-boxes") on downstream target genes. The BR is coupled to the helix-loop-helix–leucine zipper (HLH–LZ) domain that allows MYC to bind partner proteins, such as MAX, that are needed for efficient target gene activation.

The three *MYC* genes were all discovered in relation to cancer: c-*MYC* as a eukaryotic homolog of the *v-myc* avian virus oncogene; *MYCN* in neuroblastoma; and *MYCL* in lung cancer (26). The *MYC* genes are located on different chromosomes but share a simple gene structure that suggests they derive from an insertion of a v-*myc*–like, viral oncogene and later gene multiplication. Because MYC studies have primarily focused on c-*MYC*, most data exist on c-*MYC* functions, but *MYCN* and *MYCL* are equally powerful oncogenes. Considering the extensive homology between the *MYC* genes, their functional differences are in part a consequence of their differential mRNA expression during development and among tissue types. c-*MYC* is expressed throughout development and has ubiquitous expression in most– especially proliferative–tissues. c-*MYC* is the highest expressed *MYC* gene. By comparison, *MYCN* shows the highest expression during development, especially in the nervous system, which then declines considerably, but remains detectable in brain, genital tract, kidney, and stomach. *MYCL* shows restricted expression, with levels in between c-*MYC* and *MYCN*, mainly in the bladder, colon, esophagus, pancreas, and skin (for an overview, see https://www.ncbi.nlm.nih.gov/gene).

The cancer field received major new insights by the publication of the Hanahan and Weinberg reviews in 2000 and 2011 (27, 28). The authors present a list of cellular functions that a cancer cell needs to control by changing genome and gene expression. These functions are called "cancer hallmarks." *MYC* genes are unique among oncogenes in that they can achieve most, if not all, of these hallmarks. One reason for this is that *MYC* genes, as "super transcription factors," can regulate the activity of ~15% of all human genes (29). Another reason is that MYC proteins act as obligate partners of other BR–HLH–LZ transcription factors, in the MAX–MLX network (30). It has long been known that *MYC* genes boost RNA production, ribosome biogenesis, and mRNA translation. *MYC* genes thereby support the classic hallmarks of sustained proliferation and replication, evasion of growth suppression and cell death, and activation of adhesion/migration (27, 31, 32). More recently, *MYC* genes were also shown to regulate the new hallmarks of genome integrity, metabolism, immune evasion, and inflammation (28, 32–34). Importantly, *MYC* genes can activate ornithine decarboxylase 1 (*ODC1*), a sentinel gene in polyamine synthesis (12–14) suggesting that *MYC* genes are central

Table 1

***MYC* and *ODC1* aberrations in human cancer**

Public human cancer data were queried for coding mutations (Mut), copy number variations (CNV), and mRNA dysregulation (mRNA) of the (three) *MYC* and *ODC1* genes. Numbers represent % of samples with an aberration: white fields represent <1% aberrations; colored fields represent 1–5, 5–10, 10–25, and >25%. (1–5) means that 1–5% aberrations were found in specific tumor subtypes only. All datasets containing human cancer samples available on the public websites COSMIC (https://cancer.sanger.ac.uk/cosmic), cBioPortal (http://www.cbioportal.org/) (167, 168), and OncoMine (https://www.oncomine.org/) (169) were analyzed for a total of >1000 sets. Tumor types are represented by a minimum of three datasets. (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.) Aberrations from identical samples in different datasets were not over-counted. Single mutations in a dataset or datasets containing <100 samples, were not used for calculations. Presented are only the significant changes in mutant reads, CNV, or mRNA expression, according to the website default analysis parameters. Correlations between (any) *MYC* and *ODC1* mRNA expressions were calculated on the R2 website (http://r2.amc.nl/), using a 2log Pearson test on all tumor datasets on Affymetrix arrays (n = 242). Analysis was as in Ref. 17. n.d. = no data, n.s. = not significant.

Tissue	c-MYC			MYCN			MYCL			ODC1			MYC-ODC1
	Mut	CNV	mRNA	Mut	CNV	mRNA	Mut	CNV	mRNA	Mut	CNV	mRNA	Correlations
Adrenal gland			5-10			5-10						5-10	n.d.
Bladder (incl. urinary tract)		5-10	5-10	1-5	1-5	10-25	(1-5)	5-10	10-25		1-5	1-5	Positive
Brain		1-5	5-10		1-5	5-10		(1-5)	1-5		(1-5)	5-10	Positive
Breast	(1-5)	10-25	5-10		1-5	5-10		1-5	1-5		1-5	5-10	Positive
Cervix		1-5	5-10	1-5	1-5	1-5		1-5	5-10	1-5	1-5	1-5	Positive
Colon	1-5	5-10	10-25	1-5	(1-5)	5-10	(1-5)		1-5	(1-5)	(1-5)	5-10	Positive
Esophagus	(1-5)	5-10	5-10	(1-5)	(1-5)	5-10	(1-5)	1-5	5-10		1-5	10-25	n.s.
Germ Cell		1-5	1-5		1-5	5-10			1-5			5-10	n.s.
Head & Neck (incl. thyroid)	1-5	5-10	10-25	1-5	(1-5)	1-5		1-5	1-5		(1-5)	5-10	Positive
Kidney		(1-5)	5-10	1-5	10-25	5-10			10-25	(1-5)	1-5	1-5	Positive
Leukemia - Acute Lymphoid	1-5	1-5											Positive
Leukemia - Acute Myeloid	1-5		5-10			1-5			5-10			1-5	Positive
Liver (incl. biliary tract)		5-10	5-10		1-5	1-5		(1-5)	5-10		(1-5)	5-10	Positive
Lung (incl. pleura)	1-5	5-10	5-10	1-5	1-5	5-10	(1-5)	1-5	5-10		1-5	5-10	Positive
Lymphoma - Diffuse B-Cell	5-10	(1-5)	5-10			1-5			1-5				Positive
Lymphoma - T-cell	1-5												Positive
Myeloma	> 25												Positive
Ovary		> 25	5-10		1-5	5-10		10-25	5-10		1-5	5-10	Positive
Pancreas		10-25	5-10	1-5		5-10		(1-5)	1-5			5-10	Positive
PNS (incl. autonomic ganglia)			5-10		10-25	5-10			1-5			5-10	Positive
Prostate	(1-5)	5-10	5-10		(1-5)	5-10		(1-5)	5-10		1-5	5-10	Positive
Skin - Melanoma	5-10	1-5	5-10	1-5		1-5	(1-5)		1-5	1-5		5-10	n.s.
Skin - Other	5-10			5-10			5-10			5-10			n.d.
Soft tissue (incl. bone)		5-10	1-5			1-5		(1-5)	1-5		(1-5)	1-5	Positive
Stomach	1-5	10-25	5-10	(1-5)	1-5	5-10	1-5	1-5	1-5	1-5	(1-5)	5-10	n.d.
Uterus (incl. endometrium)	1-5	5-10	10-25	1-5	5-10	5-10	1-5	1-5	5-10	1-5	1-5	5-10	Positive

regulators of polyamine metabolism, as further discussed in this Minireview (see under "Polyamine synthesis and regulation").

MYC genes can fulfill these oncogenic functions by escaping their normal, strict regulation. *MYC* genes are among the most frequently deregulated oncogenes in up to 25% of tumors and in many different cancer types (Table 1) (35, 36). The *MYC* genes only rarely accumulate coding sequence–altering mutations, with a notable exception for *MYC* gene fusions in lymphomas and myelomas (37). *MYC* gene amplifications have long been considered the most common *MYC* deregulation events (35, 36) and are often accompanied by "enhancer hijacking" to upregulate *MYC* expression even further (38, 39). Tumors can contain multiple copies of one, two, or three different *MYC* genes. Occasionally, a specific *MYC* gene can govern a specific cancer subtype, for example, in brain or breast. For an overview of cancer types and *MYC* gene amplifications, see Table 1 and Refs. 26, 35, 36, 40. More recently, *MYC* gene DNA methylation and mRNA expression have also received attention as more dynamic strategies for *MYC* dysregulation (see also Table 1). As central transcription factors, *MYC* genes are prime candidates for establishing "tipping points" in cell fate (30), so that even small differences in expression could result in oncogenesis.

MYC, the "undruggable target"

The central oncogenic role of the MYC proteins in many cancer types has evoked significant effort in their targeting for novel, specific cancer therapy; however, MYC proteins have proven to be difficult to drug (hence often referred to as the "undruggable target"). The three reasons for this are as follows: 1) because the MYC proteins act as transcription factors, they lack specific active sites with a defined 3D structure, but instead they function by using larger, flexible protein domains such as the leucine zipper; 2) MYC proteins are so-called "intrinsically disordered proteins" (41), making the design of tight-binding moieties even more difficult; and 3) MYC proteins are active in the nucleus, which precludes targeting with larger molecules (*e.g.* antibodies) (42). MYC targeting has recently been excellently reviewed (31, 42, 43), and we present only a brief summary. Targeting efforts have focused on four biological processes.

MYC transcription

MYC genes contain a 5′ G-quadruplex, a G-rich folded strand DNA structure upstream of the promoter that has to be

resolved for transcription to occur. Compounds like enniantin-B, TH3, and APTO-253 that stabilize this structure decrease *MYC* transcription (44, 45). Furthermore, efficient *MYC* gene transcription needs docking of BRD proteins and other co-activating molecules, including IKZF, NME2, and CDK9 at the promoter. Especially, the inhibition of BRD4 with compounds such as JQ1 (46), OTX015 (47), and CPI-0610 (48) appears very promising for indirect blocking of MYC protein function. Drugs that target the other co-activators are also being investigated (49–51).

MYC translation

There has also been some progress in targeting *MYC* translation, in particular via specific translation inhibition by *MYC* siRNA (52) or targeting of ribosomal function using CX-5461 (53) and Inauhzin (54). Significant contributions have been made by the Ruggero group in understanding MYC-controlled protein synthesis and deregulation of translational control in cancer (55–59). MYC regulates multiple drivers in the translational machinery, including ribosomal proteins and eukaryotic initiation factors of translation (eIFs), leading to an increase in protein synthesis that is required for cell growth, cell cycle progression, and genome instability as a mechanism for cancer initiation (56).

MYC protein stability

MYC proteins are normally unstable and show cell cycle–regulated expression. Only present at low concentrations in quiescent cells, MYC protein expression is rapidly induced as cells enter the G_1 phase of the cell cycle in response to serum or mitogens. In noncancerous cells, MYC levels then decrease to a low steady-state concentration as long as the cells proliferate, as a result of timed MYC removal by the proteasome. This degradation depends upon phosphorylation of residues in MYC homology box I and subsequent ubiquitination by FBXW7, for example (60). In cancer cells, MYC degradation can be prevented by high expression of deubiquitinases (61, 62) or kinases that interfere with FBXW7, notably Aurora-A, PLK1, and proteins in the PI3K/AKT/mTOR route (63, 64). These proteins enable MYC-dependent, sustained cell cycle progression by permitting continuous high-MYC protein expression. Therefore, specific inhibition of these molecules has received much attention. In particular, Aurora-A has been targeted with inhibitors, including alisertib (65, 66), MLN8054 (67), MLN8237 (68), and CD532 (69). Also, PLK1 can be inhibited with volasertib (70) and BI2536 (71). Finally, the P22077 compound inhibits the USP7 deubiquitinase necessary for high MYCN expression in neuroblastoma (62). It is important to mention the many efforts in MYC metabolic targeting aimed at the PI3K/AKT/mTOR axis. In particular, mTOR was targeted with multiple compounds, for instance temsirolimus, everolimus, dactolisib, and INK128 (reviewed in Refs. 43, 72).

MYC DNA binding

The transcription factor function of MYC proteins depends on dimerization with proteins like MAX. The leucine zipper domain involved in this process has been targeted with several compounds, such as 10058-F4 (73), 10074-G5 (74), and Mycro3 (75). Very interesting is the recent development of compounds that can bind to multiple conformations of the leucine zipper (41). Screening of large compound libraries resulted in the characterization of additional molecules that inhibit the MYC–MAX protein interface (76–78) and in a mimic of the MYC–MAX complex E-box– binding domain (76–78) that represents interesting candidates for further development.

Although direct targeting of the *MYC* genes and proteins is the subject of intense study, it is still a relatively new field. Over the years, the indirect targeting of MYC by inhibition of one or more MYC downstream processes, including cell cycle, apoptosis evasion, and recently also tumor metabolism and immune function, has been more successful. This is due to MYC's "Achilles heel": *MYC* genes obtain their powerful oncogenic functions in part by changes in the cancer cell that, paradoxically, make the tumor more vulnerable to specific insults. Decreased MYC function, in combination with inhibition of a downstream process that is not normally toxic to the cell, can then lead to catastrophic cell death. This phenomenon called synthetic lethality (79–81) has been extensively investigated in *MYC*-addicted cancers (reviewed in Refs. 82–84). For instance, tumors with high *MYC* activity are especially sensitive to inhibition of the CHK1 cell cycle checkpoint with SB21807 or TCS2312 (85), the BCL2/BCLxl apoptotic axis using ABT-199 (86), and spliceosome activity using SD6 (87). A very promising synthetic lethal association is between *MYC* overexpression and RAS mutation, making tumors very sensitive to otherwise innocuous CDK2 inhibition (88–90).

Finally, it should be noted that we found DFMO treatment in neuroblastoma leads to significant down-regulation of MYCN protein, an indirect effect of DFMO that was further enhanced in combination with SAM486A, an AMD1 (also known as SAMDC or AdoMetDC) inhibitor (10, 11). Our observation suggests a polyamine-dependent, negative feedback loop that regulates MYC expression. Indeed, Tabib and Bachrach (91) demonstrated in Kirsten sarcoma virus–infected rat kidney cells that putrescine triggers the transcription of c-*MYC* mRNA and that inhibition of ODC activity by DFMO, which depletes putrescine, prevented the c-*MYC* transcription. The involvement of putrescine in the transcription of c-*MYC* mRNA was further confirmed by adding putrescine to cells, which resulted in the formation of c-*MYC* transcripts. These findings support the notion that putrescine (and possibly spermidine and spermine) is involved in the transcription of c-*MYC* and may in part explain our own findings that DFMO and DFMO/SAM486A treatments lead to reduced levels of MYCN protein in neuroblastoma cells (11).

Polyamine synthesis and regulation

The polyamine spermine has been first described in 1677 (92) by Antonie van Leeuwenhoek (also spelled Anthonii Lewenhoeck), preceding the discovery of DNA in 1868 by ~200 years, when the Swiss physician Friedrich Miescher isolated "nuclein" from the nuclei of white blood cells (93). Polyamines are aliphatic cations that interact with negatively charged molecules, including nucleic acids (94, 95). In 1957, Hershey (97) showed that polyamines can bind to phage DNA, and this was confirmed by Ames *et al.* in 1958 (96). The secondary structures

of polyamines in association with DNA were revealed using X-ray diffraction technology (98, 99). Polyamines were also found in association with tRNA and rRNA (100, 101) and contribute to chromatin modification and histone acetylation (102, 103). Over the years, it was discovered that polyamines are implicated in many cellular processes, including the regulation of ion channels (104) and their participation in transcriptional, translational, and post-translational events (105).

Ornithine decarboxylase (EC 4.1.1.17) is a sentinel enzyme that is mandatory to polyamine synthesis. ODC catalyzes the conversion of ornithine to putrescine (a diamine) through the release of carbon dioxide (CO_2). Putrescine is the precursor in the synthesis of spermidine (a triamine) and spermine (a tetraamine) which are formed by the action of spermidine synthase and spermine synthase, respectively (Fig. 2). Collectively, putrescine, spermidine, and spermine are referred to as polyamines. ODC has also transforming and oncogenic abilities independent of MYC (106, 107). ODC and AMD1 are rate-limiting enzymes in polyamine synthesis (108–110) and are frequently dysregulated in cancer, with a critical role in cell proliferation (108, 111–114).

It has been well documented that ODC antizyme 1–3 (OAZ1–3) proteins are negative regulators of cellular polyamine content, and OAZ expression is controlled via a unique feedback mechanism that involves a +1 frameshift during translation, induced by high cellular polyamine levels (115). ODC is functional as a homodimer, which creates two active sites at the dimer interface that contain residues contributed by each subunit. ODC monomer interactions are relatively weak and the protein cycles between monomeric and dimeric forms. Therefore, a catalytically dead ODC protein can exhibit dominant-negative properties (116). In addition, OAZ binding to ODC monomer leads to its inactivation and ubiquitin-independent proteasomal degradation (117–120). The predominant OAZ family member is antizyme 1 (OAZ1); OAZ2 is usually expressed at significantly lower concentrations, and OAZ3 is restricted to expression in testis (120, 121). Two ODC antizyme inhibitors, AZIN1 and AZIN2, further contribute to the regulation of ODC enzyme activity, a testament to the level of complexity that governs cellular ODC expression in maintaining polyamine homeostasis. Both AZINs are strikingly similar to ODC but lack any decarboxylase activity due to critical amino acid substitutions (122). Notably, AZINs bind to OAZ with greater affinity than ODC, thus leading to a natural competition and liberation of ODC from the inactive ODC–AZ heterodimer complex (123).

OAZs are the only well-established proteins that physically interact with ODC, thereby regulating its activity and degradation. We recently proposed that the ODC–OAZ–AZIN regulatory mechanism is further controlled by a new player, sepiapterin reductase (SPR), an enzyme that forms tetrahydrobiopterin (BH_4), a cofactor of nitric-oxide synthase, and together form a quartet that co-regulates both polyamine and NO biosyntheses (22). Because the predicted SPR interaction sites are close to those of ODC, there might be a competitive mechanism in which ODC and AZINs compete for binding to SPR. Sulfasalazine (SSZ) is a salicylate-based anti-inflammatory and immune-modulatory drug approved by the FDA for the treatment of ulcerative colitis and rheumatoid arthritis. SSZ is an SPR inhibitor (124), and we confirmed this finding with purified and active SPR enzyme.[3] We found that SPR knockdown by RNAi reduces endogenous ODC enzyme activity and leads to neuroblastoma tumor cell growth inhibition (22). In addition, high SPR mRNA expression in human neuroblastoma tumors samples correlated significantly with poor survival prognosis. We further showed that pharmacological interference with SSZ inhibits neuroblastoma tumor cell growth, with significant synergisms if combined with DFMO (25).

MYC–ODC axis

A number of reports in the mid-1980s hinted at a potential connection between MYC and ODC, for example through observations that included the concomitant overexpression or co-amplification of *MYC* and *ODC1* (125–128). However, a study led by George *et al.* (129) at the UK Children's Cancer Study Group reported that co-amplification of *MYCN* and *ODC1* was not detected in seven cell lines and 87 primary tumors. In contrast, more recent reports by the Hogarty group proposed that 13–20% of *MYCN*-amplified neuroblastoma tumors have co-amplification of *ODC1* (130, 131). This *MYCN* and *ODC1* co-amplification demonstrates the first example of targeted deregulation of an oncogenic transcription factor (*MYCN*) and its oncogenic target gene (*ODC1*) (130).

Although MYC transcription factors activate a large number of genes (25), *ODC1* is one of the first and best characterized *bona fide* targets. In 1992, the Cleveland group first reported that the *ODC1* gene is a direct transcriptional target of c-*MYC*, resulting in a growth factor-independent expression of *ODC1* (12, 13). The *ODC1* gene harbors canonical MYC-binding sites in its promoter that contain the conserved E-box motif. The Cleveland group later demonstrated *in vivo* in Eμ-Myc transgenic mice that *ODC1* is a critical MYC transcription target and that targeting ODC with DFMO prevents tumor formation in *MYC*-induced lymphogenesis (132). In this regard, it is noteworthy that Zell *et al.* (133) described a single nucleotide polymorphism (SNP) in the region of the *ODC1* gene E-boxes that affects MYC and MAD binding to *ODC1*, and this SNP is linked to colon cancer recurrence.

In 2004, we proposed the repurposing of DFMO for neuroblastoma in the clinic (10). This was based on the principal idea that *MYCN* amplification is a key prognostic feature in high-risk patients that confers poor prognosis (134), thus making the MYCN–ODC axis an attractive drug target. At that time, DFMO was already approved by the FDA for (intravenous) treatment of West African sleeping sickness (trypanosomiasis). DFMO was also available in oral form and had an excellent safety record, thus making it a prime candidate for pediatric cancer clinical studies. We performed a number of preclinical studies over the years to investigate polyamine pathway–associated enzymes and the impact of their inhibitors, including ODC/DFMO, SAMDC/SAM486A, DHPS/GC7, polyamine uptake receptor/AMXT-1501, SPR/sulfasalazine (8, 10, 11, 15–25), and combinations thereof in neuroblastoma. More

[3] A. S. Bachmann and D. Geerts, unpublished data.

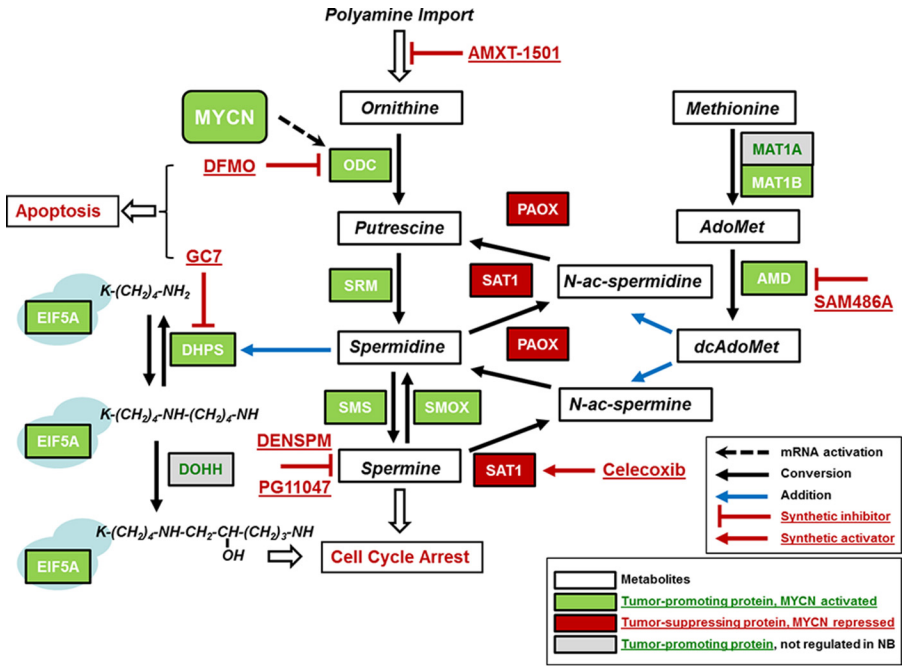

Figure 2. Hypusine–polyamine pathway in human neuroblastoma. Shown is a graphical overview of the genes involved in eIF5A hypusination and polyamine metabolism with their protein activities and metabolites. Spermidine is the sole substrate and is mandatory for the synthesis of hypusine in eIF5A. Also shown are the action sites of the inhibitors used in neuroblastoma (*NB*) studies. References for additional inhibitor studies in neuroblastoma not named in the main text are as follows: DENSPM (164) and PG11047 (165). Genes are highlighted in *green* or *red* according to their prognostic value in Zhang-498, the largest publicly available RNA-Seq dataset for human neuroblastoma (166). *Green* or *red* indicates that high mRNA expression is significantly prognostic for poor or good patient survival in Kaplan-Meier analysis, respectively. In addition, gene mRNA expression was analyzed for correlation with *MYCN* gene amplification and MYCN mRNA expression using the R2 website (http://r2.amc.nl). (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.) With the exception of *DOHH* and *MAT1B*, all other pathway genes that are prognostic for neuroblastoma also appear to be regulated by *MYCN* in this tumor. For more details on the analysis see Refs. 17, 18, 24. Data from Zhang-498 are available at the Gene Expression Omnibus database under accession number GSE62564. Reproduced with permission from Ref. 24. This research was originally published in Biochemical Journal. C. R. Schultz, D. Geerts, M. Mooney, R. El-Khawaja, J. Koster, and A. S. Bachmann. Synergistic drug combination GC7/DFMO suppresses hypusine/spermidine-dependent eIF5A activation and induces apoptotic cell death in neuroblastoma.. Biochem. J.2018; 475, 531–545. © Portland Press Ltd.

recently, we studied DFMO in osteosarcoma (135) and endometrial cancer (136). Notably, by early 2009, two excellent papers by the Hogarty and Cleveland groups had independently confirmed that DFMO inhibits tumor growth *in vivo* using the transgenic TH-*MYCN* neuroblastoma mouse model (131, 137). A landmark study with DFMO as a chemopreventive agent in colorectal cancer, in combination with sulindac, was published in 2008 by Meyskens *et al.* (7). A number of recent contributions by the Hogarty group further expanded our current knowledge on the functional relevance of ODC and polyamines in neuroblastoma (59, 130, 138–140).

Although polyamine synthesis has received widespread attention as a target for cancer therapy for close to 30 years, the focus has primarily been on epithelial tumors (carcinomas) (141). Clinical trials have predominantly been performed on breast, cervix, colon, lung, and prostate carcinomas, with a few additional studies in glioma and lymphoma (16, 141–143). Most trials with DFMO monotherapy had no or moderate success, except when used in a chemoprevention setting. However, combination therapies with NSAIDs or conventional chemotherapy agents, for example, appeared to be much more effective (7, 141, 142). Indeed, these cancer types show frequent aberration of one or more *MYC* genes, together with overexpression of *ODC1* mRNA that appears regulated by MYC activation (see Table 1). Clearly, several additional carcinomas, including head and neck, liver, ovary, pancreas, and uterus, show this MYC–*ODC1* activation pattern as well as some blastomas (for example, glioblastoma, the peripheral nervous system (PNS) tumor neuroblastoma, and the kidney tumor nephroblastoma) and sarcomas. Most of these cancer types could be selected for polyamine synthesis intervention studies, and several successful animal models have been available for a long time (144).

Neuroblastoma

Neuroblastoma appears amenable to polyamine inhibition as cancer therapy. High-stage tumors have almost 50% *MYCN* gene amplification and concomitant MYCN mRNA and protein overexpression (134), together with amplification and up-regulation of the sentinel *ODC1* polyamine synthesis gene (12, 13, 130, 131). MYCN up-regulation of *AMD1*, another sentinel polyamine synthesis gene and *bona fide* target of MYC, was also shown in neuroblastoma (145, 146). Later studies in the TH-*MYCN* neuroblastoma mouse model (131, 137), and bio-informatic analysis of large sets of human neuroblastoma samples (see Fig. 2) (17, 18, 24), have now shown that genes connected to polyamine biosynthesis (*AMD1*, *AZIN1*, *DHPS*, *EIF5A*, *MAT1B*, *ODC1*, *SMS*, and *SRM*) are all up-regulated in neuroblastomas with *MYCN* amplification/up-regulation. In contrast, the *OAZ2*, *PAOX*, and *SAT1* genes, involved in polyamine catabolism, are all down-regulated in these tumors. Strikingly, all these genes are also significantly prognostic for patient survival (18, 24, 130, 131).

Table 2
Polyamine metabolism interventions in neuroblastoma: Clinical trials
All clinical trials on neuroblastoma intervention are based on polyamine level inhibition, as listed in the National Institutes of Health clinical trial database (https://clinicaltrials.gov/; query date Aug. 8, 2018).

NCT no.	Status	Phase	Patient status	Interventions	Drugs
NCT01059071	Complete[a]	1	Refractory/relapsed	DFMO to prevent recurrence	Plus etoposide
NCT01586260	Active, not recruiting[a]	2	Remission	DFMO to prevent recurrence	Monotherapy
NCT02030964	Active, not recruiting	1	Refractory/relapsed	DFMO to prevent recurrence	Plus three agents[b]
NCT02139397	Recruiting	1/2	Refractory/relapsed	DFMO to prevent recurrence	Plus bortezomib
NCT02395666	Active, not recruiting[a]	2	Remission	DFMO to prevent recurrence	Monotherapy
NCT02559778	Recruiting	2	New diagnosis	DFMO in high-risk therapy	Plus six agents[c]
NCT02679144	Recruiting	2	Remission	DFMO to prevent recurrence	Monotherapy

[a] Results are published in Refs. 8 and 9.
[b] These are the three agents used: celecoxib, cyclophosphamide, and topotecan.
[c] These are the six agents used: bortezomib, crizotinib, dasatinib, lapatinib, sorafenib, and vorinostat.

The MYC–hypusine–polyamine network (147) appears to be central to the progression of MYC(N)-driven cancers, such as neuroblastoma, and is therefore eminently suited for novel therapeutic intervention. Hypusine-dependent activation of the eukaryotic translation initiation factor eIF5A is a unique post-translational process that exquisitely depends on the availability of spermidine, thus directly linking MYC(N), ODC, and the polyamines (spermidine) to eIF5A-controlled translational mechanisms that drive the increased biomass production necessary during tumorigenesis. In addition, eIF5A has been connected to the LIN28/let-7 pathway, which is active in neuroblastoma (148–150).

In support of this concept, the groups of Bachmann, Cleveland, and Hogarty (11, 18–25, 130, 131, 137, 147) have performed a number of preclinical studies with specific targeting of the neuroblastoma MYC–hypusine–polyamine network (see Fig. 2 for the inhibitors used. The legend lists the studies.). DFMO was a potent inhibitor of the neuroblastoma cell cycle, proliferation, and invasion (11, 20, 21). Other drugs that also target the MYC–hypusine–polyamine network, GC7, SAM486A, and AMXT-1501, were additive or synergistic with DFMO, resulting in cell cycle arrest and/or apoptosis (11, 17, 19, 21, 23, 24). In studies with the TH-*MYCN* mouse model, the Hogarty group showed that SAM486A and the NSAID celecoxib, which can activate SAT1, also potentiate DFMO activity (130). Finally, DFMO enhances the activity of standard chemotherapeutic agents such as cyclophosphamide and cisplatin in their studies (131).

Together, these preclinical studies strongly supported the use of DFMO for neuroblastoma therapy in the clinical setting. The first phase I trial with DFMO plus etoposide was launched in 2010 in neuroblastoma patients with relapsed or refractory disease, at Helen DeVos Children's Hospital (Grand Rapids, MI) and multiple consortium hospitals (coordinated through the Beat Childhood Cancer Consortium, formerly known as NMTRC). DFMO was well tolerated, and the therapy resulted in longer progression-free survival (8). Currently, several DFMO clinical trials are in progress (see Table 2) and include a recently completed DFMO phase II maintenance trial with neuroblastoma patients that are in remission but at high risk for relapse (9). Table 2 also shows that DFMO is being tested in addition to standard chemotherapy in newly diagnosed patients with high-risk disease. The results are eagerly awaited.

Considering the central role of polyamine metabolism in many cancer hallmarks, ample possibilities remain for further neuroblastoma combination therapies. One obvious strategy is to combine the inhibition of polyamine biosynthesis with targeting polyamine import, which was successful in neuroblastoma cell culture studies (23) and in a mouse melanoma xenograft model (151). Interestingly, this combined polyamine blocking treatment *in vivo* resulted in increased immune response (151). Also, *MYC* genes are involved in tumor immune response: they induce immune checkpoints like CD47 and PD-L1, regulate immune molecules like cytokines, and thereby allow tumor cells to escape immune surveillance (152). In exchange, this MYC dependence might make tumors vulnerable to immunotherapy. An interesting link between MYC and polyamines is arginase; it converts arginine to ornithine and thus directly feeds into polyamine biosynthesis. High arginase expression in neuroblastoma is prognostic for poor patient outcome and significantly is positively correlated to *MYCN* amplification and mRNA expression (results not shown and see Refs. 153,154). Indeed, arginase activity in the TH-*MYCN* mouse model was linked to decreased immune surveillance and impaired immune therapy response through a yet uncharacterized mechanism (153, 154). It is not unthinkable that combination therapies targeting MYC and polyamine metabolism will evoke a synthetic lethal response, especially in high-risk neuroblastomas.

Novel roles for polyamines

It has been well established that ODC is one of few proteins rapidly regulated by a ubiquitin-independent proteasomal degradation mechanism that requires interaction with ODC antizyme (117–120). The C-terminal destabilization region (37 amino acids) of ODC is critical for ODC degradation in the proteasome. Interestingly, it was shown that MYC interacts with OAZ2 in the nucleus and nucleolus and can also accelerate MYC degradation by the proteasome (155). Nucleolar MYC plays a key role in positively regulating ribosomal RNA (rRNA) synthesis, and therefore, OAZ2 contributes to pre-rRNA synthesis through control of nucleolar MYC levels. This suggests that OAZ2 is regulating ribosome biogenesis through MYC degradation, but the detailed mechanisms remain unclear (155). One explanation might be that OAZ2 contributes to ribosome biogenesis by controlling the proteasomal degradation of both MYC and ODC, thereby regulating available

spermidine pools needed for the following: 1) spermidine-dependent eIF5A hypusination (activation) and/or 2) spermidine-dependent, mTORC-mediated eIF4E activation, both proteins that play a role in the initiation, elongation, and termination of mRNA translation and have been connected to tumor formation (55–59). Pharmacological strategies to specifically block spermidine-dependent hypusination of eIF5A by inhibition of DHPS with GC7 alone or in combination with DFMO have been explored for neuroblastoma in our lab (17, 18, 24). Inactivation of eIF5A (and/or possibly eIF5A2) is expected to suppress mRNA translation and biomass production, a prerequisite for the survival of hyper-proliferating cells and tumorigenesis (147).

Polyamine metabolism has been linked to glycolysis in neuroblastoma cells (156). Impairment of glycolysis is able to trigger signaling events that lead to the reduction of MYCN protein levels and subsequent decrease of both ODC expression and polyamine levels, accompanied by cell cycle blockade preceding cell death (156). Moreover, c-MYC regulates a transcriptional program that stimulates mitochondrial glutaminolysis, which leads to glutamine addiction (157). Notably, c-MYC controls metabolic reprogramming upon T lymphocyte activation and links glutaminolysis to the biosynthesis of polyamines (33, 158). These findings suggest that polyamines contribute critically to the uptake and metabolism of nutrients and the addiction of cancer cells to glucose as initially described by Warburg and co-workers (159, 160).

An exciting new discovery was recently made by Zwighaft and co-workers unraveling a novel mechanism through which polyamines regulate circadian rhythms (161, 162). The authors showed that polyamine levels oscillate in a daily manner. Both clock- and feeding-dependent mechanisms regulate key enzymes of polyamine biosynthesis through engagement of BMAL1:CLOCK and core clock repressors *PER2* and *CRY1*. Notably, BMAL1:CLOCK is a heterodimeric master circadian transcription factor that has E-box–binding sites and therefore binds c-MYC and MYCN (163). Moreover, MYC alters the oscillation of glucose metabolism and perturbs glutaminolysis, thus suggesting a new link between oncogenic transformation and circadian and metabolic dysrhythmia that involves polyamines and contributes to oncogenicity (163).

Finally, our group recently discovered a novel *de novo* pathogenic variant in the *ODC1* gene in a 32-month-old girl with developmental delay, alopecia, and dysmorphic features. The mutation leads to the deletion of 14 amino acids at the ODC C terminus which causes ODC protein and putrescine accumulation in red blood cells. This is the first human case reported that presents with this new syndrome (also referred to as Bachmann–Bupp syndrome) and shows the importance of ODC in human development (170). Treatment with DFMO to counteract the increased ODC levels might be a therapeutic option for this and future patients.

Conclusions

MYC genes are master regulators and importantly regulate the development of many cancers. *ODC1* encodes the sentinel, rate-limiting enzyme ODC, which contributes to the biosynthesis of polyamines and represents an important *bona fide* gene target of *MYC* genes. Polyamines are well-studied polycationic molecules that orchestrate complex processes in both normal cell growth and cancer development. Because direct MYC inhibition continues to be a challenge, the MYC–ODC-linked polyamine synthesis pathway presents an attractive downstream target for therapy and chemoprevention. DFMO is a well-tolerated ODC inhibitor that has entered multiple clinical trials and appears to be most effective against MYC-driven cancers that display a polyamine addiction phenotype, such as neuroblastoma. The polyamine spermidine is the only available substrate to activate eIF5A via hypusination, a highly specific, post-translational modification that is instrumental in coordinating eIF5A-dependent events during protein synthesis and the translational control of cancer. Novel roles for polyamines continue to be discovered and include proteasomal degradation, the circadian clock, and immunity, all events that are controlled by MYC proteins.

Acknowledgments—We acknowledge the many excellent scientists that have made invaluable contributions to the MYC and ODC/polyamine field. We have made every attempt in this Minireview to cite the large number of important contributions of our esteemed colleagues, and we sincerely apologize for any oversight and omissions in the referenced material.

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