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Targeting the Complex Protein Network of MYCN-amplified Anaplastic Ependymoma: A Case Report

Michael P. Castro, MD[1,2,3,*]

[1]Attending Physician, Beverly Hills Cancer Center, 1735 S. Hayworth Ave., Los Angeles, 90035 USA

[2]Molecular Oncologist, Personalized Cancer Medicine, PLLC., Los Angeles, CA, USA

[3]Chief Medical Officer, Cellworks Group, Inc., USA

[*]Correspondence should be addressed to Michael P. Castro, Michael.castro@personalizedcancermedicine.us

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Abstract

The MYCN oncoprotein has been notoriously undruggable and is infamous for causing aggressive cancer with poor outcomes in children and adults. Following surgery, radiation, and chemotherapy, patients who develop progressive disease have few treatment options. An analysis of the dysregulated protein network caused by *MYCN* amplification suggested co-targeting PLK1, AURKA, CKS1, AKT, MTOR, and USP7 would be useful to take advantage of synthetic lethal vulnerabilities while overcoming redundancies and resistance mechanisms that stabilize N-Myc by preventing its proteasome degradation. Naturopathic compounds, (genistein, tanshinone, resveratrol, betulinic acid) and fluoxetine were re-purposed to target the complex protein network in a patient with *MYCN*-amplified and *PTEN*-deficient multifocal, relapsed anaplastic ependymoma following standard therapy. The patient achieved a clinically meaningful and durable response for 6 months prior to developing disease progression characterized by chromosome 11q (*YAP1, BIRC2/3*) amplification. The experience suggests molecularly-informed integration of naturopathic compounds can have utility for disease control and survival. The success, although anecdotal, suggests that the previous failure of single agent strategies could be overcome with a network targeting approach that simultaneously precipitates cell cycle arrest, rescues FBXW7 ubiquitination, and enhances oxidative stress. As such, *MYCN* may no longer be strictly unactionable but appears amenable to co-targeting key nodes in its self-sustaining disease network.

Keywords: Anaplastic ependymoma, MYCN, PLK1, AURKA, AKT, MTOR, FBXW7, USP7, miR-34a, Signaling pathway, Network targeting, Synthetic lethality, Integrative oncology

Background

Ependymoma is a rare form of glioma arising from the ependymal cells that line the ventricular surfaces of the brain and spinal canal. Different forms of the disease arise in adults and children and are classified by site of origin (e.g., supratentorial (ST), posterior fossa (PF), spinal cord (SC)), by histologic grade (WHO CNS Grade 2 or 3), and molecular drivers. Surgical resection followed by radiation therapy represents standard treatment for newly diagnosed patients. However, such rare cancers present a challenge as few if any randomized trials are available to inform care. As such, no standard of care existed for recurrent ependymoma until the recent phase II study of temozolomide and lapatinib showed antitumor benefit with some complete responses, a median progression free survival of 7.8 months, and decrease in symptom burden [1]. However, not all patients benefit from this approach and even those who respond eventually relapse and are in need of novel treatment approaches.

An especially aggressive and poor prognosis form of adult-onset anaplastic ependymoma is driven by N-myc proto-oncogene *(MYCN)* amplification [2,3]. The *MYCN* gene codes for the protein N-Myc, an E-box binding, basic helix leucine zipper (bHLH-LZ) transcription factor which orchestrates a network of signaling pathway aberrations characterized

by over-expression of key oncogenes (e.g., mouse double minute 2 homolog E3 ubiquitin-protein ligase (MDM2), aurora kinase A (AURKA), polo-like kinase-1 (PLK1), and forkhead box protein M1 (FOXM1) and takedown of a key tumor suppressors (e.g., Cellular tumor antigen p53 (p53) and F-box/WD repeat-containing protein 7 **(**FBXW7)) that together spawn tumorigenesis, aggressive disease behavior, and unsatisfactory treatment outcomes for many different kinds of pediatric and adult cancer.

N-Myc forms a series of redundant positive feedback loops [4]. By inducing transcription of FOXM1, AURKA, and PLK1 N-Myc propels movement through the cell cycle, including G1 → S phase [5,6], the G2/M checkpoint [7], and M phase to cause uncontrolled proliferation and replication stress. FOXM1 drives transcription of AURKA [8] and PLK1 [9], while PLK1 reciprocally phosphorylates FOXM1 to produce hyperactivation [10] Another feed forward loop emerges from the upregulation of *MDM2* transcription by N-Myc to promote p53 turnover [11,12]. Enhanced p53 turnover compromises transcription of the master tumor suppressor *miR-34a* [13] causing increased translation of *MYCN* and numerous oncogenes [14,15]. Turnover of p53 also enhances *PLK1* transcription [16,17] and phosphorylation of p53 by PLK1 inhibits the tumor suppressor function [18]. MDM2 upregulates MYCN mRNA [19,20], as well as activating deubiquitinase enzymes that reverse the ubiquitination of N-Myc [21].

Both AURKA and PLK1 inhibit the function of the critical tumor suppressor FBXW7 [22,23], an E3 ubiquitin kinase that promotes 26S proteasomal turnover of N-Myc, as well as other targets, including key drivers of the malignant phenotype, such as induced myeloid leukemia cell differentiation protein (MCL1), CCNE1/2, c-MYC, neurogenic locus notch homolog protein 1 and 2 (NOTCH1/2), transcription factor Jun (JUN), Krueppel-like factor 5 (KLF5), nuclear factor erythroid 2-related factor 2 protein (NRF2), Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), and mammalian Target Of Rapamicin (MTOR) [24]. FOXM1 is also a target of FBXW7 which requires glycogen synthase kinase beta (GSK3-ß) phosphorylation for turnover [25,26]. FOXM1 is responsible upregulation of DNA repair and other components of the therapy-resistant malignant phenotype [27]. Co-existing dysregulation of GSK3-ß and protein phosphatase 2 (PP2A) solidify the disease network further. Upstream phosphoinositide-3 kinase (PI3K)- protein kinase B (PKB) gene (AKT) and MTOR signaling pathways aberrations interfere with phosphorylation of the CDC4 phosphodegron (CPD) targets and impair recognition and turnover of N-Myc and other FBXW7 targets. Effectively, numerous redundant feedforward loops are contained in the protein network that stabilize N-Myc by preventing its degradation and amplifying its transcriptional program to cause a tumorigenic proteome by inhibiting the ubiquitin proteosome system [28] (**Table 1**).

For the most part, drugs have not been developed to inhibit transcription factors, including the *MYC* family oncogenes which remain undruggable. However, oncogene amplification causes exquisite sensitivity to the takedown of key proteins, referred to as synthetic lethal partners (SLP) [29]. In *MYCN*-driven cancers, cyclin kinase subunit-1 (*CKS1*) [30], polo-like kinase-1 (*PLK1*), aurora kinase A (*AURKA*) [31], aurora kinase B (*AURKB*) [32], and poly-ADP ribose phosphorylase (*PARP*) [33] are recognized SLP of the MYC family oncogenes. While no PLK1 or AURKA inhibitors have yet received regulatory approval, genistein, a soy isoflavone, has been studied in cancer prevention clinical trials [34] and inhibits PLK1 with a Kd of 7.9 μM [35], a concentration within reach of oral supplementation [6]. Genistein also upregulates *miR-34a* to reinstate translational blockade of *MYCN* [37,38]. Tanshinone derived from the rhizome of the Chinese herb *Salvia maltorrhiza* (danshen) is part of anti-cancer treatment in Traditional Chinese Medicine (TCM) [39] and inactivates *AURKA* translation by upregulating *miR-32* [40] and *Let-7a-5p* [41]. N-Myc drives the upregulation of casein kinase-1 (*CSK1*) which promotes the ubiquitin turnover of cyclin-dependent kinase inhibitor 1 protein (p21 - *CDKN1A*) and cyclin-dependent kinase inhibitor 1B protein (p27 - *CDKN1B*) the primary brakes on CDK2_CCNE driver of G1 → S phase progression in the cell cycle [42]. Fluoxetine (Prozac™) has been repurposed to block CKS1 in *MYCN*-driven neuroblastoma to precipitate cell cycle arrest. Resveratrol, a phytoalexin found in many plant species, has been utilized to target AKT, thereby reversing GSK3-ß inactivation as well as activating 5' adenosine monophosphate-activated protein kinase (AMPK) through a phosphodiesterase-4 (PDE4)-dependent mechanism to inhibit MTOR signaling [43,44]. Resveratrol also suppresses the expression of oncogenic micro-RNA 21(*miR-21)* known to promote expression of B-cell lymphoma 2 (BCL2) [45]. Lastly, the deubiquitinase enzymes also stabilize N-Myc by opposing its proteasomal fate in high grade neuroendocrine cancers [46]. Betulinic acid possesses broad activity to inhibit multiple ubiquitin-specific-processing protease (USP) deubiquitinases for this purpose [47,48].

In principle, overcoming cancer is an engineering problem whose success depends upon deciphering and targeting its underlying complex adaptive network. To embrace this complexity, co-targeting PLK1, AURKA, CKS1, AKT, MTOR, and USP7 could potentially dismantle network redundancies, precipitate proliferation arrest, and rescue FBXW7 to re-establish the normal turnover of N-Myc. A *network-targeting strategy* to attack these key nodes simultaneously was developed by combining genistein, tanshinone, resveratrol, betulinic acid, and fluoxetine for a patient with recurrent, progressive anaplastic ependymoma.

Case Report

A 58-year-old male with recurrent spinal cord

Table 1. Dysregulated protein pathways in the *MYCN* disease network provide rationale for co-targeting PLK1, AURKA, CKS1, AKT and MTOR. Numerous feed-forward, positive feedback loops perpetuate the dysregulation of the cell cycle while also sustaining N-Myc by preventing ubiquitin turnover and promoting other aspects of the malignant phenotype. Note that all pathways in the table are inhibited by the combinatorial regimen received by this patient.

PHENOTYPE	SIGNALING PATHWAY	REFERENCES
Proliferation	N-Myc → PLK1 → M_Phase	77
	N-Myc → AURKA → PLK1 → p21↓ → CDK2_CCNE↓ → S_Phase	78,79
	N-Myc → AURKA → PLK1 → CDC25 → [MYT1 and WEE1]↓ → CDK1_CCNB1 →[G2/M, M Phase progression]	80
	N-Myc → CSK1_SKP2 → [p27] ↓ → CDK1_CCNA → M_Phase	81
	N-Myc → AURKA → FBXW7↓ → CCNE1↓ → S_Phase	82
	N-Myc → AURKA → PLK1→ FBXW7↓→ CCNE1↓ → S_Phase	
	PTEN → PIP3 → AKT → GSK3ß↓ → FBXW7 → CCNE1↓ → S_Phase	
	N-Myc → AURKA → FBXW7↓ → NOTCH1	83
	N-Myc → AURKA → FBXW7↓→ KLF5↓	84,85
	N-Myc → AURKA → PLK1 → FBXW7↓ → KLF5↓	
	N-Myc → AURKA → FBXW7↓ → RICTOR↓ → OGT (O-GlcNAcylation) → [PI3K, PDK1, AKT, YAP1] → [Proliferation, Survival]	86
	N-Myc → AURKA → PLK1 → FBXW7↓ → RICTOR↓ → OGT (O-GlcNAcylation) → [PI3K, PDK1, AKT, YAP1] → [Proliferation, Survival]	
	N-Myc → AURKA → FBXW7↓ → [JUN, PSEN1]↓	87,88
	N-Myc → AURKA → PLK1 → FBXW7↓→ [JUN, PSEN1]↓	
	N-Myc → MDM2 → p53↓→ PLK1↓→ Tumorigenesis	89
	N-Myc → MDM2 → p53↓→ miR-34a → [MYCN, MDM4, BIRC5, MET, CREB, AXL, BCL2, NOTCH1, IL6R, MMP2/9, HOTAIR, SNAL, SLUG, ZEB1, MYC, AVIL, E2F1, CDK6]↓ → Tumorigenesis	90,91
	PTEN → PIP3 → AKT → MDM2 → PLK1 → Tumorigenesis	
	PTEN → PIP3 → AKT → GSK3-ß↓ → ßTrCP → ß-catenin↓ → Tumorigenesis	92
	PTEN → PIP3 → AKT → GSK3-ß↓ → ßTrCP → YAP1↓ → [MYC, AREG, CTGF] → Tumorigenesis	
	PTEN → PIP3 → AKT → GSK3-ß↓ → ßTrCP → DEPTOR↓ → MTOR↓ → [S6K, 4EBP-1, ATF4, HIF1A] → [Biosynthesis, Translation, Proliferation]	93,94
Radiation Chemotherapy Resistance	N-Myc → AURKA → FBXW7↓ → NFE2L2↓ → [GPX4, FTH1] → ROS↓ → Ferroptosis	95
	N-Myc → AURKA → PLK1 → FBXW7↓→ NFE2L2↓ → [GPX4, FTH1] → ROS↓ → Ferroptosis	
	PTEN → PIP3↓→ AKT → GSK3ß↓→ NFE2L2↓ → ßTrCP	96,97
	AURKA → PLK1 → MITOTIC_CATASTROPHE↓→ Resistance to tubulin targeting strategies	98,99
	AURKAIP1 → AURKA↓→ PLK1 → MITOTIC_CATASTROPHE↓ → Resistance to tubulin targeting strategies	
	PTEN → PIP3↓→ AKT → GSK3ß↓→ FBXW7 → FOXM1↓ → [BRCA2, RAD51] (HRR) → DNA Repair	100-102
	N-Myc → AURKA → FBXW7↓→ FOXM1↓→ [BRCA2, RAD51] (HRR) → DNA Repair	
	N-Myc → AURKA → PLK1 → FBXW7↓ → FOXM1↓ → [BRCA2, RAD51] (HRR) → DNA Repair	
	N-Myc → AURKA → PLK1 → NEK2 → GLI → MGMT → [Temozolomide, CCNU, BCNU, dacarbazine, procarbazine]_Resistance	103
	PTEN → PIP3 → AKT → GSK3-ß↓ → ßTrCP → YAP1↓ → ABCG2 → Multidrug Resistance	104,105

Cancer Stem Cells	N-Myc → AURKA → FBXW7↓ → NOTCH1↓ → CSC → Tumor_Re-population	106,107
	N-Myc → AURKA → PLK1 → FBXW7↓→ NOTCH1/2↓→ CSC → Tumor_Re-population	
	N-Myc → AURKA → PLK1 → CRAF → MEK1/2 → ERK1/2 → ZEB1 → CSC → Tumor_Re-population	
MYCN Stabilization	N-Myc → AURKA → FBXW7↓→ N-Myc↓	108-110
	PTEN → PIP3 → AKT → GSK3ß↓ → FBXW7→ N-Myc↓	
	CCNB1 → N-Myc_S62 → FBXW7 → N-Myc↓	
	N-Myc → AURKA → PLK1 → FBXW7↓→ N-Myc↓	111-113
	N-Myc → AURKA → PLK1 → FBXW7↓→ RICTOR↓ → AKT → GSK3ß↓ → FBXW7→ N-Myc↓	114,115
	N-Myc → AURKA → PLK1 → FOXM1 → AURKA → FBXW7↓→ N-Myc↓	116
	PTEN → PIP3 → AKT → GSK3ß↓ → N-Myc_T58 → FBXW7 → N-Myc↓	
	PTEN → PIP3 → AKT → GSK3ß↓ → FBXW7 → MTOR↓ → PP2A → N-Myc_S62↓ → N-Myc	117-120
	PTEN → PIP3 → AKT → TSC1/2↓ → RHEB → MTOR → PP2A → N-Myc_S62↓ → N-Myc	
	N-Myc → AURKA → FBXW7↓ → MTOR↓ → PP2A → N-Myc_S62 → N-Myc	
	N-Myc → AURKA → PLK1 → FBXW7↓ → MTOR↓→ PP2A → N-Myc_S62↓ → N-Myc	
	N-Myc → MDM2 → N-Myc	121,122
	N-Myc → MDM2 → USP7 → N-Myc	123
	N-Myc → MDM2 → p53↓→ miR-34a →N-Myc↓	124-126
	N-Myc → MDM2 → XIAP → TGFBR1_TGFBR2 → RHOA → FAK (PTK2) → SRC → AURKA → FBXW7↓ → N-Myc↓	127-129
	N-Myc → MDM2 → XIAP → TGFBR1_TGFBR2 → RHOA → FAK (PTK2) → SRC → AURKA → PLK1 → FBXW7↓ → N-Myc↓	
	PLK1 → MDM2 → p53↓	130
Apoptotic Blockade	N-Myc → AURKA → FBXW7↓ → MCL1↓ → Apoptosis↓	131
	PTEN → PIP3 → AKT → GSK3ß → FBXW7 → MCL1↓ → Apoptosis↓	
	N-Myc → AURKA → PLK1 → FBXW7↓ → MCL1↓ → Apoptosis↓	
	N-Myc → MDM2 → p53↓ → Apoptosis	132,133
	PTEN → PIP3 → AKT → GSK3-ß↓ → ßTrCP → YAP1↓ → BIRC5 → Apoptosis↓	134
Angiogenesis	N-Myc → AURKA → FBXW7↓ → MTOR↓ → HIF1A → VEGF	135,136
	N-Myc → AURKA → PLK1→ FBXW7↓→ MTOR↓ → HIF1A → VEGF	
	PTEN → PIP3↓→ AKT → GSK3ß↓→ FBXW7 → MTOR↓ → HIF1A → VEGF	
	PTEN → PIP3↓ → AKT → TSC1/2↓ → RHEB → MTOR → HIF1A → VEGF	
	N-Myc → AURKA → FBXW7↓ → MTOR↓ → HIF1A → VEGF	
	N-Myc → AURKA → PLK1→ FBXW7↓→ MTOR↓→ HIF1A → VEGF	
	PTEN → PIP3↓ → AKT → GSK3ß↓ → FBXW7 → MTOR↓ → HIF1A → VEGF	

ependymoma (CNS WHO grade 3) presented with progressive leptomeningeal metastases and parenchymal cerebral metastases. Three and one-half years previously, an MRI of the spine disclosed an intrathecal mass measuring 2.2 x 1.4 x 3.5 cm mass located at L1-2 that was occupying the entire spinal canal and displacing and deforming the conus tip of the L. posterior margin of the thecal sac. L1-L2 laminectomy and resection of an intradural tumor were performed followed by craniospinal irradiation. Subsequently, the disease remained relatively stable until indolent progression 14 months after diagnosis led to the use of temozolomide-lapatinib. A new metastatic lesion measuring 8 mm lesion in Meckel's cave was treated with gamma knife radiotherapy. The lesion was stabilized but not resolved. The patient was

hospitalized for a nearly fatal *Pneumocystis jirovecii* pneumonia (PJP) precipitated by temozolomide use.

Eight months later, chemotherapy was held for progressive pancytopenia associated with a new diagnosis of T cell large granular leukemia (LGL), an indolent chronic leukemia. At 22 months from diagnosis, progressive disseminated ependymoma was noted in the thoracic spine at T8-9 along with multiple enhancing nodules involving the cauda equina and 4 new nodules in the occipital lobe. A persistent 6 mm lesion was noted in Meckel's cave.

Genomic analysis

Next generation sequencing (NGS) was performed. (Tempus Inc.; Chicago, IL) Focal high-level amplification of the *MYCN* on chromosome 2p24 with 20 copies was observed, and a truncating frameshift mutation in the phosphatase and tensin homolog *(PTEN)* gene and loss-of-function mutation in ERCC excision repair 3 (*ERCC3)* nucleotide excision repair gene were identified. Chromosomal copy number analysis demonstrates gain of 14q, as well as losses of chromosomes 2p and 10. The combination of *PTEN* mutation and deletion implies biallelic loss of the tumor suppressor. No pathogenic mutations, focal amplifications, deep deletions, or structural variants were identified.

Course and treatment regimen

As mentioned, new metastatic lesions in the spine and brain were noted. Based on the signaling pathway impact analysis of *MYCN* and *PTEN*, the patient commenced the regimen listed in **Table 2**. Because, these naturopathic agents do not possess toxicity individually, they were combined without prior combinatorial trials to define safety and tolerability. The patient achieved disappearance of all lesions in supratentorial CNS, including the lesion in Meckel's cave. The spinal lesions regressed substantially. No toxicity was observed from the treatment.

At 6 months from inception, MRI identified a new left pre-pontine lesion. Craniotomy and sub-total resection through a retrosigmoid approach was performed, however the tumor was entangled with the 5th cranial nerve precluding complete resection. Pathology confirmed recurrent ependymoma, (CNS WHO grade 3). Repeat molecular profiling demonstrated a new chromosomal amplification of 11q22 with more than 10 copies of YES-associated protein-1(*YAP1),* baculoviral IAP repeat containing 2 (*BIRC2),* and *BIRC3*, representing a novel resistance mechanism for disease progression. Stereotactic body irradiation (SBRT) was administered to the remaining pre-pontine disease.

Discussion

In contrast to the strategies of attacking DNA with chemotherapy and radiation or blocking oncogenes with kinase inhibitors, synthetic lethal targeting capitalizes on "Achilles heel" vulnerabilities created by mutation or chromosomal copy number abnormalities, in this case *MYCN* amplification. Synthetic lethal partners define key nodes in a dysregulated protein network. As presented here, co-targeting three synthetic lethal partners in combination with takedown of PTEN-PI3K-AKT-MTOR and USP7 resistance pathways led to immediate, substantial disease regression for 6 months prior to the development of a new clone of disease sporting *YAP1* and *BIRC2/3* amplification to bypass the targeting described above.

Table 2. *MYCN* network targeting regimen.

INTERVENTION	TARGET	SOURCE
Tanshinone	[miR-32, Let-7]↑ → AURKA↓ GPX4	Aqueous extract (i.e., tea) of danshen root, 76 ounces per day (~2 L), prepared each day from approximately 6 cm danshen root boiled in water on stove top, (purchased in China Town, Honolulu, HI)
Genistein	PLK1↓ miR-34a↑	Genistein 1,000 mg three times per day, sourced from Fallon Pharmacy (Latham, NY)
Resveratrol	AKT↓ AMPK↑ → MTOR↓ miR-21↓	Resveratrol capsules utilizing 1,000 mg po q8h
Betulinic acid	USP7↓	Chaga mushroom capsules were used as a source of betulinic acid
Fluoxetine	CSK1↓	Initiated at 20 mg daily and escalated by 20 mg increments in one-week intervals to 80 mg.

Notably, the single agent use of the AURKA inhibitor alisertib failed in phase II trials to meet its prespecified endpoints in *MYCN*-driven neuroblastoma [49] and neuroendocrine prostate cancer [50].] However, the result is not surprising considering that N-Myc also upregulates *PLK1* transcription [51]. The signaling pathway analysis depicts a redundant and self-amplifying disease network where both AURKA and PLK1 impact the cell cycle and inhibit FBXW7. This redundancy suggests co-targeting PLK1 and AURKA may be essential. AURKA phosphorylates PLK1 at Threonine-210 during late in G2 to promote checkpoint recovery and cause entry into prophase. In return, PLK1 promotes the recruitment of AURKA to the centrosomes in late G2 that is important for centrosome maturation and bipolar spindle formation during metaphase [52]. In addition to their mutual activation, PLK1 cooperates with AURKA to regulate other mitotic functions, including cohesion, centrosome maturation, and kinetochore–microtubule interactions [53]. PLK1 also has non-canonical targets beyond the cell cycle, including the MAP kinase pathway through RAF1 protooncogene, serine/threonine kinase (RAF1 -*CRAF*) phosphorylation [54], while AURKA promotes tumorigenesis by participating in epithelial-mesenchymal transition (EMT), metastasis, survival, and self-renewal of cancer stem cells [55]. Both kinases perpetrate signaling cascades that activate other transcription factors, including glioma-associated oncogene zinc finger protein (GLI1), hypoxia-inducible factor 1-alpha (HIF1A), zinc finger E-box-binding homeobox 1 (ZEB1), JUN, and FOXM1 by PLK1, and YAP1, nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), Forkhead box protein O1 (FOXO1), and signal transducer and activator of transcription 3 (STAT3) by AURKA to drive malignant behavior. Not surprisingly, laboratory models show that *simultaneous* inhibition of PLK1 and AURKA produces synergistic anti-tumor activity compared to targeting either kinase alone [56,57]. Based on these contemporary signaling pathway insights, co-targeting both PLK1 and AURKA targeting may be the minimum combinatorial strategy needed for approaching *MYCN*-driven cancers. By implication, the redundancies in *MYCN* signaling network suggests the principle of establishing single agent activity before advancing a drug for combination therapy may be molecularly naïve for diseases where simultaneous takedown of multiple nodes is a prerequisite for disabling redundant signaling.

With regard to N-Myc ubiquitin turnover, FBXW7 requires dual phosphorylation of N-Myc at T58 by GSK3-ß and at S62 by G2/mitotic-specific cyclin-B1 (CCNB1), both representing the CPD site [58,59]. However, upregulation of PI3K-AKT pathway associated with the bi-allelic loss of *PTEN* in this patient leads to unopposed PI3K activation of AKT signaling to cause inhibition of GSK3-ß and loss of T58 phosphorylation. The *NCYM* transcript from the antisense *MYCN* gene (*MYCNOS*) also directly inhibits GSK3-ß [60]. In parallel, GSK3-ß mediated phosphorylation is needed for the activation of another E3 ubiquitin kinase F-box/WD repeat-containing protein 1A (ß-TrCP - *BTRC*) [61]. Inhibition of ß-TrCP function accelerates movement past G2/M checkpoint and from metaphase to anaphase to precipitate mitotic slippage [62]. When ß-TrCP is compromised, its non-canonical targets, ß-catenin, YAP1, and DEP-domain containing mTOR-interacting protein (DEPTOR) (inhibitor of MTOR), also promote cancer progression [63], including activation of PP2A to dephosphorylate MYCN at S62, thus promoting its resistance to FBXW7. GSK3ß also phosphorylates the CPD of AURKA to promote its turnover by FBXW7.

The activation of AKT caused by *PTEN* loss in this cancer also inhibits tuberous sclerosis 1 and 2, hamartin and tuberin (TSC1/2) restraint of Ras homolog enriched in brain (RHEB)-mediated activation of MTOR and subsequently PP2A, a phosphatase which reverses S62 phosphorylation. RICTOR, also a target of FBXW7 [65], creates yet another feedforward loop by upregulating AKT to redouble GSK3-ß and FBXW7 inhibition [66]. RICTOR also triggers *O*-GlcNAc transferase or N-acetylglucosaminyltransferase (OGT) to cause *O*-GluNAcylation, a post-translational modification that activates numerous oncogenes, including PI3K, AKT, and YAP1 [67], thus further compounding the feed-forward inhibition of FBXW7. Consequently, *PTEN* loss in this patient's disease resulting in inhibition of GSK3-ß causes broad dysregulation of numerous brakes on the cell cycle and the normal ubiquitin turnover of multiple oncogenes, including N-Myc. For this reason, AKT and MTOR represent critical nodes in this disease network. Because of PLK1 and AURKA also inhibit FBXW7 function, re-establishment of N-Myc ubiquitination depends upon the combination strategy with AKT and MTOR inhibition *concurrently* with PLK1 and AURKA blocking strategies. Though PLK1, AURKA, AKT, MTOR, FBXW7, and USP7 are genomically normal in this cancer, their integrated dysregulation and pleiotropic interactions in the proteome form a therapeutic imperative. Accordingly, the concomitant blockade strategy described here rescues FBXW7 ubiquitin turnover, re-establishes elimination of N-Myc by the proteasome, and precipitates cell cycle arrest, thereby achieving clinical efficacy by simultaneously overcoming key redundant resistance pathways and collapsing key driver mechanisms of the malignant phenotype (**Figure 1**).

MYCN-driven cancers also enhance cellular iron import [68]. The resulting increase in reactive oxygen species arising from the Fenton reaction threatens the cell with oxidative stress and requires glutathione peroxidase-4 (GPX4) to neutralize hydroxyl radicals. Normally, glutathione (GSH) mitigates oxidate stress and is consumed in the reaction to form oxidized glutathione (GSSG). However, GPX4 uses nicotinamide adenine dinucleotide phosphate (NADPH) to regenerate GSSG back to GSH, thus sustaining the antioxidant resistance by preventing the formation of lipid hydroperoxides that permeabilize cell membranes to cause ferroptosis (i.e., iron-dependent cell

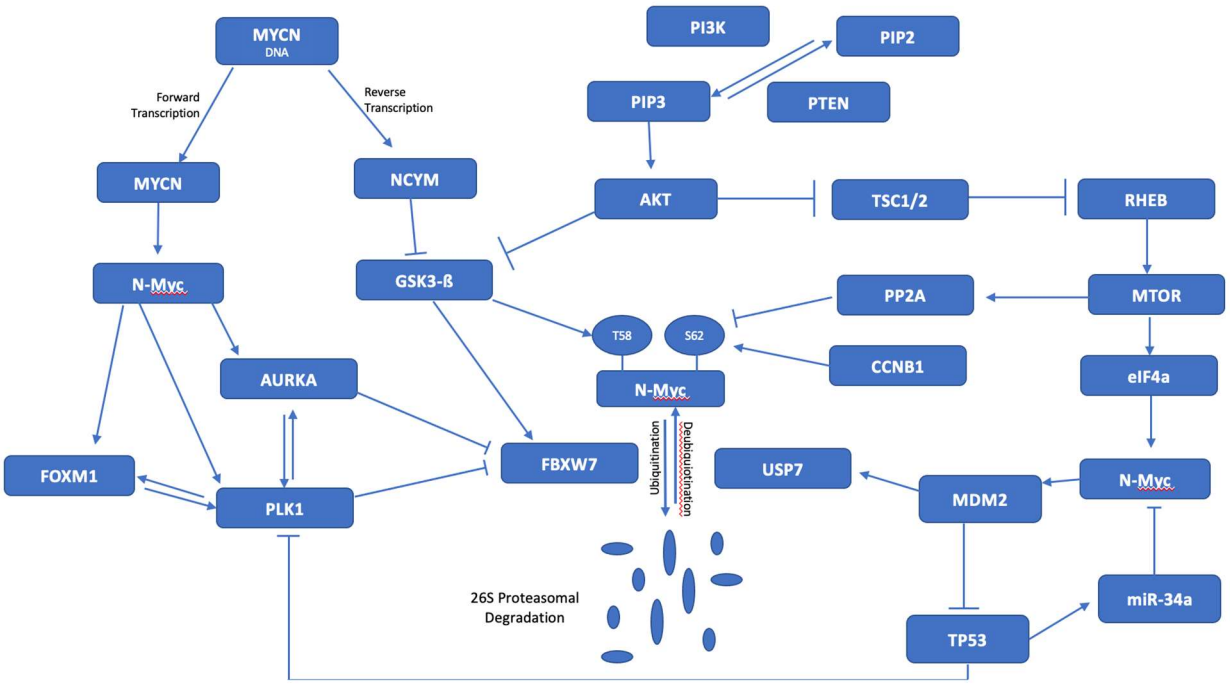

Figure 1. *MYCN* signaling network. The inhibition of FBXW7 by PLK1 and AURKA interacts with the dysregulated PTEN-AKT-GSK3-B and MTOR pathways to promote stabilization of N-Myc. MDM2 also generates stabilization of N-Myc by inhibiting TP53 and miR-34a.

death, a non-apoptotic form of cell death). Hence, GPX4 is another SLP of *MYCN*.

Notably, tanshinone has pleiotropic antitumor effects. While conceived here as an AURKA inhibitor, it also functions as a GPX4 inhibitor [69]. Tanshinone can also enhance oxidative stress by impeding expression of cystine/glutamate transporter gene *(SLC7A11)* [70], the cystine/glutamate transporter protein (Xc) antiporter that mediates the rate limiting step in GSH biosynthesis, and by silencing the expression of Ferritin heavy chain (*FTH1*) [71], which lowers oxidative stress by sequestering iron. The resulting increase in oxidative stress caused by tanshinone has been shown to accentuate the effect of chemotherapy to cause ferroptosis, perhaps accounting for 50% of chemotherapy's cytotoxic effect. Besides its effect on AURKA, a retrospective study looking at danshen use in patients with breast cancer in Taiwan showed it was associated with a marked improvement in survival (log-rank: $p < 0.001$) mediated by blocking GPX4 [72].

Notably, NRF2 (*NFE2L2*), the master regulator of antioxidant response element which prevents ferroptosis, is another FBXW7 ubiquitination target [73] rescued by the *MYCN* network. NRF2 also possesses a GSK3-ß CPD site [74,75]. Mechanistically, N-Myc upregulation of AURKA and PLK1 leads to oxidative stress resistance by inhibiting FBXW7 ubiquitination of NRF2. Conversely, GSK3-ß phosphorylation resulting from AKT inhibition prepares NRF2 for ubiquitin turnover, while rescue of FBXW7 from AURKA-mediated inhibition by tanshinone overcomes oxidative stress resistance by simultaneously enhancing NRF2 ubiquitination and GPX4 inhibition. Accordingly, tanshinone could have precipitated a lethal level of oxidative stress for this cancer, producing a cytotoxic effect to augment the collapse of the tumor network described above. As such, tanshinone could be developed as a component of a comprehensive strategy to eliminate oxidative stress resistance, including targeting GSH with sulfasalazine and thioredoxin blockade with auranofin to precipitate ferroptosis and overcome resistance to conventional therapeutic strategies [44]. Another redundant mechanism to preserve oxidative stress resistance involves GSK3-ß phosphorylation of NRF2 S338 which facilitates its ubiquitin turnover by ß-TrCp, again underscoring the importance of co-targeting AKT in the reversal of oxidative stress resistance [76]. Finally, FBXW7 rescue would enhance the proteasomal turnover of MCL1 and FOXM1, thereby reversing major chemotherapy and radiation resistance mechanisms arising from apoptotic blockade and enhanced DNA repair. For these reasons, upfront use of the network targeting in *MYCN*-amplified cancer described here could be employed to overcome multiple key resistance mechanisms to chemotherapy and radiation.

MYCN-driven cancers may be prone to chromosomal instability arising from replications stress caused by cell cycle

dysregulation, compromise of G2/M checkpoint caused by PLK1 upregulation, and p53 takedown caused by upregulation of *MDM2*. The resulting chromosomal instability, i.e., genomic entropy, drives evolutionary drug resistance, mediated by copy number aberrations that create resistance pathways. In this case, the appearance of 11q22 amplification produces YAP1 whose dysregulation is tumorigenic and BIRC2/3, the inhibitor of apoptosis proteins (IAPs) that generate cell survival in the face of chemotherapy and radiation. Therefore, it is likely that the *earliest possible* use of this network targeting approach would improve its efficacy by beating the evolution of additional resistance mechanisms. *MYCN*-driven genomic instability makes the entropy clock tick faster, and eventually, a relentlessly evolving disease complexity and heterogeneity that is either undruggable or becomes insurmountable by drugs that reasonably could be combined. For this reason, the conventional requirement for efficacy in the salvage setting before considering a drug for upfront use represents another molecularly naïve criterion prone to eliminate potentially effective approaches that might have been effective in the setting of less complex early disease management.

The achievement of a 6-month disease remission with plant-derived substances is noteworthy and provides clinical validation of synthetic lethality observed in the laboratory. While ordinary cancer prevention supplement doses of plant-derived agents may not be adequate to achieve enzyme inhibition, many naturopathic agents possess attractive enzyme affinity constants. The large doses of naturopathic compounds used in this patient may be a likely factor in the clinical efficacy observed. The absence of toxicity is also noteworthy and may be due to the relatively inconsequential impact of these substances in normal tissues possessing intact *PTEN* and diploid *MYCN*.

The prior use of radiation and temozolomide and lapatinib had limited success for this patient, echoing the frustration of treating *MYCN*-driven cancers. Additionally, the patient's compromised bone marrow function from T cell LGL was a relative contraindication to further chemotherapy. Practically, there are no clinical trials testing any PLK1, AURKA, AKT, and MTOR inhibitors in rare diseases like ependymoma, much less in the combination proposed here. The absence of a clinical trials option for this patient supported the naturopathic approach to meet the therapeutic imperatives determined by the sequencing results. But more importantly, drug development for *MYCN*-driven cancers has not caught up to the critical necessity of co-targeting multiple kinases in a complex disease network. To compound the challenge, no business model exists for co-developing a diverse group of pharmaceuticals simultaneously. But even if pharmaceuticals had been commercially available, the combination of multiple kinase inhibitors to target PLK1 (volasertib, rigosertib), AURKA (alisertib), AKT (ipatasertib), MTOR (everolimus, rapamycin), and USP7 (p5091) would have been a daunting or unfeasible proposition. Nevertheless, without embracing the complexity of the *MYCN* network and co-targeting redundancies and resistance mechanisms, single agents have no chance to achieve the desired goal. In this case, opening the therapeutic armamentarium to include plant-derived chemicals with a long track record of safety in other medical traditions permitted the design of a complex combinatorial regimen that achieves the goal of rationally co-targeting redundant disease drivers with minimal or no toxicity. By re-purposing naturopathic agents at doses suitable to achieve enzyme inhibition, the patient was able to bypass the shortcomings of drug development for the key drivers in *MYCN*-driven proteome. Potentially, the successful result presented here could re-energize the ambition of curing this notoriously lethal group of diseases and form a basis for a tumor site-independent, i.e., "basket," clinical trial design of network-directed pharmaceutical-naturopathic combinations for *MYCN*-driven cancers. While we organize clinical trials to confirm the laboratory observations that led to the success in this case report, oncologists could consider this genomically-informed, apparently safe, and relatively inexpensive combination for *MYCN* disease management.

Abbreviations

AKT: Protein kinase B (PKB) gene; AMPK: 5' Adenosine Monophosphate-activated Protein Kinase; AURKA: Aurora Kinase A; BCL2: B-cell Lymphoma 2; BIRC2/3: Baculoviral IAP Repeat Containing 2/3; βTrCP: F-box/WD repeat-containing protein 1A; C-Myc: C-myc proto-oncogene protein; CCNB1: G2/mitotic-specific cyclin-B1; CCNE1/2: G1/S-specific cyclin-E1 and 2; CDC4: Cell Division Control protein 4; CDK2: Cyclin-Dependent Kinase 2; *CDKN1:* Cyclin-Dependent Kinase Inhibitor 1 gene; *CDKN1B*: Cyclin-Dependent Kinase Inhibitor 1B gene; CKS1: Casein Kinase-1; CPD: CDC4 Phosphodegron; DEPTOR: DEP-domain containing mTOR-interacting protein; *ERCC3*: ERCC excision repair 3; FBXW7: F-box/WD repeat-containing protein 7; FOXM1: Forkhead box protein M1; FOXO1: Forkhead box protein O1; FTH1: Ferritin Heavy Chain; GLI1: Glioma-associated oncogene zinc finger protein; GPX4: Glutathione Peroxidase 4; GSH: Glutathione; GSK3-ß: Glycogen Synthase Kinase beta; GSSG: Glutathione Disulfide; HIF1A: Hypoxia-Inducible Factor 1-alpha; JUN: Transcription factor Jun; KEAP1: Kelch-like ECH-Associated Protein 1; KLF5: Krueppel-Like factor 5; MCL1: Induced myeloid leukemia cell differentiation protein; MDM2: Mouse Double Minute 2 homolog E3 ubiquitin-protein ligase; *miR-21:* micro-RNA 21; MTOR: Mammalian Target Of Rapamicin; MYCN: N-myc proto-oncogene; N-Myc: N-myc proto-oncogene protein; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; NFE2L2: Nuclear Factor Erythroid 2-related factor 2 gene; NFKB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells; NOTCH1/2: Neurogenic locus Notch homolog protein 1 and 2; NRF2: Nuclear Factor erythroid 2-related Factor 2 protein; OGT: *O*-GlcNAc transferase or N-acetylglucosaminyltransferase; p21: Cyclin-dependent kinase inhibitor 1 protein; p27: Cyclin-dependent kinase inhibitor 1B protein; p53: Cellular tumor antigen p53; PARP: Poly ADP Ribose Polymerase; PDE4:

Phosphodiesterase 4; PI3K: Phosphoinositide 3-Kinase; PLK1: Polo-Like Kinase; PTEN: Phosphatase and Tensin homolog; PP2A: Protein Phosphatase 2; RAF1: RAF1 protooncogene, serine/threonine kinase; RHEB: Ras Homolog Enriched in Brain; RICTOR: Rapamycin-Insensitive Companion of Mammalian Target of Rapamycin; SLC7A11: Cystine/glutamate transporter gene; SLP: Synthetic Lethal Partner; STAT3: Signal Transducer and Activator of Transcription 3; TSC1/2: Tuberous Sclerosis 1 and 2, hamartin and tuberin; USP7: Ubiquitin-Specific-processing Protease 7; Xc: Cystine/glutamate transporter protein; YAP1: YES-Associated Protein-1; ZEB1: Zinc finger E-box-binding homeobox 1

Conflict of Interest

I am the sole author of the manuscript and declare I have no conflicts of interest.

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Consent

Permission was obtained from the patient to publish this case report.

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