GSK3 Inhibitors Regulate MYCN mRNA Levels and Reduce Neuroblastoma Cell Viability through Multiple Mechanisms, Including p53 and Wnt Signaling

David J. Duffy1, Aleksandar Krstic1, Thomas Schwarzl1, Desmond G. Higgins1,2,3, and Walter Kolch1,2,3

Abstract
Neuroblastoma is an embryonal tumor accounting for approximately 15% of childhood cancer deaths. There exists a clinical need to identify novel therapeutic targets, particularly for treatment-resistant forms of neuroblastoma. Therefore, we investigated the role of the neuronal master regulator GSK3 in controlling neuroblastoma cell fate. We identified novel GSK3-mediated regulation of MYC (c-MYC and MYCN) mRNA levels, which may have implications for numerous MYC-driven cancers. In addition, we showed that certain GSK3 inhibitors induced large-scale cell death in neuroblastoma cells, primarily through activating apoptosis. mRNA-seq of GSK3 inhibitor–treated cells was performed and subsequent pathway analysis revealed that multiple signaling pathways contributed to the loss of neuroblastoma cell viability. The contribution of two of the signaling pathways highlighted by the mRNA-seq analysis was functionally validated. Inhibition of the p53 tumor suppressor partly rescued the cell death phenotype, whereas activation of canonical Wnt signaling contributed to the loss of viability, in a p53-independent manner. Two GSK3 inhibitors (BIO-acetoxime and LiCl) and one small-molecule Wnt agonist (Wnt Agonist 1) demonstrated therapeutic potential for neuroblastoma treatment. These inhibitors reduced the viability of numerous neuroblastoma cell lines, even those derived from high-risk MYCN-amplified metastatic tumors, for which effective therapeutics are currently lacking. Furthermore, although LiCl was lethal to neuroblastoma cells, it did not reduce the viability of differentiated neurons. Taken together our data suggest that these small molecules may hold potential as effective therapeutic agents for the treatment of neuroblastoma and other MYC-driven cancers.

Introduction
Neuroblastoma is the most common extracranial pediatric solid tumor, accounting for approximately 15% of childhood cancer deaths (1). Neuroblastoma originates from the transformation of neural crest progenitor cells during embryonic development (2). MYCN status is the strongest genetic marker for treatment failure, being amplified in 25% of neuroblastoma tumors (3, 4). Indeed, MYCN deregulation is sufficient to drive neuroblastoma formation from neural crest progenitor cells (2). The MYCN proto-oncogene regulates the expression of genes involved in many processes, including cell cycle, proliferation, differentiation, and apoptosis (5). A MYC target gene signature is emerging as a more powerful predictor of poor prognosis tumors than MYCN amplification (MNA) or MYCN expression alone, as the signature measures the ultimate response rather than identifying the driving mutation (6, 7). Although the importance of the MYC oncogenes and the benefit of downregulating MYCN to neuroblastoma outcome have long been established, clinical approaches to block transcription factors remain elusive (5). Dual PI3K/mTOR (8) or bromodomain and extraterminal domain inhibition (9) show promise as means of decreasing MYCN protein levels in neuroblastoma. However, there remains a need for additional therapeutic agents, especially those which can induce neuroblastoma cell death irrespective of the underlying abnormality driving the poor prognosis gene expression signature and phenotype.

As neuroblastoma cells are of neural crest origin, we reasoned that manipulating signaling pathways strongly associated with neural crest formation and development would likely alter neuroblastoma cell fate. Therefore, we evaluated the effect of treatment with GSK3 inhibitors in a range of neuroblastoma cell lines. GSK3 is known to be involved in a large number of signaling pathways [e.g., Wnt, Shh, receptor tyrosine kinase, Notch, mTOR, insulin,
PI3K/Akt, mitogen–activated protein kinase (MAPK), and p53], many of which are associated with the neural crest (10, 11). Canonical Wnt signaling, in particular, is known to be heavily involved in the induction, delamination, and differentiation of the neural crest (11, 12), and GSK3 is a key negative mediator of Wnt signaling. GSK3 is a master regulator of neural progenitor homeostasis, integrating multiple proliferation, and differentiation signals (13). Vertebrates possess two GSK3 genes (GSK3A and GSK3B), whereas lower metazoans have only a single GSK3 (14). However, there is redundancy between the two mammalian GSK3s in neural progenitor regulation (13, 14).

GSK3-β phosphorylates and stabilizes the MYCN protein, though this phosphorylation also enables the dephosphorylation of a separate site that can lead to MYCN degradation (5, 15). Here, we identify a novel GSK3-mediated mechanism, which regulates MYCN mRNA levels, as opposed to MYCN protein. In addition, we demonstrate that certain GSK3 inhibitors induce large-scale neuroblastoma cell death, adding weight to recent suggestions of the therapeutic potential of GSK3 inhibition for neuroblastoma treatment (16). As different GSK3 inhibitors can produce divergent phenotypes (17–21), we tested seven of them. Out of this panel, we identified LiCl and BIO-acetoxime (BIO) as the GSK3 inhibitors, which hold the most therapeutic promise.

Materials and Methods
Cell culture and inhibitor treatments
The six neuroblastoma cell lines used were received as generous gifts from Drs. Frank Westermann of DKFZ, Heidelberg, Germany, and Johannes Schulte of University Hospital Essen, Essen, Germany (2011). The lines were IMR-32 (IMR32), SH-SY5Y (SY5Y), Kelly and two patient matched lines SMS-KCN (KCN; diagnosis, primary tumor) and SMS-KCNR (KCNR; relapsed, metastatic tumor). SH-SY5Y/6TR(EU)/pTrex-Dest-30/MYCN (SY5Y-MYCN), previously generated in the Westermann laboratory (22), had stable tetracycline-inducible expression of MYCN. We also used two colorectal cancer lines obtained from the ASSET consortium by multiplex FISH karyotyping at the project’s beginning.

The following GSK3 inhibitors were used at various concentrations: 1-azakenpaullone (Sigma-Aldrich), BIO (Tocris), LiCl (Sigma-Aldrich), SB216763 (Selleck), CT90201 (Selleck), GSK3 inhibitor II (Merck), tideglusib (Selleck). Other inhibitors used were Actinomycin D (Act D; Sigma-Aldrich), NVP-BEZ235 (Selleck), PI-103 (Sigma-Aldrich), CI-1040 (Sigma-Aldrich), Pifithrin-α (Tocris), Pifithrin-μ (Tocris), and Wnt Agonist 1 (Merck), additional inhibitor details are provided in Supplementary Table S1. All inhibitors were dissolved in dimethyl sulfoxide (DMSO), except LiCl, which was dissolved in culture media. Retinoic acid (Sigma-Aldrich) was also dissolved in DMSO and used at a final concentration of 1 μmol/L. Doxycycline (Sigma) dissolved in water was used at a final concentration of 1 μg/mL to induce MYCN expression in SY5Y-MYCN. All compounds were replenished every 24 hours for any treatment longer than a 24-hour duration. Cells were imaged using an Olympus CKX41 microscope.

Western blot analysis
Total cell lysates were prepared from SY5Y-MYCN (doxycycline pretreated for 24 hours longer than inhibitors) and IMR32. Samples were resolved using 10% NuPAGE Bis-Tris Mini gels (Life technologies) and transferred to a polyvinylidene difluoride membrane. After blocking, the blots were probed overnight at +4°C with antibodies against MYCN (1/500 dilution, sc-53993, Santa Cruz Biotechnology), c-MYC (N262; 1/1,000 dilution), DKK1 (Hs00183740_m1), EGF receptor (EGFR; Hs01076078_m1), DNTD (Hs00275833_s1), DKK1 (Hs00183740_m1), NTRK1 (Hs01021011_m1), and CCND1 (Hs00765532_m1). Biological duplicates were generated for all samples at all time points. Technical replicates for every sample and time point were also performed.

Cell viability assay
Cell viability was analyzed by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega) according to manufacturer’s instructions, approximately 5,000 cells were plated per well of 96-well tissue culture plates with 100 μL of medium. To assess cell viability, rather than proliferation rate, inhibitor- and control-treated cells were assayed after the same growth time had

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elapsed. The results represent the mean ± SD of triplicate samples, expressed as a percentage of control.

**Apoptosis assays**

Activated caspase labeling was performed using CaspACE FITC-VAD-FMK In Situ Marker (Promega), which was added to harvested IMR32 cells at a final concentration of 5 μmol/L. After incubation for 30 minutes at 37°C, the cells were centrifuged, washed, and resuspended in PBS with 5 μg/mL propidium iodide (PI; Sigma-Aldrich) and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). Yo-Pro-1 dye (Invitrogen) was added to harvested and washed IMR32 cells at a final concentration of 0.1 μM to assess apoptosis based on permeability of cell membranes. After 5 min of incubation on ice, samples were analyzed immediately. Information on both assays, in accordance with ISAC recommendations (MIFlowCyt 1.0), is provided in supplemental Table S2. BIO treated samples suffered a readout shift due to BIO’s auto-fluorescence (see Table S2). Reliable gating and interpretation of results in Yo-Pro-1 staining assay was not possible for BIO samples because of the spectral overlap of BIO with propidium iodide.

**mRNA sequencing and bioinformatics analysis**

IMR32 cells were treated as follows: untreated control, 24-hour 1 μmol/L azakenpaullone, 24-hour 1 μmol/L BIO, or 24-hour 28 mmol/L LiCl with biological duplicates. Total mRNA was extracted using TRI Reagent (Sigma-Aldrich) according to manufacturer’s protocol, and DNA was digested with DNA-free Kit (Applied Biosystems). RNA quality was checked by quantitative real-time PCR (qRT-PCR; as above) and on a 2100 Bioanalyzer (Agilent) using a Eukaryote Total RNA Nano Chip (version 2.6), samples’ RIN value range 9.8 to 10. Sequencing libraries were generated from 2 μg of total RNA per sample with TruSeq RNA sample preparation Kit v2 (Illumina) according to manufacturer’s protocol. Size and purity of the libraries were analyzed on a Bioanalyzer High Sensitivity DNA chip (Agilent). Libraries were clustered using TruSeq Paired-End Cluster Kit v5-CS-GA (Illumina) and sequenced on an Illumina Genome Analyzer Ix with a TruSeq SBS Kit v5-GA (Illumina).

The sequence reads were aligned to the human reference genome GRCh37 (hg19) using TopHat (Table S3) with the parameters mate-inner-dist and mate-std-dev set to 230 and 150, respectively (23). Gene counts were summarized using the program htseq-count from the HTseq package (www-huber.embl.de/users/anders/HTSeq/). Low abundance genes were filtered as described in ref. 24: genes were removed where the number of observations with more than one read counts per million (CPM) is less than the minimal group size of 2. Multidimensional scaling (MDS) and principal component analysis (PCA) plots were made using the R/Bioconductor packages edgeR and DESeq, respectively (25–27). Differentially expressed genes were called using general linear models in edgeR (25, 26, 28). P values were adjusted for multiple testing with the Benjamini–Hochberg correction and a corrected P cutoff of 0.05 was used. The retrieved gene lists were analyzed for overrepresented pathways, biological functions, and upstream regulators using Interactive Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com). For full image legends for IPA-generated schematics, see http://ingenuity.force.com/ipa/articles/Feature_Description/Legend. P values reported for IPA results are generated by IPA using a right-sided Fisher exact test for over-representation analysis, Benjamini–Hochberg correction for multiple hypothesis testing correction, and a z-score algorithm for upstream regulator analysis. P values < 0.05 were considered significant. To make the absolute expression levels of genes comparable with each other, the read CPM were adjusted by gene length in kilobases (CPMkb). The Pearson correlation coefficient between biological replicates was >0.99 for all groups. The mRNA-seq data were deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1684. String v9.05 (http://string-db.org/newstring.cgi/show_input.page.pl?UserId=4cpYhnjW4L&sessionIId=eLViXYwKLMw) was used to generate protein interaction networks. Venn diagrams; area-proportional ones generated using BioVenn (http://www.cmbi.ru.nl/cdd/biovenn) and four-way comparisons generated using Venny (http://bioinfoogg.cnsc.es/tools/venny/index.html).

**Results**

**GSK3 inhibitors regulate mRNA levels of MYC genes**

We examined the effect of inhibiting GSK3 on the mRNA of c-MYC and MYCN, in six neuroblastomas and for comparison in two colorectal cancer cell lines. The MYCN and c-MYC mRNA expression levels varied between each untreated cell line (Fig. 1A). Certain GSK3 inhibitors strongly downregulated the MYC mRNA levels. The effect of GSK3 inhibition on the MYC genes’ mRNA expression level depended on the GSK3 inhibitor and concentration used. First, we tested seven GSK3 inhibitors in SY5Y-MYCN cells (Fig. 1B). Four GSK3 inhibitors (LiCl, azakenpaullone, SB216763, and tideglusib). Four GSK3 inhibitors reduced ectopic MYCN mRNA levels, whereas three slightly increased ectopic MYCN (BIO, GSK3 inhibitor II, and tideglusib). Four GSK3 inhibitors reduced c-MYC mRNA levels (LiCl, azakenpaullone, BIO, and SB216763), whereas the others slightly increased c-MYC or had no effect. Only BIO strongly reduced c-MYC expression at low concentrations. Changes in MYCN mRNA levels were mirrored at the protein level, though the c-MYC protein changes were less dramatic (Fig. 1C). As a proxy for the extent of GSK3 inhibition, we monitored the phosphorylation of SMAD3 (29, 30), both BIO and LiCl resulted in a more prolonged reduction of p-SMAD3 levels (Fig. 1C), this was also the case in IMR32 cells (Supplementary Fig. S1D).

As azakenpaullone and BIO differentially affect MYC gene expression in SY5Y-MYCN, cotreatments were
performed to determine whether they act through independent mechanisms. Both drugs are ATP-competitive inhibitors, with BIO binding approximately 2-fold more strongly to GSK3. When cells were cotreated, BIO prevented the normal azakenpaullone MYCN downregulation, but the reduction of c-MYC by BIO was unaltered (Fig. 1D). This indicates that BIO blocks azakenpaullone binding and that their differential regulation of MYC genes is achieved through inhibition of the same substrate (GSK3), rather than off-target effects.

To assess cell line-dependent response heterogeneity, we tested three compounds in different neuroblastoma lines (Fig. 2A). The compounds selected, because of their differential MYC response profile in induced SY5Y-MYCN, were LiCl, azakenpaullone, and BIO. All three inhibitors downregulated endogenous MYCN and c-MYC mRNA (Fig. 2A and Supplementary Fig. S1C). KCN cells were the only exception, with all three inhibitors increasing KCN endogenous MYCN, except 10 μmol/L BIO. GSK3-mediated MYCN regulation may be different in KCN cells as they are the only line tested derived from a primary tumor site. Interestingly, BIO reduces endogenous MYCN but not ectopic MYCN. In summary, these combined data suggested that GSK3 inhibition was capable of regulating MYC mRNAs in neuroblastoma cells, thus showing that GSK3 regulation of MYCN is not confined to the protein level.

**GSK3 inhibition alters MYCN mRNA stability**

If GSK3 inhibition only transcriptionally regulated MYCN, then ectopic MYCN in SY5Y-MYCN cells would not have been changed as it is not controlled by the endogenous MYCN promoter. As a control to show that the ectopic MYCN is not responsive to stimuli that alter...
endogenous MYCN levels through direct transcriptional regulation, we treated SY5Y-MYCN cells with retinoic acid. Retinoic acid is used in neuroblastoma treatment as it has an evolutionarily conserved role in neuronal cell differentiation (31, 32). Retinoic acid reduced endogenous MYCN mRNA levels in parental SY5Y cells and other neuroblastoma cell lines as expected. However, retinoic acid treatment was unable to reduce the level of ectopic MYCN mRNA (Fig. 2B and Supplementary Fig. S2A), unlike GSK3 inhibition. Retinoic acid still reduced the expression levels of c-MYC and EGFR mRNA levels as normal in SY5Y-MYCN confirming that retinoic acid was active. Thus, the effects of GSK3 inhibition are not due to transcriptional regulation of the MYCN gene, but likely to the regulation of MYCN mRNA stability.

To investigate what proportion of endogenous MYCN downregulated by GSK3 inhibition was due to altered mRNA stability, as opposed to transcriptional regulation, we used the transcriptional inhibitor Act D. As endogenous MYCN mRNA is rapidly turned over (Fig. 2C), Act D reduced MYCN mRNA levels slightly faster than azakenpaullone (Fig. 2D and Supplementary Fig. S1B). The lack of synergism with Act D and azakenpaullone cotreatment suggests that transcriptional regulation of another gene or miRNA is likely required to achieve the azakenpaullone-induced MYCN reduction. Ectopic MYCN mRNA was more stable than endogenous MYCN as it lacks the untranslated regions (Fig. 2C), yet GSK3 inhibitors still altered MYCN mRNA stability independently of these regions.

**GSK3 inhibitors alone are sufficient to decrease neuroblastoma cell viability**

Treatment with either LiCl or BIO produced a dramatic cellular phenotype in all of the neuroblastoma lines tested. By 24 hours of treatment, cells had retracted their neurites and started to round up followed by a dramatic increase in cell death by 48 hours, which was basically...
complete after 72 hours (Fig. 3A). Viability assays confirmed that LiCl and BIO reduced cell viability across all cell lines in a time- and concentration-dependent manner (Fig. 3B). Low-dosage LiCl increased viability in line with its known neuro-protective effect (33). Of note, 20 μmol/L SB216763 also reduced viability, whereas the other four GSK3 inhibitors tested did not strongly reduce the viability (Fig. 3C and Supplementary S2C).

A correlation with the cells’ MNA status and strength of LiCl-induced loss of viability was present. By 48 hours, high MYCN lines (IMR32, KCNR, and Kelly) exhibited a roughly 1.5-fold greater loss of viability than low MYCN lines (SY5Y and KCN; Fig. 3B). However, whether MYC gene regulation contributed to the phenotype is less clear. High LiCl and BIO more consistently downregulated expression of both MYCs and were the most effective drugs at inducing cell death. Also, KCN was the only cell line in which LiCl did not downregulate MYCN, and they showed the smallest LiCl-induced reduction in viability. Of note, 10 μmol/L BIO did downregulate MYCN in KCN cells and effectively reduced their viability. Conversely, low BIO concentrations induced some cell death even in cell lines in which it did not downregulate the expression of MYCN (IMR-32, Kelly, and SY5Y). Induction of ectopic MYCN in SY5Y-MYCN did not alter the viability response (Supplementary Fig. S2D). In addition, despite

Figure 3. GSK3 inhibition reduces neuroblastoma cell viability. A, images of untreated, 1 μmol/L BIO, and 28 mmol/L LiCl-treated IMR32 cells 24, 48, and 72 hours into treatment. All panels are 400× magnification. B, time course and dosage curves of the effect of LiCl (left) and BIO (right) on viability across five neuroblastoma cell lines, as measured by MTS assays. C, viability assays for five additional GSK3 inhibitors in SY5Y cells.
low concentrations of LiCl, azakenplonone, SB216763, and CT99021 downregulating MYCN, none of these inhibitors strongly reduced neuroblastoma cell viability at low concentrations.

To examine the cell death commitment profile of GSK3 inhibitor–treated cells, we performed time course inhibitor washout cell viability assays. After treatment, cells were cultured in inhibitor-free media until the end of the observation period. There was a broadly linear cell death commitment to increased duration of LiCl or BIO treatment after removal of the stimulus (Fig. 4A).

Other inhibitors (CI-1040, PI-103, and NVP-BEZ235) directed against various kinases [MAP–ERK kinase (MEK), phosphatidylinositol 3-kinase (PI3K), and mTOR] were also used in viability assays to compare with the effectiveness of the GSK3 inhibitors (Supplementary Fig. S2B and S2C). The effect of LiCl or BIO treatment was comparable with that of NVP-BEZ235 (the dual PI3K/mTOR inhibitor), the most potent of the 3 non-GSK3 inhibitors tested. NVP-BEZ235 reduced c-MYC mRNA levels only (Fig. 1B).

To assess the effect of the inhibitors on differentiated neurons, SY5Y cells were treated with 1 μmol/L retinoic acid for 4 or 10 days to induce differentiation. As differentiation progressed, the lethal effect of the inhibitors lessened (Fig. 4B and Supplementary Fig. S2B). Crucially, by 10 days of differentiation, LiCl at all concentrations (3.5–28 mmol/L) showed no reduction in cell viability. Unlike LiCl, NVP-BEZ235, BIO, and PI-103 all reduced cell viability in differentiated neurons.

**Loss of viability is primarily apoptosis mediated**

The contribution of apoptotic or necrotic cell death to the loss of viability was assayed by CaspACE (Casp), Yo-Pro-1 and propidium iodide staining, and flow cytometry.
Apoptosis levels, as measured by caspase activation (Casp3) or membrane permeability (Yo-Pro-1), were increased upon LiCl or Wnt Agonist 1 treatment (see below), whereas the level of necrosis (PI, Casp3) was not greatly altered (Fig. 4D). Conversely, BIO showed the largest increase in necrosis, whereas BclO’s autofluorescence affected the assays specificity (see Materials and Methods). Consistent with the viability data apoptosis and necrosis, levels for azakenpaullone were in line with that of control cells.

Transcriptome sequencing of GSK3 inhibitor treatments in IMR32 cells

To investigate the molecular mechanisms through which LiCl and BIO induced neuroblastoma cell death, we performed mRNA sequencing (mRNA-seq) with the MNA IMR32 cell line. The mRNA from four treatment groups was sequenced: untreated control cells, 24-hour 1 μmol/L azakenpaullone treatment, 24-hour 1 μmol/L BIO treatment, and 24-hour 28 mmol/L LiCl treatment. Azakenpaullone was used to control for genes whose expression is altered by GSK3 inhibition but which do not contribute to the cell death phenotype. Of the three inhibitors, azakenpaullone induced the fewest differentially expressed genes; BIO altered an intermediate number of genes, whereas LiCl affected the largest number (Fig. 4C). The majority of azakenpaullone-regulated genes overlapped with the other inhibitors (Fig. 5B). The Supplementary Material contains full gene expression and differential gene expression results (Supplementary Table S4). Selected sequencing results were validated by qRT-PCR as a measure of the reliability of the sequencing data (Supplementary Fig. S1A). Components of numerous pathways (including Wnt, BDNF, NGF, TGF-β, MAPK, EGF, VEGF, FGFl and Notch) were altered, highlighting GSK3’s involvement in various signaling responses and in facilitating cross-talk between pathways.

Pathway analysis of GSK inhibitor transcriptomic data

IPA was performed on the mRNA-seq data. Consistent with the phenotypes, LiCl and BIO produced similar transcriptional changes to each other, whereas azakenpaullone was more divergent. In accordance with the observed LiCl and BIO neurite shrinkage phenotype, axon growth and guidance genes were downregulated, whereas axon retraction genes were upregulated. Consistent with proliferation inhibition followed by cell death, both LiCl and BIO downregulated cell-cycle progression pathways (including p53, BRCA, MAPK, JNK, E2F, TGF-β, BMP, and c-JUN/c-FOS signaling) and upregulated proapoptotic and downregulated antiapoptotic pathway genes (including mitochondrial membrane permeabilization, p53, PTEN, Fas, JNK, MAPK, MYC, retinoic acid, and PI3K signaling). Azakenpaullone failed to induce many of these transcriptional changes. Genes involved in numerous cancer-associated pathways were differentially regulated by LiCl, as can be seen by overlaying these genes onto a summary schematic of the molecular mechanism of cancer (Fig. 5A).

When azakenpaullone was used as the reference sample, LiCl downregulated cell-cycle genes (P 6.68E-19–1.62E-02), whereas cell death genes were strongly upregulated (P 6.16E-12–1.62E-02), including neuronal cell death, apoptosis of neuroblastoma cells, and necrosis gene subsets.

IPA highlighted the interconnected p53-E2F-Cyclin signaling nexus as an important network region contributing to the cell death phenotype. First, of the 95 genes associated with p53 (TP53) signaling, 20 were differentially regulated by azakenpaullone, 40 by BIO, and 62 by LiCl. LiCl strongly upregulated genes involved in p53 signaling-mediated apoptosis and cell-cycle arrest and downregulated p53 signaling-related cell-cycle progression genes.

Second, IPA predicted upstream regulatory genes likely to be driving the observed transcriptional changes. p53 tops the LiCl list and is second on the BIO list. Furthermore, at least half of the top 14 LiCl-predicted upstream regulators are involved in the p53-E2F-Cyclin signaling axis. The top 14 are p53, HNF4A, E2F4, CDKN2A, TBX2, CCND1, E2F1, let-7, CDKN1A, CDK4, RB1, ERBB2, VEGF, and TGFβ1 (P 6.83E-29–1.02E-09), many of which are oncogenes or tumor suppressors and are associated with neuroblastoma (4, 34).

Third, even when azakenpaullone was used as the reference sample for comparison with BIO or LiCl, p53-E2F-Cyclin signaling genes were differentially expressed, with LiCl altering 58 of 95 p53 signaling genes. The top six predicted upstream regulators for LiCl and BIO were identical, when referenced to the azakenpaullone transcriptome. These were p53 (activated), E2F4, E2F1, ERBB2 (activated), c-MYC (inhibited), and TGFβ1 (P 1.55E-24–9.11E-10). Taken together, IPA suggested an important role for GSK3-induced changes to the p53-E2F-Cyclin nexus in producing the LiCl- or BIO-induced loss of viability.

Although p53-E2F-Cyclin signaling was important to the phenotype, it was not the only signaling region affected, as evidenced by the numerous p53-independent genes and pathways listed above. LiCl and BIO altered more PI3K pathway components than azakenpaullone, possibly overlapping with how dual PI3K/mTOR inhibition induced apoptosis in neuroblastoma. Another predicted upstream regulator of the LiCl transcriptome was the let-7 miRNA, which reduces MYCN levels and is often repressed in poor outcome neuroblastoma (35). let-7 was predicted to be activated upon LiCl treatment potentially contributing to the phenotype and the reduced MYCN mRNA levels. Overall, the mRNA-seq results showed that the cell death phenotype likely occurred because of cumulative effects across a range of cancer-related pathways, which lead to the loss of neuroblastoma cell viability.

GSK3 inhibition cell death phenotype is partly mediated through p53

As IPA highlighted p53 signaling (Supplementary Fig. S5A), we examined its role in the cell death phenotype. We

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compared the overlap of the mRNA-seq with the 157 gene signature predictive of neuroblastoma outcome (7) at both the gene- and IPA-predicted upstream regulator levels (Fig. 6A–C and Supplementary Fig. S3B). There was a substantial overlap for LiCl and BIO, indicating that they disrupt many of the pathways associated with poor outcome neuroblastoma, with p53 again being highlighted (Fig. 6B and C). To assess the contribution of p53–GSK3 interactions to the phenotype, we coincubated cells with LiCl or BIO and the pan-p53-function inhibitor Pifithrin-α (PFT-α). PFT-α significantly rescued a portion of the 10 μmol/L BIO-treated IMR32 cells (unpaired t test, \( P = 1.00 \times 10^{-4} \)) with 10-fold more cells surviving (Fig. 6D), equating to a partial rescue of about 50% of control viability. It was p53 transcriptional regulation that contributed to the cell PFT-α rescued BIO-treated cells, whereas a specific inhibitor of p53 mitochondrial interactions (PFTm) did not (Fig. 6D). Although KCNR cells cotreated with BIO and PFT-α also displayed partial rescue, KCN cells did not (Supplementary Fig. S4A). p53 is not the only route by which the inhibitors induce cell death, as the BIO rescue was only partial, and LiCl treatments were not rescued by...
p53 inhibition (IMR32, KCN, and KCNR; Supplementary Fig. S4A). The variability of the ability of p53 inhibition to rescue each GSK3 inhibitor may be due to varying regulation of total p53 levels, BIO reduced it and LiCl increased it (Fig. 6E). Yet only LiCl increased MDM2 mRNA levels (Supplementary Fig. S1E and Supplementary Table S4A). Despite this, both inhibitors activated p53 downstream targets (IPA). The rescue results indicate that although p53 contributes to the GSK3 inhibitor phenotype, p53-independent cell death mechanisms are also activated.

**Canonical Wnt signaling induces cell death independent of p53**

As p53 inhibition did not fully rescue the cell death response, we examined the contribution of canonical Wnt signaling to the phenotype. GSK3 inhibition mimics Wnt signaling activation, and as expected, Wnt-associated genes were detected as being differentially expressed by the mRNA-seq. Azakenpaullone altered the expression of 32 out of 170 Wnt-associated genes, BIO altered 62, and LiCl altered 78. There was heterogeneity between the inhibitors both in the specific genes altered and the direction of change (Fig. 7A). IMR32 cells were treated with Wnt Agonist 1, which activates Wnt signaling independently of GSK3 inhibition (36). The MYC mRNA regulation seen upon Wnt Agonist 1 treatment most closely resembled that of BIO treatment, with ectopic MYCN being upregulated and endogenous MYCN and c-MYC being downregulated (Fig. 7B and Supplementary Fig. S1F). Wnt Agonist 1 recapitulated the LiCl phenotype, reducing neuroblastoma cell viability (Fig. 7C) and increasing apoptosis (Fig. 7D) with differentiated neurons being less affected. p53 inhibition failed to rescue Wnt Agonist 1-treated cells (Fig. 7D), revealing that canonical Wnt activation likely contributes to the p53-independent portion of the LiCl and BIO-induced cell death. Wnt
Agonist 1 may hold potential as a neuroblastoma therapeutic in its own right, as low doses sufficient to induce neuroblastoma cell death did not cause extensive loss of viability in differentiated neurons (Fig. 7C).

Discussion

We revealed novel MYC–GSK3 interactions. First, c-MYC is known to be transcriptionally upregulated by Wnt/β-catenin signaling (37, 38), but we have shown that GSK3 inhibitors and a Wnt agonist primarily downregulated c-MYC in neuroblastoma and colorectal cancer cells. Second, although MYCN protein can be stabilized by GSK3 inhibitor II without altering MYCN mRNA levels (15), we have shown that the majority of GSK3 inhibitors do alter MYCN mRNA levels. Most GSK3 inhibitors reduced the level of MYCN independent of its endogenous promoter, likely by altering MYCN mRNA stability through transcriptional regulation of miRNAs or RBPs.

Combining previous data with our results, it seems that active GSK3 reduces MYCN protein (5, 15) while concurrently stabilizing MYCN mRNA. Such dual regulation safe guards against deleterious effects of constant elevated MYCN protein while keeping the cell primed to respond to new signaling inputs with a pool of available MYCN mRNA. Conversely, in the presence of certain signals (e.g., Wnt or PI3K), GSK3 is inhibited stabilizing MYCN protein but reducing MYCN mRNA, thereby allowing MYCN-induced proliferation but also establishing a longer term feed-back preventing continuous proliferation. Such dual regulatory strategies seem to be common safeguards, as we previously showed a similar mechanism of alternate MEK regulation of cyclin D1 protein and mRNA (S. Parodi and colleagues; submitted for publication). We have highlighted the intricate multilevel feedback inherent in cell fate signaling, and revealed that GSK3 inhibition can reduce the mRNA level of MYC oncogenes, being potentially beneficial for numerous MYC-driven tumor types.

Different GSK3 inhibitors resulted in divergent cellular responses and transcriptional changes. At the organismal level, different GSK3 inhibitors can also produce different cellular outcomes and phenotypes (17–21). Such differences are unlikely to be due to varying inhibitor affinity to GSK3-α or GSK3-β, as they occur even in basal metazoans that possess only a single GSK3 gene. Although divergent cellular phenotypes can also be caused by varying degrees of GSK3 inactivation (39), this is unlikely to be the case here as increasing azakemphaulone concentration did not recapitulate the BIO viability or MYC gene response and vice versa.

Our studies indicate the potential utility of LiCl, BIO, and Wnt Agonist 1 as therapeutic agents for neuroblastoma treatment. LiCl showed the most specificity for cancerous cells while failing to reduce the viability of differentiated neurons. LiCl also altered many of the genes and upstream regulators of the gene signature for stratification of poor prognosis neuroblastoma patients.

Although GSK3 activation is involved in neuronal apoptosis (40), GSK3 inhibition can also lead to MYC-mediated cell death (41). Three varied GSK3 inhibitors (LiCl, BIO, and SB216763) induced neuroblastoma cell death, indicating GSK3 was the responsible kinase. Supporting this, GSK3-β knockdown via short hairpin is sufficient to reduce neuroblastoma cell viability (16). Consistent with our mRNA-seq results, GSK3 inhibition (LiCl or SB415286) induced (rat and human) neuroblastoma cell-cycle arrest and increased apoptosis (16, 42), including in neuroblastoma cancer stem cells (43). Although a relatively high concentration of SB415286 (25 µmol/L) reduced cell viability (16), it did not do so as dramatically as we have shown for LiCl, BIO, or Wnt Agonist 1. Even so, SB415286 induced tumor growth delay when tested in a neuroblastoma mouse model (16). These results combined with our data strongly suggest that LiCl, BIO, or Wnt Agonist 1 would be more potent inhibitors of neuroblastoma tumor growth.

GSK3 inhibitors are gaining traction as potential therapeutics for a variety of cancers (16, 44, 45). LiCl is widely used in the treatment of neurologic disorders, and is the standard therapeutic used for bipolar disorder since the 1950s (39). Our data suggest that correct dosage of lithium would be crucial for inducing tumor cell death, needing to be at least four times higher than the 3 mmol/L used for its neuro-protective effects (33). Although BIO and Wnt Agonist 1 are more potent than LiCl, being effective in the low micromolar range, they have no history of use in patients. Given these three drugs destabilizing effect on MYCN mRNA and their induction of cell death, they may prove to have therapeutic value, particularly for high-risk MYCN-amplified neuroblastoma.

The p53-E2F-Cyclin signaling axis contributed to the GSK3 inhibitor–induced loss of viability. p53 signaling is linked directly with GSK3 (46) and Wnt signaling (47), and our data confirmed a role for p53 in the cell death phenotype. Stabilization of p53 has previously been shown to reduce neuroblastoma cell viability (48). Indeed, MYC proteins regulate apoptosis partly through interactions with p53/MDM2/CDKN2A and mutation in p53 or upstream pathway components is commonly observed in patients with relapsed neuroblastoma (5, 34, 49). Overexpression of the cyclin-related gene, CDKN2A (p14/ARF), predicted to be an activated upstream regulator of the LiCl phenotype by IPA, suppressed neuroblastoma cell viability by G1 phase arrest and increased apoptosis (34), largely recapitulating our LiCl phenotype. However, p53 inhibition only partially rescued BIO-induced cell death and showed no rescue of LiCl-treated cells. GSK3 inhibition is broad acting and also induced numerous other signaling alterations specific to LiCl and BIO, including ERBB2, TGFβ1, PTEN, and MYC.

Our data highlight the previously underappreciated involvement of Wnt signaling in neuroblastoma. Wnt Agonist 1 treatment led to altered morphology and neuroblastoma cell fate resulting in apoptosis. Although some studies have shown links between Wnt signaling and neuroblastoma patient outcome (50–52), relatively few...
Wnt-neuroblastoma studies appear in the literature when compared with other major signaling pathways. mRNA-seq profiling of neuroblastoma cell lines (unpublished) and "gene expression profiling of tumor samples" (personal communication S. Bulashevska, University of Bonn, Bonn, Germany and DKFZ, 11-06-13) is confirming that Wnt signaling components are altered in poor prognosis neuroblastoma.

In summary, we identified three novel potent therapeutic agents for neuroblastoma treatment, LiCl, BIO, and Wnt Agonist 1. The cell data suggest that these molecules will be effective even against high-risk MYCN-amplified neuroblastoma. As such these compounds should be tested in a broader panel of neuroblastoma cell lines and animal models, to confirm their utility. LiCl or BIO altered the expression of the components of numerous cancer-related pathways, ultimately shifting the balance of these pathways to favor a new cell fate, programmed cell death. The broad nature of the signaling response may make it more difficult for cancerous cells to evolve resistance to GSK3 inhibitor treatment, as resistance would likely require numerous mutations across various pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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David J. Duffy, Aleksandar Krstic, Thomas Schwarzl, et al.


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