A Novel IMP1 Inhibitor, BTYNB, Targets c-Myc and Inhibits Melanoma and Ovarian Cancer Cell Proliferation

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Lily Mahapatra[*,†], Neal Andruska[†,‡], Chengjian Mao[‡], Jeremy Le[†] and David J. Shapiro[†,§]

*Department of Molecular and Integrative Physiology, University of Illinois, Urbana, IL, 61801, USA; †College of Medicine, University of Illinois, Urbana, IL, 61801, USA; ‡Department of Biochemistry, University of Illinois, Urbana, IL, 61801, USA; §University of Illinois Cancer Center, Chicago, IL, 60612, USA

Abstract

The oncofetal mRNA-binding protein, IMP1 or insulin-like growth factor-2 mRNA-binding protein 2 (IGF2BP1), binds to and stabilizes c-Myc, β-TrCP1, and other oncogenic mRNAs, leading to increased expression of the proteins encoded by its target mRNAs. IMP1 is frequently overexpressed in cancer and is strongly correlated with a poor prognosis and reduced survival in melanoma, ovarian, breast, colon, and lung cancer. While IMP1 is an attractive anticancer drug target, there are no small molecule inhibitors of IMP1. A fluorescence anisotropy-based assay was used to screen 160,000 small molecules for their ability to inhibit IMP1 binding to fluorescein-labeled c-Myc mRNA. The small molecule, BTYNB, was identified as a potent and selective inhibitor of IMP1 binding to c-Myc mRNA. In cells, BTYNB downregulates several mRNA transcripts regulated by IMP1. BTYNB destabilizes c-Myc mRNA, resulting in downregulation of c-Myc mRNA and protein. BTYNB downregulates β-TrCP1 mRNA and reduces activation of nuclear transcriptional factors-kappa B (NF-κB). The oncogenic translation regulator, eEF2, emerged as a new IMP1 target mRNA, enabling BTYNB to inhibit tumor cell protein synthesis. BTYNB potently inhibited proliferation of IMP1-containing ovarian cancer and melanoma cells with no effect in IMP1-negative cells. Overexpression of IMP1 reversed BTYNB inhibition of cell proliferation. BTYNB completely blocked anchorage-independent growth of melanoma and ovarian cancer cells in colony formation assays. With its ability to target c-Myc and to inhibit proliferation of difficult-to-target melanomas and ovarian cancer cells, and with its unique mode of action, BTYNB is a promising small molecule for further therapeutic evaluation and mechanistic studies.

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Introduction

Insulin-like growth factor II mRNA-binding protein 1 (IGF2BP1/ IMP1), also known as the c-Myc coding region determinant-binding protein (CRD-BP) and zipcode-binding protein 1 (ZBP1), is a multifunctional RNA-binding protein that binds to diverse cancer-associated mRNAs to promote mRNA stability, localization, and translation. IMP1 stabilizes target mRNAs by shielding them from degradation by endoribonucleases and microRNAs [1,2]. While IMP1 upregulates the expression of mRNAs important in cancer, a conserved IMP1 recognition sequence has not been identified. Instead of a classical long conserved binding sequence, IMP1 exhibits high-affinity binding to weakly conserved, extended, relatively unstructured G-poor regions containing short interaction motifs [3,4]. Studies have shown that IMP1 can bind to the coding

determination sequence located in the open reading frame of several mRNAs including c-Myc (MYC), β-TrCP1 (BTRC), and PTEN [1,5–8]. IMP1 can also inhibit mRNA decay and promote translation by binding to the 3′-UTR of several transcripts [8–10].

IMP1 plays important roles in cancer. In cell culture, overexpression of IMP1 promotes enhanced cell proliferation, inflammation,

suppression of apoptosis, and resistance to taxanes and other anticancer drugs [1,11–13]. In transgenic mice, overexpression of IMP1 results in the development of mammary and colorectal tumors [14,15]. IMP enhances cell proliferation by stabilizing c-Myc mRNA, thereby increasing c-Myc mRNA and protein levels, which leads to enhanced cell proliferation. IMP1 also stabilizes the mRNA of β-TrCP1 following induction by Wnt/β-catenin signaling, which leads to ubiquitination and degradation of IκB and the release and activation of NF-κB [16]. IMP1 has also been implicated in the posttranscriptional regulation of CD24, CD44, COL5A1 (collagen, type V alpha 1), and other mRNAs involved in cell adhesion and tumor invasion [10].

IMP1 has an oncofetal pattern of expression, where it is ubiquitously expressed during development, has low expression in adult tissues, and is frequently reexpressed in cancer cells [9]. IMP1 expression is upregulated by c-Myc, β-catenin, and hypoxia, and it is a major regulatory target of *let-7* microRNA [5,11,12,17]. IMP1's aberrant reexpression and association with a poor prognosis have been implicated in a variety of cancers including melanoma and ovarian cancer [6,12,16]. Given its oncofetal pattern of expression and elevated expression in numerous cancers, targeting IMP1 with small molecule biomodulators represents a novel chemotherapeutic strategy because it allows for selected targeting of cancer cells without deleterious side effects from targeting noncancerous cells [9]. c-Myc has proven difficult to target directly; thus, reducing c-Myc levels by decreasing c-Myc mRNA stability through inhibition of the IMP1–c-Myc mRNA interaction represents a novel therapeutic strategy. RNA-binding proteins that play a role in cancer have proven challenging to target, and small molecule biomodulators of IMP1 and other cancer-related mRNA stabilizing proteins have not been reported [9].

To identify small molecule biomodulators of the RNA-binding protein IMP1, we developed a high-throughput fluorescence anisotropy/polarization microplate assay (FAMA) [18]. We screened ~160,000 small molecules and here report a small molecule, 2-{[(5-bromo-2-thienyl)methylene]amino} benzamide (BTYNB), which inhibits IMP1 binding to a specific high-affinity binding site in the coding region stability determinant of c-Myc mRNA. We show that BTYNB, identified in our *in vitro* screen, functions in cells to reduce intracellular levels of c-Myc mRNA and protein. Importantly, BTYNB inhibits cell proliferation and anchorage-independent growth of IMP1-positive cancer cells with no effect on IMP1-negative cells, making it a candidate for further therapeutic development. To our knowledge, BTYNB is the first small molecule inhibitor of an oncogenic mRNA stabilizing protein.

Materials and Methods

Plasmids, Proteins, and Fluorescein-Labeled RNA Probes

Untagged, full-length IMP1 and FLAG-PR-B were expressed and purified, as described previously [18,19]. The fluorescein-labeled c-Myc (flMyc) probe and fluorescein-labeled progesterone response element (flPRE) nucleic acid probe were produced, as we previously described [18,20]. IMP1 was overexpressed in HEK 293 cells by transiently transfecting pCMV-IMP1, which codes for full-length human IMP1. To normalize the total amount of DNA transiently transfected, carrier DNA was transfected in control transfection experiments using a pTZ18U plasmid. NF-κB luciferase experiments were carried out using a (NF-κB)$_5$-Luc reporter plasmid provided by Dr. L.F. Chen.

Chemical Libraries and Screening

The small molecule libraries screened were The ChemBridge MicroFormat containing ~150,000 small molecules, the University of Illinois Marvel library containing ~9700 small molecules [21], and the NCI Diversity Set containing ~1990 small molecules. High-throughput screening for small molecules inhibitors of IMP1 binding to flMyc was carried out using the assay we previously described [18].

Fluorescent Anisotropy Assays

FAMA was used to evaluate for small molecule inhibitors of binding of IMP1 to flMyc and inhibitors of progesterone receptor binding to flPRE, as we previously described [18,22].

Cell Lines and Cell Culture

All cells were maintained at 37°C in a humidified incubator with 5% CO_2 in growth medium containing 1% penicillin and streptomycin and fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA). All cell lines were obtained from ATCC, except for BG-1 cells provided by Dr. K. Korach, MCF10AER IN9 cells provided by Dr. B.H. Park, BT-474 cells provided by Dr. R. Schiff, and NCI/ADR-RES by NCI. Cells were maintained in the following culture media: SK-MEL2 (10% FBS, RPMI-1640); SK-MEL28 (10% FBS, EMEM); ADR-RES (10% FBS, RPMI-1640); CaOV-3 (10% FBS, DMEM); PEO4 (10% FBS, DMEM); BG-1 (5% FBS, MEM); OVCAR-3 (20% FBS, RPMI-1640); ES-2 (10% FBS, McCoy's 5A); IGROV-1 (10% FBS, RPMI-1640); ECC-1 (5% FBS, RPMI-1640); Ishikawa (5% FBS, MEM); HeLa (10% FBS, MEM); 201T (10% FBS, RPMI-1640); 273T (10% FBS, RPMI-1640); H1793 (5% FBS, DMEM/F12); A549 (10% FBS, F12 K); HepG2, (10% FBS, MEM); DU154 (10% FBS, MEM); PC-3 (10% FBS, MEM); LnCAP (10% FBS, RPMI-1640); MCF-7 (5% FBS, MEM); T47D (10% FBS, MEM); T47D-KBluc (10% FBS, RPMI-1640); HCC-1500 (10% FBS, RPMI-1640); BT-474 (10% FBS, MEM); ZR-75-1 (10% FBS, RPMI-1640); MCF10A (2% CD-FBS, DMEM/F12); MCF10A$_{ER\ IN9}$ (2% CD-FBS, DMEM/F12); MDA-MB-231 (10% FBS, MEM); and HEK 293T (10% FBS, DMEM/F12).

Transfections

siRNA knockdowns were performed using DharmaFECT Transfection Reagent and 100 nM ON-TARGETplus nontargeting pool or IMP1 siRNA SMARTpools (Dharmacon, CO). Plasmid transfections were performed using Lipofectamine 2000, according to the manufacture recommendations. IMP overexpression experiments were carried out in HEK 293T cells. Cells were plated at a density of 1000 cells/well in 96-well plates and transfected the following day with a total of 100 ng of either IMP1 or control reporter plasmid.

qRT-PCR and mRNA Decay Analyses

qRT-PCR experiments were performed after treating cells for 72 hours with a DMSO control or BTYNB. For stability experiments, SK-MEL2 cells were treated with either DMSO or 10 μM BTYNB for 24 hours. mRNA synthesis was inhibited by treatment with 5 μM Actinomycin D (mRNA decay), and the cells were harvested at the indicated time points. RNA was extracted using a QiaShredder kit (Qiagen) for cell homogenization and purified with the RNAeasy mini-kit (Qiagen, CA). cDNA was prepared from 0.5 μg of RNA by

reverse transcription using a DyNAmo cDNA synthesis kit (Finnzymes, Finland). Quantitative PCR assays were performed on samples from three independent sets of cells. Reactions contained 10 ng of cDNA and 50 nM forward and reverse primers in 15 µl and were carried out using Power SYBR Green PCR Mastermix (Applied Biosystems). The fold change in expression of each gene was calculated using the ΔΔCt method with the ribosomal protein 36B4 used as the internal control, as described previously [23].

Luciferase Reporter Assays

Estrogen receptor and glucocorticoid receptor luciferase reporter assays were carried out, as previously described [22,23]. Briefly, cells were switched to 10% CD-FBS for 4 days prior to experiments and plated at a density of 50,000 cells/well in 24-well plates. The medium was replaced the next day with medium containing BTYNB, with or without hormone, incubated for 24 hours, and luciferase assays were performed using Bright Glow reagent (Promega, WI). To measure NF-κB luciferase activity following siRNA knockdown of IMP1, IGROV-1 were plated at a density of 20,000 cells/well in 12-well plates and transfected overnight with noncoding siRNA SMARTpool or IMP1 siRNA SMARTpool. The transfection complex was replaced with complete medium for 24 hours. IGROV-1 cells were then transfected for 12 hours with pSV40 β-gal or NF-κB luciferase reporter plasmid and maintained in complete medium for 48 hours. Cells were washed with PBS and lysed with 150 µl passive lysis buffer, and luciferase activity was determined using firefly luciferase reagent (Promega, WI) according to the manufacturer's protocol. To measure NF-κB luciferase activity following BTYNB treatment, IGROV-1 cells were transfected for 12 hours with pSV40 β-gal or NF-κB luciferase reporter plasmid. The transfection medium was replaced the following day with media containing BTYNB or DMSO-control, and cells were treated for 48 hours prior to measuring luciferase activity.

Immunoblotting

Western blotting was carried out as previously described [23]. The following antibodies were used: IMP1 (#8482; Cell Signaling Technologies, MA), c-Myc (#9402; Cell Signaling Technologies, MA), eEF2 (#2332; Cell Signaling Technology, MA), and tubulin (Sigma, MO). Bound antibodies were detected using horseradish peroxidase–conjugated secondary antibodies and chemiluminescent immunodetection with an ECL Detection Kit (GE Healthcare, NJ), and were visualized using a PhosphorImager.

Protein Synthesis

Protein synthesis rates were evaluated by measuring incorporation of 35S-methionine into newly synthesized protein, as previously described [23].

Cell Proliferation Assays

Cells were suspended in the appropriate media and plated in 96-well plates at the following densities: SK-MEL2 (1000), ES-2 (1000), IGROV-1 (2000), T47D-KBluc (1000), and BG-1 cells (250). The medium was replaced with treatment medium the following day and plates were incubated at 37°C in 5% CO2 for 4 days. Treatment solutions were replaced every 2 days. Cell number was determined from MTS assays using CellTiter 96 Aqueous One Solution Reagent (Promega, WI). For each cell line, cell number was calculated from a standard curve of the number of plated cells versus A490.

Soft Agar Colony Formation Assays

Assays to assess anchorage-independent cell proliferation in soft agar were carried out as previously described, with culture medium changed every 3 days [23]. Each treatment condition was evaluated using three independent sets of cells. Colonies were visible after 1 week, and total colonies were counted at day 21 using a dissecting microscope. Photographs of colonies were taken using a Zeiss AxioImager2 imaging system at 10× magnification.

Tumor Microarray Data Analysis

Microarray data analysis was performed on publically available tumor data sets using the BRB ArrayTools (version 4.2.1) and R software version 3.2.5. Analysis of histologically normal peritoneum was compared to serous ovarian adenocarcinomas. IMP1 expression and expression of mRNA targets of IMP1 were evaluated in normal skin (GSE15605) and compared to cutaneous melanoma (GSE15605, GSE7553, GSE19234). Gene expression values from CEL files were normalized by use of the standard quantile normalization method.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean. Student's t test was used for comparison of the means between two groups. Significance was established when $P < .05$.

Results

BTYNB Is a Structure-Specific Small Molecule Inhibitor of IMP1 Binding to c-Myc mRNA

IMP1 enhances the stability of hundreds of mRNAs, but c-Myc is the most characterized IMP1 target. IMP1 stabilizes and protects c-Myc mRNA against degradation by binding to the c-Myc coding region stability determinant, which increases the levels of c-Myc mRNA and protein and promotes cell proliferation [6,7,17]. To identify small molecules that inhibit binding of IMP1 to its binding site in the coding region stability determinant of c-Myc mRNA, we developed a high-throughput FAMA. When full-length IMP1 binds to flMyc mRNA, the rotation of the flMyc-IMP1 complex slows, leading to an increase in fluorescence polarization/anisotropy. A small molecule that blocks IMP1 binding to flMyc RNA will reverse the IMP1-mediated increase in fluorescence polarization/anisotropy.

Using this screening assay, we screened ~160,000 small molecules for inhibitors of full-length IMP1. There were 981 compounds that reduced anisotropy of the IMP1-flMyc complex by >30%, which were rescreened and eliminated if they represented false positives, displayed intrinsic fluorescence, or reduced the signal of the free flMyc probe. Most of the small molecules excluded from further analysis either displayed intrinsic fluorescence or reduced the signal by a little over 30% in the initial assay and slightly less than 30% on retesting. Only 300 compounds remained after rescreening, which were further characterized using simple follow-on assays. The robust nature of our screen is shown by the fact that the lead inhibitor had a Z-score of −10.2.

From the screen, we identified a lead small molecule, 2-{[(5-bromo-2-thienyl) methylene]amino}benzamide (BTYNB), which inhibits binding of IMP1 to the coding region stability determinant of c-Myc mRNA (IC$_{50}$ = 5 µM) (Figure 1A). Specificity was evaluated using full-length progesterone receptor (PR) binding to a fluorescein-labeled progesterone response element (flPRE). Fifty micromolars BTYNB had no effect on binding of PR to flPRE
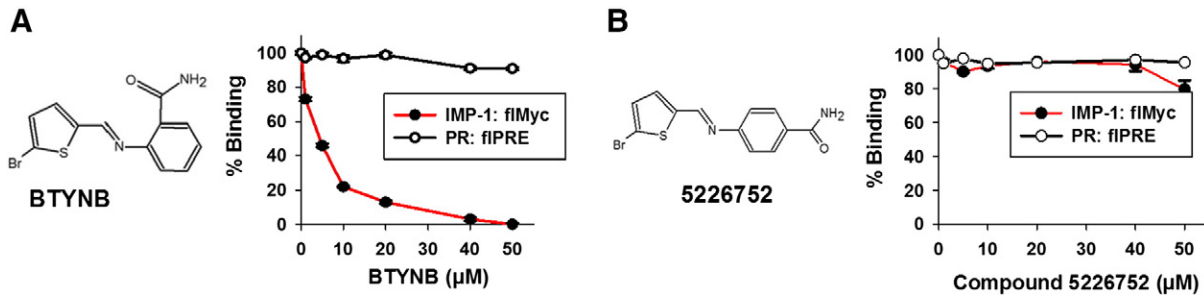
Figure 1. BTYNB is a structurally selective inhibitor of IMP1 binding to flMyc. Dose-response studies of the effect of (A) BTYNB and (B) Compound 5226752 on the binding of IMP1 to a flMyc and binding of purified full-length human PR binding to an flPRE. Compound 5226752 is a BTYNB-related compound. No inhibitor control was set to 100%. Data are mean $\pm$ SEM ($n = 5$).

(Figure 1A). To test whether BTYNB is a structure-specific inhibitor, we compared BTYNB to a close structural relative of BTYNB, Compound 5226752, which had no effect on binding of IMP1 to flMyc or on binding of PR to flPRE (Figure 1B).

BTYNB Enhances Degradation of c-Myc mRNA, Decreases c-Myc mRNA and Protein Levels, and Reduces IMP1 Protein Levels

To evaluate the efficacy of BTYNB *in vivo*, we focused on ovarian and melanoma cancers, which are difficult to target therapeutically.
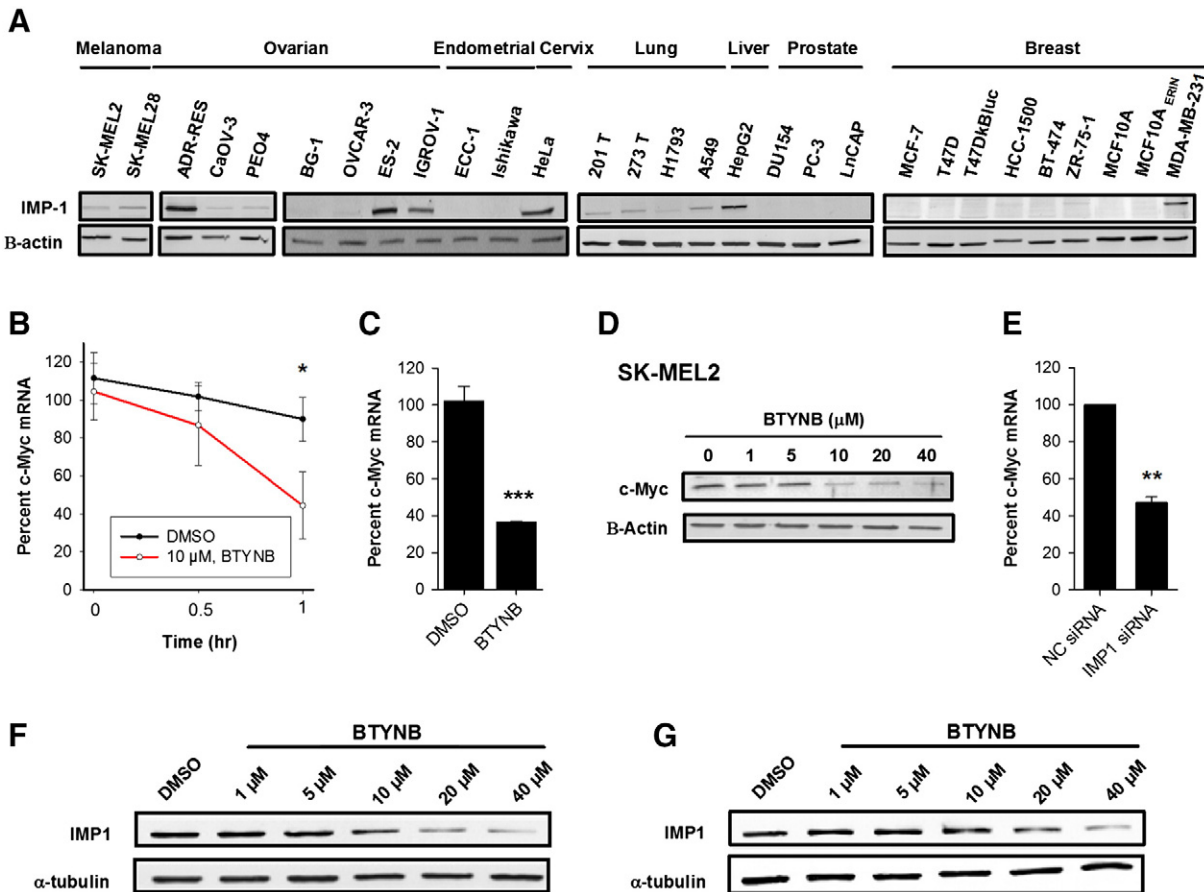

Figure 2. BTYNB decreases c-Myc protein levels by decreasing c-Myc mRNA stability, and BTYNB decreases IMP1 protein levels. (A) IMP1 protein levels in 31 cancer cell lines. (B) BYTNB enhances the degradation rate of c-Myc mRNA in SK-MEL2 cells. Cells pretreated with DMSO or 10 μM BTYNB for 72 hours, followed by treatment with actinomycin D for the indicated times. P values testing for significance between the vehicle control (DMSO)- and BTYNB-treated cells. (C) qRT-PCR analysis of c-Myc mRNA levels after treating SK-MEL2 melanoma cells for 72 hours with DMSO or 10 μM BTYNB ($n = 3$; DMSO-vehicle control set to 100%). (D) Western blot analysis of c-Myc protein levels after treating SK-MEL2 cells for 72 hours with increasing concentrations of BTYNB. (E) RNAi knockdown of IMP1 reduces c-Myc mRNA levels in SK-MEL2 cells ($n = 3$; NC siRNA set to 100%). Cells were treated with 100 nM NC or IMP1 siRNA SmartPool, and 72 hours later, c-Myc mRNA levels were quantitated *via* qRT-PCR. (F) Western blotting analysis of IMP1 protein levels in IGROV-1 cells treated for 72 hours with increasing concentrations of BTYNB. (G) Western blotting analysis of IMP1 protein levels in SK-MEL2 cells treated for 72 hours with increasing concentrations of BTYNB. Data reported as mean $\pm$ SEM. P values represent comparison to DMSO control (C, E). *$P < .05$, **$P < .01$, ***$P < .001$.

Analysis of IMP1 protein levels in 31 cancer cells showed that the majority of ovarian, melanoma, and lung cancer cells lines express IMP1 (Figure 2A). IGROV-1 and ES-2 ovarian cancer cells express high levels of IMP1, while SK-MEL2 melanoma cancer cells express much lower levels of IMP1 (Figure 2A).

If BTYNB destabilizes the c-Myc–IMP1 interaction by functioning as an allosteric inhibitor of IMP1, then BTYNB should enhance degradation and reduce total c-Myc mRNA levels. To test this, we employed the widely used technique of blocking new mRNA synthesis using actinomycin D to follow the decay of c-Myc mRNA with and without BTYNB treatment. Pretreatment of SK-MEL2 cells with 10 µM BTYNB, followed by treatment with actinomycin D, significantly increased BTYNB-mediated degradation of c-Myc mRNA (Figure 2B) and significantly reduced the level of c-Myc mRNA and protein (Figure 2, C and D). RNAi knockdown of IMP1 led to a comparable three-fold reduction in c-Myc mRNA (Figure 2E).

BTYNB may decrease cMyc mRNA levels by disrupting the ability of IMP1 to interact with cMyc mRNA and/or by decreasing intracellular levels of IMP1 protein. To address this question, we assessed whether BTYNB could reduce IMP1 protein levels. Western blot analysis showed a dose-dependent decline in IMP1 levels in IGROV-1 and SK-MEL2 cells (Figure 2, F and G). Treatment of IGROV-1 cells with 10 µM BTYNB yielded a two-fold reduction in IMP1 protein levels (Figure 2F). However, treatment of SK-MEL2 cells with 10 µM BTYNB led to minimal change in IMP1 protein

levels (Figure 2G). Thus, the decline in c-Myc mRNA and protein levels following treatment of SK-MEL2 cells with 10 µM BTYNB (Figure 2, C and D) is primarily due to disrupting the c-Myc–IMP1 interaction rather than the result of reducing total IMP1 protein levels. Further supporting this conclusion, 10 µM BTYNB inhibits binding of IMP1 to flMyc RNA by ~80% in vitro (Figure 1A).

IMP1 Stabilizes Several Oncogenic mRNA Transcripts in Melanoma and Ovarian Cancer

Knockdown of IMP1 is thought to destabilize IMP1 target mRNAs, reducing levels of cancer-related mRNAs important in cell proliferation, migration, cancer-enabling inflammation, and metabolic regulation [9]. While the IMP1 binding site on c-Myc is well established [24–26], the binding sites on many targets mRNAs remain unknown. Despite numerous studies, a comprehensive list of IMP1 target mRNAs is lacking, and most of the currently known targets have not been validated in melanoma and ovarian cancer cell lines. To validate mRNA targets of IMP1 in ovarian and melanoma cancer cells, we evaluated mRNA levels of several reported mRNA targets of IMP1 following siRNA knockdown of IMP1 in SK-MEL2 melanoma and IGROV-1 ovarian cancer cells.

We first evaluated β-TrCP1 (BTRC), which is reported to be upregulated in melanoma cancers overexpressing IMP1 [16]. Consistent with this, siRNA knockdown of IMP1 in SK-MEL2 melanoma led to a five-fold reduction in β-TrCP1 mRNA levels (Figure 3A). Similarly, RNAi knockdown of IMP1 in IGROV-1 cells
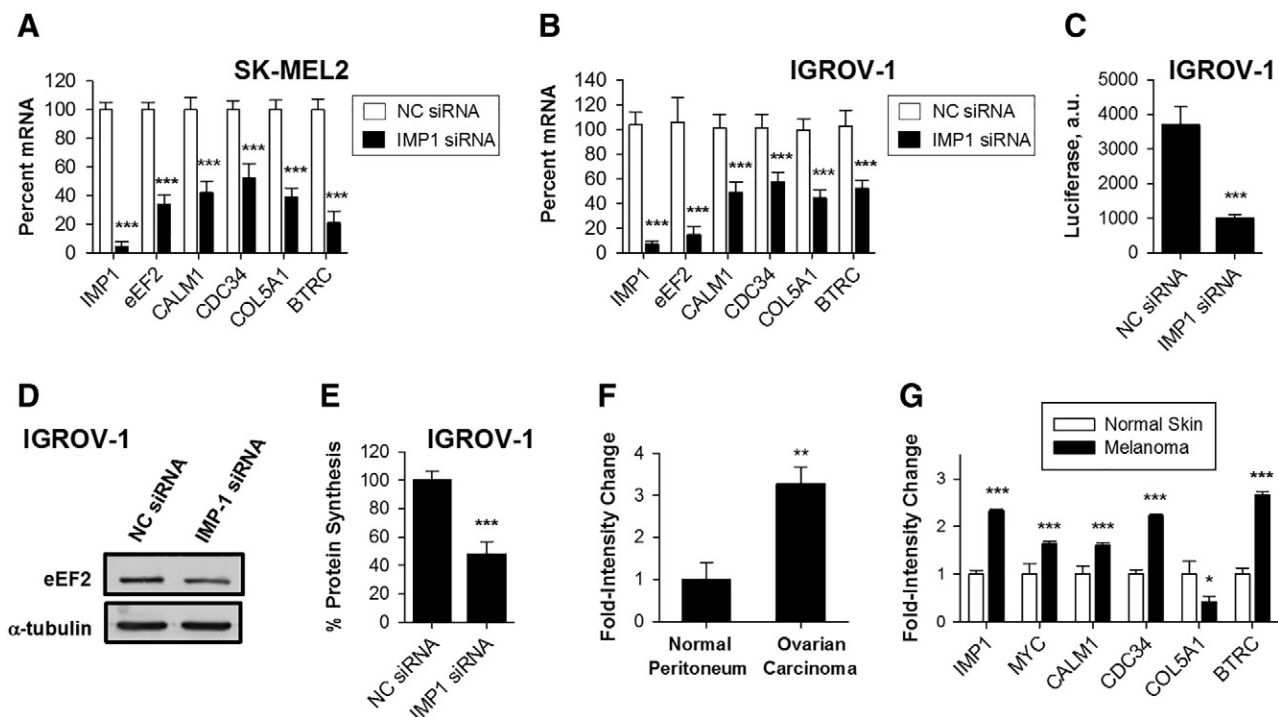

Figure 3. IMP1 stabilizes several mRNA transcripts in melanoma and ovarian cancer cells, which are upregulated in clinical tumor samples. RNAi knockdown of IMP1 decreases the mRNA levels of IMP1-regulated genes in (A) SK-MEL2 melanoma cells and (B) IGROV-1 ovarian cancer cells ($n = 4$; NC set to 100%). Cells were treated with 100 nM NC or IMP1 siRNA SmartPools. (C) NF-κB luciferase activity after treating IGROV-1 cells with 100 nM NC or IMP1 siRNA SmartPool ($n = 3$). (D) Western blot analysis of eEF2 protein levels following treatment of IGROV-1 cells with 100 nM NC or IMP1 siRNA SmartPool. (E) Protein synthesis in IGROV-1 ovarian cancer after treating with 100 nM NC or IMP1 siRNA SmartPool ($n = 5$; NC set to 100%). (F) IMP1 expression in patient samples of normal peritoneum ($n = 8$) or serous adenocarcinoma ($n = 45$). (G) Expression levels of IMP1 and IMP1-regulated genes in samples of normal skin ($n = 4$) and cutaneous melanoma ($n = 14$). Data reported as mean ± SEM. P values represent comparison to NC control (A, B, C, E) or histologically normal tissue (F, G). *$P < .05$, ** $P < .01$, ***$P < .001$. ns, not significant.

resulted in a two-fold decrease in β-TrCP1 mRNA levels (Figure 3B). To assess the regulatory role of IMP1 in induction of NF-κB–dependent transcription, IGROV-1 cells were transiently transfected with an NF-κB luciferase reporter following siRNA knockdown of IMP1. IMP1 knockdown resulted in a 50% reduction in NF-κB promoter-driven luciferase activity (Figure 3C), which demonstrates that loss of IMP1 leads to a reduction in NF-κB–dependent transcription.

Eukaryotic elongation factor 2 (eEF2), which plays an essential role in the elongation step of protein synthesis, was recently identified as the top predicted target mRNA of IMP1 in PAR-CLIP and RNA immunoprecipitation studies [4]. eEF2 enhances basal proliferation rates in cancer cells by increasing basal protein translation rates [27]. Furthermore, eEF2 is an important link between metabolic regulation in cancer and protein synthesis [28]. eEF2 is overexpressed in many cancers, including lung, colorectal, pancreatic, and breast, and is an important molecular target in these cancers [29,30]. RNAi knockdown of IMP1 resulted in a three-fold decrease and seven-fold decrease in eEF2 mRNA levels in SK-MEL2 and IGROV-1 cancer cells, respectively (Figure 3, A and B). In IGROV-1 cells, IMP1 knockdown resulted in a decrease in eEF2 protein (Figure 3D) and a decrease in overall protein synthesis (Figure 3E).

We also evaluated the role of IMP1 on several additional genes implicated in tumor cell proliferation (CDC34, CALM1 [calmodulin]) and migration (CDC34, Col5A [Collagen V, α1]) [4,5,10,13,16]. IMP1 knockdown resulted in an approximately two-fold decline in CDC34, CALM1, and COL5A in SK-MEL2 and IGROV-1 cancer cells (Figure 3, A and B). Thus, IMP1 regulates a diverse set of cancer-related mRNA targets in melanoma and ovarian cancer cell lines.

We next evaluated whether mRNA levels of IMP1 and target mRNA of IMP1 were upregulated in clinical tumors. To assess IMP1 activity early in melanoma and ovarian cancer development, we compared IMP1 activity in samples of histologically normal peritoneum with serous ovarian cancer and normal skin with melanoma, and found IMP1 levels to be significantly elevated in ovarian cancer and melanoma (Figure 3, F and G). Compared to normal skin, melanoma samples exhibited significantly higher expression of c-Myc, CALM1, CDC34, COL5A1, and BTRC (Figure 3G).

BTYNB Selectively Reduces the Levels of Cancer-Related IMP1 mRNA Targets

With a set of validated mRNA targets of IMP1 in melanoma and ovarian cancer cells, we evaluated whether BTYNB could reduce the levels of these mRNAs. Treatment of IGROV-1 and SK-MEL2 cells with 10 μM BTYNB resulted in a significant decline in β-TrCP1 levels (Figure 4, A and B). Consistent with the decrease in β-TrCP1 levels, treatment of IGROV-1 cells with 10 μM BTYNB resulted in a two-fold reduction in NF-κB luciferase reporter activity (Figure 4C). We next evaluated the effects of BTYNB on eEF2 levels and protein synthesis. Treatment of IGROV-1 and SK-MEL2 cells with 10 μM BTYNB led to ~2.5-fold decrease in eEF2 mRNA (Figure 4, A and B) and a decrease in eEF2 protein (Figure 4D). Consistent with its ability to reduce the level of eEF2, a rate-limiting protein in protein synthesis, BTYNB treatment elicited a significant decrease in protein synthesis (Figure 4E). Lastly, we evaluated the effects of BTYNB on the mRNA levels of CDC34, CALM1, and COL5A. Ten micromolars BTYNB reduced levels of CDC34, CALM1,

β-TRCP1, and Col5A1 mRNAs (Figure 4, A and B). These results demonstrate that BTYNB reduces the levels of a diverse set of cancer-related IMP1 mRNA targets.

To assess whether BTYNB was functioning as a global transcriptional inhibitor, we evaluated the effects of BTYNB on transcription by glucocorticoid receptor (GR) and estrogen receptor α (ERα) in cell lines stably expressing reporter genes. T47D-KBluc cells contain ERα and respond to addition of the estrogen 17β-estradiol (E_2) by activating expression of a stably transfected estrogen response element luciferase reporter gene [22]. Similarly, T47D/(A1-2) cells stably express GR and respond to dexamethasone by activating expression of a glucocorticoid response element (GRE) luciferase reporter gene. Treatment with BTYNB had no effect on either ERα-mediated or GR-mediated gene expression (Figure 4F). Taken together, these results suggest that BTYNB is a structurally selective small molecule biomodulator with strong selectivity for IMP1.

BTYNB Inhibits Cell Proliferation of IMP1-Positive Cancer Cells with No Effect in IMP1-Negative Cells

IMP1 is emerging as important oncogenic factor, and elevated IMP1 levels are linked to enhanced cancer cell proliferation. To assess the role of IMP1 in cell proliferation, siRNA knockdowns of IMP1 were performed in IMP1-positive and IMP1-negative cell lines. Compared to a noncoding control (NC) siRNA, RNAi knockdown of IMP1 completely blocked proliferation of SK-MEL2, IGROV-1, and ES-2 cells (Figure 5, A-C). In IMP1-negative BG-1 and T47D-KBluc cells (Figure 2A), RNAi knockdown of IMP1 had no significant effect on cell proliferation (Figure 5, D and E).

Since overexpression of IMP1 is associated with more aggressive tumors and a poor outcome in diverse cancers, and IMP1 knockdown inhibits cell proliferation, we evaluated the ability of BTYNB to inhibit proliferation of IMP1-positive cancer cells. We hypothesized that if BTYNB was disrupting IMP1 activity in the cells, treatment of the IMP1-positive cells with BTYNB should result in a decrease in cell proliferation that was similar to what we observe with RNAi knockdown of IMP1. In dose-response studies, BTYNB elicited a robust dose-dependent inhibition of cell proliferation in IMP1-positive cells with IC_{50}s of 2.3 μM, 3.6 μM, and 4.5 μM in ES-2, IGROV-1, and SK-MEL2 cells, respectively (Figure 5, F-H). Consistent with BTYNB acting by inhibiting binding of IMP1 to a target mRNA such as c-Myc, the dose-response curve for inhibition of cell proliferation (Figure 5, F-H) is similar to the dose-response curve for *in vitro* inhibition of binding of IMP1 to flMyc (Figure 1A). To test BTYNB for general cell toxicity, dose-response studies were conducted in IMP1-negative cells and demonstrated no inhibition of cell proliferation at all concentrations tested, including 50 μM (Figure 5I).

Having established that BTYNB only works in IMP1-positive cells, we next tested whether the structure specificity seen in the *in vitro* binding assay using purified IMP1 and flMyc was also observed in intact cells (Figure 1). We therefore evaluated the effect of the close structural relative of BTYNB, Compound 5226752 (Figure 1B), on proliferation of the same IMP1-positive and IMP1-negative cells. Consistent with our *in vitro* finding that Compound 5226752 did not inhibit binding of IMP1 to flMyc (Figure 1B), Compound 5226752 did not inhibit proliferation of IMP1-positive and IMP1-negative cells at all concentrations tested (Figure 5J).
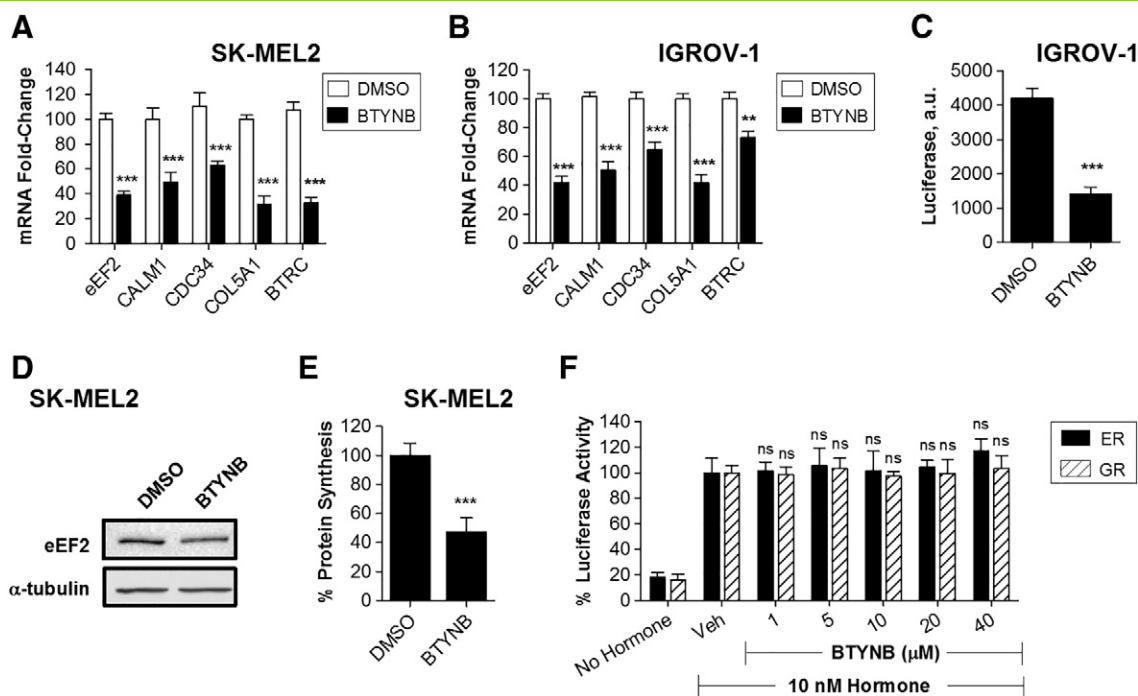
Figure 4. BTYNB selectively decreases levels of IMP1-regulated mRNAs, decreases protein synthesis, and decreases NF-κB activity. (A) qRT-PCR analysis of IMP1-regulated genes after treating (A) SK-MEL2 melanoma or (B) IGROV-1 ovarian cancer cells with DMSO-vehicle control or 10 µM BTYNB for 72 hours ($n = 4$; DMSO-vehicle control set to 100%). (C) BTYNB inhibits NF-κB luciferase activity in IGROV-1 ovarian cancer cells. Cells were treated with 10 µM BTYNB for 72 hours ($n = 3$). (D) Western blot analysis of eEF2 protein levels in SK-MEL2 cells treated with DMSO-vehicle control or 10 µM BTYNB for 72 hours. (E) Protein synthesis in SK-MEL2 melanoma cancer cells treated with DMSO-vehicle control or 10 µM BTYNB for 72 hours ($n = 6$; DMSO-vehicle control set to 100%). (F) BTYNB does not inhibit ERα or GR activity in T47D-kBluc or T47D (A1-2) cells, respectively ($n = 6$; DMSO-vehicle control set to 100%). Cells were treated for 24 hours in the presence or absence of 10 nM 17β-estradiol in T47D-KBluc cells or 10 nM dexamethasone in T47D/(A1-2) cells. Cells treated with hormone were treated with the indicated concentrations of BTYNB (or DMSO-vehicle control). P values testing for significance between the vehicle-control (DMSO) and BTYNB-treated cells in the presence of hormone. Data reported as mean ± SEM. P values represent comparison to DMSO control (A, B, C, E). *$P < .05$, **$P < .01$, ***$P < .001$. ns, not significant.

Overexpression of IMP1 in HEK 293T Cells Reverses BTYNB Inhibition of Cell Proliferation

BTYNB action through IMP1 is supported by the dose-response curves demonstrating inhibition of binding of IMP1 and flMyc RNA in *in vitro* assays as well as inhibition of cell proliferation of IMP1 cells with a concomitant lack of effect in control *in vitro* and *in vivo* assays. Thus, we postulated that if BTYNB inhibits cell proliferation by reducing binding of IMP1 to c-Myc, which plays an important role in cell proliferation, then IMP1 overexpression might reverse the effect of BTYNB on cell proliferation. Transient transfection of IMP1 in HEK 293T cells led to an increase in IMP1 protein compared to control transfection (Figure 5K). HEK 293T cells treated with 10 µM BTYNB alone or transfected with a control plasmid and treated with BTYNB demonstrated a significant decrease in cell proliferation (Figure 5K). However, overexpression of IMP1 followed by treatment with BTYNB reversed inhibition of cell proliferation (Figure 5K). This result demonstrates that BTYNB inhibits cell proliferation by targeting IMP1.

BTYNB Blocks Anchorage-Independent Growth of IMP1-Positive Cancer Cells

Anchorage-independent growth in soft agar is a hallmark of cancer cell proliferation. Growth in soft agar is often used to evaluate anchorage-independent growth of human ovarian and melanoma cancer cells. We tested the ability of BTYNB to inhibit colony formation of SK-MEL2 and ES-2 cells grown in soft agar. SK-MEL2 cells treated with medium containing DMSO formed large colonies (>0.5 mm), which were completely inhibited by 10 µM BTYNB (Figure 6A). Similarly, ES-2 cells treated with a DMSO-control formed large colonies (> 0.5 mm), which were inhibited by treating cells with 10 µM BTYNB (Figure 6B). Thus, BTYNB potently inhibits anchorage-independent growth of ovarian and melanoma cancer cells.

Discussion

IMP1 contributes to carcinogenesis by stabilizing the mRNA of oncogenes, and therefore, finding inhibitors of IMP1 could contribute to the development of new cancer therapy. Our development of the first selective small molecule IMP1 biomodulator is significant on several levels. Despite their ubiquitous roles in diverse cell processes, small molecule biomodulators of mammalian mRNA-binding proteins have rarely been described. Thus, BTYNB serves as a prototype for this class of molecule. IMP1's role in cancer, and the cancer-related proteins and microRNAs (miRNAs) that control IMP1 expression [11], makes targeting IMP1 especially attractive. IMP1 is induced by the oncogene c-Myc, and IMP1 increases the level of c-Myc mRNA and protein by stabilizing c-Myc mRNA [24,31]. This sets up a tumor-promoting feed-forward regulatory loop in which c-Myc induces IMP1 and IMP1 increases c-Myc protein levels, enabling c-Myc to further induce IMP1.
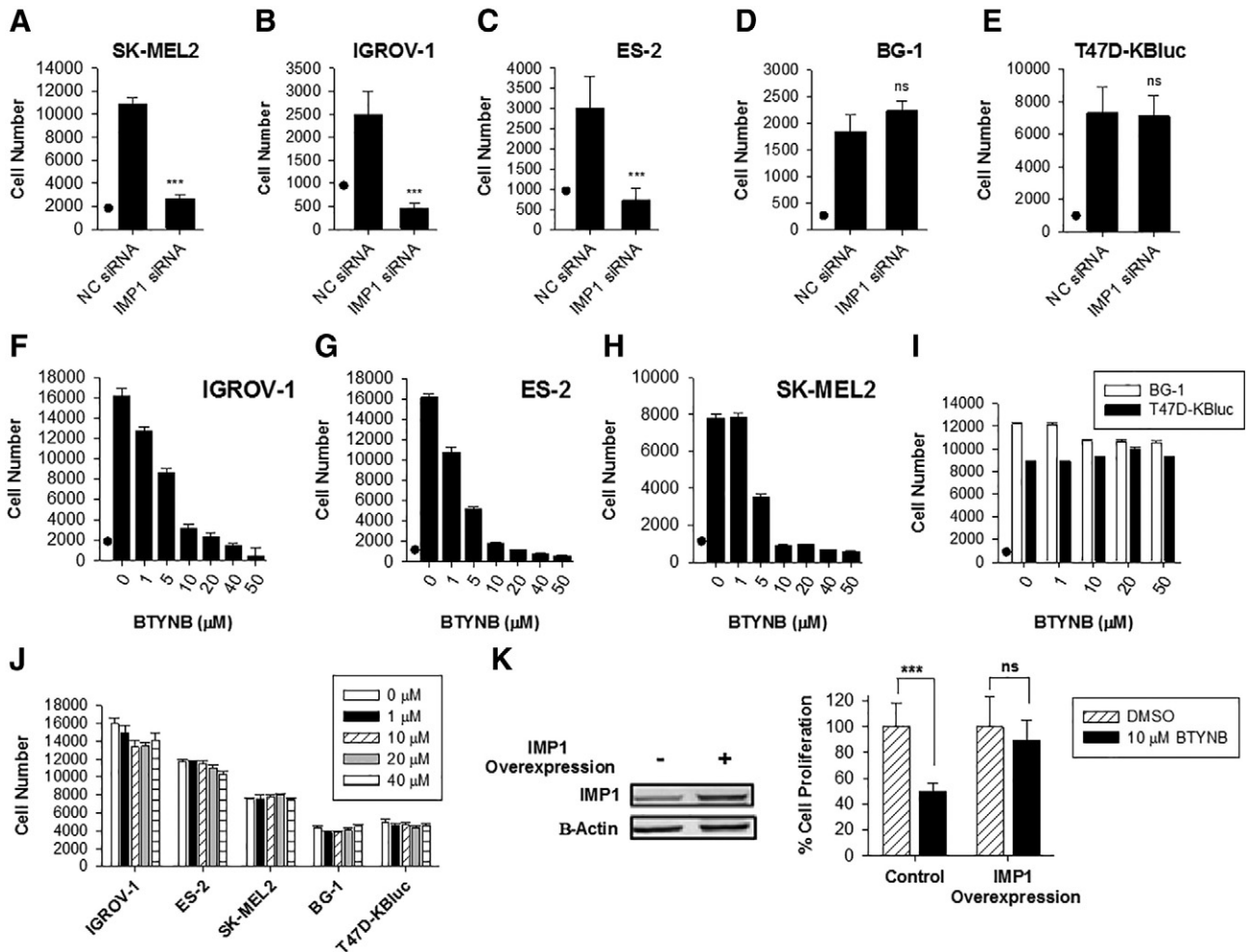
Figure 5. IMP1 depletion and BTYNB block proliferation of IMP1-positive cancer cells, and overexpression of IMP1 is sufficient to reverse BTYNB-mediated inhibition of cell proliferation. siRNA knockdown of IMP1 blocks proliferation of IMP1-positive (A) SK-MEL2 melanoma, (B) IGROV-1 ovarian, and (C) ES-2 ovarian cancer cells with no effects on IMP1-negative (D) BG-1 ovarian and (E) T47D-KBluc breast cancer cells. Cells were treated with 100 nM NC or IMP1 siRNA SmartPool for 72 hours prior to evaluating cell number ($n = 6$). "•" denotes number of cells plated at start of experiment. BTYNB blocks proliferation of (F) IGROV-1, (G) ES-2, and (H) SK-MEL2 cancer cells with no effects on (I) IMP1-negative BG-1 ovarian and T47D-KBluc breast cancer cells. Cells were treated with a DMSO-control or increasing concentrations of BTYNB for 72 hours prior to evaluating cell number ($n = 6$). (J) The biologically inactive compound, 5226752, has no effects on proliferation of IMP1-positive and IMP1-negative cancer cell lines. Cells were treated for 72 hours with increasing concentrations of compound 5226752 ($n = 6$). (K) Overexpression of IMP1 is sufficient to block BTYNB-mediated inhibition of cell proliferation ($n = 6$). IMP1-positive HEK 293T cells were transiently transfected with 100 ng of a control-plasmid (Control) or CMV-IMP1 expression plasmid (IMP1 overexpression). Western blot shows increased IMP1 protein in the cells transfected with CMV-IMP1. Twenty four hours after transfection, cells were treated with the control (DMSO) or 10 μM BTYNB for 48 hours before evaluating cell number. Proliferation rates were normalized to cells treated with DMSO-control. Data are reported as mean $\pm$ SEM and P values represent comparison to NC controls (A-E). *** $P < .001$. ns, not significant.

Breaking this oncogenic feed-forward regulatory loop (c-Myc→IMP1 ↑→c-Myc↑) using c-Myc inhibitors is challenging. Despite considerable effort, its important role in mediating cell proliferation in both normal and cancerous cells [19,32] has made c-Myc difficult to target. Targeting IMP1 with small molecule inhibitors, such as BTYNB, presents an alternative method for targeting c-Myc and breaking the c-Myc-IMP1 feed-forward regulatory loop.

RNAi knockdown of IMP1 showed that IMP1 plays an important role in cell proliferation and that loss of IMP1 reduces levels of c-Myc mRNA. Moreover, the proliferation phenotype of cancer cells in which IMP1 or c-Myc has been knocked down is similar, suggesting that c-Myc is an important IMP1 target [17]. Based in part on data from an endonuclease protection assay, a high-affinity binding site for

recombinant IMP1 was identified in the coding region instability determinant of c-Myc RNA [24]. For our high-throughput screen, we used recombinant DNA technology and enzymatic coupling to synthesize a 93-nucleotide fluorescein-labeled RNA probe containing the c-Myc mRNA coding region instability determinant that binds IMP1 and purified untagged IMP1 [18]. BTYNB was the most potent and selective compound to emerge from our screen of ~160,000 compounds.

Our data support the view that the intracellular actions of BTYNB are based on its ability to inhibit IMP1. BTYNB exhibits similar dose-response curves for inhibition of binding of IMP1 to flMyc *in vitro* and for inhibition of cell proliferation. BTYNB inhibits proliferation of IMP1-positive cells in which IMP1 knockdown
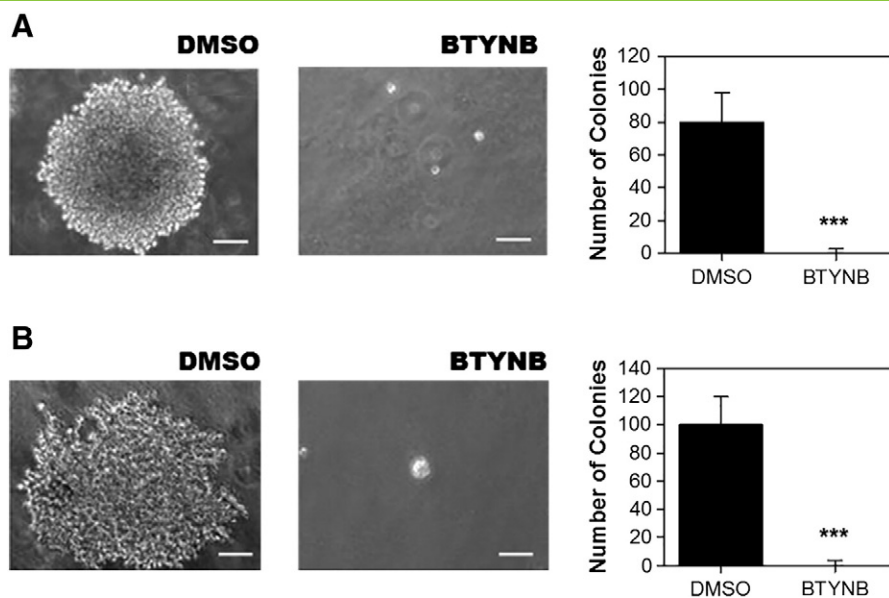
Figure 6. BTYNB inhibits anchorage-independent growth of IMP1-positive cancer cells. (A) SK-MEL2 cells treated for 21 days with medium containing DMSO-vehicle control or 10 μM BTYNB. (B) ES-2 cells treated for 14 days with medium containing DMSO-vehicle control or 10 μM BTYNB. Photographs are at 10$\times$ magnification and are representative of the entire plate and of triplicate experiments. The bar graphs represent the average of the total number of colonies counted after treating with DMSO-control or 10 μM BTYNB. ***$P < .001$.

similarly inhibits cell proliferation and has no effect in IMP1-negative cells in which IMP1 siRNA has no effect. Overexpression of IMP1 abolishes BTYNB inhibition of cell proliferation. We also found that the BTNYB derivative, Compound 5226752, did not inhibit binding of IMP1 to flMyc *in vitro* and did not downregulate the IMP1 mRNA target, c-Myc.

Here we describe a broadly applicable HTS system for identifying small molecules that inhibit interaction of RNA-binding proteins with their RNA recognition sequences, and demonstrate that a small molecule identified using this simple *in vitro* assay with isolated components destabilizes c-Myc mRNA in cells, leading to decreases in steady-state c-Myc mRNA and protein levels. Moreover, the small molecule identified using this system is broadly effective against diverse targets of the mRNA-binding protein. BTYNB's selectivity and low toxicity, effectiveness against diverse cancer-related IMP1 mRNA targets, and ability to abolish anchorage-independent growth of IMP1 containing cancer cells make it a strong candidate for further therapeutic testing and development.

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