Combination therapy with the CDK7 inhibitor and the tyrosine kinase inhibitor exerts synergistic anticancer effects against *MYCN*-amplified neuroblastoma

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Short title: THZ1 and TKIs induce synergistic anticancer effects

Key words: Neuroblastoma, N-Myc, CDK7, THZ1, tyrosine kinase inhibitors, PNUTS

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Novelty and Impact: It is unknown which anticancer agents should be combined with CDK7 inhibitors in clinical trials. In this study, the tyrosine kinase inhibitors (TKIs) ponatinib and lapatinib

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were found to be the approved oncology drugs exerting the best synergistic anticancer effects with the CDK7 inhibitor THZ1 in *MYCN* oncogene-amplified neuroblastoma cells. THZ1 and the TKIs synergistically blocked N-Myc protein expression by suppressing PNUTS expression. Our data suggest the addition of the TKIs in CDK7 inhibitor therapy.

Abbreviations: AOD, approved oncology drug; CDK7, cyclin dependent kinase 7; CI, combination index; KRCC1, lysine rich coiled-coil 1; PI3K, phosphatidylinositol 3-kinase; PNUTS, phosphatase 1 nuclear targeting subunit; RT-PCR, reverse transcription PCR; TKI, tyrosine kinase inhibitor

Abstract

Patients with neuroblastoma due to *MYCN* oncogene amplification and consequent N-Myc oncoprotein overexpression have very poor prognosis. The CDK7/super-enhancer inhibitor THZ1 suppresses *MYCN* gene transcription, reduces neuroblastoma cell proliferation, but does not cause significant cell death. The protein kinase PNUTS has recently been shown to interact with c-Myc protein and suppresses c-Myc protein degradation. Here we screened the US Food and Drug Administration-Approved Oncology Drugs Set V from the National Cancer Institute, and identified tyrosine kinase inhibitors (TKIs), including ponatinib and lapatinib, as the Approved Oncology Drugs exerting the best synergistic anticancer effects with THZ1 in *MYCN*-amplified neuroblastoma cells. Combination therapy with THZ1 and ponatinib or lapatinib synergistically induced neuroblastoma cell apoptosis, whilst having little effects in normal non-malignant cells. Differential gene expression analysis identified *PNUTS* as one of the genes most synergistically reduced by the combination therapy. RT-PCR and immunoblot analyses confirmed that THZ1 and the TKIs synergistically downregulated PNUTS mRNA and protein expression and reduced N-Myc protein but not N-Myc mRNA expression. In addition, PNUTS knockdown resulted in decreased N-Myc protein but not mRNA expression and decreased *MYCN*-amplified neuroblastoma cell proliferation and survival. As CDK7 inhibitors are currently under clinical evaluation in patients, our data suggestion the addition of the TKI ponatinib or lapatinib in CDK7 inhibitor clinical trials in patients.

Introduction

The most common cause of death from diseases in children is cancer. Neuroblastoma is accountable for approximately 8% of all paediatric cancers but 15% of all paediatric cancer mortality.[1-2] Neuroblastoma arises from embryonic sympathoadrenal lineage of neural crest cells and is commonly localized within the adrenal medulla, head, chest, neck and pelvis.[3] *MYCN* oncogene amplification occurs in approximately 25% of human neuroblastoma tissues and correlates with poor patient prognosis.[1-2]

MYCN oncogene encodes N-Myc oncoprotein, a Myc family transcription factor important for cell cycle progression, cell proliferation, resistance to apoptosis *in vitro* as well as neuroblastoma formation and progression *in vivo*.[4-5] While N-Myc presents as a therapeutic target, Myc oncoproteins are considered "undruggable" as they have no active site for ligand binding by small molecule compounds.[6-7] As such, targeting N-Myc down-stream targets, N-Myc protein stability or *MYCN* gene transcription provides an attractive approach for inhibiting the N-Myc oncogenic pathway.

MYCN oncogene transcription is regulated by super-enhancers and cyclin dependent kinase 7 (CDK7).[8] CDK7 binds to Transcription Factor II Human and regulates transcriptional initiation, pausing and elongation by phosphorylating the serine 5 and serine 7 residues of the C-terminal domain of RNA polymerase II at super-enhancer-associated oncogene loci.[8-10]. The covalent CDK7 inhibitor THZ1 binds to the cysteine 312 residue adjacent to the ATP cleft of the canonical kinase domain of CDK7 protein, suppresses CDK7 function, RNA polymerase II phosphorylation and the transcription of super-enhancer-associated oncogenes including *MYCN*, *MYC* and *BLC2*, and induces cancer cell growth inhibition.[8-13]

Receptor tyrosine kinases are a family of enzymes which are key regulators of cell proliferation, differentiation, metabolism, survival and apoptosis.[14] Tyrosine kinases are activated due to ligand binding or constitutively activating mutations and induces phosphatidylinositol 3-kinase (PI3K) activation, leading to glycogen synthase kinase 3β inactivation, c-Myc and N-Myc protein dephosphorylation at threonine 58, and c-Myc and N-Myc protein stabilization.[4]

In this study, we have performed drug screening and identified tyrosine kinase inhibitors (TKIs), including ponatinib and lapatinib, as the approved oncology drugs which exerted the best synergistic anticancer effects with the CDK7 inhibitor THZ1 against *MYCN*-amplified neuroblastoma cells. Combination therapy with THZ1 and ponatinib or lapatinib synergistically reduced gene expression of the protein kinase *PNUTS* and thereby reduced N-Myc protein but not mRNA expression, and synergistically induced *MYCN*-amplified neuroblastoma cell apoptosis.

Materials and Methods

Cell culture

BE(2)-C (RRID:CVCL_0529), LAN-1 (RRID:CVCL_1827), SK-N-SH (RRID:CVCL_0531), SK-N-AS (RRID:CVCL_1700), WI38 (RRID:CVCL_0579), Kelly (RRID:CVCL_2092) and CHP134 (RRID:CVCL_1124) cells were cultured in Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute Medium 1640, supplemented with 10% fetal calf serum. BE(2)-C cells were obtained from Barbara Spengler (Fordham University, NY) 20 years ago, and the other cells were purchased from the European Collection of Cell Cultures in 2010 (Sigma, Sydney, Australia). Cell line identity was confirmed in the last 3 years by short tandem repeat profiling at Garvan Institute of Medical Research or Cellbank Australia, and all cell lines were confirmed to be mycoplasma free.

Alamar blue assays

Alamar blue cell viability assays were performed as we described.[15-17] Briefly, cells were transfected with control or PNUTS siRNAs for 96 hours, or treated with vehicle control or anticancer agents for 72 hours. The cells were incubated with Alamar blue (Invitrogen, Carlsbad, CA) for the last five hours, and cell culture plates were read on a microplate reader at 570/595 nm. Results were quantified according to the optical density absorbance units and expressed as percentage changes in the number of cells, relative to control siRNA-transfected or vehicle control-treated samples.

Drug screening

For primary screening, *MYCN* oncogene-amplified BE(2)-C neuroblastoma cells were seeded into 96-well plates and treated in triplicates with vehicle control, the CDK7 inhibitor THZ1 at 16nM which reduced the number of BE(2)-C cells by approximately 30% on its own, the US Food and Drug Administration-Approved Oncology Drugs (AODs) Set V from National Cancer Institute at 1μM, or combination for 72 hours. Cell viability was determined by Alamar blue assays. Synergistic/additive interaction between THZ1 and the AODs was examined by fractional product method and expressed as R values.[18]

AODs which reduced the number of viable BE(2)-C cells by ≥ 90% on their own and AODs which synergized with THZ1 in reducing the number of viable BE(2)-C cells with a R value of less than 0.7 were shortlisted for secondary drug screening. BE(2)-C cells were treated with a range of doses of THZ1, the shortlisted AODs, or combination of THZ1 and the AODs for 72 hours, followed by Alamar blue assays. To determine if the effect of THZ1 and the AOD were synergistic or additive, Bliss-additivity was calculated with Bliss-additive formula.[19] If the actual combination effect was greater than that of the predicted additivity, THZ1 and the AOD exerted synergistic anticancer effect. Synergy was further validated by calculating combination indexes (CIs) for effective doses for 75% and 90% cell number reduction with the Chou-Talalay method[20] and CompuSyn software (Combosyn Inc, http://www.combosyn.com/), where CI <1, 1 or >1 indicates synergistic, additive and antagonistic effect, respectively.

Apoptosis analysis

Neuroblastoma cells were transfected with control siRNA, PNUTS siRNA-1 or PNUTS siRNA-2 for 72 hours, or treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 72 hours. Cells were then stained with Annexin V and **7**-amino-actinomycin D (7-AAD), followed by flow cytometry analysis of cells positively stained by Annexin V and/or 7-AAD, using FACS Canto Flow Cytometer (BD Biosciences, Franklin Lakes, NJ). The percentage of Annexin V and/or 7-AAD positively stained cells was analyzed with FlowJo Version 10 (TreeStar Inc., Ashland, OR) as we described.[16, 21]

siRNA transfection

Neuroblastoma cells were plated into 6 well plates or T25 flasks and transfected with siRNAs using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and as we described previously.[22-23] RNA or protein were harvested for RT-PCR and immunoblot analyses.

Plasmid transfection

pCMV6-entry empty vector and pCMV6-entry PNUTS expression constructs were purchased from Origene (Origene, Rockville, MD). Neuroblastoma cells were transfected with the constructs using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol and as we described.[15-16]

Real time reverse transcription PCR (RT-PCR)

RNA was extracted from cells with RNeasy Plus Mini kit (Qiagen, Hamburg, Germany) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. RT-PCR was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) for cDNA synthesis and gene specific primers and Power SYBR Green Master Mix (Invitrogen) as the fluorescent dye for PCR in Applied Biosystems 7900 (Applied Biosystems, Grand Island, NY). The sequences of RT-PCR primers were: 5'-CGACCACAAGGCCCTCAGTA-3' (forward) and 5'-CAGCCTTGGTGTTGGAGGAG-3' (reverse) for N-Myc; 5'-GCAGCTGAGCAAGTCAAGTG-3' (forward) and 5'-TTCTCAGCAGGCTGGGTACT-3' (reverse) for PNUTS; 5'-TTGACAAAACGGGATTTTCC-3' (forward) and 5'-TGGAGAATTGGACAAAAATTG-3' (reverse) for KRCC1; 5'-AGGCCAACCGCGAGAAG-3' (forward) and 5'-ACAGCCTGGATAGCAACGTACA-3' (reverse) for Actin. All primers were synthesized by Sigma (Sigma). The comparative threshold cycle ($\Delta\Delta$Ct) method[24] was used to evaluate fold changes in target genes, relative to the housekeeping gene actin.

Immunoblot

Neuroblastoma cells were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 7.5) containing protease inhibitors (Sigma) and phosphatase inhibitors (Roche, Penzberg, Germany). Protein was extracted, quantified with the Bicinchoninic Acid Assay kit (Pierce, Rockford, IL), and loaded onto sodium dodecyl sulphate-polyacrylamide gels, followed by electrophoresis and transfer to nitrocellulose membranes. After blocking, membranes were probed with rabbit anti-PARP (1:1000) (Cell Signaling, Danvers, MA), rabbit anti-cleaved PARP (1:1000) (Cell

Signaling), rabbit anti-PNUTS (1:1,000) (Bethyl Laboratories, Montgomery, TX) and mouse anti-N-Myc (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The membranes were then incubated with a goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (1:10000) (Santa Cruz Biotechnology), and protein bands were visualized with SuperSignal (Pierce). The membranes were finally probed with an anti-actin antibody (1:30000) (Sigma) as loading controls.

Affymetrix microarray differential gene expression study

BE(2)-C neuroblastoma cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib. Six hours later, RNA was extracted from the cells with RNeasy Mini kit (Qiagen), and differential gene expression was investigated using Affymetrix Arrays (Affymetrix, Santa Clara, CA), as we described.[15-16, 22] Microarray data were analyzed in R (http://www.r-project.org/) with bioconductor package (http://www.bioconductor.org/), and normalized with GenePattern software (Version 3.9.0 Broad Institute) and LimmaGP modules (Version 20.0) available at (https://pwbc.garvan.org.au/gp/), and deposited at Gene Expression Omnibus website (Series GSE128649).

Statistical analysis

Experiments for statistical analysis were performed at least 3 times. Data were examined with Graphpad Prism 6 program and expressed as mean ± standard error. Differences were analyzed for statistical significance with one-way ANOVA among groups or two-sided unpaired *t* test for two groups.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Screening of the Approved Oncology Drugs Set identifies TKIs that synergize with the CDK7 inhibitor THZ1

While showing promising anticancer effects, CDK7 inhibitors do not cause considerable tumor cell death *in vitro* and tumor regression *in vivo*.[8-9, 11-12] To identify anticancer agents which exert the best synergistic anticancer effects with the CDK7 inhibitor THZ1 against *MYCN*-amplified neuroblastoma cells, we performed a primary screen of the AOD Set V from the US National Cancer Institute. The AOD Set V included 114 representatives of all classes of the US Food and Drug Administration-approved anticancer agents. *MYCN*-amplified BE(2)-C neuroblastoma cells were treated with vehicle control, 16nM THZ1 alone which reduced the number of BE(2)-C cells by approximately 30% on its own, 1μM AODs alone, or combination. Cytotoxicity testing using Alamar blue assays showed that 17 compounds reduced the number of viable BE(2)-C cells by ≥ 90% on their own, and also reduced the number of viable BE(2)-C cells by ≥ 90% when combined with THZ1 (Fig. 1*a*, Table 1 and Supporting Information Table S1). The 17 AODs included the DNA topoisomerase II inhibitors daunorubicin, doxorubicin, idarubicin and mitoxantrone; microtubule inhibitors vinblastine, vincristine, docetaxel, cabazitaxel, ixabepilone and paclitaxel; the MEK inhibitor trametinib; the gene transcription/histone deacetylase/RNA synthesis inhibitors dactinomycin, romidepsin and plicamycin; the proteasome inhibitors carfilzomib and bortezomib and the protein translation inhibitor omacetaxine mepesuccinate. Due to high cytotoxicity on their own, it was not possible to calculate synergism of these compounds in combination with THZ1. In addition, 5 out of the remaining 97 compounds, including the TKIs

ponatinib, lapatinib and nilotinib as well as the antimetabolites pralatrexate and methotrexate, at 1μM showed significant synergistic anticancer effects with THZ1 [$R < 0.7$, fractional product method[25]] (Fig. 1*b*, Table 1 and Supporting Information Table S1).

The 17 AODs which reduced cell viability by ≥ 90% on their own at 1μM and the 5 AODs which synergized with THZ1 at 1μM were then subjected to secondary screen. BE(2)-C cells were treated with vehicle control, a range of dosages of the AODs (2 fold serial dilution of the highest dose which reduced cell viability by ≤ 80%), THZ1, or combination, followed by Alamar blue assays. Synergy or additivity of the THZ1 and AOD combination therapies was calculated using the Bliss-additivity model.[19] The synergy/additivity analysis showed that vincristine and doxorubicin, two first line chemotherapy drugs for treating neuroblastoma patients, did not synergize with THZ1 (Fig. 1*c* and Supporting Information Fig. S1*a*); that the other DNA topoisomerase II inhibitors, the other microtubule inhibitors, the MEK inhibitor, the gene transcription/histone deacetylase/RNA synthesis inhibitors, the proteasome and protein translation inhibitors as well as the antimetabolites did not considerably synergize with THZ1 (Supporting Information Fig. S2 and Fig. S3); and that the TKIs ponatinib, lapatinib and nilotinib synergized with THZ1 (Fig. 1*d* and Supporting Information Fig. S1*b*). The primary and secondary screens therefore identify the TKIs as the AODs exerting the best synergistic anticancer effects with THZ1 against *MYCN*-amplified neuroblastoma.

THZ1 and TKIs synergistically induce neuroblastoma cell apoptosis with little toxicity against normal cells

We next examined whether TKIs and THZ1 exerted synergistic anticancer effects in a range of *MYCN*-amplified neuroblastoma cell lines with little toxicity to normal cells. *MYCN*-amplified Kelly and CHP134 neuroblastoma cells were treated with vehicle control, a range of doses of THZ1, ponatinib, lapatinib, nilotinib, THZ1 plus ponatinib, lapatinib or nilotinib for 72 hours, followed by Alamar blue assays. Combination effect analysis with the Bliss-additivity model demonstrated that combination therapy with THZ1 and ponatinib, lapatinib or nilotinib synergistically reduced the number of viable Kelly and CHP134 cells (Fig. 2*a*-2*c*). In addition, combination index (CI) values for reducing BE(2)-C, Kelly and CHP134 neuroblastoma cell viability by 75% and 90% by the combination therapies were generated by Compusyn[26], and the CI values further confirmed that combination therapies with THZ1 and ponatinib, lapatinib or nilotinib synergistically reduced the number of viable *MYCN*-amplified BE(2)-C, Kelly and CHP134 cells with CI values < 0.6 in all cases (Figs. 2*d*-2*f*).

Ponatinib and nilotinib both target the tyrosine kinase Bcr-ABL[27] while lapatinib targets the tyrosine kinase ERBB2 and EGFR.[28] As the concentrations for achieving considerable synergistic anticancer effects with THZ1 was higher for nilotinib (up to 8μM) than ponatinib (up to 1μM) and lapatinib (up to 2μM) (Figs. 2*a*-2*c*), we used ponatinib and lapatinib for further studies. To examine whether THZ1 and TKIs exerted synergistic anticancer effects selectively against *MYCN*-amplified neuroblastoma cell lines, we treated the *MYCN*-amplified LAN-1 cells with vehicle control, a range of doses of THZ1, ponatinib, lapatinib, THZ1 plus ponatinib or lapatinib for 72 hours. Alamar blue assays and combination effect analysis with the Bliss-additivity model confirmed that combination therapies with THZ1 and ponatinib or lapatinib synergistically reduced the number of viable *MYCN*-amplified LAN-1 cells (Supporting Information Fig. S4*a*). In addition, *MYCN*-non-amplified SK-N-AS and SK-

N-SH cells were treated with vehicle control, a range of doses of THZ1, ponatinib, or THZ1 plus ponatinib for 72 hours. Alamar blue assays and combination effect analysis with the Bliss-additivity model confirmed that combination therapies with THZ1 and ponatinib did not show synergistic anticancer effects against the *MYCN*-non-amplified neuroblastoma cell lines (Supporting Information Fig. S4*b*).

BE(2)-C, Kelly and CHP134 neuroblastoma and WI-38 fibroblast cells were next treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, or combination of THZ1 and ponatinib or lapatinib for 72 hours, followed by staining with the apoptosis marker Annexin V. Flow cytometry analysis showed that THZ1 and ponatinib, as well as THZ1 and lapatinib, synergistically induced apoptosis in 80% and 83% BE(2)-C, 53% and 67% Kelly, and 63% and 87% CHP134 neuroblastoma cells but not in WI38 fibroblasts (Fig. 2*g*). In addition, BE(2)-C and Kelly cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, or combination of THZ1 and ponatinib or lapatinib for 48 hours. Immunoblot analysis showed that THZ1 alone induced minor PARP cleavage, that TKIs alone did not show any effect, and that THZ1 and TKIs synergistically induced PARP cleavage (Supporting Information Fig. S5). Taken together, the data demonstrate that THZ1 and the TKIs ponatinib and lapatinib synergistically induce apoptosis in *MYCN*-amplified neuroblastoma but not normal non-malignant cells.

THZ1 and the TKI ponatinib and lapatinib synergistically reduce N-Myc protein but not mRNA expression

THZ1 suppresses *MYCN* gene transcription by blocking CDK7 binding to the *MYCN* gene promoter.[8] We examined whether THZ1 and the TKI ponatinib and lapatinib synergistically regulate N-Myc mRNA and protein expression. BE(2)-C and Kelly cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours. RT-PCR and immunoblot analyses showed that THZ1 alone reduced N-Myc mRNA and protein expression, and that combination therapy with THZ1 and the TKIs did not co-operatively reduce N-Myc mRNA expression, but synergistically blocked the expression of N-Myc protein (Figs. 3*a*-3*b*). Consistent with these data, combination therapy with THZ1 and the TKI ponatinib or lapatinib also synergistically reduced N-Myc protein expression in CHP134 cells (Supporting Information Fig. S6). The data suggest that THZ1 and TKI combination therapy synergistically blocks N-Myc protein expression through a post-transcriptional mechanism.

THZ1 and the TKI ponatinib and lapatinib synergistically reduce *PNUTS* gene and protein expression

To examine the mechanism through which THZ1 and the TKI ponatinib and lapatinib synergistically reduce N-Myc protein expression and neuroblastoma cell proliferation and survival, we performed Affymetrix microarray gene expression experiments in BE(2)-C cells after treatment with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib or THZ1 plus lapatinib for 6 hours. Using two fold change as the cut-off point, 34 genes were found to be commonly down-regulated and two gene commonly up-regulated by both THZ1 and ponatinib as well as THZ1 and lapatinib combinations (Supporting Information Table S2). Lysine rich coiled-coil 1 (KRCC1) and

phosphatase 1 nuclear targeting subunit (PNUTS, also known as PPP1R10), the genes most synergistically downregulated by THZ1 and TKI combination therapies were selected for validation.

BE(2)-C and Kelly cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours. RT-PCR analysis showed that monotherapy with THZ1 reduced KRCC1 and PNUTS mRNA expression, that monotherapy with ponatinib or lapatinib showed no effect, and that combination therapy with THZ1 and ponatinib or lapatinib synergistically and dramatically reduced KRCC1 (Fig. 4*a*) and PNUTS (Fig. 4*b*) mRNA expression. Consistent with the RT-PCR data, immunoblot analysis showed that combination therapy with THZ1 and ponatinib or lapatinib synergistically blocked PNUTS protein expression (Fig. 4*c*). Taken together, the data demonstrate that the THZ1 and TKI combination therapy synergistically reduces KRCC1 and PNUTS expression in *MYCN*-amplified neuroblastoma cells.

PNUTS is required for N-Myc protein expression and *MYCN*-amplified neuroblastoma cell proliferation and survival

PNUTS has recently been shown to directly bind to Aurora kinase A and c-Myc proteins and to block c-Myc protein degradation.[29-30] We examined whether PNUTS regulates N-Myc protein expression and neuroblastoma cell proliferation and survival. Real-time RT-PCR and immunoblot analyses showed that transfection of BE(2)-C and Kelly cells with two independent PNUTS siRNAs, PNUTS siRNA-1 or PNUTS siRNA-2, efficiently knocked down PNUTS mRNA and protein expression, and considerably reduced N-Myc protein but not N-Myc mRNA expression (Figs. 5*a*-5*c*). BE(2)-C and

Kelly cells were next transfected with an empty vector or PNUTS expression construct. RT-PCR and immunoblot analysis demonstrated that PNUTS over-expression up-regulated N-Myc protein but not mRNA expression (Fig. 5*d-f*).

BE(2)-C and Kelly cells were then transfected with control siRNA or PNUTS siRNAs for 72 hours, followed by Alamar blue assays or staining with Annexin V for flow cytometry analysis of Annexin V positively stained apoptotic cells. Alamar blue assays showed that knocking down PNUTS expression significantly reduced the number of viable BE(2)-C and Kelly neuroblastoma cells (Fig. 5*g*), and flow cytometry analysis of Annexin V stained cells showed that knocking down PNUTS expression led to BE(2)-C and Kelly cell apoptosis (Fig. 5*h*). Taken together, the data demonstrate that PNUTS is important for N-Myc protein expression and neuroblastoma cell proliferation and survival.

Discussion

CDK7 plays a critical role in the transcriptional activation of super-enhancer-associated oncogenes such as *MYCN* and *MYC*,[8-10, 12] and several CDK7 inhibitors, including SY-1365 of Syros Pharmaceuticals and CT7001 of Carrick Therapeutics, are currently in clinical trials. However, monotherapy with CDK7 inhibitors does not considerably induce cancer cell death, and it is unknown which drugs should be combined with CDK7 inhibitors in patients. In this study, we have screened the AOD Set from the US National Cancer Institute for anticancer agents which exert strong synergistic anticancer effects with the CDK7 inhibitor THZ1 against *MYCN* oncogene-amplified neuroblastoma cells. TKIs including ponatinib, lapatinib and nilotinib have been identified as the class of AODs which exert the best synergy with THZ1.

The TKIs ponatinib and nilotinib mainly target Bcr-ABL while lapatinib targets ERBB2 and EGFR.[27-28, 31] Treatment with ponatinib, nilotinib or lapatinib blocks tyrosine kinase activity and consequently suppresses RAS-MAP kinase pathway and AKT pathway, leading to ERK and p38 protein dephosphorylation, cancer cell growth inhibition and cell death.[32-34] While nilotinib and laptinib are currently used in the clinic to treat chronic myelogenous leukemia and ERBB2 positive breast cancer respectively, many chronic myelogenous leukemia patients intolerant to nilotinib respond well to ponatimib.[35]

Unlike leukemia and breast cancer, human neuroblastoma is not characterized by Bcr-ABL, ERBB2 or EGFR abnormalities. Interestingly, in a recent screen of 349 compounds, ponatinib has been found to be one of the approved drugs showing the most promising anticancer effects against *MYCN*-

amplified neuroblastoma cells.[36] In a pairwise drug combination screen, lapatinib was found to be one of the compounds exerting the best synergistic anticancer effects with the survivin inhibitor YM155 in neuroblastoma cells.[37] In a *MYCN* transgenic zebrafish model, YM155 and lapatinib synergistically suppressed neuroblastoma progression.[37] In this study, we have found that monotherapy with THZ1, ponatinib, lapatinib or nilotinib reduces the number of *MYCN*-amplified neuroblastoma cells, but does not significantly induce apoptosis. Importantly, combination therapy with THZ1 and the TKIs synergistically induces considerable apoptosis in *MYCN*-amplified neuroblastoma but not normal non-malignant cells.

Ponatinib and lapatinib show dramatic initial clinical responses in patients with leukemia and ERBB2 over-expressing breast cancer, however patients frequently relapse because the expression of other oncogenic protein kinases changes in cancer cells due to transcriptional activation.[38-40] Ponatinib and lapatinib have recently been investigated in combination with the BET bromodomain/super-enhancer inhibitor JQ1. Combination therapies with JQ1 and the TKI show strong synergistic anticancer effects against leukaemia and breast cancer through blocking the expression of other oncogenes important for kinome reprogramming.[39-41] In the current study, we have found that monotherapy with THZ1 but not ponatinib or lapatinib reduces N-Myc mRNA and protein expression, and that combination therapy with THZ1 and ponatinib or lapatinib synergistically block N-Myc protein but not mRNA expression. Differential gene expression analysis reveals that THZ1 and TKIs synergistically reduce the transcription of target genes including the protein phosphatase PNUTS, a proto-oncogene.[42] PNUTS directly interacts with the lipid-binding domain of PTEN, sequesters PTEN protein and blocks PTEN-mediated PI3K/AKT pathway inactivation. Depletion of PNUTS leads to

cancer cell growth inhibition and apoptosis, and forced PNUTS over-expression in normal cells leads to tumorigenic capacity.[42] In addition, PNUTS forms protein complexes with Aurora kinase A/B and c-Myc, activates Aurora kinase A/B, blocks FBXW7 E3 ligase-mediated c-Myc protein ubiquitination and proteasomal degradation, and thereby increases c-Myc protein expression.[29-30] In the current study, we have confirmed that PNUTS knockdown reduces N-Myc protein but not N-Myc mRNA expression, that forced PNUTS over-expression up-regulates N-Myc protein expression, and that PNUTS knockdown leads to *MYCN*-amplified neuroblastoma cell growth inhibition and apoptosis. Our data suggest that combination therapy with THZ1 and the TKIs synergistically blocks N-Myc protein expression and induces apoptosis at least partly through down-regulating *PNUTS* gene expression.

In summary, our screening of AODs identifies TKIs ponatinib, lapatinib and nilotinib as the AODs exerting the best synergistic anticancer effects with THZ1 against *MYCN* gene-amplified neuroblastoma cells. TKIs exert synergistic anticancer effects with THZ1 through a non-canonical function, blocking *PNUTS* gene expression, and THZ1 and TKIs synergistically induce apoptosis in *MYCN*-amplified neuroblastoma but not normal non-malignant cells. PNUTS up-regulates N-Myc protein expression, and PNUTS knockdown induces *MYCN*-amplified neuroblastoma cell growth inhibition and apoptosis. As CDK7 inhibitors are currently under clinical evaluation in patients, our data suggest the addition of the TKIs in CDK7 inhibitor clinical trials in patients.

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Figures Legends

Figure 1. Screening of the Approved Oncology Drugs Set identifies TKIs that synergize with the CDK7 inhibitor THZ1. (*a*-*b*) BE(2)-C cells were treated with vehicle control, 16nM THZ1, 1μM Food & Drug Administration-Approved Oncology Drugs (AODs) Set V or combination for 72 hours, followed by Alamar blue assays. Among the 114 AODs, 17 AODs reduced the number of viable BE(2)-C cells by ≥ 90% on their own (inside the dotted rectangle) (*a*), and five AODs reduced the number of viable BE(2)-C cells by more than 65% when combined with THZ1 and showed R values of < 0.7 (*b*). R values of < 1, = 1, or > 1 indicate synergistic, additive and antagonistic effects respectively. (*c*-*d*) BE(2)-C cells were treated with vehicle control, a range of dosages of vincristine or doxorubicin (*c*), two first line chemotherapy drugs for treating neuroblastoma patients, a range of dosages of the TKI ponatinib, lapatinib or nilotinib (*d*), or combination for 72 hours, followed by Alamar blue assays. Cell viability graph showed percentage changes in the number of viable cells after treatment with THZ1 alone, the AODs alone, the predicted additivity line according to the Bliss-additivity model (the dotted line), and the actual percentage change in the number of viable cells after combination therapies. Error bars represented standard error.

Figure 2. THZ1 and TKIs synergistically induce neuroblastoma cell apoptosis with little toxicity against normal cells. (*a*-*c*) Kelly and CHP134 neuroblastoma cells were treated with vehicle control, various dosages of THZ1 or the TKI ponatinib (*a*), lapatinib (*b*) or nilotinib (*c*), or combination of THZ1 and the TKIs for 72 hours, followed by Alamar blue assays. Cell viability graph showed

percentage changes in the number of viable cells after treatment with THZ1 alone, the TKIs alone, the predicted additivity line according to the Bliss-additivity model (the dotted line), and the actual percentage change in the number of viable cells after the combination therapies. (*d-f*) Combination effects of THZ1 and the TKI ponatinib (*d*), lapatinib (*e*) or nilotinib (*f*) were further examined by combination indexes (CIs), and CIs for 75% and 90% of BE(2)-C, Kelly and CHP134 cell number reduction were calculated with CalcuSyn. (*g*) BE(2)-C, Kelly and CHP134 neuroblastoma and WI-38 embryonic fibroblast cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 72 hours. Cells were then stained with Annexin V and analysed with flow cytometry. The percentage of cells positively stained by Annexin V was quantified. Error bars represented standard error. *** indicated $P < 0.001$.

Figure 3. THZ1 and the TKI ponatinib and lapatinib synergistically reduce N-Myc protein but not mRNA expression. *MYCN*-amplified BE(2)-C and Kelly neuroblastoma cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours, followed by RNA extraction and real-time RT-PCR analyses of N-Myc mRNA expression (*a*), or followed by protein extraction and immunoblot analysis of N-Myc protein expression (*b*). Error bars represented standard error. *** indicated $P < 0.001$.

Figure 4. THZ1 and the TKI ponatinib and lapatinib synergistically reduce *PNUTS* gene and protein expression. (*a-b*) *MYCN*-amplified BE(2)-C and Kelly neuroblastoma cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus

lapatinib for 48 hours, followed by RNA extraction and real-time RT-PCR analyses of KRCC1 (*a*) and PNUTS (*b*) mRNA expression. (*c*) BE(2)-C and Kelly cells were treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours, followed by protein extraction and immunoblot analysis of PNUTS protein expression. Error bars represented standard error. * and ** indicated $P < 0.05$ and 0.01 respectively.

Figure 5. PNUTS is required for N-Myc protein expression and *MYCN*-amplified neuroblastoma cell proliferation and survival. (*a-c*) BE(2)-C and Kelly neuroblastoma cells were transfected with control siRNA, PNUTS siRNA-1 or PNUTS siRNA-2 for 48 hours, followed by RT-PCR analysis of PNUTS (*a*) and N-Myc (*b*) mRNA expression or PNUTS and N-Myc protein expression (*c*). (*d-f*) BE(2)-C and Kelly cells were transfected with an empty vector or PNUTS expression construct for 48 hours, followed by RT-PCR and immunoblot analysis of PNUTS mRNA (*d*) and N-Myc mRNA (*e*) and PNUTS and N-Myc protein (*f*) expression. (*g-h*) BE(2)-C and Kelly neuroblastoma cells were transfected with control siRNA, PNUTS siRNA-1 or PNUTS siRNA-2. Cells were incubated with Alamar blue for Alamar blue assays of the number of viable cells 96 hours after siRNA transfections (*g*), or stained with Annexin V for flow cytometry analysis of apoptotic cells 72 hours after siRNA transfections (*h*). Error bar represented standard error. *, ** and *** indicated $P < 0.05$, 0.01 and 0.001 respectively.

Table 1. Primary screening of Approved Oncology Drugs (AODs) Set V from the US National Cancer Institute for THZ1 enhancers. BE(2)-C neuroblastoma cells were treated with vehicle control, 16nM of THZ1, 1μM of 114 AODs or combination for 72 hours, followed by Alamar blue assays. Among the 114 AODs, 17 AODs reduced the number of viable BE(2)-C cells by ≥ 90% on their own, and five AODs showed R values of < 0.7 when combined with THZ1. R values of < 1, = 1, or > 1 indicate synergistic, additive and antagonistic effects respectively.

AOD Name	% change THZ1/Control	% change AOD/control	% change THZ1+AOD/control	R value
Romidepsin	63.394	-0.199	0.22	-1.744
Dactinomycin	69.72	-0.073	0.146	-2.858
Omacetaxine	63.394	0.065	0.495	12.086
Idarubicin HCl	63.394	0.166	0.298	2.83
Vinblastine sulfate	65.073	0.291	0.243	1.286
Carfilzomib	65.073	0.402	0.291	1.111
Bortezomib	69.743	0.603	0.246	0.586
Vincristine sulfate	65.073	0.901	0.649	1.107
Ixabepilone	63.394	1.174	1.526	2.051
Daunorubicin HCl	70.94	1.414	1.444	1.439
Plicamycin	69.72	1.771	1.88	1.523
Docetaxel	65.073	2.172	2.316	1.639
Cabazitaxel	65.073	2.974	2.79	1.442
Paclitaxel	65.073	3.239	3.389	1.608
Mitoxantrone	71.693	5.863	5.236	1.246
Trametinib	63.376	6.595	5.281	1.264
Doxorubicin HCl	70.94	7.766	9.324	1.692
Epirubicin HCl	70.94	8.558	10.315	1.699

Ponatinib	70.94	21.725	0.258	0.017
Pralatrexate	70.881	37.004	15.341	0.585
Lapatinib	63.376	83.035	32.401	0.616
Nilotinib	63.394	83.044	33.184	0.63
Methotrexate	71.693	37.451	18.482	0.688

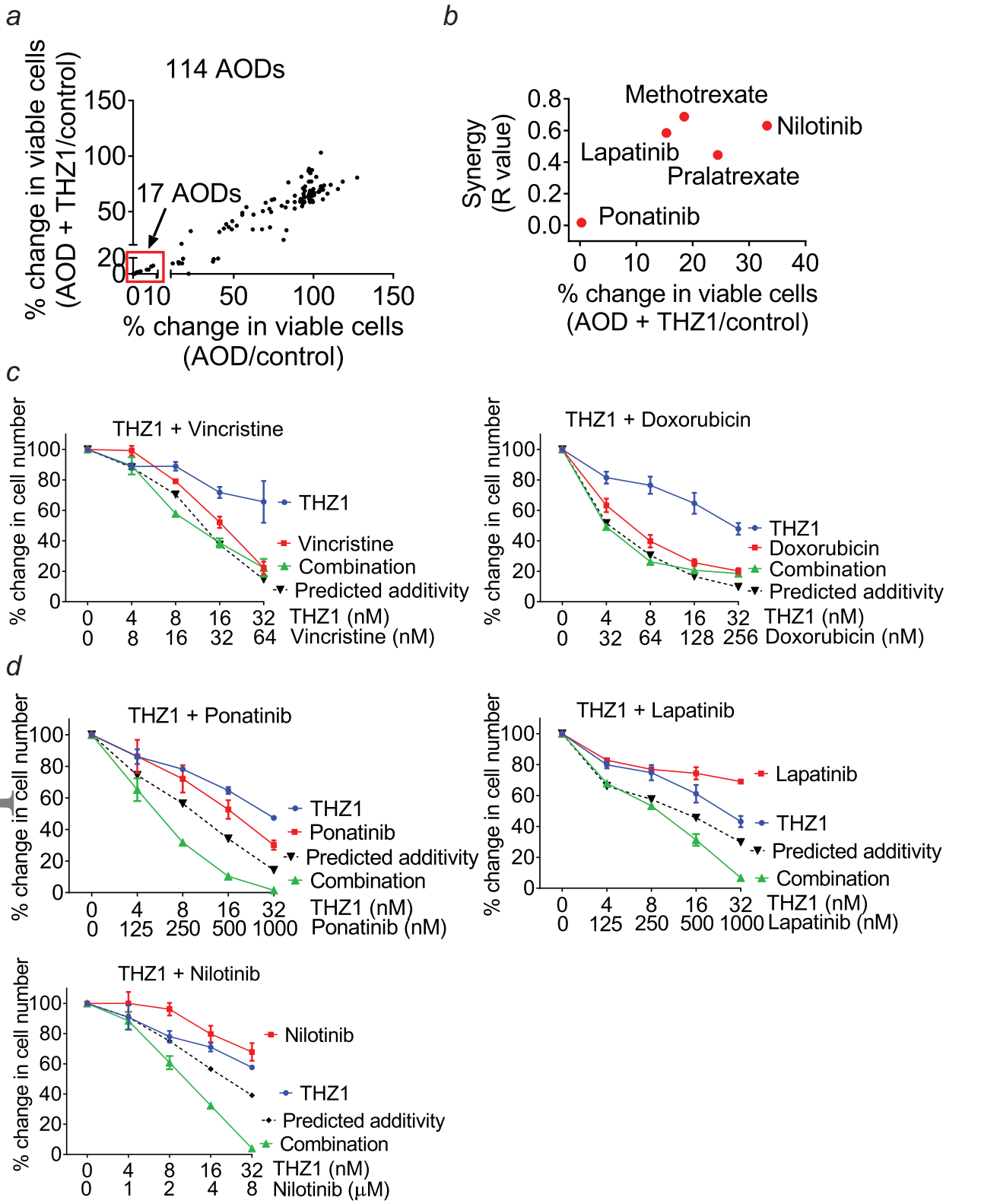
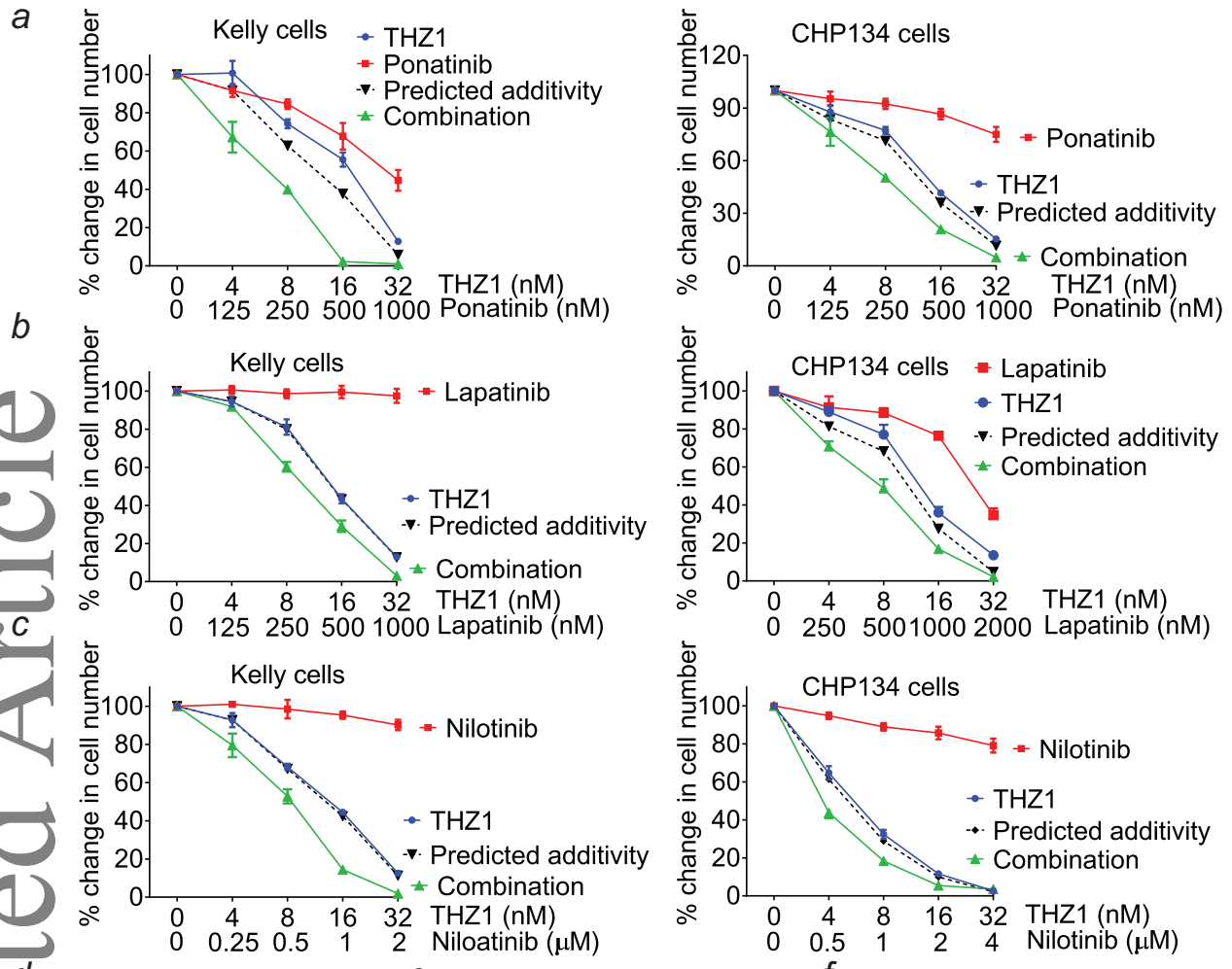


Figure 1



d

Ponatinib			
	CI at inhibition of		
Cell line	75%	90%	r
BE(2)-C	0.40	0.23	0.99
Kelly	0.37	0.40	0.99
CHP134	0.35	0.43	0.99

e

Lapatinib			
	CI at inhibition of		
Cell line	75%	90%	r
BE(2)-C	0.31	0.16	0.97
Kelly	0.37	0.44	0.99
CHP134	0.27	0.27	0.99

f

Nilotinib			
	CI at inhibition of		
Cell line	75%	90%	r
BE(2)-C	0.52	0.53	0.99
Kelly	0.38	0.46	0.99
CHP134	0.34	0.40	0.99

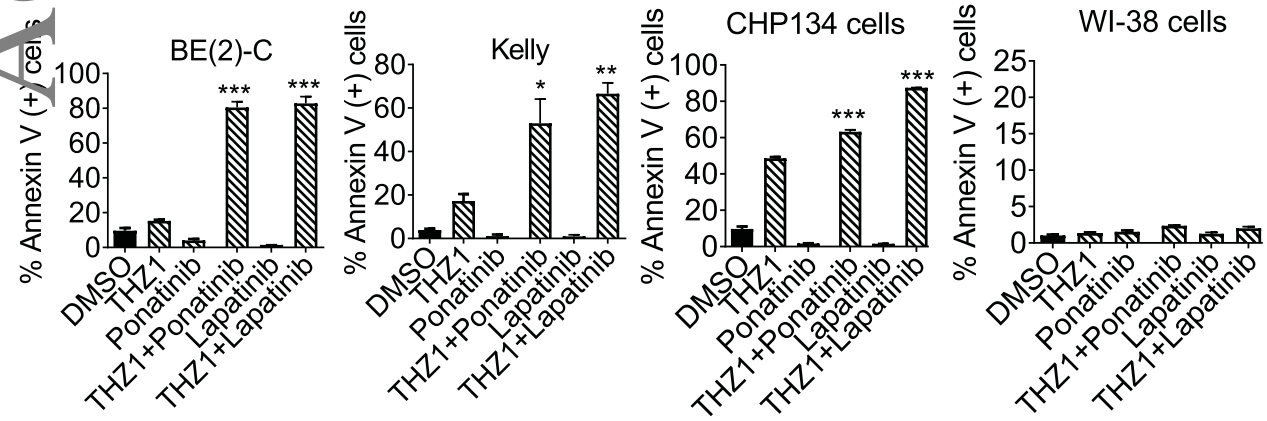


Figure 2

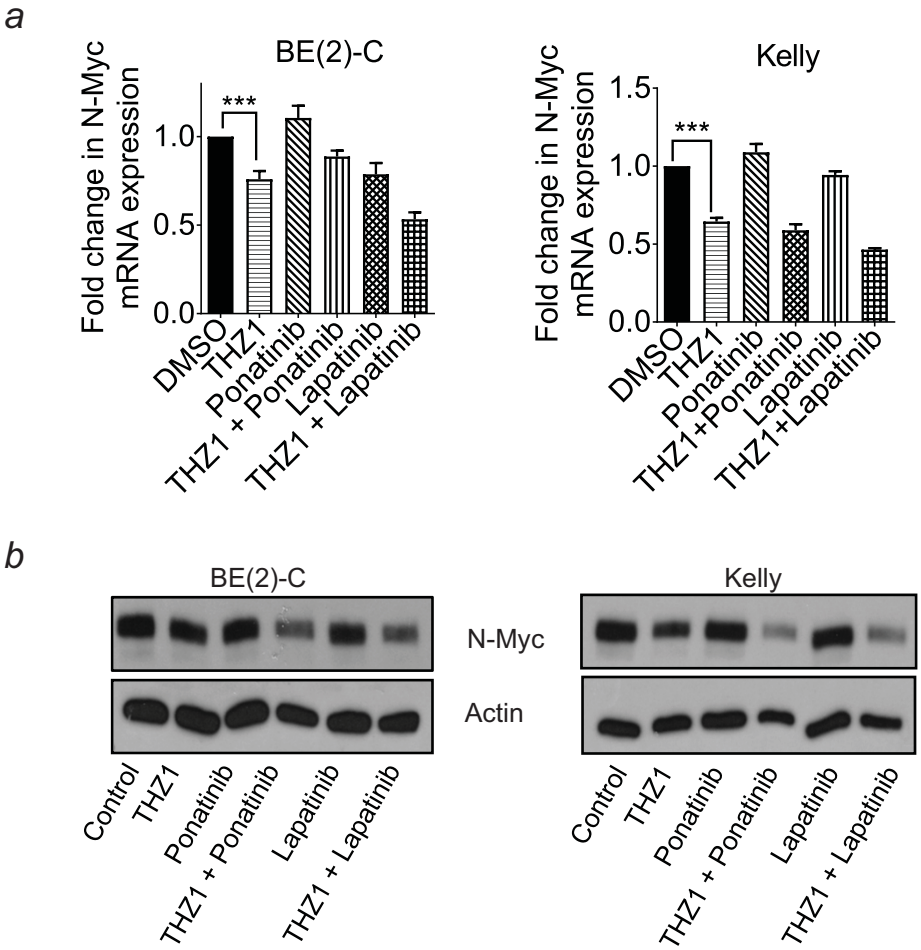


Figure 3

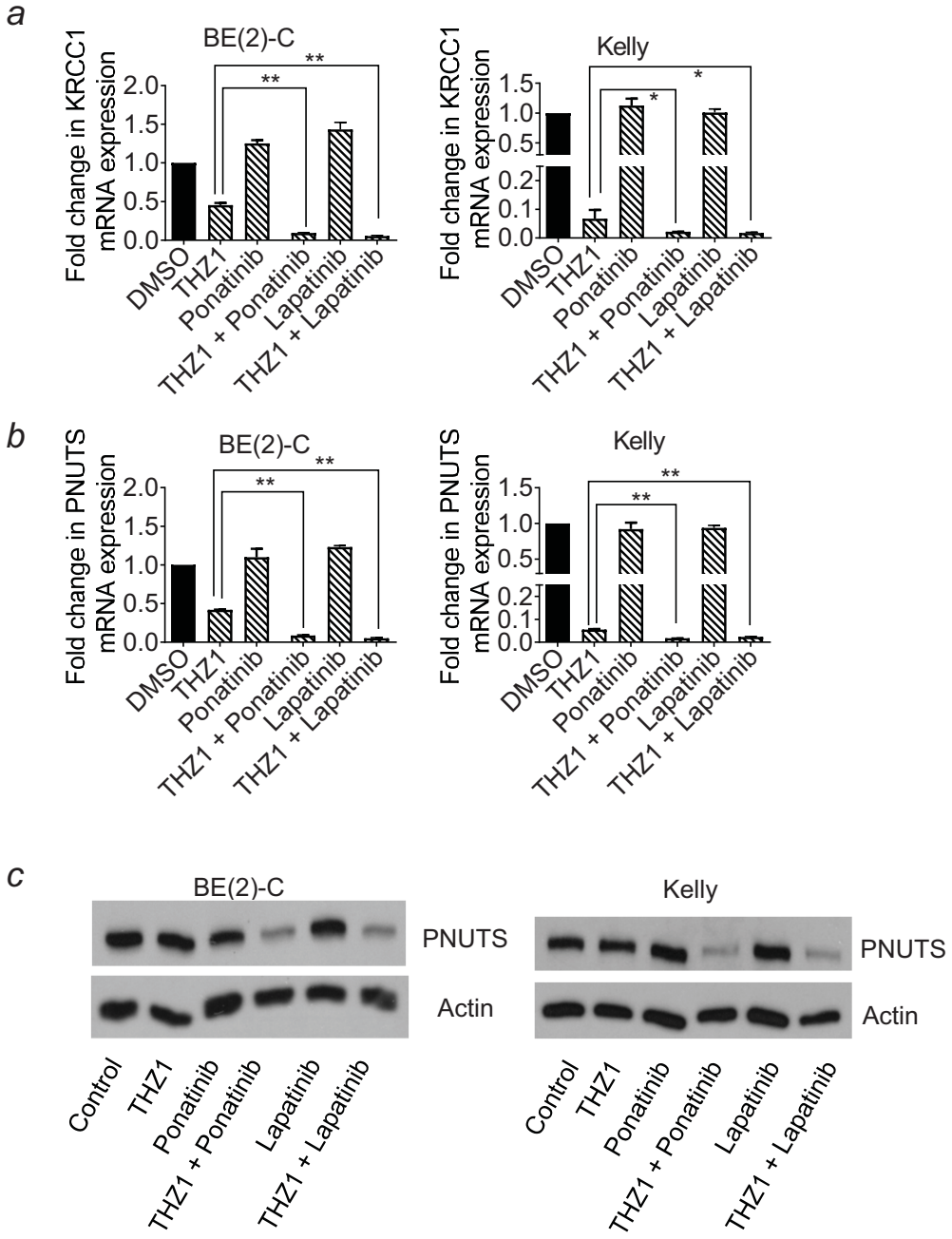


Figure 4

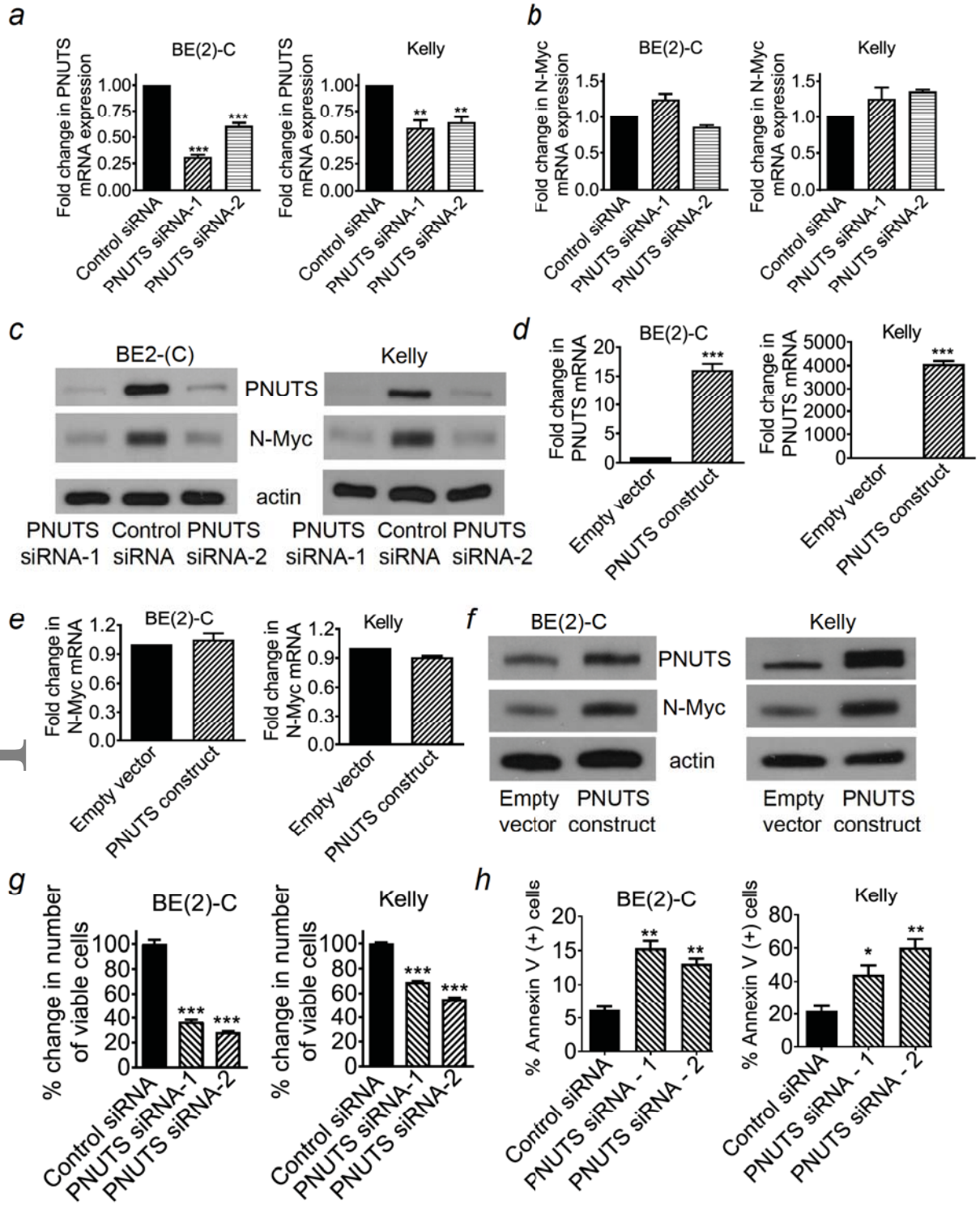


Figure 5